Submission of petition for Evaluation of Substances for Inclusion on the National List of Substances Allowed in Organic Production and Handling

1. Seed Mold (Dry mash culturing starter)

2. Name : Kikkoman Corporation
   Address : 250 Noda Noda City Chiba Pref. 278-8601 , Japan
   Telephone: (81)-4-7123-5145

3. In soy sauce production process, soybeans and wheat are treated with heat under controlled conditions and then blended. The resulting mixture is inoculated with Kikkoman aspergillus and cultured. The solid mass obtained from this process is called koji. This Kikkoman aspergillus to make koji is seed mold (dry mash culturing starter).

4. Aspergillus oryzae or Aspergillus sojae, Wheat bran as medium

   The following guidelines should be followed in koji cultivation:
   a) Grow as much mold mycelia and mold enzymes as possible.
   b) Prevent the inactivation of the enzymes produced.
   c) Minimize the carbohydrate consumption in raw materials during cultivation.
   d) Avoid as much bacterial contamination in the starting materials and during the cultivation of mold as possible.
   e) Shorten the cultivation time with minimum use of water, electricity, and fuel oil.

5. The mixture of cooked soybeans and roasted crushed wheat kernels is mixed with 0.1-0.2% of starter mold. Aspergilus oryzae or A. sojae. The mixed materials are usually cultured for 3 days in warm room, in which the temperature is controlled.
   The materials are cooled twice or more by mixing when their temperature rises to about 35 C or more because of the growth of molds. This allows the mold to grow throughout the mass and provide the amylase and proteases needed to hydrolyze proteins and starch in the raw materials. This material is called Koji.

6. yeast for bread、yeast for fermentation

7. Scientific data and information for the select committee on GRAS substances relating to fermented soy sauce.

APPENDIX 6.5

Personal History

Harumi Kashima
Kikkoman Foods, Inc.
Vice President of Manufacturing
Walworth, Wisconsin 53184, U. S. A.

Born in Japan, 1934 (Oami Oamishirasatomachi Chiba Prefecture)

Graduated, Hokkaido University, Laboratory of Applied Microbiology and School of Agriculture, (1957)

Entered, Kikkoman Shoyu Co., Ltd., Nodashi, Japan, (1957), Research in microbiology at Noda Institute for Scientific Research and Central Research Laboratories, (1957-63)

Worked at #7 Plant, Kikkoman Shoyu Co., Ltd., (1963-1970), Assistant Manager, (1970)

Production Control Department of Kikkoman Shoyu Co., Ltd., (1971)

Kikkoman Foods, Inc., Vice President of Manufacturing, (1972-present)

8. None

9. Scientific data and information for the select committee on GRAS substances relating to fermented soy sauce

10.None

11. # Scientific data and information for the select committee on GRAS substances relating to fermented soy sauce
    # ADVANCES IN FOOD RESEARCH (Vol, 30) ACADEMIC PRESS, INC (1986)

12. Soy sauce is prepared by digesting mixtures of soybeans and wheat with enzymes produced by Koji molds, Aspergillus sojae or Aspergillus oryzae, in the presence of 16 to 18% salt. The salt tolerant Pediococcus halophilus and Saccharomyces rouxii grow during the process and produce lactic acid, alcohol and carbon dioxide. In these soy sauce production process, seed mold is necessary substance.

13. None

APPENDIX 6.4

Personal History

Akira Okuhara
Kikkoman Shoyu Co., Ltd.
Manager, Products Analysis Section
Kikkoman Central Research Laboratories
399 Noda, Noda-shi, Chiba-Ken, Japan

Born, September 1, 1931

Graduated, Tohoku University, Agricultural Chemistry, (1956)

Entered, Kikkoman Shoyu Co., Ltd., (1956)

Doctor of Agriculture, Tohoku University, (1972)

Kikkoman Shoyu Co., Ltd., Manager, Products Analysis Section, (1974-present)

Major Research Publications (including co-research)

1. Studies on the Flavorous Substances in Soy Sauce, (21,22), J. of Agricultural Chemical Society of Japan, (1958-1963).
2. Analytical Method of Soy Sauce, (1,3,4), J. of Agricultural Chemical Society of Japan, (1958-1963), Seasoning Science, (1971).
3. Color of Soy Sauce, (1-7), J. of Fermentation Technology, (1969-1971), J. of Agricultural Chemical Society of Japan, (1972), J. of Society of Brewing, Japan, (1976).

SOUTH RIVER MISO COMPANY
Certified Organic & Unpasteurized
WOOD-FIRED HANDMADE MISO SINCE 1979

Toni Strother
USDA/AMS/TM/NOP
1400 Independence Avenue SW
Room 4008-SO, AG STOP 0268
Washington, DC 20250

August 16, 2002

Dear Toni,

I am writing on behalf of our company to inquire if we need to petition the NOSB to place the *Aspergillus oryzae*, koji spore powder on the National List. I am writing also on behalf of Barry Evans of the American Miso Company, who shares the same concern.

We use *Aspergillus oryzae* (hereafter called "spore powder") as a processing aide to make miso, a fermented food product made from the combination of cultured grain (called "koji", the Japanese term), cooked beans, sea salt, and water.

The spore powder originates in Japan. It is used to make koji, which is used not only for miso making but as the "starter" for a number of traditional fermented foods including sake, amasake, rice vinegar, and mirin.

At South River Miso Company we currently use the spore powder produced and packaged by Nihon Jozo Kogyo Co. (NJK) and exported to the USA by Mitoku Co., Ltd. A declaration from the producer of this spore powder is attached. There are other Japanese producers of koji spore powder as well. For example, American Miso Co. uses spore powder produced by Bioc Co., Ltd., also exported by Mitoku. The Bioc Company is presently on vacation. For that reason Barry Evans has been unable to obtain a declaration from them at this time.

We obtain the NJK spore powder in sealed foil packets, each containing 40 grams Net WT of spore powder. We ourselves use their

APPENDIX 6.3

Personal History

Shinichi Sugiyama
Kikkoman Shoyu Co., Ltd.
Manager of Life Science Division
Central Research Laboratories
399 Noda, Noda-shi, Chiba-Ken, Japan

Born, February 2, 1928

Graduated, University of Tokyo, Agricultural Biology, (1953)

University of Tokyo, Graduate School of Agricultural Chemistry (Microbiological Chemistry), (1953-1957)

Received Doctor of Agriculture, University of Tokyo for studies on metabolism of aromatic compounds by microbes, (1961)

Entered, Kikkoman Shoyu Co., Ltd., Research staff at the Central Research Laboratories, (1957), Research on amino acid and nucleic acid metabolism, (1957-1966)

Associate scientist, City of Hope Medical Center in California (Experimental Pathology), for studies on mycotoxins, (1967-1969)

Kikkoman Shoyu Co., Ltd., Chief of Food Hygienic Research Section, Central Research Laboratories, (1970-present), Manager of Life Science Division, Central Research Laboratories, (1977)

Major Research Publications (including co-research).

1. Metabolism of Aromatic Compounds by Microbes, (Part 2-9), Bulletin of the Agricultural Chemical Society of Japan and J. of General and Applied Microbiology, (1953-1960).

2. The Production of Dihydroxy Acetone by Bacteria, Agricultural and Biological Chemistry, (1964).

3. D-Ribose Formation by Bacteria, Agr. Biol. Chem., (1966).

4. Mycotoxins in Fermented Food, Cancer Research, 28, 2296, (1968).

(page 2)

products #301A and #301C as listed in the ingredients panel of the declaration attached.

Miso making involves a double fermentation process. The first step is to make the cultured grain koji. To do this we mix the spore powder with steamed grain, usually rice or barley.

The *Aspergillus* mold then grows on the steamed grain for about 48 hours. During this time the starches of the grain are converted into simple sugars and an abundance of digestive enzymes are created, which are needed for the second stage of miso fermentation. [Please note that by this ancient process we are "growing" our own enzymes on organic grain substrates and not purchasing outside enzyme inputs such as might be referred to on the National List, section 205.604 (8).]

After 48 hours the whole grain koji is harvested and mixed with sea salt and cooked beans (usually soybeans). This mixture of raw miso is put into fermentation vats for a period of three weeks to three years depending on the variety of miso, the salt content, and other factors such as temperature. After fermentation the finished miso is packaged and distributed for sale and consumption.

Miso itself is an ancient food and seasoning, developed through many generations of miso makers in Japan. The word miso first appeared in the Japanese script in 800 AD.

In terms of quantity used per batch of koji, 3 Tablespoons (about ½ ounce) of spore powder are used to inoculate 350-360 pounds of steamed grain.

In terms of quantity used per weight of finished product, this amounts to a range of .00204% to .0039% of the finished product by weight. (For example, about 2.5 ounces of spore powder are used to make 1750 lbs. of barley koji, which goes into making 7650 lbs. of Barley Miso.)

As you can read on the declaration, NJK mixes the pure spores of *Aspergillus oryzae* with non-GMO soluble potato starch as an extender, or substance used to carry the microscopic spores, which would otherwise be difficult, if not impossible to handle effectively. The NJK spore powder is fortified with yeast and lactic acid bacteria as

Ribelin, W.E., Masri, M.S. and DeEds, F.: Fluorescence of bone after Quercitin ingestion. Proc. Soc. Exp. Biol. Med., 103:271-272, 1960.

Ribelin, W.E.: Fat necrosis in man and animals. J. Am. Vet. Med. Assoc., 136: 135-139, 1960.

Ribelin, W.E. and Bailey, W.S.: Esophageal sarcomas associated with Spirocerca lupi infection in the dog. Cancer, 11:1242-1246, 1958.

Ribelin, W.E.: The cytopathogenesis of vesicular stomatitis virus infection in cattle. Am. J. Vet. Res., 19:66-73, 1958.

Frenkel, H.S. and Ribelin, W.E.: Cultivation of the foot-and mouth disease virus in explanted epithelium of the bovine tongue. IX. Growth characteristics of the virus in large scale tissue cultures. Am. J. Vet. Res., 17:40-44, 1956.

Ribelin, W.E. and Kintner, L.D.: Lipodystrophy of the central nervous system in a dog. A disease with similarities to Tay-Sachs disease of man. Cornell Veterinarian, 46:532-537, 1956.

Ribelin, W.E.: The incidence of distemper in canine encephalitis cases. Am. J. Vet. Res., 14:96-104, 1953.

Ribelin, W.E.: Azpturia and the Crush syndrome. J. Am. Vet. Med. Assoc., 119: 284-288, 1951.

Cordy, D.R. and Ribelin, W.E.: Six congenital cardiac anomalies in animals. Cornell Veterinarian, 40:249-256, 1950.

Languages: Read--English, German, French, Spanish, Dutch
Spoken--English, Spanish

(page 3)

specified in the footnote of the declaration. [I see that yeast and lactic acid are on the National List, section 205.605 (1.iii) and (20) respectively. Please note that the yeast is not cultured on petrochemical or sulfite waste liquor substrates as prohibited on the National List 205.605(20).]

We would like to know as soon as possible, if we need to petition the NOSB to put the *Aspergillus oryzae* spore powder on the National List. If such a petition were required, under which category would it be classified for inclusion, since none of the categories under ITEM A of the sheet entitled "INFORMATION TO BE INCLUDED IN A PETITION" seem to apply?

If any further information is needed from me in order for the committee to make a determination, I can be reached at our company phone listed below and at my home phone 413-369-4066.

Thanking you very much for your prompt attention to this, I am

Sincerely Yours,

Christian Elwell

Christian Elwell
South River Miso Company
(413) 369-4057 work
(413) 369-4066 home

cc. Barry Evans, American Miso Company (tel 828-665-7790)
Don Franczyk, NOFA Mass. (tel 978-297-4171)

Hsu, I-C., Smalley, E.B., Strong, F.M. and Ribelin, W.E.: Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. Appl. Microbiol., 24(5):684-690, 1972.

Still, P.E., Macklin, A.W., Ribelin, W.E. and Smalley, E.B.: Relationship of Ochratoxin A to fetal death in laboratory animals and domestic animals. Nature, 234(5531):563-564, 1971.

Walsh, A.H. and Ribelin, W.E.: Fish: Experimental models for human disease. Comp. Path. Bull. (A.F.I.P.), III(4):1-4, 1971.

Leswing, R.J. and Ribelin, W.E.: Physiologic and pathologic changes in acrylamid neuropathy. Arch. Environ. Health, 18:22-29, 1969.

Ribelin, W.E. and Leswing, R.J.: Conduction velocities of peripheral nerves in cats and Cebus albifrons monkeys. Am. J. Vet. Res., 29:2401-2405, 1968.

Thomas, J.O., Ribelin, W.E., Wilson, R.H., Keppler, D.C. and DeEds, F.: Chronic toxicity of diphenylamine to dogs. Toxicol. Appl. Pharm., 11:184-194, 1967.

Ribelin, W.E., Levinskas, G.J., Owen, G. and Rubin, L.: Cataracts produced in dogs and rats by prolonged feeding of Sulfaethoxypyridazine. Toxicol. Appl. Pharm., 10:557-564, 1967.

Thomas, J.O., Ribelin, W.E., Wilson, R.H., Keppler, D.C. and DeEds, F.: Chronic toxicity of diphenylamine to albino rats. Toxicol. Appl. Pharm., 10:362-373, 1967.

Levinskas, G.J., Ribelin, W.E. and Shaffer, C.B.: The acute and chronic toxicity of Pimaricin. Toxicol. Appl. Pharm., 8:97-109, 1966.

Ribelin, W.E. and Levinskas, G.J.: Diverticulitis and neoplasia of the small intestine of rats fed 2-(p-Dimethylaminostyryl)-1-methylquinolinium methyl sulfate. Toxicol. Appl. Pharm., 7:619-626, 1965.

Booth, A.N., Robbins, D.J., Ribelin, W.E., DeEds, F., Smith, A.K. and Rackis, J.J.: Prolonged pancreatic hypertrophy and reversibility in rats fed raw soybean meal. Proc. Soc. Exper. Biol. Med., 116:1067-1069, 1964.

Ribelin, W.E., Shaffer, C.B. and Levinskas, G.J.: Tumorigenic properties of 2-Cyano-4-Aminostilbene in rats. Toxicol. Appl. Pharm., 5:344-349, 1963.

Ribelin, W.E.: Atrophy of rat testis as index of chemical toxicity. Arch. Path., 75:229-235, 1963.

Ribelin, W.E., Booth, A.N. and Robbins, D.J.: Pancreatic hyperplasia in rats induced by soybean meal diets. Fed. Proc., 19(1):Part I:5, 1960.

Booth, A.N., Robbins, D.J., Ribelin, W.E. and DeEds, F.: Effects of raw soybean meal and amino acids on pancreatic hypertrophy in rats. Proc. Soc. Exp. Biol. Med., 104:681-683, 1960.

MITOKU CO LTD

Sunshine Bldg. 5-31-10 Shiba, Minato-ku, Tokyo 108-0014, JAPAN
Tel +81-3-5444-6701 Fax +81-3-5444-6702
E-mail export@mitoku.co.jp

To whom it may concern,

DECLARATION

I hereby declare that the product mentioned below is not derived from o r produced using GMOs or their derivatives, and that all reasonable steps have been taken to avoid contamination from GMOs or their derivatives.

1. Ingredients

Item code/name	substrate	extender	fortifier
301A / Koji Spores*, Rice Miso	barley	soluble potato starch	yeast**, lactic acid***
301C / Koji Spores, Light Rice Miso	barley	soluble potato starch	yeast, lactic acid
301D / Koji Spores, Barley Miso	barley	not added	not added
301E / Koji Spores, Soybean Miso	barley	soluble potato starch	not added
309A / Koji Spores, Shoyu	wheat, wheat bran	soluble potato starch	yeast, lactic acid

note) * : Aspergillus oryzae
** : Zygosaccharomyces rouxii
*** : Tetragenococcus halophilus

yeast)
No petrochemical substrate or sulfite waste liquor is used.
processing flow : make culture medium → inoculate yeast → cultivation → mix to dispersion medium → freeze drying → added to koji spores

GMO barley and GMO wheat is not allowed in the Japanese market.
The soluble potato starch is made with potato of Japanese or EU origin. GMO potato is not allowed in both areas.

William E. Ribelin, D.V.M., Ph.D. Curriculum Vitae

RESEARCH INTERESTS:

Comparative Pathology Between Animal Species, Particularly Tumors.
Diseases in Animals and Man Caused by Mycotoxins, Pesticides, Heavy Metals.
Diseases of Laboratory Animals.
Pathology of Infectious and Nutritional Diseases

APPOINTMENTS:

Instructor in Veterinary Pathology, Washington State University, 1949-52.
Research Veterinarian, U.S.D.A., Foot-Mouth Disease Mission, Amsterdam, Holland, 1952-54.
American Veterinary Medical Association Fellow, University of Wisconsin, 1954-56.
Professor of Veterinary Pathology, Auburn University, 1956-58.
Head, Pathology Investigations U.S.D.A., Pharmacology Laboratories, Albany, Calif., 1958-62.
Senior Scientist, American Cyanamid Company Environmental Health Laboratories, Princeton, New Jersey, 1962-68.
Professor of Medical Pathology, and Professor of Veterinary Science, University of Wisconsin, Madison, 1968 to present.
Director, U.W. Research Animals Resources Center

BOOKS:

Ribelin, W.E. and McCoy, J.R.: The Pathology of Laboratory Animals. Charles Thomas Company, Springfield, Illinois, 1965.

Ribelin, W.E. and Migaki, G.: The Pathology of Fish. University of Wisconsin Press, 1975.

ARTICLES:

Ribelin, W.E.: Effect of ochratoxin and trichothecene mycotoxins on cattle. In: Handbook of Mycotoxins. Edited by T.D. Wyllie and L.G. Morehouse. Elsevier Publishers, in press.

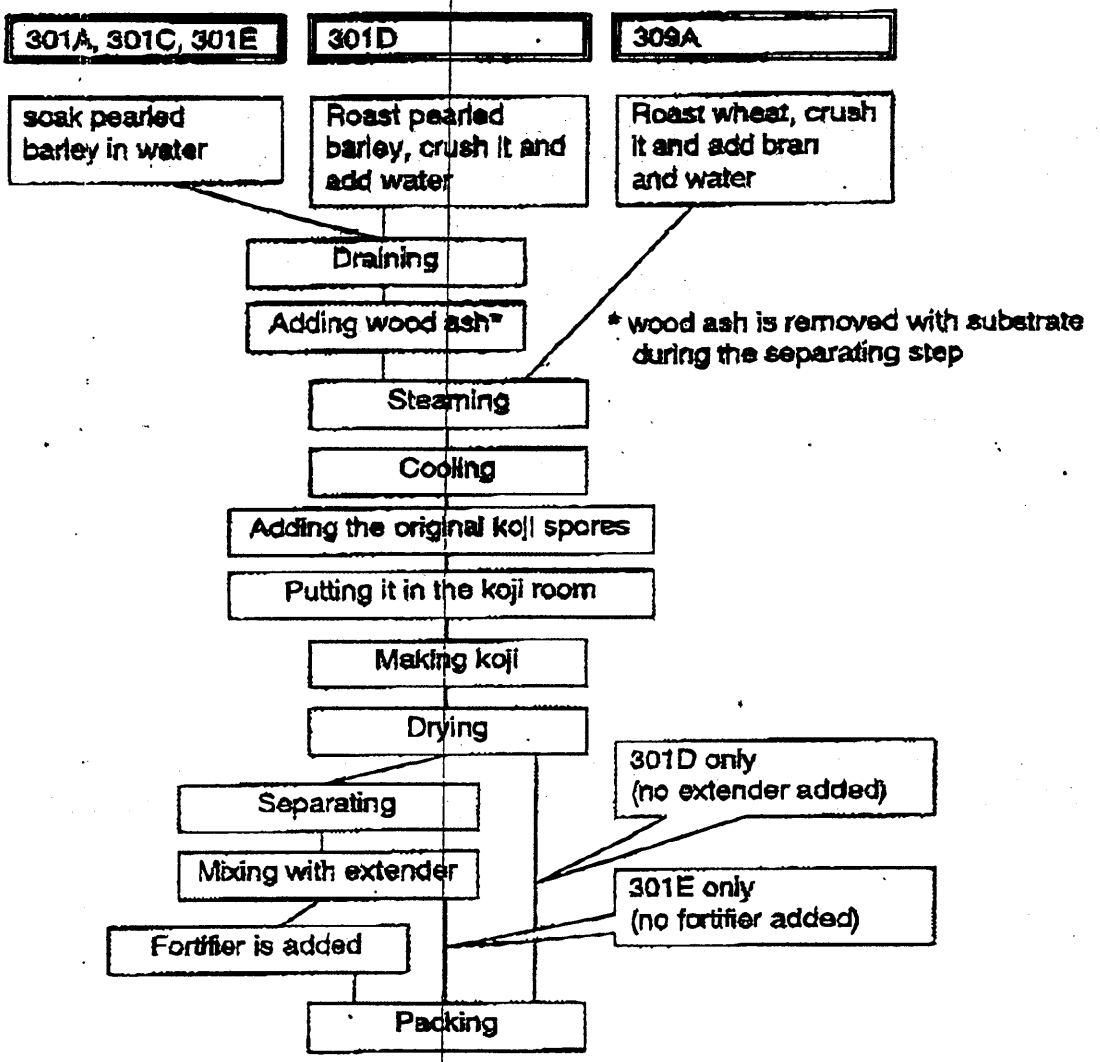
Anslow, R.O. and Ribelin, W.E.: Recognition and control of mouse pox in dispersed mouse facilities. Proc. 25th Ann. Mtg. Am. Assoc. Lab. An. Sci., #69, 1974.

Ribelin, W.E.: Use of fish as laboratory animals. Proc. 24th Ann. Mtg. Am. Assoc. Lab. An. Sci., #7, 1972.

Ribelin, W.E., Smalley, E.N. and Strong, F.M.: Effect of ochratoxin and other mycotoxins on cattle. 2nd Internat. Congress Plant Path. (Minneapolis), Abstract #0865, 1973.

Walsh, A.H. and Ribelin, W.E.: The pathology of pesticide and herbicide poisoning in fish. In: Pathology of Fish. Edited by W.E. Ribelin. Universtiy of Wisconsin Press, 1974.

2. processing flow



Signature Masaaki Suzuki Date Aug. 7, 2002.

Name Masaaki Suzuki

Company name and address Nihon Jozo Kogyo Co. Ltd.
3-18-9 Koishikawa Bunkyo-ku, Tokyo Japan

APPENDIX 6.2

April 1976

CURRICULUM VITAE

William E. Ribelin, D.V.M., Ph.D. Male

Professor

October 1, 1924 (Birthdate)

Riverside, California (Birthplace)

5701 Pheasant Hill Rd. (Home)
Monona, Wisconsin 53716

Business Address:
Department of Veterinary Science
University of Wisconsin-Madison
Madison, Wisconsin, U.S.A. 53706

EDUCATION:

Degree	Institution Conferring	Year
D.V.M.	Iowa State University	1949
M.Sc. (Animal Pathology)	Washington State University	1952
Ph.D. (Pathology)	University of Wisconsin	1957

HONORS; SOCIETIES:

Founding Member, Northern California Chapter American Assoc. Lab. Animal Science, 1962.
Chairman, Committee on Education, American College of Veterinary Pathology, 1966-68.
Co-chairman, New York Academy of Medicine Conference on the Pathology of Laboratory Animals, 1964.
Co-chairman, Armed Forces Institute of Pathology Conference on the Pathology of Fish, 1972.
Fellow, American Association for Advancement of Science.
Member, Executive Committee, University of Wisconsin Center for Environmental Toxicology.
Phi Kappa Phi.
Phi Zeta.
Alpha Zeta.
Lecturer, Armed Forces Institute of Pathology Short Course on Pathology of Laboratory Animals, 1964 & 1966.
Co-founder, New York University Annual Conference on Use of Primates in Biology and Medicine, 1964.
Lecturer and Consultant, Armed Forces Institute of Pathology, 1974.
Member, Advisory Committee to Surgeon General, U.S.P.H.S. on Use of Phosphates and Chelating Agents in Detergents, 1972.
Member, Advisory Board, Smithsonian Institute Registry of Tumors in Lower Vertebrates

SOCIETIES:

American College of Veterinary Pathologists
Society of Experimental Pathology
International Academy of Pathology
Society of Toxicology
American Fisheries Society
American Veterinary Medical Association
American Association for Laboratory Animal Science

Toni Strother
USDA/AMS/TM/NOP
1400 Independence Avenue SW
Room 4008-SO, AG STOP 0268
Washington, DC 20250

October 29, 2002

Dear Toni,

I write to you today on behalf of our company, the American Miso Company, to inquire whether or not we must petition the NOSB to place the spores we use in miso production, *Aspergillus oryzae* in the form of koji spore powder, on the National List of substances approved for use in certified organic production. I also write in support of the earlier inquiry submitted to you on August 16, 2002, by Christian Elwell on behalf of the South River Miso Company. I consulted with Christian on that inquiry and agree with every point he made at that time. Although we use a spore powder produced by the Bio'c Co., Ltd., a different supplier of a different product, both spore powders are similar in that both are commercial forms of dried *Aspergillus oryzae* produced in Japan and exported to the US by the Mitoku Co., Ltd.

We use the spore powder to make miso, a traditional Japanese fermented food product made from a combination of cultured grain, usually rice or barley, called "koji" by the Japanese, cooked beans, usually soybeans, sea salt, and water. We obtain the Bio'c spore powder in sealed foil packets of 20 grams each. We use 10 of the 20-gram packets per 36 "mixings" of miso. Since each mixing weighs from 47.5 kilos to 96 kilos, depending on the variety of miso we are making, the proportion of spore powder to finished miso product ranges from 0.00583% to 0.01179%. Our spore powder proportion is, therefore a little more than one in 20,000 on the low side to a little more than one in 10,000 on the high side.

Our proportion of spore powder used is two to three times as high as that used by Christian Elwell at South River. I have discussed this issue with Bill Shurtleff, author of The Book Of Miso and director of The Soy Foods Institute. Bill believes that South River does not have to use as much spore powder because their spore powder is fortified with yeast and lactic acid bacteria, while our spore powder is not so fortified. Bill informed me in a recent conversation that the Nihon Jozo Kogyo Company, South River's supplier, is one of the oldest spore companies in Japan, dating from the 1700's, and that this process of fortifying spore powder with a particular strain of yeast and a particular type of bacteria in order to promote thorough and appropriate fermentation of miso was developed by this company and others through a lengthy process of miso making and observation of that process.

BIOGRAPHICAL SKETCH

Professor E. M. Foster, University of Wisconsin, Madison, Wisconsin

A native of Texas, Professor Foster earned his Ph.D. degree in Bacteriology at the University of Wisconsin in 1940. He joined the faculty there after World War II and has been a professor of food bacteriology since that time. Currently Dr. Foster is Director of the Food Research Institute and Chairman of the new Department of Food Microbiology and Toxicology.

Professor Foster has been President of both the American Society for Microbiology and the American Academy of Microbiology.

He has accepted numerous assignments for the National Academy of Sciences--National Research Council including Member of the Agricultural Board, Member of the Food and Nutrition Board, Chairman of the Committee on Salmonella and Chairman of the Committee on Food Protection. He has served on the National Advisory Food and Drug Committee of the FDA, the Expert Advisory Panel on Food Hygiene of the World Health Organization, the Expert Panel on Food Safety and Nutrition of the Institute of Food Technologists, and the Advisory Committee on Regulatory Programs of the U.S. Department of Agriculture.

Dr. Foster is a Charter Fellow of the Institute of Food Technologists. He received the Distinguished Alumnus Award from North Texas State University; the Pasteur Award from the Illinois Society for Microbiology; and the Nicholas Appert Award from the Institute of Food Technologists.

At The American Miso Company, during our first years of operation, we always added a small quantity of Onozaki Miso, a traditionally made miso imported from Japan, to each batch of our own miso as "seed miso", much as bakers add sourdough "mother" to their dough. Through this process, we gradually colonized our entire plant with lactobacillus bacteria and yeast and we have never used fortified spore powder to produce our miso. We have always purchased our unfortified spore powder from the Bio'c Company, an organization with a less ancient lineage than NJK.

I won't repeat the description of miso making and its double fermentation process that Christian delivered so well in the first four paragraphs of the second page of his inquiry letter except to say that our procedure differs only in details from his. I also want to re-emphasize his comments on "growing" our own enzymes in his second paragraph and add that the enzyme section of the National List says such enzymes must be "derived from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria" in order to be approved. Our point is that our spores are a nonpathogenic fungi used in traditional food production for centuries and if "enzymes", whatever can even be meant by such a broad term, derived from "nonpathogenic fungi" are allowed, then it follows that the nonpathogenic fungi themselves must be allowed.

It is furthermore our contention that koji spores are a "nonorganically produced agricultural product" that "may be used in accordance with the restrictions specified in this section and when the product is not commercially available in organic form" as stated in section 205.606. Granted that koji spores are not the Old MacDonald's Farm style of agricultural product in the same way that rutabagas, ears of corn, or pork chops are, but in a broadly considered, non-Occidentalised view of agriculture, koji spores are every bit as much of an agricultural product ("any" of which are acceptable) as Mom's umeboshi plum pie. We, therefore, contend that the NOSB or its staff should rule that koji spore powder, as an agricultural product not available in organic form, is ipso facto accepted for use in certified organic products and does not need to be put on the National List.

We have included a declaration from our supplier averring that they do not use GMO's or their derivatives, sewage sludge, irradiation, synthetic preservatives, or synthetic stabilizers. Furthermore, our supplier notes on their accompanying flow chart that the substrate upon which the spores are cultured is composed of non-GMO rice only, while the necessary extender added to the microscopic spores for handling is composed only of non-GMO potato. No fortifiers are added. Our supplier also states that they maintain their sterile lab environment by computer control of temperature, humidity, and filtered air flow only without use of chemicals.

Member, Food Safety Task Force, U.S. Department of Agriculture.

Member, Advisory Committee on Radiation Pasteurization of Foods, U.S. Atomic Energy Commission.

Consultant on Food Safety, School of Aviation Medicine, U.S. Air Force.

Member, Advisory Council on Microbiology, Association of Food and Drug Officials of the U.S.

Member, Advisory Committee on Microbiology of Frozen Foods, Association of Food and Drug Officials of the U.S.

Member, Intersociety/Agency Committee on Microbiological Methods for Foods, American Public Health Association.

Chairman, Panel 10 (Research Needs) National Conference on Food Protection, American Public Health Association, 1971.

Member, Expert Committee on Food Hygiene, World Health Organization.

Chairman, Subcommittee on Microbiological Safety, Citizens' Commission on Science, Law, and the Food Supply.

8. Awards

Distinguished Alumnus Award, North Texas State University, 1967.
Nicholas Appert Award, Institute of Food Technologists, 1969.
Pasteur Award, Illinois Society for Microbiology, 1969.
Charter Fellow, Institute of Food Technologists.

9. Club Memberships

Blackhawk Country Club, Madison, Wisconsin
Cosmos Club, Washington, D.C.

10/1/76

In closing let me note that both South River Miso and The American Miso Company have been faithfully practicing organic processing since their founding over twenty years ago and have been certified as long as certification for processors has been available. Neither company has ever used any non-organic ingredient except koji spore powder. We have repeatedly entreated our spore suppliers to produce organic spores for us, which could easily be done and in which case we would not be bothering you, but our pleas have fallen on deaf capitalist ears. Our volume is too small for our producers to bother with organic spores. We were inclined to simply take for granted that our spores were an agricultural product under section 205.606, but our certifier felt that we needed to obtain your written assessment of this minor ingredient or processing aid.

Please let us know if you require any more information from us for consideration o0f this issue at your March meeting. We can be reached at the phone numbers, email address, and address below.

Yours truly,

Barry Evans

Barry Evans
The American Miso Company
92 McIntosh Rd.
Asheville, NC 28806-1406
Office – 828-285-0723
Cel – 828-275-2032
Email – generalmgr@great-eastern-sun.com

Institute of Food Technologists

President, Wisconsin Section 1963-64

6. Current Professional Activities

Member, Advisory Committee on Regulatory Programs, U.S. Department of Agriculture.

Member, National Advisory Food and Drug Committee, Food and Drug Administration.

Member, Expert Advisory Panel on Food Hygiene, World Health Organization.

Member, Expert Panel on Food Safety and Nutrition, Institute of Food Technologists.

Member, Food and Nutrition Liaison Committee, The Nutrition Foundation.

7. Past Professional Assignments

Member, Food and Nutrition Board, National Academy of Sciences -- National Research Council.
Member, Executive Committee.

Chairman, Food Protection Committee, National Academy of Sciences -- National Research Council.
Member, Subcommittee on Food Microbiology.

Chairman, Committee on Salmonella, National Academy of Sciences -- National Research Council.

Member, Agricultural Board, NAS-NRC. (Member, Executive Committee 1970-72).

Member, Subcommitte on Food and Nutrition, Research Advisory Committee to the Secretary of Agriculture, NAS-NRC.

Chairman, Committee on Microbiology of Food, Advisory Board on Military Personnel Supplies, NAS-NRC.

Member, General Committee on Department of Defense Food Program, Advisory Board on Military Personnel Supplies, NAS-NRC.

Member, Advisory Committee on Botulism Hazard, Food and Drug Administration.

Chairman, Interagency-Industry Committee on Classification of Foods According to Risk (for Food and Drug Administration).

Consultant, Protocols for Safety Evaluation Advisory Committee, Food and Drug Administration.

Bio'c CO.,LTD.
Toyohashi Japan

DECLARATION

I hereby declare that our Koji spores(Aspergillus oryzae) sold to Mitoku and its contract processors is not derived from or produced using GMOs or their derivatives, and that all reasonable steps have been taken to avoid contamination from GMOs or their derivatives. Furthermore, these products are produced and handled without irradiation or sewage sludge. No synthetic stabilizers or synthetic preservatives are added.

We are taking the following steps to incubate our Koji spores(Aspergillus oryzae).
1. Incubates all of its koji spores from original koji spores of this species, which have existed in nature for many centuries, detected, classified, and gathered by us from their natural state. These original koji spores are kept in test tubes and used to produce new offspring spores as needed. These original incubated koji spores can be called "parent" spores, new offspring spores can be called f1, or "child" spores.
2. At step 5 of the processing flow below, f1-koji spores are added to the substrate. The resulting finished product can be called f2 or "grandchild" spores. We then repeats this parent-f1-f2 process each time we receive an order to produce a new batch for sale. Our koji spores are always produced fresh, not left over from the previous batch.

We use computers to control the clean room in order to keep the temperature and humidity at the same level at all times. We also circulate filtered air to keep various troublesome bacteria under control. These measures allow us to maintain an uncontaminated environment for our spores without using chemicals.

Signature: N. Kamiya Date: 2002. 9. 26.
Name and title: Naotaka Kamiya, Techno-service dept.

(continue to next sheet)

株式会社 ビオック 〒441-8087 豊橋市牟呂町内田111-1 ☎0532-31-9204 FAX 0532-31-0316

APPENDIX 6.1

Curriculum Vitae

Professor E. M. Foster
Director, Food Research Institute
1925 Willow Drive, University of Wisconsin
Madison, Wisconsin 53706
Area Code 608/263-7777

1. Personal: Born January 1, 1917, Alba, Texas; married Winona Lively, 1941; one child, Michael, born 1947.

2. Education: B.A. (Biology), North Texas State College, 1936; M.A., 1937; Ph.D. (Bacteriology), University of Wisconsin, 1940.

3. Professional Experience: Instructor, Bacteriology, University of Wisconsin, 1940-41; Instructor, Bacteriology, University of Texas, 1941-42; 1st Lt. and Captain, U.S. Army, 1942-45; Assistant Professor, Bacteriology, University of Wisconsin, 1945-46; Associate Professor, 1946-52; Professor, 1952--. Also Director, Food Research Institute, 1966--; Professor and Chairman, Food Microbiology and Toxicology, 1975--.

4. Research and Teaching Interests: Microbiology of foods, especially public health aspects.

5. Professional and Scientific Societies:

   a. Member: American Society for Microbiology (since 1946)
   American Academy of Microbiology (Charter Fellow)
   Institute of Food Technologists
   American Meat Science Association
   International Association of Milk and Food Sanitarians
   Sigma Xi
   Phi Kappa Phi (Honorary)
   Phi Beta Kappa (Honorary)

   b. Offices and Committees:

   American Society for Microbiology

   President 1969-70
   Vice President 1968-69
   Secretary 1957-61
   Member Council Policy Committee, 1957-65; 1968-71
   Chairman, A and I Division, 1951
   President, North Central Branch, 1962-63
   Director, Am. Soc. for Microbiology Foundation, 1972-73

   American Academy of Microbiology

   President, 1965-66
   Member, Board of Governors 1962-68
   Secretary-Treasurer 1962-65
   Member, Joint ASM-AAM Committee on Future of Microbiology 1964-67 (Chairman 1964-65)

Bio'c CO.,LTD.
Toyohashi Japan

種麹製造工程	Koji spores production process
原料米（96～97%精米）	Rice* (96-97% milled), as substrate (sterilized by high temperature and pressure)
↓	* GMO rice is not commercially available in Japan yet.
浸漬	Soaking
↓	
蒸煮	Boiling
↓	
放冷	Cooling
↓	
麹菌接種	Adding Koji spores (Aspergillus oryzae)
↓	
培養	Incubation
↓	
乾燥	Drying
↓	
胞子回収	Collecting Koji spores
↓	
品質検査	Quality checking
↓	
製品調整・袋詰め	Mixing spores and α-starch* (pregelatinized starch), and packing
↓	*Starch is made with potato of Japanese or EU origin. No fortifier is added.
製品検査	Product checking

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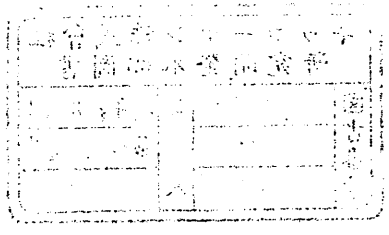
APPENDIX 6

PERSONAL HISTORIES OF PERSONS PARTICIPATING IN THE RESEARCH CONTAINED IN THIS REPORT AND IN THE EDITING AND COMPILATION



ADVANCES IN FOOD RESEARCH

VOLUME 30

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1986

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
Orlando San Diego New York Austin
London Montreal Sydney Tokyo Toronto

STATE OF WISCONSIN

DEPARTMENT OF AGRICULTURE

SAMPLE RECORD

PAGE 1 OF 1

MPLED AT: Kikkoman Foods, Inc.

DDRESS: Hwy 14

STATE: Walworth, Wis. ZIP:

NUFACTURER – DISTRIBUTOR – PRODUCER:

DDRESS:

TY: 5 STATE: ZIP:

NO. OF SAMPLES: 3 SAMPLES OF: Processed products

WITNESSED SAMPLING: INSPECTOR: R. Kurtzweil

ANALYSIS REQUESTED: Salmonella

INFORMATION: C

TE SAMPLED: 4/18/74 SHIPPED VIA: DATE RECEIVED: 4-24-74

DATE REPORTED: 5-6-74 REPORTED TO: Food & Standards

LAB NO	INSPECTOR NO	DESCRIPTION	RESULTS OF ANALYSIS
	1		
	2		RR
409	3 15-27	Teriyaki Sauce (bottled)	No Salmonella detected (1-114)
	4		
	5		
410	6 15-28	Soy Sauce (bottled)	No Salmonella (1-114)
	7		
	8		
411	9 15-29	Unpasteurized Soy Sauce	No Salmonella detected (1-114)
	10		
	11		
	12		
	13		
	14		
	15		
	16		
	17		
	18		
	19		
	20		

MILWAUKEE

MAY 8 1974

WIS. DEPT. OF AGRIC.

LABORATORY ADMINISTRATOR

John G. McClellan

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 48-7808

ISBN 0–12–016430–2

PRINTED IN THE UNITED STATES OF AMERICA

86 87 88 89 9 8 7 6 5 4 3 2 1

CONTENTS

STATE OF WISCONSIN
DEPARTMENT OF AGRICULTURE

SAMPLE RECORD

PAGE 1 OF

AT: Wyman Foods, Inc.
Hwy 14
Luxath(?) STATE Wisconsin ZIP

NO. OF SAMPLES: 3
SAMPLES OF: Grain Ingredients
WITNESSED SAMPLING
INSPECTOR: [illegible]
ANALYSIS REQUESTED: Salmonella – Aflatoxin

TURER – DISTRIBUTOR – PRODUCER
INFORMATION

STATE ZIP

SAMPLED	SHIPPED VIA	DATE RECEIVED	DATE REPORTED	REPORTED TO
/18/74		4-24-74	5-22-74	Food & Standards Division

	INSPECTOR NO.	DESCRIPTION	RESULTS OF ANALYSIS
	1		
	2		
6	3 15-24	Raw Wheat	No Salmonella detected (1-114)
			Mold = 2100 per gram (#1-90)
	4		Aflatoxin B_1 - B_2 - G_1 - G_2 --
	5		None detected - (214)
7	6 15-25	Soya meal	No Salmonella detected
			Mold = 70 per gram (1-90)
	7		Aflatoxin B_1 - B_2 - G_1 - G_2 --
	8		None detected - (214)
8	9 15-26	bran	No Salmonella detected (1-114)
	10		Mold = 1500 per gram (1-90)
			Aflatoxin B_1 - B_2 - G_1 - G_2 --
	11		None detected - (214)
	12		
	13		
	14		
	15		
	16		
	17		
	18		
	19		
	20		

RRK

MILWAUKEE
JUN 1 ? 1974
WIS. DEPT. OF AGRIC.

LABORATORY ADMINISTRATOR
John H. McClellan

SOY SAUCE BIOCHEMISTRY

TAMOTSU YOKOTSUKA

Kikkoman Corporation,
Noda-shi, Chiba-ken 278, Japan

STATE OF WISCONSIN
DEPARTMENT OF AGRICULTURE

SAMPLE RECORD

REGION IV / 84 | Page 1 | Of

Sampled at: KIKKOMAN FOODS, Inc.
Address P.O. Box 69
City WALWORTH State Wis. Zip 53184
Manufacturer–Distributor–Producer SAME
Address
City State Zip

No. Samples 2, 4/5 subs. ea | Samples of CANNED GOODS (SOY SAUCE)
Witnessed Sampling Haruni Kashima | Inspector R.C. WASHBURN | Insp. No. 84
Analysis Requested BACTERIA, AFLATOXIN, EXTRANEOUS
Information (Rodent & INSECT FRAGMENTS)

Holding order | H. O. Number

1	X	SURVEILLANCE
2		COMPLIANCE
3		CONSUMER COMPLAIN
4		INVESTIGATION
5		SUPERVISORY
6		
R		RESTAURANT

Date Sampled 11-12-75 | Date Received 11-19-75 | Date Reported 12-1-75 | Reported To Food & Standards Div.

Lab No.	Inspector No.	Description Size – Lot – Code	Computer Product Code	1–136 SPC/ml PLC	1–76 I.S.	1-90 Mold pc/ml	1-160 Yeast pc/ml	
1								
2	19631	84-126 SOY SAUCE, 5 5oz bottles			A	<1	<1	
3		(5 subs) Kikkoman, Naturally Brewed,			B	<1	<1	Extraneous -- (5) None detected in any of the 5 bottles.
4		All purpose seasoning, brewed			C	<1	<1	No Aflatoxins B_1, B_2, G_1 or G_2
5		by KIKKOMAN, Coded 11075,			D	<1	<1	detected. (4-214)
6		Water, Soybean, Wheat, Salt, Sodium Benzoate			E	<1	3	
7	19632	84-127 TERIYAKI, 5 5oz bottles,			A	<1	1	Extraneous -- (526)
8		(5 subs) KIKKOMAN BARBECUE MARINADE			B	<1	<1	Three pieces of wax-like matter found in Sub E. All other
9		& SAUCE, Coded 11125,			C	<1	<1	bottles -- none detected.
10		Ingredients, Same as above			D	<1	1	No Aflatoxins B_1, B_2, G_1, or G_2
11		Plus Wine, Vinegar, Sugar, dist'd			E	<1	<1	detected. (4-214)
12		Spirits, Spices, Succinic Acid, Onion & Garlic powders						

Remarks: 1/

MILWAUKEE, WIS.
DEC 2 1975
WISCONSIN DEPT. OF AGRIC.

TABLE I

HISTORY OF FERMENTED SOYBEAN FOODS[a]

China	Japan
Shu-Ching (700 B.C.)[b]	
Chu[c]	Manyo-shu (350–759)[b]
Chiang (made from fish, bird, or meat)	Koji (same as chu)
Chi-Min-Yao-Shu (532–549)[b]	Hishio (same as chiang, made from fish, meat, or soybeans)
Chu (made from crushed wheat, or wheat flour made into balls or cakes, or cooked rice)	Koma-hishio and miso
Chiang (made from soybeans or wheat)	Taiho-Law (701)[b]
Shi and shi-tche	Soybean-hishio, miso, kuki (same as shi)
Tang dynasty (618–906)	taremiso, usudare, misodamari
Ben-Chao-Gong-Mu (1590)[b]	Ekirinbon-Setsuyoshu (1598)[b]
Chiang-yu	Shoyu (same chinese characters as chiang-yu)
Tao-yu	Honcho-Shokukan (1962)[b]
	Shoyu, miso, tamari
	Industrial production of koikuchi-shoyu in Noda (1561) and Choshi (1616), that of usukuchi-shoyu in Tatsuno (1666), that of miso in Sendai (1645); export of shoyu from Nagasaki, Japan (1668); visit of C. Thunberg to Japan from Sweden (1775)

[a] Arranged from "35 Years History of Noda Shoyu Company (1955)"; The History of Kikkoman (1977); Sakaguchi Kinichiro (1981); Wang and Fuang (1981); and Bo (1982, 1984).

[b] Names of old references.

[c] Note: Chu: mold-cultured cereals; chiang; a mixture of chu, proteinous foodstuffs, and salt; shi: mold-cultured soybeans with or without salt; shi-tche: the saltwater extract of shi; chiang-yu: the liquid separated from chiang; tao-yu: the liquid separated from soybean chiang.

The koikuchi mash is subjected to vigorous lactic and alcohol fermentations, and the finished product is pasteurized at a rather high temperature (about 80°C) to give it a characteristic dark reddish brown color and strong heat flavor.

Good-quality koikuchi shoyu contains 1.5–1.8% (grams per volume) total nitrogen, 3–5% reducing sugar (mainly glucose), 2–2.5% ethanol, 1–1.5% polyalcohol (primarily glycerol), 1–2% organic acid (predominantly lactic acid of pH 4.7–4.8), and 17–18% sodium chloride. In order for a shoyu to have palatable taste, about one-half of its nitrogenous compounds must be free amino acids, and more than 10% of the nitrogenous compounds must be free glutamic acid.

Usukuchi shoyu is made from a mixture containing more soybeans and less wheat than the koikuchi type. The saccharified rice koji with water, which is called amasake, or enzymatically saccharified starch or glucose, is sometimes

TABLE II

AMOUNT AND KINDS OF SHOYU IN JAPAN (1982)

Total production:	1,187,148[a]	
Total sales:	1,184,306[a]	
Koikuchi	902,862	(84.4)[b]
Usukuchi	138,261	(12.9)
Tamari	20,885	(2.0)
Saishikomi	3,130	(0.3)
Shiro	5,042	(0.5)
Total	1,070,180	(100.1)

[a] Bureau of Foods, Japan.

[b] In kiloliters. Numbers in parentheses are percentages. From Japan Shoyu Inspection Association. This amount was checked by Japan Agricultural Standards.

TABLE III

CHEMICAL ANALYSES OF GENUINE FERMENTED AND SPECIAL GRADE OF SHOYU IN JAPANESE MARKET (JANUARY, 1983)[a]

Kind of shoyu	Kind of special grade	Number of sample	Baumé	NaCl	TN[b]	FN	RS	Alc	pH	Ex	Col
Koikuchi	Ordinary[c]	6	21.8	17.1	1.56	0.90	3.10	2.23	4.85	19.2	11
	Super[c]	3	22.4	17.1	1.69	0.96	3.81	2.17	4.84	21.1	9
	Ultrasuper[c]	1	23.5	17.4	1.83	0.98	3.87	1.84	4.79	22.2	3
	Less salt[d]	5	19.9	13.5	1.57	0.91	3.53	3.20	4.88	21.4	9
	Reduced salt[d]	2	16.3	8.9	1.55	0.87	3.41	3.41	4.86	22.1	8
Usukuchi	Ordinary[e]	5	22.2	18.5	1.19	0.72	4.04	2.57	4.89	16.1	28
	Super[e]	1	22.3	18.1	1.49	0.92	3.83	2.65	3.97	18.8	26
	Less salt[d]	1	19.5	14.9	1.20	0.71	4.79	3.73	4.95	18.0	28
Tamari		1	23.1	17.1	1.89	0.99	3.05	3.03[f]	4.97	22.1	7
Saishikomi		2	27.5	14.1	2.24	1.06	9.43	1.47	4.89	34.9	2>
Shiro		1	24.9	17.9	0.53	0.29	16.7	0.08	4.74	20.5	46<

[a] Arranged from *J. Japan Soy Sauce Res. Inst.* 9(2), 90 (1983).

[b] TN: Total nitrogen (%) (g/100 ml); FN: formyl nitrogen; RS: reducing sugar; Alc: alcohol; Ex: extract without salt; Col: number of shoyu color standard issued by Japan Shoyu Inspection Association, the smaller the darker.

[c] Total nitrogen content: Ordinary, more than 1.50%; super, more than 1.65%; ultrasuper, more than 1.80%.

[d] Salt content: Less salt, less than 20%; reduced salt, less than 50% of the standards, which are koikuchi, 17.5%; usukuchi, 19.9%; tamari, 17.9%; saishikomi, 15.6%; and shiro, 17.9%.

[e] Extract without salt: Ordinary, more than 14.0%; super, more than 15.4%. The classification of special grade is provided in the bylaws of the Japan Soy Sauce Association.

[f] The too high content of alcohol for tamari (underlined) may be the one added after fermentation.

Aspergillus oryzae (NRRL Strain 1988)

The technical comment "*Aspergillus oryzae* (NRRL strain 1988): A clarification" by Fennell (*1*) apparently requires further clarification, since Morse has expressed the opinion that "the question remains open" (*2*).

We offer the following additional information that may help settle the question of contaminant versus variant. Our laboratory was one to which Morse sent a subculture of their NRRL 1988 "variant." We confirmed its capability for producing aflatoxins and characterized the mold as typical of *Aspergillus parasiticus*. Since El-Hag and Morse had reported receiving a similar variant from the American Type Culture Collection subculture (ATCC 9362) of the same strain of *A. oryzae* they had received from the USDA Northern Regional Research Laboratory (NRRL), we obtained ATCC 9362 directly from ATCC. This subculture did not produce aflatoxin on any of the substrates usually employed for this purpose, and its culture characteristics were typical of *A. oryzae*. The "variant" was therefore not present in the ATCC master culture, nor, as Fennell reports, was it present in the NRRL master culture. Why should the aflatoxin-producing variant show up in New Brunswick in three separate transfers (two from NRRL and one from ATCC) and not in Washington or Peoria? We suggest the New Brunswick laboratory should have paid more attention to Fennell's admonition that the subculture sent to her from New Brunswick was "heavily infested with culture mites."

Mites are notorious for cross-contaminating mold cultures when infestations become heavy, and their populations spread from either culture or commodity habitats.

LEONARD STOLOFF
PHILIP MISLIVEC, A. F. SCHINDLER
Bureau of Foods, Food and Drug Administration, Washington, D.C. 20204

References

1. D. I. Fennell, *Science* 194, 1188 (1976).
2. R. E. Morse, *ibid.*, p. 1188.

20 December 1976

I would not wish to enter into the fray of the El-Hag and Morse (*1*) versus Fennell (*2*) debate over the identity of the supposed "variant of *Aspergillus oryzae* NRRL 1988" were it not that the implications are frightening. Morse (*3*) chooses to quote paragraphs from a text by Raper and Fennell (*4*) that do nothing for his case. As every mycological taxonomist knows, diversity and variability is one thing, delimitation of species is another. Morse is essentially questioning our taxonomic expertise and our success at applying the concept of species. Raper and Fennell do not remotely suggest that a given specific entity can vary and mutate to become another recognizable species. It is hard to believe that anyone could convince himself that *A. oryzae* could become *Aspergillus parasiticus*, which Morse would have to do in order to make his case watertight. Morse expresses a wish to have the matter closed but yet maintains that the question remains open. There is only one way to close the matter—El-Hag and Morse should realize that from the published evidence the cultures they received became contaminated in their laboratory.

G. MORGAN-JONES
Department of Botany and Microbiology, Auburn University, Auburn, Alabama 36830

References

1. N. El-Hag and R. E. Morse, *Science* 192, 1345 (1976).
2. D. I. Fennell, *ibid.* 194, 1188 (1976).
3. R. E. Morse, *ibid.*, p. 1188.
4. K. B. Raper and D. I. Fennell, *The Genus Aspergillus* (Krieger, Huntington, N.Y., 1965).

13 January 1977

SCIENCE, VOL. 196
June 17, 1977

added to usukuchi mash to ameliorate the salty taste. The nitrogen content of the finished product does not exceed 1.2%. Usukuchi shoyu is used mainly for cooking when one wishes to preserve the original color and flavor of the foodstuff. Koikuchi shoyu imparts a strong aroma and dark color during cooking.

Tamari shoyu is made mostly from soybeans with only a small amount of wheat. Its nitrogen content is sometimes more than 2% and there is only a trace of alcohol.

Shiro shoyu is very light in color and is made mostly from wheat with very little soybean. It is said that shiro shoyu was invented about 150 years ago in Aichi Prefecture, in central Japan.

Saishikomi shoyu is made by enzymatically hydrolyzed soybeans and wheat in shoyu instead of the commonly used salt water. It was prepared for the first time about 200 years ago in Yamaguchi Prefecture, in the western part of Japan. Its original name was kanro shoyu, and it is characterized by its heavy taste due to its high content of extractable materials, nitrogenous compounds, and sugars. It is favored by some consumers as a dipping shoyu for some typical Japanese dishes such as raw fish and broiled eel.

The JAS specifies three grades for each variety of shoyu: special, upper, and standard. The grade is determined by organoleptic evaluation, total nitrogen content, soluble acids (without sodium chloride), and alcohol content. Only high-quality shoyu made by fermentation can qualify for the special grade. About 65% of Japanese shoyu was qualified as special grade in 1980. The JAS for the special grade of koikuchi shoyu is more than 1.5% total nitrogen, more than 16% extract, and more than 0.8% alcohol.

Blending fermented shoyu with 50% or less of chemical hydrolysate of plant protein or 30% or less of enzymatic hydrolysate of plant protein on a nitrogen basis is permitted for making products of upper and standard grades as long as the characteristic flavor of fermented shoyu is maintained.

The yearly consumption of shoyu per capita in Japan is about 10 liters; 4.4 out of 10 liters is consumed in homes and the remaining 5.6 liters is consumed by institutions and industry. The shoyu producers in Japan are assumed to be less than 3200 in number; the five largest manufacturers produce 50% of the total and some 50 other companies contribute 25% of the total produced.

The Japanese consume 34.1 g shoyu daily, which contains 14.0 g carbohydrate, 2.4 g protein, 0.2 g fat, and 5.8 g salt. Since average daily intake of protein in Japan is about 80 g, the role of shoyu as a source of protein or amino acid is not significant (Bureau of Foods, Japan, 1976).

The primary role of shoyu in the Japanese diet is as a source of salt, flavor, and color, especially for a bland and basic diet of rice, fish, bean curd (tofu), fermented beans, and boiled vegetables.

Worldwide, shoyu has long been recognized as a flavorful compliment to meat

TABLE IV

COMPARISON BETWEEN FERMENTED SOY SAUCE AND PROTEIN CHEMICAL HYDROLYSATE

Item	Fermented soy sauce	Protein chemical hydrolysate
Amino acid N/total N	45–50%	60–65%
Amino acid contents	Tryptophane (+), methionine, cystine; higher	Tryptophane (−), glutamic acid, aspartic acid; higher
Dominant organic acids	Lactic acid (1–1.5%), acetic acid (0.1–0.2%)	Levulinic acid (1.2–1.4%), formic acid (0.1–0.5%)
Ethanol	1–3%	ND[a]
Higher alcohols	Isobutyl, *n*-Butyl, isoamyl alcohols	ND
Volatile S compounds	Trace amount	+++
CH_3SH, $(CH_3)_2S$	0.08, 0.02 mg/total N g	0.22, 2.10 mg/total N g
Buffer capacity	Usually higher	Usually lower
Total polyol (glycerol)	+++	+
α-Diketone compounds	Diacetyl, acetylpropionyl, acetylbutyryl	Diacetyl
Pyrazines	Less	More
Characteristic volatile flavor components	Ethyl lactate, HEMF, methionol, 4-ethylguaiacol, 2-phenylethanol, etc.	Methional, γ-valerolactone, methyl sulfide, etc.
Color stability	Lower	Higher

[a] ND, No data.

and high-fat dishes. It is thought by some to increase the appetite and to promote digestion; others claim that it has beneficial and medicinal effects. Its role in promoting the secretion of gastric juice has been compared with caffeine and histamine by some physicians.

The original soy sauce product is considered to have been originated from Asian continents, but there are some aspects of production in Japan that differentiate the current Japanese shoyu from other oriental soy sauce products:

1. Greater amounts of wheat are mixed with soybeans as raw materials.
2. Protein from the raw materials is highly degradated by the enzymes from *A. soyae* or *A. oryzae*.
3. Mash is subjected to vigorous lactic and alcoholic fermentations.
4. Pasteurization is done at higher temperatures to give strong aroma, flavor, and color to the product.

These characteristics of Japanese shoyu production also explain the differences between chemical hydrolysate of plant protein, or so-called HVP, and Japanese genuine fermented shoyu in terms of the chemical components and the organoleptic evaluations, as indicated in Table IV.

Chelone midas and the snakes *Thamnophis sirtulis* and *Natrix sipedon*. Papez considered this nucleus to be similar to nucleus Z in the alligator as described by C. C. Huber and E. C. Crosby [*J. Comp. Neurol.* 40, 97 (1926)].

10. The third and fourth cranial nerve nuclei are located below the floor of the cerebral aqueduct ventral to the central gray in the caudal midbrain. The third nerve nucleus is split by the fibers of the medial longitudinal fasciculus into a dorsal and ventral division. The sixth cranial nerve nucleus is located just below the central gray at the level of attachment of the fifth cranial nerve to the brainstem. The neurons of the third and fourth cranial nerve nuclei accumulate a much greater amount of HRP than the neurons of the sixth cranial nerve nucleus, as judged from the intensity of the reaction in the third and fourth nerve nuclei and the relative paucity of reaction product in the sixth nerve neucleus.
11. F. Scalia and D. R. Colman, *Brain Res.* 79, 496 (1974).
12. H. A. Hansson, *Exp. Eye Res.* 16, 377 (1973).
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23 July 1976

Aspergillus oryzae (NRRL Strain 1988): A Clarification

El-Hag and Morse (*1*) chose to ignore two opinions regarding the identity of their aflatoxin-producing "variant of *Aspergillus oryzae* NRRL 1988," and reported incorrectly the two opinions they acknowledged (*2*). The culture, as eventually sent to the Food and Drug Administration by Morse and to NRRC by El-Hag, proved to be a strain of *Aspergillus parasiticus* contaminated with a yeast. The subculture sent to me also was heavily infested with culture mites. This information was made available to El-Hag well in advance of publication.

We had supplied El-Hag with two freeze-dried preparations of *A. oryzae* NRRL 1988 (lyophilized on two different dates) prior to his first report of aflatoxin production on millet by this strain (*3*). Immediately following this report, I opened one preparation from each of the two dates and compared the subculture with the stock culture in our collection. All three were identical and were *A. oryzae* as it is known to me. Subcultures from each source were tested for production of aflatoxin on wheat, corn, millet, and rice. Later, when El-Hag stated that the aflatoxin production on millet was an "artifact" (*4*) and changed the substrate, the stock culture was also tested on cowpeas and soybeans. No aflatoxin was produced on any of these substrates by any of the three subcultures. El-Hag was informed of these results.

I believe it most unlikely that the culture distributed by El-Hag arose as a variant of NRRL 1988, a strain of *A. oryzae* that has remained unchanged in our collection through 30 years of maintenance by periodic transfer. A more likely explanation, in my opinion, would be that *A. oryzae* NRRL 1988 has been replaced by their *A. parasiticus* through mite infestation. This opinion of its origin was transmitted to El-Hag. [A complete summary of our contacts with their investigations will be sent to any interested person (or persons) on request.]

The variability in aflatoxin production reported by El-Hag and Morse, and attributed by them to instability of the culture, probably reflects varying degrees of contamination of their fermentations. I am not surprised that the culture they returned to me produces aflatoxins B_1, B_2, G_1, and G_2. *Aspergillus parasiticus* is known for this ability.

In my opinion, manufacturers of industrial enzymes from *A. oryzae* and users of their products need not be disturbed. Strain NRRL 1988 and other strains of *A. oryzae* used in the food and enzyme industries have failed repeatedly to produce aflatoxin when tested at this center on corn, wheat, rice, millet, cowpeas, soybeans, and a liquid medium. Our results corroborate the extensive Japanese work (*5–8*) demonstrating that none of hundreds of industrial strains of *A. oryzae* produce this mycotoxin.

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28 June 1976; revised 13 September 1976

We have read the comments by Fennell and think it is interesting to compare them with a text by the same author (*1*).

> The Aspergilli are characterized by great diversity and variability as they are isolated from nature, and these differences may be interpreted as having emerged by processes of variation and mutation similar to those that occur in the laboratory. . . .
>
> The term variant is applied to strains arising through gradual change from normal members of identifiable species. The characters of a variant are generally not stable but subject to continued change and further variation.

This statement appears to be at variance with the position taken by Fennell.

When we first encountered rat mortality, followed by isolation of aflatoxins, we were anxious to identify the cause. We sent samples of the culture to two collaborators and to the Northern Regional Research Center and the Food and Drug Administration. The two collaborators gave the identification described in our report. Prepublication copies of the report were sent to collaborators and to the Northern Regional Research Center. Collaborators acquiesced to the report as written; but phone calls from Fennell produced the reversal described in Fennell's comment.

All in all it has been an episode that we would like to have closed. In our view the question remains open.

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4 October 1976

10 Dec. 1976

B. THE SOY SAUCE PRODUCED IN OTHER ORIENTAL COUNTRIES

The fermented soy sauce industrially produced in Korea is of the Japanese koikuchi type, and in 1970, the annual industrial production was estimated to be about 220,000 kl. Assuming that the per capita daily consumption is about 20 ml, the amount cited above is equivalent to one-half the demand for soy sauce in Korea, and it is estimated that the same amount is produced at home (Li, 1970). The homemade soy sauce is prepared by a traditional method in which cooked soybeans are smashed and made into small balls, then subjected to natural inoculation of *Aspergillus* and *Rhizopus* molds, a process taking several months in winter. When spring comes, these mold-cultured materials are extracted with salt water. The liquid part is boiled and fermented under the sun to make soy sauce. The residue of extraction is mixed with salt and stored to make miso, sometimes along with red pepper.

The annual production of soy sauce in Taiwan was estimated to be 130,000 kl in 1976, which is equivalent to 10 liters per capita comsumption per year. The largest four producers manufacture about 60% of the total produced. Of Taiwan soy sauce, 5–10% is estimated to be inyu, which is made only from soybeans and very much resembles Japanese tamari. The remarkable characteristics of inyu are that it is prepared from black soybeans instead of yellow soybeans, and that the black bean koji is washed with water before it is mixed with salt water to make mash. There are three national standard grades of soy sauce in Taiwan, and in 1980, their total nitrogen percentages were 1.4, 1.2, and 1.0%, respectively.

