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Fermentation of meat with koji and commercial enzymes, and properties of its extract

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Abstract: This study was carried out in order to make the Japanese fermented meat sauce *shishibishio*. The fermentation mixtures (*moromi*) were prepared by mixing ground pork with salt at one of three levels (15, 20 or 25%), koji (rice fermented with *Aspergillus oryzae*) and pepper. Commercial enzymes, ie Alcalase or Pectinase 3S, were added in order to accelerate the proteolysis of the *moromi*. After 3 months of fermentation, counts of viable bacteria were below 300 cfu g^{-1}, and no coliforms was detected in any *moromi*. *Shishibishio* obtained after 3 months had an acceptable seasoning with high peptide and free amino acid content, and good hygienic quality; in particular, no unpleasant smell and taste was found. The addition of Alcalase or Pectinase 3S appreciably increased yield of *shishibishio* and protein recovery from *moromi* by accelerating the liquefaction and the proteolysis, resulting in the improvement of the sensory quality of the products. The highest yields were, respectively, 49.8 to 50.6%, collected from *moromi* in which we used 15% salt. *Shishibishio* with Alcalase had a higher peptide content but a lower total free amino acid content than that with Pectinase 3S. However, there was not much difference in the sensory evaluations for two enzyme treatments.

Keywords: meat; fermented sauce; koji; commercial enzymes; *shishibishio*

INTRODUCTION

In Asia, there are two popular sauces that are used not only mainly as condiments but also as additional sources of protein for humans. They are the soy sauce that is made from fermented soybeans, and the fish sauce made from fermented fishes. In Japan, soy sauce is a dominant seasoning. Although not as popular as soy sauce, fish sauce is widely consumed and three kinds of fish sauce, ie *ishiru*, *shottsuru* and *ikanago-shoyu* are very well-known.[1,2] In addition, fermented meat sauce '*shishibishio*' has been used since ancient times.[3]

There is very little literature concerning the production and properties of *shishibishio*, while publications about fish sauces and soy sauces are readily available. In the production of fish sauce, traditional methods take about 9–18 months.[1] In order to suppress the growth of harmful microorganisms during the fermentation period, salt is added at more than 20%. So as to shorten the fermentation period, additives containing lactic acid bacteria (LAB) such as koji (rice fermented with *Aspergillus oryzae*) have recently been commonly used in Japan and Korea.[1,4,5] The use of proteolytic enzymes, such as papain, bromelain and Neutrase, to accelerate the proteolysis of meat protein during the fermentation period has also been reported by several authors.[6–8]

As a part of the efforts to diversify meat products, Nakamura *et al*[9] and Yano *et al*[10] have studied the production of fermented meat sauce from lean beef, beef defatted tissue and porcine red blood cell. In their studies, the procedure of soy sauce production was applied in which koji was used to accelerate the fermentation process. However, there has not been any report on the use of commercial enzymes in fermented meat sauce production.

In our study, the fermented meat sauce *shishibishio* was made from ground pork as a main material with additions of salt, koji, pepper and commercial enzymes. The present study aimed to examine the properties of this product, and to examine the effects of addition of Alcalase or Pectinase 3S on accelerating the proteolysis of its fermentation mixtures in order to shorten production period.

MATERIALS AND METHODS

Materials

Ground pork was obtained from a meat packer 1 day after slaughter, and stored at −30 °C until

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used for making *moromi*. Koji was obtained from a koji shop in Obihiro, Japan. Alcalase 2.4L (EC 3.4.21.62), produced from *Bacillus licheniformis* by Novozymes A/S, Denmark, had a declared activity of 2.4 Anson Unit g^{-1}, which is equivalent to 2736 IU (international units) when soya isolate at pH 8.0 and 50 °C was used as substrate. One IU of proteolytic activity is defined as that which can cleave 1 micromol peptide bond per minute (initial reaction rate). The authors recognise that pectinases, in general, are a group of polysaccharide degrading enzyme complexes, commonly used for the clarification of apple, orange and grape juices as well as fruit wines. However, Pectinase 3S (EC 3.2.11.5), produced from *Aspergillus* sp by Yakult Pharmaceutical Industry Inc, Japan, also possesses proteolytic functions with a declared activity of 25 000 unit per gram, when casein at pH 4.0 and 40 °C was used as substrate. Therefore, in view of this high level of protease activity, it has been used as a proteolytic enzyme in this study.

