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Nutritional Value and Sensory Acceptance of Fermented Meat Sauce "Shishibishio" with Neutrase and Flavourzyme Enzyme under Different Salt Concentrations

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Abstract: Shishibishio, a Japanese traditional seasoning, is made from fermented meat. The study aimed to evaluate the effect of neutrase 0.5 L alone or flavourzyme 500 L + neutrase 0.5 L on the acceleration of fermentation period and the improvement of nutritional value and sensory acceptance of the final product. The fermentation mixtures (moromi) were prepared by mixing ground pork with salt at one of three concentrations (15%, 20% or 25%), koji (rice fermented with *Aspergillus oryzae*) and pepper. To accelerate the fermentation of moromi, all treatments were added with neutrase at the begining, then adding with or without flavourzyme after one month. The results showed that neutrase or neutrase + flavourzyme mixture accelerated hydrolysis of meat protein during the fermentation of moromi. Yield, protein recovery, total nitrogen and free amino acid in shishibishio treated with enzymes were significantly greater ($P < 0.05$) than that of control, especially at 15% salt. The treatment treated with neutrase + flavourzyme was significantly increased ($P < 0.05$) in free amino acid as compared with treatment with neutrase alone, resulting a better sensory taste and smell, which was mostly accepted. Almost the shishibishio obtained after six fermentative months was acceptable seasonings having a good taste and no unpleasant smell.

Key words: Fermented meat sauce, flavourzyme, neutrase, shishibishio, nutritional value, sensory acceptance.

1. Introduction

In Asia, there are two popular sauces that are not only used mainly as condiments, but also as additional sources of protein for human consumers. They are the soy sauce made from fermented soybeans, and the fish sauce made from fermented fishes. In Japan, soy sauce is the dominant seasoning. Although fish sauce is as not popular as soy sauce that has a much milder taste and smell, it has been consumed since ancient times. In addition, meat and meat by-products can be considered as alternative sources, because they had been used for hundreds of years to make the fermented sauce called shishibishio [1].

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There is very little literature regarding the production and properties of shishibishio, while that of fish sauce and soy sauce are very available. In fish sauce production, traditional methods take about 9-18 months. To accelerate the fermentation process, additives containing proteolytic enzymes are also used. Neutrase and flavourzyme are the commercially available enzymes that have been widely used in the production of protein hydrolysates [2-7].

Generally, flavor of the protein hydrolysates has to meet the consumer preference. The extensive hydrolysis of proteins normally produces a strong meaty flavor. On the other hand, partial hydrolysis of some proteins, especially soybean, casein protein, can produce a bitter taste by the formation of bitter peptides [2]. To control the bitterness of partially

hydrolyzed protein, enzymatic hydrolysis is applied. The mixture of endo-peptidase and exo-peptidase has been used for food protein hydrolysis in order to reduce the bitterness of the hydrolysates, as well as to increase the degree of hydrolysis [2, 6]. Chae et al. [8] suggested that if the first hydrolysis of animal proteins is carried out using neutrase, the hydrolysates produce in this step may be relatively bitter in some case due to the formation of bitter peptides, and this bitterness will disappear during the subsequent hydrolyse with flavourzyme, as the bitter peptide is degraded. The application of neutrase and flavourzyme has been reported to hydrolyse animal and vegetable protein and their by-products [2-4, 7].

Shishibishio made from ground pork using commercial enzymes, i.e., Alcalase 2.4L or Pectinase 3S, Alcalase 2.4L produced a higher peptide contents, but lower total free amino acid contents in comparison with Pectinase 3S. Thus, acceleration of hydrolysis of peptides to free amino acids during fermentation period would also be an advantage for improving the sensory quality of shishibishio [9]. In the current study, ground pork mixed with some ingredients: salt, koji and pepper was used to make shishibishio. Neutrase 0.5L alone or flavourzyme 500L + neutrase 0.5L were added into fermentation mixtures to evaluate their effect on the acceleration of fermentation period and the improvement of nutritional value and sensory acceptance of the final product.

2. Materials and Methods

Ground pork was obtained from a meat packer at 1 d after slaughter for making moromi. Koji was obtained from a koji shop in Obihiro, Japan. Neutrase 0.5L (EC 3.4.24.28) with activity of 0.5 Anson units per gram (AU/g) and flavourzyme 500L (EC 3.4.11.1) with activity of 500 leucine amino peptidase units per gram (LAPU/g) were obtained from Novozymes A/S, Bagsvaerd, Denmark.

2.1 Preparation of Fermented Meat Sauce (Shishibishio)

The shishibishio production scheme was shown in Fig. 1. It was divided into two steps: the first was to prepare fermentation mixture (moromi) and the second was to collect shishibishio.

For moromi fermentation, ground pork was thoroughly mixed with koji, salt, pepper and neutrase 0.5L in the composition as shown in Table 1. In these mixtures, percentage (w/w) of koji was 10%, the salt was 15%, 20% or 25% in different treatments and enzyme was 0.5% neutrase 0.5L. For control, commercial enzyme was not added into moromi, and the ingredients ratio was same as above. The moromi was packed into plastic bags (800 g/bag), carefully sealed and kept in incubator (30 ± 0.2 °C) for fermentation. After one month, the moromi of enzyme treatments were divided into two parts, and flavourzyme 500L (0.5% w/w) was added into one part. Right after moromi prepared (0 month), and then right after one, two, three and six months fermented, samples of about 60 g were taken from the control and enzyme treatments groups for microbial analysis and collection of shishibishio. The samples were centrifuged for 30 min at 28,000 ×g and 0 °C, then filtered with Toyo filter paper No. 5C to obtain the liquid called shishibishio. Samples of shishibishio were kept in the refrigerator at 4 °C until the chemical analysis was carried out. All data was presented as the averages of triplicate experiments.