Fermented soy sauce similar to inyu and tamari is still being produced in the southern part of China, and it seems to be the prototype of the soy sauce prepared only from soybeans. In Japan, tamari mash is usually fermented in wooden kegs, but the soy sauce in Taiwan, Singapore, and the southern part of China is fermented in big china pots placed in the sun. However, most of the soy sauce made in Peking and Shanghai today is prepared differently: The koji is prepared by using a large-scale method to culture *A. oryzae* with a mixture of steamed soybeans and wheat or wheat bran (6:4), and the koji is mixed with salt water to make hard mash, the moisture content of which is about 80% and the salt concentration about 6–7%. This hard and low-salt mash is kept at 45–50°C for about 3 weeks for enzymatic digestion. The digested mash is extracted with hot salt water and then with plain hot water. The residue without salt is good for animal feed. There is no alcoholic fermentation of mash or the pressing of mash as there is in the case of Japanese shoyu manufacture. The nitrogen basis of the soy sauce yield in 1979 was 75–80% because the defatted soybean as raw material was cooked by the NK method. The highest governmental standard of soy sauce is as follows: total nitrogen, 1.6%; reducing sugar, 4%; and sodium chloride, 19% or more.

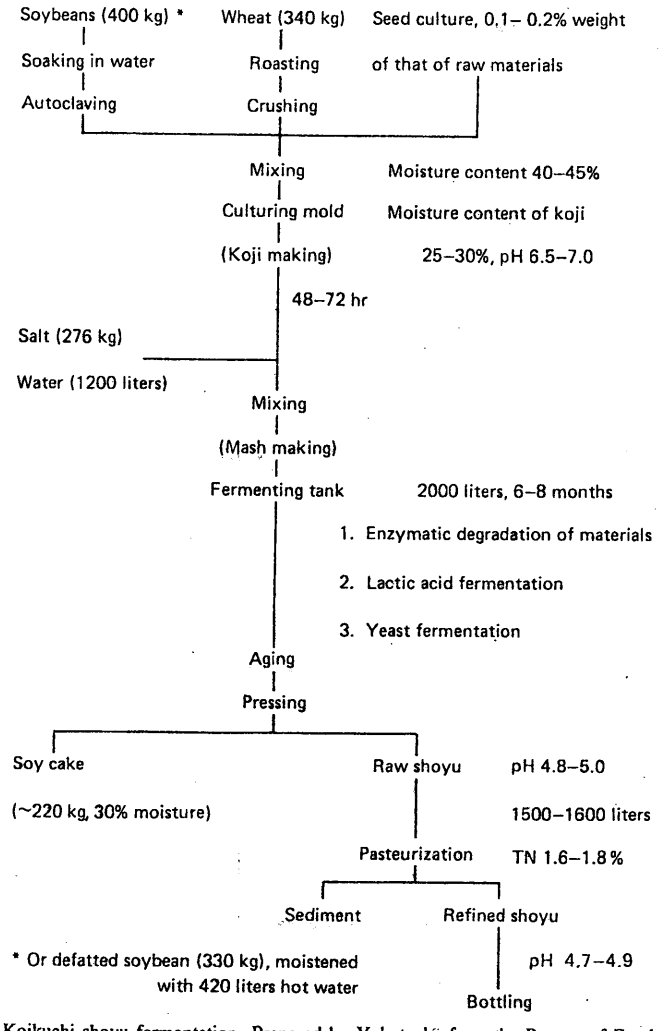

FIG. 1. Koikuchi shoyu fermentation. Prepared by Yokotsuka from the Bureau of Foods, Japan (1976); Fukuzaki (1972) and others.

nvestigators also observed aflatoxin production by an isolate of strain 1988 obtained from other sources but were unable to repeat the finding. The culture possessed the gross colony appearance, ie morphology and size of the conidial structures, and the cultural response on Czapek's medium characteristic of *A. oryzae*. However, 11-day-old cultures of this microorganism exhibited a deep green color and spore dimensions and patterns that are not characteristic of *A. oryzae*.

In summary, it has been shown that aflatoxin accumulation results from the growth of a variant strain of *A. oryzae* (NRRL strain 1988) on cowpeas. Aflatoxin was also produced by this organism growing on rice, but not on soybean. These findings lead us to conclude that this strain is a variable one. While NRRL 1988 does not produce aflatoxin during fermentation of soy sauce, it appears to have the capability of toxin production on other substrates. The use of this strain of *Aspergillus oryzae* for food fermentation and production of enzymes as food processing aids should be reexamined in light of these findings.

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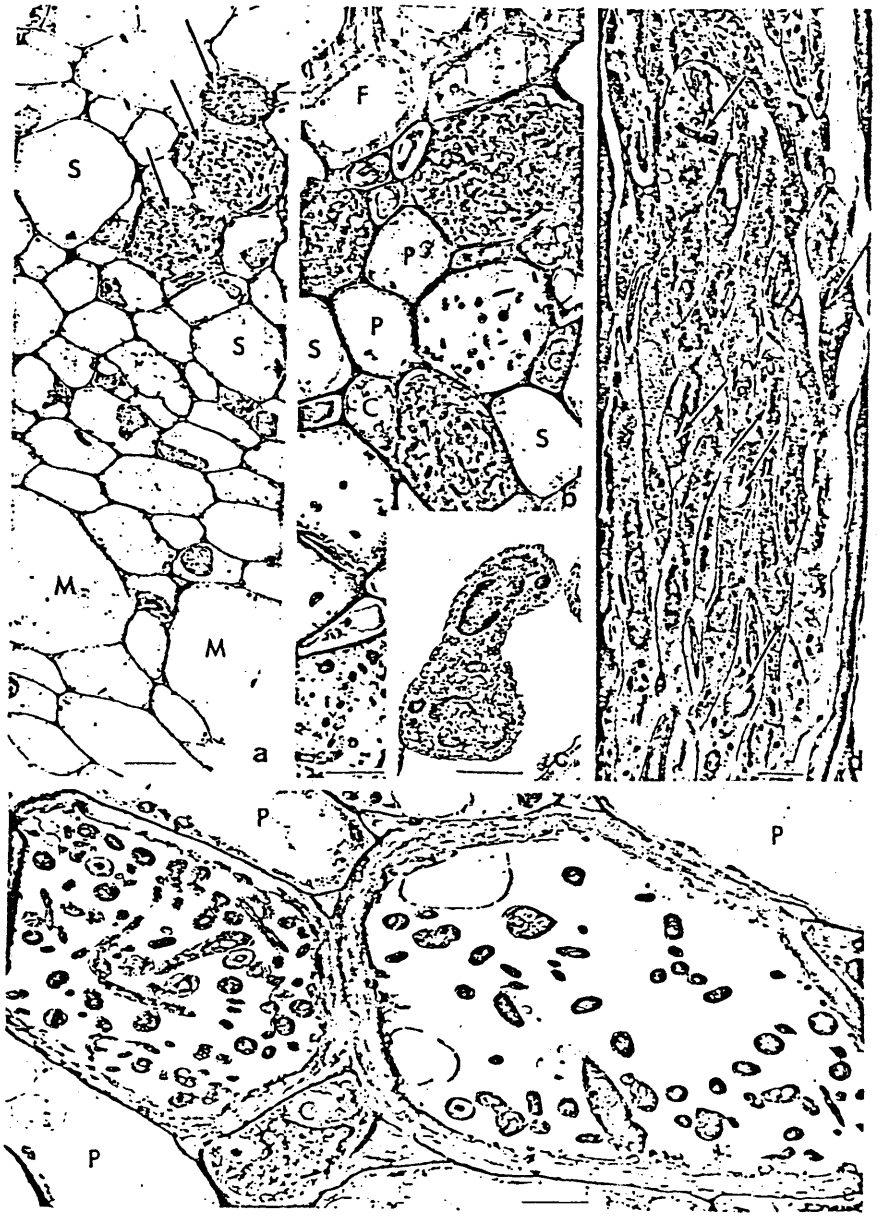
21 January 1976; revised 8 March 1976

Trypanosomatid Flagellate in the Phloem of Diseased Coconut Palms

Abstract. *Ultrastructural observations of the phloem of coconut palms affected by "hartrot" disease in Suriname have revealed the presence of the plant-infecting flagellate* Phytomonas *in mature sieve tubes. The occurrence of these flagellates during the earliest symptoms of the disease and the correlated increase and spread of the flagellates in the phloem as the disease progresses suggest that the organisms may be pathogenic to the palms.*

Although the existence of the plant-infecting trypanosomatid flagellate *Phytomonas* (*1*) has been known for nearly 70 years, relatively little is known about the flagellate's relationship to its hosts. The flagellate has been found chiefly in laticiferous plants (*2*), and in those plants it is apparently confined to the latex-bearing cells—the laticifers (*3*). Most investigators suggest that the flagellate is nonpathogenic to its laticiferous plant hosts (*2*, *4*). However, a few reports from Europe and elsewhere do suggest that the flagellate may be pathogenic to some of its latex-bearing hosts (*5*). The lygaeid hemipteran *Oncopeltus* is known to be



II. MANUFACTURE

A. KOIKUCHI SHOYU

Japanese-fermented shoyu of the koikuchi type involves five main processes: the treatment of raw materials, the making of koji, the making and aging of mash, pressing, and refining. One example of the preparation of koikuchi shoyu is schematically indicated in Fig. 1.

1. Treatment of Raw Materials

Whole soybeans, or more commonly, defatted soybean grits, are moistened and cooked with steam under pressure. This process greatly influences the digestibility of soybean protein. Details will be provided in a later section. Wheat kernels, the other half of the raw materials, are roasted at 170–180°C for a few minutes, then coarsely crushed into four or five pieces.

2. The Making of Koji

These two materials are inoculated with a small amount of seed mold or pure culture of *A. oryzae* or *A. sojae*. This mixture is spread to a depth of 30–40 cm on a large perforated stainless-steel plate having a rectangular shape that is 5 m in width and 12 m in length, for example, or a doughnut shape with a diameter of 15–30 m. The heat-treated raw materials are aerated for 2–3 days with controlled temperatures and moisture-controlled air, which comes up from the bottom holes through the ingredients to create the proper conditions for mold cultivation and enzyme formation. The temperature of the materials is kept at ~30°C, and the moisture content of the materials, which is 40–43% at the beginning of cultivation, decreases to 25–30% after 2 or 3 days. This allows the mold to grow throughout the mass and provides the enzyme necessary to hydrolyze the protein, starch, and other constituents of the raw materials. This mold-cultured material is called koji.

3. The Making and Aging of Mash

In making mash, the koji is mixed with saline water which has a 22½3% salt content and a volume 120–130% that of raw materials. The mash, or "moromi, is transferred to deep fermentation tanks. Approximately 5- to 10-kl wooden kegs or 10- to 20-kl concrete tanks for shoyu fermentation are now being replaced by resin-coated iron tanks of 50–300 kl. The moromi is held for 4–8 months, depending upon its temperature, with occasional agitation with compressed air to mix the dissolving contents uniformly and to promote the microbial growth. During the fermentation period, the enzymes from koji mold hydrolyze most of the protein to amino acids and low-molecular-weight peptides. Approximately 20% of the starch is consumed by the mold during koji cultivation, but almost all of the remaining starch is converted into simple sugars. More than half of this is fermented to lactic acid and alcohol by lactobacilli and yeasts, respectively. The initial pH value drops from 6.5–7.0 to 4.7–4.9. The lactic acid fermentation produced in the beginning stage is gradually replaced by yeast fermentations. Pure-cultured *Pediococcus halophylus* and *Saccharomyces rouxii* are sometimes added to the mash. The salt concentration of mash remains at 17–18% (weight per volume) after 1 or 2 months. The high concentration of mash effectively limits the growth to only a few desirable types of microorganisms.

4. Pressing of Mash

An aged mash is filtered through cloth under high hydraulic pressure. Usually 12–13 liters of shoyu mash is put on a square sheet of cloth, 100 × 100 cm, which is then folded into a square, 70 × 70 cm. A second, smaller square sheet of cloth, 65 × 65 cm, is placed on top to wrap the mash. Successive layers are added and placed in a wooden box until there are 300–400 sheets of folded cloth containing the mash. These are then pressed for 2–3 days under hydraulic pressure. The pressure is increased in two or three steps, sometimes reaching 100 kg/cm^2 in the final stage, making the moisture content of the presscake less than 25%. A diaphragm-type of pressing machine has recently been used for shoyu mash filtration instead of the batch-type hydraulic press, resulting in a presscake with a moisture content of more than 30%. The residue from the pressing of the shoyu mash, or shoyu cake, is used for animal feeds for cows and ducks.

5. Refining

The liquid part of the mash obtained by pressing is stored in a tank and divided into three layers: the sediment on the bottom, the clear supernatant of the middle layer, and the oil layer floated on top. The middle layer is sometimes further clarified by filtration with Kieselgel as a filter aid in order to get the raw shoyu. After adjusting the salt and nitrogen concentrations to the standard, the clarified raw shoyu is pasteurized at 70–80°C and stored in a semiclosed tank. The clear middle layer is bottled or canned, or sometimes spray dried. The oil layer separated from the heated shoyu consists of free fatty acids, and their ethyl esters derived from the yeat metabolism of soybean and wheat oils, and it is sometimes mixed with paint as a antifreezing agent.

Aflatoxin Production by a Variant of *Aspergillus oryzae* (NRRL Strain 1988) on Cowpeas (*Vigna sinensis*)

Abstract. *Aflatoxin B_1, B_2, G_1, and G_2 are produced when a variant of* Aspergillus oryzae *(NRRL strain 1988) is grown on cowpeas or rice. The present study indicates that a strain of* Aspergillus oryzae *approved for use in food processing is variable and the resulting variant, unlike the parent strain, has a propensity to produce aflatoxin.*

Aspergillus oryzae is widely used for preparation of koji, a starter in the production of soy sauce, and miso, a fermented soybean paste used in Japanese cooking (annual use, 900,000 metric tons) (*1*). Miso is prepared by first inoculating rice with *A. oryzae*, a combination called koji-rice, which produces an enzyme source for subsequent admixture with soybeans (*2*). The process is analogous to that in which malt is used in brewing. The subsequent soybean-koji-rice fermentation is conducted at 27°C for hours. Since the widespread use of this fungus in food processing was known, the organism was thoroughly investigated for production of toxin. Hesseltine (*3*) tested 52 strains of *A. oryzae* known to be in commercial use and found no aflatoxin production. He also checked NRRL strain 1988 for aflatoxin production using wheat, corn, rice, and millet as substrates, and obtained negative results. Matssura (*4*) did an even broader study including the testing of 128 samples of miso, 28 samples of koji-rice, and 238 strains of industrial koji inoculum (*A. oryzae*), but he also found no aflatoxin production. Mislivec *et al.* (*5*) examined this fungus on a variety of substrates and found no aflatoxins. Other substrates that were used include sterile peanuts (*6*), corn, oats, rye, rice, and soybeans (*7*). From this total effort it was concluded that *A. oryzae* was not an aflatoxin producer.

However, when we inoculated cowpeas (*Vigna sinensis*) with *A. oryzae* (NRRL strain 1988) significant quantities of aflatoxin B_1, B_2, G_1, and G_2 were produced. These results are surprising because this microorganism had been tested extensively for production of aflatoxin and none had been found.

It has long been known that the substrate affects aflatoxin yields (*7*) and that there are variations in this yield even on the same substrate (*8*). Growth conditions, including time, temperature, *p*H, and substrate moisture level, also affect the production of this toxin (*9*).

The cowpeas for the diet were prepared by soaking them (2 kg) overnight at 25°C; they were dehulled by hand and then cooked for 12 minutes at 121°C. After draining, the peas were spread out for inoculation in a previously sterilized hood. The inoculum was prepared from cultures of *A. oryzae* (NRRL strain 1988) grown on potato-dextrose agar slants and incubated for 7 days at 25°C. Spores were harvested by adding 0.8 ml of sterile distilled water to each slant, followed by dislodgment with a sterile needle. The spore crop from two agar slants was sufficient for 100 g of cowpeas. Inoculated, partially dried cowpeas were packed tightly into petri dishes and incubated for 42 hours at 33°C and 50 percent relative humidity. Fermented cowpeas were then dried in an air oven for 12 hours at 50°C and ground in a Wiley mill through a 20-mesh screen.

We fed male weanling Sprague-Dawley rats (ARS—Sprague-Dawley, Madison, Wis.) diets containing 23 percent fermented cowpeas that had been dried after fermentation. (We used 23 percent cowpeas in order to provide a diet containing 10 percent protein.) The remainder of the diet was prepared according to the method of the Association of Official Agricultural Chemists (AOAC) (*10*) and was composed of cornstarch, salt mixture, vitamin mixture, cottonseed oil, and nonnutritive cellulosic fiber.

Diets were fed to rats in groups of six. All six animals on the fermented cowpea diet died within 7 days. The other three groups grew well, including a control fed on casein and others fed on unfermented cooked peas and peas inoculated with *Rhizopus oligosporus*. Growth curves for the test animals are shown in Fig. 1.

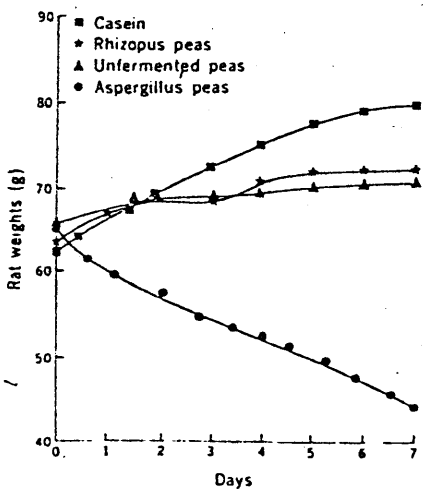


Fig. 1. Weights of rats on diets with fermented cowpeas as the source of protein.

Subsequent analysis of the cowpea powder by the AOAC method (*10*), in which thin-layer chromatography (TLC) is used, showed the presence of B_1, B_2, G_1, and G_2 aflatoxins. Confirmation of this was obtained by further analysis of the substance from the B_1 spot on the TLC plate according to the AOAC suggested method (*10*), employing the acetate derivative.

The culture of *A. oryzae* originally obtained from the Northern Regional Research Laboratory (their strain NRRL 1988) produced substantial levels of aflatoxin on cowpeas (Table 1), although a second culture from this laboratory (now the Northern Utilization Research and Development Division) showed no detectable aflatoxin production. A third culture of NRRL strain 1988, obtained from the American Type Culture Collection (ATCC 9362), also produced aflatoxin on cowpeas but at lower levels than the original NRRL strain 1988 (Table 1). The inconsistent behavior of different isolates of *A. oryzae* NRRL strain 1988 indicates the existence of subtle differences in or variants of this organism. Independent examinations of the original NRRL strain 1988 were performed by the Department of Plant Pathology, Rutgers University, and the Department of Food Science, University of Georgia. Independent experiments at the University of Georgia showed that *A. oryzae* from our source (NRRL) produced aflatoxin (*11*). These

Table 1. Aflatoxin production by a strain of *Aspergillus oryzae* on cowpeas. *Aspergillus oryzae*, NRRL strain 1988, was obtained from the Northern Utilization Research and Development Division 4 April 1974. This strain produced aflatoxin on rice (expressed as grams per kilogram: B_1, 930; B_2, 89; G_1, 647; and G_2, 143). The culture grew with difficulty on soybeans. However, a second culture obtained from the same laboratory 14 June 1974 did not produce aflatoxin on cowpeas or rice. Data are the average of five analyses. We also obtained *A. oryzae*, NRRL strain 1988, from the American Type Culture Collection (ATCC 9362) in June 1974. These data are also the average value of five analyses.

Type of aflatoxin	Aflatoxin (g/kg) from *A. oryzae*	
	Strain 1988 from NRRL	Strain 1988 from ATCC (ATCC 9362)
B_1	9400 ± 200	150 ± 40
B_2	1500 ± 200	50 ± 10
G_1	6600 ± 350	125 ± 10
G_2	1400 ± 400	100 ± 30

B. USUKUCHI SHOYU

The principles of usukuchi shoyu preparation are almost the same as those of koikuchi shoyu, except that all of the procedures are directed at getting lighter color and aroma in the final product (Fukuzaki, 1972) by the following:

1. Making a mixture containing more soybeans and less wheat than koikuchi shoyu;
2. Using a strain of mold belonging to *A. oryzae*, which is a better producer of α-amylase, whereas the strain for koikuchi shoyu is *A. sojae* or *A. oryzae*, which is a good producer of both protease and α-amylase. *Aspergillus oryzae* tends to impart milder aroma and flavor and lighter color to the final product as compared to *A. sojae;*
3. Making a more diluted mash with a lower nitrogen content. The volume of water used to made koikuchi mash is 120–130% that of the raw ingredients, while that of usukuchi shoyu is 130–150%;
4. Keeping the higher salt concentration of mash. With usukuchi shoyu, the salt is about 17–18% weight per volume, while in the case of koikuchi, it is about 16–17%;
5. Culturing the koji and fermenting the mash for a shorter period of time than is used for koikuchi shoyu;
6. Avoiding excessive heat in treating raw materials during mash fermentations and aging of mash, and in pastuerizing the final product in the preparation of usukuchi shoyu; and
7. Adding a dextrin-like substance, such as enzymatically hydrolyzed rice koji, in order to make the color stable and to ameliorate the salty taste.

C. TAMARI SHOYU

The basic principles of tamari shoyu preparation are almost the same as those of koikuchi and usukuchi, except for the following items (Yoshii, 1960):

1. Autoclaved soybeans or defatted soybean grits with a small amount of roasted and crushed wheat (20:3) are treated with an extruder to make pellets 12–16 mm in diameter. These pellets are inoculated with the seed mold and the powder of roasted barley, the amount of which is less than 1.5% that of the raw materials.
2. The strain used for tamari koji is originally *Aspergillus tamarii*, but *A. sojae, A. oryzae*, or the mixture of these strains are also used. The raw material used for tamari contains smaller amounts of carbohydrate material than those used for koikuchi. The strain of mold may not necessarily be the best producer of amylase, but a good producer of protease and lipase is required.

3. Tamari koji is usually dried for several days so as to decrease the weight of koji by 7–8% before preparing mash. This dried koji is mixed with salt water, the volume of which is 50–80% that of the raw material. Tamari mash cannot be agitated with compressed air, as can koikuchi or usukuchi mash; only the liquid part of mash is repeatedly siphoned off and poured onto the surface of mash.
4. Major fermentation that occurs in mash is lactic acid formation by *P. halophylus*, and there is almost no alcoholic fermentation by yeasts. The digestibility of nitrogenous materials in tamari mash is much less than in koikuchi or usukuchi.
5. The liquid part of mash cannot be separated by the pressing of mash, but is obtained by dripping, followed by one or two extractions with salt water to get lower grade products. Tamari shoyu usually is not pasteurized and is usually heated at a low temperature to avoid the burnt odor derived from its high concentration of extractable substances with heating.
6. The residue from dripping the tamari mash used to be sold as tamari miso for making soup, but consumers have come to prefer traditional miso or fermented soybean paste, which, because of its preparation, retains the delicious liquid part of the mash. Consequently, there is a tendency to prepare tamari mash by mixing a 100–130% volume of salt water with raw materials to shorten the fermentation period, to increase lactic fermentation, and to make it possible to get the liquid part of mash by pressing instead of dripping.

A flowchart for the preparation of tamari shoyu is shown in Fig. 2.

D. SHIRO SHOYU

Shiro shoyu is made mostly from wheat with very little soybean, the volume of which is 10–20% that of wheat. The specific characteristics of shiro shoyu preparation are as follows: Wheat is polished to remove about 5% of the outer layer of grain, which is rich in pentose; the soybeans are roasted and then crushed, followed by dehulling to decrease the pentose content; these components are then steamed under an atmospheric pressure. The light-colored product is derived from the decreased pentose levels of the raw materials.

The recommended strain of mold for shiro shoyu koji is *A. oryzae* which has a long stalk and was originally used for making miso. The amount of salt water needed to make mash is 120–130% of the volume of the raw materials. The nitrogen content of shiro shoyu is about one-half that of koikuchi shoyu, and the reducing sugar content is very high, ranging from 15 to 20%. The salt content is usually 18–19%.

Like usukuchi shoyu, shiro shoyu is used mainly for cooking, and a gradual increase in the production level of shiro shoyu has recently been observed in

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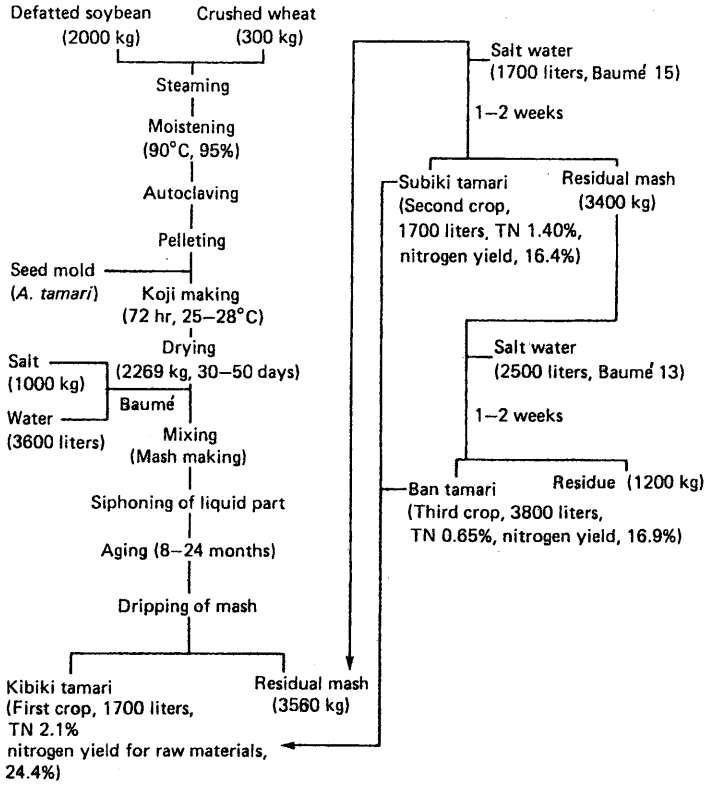

FIG. 2. Tamari shoyu fermentation. From H. Yoshii (1960), *Brewing Industry*, p. 198.

Japan. There are good introductions to shiro shoyu by H. Yoshii (1960) and K. Fukuzaki (1972).

E. SAISHIKOMI SHOYU

Saishikomi shoyu is made by enzymatically degrading koikuchi koji in shoyu instead of salt water. The volume of shoyu used for this purpose is 110–120% that of the raw material of koji. Mash is stored for 3 months at 26–28°C, followed by 5–6 months at room temperature. There is almost no microbial fermentation during mash storage. Average chemical composition of six marketed products in 1960 were Baumé, 29.26%; sodium chloride, 15.3%; total nitrogen, 2.250%; reducing sugar, 10.76%, and pH, 4.6 (Bureau of Foods, Japan, 1976.)

III. RECENT RESEARCH AND TECHNOLOGICAL ADVANCES IN SHOYU MANUFACTURE

Recent technological improvements in shoyu industry are summarized as follows:

1. The use of more defatted soybean instead of whole beans.
2. Increase in protein digestibility of raw materials from 65 to 90% as the result of improved methods of cooking soybeans and wheat, the selection and mutation of starter molds, improved conditions for culturing molds or making koji, and the control of mash in terms of the temperature, pH, types, and behavior of lactobacilli and yeasts, and the chemical components.
3. Reduction of the time for koji cultivation from 72 hr to 48 hr.
4. Decrease of fermentation period of mash from 1 to 3 years to about 6 months.
5. The use of pure cultured starters of lactobacilli and yeasts.
6. Mechanization of the equipment and expanding of the production scale.
7. Improvement of quality and reduction of cost.

A. COMPARISON BETWEEN WHOLE AND DEFATTED SOYBEANS AS RAW MATERIALS

Until 50 years ago, only whole soybeans were used as the raw material for shoyu. Today, defatted soybean grits are prepared by extracting the dehulled and crushed whole soybean with a solvent. Hexane at a lower boiling point is widely used for this purpose. In Japan during 1978, of the total number of soybeans used for the production of shoyu, only 3.2% were whole beans. Years ago, the yield and the quality of shoyu made from defatted soybean used to be inferior to those produced from whole beans, but today the disadvantages of using defatted soybeans as the raw material of fermented shoyu, compared with whole beans, have been largely overcome by advances in technology. Yokotsuka (1972) compared whole and defatted soybeans with respect to cost, enzymatic digestibility of the protein, fermentation period, the relative difficulty in manufacture (especially in koji making, mash controlling, and mash pressing), and the quality of the shoyu produced in terms of its chemical components, organoleptic properties, and stability:

The defatted soybeans used years ago were much more difficult for enzymatic digestion than those of today, and the quality of the defatted soybean used in the fermentative production of shoyu has been much improved. This is due to the improvement in the pressing method which uses an expeller with a battery system or a continuous extractor to remove the solvent. Furthermore, the extract-

Chloroform soluble fluorescent compounds produced by koji-molds. J. Food Sci. and Technol., 15, 341-346, (text in Japanese).

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ing temperature has been reduced by using hexane instead of benzene as the solvent. These changes have only slightly reduced the quality of the protein, altered during processing. A nitrogen solubility index (NSI) value of about 20 for defatted soybean is generally believed to be adequate for shoyu production.

The enzymatic digestibility of the proteins contained in whole and defatted beans in the course of shoyu production is reported to be 62 and 60%, respectively (Kawamori, 1940), but these figures have been almost the same, and sometimes higher, for defatted beans by 1–2% since the invention of the NK method of cooking, which will be discussed later.

Based on protein content, the cost of whole soybean is 10% higher than defatted soybeans.

Whole soybeans are reported to have a slower rate of fermentation, about 15 months for whole beans at room temperature versus only 10 months for defatted beans (Yokotsuka, 1960). The fermentation period of shoyu mash is principally dependent upon the enzymatic digestibility of cooked soybeans and on the enzymatic activities of koji. These two factors are now considered to be associated with the manufacturing technology and not with the differences between whole and defatted soybeans. The average fermentation period of shoyu nowadays is about 6 months.

It has been traditionally easier to make good koji with whole beans than with defatted ones, since the conventional method involves cooling the materials by hand mixing, and the larger particles of whole beans are cooled more easily than are the smaller particles of the defatted bean. Recently, it has become easy to prepare a good koji from defatted beans by using mechanical koji equipment in which the temperature is controlled by mechanical aeration.

Shoyu made from whole beans has been reported to have a lighter color and better color stability, a higher alcohol and glycerol content, a smaller amount of lactic acid and reducing sugar, and a better organoleptic evaluation than the shoyu made from defatted beans (Okuhara and Yokotsuka, 1958; Moriguchi and Ishikawa, 1960a,b; Moriguchi and Kawaguchi, 1961; Moriguchi and Ohara, 1961). The glycerol contents of shoyu made from whole and defatted beans were reported to be 1–1.2% and 0.6–0.7%, respectively (Okuhara and Yokotsuka 1958, 1962, 1963). The amount of glycerol in a shoyu mainly derived from the degradation of soybean oil is calculated to be about 0.5%. However, glycerol has also been produced in mash by the yeast fermentation of glucose in the presence of high salt concentrations.

Shoyu mash is now subjected to much more vigorous yeast fermentation than before, resulting in a higher concentration of glycerol, sometimes reaching 1.5–1.7%. Thus, the advantage of using whole soybeans to give shoyu a higher glycerol content has diminished (Sakurai and Okuhara, 1977). The lactic acid content of shoyu is now easily adjusted by controlling the degree of lactic acid

fermentation in mash regardless of the kind of soybean used. Almost the same can be said for the alcohol content of shoyu, although the aerobic condition of the mash made from the whole beans was in the past thought to make alcoholic fermentation easier.

The differences between the chemical components of those shoyus made from whole beans and those derived from defatted beans can be minimized by making the physical structure of whole beans similar to that of defatted beans by pressing (Okuhara and Yokotsuka, 1963).

Both the color intensity and the color stability of a shoyu seem to be fundamentally related to the degree of digestion of raw materials and not to the kind of soybean used.

Because whole soybeans have a higher protein content and are therefore more costly than defatted beans, a shoyu made from defatted beans of the same price has a higher content of free amino acids, including glutamic acid, which gives it a more delicious taste. Nevertheless, it is true that a shoyu made from whole beans has some characteristic flavor. This may account for the fact that some shoyu producers are still making their products from the mixture of whole and defatted soybeans.

From environmental viewpoints, whole beans have several waste problems, e.g., soaking water and sticky liquid from cooking. Both contain carbohydrates and proteins from whole beans, which should be removed before disposing of the wastes.

A large portion of the oil contained in soybeans and wheat is metabolized into the ethyl esters of higher fatty acids and glycerol in the course of yeast fermentation. The ethyl esters make the pressing of mash difficult, as these must then be separated from the upper layer of the liquid obtained by pressing. The separated oil is called shoyu oil, and its major chemical constituents are ethyl linolate and ethyl oleate accompanied by free higher fatty acids and sitosterols. This by-product is troublesome to shoyu producers.

B. TREATMENT OF RAW MATERIALS

1. Soybeans

The protein in raw soybeans is present in an undenatured state and is not hydrolyzed by the enzymes of koji mold. Therefore it is necessary to denature the soybean protein so that it can be digested by the enzymes of koji mold to make shoyu. Steam cooking has generally been used to denature the soybean protein. Years ago, soybeans were steamed or boiled at atmospheric pressure, but Kawano (1938) found that when the soybeans were cooked at the gauge pressure of 0.5 kg/cm as compared with 0, 1.0, 1.5, and 2.0 kg/cm, the highest enzymatic

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TABLE V

NK COOKING METHOD OF SOYBEANS AS COMPARED TO THE CONVENTIONAL METHOD[a]

Cooking method	Digestibility (%) of proteins in mash, salt 18%, room temperature, 1 year	Ratio between formyl N and total N (%)	Ratio between glutamic N and total N (%)
Conventional[b]	68.7	49.4	5.5
NK method[c]	73.1	53.8	7.3
Increasing ratio	106.4	108.8	135.4

[a] From Tateno and Uneda (1955). Kikkoman Shoyu Co., Ltd.
[b] Cooked at 0.8 kg/cm^2 for 1 hr, soybeans left in autoclave for additional 12 hr.
[c] Cooked at 0.8 kg/cm^2 for 1 hr, soybeans taken out of autoclave immediately.

digestibility of cooked soybeans and the highest free amino acid content of the shoyu prepared from cooked soybeans were obtained.

Until 25 years ago, soybeans were cooked at a gauge pressure of 0.8 kg/cm for several hours. Since then, the time has been shortened to less than 1 hr under the same pressure. Thoroughly moistened soybeans were cooked in a rotary cooker and the materials immediately cooled to below 40°C by reducing the inside pressure with the aid of a jet condenser. This method was called the NK method (Tateno *et al.*, 1955) and is given in Table V.

The protein digestibility in shoyu manufacture (i.e., the ratio between the total nitrogen of a shoyu and that of the raw materials) was increased from 69 to 73% by the NK method. In the conventional cooking method, the soybeans are cooked at the same pressure and for the same time as in the NK method, but the cooked soybeans remain in the autoclave after steaming for an additional 12 hr without opening the seal. It is important that there be enough water in cooked soybeans (about 58% of the volume for whole beans and about 62% for defatted beans) because the utilization of total and amino nitrogen increases with an increase in the moisture content of the beans. It is also important that the steaming soybeans be uniform and that no undenatured protein is left in the cooked soybeans.

The treatment of soybeans with water containing methanol, ethanol, or propanol was found to markedly increase the enzymatic digestibility of protein (Yamaguchi, 1954; Fukushima *et al.*, 1955, 1957). These treatments are given in Tables VI and VII, respectively. These methods have not been employed industrially mainly because of the difficulty of making koji and the possibility of bacterial contamination during koji cultivation, which results in a final shoyu product of inferior organoleptic quality.

TABLE VI

CHEMICAL ANALYSES OF SHOYU FERMENTED FROM SOYBEANS DENATURED BY METHANOL AND BY CONVENTIONAL COOKING[a]

Denaturing method	Total N (%)	Amino N (%)	NaCl (%)	Reducing sugar (%)	Protein digestibility (%)
Methanol[b]	1.92	0.98	17.50	2.92	90.69
Conventional cooking[c]	1.39	0.63	17.63	4.53	65.16

[a] Yamaguchi (1954). Japanese patent 219,545. Kikkoman Shoyu Co., Ltd.
[b] Boiled with methanol for 2 hr, methanol removed, and then steamed for 1 hr without pressure.
[c] Steamed with 1 kg/cm^2 pressure for 1 hr and kept in the autoclave without reducing pressure for several hours.

Yokotsuka *et al.* (1966) found it useful to increase the enzymatic digestibility by cooking at a higher temperature for a shorter time than the NK method, as given in Table VIII. This method indicated the possibility of having 92–93% protein digestion in shoyu production, with a final product of better organoleptic quality (Yasuda *et al.*, 1973a,b). Similar research results were reported by Harada *et al.* (1968) in which defatted soybean was cooked at the elevated pressure of 4 kg/cm for 3 min.

In the above cases, thoroughly moistened soybeans were cooked by using saturated steam. Aonuma *et al.* (1970) reported a new method of cooking soybeans and wheat used for brewing without adding or with adding 10–20% of moisture before cooking by using superheated steam at a gauge pressure of 4–8

TABLE VII

ANALYSES OF LIQUID PART OF SHOYU MASH AFTER 40 DAYS PREPARED FROM DEFATTED SOYBEAN DENATURED BY ETHANOL, ISOPROPANOL, OR NK COOKING[a]

Treating method of defatted soybean	Total N (%) (w/v)	Amino N (%) (w/v)	Amino N/ total N	Protein digestibility (%)
Boiling with 85% (w/v) ethanol, 40 min	1.80	1.80	59.6	89.3
Boiling with 70% (w/v) isopropanol, 60 min	1.77	1.00	56.5	84.3
Control 1: NK cooking	1.62	0.90	55.3	80.6
Control 2: NK cooking	1.69	0.93	55.6	80.8

[a] Fukushima *et al.* (1955, 1957). Japanese patent 236,368,237,805 (1955) 248,103 (1957). Kikkoman Shoyu Co., Ltd.

ACKNOWLEDGEMENT

The authors wish to express their thanks to Dr. K. Aibara, Director of Department of Biomedical Research on Food, National Institute of Health, Tokyo, for his helpful suggestion, and also express their appreciation to Mr. K. Okada, T. Manaka, Mr. M. Furuya and Mr. H. Katsuta, for proficient technical assistance. Editorial clarification was provided by Dr. Wm. E. Ribelin, Department of Veterinary Science, University of Wisconsin - Madison.

TABLE VIII
EFFECT OF COOKING CONDITIONS ON SOYBEANS ON ENZYMATIC DIGESTIBILITY OF PROTEIN[a]

Steam pressure (kg/cm^2)	Cooking time (min)	Digestibility of protein in enzyme solution (salt 0%, 37°C, 7 days)
0.9	45	86%
1.2	10	91
1.8	8	91
2.0	5	92
3.0	3	93
4.0	2	94
5.0	1	95
6.0	½	95
7.0	¼	95

[a] Yokotsuka *et al.* (1966). Japanese patent 929,910. Kikkoman Shoyu Co., Ltd.

kg/cm or at 200–289°C for not less than 15 sec. They confirmed almost the same protein digestibility as that obtained by saturated steam under the above-mentioned conditions. This method has the advantages of making it possible to stock the heat-treated raw materials.

This new high temperature–short time (HTST) method of cooking raw materials for shoyu brewing spurred the development of several types of continuous cookers, shown in Fig. 3.

At the same time, the NK method was also greatly improved. Protein digestibility of 87.80% was achieved by cooking soybeans at 1.7 kg/cm for 8 min by using an NK cooker compared with 81.80% obtained by the conventional NK cooking conditions at 0.9 kg/cm for 40 min (Iijima *et al.*, 1973). The time for cooling of autoclaved soybeans in an NK cooker is greatly associated with their proteolytic digestibility, which is given in Table IX (Yasuda *et al.*, 1973a).

By enlarging the diameter of both the entry and exhaust steam pipes of an NK cooker to make the cooking time precise and to make the cooling time of cooked soybeans as fast as possible, the protein digestibility increased by about 3% under the same conditions (Eguchi, 1977).

If portions of soybean protein remain undenatured, they mix with the final shoyu product which becomes turbid when it is diluted with water and heated, thus diminishing its commercial value. The relationship between steaming pressure and time for soybean cooking to the denaturation of soybean protein is indicated in Fig. 4 (Yokotsuka *et al.*, 1966a). The influence of the cooling speed

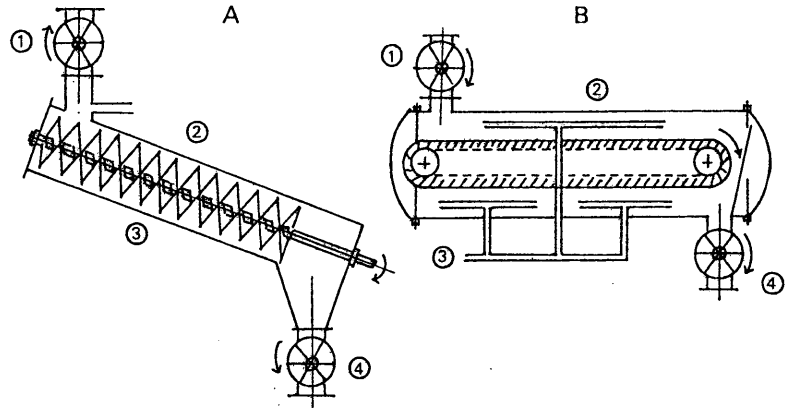


FIG. 3. Continuous soybean cooker. (A) Screw type; (B) net conveyer type. (1) Rotary valve (charge), (2) steam, (3) cooker, (4) rotary valve (discharge).

is not great at low cooling temperatures, but slow cooling following higher cooking temperatures and shorter cooking time gives rise to overdenaturation.

The recent trends of raw material treatment for fermented foods are toward the application of very high gauge pressure of 20–90 kg/cm^2 for less than 5 sec and that of intermediate moisture contents of materials, which are between the wet and dry methods. These are summarized in Table X.

Yokotsuka *et al.* (1965) and Hayashi *et al.* (1968a,b) demonstrated a 2–3% increase of enzymatic digestibility of soybean protein by treating it with a small

TABLE IX
COOLING SPEED AND DIGESTIBILITY OF PROTEIN[a]

Experiment no.	Cooking		Cooling time (min)[b]	Digestibility (%)
	Pressure (kg/cm^2)	Time (min)		
1	2.0	5	1	91.65
2	2.0	5	5	91.32
3	2.0	5	20	85.38
4[c]	1.0	45	1	87.25

[a] From Yasuda *et al.* (1973a).
[b] Time required to attain atmospheric pressure after cooling.
[c] Control.

in the case of the sodium chloride treatment. Changes in animals fed shoyu at the high dose levels were accounted for by the changes resulting from the treatment of same concentration of sodium chloride.

There was no indication of carcinogenic effect at any of the levels of feeding of shoyu.

TABLE X

IMPROVED COOKING METHOD OF SOYBEANS (AND WHEAT) TO BE USED FOR FERMENTED FOODS

Inventor (year)	Moisture of raw materials (%)	Kind of steam	Pressure (temperature)	Cooking time	Total digestibility (%)
Tateno and Ueda (1955), Kikkoman	Saturated (60)	Saturated	0.8 kg/cm^2	40–60 min	73
Yokotsuka *et al.* (1966), Kikkoman	Saturated (30–70)	Superheated → superheated	6–7 kg/cm^2 (~170°C)	20–30 sec	92
Aonuma *et al.* (1970), Kikkoman	10–20	Superheated	4–8 kg/cm^2 (200–289°C)	Less than 5 sec	92
Matsumoto *et al.* (1977), Nisshin	20–50	Saturated	More than 20 kg/cm^2 (120–150°C)	2–10 sec	89
Ohno *et al.* (1979), Ajinomoto	10–35	Saturated	10–90 kg/cm^2 (160–200°C)	1–5 sec	89
Akao *et al.* (1980), Kikkoman	10–20	Superheated → saturated	3–10 kg/cm^2 (160–210°C) → 3–10 kg/cm^2 (143–183°C)	Less than 5 sec	92.5
Yamanaka *et al.* (1982), Kikkoman	30–45	Saturated	More than 4 kg/cm^2	15–30 sec	94

FIG. 4. Denaturation of soybean protein by steaming at 130% moistening. (A) Overdenaturation region; (B) underdenaturation region; (C) proper denaturation region for shoyu production. From Yokotsuka *et al.* (1966).

amount of sulfite (0.04–0.4%), peroxide (0.1–0.2%), or perchlorite (0.2–0.4%) before or after cooking the soybeans. These procedures were presumed to destroy the S–S linkages of hydrophobic bonds in the remaining protein molecules of cooked soybeans or those newly produced during cooking, which do not interact with the proteases, thereby hindering digestion.

2. *Wheat*

Wheat is the major source of carbohydrate among the raw materials used to make shoyu, but its protein content cannot be overlooked because it constitutes about one-fourth of the protein in shoyu. If wheat is insufficiently roasted, its raw starch or β-starch cannot be digested by the mold amylase and becomes white particles in the presscake of mash. However, if wheat is overroasted, the protein digestibility decreases. The β-starch in wheat kernels must be changed

CONCLUSION

The acute toxicity of shoyu was accounted for by the toxicity of sodium chloride in the shoyu. The LD_{50} values of shoyu were 20.6 ml/kg in rats and 27.3 ml/kg in mice.

In the long-term feeding test, food intakes of animals given diets containing shoyu had few differences from the controls, even in the animals given diets containing 10% P-shoyu (corresponding to approximately 25% shoyu). Shoyu did not appear to be an unpalatable food for animals.

The animals fed shoyu were clearly smaller than the controls; however, no significant differences occurred between treated and control animals in the mortality. In the case of rats fed for 6 months, the growth rate of males given shoyu (diet containing 5% or 2% P-shoyu) was faster than that of rats given sodium chloride which is equivalent to the sodium chloride in shoyu (diets containing 2.25% or 0.9% sodium chloride).

At the highest dose level (10% P-shoyu), there were significant differences between treated and control animals in the urinary system. Increased relative weights of kidneys and urinary bladder in mice and rats, higher concentration of serum-urea in rats, and hydronephrosis in mice for 1.5 years were observed. These differences were observed similarly

into α-starch by adequate roasting in order to be digested by mold amylase. The content of α-starch in roasted wheat is determined by calculating the ratio of the amount of glucose digested from roasted wheat to the glucose digested from wheat throughly boiled with amylase produced by *Aspergillus* mold, which serves as the control.

Canadian wheat was mainly utilized in the Japanese shoyu industry previously because of its relatively high protein content. Haga *et al.* (1970) found almost no differences between Japanese wheat and wheat imported from Canada, the United States, and Australia with regard to the content of α-starch after roasting, the loss of carbohydrate during koji cultivation, the difficulty of pressing of mash, and the sensory evaluation of the final product.

Wheat is continuously roasted in a rotary oven with sand which recycles in the oven and is kept separate from the roasted wheat. The content of α-starch in the roasted wheat is used as the criterion for the extent of roasting. The preferred amounts of α-starch in roasted wheat used for the preparation of koikuchi and usukuchi shoyu were reported to be 40% and 20–30%, respectively, by Moriguchi and Nishiyama (1960).

The factors to increase the enzymatic digestibility of starch and protein, respectively, of roasted wheat by recycling heated sand are contradictory to each other, which is indicated in Fig. 5 (Aiba, 1982). It is effective to increase the α-starch content of roasted wheat kernels by making the moisture content of wheat before roasting 15–25% (Yamaguchi *et al.*, 1961). The same HTST method used with soybeans, adding some 10% moisture, gives good results as well for roasting wheat which has the same amount of water added (Uchiyama and Matsumura, 1974). According to Aiba (1982), good results were obtained when the wheat containing more than 8% moisture was treated with hot air of more than 150°C for less than 45 sec at atmospheric pressure. The highest digestibilities of starch and protein of the roasted wheat were 86 and 97%, respectively. In actual shoyu production, the utilization of starch in raw materials was improved by 2%.

When roasted wheat is crushed into four or five pieces accompanied by smaller particles of wheat flour, the preferred amount of wheat flour which passes through the 32 meshes is about 30%, which covers the cooked soybeans to reduce its surface moisture content and to minimize the bacterial contamination during koji cultivation (Umeda, 1967).

According to Aouma *et al.* (1971), the wheat treated in a current of superheated steam having a gauge pressure of 8.0 kg/cm and a temperature of 260°C for 8 sec followed by rapid cooling by explosion puffing produced a higher content of α-starch, a superior ability to absorb and retain moisture during koji cultivation, and a better yield of shoyu compared with the roasted wheat obtained by the conventional method. They also reported on a process of heating the

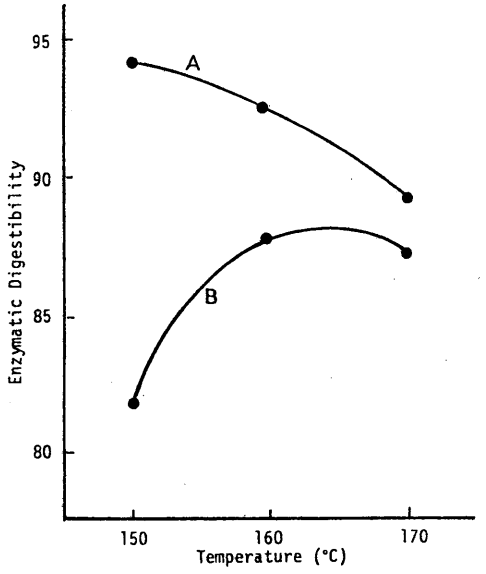


FIG. 5. Conventional roasting wheat with recycling heated sand. (A) Total nitrogen; (B) starch. From Aiba (1982).

mixture of crushed soybeans and wheat by explosion puffing for the use of shoyu manufacture.

Fujita and Kishi (1975), reported that during the milling process, a mixture of wheat flour and wheat bran was produced. This mixture was moistened and subjected to extrusion steam cooking to obtain a heat-denatured wheat product for shoyu manufacture. The α-starch content in this product ranged from 30 to 42%, while that of roasted wheat from 12 traditional shoyu producers ranged from 17 to 58%. The heat-denatured wheat for koikuchi and usukuchi shoyu production was prepared by adjusting the ratio of wheat flour to wheat bran. The greater the wheat bran, the higher the nitrogen content of shoyu and the darker its color, making it suitable for koikuchi shoyu. This heat-treated wheat was moistened and then cultured with *A. oryzae* to make koji. The protein digestibility of this koji in 18% salt water was 93.6%, while that of the wheat roasted by the conventional method was 79.7%. This difference in protein digestibility as a result of this preparation is equivalent to a 3–4% increase in the protein digestibility of final shoyu produced from the mixture of wheat and soybeans. This heat-treated wheat also has the advantage of being better able to absorb and retain moisture, which is good for koji cultivation.

Table 9. Histopathological lesions in rats given each experimental diet for 6 months.

Tissue and finding	Group No.	Incidence of lesion																			
		Male											Female								
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	I	II	III	IV	V	VI	VII	VIII	IX	X
	No. examined	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
alivary glands																					
Hyperplasia of serous alveoli		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
hyroid																						
Suppurative foci		0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0
ungs																						
Perivascular leucocytic infiltration		5	5	6	6	5	5	7	6	5	7	5	4	4	6	5	3	4	4	5	4	3
Bronchopneumonia		0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0
iver																						
Granuloma		4	3	3	2	3	3	2	3	4	3	2	5	6	6	7	5	6	5	5	6	6
Localized fatty change or vacuolation		0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	1	0
Focal necrosis of infarction		0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Bile-duct hyperplasia		0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0
Degenerative changes		1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
ancreas																						
Fibrosis of islets		0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pancreatic duct hyperplasia		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Leucocytic infiltration		1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
idneys																						
Interstitial leucocytic infiltration		0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Cast formation		2	3	4	1	1	1	3	0	2	5	3	0	0	0	0	0	0	0	0	0	0
tomach (fore)																						
Submucosal edema		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
estes																						
Atrophy (bilateral)		0	1	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-

C. KOJI MOLDS

Mold strains used for food fermentation are selected on the basis of the following characteristics:

1. Providing good flavor to the final products
2. Readiness with which they become mold starters with a sufficient amount of spores
3. Ease and speed of growth, making them easy to handle in koji making
4. Providing enzymatic activity, especially high proteolytic activity and macerating power to decompose the tissues of soybeans and wheat
5. Consuming a small quantity of carbohydrate in the raw materials during koji cultivation, yielding more sugar and alcohol in the mash
6. Having a shorter stalk (conidiophore), which makes possible the mechanical cultivation of materials with greater thickness. It is known that a long-stalk koji mold tightens the materials during koji cultivation and makes the aeration of the materials difficult.
7. Having genetic stability with little back mutation
8. Providing final products with desirable color (light or dark) as required.
9. Producing no toxic substances such as aflatoxins, cyclopiazonic acid, aspergillic acid, koji acid, β-nitropionic acid, oxalic acid, and other kinds of so-called mycotoxins
10. Yielding a mash that is easy to press.

According to Murakami (1973), among 327 strains of *Aspergillus* mold used in food fermentation in Japan, 159 were used for sake brewing. These included 157 strains of *A. oryzae;* 43 strains were used for miso brewing, which included 1 strain of *A. sojae,* 2 strains of *A. tamarii,* and 38 strains of *A. oryzae;* and 125 strains were used for shoyu brewing, which included 29 strains of *A. sojae* and 92 strains of *A. oryzae.*

Shoyu koji cultured with *A. oryzae* has a lower pH value, lower carbohydrate content, higher activity of α-amylase, higher activity of acid proteases, high activity of acid carboxypeptidase, and lower activity of polygalacturonase than does koji cultured with *A. sojae* (Tereda *et al.,* 1980, 1981). Furthermore, Hyashi *et al.* (1981) reported that koji cultured with *A. sojae* had a higher pH value because it contains less citric acid, and more carbohydrate because of lower consumption during koji cultivation. Shoyu koji cultured with *A. sojae* resulted in lower viscosity of mash, a higher content of sugar, lactose, and ammonia, a lower pH value of raw shoyu, and less coagulant produced by pasteurization of raw shoyu because of fewer active enzymes derived from koji.

Rhizopus molds are widely used in food fermentations in China, Taiwan, and Indonesia. In Japan, the koji is sometimes contaminated with *Rhizopus* molds such as *R. nigricans* when the temperature is too low.

Ebine *et al.* (1968) compared the proteolytic activities produced by 36 strains of *Rhizopus* with that of *Aspergillus* molds. *Rhizopus tamari* and *R. thermosus* were found to grow well on wheat and soybeans and to provide a very high proteolytic activity at pH 3.0, but almost none at pH 6.0. In this regard, the *Rhizopus* molds were distinctly different from the *Aspergillus* molds in that they gave a somewhat lower protein digestibility than did the *Aspergillus* molds on a small scale of experimental brewing. Improvements in the proteolytic activity of koji molds have been achieved by induced mutation, crossing, or cell fusion (Nasuno *et al.,* 1971, 1972; Nasuno and Nakadai, 1971; Nasuno and Ohara, 1971, 1972a,b). In one case, a 2–6% increase in protein digestibility in shoyu production was produced by the use of an induced mutant of *A. sojae* in which protease increased six times above that of the mother strain.

Furuya *et al.* (1983) achieved a strain of koji mold having both strong proteolytic activity and good spore formation through protoplast fusion, and Ushijima and Nakadai (1983, 1984) achieved a strain having strong proteolytic activity and good glutaminase formation through the same method as above, since these two factors in each case tended to be contradictory to each other.

It is generally recognized that the total proteolytic activity of koji is well correlated with its alkaline protease activity, i.e., the major protease produced by koji molds is an alkaline protease. But besides this, three kinds of acid protease, two kinds of neutral protease, and one semialkaline protease have been isolated (Nakadai, 1977; Nasuno and Nakadai, 1977), as presented in Table XI.

TABLE XI

FRACTIONATION OF PROTEASES PRODUCED BY *Aspergillus sojae* THROUGH SEPHADEX G-100[a]

Protease	MW	Units/g koji
Acid I	39,000	41.1[b]
Acid II	100,000	10.0[b]
Acid III	31,000	4.6[b]
Neutral I	41,000	80.0[c]
Neutral II	19,300	8.7[c]
Semialkali	32,000	55.4[c]
Alkali	23,000	929.0[c]

[a] From Nakadai *et al.* (1977). *J. Japan Soy Sauce Res. Inst.* 3(3), 99.

[b] Activity on casein at pH 3.0.

[c] Activity on casein at pH 7.0.

Table 8. Systolic blood pressures at week 26 in rats given each experimental diet

Experimental group No.	Systoli blood pressure (mm Hg) Male	Female
I (10.0 % P-shoyu)	143.3(7.5)**	123.3(11.4)
II (5.0 % P-shoyu)	133.1(7.0)	127.4(6.1)
III (2.0 % P-shoyu)	141.4(10.3)	122.1(8.3)
IV (1.0 % P-shoyu)	140.8(14.7)	125.6(8.2)
V (0.4 % P-shoyu)	147.8(10.3)**	127.4(8.0)
VI (4.5 % NaCl)	144.4(14.7)	126.8(6.1)
VII (2.25% NaCl)	147.9(14.9)*	124.3(9.9)
VIII(0.9 % NaCl)	142.4(10.2)	120.5(6.4)
IX (0.45% NaCl)	145.9(13.7)*	128.5(12.7)
X (0.18% NaCl)	143.9(14.5)	128.3(12.0)
XI (Control)	133.6(7.2)	125.0(9.2)

The figures are means and standard deviation in parentheses and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

The strong soybean protein digesting ability of the neutral protease I and II, and especially of the former, has been reported (Sekine, 1972a,b; 1976), but other investigators have observed that the proportion of neutral protease to the total protease produced by 109 kinds of koji mold ranges from 10 to 20% (Tagami *et al.*, 1977). Three kinds of aminopeptidase and four kinds of carboxypeptidase have also been isolated; it is these peptidases, especially leucine aminopeptidase, which are greatly associated with the enzymatic formation of formol nitrogen and glutamic acid in shoyu mash (Iguchi and Nasuno, 1978).

D. KOJI MAKING

The following guidelines should be followed in koji cultivation:

1. Grow as much mold mycelia and mold enzymes as possible.
2. Prevent the inactivation of the enzymes produced.
3. Minimize the carbohydrate consumption in raw materials during cultivation.
4. Avoid as much bacterial contamination in the starting materials and during the cultivation of mold as possible.
5. Shorten the cultivation time with minimum use of water, electricity, and fuel oil.

The mixture of cooked soybeans and roasted crushed wheat kernels is mixed with 0.1–0.2% of starter mold, *A. oryzae* or *A. sojae*. The mixed materials are usually cultured for 72 hr in small boxes or trays in a warm room, in which the temperature is controlled by windows. About 1 ton of raw material is divided into about 1000 wooden trays with a thickness of 3–5 cm.

The materials are cooled twice by hand mixing when their temperature rises to about 35°C or more because of the growth of molds. One example of the temperature change during koji cultivation in wooden trays is presented in Fig. 6.

The temperature for culturing mold on raw materials is lowered from the traditional level with protease formation in the koji, although 35°C or more is

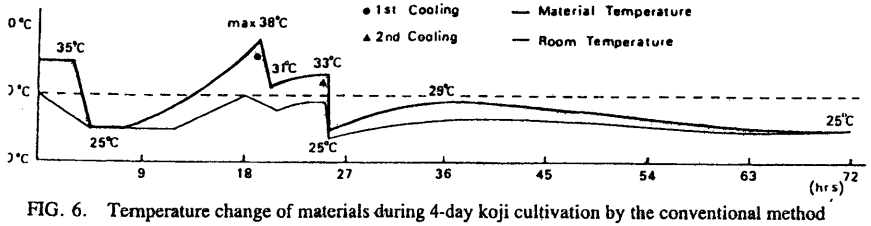


FIG. 6. Temperature change of materials during 4-day koji cultivation by the conventional method using wooden trays. From Shibuya (1969).

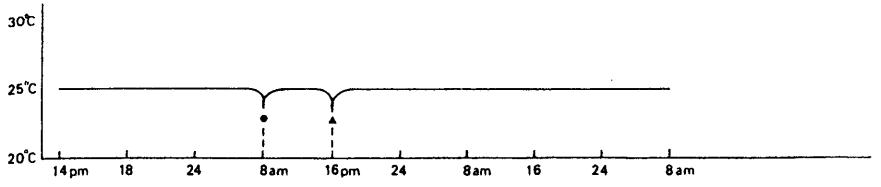


FIG. 7. Four-day koji cultivation, keeping the temperature of materials at 25°C. (●), First cooling; (▲), second cooling. From Haga (1968).

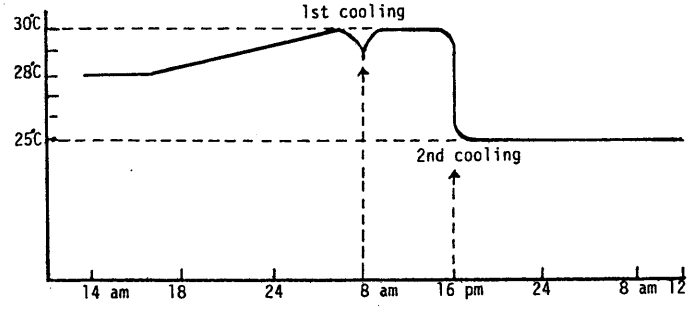


FIG. 8. Preferable temperature change of materials during 3-day koji cultivation. From Haga (1968).

considered to be adequate (Yamamoto, 1957). Temperatures as high as 30–35°C have been found to be preferable for the growth of mycellium and the prevention of bacillus as a contaminant in the beginning stages of koji cultivation. A lower temperature, 20– 25°C, is necessary both before and during spore formation in the latter stage or after the second cooling when protease develops in the koji (Ohara *et al.*, 1959). It has been suggested that koji be prepared at a constant temperature of 23–25°C for 66 hr and cooled twice (Fig. 7) to produce more protease and to avoid the inactivation of peptidase, which occur above 25°C (Miyazaki *et al.*, 1964; Tazaki *et al.*, 1966; Imai *et al.*, 1967). The preferred temperature change during koji cultivation is shown in Fig. 8, but it is difficult to maintain this temperature change in the conventional hand-operated method of koji making. However, development of mechanical equipment for koji cultivation has made it possible to provide the desired temperature and humidity of the materials to be cultured with koji mold, to reduce the time required for koji cultivation from 72 to 48 hr, to increase the enzymatic activities of koji, and to reduce the undesirable bacterial contamination in koji. The typical temperature change of materials during mechanical koji cultivation with a throughflow system of aeration is shown in Fig. 9.