Fermented meat sauce (*shishibishio*) preparation

The *shishibishio* production scheme is shown in Fig 1. It can be divided into two steps: the first is the preparation of fermentation mixture (*moromi*) and the second is the collection of *shishibishio*. For *moromi* fermentation, ground pork was thoroughly mixed with koji, salt, pepper and Alcalase 2.4L or Pectinase 3S according to the proportions shown in Table 1. In these mixtures, the percentage of koji was 10%; that of salt was 15, 20 or 25%, and the levels of the enzymes were 0.5% Alcalase or 0.25% Pectinase 3S. The *moromi* were placed into plastic bags (800 g per bag), carefully sealed, and kept in an incubator (30 ± 0.2 °C) for fermentation. At the end of 0, 1, 2 and 3 months, samples of about 60 g were taken from each *moromi* for microbial analysis and collection of *shishibishio*. The samples were separately centrifuged for 30 min at 28 000 × *g* and 0 °C, and then filtered with Toyo filter paper No 5C (Toyo Roshi Kaisha Ltd, Tokyo, Japan) to obtain the liquid called *shishibishio*. Yield of *shishibishio* was calculated as the percentage of weight of the *shishibishio* divided by weigh of *moromi*. Samples of *shishibishio* were kept in the refrigerator at 4 °C until chemical analysis.

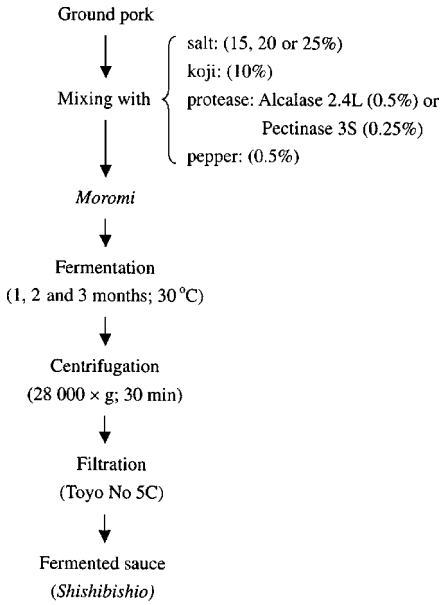

Figure 1. *Shishibishio* production scheme.

Microbiological analysis

Moromi (5g) was homogenized for 3 min with 45 ml of sterilized saline (0.85% NaCl) in a Stomacher (BA 7021, Seward, London, UK). Suitable serial dilutions (10^{-1}–10^{-5}) of the homogenate were prepared, and a 1-ml sample of each dilution was pipetted into sterile Petri dishes. Fifteen millilitres of the appropriate growth medium that had cooled to 37–38 °C were then poured into each dish and mixed gently. The presence of common bacteria was determined on standard agar (Eiken chemical Ltd, Tokyo, Japan). De Man, Rogosa, Sharpe agar (MRS agar) (Oxoid Ltd, Hampshire, UK) was used for growth of lactic acid bacteria (LAB). The presence of LAB was identified by a catalase test using 3% H_2O_2. Chromocult coliform agar (Merch KGaA 64271 Darmstadt, Germany) was used for the growth of *E coli* and other coliforms. Plates were incubated at 37 °C for 24 h for *E coli* and other coliforms, 48 h for common bacteria, and 72 h for LAB. Colonies were counted by direct dilution from plates of each selective medium containing discrete colonies (30–300), and the total population of bacteria in *moromi* was calculated as cfu per gram (cfu g^{-1}).

Chemical analysis

pH values were measured with a pH meter (HM-5S, TOA Electronics Ltd, Tokyo, Japan). NaCl content

Table 1. Composition of *moromi*

	15% Salt			20% Salt			25% Salt		
	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase
Ground pork (g)	596	592	594	556	552	554	516	512	514
Salt (g)	120	120	120	160	160	120	200	200	200
Koji (g)	80	80	80	80	80	80	80	80	80
Pepper (g)	4	4	4	4	4	4	4	4	4
Enzyme (g)	0	4	2	0	4	2	0	4	2
Total (g)	800	800	800	800	800	800	800	800	800

There were three replications of each analysis of *moromi* to give each of the values.

was determined with a salt meter (Model TM-25, Takemura Electric Works Ltd, Tokyo, Japan). The salt meter was adjusted with 3% NaCl solution as a standard. A 25-fold dilution of each *shishibishio* was prepared for measurement. NaCl content was calculated as the measured value multiplied by 25. Total nitrogen was determined by Kjeldahl's method,[11] and crude protein was calculated as N × 6.25. Protein recovery was calculated as the percentage of crude protein of *shishibishio* divided by crude protein of *moromi*. Samples for determination of peptides and free amino acids were prepared according to the procedures described by Mikami *et al*[12] as follows. A 2% trichloroacetic acid (TCA) solution was prepared by mixing 4 g of *shishibishio* with the same weight of 4% TCA solution. It was incubated for 30 min at 37 °C and centrifuged at 4500 × *g* for 10 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[13] with bovine serum albumin as a standard. Free amino acid content was determined by the *O*-phthalaldehyde reagent with an amino acid analyzer (Model 8000, JASCO Corporation, Tokyo, Japan) using the lithium buffer system.

