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Note

Changes in Proteins during Nyufu Production

Chiyoko TOKUE[1] and Eiko KATAOKA[2]

[1]*Department of Nutritional Science, Faculty of Applied Bio-Science,* [2]*Department of Nutrition, Junior College of Tokyo University of Agriculture, 1-1-1, Sakuragaoka, Setagayaku, Tokyo 156-8502, Japan*

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The aging rate of fungi-inoculated tofu was found to be 34.5% and that of 1-month-old and 3-month-old nyufu fermented milk 56.2% and 71.4%,respectively. The quantity of free amino acids in nyufu increased to approximately 10 times the level in fungi-inoculated tofu. Because solubilization of protein was shown to be associated with the duration of nyufu aging, changes in the molecular weight of the water soluble nitrogen fraction were assessed using HPLC gel filtration chromatography. Almost no peptides with a molecular weight over 5000 were found in the 3-month-old nyufu; approximately 57% had a molecular weight of 1800, and 38% existed at peaks below a molecular weight of 255, indicating a decreased molecular size and a noticeable difference in molecular weight distribution. Water soluble peptides were separated into acidic, basic, and neutral fractions. The neutral fraction had the highest score of sensory evaluation. The concentration of glutamic acid and aspartic acid was high in neutral and acidic fractions, reflecting the palatability. Approximately 50 of the peptide chromatograms were detected in peaks 2 and 3 of the neutral fraction, which was high in the sensory evaluation. This agreement between the sensory evaluation results and chromatographic analysis indicates that peptides influence the taste of nyufu.

Keywords: Nyufu, soybean cheese, protein, water soluble peptides, amino acids, cheese-like taste, aging rate

Nyufu (lit. "fermented milk", in Chinese: Rufu) is a fermented soybean food that has been produced for many years in China and Taiwan, where itis known as Tofunyu (orig. Tofu-ryu) or Funyu (orig. Furu), respectively. Nyufu is a seasoned food in which a *Mucor* fungus is grown on the surface of the tofu, after which the tofu is pickled and then aged by soaking invarious unrefined soy sauces. This process produces a soft, moderately sticky and elastic structure with a smooth cross section. A variety of flavors can be obtained by addition of various seasonings to the unrefined soy sauce. The entire surface of the tofu is covered with fungal mycelia through the propagation of fungi such as *Actinomucor repens* (Ho, 1979), *Mucor hiemalis*, and *Mucor prainii* (So, 1979) during the production process. This prevents the tofu from losing its shape during the aging process. The fungi used in this process demonstrate weak decomposition activity for the tofu component while promoting rapid growth of the mycelia. In addition, the fungi have long, pure white mycelia that are very strong. Tofuyo, a product from Okinawa that is similar to nyufu, is produced using a partial improvement of the nyufu process (Katsura & Fukuba, 1980; Yasuda, 1990). However, despite its long history, the biochemical processes involved in basic nyufu production remain unclear. Previous studies have concentrated on the soft structureand cheese-like taste of aged nyufu. These studies have also determined the composition of the tofu during nyufu production, as well as changes in both the volatile acid content (Okada *et al.*, 1974) and lipid component (Tokue & Kataoka, 1997).

The present study investigated the changes in proteins thought to influence the properties and taste of nyufu in an attempt to understand aging in the production process.

Materials and Methods

Process of nyufu production Tofu of maximum firmness was produced as described (Fig. 1) (Tokue & Kataoka, 1997), and designated raw tofu. Dried tofu was then created by cutting the raw tofu into 2 cm cubes and circulating warm air at approximately 50°C over the cubes for 30 min. The dried tofu was inoculated with *Actinomucor repens* IAM 6160 so that white mycelia covered the entire surface, this was designated fungi-inoculated tofu. The fungi-inoculated tofu was then pickled in a 10% brine for 3 days to produce salted tofu. Next, the salted tofu was pickled in unrefined soy sauce for 1 or 3 months. (The unrefined soy sauce used in the present study was made by grinding six-month-old unrefined soy manufactured by Kubota Miso and Syoyu, Ltd., Chiba, using a MASUKOROIDA, MKZB-15-40).

