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Pressure Inactivation of Yeasts, Molds, and Pectinesterase in Satsuma Mandarin Juice: Effects of Juice Concentration, pH, and Organic Acids, and Comparison with Heat Sanitation

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Satsuma mandarin juice, the concentrated juice and the acid-free juice were prepared and treated with pressures of 1000–6000 bar after mixing with organic acids, various yeasts and molds, or pectinesterase, to study the effects of pressure on inactivation of microorganisms and the enzyme. The effects of heat treatment were also studied in similar conditions. As results, the inactivation effect of pressure on microorganisms and the enzyme was decreased by increasing juice concentration. Pressure-induced inactivation of microorganisms was not affected by either juice pH (between 2.5 and 4.5) or kinds of organic acids (citric, tartaric, lactic, or acetic acid). With 9 species of yeasts and molds tested, pressurization at 3500 bar for 30 min or at 4000 bar for 5 min was required to reduce them to $1/10^5$ or below $1/10^5$. Microorganisms resistant to heat tend to be highly resistant to pressure. Although no complete inactivation of pectinesterase was attained after pressurization at 3000 or 4000 bar for 10 min, the partly inactivated enzyme did not recover under ordinary conditions of storage and transportation.

High hydrostatic pressure was first attempted for the preservation of milk, fruits, and vegetables by Hite, Giddings, and Weakley from 1899 to 1914.[1] In 1914, Bridgman examined the effects of pressure on coagulation of "albumen"[2] and in 1965, Timson and Short studied the effects of pressure on microorganisms in milk.[3] However, high pressure technology has not been established as a way of food preservation until the present time. Recently, Hayashi has proposed the application of high pressure technology to cooking, sterilization, processing, and storage of food.[4,5]

In previous papers,[6,7] we reported that high pressure treatment could sterilize microorganisms in juices from various citrus fruits without major changes in their nutritive components and natural flavor and taste.

To open a way for pressure-sterilization in juice manufacture, we have been precisely examining the influence of high pressure treatment on inactivation of microorganisms and pectinesterase contained in Satsuma mandarin (*Citrus unshiu*) juice. Here the effects of pH, juice concentration, and organic acids are described and the relationships between pressure and heat resistances of microorganisms and enzyme are discussed.

Materials and Methods

Test microorganisms. Six species, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Hansenula anomala*, *Rhodotorula glutinis*, *Aspergillus awamori*, and *Mucor plumbeus* were supplied from the Department of Fermentology, Hiroshima University, and two species, *Saccharomyces bayanus* and *Pichia membranaefaciens*, from the Fermentation Research Institute, Osaka. These species grow well in juice, and some of them are highly heat-resistant while others are less heat-resistant.[8]

Wild yeasts which were highly heat-resistant were isolated as follows; microorganisms collected at a juice plant were added to Satsuma mandarin juice, which was then packed in 200-g cans. The cans were immersed in a thermostat and warmed for 10 min, maintaining the tem-

perature of the can center at 60°C. After several days of storage, swollen cans and cans with putrefied contents were selected. Juices thus selected were plated onto potato dextrose agar,[9] and unknown microorganisms were isolated from the cultures.

Preparation of test solutions of microorganism. Each test microorganism was plated onto potato dextrose agar with the pH adjusted to 3.5 with 10% tartaric acid solution. After the plate was incubated at 27°C for 10 days, colonies on the plate surface were collected with a platinum spatula. Sporulation was confirmed by staining and microscopy in the case of ascospore yeast culture. Collected colonies were inoculated into a small amount of juice and suspended uniformly using a glass homogenizer. The suspension was then mixed with various juice specimens to obtain test solutions in which the count of initial microorganism was 10^4–10^6/ml.

Of the test microorganisms, *Saccharomyces cerevisiae, Saccharomyces bayanus*, and wild yeast were separately incubated with MY20 liquid medium[10] at 20°C for 2 days. The cultures were then plated onto *Gorodokowa* agar[10] and incubated at 30°C for 8 days. The colonies were collected and inoculated into juice specimens as described above.