2.2 Microbiological Analysis

Five grams of moromi was taken from fermentatived plastic bag, placed in a sterile stomacher bag and homogenized for 3 min with 45 mL of sterilized saline (0.85% NaCl) in a Stomacher laboratory mixer (BA 7021, Seward, Lab Blender, England). Then this homogenate was diluted from 10^{-1} to 10^{-5} dilution, and 1 mL samples of the diluted mixture were pipetted out into sterile Petri dishes. Fifteen milliliters of the appropriate growth medium cooled about 37-38 °C were then poured into each dish and mixed gently. The presence of total bacteria was determined on standard agar (Eiken, Japan). De Man, Rogosa and Sharpe agar (MRS agar, Oxoid, England)

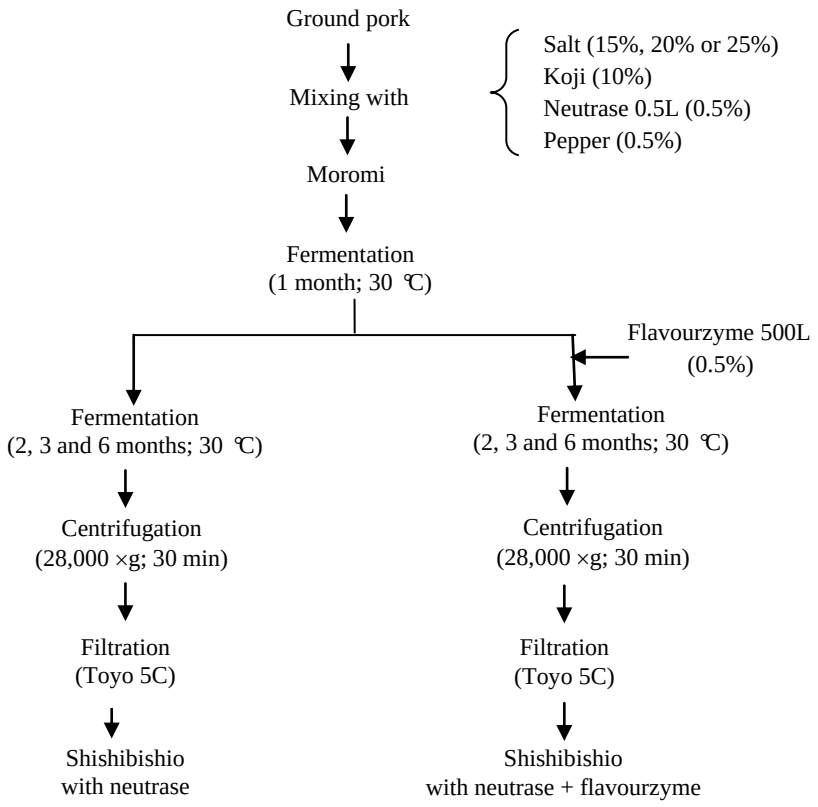

Fig. 1 Shishibishio production scheme.

Table 1 Composition of moromi[*].

Composition	Composition of moromi (g)					
	15% salt		20% salt		25% salt	
	Control	Neutrase	Control	Neutrase	Control	Neutrase
Ground pork	596	592	556	552	516	512
Salt	120	120	160	160	200	200
Koji	80	80	80	80	80	80
Pepper	4	4	4	4	4	4
Neutrase	0	4	0	4	0	4
Total	800	800	800	800	800	800
	Neutrase-treated moromi after one fermentative month[**]					
	Without flavourzyme	With flavourzyme	Without flavourzyme	With flavourzyme	Without flavourzyme	With flavourzyme
Moromi	400	398	*400*	398	400	398
Flavourzyme	0	2	0	2	0	2

[*] There were three replications of each moromi.

[**] Each moromi of neutrase bag was divided into two parts, then flavourzyme was added to one part.

was used for growth of lactic acid bacteria (LAB). The present of LAB was identified by a catalase test using 3% H_2O_2. Chromocult coliform agar (Merck, Germany) was used for the growth of *Coliform* group. Plates were incubated at 37 °C for 24 h for *Coliform* group, 48 h for total bacteria and 72 h for LAB. Colonies were counted from plates containing 30-300 colonies, the plates containing less than 30 colonies

were not counted and subsequently considered less than 300 CFU/g.

2.3 Chemical Analysis

Yield of shishibishio from moromi was calculated as the percentage of weight of shishibishio divided by weight of moromi.

Total nitrogen was determined by Kjeldahl's method, and crude protein was calculated as N × 6.25.

Protein recovery of shishibishio from moromi was calculated as the percentage of crude protein of shishibishio divided by crude protein of moromi.

Samples determining peptides and free amino acids were prepared according to the procedures described by Mikami et al. [10]. The 2% trichloroacetic acid (TCA) solution was prepared by mixing 4 g of shishibishio with the same weight of 4% TCA solution. It was incubated at 37 °C for 30 min. The supernatant was then filtered with Toyo filter paper No. 5C. The filtrate was used for analysis of peptides and free amino acids.

Peptide content was determined by the Lowry-Folin method [11] with bovine serum albumin (Seikagaku Corporation Co., Tokyo, Japan) as a standard.

Free amino acid content was determined by the O-phthalaldehyde reagent with amino acid analyzer (JASCO, Model 8000) using the lithium buffer system.