Table 7. (continued)

Experimental group No.	Organ					
	Spleen	Stomach	Ovary+ (L)	Ovary+ (R)	Uterus	Bladder+
	Organ weight (g)					
I (10.0 % P-shoyu)	0.440(0.036)	1.12(0.05)	39.4(3.6)	34.7(4.5)	0.656(0.120)	103(10)**
II (5.0 % P-shoyu)	0.450(0.041)	1.11(0.06)	35.4(5.4)	35.3(3.0)	0.656(0.093)	87(12)
III (2.0 % P-shoyu)	0.450(0.050)	1.10(0.04)	34.9(4.8)	35.3(7.6)	0.645(0.103)	81(9)
IV (1.0 % P-shoyu)	0.439(0.067)	1.08(0.06)	35.3(4.5)	34.7(3.8)	0.703(0.093)	75(12)
V (0.4 % P-shoyu)	0.450(0.039)	1.13(0.08)	40.3(5.9)	38.7(7.3)	0.727(0.116)	92(20)
VI (4.5 % NaCl)	0.452(0.043)	1.14(0.06)	37.0(5.9)	35.5(5.6)	0.677(0.123)	125(18)**
VII (2.25% NaCl)	0.437(0.023)	1.09(0.06)	35.6(6.0)	32.9(4.6)*	0.720(0.180)	95(16)*
VIII(0.9 % NaCl)	0.439(0.025)	1.10(0.04)	35.9(3.0)	34.9(4.6)	0.663(0.103)	88(21)
IX (0.45% NaCl)	0.439(0.037)	1.08(0.07)	34.3(7.4)	33.1(5.2)*	0.641(0.121)	77(8)
X (0.18% NaCl)	0.431(0.037)	1.09(0.08)	35.6(5.6)	32.4(5.1)*	0.715(0.206)	84(12)
XI (Control)	0.446(0.029)	1.12(0.06)	37.2(4.4)	37.7(5.7)	0.755(0.185)	82(17)
	Relative organ weight (g/100 g body weight)					
I (10.0 % P-shoyu)	0.223(0.016)	0.570(0.035)*	20.0(2.1)	17.6(2.5)	0.332(0.058)	52.2(4.5)**
II (5.0 % P-shoyu)	0.223(0.017)	0.550(0.036)	17.4(2.3)	17.5(1.2)	0.325(0.046)	42.9(5.2)
III (2.0 % P-shoyu)	0.222(0.022)	0.543(0.039)	17.2(2.3)	17.4(3.5)	0.318(0.052)	40.1(4.5)
IV (1.0 % P-shoyu)	0.217(0.032)	0.536(0.030)	17.4(2.0)	17.2(2.2)	0.350(0.058)	37.1(5.7)
V (0.4 % P-shoyu)	0.216(0.018)	0.542(0.046)	19.4(3.0)	18.7(3.8)	0.351(0.066)	44.1(9.4)
VI (4.5 % NaCl)	0.220(0.019)	0.552(0.028)	18.0(2.8)	17.3(2.5)	0.331(0.066)	60.8(9.7)**
VII (2.25% NaCl)	0.219(0.013)	0.545(0.034)	17.8(2.9)	16.6(2.6)	0.360(0.092)	47.6(7.8)*
VIII(0.9 % NaCl)	0.215(0.015)	0.540(0.030)	17.6(1.6)	17.1(2.4)	0.325(0.053)	42.8(8.7)
IX (0.45% NaCl)	0.220(0.015)	0.543(0.031)	17.3(3.9)	16.6(2.5)	0.320(0.055)	38.8(4.3)
X (0.18% NaCl)	0.213(0.013)	0.537(0.029)	17.6(2.9)	16.0(2.6)*	0.354(0.108)	41.5(7.4)
XI (Control)	0.217(0.011)	0.542(0.023)	18.0(2.1)	18.3(2.6)	0.364(0.086)	39.7(8.0)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively. The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$p < 0.01$.

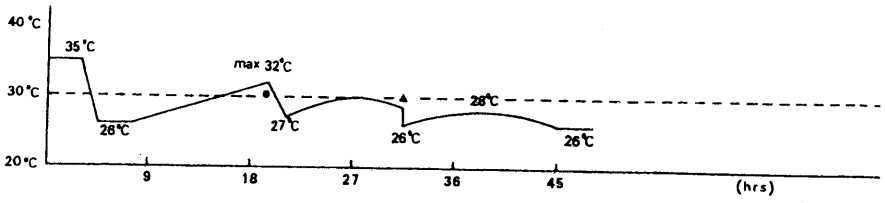


FIG. 9. Temperature change of materials during 3-day mechanical koji cultivation with throughflow system of aeration. (●), First cooling; (▲), second cooling. From Shibuya (1969).

The appropriate mixing ratio of soybeans and wheat to be cultured with mold generally ranges between (4:6) and (6:4). According to Shibata *et al.* (1967), a lower C/N ratio results in a smaller amount of mycelia and a greater amount of alkaline protease in koji, while a higher C/N ratio gives a greater amount of mycelia and a predominance of acid protease in koji. There appears to be no correlation between the C/N ratio of materials to be cultured with the mold and the formation of neutral protease. At a higher cultivation temperature, the formation of mycelia and acid protease increases and that of alkaline protease decreases.

The average consumption of starch in raw materials during koji cultivation is about 20%, depending upon the moisture content of the materials. According to Abe *et al.* (1975) and Katagiri *et al.* (1976), a remarkable decrease in the moisture content of materials takes place, sometimes as much as 50%, from hour 17 of cultivation (first cooling) to hour 30. During that time, a remarkable increase in the activities of protease and amylase, and of NSI (water-soluble protein/total nitrogen), sometimes reaching 50%, are observed, and the water-soluble protein becomes 31% as compared with that of the final koji.

Although the level of bacterial contamination in koji has reached as high as 10^{7-9}/g in Japan, this level does not constitute a health hazard. The major bacterial contamination of koji cultured in wooden trays years ago was from the genus *Bacillus* because of the difficulty in cooling the materials by hand, but in the modern throughflow system of mechanical cultivation, the dominant bacterial contaminant is from the genus *Micrococcus*, which is more aerobic and grows at a lower temperature than *Bacillus*. In addition, *Leuconostoc* and *Lactobacillus* are sometimes found in Koji. Too much *Bacillus* contamination in koji not only reduces the proteolytic activities of koji, but also makes the flavor of the shoyu inferior. The presence of too much *Micrococcus* also lowers its pH value, which leads to inferior protein digestion in mash; and if the dead cells of *Micrococcus* remain in shoyu, its filtration is sometimes more difficult. It is possible to reduce the bacterial contamination in koji to 10^6 or less by starting with a bacterial count of 10^2 or less in the materials in the beginning stage of koji

cultivation. To avoid the bacterial contamination during koji cultivation, the total moisture content of the starting materials should be 40–50% at most. For the same reason, it is advisable to reduce the moisture content of the surface of the cooked soybeans by initially wrapping them with roasted, then with finely crushed wheat kernels. Some investigators have found that a temperature below 34°C is best for avoiding bacterial contamination (Ishigami *et al.*, 1965, 1967; Fujita *et al.*, 1977).

The inhibition effects of acetic acid, lactic acid, citric acid, and hydrochloric acid on the growth of various microorganisms were reported by Hayashi *et al.* (1979). By keeping the acetic acid concentration beyond the range of 0.4–0.8%, based on the water content of the koji substrate, the growth of a strain of *Micrococcus* sp., which had been isolated from shoyu koji, was effectively suppressed, but the growth of various strains of koji molds was not inhibited. Acetic acid had a remarkable inhibition effect on the growth of some strains of bacteria belonging to *Staphylococcus* species, Gram-negative aerobes, and Enterobacteriaceae which were artificially added to the koji substrate, but the growth of lactic acid bacteria was not retarded or was retarded only slightly. In addition, the use of sulfite, glycine, ammonium acetate, or a combination of these substances to retard bacterial contamination has been reported by some researchers.

The following is a list of the mechanical equipment used in koji cultivation:

1. Throughflow system of aeration (Fig. 10)
   a. Batch-type with a rectangular, perforated plate
   b. Batch-type with a doughnut-shaped moving perforated plate
   c. Continuous-type with a doughnut-shaped moving perforated plate
2. Rotary drum
3. Surface-flow system of aeration: The temperature- and moisture-controlled air flows over the materials which are placed in numerous trays
4. Liquid cultivation

Systems 1a and 1b can handle about 5–10 tons of raw materials in one batch. When System 1b is used, the plate is moved only when the raw materials are ready to begin cultivation, the materials are mixed, and the finished koji is taken out.

A new system for continuous koji cultivation in the shoyu industry (System 1c above) has been developed by Akao *et al.* (1972). The principal apparatus consists of a perforated and doughnut-shaped circular plate with an outer diameter of 38 m. The plate rotates slowly once every 48 hr. The temperature of the solid mixture of cooked soybeans and roasted wheat, which spreads thickly over the plate, can be controlled by circulating humid air through the culture bed and the housing area in which the apparatus is installed. With continuous production,

Table 7. (continued)

Experimental group No.	Organ					
	Heart	Liver	Kidney (L)	Kidney (R)	Adrenal+ (L)	Adrenal+ (R)
	Organ weight (g)					
I (10.0 % P-shoyu)	0.689(0.042)	5.48(0.43)	0.765(0.054)**	0.763(0.050)**	29.6(3.4)	27.5(2.7)
II (5.0 % P-shoyu)	0.667(0.042)	5.34(0.45)	0.708(0.051)**	0.699(0.043)**	28.2(2.6)	27.0(2.3)
III (2.0 % P-shoyu)	0.647(0.039)*	5.24(0.40)	0.639(0.059)	0.652(0.044)	29.2(2.9)	27.0(2.3)
IV (1.0 % P-shoyu)	0.638(0.039)**	5.19(0.35)	0.668(0.040)	0.660(0.042)	29.7(2.6)	27.8(2.5)
V (0.4 % P-shoyu)	0.665(0.048)	5.30(0.26)	0.657(0.029)	0.662(0.034)	29.5(3.9)	28.6(2.5)
VI (4.5 % NaCl)	0.742(0.051)**	5.55(0.43)*	0.780(0.066)**	0.793(0.059)**	30.3(3.9)	27.9(2.6)
VII (2.25% NaCl)	0.702(0.050)	5.20(0.37)	0.687(0.045)**	0.694(0.052)*	29.4(2.3)	26.7(2.5)
VIII(0.9 % NaCl)	0.691(0.045)	5.29(0.41)	0.659(0.044)	0.656(0.046)	29.5(3.6)	27.8(2.8)
IX (0.45% NaCl)	0.676(0.033)	5.25(0.24)	0.636(0.033)	0.635(0.043)	31.1(4.5)	28.0(3.0)
X (0.18% NaCl)	0.676(0.038)	5.25(0.75)	0.638(0.050)	0.639(0.047)	28.8(2.9)	27.0(1.9)
XI (Control)	0.677(0.021)	5.21(0.25)	0.642(0.040)	0.647(0.041)	30.3(2.8)	28.3(2.8)
	Relative organ weight (g/100 g body weight)					
I (10.0 % P-shoyu)	0.349(0.015)**	2.77(0.12)**	0.387(0.024)**	0.386(0.016)**	15.0(1.5)	13.9(1.0)
II (5.0 % P-shoyu)	0.326(0.018)	2.62(0.20)	0.349(0.017)**	0.346(0.013)**	14.0(1.0)	13.4(1.0)
III (2.0 % P-shoyu)	0.320(0.018)	2.59(0.18)	0.316(0.032)	0.322(0.022)	14.5(1.5)	13.3(1.0)
IV (1.0 % P-shoyu)	0.315(0.012)*	2.57(0.12)	0.330(0.012)**	0.326(0.019)	14.7(1.3)	13.7(1.3)
V (0.4 % P-shoyu)	0.320(0.018)	2.55(0.10)	0.316(0.020)	0.318(0.019)	14.2(1.6)	13.8(1.2)
VI (4.5 % NaCl)	0.361(0.026)**	2.70(0.16)**	0.380(0.033)**	0.385(0.024)**	14.7(1.7)	13.6(1.3)
VII (2.25% NaCl)	0.351(0.031)*	2.60(0.10)*	0.343(0.015)**	0.347(0.019)**	14.7(1.3)	13.3(1.3)
VIII(0.9 % NaCl)	0.338(0.024)	2.58(0.12)	0.322(0.019)	0.321(0.022)	14.4(1.4)	13.6(1.1)
IX (0.45% NaCl)	0.340(0.024)	2.64(0.10)**	0.319(0.011)	0.319(0.015)	15.7(2.8)	14.1(1.6)
X (0.18% NaCl)	0.334(0.026)	2.59(0.26)	0.315(0.019)	0.314(0.015)	14.2(1.3)	13.3(0.8)
XI (Control)	0.329(0.015)	2.53(0.08)	0.311(0.014)	0.314(0.015)	14.7(1.4)	13.8(1.3)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively.
The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

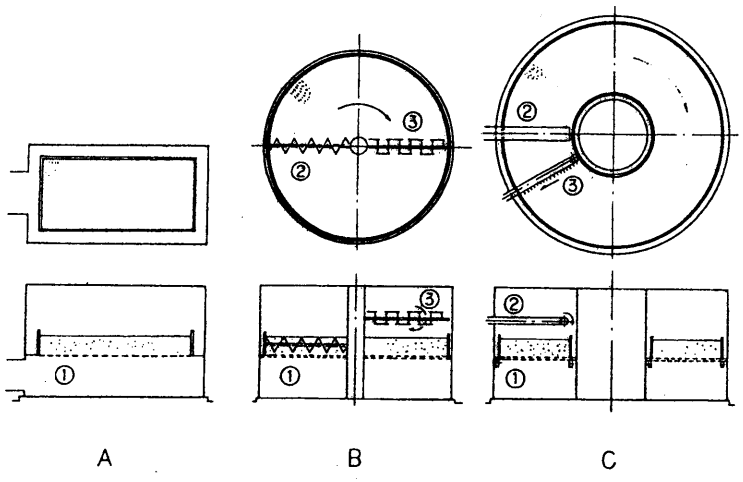


FIG. 10. Koji culturing machines with throughflow system of aeration. (A) Rectangular type; (B) circular type (batch); (C) circular type (continuous). (1) Perforated plate; (2) feeding conveyer; (3) discharging conveyer.

any desired decrease in the pressure through the bed can be established in the direction of plate rotation, and the product can be discharged without difficulty. A washer and a dryer are located near the place where the koji product discharges. An annular space bounded by the concentric and cylindrical walls of the rotary disk is subdivided by radial plates into a total of 96 compartments, similar to an echelon in shape. The lower edges of the two walls and the radial plates slide on a stationary floor. The perforated plate covers the upper surface of the disk, serving as the bottom of the solid culture spread. The stationary floor, supporting the radial plates and the walls of the disk, has 85 vents which permit the inflow of air; the remaining compartments are used to discharge the koji product and to clean the disk.

The rate of airflow through the culture bed is checked easily by measuring the change in pressure. The culture becomes solidified considerably by water evaporation due to the heat released from the mold growth and by an inextricable network of the mycelia. As solidification continues, the airflow resistance through the culture medium increases and causes a rise in the temperature of the solid culture, suggesting the possibility that the mold will become nonviable. By crushing the solidified culture, airflow resistance can be reduced without inflicting serious damage to the mycelia. Four cutters and two mixers are used for this purpose.

Table XII presents one example of an air supply arrangement used during koji

TABLE XII

CONDITIONS OF AIR SUPPLIED DURING CONTINUOUS KOJI CULTIVATION[a]

Group no.	Number of components	Temperature (°C)	Humidity (%)	Superficial air velocity	Pressure drop (max) (cm H_2O)
1	20	34	90	11	10
2	10	34	90	13	30
3	10	27	97	17	25
4	20	25	97	15	20
5	15	20	97	11	15

[a] From Akao *et al.* (1972).

cultivation. In miso manufacturing, the rotary drum is widely used only for rice koji cultivation. The surface-flow system of aeration used in shoyu koji cultivation (System 3 above) is not popular today. The use of liquid cultivation of koji mold for shoyu production has not been successful yet because of the high cost of facilities and the resulting lack of flavor of the final product.

Akao and Okamura (1983) reported on the cultivation of *A. sojae* in an air–solid fluidized bed. By use of a bench scale and a pilot plant, ground wheat bran having a 40% moisture content was fluidized by sterile air at 33°C for 50 hr. Cell yield in this method increased two- or threefold, and activities of alkaline protease and peptidase increased 5- to 15-fold, as high as those obtained in conventional solid culture.

E. CONTROL OF MASH

1. Temperature of Mash

Based on many years of experience, the Japanese have long known that the best quality shoyu results when koji and mash are prepared in February or March at a room temperature of 5–15°C, and the mash is fermented and aged from spring until autumn. Alcohol fermentation takes place in summer when the room temperature rises to around 30°C. It has also been known that shoyu prepared during the summer has less total nitrogen, amino nitrogen, and glutamic acid, a high level of organic acids, and an inferior organoleptic evaluation than shoyu prepared during the winter. In Japan, the ideal change in temperature for shoyu fermentation occurs during the 8-month period from winter to autumn. Today mash is usually made by mixing koji with brine solution of about 0°C, keeping the temperature of the new mash below 15°C for several days, and gradually raising it to 28–30°C after 20–30 days (Ebine *et al.*, 1976). A 1–3% increase in the protein digestibility of the new mash is expected with cooling because the

Table 7. Organ weights and relative organ weights of female rats given each experimental diet for 6 months

Experimental group No.	No. of rats examined	Organ				
		Brain	Pituitary+	Thyroid+	Thymus+	Lungs
		Organ weight (g)				
I (10.0 % P-shoyu)	14	1.85(0.04)	14.1(1.8)	13.9(1.9)	141(19)	0.854(0.034)
II (5.0 % P-shoyu)	14	1.87(0.04)	14.0(1.9)	15.5(2.8)	140(19)	0.843(0.030)
III (2.0 % P-shoyu)	14	1.85(0.04)	14.5(1.7)	14.9(2.5)	136(17)	0.864(0.032)
IV (1.0 % P-shoyu)	14	1.87(0.04)	14.7(1.2)*	14.0(2.8)	135(11)	0.844(0.046)
V (0.4 % P-shoyu)	14	1.85(0.04)	14.8(1.6)*	14.1(2.6)	140(22)	0.870(0.037)
VI (4.5 % NaCl)	14	1.83(0.04)	13.0(3.0)	14.8(2.4)	141(14)	0.827(0.061)
VII (2.25% NaCl)	14	1.83(0.04)	13.3(2.0)	14.2(2.1)	143(23)	0.837(0.062)
VIII(0.9 % NaCl)	14	1.83(0.04)	13.4(2.8)	14.5(2.5)	145(23)	0.837(0.038)
IX (0.45% NaCl)	14	1.81(0.04)	13.4(1.5)	15.1(2.8)	133(35)	0.829(0.053)
X (0.18% NaCl)	14	1.83(0.04)	12.8(1.8)	13.9(1.7)	136(15)	0.847(0.070)
XI (Control)	14	1.83(0.03)	13.0(2.2)	14.5(2.5)	143(20)	0.851(0.037)
		Relative organ weight (g/100 g body weight)				
I (10.0 % P-shoyu)	14	0.940(0.052)**	7.10(0.71)*	7.01(0.94)	71.1(7.7)	0.433(0.027)*
II (5.0 % P-shoyu)	14	0.926(0.051)*	6.96(1.01)	7.67(1.43)	68.9(6.8)	0.418(0.027)
III (2.0 % P-shoyu)	14	0.917(0.050)	7.14(0.85)*	7.31(1.07)	70.7(10.6)	0.427(0.027)
IV (1.0 % P-shoyu)	14	0.925(0.051)*	7.28(0.47)**	6.90(1.25)	67.0(4.3)	0.418(0.025)
V (0.4 % P-shoyu)	14	0.892(0.053)	7.04(0.86)*	6.82(1.18)	67.2(8.7)	0.418(0.028)
VI (4.5 % NaCl)	14	0.891(0.054)	6.28(1.30)	7.12(1.09)	68.6(7.4)	0.402(0.025)
VII (2.25% NaCl)	14	0.916(0.060)	6.65(0.75)	7.07(0.74)	71.4(11.4)	0.420(0.037)
VIII(0.9 % NaCl)	14	0.899(0.049)	6.51(1.19)	7.07(1.02)	66.9(6.9)	0.411(0.023)
IX (0.45% NaCl)	14	0.911(0.041)	6.73(0.82)	7.58(1.39)	70.2(10.6)	0.419(0.030)
X (0.18% NaCl)	14	0.903(0.057)	6.30(0.83)	6.84(0.72)	66.8(7.7)	0.418(0.034)
XI (Control)	14	0.890(0.035)	6.28(0.97)	7.06(1.27)	69.1(8.8)	0.408(0.024)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively. The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

lower temperature prevents a rapid decrease in the pH value caused by too rapid lactic fermentation and the inactivation of alkaline protease (Komatsu *et al.*, 1968; Tazaki *et al.*, 1969; Goan, 1969; Ueda *et al.*, 1958; Haga *et al.*, 1967; Imai *et al.*, 1969).

According to Machi (1966), the volume of glutamic acid content per 1g of total nitrogen in two kinds of shoyu was 0.82 mg and 0.66 mg, respectively. In the first instance, the shoyu was prepared over a period of 330 days, beginning in January when the temperature was 10°C under naturally occurring temperature changes; the other was prepared over a period of 220 days, also beginning in January, but with the warming of mash artificially to a constant temperature of 20°C. Kuroshima *et al.* (1969) have pointed out that glutaminase, which is derived from koji molds, is very sensitive to heat, and its activity rapidly decreases in new mash. Glutamine is converted into glutamic acid due to the action of glutaminase, but when the glutaminase is inactivated, glutamine nonenzymatically changes into pyroglutamic acid, which is not flavorful compared to glutamic acid. Kuroshima reported that glutamic acid present in the average shoyu on the market consists of 60% free glutamic acid, 10% pyroglutamic acid, and 30% a conjugated form. Shikata *et al.* (1978) separated the glutaminase in koji molds into two fractions, water soluble and insoluble. The latter, which remains in the cells, is more resistant to heat and salt and is the major contributor to the production of free glutamic acid in shoyu mash. Adding glutaminase resistant to heat and salt produced by some yeasts to the new mash has been found to be effective in increasing the glutamic acid content of the final product as long as the temperature of the mash is below 60°C (Yokotsuka *et al.*, 1968d, 1970, 1972; Iwasa *et al.*, 1972a,b.).

2. *Period of Mash Fermentation*

The remarkable increase in protein digestibility of shoyu due to recent improvements in the process of soybean cooking and to koji cultivation has also helped to reduce the fermentation period from the 1–3 years required in the past to less than 1 year.

According to Udo (1931), the time needed to produce the highest level of glutamic acid in shoyu mash prepared from wheat and soybeans and fermented under natural temperature was 15 months. Umeda (1953) reported a period of 10 months is required for mash prepared from wheat and defatted soybean. Today, however, 3–4 months are required, although a few more months are necessary to complete fermentation and aging. Completing the fermentation process within 6 months without damaging the final product can be accomplished by keeping the shoyu mash at a temperature of about 30°C, but heating it to temperatures of 35–40°C reduces its organoleptic qualities. In addition, the amount of water to be

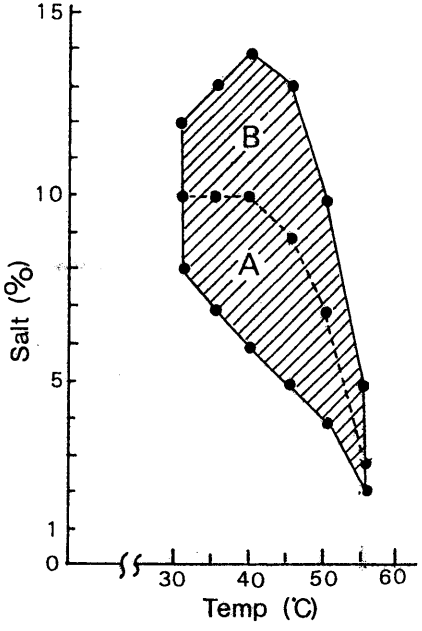


FIG. 11. Safety zone for enzymatic digestion of shoyu koji. Protein digestibility and amino acid content in Zone A are better than those in Zone B. Anaerobic bacteria: less than 10^6/g in koji, 10^2/g in final koji; aerobic bacteria: less than 10^8/g in koji, $10^2/10^3$/g in final broth. From Yokotsuka *et al.* (1977); Japanese patent No. 1,042, 917; Takamatsu *et al.* (1975): Japanese patent No. 1,120,428.

mixed with koji and the salt content of the mash are other major factors which determine the fermentation period. A ratio of 1.2–1.3 parts of water to 1 part of raw materials and 17–18% (w/v) of salt in the mash after 1 or 2 months seems to be the average figures in actual industrial production. By keeping the mixture of shoyu koji and water with 0% salt at 55°C for 24 hr or by keeping the same mixture with 8% salt at 43°C for 48 hr, in both cases with strong agitation and in the presence of heat and salt-resistant glutaminase, about a 90% protein digestibility with a more than 5.5 pH value and with a higher glutamic acid content than that of average fermented shoyu was achieved without microbial contamination. The relationship between the salt concentrations and the temperature to avoid microbial contamination in the enzymatic degradation of shoyu koji is indicated in Fig. 11 (Takamatsu *et al.*, 1975; Yokotsuka *et al.*, 1977). It usually takes 2–3 months to finish the lactic and yeast fermentations of salty shoyu mash at 20–30°C, but these fermentations of the liquid separated from the enzymatic degradate of shoyu koji at elevated temperatures can be finished within 5 to 10

Table 6. (continued)

Experimental group No.	Epididymis (L)	Epididymis (R)	Seminal vesicle	Prostate	Bladder+
			Organ weight (g)		
I (10.0 % P-shoyu)	0.492(0.027)	0.480(0.026)**	1.54(0.22)	0.783(0.123)	165(19)**
II (5.0 % P-shoyu)	0.500(0.025)	0.493(0.033)	1.55(0.15)	0.810(0.078)	148(12)
III (2.0 % P-shoyu)	0.498(0.026)	0.495(0.022)	1.59(0.15)	0.827(0.090)	134(23)
IV (1.0 % P-shoyu)	0.494(0.016)	0.486(0.036)*	1.60(0.21)	0.803(0.104)	130(25)
V (0.4 % P-shoyu)	0.498(0.034)	0.473(0.028)**	1.63(0.12)*	0.802(0.073)	120(26)*
VI (4.5 % NaCl)	0.508(0.027)	0.524(0.043)	1.59(0.13)	0.838(0.067)	175(35)**
VII (2.25% NaCl)	0.490(0.023)	0.503(0.033)	1.62(0.13)	0.837(0.070)	149(33)
VIII(0.9 % NaCl)	0.500(0.021)	0.497(0.023)	1.59(0.23)	0.798(0.127)	140(30)
IX (0.45% NaCl)	0.492(0.031)	0.496(0.037)	1.49(0.15)	0.744(0.128)	140(28)
X (0.18% NaCl)	0.496(0.038)	0.500(0.038)	1.57(0.12)	0.848(0.110)	136(23)
XI (Control)	0.489(0.027)	0.512(0.029)	1.51(0.16)	0.791(0.090)	140(24)
			Relative organ weight (g/100 g body weight)		
I (10.0 % P-shoyu)	0.138(0.007)**	0.135(0.008)	0.434(0.066)	0.220(0.034)	46.2(4.6)**
II (5.0 % P-shoyu)	0.135(0.009)*	0.133(0.009)	0.418(0.057)	0.217(0.025)	39.7(2.8)
III (2.0 % P-shoyu)	0.136(0.010)**	0.135(0.007)	0.434(0.053)	0.225(0.028)	36.6(6.6)
IV (1.0 % P-shoyu)	0.129(0.011)	0.128(0.010)	0.402(0.096)	0.211(0.031)	34.2(6.4)
V (0.4 % P-shoyu)	0.133(0.008)	0.126(0.006)**	0.438(0.047)*	0.212(0.016)	32.1(6.4)
VI (4.5 % NaCl)	0.142(0.008)**	0.146(0.011)**	0.446(0.035)**	0.234(0.018)**	48.7(9.3)**
VII (2.25% NaCl)	0.135(0.009)*	0.138(0.009)	0.444(0.044)**	0.231(0.023)*	40.8(8.8)
VIII(0.9 % NaCl)	0.137(0.010)**	0.136(0.006)	0.414(0.055)	0.218(0.033)	38.3(7.4)
IX (0.45% NaCl)	0.131(0.011)	0.132(0.010)	0.381(0.095)	0.198(0.037)	37.5(8.6)
X (0.18% NaCl)	0.135(0.012)*	0.136(0.010)	0.425(0.035)	0.231(0.033)	36.7(5.8)
XI (Control)	0.128(0.006)	0.135(0.010)	0.395(0.046)	0.208(0.029)	37.0(6.5)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively.

The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

days (Yokotsuka and Asao, 1969). Moreover, these fermentations of the liquid degradate of shoyu koji containing more than 8% salt of pH 3.0–7.0 can be finished within 2 or 3 days by passing the liquid through two or three columns which are packed with immobilized lactobacilli, *Saccharomyces rouxii* and *Candida versatillis* if necessary, respectively (Akao *et al.*, 1982).

3. *Microbes in Mash*

In newly produced mash, salt-intolerant lactobacilli and wild yeasts derived from koji are destroyed rapidly, and *Bacillus subtilis* remains only as spores. Salt-tolerant Micrococci also rapidly disappear because of anaerobic conditions of mash. The predominant active microbes in shoyu mash are salt-tolerant lactobacilli and yeasts such as *Saccharomyces rouxii* and *Candida* (*Torulopsis*) *versatillis* or *C. etchellsii*.

Sakaguchi Kenji (1958) found that major lactobacilli were *Pediococcus soyae* and Buchanan *et al.* (1974) determined that they were *P. halophylus* morphologically. Good results have also been obtained by adding pure cultured lactobacilli to the new mash (Watanabe *et al.*, 1970; Nagase *et al.*, 1971; Jose and Sugimori, 1973). In one typical lactic fermentation of shoyu mash, the intitial inoculum of 10^2–10^3 of lactobacilli reached 10^8 after 3 months (Jose *et al.*, 1976). Lactic and alcohol fermentations of shoyu mash are presented in Fig. 12. Caution must be taken not to add too much lactic starter as this is correlated with a decrease in the pH value and a decrease in protein digestibility. Some researchers have noted that the diversity of lactobacilli in shoyu mash relates to the aroma, pH, and color of shoyu (Fujimoto *et al.*, 1980), to the metabolic roles of organic acids (Terasawa *et al.*, 1979), and to the presence of sugars and some amino acids, such as arginine, histidine, tyrosine, and aspertic acid (Uchida, 1978). Various metabolic patterns by lactobacilli in shoyu mash are summarized in Table XIII.

The initial pH value of mash, 6.5–7.0, gradually decreases as the raw materials are degraded and lactic acid fermentation proceeds, and at around pH 5.5, yeast fermentation takes the place of lactic acid fermentation. The predominant yeast of shoyu fermentation, *S. rouxii*, grows and reaches to a viable count of 10^6–10^7/ml. To accelerate the alcoholic fermentation and to shorten its development time, pure cultured yeasts, *S. rouxii*, are sometimes added to the shoyu mash when its pH value reaches about 5.3, usually 3–4 weeks after the mash making (Watanabe *et al.*, 1970). The addition of *Torulopsis* yeasts along with *S. rouxii* have been recommended as a way of obtaining good volatile flavors in the finished product (Suzuki *et al.*, 1972). The changes in the viable counts of these two kinds of yeast in a natural shoyu mash kept at room temperature are presented in Table XIV (Keitaro *et al.*, 1968).

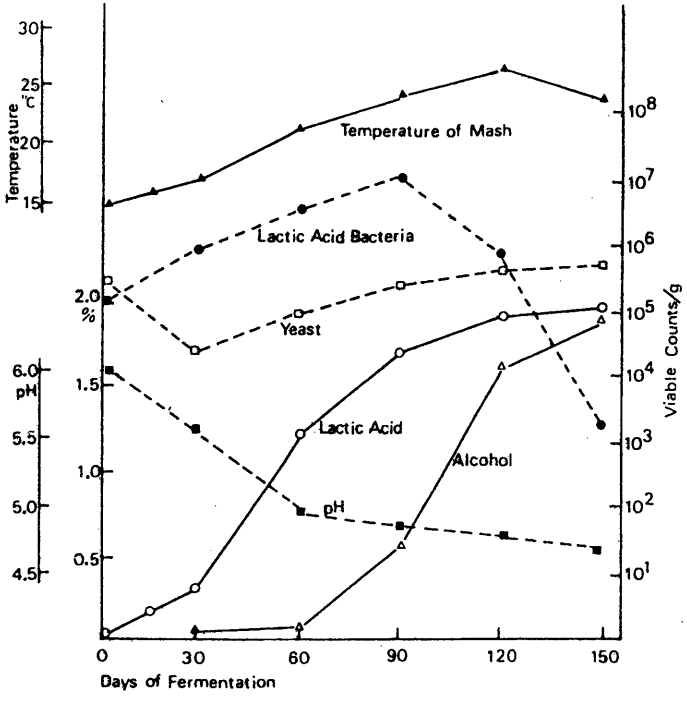


FIG. 12. Lactic and alcoholic fermentation of shoyu mash. From Jose *et al.* (1976).

TABLE XIII

VARIOUS METABOLIC PATTERNS BY LACTOBACILLI IN SHOYU MASH[a]

1. Homofermentation: Glucose → 2 mol lactic acid
2. Heterofermentation: Glucose → 1 mol lactic acid, ethanol, acetic acid, CO_2, H_2, acetone, butanol
3. 67 patterns of metabolic manners for arabinose, lactose, melibiose, manitol, and sorbitol
4. Metabolic manners for amino acids and citric acid:
   - Histidine → Histamine + CO_2
   - Tyrosine → Tyramine + CO_2
   - Arginine → Ornithine + 2 NH_3 + CO_2
   - Citric acid → Acetic acid + malic acid → Lactic acid + CO_2
   - Aspartic acid → Alanine + CO_2

[a] Adapted from Fujimoto *et al.* (1978, 1980); Iizuka (1973); Terasawa (1979); and Uchida (1978, 1982).

Table 6. (continued)

Experimental group No.	Organ			
	Spleen	Stomach	(L) Testis (R)	
	Organ weight (g)			
I (10.0 % P-shoyu)	0.643(0.059)	1.57(0.12)	1.51(0.10)	1.46(0.14)
II (5.0 % P-shoyu)	0.642(0.055)	1.54(0.06)	1.58(0.09)	1.52(0.09)
III (2.0 % P-shoyu)	0.629(0.054)*	1.54(0.09)	1.57(0.08)	1.51(0.09)
IV (1.0 % P-shoyu)	0.654(0.042)	1.51(0.11)	1.58(0.11)	1.53(0.09)
V (0.4 % P-shoyu)	0.628(0.069)	1.52(0.09)	1.54(0.12)	1.48(0.14)
VI (4.5 % NaCl)	0.600(0.037)**	1.54(0.08)	1.57(0.05)	1.52(0.07)
VII (2.25% NaCl)	0.601(0.073)**	1.51(0.09)	1.49(0.06)**	1.46(0.05)*
VIII(0.9 % NaCl)	0.599(0.058)**	1.47(0.06)*	1.55(0.06)	1.50(0.07)
IX (0.45% NaCl)	0.620(0.045)*	1.52(0.10)	1.53(0.10)	1.49(0.08)
X (0.18% NaCl)	0.631(0.052)*	1.51(0.09)	1.55(0.10)	1.47(0.11)
XI (Control)	0.685(0.082)	1.55(0.09)	1.57(0.09)	1.54(0.13)
	Relative organ weight (g/100 g body weight)			
I (10.0 % P-shoyu)	0.180(0.016)	0.440(0.021)**	0.424(0.027)	0.409(0.034)
II (5.0 % P-shoyu)	0.172(0.014)	0.413(0.020)	0.427(0.022)	0.410(0.025)
III (2.0 % P-shoyu)	0.172(0.013)	0.419(0.024)	0.428(0.025)	0.413(0.026)
IV (1.0 % P-shoyu)	0.172(0.009)	0.396(0.022)	0.415(0.023)	0.401(0.023)
V (0.4 % P-shoyu)	0.168(0.021)	0.407(0.021)	0.411(0.029)	0.395(0.037)
VI (4.5 % NaCl)	0.168(0.010)	0.431(0.020)**	0.448(0.027)**	0.426(0.019)*
VII (2.25% NaCl)	0.165(0.018)*	0.414(0.023)	0.408(0.018)	0.402(0.012)
VIII(0.9 % NaCl)	0.164(0.013)*	0.403(0.020)	0.423(0.015)	0.409(0.015)
IX (0.45% NaCl)	0.166(0.017)*	0.403(0.021)	0.408(0.028)	0.399(0.023)
X (0.18% NaCl)	0.171(0.014)	0.408(0.022)	0.419(0.028)	0.399(0.028)
XI (Control)	0.179(0.017)	0.404(0.019)	0.411(0.022)	0.403(0.030)

The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

TABLE XIV
CHANGES OF VIABLE COUNTS OF YEAST IN SHOYU MASH[a]

Months of aging	*Saccharomyces rouxii*	Other kinds (*Torulopsis*)
0.3	0×10^4	436×10^4
0.6	136	70
1	381	323
1.5	530	100
3	221	131
6	0	399
7	0	182
8	21	96
10	0	66
12	0	3

[a] From Mogi Keitaro *et al.* (1968). *J. Agric. Chem. Soc. Japan* 42(8), 466.

The factors that most hinder the activities of lactobacilli and yeasts in shoyu mash are, in the case of lactobacilli, its salt content and, in the case of yeasts, ingredients such as guaiacol and vanillin and alcohol, which can be extracted with ether (Sakasai *et al.*, 1975a,b; Noda *et al.*, 1975, 1976a–c). Lactic acid fermentation is affected by the yeasts derived from koji and others (Kusumoto *et al.*, 1977; Fujimoto *et al.*, 1980).

The effect of oxygen supply, initial pH, and inoculum size on growth and fermentation of *P. halophylus* and *S. rouxii* was examined. In mixed culture with an initial pH of 6.0, the growth of *P. halophylus* was inhibited by *S. rouxii* under aerobic conditions, and the growth of *S. rouxii* was inhibited by *P. halophylus* under anaerobic conditions. With an initial pH of 5.6, the growth of *P. halophylus* declined irrespective of the aeration condition (Inamori *et al.*, 1984).

4. *Ingredient Change during Mash Fermentation*

Proteins, carbohydrates, and oil from soybeans and wheat are degraded by protease, peptidase including glutaminase, amylase, and lipase, pectinase, and phosphatase derived from koji. The activities of protease and amylase remaining in mash as relates to the progress of fermentation are shown in Table XV.

The quantities of glycine, alanine, valine, and leucine increase as mash fermentation advances, and the quantities of aspertic acid, serine, proline, hystidine, arginine, and tyrosine decrease, mostly because of decarboxylation by lactobacilli. The quantity of glutamic acid decreases after reaching its peak

TABLE XV
ENZYME ACTIVITIES REMAINING IN SHOYU MASH[a]

Days of mash fermentation	Protease[b] pH 3 (15°C)[c]	pH 3 (25°C)	pH 7 (15°C)	pH 7 (25°C)	pH 9 (15°C)	pH 9 (25°C)	Amylase pH 5 (15°C)	Amylase pH 5 (25°C)
7	100%	100%	100%	100%	100%	100%	100%	100%
20	132	103	88	91	94	100	82	107
40	106	78	77	72	82	80	94	55
60	86	73	72	60	76	59	81	73
90	79	78	41	24	42	24	63	71
135	68	65	24	19	21	11	34	34
150	74	59	17	16	12	11	33	29
180	79	63	18	17	15	14	44	33

[a] Arranged from Komatsu (1968). *Season. Sci.* 15(2), 18.
[b] Determined at pH values indicated.
[c] Starting temperature of mash.

because of its nonenzymatic conversion into pyroglutamic acid. Citric acid and malic acid are derived from the raw materials after ~60 days. In a shoyu mash for which salt concentration is less than 15%, some lactobacilli such as *Lactobacillus pluntarium* grow and totally decompose glutamic acid by decarboxylation (Hanaoka, 1976). Mono- and disaccharides rapidly decrease as a result of

TABLE XVI
MAJOR INGREDIENT CHANGE IN SHOYU MASH FERMENTATION[a]

Days of mash	NaCl (g/100 ml)	TN[b] (g/100 ml)	FN (g/100 ml)	NH_3—N (g/100 ml)	pH	FN/TN (%)	TNUR (%)
7	17.7	0.98	0.36	0.06	5.7	37.1	44.7
20	17.4	1.29	0.53	0.09	5.6	41.0	55.4
40	16.9	1.55	0.73	0.15	5.0	46.7	74.4
60	16.7	1.61	0.80	0.20	4.8	49.3	78.1
90	16.6	1.67	0.85	0.21	4.7	51.1	81.4
135	16.6	1.69	0.89	0.21	4.7	52.5	82.4
150	16.5	1.96	0.91	0.21	4.7	55.7	82.7
180	16.4	1.69	0.94	0.20	4.7	55.7	83.1

[a] Arranged from Komatsu (1968). *Season. Sci.* 15(2), 13.
[b] Note: TN, total nitrogen; FN, formyl nitrogen; TNUR, TN in shoyu/TN in raw materials. Temperature of mash: 15°C (0–30 days); 25°C (31–150 days); 25°C (31–150 days); and 28°C (151–180 days).

Table 6. (continued)

Experimental group No.	Organ					
	Heart	Liver	Kidney (L)	Kidney (R)	Adrenal+ (L)	Adrenal+ (R)
	Organ weight (g)					
I (10.0 % P-shoyu)	1.01(0.05)	10.15(0.63)	1.24(0.09)**	1.21(0.06)**	23.5(2.7)	21.9(2.2)
II (5.0 % P-shoyu)	0.99(0.06)	10.23(0.96)	1.17(0.07)**	1.17(0.07)**	24.0(2.2)	22.7(2.0)
III (2.0 % P-shoyu)	0.97(0.05)	9.92(0.92)	1.08(0.07)	1.08(0.06)	25.1(2.3)*	23.6(2.4)
IV (1.0 % P-shoyu)	0.99(0.04)	9.81(0.83)	1.08(0.07)	1.11(0.15)	24.0(2.2)	23.2(2.4)
V (0.4 % P-shoyu)	0.98(0.07)	9.67(0.71)	1.09(0.07)	1.07(0.07)	24.1(2.3)	22.9(1.5)
VI (4.5 % NaCl)	1.10(0.11)*	9.65(0.68)	1.19(0.05)**	1.17(0.05)**	23.1(1.3)	21.9(2.8)
VII (2.25% NaCl)	1.02(0.06)	9.60(0.64)	1.10(0.06)	1.11(0.04)**	23.1(1.3)	22.5(1.5)
VIII(0.9 % NaCl)	1.01(0.07)	9.44(0.46)*	1.06(0.06)	1.05(0.06)	22.9(2.2)	22.2(3.8)
IX (0.45% NaCl)	1.05(0.10)	9.87(0.84)	1.08(0.08)	1.06(0.07)	22.9(2.9)	22.5(2.2)
X (0.18% NaCl)	1.04(0.13)	9.79(0.68)	1.05(0.06)	1.04(0.07)	23.4(2.3)	22.0(2.0)
XI (Control)	1.02(0.09)	10.10(0.81)	1.07(0.07)	1.05(0.06)	23.2(1.5)	22.4(1.8)
	Relative organ weight (g/100 g body weight)					
I (10.0 % P-shoyu)	0.285(0.017)**	2.84(0.09)**	0.349(0.024)**	0.340(0.016)**	6.60(0.70)*	6.15(0.67)
II (5.0 % P-shoyu)	0.268(0.012)	2.75(0.11)**	0.313(0.015)**	0.312(0.015)**	6.42(0.48)*	6.09(0.54)
III (2.0 % P-shoyu)	0.262(0.011)	2.70(0.12)	0.296(0.015)**	0.293(0.016)**	6.86(0.58)**	6.45(0.63)*
IV (1.0 % P-shoyu)	0.259(0.012)	2.57(0.10)	0.284(0.013)	0.291(0.038)	6.29(0.65)	6.08(0.67)
V (0.4 % P-shoyu)	0.261(0.014)	2.58(0.07)	0.290(0.010)*	0.286(0.011)*	6.42(0.39)*	6.13(0.42)
VI (4.5 % NaCl)	0.306(0.026)**	2.70(0.15)	0.332(0.013)**	0.328(0.012)**	6.47(0.39)**	6.10(0.63)
VII (2.25% NaCl)	0.281(0.023)*	2.63(0.10)	0.303(0.012)**	0.304(0.010)**	6.33(0.39)	6.16(0.41)
VIII(0.9 % NaCl)	0.276(0.017)	2.59(0.09)	0.289(0.012)	0.285(0.012)	6.26(0.55)	6.07(1.02)
IX (0.45% NaCl)	0.281(0.031)	2.62(0.09)	0.288(0.011)	0.281(0.011)	6.09(0.69)	6.00(0.54)
X (0.18% NaCl)	0.280(0.032)	2.65(0.09)	0.285(0.013)	0.282(0.011)	6.33(0.57)	5.92(0.51)
XI (Control)	0.266(0.014)	2.64(0.13)	0.279(0.016)	0.276(0.014)	6.08(0.36)	5.87(0.52)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively. The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *P < 0.05; **P < 0.01.

the lactic and yeast fermentations. The changes in the major chemical ingredients in shoyu mash are presented in Table XVI.

5. *Agitation of Mash*

Shoyu mash is occasionally agitated with compressed air for the following purposes:

1. To control the uniform salt concentration and pH value of mash as relates to the enzymatic solubilization of the raw materials in order to prevent bacterial putrification by salt-intolerant bacteria in those parts of mash where there is a low concentration of salt. Accordingly, frequent agitation becomes necessary, especially in newly produced mash.
2. To provide sufficient oxygen to promote the growth of yeasts in the middle stage of fermentation.
3. To mix the *Torulopsis* yeasts which grow aerobically on the surface of the mash in the latter stage of fermentation.
4. To prevent the growth of too many film-forming yeasts on the surface of mash in the middle and latter stages of fermentation, since overabundance adversely affects the aroma of the final shoyu.

Koizumi and Takahashi (1974) studied the effects of varying frequencies of mash agitation during fermentation. In the control group, the newly produced mash was agitated once every 4 days for 1 month, once every 3 days for the next month, and then three or four times a month. The frequency of agitation in the experimental group was made about one-half that of the control group. The experimental group's frequency of agitation resulted in shoyu with better total nitrogen solubility, better alcoholic fermentation, and a deeper shade of red. By contrast, mixing mash by syphoning the liquid part from the bottom and then pouring it on the surface, as is done in the preparation of tamari shoyu, yields far inferior products based on the above quality characteristics.

The salt content in water greatly affects its oxygen solubility, which decreases to 60% in 10% salt water and to 40% in 18% salt water as compared with that in fresh water. There was no oxygen solubilized in shoyu mash 15 min after its agitation with compressed air. Yeasts were found surviving within 10 cm, but not within 40 cm from the surface of the shoyu mash 1 month after mash making (Miyauchi *et al.*, 1981).

The balance between lactic and yeast fermentation is also greatly affected by the water-absorbing ability of the solid in new mash, which relates to the kinds of koji and to the initial frequency of mash agitation. When a new mash absorbs much water and a smaller water layer in the mash remains, lactic fermentation tends to increase and yeast fermentation is suppressed. A too frequent agitation makes mash too sticky and promotes lactic fermentation but suppresses yeast fermentation. The salty mash made from a koji of less water-absorbing ability tends to make more yeast fermentation and less lactic fermentation, if the mash is not so severely agitated (Inamori *et al.*, 1977).

F. REFINING

1. *Pressing of Mash*

An aged mash is press-filtered through cloth under increasing hydraulic pressure, sometimes reaching 100 kg/cm^2, for 1–3 days. The difficulty of pressing shoyu mash has lessened with its increased protein digestibility in recent years. The viscosity of aged shoyu mash used to be more than 3000 cp; today it is generally less than 2000 cp. The activity of plant tissue-degrading enzymes in koji is highly correlated with a decrease in both the viscosity and the amount of shoyu mash presscake.

Tazaki *et al.* (1962) and other researchers have reported on the effectiveness of cellulase in increasing the extractibility and digestibility of soybean proteins. Further, according to Nakayama *et al.* (1965) and Harada *et al.* (1966), the addition of macerating enzymes to cellulase enhances the efficacy.

Ishii *et al.* (1972) isolated *A. sojae* no. 48, which processes strong degradating activity in the tissues of soybeans and wheat. Two kinds of shoyu koji were cultured using two different molds: (1) *A. sojae* no. 48, and (2) *A. sojae* X816, which was used as the control, and produced the stongest proteolytic activity among the available strains of mold. Both of these koji were digested in 25% saltwater solution. As indicated in Table XVII, the Koji cultured with *A. sojae* no. 48 exhibited a 3.7% higher protein digestibility and yielded twice as much reducing sugar as compared to the control mold. A significant difference in viscosity was observed in the two mashes after 3 months, and the mash of *A. sojae* no. 48 was much easier to filtrate. The enzyme activities produced by these two molds in shoyu koji are compared in Table XVIII. Remarkable differences exist in the activities of cellulase C_1 and the pectinase system such as macerating activity, pectin transeliminase, and endopolygalacturonase. While the cellulase C_1 preparation from *Tricoderma viride* which was added to shoyu koji exhibited almost no effect on protein digestibility and only a slight increase in the yield of reducing sugar, these results suggest that the pectolytic enzyme of *A. sojae* no. 48 in the presence of hemicellulose is effective in promoting the degradation of soybean and wheat tissues and in lowering the viscosity of shoyu mash. But the activity of pectin transeliminase is remarkably hindered in a 17–18% salt solution.

The relationships between enzyme activities of shoyu koji and mash viscosity

Table 6. Organ weights and relative organ weights of male rats given each experimental diet for 6 months

Experimental group No.	No. of rats examined	Organ				
		Brain	Pituitary+	Thyroid+	Thymus+	Lungs
		Organ weight (g)				
I (10.0 % P-shoyu)	14	1.98(0.05)	10.7(0.8)**	20.3(3.3)	150(21)*	1.16(0.16)
II (5.0 % P-shoyu)	14	2.01(0.05)	10.3(0.6)**	21.7(3.0)	153(22)	1.18(0.09)
III (2.0 % P-shoyu)	14	2.00(0.04)	10.1(0.7)	20.8(1.6)	137(21)**	1.18(0.06)*
IV (1.0 % P-shoyu)	14	2.00(0.05)	10.2(0.8)	22.0(2.6)	161(25)	1.20(0.06)**
V (0.4 % P-shoyu)	14	2.00(0.05)	10.0(0.5)	21.1(2.7)	141(25)**	1.16(0.09)
VI (4.5 % NaCl)	14	1.98(0.03)	10.1(1.2)	20.8(2.8)	160(23)	1.14(0.06)
VII (2.25% NaCl)	14	1.96(0.07)	9.7(0.8)	19.4(2.5)	156(14)	1.10(0.05)
VIII(0.9 % NaCl)	14	1.94(0.06)*	9.5(0.8)	19.6(2.2)	155(19)	1.13(0.10)
IX (0.45% NaCl)	14	1.97(0.04)	9.5(0.7)	22.0(2.8)	152(24)	1.16(0.05)
X (0.18% NaCl)	14	1.97(0.04)	9.8(1.1)	20.0(3.3)	161(19)	1.15(0.08)
XI (Control)	14	1.98(0.05)	9.4(0.9)	21.5(2.4)	173(30)	1.14(0.06)
		Relative organ weight (g/100 g body weight)				
I (10.0 % P-shoyu)	14	0.557(0.029)**	2.98(0.18)**	5.67(0.83)	44.2(5.9)	0.324(0.038)*
II (5.0 % P-shoyu)	14	0.543(0.034)	2.77(0.20)**	5.81(0.72)	41.1(5.9)	0.319(0.024)**
III (2.0 % P-shoyu)	14	0.545(0.033)*	2.76(0.17)**	5.66(0.33)	37.2(4.6)**	0.322(0.014)**
IV (1.0 % P-shoyu)	14	0.526(0.033)	2.68(0.21)	5.79(0.69)	42.0(5.3)	0.315(0.018)*
V (0.4 % P-shoyu)	14	0.535(0.025)	2.68(0.15)	5.64(0.66)	37.8(6.5)**	0.311(0.022)
VI (4.5 % NaCl)	14	0.554(0.018)**	2.82(0.38)**	5.81(0.66)	44.6(5.5)	0.318(0.020)**
VII (2.25% NaCl)	14	0.535(0.034)	2.67(0.24)	5.33(0.67)	42.8(3.6)	0.303(0.015)
VIII(0.9 % NaCl)	14	0.531(0.025)	2.59(0.16)	5.36(0.55)	42.4(4.3)	0.309(0.019)
IX (0.45% NaCl)	14	0.526(0.037)	2.54(0.17)	5.86(0.75)	40.7(6.5)	0.308(0.015)
X (0.18% NaCl)	14	0.534(0.024)	2.66(0.29)	5.42(0.91)	43.6(4.6)	0.311(0.018)*
XI (Control)	14	0.520(0.030)	2.46(0.19)	5.64(0.64)	45.3(7.1)	0.298(0.015)

+Values for organ weights and relative organ weights expressed in mg and mg/100 g body weight respectively. The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

TABLE XVII

ENZYME ACTIVITIES PRODUCED BY *Aspergillus sojae* X-816 AND *Aspergillus sojae* NO. 48 IN SHOYU KOJI[a]

Enzyme system	Activity (units/ml of extract)	
	X-816	No. 48
β-Glucanase		
Cellulase C_1	0	71.4
CM-cellulase	4.38	5.69
β-Glucosidase	2.77	14.01
β-1,3-Glucanase	2.10	6.50
Pectinase		
Macerating activity	>0	85.7
Pectin transeliminase	0.53	26.84
Endopolygalacturonase	10.3	104.5
Hemicellulase		
Xylanase	26.56	30.90
Arabanase	0.52	0.92
Galactanase	1.70	1.70
Protease		
Acid protease	1.35	1.32
Alkaline protease	4.99	2.04

[a] From Ishii *et al.* (1972). Each strain was grown on a culture medium which was composed of 15 g of defatted soybean and 15 g of wheat in an Erlenmeyer flask at 30°C. After 3 days the enzyme in shoyu koji was extracted with 150 ml of water.

TABLE XVIII

DEGRADATION OF SHOYU KOJI OF *Aspergillus sojae* X-816 AND *Aspergillus sojae* NO. 48 IN THE PRESENCE OF A HIGH CONCENTRATION OF NaCl[a]

	A. sojae X-816	*A. sojae* No. 48
NaCl	17.2%	16.9%
Total N/ml	1.66	1.70
Degradation rate of total N	82.8	86.5
Reducing sugar/ml	0.46	0.90

[a] From Ishii *et al.* (1972). Shoyu koji (30 g) of each strain was allowed to stand with 60 ml of 25% NaCl solution at 30°C for 37 days.

and filtration rates were analyzed by using the stepwise multiple regression analysis method. The correlation coefficient between mash viscosity and filtration rate was −0.772 (significant at 1% level). Contributing proportions of pectin-liquifying activity, pectin lyase activity, and carboxymethyl cellulose (CMC) saccharifying activity for mash viscosity were 21.3, 19.7, and 17.4%, respectively. Contributing proportions of pectin-liquifying activity, pectin lyase activity, pectic acid-liquifying activity, and ACM saccharifying activity for the filtration rate were 22.0, 18.3, 9.7, and 8.4%, respectively.

Kikuchi *et al.* (1975, 1976, 1977; Kikuchi, 1976) investigated those chemical compounds in shoyu mash which make the pressing of shoyu mash difficult and concluded that the problem is largely due to the presence of acidic polysaccharide. The insoluble solid contained in the presscake made from shoyu mash was estimated to consist of 10% microbial cells, 30% protein, and 20–30% nonproteinous substances derived from soybeans and wheat, respectively. Among these, the content of noncellulose polysaccharides was 7%, but its contribution to the filtration resistance of the insoluble solid was 70%, more than 40% of which was attributed to the acidic polysaccharides. One kind of acidic polysaccharide found in the shoyu contained more than 90% glacturonic acid, which forms a strong gel in high concentrations of aqueous salt solution and is presumed to make the filtration of shoyu mash difficult. Among the three kinds of polysaccharides present in the cell wall of soybeans (arabinogalactan, cellulose, and acidic polysaccharide), acidic polysaccharide goes into shoyu presscake at the ratio of 2:1. The amount of acidic polysaccharide in shoyu was determined to be only 0.7%, but its contribution to the viscosity of shoyu was 20%.

2. *Pasteurization*

The filtrate of an aged mash is heated at 70–80°C in order to retard the greater part of microbial and enzymatic reactions. The major changes resulting from this heating are the formation of an agreeable flavor and dark brown color, the separation of heat-coagulant substances, an increase in acidity, clarity, and anti-yeast potency, a decrease in the reducing sugar and amino acid content, and the evaporation of volatile compounds (Yokotsuka, 1954; Yokotusuka *et al.*, 1956, 1958; Okuhara *et al.*, 1961; Onishi 1970a, 1971, 1972, 1975, 1976). It is sometimes necessary to remove or destroy the heat-tolerant bacterial spores either by the HTST method or by filtration.

Retarding alcohol evaporation during pasteurization of shoyu improved its organoleptic acceptance, but only when the coverage is tightly attached to the surface of shoyu, and caution must be exerted to avoid producing too much heat flavor. Adequately cooling the shoyu after heating is necessary. Until recently, the heated shoyu was stored in open tanks for 6–7 days to promote clarification, and it was generally believed that covering the containers of heated shoyu during

Table 5. (continued).

Experimental group No.	Bilirubin (mg/100 ml)	Cholesterol (mg/100 ml)
	Male	
I (10.0 % P-shoyu)	0.35(0.10)	67(8)
II (5.0 % P-shoyu)	0.33(0.06)	67(10)
III (2.0 % P-shoyu)	0.35(0.15)	64(10)
IV (1.0 % P-shoyu)	0.36(0.15)	64(10)
V (0.4 % P-shoyu)	0.33(0.08)	61(12)
VI (4.5 % NaCl)	0.30(0.07)	61(7)
VII (2.25% NaCl)	0.33(0.07)	58(7)
VIII(0.9 % NaCl)	0.36(0.12)	62(7)
IX (0.45% NaCl)	0.37(0.13)	62(11)
X (0.18% NaCl)	0.33(0.14)	61(8)
XI (Control)	0.32(0.09)	63(7)
	Female	
I (10.0 % P-shoyu)	0.38(0.19)	97(19)
II (5.0 % P-shoyu)	0.39(0.18)	96(12)
III (2.0 % P-shoyu)	0.39(0.14)	99(9)
IV (1.0 % P-shoyu)	0.36(0.12)	96(11)
V (0.4 % P-shoyu)	0.36(0.15)	98(10)
VI (4.5 % NaCl)	0.42(0.20)	98(15)
VII (2.25% NaCl)	0.40(0.19)	94(12)
VIII(0.9 % NaCl)	0.39(0.15)	96(10)
IX (0.45% NaCl)	0.39(0.17)	103(11)*
X (0.18% NaCl)	0.34(0.09)	96(15)
XI (Control)	0.37(0.21)	94(12)

The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

Blank

Table 5. (continued)

Experimental group No.	GOT (Ka. Unit)	GPT (Ka. Unit)	Al-P (K-A Unit)	LAP (G-R Unit)	Glucose (mg/100 ml)	BUN (mg/100 ml)
			Male			
I (10.0 % P-shoyu)	109.2(18.3)	50.1(10.8)	11.1(1.6)	101(10)	150(21)	18.3(2.9)**
II (5.0 % P-shoyu)	101.6(13.5)*	42.1(8.1)**	10.1(1.4)	103(7)	143(12)	16.7(1.2)**
III (2.0 % P-shoyu)	102.2(17.4)	44.4(11.6)**	11.3(1.4)	105(7)	148(13)	14.9(1.8)
IV (1.0 % P-shoyu)	97.6(13.9)**	45.4(11.3)**	10.7(1.4)	103(7)	148(14)	14.9(1.3)
V (0.4 % P-shoyu)	110.5(20.7)	48.2(13.8)*	11.2(1.8)	100(6)	150(17)	15.4(0.8)
VI (4.5 % NaCl)	108.4(11.7)	49.4(8.4)*	10.8(1.5)	105(10)	145(18)	19.6(3.2)**
VII (2.25% NaCl)	107.1(15.3)	46.2(9.5)**	11.0(1.1)	108(7)	144(15)	17.1(2.3)**
VIII(0.9 % NaCl)	112.2(21.1)	45.7(16.9)*	11.4(1.2)	107(7)	143(19)	15.4(1.3)
IX (0.45% NaCl)	108.6(13.9)	47.2(8.4)**	11.8(1.9)	105(10)	149(16)	15.2(1.8)
X (0.18% NaCl)	105.9(20.3)	46.6(10.8)**	10.8(1.4)	102(8)	141(13)	15.4(2.2)
XI (Control)	115.7(19.0)	58.7(11.2)	11.2(1.1)	103(5)	145(8)	14.7(1.5)
			Female			
I (10.0 % P-shoyu)	102.3(17.1)	44.0(13.8)	8.3(1.7)	100(10)	127(13)**	20.6(3.8)**
II (5.0 % P-shoyu)	102.3(14.6)	42.4(13.9)	8.8(1.2)	100(10)	118(15)**	19.2(2.0)**
III (2.0 % P-shoyu)	101.3(8.8)	38.5(8.7)	9.0(1.1)	101(10)	111(13)	16.9(2.0)
IV (1.0 % P-shoyu)	98.0(14.3)	38.3(8.3)	9.2(2.8)	99(8)	123(16)**	17.6(2.1)
V (0.4 % P-shoyu)	102.2(15.8)	42.2(11.6)	9.1(1.4)	100(12)	109(15)	16.5(2.2)
VI (4.5 % NaCl)	100.1(11.2)	43.3(8.9)	7.9(1.1)	102(11)	115(13)*	19.7(2.5)**
VII (2.25% NaCl)	102.6(11.1)	42.1(14.3)	8.6(1.5)	105(12)	117(15)**	19.4(2.7)**
VIII(0.9 % NaCl)	103.3(15.0)	44.0(15.8)	8.9(1.4)	100(12)	118(19)*	18.5(3.1)*
IX (0.45% NaCl)	104.5(8.5)	44.7(10.5)	8.7(1.5)	102(14)	112(9)*	16.7(2.6)
X (0.18% NaCl)	99.4(13.3)	41.0(10.4)	9.0(1.3)	100(13)	111(19)	16.7(2.4)
XI (Control)	94.8(16.1)	39.6(8.4)	9.1(2.4)	103(14)	104(9)	16.3(2.3)

GOT = Glutamic - oxalacetic transaminase (Karmen Unit), GPT = Glutamic - pyruvic transaminase (Karmen Unit), Al-P = Alkaline phosphatase (King-Armstrong Unit), LAP = Leucine aminopeptidase (Goldbarg-Rutenburg Unit), BUN = Blood urea nitrogen

The figures are means and standard viation in parentheses for the numb of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *P < 0.05; **P < 0.01.

this period produced shoyu of an inferior quality. In Japan, it is legal to add benzoic acid or butyl-*p*-hydroxybenzoate to the refined shoyu as a preservative, but the trend seems to be toward using aseptic bottling or the addition of ethanol as a preservative. There is a general tendency in Japan to lower the heating temperature of shoyu in the final stage of production in order to produce a product with a milder flavor and a lighter color.

According to Hashimoto *et al.* (1971, 1972, 1973, 1974, 1976), the heat-coagulating substances produced by heating raw shoyu are equivalent to 10% of its volume and 0.025–0.05% of its weight. They consist of 89.1% protein, 9.7% carbohydrate, and 1.2% ash. Their nitrogen content is 0.2–0.4% that of the shoyu. The amino acid composition of these heat-coagulating substances in shoyu is significantly different from that of soybeans or wheat in the ratio of aspartic acid to glutamic acid or of proline to leucine. The major ingredients of the heat-coagulating substances in shoyu, determined by immunological identification, are the undenatured proteins derived from the enzymes produced by koji mold.

The speed with which coagulation occurs is inversely related to the heating temperature and is thought to be due to the inactivation of proteases which do not tolerate heat, such as acid proteases, neutral protease, and alkaline protease, and to heat-tolerant neutral protease II, which is relatively stable at 60 or 80°C. This fact may explain why the coagulation of shoyu achieved by heating is not caused merely by the coagulation of the undenatured protein derived from the raw materials. Adding a small amount of raw shoyu to pasteurized shoyu remarkably promotes coagulation, which means that there are some factors in raw shoyu which promote coagulation when heated. Coagulation of shoyu is also promoted by the addition of protease isolated from koji, especially when the optimal pH value is 5.0, particularly at higher heating temperatures. The addition of the heat-resistant acid protease, which is produced by *Penicillium duponti* K1014, is stable at 60°C, and exhibits its highest activity at pH 4.6 and 75°C, remarkably promoted the coagulation of heated shoyu. It has been suggested that the protein molecules associate with each other through hydrophobic bonds by the action both of heat and proteases.