Samples The raw tofu, dried tofu, fungi-inoculated tofu, salted tofu, 1-month-old nyufu, 3-month-old nyufu, unrefined soy sauce, unrefined soy sauce from 1-month-old nyufu, and unrefined soy sauce from 3-month-old nyufu samples specified above were analyzed in this study.

Analytical methods The chemical composition of samples was measured as described (Tokue *et al.*, 1987).

The total nitrogen content was measured by the Kjeldahl method in accordance with the Food Analysis Method (Nagahara *et al.*, 1966). This measurement was performed 5 times for each sample and the average total nitrogen content calculated. The water soluble nitrogen content was measured in accordance with the method normally used in cheese analysis (Tokyo University, Faculty of Agriculture, Agricultural Chemistry Lectures, 1960). Briefly, 10 g of each sample

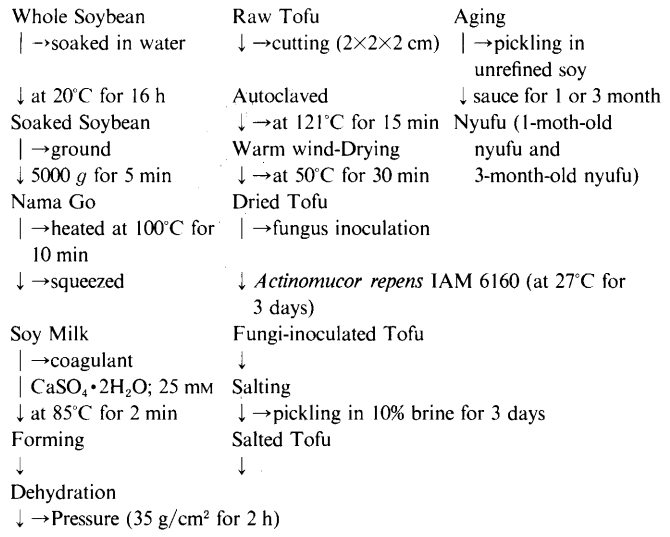

Fig. 1. Process for nyufu production.

was pulverized and thoroughly mixed with demineralized distilled water that had been heated to approximately 50°C. The mixture extraction was then shaken for 20 min, and then was centrifuged at 3000 rpm for 15 min and the resultant supernatant was diluted to 200 ml. Next, an aliquot of the diluted solution was used to measure the water soluble nitrogen content.Five measurements were taken for each sample and the average water soluble nitrogen content was calculated.

Free amino acids were measured according to the method of Tamura and Suzuki (1972). Demineralized distilled water (20 ml) was added to 2 g of each sample, and the mixtures homogenized. Next, 70 ml of 95% ethanol was added, bringing the total volume of each solution to 100 ml. After being kept at 5°C for 3 h, the solutions were filtered and the ethanol was removed using an evaporator. Finally, 10 ml of pH 2.2 N citric acid buffer solution was added to each mixture. The mixtures were then filtered and the resulting filtrates were used for analysis. The total amino acid content was measured as follows: 10 ml of each sample was demineralized using a cartridge (TOYOPAK ODS) and dehydrated with a centrifugal evaporator. Then, 6 N hydrochloric acid was added for hydrolysis to take place at 110°C for 24 h. The amino acid component of each sample was measured using a Hitachi 835 amino acid analyzer and a Hitachi custom ion-exchange resin.