2.4 Sensory Evaluation

Shishibishio obtained after six months fermentation was used for sensory evaluation. Sixteen panelists whose ages ranged from 18 to 50 years were asked to evaluate the colour, smell, taste and overall of the products according to three scales ("very good", "good" or "bad") of preference. Samples were evaluated in random order, and the panelists were not allowed to talk to each other during testing. For the assessment of smell, panelists sniffed directly each sample containing in a test tube (1 cm × 10 cm). For the taste test, about 1 mL of each sample was given to the panelists one after the other and they were asked to rinse their mouth with water between samples. Along with smell and taste, the colour was judged by visual observation.

2.5 Statistical Analysis

Analysis of variance (ANOVA) was calculated with Statistical Analysis System (SAS Institute Inc.) according to the general linear model (GLM) procedures using Duncan's multi-range test ($P < 0.05$).

3. Results and Discussion

3.1 Changes in Microbial Counts in Moromi during Fermentation

Changes in microbial counts from moromi were presented in Table 2. Initial counts of total bacteria ranged from 5.4×10^5 CFU/g to 7.4×10^5 CFU/g in all samples. As the fermentation advanced, the numbers of bacteria decreased rapidly, and only a smaller number of viable count were detected (less than 300 CFU/g) in any moromi after three months. The decrease in the viable microbial counts was caused by high salt concentration and reduced pH [7, 12, 13]. It has been reported that total viable microbial counts was decreased as increased fermentation time during the production of fermented fish sauces [12, 14].

LAB was presented in all samples of moromi, and the counts ranged from 1.6×10^4 CFU/g to 2.6×10^4 CFU/g at start. LAB dropped to below 300 CFU/g in the control after one month, while it still remained 3.5×10^2 to 1.5×10^3 CFU/g in enzyme treatments after two months, then dropped to below 300 CFU/g after three months in any moromi. The higher number LAB in moromi with enzyme treatments was probably due to that the addition of enzymes immediately accelerated proteolysis, resulting in more available amino acids for LAB growth. Optimum growth of LAB depended on salt concentration. Paludan-Muller et al. [15] showed that increasing the salt concentration delayed or inhibited LAB growth in

Table 2 Changes in microbial counts in moromi during fermentation.

Months	15% salt			20% salt			25% salt		
	Control	Neutrase	N + F	Control	Neutrase	N + F	Control	Neutrase	N + F
Common bacteria (CFU/g)									
0	7.4×10^5	6.3×10^5	6.5×10^5	5.8×10^5	6.3×10^5	6.4×10^5	5.6×10^5	6.5×10^5	5.4×10^5
1	5.1×10^3	4.6×10^3	4.8×10^3	2.8×10^3	2.6×10^3	2.4×10^3	1.5×10^3	2.4×10^3	2.5×10^3
2	4.0×10^2	3.7×10^3	3.5×10^3	3.2×10^2	3.4×10^3	2.5×10^3	3.6×10^2	1.8×10^3	1.5×10^3
3	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300
6	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300
Lactic acid bacteria (CFU/g)									
0	2.6×10^4	2.3×10^4	2.2×10^4	2.0×10^4	2.4×10^4	2.1×10^4	1.7×10^4	1.6×10^4	1.8×10^4
1	< 300	2.2×10^3	2.4×10^3	< 300	1.8×10^3	1.6×10^3	< 300	8.3×10^2	8.7×10^3
2	< 300	1.5×10^3	1.6×10^3	< 300	1.4×10^3	2.3×10^3	< 300	3.5×10^2	3.2×10^2
3	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300
6	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300
Coliform group (CFU/g)									
0	5.2×10^3	4.2×10^3	4.2×10^3	4.6×10^3	5.0×10^3	5.0×10^3	3.4×10^3	2.6×10^3	2.9×10^3
1	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300	< 300
2	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values were expressed as mean for $n = 3$; N + F: neutrase was combined with flavourzyme; ND: not detected.

Thai fermented plaa-som. In the current study, the higher salt concentration of the moromi was expected so that it would suppress LAB growth, resulting in a reduced count.

At initial time, *Coliform* group counts ranged from 2.6×10^3 CFU/g to 5.2×10^3 CFU/g, but dropped to below 300 CFU/g after one month, and no viable counts were detected in any moromi after two months. The decrease in the viable *Coliform* group counts was probably related to the high salt concentration of the moromi. Salt was known to play an important role in preventing the growth of spoilage and pathogenic bacteria in fermentation [13, 14]. This suggested that even 15% salt addition could inhibit the growth of *Coliform* group in these moromi.

3.2 Change in Shishibishio Yield during Fermentation

Shishibishio yield obtained from moromi was presented in Fig. 2. At start, yield ranged from 10.58% to 14.05%. During the first months, yield increased sharply and thereafter increased gradually. The amount of yield obtained from moromi in the enzyme treatments was significantly higher than that in control ($P < 0.05$), suggesting that the addition of enzymes to the moromi increased the rate of liquefaction of moromi during fermentation. Neutrase and flavourzyme activity were reported to be high in hydrolysis of fish, meat and soybean [2, 4, 6, 7]. After six months, shishibishio yield with 15% salt reached to 35.05%, 63.25% and 64.75% for the control, neutrase and neutrase + flavourzyme, respectively. In case with 20% and 25% salt, yield were 28.5%, 56.12%, 58.97% and 26.74%, 52.35%, 54.78% for the control, neutrase and neutrase + flavourzyme, respectively. This suggested that the use of flavourzyme increased the amount of shishibishio. Lee et al. [2] studied with the enzymatic hydrolysis of defatted soybean flour and reported that the combination of alcalase + flavourzyme mixture gave higher yield of soluble protein and its derivatives than the use of a single enzyme. Among three levels of salt concentration, yield with 15% salt was the highest, followed by that with 20% salt and 25% salt. Reduction of yield with 20% or 25% salt was probably