The microorganisms used here were a mixture of nutritive cells and spores. Test solutions thus obtained were stored at 0°C for 12–24 hr until use for the pressure treatment.

Preparation of juice specimens.

A. *Squeezed and concentrated juice of Satsuma mandarin.* Unsterilized squeezed juice of Satsuma mandarin was prepared as described previously,[6] and concentrated by vacuum evaporation, frozen, and stored. It was thawed immediately before use. To prepare juice specimens with different soluble solid concentrations, the concentrated juice was diluted with water, or mixed with the original unsterilized squeezed juice and water.

B. *Preparation of pH-adjusted juices.* The concentrated juice was diluted with water to the concentration of natural juice (10°Bx). The diluted juice was then treated with an anion exchange resin[11] (Mitsubishi Kasei Co., Diaion WA30) to remove acids. The acid-free juice (pH 6.0) was sterilized by heat-treatment at 95°C for 2 min. Different amounts of sterilized solution of citric acid were added to the sterilized and acid-free juice to adjust the pH to 2.5, 3.5, or 4.5.

C. *Mixing of juice and organic acids.* The sterilized acid-free juice was mixed with the sterilized solution of citric, malic, tartaric, lactic, or acetic acid to a concentration of 0.7%.

Experiments on microorganisms.

A. *Test of heat resistance.* Juice (4 ml) inoculated with one of the test microorganism was placed into a hard glass tube (10 mm in internal diameter and 12 mm in external diameter). The orifice was covered with aluminum foil and the upper part of the tube was sterilized. The tube was then heated at 50, 53, 58, 60, or 62°C (temperature variance within ±0.2°C) in a thermostat. It took 2.5 to 3.5 min to reach the desired temperature. After reaching the desired temperature, the heat treatment was performed for 0, 0.5, 2, 5, 10, or 30 min (excluding heating and cooling times).[12] After the heat treatment, the glass tubes were cooled and specimens were incubated to count living microorganisms.

B. *Measurement of the survival of microorganisms.* The number of surviving yeasts and molds in each juice specimen was measured by a colony count after incubation at 25°C for 5 days on the plate of potato dextrose agar acidified to pH 3.5 with 10% tartaric acid.

Method of high pressure treatment. High pressure treatment of 1000 to 6000 bar was applied to juices using a high-pressure generator (Mitsubishi Heavy Industries, Ltd., Type MFP-7000) as described before.[6] For pressurizing up to 3,000, 4,000, and 6,000 bar, the times required were about 30, 40, and 60 sec, respectively. The time required to depressurize to atmospheric pressure was about 10 sec.

Analytical method. Pectinesterase activity was measured as described previously.[6]

Results and Discussion

Effects of juice concentration on the inactivation of pectinesterase and microorganisms

Concentrated juices with different soluble solid levels were pressurized for 10 min and the resulting pectinesterase activities were measured (Fig. 1, A). Pressurization at 1000 and 2000 bar caused hardly any inactivation of pectinesterase, but pressurization at 3000 or 4000 bar decreased the activity. Purified pectinesterase was also inactivated at 3000 bar or higher (Fig. 1, B). However, the difference in activity between 1000 and 4000 bar decreased with an increase in soluble solid concentrations. This result shows that soluble solids protect pectinesterase from pressure inactivation.

Marshall *et al.*[13] described how pectinesterase activity in citrus fruit juice is protected from heat treatment when soluble solid levels are increased. Therefore, increased soluble solids protect the enzyme from pressure as well as heat inactivations.