Measurement of the molecular weights of water soluble nitrogen fractions during nyufu production was performed by gel-chromatography (Kato, 1990; Souda, 1991). Based on a calibration curve prepared using molecular weight markers, the molecular weight of each peak top was determined, and the peak area was calculated by the area percentage. The extracts were then centrifuged at 10,000 rpm for 30 min and the resulting supernatants were suction filtered by a membrane filter (Cellulose Nitrate, 0.45 μm, Toyo Roshi, Tokyo) in order to obtain the water soluble nitrogen fractions of each sample. The filtrates were then adjusted to 50 ml with demineralized distilled water and analyzed using a high performance liquid chromatography (HPLC) (Shimadzu LC-6A, SCL-6B, Kyoto) under the following conditions: column: TSK-GEL, G3000 SW (7.5 mmID×30 cm, manufactured by Toso Co., Tokyo), column temperature: 25°C, eluant: 50 mM Phosphoric acid buffer solution +0.3 N NaCl (pH 7.0), flow rate: 0.5 ml/min, detection wavelength: UV (220 nm).

Separation and reversed-phase HPLC of the water soluble peptides To extract the water soluble peptide fractions from a 10 ml sample of 1-month-old nyufu, the sample was loaded onto a column containing three types of preprocessing cartridges: one for the acid fraction (TOYOPAK DEAE), one for the basic fraction (TOYOPAK IC-SP), and one for the neutral fraction (TOYOPAK ODS) (Yasuhara *et al.*, 1995). The bound peptides were then eluted using 5 ml of 1 M acetic acid for the acid fraction (DEAE), 5 ml of 0.1 M triethylamine for the basic fraction (IC-SP), and 5 ml of methanol for the neutral fraction (ODS). The fractions containing the adsorbed material were isolated and each portion was lyophilized (EYELA FD-81, Tokyo Rika) after desalination to produce acidic, basic, and neutral fractions. The fractions were then analyzed by gel-chromatography. Four peaks were isolated from the acidic fraction,four from the basic fraction, and three from the neutral fraction.

Reversed-phase HPLC (Ikenaka & Sakiyama, 1990) was performed on peak 2 of the neutral fraction and a peptide chromatogram obtained. The measurement conditions for the reversed-phase HPLC were: column: TSK-GEL ODS-80TS (4.6 mmID×25 cm, Toso Co.), eluant: solvent A was 0.1% trifluoroacetic acid (TFA, Pierce Co.) in demineralized distilled water and solvent B was 0.1% TFA in 100% acetonitrile. gradient: A/B (85/15)→B (90 min linear gradient), flow rate: 1.0 ml/min, column temperature: 25°C, detection wavelength: UV (220 nm).

Sensory evaluation was performed by ten trained panels (aged 25 to 48 years) at the Department of Nutrition, Tokyo University of Agriculture. Sensory evaluation (Shimada *et al.*, 1975) for palatability (including sweetness), bitterness, and body was performed by the scoring method (−2∼+2) using de-mineralized distilled water as a contrast.

Results and Discussion

Changes in protein, aging rate, and amino acids during nyufu production The changes observed in composition during nyufu production are shown in Table 1. The protein content of the dried tofu was 10.3%, whereas that of the fungi-inoculated tofu was 8.3%, thus confirming that the fungi-inoculation process decomposes proteins. The protein content of salted tofu was even lower at 7.3%. Although the protein content of the nyufu was higher than that of salted tofu, the composition of proteins in the dry matter was 49% in the dried tofu and 32.1% in the 1-month-old nyufu. This shows that proteins are decomposed during the aging process. The water content was 79% at the dried tofu stage but decreased to 65.4% for the 1-month-old nyufu. The lipid content continually decreased throughout the aging process, however, the sugar, fiber, and ash content all increased.

Because proteins appear to be decomposed during the production of nyufu, the aging rate (the ratio of soluble

Table 1. Chemical composition of each samples during nyufu production. (g/100 g)