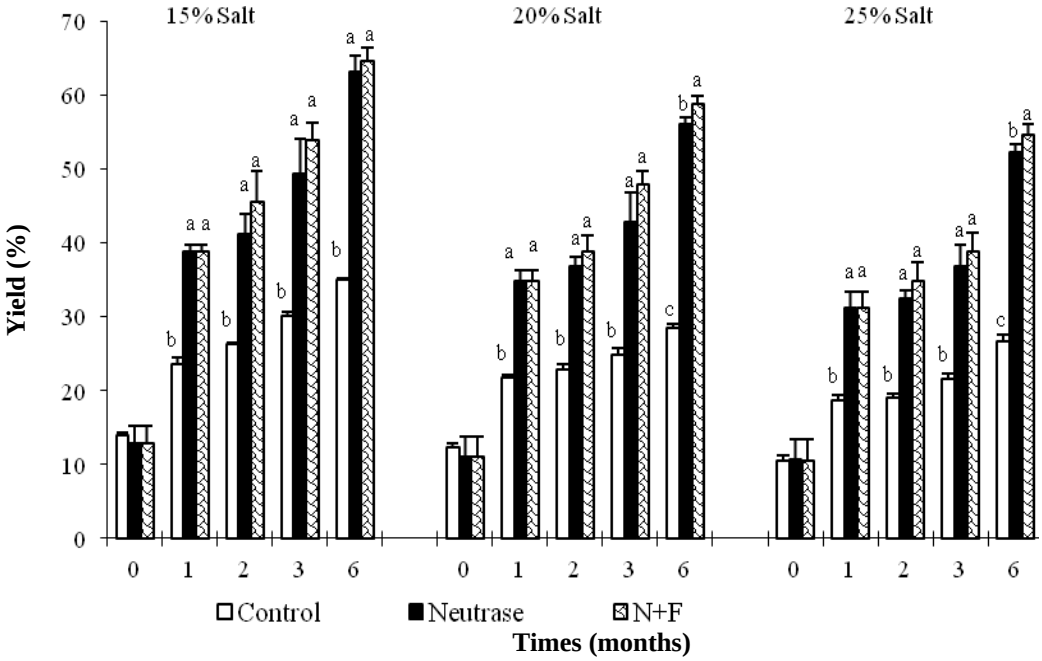

Fig. 2 Changes in shishibishio yield during fermentation.
Values were expressed as mean ± standard deviation (n = 3). [a-c] Values in the same group with no common superscript were significantly different ($P < 0.05$).

caused by the lower amount of meat than that with 15% salt at initial time (Table 2). This result agrees with the study of Aquerreta et al. [3], who reported that a decrease in the level of salt from 25% to 5% increased the yield of Roman fish sauce garum.

3.3 Change in Total Nitrogen of Shishibishio during Fermentation

Changes in total nitrogen of shishibishio during fermentation were shown in Fig. 3. Total nitrogen ranged from 0.52 g/100 mL to 0.83 g/100 mL in shishibishio at start. Total nitrogen increased rapidly during first month, and thereafter only a slight increase was observed in any shishibishio. After six months, total nitrogen of shishibishio reached to 1.87-2.24 g/100 mL in control and 2.71-2.91 g/100 mL in enzyme treatments. The release of water-soluble protein from muscle cells caused by osmotic pressure resulted in an total nitrogen increase during fermentation [12]. The proteolytic activity of moromi with enzyme treatments was faster than that of control during fermentation, resulting that the total nitrogen in enzyme treatments was significantly higher than that of control throughout the fermentation ($P < 0.05$). This might be caused by less of proteolytic enzymes in control to hydrolyze meat protein to peptides and free amino acids. However, no significant changes were observed in total nitrogen between neutrase and neutrase + flavourzyme treatments. This might be due to the effects in the hydrolysis functionality of enzyme. Flavourzyme was aminopeptidase, which hydrolysed peptides to free amino acids, while neutrase was exoproteinase that was widely used in the production of protein hydrolysates. Park et al. [16] indicated that total nitrogen of fish sauce from seven countries of Southeast and East Asia changed from 0.35 g/100 mL to 2.59 g/100 mL. On the other hand, Nakamura et al. [17], Yano et al. [18] and Trang et al. [9] reported that the total nitrogen of fermented meat sauce reached to 1.9-2.6 g/100 mL after three months of fermentation. According to Thai Industrial Standard Institute, high quality nampla had a total nitrogen more than 1.5 g N/100 mL [19]. Total nitrogen, equivalent to or higher