It is interesting that pectinesterase activity

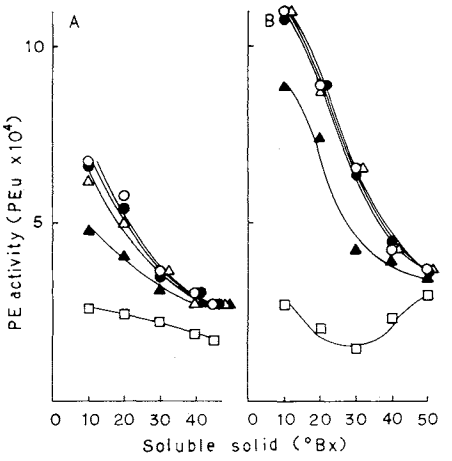

Fig. 1. Effects of Juice Concentration on Pressure-induced Inactivation of Pectinesterase (PE).

A: Freshly-squeezed and non-pasteurized Satsuma mandarin juice was blended with concentrated juice or water to make each Brix indicated, and pressurized.

B: Pectinesterase purified from orange peel (Sigma Chemicals) was mixed with the concentrated juice or water as above and pressurized.

Pressurization was done at 1 (○), 1000 (●), 2000 (△), 3000 (▲), and 4000 bar (□) and at room temperature (about 23°C) for 10 min.

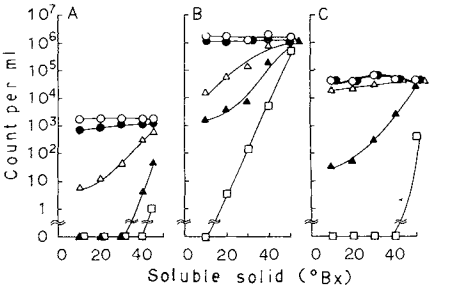

Fig. 2. Effects of Juice Concentration on Pressure Inactivation of Yeast and Mold.

Freshly-squeezed and non-pasteurized juice (A) and the juices which were inoculated with *Saccharomyces cerevisiae* (B) and *Aspergillus awamori* (C) were pressurized at 1 (○), 1000 (●), 2000 (△), 3000 (▲), and 4000 bar (□) and at room temperature for 10 min.

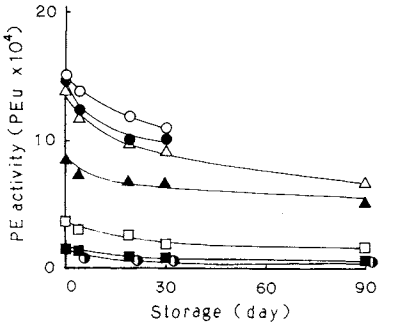

Fig. 3. Changes in Pectinesterase (PE) Activity of Pressurized Juices during Storage.

Freshly-squeezed and non-pasteurized Satsuma mandarin juice treated with a finisher was pressurized at 1 (○), 1000 (●), 2000 (△), 3000 (▲), 4000 (□), 5000 (■), and 6000 bar (◑) at room temperature for 10 min, and stored at 0°C.

decreased with an increase in soluble solids (see Fig. 1), though the same level of pectinesterase activity was mixed with the test juice before pressure treatment. It is unknown whether this is due to the absorption of the enzyme on the increased amounts of pectin or pectic acid, or due to the inactivation of the enzyme by juice components.

Figure 2 shows the influence of juice concentration on the pressure inactivation of yeast and mold in unsterilized and squeezed juice (Fig. 2, A). The inactivation effect of pressure at 2000 bar or higher decreased as juice concentrations increased. A similar decrease was also observed with the concentrated juices inoculated with *Saccharomyces cerevisiae* (Fig. 2, B) and *Aspergillus awamori* (Fig. 2, C). *Saccharomyces bayanus, Pichia membranaefaciens*, and *Mucor plumbeus* were also inactivated similarly, but the inactivation was prevented in concentrated juice (data not shown).

Sugars (such as sucrose), protein, and lipid exert a protective action against heat inactivation of microorganisms.[14] This is also the case of the pressure-treatment of the concentrated juice which chiefly contains sucrose as sugars.