Contributions to the sedimentation of coagula during the shoyu pasteurization process were investigated by measuring the amount, density, and particle sizes of the coagula. The results indicated that α-amylase had no effect, acidic protease had some promoting effect, and alkaline protease had a remarkable retarding effect on the sedimentation of coagula in shoyu.

3. *Chemical Composition of Koikuchi Shoyu*

Koikuchi shoyu produced at the United States Kikkoman plant was analyzed by Okuhara and Yokotsuka (1977), with results presented in Table XIX. The

TABLE XIX

DETAILED COMPOSITION OF FERMENTED SHOYU[a]

Component	Percent (w/w) of shoyu, "as is"	Percent (w/w) of shoyu, "dry" basis
Soluble solids (dry matter)	34.00	
Alcohol	1.47	
Water (by difference)	64.53	
Inorganic components		
Sodium	6.10	17.94
Chlorine	8.82	25.94
Calcium	0.02	0.06
Potassium	0.40	1.17
Phosphorus	0.15	0.44
Magnesium	0.07	0.21
Sulfur	0.06	0.17
Iron	0.002	0.006
Manganese	0.001	0.003
Total	15.60	45.94
Organic components		
Polyols		
Glycerol	1.50	4.41
Mannitol	0.17	0.50
Total	1.67	4.91
Ether-soluble compounds	0.14	0.41
Ether-soluble volatile matter	0.005	0.01
Amino acids		
Lysine	0.56	1.65
Histidine	0.21	0.62
Cystine	0.07	0.21
Arginine	0.22	0.65
Aspartic acid	0.90	2.65
Threonine	0.36	1.06
Serine	0.45	1.32
Glutamic acid	1.92	5.65
Proline	0.59	1.74
Glycine	0.34	1.00
Alanine	0.38	1.12
Valine	0.47	1.38
Methionine	0.12	0.35
Isoleucine	0.41	1.21
Leucine	0.62	1.82
Tyrosine	0.08	0.24
Phenylalanine	0.36	1.06
Ornithine	0.49	1.44
Total	8.55	25.17

(*continued*)

Table 5. Results of analysis of the serum of rats given each experimental diet for 6 months

Experimental group No.	No. of rats examined	Total protein (g/100 ml)	Albumin (g/100 ml)	A/G ratio	ZTT (Ku. Unit)	TTT (Ma. Unit)
				Male		
I (10.0 % P-shoyu)	14	5.67(0.23)*	3.51(0.25)	1.68(0.41)	0.45(0.17)	0.33(0.09)
II (5.0 % P-shoyu)	14	5.89(0.27)	3.65(0.30)	1.70(0.35)	0.43(0.14)	0.39(0.14)
III (2.0 % P-shoyu)	14	5.85(0.29)	3.64(0.27)	1.67(0.25)	0.43(0.16)	0.35(0.10)
IV (1.0 % P-shoyu)	14	5.66(0.30)*	3.57(0.33)	1.74(0.31)	0.46(0.15)	0.35(0.15)
V (0.4 % P-shoyu)	14	5.79(0.30)	3.61(0.25)	1.68(0.21)	0.41(0.11)	0.29(0.10)
VI (4.5 % NaCl)	14	5.83(0.23)	3.61(0.28)	1.66(0.32)	0.36(0.10)	0.29(0.11)
VII (2.25% NaCl)	14	5.89(0.19)	3.61(0.26)	1.62(0.27)	0.41(0.10)	0.34(0.14)
VIII(0.9 % NaCl)	14	5.87(0.21)	3.76(0.29)	1.71(0.27)	0.45(0.17)	0.38(0.12)
IX (0.45% NaCl)	14	5.87(0.23)	3.72(0.32)	1.72(0.37)	0.46(0.20)	0.39(0.17)
X (0.18% NaCl)	14	5.81(0.22)	3.59(0.32)	1.65(0.29)	0.40(0.16)	0.36(0.12)
XI (Control)	14	5.86(0.14)	3.64(0.17)	1.66(0.19)	0.39(0.15)	0.34(0.10)
				Female		
I (10.0 % P-shoyu)	14	5.87(0.41)	3.57(0.45)	1.58(0.31)	0.37(0.11)	0.23(0.06)
II (5.0 % P-shoyu)	14	6.00(0.28)	3.74(0.28)	1.69(0.30)	0.42(0.11)*	0.27(0.11)
III (2.0 % P-shoyu)	14	5.90(0.33)	3.55(0.32)	1.53(0.23)	0.41(0.14)	0.25(0.08)
IV (1.0 % P-shoyu)	14	6.14(0.25)*	3.86(0.28)	1.73(0.34)	0.35(0.11)	0.24(0.09)
V (0.4 % P-shoyu)	14	5.98(0.24)	3.76(0.36)	1.76(0.45)	0.34(0.12)	0.21(0.06)
VI (4.5 % NaCl)	14	5.95(0.34)	3.76(0.30)	1.80(0.46)	0.36(0.15)	0.25(0.07)
VII (2.25% NaCl)	14	6.09(0.34)	3.74(0.38)	1.61(0.26)	0.36(0.09)	0.21(0.05)
VIII(0.9 % NaCl)	14	6.00(0.33)	3.81(0.22)	1.76(0.27)	0.41(0.14)	0.25(0.09)
IX (0.45% NaCl)	14	6.11(0.32)	3.91(0.23)*	1.81(0.34)	0.39(0.11)	0.25(0.06)
X (0.18% NaCl)	14	6.08(0.33)	3.79(0.33)	1.75(0.42)	0.37(0.13)	0.27(0.16)
XI (Control)	14	5.87(0.38)	3.65(0.36)	1.66(0.25)	0.34(0.09)	0.26(0.12)

A/G ratio = Albumin/Globurin ratio, ZTT = Zinc Turbidity Test (Kunkel Unit), TTT = Thymol Turbidity Test (Maclagan Unit
The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *P < 0.05; **P < 0.01.

TABLE XIX (*Continued*)

Component	Percent (w/w) of shoyu, "as is"	Percent (w/w) of shoyu, "dry" basis
Ammonia	0.30	0.88
Organic acids		
Formic	0.02	0.06
Acetic	0.16	0.47
Citric	0.04	0.12
Succinic	0.05	0.15
Lactic	0.68	2.00
Total	0.95	2.80
Sugars		
Monosaccharides		
Mannose	0.06	0.18
Arabinose	0.08	0.24
Galactose	0.17	0.50
Xylose	0.06	0.18
Glucose	2.05	6.03
Unidentified	0.23	0.68
Total	2.65	7.81
Disaccharides	0.65	1.91
Oligosaccharides	—	—
Polysaccharides	1.15	3.38
Total sugars (as glucose)	4.45	13.10
Total organic components	16.1	47.3
Solids accounted for	31.7	93.2
With ammonia calculated as amino acids	32.69	96.1

[a] From Okuhara and Yokotsuka (1977).

middle column gives the component percentages based on the liquid condiment as it is used by the consumer. The right-hand column lists the components on a dry basis. The soluble solids were divided almost equally between inorganic (46%) and organic components (47%). Sodium and chlorine were the principal inorganic constituents. Polyalcohols comprised almost 5% of the soluble solids, amino acids 25%, organic acids nearly 3%, and carbohydrates 13%. Amino acids were determined both before and after acid hydrolysis to obtain free and total values for each. Table XIX gives the values after hydrolysis except for methionine and tyrosine, which are unstable in acid hydrolysis. The values for these two amino acids were obtained before acid hydrolysis and therefore do not reflect any methionine and tyrosine that might have been bound in peptides. Furthermore, Table XIV shows no values for tryptophan. Recently, Hugli and Moore (1972) found 0.002% by a different method. Even with these deficiencies

the values reported in the table account for 93.2% of the soluble solids in shoyu. The ammonia found after acid hydrolysis probably resulted from decomposition of amino acids and should be calculated as amino acid. When this is done, the figure for total amino acids becomes 9.8% on a wet basis. This calculation accounts for 32.69 g, or 96.1% of the soluble solids in the sample shoyu. However, shoyu also contains browning pigments in addition to the compounds described in the analytical tables. The components listed do not include those many compounds present in trace amounts. Approximately 300 components of the ether-soluble volatile fraction, which constitutes less than 0.005% of fermented shoyu, have been identified to date.

Among the components, the free amino acids have been of most interest because of their characteristic taste and appreciable quality. The free amino acids usually account for 40–50% of the total soluble nitrogen and about 40% of the residual nitrogenous substances which can be hydrolyzed by acid to form additional free amino acids (Oka and Nagata, 1974a). As the latter nitrogenous substances are oligopeptides, it has long been thought that some peptides may contribute to the flavor of fermented food products, although few concrete data have been reported.

Oka and Nagata (1974a,b) fractionated a shoyu sample by gel filtration on a Shephadex G-15 column, with subsequent subfractionation on the basis of acidity by ion-exchange chromatography. After preliminary fractionation, the components in the subfractions were transformed into copper salts, and these chromatographed to separate out neutral peptide subfractions. The peptide fractions were further fractionated on a preparative amino acid analyzer and by paper chromatography. Thus, three glycopeptides and eight dipeptides were isolated and characterized as the major neutral peptide components in shoyu. However, the practical contributions of these components to the flavor of shoyu were judged to be negligible. Four dipeptides and sugar derivatives of ten dipeptides and two tripeptides were isolated by further fractionation of the acidic subfractions and characterized as the major acidic peptides in shoyu. However, it was difficult to attribute any direct contribution of these peptides to the flavor of shoyu on the basis of their quantity and taste response.

IV. COLOR OF SHOYU

The color of shoyu is an important attribute to Japanese dishes, although it has become lighter in recent years. The color and flavor of shoyu are very closely related, as both are affected by the aging of mash and the pasteurization of raw shoyu. During the brewing process, the development of shoyu color derives mainly from nonoxidative and nonenzymatic browning reactions. Enzymatic

Table 4. Hematological findings at week 26 in rats given each experimental diet

Experimental group No.	No. of rats examined	PCV (%)	Hb (g/100 ml)	RBC ($10^4/mm^3$)	WBC ($10^2/mm^3$)
			Male		
I (10.0 % P-shoyu)	14	45.9(2.5)*	14.9(0.6)	770.1(52.7)	46.8(14.9)**
II (5.0 % P-shoyu)	14	46.1(1.0)**	15.0(0.4)**	793.1(56.5)	42.2(12.3)*
III (2.0 % P-shoyu)	14	46.2(1.5)**	14.8(0.4)**	782.2(55.9)	45.7(10.8)**
IV (1.0 % P-shoyu)	14	45.8(1.9)**	14.9(0.4)*	790.9(54.9)	43.4(9.7)**
V (0.4 % P-shoyu)	14	46.2(1.2)**	15.1(0.5)**	816.2(51.4)	50.1(18.2)**
VI (4.5 % NaCl)	14	45.0(1.9)	14.7(0.3)	761.9(67.9)	35.1(9.5)
VII (2.25% NaCl)	14	44.7(2.5)	14.7(0.4)	788.2(52.1)	35.8(9.3)
VIII(0.9 % NaCl)	14	44.7(2.4)	14.8(0.4)*	789.4(65.0)	36.3(9.5)
IX (0.45% NaCl)	14	45.3(1.5)*	14.8(0.4)*	784.8(42.1)	38.5(11.4)
X (0.18% NaCl)	14	44.5(1.5)	14.6(0.3)	786.8(62.6)	39.5(11.4)
XI (Control)	14	43.9(1.3)	14.5(0.3)	800.4(59.6)	33.5(8.5)
			Female		
I (10.0 % P-shoyu)	14	45.5(1.7)*	15.1(0.4)	760.0(38.1)	24.6(8.2)
II (5.0 % P-shoyu)	14	44.6(1.5)	14.9(0.4)	768.1(39.7)	24.1(6.5)
III (2.0 % P-shoyu)	14	44.3(1.1)	14.8(0.4)	758.4(33.4)	23.4(7.0)
IV (1.0 % P-shoyu)	14	44.9(2.0)	15.0(0.4)	762.2(31.9)	27.9(5.9)**
V (0.4 % P-shoyu)	14	44.3(2.5)	14.9(0.3)	768.0(41.7)	27.0(6.9)*
VI (4.5 % NaCl)	14	43.5(2.2)	14.7(0.5)	729.6(50.0)	24.6(6.3)
VII (2.25% NaCl)	14	44.0(2.0)	14.8(0.4)	752.9(33.9)	24.4(5.9)
VIII(0.9 % NaCl)	14	43.0(1.5)	14.7(0.3)	753.1(33.1)	22.7(5.6)
IX (0.45% NaCl)	14	43.5(2.0)	14.7(0.3)	754.5(32.7)	24.1(5.4)
X (0.18% NaCl)	14	43.3(2.1)	14.7(0.5)	742.9(29.2)	24.0(7.6)
XI (Control)	14	43.9(1.9)	14.8(0.4)	760.6(45.5)	22.1(3.9)

PCV = Packed cell volume, Hb = Hemoglobin, RBC = Red blood cells, WBC = White blood cells
The figures are means and standard deviation in parentheses for the number of rats shown and those marked with an asterisk differ significantly (Student's t-test, from those of controls: *P < 0.05; **P < 0.01.

reactions, which occur between amino compounds and sugars, are rare. When koikuchi or usukuchi shoyu is packed in glass bottles or cans, the color is relatively stable, but it darkens rather quickly after the seal is broken due to the oxidative and nonezymatic browning reaction. These reactions cause the organoleptic quality of shoyu to be inferior. In the preparation of usukuchi shoyu, considerable effort is directed toward minimizing the intensity of color development by decreasing the amount of protein and total solid in the mash, increasing its salt concentration, and by avoiding too long a period of fermentation and aging as well as extended heating of the raw shoyu during pasteurization. In these respects, it differs from the production of koikuchi shoyu.

A. COLOR COMPOUNDS OF SHOYU

Kurono (1927) reported that the color of shoyu was a type of melanoidin pigment and consisted mainly of two compounds: $C_{27}H_{17}N_3O_{13}$ and $C_{27}H_{15}N_3O_{12}$. Omata *et al.* (1955d) separated the color substances of shoyu into two fractions, acidic and basic, by column and paper chromatography and then spectrometrically determined the increases of these fractions during the brewing of mash and the storage of shoyu. The quantitative increase in the acidic fraction was higher than that in the basic fraction. Mitsui and Kusaba (1957) also isolated two kinds of shoyu pigment, one of which was the same as that isolated by Kurono.

Hashiba (1971) isolated the browning compounds present in shoyu by gel filtration with Sephadex G-25 into three peaks, PI, PII, and PIII, according to the rate of elution. The quantity of PI increased during oxidative storage, while the quantity of PIII increased remarkably during the pasteurization process. The increase of PI gave the shoyu a dark brown color, while that of PIII gave it a red tone. Hashiba (1973a) purified the melanoidin produced during the storage of shoyu at 37°C for 50 days by dialysis, DEAE-cellulose chromatography, and Sephadex G-100 gel filtration until a single band on the disc electrophoresis appeared. The color of melanoidin thus obtained was not affected by heating or oxidation. When hydrolyzed, the melanoidin liberated sugars such as glucose, xylose, galactose, and arabinose, and all of the amino acids found in shoyu.

Motai *et al.* (1972) and Motai and Inoue (1974a) fractionated the material which gives shoyu its color into eight color components by DEAE-cellulose chromatography with stepwise elution. The color intensity of each peak became darker, and E_{450} and molecular weight became higher with successive orders of elution. When the shoyu was heated, the color components became brighter, while with oxidation they became darker in tone. The melanoidin pigments prepared by heating an aqueous solution of glycine and xylose at 100°C for 2 hr were chromatographically fractionated into eight color components. The fractionated color components from the glycine–xylose model system exhibited

similar changes when heated and oxidized, as did those of shoyu. Based on spectral measurements, elemental analysis, and amino acid analysis, all the color components appeared to be very similar in chemical structure, having stepwise different molecular weights. The infrared absorption spectra of eight peaks had the same pattern, suggesting that they were melanoidins. These results indicate that shoyu is made up of at least eight kinds of melanoidin pigment having different degrees of polymerization.

B. MEASUREMENT OF SHOYU COLOR

The color of shoyu represented by the International Commission on Illumination (CIE) system was reported by Omata and Ueno (1953a) to have a dominant wavelength of 590–620 nm, an excitation purity of 86–88%, and a luminous transmittance of 0.14–0.17. Using the CIE system, Umeda and Saito (1956) analyzed the color of shoyu prepared from mash aged for different periods of time. The color standard of shoyu was prepared from known chemical pigments so as to match the color changes described above, which consisted of 30 degrees of color of the same visual distance. This method of using a color standard to assess the color of a shoyu is very simple and convenient when the shoyu has a single or consistent color tone (i.e., when it is separated from mash or just after pasteurization). However, shoyu having different color tones produced by oxidation during storage is difficult to analyze by this method.

Motai (1976) has reported a linear relationship between the logarithm of absorbance (log A) and the wavelength (450–650 nm) in the color distribution of shoyu and melanoidin prepared from the model system. (A similar relationship has been observed in whiskey, cola drinks, beer, caramel, and miso.) There was no change in log A per 100 nm (designated as ΔA) in each of eight pigments which were fractionated from shoyu or melanoidin using the model system, either during heating or oxidation. Therefore, the log A per 100 nm in this case can be used as the parameter for expressing the color tone of shoyu.

That shoyu becomes more red in color when heated and takes on a darker brown tone when oxidized is generally acknowledged (Okuhara *et al.*, 1969).

As shown in Fig. 13, Motai (1976) observed three types of browning reactions of shoyu in relation to the increase in color intensity (E_{450}). His findings are summarized below:

Type a: The color tone darkens along with an increase of ΔA, which occurs during the storage of shoyu in the open air as a result of nonenzymatic oxidative browning.

Type b: The color tone is unchanged along with the unchanged ΔA, which occurs in bottled or canned shoyu as a result of the heat-dependent browning of shoyu.

Table 3. Food and water consumptions and shoyu intake of rats given each experimental diet for 6 months

Experimental group No.	Food consumption (g/rat/day)	Water consumption (ml/rat/day)	Intake of shoyu+ (ml/rat/day)	Intake of shoyu+ (ml/kg/day)
		Male		
I (10.0 % P-shoyu)	19.4	55.3**	5.04	16.64
II (5.0 % P-shoyu)	19.3	38.3**	2.51	7.96
III (2.0 % P-shoyu)	19.0	30.4	0.99	3.15
IV (1.0 % P-shoyu)	19.0	26.3	0.49	1.53
V (0.4 % P-shoyu)	19.1	27.4	0.20	0.62
VI (4.5 % NaCl)	19.2	52.6**	(0.86)	(2.83)
VII (2.25% NaCl)	18.7	36.8**	(0.42)	(1.37)
VIII(0.9 % NaCl)	19.1	28.8	(0.17)	(0.55)
IX (0.45% NaCl)	19.1	28.3	(0.09)	(0.27)
X (0.18% NaCl)	18.9	26.2	(0.03)	(0.11)
XI (Control)	18.9	28.3	--	--
		Female		
I (10.0 % P-shoyu)	13.8	44.7**	3.59	19.76
II (5.0 % P-shoyu)	13.2	30.2**	1.71	9.41
III (2.0 % P-shoyu)	13.0	23.1	0.68	3.69
IV (1.0 % P-shoyu)	13.2	23.9	0.34	1.84
V (0.4 % P-shoyu)	13.4	24.5	0.14	0.74
VI (4.5 % NaCl)	14.0	42.8**	(0.63)	(3.37)
VII (2.25% NaCl)	12.9	29.0**	(0.29)	(1.59)
VIII(0.9 % NaCl)	13.5	24.7	(0.12)	(0.66)
IX (0.45% NaCl)	13.0	22.3	(0.06)	(0.33)
X (0.18% NaCl)	13.4	22.3	(0.02)	(0.13)
XI (Control)	13.5	23.0	--	--

+The figures for shoyu intake are calculated from data on food consumption, body weight and sodium chloride contents in shoyu. Values in parentheses show sodium chloride intake expressed in g/rat/day and g/kg/day.

The figures marked with asterisks differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

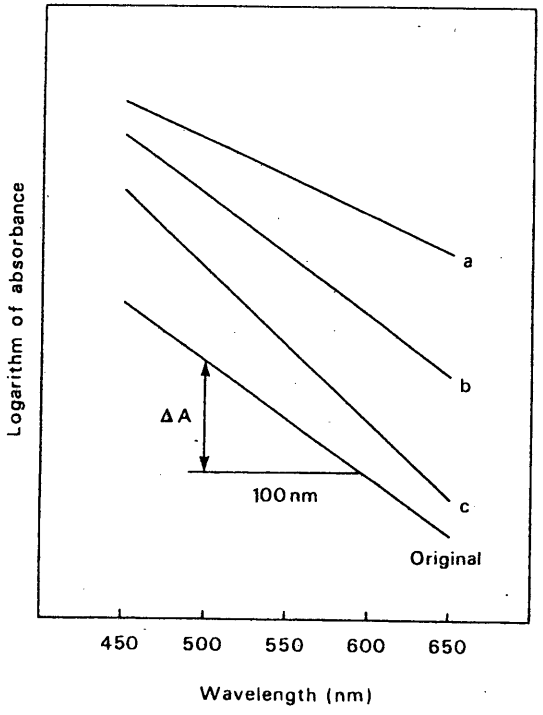


FIG. 13. Change of ΔA during the browning reaction. From Motai *et al.* (1972).

Type c: The color tone becomes bright along with an increase in ΔA during the aging of the mash or pasteurization of shoyu, which occurs as a result of the heat-dependent browning.

The ΔA values of koikuchi and usukuchi shoyu available on the market were reported to be 0.63–0.70 and 0.56–0.60, respectively. The smaller ΔA value of usukuchi shoyu is due to the shorter aging period of the mash and to the shorter pasteurization time used in the preparation of usukuchi as compared to koikuchi shoyu.

C. BROWNING MECHANISM OF SHOYU

1. Color Formation during the Brewing Process

About 50% of the color of koikuchi shoyu is formed during the fermentation and aging of mash, and the remaining 50% during pasteurization of shoyu. Both

TABLE XX
COLOR FORMATION DURING PREPARATION OF SHOYU[a]

Period of mash fermentation (months)	ΔA	Color degree (E_{450})	Percentage of color formation
1[b]	0.45	2.48	13.7
2[b]	6.08	6.08	33.7
6[b]	8.54	8.54	47.2
6 (pasteurized)[c]	18.07	18.07	100.0

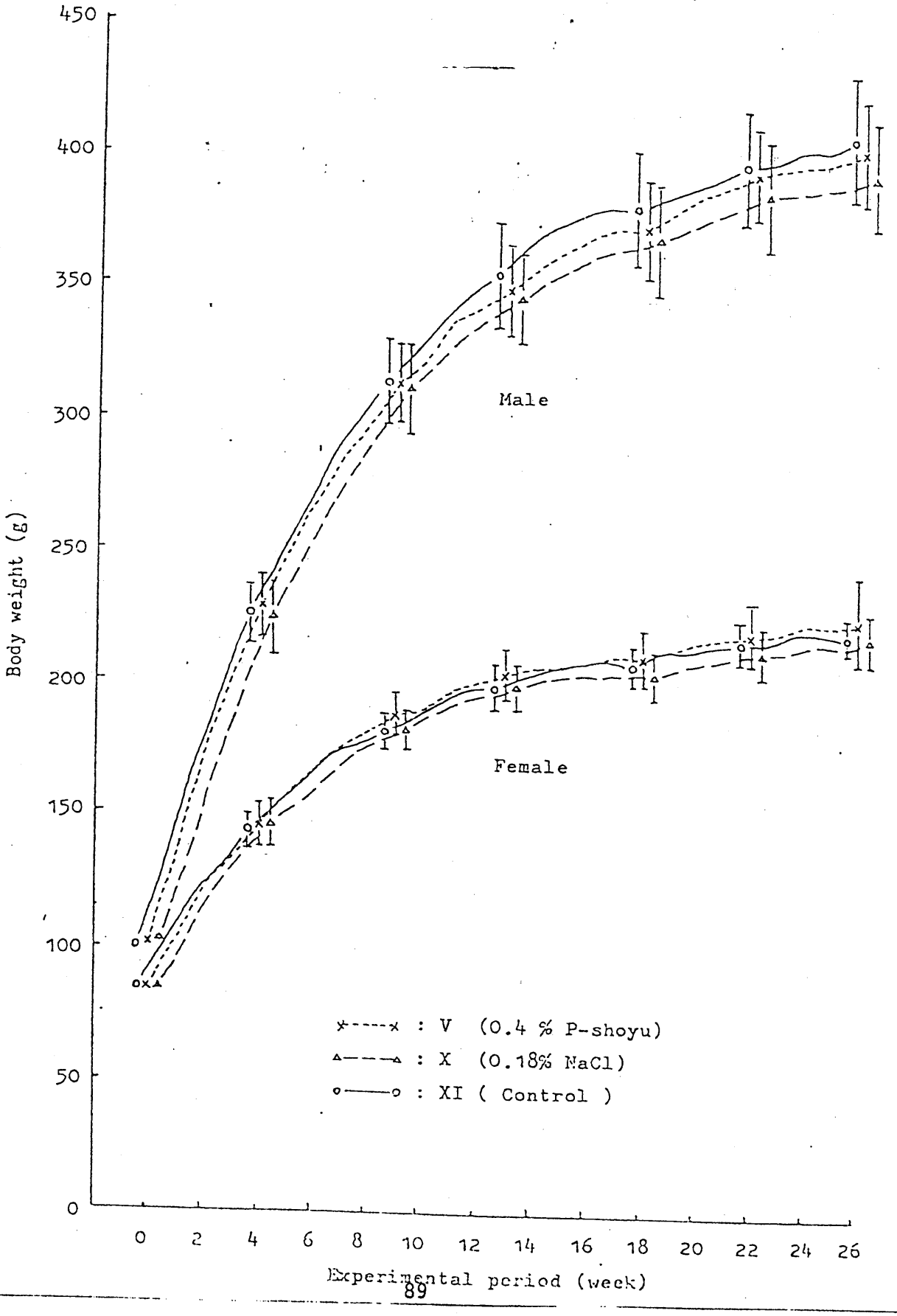
[a] From Motai (1976).
[b] The color of the liquid part of mash was determined.
[c] The liquid part of mash was pasteurized and the color was determined.

are considered to be due primarily to heat-dependent browning, or the so-called Maillard browning reaction between amino compounds and sugars. One example of the development of color during shoyu preparation is given in Table XX (Motai, 1976). The amount of hexose in shoyu, which is mainly glucose, is from 6 to 10 times greater than the proportions of pentose. However, some researchers have considered that the sugar, which is involved primarily in the browning of shoyu, is a pentose such as xylose and arabinose (Kamata and Sakurai, 1964; Kato and Sakurai, 1962; Shikata *et al.*, 1971a).

About 30% of the pentosan contained in the raw materials is degradated into the water-soluble form, and the resultant color intensity of the mash is proportional to the amount of pentosan dissolved in the mash. The degradation of pentosan in the course of koji cultivation increases with the elevation of the temperature of koji, which results in an increase in the color intensity of the shoyu obtained. According to Okuhara *et al.* (1969), to three kinds of shoyu were added 0.025, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0% xylose, respectively, and these were heated at 80°C for 5 hr. The results are presented in Fig. 14. There was a linear relationship between color intensity and the utilization of pentose, but the degree of color change per 1 mg utilization of xylose for the three kinds of shoyu was different. When the mixture of xylose, glucose, glycine, lactic acid, 18% salt water, and shoyu was heated after adjusting its pH to 4.8, the increase in color intensity was smaller and occurred more slowly than when the shoyu or chemical protein hydrolysate was heated. The participation of xylose in the heat-dependent browning of shoyu was calculated to be only 10–20% (Okuhara *et al.*, 1975).

Okuhara *et al.* (1970) statistically analyzed the relationship between the composition of raw shoyu separated from mash and its rate of browning. The correlation and multiregression models were calculated as indicated in Table XXI. In order to obtain various concentrations of individual shoyu components, in Pro-

Fig. 5. Growth curve of rats given 0.4 % P-shoyu, 0.18 % NaCl and control diets for 26 weeks (mean ± SD)



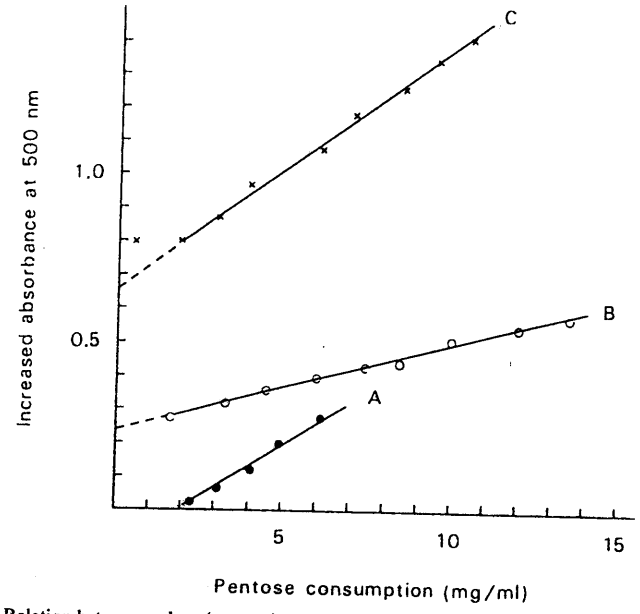


FIG. 14. Relation between xylose (pentose) consumption and pigment formation. From Okuhara *et al.* (1969).

portion I, varying weights were given to the koji per unit amount of raw materials, and in Proportion II, small differences were given to the concentration of salt mash. Those components that correlated significantly with the browning rate were the concentration of koji in mash (CK), the weight of Koji from a unit amount of raw material (WK), the amount of shoyu obtained from a unit amount of raw material (L), nonamino nitrogenous compounds (TN − FN), the titratable acidity (TA), reducing sugar of shoyu (RS), the reducing power of raw shoyu [R(N)], and above all (TN − FN) and (RS). Such correlations indicate that the intensity of browning pigment can be calculated from (TN − FN) and (RS). Okuhara *et al.* (1971) also speculated that (TN − FN) might be significantly correlated with the browning of shoyu, based on the following experimental findings:

1. The browning of shoyu takes place more rapidly than does a solution of sugar and amino acids.
2. The browning of shoyu is dependent upon the degree of mash fermentation and the amount of (TN − FN).

TABLE XXI

SIGNIFICANTLY CORRELATED COMPONENTS RELATED TO BROWNING OF SHOYU[a]

Variable[d]	C(HF)[b]	C(OXH)		C(HN)		C(N)	
	II[c]	I	II	I	II	I	II
CK			**[e]	*	**		**
WK	*		**	*	**	**	**
L			*		**		**
TN − FN		*	*	**(++)	**	**(+)	**(++)
FN − NH_3 − N		−*	**	(+)			*
NH_3 − N				*			
TA	(−)			**(+)	**	**	**(+)
RS		*	*	**	**(++)	**(++)	**
Alcohol				−**		−**	
Org. A		−**		−*		−**	
pH						−**(− −)	
RN				**	**	**	**
R(HN)		−**		*	*		
Red(N)			**(+)				
Red(HN)	*				**		
NaCl		(−)		*			
C(N)[b]		**(++)	**	**			
C(H)[b]			*(−)		**		
C(HN)[b]		(− −)					

[a] From Okuhura *et al.* (1970).

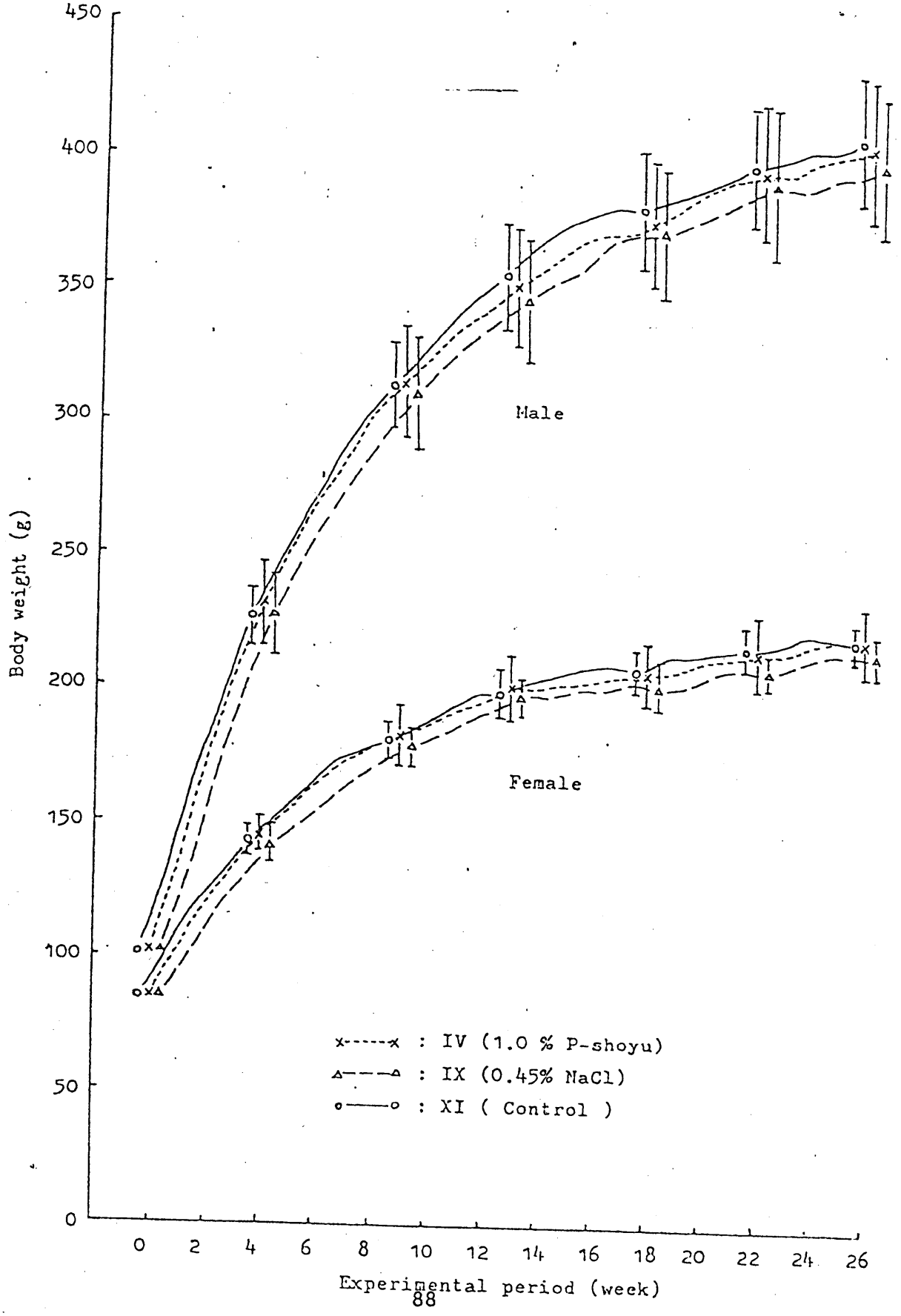
[b] C(N): Color intensity of raw shoyu; C(H): color intensity of pasteurized shoyu; C(HN): subtracted C(N) from C(H); C(OXH): subtracted C(H) from color intensity of oxidized shoyu; C(HF): subtracted color intensity of faded shoyu from C(H).

[c] Shoyu types I or II.

[d] CK: The ratio of koji to mash water; WK: weight of koji (kg) per material unit; L: volume of shoyu per material unit; TN − FN: subtracted formol nitrogen (%) from total nitrogen (%); FN − NH_3 − N: subtracted ammonium nitrogen (%) from folmol nitrogen; NH_3 − N: ammonium nitrogen (%); TA: titratable acidity (meq/10 ml); RS: residual reducing sugar (%); Alcohol: % (v/v); Org. A: organic acid (meq/ml); pH: pH of raw shoyu; RN: reducing power of raw shoyu; R(HN): subtracted RN from reducing power of pasteurized shoyu; Red(N): reduction (μg/ml) of raw shoyu; Red(HN): subtracted Red(N) from reductone (μg/ml) of pasteurized shoyu; NaCl: % (w/v).

[e] *, Linear correlation is significant at 5% level; **, linear correlation is significant at 1% level; (+) or (−), partial correlation is significant at 5% level; (++) or (− −), partial correlation is significant at 1% level; +, positive correlation; −, negative correlation.

Fig. 4. Growth curve of rats given 1 % P-shoyu, 0.45 % NaCl and control diets for 26 weeks (mean ± SD)



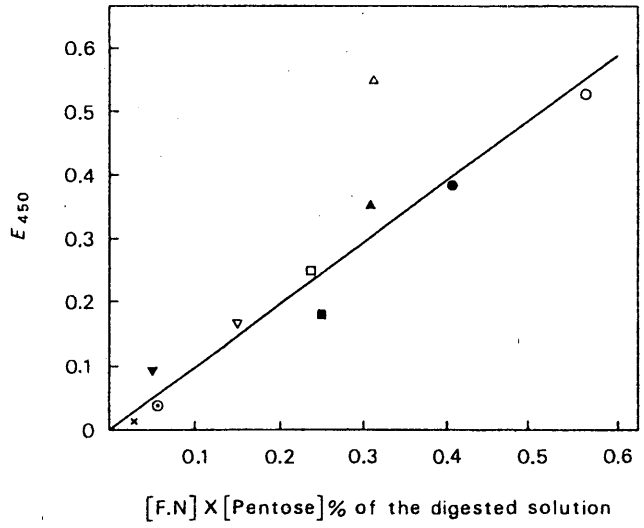


FIG. 15. The relation between (FN) × (pentose) and color degree. (x), Polished rice; (⊙), corn; (▼), corn gluten; (▽), domestic wheat; (■), wheat gluten; (□), dehulled domestic wheat; (▲), imported wheat; (●), Whole soybeans; (△), wheat bran; (○), defatted soybean. From Motai *et al.* (1975).

3. The browning of a solution of xylose and glycine is accelerated by adding a small amount of shoyu.
4. The browning of a solution of xylose and glycine is accelerated by adding the enzymatic hydrolysate of soybean protein formed by using the crude extract of shoyu koji as an enzyme source.

Shikata *et al.* (1971b) enzymatically hydrolyzed the raw materials of shoyu with an enzyme mixture of cellulase, diasatase, and protease and observed that the color intensity of the hydrolysate solution was highly correlated with formol nitrogen × pentose% (Fig. 15).

According to Motai *et al.* (1975), when shoyu was brewed varying the ratio of concentration of soybeans and wheat, the heated shoyu became darker in color with greater concentrations of soybean. The color tone of shoyu formed by heating became lighter with an increasing ratio of the soybean concentration; shoyu produced from wheat alone exhibited a darker color. These investigators isolated the amino fraction and the sugar fraction from the enzymatic hydrolysate of defatted soybean and wheat, respectively, and determined the contributions of these fractions to the browning of shoyu under heat. It was suggested that the contributions of the amino fractions of soybeans and wheat were 75 and 25%,

TABLE XXII
FORMATION OF 3-DEOXY-D-GLUCOSONE (3DG) DURING PREPARATION OF SHOYU[a]

Sample	3DG (mg%)
Koji (72 hr cultivation)	0
Liquid part of: 3-day mash	0.9
6-day mash	3.2
10-day mash	10.0
33-day mash	14.0
Raw shoyu after 4 months	20.0

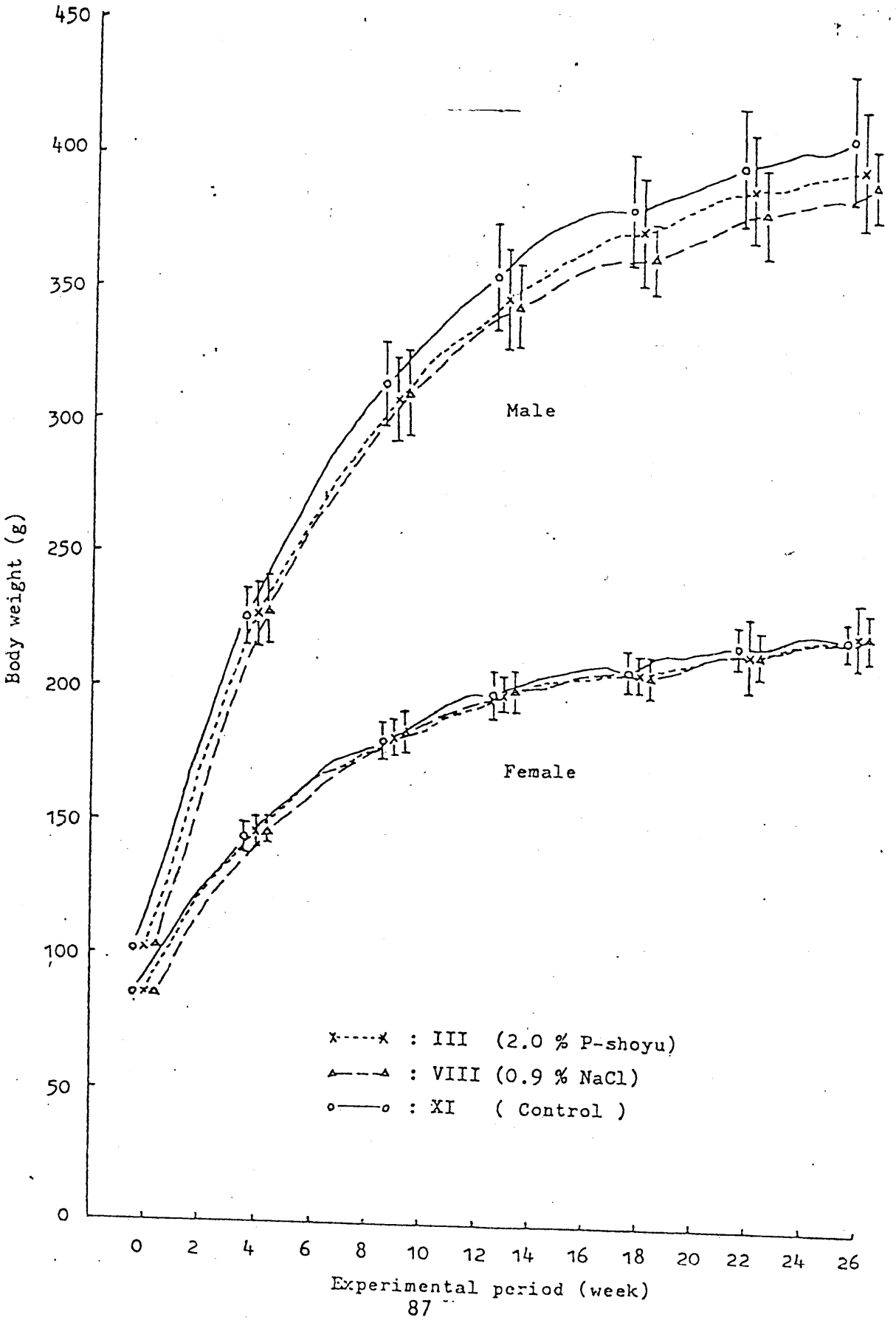
[a] From Kato *et al.* (1961).

respectively, and those of the sugar fractions were 44 and 56%, respectively. These data suggest that the contribution of soybeans and wheat to changes in color observed in shoyu when subjected to heat is 60 and 40%, respectively.

Omata *et al.* (1955a,b) found that some ether-soluble carbonyl compounds of shoyu, including furfural and acetaldehyde, darken the color of shoyu.

Kato (1958, 1959) concluded that aromatic amine-*N*-xylosides decompose to form red pigments of melanoidin when catalyzed by a weak acid and that furfural is not an intermediate in melanodin production. Kato (1960) isolated 3-deoxy-xylosone and 3-deoxy-D-glucosone from this reaction mixture as bis-2,4-dinitrophenylhydrazones and pointed out the significance of their role as intermediates in the browning reaction which occurs during the development of melanoidin. Kato *et al.* (1961) also identified 3-deoxy-D-glucosone in fermented shoyu. Its quantities were 8 mg% in koikuchi shoyu, 3 mg% in usukuchi shoyu, and 17 mg% in tamari shoyu, respectively, but it was not found in the chemical hydrolysate of plant protein. These amounts of 3-deoxy-D-glucosone in fermented shoyu are much greater than that of furfural in shoyu, which was reported to be 0.2–0.7 mg% by Omata (1955b). Moreover, it was pointed out that 3-deoxy-D-glucosone is more reactive with amino acids than with furfural. The amount of 3-deoxy-D-glucosone formed during the preparation of shoyu is presented in Table XXII (Kato *et al.*, 1961). 3-Deoxypentosone has also been isolated from pasteurized shoyu, but in smaller quantities than 3-deoxy-D-glucosone, a finding which has been attributed to its lack of stability. When xylose was added to pasteurized shoyu and kept at 37°C, a rapid increase of 3-deoxypentosone content was observed. When pasteurized shoyu was kept at 40°C or heated at 80°C, the amount of hexose and pentose of shoyu decreased, the intensity of the color (as determined by measuring the absorbance of 470 nm) increased, and the 3-deoxy-D-glucosone gradually increased and then decreased after reaching a peak. This

Fig. 9. Growth curve of rats given 2 % P-shoyu, 0.9 % NaCl and control diets for 26 weeks (mean ± SD)



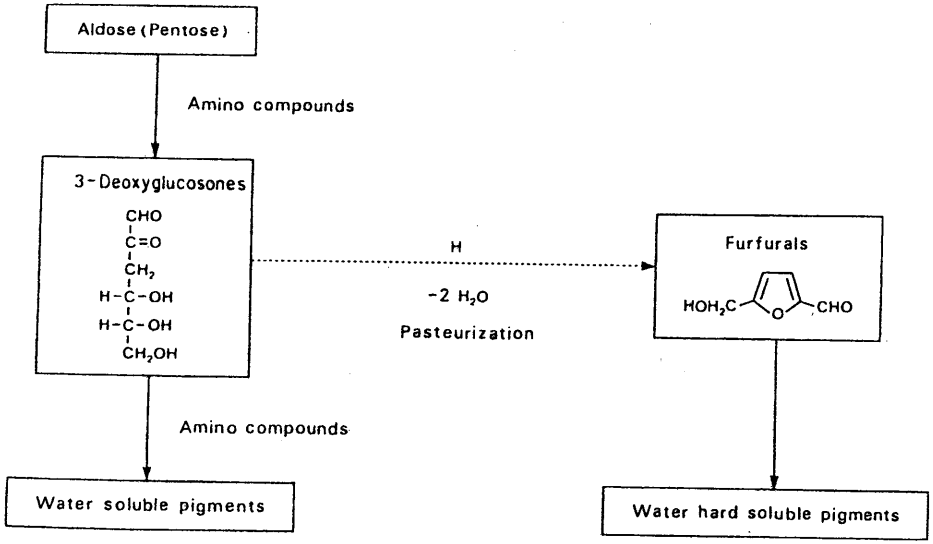


FIG. 16. The mechanisms of heat-dependent color formation of shoyu. From Kato and Sakurai (1963).

reaction was accelerated by the oxygen in air, but also proceeded in the absence of air (Kato and Sakurai, 1962). From these results, the reaction mechanism by which the color intensity of pasteurized shoyu increases is presumed to proceed as follows: aldose → 3-deoxyosones → color pigments.

When the chemically synthesized 3-deoxyosones, including 3-deoxypentosone, 3-deoxy-D-glucosone, and 3-deoxygalactosone, were added to shoyu, large amounts of water-soluble browning color substances were produced, but only a small amount of browning color substances was produced from furfural. Kato and Sakurai (1963) concluded that in amino–carbonyl reactions most 3-deoxyosones are reacted with the excess amount of amino acids instead of being converted into hydroxymethylfurfural (HMF), resulting in the formation of water-soluble pigments (see Fig. 16).

According to Burton *et al.* (1963), almost all carbonyl compounds react with amino radicals, and α-ketoaldehydes (pyruvic aldehyde, 3-deoxyosones), diketons (diacetyl), and α-β-unsaturated aldehydes (crotonaldehyde, furfurals) are the most reactive species among them. α-Hydroxyaldehydes change into α-β-unsaturated aldehydes after dehydration and react with amino compounds. Reducing sugars react with amino radicals and produce α-ketoaldehydes (3-deoxyosones) and α-β-unsaturated carbonyls (unsaturated osones) and take part in the browning reactions. Xylose is 10 times more reactive with amino radicals than glucose.

According to Motai (1976), there is a linear relationship between the increase in intensity of color and the elevation of temperature during pasteurization of shoyu: This relationship is expressed by the following equation:

$$D = \alpha \times 10^{-0.04t}$$

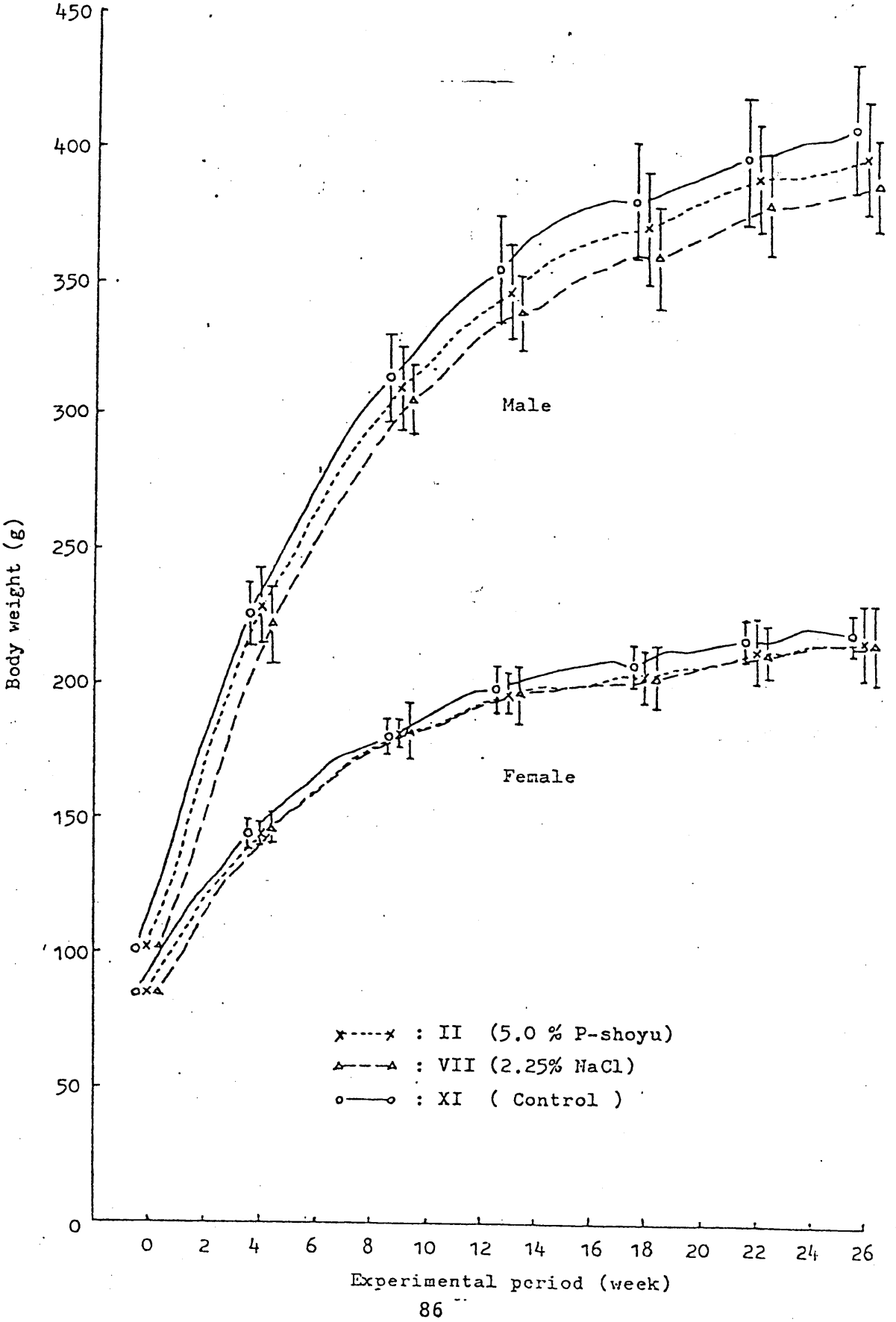
where D is the time attaining to definite color intensity, α is a constant, and t is the temperature of pasteurization. The α value varies with the concentrations of pentose and total nitrogen in shoyu; generally, the higher the pentose × total nitrogen, the lower the α value. The intensity of browning increased 2.5–3 times for koikuchi shoyu, corresponding to a 10°C elevation of temperature within the range of 50–90°C (Motai, 1976; Onishi, 1970b). Generally, the higher the pH value tends to be, the greater the extent of the browning reaction, but within the average range of pH values of shoyu (4.6–4.9), there is no practical difference in the extent of heat-dependent browning.

Moriguchi and Ohara (1961) observed that when soybeans to which 0.8% $K_2S_2O_3$ had been added were steamed and then subjected to enzymatic digestion, as in the usual method of usukuchi shoyu preparation, the color of shoyu obtained was lighter by 37% than a control group. Okuhara and Saito (1970) reported a slight effect on the depression of heat-dependent browning of shoyu when it was decolorated by heating with reducing agents such as ascorbic acid or systine, metals such as Zn, Al, Fe, Na, and Mg, or by electrolysis.

Shoyu made from mash which has been well fermented with yeast is less susceptible to heat-dependent browning because the pentose is assimilated by the yeast along with glucose, and the decrease in rH value caused by yeast fermentation prevents the browning reaction (Okuhara *et al.*, 1975).

The color of shoyu is also dependent on the kinds of lactobacilli or *P. halophylus* in salty mash (Fujimoto *et al.*, 1980). Kanbe and Uchida (1984) reported that the rH value of the shoyu mash naturally inoculated with lactobacilli decreased to 7.5 around the time of maximum growth, about 50 days from mash making, while the shoyu mash inoculated with 1×10^4/g of *P. halophylus* no. 34, which was isolated as having a strong reducing potency, showed an rH value of 6.0 at the peak of its growth. The color of the shoyu mash inoculated with no. 34 after 180 days of storage was more than 35% lighter than that of the naturally inoculated control mash. The raw shoyu was separated from these two kinds of salty mashes. They were pasteurized under the same conditions and their heat-dependent and oxidative browning rates were determined on the same basis of NaCl at 17.2% and TN at 1.57% of shoyu. These rates were 24 and 18% less, respectively, than those of the shoyu from the control mash. It was also observed with the test shoyu that the reducing power for potassium ferricyanide and the contents of hydroxymethylfurfural, reductones, and 3-deoxyosones, all of which belong to the so-called browning intermediate compounds, were 73, 73, 78, and 91%, respectively, of those of the control shoyu.

Fig. 2. Growth curve of rats given 5 % P-shoyu, 2.25 % NaCl and control diets for 26 weeks (mean ± SD)



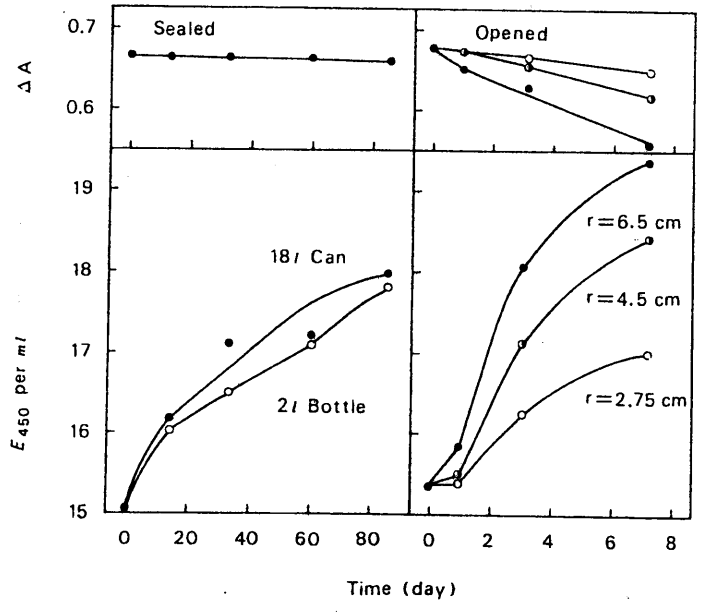


FIG. 17. Change of shoyu color on storage at 30°C. Opened condition: 300 ml of shoyu was placed in the beaker with the same height, but different surface area, covered with cellophane film, and stored at 30°C. From Motai and Inoue (1974).

2. *Color Formation on the Storage Shelf after Opening the Seal*

According to Omata and Ueno (1953b), the change in color which occurs in pasteurized shoyu during storage is not due to enzymatic reactions, but is greatly affected by the action of air and, to a lesser degree, temperature and light. When the color of shoyu is deepened by aeration, its transmittance curve does not change significantly.

When the pasteurized shoyu is sealed and stored in a glass bottle or a can, the color intensity increases as the result of browning by heat, but without a change in the ΔA value. The increase in color intensity of shoyu stored open to the air is much greater and is caused by nonenzymatic oxidative browning in which the ΔA value decreases as the ratio between the surface area and the volume of the shoyu increases. These changes are shown in Fig. 17 (Matai and Inoue, 1974).

The effective participation of reductones in the oxidative browning reactions was pointed out by Hodge (1953). The nonenzymatic oxidative browning of shoyu which occurs during storage has been attributed to the participation of such intermediates of Maillard reaction as reductones, Amadori reaarangement compounds, and melanoidins. The ascorbic acid in shoyu, which belongs to the catalog of reductones, changes into dehydroascorbic acid with oxidation, which in turn reacts with amino acids to deepen the color of shoyu (Omata *et al.*, 1955c). Okuhara *et al.* (1972) heated raw shoyu at 60, 70, and 80°C, respectively, to obtain different intensities of color. These pasteurized shoyu were then shaken in the air for 24 hr at 40°C. The oxidative browning and the reductone formation which took place during the heat treatment was almost proportional to the starting color intensity of each pasteurized shoyu. The oxidative browning reaction took place simultaneously with the utilization of the reductones during the oxidation. It seems likely that the pigment formed from the oxidative browning reaction was not from an amino–carbonyl reaction of the dehydro compounds of the reductones, but that it was an oxide of the reductones. The oxidative browning mechanism was analyzed by multiple correlation analysis; the results confirmed the correlation between the oxidative browning and the oxidation of reductones formed during the heating of shoyu and the Baumé of the shoyu.

The amount of oxidative browning determined by OD at 600 nm with both raw and pasteurized shoyu was correlated only with the initial color intensities and not with the temperature of heating, as shown in Fig. 18. The shoyu of the same color intensities browned by oxidation to the same color degree, regardless of the contents of reducing sugars, peptides, amino acids, and other compounds, if the shoyu was made from the same raw materials and the concentrations of both total nitrogen and sodium chloride were adjusted to be the same (Okuhara *et al.*, 1977).

Hashiba (1973b) separated shoyu into three fractions—cationic, neutral, and anionic—by using ion-exchange resins. When these three fractions were stored separately, only the cationic one darkened considerably. When they were combined and stored, the color of the mixture increased at nearly the same rate as that of the original shoyu. The effects of the anionic fraction containing organic acids and the ashed cationic fraction on the overall browning of shoyu were calculated

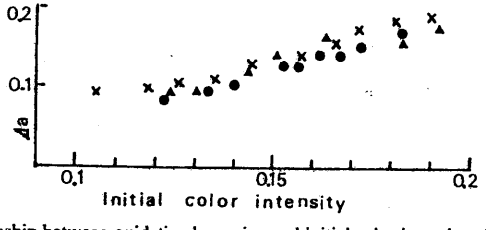
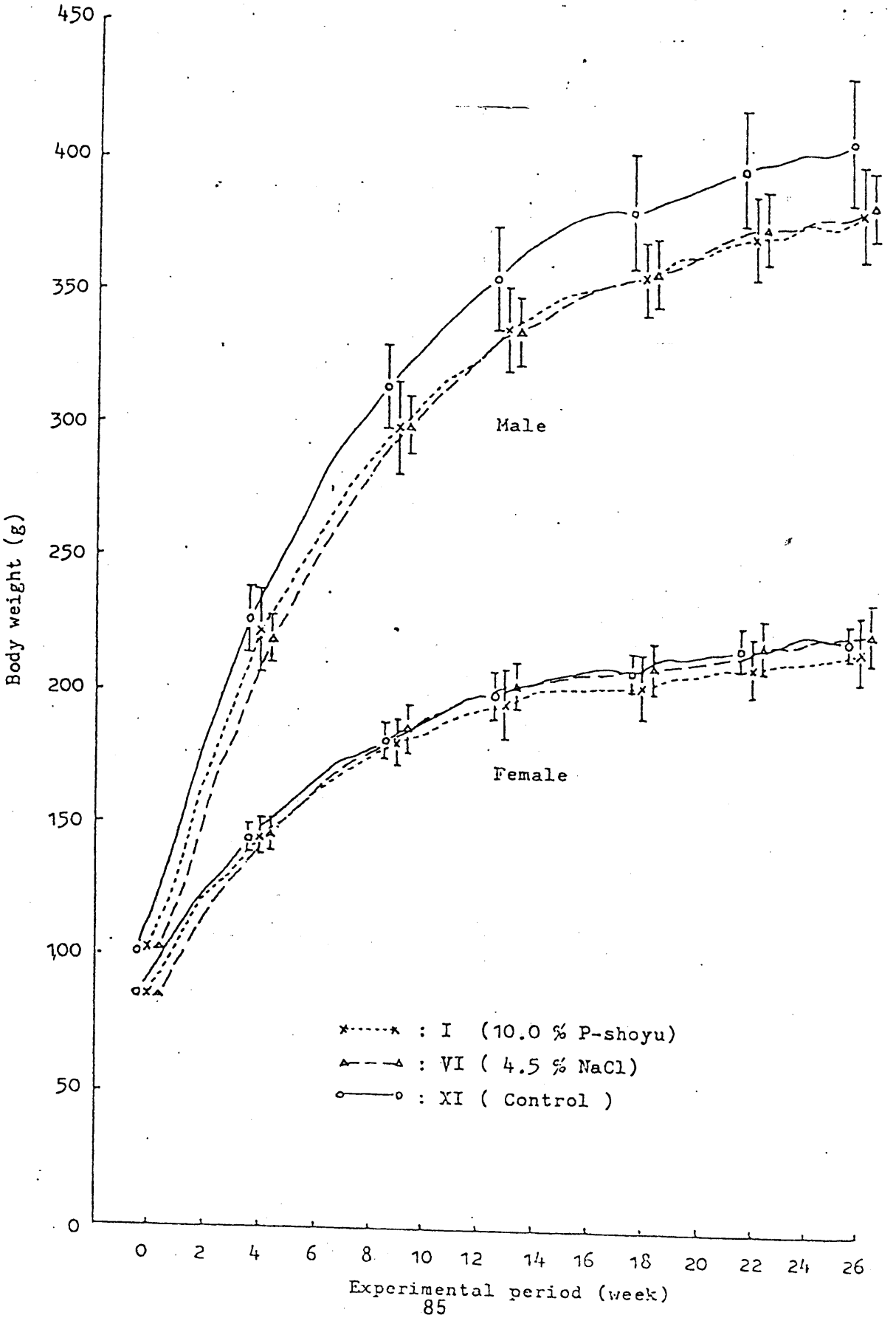


FIG. 18. Relationship between oxidative browning and initial color intensity of shoyu. Pasteurizations were carried out at (×) 80°C, (▲) 70°C, and (●) 60°C. Pasturized shoyu was oxidized by shaking at 40°C for 24 hr. Δa, Increment in OD at 600 nm by oxidation. From Okuhara *et al.* (1977).