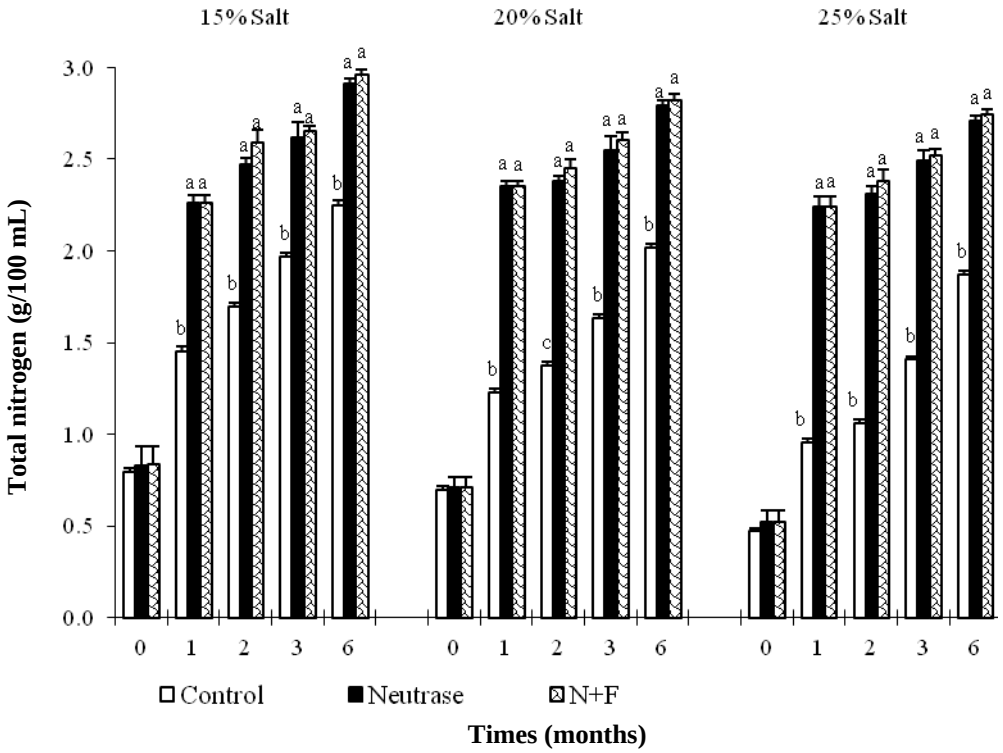

Fig. 3 Changes in total nitrogen of shishibishio during fermentation.

Values were expressed as mean ± standard deviation for $n = 3$. [a-c] Means in the same group with no common superscript were significantly different ($P < 0.05$).

than this level (1.5 g N/100 mL), was obtained from shishibishio after one month for enzyme treatments. Base on the current study, it was suggested that addition of commercial enzymes could increase the conversion of insoluble to soluble protein derivatives and could accelerate the fermentation period in the production of shishibishio. In comparing total nitrogen among three salt concentrations, shishibishio with 15% salt had the highest value, followed by that with 20% and then 25% at every time of fermentation. It was considered that the amount of meat in moromi with 15% salt was larger than with 20% or 25% salt (Table 2), and therefore the level of total nitrogen in shishibishio was influenced by the salt concentration in moromi.

3.4 Change in Protein Recovery of Shishibishio during Fermentation

Changes in protein recovery of shishibishio during fermentation were shown in Fig. 4. Protein recovery in control and enzyme treatments was significant difference ($P < 0.05$). The higher protein recovery was obtained from moromi treated with enzyme in comparison with control during fermentation. Protein recovery of shishibishio increased sharply during the first month, then increased gradually and reached to 28.55%, 65.54% and 68.72%, respectively, for the control, neutrase and neutrase + flavourzyme of shishibishio with 15% salt after six months. In case of shishibishio with 20% and 25% salt, these values were 21.69%, 58.42%, 62.73% and 17.36%, 54.22%, 57.56%, respectively. In the current study, the percentage of protein recovery for enzyme treatments was around two or three folds in comparison with control, and the value of protein recovery with neutrase + flavourzyme treatments was higher than that of neutrase treatments with the same level of salt concentration. The result indicated that addition of commercial enzymes increased the conversion of protein from insoluble to soluble forms. Among three

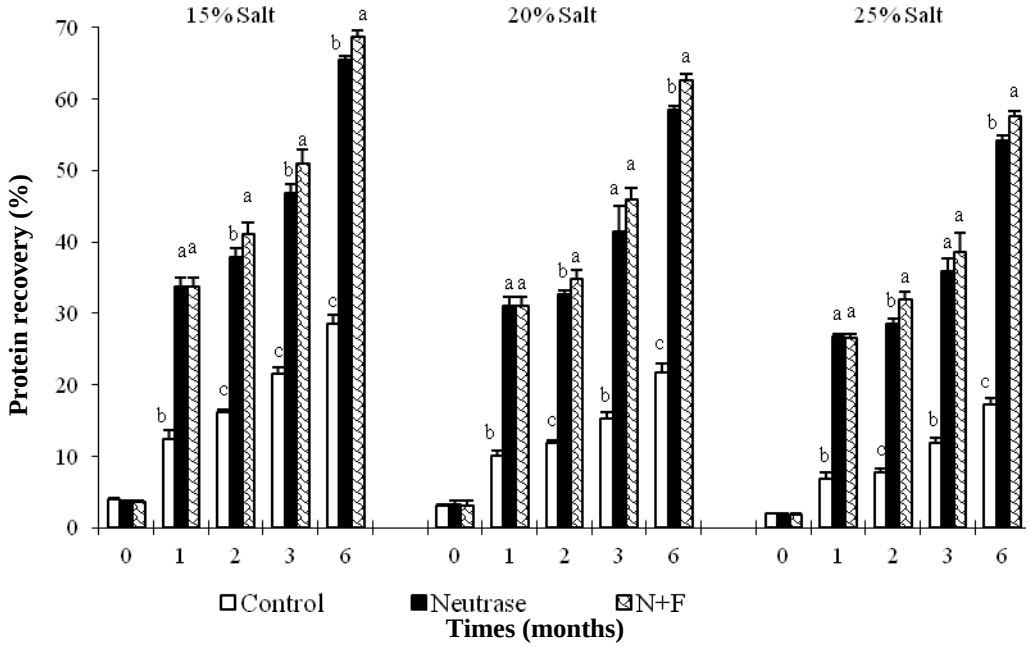

Fig. 4 Changes in protein recovery of shishibishio during fermentation.
Values were expressed as mean ± standard deviation (n = 3). [a-c] Values in the same group with no common superscript were significantly different ($P < 0.05$).