Component	A	B	C	D	E	F
Moisture	79.0	79.1	75.0	65.4	62.1	66.4
Protein	10.3 (49.0)	8.3 (39.7)	7.3 (29.1)	11.3 (32.1)	12.4 (32.6)	9.9 (29.5)
Lipid	6.1 (29.0)	5.9 (28.2)	5.3 (21.2)	5.6 (16.2)	5.8 (15.3)	0.3 (0.9)
Total-sugar	3.3 (15.8)	4.9 (23.5)	5.8 (23.3)	8.6 (24.8)	14.2 (24.4)	7.2 (22.6)
Fiber	0	0.5 (2.4)	0.6 (2.4)	0.9 (2.6)	1.1 (2.9)	2.2 (6.5)
Ash	1.3 (6.2)	1.3 (6.2)	6.0 (24.0)	8.4 (24.3)	9.4 (24.8)	13.6 (40.5)

A, Dried tofu; B, Fungi-inoculated tofu; C, Salted tofu; D, 1-month-old nyufu; E, 3-month-old nyufu; F, Unrefined soy sauce. ( ), In parenthesis, dry base percentage is shown.

Table 2. Changes in aging rate during nyufu production. (%)

Aging rate	A	B	C	D	E	F
Aging rate (WSN/TN)	4.4	34.5	32.8	56.2	71.4	69.9

A, Dried tofu; B, Fungi-inoculated tofu; C, Salted tofu; D, 1-month-old nyufu; E, 3-month-old nyufu; F, Unrefined soy sauce. WSN: Water soluble nitrogen, TN: Total nitrogen.

Table 3. Free amino acids content of each samples during nyufu production. (mg/100 g)

Amino acid	A	B	C	D	E	F	G	H
Asp	7	57	66	425	470	490	450	509
Thr	2	15	18	197	241	236	231	263
Ser	2	20	27	265	318	335	297	359
Glu	9	107	100	986	1104	1038	1063	1224
Pro	ND	ND	ND	275	276	282	238	352
Gly	4	8	8	158	181	181	161	191
Ala	10	45	19	225	238	250	231	261
Cys	ND	7	tr	98	112	99	93	115
Val	5	22	23	313	342	226	307	172
Met	ND	6	tr	95	103	98	91	106
Ile	ND	11	13	261	193	281	255	300
Leu	5	30	37	443	478	457	423	497
Tyr	ND	29	34	116	110	254	267	320
Phe	8	36	42	337	342	317	300	365
Lys	9	19	18	296	319	290	298	341
His	4	12	tr	106	116	98	108	123
Arg	14	tr	tr	290	306	355	340	332
GABA	1	52	9	50	29	52	51	33
Orn	ND	14	11	10	14	ND	6	14
NH_3	ND	42	29	149	154	150	142	169
Total	80	532	454	5095	5446	5489	5352	6046

A, Dried tofu; B, Fungi-inoculated tofu; C, Salted tofu; D, 1-month-old nyufu; E, 3-month-old nyufu; F, Unrefined soy sauce; G, Unrefined soy sauce from 1-month-old nyufu; H, Unrefined soy sauce from 3-month-old nyufu; ND, not detected; tr, trace.

nitrogen to total nitrogen) of each sample was determined (Table 2). Aging rate is an important indicator for the aging of miso, cheese, and fermented soybean food product (Fuke & Matsuoka, 1987; Yasuda *et al.*, 1993). The aging rate of fungi-inoculated tofu was found to be 34.5%, whereas that of salted tofu was 32.8% due to the dissolution of soluble nitogen in the brine. The aging rate of the 1-month-old and 3-month-old nyufu was 56.2% and 71.4%, respectively. However, the concentration of insoluble nitrogen was the highest in the salted tofu while that in the 1-month-old nyufu was decreased. As a result, increases in the aging rate can be explained in terms of the decomposition of protein by aging and the shift of proteins from the unrefined soy sauce. The aging rate of the 3-month-old nyufu was higher than that of unrefined soy sauce, suggesting that the components of the two substance can move both ways. The changes in free amino acids during nyufu production are shown in Table 3. The free amino acid content of dried tofu was 80 mg/100 g, while that of fungi-inoculated tofu was 532 mg/100 g. The large increase in the value of amino acids in these samples appears to be caused by the generation of free amino acids by *Actinomucor repens* IAM 6160 generated proteases or by bacteria present in the tofu. The quantity of free amino acids in both 1-month-old and 3-month-old nyufu increased to approximately 10 times the level in fungi-inoculated tofu, and the quantity present in 3-month-old nyufu increased more than that in 1-month-old nyufu. This increase is clearly due to the absorption of amino acids into the nyufu from the unrefined soy sauce. This conclusion is also supported by the fact that the quantity of amino acids in unrefined soy sauce after three months of pickling was higher than that in the nyufu. Furthermore, because yeast proteases are present in unrefined soy sauce (Takeda *et al.*, 1967; Hosokawa & Yoshii, 1967), the increase in amino acid content appears to be linked to the degradation of protein in the nyufu by these proteases. Although no difference between the amino acid content of the unrefined soy sauce after one month of pickling and that before pickling were evident, after three month of pickling the free amino acid content was higher than that before pickling, thus indicating that the soybean protein in nyufu is converted to amino acids and released into the unrefined soy sauce. Therefore, amino acids appear to move reciprocally between unrefined soy sauce and nyufu. The high content of glutamic acid and aspartic acid as palatability indicators, as well as the high content of the amino acids proline, serine, alanine, and threonine as sweetness indicators, greatly influence the taste of nyufu.