Fig. 1. Growth curve of rats given 10 % P-shoyu, 4.5 % NaCl and control diets for 26 weeks (mean ± SD)



to be 10–20% and 20%, respectively. The sum of the contribution rates on the anionic fraction, the neutral fraction, the amino acids, and the ashed cationic fraction in the browning of shoyu was calculated to be ~40%. Compounds responsible for the residual 60% are thought to be present in the cationic fraction. It was suggested that such compounds have strong reducing powers and oxygen-uptaking ability.

Hashiba (1974) prepared a simulated shoyu, which was an amino acid solution containing glucose (5%), xylose (1%), NaCl (17%), and lactic acid (2%), and adjusted the final pH to 5.0. This sugar–amino acid model system was stored for aging for 3 months at 30°C under anaerobic or aerobic conditions and subsequently for another 2 weeks at 37°C under aerobic conditions in order to analyze the extent of oxidative browning. The oxidative browning of the model systems increased as the length of the aging period increased; the model system aged under anaerobic conditions darkened less than did those aged under aerobic conditions. Adding 40 ppm Fe^{2+} to the model system, which is the average amount of Fe^{2+} in shoyu, accelerated this oxidative browning reaction. An Amadori product, 1-deoxy-1-glycine-D-fructose, was isolated from the aged glucose–glycine model system and played a role in causing a marked increase in the rate of oxidative browning. Hashiba (1975) also isolated an Amadori compound, 1-deoxy-1-diglycine-D-fructose, from the glucose–diglycine model system. This Amadori compound promoted the oxidative browning of the aqueous solution of glucose and diglycine, which was further accelerated 30–40 times by the presence of Fe^{2+}. In the browning reaction between glucose and triglycine, similar intermediates were detected.

The Amadori compounds, composed of aromatic or heterocyclic amino acids such as fructose-tyrosine, fructose-phenylalanine, fructose-histidine, and fructose-tryptophan, were especially reactive in oxidative browning, which was synergistically accelerated by the presence of Fe^{2+} and Mn^{2+}. Oxygen is thought to accelerate the breakdown of Amadori compounds to liberate amino acids and glucose (Hashiba, 1976). The Amadori compounds derived from pentose, such as xylose-glycine, browned more rapidly than those from hexose, such as fructose-glycine. In a reaction between glucose and seven peptides, the liberation of C-terminal amino acids by the cleavage of peptide bonds was observed. There is some evidence that amino acids are liberated from the peptide in Amadori compounds, as the peptide bond in Amadori compound has been found to be more labile than that of free peptide (Hashiba *et al.*, 1977).

Amadori compounds have been isolated from shoyu by ion-exchange chromatography, gel filtration, and paper chromatography (Hashiba, 1978). Five compounds—fructose-glycine, fructose-alanine, fructose-valine, fructose-isoleucine, and fructose-leucine—were identified and their relative quantities in shoyu estimated to be approximately 0.2, 0.3, 1.2, 1.3, and 1.5 m*M*, respectively. These Amadori compounds caused increases in browning with the presence of oxygen and iron (Table XXIII). In addition, amino acids promoted the oxidative browning process. Amadori compounds from pentose or peptides were considered to be so unstable that they would have decomposed while passing through the chromatographic resin. For this reason they were not isolated from shoyu. Amadori compounds reacted with iron and produced red pigments, from which colorless compounds (Fig. 19), were separated (Hashiba and Abe, 1984).

TABLE XXIII

OXIDATIVE BROWNING OF AMADORI COMPOUNDS ISOLATED FROM SHOYU[a]

Amadori compound[b]	Browning after 14 days at 37°C (E_{559})		Content in shoyu (mm mol[c]/liter)
	Nonoxidative	Oxidative	
F-Gly	0.012	0.266	0.2
F-Ala	0.009	0.360	0.3
F-Val	0.009	0.360	1.2
F-isoLeu	0.006	0.400	1.3
F-Leu	0.010	0.310	1.5
Mixture of amino acids and sugar[d]	0.000	0.003	—

[a] From Hashiba (1978).
[b] 0.1 *M* Amadori compounds were added to amino acid mixture; F, fructose.
[c] 0.1 *M* glucose was added to amino acid mixture.
[d] Contents of Amadori compounds in shoyu.

According to Hashiba (1981), the participation of peptides in the browning process during the aging of shoyu mash was remarkable, but amino acids are more active than peptides in the oxidative browning of shoyu. The respective contributions of pentose and hexose to oxidative browning were estimated to be 75 and 25%.

According to Motai and Inoue (1974b), the color compounds of shoyu consist of polymerized melanoidins at different degrees, and the oxidative color increase in shoyu as a result of this polymerization occurs according to the following equation:

$$E = K \times M^{\alpha}$$

where E is the color intensity determined by $E_1^{1\%}{}_{cm}$ (450 nm), M is the molecular weight, and K and α are constants. The α value is almost totally independent of the length of heating and the kinds of sugars used in the melanoidin reaction, but is dependent on the kinds of amino compounds used and, above all, on their molecular weights. Table XXIV presents a comparison between K and α values

Table 2. Proximate analyses of each experimental diet

Experimental group No.	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	NaCl (%)	N-free extract* (%)
I (10.0 % P-shoyu)	9.62	21.25	4.29	3.62	10.11	4.68	51.11
II (5.0 % P-shoyu)	8.53	21.25	4.17	3.84	7.98	2.20	54.23
III (2.0 % P-shoyu)	7.33	22.44	4.32	3.75	6.67	1.63	55.49
IV (1.0 % P-shoyu)	7.96	21.31	4.31	3.75	6.41	0.70	56.26
V (0.4 % P-shoyu)	10.76	21.31	4.22	3.56	6.02	0.30	54.13
VI (4.5 % NaCl)	7.71	21.31	4.23	3.50	10.33	4.73	52.92
VII (2.25% NaCl)	7.96	20.75	4.48	3.25	8.06	2.43	55.50
VIII(0.9 % NaCl)	9.87	20.50	4.32	3.42	6.60	1.09	55.29
IX (0.45% NaCl)	8.54	20.63	4.26	3.04	6.08	0.69	57.45
X (0.18% NaCl)	8.61	20.88	4.35	3.40	6.02	0.38	56.74
XI (Control)	6.11	21.13	4.46	3.74	5.97	0.30	58.59

*Nitrogen free extract

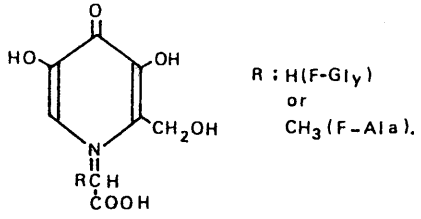


FIG. 19. Colorless compounds separated from red pigments which were produced by oxidative browning between F-Gly or F-Ala and iron. From Hashiba and Abe (1984).

of miso color, shoyu color, and various other melanoidins, and further suggests that the melanoidins of shoyu originate from di- or tripeptides.

It is generally ackowledged that deterioration of the flavor of shoyu is related to its oxidative browning; it is more highly correlated with increased darkening (or a decrease in ΔA caused by oxidative browning) than with increased color intensity (or the heightened red color which occurs with heating). Changes in volatile flavor components, especially a decrease of ethyl acetate and an increase of acetaldehyde, have been observed with oxidative browning (Onishi, 1970).

3. *Other Factors Which Affect Browning*

The elevation of temperature (but only up to 65°C) promotes oxidative browning of shoyu (Motai, 1976). Lactic acid and citric acid also promotes the process (Hashiba, 1973). However, phosphoric acid has no effect, although it does

TABLE XXIV

K AND α VALUES OF MISO COLOR COMPARED WITH SHOYU COLOR AND VARIOUS MELANOIDINS[a]

Melanoidin	*K*	α
Miso	4.57×10^{-4}	1.32
Shoyu	4.47×10^{-4}	1.30
Gly–xylose system	2.75	0.29
Lys–	1.45	0.39
Glu–	0.30	0.56
Gly_2–	0.11	0.70
Gly–Leu–	0.0115	0.95
Gly_3–	2.70×10^{-4}	1.45

[a] From Motai and Inoue (1974).

promote heat-dependent browning in the Maillard browning reaction (Kato, 1956).

The influence of iron on the browning reaction has long been known. Furuta and Ohara (1954) added 30–60 ppm Fe^{3+} to shoyu mash and observed an immediate 12–20% increase in browning. By comparison, a 21–30% increase was noted in a control sample after storage for 20 days at 30°C. Adding Fe^{3+} to shoyu heated to temperatures of 80–100°C had little effect, unlike its addition to mash. According to Hashimoto *et al.* (1970), the average amount of iron in shoyu is 20–30 ppm and is calculated to be derived from raw materials: soybeans, wheat, salt, and water. Most of the iron in raw and heated shoyu is in the form of Fe^{2+}. The addition of tannic acid or potassium ferrocyanide effectively removed 60–70% of the iron in shoyu without affecting its organoleptic quality. When these chemicals were added during shoyu heating, about 93% of the iron was removed. The influence of Fe^{2+} on darkening during storage was less than that of Fe^{3+} and Cu^{2+}. However, the rate of browning during storage of shoyu containing 7–10 ppm Fe and which had been prepared by the above procedure was about equal to that of shoyu that typically contains 20–23 ppm Fe. The oxidative browning increase in the color intensity of shoyu containing 2 ppm Fe was slightly less than that of untreated shoyu containing 32 ppm Fe.

According to Hashiba *et al.* (1970), Fe^{2+} and Mn^{2+} contribute to shoyu's increased color intensity during oxidation, while the other trace metal ions, Cu^{2+}, Zn^{2+}, Co^{2+}, and Cd^{2+}, do not. Iron ions (Fe^{2+}, Fe^{3+}), however, have no effect on the darkening of shoyu with treatment.

According to Hashiba (1973c), when raw shoyu was ultrafiltrated, it lost 10–40% of its initial color; the color intensity of treated shoyu when heated was 33–50% that of untreated shoyu. No sedimentation was found in the course of heating the treated raw shoyu. When pasteurized shoyu was ultrafiltrated, the intensity of its color decreased to about half that of untreated shoyu. The substances related to the browning of shoyu, such as Fe^{2+}, 3-deoxyglucosone, hydroxymethylfurfural, reductone, carbonyl compounds, and ferricyanide-reducing substance, were removed by ultrafiltration; 5–7% of the total nitrogen and 20% of the reducing sugar contained in shoyu were removed by the same procedure.

V. FLAVOR EVALUATION OF KOIKUCHI SHOYU

Using a multivariate analysis, Tanaka *et al.* (1969a) indicated that among the factors by which preference for a given shoyu was formed, its chemical composition as a whole contributed only 46.3%. Among the individual chemical components, listed in descending order of their degree of correlation to a "preferred"

Table 1. Experimental groups and dose levels of samples in each experimental diet—

Experimental group No.	Sample	Dose levels* (Sample in diet) (%)
I	P-shoyu	10.0**
II	P-shoyu	5.0
III	P-shoyu	2.0
IV	P-shoyu	1.0
V	P-shoyu	0.4
VI	NaCl	4.5***
VII	NaCl	2.25
VIII	NaCl	0.9
IX	NaCl	0.45
X	NaCl	0.18
XI	(Control)	0

*Dose levels were decided on the basis of the mean value of daily intake of shoyu in Japanese.

**Diet containing 10% P-shoyu was equal to 25 times the mean daily human intake on a mg/kg basis.

***Same NaCl concentration as diet containing 10% P-shoyu.

TABLE XXV
RELATIONSHIP BETWEEN PREFERENCE FOR A SHOYU AND CHEMICAL COMPONENTS[a]

Component	Partial correlation coefficients[b]
Alcohol	0.35
Baumé	−0.30
NaCl	0.21
Reducing sugar	0.21
Color	−0.19
Formyl nitrogen	0.11
Total nitrogen	0.09
Glutamic acid	0.09
Titratable acidity	0.07
pH	0.05
Ammonium nitrogen	0.02

[a] From Tanaka *et al.* (1969a).
[b] Contributing proportion: 46.3%.

rating, are alcohol, Baumé, sodium chloride, reducing sugar, color, formol nitrogen, total nitrogen, glutamic acid, titratable acidity, pH, and ammonium nitrogen (see Table XXV). The 17 aspects relating to odor contributed 96.5%. There were no predominant factors, but fragrance and the aroma of alcohol were the major desirable factors; the major negative factors were the smell of chemically hydrolyzed proteins, an oily smell, a Natto smell, an abnormal smell, a butyric acid smell, a warm brewing smell, a steamed soybean smell, and a moldy smell (see Table XXVI). Factors related to taste contributed 97.6% to preference judgments. A good aftertaste, a pure, a palatable, and a moderate salty taste were the major desirable factors; a too sweet, too sour, and an abnormal taste were the major negative factors (see Table XXVII).

The relationship between the organoleptic evaluation of a shoyu and its chemical constituents was investigated with 59 brands of shoyu available on the Japanese market (Tanaka *et al.*, 1970). Nineteen kinds of chemical and physical analyses were conducted. The results indicated that the fragrance of a fermented shoyu was roughly proportional to its ethanol and extract content ($r = +0.700$ and $+0.425$, respectively). The correlation coefficient in a linear regression estimation between the smell of the chemical hydrolysate of defatted soybean or some other plant protein and the levulinic acid content of fermented shoyu blended with the chemical hydrolysate was found to be $+0.942$. The preferred

TABLE XXVI
RELATIONSHIP BETWEEN PREFERENCE FOR A SHOYU AND ODOROUS COMPONENTS[a]

Component	*T* ratio of major partial correlation coefficients (11 out of 17)[b]
Smell of chemical hydrolysate of proteins	−1.59
Oily smell	−1.34
Deteriorated smell	1.29
Fragrance	1.18
Natto smell	−0.97
Abnormal smell	−0.97
Butyric acid smell	−0.76
Warm brewing smell	−0.73
Steamed soybean smell	−0.67
Alcohol smell	0.66
Moldy	−0.61

[a] From Tanaka *et al.* (1969a).
[b] Contributing proportion: 96.5%.

TABLE XXVII
RELATIONSHIP BETWEEN PREFERENCE FOR A SHOYU AND TASTE COMPONENTS[a]

Component	*T* ratio of partial correlation coefficients[b]
Aftertaste	2.98
Pure	2.46
Sweet	−1.86
Sour	−1.34
Salty	1.30
Palatable	1.25
Abnormal	−1.14
Harmony	−1.11
Good body	−0.53

[a] From Tanaka *et al.* (1969a).
[b] Contributing proportion: 97.6%.

chloride. The increases of relative weights of the heart and liver by shoyu or sodium chloride treatment were regarded as clearly attributed to the intake of sodium chloride. The higher values for relative liver weights in males on 10% or 5% P-shoyu were observed; however, these higher values were not associated with any particular histopathological changes and occurred without any noticeable changes in liver function, as judged by serum levels of total protein, albumin, ZTT, TTT, bilirubin, cholesterol and activities of GOT, GPT, Al-P and LAP. Therefore, it seems likely that these changes were minute changes. As to the alterations of relative weights of other organs such as brain, lungs, stomach, genital organs in males, there was no histopathological evidence to suggest any damage to these organs so that these changes are likely to be a reflection of the body weight differences rather than of a treatment-mediated change. The other organs that differed significantly from the controls were not considered to be indicative of a test-related response.

Although in rats fed shoyu at the highest dose level, many changes such as alterations of relative weights of the kidneys, urinary bladder, heart and higher concentration of BUN were observed, most of these changes were accounted for by the changes resulting from the excessive intakes of sodium chloride.

aroma of a shoyu was negatively correlated with the levulinic acid content ($r = -0.538$). The preferred pH value of a shoyu in terms of aroma was found to be between 4.6 and 4.8. The higher pH value of a shoyu suggests the possible blending of some quantity of chemical hydrolysate of plant proteins or an undesirable bacterial contamination during koji cultivation and/or mash fermentation, all of which relate to the fact that few of these samples had a good aroma. On the basis of these findings the investigators concluded that in order for shoyu to have a good aroma it should (1) be prepared by a genuine fermentation process without the addition of the chemical hydrolysate of plant proteins; (2) be free from undesirable bacterial contamination during koji and mash fermentations; (3) be made from mash thoroughly fermented with yeasts; (4) have the pH value 4.6–4.8; and (5) be appropriately balanced in terms of its chemical components.

The palatability of shoyu as a function of its salt and total nitrogen content was investigated by Tanaka *et al.* (1969b). Nine kinds of shoyu were prepared by the combination of total nitrogen, 1.5, 1.6, and 1.7% (w/v), and sodium chloride, 14, 16, and 18%. Degree of saltiness, palatability, and overall taste preferences were organoleptically evaluated. While it is expected that the degree of perceived saltiness would be proportional to actual salt concentrations, it is interesting that this perception of saltiness also increases with an increase in the total nitrogen content; however, this is readily understandable as this relates to an increase in the free amino acid content. Palatability also increases with decreases in salt concentrations, but the greatest palatability was correlated with a salt concentration of 16%.

The interrelationships among salt, sugar, and organic acid in shoyu were also investgated. The 27 different test samples were prepared by adding (1) salt, 1 or 2%, (2) glucose, 0.5 or 1.0%, and (3) sodium lactate, 90 or 180 meq/liter to a shoyu which contained total nitrogen 1.535%, sodium chloride 15.5%, reducing sugar 4.0%, and organic acid (90% of which is lactic acid) 150 meq/liter. The degree of saltiness, sweetness, sourness, and overall taste preferences were determined by a series of sensory tests. At a 4.5% sugar level, the degree of perceived saltiness increased with an increase in organic acid content. The shoyu was rated as saltier with an increase in the sugar content when the salt concentration was about 15%, but as less salty with an increase in the sugar content when the salt concentration was about 18%. An increased salty taste was also reported with an increase in organic acid concentration from 105 to 375 meq/liter when the sugar content was less than 4.25% and the salt content was 16.5%. Shoyu was judged to be more sour in taste with higher salt concentrations given a 4.5% sugar content. The highest preference rating was awarded to shoyu with 16% salt, 4.25% sugar, and 240 meq/liter organic acid. But irrespective of the sugar level (3.5, 4.5, and 5.5%, respectively), preference in taste increased with increased amounts of organic acid.

The results of multivariate analysis indicated that both a harmonious balance of various taste components, such as salty, acidic, sweet, bitter, and delicious, and a good aroma are important to an organoleptically preferred shoyu. According to Mori (1979), 4 out of 12 factors which contribute 60% to judgments about the quality and taste of shoyu, in decreasing order of importance, are as follows: (1) nitrogenous constituents, such as amino nitrogen, formyl nitrogen, glycine, total nitrogen, and glutamic acid; (2) sugar constituents, such as extract, total sugars, glucose, and reducing sugar; (3) potency of delicious taste; salty, acidic, and bitter taste in positive direction, and sweet and delicious taste in a negative direction; and (4) taste factors relative to lactic acid fermentation and others; lactic acid, acetic acid, and ammonium nitrogen contribute in a positive direction, whereas malic acid and citric acid contribute in a negative direction.

Since a linear correlation was found between the sensory test used in assessing shoyu flavor and the gas–liquid chromatographic (GLC) data from a stepwise multiple regression analysis, an effort was made to use the GLC data to correlate an objective evaluation of shoyu flavor. The multiple correlation coefficient (r) increased with higher step numbers, exceeded 0.9 at step 10, and reached 0.968 at the last step, 43. The standard error of estimate reached a minimum value at step 28 and then increased gradually. The regression model most predictive of the test panel's sensory ratings was calculated for each step, and the resulting calculated models were tested by substituting the GLC data. The results indicated that GLC data provide a reliable estimate of quality ratings obtained by subjective sensory tests (Aishima *et al.*, 1976, 1977).

Next, the contributing proportions of all the peaks of a GLC pattern were calculated to determine the importance of each peak for the whole aroma of shoyu. In one study, eight principal components were identified from 39 GLC peaks as significant factors in shoyu aroma, contributing a cumulative proportion of 87% to the total variance. The second peak contributed the greatest proportion, 57.6% (Aishima, 1979a–c).

VI. VOLATILE FLAVOR INGREDIENTS OF KOIKUCHI SHOYU

More than 20 Japanese investigators had isolated about 130 flavor compounds from fermented shoyu by the time Goto first introduced the gas chromatography mass spectrometry (GCMS) method into this area of research in 1973, adding six new volatile compounds using this method. Instrumental analysis, which involved using gas or liquid chromatography with ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectrometry (MS), rapidly increased the number of volatile flavor constituents isolated from shoyu. Nearly 300 kinds of such compounds have been identified to date as contributors to the

intermediate dose levels, the body weight gain of males given shoyu was larger than those given sodium chloride. It seems likely that the growth inhibition by sodium chloride was reduced by shoyu. Recently, MacDonald et al. (14) reported that the rats on shoyu were clearly smaller than those on the control diet; however, the longest lived, most active and apparently most healthy rats were those given diets containing shoyu. In females, there were few differences between treated and control rats in body weight gain even in the rats on the highest dose level of shoyu or sodium chloride in the present experiment. It seems likely that the tolerance or adaptability against sodium chloride varies with sexes.

The urinary bladders in both sexes given diets containing shoyu or sodium chloride at the highest dose level were thickened macroscopically. These changes were considered to have occurred by work hypertrophy because these rats had polydipsia and polyuria due to the excessive intake of sodium chloride (13). The increases of both the weights and relative organ weights of the kidneys in both sexes on shoyu or sodium chloride were considered similarly to relate to the excessive intake of sodium chloride. These increases were exactly parallel with the increases of serum-urea (BUN) concentration. Therefore, it seems likely that the kidneys in both sexes of these groups have mild effects without histopathological changes by sodium

fragrance of koikuchi shoyu. These include 37 hydrocarbons, 32 alcohols, 41 esters, 15 aldehydes, 4 acetals, 19 ketones, 24 acids, 17 phenols, 16 furans, 8 lactones, 6 furanones, 5 pyrones, 27 pyrazines, 7 pyridines, 6 other nitrogenous compounds, 16 sulfur-containing compounds, 4 thiazoles, 3 terpenes, and 3 others (Yokotsuka, 1953a, 1975; Asao *et al.*, 1958a,b, 1967; Sasaki, 1975; Asao and Yokotsuka, 1977; Sasaki and numomura, 1978, 1981; Nunomura *et al.*, 1976a,b, 1977a,b, 1978, 1979, 1980; Nunomura and Sasaki 1981, 1982; Yokotsuka *et al.*, 1980).

Solvent extraction or steam or vacuum distillation of shoyu or shoyu cake (the residue from the pressing of shoyu mash) was applied to the concentration of volatile flavor constituents of shoyu in the past when a fairly large number of isolated flavor compounds was necessary for the determination of their chemical structures.

Sasaki (1975) investigated methods of obtaining a volatile flavor concentrate of shoyu which is most similar to that identified by a sensory evaluation of a given shoyu. The best way, he concluded, is by distilling the shoyu under vacuum of 15 mm Hg at 40°C, collecting the distillate through successive cold traps consisting of a mixture of ice and sodium chloride, dry ice, and ethanol, and liquid nitrogen, extracting the distillate with dichloromethane, and then evaporating the solvent.

Three popular brands of koikuchi shoyu, A, B, and C, obtained on the Japanese market, were treated by this method. Fifty milliliters of each were divided into the volatile flavor concentrates (40 ml) and the distillate and were filled to 50 ml with distilled water. Three kinds of original shoyu, the volatile flavor concentrates, and the residue, and nine mixtures of each flavor concentrate and residue were subjected to a sensory evaluation of volatile flavors by a ranking Hedonic method (see Table XXVIII). The average scores of samples A and B were not markedly different from each other, but sample C was judged to be distinctly inferior to both A and B. The relative rankings of the flavor concentrates a, b, and c closely paralleled those of the original shoyus, but minor differences were observed among the three distillates, a′, b′, and c′. The flavor concentrate c, obtained from the sample shoyu receiving the lowest score (C), was also ranked last when added to the distillation residues a′, b′, or c′. These findings suggest that volatile flavor concentrates prepared in this way are nearly identical to the volatile flavors of the original shoyus. Further evidence is the importance of a shoyu's distillate to its ultimater favor, lending support to the validity of determining the quality of one's preference for a particular shoyu by its aroma based organoleptically on checking its volatile odor.

Japanese researchers of shoyu flavor have endeavored to identify the following:

1. As many volatile flavor constituents as possible
2. The nature of those compounds which impart flavor to fermented shoyu

TABLE XXVIII
DATA OF SENSORY EVALUATION[a]

Code no.[b]	Ranking 1	2	3
1	B	A	C
	1.36[c]	1.64	3.00
2	a	b	c
	1.46	1.53	3.00
3	a′	b′	c′
	1.75	2.06	2.19
4	a	b	c
	a′	b′	c′
	1.44	1.66	3.00
5	a	b	c
	b′	b′	b′
	1.11	1.88	3.00
6	b	a	c
	c′	c′	c′
	1.33	1.67	3.00
7	a	a	b
	b′	a′	c′
	1.27	1.47	2.47

[a] From Sasaki (1975).
[b] Code 1: Shoyu (A, B, C) (samples are koikuchi heated shoyu); code 2: distillate (a, b, c); code 3: residue (a′, b′, c′); codes 4–6: distillate + residue; code 7: comparison among the highest in code numbers.
[c] Average of ranking.

3. Some of the chemical flavor constituents which improve the flavor of fermented shoyu as well as those which have a negative effect
4. The preferred combination of flavor constituents
5. The differences between the flavor constituents of raw and pasteurized shoyu
6. The stability of volatile flavor compounds in shoyu
7. The differences between the volatile flavor constituents of the chemical hydrolysate of plant protein and fermented shoyu

The most important component of the flavor of fermented shoyu seems to exist in its weak acidic fraction, which is recognized in the following ways:

1. When the volatile fraction of a shoyu is further fractionated into its functional groups, the strongest flavor is observed in its phenolic fraction.

examination (Table 9) were similar in treated control rats. The commonly noticeable lesions in rats of all experimental groups were perivascular leucocytic infiltration in the lungs and granuloma in the liver in both sexes and cast formation in the kidneys in males. In the organs showning statistically significant differences in organ weights or relative organ weights compared with the controls, the kidneys, urinary bladder, heart, pituitary, liver, brain, stomach and genital organs of male, no particularly noticeable lesions were observed. In conclusion, no histopathological changes or lesions which were considered to be indicative of a test-related response were observed in any of the tissues.

Discussion

None of the rats were dead and no abnormalities in appearance or behaviour were noted in treated and control rats throughout the study.

The significant reduction in body weight gain in males given shoyu or sodium chloride at the highest dose level was observed in comparing with the controls; however, there were no significant differences between the males given shoyu and those given sodium chloride. The reduction in body weight gain was clearly due to the excessive intake of sodium chloride. At

2. When a shoyu is neutralized with alkali, its flavor immediately disappears and does not return in full strength when it is acidified.

3. At lower pH value, i.e., within the range of 4.6–5.0, sensory tests of shoyu flavor yield better ratings.

4. Some of the most important flavor compounds, such as maltol and 4-hydroxyfuranones, are in weak acidic fraction. These were isolated from the peaks of gas chromatography. The flavor characteristic of fermented shoyu was strongest among all of the peaks obtained.

5. Some other isolated flavor ingredients, such as phenols, lactones, cyclothene, and phenol esters, seem to be essential to the flavor of fermented shoyu.

A. ORGANIC ACIDS

The organic acids found in shoyu are presented in Tables XXIX and XL. The pasteurized shoyu (I) in Table XXIX appears to contain more levulinic and formic acid and less succinic acid than does (II), a genuine fermented shoyu, while (I) is blended with a fairly large amount of a chemical hydrolyzed of plant protein, usually from defatted soybean.

Most of the organic acids found in shoyu have fairly high threshold values, but

TABLE XXIX

CONTENT OF MAJOR ORGANIC ACIDS IN SHOYU CORRESPONDING TO 1% PROTEIN (mg/100 ml)[a]

Organic acid	Unpasteurized shoyu	Chemical hydrolysate of plant protein	Pasteurized shoyu (I)	Pasteurized shoyu (II)
n-Butyric	0.1	3.0	1.4	0.5
Isobutyric	0.5	0.8	2.4	—
Unknown	0.9	—	0.3	—
Propionic	5.2	1.7	13.0	4.0
Levulinic	25.4	841.1	237.6	4.4
Acetic	87.9	82.9	134.0	126.2
Pyruvic	36.5	—	10.5	11.9
Formic	2.3	194.6	53.7	6.2
α-Ketobutyric	1.9	0.7	1.0	0.2
Lactic	887.8	20.0	852.6	1156.6
Succinic	25.2	—	27.2	49.8
Pyroglutamic	30.0	13.8	44.3	110.6
Glycolic	8.8	4.2	4.4	9.9
Malic	Trace	3.3	1.7	Trace
Citric	Trace	25.3	8.8	Trace
Total	1112.5	1191.4	1392.8	1480.3

[a] From Ueda *et al.* (1958).

that of isovaleric acid is relatively low—0.7 mg/liter in water (Stahl, 1973)—and its high content is derived from the contamination of *Bacillus natto* during koji cultivation. Adding cinnamic acid to shoyu gives it a foul odor, the result of bacterial contamination in shoyu mash; however, this compound has not as yet been isolated from shoyu. It is difficult to estimate the contribution of each organic acid to the flavor because, as Salo *et al.* (1972) pointed out, they react synergistically with each other.

α-Ketobutyric acid is sometimes isolated from the chromatographic fraction of shoyu having a strong shoyu-like aroma, and the chemically synthesized α-ketobutyric acid also has a strong aroma reminiscent of a very important component of shoyu flavor. This compound was found in the chemical hydrolysate of a protein containing threonine (Wieland and Wiegandt, 1955) and was recognized as an important flavor component (Blockman and Frank, 1955). Sulser *et al.* (1967) reported that newly synthesized α-ketobutyric acid has no taste or odor, and that α-hydroxy-*p*-methyl-Δ,α,β-hexenolactone (an isomer of α-keto-β-methyl-γ-carprolactone which is produced by the condensation of two molecules of α-ketobutyric acid, followed by cyclization and decarboxylation) has a very strong odor, characteristic of chemical protein hydrolysate. The threshold value of α-ketobutyric acid was reported to be 0.04 ppm; its odor easily attaches to skin and clothes, and its taste remains for several hours.

B. ALCOHOLS

The kinds of alcohols isolated from shoyu and their relative quantities are indicated in Tables XXXIX and Table XL, respectively. The ethanol content of shoyu ranges from 1 to 3.5% (v/v). The important alcohols, because of their relative threshold values and high proportion, are *n*- and isobutyl alcohol, isoamyl alcohol, 2-phenyl alcohol, and furfuryl alcohol. The latter three are found in typical alcoholic beverages and resemble yeasts in their metabolic action and synthesis of amino acids. The so-called fusel alcohols are mainly derived from the fermentation of hexoses and, to a lesser degree, from the degradation of the corresponding amino acids following Ehrlich's pathway (Webb *et al.*, 1969).

Since the amount of furfuryl alcohol increases during pasteurization of shoyu in the final processing, its quantity indicates the degree of pasteurization. Hexyl alcohol is derived from raw soybeans (Nakajima and Takei, 1949), and methylnonylcarbinol is derived from soybean oil (Shoji, 1936).

The differences in the contents of the major flavor ingredients and various alcohols of eight brands of shoyu selected from the Japanese market in 1980 are indicated in Table XXX (Nunomura and Sasaki, 1981); their relative proportions in four brands are compared schematically in Fig. 20 (Sasaki, 1975).

Higher values for the relative organ weights but no differences for the organ weights were found in the case of the brain in both sexes at 10% P-shoyu and in males at 4.5% sodium chloride, pituitary in males at 4.5% sodium chloride and in females at 10% P-shoyu, lungs in both sexes at 10% P-shoyu and in males at 5% P-shoyu or 4.5% sodium chloride, heart in both sexes at 10% P-shoyu or 2.25% sodium chloride, liver in both sexes at 10% P-shoyu and in males at 5% P-shoyu and in females at 2.25% sodium chloride, kidneys in males at 2% P-shoyu, stomach in both sexes at 10% P-shoyu and in males at 4.5% sodium chloride, genital organs in males (testes, epididymides, seminal vesicle and prostate) at 4.5% sodium chloride. The alterations of weights of other organs were not considered to be indicative of a test-related response.

The results of systolic blood pressures at week 26 in both sexes are shown in Table 8. In males at 10%, 0.4%, P-shoyu or 2.25%, 0.45% sodium chloride, these values were significantly higher than the controls without the dose-related increase. No statistically significant differences of those values were found between treated and control rats in females. Therefore, no influence in the rats was considered for systolic blood pressure by intake of shoyu or sodium chloride in the study at week 26.

The lesions encountered in the histopathological

TABLE XXX

CONTENTS OF MAJOR FLAVOROUS INGREDIENTS IN EIGHT KINDS OF SHOYU[a]

Peak no.	Compound	Content (ppm)
1.	2-Methyl-1-propanol (isobutyl alcohol)	3.07–18.35
2.	1-Butanol (*n*-butyl alcohol)	1.41–11.48
3.	3-Methyl-1-butanol (isoamyl alcohol)	4.47–22.45
4.	3-Hydroxy-2-butanone (acetoin)	5.05–8.44
5.	Ethyl 2-hydroxypropionate (ethyl lactate)	7.35–27.12
6.	Furfuryl alcohol	4.35–10.07
7.	3-(Methylthio)-1-propanol (methionol)	2.60–4.47
8.	2-Phenylethanol	3.71–10.25
9.	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF)	1.83–5.39
10.	4-Ethyl-2-methyoxyphenol (4-ethylguaiacol) (4-EG)	1.12–3.67
11.	4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF)	177.78–418.67
12.	4-Hydroxy-5-methyl-3(2*H*)-furanone (HMMF)	84.54–153.58

[a] From Nunomura and Sasaki (1981), unpublished. Kikkoman Corporation, Japan.

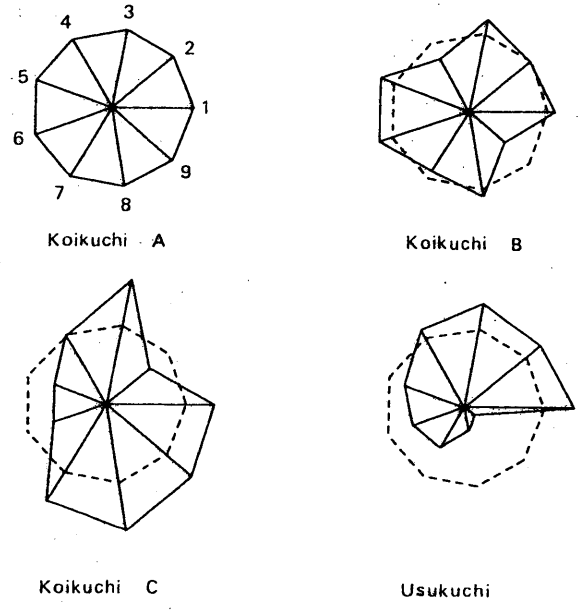


FIG. 20. Contents of major flavorous ingredients in four brands of shoyu in Japan as compared to (A) as the standard. (1), Isobutyl alcohol; (2), *n*-butyl alcohol; (3), isoamyl alcohol; (4), acetoin; (5), ethyl lactate; (6), furfuryl alcohol; (7), methionol; (8), 2-phenylethanol; (9), 4-ethylguaiacol. From Sasaki (1975).

TABLE XXXI

CONTENT OF ESTERS IN PASTEURIZED SHOYU[a]

Compound	Pasteurized shoyu (A)	Pasteurized shoyu (B)
Ethyl acetate	1.3	1.80
Ethyl propionate	0.14	—
Ethyl isovalerate	0.08	—
Butyl acetate	0.31	Trace
Isoamyl acetate	0.06	Trace
Ethyl lactate	3.60	3.90
Ethyl malonate	4.00	1.94
Ethyl levulinate	3.36	1.30
Ethyl benzoate	3.00	0.99
Ethyl succinate	1.89	0.34
Ethyl maleate	1.44	Trace

[a] From Morimoto and Murakami (1966). *J. Ferment. Technol.* 44(8), 467–475.

C. ESTERS

Forty various types of esters have been isolated from shoyu; the results of qualitative analyses of 11 major ones are listed in Table XXXI. Almost all, except from butyl acetate and isoamyl acetate, are ethyl esters, but the existence of many others, produced by the combination of alcohols and organic acids found in shoyu, is also considered. *N*-, and isobutyl benzoate or vanillate have been found to be more important to shoyu flavor than either ethyl benzoate or ethyl vanillate. The presence of different amounts of these esters lent a distinctive character to different kinds of shoyu (Yokotsuka, 1975). Since ethyl levulinate is produced by the metabolism of yeasts in the shoyu mash to which the chemical hydrolysate of defatted soybean is blended, the amount of ethyl levulinate is an indicator of the amount of chemical hydrolysate blended to genuine shoyu mash.

D. CARBONYLS AND RELATED COMPOUNDS

1. Maillard Reaction and Strecker Degradation

It is generally acknowledged that heating raw shoyu increases the proportion of acetaldehyde and other lower aldehydes. The so-called caramel reaction only takes place in the course of heating sugars, but this reaction is accelerated in the presence of amino residues, which is generally known as the Maillard reaction.

found in females. The results of analyses of serum are shown in Table 5. At the two higher dose levels of shoyu and sodium chloride (10%, 5% P-shoyu, or 4.5%, 2.25% sodium chloride), the blood urea nitrogen (BUN) was significantly higher than the control value in both sexes (Table 5). The glutamic-pyruvic transaminase (GPT) activity was lower than the control value at all dose levels except the highest dose level of shoyu in males (Table 5). No dose-related reduction in this activity was found, so this reduction was not accounted for by the intake of shoyu or sodium chloride. The glucose concentration was significantly higher than the controls in 7 groups of females without the dose-related increase (Table 5). Except the BUN, analyses of serum from both sexes showed no differences between treated and control rats in both sexes.

The weights of all organs and relative organ weights are shown in Table 6 and 7. As shown in Table 6 and 7, both the weights and relative organ weights of the kidneys in both sexes fed the diet containing 10%, 5% P-shoyu or 4.5%, 2.5% sodium chloride were higher than the controls. The urinary bladder in both sexes on 10% P-shoyu or 4.5% sodium chloride, urinary bladder in females on 2.25% sodium chloride, heart in both sexes on 4.5% sodium chloride, pituitary in males on 10% or 5% P-shoyu and liver in females on 4.5% sodium chloride showed similar increases in the weights and relative organ weights.

TABLE XXXII

ALDEHYDES PRODUCED FROM STRECKER DEGRADATION[a]

Amino acid		Corresponding aldehyde	
Glycine	$CH_2(NH_2)COOH$	Formaldehyde	HCHO
α-Alanine	$CH_3CH_2(NH_2)COOH$	Acetaldehyde	CH_3CHO
α-Aminobutyric acid	$CH_3CH_2CH_2(NH_2)COOH$	Propionaldehyde	CH_3CH_2CHO
Valine	$(CH_3)_2CHCH_2CH(NH_2)COOH$	Isobutyraldehyde	$(CH_3)_2CHCHO$
Leucine	$(CH_3)_2CHCH_2CH(NH_2)COOH$	Isovaleraldehyde	$(CH_3)_2CHCH_2CHO$
Isoleucine	$CH_3CH_2CH(CH_2)CH(NH_2)COOH$	2-Methylbutyraldehyde	$CH_3CH_2CH(CH_3)CHO$
Serine	$HOCH_2CH(NH_2)COOH$	Glycol aldehyde	$HOCH_2CHO$
Threonine	$CH_2CH(OH)CH(NH_2)COOH$	Lactic aldehyde	$CH_3CH(OH)CHO$
Methionine	$CH_3SCH_2CH_2CH(NH_2)COOH$	Methional	$CH_3SCH_2CH_2CHO$
Cysteine	$HSCH_2CH(NH_2)COOH$	Mercaptoacetaldehyde	$HSCH_2CHO$
Cystine	$(—SCH_2CH(NH_2)COOH)_2$	Dithioacetaldehyde	$OHCCH_2SSCH_2CHO$
Glutamic acid	$HOOCCH_2CH_2CH(NH_2)COOH$	Succinic monoaldehyde	$HOOCCH_2CH_2CHO$
Phenylglycine	$C_6H_5CH(NH_2)COOH$	Benzaldehyde	C_6H_5CHO
Phenylalanine	$C_6H_5CH_2CH(NH_2)COOH$	Phenylacetaldehyde	$C_6H_5CH_2CHO$

[a]From Yokotsuka (1975).

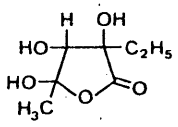
α-Dicarbonyl compounds which are produced by this reaction degradate into one carbon-less aldehydes or ketones by Strecker degradation (see Table XXXII). These compounds further degradate into other odorous compounds: acetaldehyde and propionaldehyde from lactic aldehyde, dimethyl sulfide from methional, and sulfur dioxide from dithioacetaldehyde, for example. These compounds are clearly rich in fermented shoyu which contains a large amount of free amino acids.

2. *α-Dicarbonyl Compounds*

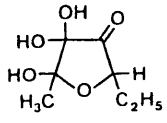
Diacetyl, $CH_3COCOCH_3$, is produced by the oxidation of acetoin, $CH_3CH(OH)COCH_2CH_3$ (Yamada, 1928, 1929), and acetylpropionyl, $CH_3COCOCH_2CH_3$, is derived from acetylethylcarbinol, $CH_3CH(OH)COCH_2CH_3$ (Asao and Yokotsuka, 1963). However, acetylbutyryl, $CH_3COCOCH_2CH_2CH_3$, which was first isolated from shoyu (Asao and Yokotsuka, 1961a), was reported to be produced by heating $C_7H_{12}O_5$, a lactol compound isolated from shoyu (Asao and Yokotsuka, 1961b; Yokotsuka and Asao, 1961). The 3% sodium chloride-soluble part of the chloroform extract of unpasteurized shoyu was adjusted to pH 7.2 and then extracted with ether. From this ether extract, 4-ethylguaiacol and tyrosol were isolated, but the residue still had a very strong shoyu-like flavor. It was then subjected to column chromatography of aluminum oxide, and the crude crystals

obtained were purified by sublimation until the mp was 114–115°C. The yield of the crystals was 20 mg from 40 liters of shoyu.

By elementary analysis and molecular weight, the molecular formula $C_7H_{12}O_5$ was derived. The oxidation of this compound by periodate yielded α-ketobutyric acid, pyruvic acid, propionaldehyde, and carbon dioxide. Oxidation by potassium permanganate yielded acetic acid and propionic acid, and when oxidized by chromic acid, it was suggested that the sample has one —CH_3 and one —CH_2CH_3 at the end of the structure. The sample was very unstable at room temperature and decomposed into propionaldehyde, pyruvic acid, acetaldehyde, carbon dioxide, α-hydroxybutyric acid, and acetylbutyryl. When heated with diluted sulfuric acid, the sample decomposed into 1 mol carbon dioxide, 1 mol α-hydroxybutyric acid, and acetic acid, yielding a good amount of acetylbutyryl. The acetylbutyryl was identified as 2,4-dinitrophenylhydrazone (mp 242–243°C), which was compared with the authentic compound. The IR spectrum of the sample indicated the existence of a lactol linkage and OH in its structure. Based on these experimental results, the chemical structure (**A**) was tentatively assigned to the $C_7H_{12}O_5$ compound, but it was later corrected to be (**B**) by Nunomura *et al.* (1976).



(A)



(B)

Acetylbutyrl lends a fruity fragrance, which is different from diacetyl, and is detected by a sensory test at the concentration of ($1:10^{-7}$). However, the fragrance of acetylpropionyl resembles that of fermented rice wine. The total α-diketone compounds increase from 0.05–0.1 to 0.2–0.3 mg% during the pasteurization of shoyu conducted at 80°C for 5 hr. This increase was calculated to be much greater than that produced by the degradation of the $C_7H_{12}O_5$ compound contained in shoyu. The total α-diketon compounds in pasteurized shoyu was first isolated by steam distillation and then converted into dioxime derivatives, which were fractionated through column chromatography containing Dowex 1.X-8 of borate type into three peaks, P1, P2, and P3. These three peaks were purified by sublimation in a vacuum, followed by rechromatography and recrystallization. Three kinds of purified crystal thus obtained were compared with authentic samples with respect to their melting points, their IR spectra, and so on. P1, P2, and P3 were identified as dioximes of diacetyl, acetylpropionyl, and acetylbutyryl, respectively. Their proportions in shoyu were found to be in the ratio of 100:20:3. The total glyoxal content, including glyoxal and meth-

(Fig. 4 and 5).

The food intakes (Table 3) of rats given shoyu and sodium chloride at all dose levels showed few differences from the controls within each sex. The mean daily food intakes were approximately 19 g per rat in males and 13 g per rat in females. Therefore, the mean daily intakes of shoyu in males were approximately 16.6, 8.0, 3.2, 1.5 and 0.6 ml/kg/day in rats at each dose level (10%, 5%, 2%, 1%, and 0.4% P-shoyu), and those in females were approximately 19.8, 9.4, 3.7, 1.8 and 0.7 ml/kg day in rats at each dose level.

The water intakes (Table 3) of rats given 10%, 5% P-shoyu or 4.5%, 2.25% sodium chloride were significantly increased compared with the controls within each sex. The mean values of water intakes of males and females given 10% P-shoyu or 4.5% sodium chloride were about 55 ml/male/day and 45 ml/ female/day, respectively. These values were approximately twice those of the controls. And those of males and females on 5% P-shoyu and 2.25% sodium chloride were approximately 40 ml/male/day and 30 ml/female/day, respectively. These values were approximately 1.3 times those of the controls.

The hemoglobin concentrations and the number of leucocytes in all the males given shoyu were increased compared with the controls. This was accompanied by increased values for the packed cell volume (Table 4). No similar changes were

ylglyoxal, also increased during the pasteurization of shoyu and was equivalent to about one-third of the total diketone compounds.

Asao and Yokotsuka (1963) investigated the formation of these α-diketone compounds in shoyu mash. They reported that small amounts of methylglyoxal and diacetyl were produced by the cooking of soybeans, and that some increase in diacetyl and in trace amounts of acetylpropionyl occurred during koji cultivation. *Pediococcus halophylus* did not affect the formation of dicarbonyl compounds, but their presence doubled with the fermentation of mash by *S. rouxii*. However, the increase in α-diketone compounds during the heating of shoyu was positively correlated with the amount of reducing sugar present in the shoyu. It was demonstrated that glyoxal and methylglyoxal are produced from xylose in the course of heating shoyu, while acetylpropionyl is oxidatively produced from acetylethylcarbinol, which was identified in shoyu. The other precursor of acetylpropionyl corresponding to the $C_7H_{12}O_5$ compound, which is the precursor of acetylbutyryl, could not be detected.

The formation of acetylmethylcarbinol and acetylethylcarbinol in shoyu mash takes place a little earlier than that of alcohol, and both correspond to a decrease in the amount of reducing sugar in mash which results from yeast fermentation. The researchers also noted the conversion of acetylmethylcarbinol and of acetylethylcarbinol from pyruvic acid and α-ketobutyric acid, respectively, by the action of yeasts.

Saccharomyces rouxii demonstrated the strongest conversion ability among the yeasts belonging to the *Saccharomyces*. Also noted was an inverse correlation between the ability of different yeasts to produce alcohol and to produce acetylethylcarbinol. The good producers of alcohol, such as *Saccharomyces cereviciae*, had the tendency to be poor producers of acetylethylcarbinol; the good producers of acetylethylcarbinol, such as *S. rouxii*, were poor producers of alcohol. α-Diketone compounds and glyoxal consisting of from four to eight carbons were chemically synthesized, and their contributions to the flavor constituents of some fermented foods were organoleptically evaluated. α-Diketones exhibited a flavor resembling shoyu and rice wine, while glyoxals resembled vinegar when detected at a concentration of 10^{-6}–10^{-8} (Yokotsuka and Asao, 1961).

Various investigators have reported the isolation of some dicarbonyl compounds of tentative chemical structures from the steam distillate or the distillate of shoyu: $C_8H_{14}O_2$ (Ikeda and Kawaguchi, 1922), $C_5H_6O_2$ and $C_6H_8O_2$ (Kodama, 1922), and $C_6H_{10}O_2$ (Nakaiima and Takei, 1949). Yokotsuka and Asao (1961) pointed out the close resemblance of these compounds to acetylpropinoyl or acetylbutyryl with regard to the characteristic yellow color of the liquid sample, the results of chemical analyses and the melting by silver oxide, the color reaction with 2,4,-dinitrophenylhydrazine, and their absorption of Br^2 (see Table XXXIII).

TABLE XXXIII
DICARBONYLS ISOLATED FROM SHOYU

Researchers[a]	Isolation procedures	Boiling point	Molecular formula	Structural formula
Ikeda and Kawaguchi (1922)	Steam distillation, fractional distillation	50–55°C/1 mm	$C_8H_{14}O_2$	
Kodema (1922)	Steam distillation, fractional distillation	50–60°C/15 mm	$C_5H_6O_2$, $C_6H_8O_2$	$CH_3COCH{=}CHCHO$[b] $CH_3CH_2COCH{=}CHCHO$[b] $CH_3COC(CH_3){=}CHCHO$[b] $CH_3COCH{=}C(CH_3)CHO$[b]
Kurono (1928)	Steam distillation, dimedon, decomposition, fractional distillation		$C_6H_{10}O_2$	$CH_3COCH{=}CH_2CH_2CHO$[b]
Nakajima and Takei (1949)	Extraction with ether, fractional distillation, 2,4-DNP	71–100°C/6 mm	$C_6H_8O_2$	$CH_3COC(CH_3){=}CHCHO$[b]
Yokotsuka and Asao (1961)	Decomposition of $C_7H_{12}O_5$	128°C	$C_6H_{10}O_2$	$CH_3COCOCH_2CH_2CH_3$
Asao and Yokotsuka (1961)	Steam distillation, oxim	108°C	$C_5H_8O_2$	$CH_3COCOCH_2CH_3$

[a] Ikeda *et al.* (1922), *J. Japan Chem. Soc.* **43**, 956; Kodama (1922), *J. Japan Chem. Soc.* **43**, 956; Kurono and Fukai (1928), *J. Japan Chem. Soc.* **4**, 361, 415; Nakajima and Takei (1949), *J. Japan Chem. Soc.* **70**(3), 47; Yokotsuka and Asao (1961), *J. Agric. Chem. Soc. Japan* **35**, 837–845; Asao and Yokotsuka (1961), *J. Agric. Chem. Soc. Japan* **35**, 1211–1218.

[b] Tentative identification.

glutamic-pyruvic transaminase, alkaline phosphatase and leucine aminopeptidase.

Systolic blood pressure was measured by the pulse-pickup method (method of indirect measurement of blood pressure) in tail prior to autopsy at week 26.

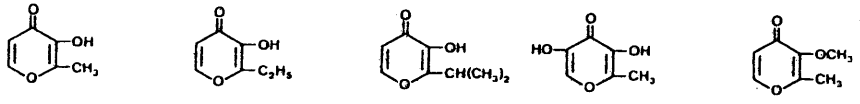
Results

The growth curves of the 11 groups of both sexes are shown in Fig. 1, 2, 3, 4 and 5. In females, there were few differences between treated and control rats in the rate of body weight gain for 26 weeks. In the case of males at the highest dose level (10% P-shoyu), the rats given shoyu were clearly smaller than the controls; however, there were few differences between the rats on shoyu and those on sodium chloride (4.5% sodium chloride) in the rate of body weight gain for 26 weeks (Fig. 1). At the intermediate dose levels (5% P-shoyu or 2% P-shoyu), the rats given shoyu were smaller than the controls, but rats given shoyu were larger than those given sodium chloride (2.25% sodium chloride or 0.9% sodium chloride) in the rate of body weight gain for 26 weeks (Fig. 2 and 3). At the lower dose level of shoyu (1% P-shou or 0.4% P-shoyu), the rate of body weight gain showed few differences from the controls and rats given sodium chloride (0.45% sodium chloride or 0.18% sodium chloride)

3. γ-Pyrones

Kihara (1940) first isolated maltol (**1**) from the chemical hydrolysate of defatted soybean, then 20 mg crude crystals of maltol were isolated from 2 liters of shoyu (1983). Maltol has been known as a characteristic flavor constituent of malt (Brand, 1894). Kihara found that maltol exists in soybeans in a conjugated form with polysaccharide, from which maltol is separated during the heating of soybeans or shoyu.

Maltol is produced by the caramelization of maltose, or the sugars containing maltose, and only in minimal amounts from glucose and starch (Baker *et al.*, 1953; Diemer and Hara, 1959). The formation of maltol is promoted in the presence of amino radicals at lower temperatures in neutral conditions (Patton, 1950; Hodge *et al.*, 1963). Maltol is a typical caramel flavor compound and synergistically enhances sweetness at a concentration of 30–250 ppm. Maltol also synergistically enhances the flavor of vanillin, glutamic acid, and some other amino acids (Hayashi and Kawase, 1970). It is a typical cyclicenolone, having an enolic radical in its molecule and weak acidity. Ito (1972) observed that the aromatic flavor of the weak acidic fraction of foods in general is often due to maltol. Ethylmaltol (**2**) has not been isolated yet from nature, although it has four to six times the flavor intensity of maltol. Isopropylmaltol (**3**) was reported to have a shoyu-like flavor, but it has not been isolated from shoyu. 5-Hydroxymaltol (**4**) has a weak maltol-like flavor and was isolated from roasted barley (Shimizu *et al.*, 1970) and from shoyu (Nunomura *et al.*, 1980). 3-Methoxy-2-methyl-4*H*-pyran-4-one (**5**) was isolated from shoyu, but has no aroma (Nunomura *et al.*, 1980).



(1) (2) (3) (4) (5)

4. 4-Hydroxy-3-furanones and the Related Compounds

a. Isolation of HEMF. The flavor concentrate from the chloroform extract of unpasteurized shoyu was directly subjected to gas chromatography (Nunomura *et al.*, 1976b). The results are shown in Fig. 21. Each peak that was fractionated by gas chromatography equipped with a TCD detector was also subjected to a sensory test of its aroma. Peaks 36–39, and especially no. 39, had the aroma most resembling that of shoyu. GCMS analyses proved that peak 36 was 2-phenylethanol, and peak 37 was a mixture of three compounds: 1-(2-pyrrolyl)-1-ethanone (2-acetylpyrrole) (odorless), maltol, and 3-methoxy-2-methyl-4*H*-pyran-4-one (odorless) (Nunomura *et al.*, 1980). One example of gas chromatograms of shoyu flavor concentrate by GCMS is shown in Fig. 22. The

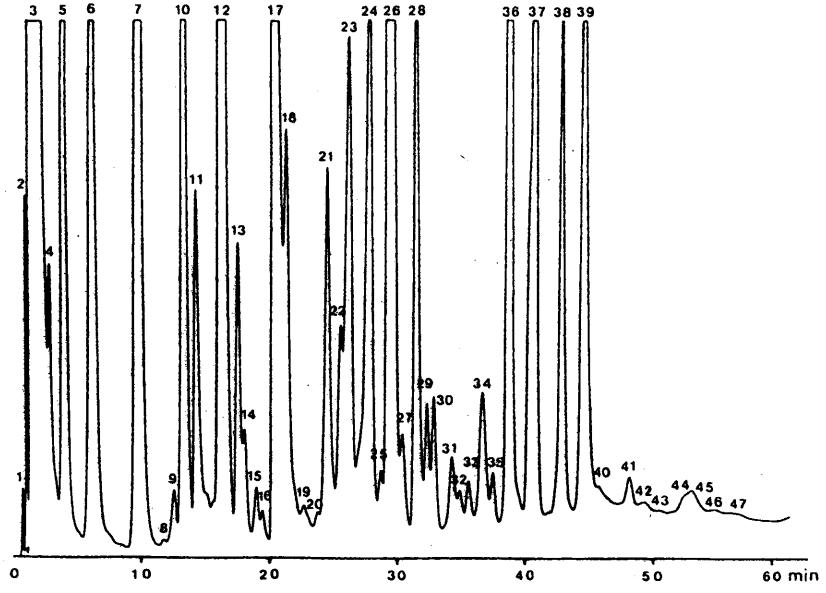
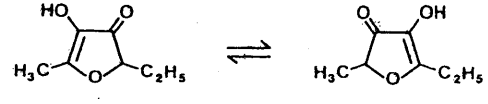


FIG. 21. Gas chromatograph of shoyu flavor concentrate. Instrument: Shimazu 4BM-PF(FDI). Conditions: Column: FFAP 10%, 3 mm i.d. × 2 m (glass). Injector: 240°C; detector: 240°C. Column oven: 50–180°C (3°C/min). Carrier gas: N_2, 30 ml/min. From Nunomura *et al.* (1976).

major constituent of peak 39 was first isolated by distillation in a vacuum from 760 liters of shoyu, then concentrated by extraction with CH_2Cl_2, followed by fractionation and purification through gas chromatography, which gave 176 mg of oil substance. This compound, which was chemically synthesized by Luciano Re *et al.* in 1973, was designated 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*) furanone (HEMF) as the result of UV, proton magnetic resonance (PMR), C-NMR, and high-resolution MS determinations (Nunomura *et al.*, 1976b, 1980). HEMF was first isolated from fermented shoyu, but was not detected in the chemical hydrolysate of plant protein. It exists in the form of a tautomer, (**A**):(**B**) = 3:2, which was determined by PMR as follows:



(A) (B)

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2 H)-furanone (HEMF)

The animals were weighed individually at one week intervals. The quantities of food and water consumed by each rat were measured at the same intervals. Animals that died during the study were subjected to post-mortem examination as soon as possible. All animals surviving at week 26 were killed by an ip injection of barbiturate, blood was collected from the aorta for hematological examination and examined for macroscopic abnormalities, while samples of brain, pituitary, thyroid, thymus, lungs, heart, liver, kidneys, adrenals, spleen, stomach, testes, epididymides, seminal vesicle, prostate, ovaries, uterus and urinary bladder were weighed. Samples of above organs and salivary glands, trachea, esophagus, various lymph nodes, pancreas, small intestine, caecum, colon, rectum, bone marrow together with any other tissues that appeared to be abnormal were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with hematoxylin and eosin for histopathological examination.

The examination of the blood was conducted to measure the hemoglobin concentration and packed cell volume and number of the total erythrocytes and leucocytes. Serum samples collected from the rats killed at week 26 were analyzed for levels of total protein, albumin, zinc turbidity test, thymol turbidity test, glucose, blood urea nitrogen, bilirubin and cholesterol and for the activities of glutamic-oxaloacetic transaminase,

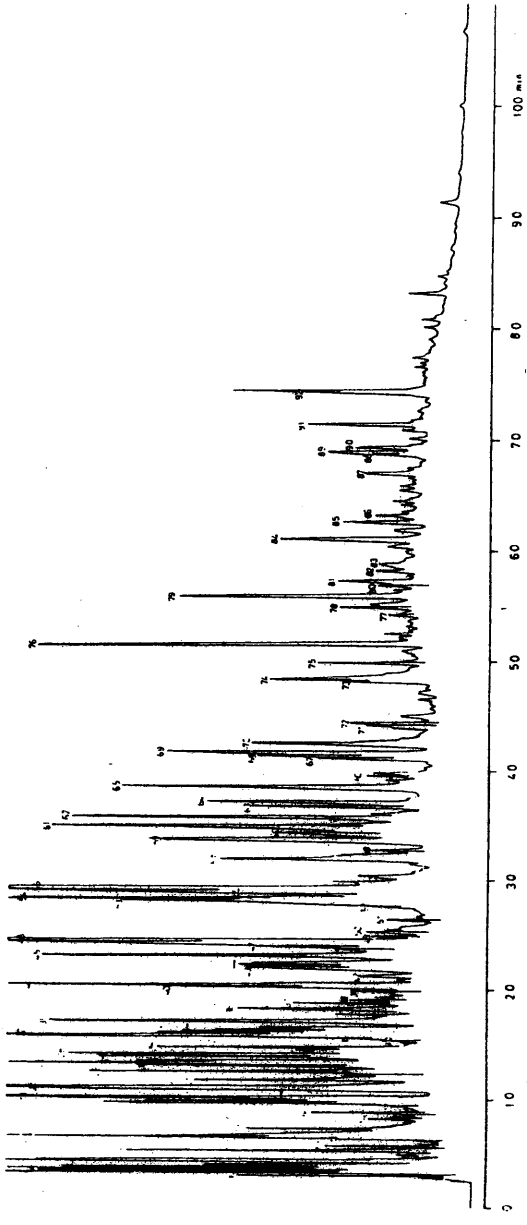
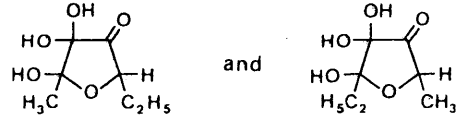


FIG. 22. Gas chromatograph (capillary column) of shoyu flavor concentrate (GCMS). Instrument: RMU-6MG. Column: FFAP, glass, 0.25 mm i.d. × 30 m. Oven temperature: 60–220°C, 2°C/min. He: 0.2 kg/cm². Ionizing voltage: 20 eV. Ion source temperature: 200°C. From Nunomura *et al.* (1980).

HEMF seems to be the most important flavor ingredient and characteristic component of fermented shoyu in view of its high proportion (about 100–200 ppm) and its very low threshold value (less than 0.04 ppb) in water (Ohloff, 1978). Adding 0.01 ppm of HEMF to shoyu is very effective in ameliorating shoyu's otherwise salty taste.

b. OX-HEMF. HEMF is quite stable in shoyu, but is unstable in alkali and acid. Under the basic condition, it changes into the odorless compound 4,4,5-trihydroxy-2-ethyl(or methyl)-5-methyl(or ethyl)-3-tetrahydrofuranone (OX-HEMF).



OX-HEMF

The IR spectrum of OX-HEMF coincided with that of a very unstable sublimatic compound, $C_7H_{12}O_5$, which was isolated from a weak acidic fraction of unpasteurized shoyu and tentatively identified as 2-furanone, the structure of which was given previously (Asao and Yokotsuka, 1961a,b; Yokotsuka and Asao, 1961). It is presumed that the conversion of HEMF into OX-HEMF occurred in the course of the alkali treatment of the chloroform extract of unpasteurized shoyu with 5% Na_2CO_3. OX-HEMF degradates by heating or by autooxidation into such compounds as acetylbutyryl (2,3-hexandione), α-ketobutyric acid, α-ketopropionic acid, acetaldehyde, and other compounds, as shown in Fig. 23.

c. HDMF, HMMF, and the Other 4-Hydroxy-3(2H)-furanones. The quantity of 4-hedroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) in shoyu was reported to be about 10 ppm (Nunomura *et al.*, 1980), with a threshold value of 0.04 ppb in water (Ohloff, 1976). This compound was first isolated from pineapple and was reported to have a pineapple-like flavor, having a threshold value of 0.1–0.2 ppm (Rodin *et al.*, 1965).

The quantity of 4-hydroxy-5-methyl-3(2*H*)-furanone (HMMF) (Nunomura *et al.*, 1979) is small, but increases when shoyu is heated, reaching about 200 ppm. HMMF was reported to have a caramel flavor similar to roasted chestnuts. HEMF, HMMF, and HDMF resemble each other in chemical structure, but have different patterns of development. HEMF is produced by the yeast fermentation of shoyu mash, while HMMF and HDMF are typical browning compounds.

Materials and Methods

Animals : The rats of both sexes of a STD-Wistar strain obtained from a conventional colony were housed individually in a room maintained at 23 ± 1° C with a relative humidity of 55 ± 5%. The rats were divided into 11 groups of 14 males and 14 females 5 weeks of age and have been fed with each experimental diet for 26 weeks.