salt concentrations, the recovery rate of protein at 15% salt was higher than that at 20% or 25% salt. This was probably the same reason in case of total nitrogen. Moreover, the numbers of microoganisms and enzyme activity were also higher at the lower saline concentration. Gildberg and Thongthai [20] reported that both autolytic and microbial activities increased, when salt content was reduced to below 20%. In the current study, these values for enzyme treatments after six months were similar to that in Chinese, Vietnamese, Thai, Korean and Japanese fish sauces (57.8%, 61.6%, 64.3%, 68.5% and 70.4%, respectively) [16], but higher than that in fermented seasoning (45.8%) made from lean beef after four months [17]. Gildberg [21] reported that the best protein recovery of fish sauce was made from fish by-products (male Arctic capelin and Atlantic cod intestines).

3.5 Change in Peptide Content of Shishibishio during Fermentation

The change in the peptide content of shishibishio during fermentation was presented in Fig. 5. Peptide content increased to 2.44-2.58 g/100 mL in control and to 6.62-7.46 g/100 mL in enzyme treatments at the first month. However, peptide content decreased in neutrase and neutrase + flavourzyme, but increased at two months in control. This was probably that commercial enzymes might contain proteinase and aminopeptidase, therefore, peptide content increased at initial time due to the hydrolysis of protein by proteinase, and then peptides were hydrolyzed to amino acids by aminopeptidase at two months. At six months, the peptide content reached to 6.81-7.23 g/100 mL in control, 8.79-9.41 g/100 mL in neutrase and 7.64-8.16 g/100 mL in neutrase + flavourzyme, respectively. The release of higher amount of peptide contents in enzyme treatments was mainly due to breakdown of the meat protein by commercial enzymes. In contrast, peptide contents of neutrase + flavourzyme treatments were lower than that of neutrase treatments from two months to six months ($P < 0.05$). According to Chae et al. [8], flavourzyme was a peptidase enzyme and therefore accelerated the hydrolysis

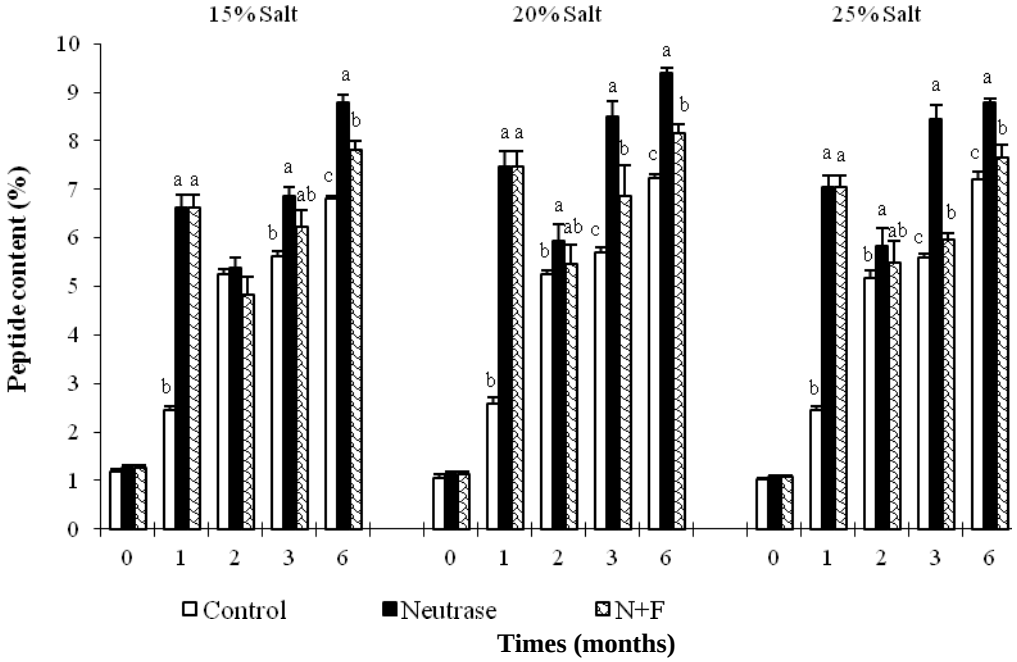

Fig. 5 Changes in peptide content of shishibishio during fermentation.
Values were expressed as mean ± standard deviation (n = 3). [a-c] Values in the same group with no common superscript were significantly different ($P < 0.05$).

of peptides into amino acids.