Changes in molecular weight distribution in water soluble nitrogen fraction during nyufu production Because solubilization of protein was shown to be associated with the duration of nyufu aging, changes in the molecular weight in the water soluble nitrogen fraction were assessed using HPLC gel filtration chromatography (Fig. 2). For raw tofu, 6 peaks occurred at >285,000, 20,000, 7800, 3400, 700 and <255. However, the 7800 and 3400 peaks dissipated to 5400 and 1800, respectively, for fungi-inoculated tofu, indicating a lower molecular weight. Approximately 12% of the water soluble peptide of the fungi-inoculated tofu had a molecular weight of 20,000, 24% had a weight of 5400, 38% had a weight

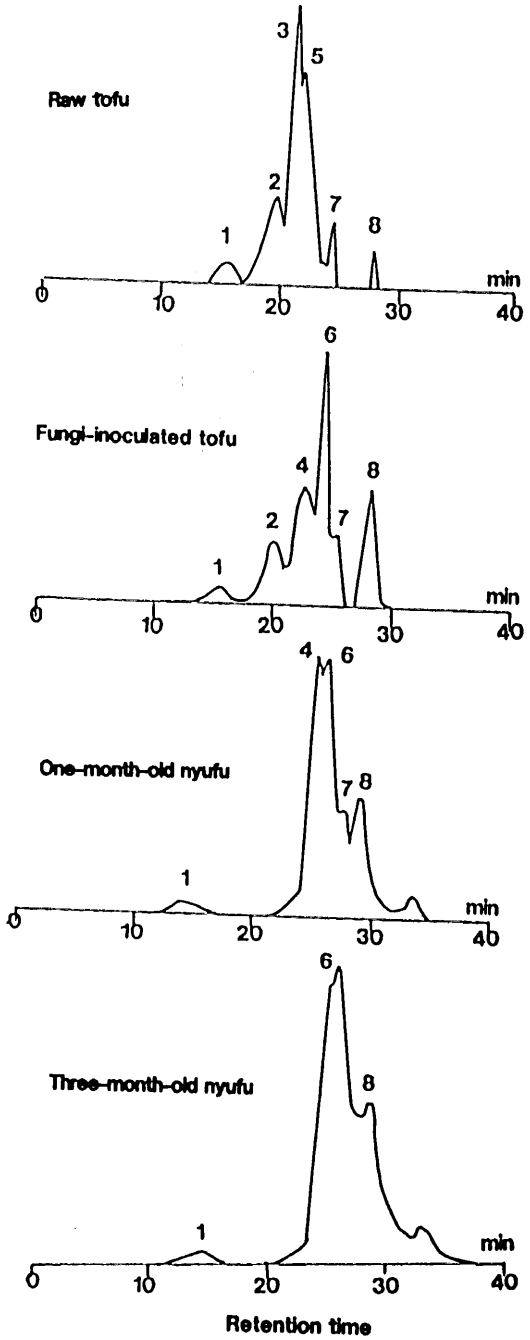

Fig. 2. Gel-filtration chromatogram by HPLC of the water soluble fractions during nyufu production. Peak 1, M.W. more 285,000; 2, M.W. 20,000; 3, M.W. 7800; 4, M.W. 5400; 5, M.W. 3400; 6, M.W. 1800; 7, M.W. 700; 8, M.W. less 255.