Materials and Procedure : The powder of Kikkoman-brand shoyu was used as sample (contains 45% sodium chloride) (P-shoyu). Standard diets to which were added the sample at each provided rate were used as experimental diets. Furthermore, the diets containing sodium chloride at the same concentration as each diet containing shoyu were used as experimental diets. Funabashi Farm's Laboratory Small Animal Diet was used as standard diet. All the diets were solidified and given to animals and tap-water were provided ad lib.

The experimental groups are shown in Table 1. The dose levels were decided on the basis of the mean daily intake of shoyu in Japan. In 1971 that value was 33 ml per person per day. The highest dose was worth 25 times as much as the mean human value and the lowest dose was worth as much as the mean value. Proximate analyses of experimental diets are shown in Table 2.

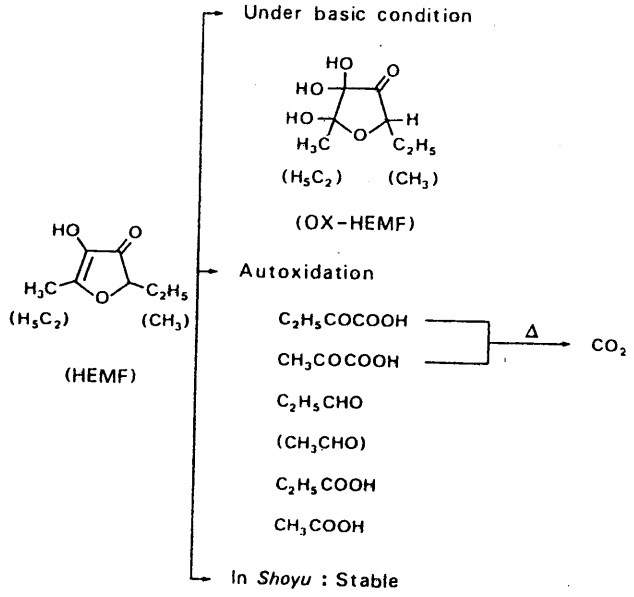
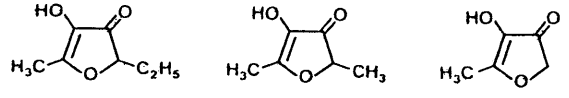


FIG. 23. Oxidation of process of HEMF. From Nunomura *et al.* (1976b).

A total of 257 strains of yeast isolated from 11 brewing houses of Kikkoman Company were cultured at 30°C for 40 days in an aseptic filtrate of shoyu mash fermented for 25 days at pH 4.8 and adjusted with lactic acid in a flask with shaking once a day. All the strains tested produced HEMF, on the average 129.6 ppm, with ranges from trace to 28.4 ppm (Sasaki *et al.*, 1984).

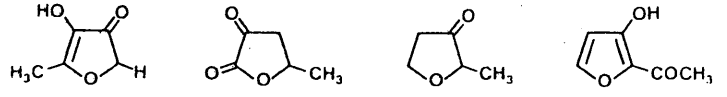


(HEMF) (HDMF) (HMMF)

d. Other Similar Compounds. In 1970, the Ajinomoto Company got a Japanese patent for improving the flavor of foods and condiments by adding 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (**6**). This compound was purified by sublimation from the heated product of sugar and amino acids, and was reported to form colorless, needle-shaped cyrstals, mp 126–127°C, to have maltol-like flavor, and to turn dark blue in color when mixed with $FeCl_3$.

Yokotsuka (1958) isolated the compound $C_5H_6O_3$ from a weak acidic fraction of ether extract of shoyu with a yield of 10 mg from 10 liters of shoyu. It formed needle-shaped crystals, with an mp of 126–128°C, was sublimatic, had a strong ricecracker-like flavor, and turned a dark green color when mixed with $FeCl_3$. These two compounds would seem to be identical judging from the descriptions given above; however, Sulser *et al.* (1967) presumes that the compound $C_5H_6O_3$ has the structure of α-keto-γ-valerolactone (**7**).

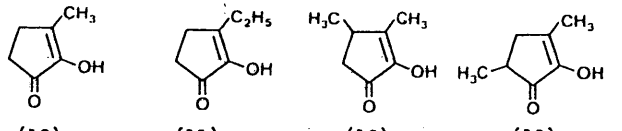
Nunomura *et al.* (1980) isolated 2-methyl-3-tetrahydrofuranone (**8**) from shoyu, which had been previously isolated from coffee by Gianturco *et al.* (1964), with no description of its aroma. Isomaltol (**9**) has also been isolated from shoyu and has the fragrance of burnt sugar (Nunomura *et al.*, 1980).



(6) (7) (8) (9)

5. *Alkylcyclopentadiones*

Several kinds of alkylcyclopentadiones, such as 2-hydroxy-3-methyl-2-cyclopentene-1-one (**10**), 3-ethyl-2-hydroxy-2-cyclopentene-1-one (**11**), 2-hydroxy-3,4-dimethyl-2-cyclopentene-1-one (**12**), and 3-hydroxy-3,5,-dimethyl-2-cyclopentene-1-one (**13**), have been isolated from heat-treated foodstuffs, such as coffee beans, and the compounds of fructose degradated by heat (Gianturco *et al.*, 1963, 1964; Gianturco and Friedel, 1963). Gyclotene is only one of the compounds belonging to this group which was isolated from shoyu (Nunomura *et al.*, 1980). All of these alklycyclopentadiones were reported to have a flavor similar to caramel, roasted sugar, or maple syrup.



(10) (11) (12) (13)

6. *Acetals*

Yokotsuka (1950) identified a great amount of isovaler-aldehyde-diethylacetal, $(CH_3)_2CHCH_2CH(OC_2H_5)_2$, in the steam distillate of shoyu or shoyu cake (the press residue of shoyu mash). Also identified in the same distillate, but with less certainty, were α-hydroxyisocaproaldehyde-diethylacetal, $(CH_3)_2CHCH_2$

LONG-TERM (6 MONTHS) FEEDING TEST IN RATS

Abstracts

Groups of 30 rats of each sex were given diets containing 0, 0.4, 1, 2, 5 or 10% shoyu, 0.18, 0.45, 0.9, 2.25 or 4.5% sodium chloride for 6 months. At the higher dose levels, the rate of body weight gain of male rats given shoyu or sodium chloride was clearly lower than the controls; however, it seems likely that the growth inhibition by shoyu was smaller than that by sodium chloride. In females, there were few differences between the rats on shoyu or sodium chloride and the controls in the rate of body weight gain.

At the highest dose level of shoyu or sodium chloride, extremely increased water intake, alterations of relative weights of kidneys, heart and urinary bladder, higher concentration of serum-urea were observed. These changes were accounted for by the changes resulting from the treatment of a high concentration of sodium chloride. In spite of an increase in relative liver weight, histopathological changes and changes in liver function were not observed.

$CHOHCH(OC_2H_5)_2$, and/or α-ketoisoaldehyde-diethylacetal, $(CH_3)_2CHCH_2COCH(OC_2H_5)_2$. Inasmuch as these compounds were not identified in the ether extract of shoyu, and since acetals in general are unstable in an acidic condition like that of shoyu, these acetals were presumed to be synthesized in the stream of shoyu and to constitute an important part of the flavor of shoyu vapor while cooking. On the other hand, chemically synthesized *n*-, and isobutyracetal and isovaleracetal were claimed to have as important a volatile flavor as is organoleptically detectable in shoyu or rice wine.

Fujita (1960, 1961) reported that the diethylacetals of phenylglyoxal, $C_6H_5COCH(CO_2H_5)_2$—benzylglyoxal, $C_6H_5CH_2COCH(OC_2H_5)_2$, methionylglyoxal, $CH_3SCH_2CH_2COCH(OC_2H_5)_2$, and *sec*-butylglyoxal, $(CH_3)_2CHCOCH(OC_2H_5)_2$—exhibited the characteristic aroma of shoyu.

Yoshida *et al.* (1980) analyzed the topnote of aroma concentrate from shoyu and identified ethanol, ethyl acetate, isobutyraldehyde, the diethylacetals of these aldehydes, isoamyl alcohol, and a trace amount of dimethyl sulfide.

E. PHENOLIC COMPOUNDS

4-Ethylguaiacol (4EG) (Yokotsuka, 1953) and *p*-ethylphenol (Asa and Yokotsuka, 1958) are important flavor ingredients of shoyu belonging to its weak acidic fraction, which had been isolated from shoyu prior to the finding of other weak acidic constituents of flavor. The formation of phenolic compounds in shoyu production has been studied (Yokotsuka *et al.*, 1967a,b; Asao *et al.*, 1967, 1969). These phenolic compounds were reported to derive for the most part from wheat. The phenolic fraction of wheat was observed to increase in the course of roasting, and vanillin, ferulic acid, and vanillic acid were identified as the major constituents of this phenolic fraction. Shakuchirin (Sahia and Shaw, 1961), which was found in the seed leaves of wheat and in coniferyl alcohol as a part of its lignin structure, were identified as the possible precursors of these phenolic compounds (see Fig. 24). The formation of vanillin and vanillic acid from a part of ferulic acid and the formation of *p*-hydroxycinnamic acid and its conversion into *p*-hydroxybenzoic acid have been observed during the growth of *Aspergillus* molds during koji cultivation (Asao and Yokotsuka, 1958). The greatest amount of phenolic was reported after the first 24 hr of the 72-hr period of koji cultivation, which coincided with the maximum mycelial growth of koji mold. The major constituent of the phenolic fraction of koji was identified as ferulic acid. Ferulic acid and *p*-hydroxycinnamic acid are metabolized into 4EG and *p*-ethylphenol, respectively, in the latter half of the period of yeast fermentation of shoyu mash ("moromi") by the action of a *Candida* (*Torulopsis*) yeast, wuch as *C. versatillis* or *C. etchellsii*, and not by *S. rouxii*, which is generally considered to be the predominant yeast in shoyu fermentation. The various kinds

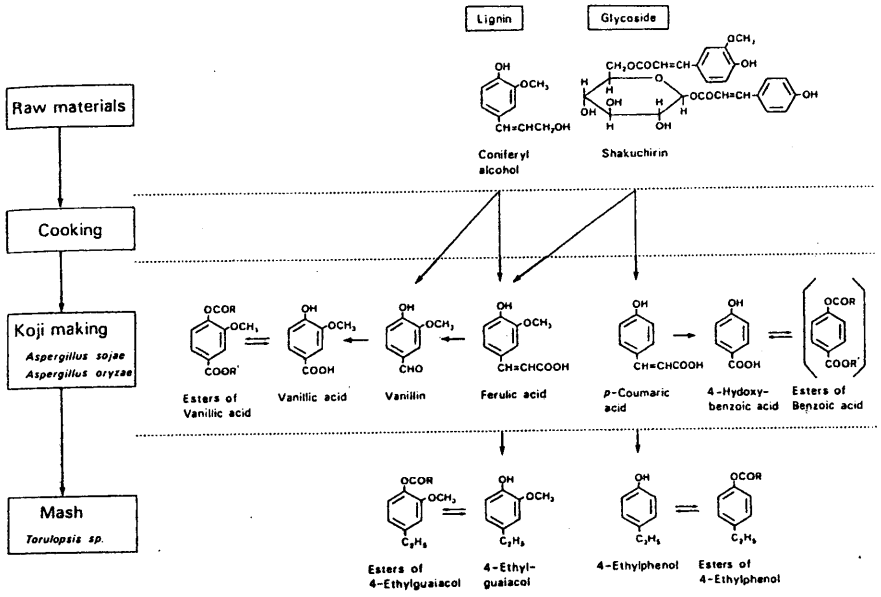


FIG. 24. Formation of alkylphenols during the manufacturing process of shoyu. Structures in brackets have not been identified. From Yokotsuka *et al.* (1967a,b); Asao *et al.* (1967).

of yeast that convert ferulic acid into 4EG are listed in Table XXXIV. The yield of a fractional distillate with a bp of ~185°C from the steam distillate of shoyu cake was small, but it had a strong shoyu flavor. Organic acids, phenols, carbonyls, and sulfur-containing compounds were removed from this fraction, and the residue slightly hydrolyzed to obtain acetic acid, benzoic acid, 4EG, ethyl vanillate, and at least two kinds of unknown phenols by paper chromatography. These findings suggest that the unstable phenolic esters between phenols, such as 4EG and ethyl vanillate, and organic acids, such as acetic acid and benzoic acid, are present in shoyu and are the precursors of free phenolic compounds which increase during the pasteurization of shoyu. The quality of free phenols, including 4EG, doubled when shoyu was pasteurized at 80°C for 5 hr.

About 25% of the 50–70 samples of shoyu tested in 1964, 1965, and 1966 contained 0.5–2.0 ppm of 4EG. The organoleptically best 10 samples among 50 and only 1 sample among the remaining 40 contained 4EG (Yokotsuka *et al.*, 1967a,b). Thus, 4EG is a very important ingredient of fermented shoyu, as the difference of 0.5% of 4EG in shoyu is easily detected by a sensory test and characterizes a given brand of shoyu. Moreover, it was observed that 4EG tasted

Table 8. Incidence of tumours in mice given each experimental diet for 1.5 years

Tissue and tumour	Group No. ...	Incidence of tumour											
		Male						Female					
		I	II	III	IV	V	VI	I	II	III	IV	V	VI
	No. examined ...	32	36	29	32	33	31	33	28	32	31	35	34
Lungs													
Adenoma		5	4	5	6	9	12	2	5	4	2	2	5
Liver													
Hepatoma (probably benign)		3	10	9	2	12	11	0	0	0	0	0	0
Adrenals													
Adenoma		0	0	1	0	1	1	0	0	0	0	0	0
Testes													
Interstitial-cell tumour		0	0	4	0	0	2	–	–	–	–	–	–
Prostate													
Adenoma		0	0	0	2	0	1	–	–	–	–	–	–
Ovaries													
Cystoma		–	–	–	–	–	–	0	1	0	0	0	1
Hematoma		–	–	–	–	–	–	0	0	0	0	1	1
Uterus													
Myoma		–	–	–	–	–	–	2	0	0	4	0	5
Hemo- lymphopoietic system													
Leukemia or Lymphoma		6	4	8	4	0	5	4	4	7	8	6	7

TABLE XXXIV

KINDS OF YEAST ISOLATED FROM SHOYU MASH AND THEIR ABILITIES TO CONVERT FERULIC ACID INTO 4-ETHYLGUAIACOL (4EG)[a]

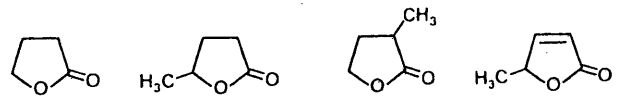
Stage of fermentation of moromi	Strain of yeast	Stock number	Formation of 4EG	Assimilation of nitrate
Beginning	*Torulopsis famata*	E29a	–	–
	Pichia farinosa	A6	–	–
	Trichosporon behrendii	E-3A	–	–
	Candida porimorpha	EK	–	–
Fermentative	*Saccharomyces rouxii*	E-7 No. 210	–	–
	Saccharomyces rouxii var. *halomembranis*	J3	–	–
	Saccharomyces acidifaciens	S9	–	–
	Saccharomyces acidifaciens var. *halomembranis*	R6	–	–
Aging	*Torulopsis halophylus*	N-24, 10A-40	+ or –	+
	Torulopsis nodaensis	N-21, 29B-45	+	+
	Torulopsis versatilis	N552, 2C-5	+	+
	Torulopsis etchellsii	15A-26, 19C-7	+	+
	Torulopsis anomala	3B-42, 17C-28	+	+
	Torulopsis sake	5C-5, 22B-2	+	+

[a] From Asao and Yokotsuka (1958).

like fermented shoyu and ameliorated its salty taste. Noda and Nakano (1979) determined the quantity of 4EG in the three popular brands of koikuchi shoyu in Japan to be 1.0, 1.8, and 2.1 ppm, and in the three brands of usukuchi shoyu to be 0.5, 1.3, and 0.3 ppm, respectively. The yeast flora in 35 kinds of shoyu mash obtained in Hokkaido (northernmost island of Japan) was studied in 1960, and it was found that organoleptically good mashes contained large amounts of *Candida* (*Torulopsis*) *etchellsii* and *C. versatilis* (Sasaki *et al.*, 1964, 1966a,b; Yoshida, 1979). Among 257 strains of yeast isolated from shoyu mash, 17 produced 4.51 ppm of 4EG on an average, ranging from 0.31 to 8.99 ppm (Sasaki *et al.*, 1984).

F. LACTONES

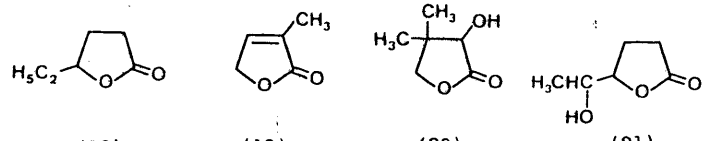
In general, aliphatic lactones are important among the flavor ingredients of foods because of their strong and characteristic flavor. Many varieties and large amounts of γ-lactones are found in animal foods and greatly contribute to the dairy flavor, for example. Many kinds of γ-lactones are also present in vegetables. Four kinds of γ-lactones have been identified in Japanese fermented shoyu (Nunomura *et al.*, 1980): 4-butanolide (γ-butyrolactone) (**14**), 4-pentanolide (γ-valerolactone) (**15**), 2-methyl-4-butanolide (**16**), and 2-pentene-4-olide (**17**). A schematic presentation of their chemical structures is presented here.



(14) (15) (16) (17)

There is a very small amount of 4-pentanolide in fermented shoyu, but a large amount in the chemical protein hydrolysate or its yeast-fermented product, which in Japan is called semichemical shoyu, as well as the ethyl levulinate, which is also a characteristic ingredient of semichemical shoyu (Nunomura *et al.*, 1977a).

Liardon and Phillipossian (1978, 1980) cultured koji *A. oryzae* with a mixture of cooked soybeans and wheat, and combined the koji with 18% saline water to make the mash, which was adjusted to pH 4.5 and then fermented with *S. rouxii* at 38–40°C for 30 days to make shoyu. This process might be slightly different from the average shoyu produced in Japan in that the mash is not fermented with, but is fermented by yeasts at a very high temperature. From this shoyu, eight kinds of lactones were isolated, including the four previously cited (**14–17**), and four more recently isolated γ-lactones: 4-hexanolide (γ-caprolactone) (**18**), 2-methyl-2-buten-4-olide (**19**), 2-hydroxy-3,3-dimethyl-4-butanolide (**20**), and 5-hydroxy-4-hexanolide (**21**), presented here.



(18) (19) (20) (21)

All of these γ-lactones have been widely identified in many foodstuffs, including black tea, cocoa, coffee, pineapple, tomato, peach, apricot, strawberry, plum, tobacco, fried onion, roasted peanut, beef tallow, lard, mushroom, and sherry wine.

G. PYRAZINES

Pyrazines are typical components of the so-called browning flavors—corn, nut, and bread—and they play an important role in the flavor of heat-treated foodstuffs (Hodge, 1972).

Table 7. Histopathological lesions (excluding tumours) in mice sacrified at year 1.5

Tissue and finding	Group No.	Incidence of lesion					
		Male			Female		
		I	IV	VI	I	IV	VI
	No. examined	16	14	16	10	10	11
Salivary glands							
Leucocytic infiltration		4	4	7	0	0	4
Other degenerations		2	2	2	2	3	1
Thyroid							
Hypertrophy of follicle		0	0	3	0	2	2
Other degenerations		2	0	1	0	2	0
Lungs							
Leucocytic infiltration		3	2	2	2	0	3
Degeneration of bronchi		0	0	0	0	0	1
Pneumonia		0	0	1	0	2	0
Heart							
Myocardial fibrosis		6	6	5	2	2	0
Degeneration of auricle		0	0	3	0	0	0
Liver							
Fatty change		0	0	0	0	2	0
Middle lobular degeneration		0	3	2	0	0	3
Leucocytic infiltration		3	2	2	3	2	3
Vacuolar degeneration		0	0	1	0	0	0
Bile-duct hyperplasia		0	0	3	0	0	0
Other degenerations		3	2	1	2	4	3
Spleen							
Hyperplasia of red pulp		0	0	5	0	2	2
Hyperplasia of white pulp		3	2	2	3	2	2
Pancreas							
Leucocytic infiltration		2	2	4	0	2	2
Stomach							
Hyperplasia of mucosal epithelium		0	0	1	0	0	0
Other degenerations		0	0	0	0	0	1
Adrenals							
Leucocytic infiltration		0	0	5	4	2	5
Other degenerations		0	0	5	2	5	5
Kidneys							
Glomerular nephritis		4	2	5	0	0	1
Leucocytic infiltration		4	3	6	4	4	2
Hydronephrosis		6	4	4	4	4	2
Other degenerations		0	2	3	0	2	4
Testes							
Atrophy		0	2	2	-	-	-
Increase of interstitial cells		0	2	4	-	-	-
Ovaries							
Cystic		-	-	-	3	0	4
Atrophy (Fibrosis)		-	-	-	2	4	0
Uterus							
Cystic glandular proliferation		-	-	-	2	2	5
Urinary bladder							
Dilatation and hypertrophy		9	6	3	8	8	0
Mucosal degeneration		0	2	0	0	4	4

TABLE XXXV
CONCENTRATIONS OF MAJOR PYRAZINES
BEFORE AND AFTER PASTEURIZATION OF KOIKUCHI SHOYU[a]

Compound	Concentration (mg/liter)		Ratio (heated/raw)
	Raw shoyu	Pasteurized shoyu	
2-Methylpyrazine	0.024	0.075	3.1
Dimethylpyrazine	0.184	0.746	4.1
Ethylmethylpyrazine	0.338	0.746	1.9
Trimethylpyrazine	0.040	0.050	1.3

[a] From Nunomura *et al.* (1978).

The greater part of pyrazines in foods is produced by the heat degradation of proteins and amino acids or by the chemical reactions between sugar and protein, although some are biosynthesized in plant tissues, such as 2-isobutyl-3-methoxypyrazine in bell pepper (Buttery *et al.*, 1969). Approximately 70 pyrazines have been identified in foods to date, but it is only since 1970 that the importance of pyrazines as food flavor ingredients has been generally recognized and utilized in the manufacture of artificial food flavorings. It is likely that shoyu contains many kinds of pyrazine compounds, but until recently, tetramethylpyrazine is the only one that has been isolated. Nunomura *et al.* (1978, 1980) identified 27 pyrazines in the basic fraction of shoyu by GCMS analysis. The flavor of pyrazines in shoyu is weakened by the weak acidic pH value of shoyu (4.7–4.9), but becomes dominant when the pH of shoyu is neutralized by dilution with water in cooking. When shoyu is heated, there is a substantial increase in the quantity of pyrazines (as indicated in Table XXXV), suggesting that they are one of the characteristic flavor components of pasteurized shoyu.

H. SULFUR-CONTAINING COMPOUNDS

When an aqueous solution of mercuric chloride ($HgCl_2$) is added to shoyu, part of the characteristic volatile flavor disappears at once, perhaps evidence of the fact that some sulfur-containing compounds play an important role in the volatile flavor. Methionol (3-methylthio-1-propanol), which was first isolated from shoyu by Akabori and Kaneko (1936), and methional (3-omethylthio-1-propanal), synthesized by these researchers (1937), are claimed to be important ingredients of shoyu flavor. Yokotsuka (1953) identified lower mercaptans and mercaptals in the steam distillate of shoyu cake. It is believed that these compounds are produced not only by fermentation, but by the heating of sulfur-containing compounds during distillation. It is generally known that methylmercapto radicals, methyl mercaptan, and hydroxysulfide are produced by microbial metabolism or by the heat degradation of sulfur-containing compounds. The chemical hydrolysate of plant protein contains more amounts of lower boiling sulfur compounds than does fermented shoyu, as shown in Table XXXVI (Ueno, 1963).

TABLE XXXVI
CONTENT OF VOLATILE SULFUR-CONTAINING COMPOUNDS IN SHOYU
AND THE CHEMICAL HYDROLYSATE OF PLANT PROTEIN (ppm)[a]

Sample	H_2S	CH_2SH	$(CH_3)_2S$
Fermented shoyu	3.40	1.20	0.22
Semichemical shoyu[b]	3.10	1.90	4.40
Chemical hydrolysate of plant protein	5.30	4.70	44.60

[a] From Ueno (1963), Report of Kikkoman Shoyu Co., Ltd., Vol. 5.
[b] The mixture of chemical hydrolysate of defatted soybean, soybean and wheat koji, and salt water is fermented with *Saccharomyces rouxii* for 1 week at 30°C in the presence of 18% salt.

Dimethyl sulfide, present in the chemical hydrolysate of defatted soybean, is produced by the degradation of methionine methyl sulfonium, $Me(CH_3)SCH_2CH_2CH(NH_2)COOH$, itself produced by the reaction between methionine and methyl chloride. Methyl chloride is decomposed from the methoxy group of soybeans by the action of HCl (Ogasawara, 1963). Guadagni *et al.* (1963) reported threshold values of dimethyl sulfide and methyl mercaptan (methanthiol) to be 0.02 and 0.33 ppb, respectively.

I. TERPENES

Several kinds of terpenes have been isolated from whiskey, brandy, rum, and fusel oil. It is interesting to note that borneol, bornyl acetate, and *cis*-rose oxide [4-methyl-2-(2-methyl-1-propenyl)-tetrahydropyran] were isolated from shoyu (Nunomura *et al.*, 1976a, 1979).

J. FLAVOR CONSTITUENTS OF THE TOPNOTE AROMA OF THE PASTEURIZED SHOYU

Newly pasteurized fermented shoyu has a characteristic pleasant odor, most of which disappears in a short time by natural evaporation. Sasaki and Nunomura (1979) directly analyzed the topnote flavor concentrate of pasteurized shoyu by the GCMS method. The sample to be analyzed was prepared by passing helium

Table 6. (continued)

Experimental group No.	Organ			
	Stomach	Ovary+ (L)	Ovary+ (R)	Bladder
	Organ weight (mg)			
I (11.0 % P-shoyu)	346(46)	20.1(6.2)*	10.7(3.8)	177(79)**
II (1.1 % P-shoyu)	417(49)	14.0(2.7)	11.7(4.6)	42(15)
III(0.11% P-shoyu)	370(83)	11.4(6.3)	9.4(5.2)	42(9)
IV (5.0 % NaCl)	377(73)	12.0(5.2)	8.9(2.3)	159(97)**
V (0.5 % NaCl)	416(42)	13.6(4.6)	9.4(3.0)	43(13)
VI (Control)	372(61)	12.4(3.5)	9.6(3.3)	40(11)
	Relative organ weight (g/100 g body weight)			
I (11.0 % P-shoyu)	1.087(0.216)	60.0(15.3)**	34.8(11.5)	0.558(0.268)**
II (1.1 % P-shoyu)	0.892(0.316)	28.4(7.6)	25.2(13.7)	0.085(0.027)
III(0.11% P-shoyu)	0.962(0.172)	24.2(14.5)	18.2(5.4)	0.109(0.022)
IV (5.0 % NaCl)	1.097(0.106)	32.5(9.2)	21.0(7.0)	0.410(0.250)**
V (0.5 % NaCl)	1.017(0.138)	31.3(11.9)	22.7(7.9)	0.104(0.020)
VI (Control)	1.013(0.215)	31.0(11.9)	25.8(15.8)	0.105(0.032

+Values for relative organ weights expressed in mg/100 g body weight.

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisks differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

TABLE XXXVII
QUANTITATIVE ANALYSIS OF HEADSPACE GAS FROM SHOYU[a]

Compounds	Concentrations (ppm) (x, n = 10)	Coefficient of variation (%)
Methanol	9.45	4.43
Acetaldehyde	3.76	9.58
Ethanol	5605.18	3.50
Propionaldehyde	1.70	8.52
Acetone	2.09	3.75
Ethyl formate	1.66	3.02
n-Propyl alcohol	0.82	5.64
Isobutyraldehyde	6.38	3.16
Ethyl acetate	33.41	1.83
Isobutyl alcohol	3.79	1.75
n-Butyl alcohol	0.69	10.75
Isovaleraldehyde	8.17	2.88
2,3-Pentanedione	0.76	8.25
Isoamyl alcohol	2.36	9.38

[a] From Sasaki and Nunomura (1978).

gas through the shoyu at 20°C and then trapping the vapor by dry ice–ethanol, liquid nitrogen, and activated carbon, in succession. A total of 24 compounds, 3 of which were isolated for the first time, were identified; of them, 14 are listed in Table XXXVII. The respective constituents of odor in three isolated compounds were calculated, and the aroma of the headspace gas from fresh fermented shoyu was attributed primarily to isovaleraldehyde, ethanol, and isobutyraldehyde, as

TABLE XXXVIII
ODOR UNITS OF 6 OF 14 CONSTITUENTS OF HEADSPACE GAS FROM SHOYU[a]

Compound	Concentration (ppm)	Threshold (ppm in water)	Odor units	Relative odor units (%)
Ethanol	5605.18	1.83×10^{-1}	30,629.40	33.05
Ethyl acetate	33.41	6.0×10^{-1}	55.68	0.06
Isovaleraldehyde	8.17	1.5×10^{-4}	54,466.67	58.77
Isobutyraldehyde	6.38	9.0×10^{-4}	7,088.89	7.65
Acetaldehyde	3.76	1.5×10^{-2}	250.67	0.27
Propionaldehyde	1.70	9.5×10^{-3}	178.95	0.19

[a] From Sasaki and Nunomura (1978).

shown in Table XXXVIII. The researchers recognized the presence and importance of volatile sulfur compounds, but could not identify them.

Yoshida *et al.* (1980) analyzed the topnote aroma concentrate of shoyu and identified ethanol, ethyl acetate, isobutyraldehyde, isovaleraldehyde, the diethylacetals of these aldehydes, isoamyl alcohol, and a trace amount of dimethyl sulfide.

Sasaki and Nunomura (1979) measured the loss of the aroma constituents due to the evaporation of shoyu at 23°C. Propionaldehyde disappeared completely in 15 min and half of the ethanol and isovaleraldehyde dissipated in 30 min, indicating the freshness of a pasteurized shoyu rapidly exposed to the open air.

K. METHODS OF QUANTITATIVE ANALYSES OF THE VOLATILE FLAVOR CONSTITUENTS OF SHOYU

The accuracy of the quantitative analyses is particularly critical in an investigation of the relationship between the gas-chromatographic pattern of a shoyu and its organoleptic evaluation. Sasaki *et al.* (1980) compared the coefficients of variation (CV) and the recoveries of flavor compounds of shoyu using three analytical procedures:

1. Shoyu (50 ml) was distillated in a vacuum at 40°C and the distillate (35 ml) saturated with NaCl and then extracted with CH_2Cl_2, which had been concentrated into 2 ml by evaporation of the solvent.
2. Shoyu (50 ml) was saturated with NaCl and then extracted with CH_2Cl_2, which was concentrated into 2 ml.

TABLE XXXIX
COMPARISON OF COEFFICIENTS OF VARIATION (CV) AND RECOVERIES USING THREE METHODS[a]

	Method 1		Method 2		Method 3	
Compound	CV (%)	% (10 ppm)[b]	CV (%)	% (10 ppm)	CV (%)	% (10 ppm)
Isobutyl alcohol	15.19	27.90	8.1	38.00	1.39	102.00
n-Butyl alcohol	9.68	31.20	6.4	44.93	1.44	103.90
Isoamyl alcohol	7.66	31.50	4.2	54.12	1.39	108.10
Acetoin	10.00	15.10	4.6	49.73	2.23	96.20
Ethyl lactate	12.67	6.50	6.4	43.13	1.29	102.30
Furfuryl alcohol	13.95	25.90	12.8	61.58	5.88	92.70
Methionol	6.85	26.30	16.1	66.22	1.86	88.40
2-Phenylethanol	14.29	56.10	26.7	71.85	3.37	97.30
4-Ethylguaiacol	16.90	64.70	17.0	82.82	1.48	97.90

[a] From Sasaki *et al.* (1980).
[b] Recoveries.

Table 6. (continued)

Experimental group No.	Organ					
	Liver++	(L) Kidney	(R)	(L) Adrenal+	(R)	Spleen
			Organ weight (mg)			
I (11.0 % P-shoyu)	1.84(0.38)	363(42)	383(60)	4.8(0.7)	4.0(0.4)	183(132)
II (1.1 % P-shoyu)	2.31(0.67)	340(51)	364(54)	5.4(1.3)	4.8(1.3)	218(151)
III(0.11% P-shoyu)	2.19(1.03)	317(81)	331(65)	5.4(1.3)	5.3(0.9)	193(100)
IV (5.0 % NaCl)	2.25(0.94)	400(71)*	421(95)*	4.5(1.1)	4.3(1.0)	177(51)
V (0.5 % NaCl)	2.29(0.39)	333(61)	352(70)	5.8(1.0)	5.3(1.0)	218(52)
VI (Control)	2.23(0.67)	326(68)	340(75)	5.8(1.2)	5.1(1.0)	193(75)
			Relative organ weight (g/100 g body weight)			
I (11.0 % P-shoyu)	5.66(0.67)	1.124(0.051)*	1.180(0.096)*	14.7(0.7)	12.3(1.0)	0.592(0.414)
II (1.1 % P-shoyu)	4.74(1.04)	0.725(0.187)	0.771(0.217)	12.3(3.9)	11.1(4.0)	0.486(0.411)
III(0.11% P-shoyu)	4.97(0.76)	0.827(0.201)	0.869(0.193)	14.2(4.8)	13.9(4.3)	0.535(0.255)
IV (5.0 % NaCl)	5.46(0.59)	1.089(0.110)*	1.148(0.203)*	12.9(5.2)	12.0(3.7)	0.458(0.071)
V (0.5 % NaCl)	5.74(0.74)	0.817(0.091)	0.863(0.110)	14.4(3.1)	13.3(3.1)	0.541(0.131)
VI (Control)	5.37(1.11)	0.845(0.195)	0.877(0.190)	13.9(3.1)	12.5(3.5)	0.529(0.233)

+Values for relative organ weights expressed in mg/100 g body weight.

++Values for organ weights expressed in gram.

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisks differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

TABLE XL
RESULTS OF QUANTITATIVE ANALYSIS OF FLAVOR CONSTITUENTS IN KOIKUCHI SHOYU (ppm)[a]

Ethanol	31,501.10	Furfuryl alcohol	11.93
Lactic acid	14,346.57	Isoamyl alcohol	10.01
Glycerol	10,208.95	Acetoin	9.78
Acetic acid	2,107.74	*n*-Butyl alcohol	8.69
HMMF	256.36	HDMF	4.83
2,3-Butanediol	238.59	Acetaldehyde	4.63
Isovaleraldehyde	233.10	2-Phenylethanol	4.28
HEMF	232.04	*n*-Propyl alcohol	3.96
Methanol	62.37	Acetone	3.88
Acetol	24.60	Methionol	3.65
Ethyl lactate	24.29	2-Acetylpyrrole	2.86
2,6-Dimethoxyphenol	16.21	4-Ethylguaiacol	2.77
Ethyl acetate	15.13	Ethyl formate	2.63
Isobutyraldehyde	14.64	γ-Butyrolactone	2.02
Methyl acetate	13.84	4-Ethylphenol	Trace
Isobutyl alcohol	11.96		

[a] From Yokotsuka *et al.* (1980).

3. Shoyu (5 ml), 2 ml methyl acetate, and 1 g NaCl were shaken in a closed test tube and then centrifuged at 3400 rpm for 10 min at 5°C. The methyl acetate layer was directly subjected to gas-chromatographic analysis.

As is indicated in Table XXXIX, procedure 3 gave the most reliable results. One example of an analysis of the flavor constituents of shoyu is indicated in Table XL.

L. CONTRIBUTION OF VOLATILE FLAVOR CONSTITUENTS TO OVERALL FLAVOR EVALUATION

The content of 4EG and the sensory evaluation of shoyu are in a parabolic relationship, and both too great and too small an amount were not liked by consumers. The optimum content of 4EG was roughly claimed to be less than 0.5 ppm (Yokotsuka, 1967c).

Mori *et al.* (1982, 1983) confirmed the correlation coefficients between each of 27 kinds of odor components and the sensory evaluation of their company's shoyu to be 0.313 at the highest. This fact suggested that it was difficult to predict the scale value of the shoyu by only one kind of odor component. By checking the effects of all combinations of each of two components, the combination of 4EG and methionol was found chiefly to influence the variation of

sensory data. The optimum sum of two components was first determined to be 4.5 ppm, then the optimum content of each component was found to be 0.3 ppm for 4EG and 3.9 ppm for methionol, respectively, which was confirmed both by mathematical calculation and by an addition test with shoyu. The relationship between the content of 4EG or methionol and the sensory evaluation of shoyu was parabolic. The same authors (1984) conducted a similar experiment with 30 brands of shoyu on the Japanese market. The content of each flavor constituent of the samples ranged wider than that of a simple brand of product. The highest correlation coefficient was found for ethyl acetate to be ($r = -0.551$). Ethyl acetate was found to give a kind of freshness to shoyu. Both *n*-butyric acid and HEMF were found to be in a parabolic relationship to sensory evaluation. Most shoyu tested contained about 1 ppm of *n*-butyric acid, and generally, a content of more than 3 ppm of *n*-butyric acid yielded an inferior sensory evaluation. The average content of HEMF of the shoyu tested ranged from 100 to 200 ppm, and an inferior sensory evaluation was given to the shoyu that contained less than 50 ppm of HEMF. A total of 595 combinations of each 2 among 35 flavor components was checked for their contents. Many were found to have the sum of contents of 4EG and methionol at more than 4.5 ppm, which was reported to be optimum. The sum of contents of acetoin and isobutyric acid was found in this case to be highly associated with sensory evaluation of shoyu. Sasaki *et al.* (1984) compared Japanese fermented shoyu (I) and Southeast Asian soy sauces (II) in terms of flavor constituents of headspace gas and solvent extract as follows:

1. The content of HEMF was 150–400 ppm for I, but 0–trace for II.
2. The sum of isobutyl alcohol, *n*-butyl alcohol, isoamyl alcohol, methionol, and 2-phenylethanol of II was 0–20 ppm, which was about one-half of I.
3. Methional [3-(methylthio)propanal] was distributed widely in both I and II, with the contents of 0.2–2.0 ppm.
4. More pyrazines were found in accordance with an increase of HVP, which was blended with fermented soy sauce.

VII. SAFETY PROBLEM OF SHOYU

A. NONPRODUCTIVITY OF MYCOTOXINS BY JAPANESE INDUSTRIAL MOLDS

The capability of some strains of mold to produce mycotoxins has been reported. Examples are aflatoxins, ochratoxins, sterigmatocystin, patulin, penicillic acid, islanditoxin, cyclopiazonic acid, and zearalenones, including T-2 toxin. Among these, aflatoxins seem to be the most important because of their

Table 6. Organ weights and relative organ weights of female mice given each experimental diet for 1.5 years

Experimental group No.	No. of mice examined	Organ				
		Brain	Pituitary+	Thyroid+	Lungs	Heart
		Organ weight (mg)				
I (11.0 % P-shoyu)	8	553(51)	2.9(0.7)	4.1(0.3)*	327(55)	212(15)
II (1.1 % P-shoyu)	16	548(36)	3.4(1.2)	6.3(1.5)	341(70)	197(16)
III(0.11% P-shoyu)	16	574(36)	3.9(1.0)	5.1(1.4)	307(61)	196(42)
IV (5.0 % NaCl)	8	551(41)	3.5(1.2)	5.7(1.8)	333(60)	220(37)
V (0.5 % NaCl)	18	547(35)	4.3(0.8)	7.8(1.3)**	336(91)	198(28)
VI (Control)	11	570(47)	3.8(0.8)	5.4(1.0)	322(81)	197(35)
		Relative organ weight (g/100 g body weight)				
I (11.0 % P-shoyu)	8	1.72(0.22)	8.91(2.21)	12.6(0.6)	1.019(0.207)	0.658(0.051)*
II (1.1 % P-shoyu)	16	1.16(0.35)	7.48(2.20)	13.4(5.1)	0.723(0.169)	0.426(0.131)
III(0.11% P-shoyu)	16	1.56(0.44)	10.34(3.10)	13.2(3.8)	0.779(0.235)	0.518(0.134)
IV (5.0 % NaCl)	8	1.55(0.38)	9.88(3.76)	13.6(4.2)	0.906(0.169)	0.608(0.114)
V (0.5 % NaCl)	18	1.37(0.24)	10.70(2.10)	19.1(3.7)*	0.871(0.328)	0.490(0.056)
VI (Control)	11	1.55(0.62)	9.73(2.22)	13.5(3.6)	0.811(0.300)	0.513(0.118)

+Values for relative organ weights expressed in mg/100 g body weight.

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisks differ significantly (Student's t-test) from those of controls: *P < 0.05; **P < 0.01.

acute toxicity and significant carcinogenicity. Moreover, according to Sargeant *et al.* (1961), aflatoxins are produced by the *Aspergillus flavus* group, which include Japanese koji molds, such as *A. oryzae* and *A. sojae*, classified by Sakaguchi and Yamada (1944), used for food fermentation. According to the classification by Raper and Fennel (1965), the *A. flavus* group includes *A. flavus*, *A. parasiticus*, and *A. oryzae*, while aflatoxin producers are found in *A. flavus* and *A. parasiticus*. The question of whether *Aspergillus* molds used for food preparation produce aflatoxins follows logically. Murakami (1971) studied the taxonomic classification of *Aspergillus* molds. He reported that industrial mold mostly belongs to the *A. oryzae* group, *A. sojae*, and *A. tamarii*, while all of the aflatoxin-producing molds belong to *A. parasiticus* and *A. toxicurius* Murakami, which are clearly distinguishable from industrial molds. Nevertheless, it is important to note that these *Aspergillus* molds are morphologically continuous with regard, for example, to the roughness of their stalks or color and surface conditions of their spores. Therefore, it is sometimes difficult to classify these molds definitively using only their morphological features. This is especially true in differentiating between *A. sojae* and *A. parasiticus*, both of which are good producers of proteolytic enzymes. Several classifications of Aspergilli are summarized in Table XLI. Among the 125 strains of mold used for shoyu production in Japan, there are 29 *A. sojae* and 92 *A. oryzae* (Murakami, 1973). Accordingly, from the viewpoint of the food industry, it becomes extremely important to confirm by means of chemical analyses that the molds to be used do not produce aflatoxin.

Some investigators have reported negative findings in studies of the use of Japanese industrial molds in fermentation and the production of aflatoxin. Hesseltine *et al.* (1966) studied 53 cultures at the Northern Regional Research Laboratory in the United States, but tests of miso, shoyu, and sake, all made with strains of *A. oryzae*, were negative. Aibara and Miyaki (1965) examined 180 strains, including those used in the preparation of miso and cheese, but analyses with UV absorption, excitation, and fluorescence spectra revealed no producer of aflatoxin. Masuda *et al.* (1965) studied 21 strains of industrial mold with the same results. Murakami *et al.* (1967, 1968) examined 214 kinds of *Aspergillus* mold by fluorometry and thin-layer chromatography (TLC), including 176 industrial strains, for their aflatoxin-producing ability. Thirteen strains gave fluorescent spots on TLC corresponding to aflatoxin, but their UV absorption spectra were different from those of aflatoxin. Manabe *et al.* (1968) observed that 49 strains among 212 koji molds exhibited aflatoxin-like fluorescent spots on TLC, but that all of their UV absorption spectra were different from those of aflatoxins. Kinoshita *et al.* (1968) concluded from their results of TLC and UV absorption spectra that of 37 strains of mold isolated from Japanese katsuobushi (dried bonito used for seasoning), shoyu, and miso, none produced aflatoxin.

TABLE XLI

CLASSIFICATION OF ASPERGILLI

1. Sakaguchi and Yamada (1944)	
Koji molds:	*Aspergillus oryzae* (Ahlburg) Corn
	A. sojae, Sakaguchi et Yamada, prominently echinulate conidia and smooth-walled conidiophore
2. Raper and Fennel (1965)	
A. flavus groups:	*A. flavus* L.
	A. flavus var columnaris R. et F.
	A. parasiticus A (include *A. sojae*)
	A. oryzae (A) C.
	A. tamari K
	unnamed species
3. Murakami (1971)	
A. oryzae group:	*A. sojae* S. et Y.
	A. tamari K
	A. oryzae (A) C.
	A. oryzae var. viride M.
	A. oryzae var. *brunneus* M.
A. flavus group:	*A. parasiticus* S.
	A. toxicallius M.
	A. flavus L.
4. American Type Culture Collection (1982)	
Recognized *A. sojae* S. et Y. as a new species, and separated from *A. parasiticus*	
5. Kurtzman (1983)	
A 90% or more relatedness of *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* regarding DNA structures[a]	

[a] Kurtzman (1983).

The research techniques used in these investigations and the kinds of data thus generated limit the analysis largely to a comparison of R_f values of TLC and fluorescence spectra of the spots. Fluorescent compounds with violet-to-green fluorescence resembling aflatoxin B or G, which have been reported to be produced by *Aspergillus* molds, are flavacol (Dunn *et al.*, 1949), isoxanthopterin (Kaneko, 1965), ferulic acid (Asao and Yokotsuka, 1958a), aflatoxin B and G (Sargeant *et al.*, 1961), some degradated products of ergosterol (Yokotsuka *et al.*, 1966), and others (Kihara *et al.*, 1944). Among the fluorescent compounds produced by *Aspergillus* molds, aflatoxin clearly differs from the others with respect to its R_f value on TLC. Some investigators, however, have found that a fairly large number of strains of *Aspergillus* mold do produce aflatoxin-like fluorescent compounds having R_f values on TLC, similar to the aflatoxins, but with different UV maximum absorptions. These include seven kinds of pyrazine compounds, isocoumarin compounds, lumichrome, and unknown compounds

Table 5. (continued)

Experimental group No.	Organ					
	Stomach	(L) Testis	(R)	(L) Epididymis	(R)	Bladder
			Organ weight (mg)			
I (11.0 % P-shoyu)	357(84)	128(27)	133(27)	53(11)	56(8)	328(102)**
II (1.1 % P-shoyu)	385(54)	143(21)	146(29)	64(14)	64(14)	78(27)
III(0.11% P-shoyu)	336(61)	132(34)	138(36)	63(14)	64(16)	76(29)
IV (5.0 % NaCl)	309(30)*	110(28)	114(44)	50(11)	60(23)	221(111)**
V (0.5 % NaCl)	398(46)	119(38)	146(25)	62(12)	65(12)	90(32)
VI (Control)	381(59)	126(31)	133(33)	62(12)	71(22)	84(39)
			Relative organ weight (g/100 g body weight)			
I (11.0 % P-shoyu)	0.814(0.076)	0.300(0.072)	0.313(0.080)	0.13(0.03)	0.13(0.03)	0.761(0.139)**
II (1.1 % P-shoyu)	0.765(0.125)	0.284(0.061)	0.289(0.078)	0.12(0.03)	0.13(0.03)	0.151(0.048)
III(0.11% P-shoyu)	0.769(0.088)	0.291(0.065)	0.303(0.068)	0.14(0.03)	0.14(0.03)	0.168(0.063)
IV (5.0 % NaCl)	0.803(0.121)	0.288(0.080)	0.301(0.128)	0.13(0.03)	0.15(0.05)	0.577(0.294)**
V (0.5 % NaCl)	0.696(0.060)	0.207(0.075)	0.252(0.048)	0.11(0.02)	0.11(0.03)	0.153(0.053)
VI (Control)	0.732(0.116)	0.260(0.081)	0.278(0.096)	0.13(0.03)	0.14(0.04)	0.170(0.081)

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisks differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

with aflatoxin G-like green fluorescence. The existence of these fluorescent compounds makes it difficult to determine the capability of some strains of mold to produce aflatoxin by TLC alone.

B. FLUORESCENT COMPOUNDS PRODUCED BY *Aspergillus* MOLDS WITH R_f VALUES RESEMBLING THOSE OF AFLATOXINS

Yokotsuka *et al.* (1966b, 1967c, 1968a,b) and Sasaki *et al.* (1967, 1968a,b) examined 73 industrial strains of *Aspergillus* mold used either for the production of shoyu, miso, and rice wine, or found in the stock cultures, for their production of fluorescent compounds after being cultured in a zinc-containing Czapek Dox medium (Nesbitt *et al.*, 1962). About 30% of these strains showed fluorescent spots resembling those of aflatoxin B or G. Ultraviolet absorption spectra of the eluants of 14 strains whose R_f values were similar to aflatoxin B1 were divided into two groups having UV absorption maxima at 320–330 and 310–315 nm, respectively. However, no eluant had a UV absorption maximum of 363 nm, which is characteristic of aflatoxin B_1. Likewise, eluants of the spots of eight strains whose R_f values were similar to that of aflatoxin G_1 did not show the UV absorption maximum of aflatoxin G_1 (Yokotsuka *et al.*, 1968c).

The best producing strain of the fluorescent compounds whose UV absorption spectrum was at 320–330 nm was *A. sojae* X-1, a wild strain cultured in peptone-enriched Czapek Dox medium (modified Mayer's medium). Eight fluorescent spots were observed on TLC, but their R_f values were different from those of aflatoxin B_1 using 11 kinds of solvent systems (see Fig. 25). Eight fluorescent compounds, including flavoacol, and eight nonfluorescent compounds, including aspergillic acid and hydroxyaspergillic acid, were isolated in cyrstalline form from cultured broth according to the method depicted in Fig. 26. The chemical structure of each compound was determined by elemental analysis, melting point, NMR, UV spectrum, IR spectrum, and so forth. Aflatoxin B-like compounds were related to each other, with a pyrazine ring common to their structure. This was indicated by similar UV spectra, with absorption at 310–320 nm, and similar IR spectra, with absorption at 1600 cm^{-1}.

Also confirmed was the finding that when these isolated compounds have 2-hydroxypyrazine rings, they give fluorescence, but when the first nitrogen has an oxide structure, they give no fluorescence. The maximum absorption IR spectra at ~950 cm^{-1} seemed to be associated with those differences. Identified fluorescent and nonfluorescent pyrazine compounds are listed in Tables XLII and XLIII, respectively (Sasaki *et al.*, 1967, 1968a–c; Yokotsuka *et al.*, 1968b,c).

These compounds are considered to be condensation products of two molecules of amino acid, as was suggested by J. C. MacDonald *et al.* (MacDonald, 1961, 1962, 1965, 1967; Micetich and MacDonald, 1965). Examples include

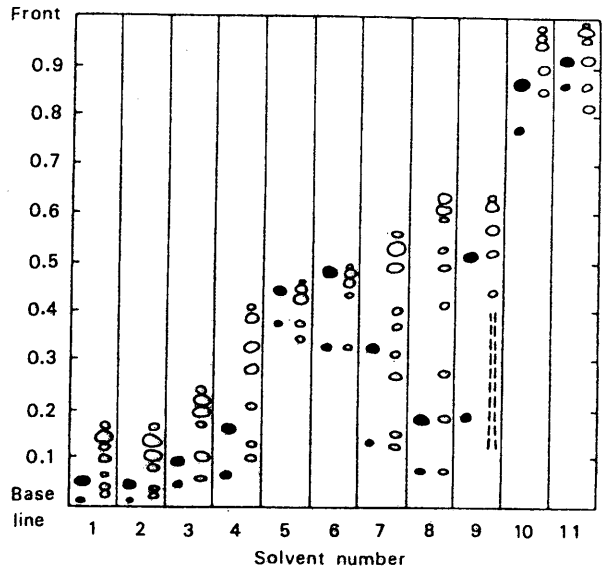


FIG. 25. Variation of R_f values of aflatoxin B-like compounds of *Aspergillus sojae* X-1 with different solvent systems. Aflatoxin B_1 and G_1; Black spots on left side of each column. Aflatoxin B-like compounds: B0 to B8 from the top on right side. Absorbent: Kieselgel G, 0.5 mm. Solvent 1: Benzene–ethyl acetate (3 + 1); 2: benzene–acetone (3 + 1); 3: chloroform–ethyl acetate (3 + 1); 4: benzene–ethyl acetate–ethanol (30 + 19 + 1); 5: chloroform–methanol (97 + 3); 6: benzene–ethanol (9 + 1); 7: chloroform–ethyl acetate–ethanol (30 + 19 + 1); 8: ethyl acetate–hexane (3 + 1); 9: chloroform–acetone (3 + 1); 10: ethyl acetate–methanol (3 + 1); 11: acetone–hexane (3 + 1). From Yokotsuka *et al.* (1966).

leucine and leucine, isoleucine and isoleucine, isoleucine and leucine, and valine and leucine, in our case. In view of the fact that thin-layer chromatographs have exhibited many other faint spots of possible fluorescent pyrazine compounds, it is reasonable to suspect that many of the pyrazine compounds produced by molds from two molecules of amino acid (e.g., from valine and valine or from valine and isoleucine) exist in nature.

Importantly, these are not limited to *A. sojae* X-1, but are equally applicable to the production of *A. sojae* and *A. oryzae*, which are actually used in the preparation of fermented foods. The crystals of fluorescent pyrazine compounds B0 to B6 (excluding B5 and B7) were injected intraperitoneally into mice. Because of the shortage of test samples, only three mice were tested for each dosage, 250 mg and 500 mg/kg. These compounds exhibited no acute toxicities of more than 250 mg/kg (Sasaki *et al.*, 1968a–c). The same test for toxicity was applied to the

Table 5. (continued)

Experimental group No.	Organ					
	Liver++	(L) Kidney (R)		(L) Adrenal+ (R)		Spleen
	Organ weight (mg)					
I (11.0 % P-shoyu)	2.55(0.38)	544(94)	583(138)	2.6(0.8)	2.5(0.6)	154(90)
II (1.1 % P-shoyu)	2.99(0.67)	466(55)	485(59)	3.1(0.4)	2.9(0.6)	158(68)
III(0.11% P-shoyu)	2.59(0.45)	477(53)	499(65)	3.2(0.7)	2.7(0.6)	137(44)
IV (5.0 % NaCl)	1.91(0.41)	480(54)	526(58)	3.1(0.8)	2.7(0.5)	139(39)
V (0.5 % NaCl)	3.19(0.54)	557(74)	568(94)	3.2(0.8)	2.8(0.8)	151(48)
VI (Control)	2.69(0.88)	480(84)	505(86)	3.5(0.8)	3.2(0.7)	137(71)
	Relative organ weight (g/100 g body weight)					
I (11.0 % P-shoyu)	5.93(0.92)	1.261(0.216)**	1.339(0.221)**	5.20(0.69)	5.36(1.27)	0.247(0.030)
II (1.1 % P-shoyu)	5.74(0.97)	0.910(0.103)	0.950(0.127)	6.09(1.24)	5.73(1.54)	0.289(0.142)
III(0.11% P-shoyu)	5.44(1.03)	1.062(0.154)	1.113(0.192)	7.11(1.85)	5.99(1.64)	0.309(0.143)
IV (5.0 % NaCl)	4.88(0.71)	1.240(0.134)**	1.357(0.119)**	7.96(1.73)	6.82(0.95)	0.361(0.106)
V (0.5 % NaCl)	5.15(0.56)	0.975(0.117)	1.006(0.170)	5.45(1.30)	4.74(1.17)	0.252(0.076)
VI (Control)	5.07(0.99)	0.929(0.118)	0.982(0.146)	6.58(1.61)	6.03(1.52)	0.258(0.125)

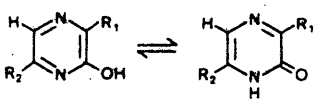
+Values for relative organ weights expressed in mg/100 g body weight.

++Values for organ weights expressed in gram.

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisks differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

TABLE XLII

IDENTIFIED FLUORESCENT PYRAZINE COMPOUNDS PRODUCED BY *Aspergillus sojae* X-1 SIMILAR TO AFLATOXIN B_1 WITH RESPECT TO THEIR R_f VALUES ON TLC[a]



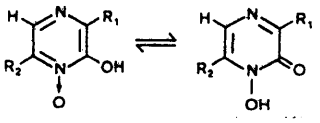
I. $-CH(CH_3)C_2H_5$
II. $-CH_2CH(CH_3)_2$
III. $-CH(CH_3)_2$
IV. $-C(OH)(CH_3)C_2H_5$
V. $-CH(OH)CH(CH_3)_2$
VI. $-C(OH)(CH_3)_2$

Abbreviated mark	R′	R″	Name of compound
B0	I	I	2-Hydroxy-3,6-di-*sec*-butylpyrazine[b]
B1	II	I	Deoxyaspergillic acid
B2	II	II	Flavacol
B2′	II	III	Deoxymutaaspergillic acid
B3	I	IV	2-Hydroxy-6-(1-hydroxy-1-methylpropyl)-3-*sec*-butylpyrazine[b]
B4	II	IV	Deoxyhydroxyaspergillic acid
B5	II	V	2-Hydroxy-6-(1-hydroxy-2-methylpropyl)-3-isobutylpyrazine[b]
B6	II	VI	2-Hydroxy-6-(1-hydroxyisopropyl)-3-isobutylpyrazine

[a] From Yokotsuka *et al.* (1967), Sasaki *et al.* (1967, 1968a).
[b] Uncertain identification.

TABLE XLIII

IDENTIFIED NONFLUORESCENT PYRAZINE COMPOUNDS FROM CULTURE OF *Aspergillus sojae* X-1 AND OTHER SOURCES[a]



I. $-CH(CH_3)C_2H_5$
II. $-CH_2CH(CH_3)_2$
III. $-CH_2(CH_3)_2$
IV. $-C(OH)(CH_3)C_2H_5$
V. $-CH(OH)CH(CH_3)_2$
VI. $-C(OH)(CH_3)_2$

Abbreviated mark	R′	R″	Name of compound
A0	I	I	2-Hydroxy-3,6-di-*sec*-butylpyrazine-1-oxide
A2	II	I	Aspergillic acid[b]
	II	II	Neoaspergillic acid[c]
A3	II	III	2-Hydroxy-3-isobutyl-6-isopropylpyrazine 1-oxide
A4	I	IV	2-Hydroxy-6-(1-hydroxy-1-methylpropyl)-3-*sec*-butylpyrazine-1-oxide
A5	II	IV	Hydroxyaspergillic acid[d]
	II	V	Neohydroxyaspergillic acid[e]
	II	IV	Mutaaspergillic acid[f]

[a] From Yokotsuka *et al.* (1968b) and Sasaki *et al.* (1968b).
[b] White (1943); Dutcher (1957); from *A. flavus*.
[c] MacDonald *et al.* (1964); from *A. scleotiorum*.
[d] Menzel (1943); from *A. flavus*.
[e] Weiss (1958).
[f] Nakamura (1960) and (1961); from *A. oryzae*.

Table 5. Organ weights and relative organ weights of male mice given each experimental diet for 1.5 years

Experimental group No.	No. of mice examined	Organ				
		Brain	Pituitary+	Thyroid+	Lungs	Heart
		Organ weight (mg)				
I (11.0 % P-shoyu)	14	530(37)	2.4(0.3)	4.5(0.7)**	332(81)	293(56)
II (1.1 % P-shoyu)	14	551(35)	3.0(0.7)	5.5(0.9)	323(94)	278(39)
III(0.11% P-shoyu)	16	553(38)	2.5(0.8)	6.7(1.5)	333(78)	250(33)
IV (5.0 % NaCl)	12	517(29)	2.9(0.7)	6.7(2.3)	441(88)	308(15)
V (0.5 % NaCl)	16	549(44)	2.5(0.7)	7.3(1.4)	382(96)	290(24)
VI (Control)	16	550(40)	2.9(0.9)	6.9(1.4)	381(66)	262(45)
		Relative organ weight (g/100 g body weight)				
I (11.0 % P-shoyu)	14	1.26(0.29)	5.56(1.27)	10.5(2.4)*	0.772(0.097)	0.681(0.116)**
II (1.1 % P-shoyu)	14	1.09(0.19)	5.18(0.96)	11.1(1.7)*	0.635(0.197)	0.546(0.091)
III(0.11% P-shoyu)	16	1.20(0.14)	5.63(1.82)	14.9(3.3)	0.745(0.195)	0.554(0.071)
IV (5.0 % NaCl)	12	1.34(0.14)	7.36(1.67)	17.0(5.1)	1.045(0.193)	0.798(0.073)**
V (0.5 % NaCl)	16	0.95(0.10)	4.33(1.30)	12.9(3.5)	0.656(0.169)	0.527(0.074)
VI (Control)	16	1.14(0.29)	5.89(1.73)	14.1(2.7)	0.767(0.231)	0.518(0.111)

+Values for relative organ weights expressed in mg/100 g body weight.

The figures are means and standard deviation in parentheses for the number of mice shown and those marked with an asterisk differ significantly (Student's t-test) from those of controls: *$P < 0.05$; **$P < 0.01$.

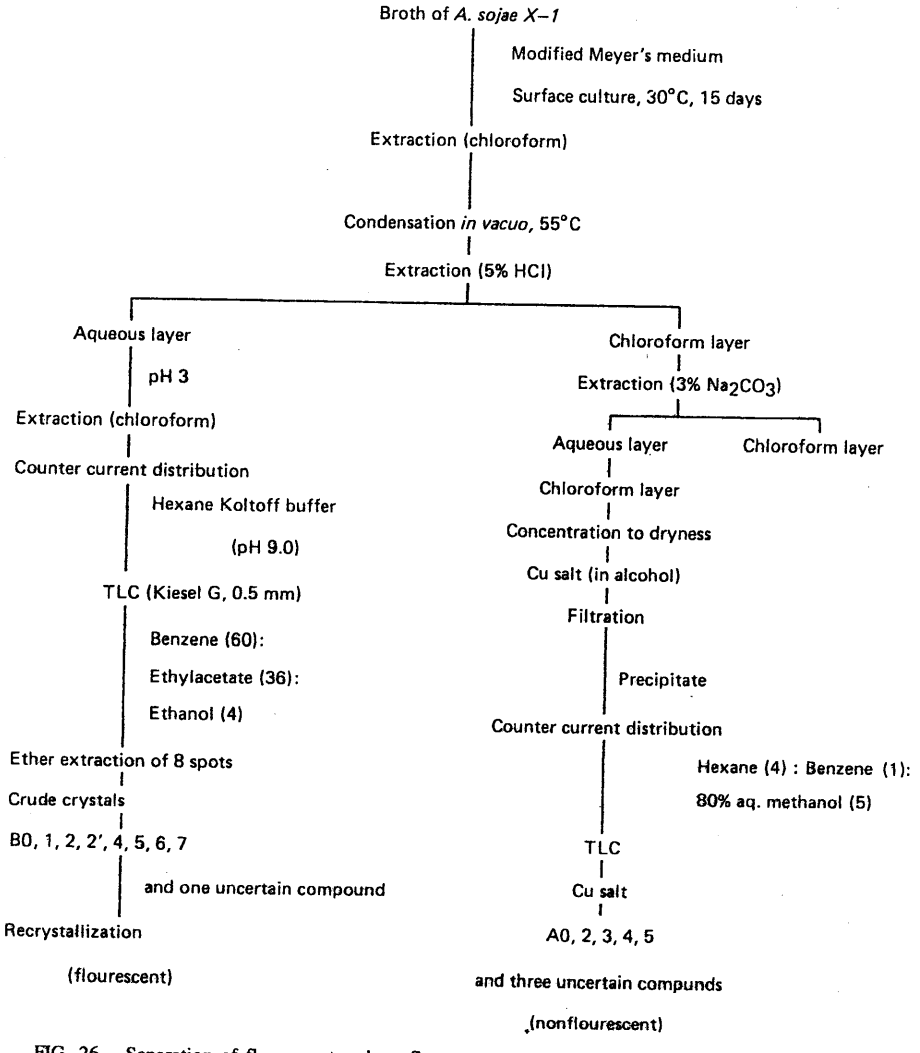
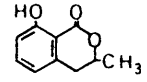
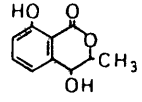
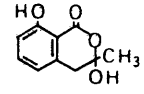
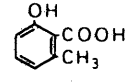


FIG. 26. Separation of fluorescent and nonfluorescent pyrazine compounds from culture of *Aspergillus sojae* X-1. From Yokotsuka *et al.* (1966, 1967, 1968a,b) and Sasaki *et al.* (1967, 1968a).