Inactivation and reactivation of pectinesterase after pressure treatment

After unsterilized squeezed juices of Satsuma mandarin were pressurized at 1000–6000 bar for 10 min, the pressurized juices were stored at 0°C, room temperature (about 23°C), or 37°C for three months. Occasionally, samples were taken out and pectinester-

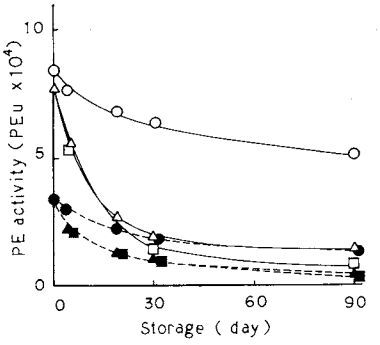

Fig. 4. Changes in Pectinesterase (PE) Activity of Pressurized Juices during Storage.

Pressure treatment was done at 3000 (——) and 4000 bar (------), and at room temperature for 10 min. Pressurized juice was stored at 0°C (○, ●), room temperature (△, ▲), and 37°C (□, ■). See Fig. 3 for the other experimental conditions.

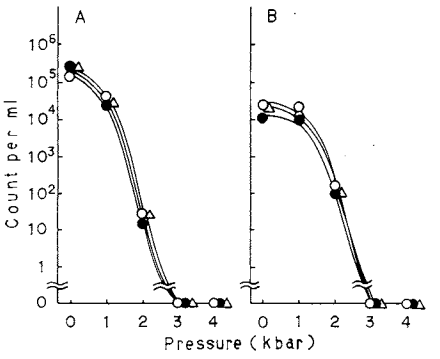

Fig. 5. Effects of pH of Juice on Pressure Inactivation of Microorganism.

Saccharomyces bayanus (A) and *Mucor plumbeus* (B) were inoculated in juice adjusted to pH 2.5 (○), pH 3.5 (●), and pH 4.5 (△), and pressurized as indicated in the figure for 10 min. After the pressure-treatment the survival of microorganisms was measured.

ase activities were measured (Figs. 3 and 4).

Treatment at different pressures resulted in different degrees of reduction in pectinesterase activity. This result was also described previously.[6] The reduced pectinesterase activity did not recover during the storage at 0°C (Fig. 3). In the storage at higher temperature (room temperature or 37°C), pectinesterase activity also decreased gradually without showing any tendency to recover (Fig. 4). Juice pressurized at 1000 or 2000 bar putrefied during storage at room temperature and 37°C.

These results show that high pressure treatment partly and irreversibly inactivates pectinesterase, not to be reactivated during storage and transportation.

Versteeg *et al.* observed the different heat resistance among pectinesterase isozymes found in various citrus fruits (Navel orange, Valencia orange, *etc.*).[15] The pectinesterase isozymes with high heat resistance may also remain active after high pressure treatment, because no complete inactivation of the enzyme was attained at any pressure level used here.

Effects of pH on the inactivation of microorganisms

Satsuma mandarin juices adjusted to various pHs (pH 2.5 to 4.5) were inoculated with various species of yeast and mold, and were pressurized at 1000 to 4000 bar for 10 min.

As shown in Fig. 5, no pH dependency was observed with the inactivation of *Saccharomyces bayanus* and *Mucor plumbeus* by the pressurization. Similar results were also obtained for other species (*Schizosaccharomyces pombe, Saccharomyces cerevisiae, Hansenula anomala, Rhodotorula glutinis, Pichia membranaefaciens,* and *Aspergillus awamori*).

The pH-effects on the pressure inactivation were tested with the juices pH adjusted with HCl or NaOH. No difference in pressure-induced inactivation was observed with yeasts and molds. Thus, it is concluded that the inactivation effect of pressure does not depend on the pH of the juice.

In heat sterilization, the influence of pH has been studied and it is well known that the heat resistance of bacteria decreases as the pH becomes lower. However, there are only few and controvertible studies regarding the relationship between pH and heat resistance of yeasts and molds which prefer relatively acidic conditions.