of 1800, and 16% were considered to be free amino acids having a peak below a molecular weight of 255. Almost no peptides with a molecular weight over 5000 were found in the 3-month-old nyufu; approximately 57% had a molecular weight of 1800, and approximately 38% existed at peaks below a molecular weight of 255, indicating a decreased molecular size and a noticeable difference in molecular weight distribution. This confirmed that an abundance of low molecular peptides existed in the water soluble fraction of 1-month-old and 3-month-old nyufu.

Taste of water soluble peptide fraction Previous studies (Okada *et al.*, 1974; Tokue & Kataoka, 1997) have concentrated on the soft structure and cheese-like taste of aged nyufu. A large number of compounds such as nucleic acids, free amino acids, sugars, organic acids, and minerals are known to produce taste in food. Peptides are also considered to be such compounds. Although they do not function as the main element of taste, peptides provide body and smoothness and are thus viewed as indispensable to flavor (Fujimaki *et al.*, 1968a; b; Kirimura *et al.*, 1969; Shimizu & Kataoka, 1970; Nishimura *et al.*, 1988; Ishii *et al.*, 1994; Ishii, 1996). Sensory evaluation was therefore made of the acidic, basic, and neutral fractions in water soluble peptide fractions of 1-month-old nyufu. The neutral fraction had the highest score with an average palatability (including sweetness) of 0.8, an average body of 0.4 and an average bitterness of 0. The acid fraction received the next best positive score, with palatability (including sweetness), bitterness, and body all achieving scores of 0.2. The basic fraction received the lowest score, with an average of −0.2 for each category. The amino acid composition of these three fractions is shown in Table 4. The concentration of glutamic and aspartic acids was high in the neutral and acidic fractions, reflecting the palatability, and the concentration of proline, glycine, and alanine was also high in these fractions, reflecting the sweetness. The basic amino acids valine, methionine, arginine, and lysine which reflect bitterness were abundant in the basic fraction. Comparatively weak bonds, which are partially comprised of the phospholipid phosphatidylcholine, exist between soybean proteins and soybean lipids. These hydrophilic proteins have a similar amino acid composition to 7S and 11S globulin (Kito *et al.*, 1979). The unique properties and taste of nyufu are generated by soluble protein, which, together with the neutral lipids and phospholipids, is emulsified and dispersed throughout the nyufu. Gel filtration was also performed on these three fractions, with four peaks being isolated from the acidic fractions, four from the basic fraction, and three from the neutral fraction. The results of sensory evaluation are

Table 4. Amino acids composition of whole, acidic, basic and neutral water soluble fractions in 1-month-old nyufu. (mol%)

Amino acid	Whole	Acidic	Basic	Neutral
Asp	10.4	10.7	2.1	9.9
Thr	2.8	5.5	5.1	6.5
Ser	2.9	6.3	4.9	3.8
Glu	13.6	15.7	7.0	17.2
Pro	8.1	9.8	2.2	8.5
Gly	5.6	8.9	8.1	8.9
Ala	7.2	8.7	5.0	7.9
Cys	3.3	3.0	3.3	6.3
Val	7.6	6.3	11.6	6.2
Met	4.2	5.7	11.9	3.5
Ile	3.0	3.9	7.7	5.2
Leu	8.6	7.3	5.0	8.1
Tyr	4.0	1.8	2.0	1.4
Phe	6.0	2.6	6.1	2.4
Lys	4.5	3.8	5.2	2.6
His	2.5	ND	2.0	ND
Arg	1.9	ND	10.8	ND
NH_3	3.8	ND	ND	1.6

ND, not detected.