TABLE XLIV
ISOLATED ISOCOUMARIN AND RELATED COMPOUNDS
FROM THE CULTURE OF *Aspergillus oniki* 1784[a]

Compound number and structure	Melting point (°C)	UV spectra, λ_{max}^{EtOH} nm	Fluorescence on TLC	Acute toxicity on mice (LD_{50} mg/kg) (ip)
BV-1 (Mellein) 	56	246, 315	+	550–1250
BV-2 	121–121.5	244.5, 315	+	1000–1500
BV-3 	109–109.5	246, 315	—	262
BV-4 	171	—	—	—

[a] From Sasaki *et al.* (1970).

nonfluorescent compounds, for the total mixture, and for A0, 2, 3, 4, and 5. The toxicities of aspergillic acid and hydroxyaspergillic acid have been previously reported in the literature (Nakamura and Shiro, 1960, 1961). The results suggest the toxicity of these compounds is similar to that of aspergillic acid, LD_{50}, which is ~100 mg/kg.

From the culture of the strains which produce aflatoxin B-like, bluish violet fluorescent compounds of Group 2, three isocoumarin compounds, including mellein (Nishikawa, 1933), 4-hydroxymellein, and 3,4-dihydro-3,8-dihydroxy-3-methylisocoumarin, were isolated (see Table XLIV). These isocoumarin compounds were found to be produced by some strains of *Aspergillus*

Table 4. Food and water consumptions and shoyu intake of mice given each experimental diet for 70 weeks

Experimental group No.	Food consumption (g/mouse/day)	Water consumption (ml/mouse/day)	Intake of shoyu+ (ml/mouse/day)	Intake of shoyu+ (ml/kg/day)
		Male		
I (11.0 % P-shoyu)	6.4	39.7**	1.83	43.10
II (1.1 % P-shoyu)	5.4	7.3	0.15	3.33
III(0.11% P-shoyu)	5.0	6.2	0.014	0.31
IV (5.0 % NaCl)	6.1	32.2**	(0.304)	(7.42)
V (0.5 % NaCl)	6.0	8.0	(0.030)	(0.61)
VI (Control)	5.6	6.6	--	--
		Female		
I (11.0 % P-shoyu)	6.4	58.6**	1.83	53.51
II (1.1 % P-shoyu)	5.5	10.2	0.16	3.87
III(0.11% P-shoyu)	5.5	7.2	0.016	0.42
IV (5.0 % NaCl)	6.3	48.9**	(0.315)	(8.75)
V (0.5 % NaCl)	5.0	7.9	(0.025)	(0.67)
VI (Control)	5.4	7.6	--	--

+The figures for shoyu intake are calculated from data on food consumption, body weight and sodium chloride contents in shoyu. Values in parentheses show sodium chloride intake expressed in g/mouse/day and g/kg/day.

The figures marked with asterisks differ significantly (Student's t-test) from those of control: *P < 0.05; **P < 0.01.

TABLE XLV

PHYSICAL DIFFERENCES OF COMPOUND G_3 AND AFLATOXIN G_1[a]

Compound	Melting point (°C)	Color of fluorescence on TLC (365 nm)	UV spectra (λ_{max}^{MeOH} nm)	Excitation spectra ($\lambda_{max}^{C_6H_5Cl_3}$ nm)	Fluorescence spectra ($\lambda_{max}^{C_6H_5Cl_3}$ nm)
G_3	266	Bluish green	342	350	410
Aflatoxin G_1	247–250	Green	363	365	450

[a] From Yokotsuka *et al.* (1968c).

ochraceus. Although *A. ochraceus* is not used in food industries, it is found in foodstuffs as a contaminant. Under certain experimental conditions, these compounds also exhibit fluorescence and R_f vlaues resembling those of aflatoxin B (Sasaki *et al.*, 1970).

Regarding green fluorescent compounds produced by *Aspergillus* molds, 7 out of 72 strains tested exhibited four kinds of green fluorescent spots on TLC. From 400 liters of cultured broth of *Aspergillus* M4-1, which is used in making miso, four kinds of green fluorescent compounds were isolated: three kinds of crude crystals, and 6.2 mg of purified crystals with mp 266°C. TLC yielded R_f values resembling that of aflatoxin G_1 under certain experimental conditions, but analyses with 15 kinds of solvent systems confirmed their difference from aflatoxin G_1. Other physical properties, including UV absorption, were also different from those of aflatoxin G_1 (Yokotsuka *et al.*, 1968c), as shown in Table XLV.

Approximately 200 strains of mold, including 126 newly added to the previous 73 strains, were reexamined for their productivity of aflatoxins. It was found that none produced aflatoxins. However, almost all newly tested strains produced lumichrome, $C_{12}H_{10}N_4O_2$ (Karrer *et al.*, 1934). This compound displays green fluorescence and an R_f value similar to that of aflatoxin G under certain experimental conditions (Sasaki *et al.*, 1974).

Two strains of *A. flavus* Link were reported to produce aflatoxin B_1 (Kurata *et al.*, 1968, 1969). One was isolated from wheat flour imported into Japan, and the other was isolated from home-made rural miso. Sasaki *et al.* (1975) reconfirmed that aflatoxin was produced from the former strain, but aflatoxin B_1 was not detected in the latter strain. The purified fluorescent sample isolated from 300 liters of cultured broth exhibited the same R_f value as did aflatoxin B_1 using TLC with chloroform, methanol 97:3, but a different R_f value from that obtained when aflatoxin B_1 was analyzed with a solvent system composed of benzene:acetone, 3:1. The sample was further purified into two compounds with a different UV absorption from that of aflatoxin B_1.

From these data it is evident that some *Aspergillus* molds produce fluorescent compounds with R_f values resembling those of aflatoxins. Indetecting and characterizing samples that are contaminated with aflatoxins, R_f values should be determined with two or more solvent systems, and UV and IR spectral data should also be used. This implies that the compounds must be chemically isolated and identified.

C. MYCOTOXINS OTHER THAN AFLATOXINS

A total of 69 strains of Japanese industrial mold was tested for their productivity of aspergillic acid, kojic acid, β-nitropropionic acid, and oxalic acid, although these acids are not carcinogenic and their toxicity is not as great as aflatoxins (Yokotsuka *et al.*, 1969). The following are the respective numbers of non-acid-producing strains among the 69 tested in liquid media: aspergillic acid ($N = 40$); kojic acid ($N = 32$); β-nitropropionic acid ($N = 48$); and oxalic acid ($N = 37$). Some strains of mold that proved to be good producers of aspergillic acid and kojic acid in liquid media did not produce these acids on a solid substrate composed of soybeans and wheat, at least not within the usual 2-day period required for koji cultivation. These tests confirmed that koji, or a mixture of soybeans and wheat cultured with these moles, does not contain a sufficient amount of these weak toxic compounds to constitute a hazard to humans who consume shoyu, even when it is prepared from koji cultured with the strongest acid producer of these toxic compounds among the strains tested.

Yokotsuka *et al.* (1977) were unable to detect aflatoxin, patulin, ochratoxin, or sterigmatocystin in the culture of *A. sojae*, which is used for shoyu production. Sasaki (1980) checked the ability of 33 kinds of industrial *Aspergillus* mold to produce aflatoxin, sterigmatocystin, ochratoxin, patulin, cyclopiazonic acid, and penicillic acid. None of the strains tested produced these compounds, with the exception of a very few strains which produced cyclopiazonic acid. Sasaki concluded that it is feasible to avoid mycotoxin contamination from a purely cultured starter mold if the strains which do not produce these mycotoxins are selected. Manabe *et al.* (1985) observed that some koji molds belonging to *A. oryzae* or *A. sojae* produced cyclopiazonic acid. Shinshi *et al.* (1985) found that cyclopiazonic acid added to salty shoyu mash was decomposed by *S. rouxii* or *Candida* (*Torulopsis*) *versatillis*, especially by the latter. They did not find shoyu on the market which contained cyclopiazonic acid.

According to Yokotsuka (1977), Kikkoman's koji culture of *A. sojae* does not contain patulin, ochratoxin, or sterigmatocystin. Attempts to detect lysinoalanine in fermented shoyu have been unsuccessful. Five lots of shoyu from Kikkoman's Wisconsin plant in the United State were analyzed for heavy metal. Arsenic, mercury, and selenium were not detected, lead and copper were found in trace amounts, and the figure for total heavy metals was less than 2 ppm.

Table 3. Cumulative mortality of mice given each experimental diet for 70 weeks

Experimental group No.	No. of mice dead at week												
	10	15	20	25	30	35	40	45	50	55	60	65	70
							Male						
I (11.0 % P-shoyu)	0	0	0	0	0	0	0	0	0	4	8	10	12
II (1.1 % P-shoyu)	0	1	2	2	2	2	2	3	4	4	6	8	12
III(0.11% P-shoyu)	0	0	0	0	2	5	6	6	6	6	6	9	10
IV (5.0 % NaCl)	0	2	2	3	4	6	6	7	8	9	12	14	14
V (0.5 % NaCl)	0	0	0	0	2	4	8	8	8	8	8	10	10
VI (Control)	0	0	0	0	0	0	0	1	2	6	8	11	12
							Female						
I (11.0 % P-shoyu)	0	0	0	2	6	6	6	9	12	14	16	16	18
II (1.1 % P-shoyu)	0	0	0	0	0	0	0	0	2	4	4	7	10
III(0.11% P-shoyu)	0	0	0	0	0	1	4	4	4	5	8	8	10
IV (5.0 % NaCl)	0	0	0	0	0	2	4	8	8	10	12	14	18
V (0.5 % NaCl)	2	2	2	2	2	2	2	2	4	4	4	5	8
VI (Control)	0	0	1	1	3	4	6	8	8	8	11	13	17

The figures are the total number of animals dead or killed in extremis from group of 40.

The figures are the total number of animals dead or killed in extremis from group of 32 after 30 weeks, as 8 mice of each group were killed at week 26.

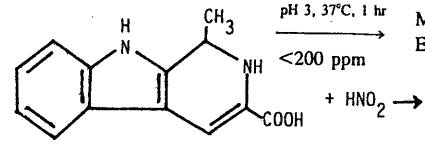
D. MUTAGENIC SUBSTANCES IN SHOYU

Although the noncarcinogenicity of fermented shoyu has long been known from long-term animal studies, the mutagenicity of heated products of amino acids or proteins such as Trip-P-1, Trip-P-2, Glu-P-1, and Glu-P-2 has been established more recently. Secliff and Mower (1977) reported that soy sauce produces mutagens upon the heating of glucose, galactose, and arabinose in shoyu.

Using the salmonella/mammalian microsome mutagenicity test, Lin *et al.* (1978) found that when treated with nitrite at 2000 ppm, soybean sauce produced a mutagenic substance. As fermented shoyu sometimes contains a small amount of amines (e.g. histamine and tyramine), the formation of mutagenic substances as a result of the reaction between amines and an abundance of nitrite is possible. Shibamoto (1983) mixed soy sauce with 100, 500, 1000, and 2000 ppm of sodium nitrite, adjusting pH at 3.0, and heated the mixture for 2 hr at 25°C and then for an additional 30 min at 80°C. Only at the highest concentration, 2000 ppm, was mutagenicity exhibited in the Ames test. Shibamoto concluded that the formation of nitrosamines may not be significant because the quantity of nitrite used in the study was excessive compared with actual food systesm. It is generally reported that the nitrite concentration remaining in the human stomach after a meal is estimated to be about 5 ppm, or about 15 ppm at most. It was also reported that just after ingestion of cured ham, the concentration in the stomach is about 70 ppm. According to Nagahara *et al.* (1984), shoyu itself did not represent mutagenicity. 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) decreased in a buffer solution when treated with more than 10 ppm of nitrite for 1 hr at 37°C and pH 3.0, but in shoyu, it decreased with more than 250 ppm of nitrite. Tyramine decreased in a buffer solution when treated with more than 50 ppm of nitrite for 1 hr at 37°C and pH 1.0, but in shoyu, it did not decrease even when treated with 2300 ppm of nitrite. Nagahori *et al.* (1980) reported that the addition of 5–7% fermented shoyu to a mixture of dimethylamine and nitrite at pH 3.6 suppressed the formation of *N*-nitrosodimethylamine by 60–80%. Moreover, the quantity of nitrosamine formation hindering substances in fermented shoyu increased with the advance of fermentation and aging of the mash. These substances were identified as the amino acids present in shoyu, which react more easily with nitrite than with dimethylamine. Ochiai *et al.* (1982) and Wakabayashi *et al.* (1983) isolated a nitrosable precursor of mutagens from shoyu. Its chemical structure was confirmed to be 1,2,3,4-tetrahydroharman-3-carboxylic acid (1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, MTCA). When this compound was treated with 3450 ppm of nitrite for 1 hr at 37°C and pH 3.0, the nitration product was strongly mutagenic to *Salmonella typhimurium* TA 100. Wakabayashi *et al.* (1983) determined the tyramine content of shoyu to be 17–

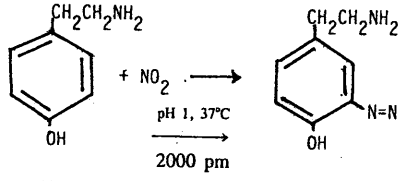
TABLE XLVI

NITROSABLE PREMUTAGENS ISOLATED FROM SHOYU



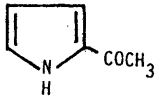
Mutagenic (*Salmonella tryphimurium* TA 100) Eighty percent of nitrosation product is not mutagenic. From Ochiai *et al.* (1982)

(−) − (1*R*,3*S*)-MTCA and its isomer
Content in shoyu is 0.03–0.7 ppm.



Reaction is one-third at pH 3. No reaction at pH 5–6. From Nagao *et al.* (1983)

Tyramine 3-Diazotyramine
Content in shoyu is 0–1000 ppm.



Yan and Lee (1984)

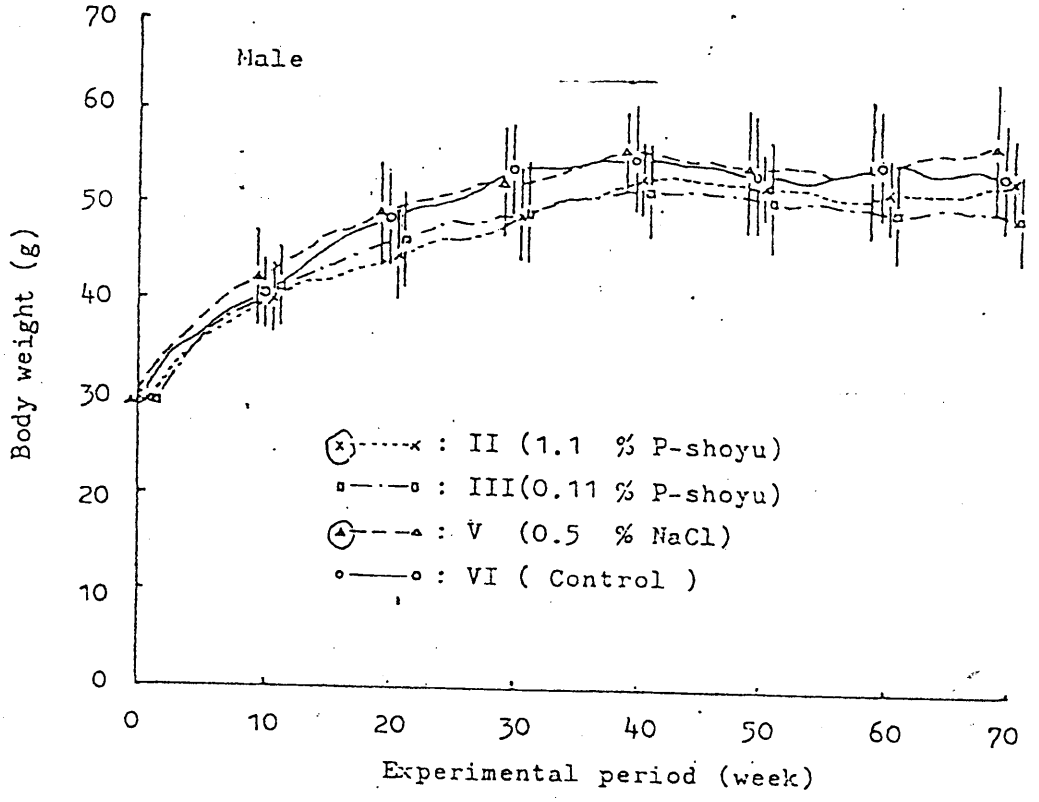
2-Acetylpyrrole
Content in shoyu is 3 ppm.

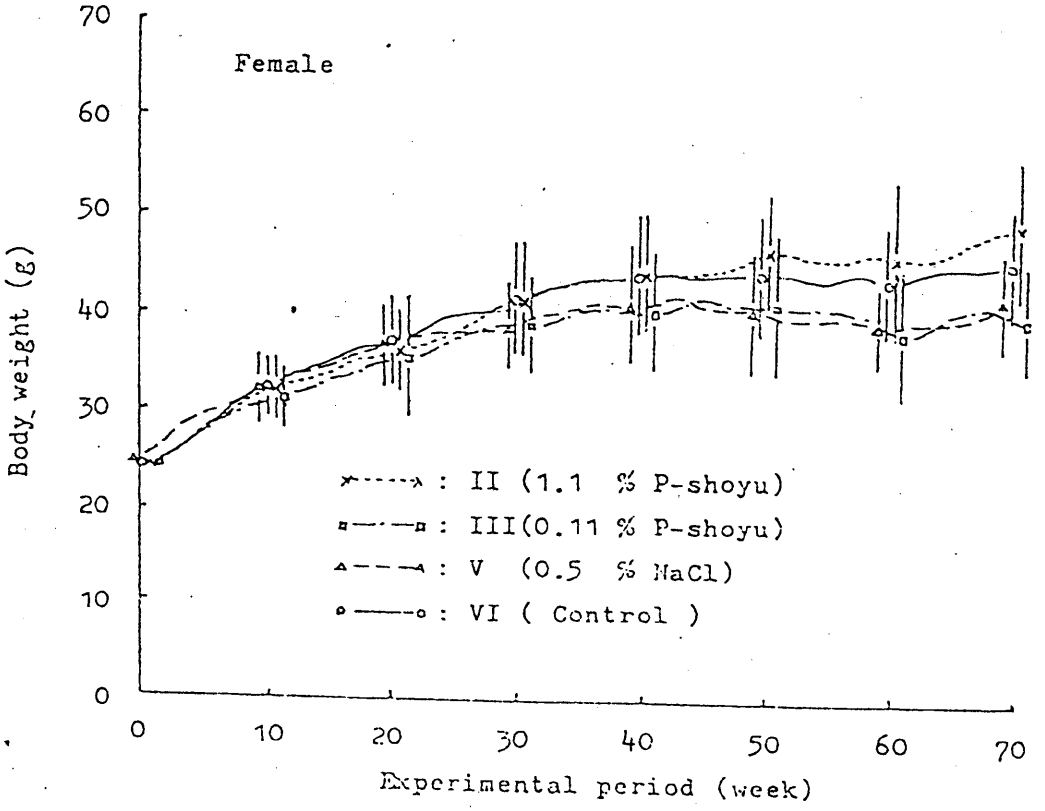
2250 ppm, and when tyramine was treated with 2300 ppm of nitrite for 1 hr at 37°C and pH 1.0, strong mutagenicity to TA 100 was observed. Yen and Lee (1984) isolated 2-acetylpyrrole as a nitrosable premutagen from shoyu. These are given in Table XLVI.

E. BACTERICIDAL ACTION OF SHOYU

Ujiie *et al.* (1956) identified the bactericidal nature of a commercial fermented shoyu with respect to nine kinds of intestinal pathogenic bacteria, such as *Escherichia coummunis, Shigella flexneria, Vibrio cholerate* Inaba, *Salmonella typhimurium* Shikata, *Bacillus subtilis* (B-31). The kinds of bacteria present were attributed to the acidity, high osmotic pressure, and some of the chemicals contained in the shoyu. To the sample, 0.005% of butyl-*p*-hydroxybenzoate was added as a preservative. Sakaguchi *et al.* (1975) tested the fate of staphylococci during incubation in a normal shoyu and in a milder shoyu, containing 17% (w/v) and 9% (w/v) of sodium chloride, respectively. No chemical preservatives

Fig. 2. Growth curve of mice given 1.1 % P-shoyu, 0.11 % P-shoyu, 0.5 % NaCl and control diets for 70 weeks (mean ± SD)





were added to either sample. The normal shoyu which initially contained 10^6 staphylococci per milliliter was nearly free of viable staphylococci within 3 hr. In the milder shoyu, over 90% of the cells were destroyed within 22–30 min, while in the normal shoyu, only 13–14 min were required. That sodium chloride contributes to the destruction of staphylococci in soy sauce is evident because the rate of killing in normal shoyu is greater than in milder shoyu. The fate of staphylococci in phosphate buffer saline solutions with a pH level of 4.7 containing 10 and 17% sodium chloride, respectively, was tested under the same conditions. The time taken to destroy over 90% of the cells in the 10% solution and in the 17% solution was 980–1440 min and 460–530 min, respectively. These results suggest the participation of some factor other than sodium chloride in the destruction of staphylococci in shoyu. The activity of *Clostridium botulinum* in shoyu was also tested during months of storage at 30°C. Neither *C. botulinum* 62A (Type A) nor *C. botulinum* Okre (Type B) grew during this time. The number of Type A spores remained the same, but those of Type B decreased slightly in number after the 3 months.

According to Yamanoto *et al.* (1978), the time needed for the total destruction of *Escherichia coli* 215 or *Staphylococcus aureus* 209P (ATCC 11522) inoculated in fermented shoyu was dependent upon the initial number of cells in these bacteria; 4–6 hr for 10^3/ml, 24–48 hr for 10^5/ml, and 5–7 hr for 10^7/ml. A high salt content was the dominant factor in accelerating the speed of sterilization; the pH value and amount of alcohol, total nitrogen, and ether-soluble compounds were judged to be supplementary factors.

F. BIOLOGICAL TESTS OF SHOYU

The long-term effects of Japanese shoyu (Kikkoman) on the gastric mucosa of intact rats and those with a fundasectomy were studied by MacDonald and Dueck (1976) in Canada. At the end of the test period, the animals that had been fed shoyu were smaller than the controls; the 15 intact rats that received the shoyu were healthier, more active, and lived 33 months longer than did the 7 controls. Breast tumors developed in 10 control rats, but in none of the experimental animals given shoyu. These findings suggest that shoyu does not appear to be carcinogenic in rats; its prolonged use impaired neither health nor longevity. Oshita *et al.* (1977) studied the acute and long-term effects of large amounts of Kikkoman shoyu on mice and rats. The acute toxicity of shoyu was attributed to the toxicity of its sodium chloride component. The oral LD_{50} values for shoyu were 20.6 ml/kg for rats and 27.3 mg/kg for mice. In long-term feeding tests (1.5 years for mice and 6 months for rats), the food intake of animals given a diet containing shoyu was otherwise comparable to that of the control group. This was true even for animals given a diet containing 10% powdered shoyu (corresponding to ~25% liquid shoyu). Although the animals that were fed shoyu were smaller than the controls, no significant differences in mortality were observed between the two groups. In addition, male rats given a diet containing 5% or 2% powdered shoyu grew faster than rats fed an equivalent amount of sodium chloride alone (i.e., diets containing 2.25% or 0.9% sodium chloride). At the highest dose level, 10% powdered shoyu, there were significant differences in the urinary systems of experimental and control animals. While both rats and mice developed enlarged kidneys and bladders, rats developed higher concentrations of in serum, and mice gave evidence of hydronephrosis after 1.5 years. The same effects were observed in animals who received sodium chloride in the same concentrations as those fed the highest level of shoyu. There was no indication of carcinogenic effects at any level of shoyu feeding.

VIII. RESEARCH NEEDS

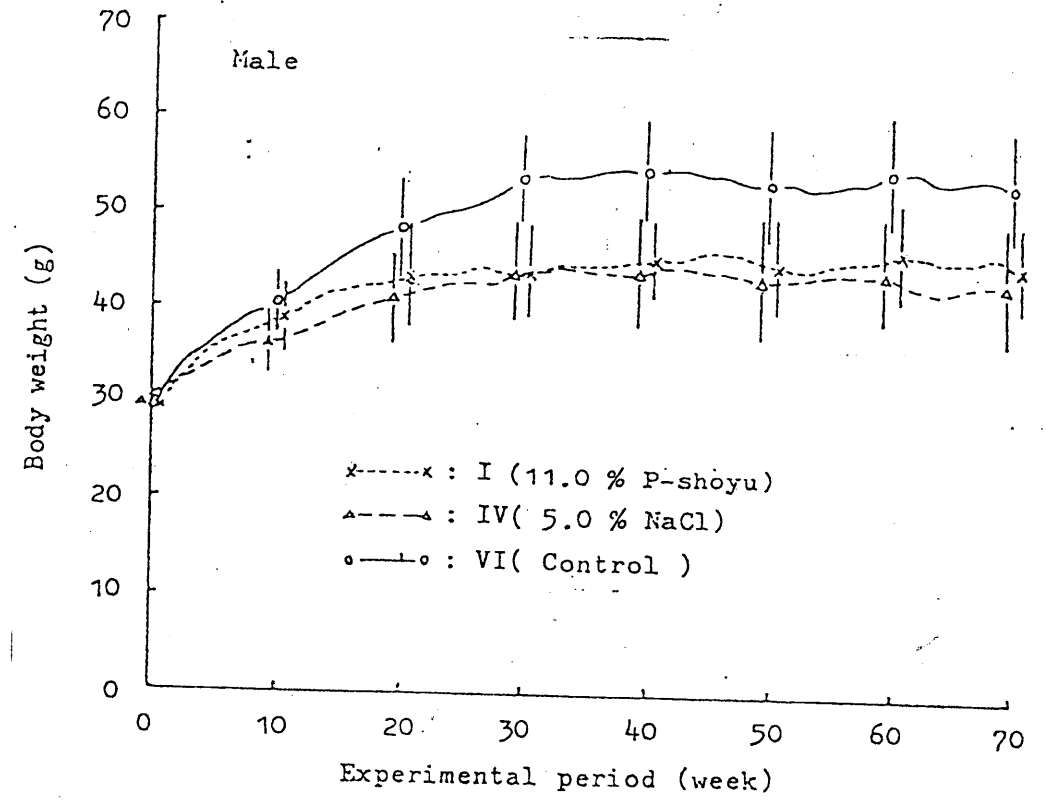
A. RAW MATERIALS

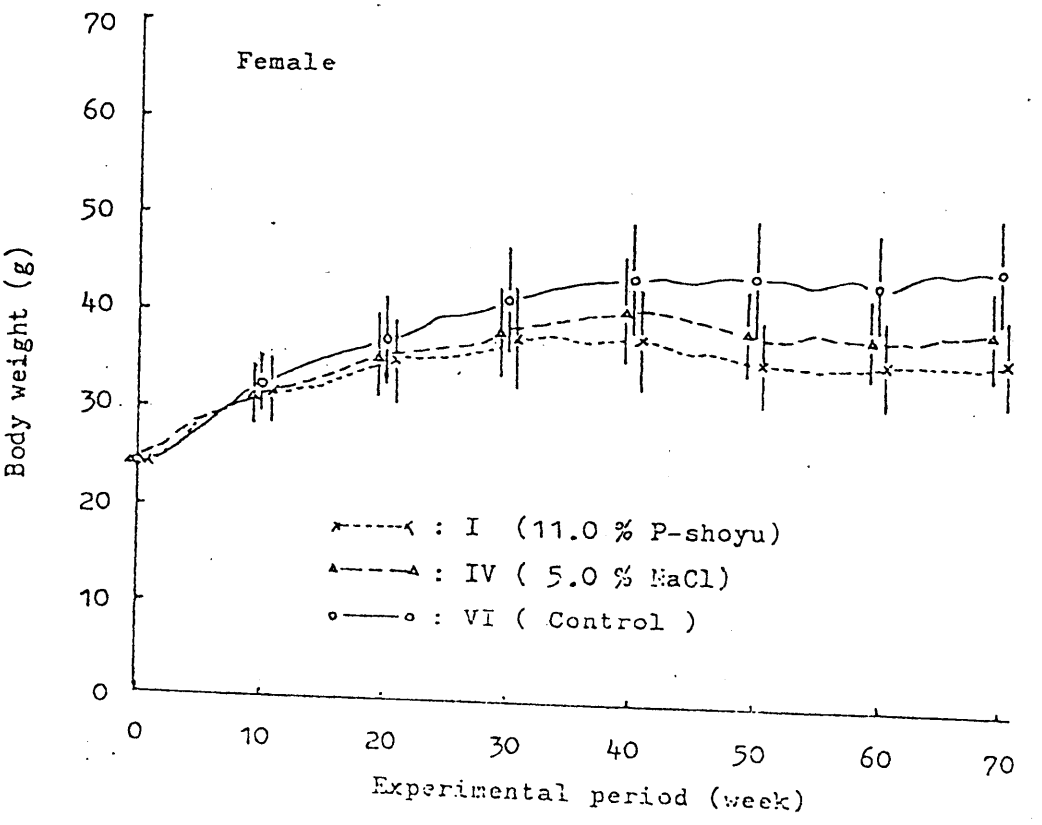
The precise mixture of soybeans and wheat used as the raw materials in shoyu production is the result of technological know-how developed over hundreds of years. But the shoyu-like seasonings can be prepared from a mixture of plant proteins and starches other than soybeans and wheat, and these are available worldwide. The by-products of oil pressing and extraction, such as peanut cake, copra meal, cottonseed meal, rapeseed protein, and sesame protein, have been experimentally substituted, with good results. Many kinds of mung beans also seem to have good potential. On the other hand, wheat kernel is considered to be the best starch raw material for shoyu, but barley, rye, oats, rice, and corn are sometimes used. The superiority of the wheat kernel lies in its high source of protein (glutamine) and its high concentration of glucosides which give koikuchi its destructive bran flavor. The use of a mixture of wheat bran and starches other than wheat, such as rice, corn, and potato, would be worth exploring for those nations that do not have wheat. Steaming and puffing corn kernels, mung beans, and other raw materials which have hard plant tissues or extruding these moistened powders may also give good results in shoyu preparation.

B. KOJI MOLDS

Although the amount of total nitrogen extracted from the raw materials for use in shoyu production has reached ~90%, the possibility of increased protein digestibility seems to exist. A systematic effort to identify better strains of koji mold which have a greater capability to produce protease and enzymes that

Fig. 1. Growth curve of mice given 11 % P-shoyu, 5 % NaCl and control diets for 70 weeks (mean ± SD)





degrade plant tissue in general is needed. Such strains of mold will not only increase protein digestibility, but will shorten the fermentation period and enhance the flavor of shoyu.

A strain of koji mold having enzymes which readily degrade plant tissue in the presence of high salt concentrations would reduce the viscosity of mash. If its viscosity could be reduced, shoyu mash could be press-filtered by a much simpler and more economic apparatus than is now in use.

The relationship between various kinds of koji mold and the volatile flavor constituents of shoyu should be studied. While it is clear that the volatile flavor of the final shoyu is influenced by the kind of koji mold used, its biochemical details are not yet known. To date, the selection of koji mold for this purpose has been conducted only by means of a sensory test.

C. REDUCTION OF FERMENTATION PERIOD OF MASH

Although the fermentation period of shoyu mash has been shortened by 6–8 months as a result of technological developments, a much greater reduction in time is necessary for economic reasons. The time needed for the enzymatic degradation of the raw materials and for lactic and yeast fermentation is about 3 months, but at least another 3 is required to complete the aging process. During these 3 months, the color deepens and the flavor develops fully. Gas-chromatographic patterns reveal that the major chemical changes that take place during the last 3 months of aging are mostly due to heat-dependent reactions. But the temperature cannot be raised too high to accelerate the aging process (e.g., to more than 35°C), as this generates an unpleasant odor. In order to shorten the fermentation period, the chemical changes occurring during the aging of shoyu mash must first be fully understood.

To shorten the early stage of mash fermentation, which usually takes 3 months, lactic and yeast fermentation can be accelerated by adding a sufficient number of pure cultured cells of lactobacilli or *Saccharomyces* yeast to the mash and increasing the temperature of the mash 30°C or more. The physiology of lactobacilli and shoyu yeasts in the presence of low and high salt concentrations should be studied, especially with respect to the chemical processes by which flavor develops.

If shoyu mash is enzymatically degraded at more than 50°C by using heat-tolerant strains of mold, no salt needs to be added to the mash, and degradation of the protein is completed within 1 or 2 days, yielding a mash whose protein is highly digestible. Caution should be taken not to reduce the free amino acid content, especially that of glutamic acid, by sapplying heat-tolerant peptidases such as glutaminase. To avoid the development of the unpleasant odor which develops when mash is subjected to high temperatures, the salt concentration can

be reduced to less than 10% and the temperature lowered to 40°C in order to complete the enzymatic degradation of starch and proteins with good yields. Thus, an enzymatic protein hydrolysate of good chemical composition in terms of amino acids and sugars can be produced. However, the enzymatic degradate must undergo lactic and yeast fermentation for ~3 months (comparable to the normal shoyu fermentation period) so that it acquires a shoyu-like flavor in a slurry or pasty condition of shoyu mash, but as for the liquid separated from the enzymatic degradate, lactic and yeast fermentations can be finished in about a week in batch-type fermentation tanks, or in 2 or 3 days when the liquid is passed through the columns packed with the immobilized cells of these microbes. Sensory quality improvements still need to be studied with regard to this method.

D. APPLICATION OF ENZYME PREPARATIONS

The solid koji method of shoyu manufacture seems to be the simplest, most economic, and best quality-producing method of degrading protein by the use of enzymes. Research efforts directed at substituting enzyme preparations from microbial sources other than koji molds have not yet succeeded. It is well established that the enzyme systems involved in koji molds are much better than those of *Rhizopus* and *Bacillus* with regard to their ability to decompose soybean protein in the presence of high salt concentrations. Moreover, the safety of koji molds has been proved by hundreds of years of consumer use. The search for better strains of mold for shoyu production should begin with the exploration of koji molds *A. oryzae* or *A. sojae*. Nevertheless, supplementing the enzyme systems of koji molds with other microbial sources would also help to increase the protein digestibility and amino acid content of shoyu and to reduce both the fermentation period and the viscosity of mash.

E. REFINING AND PASTEURIZATION

The heat-coagulant substances produced by pasteurization during the industrial manufacture of shoyu today pose a difficult problem. Centrifugation is not an effective means of removing these substances from shoyu because of the high gravity of shoyu which contains salt and other solids. Filtration with some aid, such as Celite, is the best method for clarifying the clear upper layer of heated shoyu after sedimentation. But it is very difficult to recover shoyu from the sediment layer which contains more than 95% shoyu after filtration centrifugation. A method using sedimentation-accelerating substances has been tried without success.

It has also been established that heat coagulation in pasteurized shoyu is positively correlated with the amount of protease in raw shoyu. In addition, the

Table 2. Proximate analyses of each experimental diet

Experimental group No.	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	NaCl (%)	N-free extract* (%)
I (11.0 % P-shoyu)	9.00	22.56	4.43	3.32	10.57	4.97	50.12
II (1.1 % P-shoyu)	9.09	20.86	4.89	3.43	5.68	0.56	56.05
III(0.11% P-shoyu)	9.13	20.85	4.35	3.17	5.35	0.11	57.15
IV (5.0 % NaCl)	9.08	20.38	4.43	3.27	10.56	4.90	52.26
V (0.5 % NaCl)	9.03	20.81	4.49	3.49	5.77	0.51	56.41
VI (Control)	8.97	20.81	4.45	3.22	5.33	0.15	57.22

*Nitrogen free extract

ongoing protein activity in shoyu is positively associated with the protease activity of koji, the alcohol content of shoyu mash which inhibits the protease activity, the period of fermentation of mash during which protease activity gradually decreases, and with the pH value of shoyu mash, which at lower levels inactivates protease. Consequently, some method of reducing the protease activity remaining in mash needs to be found. Decomposing or removing the precursor of heat coagulation in shoyu before pasteurization, keeping the mash or raw shoyu at higher temperatures, or subjecting raw shoyu to ultrafiltration have been tried.

F. FLAVOR

The volatile and nonvolatile flavor constituents of koikuchi shoyu are mostly products derived from the metabolism of raw materials by koji molds, lactobacilli, and yeasts, and from their mutual chemical reactions during the pasteurizing and aging of mash. Although it is difficult to make the koikuchi flavor stronger without increasing its color intensity, it would be interesting to try to produce the flavor compounds of koikuchi shoyu biochemically for the purpose of a wide application to food preparation other than shoyu, or to prepare a shoyu lighter in color, but with the characteristic koikuchi flavor, or to prepare a shoyu having the color of koikuchi, but weaker in taste.

There is also a need to develop a method for evaluating the quality of a shoyu from the contents of some key flavor components to supplement the current sensory test (see Table XLVII).

G. COLOR

Fermented shoyu consists of many kinds of enzymatic intermediate degradates of raw materials which are unstable under heat or oxidation and which react with each other. By comparison, the chemical hydrolysate of plant protein, to which almost all materials are ultimately degraded by strong hydrolysis with HCl at more than 100°C, is more stable under these conditions. The stabilization of fermented shoyu under heat and oxidation is therefore a very difficult but important problem.

One of the best ways to prevent fermented shoyu from deteriorating chemically is to convert it into a powdered form by dehydration. However, its low boiling point, labile chemical components, and nonvolatile hygroscopic components, such as glycerol and lactic acid, make dehydration a difficult research problem.

TABLE XLVII

FLAVOR COMPONENTS FOUND IN SHOYU

Compound	Molecular weight	Molecular formula	Reference number[a]
I. Hydrocarbons (37)			
1. Benzene	78	C_6H_6	1
2. Toluene	92	C_7H_8	1
3. Styrene	104	C_8H_8	1
4. *o*-Xylene	106	C_8H_{10}	1
5. *m*-Xylene	106	C_8H_{10}	1
6. *p*-Xylene	106	C_8H_{10}	1
7. Ethylbenzene	106	C_8H_{10}	1
8. Mesitylene	120	C_9H_{12}	1
9. 1,2,3-Trimethylbenzene	120	C_9H_{12}	1
10. 1,2,4-Trimethylbenzene	120	C_9H_{12}	1
11. 1-Ethyl-2-methylbenzene	120	C_9H_{12}	1
12. Cumene	120	C_9H_{12}	1
13. Naphthalene	128	$C_{10}H_8$	1
14. 4-Methylindan	132	$C_{10}H_{12}$	1
15. 5-Methylindan	132	$C_{10}H_{12}$	1
16. 1,2,3,4-Tetrahydronaphthalene	132	$C_{10}H_{12}$	2
17. 1-Ethyl-2,3-dimethylbenzene	134	$C_{10}H_{14}$	1
18. 1-Ethyl-2,4-dimethylbenzene	134	$C_{10}H_{14}$	1
19. 1-Ethyl-3,5-dimethylbenzene	134	$C_{10}H_{14}$	1
20. 2-Ethyl-1,3-dimethylbenzene	134	$C_{10}H_{14}$	1
21. 2-Ethyl-1,4-dimethylbenzene	134	$C_{10}H_{14}$	1
22. 2-Ethyl-1,2-dimethylbenzene	134	$C_{10}H_{14}$	1
23. 1-Methyl-2(or 4)-propylbenzene	134	$C_{10}H_{14}$	1
24. 1,2,3,5-Tetramethylbenzene	134	$C_{10}H_{14}$	1
25. 1,2,4,5-Tetramethylbenzene	134	$C_{10}H_{14}$	1
26. 1,2-Diethylbenzene	134	$C_{10}H_{14}$	1
27. 1,3-Diethylbenzene	134	$C_{10}H_{14}$	1
28. 1,4-Diethylbenzene	134	$C_{10}H_{14}$	1
29. Butylbenzene	134	$C_{10}H_{14}$	1
30. Cyclohexylcyclohexane	134	$C_{10}H_{14}$	1
31. 1-Methylnaphthalene	142	$C_{11}H_{10}$	1
32. 2-Methylnaphthalene	142	$C_{11}H_{10}$	1
33. 2,3,5(or 6)-Trimethylnaphthalene	170	$C_{13}H_{14}$	1
34. Tetradecane	198	$C_{14}H_{30}$	1
35. Pentadecane	212	$C_{15}H_{32}$	1
36. Hexadecane	226	$C_{16}H_{34}$	1
37. 5-Phenyldodecane	246	$C_{18}H_{30}$	1
II. Alcohols (30)			
1. Methanol	32	CH_4O	2, 3
2. Ethanol	46	C_2H_6O	

(*continued*)

Table 1. Experimental groups and dose levels of samples in each experimental diet

Experimental group No.	Sample	Dose levels* (Sample in diet) (%)
I	P-shoyu	11.0**
II	P-shoyu	1.1
III	P-shoyu	0.11
IV	NaCl	5.0***
V	NaCl	0.5
VI	(Control)	0

*Dose levels were decided on the basis of the mean value of daily intake of shoyu in Japanese citizens.

**Diet containing 11 % P-shoyu was worth 100 times as much as the mean value.

***Same NaCl concentration as diet containing 11 % P-shoyu.

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
II. Alcohols (30) (*continued*)			
3. 1-Propen-3-ol	58	C_3H_6O	4
4. 2-Propen-1-ol (allyl alcohol)	58	C_3H_6O	9
5. 1-Propanol	60	C_3H_8O	2
6. 2-Propanol	60	C_3H_8O	5, 6
7. 2-Methyl-1-propanol	74	$C_4H_{10}O$	5, 3
8. 1-Butanol	74	$C_4H_{10}O$	2
9. 2-Methyl-2-buten-1-ol	86	$C_5H_{10}O$	1
10. 1-Penten-3-ol	86	$C_5H_{10}O$	2
11. 3-Penten-2-ol	86	$C_5H_{10}O$	1
12. 2-Methyl-1-butanol	88	$C_5H_{12}O$	2, 7
13. 3-Methyl-1-butanol	88	$C_5H_{12}O$	2, 3
14. 1-Pentanol	88	$C_5H_{12}O$	2
15. 3-Pentanol	88	$C_5H_{12}O$	2
16. 3-Buten-1,2-diol	88	$C_4H_8O_2$	1
17. 2-Ethoxyethanol	90	$C_4H_{10}O_2$	8
18. L-2,3-Butanediol	90	$C_4H_{10}O_2$	2, 10, 11
19. *meso*-2,3-Butanediol	90	$C_4H_{10}O_2$	2, 10, 11
20. (*E*)-2-Hexen-1-ol	100	$C_6H_{12}O$	8
21. 1-Hexanol	102	$C_6H_{14}O$	3
22. Benzyl alcohol	108	C_7H_8O	5
23. 2,3-Dimethyl-2-pentanol	116	$C_7H_{16}O$	1
24. 2,4-Dimethyl-3-pentanol	116	$C_7H_{16}O$	1
25. 3-Methyl-3-hexanol	116	$C_7H_{16}O$	1
26. 2-Phenylethanol	122	$C_8H_{10}O$	5, 12
27. 1-Octen-3-ol	128	$C_8H_{16}O$	8
28. 5-Nonanol	144	$C_9H_{20}O$	1
29. 2-Phenyl-1-butanol	150	$C_{10}H_{14}O$	1
30. 2-Undecanol	172	$C_{11}H_{24}O$	3
III. Esters (41)			
1. Methyl acetate	74	$C_3H_6O_2$	4
2. Ethyl formate	74	$C_3H_6O_2$	13, 4
3. Ethyl acetate	88	$C_4H_8O_2$	5, 14, 15
4. 2-Oxopropyl acetate (acetol acetate)	100	$C_5H_8O_2$	2
5. Ethyl propionate	102	$C_5H_{10}O_2$	2
6. Butyl formate	102	$C_5H_{10}O_2$	8
7. 1-Methylpropyl acetate	116	$C_6H_{12}O_2$	2
8. 2-Methylpropyl acetate	116	$C_6H_{12}O_2$	1
9. Butyl acetate	116	$C_6H_{12}O_2$	6
10. Ethyl 2-hydroxypropanoate (ethyl lactate)	118	$C_5H_{10}O_3$	5, 16
11. 3-Methylbutyl acetate	130	$C_7H_{14}O_2$	17, 5
12. Pentyl acetate	130	$C_7H_{14}O_2$	18

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
III. Esters (41) (*continued*)			
13. 2-Methylpropyl propionate	130	$C_7H_{14}O_2$	1
14. Ethyl 3-methylbutanoate	130	$C_7H_{14}O_2$	19, 20
15. Ethyl 2-methylbutanoate	130	$C_7H_{14}O_2$	9
16. Ethyl pentanoate	130	$C_7H_{14}O_2$	17
17. 2-Ethyoxyethyl acetate	132	$C_6H_{12}O_3$	2
18. Ethyl 4-oxopentanoate (ethyl levulinate)	144	$C_7H_{12}O_3$	6
19. Ethyl hexanoate (ethyl caproate)	144	$C_8H_{16}O_2$	19
20. Diethyl oxalate	146	$C_6H_{10}O_4$	48
21. 2-Phenylethyl formate	150	$C_9H_{10}O_2$	1
22. Ethyl benzoate	150	$C_9H_{10}O_2$	5, 19, 20
23. Diethyl malonate	160	$C_7H_{12}O_4$	6
24. 2-Phenylethyl acetate	164	$C_{10}H_{12}O_2$	5
25. Ethyl phenylacetate	164	$C_{10}H_{12}O_2$	5
26. 3-Methylbutyl 3-methylbutanoate	172	$C_{10}H_{20}O_2$	17
27. Ethyl octnoate (ethyl caprylate)	172	$C_{10}H_{20}O_2$	49
28. Diethyl maleate	172	$C_8H_{12}O_4$	6
29. Diethyl succinate	174	$C_8H_{14}O_4$	5, 6
30. Ethyl 3-phenylpropenoate (ethyl cinnamate)	176	$C_{11}H_{12}O_2$	1
31. Pentyl hexanoate (amyl caproate)	186	$C_{11}H_{22}O_2$	17
32. Ethyl nonanoate (ethyl pelargonate)	186	$C_{11}H_{22}O_2$	17
33. 2-Phenylethyl butanoate	192	$C_{12}H_{16}O_2$	1
34. 4-Formyl-2-methoxyphenyl acetate (vanillin acetate)	194	$C_{10}H_{10}O_4$	2
35. Ethyl 4-hydroxy-3-methoxybenzoate (ethyl vanillate)	196	$C_{10}H_{12}O_4$	2, 7
36. Ethyl dodecanoate (ethyl laurate)	228	$C_{14}H_{28}O_2$	17
37. Ethyl tetradecanoate (ethyl myristate)	256	$C_{16}H_{32}O_2$	17, 5
38. Ethyl hexadecanoate (ethyl palmitate)	284	$C_{18}H_{36}O_2$	21, 22
39. Ethyl 9,12-octadecadienoate (ethyl linoleate)	308	$C_{20}H_{36}O_2$	22
40. Ethyl 9-octadecenoate (ethyl oleate)	310	$C_{20}H_{38}O_2$	22
41. Ethyl octadecanoate (ethyl stearate)	312	$C_{20}H_{40}O_2$	21
IV. Aldehydes (15)			
1. Acetaldehyde	44	C_2H_4O	5, 14, 23, 24
2. Propanal	58	C_3H_6O	5, 24
3. 2-Methylpropanal	72	C_4H_8O	5, 25, 24
4. Butanal	72	C_4H_8O	24, 26
5. 2-Methylbutanal	86	$C_5H_{10}O$	1
6. 3-Methylbutanal	86	$C_5H_{10}O$	5, 14, 25, 24
7. Pentanal	86	$C_5H_{10}O$	27
8. Hexanal	100	$C_6H_{12}O$	17, 2

(*continued*)

in other groups and in these mice caused polydipsia and polyuria by the excessive intake of sodium chloride (13). Higher values for relative weights of heart and kidneys in both sexes given 11% P-shoyu or 5% sodium chloride were considered to relate to the slightly higher incidence of myocardial fibrosis or hydronephrosis which appear to be produced by the excessive intake of sodium chloride. The decreases of weights and relative weights of thyroid in males given 11% P-shoyu were considered to relate to the lowered body weight. The decreases of stomach weights in males given 5% sodium chloride and thyroid weights in females given 11% P-shoyu were regarded as related to the lowered body weight in these animals as evidenced by the disappearance of any significant differences when the weights were expressed relative to body weight.

Most of the tumours encountered occurred with a similar or higher frequency in control than in treated mice. It is clear that there was no indication of carcinogenic effect at any of the levels of treatment. This is in agreement with MacDonald and Dueck who found no tumours induced in rats fed shoyu for 29 months (14).

No adverse effects, other than an enlargement of the bladder, kidneys, heart and a reduction in body weight gain (that were attributed to sodium chloride in shoyu) were seen in mice given shoyu corresponding to the amounts of 100 times the daily intake of Japanese citizens.

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
IV. Aldehydes (15) (*continued*)			
9. Benzaldehyde	106	C_7H_6O	5, 20
10. 2,3-Dihydro-4*H*-pyran-2-carbaldehyde	112	$C_6H_8O_2$	1
11. Phenylacetaldehyde	120	C_8H_8O	5
12. 3-Phenyl-2-propenal (cinnamaldehyde)	132	C_9H_8O	17
13. 2,5-Dimethyl-2,3-dihydro-5*H*-pyran-2-carbaldehyde	140	$C_8H_{12}O_2$	2
14. 2-Methyl-3-phenyl-2-propenal (α-methylcinnamaldehyde)	146	$C_{10}H_{10}O$	1
15. 4-Hydroxy-3-methoxybenzaldehyde (vanillin)	152	C_8H_8O	3, 26
V. Acetals (4)			
1. 1,1-Diethoxyethane	118	$C_6H_{14}O_2$	2, 28
2. 1,1-Diethoxy-3-methylbutane	160	$C_9H_{20}O_2$	29
3. 1,1-Diethoxy-2-methylbutane	160	$C_9H_{20}O_2$	9
4. 1,1-Diethoxy-4-methyl-2-pentanol	190	$C_{10}H_{22}O_3$	29
VI. Ketones (19)			
1. Acetone	58	C_3H_6O	5
2. 2-Butanone	72	C_4H_8O	1
3. Hydroxyacetone (acetol)	74	$C_3H_6O_2$	2
4. 2,3-Butanedione (diacetyl)	86	$C_4H_6O_2$	2, 30
5. 3-Hydroxy-2-butanone (acetoin)	88	$C_3H_8O_2$	5, 14, 11
6. 2-Cyclohexin-1-one	96	C_6H_8O	33
7. 4-Methyl-3-penten-2-one	98	$C_6H_{10}O$	1
8. 4-Methyl-2-pentanone	100	$C_6H_{12}O$	1
9. 2-Hexanone	100	$C_6H_{12}O$	5
10. 2,3-Pentanedione	100	$C_5H_8O_2$	2, 30
11. 3-Hydroxy-2-pentanone	102	$C_5H_{10}O$	31
12. 2-Hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene)	112	$C_6H_8O_2$	2
13. 5-Methyl-2-hexanone	114	$C_7H_{14}O$	1
14. 2,3-Hexanedione	114	$C_6H_{10}O_2$	5, 30
15. Acetophenone	120	C_8H_8O	2
16. 3-Octanone	128	$C_8H_{16}O$	2
17. 2,6-Dimethyl-4-heptanone	142	$C_9H_{18}O$	1
18. 2-Methyl-3-octanone	142	$C_9H_{18}O$	1
19. 3-Methyl-3-decen-2-one	168	$C_{11}H_{20}O$	33
VII. Acids (24)			
1. Formic acid	46	CH_2O_2	19
2. Acetic acid	60	$C_2H_4O_2$	5, 32, 35
3. Propionic acid	74	$C_3H_6O_2$	2, 19
4. (*E*)-2-Butenoic acid (crotonic acid)	86	$C_4H_6O_2$	2
5. 2-Methylpropanoic acid (*iso*-butyric acid)	88	$C_4H_8O_2$	17, 5

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
VII. Acids (24) (*continued*)			
6. Butanoic acid (*sec*-butyric acid)	88	$C_4H_8O_2$	5, 19
7. 2-Oxopropanoic acid (pyruvic acid)	88	$C_3H_4O_3$	24
8. 2-Hydroxypropanoic acid (lactic acid)	90	$C_3H_6O_3$	17
9. 2-Methyl-2-butenoic acid	100	$C_5H_8O_2$	2
10. 2-Methylbutanoic acid	102	$C_5H_{10}O_2$	2
11. 3-Methylbutanoic acid	102	$C_5H_{10}O_2$	5, 19
12. Pentanoic acid (*n*-valeric acid)	102	$C_5H_{10}O_2$	17, 2
13. 2-Oxobutanoic acid (2-ketobutyric acid)	102	$C_4H_6O_3$	34
14. 4-Methylpentanoic acid	116	$C_6H_{12}O_2$	17, 2
15. Hexanoic acid (caproic acid)	116	$C_6H_{12}O_2$	2, 19
16. 4-Oxopentanoic acid (levulinic acid)	116	$C_5H_8O_3$	17
17. Butanedioic acid (succinic acid)	118	$C_4H_6O_4$	17
18. Benzoic acid	122	$C_7H_6O_2$	5, 19
19. Phenylacetic acid	136	$C_8H_8O_2$	17, 2, 19
20. Octanoic acid (caprylic acid)	144	$C_8H_{16}O_2$	17
21. Dodecanoic acid (lauric acid)	200	$C_{12}H_{24}O_2$	17
22. Hexadecanoic acid (palmitic acid)	254	$C_{16}H_{32}O_2$	35, 36, 22
23. 9,12-Octadecadienoic acid (linoleic acid)	280	$C_{18}H_{32}O_2$	22
24. 9-Octadecenoic acid (oleic acid)	282	$C_{18}H_{34}O_2$	22
VIII. Phenols (17)			
1. Phenol	94	C_6H_6O	2
2. 1,2-Benzenediol (pyrocatechol)	110	$C_6H_6O_2$	2
3. 4-Vinylphenol	120	C_8H_8O	33
4. 4-Ethylphenol	122	$C_8H_{10}O$	5, 12
5. 2-Methoxyphenol (guaiacol)	124	$C_7H_8O_2$	5
6. 4-(2-Hydroxyethyl)phenol (tyrosol)	138	$C_8H_{10}2$	37
7. 4-Ethyl-1,3-benzenediol (4-ethylresorcinol)	138	$C_8H_{10}2$	1
8. 4-Hydroxybenzoic acid	138	$C_7H_6O_3$	12
9. 2-Methoxy-5-vinylphenol	150	$C_9H_{10}2$	2
10. 4-Ethyl-2-methoxyphenol (4-ethylguaiacol)	152	$C_9H_{12}2$	5, 35
11. 2,6-Dimethoxyphenol	154	$C_8H_{10}3$	5
12. 3,4-Dihydroxybenzoic acid (protocatechuic acid)	154	$C_7H_6O_4$	19
13. 3-(4-Hydroxyphenyl)propenoic acid (*p*-coumaric acid), (*p*-hydroxycinnamic acid)	164	$C_9H_8O_3$	12
14. 4-Hydroxy-3-methoxyacetophenone (acetovanillon)	166	$C_9H_{10}3$	2, 19
15. 4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	168	$C_8H_8O_4$	35, 38

(*continued*)

reduction in body weight gain was clearly due to the excessive intake of sodium chloride.

Generally, the deaths of mice for up to the first 30 weeks of the study were attributed to pulmonary infection or inflammation that was found at autopsy. These were probably not related to treatment. After 30 weeks, although there were no marked differences among the groups in mortality, the tendency of slightly higher mortality in mice given shoyu or sodium chloride at the highest dose level was observed; compared with the other groups, it was clear in females. This was attributed to severe hydronephrosis and cachectic edema and was due to the excessive intake of sodium chloride (12). In the other groups, the general anemia, pulmonary failures and enlargements of thymus, spleen and various lymph nodes (probably lymphoma or leukemia) were found at autopsy in the mice which died during the study. These lesions were probably not related to treatment.

The increases of both the weights and relative organ weights of urinary bladder in both sexes fed the diet containing 11% P-shoyu and 5% sodium chloride were histopathologically accounted for by the incidence of dilatation and hypertrophy in these groups. These lesions were considered to have occurred by work hypertrophy, because the water intakes in these mice were 30-60 ml/mouse/day, these values were 5-8 times of those

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
VIII. Phenols (17) (*continued*)			
16. 3-(4-Hydroxy-3-methoxyphenyl) propenoic acid (ferulic acid)	194	$C_{10}H_{10}O_4$	39
17. 4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid)	198	$C_9H_{10}O_5$	39
IX. Furans (16)			
1. Furan	68	C_4H_4O	4
2. 2-Methylfuran	82	C_5H_6O	4
3. 2-Furfural	96	$C_5H_4O_2$	5, 14, 40
4. Furfuryl alcohol	98	$C_5H_6O_2$	5, 41
5. Tetrahydrofurfuryl alcohol	102	$C_5H_{10}O_2$	2
6. 1-(2-Furyl)-1-ethanone (2-furyl methyl ketone)	110	$C_6H_6O_2$	8, 5
7. 5-Methyl-2-furfural	110	$C_6H_6O_2$	2
8. 1-(2-Tetrahydrofuryl)-1-ethanone (2-tetrahydrofuryl methyl ketone)	114	$C_6H_{10}O_2$	2
9. 1-(2-Furyl)-1-propanone (ethyl 2-furyl ketone)	124	$C_7H_8O_2$	1
10. 2-furfuryl acetate	126	$C_6H_6O_3$	5
11. 1-(3-Hydroxy-2-furyl)-1-ethanone (isomaltol)	126	$C_6H_6O_3$	2
12. 5-Hydroxymethyl-2-furfural	126	$C_6H_6O_3$	42
13. 1-(2,5-Dimethyl-3-furyl)-1-ethanone	138	$C_8H_{10}O_2$	1
14. Ethyl 2-furoate	140	$C_7H_8O_3$	1
15. 3-Phenylfuran	144	$C_{10}H_8O$	1
16. 2-Propenyl 2-furoate	152	$C_8H_8O_3$	2
X. Lactones (4)			
1. 4-Butanolide	86	$C_4H_6O_2$	2
2. 2-Penten-4-olide [5-methyl-2(5*H*)-furanone] (β-angelicalactone)	98	$C_5H_6O_2$	2
3. 2-Methyl-4-butanolide	100	$C_5H_8O_2$	2
4. 4-Pentanolide	100	$C_5H_8O_2$	5
XI. Furanones (6)			
1. 3-Methyl-2(5*H*)-furanone	98	$C_5H_6O_2$	33
2. 2-Methyl-3-tetrahydrofuranone	100	$C_5H_8O_2$	5
3. 4-Hydroxy-5-methyl-3(2*H*)-furanone	114	$C_5H_6O_3$	43
4. 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	128	$C_6H_8O_3$	2
5. 4,5-Dihydro-5-(1-hydroxyethyl)-2(3*H*) furanone	130	$C_6H_{10}O_3$	33
6. 4-Hydroxy-2(or 5)-ethyl-5-(or 2)-methyl-3(2*H*)-furanone	142	$C_7H_{10}O_3$	4

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
XII. Pyrones (5)			
1. 3-Hydroxy-2-methyl-4*H*-pyran-4-one (maltol)	126	$C_6H_6O_3$	5, 13
2. 5-Hydroxy-2-methyl-4*H*-pyran-4-one	126	$C_6H_6O_3$	2
3. 3-Methoxy-2-methyl-4*H*-pyran-4-one	140	$C_7H_8O_3$	2
4. 3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one	142	$C_6H_6O_4$	2
5. 3,5-Dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one	144	$C_6H_8O_4$	2
XIII. Pyrazines (27)			
1. Pyrazine	80	$C_4H_4N_2$	28
2. 2-Methylpyrazine	94	$C_5H_6N_2$	5, 28
3. 2,3-Dimethylpyrazine	108	$C_6H_8N_2$	5, 28
4. 2,5-Dimethylpyrazine	108	$C_6H_8N_2$	28
5. 2,6-Dimethylpyrazine	108	$C_6H_8N_2$	5, 28
6. 2-Ethylpyrazine	108	$C_6H_8N_2$	28
7. 5*H*-Cyclopenta[*b*]pyrazine	118	$C_7H_6N_2$	28
8. 2-Methyl-6-vinylpyrazine	120	$C_7H_8N_2$	2
9. 6,7-Dihydro-5*H*-cyclopenta[*b*]pyrazine	120	$C_7H_8N_2$	28
10. 2,3,5-Trimethylpyrazine	122	$C_7H_{10}N_2$	28
11. 2-Ethyl-5-methylpyrazine	122	$C_7H_{10}N_2$	28
12. 2-Ethyl-6-methylpyrazine	122	$C_7H_{10}N_2$	5, 28
13. 2(or 3)-Methyl-5*H*-cyclopenta[*b*]-pyrazine	132	$C_8H_8N_2$	28
14. 6-Methyl-5*H*-cyclopenta[*b*]pyrazine	132	$C_8H_8N_2$	28
15. 7-Methyl-5*H*-cyclopenta[*b*]pyrazine	132	$C_8H_8N_2$	28
16. Pyrrolo[1,2-*a*]-3-methylpyrazine	132	$C_8H_8N_2$	33
17. 2-Methyl-6,7-dihydro-5*H*-cyclopenta[*b*]-pyrazine	134	$C_8H_{10}N_2$	28
18. Tetramethylpyrazine	136	$C_8H_{12}N_2$	5, 28
19. 3-Ethyl-2,5-dimethylpyrazine	136	$C_8H_{12}N_2$	5, 28
20. 2,3-Diethylpyrazine	136	$C_8H_{12}N_2$	28
21. 2,6-Diethylpyrazine	136	$C_8H_{12}N_2$	28
22. 2(or 3),6(or 7)-Dimethyl-5*H*-cyclopenta[*b*]pyrazine	146	$C_9H_{10}N_2$	28
23. Pyrrolo[1,2-*a*]-3,4-dimethylpyrazine	146	$C_9H_{10}N_2$	33
24. 2-Ethyl-6,7-dihydro-5*H*-cyclopenta[*b*]-pyrazine	148	$C_9H_{12}N_2$	28
25. 2-Ethyl-3,5,6-trimethylpyrazine	150	$C_9H_{14}N_2$	28
26. 2,6-Diethyl-3-methylpyrazine	150	$C_9H_{14}N_2$	28
27. 2,3,5-Trimethyl-6,7-dihydro-5*H*-cyclopenta[*b*]pyrazine	162	$C_{10}H_{14}N_2$	28

(*continued*)

of all other lesions were almost similar in all groups within each sex, and many of the lesions were chronic inflammation or degenerative changes that normally are found in ageing mice.

The tumours were found in the lungs, liver, adrenals, testes, prostate, ovaries, uterus and hemo-lymphopoietic system. The main tumours were pulmonary adenoma, hepatoma (probably benign) and leukemia or lymphoma. A survey of the tumour incidences of all the mice (including animals that died during the study) is summarized in Table 8. The incidences of these tumours were similar in all groups within each sex except hepatoma in males. The incidence of hepatoma in males given 11% P-shoyu or 5% sodium chloride was lower than in controls. The other tumours found in the study were adrenal adenoma, testicular interstitial-cell tumour, prostatic adenoma, ovarian cystoma or hematoma, uterine myoma and others. These tumours were present with a similar incidence in all groups within each sex.