3.6 Total Free Amino Acid of Shishibishio after Six Months

Total free amino acid of shishibishio at six months was shown in Table 3. Total free amino acid was approximately 4,247-5,943, 6,361-7,482 and 7,841-8,817 mg/100 mL in control, neutrase and neutrase + flavourzyme, respectively. The total free amino acid in enzyme treatments was higher in comparison with control ($P < 0.05$). This resulted from the more proteolytic activity contributed by enzyme addition. Total free amino acid was higher in the treatment treated with neutrase + flavourzyme (1,334-1,481 mg/100 mL) than the neutrase treatment. These results are consistent with the fact that the peptide contents detected in treatments with neutrase + flavourzyme were lower than that with neutrase alone (Fig. 5). Comparing the results obtained for samples with and without enzymes at the three levels of salt, it could be observed that a decrease in the level of salt would increase the proteolytic, resulting in the higher total free amino acid, especially when enzymes were added. According to Ijong and Ohta [22], different salt concentrations had a great effect on the contribution of amino acid in bakasang. The individual free amino acid varied among the shishibishio, however glutamic acid, alanine, valine, leucine, lysine and arginine were the predominant free amino acids in any shishibishio. The relationship between the individual amino acid and the flavor has been reported by Aquerreta et al. [3], Herpandi et al. [6] and Shih et al. [23]. All these free amino acids could contribute to the final taste of the product. Especially, the good taste of sauce might be attributed to the high concentration of glutamic acid [24]. In the current study, glutamic acid in shishibishio ranged from 594 mg/100 mL to 1,302 mg/100 mL. The difference of glutamic acid among shishibishio might contribute an important part to the difference of sensory evaluation of products (Table 3).

3.7 Sensory Evaluation for Shishibishio at Six Months

The sensory evaluation of shishibishio after six

Table 3 Free amino acids (mg/100 mL) in shishibishio after six months.

Amino acid	15% salt			20% salt			25% salt		
	Control	Neutrase	N + F	Control	Neutrase	N + F	Control	Neutrase	N + F
Asp	496.7 ± 29.9^{b}	519.4 ± 19.8^{b}	750.9 ± 28.2^{a}	311.4 ± 15.9^{c}	425.4 ± 11.6^{b}	657.4 ± 14.3^{a}	252.7 ± 15.9^{c}	394.3 ± 10.2^{b}	613.3 ± 16.5^{a}
Thr	246.5 ± 7.4^{c}	355.4 ± 8.2^{b}	500.4 ± 14.2^{a}	252.6 ± 20.4^{c}	308.7 ± 11.2^{b}	480.8 ± 19.3^{a}	177.3 ± 17.0^{c}	291.7 ± 22.4^{b}	390.3 ± 9.9^{a}
Ser	326.2 ± 22.3^{c}	435.2 ± 1.8^{b}	471.5 ± 19.4^{a}	260.8 ± 11.6^{c}	361.7 ± 4.4^{b}	429.9 ± 6.5^{a}	247.9 ± 18.1^{c}	307.2 ± 16.8^{b}	437.9 ± 21.1^{a}
Asn	182.2 ± 19.4^{c}	291.5 ± 8.3^{b}	384.7 ± 34.5^{a}	159.5 ± 10.0^{b}	200.1 ± 19.1^{b}	340.8 ± 24.2^{a}	143.8 ± 4.8^{b}	207.3 ± 5.5^{a}	226.6 ± 18.3^{a}
Glu	827.0 ± 10.6^{b}	1,197.8 ± 82.4^{a}	1,301.9 ± 59.3^{a}	677.0 ± 17.5^{c}	1,076.5 ± 17.3^{b}	1,266.1 ± 11.3^{a}	594.0 ± 17.2^{c}	993.6 ± 10.1^{b}	1,263.2 ± 38.0^{a}
Gln	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pro	157.0 ± 10.2	189.3 ± 62.7	219.6 ± 19.8	91.0 ± 6.3^{b}	89.3 ± 8.7^{b}	207.3 ± 16.3^{a}	54.5 ± 12.0^{b}	173.6 ± 17.0^{a}	197.6 ± 6.1^{a}
Gly	164.5 ± 21.0^{b}	195.7 ± 9.2^{b}	251.5 ± 22.3^{a}	111.7 ± 8.3^{b}	163.6 ± 11.1^{b}	221.2 ± 18.7^{a}	111.1 ± 12.4^{c}	131.8 ± 3.7^{b}	199.5 ± 8.4^{a}
Ala	517.1 ± 11.8^{b}	642.6 ± 12.3^{a}	691.3 ± 24.4^{a}	404.9 ± 25.2^{b}	635.4 ± 5.7^{a}	657.7 ± 24.7^{a}	327.2 ± 8.7^{b}	619.1 ± 28.8^{a}	619.6 ± 11.1^{a}
Val	322.4 ± 13.2^{c}	507.8 ± 5.2^{b}	608.8 ± 13.8^{a}	311.4 ± 26.3^{c}	466.8 ± 13.9^{b}	640.6 ± 20.3^{a}	263.4 ± 13.4^{c}	435.5 ± 10.3^{b}	558.6 ± 16.7^{a}
Cys	ND	ND	ND	ND	ND	ND	ND	ND	ND
Met	213.1 ± 6.8^{b}	201.7 ± 17.1^{b}	230.5 ± 9.0^{a}	158.3 ± 10.2^{b}	219.0 ± 4.8^{a}	176.4 ± 24.1^{ab}	167.0 ± 10.1	179.2 ± 3.3	146.4 ± 13.8
Ile	313.1 ± 22.5^{b}	364.9 ± 27.1^{a}	406.4 ± 14.6^{a}	317.7 ± 18.7^{b}	363.7 ± 21.6^{a}	390.0 ± 0.3^{a}	249.3 ± 19.6^{b}	335.4 ± 8.4^{a}	350.3 ± 12.8^{a}
Leu	604.5 ± 13.4^{a}	513.3 ± 9.8^{b}	424.1 ± 6.1^{c}	490.8 ± 24.4^{a}	515.5 ± 14.0^{a}	424.6 ± 10.8^{b}	467.1 ± 19.1	477.4 ± 42.7	442.6 ± 16.0
Tyr	151.6 ± 3.4^{b}	213.5 ± 17.7^{a}	155.2 ± 8.9^{b}	158.1 ± 15.4^{b}	216.6 ± 2.7^{a}	176.8 ± 10.3^{b}	117.7 ± 13.8^{b}	168.2 ± 18.9^{a}	158.3 ± 9.8^{ab}
Phe	207.5 ± 9.7^{c}	341.0 ± 29.4^{b}	409.4 ± 9.9^{a}	203.7 ± 24.7^{c}	348.2 ± 10.2^{b}	383.1 ± 17.1^{a}	154.1 ± 5.8^{c}	316.3 ± 3.0^{b}	391.6 ± 12.9^{a}
Lys	683.9 ± 20.2^{c}	887.2 ± 21.5^{b}	1098.6 ± 55.6^{a}	612.2 ± 29.9^{c}	859.7 ± 78.1^{b}	948.7 ± 23.3^{a}	529.4 ± 27.2^{c}	782.8 ± 20.4^{b}	1026.7 ± 15.5^{a}
His	89.3 ± 10.6^{b}	112.4 ± 9.3^{b}	187.9 ± 9.6^{a}	62.9 ± 9.8^{c}	104.1 ± 5.8^{b}	169.4 ± 2.5^{a}	74.0 ± 10.6^{b}	88.0 ± 12.3^{b}	155.6 ± 11.5^{a}
Arg	440.8 ± 30.3^{c}	513.8 ± 9.6^{b}	723.9 ± 36.7^{a}	314.7 ± 13.7^{c}	527.5 ± 26.1^{b}	762.6 ± 15.9^{a}	316.1 ± 21.4^{c}	459.3 ± 12.4^{b}	636.0 ± 36.7^{a}
Total	5,943.4 ± 63.6^{c}	7,482.4 ± 149.1^{b}	8,816.7 ± 84.8^{a}	4,898.8 ± 65.3^{c}	6,881.7 ± 55.8^{b}	8,333.5 ± 35.6^{a}	4,246.5 ± 55.8^{c}	6,360.7 ± 85.6^{b}	7,841.2 ± 30.3^{a}