Effects of organic acids on the inactivation of microorganisms

Various organic acids were mixed with the

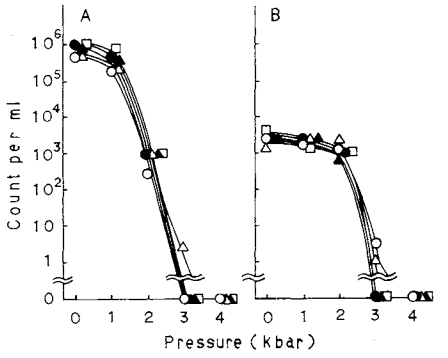

Fig. 6. Effects of Organic Acids on Pressure Inactivation of Microorganisms.

Citric acid (pH 3.5) (○), malic acid (pH 3.5) (●), tartaric acid (pH 3.2) (△), lactic acid (pH 3.6) (▲), or acetic acid (pH 4.0) (□) was mixed with the acid-free juices (pH shown in parentheses is pH of the mixture.) and *Saccharomyces bayanus* (A) and *Mucor plumbeus* (B) were inoculated in the mixture. After pressurization was done at 1000 to 4000 bar for 10 min, the survival of microorganisms was measured.

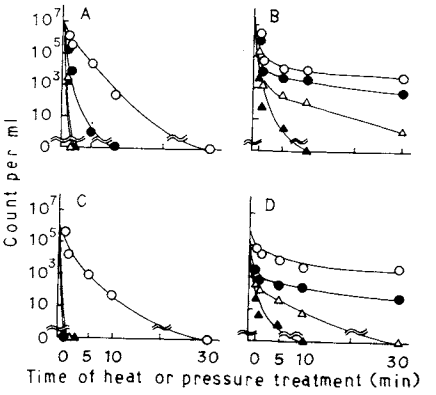

Fig. 7. Effects of Pressure or Heat Treatments on *Saccharomyces cerevisiae* in Juice.

The juice inoculated by *Saccharomyces cerevisiae* was pressurized at 2500 (○), 3000 (●), 3500 (△), and 4000 bar (▲) and at room temperature (A and B), or heated at 53°C (○), 58°C (●), 60°C (△), and 62°C (▲) and 1 bar (C and D). *Saccharomyces cerevisiae* inoculated was previously cultured on potato dextrose agar medium (A and C) or *Gorodokowa* medium (B and D).

juice, acids of which were previously removed, and microorganisms inoculated into the mixture. These juices were subjected to pressure treatment to measure the influence of organic acids on the pressure inactivation of microorganisms.

Figure 6, A and B show the results with *Saccharomyces bayanus* and *Mucor plumbeus* inoculated, respectively. Neither stimulation nor suppression of pressure inactivation was observed with organic acid mixed in, and no great difference in sterilizing effects was observed among the different organic acids. A similar result was also obtained for the other 6 species (*Schizosaccharomyces pombe, Saccharomyces cerevisiae, Hansenula anomala, Rhodotorula glutinis, Pichia membranaefaciens,* and *Aspergillus awamori*), except that acetic acid stimulated the sterilizing effects on *Rhodotorula glutinis.*

As pH varied from pH 3.2 to 4.0 among juices containing different organic acids, the result also shows that pH does not influence the pressure inactivation of these tested microorganisms.

Comparison of pressure and heat effects on the inactivation of yeasts and molds

To study the effects of heat and pressure on microorganisms, Satsuma mandarin juice inoculated with yeasts or molds was treated with heat (50–62°C) or pressure (2500–4000 bar) and the courses of the inactivation were measured.

Figure 7 shows the results with *Saccharomyces cerevisiae.* The sterilizing effect increased as the applied pressure or heat and the duration of the treatments were increased. However, the effects were different between two culture media, potato dextrose agar medium and *Gorodokowa* medium. As *Gorodokowa* medium is served as a medium for sporulation, the results may be due to a difference in pressure or heat resistance of the nutritive cells and spores of yeast. That is, spores of yeast are more resistant to pressure as well as heat treatment than the nutritive cells.