Discussion

At the highest dose level of shoyu or sodium chloride (11% P-shoyu or 5% sodium chloride), a significant reduction in body weight gain was observed when compared with the controls in both sexes; however, there were no significant differences between the mice on shoyu and those on sodium chloride. The

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
XIV. Pyridines (7)			
1. Pyridine	79	C_5H_5N	28
2. 3-Methylpyridine	93	C_6H_7N	28
3. 2,6-Dimethylpyridine	107	C_7H_9N	28
4. 2-Ethylpyridine	107	C_7H_9N	28
5. 2-Pyridylmethanol	109	C_6H_7NO	1
6. 3-Methoxypyridine	109	C_6H_7NO	28
7. Ethyl 3-pyridinecarboxylate (ethyl nicotinate)	151	$C_8H_9NO_2$	28
XV. Miscellaneous nitrogen-containing compounds (8)			
1. 1-Methyl-2-pyrrolidinone	99	C_5H_9NO	1
2. 1-(2-Pyrrolyl)-1-ethanone	109	C_6H_7NO	8, 5, 28
3. Benzoxazole	119	C_7H_5NO	28
4. 1-(5-Methyl-2-pyrrolyl)-1-ethanone	123	C_7H_9NO	1
5. 1,5-Dimethyl-2-pyridone	123	C_7H_9NO	1
6. 2-Methylbenzoxazole	133	C_8H_7NO	2, 28
7. Ethyl 2-pyrrolidone-5-carboxylate	157	$C_7H_{11}NO_3$	33
8. Ethyl 2-(acetylamino)-4-methyl-pentanoate (*N*-acetylleucine ethyl ester)	201	$C_{10}H_{19}NO_3$	33
XVI. Sulfur-containing compounds (16)			
1. Hydrogen sulfide	34	H_2S	46
2. Methanethiol	48	CH_4S	29
3. Dimethyl sulfide	62	C_2H_6S	4
4. Ethanethiol	62	C_2H_6S	24
5. 2-Propene-1-thiol	74	C_3H_6S	17
6. Thiophene	84	C_4H_4S	33
7. Dimethyl disulfide	94	$C_2H_6S_2$	2
8. 4-Methyl-1,3-oxathiolane	104	C_4H_8OS	33
9. 3-Methylthiopropanal (methional)	104	C_4H_8OS	46
10. 3-(Methylthio)-1-propanal (methionol)	106	$C_4H_{10}OS$	5, 16, 47
11. Phenylmethanethiol	124	C_7H_8S	1
12. Dimethyl trisulfide	126	$C_2H_6S_3$	1
13. 3,4-Dimethyl-2,5-dihydrothiophen-2-one	128	C_6H_8OS	33
14. 2-Ethyl-6-methyl-1,3-oxathiane	146	$C_7H_{14}OS$	1
15. 3-(Methylthio)propyl acetate	148	$C_6H_{12}O_2S$	1
16. 1,1-Bis(methylthio)-2-methylpropane	150	$C_6H_{14}S_2$	24
XVII. Thiazoles (4)			
1. 2-Ethoxythiazole	129	C_5H_7NOS	1
2. 2-Butoxythiazole	157	$C_7H_{11}NOS$	1
3. *N*-Acetyl-1*H*-benzothiazol	179	C_9H_9NOS	33
4. 2-(Methylthio)benzothiazole	181	$C_8H_7NS_2$	1
XVIII. Terpenes (3)			
1. Borneol	154	$C_{10}H_{18}O$	5

TABLE XLVII (*Continued*)

Compound	Molecular weight	Molecular formula	Reference number[a]
XVIII. Terpenes (3) (*continued*)			
2. 4-Methyl-2-(2-methyl-1-propenyl)-tetrahydropyran (*cis*-rose oxide)	154	$C_{10}H_{18}O$	1
3. Bornyl acetate	196	$C_{12}H_{20}O_2$	5
XIX. Miscellaneous compounds (3)			
1. 1,4-Dioxane	88	$C_4H_8O_2$	33
2. β-Methoxystyrene	134	$C_9H_{10}O$	1
3. 1,5-Dimethoxynaphthalene	188	$C_{12}H_{12}O_2$	1

[a] References: 1: Nunomura *et al.* (1979); 2: Nunomura *et al.* (1980); 3: Shoji (1936); 4: Sasaki and Nunomura (1979); 5: Nunomura *et al.* (1976a); 6: Morimoto and Murakami (1966); 7: Yamada and Goan (1969); 8: Goto (1973); 9: Sasaki and Nunomura (1981); 10: Taira (1926a); 11: Tomiyasu (1927); 12: Asao *et al.* (1967); 13: Kihara (1940); 14: Akabori (1936); 15: Taira (1926b); 16: Akabori and Kaneko (1936); 17: Ishizu (1969); 18: Yokotsuka (1951a); 19: Yokotsuka (1953b); 20: Yokotsuka (1951c); 21: Fukai (1929); 22: Yokotsuka (1951b); 23: Yamada (1928); 24: Yokotsuka (1953c); 25: Nakajima and Takei (1949); 26: Yokotsuka (1954); 27: Ikeda and Kawaguchi (1922); 28: Nunomura *et al.* (1978); 29: Yokotsuka (1950); 30: Asao and Yokotsuka (1961b); 31: Asao and Yokotsuka (1963); 32: Matsumoto (1921); 33: Nunomura and Sasaki (1981); 34: Yokotsuka and Asao (1961); 35: Yokotsuka (1953a); 36: Yokotsuka (1953d); 37: Yukawa (1917); 38: Yokotsuka (1956); 39: Asao and Yokotsuka (1958a); 40: Shoji and Onuki (1932); 41: Morimoto and Murakami (1967); 42: Yokotsuka (1949); 43: Nunomura *et al.* (1979); 44: Nunomura *et al.* (1976b); 45: Kosuge *et al.* (1971); 46: Nunomura and Sasaki (1982); 47: Morimoto and Murakami (1966); 48: Ishizu (1963).

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to his colleagues, Dr. M. Sasaki, Mr. N. Nunomura, Dr. Y. Asao, Dr. A. Okuhara, Dr. H. Hashimoto, Mr. T. Iwasa, Mr. T. Sakasai, Dr. F. Noda, Dr. S. Ishi, Dr. T. Kikuchi, Dr. S. Sugiyama, Mr. K. Oshita, Mr. A. Yasuda, and Dr. K. Hayashi for their valuable contributions and cooperation in the preparation of this work. I am also grateful to Dr. K. Sakaguchi, Professor Emeritus of Tokyo University, and Dr. T. Obara, Professor Emeritus of Tokyo University of Education, for their kind guidance and encouragement. Finally, I wish to express my hearty thanks to Chancellor Emeritus Dr. E. M. Mrak, Professor F. S. Stewart, and Professor B. S. Schweigert of the University of California at Davis, and to Dr. C. O. Chichester, Vice President of the Nutrition Foundation, Inc., New York, for their assistance in publishing this review.

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5 and 6. Both the weights and relative organ weights of the urinary bladder in both sexes fed the diet containing 11% P-shoyu or 5% sodium chloride and kidneys in females on 5% sodium chloride were higher than the control values. Both the weights and relative organ weights of the thyroid in males fed the diet containing 11% P-shoyu were lower than the control values. Higher values for the relative organ weights but no differences for the organ weights were found in the case of the heart and kidneys in both sexes at 11% P-shoyu and in males at 5% sodium chloride. Lower values for the organ weights but no differences for the relative organ weights were found in the case of the stomach in males at 5% sodium chloride and thyroid in females at 11% P-shoyu. Both the weights and the relative weights of other organs showed few differences from the controls.

The lesions encountered in the histopathological examination of mice at week 70 are shown in Table 7. In this Table 7 are representatively shown the findings in both sexes fed the diets containing 11% P-shoyu (Group I), 5% sodium chloride (Group IV) and control (Group VI). The urinary bladder in both sexes given 11% P-shoyu or 5% sodium chloride was observed markedly higher frequency of dilatation and hypertrophy than in the controls. There was a slightly high incidence of myocardial fibrosis and hydronephrosis in both sexes on 11% P-shoyu or 5% sodium chloride compared with the controls. The incidences

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on 1.1%, 0.11% P-shoyu and the mice on 0.5% sodium chloride showed few differences from the control in the rate of body weight gain.

The cumulative mortality of the mice in each group is shown in Table 3. The figures show all except the number of the mice sacrified during the study. As is usual in long-term study, there were deaths in all the groups but in the mortality, no differences between treated and control mice were observed.

The food intakes (Table 4) of both sexes given shoyu and sodium chloride at all dose levels showed few differences from the controls. The mean daily food intakes were approximately 5-6 g per mouse in both sexes. Therefore, the mean daily intakes of shoyu in males were approximately 43.1, 3.3 and 0.3 ml/kg/day at each dose level (11%, 1.1% and 0.11% P-shoyu), and those in females were approximately 53.5, 3.9 and 0.4 ml/kg/day at each dose level. The water intakes (Table 4) of the both sexes given 11% P-shoyu or 5% sodium chloride were extremely increased compared with the controls, the differences being statistically significant at most intervals throughout the study. The mean values of water intakes of these groups were 30-60 ml/mouse/day in both sexes (Table 4). These values were 5-8 times of those in the other groups.

The weights of all organs and relative organ weights which were expressed relative to body weight are shown in Table

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Animals that died during the study were autopsied as soon as possible unless this was precluded by advanced autolysis. Animals found to be *in extremis* during the study, and those surviving at week 70 were killed by chloroform anaesthesia and examined for macroscopic abnormalities, while samples of brain, pituitary, thyroid, lungs, heart, liver, kidneys, adrenals, spleen, stomach, testes, epididymides, ovaries and urinary bladder were weighed. Samples of these organs together with samples of salivary glands, trachea, esophagus, various lymp nodes, thymus, pancreas, small intestine, caecum, colon, rectum, seminal vesicle, prostate, uterus, bone marrow, spinal cord and any other tissues which appeared abnormal were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with hematoxylin and eosin for histopathological examination.

Results

The growth curves of male and female mice in each group are shown in Fig 1 and 2. The mice given diets containing 11% P-shoyu and those given diets containing 5% sodium chloride were smaller than the control mice in both sexes. However, there were few differences between the mice on shoyu and those on sodium chloride in the rate of body weight gain. The mice

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a closed colony in this laboratory were housed in a room maintained at 23 ± 1° C with a relative humidity of 55 ± 5%. The mice were divided into 6 groups 40 males and 40 females at 5 weeks of age and have been fed with each diet for 70 weeks.

Materials and procedure : The powder of commercial shoyu (Kikkoman-brand shoyu) was used as shoyu sample. The sample (P-shoyu) contains 45% sodium chloride. Standard diets to which was added the sample at each provided rate were used as experimental diets. Furthermore, the diets containing sodium chloride at the same concentration as each diet containing P-shoyu were used as experimental diets. Funabashi Farm's Laboratory Small Animal Diet was used as standard diet. The experimental groups are shown in Table 1. The dose levels were decided on the basis of the mean value of daily intake of shoyu in Japan. The highest dose (diet containing 11% P-shoyu) was worth 100 times as much as the mean value. All the diets were solidified and given to mice and tap-water was provided ad lib. The values of proximate analyses of each diet are shown in Table 2.

Animals were observed for abnormalities every day, and weighed individually at one week intervals. The quantities of food and water consumed by individual mice were measured at the same intervals.

At month 6, 8 mice of each sex from each group were killed for post-mortem examination.

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LONG-TERM (1.5 YEARS) FEEDING TEST IN MICE

Abstract

Groups of 40 mice of each sex were given diets containing 0, 0.11, 1.1 or 11.0% shoyu, 0.5% or 5.0% sodium chloride for 1.5 years. At the highest dose level of shoyu or sodium chloride (11% P-shoyu or 5% sodium chloride), the rate of body weight gain of mice given shoyu or sodium chloride was lower than the controls. There were no differences between the mice on shoyu and the controls in the mortality. At the highest dose level of shoyu or sodium chloride, the mice consumed large amounts of water throughout the experiment and the mean values of water intake of both groups were from 5 to 8 times of those in other groups. Alterations of relative organ weights of the kidneys and urinary bladder, hydronephrosis, high frequence of dilatation in urinary bladder were observed at the highest dose level of shoyu or sodium chloride.

There was no indication of a carcinogenic effect at any level of treatment.

Materials and Methods

Animals : Mice of both sexes of a ICR-JCL strain obtained from

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Table 6. The LD_{50} values of the Shoyu, P-shoyu and sodium chloride in rats and mice

Sample	LD_{50} values in	
	Rat	Mouse
Shoyu	20.6 ml/kg (19.3 - 22.0 ml/kg)*	27.3 ml/kg (25.6 - 29.2 ml/kg)
(as NaCl)	3.6 g/kg (3.4 - 3.8 g/kg)	4.8 g/kg (4.5 - 5.1 g/kg)
17.4 % NaCl solution	18.5 ml/kg (16.8 - 20.4 ml/kg)	28.3 ml/kg (26.0 - 30.9 ml/kg)
(as NaCl)	3.2 g/kg (2.9 - 3.6 g/kg)	5.0 g/kg (4.6 - 5.4 g/kg)
52.3 % P-shoyu solution	16.4 ml/kg (15.4 - 17.7 ml/kg)	19.9 ml/kg (18.3 - 21.7 ml/kg)
(as P-shoyu)	8.6 g/kg (8.0 - 9.1 g/kg)	10.4 g/kg (9.5 - 11.3 g/kg)
(as NaCl)	3.9 g/kg (3.6 - 4.1 g/kg)	4.7 g/kg (4.3 - 5.1 g/kg)
23.5 % NaCl solution	16.5 ml/kg (15.3 - 17.7 ml/kg)	20.4 ml/kg (19.5 - 21.4 ml/kg)
(as NaCl)	3.9 g/kg (3.6 - 4.2 g/kg)	4.8 g/kg (4.6 - 5.1 g/kg)

*Values in parentheses show 95 % confidence limits of LD_{50} value.

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Table 5. pH value and osmotic pressure of samples

Sample (aqueous solution)	pH	Osmotic pressure (Osm/kg)
Shoyu	4.7	6.9
17.4 % NaCl	4.7	5.6
52.3 % P-shoyu	4.4	7.9
23.5 % NaCl	4.4	6.3

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Table 4. Dose level and number of animals dying following oral administration of 52.3% solution of P-shoyu and 23.5% saline solution

Sample	Rat					Mouse				
	Dose			No. of animals		Dose			No. of animals	
	(ml/kg)	as P-shoyu (g/kg)	as NaCl (g/kg)	Used	Dead	(ml/kg)	as P-shoyu (g/kg)	as NaCl (g/kg)	Used	Dead
52.3 % P-shoyu solution	19.1	10.0	4.5	10	10	23.3	12.2	5.5	10	10
	17.0	8.9	4.0	10	7	21.2	11.1	5.0	10	7
	14.9	7.8	3.5	10	1	19.1	10.0	4.5	10	4
	12.8	6.7	3.0	10	0	17.0	8.9	4.0	10	0
23.5 % NaCl solution	19.1	--	4.5	10	10	23.3	--	5.5	10	10
	17.0	--	4.0	10	6	21.2	--	5.0	10	7
	14.9	--	3.5	10	2	19.1	--	4.5	10	2
	12.8	--	3.0	10	0	17.0	--	4.0	10	0

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Table 3. Dose level and number of dead animals following oral administration of Shoyu and 17.4% saline solution

	Rat				Mouse			
	Dose		No. of animals		Dose		No. of animals	
	(ml/kg)	as NaCl (g/kg)	Used	Dead	(ml/kg)	as NaCl (g/kg)	Used	Dead
Shoyu	26	4.5	10	10	35	6.2	10	10
	23	4.0	10	8	30	5.3	10	8
	20	3.5	10	4	25	4.4	10	2
	17	3.0	10	0	20	3.5	10	0
17.4 % NaCl solution	23	4.0	10	10	35	6.2	10	10
	20	3.5	10	7	30	5.3	10	7
	17	3.0	10	3	25	4.4	10	1
	14	2.5	10	0	20	3.5	10	0

SCIENTIFIC DATA AND INFORMATION FOR THE SELECT COMMITTEE ON GRAS SUBSTANCES RELATING TO FERMENTED SOY SAUCE

July 26, 1977

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Table 2. The proximate analyses of materials

	Shoyu (g/100 ml)	P-shoyu (g/100 g)
Moisture	61.03	4.05
Total nitrogen	1.58	4.15
Crude fat	0.15	0.61
Crude fiber	0	0
Ash (include NaCl)	18.61	48.24
NaCl	17.40	45.00

CONTENTS

APPENDICES

(continued on next page)

Table 1. Total amino acid* composition of materials

Amino acid	Shoyu (g/100 ml)	P-shoyu (g/100 g)
Lysine	0.52	1.33
Histidine	0.14	0.35
Arginine	0.22	0.54
Aspartic acid	0.58	1.50
Threonine	0.24	0.52
Serine	0.31	0.60
Glutamic acid	1.33	3.41
Proline	0.35	0.88
Glycine	0.25	0.61
Alanine	0.31	0.79
Cystine	--	--
Valine	0.30	0.76
Methionine	0.01	0.02
Isoleucine	0.30	0.77
Leucine	0.44	1.12
Tyrosine	0.05	0.11
Phenylalanine	0.27	0.70
Ornithine	0.35	0.90
Total nitrogen	1.58	4.15

*Total amino acid means the value of chemical analysis after each material was hydrolyzed by 6N-HCl at 110 ^{o}C for 18 hr.

APPENDICES continued

chloride varies with species of animals. The published LD_{50} of sodium chloride for rats is 3000 mg/kg, mice 2600 mg/kg and man 8200 mg/kg (15).

As to the pathological changes, certain changes of the stomach caused by shoyu in dogs were reported by Kajimoto (10). Enlargement and exfoliation of epithelial cells or vasodilatation in the mucosa and submucosa were shown in the stomach following oral administration of shoyu in dogs. On the other hand, dilatation and hemorrhage in the digestive tract and hemorrhage in the cranial cavity, vacuolation in various parts of the brain, and degeneration of the nerve cells were caused by sodium chloride in rats as described by Shimizu et al. (11). The pathological changes from oral administration of the shoyu or sodium chloride obtained here were similar to changes reported by Kajimoto (10) and Shimizu et al. (11). However, a vacuolation in various parts of the brain and a degeneration of the nerve cells observed by Shimizu et al. (11) were not found in the present experiment.

"SHOYU"

Japanese Fermented Soy Sauce

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Definition

Shoyu is the Japanese name for soy sauce, a popular liquid condiment used in Oriental cuisine. Many varieties of shoyu are produced in Japan and other Oriental countries. Their characteristics depend on the kinds and ratios of raw materials used, the kinds of microbes employed, and the conditions of preparation. Though most varieties are made from vegetable materials, fish soy is popular in South-East Asian countries and is even produced in Japan in small amounts. Fish soy is not included in the Japan Agricultural Standard (JAS) definition of shoyu, however.

The Japan Agricultural Standard (JAS) recognizes five kinds of shoyu: Koikuchi, Usukuchi, Tamari, Shiro and Saishikomi (52). All of these are prepared by digesting a mixture of soybeans and wheat with enzymes produced by so-called koji molds (Aspergillus

hemorrhages in the leptomeninges and mucosal layer of the fundic stomach and duodenum were found, and hyperemic and hemorrhagic changes were observed in the lungs.

All the dead animals given shoyu, P-shoyu and sodium chloride in the lower dose level showed similar symptoms and clinical course with death within 18 hr after dosage. Pathologically, the same changes were observed.

All the animals surviving for 7 days showed depression shortly after dosage; however, they recovered gradually in 5 to 18 hr. Neither macroscopic nor microscopic changes were found in the animals at day 7.

Discussion

The LD_{50} value of shoyu agreed with that of the saline solution used as the control. Similar symptoms and similar pathological changes were observed in the animals given shoyu or sodium chloride. These findings suggest that the acute toxicity of shoyu is accounted for by the toxicity of sodium chloride in the shoyu.

In the present experiment, differences between rats and mice in the LD_{50} value were observed. Kajimoto (10) reported that rabbits given shoyu at the dose of 20 ml/kg died within 3 to 10 hr. It seems likely that the tolerance against sodium

sojae or A. oryzae) in the presence of 17 to 18% (w/v) salt. Concurrently the digesting mixture undergoes lactic and alcoholic fermentations by Pediococcus halophilus and Saccharomyces rouxii, respectively.

Eighty-six percent of all shoyu consumed in Japan (53) is of the Koikuchi type, which means dark in color. In fact, the word shoyu has become synonymous with Koikuchi in common parlance. It is made from approximately equal parts of wheat and soybeans and is characterized by its rather dark reddish brown color and strong flavor. These qualities derive from vigorous lactic and yeast fermentations followed by pasteurization at a relatively high temperature (about 80 C for one hour).

About 10% of Japanese shoyu is classified as Usukuchi type, which means light in color. It is made from a mixture containing more wheat and less soybeans than Koikuchi type. Sometimes a little rice is added to the soybeans and wheat. The nitrogen content of finished product does not exceed 1.2%. Usukuchi shoyu is used mainly for cooking when one wishes to preserve the original color and flavor of the foodstuff. Koikuchi shoyu imparts a strong aroma and reddish brown color during cooking.

Tamari shoyu is made mostly from soybeans, with only a small amount of wheat. Its nitrogen content is sometimes more than 2.0% and there is only a trace of alcohol.

in each animal. However, the $\overline{LD_{50}}$ value in rats was significantly smaller than that in mice. On the other hand, the LD_{50} values of 52.3% P-shoyu solution were 16.4 ml per kg of body weight (8.6 g/kg as P-shoyu, 3.9 g/kg as sodium chloride) in rats, 19.9 ml per kg of body weight (10.4 g/kg as P-shoyu, 4.7 g/kg as sodium chloride) in mice respectively, and those of 23.5% saline solution were determined as 16.5 ml per kg of body weight (3.9 g/kg as sodium chloride) in rats, and 20.4 ml per kg of body weight (4.8 g/kg as sodium chloride) in mice. There were few differences between the P-shoyu solution and 23.5% saline solution in the LD_{50} values.

From the above data, the oral LD_{50} values of shoyu or P-shoyu solution were approximately those of saline solutions containing the equivalent of sodium chloride in the shoyu or the P-shoyu solution.

Clinical signs and course : The rats and mice administered the highest dose level of shoyu, P-shoyu and sodium chloride showed a marked depression shortly after dosage. Subsequently, they were unresponsive to painful and sound stimuli, and their hair erected and lost brightness. All the animals which died during 7 days did so within 18 hr after dosage. The gross lesions of these animals were hemorrhages in the cranial cavity, reddening the lungs, and dilatation and hemorrhage of the stomach and small intestine (mainly duodenum). Microscopically,

Shiro shoyu is very light in color and is made mostly from wheat with very little soybeans.

Saishikomi shoyu is made by enzymatically degrading soybeans and wheat cultured with Aspergillus sojae or A. oryzae (Koji) in shoyu instead of the usual salt water.

Tamari, Shiro and Saishikomi shoyu are produced only in small amounts and are consumed in localized areas of Japan.

Table 1 gives typical composition figures for the five varieties mentioned above.

Table 1. Typical Composition of Different Kinds of Shoyu.

Kind	Be	Nacl% (w/v)	Total nitrogen% (w/v)	Formol nitrogen% (w/v)	Reducing Sugar% (w/v)	Alcohol% (v/v)	pH
Koikuchi	22.5	17.6	1.55	0.88	3.8	2.2	4.7
Usukuchi	22.8	19.2	1.17	0.70	5.5	0.6	4.8
Tamari	29.9	19.0	2.55	1.05	5.3	0.1	4.8
Shiro	26.9	19.0	0.50	0.24	20.2	trace	4.6
Saishikomi	26.9	18.6	2.39	1.11	7.5	trace	4.8

The Japan Agricultural Standard (JAS) establishes three grades for each variety of shoyu: Special, Upper and Standard. The grade is determined by organoleptic evaluation, total nitrogen content, soluble solids without sodium chloride content and alcohol content. Only high quality shoyu made by fermentation can qualify for the Special Grade.

No diet and water were given to the animals after dosing, and feeding was recommenced with a normal diet after 3 hr. The animals were observed for abnormalities during 7 days. The LD_{50} value was calculated according to the method of Litchfield and Wilcoxon (9). The animals that died within 7 days were autopsied as soon as possible, those surviving at day 7 were killed and were subjected to post-mortem examination. Organs were fixed in 10% buffered formalin. Paraffin-wax sections of tissues were stained with hematoxylin and eosin for histopathological examination.

Results

The LD_{50} values : The number of animals dying within 7 days are shown in Table 3, 4, and the LD_{50} values were calculated from the results shown in Table 3, 4. The oral LD_{50} values of the shoyu, P-shoyu solution and saline solution are shown in Table 6. As shown in Table 6, the oral LD_{50} values of the shoyu were determined as 20.6 ml per kg of body weight in rats, 27.3 ml per kg of body weight in mice respectively, and those of 17.4% saline solution were determined as 18.5 ml per kg of body weight in rats, 28.3 ml per kg of body weight in mice. There were no statistically significant differences between shoyu and 17.4% saline solution in the LD_{50} value with-

Blending fermented shoyu with chemical hydrolysate of plant protein is permitted for Upper and Standard grades as long as the characteristic flavor of fermented shoyu is not spoiled. Overall, about 30% of the nitrogen in Japanese shoyu derives from chemical hydrolysate of plant protein. Individual samples may range from nearly 100% chemical hydrolysate to 100% fermented shoyu.

The amount of chemical hydrolysate present in a blend with fermented shoyu can be determined by the content of levulinic acid, which is present initially only in the chemical hydrolysate. Chemical hydrolysate made from defatted soybean grits contains, on the average, 1.4% (w/v) levulinic acid when the nitrogen content of the hydrolysate is 2% (w/v).

The amount of chemical protein hydrolysate in a mixture with fermented shoyu also is judged organoleptically by intensity of the characteristic odor of chemical hydrolysate.

Lactic acid, acetic acid and ethanol are sometimes added when chemical hydrolysate is mixed with fermented shoyu.

The fermented soy sauce consumed in the United States is almost entirely Koikuchi type Special grade. Our company is the major producer and we do not blend fermented shoyu with chemical hydrolysate.

Historians estimate that shoyu has been produced in Japan for more than 1500 years. The original product likely

chloride in the shoyu.

Materials and Methods

Animals : Male STD-Wistar rats aged 5 weeks and male ddY-F mice aged 4 weeks were divided into 16 groups of 10 animals each. The animals were fasted and given only water for 18 hr before administration.

Materials : Kikkoman-brand shoyu and shoyu powder prepared by spray drying were used as samples. The amino acid composition and the proximate analyses of two samples are shown in Table 1 and 2.

Procedure : Shoyu was given to animals at 4 dose levels as shown in Table 3 by a gastric tube. As the shoyu contains 17.4% (g/100 ml) sodium chloride, 17.4% saline solution was given to animals at the same dose levels as the shoyu. A powder of shoyu (P-shoyu) was dissolved in water in a concentration of 52.3% (g/100 ml) and was given to animals at 4 dose levels as shown in Table 4. The P-shoyu was hardly soluble in water at more than this concentration. As the P-shoyu solution contains 23.5% (g/100 ml) sodium chloride, so 23.5% saline solution was given to animals at same dose levels as P-shoyu solution. pH values and osmotic pressure of the shoyu and P-shoyu solution are shown in Table 5.

was introduced from mainland China, but changes have been made over the years. Features that differentiate Japanese fermented shoyu from similar Oriental products include:

1. More wheat and less soybeans are used as raw materials;

2. Protein is highly degraded by the enzymes of Aspergillus sojae or A. oryzae;

3. The mash is subjected to vigorous lactic and alcoholic fermentations;

4. The finished product is pasteurized to give strong aroma, flavor and color.

ACUTE TOXICITY BY ORAL ADMINISTRATION OF SHOYU IN RATS AND MICE

Abstract

The oral LD_{50} values of liquid shoyu (Kikkoman-brand) and powdered shoyu (P-shoyu) were determined in rats and mice. The LD_{50} values of shoyu (containing 17.4%, w/v, sodium chloride) were determined as 20.6 ml/kg in rats and 27.3 ml/kg in mice. The LD_{50} values of 17.4% (w/v%) saline solution were determined as 18.5 ml/kg in rats and 28.3 ml/kg in mice. The LD_{50} values of P-shoyu solution (52.3% w/v%) which contains 23.5% (w/v%) sodium chloride were determined as 16.4 ml/kg in rats and 19.9 ml/kg in mice. The LD_{50} values of 23.5% (w/v%) saline solution were determined as 16.5 ml/kg in rats and 20.4 ml/kg in mice. The oral LD_{50} values of shoyu or P-shoyu solution thus did not differ significantly from those of saline solutions containing the equivalent of sodium chloride in the shoyu or the P-shoyu solution. The LD_{50} values of P-shoyu were calculated being based on the LD_{50} values of P-shoyu solution as 8.6 g/kg in rats and 10.4 g/kg in mice. Hemorrhages in the leptomeninges, the mucosal layer of the fundic stomach, the duodenum, and the lungs were observed in all dead animals given shoyu, P-shoyu or sodium chloride alone. The acute toxicity of shoyu was accounted for by the toxicity of sodium

Manufacture

Figure 1 gives the steps in making fermented shoyu.

(1) Koji Making. Defatted soybean grits are moistened in hot water and cooked with steam under pressure (see Appendix 2 for times and temperatures). Wheat kernels are roasted at 170 to 180 C for a few minutes, then crushed into 4 or 5 pieces. Approximately equal quantities of the two ingredients are cooled and inoculated with a pure culture of Aspergillus sojae or A. oryzae growing on a medium of moistened cooked soybean grits and roasted crushed wheat.

The inoculated mixure is spread evenly to a depth of 15 inches in special rectangular vessels with perforated bottoms. Humidified air is passed through the mixture for 2 or 3 days during which time the mold grows vigorously, producing various protease and amylase enzymes. The material at this stage is called Koji. The temperature of cultivation is about 30 C for best protease formation (18)-(22). Six kinds of protease have been found in shoyu koji cultured with A. sojae (27, 28). Alkaline protease is present in greatest amount but neutral protease is the most important for shoyu production (29).

Commercial strains of aspergilli vary a great deal in protease activity. Protease production by a widely used strain of A. sojae has been increased four to six fold through successive

INTRODUCTION

Shoyu is one of the most characteristic foodstuffs which is consumed daily in significant quantities by almost all peoples in Japan. Shoyu has recently became popular among many peoples in the world for its excellent flavor and taste.

Although it has been elucidated that the fungal strains used in shoyu production do not elaborate common mycotoxins (1, 2, 3, 4, 5, 6, 7, 8), the safety evaluation of these popular foodstuffs has not been fully explored.

In order to clarify the safety of shoyu as foodstuff, a series of investigations such as acute toxicity tests and long-term feeding tests have been carried out by using mice and rats.

Commercial shoyu generally contains at least 17% (g/100 ml) sodium chloride. Therefore, the present investigations were also undertaken to find positive differences in biological effects on experimental animals between shoyu and sodium chloride alone.

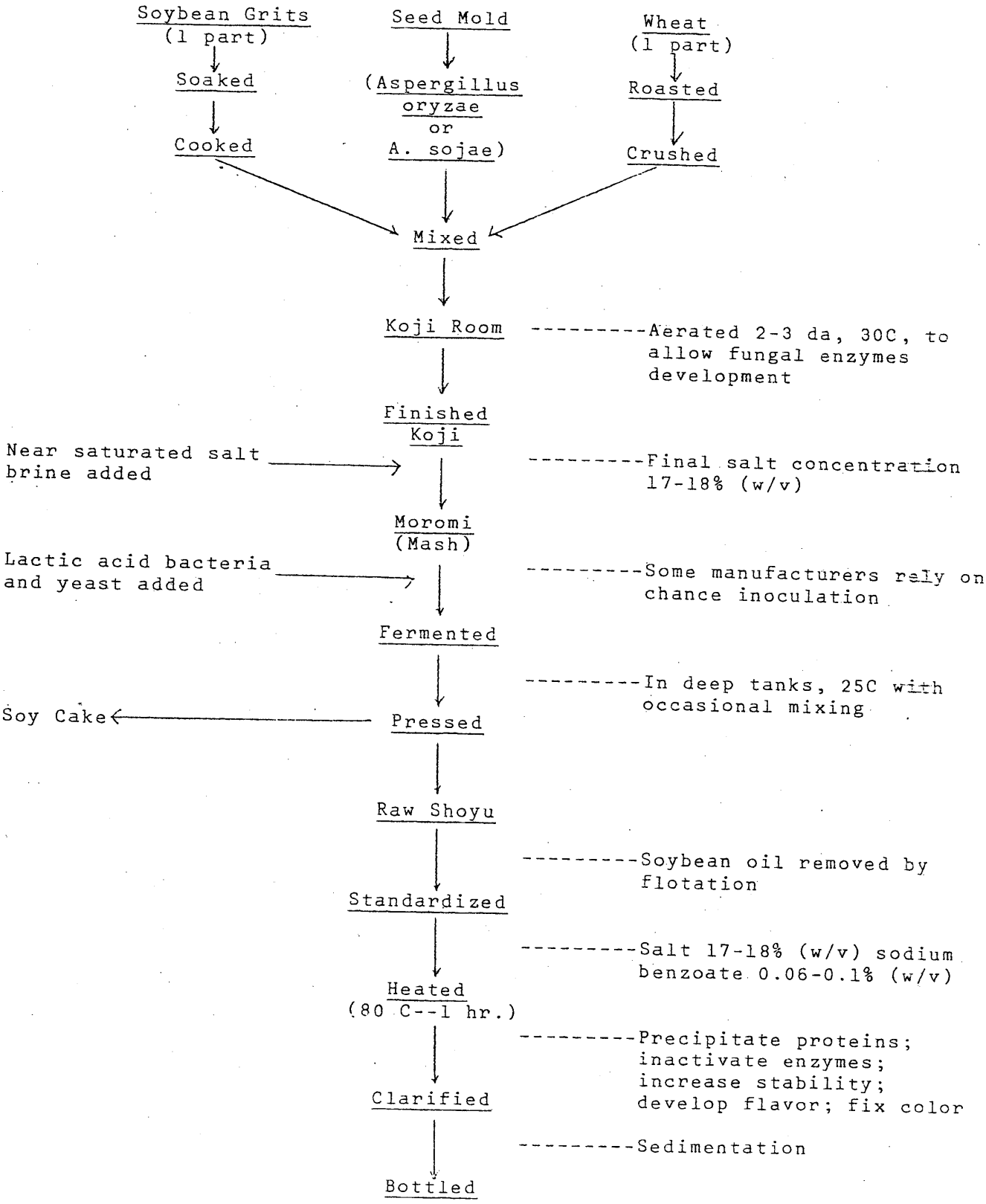

Figure 1. FLOW SHEET FOR MANUFACTURE OF FERMENTED SOY SAUCE (SHOYU)

CONTENTS

isolation of protease hyperproductive mutants induced by X-ray and UV-ray exposure. In small scale trials the total nitrogen content of shoyu was increased from 2 to 6% by applying the hyperproductive mutant (41).

(2) Fermentation. Koji is mixed with strong salt solution to produce a mash or moromi having a final salt concentration of 17 to 18% in the mash liquor. The moromi is transferred to deep fermentation tanks, inoculated with a pure culture of Pediococcus halophilus, and stored at 25 C. After about one month a yeast culture, Saccharomyces rouxii, is added.

Three or four times each month the mash is agitated with compressed air to promote microbial growth and maintain a uniform salt concentration. Total time for the lactic and alcoholic fermentations with subsequent aging is 6 to 8 months.

The initial pH of moromi is about 6.5 to 7.0. Salt tolerant bacteria (Pediococcus sojae) soon begin to grow and produce lactic acid and acetic acid. From the initial inoculum of 10^2 - 10^3 /ml they reach numbers of 10^8 - 10^9 /ml by the fourth month. These organisms are largely responsible for reducing the pH to its final value of 4.7 to 4.8 (30)-(39).

The predominant yeast of shoyu fermentation, Saccharomyces rouxii, grows slowly and reaches a viable count of 10^6 to 10^7

APPENDIX 3

SAFETY EVALUATION OF SHOYU

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/ml. Torulopsis yeasts, such as T. etchellsii, have been found in the last stage of aging some kinds of moromi in Japan (34)-(39).

The total nitrogen content of shoyu does not decline during manufacture but total carbohydrate decreases as fermentation proceeds. In all, about 25% of the carbohydrate is consumed by the end of cultivation (22).

Table 2 shows some of the major changes in shoyu during the fermentation period (23). Nitrogenous compounds solubilization proceeds slowly while reducing sugars are used up. This change is reflected by the decline in pH.

Table 2. Major Changes in Moromi Liquid During Fermentation

Days	Baume	NaCl	Total nitrogen	Formol nitrogen	Reducing sugar	pH	Utilization percentage of total nitrogen*
			(g/100 ml)				
20	23.4	16.8	1.40	0.66	9.41	5.68	68.4
60	23.9	16.3	1.57	0.76	9.92	5.38	78.8
105	21.4	16.5	1.66	0.86	2.84	4.89	82.2
150	21.8	16.9	1.74	0.91	2.51	4.82	84.2

* $\frac{\text{Moromi Liquid (Volume) x Total Nitrogen in Moromi Liquids}}{\text{Total Nitrogen in Materials Used}} \times 100 =$ Utilization percentage of total nitrogen

About 60% of the cooked soybean protein consumed by humans is digestible (40). In contrast, almost 90% of the soy protein in shoyu is digested during the long, slow fermentation. About half of the nitrogenous component of shoyu appears as

APPENDIX 2

Effect of Cooking Conditions on Enzymatic Digestibility of Soy Protein

Steam Pressure (Kg/cm^2)	Cooking Time (minutes)	Digestibility of Protein in Enzyme Solution* (%)
0.9	45	86
1.2	10	91
1.8	8	91
2.0	5	92
3.0	3	93
4.0	2	94
5.0	1	95
6.0	0.5	95
7.0	0.25	95

*0% salt, 37 C, 7 days

Note: Moistened and defatted soybeans or soybean grits usually are cooked at a pressure less than 1 Kg/cm^2 for one hour. Cooking at higher temperature for shorter time seems to be the current trend because it increases the enzymatic digestibility of soybean protein (16, 17). Autoclaves for continuous cooking under high pressure for shorter periods of time have been developed recently (12)--(14).

amino nitrogen. From these figures it is clear that shoyu is easily digested and absorbed by the body. The same is true of the enzymatically degraded carbohydrates and oils of soybeans and wheat.

With a few exceptions, the amino acids in shoyu occur in the same amounts and proportions as those found in the soybean-wheat mixture from which shoyu is made (Table 3, columns C and D). Much of the arginine is converted to ornithine. The concentrations of tryptophan, cystine and arginine decline during fermentation because of their instability. The tyrosine content decreases because of its low water solubility.

Additional minor changes result from deamination, amino-carbonyl reactions, and other chemical changes involved in the formation of characteristic color and aroma of shoyu. These reactions continue during storage of shoyu as shown by continued darkening with time.

The amino acid imbalance in shoyu, as compared with the raw material, has no nutritional consequence because shoyu is used as a condiment and its intake is very small. Even with a Japanese, the world's greatest soy sauce consumer, shoyu accounts for only 2.4 g of an average daily protein intake amounting to 80 g. Shoyu is always consumed with many kinds and amounts of proteinaceous foodstuffs. Thus any amino acid imbalance

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Table 3. Amino Acid Composition of the Proteins in Soybean, Wheat, the Mixture Used as Raw Material for Shoyu, and Finished Shoyu.

Amino acid	Amino acids in Soybean Protein (%) (A)	Amino acids in Wheat Protein (%) (B)	Amino acids in Protein of Raw Materials of Shoyu (%) (C)	Total Amino acids in Shoyu (%) (D)
Arginine	8.42	4.71	7.58	2.6
Histidine	2.55	2.12	2.45	2.5
'sine	6.86	2.67	5.90	6.5
,yrosine	3.90	3.19	3.74	1.0
Tryptophan	1.28	1.13	1.25	-
Phenylalanine	5.01	4.43	4.88	4.2
Cystine	1.58	1.80	1.63	0.9
Methoinine	1.56	1.74	1.60	1.4
Serine	5.57	5.22	5.49	5.3
Threonine	4.31	2.76	3.96	4.2
Leucine	7.72	6.52	7.45	7.3
Isoleucine	5.10	3.78	4.80	4.8
Valine	5.38	4.69	5.22	5.5
Glutamic acid	21.00	29.30	22.89	22.5
Aspartic acid	12.01	4.85	10.38	10.5
Glycine	4.52	3.94	4.39	3.9
Alanine	4.51	3.37	4.25	4.4
Proline	6.28	9.94	7.11	6.5
Ornithine	-	-	-	5.7
tal	106.56	96.16	104.97	100.1

(A) Values from A. K. Smith and S. J. Circle, in "Soybeans, Chemistry and Technology", Vol. 1, page 242, The Avi Publishing Co., Inc. 1972.

(B) Values from F. N. Hepburn et al., Cereal Chemistry, 37, page 749, 1960

(C) Values calculated by the formula $(C)=\frac{(3.4\ A+B)}{4.4}$

on the assumption that protein content of defatted soybean meal is 3.4 times that of wheat, and the raw materials of shoyu consist of equal quantities of soybeans and wheat.

(D) Analytical values from Kikkoman Shoyu Co.

Major research publications (including co-research).

Tamotsu Yokotsuka
May, 1977

1. Studies of flavorous compounds in Shoyu (1-28), Agricultural Chemical Society, Japan, (1949-1967)

2. Aroma and Flavor of Japanese Soy Sauce (50 pages), Advances in Food Research, Vol. 10, (1961)

3. Shoyu, Conversion and Manufacture of Foodstuffs by Micro-organisms, IUFoST and IFT, Kyoto, Japan, Dec. (1971)

4. Some Recent Technological Problem Related to the Quality of Japanese Shoyu, 4th International Fermentation Symposium, Kyoto, Japan, (May, 1972)

5. Studies on compounds produced by molds, having fluorescence similar to that of aflatoxins, (1-8)
   4th Annual Meeting, IFT, Warsaw, Poland, (1966)
   American Chemical Society, Annual Meeting, New York, (1966)
   US-Japan Cooperate Program in Natural Resources, Honolulu, Hawaii, (Oct. 1968)

6. Analysis of shoyu, (1-8), Agr. Chem. Soc. Japan, (1958-1966)

7. Studies on color of shoyu, (1-7), Fermentation Technology, Japan, (1962-1972)

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10. Clarification of fruit juice by Pectin trans-Eliminase, J. Agricultural and Food Chemistry, Vol 19, No 5, (1971), and Vol 20, No 4, (1972), Vol 21, No 2, 1973.

11. Production and Purification of Acid Protease produced by Penicillium duponti K1014, J. American Society of Microbiology, Vol 24, No 6, (1972), and others.

in shoyu is easily adjusted by other components of the meal.

(3) Refining. When fermentation is complete the moromi or mash is filtered through cloth under hydraulic pressure as high as 100 Kg/cm^2. The salt content of the filtrate is adjusted if necessary, sodium benzoate (up to 0.1%) sometimes is added as a preservative, and the raw shoyu is heated to 70-80 C for an hour or so. This heat treatment has great influence on the flavor, color and stability of the finished product. Changes that occur during heating include:

1. Increased clarity resulting from the removal of oil droplets mixed with heat coagulable substances.
2. Flavor changes resulting from (a) an increase in phenolic compounds, aldehydes and acetals, mercaptans and mercaptals, organic acids, pyrazines, furfurals, alpha-diketon compounds, and other kinds of so-called brown flavor compounds; and (b) a decrease in volatile compounds of low molecular weight (including ethyl alcohol), amino acids and reducing sugars (1)--(6), (24, 25).
3. Greater resistance to growth of film-forming yeasts mainly resulting from an increase in organic acids and phenolic compounds (26).
4. Color intensity doubles.
5. Most enzymes are inactivated.

Materials that precipitate during heating are allowed to settle out and the clear supernatant is decanted. It is now finished shoyu and ready for packaging. In Japan about

APPENDIX 1

Personal History

Tamotsu Yokotsuka
Kikkoman Shoyu Co., Ltd.
Senior Vice President
Head of Production, and in charge of Research and Engineering.
339 Noda, Noda-shi, Chiba-Ken, Japan.

Kikkoman Foods Inc.
Director
Walworth, Wisconsin 53184, U.S.A.

Born, Mar. 25, 1917

Graduated, Tokyo University, Agricultural Chemistry (Food Chemistry), (1941)

Mitsubishi Trading Co., (1941-1943)

Military Quartermaster, captain, (1945)

Degree of Agriculture, Tokyo University, for studies on shoyu flavor, (1953)

Award of Agricultural Chemical Society, for above research, (1951)

Entered, Kikkoman Shoyu Co., Ltd., (1945)
Director of Central Research, (1956-1976), Vice President, (1969), Senior Vice President, (1973)

Assistant Professor, Tokyo University, doctor course of food chemistry, (1973-1975)

Vice Chairman, Japan Agricultural Chemical Society, (1977-1979)

Member, Governmental Committee for Establishment of Japan Agricultural Standard (JAS) for Soy Sauce, (1962) and (1972)

Member, Governmental Committee for Export of Soy Sauce, Ministry of Commerce, (1975-present)

Member, Governmental Committee for Investigation of Japanese Dietary Life, Ministry of Agriculture and Forest, (1974-1976)

60% of the shoyu is packed in 2-liter glass bottles, 30% in 1-liter plastic bottles (9).

Liquid shoyu can be spray-dried if the powdered form is desired.

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Chemical Composition

Although fermented soy sauce and hydrolyzed vegetable protein are widely used as condiments and flavoring agents, their composition and characteristics differ substantially.

(1) Chemical hydrolyzate contains more free amino acids but less sugars and alcohols than fermented shoyu. Major organic acids of fermented shoyu are lactic acid and acetic acid, while those of chemical hydrolyzate are levulinic acid and formic acid.

(2) Levulinic acid is not present in fermented shoyu. Its concentration in a blended soy sauce provides a measure of the amount of chemical hydrolyzate in the mixture.

(3) Chemical hydrolyzate has a characteristic and distinctive odor. This is partly because it contains more volatile sulphur compounds than fermented shoyu. This odor may be removed but it returns on storage.

These differences are not surprising when one recognizes that fermented shoyu is made by mixed enzyme reactions on very complex raw materials (whole wheat and defatted soybeans).

As a generalization we can say that good quality Koikuchi shoyu contains about:

1.5% Total Nitrogen (w/v). Of this:

45% is found in lower peptides
45% is found in amino acids
10% is found in ammonium compounds

2-5% Reducing sugars (w/v). Of this 80% is glucose.

1-2% Organic acids (w/v). Of this 70% is lactic acid.

1-1.5% Polyalcohols (w/v). Of this 80-90% is glycerol.

2-2.5% Ethanol (v/v)

17-18% Salt (w/v)

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4.7-4.8 pH

For more precise identification of the components we have analyzed a sample of Koikuchi shoyu produced at Kikkoman's U.S. plant with the results in Table 4 (54). Percentages are given on both wet and dry bases to facilitate comparision with other materials.

Fermented shoyu of the type analyzed contained about one-third soluble solids (34%). Of this almost one-half consisted of inorganic components, primarily sodium chloride.

Amino acids comprised about 25% of the soluble solids, sugars 13%, polyhydric alcohols 5% and organic acids almost 3%. Ether soluble matter accounted for less than one-half of one per cent of the soluble solids in shoyu.

Amino acids were determined both before and after acid hydrolysis. The latter values were used in Table 4 except for methionine and tyrosin, which are not stable to acid hydrolysis. For these two compounds the values obtained before hydrolysis, which gave only the "free" forms of the amino acids, were used in the table.

Tryptophan and cysteine are decomposed during acid hydrolysis and do not appear in Table 4. Recently we found 0.002% tryptophan by a different method (55).

The values in Table 4 account for 93.2 % of the soluble solids in shoyu. The ammonia found after acid hydrolysis probably resulted from decomposition of amino acids and should be calculated as amino acid. When this is done the figure for

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Table 4

DETAILED COMPOSITION OF FERMENTED SOY SAUCE (SHOYU)

Component	Per cent (w/w) of soy sauce "as is basis"		Per cent (w/w) of soy sauce "dry basis"	
Soluble solids (dry matter)	34.00			
Alcohol	1.47		100.00	
Water (by difference)	64.53			
INORGANIC COMPONENTS				
Sodium	6.10		17.94	
Chlorine	8.82		25.94	
Calcium	0.02		0.06	
Potassium	0.40		1.17	
Phosphorus	0.15		0.44	
Magnesium	0.07		0.21	
Sulfur	0.06		0.17	
Iron	0.002		0.006	
Manganese	0.001		0.003	
TOTAL INORGANIC COMPONENTS		15.6		45.94
ORGANIC COMPONENTS				
Polyols				
Glycerol	1.50		4.41	
Mannitol	0.17		0.50	
TOTAL POLYOLS		1.67		4.91
Ether soluble matter		0.14		0.41
Ether soluble volatile matter		0.005		0.01
Amino acids:				
Lysine	0.56		1.65	
Histidine	0.21		0.62	
Cystine	0.07		0.21	
Arginine	0.22		0.65	
Aspartic acid	0.90		2.65	
Threonine	0.36		1.06	
Serine	0.45		1.32	
Glutamic acid	1.92		5.65	
Proline	0.59		1.74	
Glycine	0.34		1.00	
Alanine	0.38		1.12	
Valine	0.47		1.38	
Methionine	0.12		0.35	
iso-Leucine	0.41		1.21	
Leucine	0.62		1.82	
Tyrosine	0.08		0.24	
Phenylalanine	0.36		1.06	
Ornithine	0.49		1.44	
TOTAL AMINO ACIDS		8.55		25.17
[illegible]	0.002			

(continued on next page)

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Table 4 continued

DETAILED COMPOSITION OF FERMENTED SOY SAUCE (SHOYU)

Component	Per cent (w/w) of soy sauce "as is basis"		Per cent (w/w) of soy sauce "dry basis"	
Ammonia		0.30		0.88
Organic acids				
Formic	0.02		0.06	
Acetic	0.16		0.47	
Citric	0.04		0.12	
Succinic	0.05		0.15	
Lactic	0.68		2.00	
TOTAL ORGANIC ACIDS		0.95		2.80
Sugars				
Monosaccharides				
Mannose	0.06		0.18	
Arabinose	0.08		0.24	
Galactose	0.17		0.50	
Xylose	0.06		0.18	
Glucose	2.05		6.03	
Unidentified	0.23		0.68	
TOTAL MONOSACCHARIDES	2.65		7.81	
Disaccharides	0.65		1.91	
Oligosaccharides	----		----	
Polysaccharides	1.15		3.38	
TOTAL SUGARS (as glucose)		4.45		13.1
TOTAL ORGANIC COMPONENTS		16.1		47.3
Solids accounted for		31.7		93.2
With ammonia calculated as amino acids		32.69		96.1

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total amino acids becomes 9.8% (wet basis).

This calculation accounts for 32.69 grams or 96.1% of the soluble solids in fermented shoyu.

Considering the widespread interest in safety of monosodium glutamate, it should be mentioned that the glutamic acid in fermented soy sauce occurs as the free acid or its ammonium salt, not as monosodium glutamate.

Soy sauce contains browning pigments (melanoidins) besides the compounds described in the analytical tables. The browning pigments are formed by amino-carbonyl reactions in which most of the amino compounds are peptides and the carbonyl compounds are sugars and sugar derivatives. We are conducting further studies to identify the browning pigments.

The components listed in Table 4 do not include the many compounds present in trace amounts. Japanese investigators have identified at least 150 components of the ether soluble volatile fraction, which in toto constitutes less than 0.005% of fermented soy sauce. These materials include alpha-diketon compounds such as acetyl propionyl and acetyl-butyryl; phenolic compounds such as vanillin, vanillates, p-ethylphenol and 4-ethyl guaiacol; cyclic-enolones such as maltol; hydroxy-furanones such as 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-2(H)-furan-3-one; terpenes such as borneol; various aliphatic alcohols such as n- and iso-butyl alcohol, iso-amyl alcohol; 2-phenyl ethanol; aromatic carbonyls and esters; volatile sulphur compounds; ketons and acetals; and various pyrazines. Compounds such as these are considered to be especially important components of the flavor and aroma of Koikuchi shoyu (1)-(8).

Table 5 shows the degree of variability of shoyu produced at Kikkoman's plant in the U.S.

Table 5. Monthly Average of Soy Sauce Analysis (After Sedimentation)

Month	NaCl (%w/v)	TN (%w/v)	Color	pH	Be	Alc (%v/v)	RS (%w/v)
May, 1976	17.30	1.651	963	4.73	23.18	1.72	
June	17.20	1.649	920	4.73	23.07	2.00	
July	17.27	1.651	884	4.75	23.13	1.78	4.64
August	17.22	1.649	964	4.76	23.04	1.94	5.09
September	17.27	1.656	939	4.77	23.00	2.10	
October	17.27	1.662	786	4.79	22.68	2.27	
November	17.30	1.652	880	4.78	22.52	2.57	3.42
December	17.24	1.656	890	4.74	22.53	2.55	3.59
January, 1977	17.28	1.655	1001	4.72	22.80	2.25	
February	17.31	1.650	875	4.70	22.67	2.43	3.90
March	17.26	1.655	810	4.69	22.67	2.30	
April	17.26	1.657	846	4.67	22.54	2.17	3.81
$\bar{x}$ [1]	17.27	1.654	897	4.74	22.82	2.17	4.08
s [2]	0.031	0.004	61.5	0.035	0.240	0.266	0.594

1) Average
2) Standard Deviation

TN - Total Nitrogen
Be - Baume
RS - Reducing Sugars
Color - by Kikkoman Standard

APPENDIX 5 reports negative results for aflatoxin in samples collected by the Wisconsin Department of Agriculture.

More recently we have had WARF Institute test the Koji cultures that we regularly use in our Wisconsin plant. None produced aflatoxin.

To the contrary, extensive metabolic studies have shown that certain cultures of Aspergillus sojae are able to produce aspergillic acid, kojic acid, Beta-nitro-propionic acid or oxalic acid under special conditions of culture (56). These compounds offer little or no hazard to the consumer, however, because (1) cultures of A. sojae in commercial use produce little if any of the toxic metabolites under any condition of growth; (2) wheat and soybeans are poor media for production of toxic metabolites; (3) toxins are not formed in the 2 or 3 days used for koji making; and (4) toxicity of the metabolites is very low (LD_{50} i.p. about 100 mg/kg).

In a continuing check for mycotoxins we have injected the ether extractable components from 12 liters of shoyu, or the equivalent amount of finished koji, into a single mouse without harm (50). This dose is equivalent to a full year's soy sauce consumption by the average Japanese.

Recent tests of Kikkoman's Koji cultures of A. sojae have been negative for patulin, ochratoxin and sterigmatocystin.

7. Lysinoalanine. Attempts to detect lysinoalanine in fermented shoyu have been unsuccessful.

Heavy Metal Contents

Five lots of shoyu from our Wisconsin plant were analyzed recently for arsenic, lead, mercury, copper and total heavy metals, with the results in Table 6.

Table 6. Heavy Metals in Fermented Soy Sauce

Item	Sample size (g)	Method of analysis	Min. Amt. detectable	Codex limit (ppm)	Amount found (ppm)
Heavy Metals Test	9.52	Codex (1)	-	-	1.87
Arsenic Test	2.38	Codex	0.42 ppm	3	N.D.**
Arsenic	5.95	AOAC (2)	0.1 ppm	-	N.D.
Lead Limit Test	2.39	Codex	0.42 ppm	1	N.D.
Lead	23.7	AOAC	0.2 ppm	-	Trace
Mercury Limit Test	6.01	Codex	1.6 ppm	*	N.D.
Mercury	23.8	AOAC	0.01 ppm	-	N.D.
Selenium Limit Test	1.18	Codex (3)	30.0 ppm	30	N.D.
Copper	23.9	AOAC	-	-	0-0.15

*No figures for soy sauce **Not Detected

(1) Food Chemicals Codex II NAS-NRS 1972

(2) Methods of Analysis A.O.A.C. Twelfth Edition (1975)

(3) First Supplement to Food Chemical Codex

Arsenic, mercury and selenium were not detectable. Lead and copper were found in trace amounts, and the figure for total heavy metals was less than 2 ppm.

early 1960s naturally stimulated interest in the aspergilli and other molds of industrial importance.

Japanese investigators failed to find a single aflatoxin producer among the organisms used for Oriental food production. These included 140 cultures in one study (44) and 73 in another (45, 46, 50). These findings were confirmed by investigations of 53 cultures at the Northern Regional Research Laboratory in the U.S. (42). Tests on Miso, Shoyu and Sake in Japan, all made with strains of Aspergillus oryzae were negative (43). Other investigations gave similar results (45)-(48)-(57).

In the course of these early studies it was learned that some cultures produced fluorescent compounds having Rf values resembling those of aflatoxin. Further investigation showed the fluorescent spots to be non-toxic pyrazine compounds (46). Clearly they were not aflatoxin.

Meanwhile, taxonomic studies showed clear differences between Japanese food industrial molds and species that produce aflatoxin (49).

Note: A report alleging aflatoxigenesis by a variant of Aspergillus oryzae NRRL 1988 appeared in Science magazine last year (Vol. 192, p. 1345, 1976). The findings have been disputed by other investigators who claim that the original authors used a culture contaminated with Aspergillus parasiticus (APPENDIX 4)

Repeated tests in both Japan and the U.S. have consistently failed to demonstrate aflatoxin in Kikkoman shoyu.

Consumption

Shoyu has long been recognized as a pleasant condiment for meat and oily dishes all over the world. It adds a delicious and spicy flavor to meals, it is believed to promote digestion, and it may even have some kind of pharmacological action. The ability of shoyu to promote secretion of gastric juice has been compared with that of caffein and histamine (10).

The total amount of shoyu produced in Japan is about 120,000,000 kiloliters per year. This figure has remained almost unchanged since 1974. There are nearly 3,600 shoyu producers in Japan, with the biggest five accounting for half the total. Their brands are Kikkoman, Yamasa, Higashimaru, Higeta and Marukin. The market share of the biggest, Kikkoman, is about 30%.

Per capita consumption of shoyu in Japan averages 12 liters per year. Of this 63% is consumed in homes and 37% in food industries and restaurants (9).

A typical Japanese takes 34.1 g of shoyu daily. This corresponds to 14.0 calories, 2.4 g of protein, 0.2 g of fat, and 5.8 g of salt (9). The average daily protein intake by a Japanese is about 80 g, according to the Government. Thus shoyu plays a minor role as a source of protein or amino acids.

given a diet containing 5% or 2% powdered shoyu grew faster than rats fed an equivalent amount of sodium chloride alone (that is, diets containing 2.25% or 0.9% sodium chloride).

At the highest dose level, 10% powdered shoyu, there were significant differences in the urinary system between treated and control animals. Both species showed increased relative weights of kidneys and urinary bladder, rats developed higher concentrations of serum urea, and mice gave evidence of hydronephrosis after 1.5 years. These same effects were observed in animals who received sodium chloride in the same concentrations as those fed the highest level of shoyu.

There was no indication of carcinogenic effect at any level of shoyu feeding.

5. Long-term effect of shoyu on rats with fundusectomy. MacDonald and Dueck (51) fed rats a standard laboratory diet without and with Kikkoman soy sauce (50 ml/100 g of feed). Half of the animals on each diet had undergone a fundusectomy before the experiment. Rats fed shoyu were somewhat smaller than the controls but they were healthier, more active and longer lived. There was no evidence that shoyu is carcinogenic. In fact, the control animals developed 18 tumors, the shoyu fed animals only 5. The experiment was terminated when the rats reached 33 months of age.

6. Mycotoxins. The discovery of aflatoxin in the

Shoyu provides salt, flavor and color in the Japanese diet. This is especially important for bland foods such as rice, fish, beancurd, fermented beans, boiled vegetables, and so on.

Table 7 shows the estimated consumption of fermented shoyu in the United States during 1974-76. Figures for Japan are included for contrast.

Average per capita consumption of fermented soy sauce is less than 1/200 of that in Japan. Virtually all of this is used as a condiment by the individual consumer.

Table 7. Consumption of Fermented Shoyu in the U.S. and Japan

Year	Gallons of liquid shoyu consumed		Pounds of shoyu solids consumed	
	United States*	Japan	United States*	Japan
1974	2,600,000	311,676,000	8,840,000	1,059,698,000
1975	2,900,000	293,331,000	9,860,000	997,325,000
1976	3,300,000	320,728,000	11,220,000	1,090,475,000

Annual per capita consumption in:	Japan	12,000 ml
	U.S.	57 ml

*Domestic production plus imports from the Orient.

In females there was little difference between the body weight gain rates for animals fed shoyu or sodium chloride and those on the control diet.

At the highest dose level of shoyu or sodium chloride there were marked increases in water intake, alterations in relative weights of kidney, heart and urinary bladder, and higher concentrations of serum urea. These changes were attributable to the high salt intake.

No histopathological changes or alterations in liver function were observed in spite of the increased relative liver weights.

4. Conclusions from animal feeding tests. The acute toxicity of shoyu was accounted for by the toxicity of its sodium chloride component. The oral LD_{50} values for shoyu were 20.6 ml/kg for rats and 27.3 ml/kg for mice.

In the long-term feeding tests, food intakes of animals given a diet containing shoyu were scarcely different from the controls. This was true even with animals given a diet containing 10% powdered shoyu (corresponding to approximately 25% liquid shoyu). Shoyu did not appear to be unpalatable to the animals.

The animals fed shoyu clearly were smaller than the controls; however, no significant differences in mortality were observed between treated and control animals. Male rats

Safety of Fermented Shoyu

Large amounts of Kikkoman shoyu have been fed to mice and rats to observe acute and long-term effects. A detailed report of these tests is attached as APPENDIX 3.

Similarly, much effort has been expended to be sure there are no mycotoxins of significance in fermented shoyu. The results will be reported later in this section.

1. Acute toxicity. Table 8 gives LD_{50} values for Kikkoman shoyu in mice and rats. There were no significant differences between the figures for shoyu and for saline solutions of the same salt concentration.

Table 8. Acute Toxicity of Liquid and Powdered Shoyu Compared with Saline Solutions of the Same Salt Concentrations

Test Material	NaCl content (% w/v)	Acute toxicity (LD_{50}/kg)	
		Rats	Mice
Liquid shoyu	17.4	20.6 ml	27.3 ml
Saline solution	17.4	18.5 ml	28.3 ml
Powdered shoyu, 52.3% solution	23.5	16.4 ml	19.9 ml
Saline solution	23.5	16.5 ml	20.4 ml
Powdered shoyu (calculated from above)	45 (w/w)	8.6 g	10.4 g

2. Long-term (1.5 years) feeding test in mice.

Forty mice of each sex per group were fed diets containing 0, 0.11, 1.1 or 11.0% shoyu, 0.5 or 5.0% sodium chloride for 1.5 years. Mice receiving the highest dose levels (11% powdered shoyu or 5% sodium chloride) exhibited lower rates of body weight gain than did the controls. There was no difference in mortality, however, between the mice fed shoyu and the controls. At the highest dose levels of both shoyu and sodium chloride the mice consumed large amounts of water throughout the experiment, with mean water intake values for both groups from 5 to 8 times those in other groups. Increased relative weights of kidneys and urinary bladder, hydronephrosis and high frequency of bladder dilatation were observed at the highest dose levels of shoyu and sodium chloride.

No treatment level showed evidence of a carcinogenic effect for shoyu.

3. Long-term (6 months) feeding test in rats. Groups of 30 rats of each sex were given diets containing 0, 0.4, 1, 2, 5 or 10% shoyu or 0.18, 0.45, 0.9, 2.25 or 4.5% sodium chloride for six months. At the higher dose levels for both shoyu and sodium chloride the body weight gain rates among male rats clearly were lower than the controls. However, the extent of growth inhibition caused by shoyu appeared to be less than that caused by the equivalent level of sodium chloride.