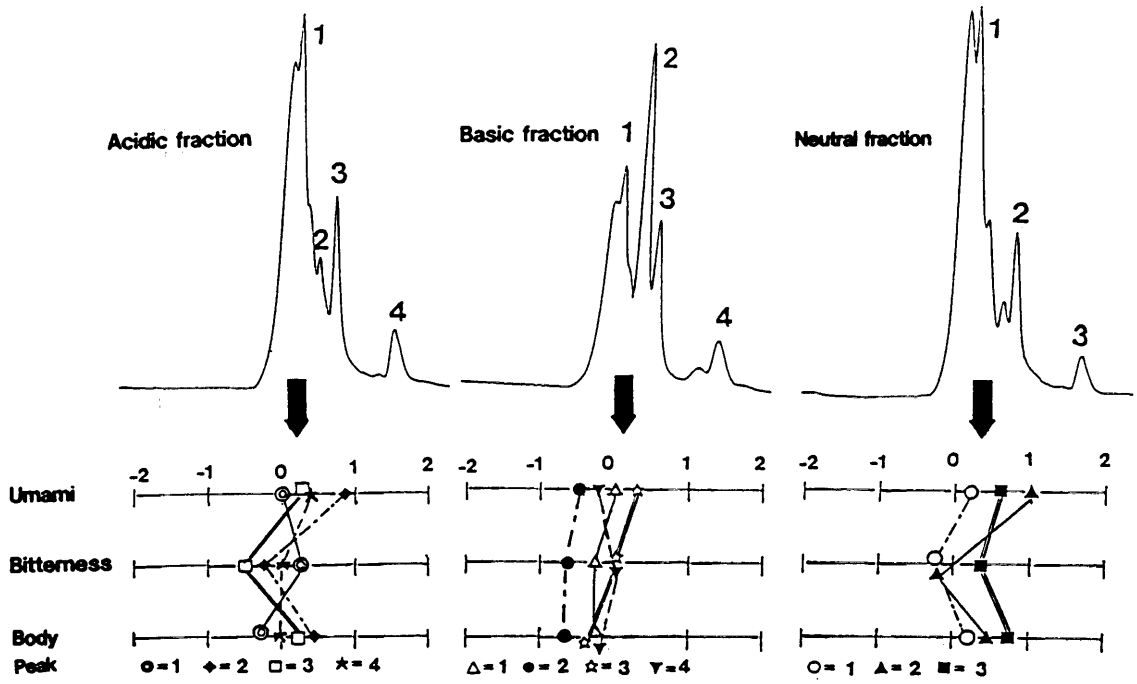

Fig. 3. Sensory evaluation was performed the 4 peaks isolated from the acidic fraction, the 4 peaks from the basic fraction, and the 3 peaks from the neutral fraction of the water soluble peptide fractions in 1-month-old nyufu.

shown in Fig. 3. The acidic fraction obtained a high score for peak 2 (M.W. 1300~255) and the neutral fraction did so for peaks 2 (M.W. 1000~255) and 3 (M.W. below 255). The overall flavor of the basic fraction was weak and had a strong bitterness component. Thus, low molecular weight peptides are the components responsible for the unique taste and flavor of nyufu. To identify these peptides, the aforementioned peaks were isolated and reversed-phase HPLC was performed after lyophilization. Only a few peaks on chromatograms of the basic fraction, which had bean scored low in the sensory evaluation, were detected. However, approximately 45 of the peptide chromatograms were detected in peak 2 of the acidic fraction, which scored high in the sensory evaluation, and almost 50 of the chromatograms were detected in peaks 2 and 3 of the neutral fraction. This agreement between the sensory evaluation results and chromatographic analysis indicates that peptides influence the taste of nyufu. In the future, we plan to identify the peptides involved in taste perception.

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