Figure 8 shows the results with the highest heat-resistant wild yeast which was isolated in the juice factory.

These strains were resistant to not only heat treatment but also pressure treatment.

Figure 9 shows the results with *Rhodotorula*

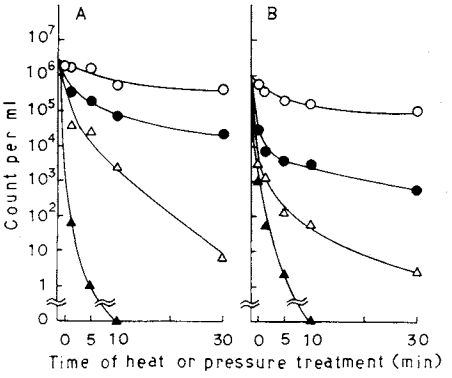

Fig. 8. Effects of Pressure or Heat Treatments on Heat-Resistant Yeast in Juice.

The juice inoculated with wild yeast was pressurized at 2500 (○), 3000 (●), 3500 (△), and 4000 bar (▲), and at room temperature (A), or heated at 53°C (○), 58°C (●), 60°C (△) and 62°C (▲) and at 1 bar (B). Wild yeast isolated in the juice factory was previously cultured on the *Gorodokowa* medium.

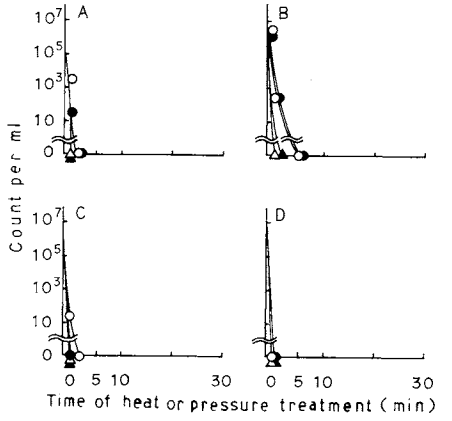

Fig. 9. Effects of Pressure or Heat Treatment on *Rhodotorula glutinis* and *Hansenula anomala* in Juice.

Juice inoculated by *Rhodotorula glutinis* (A and C) or *Hansenula anomala* (B and D) was pressurized at 2500 (○), 3000 (●), 3500 (△), and 4000 bar (▲) and at room temperature (A and B), or heated at 53°C (○), 58°C (●), 60°C (△) and 62°C (▲) and at 1 bar (C and D). *Rhodotorula glutinis* and *Hansenula anomala* were previously cultured on potato dextrose agar medium.

glutinis and *Hansenula anomala*. These species were poorly resistant to pressure and heat treatments.

The results with *Aspergillus awamori* and *Mucor plumbeus* (Fig. 10) also show the parallelism in heat and pressure resistance.

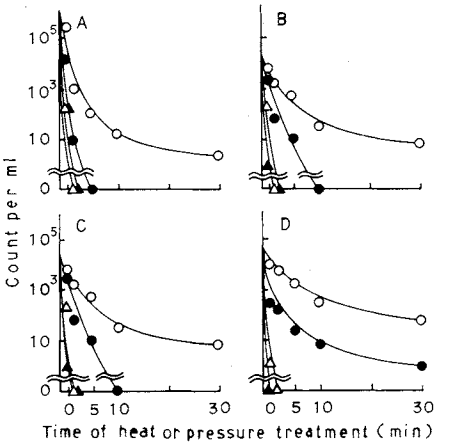

Fig. 10. Effects of Pressure or Heat Treatments on *Aspergillus awamori* or *Mucor plumbeus* in Juice.

Juice inoculated by *Aspergillus awamori* (A and C) or *Mucor plumbeus* (B and D) was pressurized at 2500 (○), 3000 (●), 3500 (△), and 4000 bar (▲) and at room temperature (A and B), or heated at 50°C (○), 53°C (●), 58°C (△) and 60°C (▲) and at 1 bar (C and D). *Aspergillus awamori* or *Mucor plumbeus* were previously cultured on potato dextrose agar medium.