Values were expressed as mean ± standard deviation (n = 3); N + F: neutrase combined with flavourzyme; ND: not detected.
$^{a-c}$ Values in a row of the same group with no common superscript were significantly different (P < 0.05).

Table 4 Sensory evaluation for shishibishio after six months.

Shishibishio		15% salt			20% salt			25% salt		
		Control	Neutrase	N + F	Control	Neutrase	N + F	Control	Neutrase	N + F
Colour	Very good	4	11	15	9	10	14	8	12	13
	Good	8	5	1	7	6	2	6	4	3
	Bad	4	0	0	0	0	0	2	0	0
Smell	Very good	4	10	15	3	10	11	7	8	10
	Good	11	5	1	13	4	5	8	6	6
	Bad	1	1	0	0	2	0	1	2	0
Taste	Very good	4	11	15	4	11	10	4	7	5
	Good	5	5	1	4	4	6	5	7	10
	Bad	7	0	0	8	1	0	7	2	1
Overall	Very good	5	10	16	4	9	14	4	5	5
	Good	5	6	0	7	6	2	4	6	10
	Bad	6	0	0	5	1	0	8	5	1

Total panelists were 16 people and people were in each parameter; N + F: neutrase combined with flavourzyme.

months was shown in Table 4. The color of shishibishio treated with enzymes was highly "very good" and "good", while that of control was accepted at the least. A clear lightly brown liquid was produced in control; however, a deep brown color developed in treatments with neutrase and neutrase + flavourzyme. The darkening of color in those samples might be due to browning reactions, formed in aqueous solutions of

reducing sugars and amino acids [14, 24]. Due to the higher amino acid in enzyme treatments, shishibishio produced from moromi with neutrase or neutrase + flavourzyme was darker and more brown, and shishibishio production was faster than in control. The treatments treated with neutrase + flavourzyme showed a difference in the smell and taste as compared to that in neutrase treatment and control. The difference might be attributed to the difference in the concentration of salt and free amino acid content. Most of the panelists evaluated "very good" and "good" on the smell of treatments treated with neutrase + flavourzyme, while 1-2 panelists rejected that of control and neutrase. Similarly, the taste of treatments treated with neutrase + flavourzyme was evaluated mostly "very good" and "good", except for a few panelists evaluated "bad" in 25% initial salt due to their saltier taste. These samples also were low content of total free amino acid. In general, overall quality in enzyme treatments with 15% and 20% salt was mostly accepted, but that with 25% initial salt was accepted at the least.

4. Conclusions

In general, salt, koji, commercial proteases (neutrase and flavourzyme) and minor ingredients have significant effects on shishibishio quality. In the current study, salt concentrations were applied with 15%, 20% and 25%, and the viable bacteria counts after three fermentative months were low (< 300 CFU/g). The addition of commercial proteases (neutrase and flavourzyme) increased the autolytic release from protein to peptide and free amino acids, especially in shishibishio with low salt (15%). Shishibishio made from the fermentation of ground pork is high in total protein, peptide and amino acid. Shishibishio with add of neutrase has higher peptide, but lower total amino acid as compared with that added with neutrase + flavourzyme. In addition, amino acid is higher in shishibishio treated with flavourzyme than that without flavourzyme. Protein recovery of shishibishio and yield obtained from moromi are also high after six fermentative months. Although in the current study, organoleptic testing is not showed, a brief judgement by members of the laboratory indicates that shishibisio is as tasty as high quality commercial fish sauces, especially it has slightly aroma and flavor. Among shishibishio, addition with 25% NaCl leads to stronger salty in taste, and 15% better in taste, aroma and flavor.

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