Other than the six species tested in Figs. 7–10, three reference species (*Pichia membranaefaciens, Saccharomyces bayanus*, and *Schizosaccharomyces pombe*) also showed paralleled resistance to heat and pressure (data not shown). Thus, heat-resistant yeasts and molds are judged to be pressure-resistant.

Consideration of pressure-sterilization of juices

Conditions required for heat-sterilization at a given temperature are usually expressed as nD (n is a safety index and usually 5–6, and D means decimal reduction time at a certain temperature).[16] This method may also be applicable to pressure-sterilization. As judged from Fig. 8, pressure-treatment at 3500 bar for 30 min or at 4000 bar for 5 min reduces the count of highly pressure-resistant yeast to $1/10^5$.

Pressure of several to ten thousand bar is not enough to inactivate highly heat-resistant bacterial spores.[3] High pressure is also required to inactivate enzymes in juice. These situations are similar to heat-treatment: higher temperature is required to inactivate bacterial

spores and enzymes than to achieve commercial sterility. These similarities of temperature and pressure effects indicate pressure treatment is as useful in inactivation of microorganisms and enzymes as heat treatment. However, the merits of pressure use to keep natural flavor and taste should be valuable in juice manufacturing.

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References

1) B. H. Hite, N. J. Giddings and C. E. Weakley, *Bull. West Virginia Univ. Agric. Exp. Sta.*, **146**, 1 (1914).
2) P. W. Bridgman, *J. Biol. Chem.*, **19**, 511 (1914).
3) W. J. Timson and A. J. Short, *Biotechnol. Bioeng.*, **7**, 139 (1965).
4) R. Hayashi, *Shokuhin to Kaihatu*, **22**(7), 55 (1987).
5) R. Hayashi, in "Engineering and Food," ed. by W. E. L. Spiess and H. Schubert, Elsevier Appl. Su., England, 1989, p. 815.
6) H. Ogawa, K. Fukuhisa, H. Fukumoto, K. Hori, and R. Hayashi, *Nippon Nōgeikagaku Kaishi*, **63**, 1109 (1989).
7) H. Ogawa, K. Fukuhisa, H. Fukumoto, in "Use of High Pressure in Food," ed. by R. Hayashi, San-ei Shuppan Co., Kyoto, 1989, p. 57.
8) T. Takahashi, I. Yashiro, H. Tokumura, *Aichi-ken Shokuhin Kogyo Shikenzyo Nenpo*, **16**, 6 (1975).
9) T. Itou, S. Kawabata, H. Kurusu, N. Nagumo and M. Matsumoto, in "Standard Methods of Analysis for Hygienic Chemists-With Commentary," authorized by the Pharmaceutical Society, Kanehara Shuppan Co., Tokyo, 1980, p. 132 and p. 258.
10) F. Sakabe, in "Shokuhin Eisei Kensa Shishin," Vol. 1, ed. by Environmental Health Bureau, Ministry of Health and Welfare, Nippon Shokuhin Eisei Kyokai, Tokyo, 1973, p. 161 and p. 162.
11) H. Kitagawa, S. Kamei, K. Kawada and T. Tarutani, *Engei Gakkai Zasshi*, **52**, 189 (1983).
12) N. Matsuda, M. Komaki, and K. Matsunawa, *Kanzumeziho*, **59**, 785 (1980).
13) M. R. Marshall, J. E. Marcy, and R. J. Braddock, *J. Food Sci.*, **50**, 220 (1985).
14) T. Kawabata, "Shokuhin Biseibutsugaku," ed. by K. Aiso, Ishiyaku Shuppan, Tokyo, 1976, p. 154.
15) C. Versteeg, F. M. Rombouts, C. H. Spaansen, and W. Pilnik, *J. Food Sci.*, **45**, 970 (1980).
16) N. Matsuda, *Boukin Boukabi*, **7**, T577 (1979).