Sensory evaluation

Shishibishio obtained after 3 months of fermentation were used for sensory evaluation. Sixteen panelists whose age ranged from 18 to 50 years were asked to evaluate the colour, aroma, flavour and overall characteristics of the products according to three-point scales ('very good', 'good' and 'bad') of their preference. They were given the samples in random order and not allowed to talk to each other during testing. For the assessment of aroma, panelists sniffed directly each sample containing in a test tube (1 cm × 10 cm). For the flavour 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. At the same time as the aroma and flavour tests, the colour was judged by visual observation.

Statistical analysis

Significant difference between means were analyzed with Statistical Analysis System (SAS)[14] by analysis of variance (ANOVA) according to the General Linear Model (GLM) procedures using Duncan's multi-range test ($p < 0.05$).

RESULTS AND DISCUSSION

Table 2 shows changes in the microbial counts of *moromi*. At month 0, the common bacterial counts ranged from 4.6×10^5 to 9.3×10^5 cfu g^{-1}. However, the numbers of bacteria decreased rapidly as fermentation advanced and, by 3 months, had dropped to below 300 cfu g^{-1} in all *moromi*. Lopetcharat *et al*[15] reported a similar tendency of of total viable count to decline with increased fermentation time during the production of fermented fish sauces such as bakasang, nampla and aekieot in Indonesia, Thailand and Korea, respectively.

In *moromi* with 15% salt, LAB count at month 0 was 2.8×10^4, 2.1×10^4 and 2.3×10^4 cfu g^{-1} in the control, Alcalase and Pectinase 3S respectively. It dropped to below 300 cfu g^{-1} in the control after 1 month, while still at 1.1×10^3 and 1.5×10^3 cfu g^{-1} in Alcalase and Pectinase 3S *moromi*, respectively, after 2 months; it then dropped to below 300 cfu g^{-1} after 3 months in any *moromi*. The similar tendency was also observed in *moromi* with 20 and 25% salt. The higher number of LAB in *moromi* with enzyme treatments was probably due to the addition of enzymes, which

Table 2. Changes in microbial counts in *moromi* during fermentation (cfu g^{-1})

Months	15% salt			20% salt			25% salt		
	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase
Common bacteria									
0	6.4×10^5	9.3×10^5	7.6×10^5	4.8×10^5	6.4×10^5	5.3×10^5	4.6×10^5	6.0×10^5	5.1×10^5
1	2.1×10^3	5.8×10^3	5.8×10^3	1.8×10^3	2.5×10^3	3.1×10^3	1.6×10^3	2.2×10^3	2.9×10^3
2	3.9×10^2	3.8×10^3	1.5×10^3	3.0×10^2	3.1×10^3	1.3×10^3	3.7×10^2	1.9×10^3	6.7×10^2
3	<300	<300	<300	<300	<300	<300	<300	<300	<300
Lactic acid bacteria									
0	2.8×10^4	2.1×10^4	2.3×10^4	2.1×10^4	2.2×10^4	1.8×10^4	1.5×10^4	1.9×10^4	1.6×10^4
1	<300	2.5×10^3	2.5×10^3	<300	1.7×10^3	1.9×10^3	<300	9.4×10^2	1.1×10^3
2	<300	1.1×10^3	1.5×10^3	<300	1.2×10^3	1.5×10^3	<300	3.6×10^2	6.3×10^2
3	<300	<300	<300	<300	<300	<300	<300	<300	<300
Coliform group									
0	6.2×10^3	5.2×10^3	1.3×10^4	4.8×10^3	5.0×10^3	6.8×10^3	3.2×10^3	2.8×10^3	4.5×10^3
1	<300	<300	<300	<300	<300	<300	<300	<300	ND
2	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values are expressed as mean for $n = 3$; ND: not detected.

caused immediate acceleration of proteolysis, resulting in more available amino acids for LAB synthesis. The slightly lower LAB counts in *moromi* with 20 and 25% salt could have resulted from increased the salt level. The data of Paludan-Muller *et al*[16] showed that increasing the salt concentration delayed or inhibited LAB growth in Thai fermented plaa-som.

Coliform group counts ranged from 2.8×10^3 to 1.3×10^4 cfu g^{-1} at month 0, but dropped to below 300 cfu g^{-1} after 1 month and no viable count was detected in any *moromi* sample after 2 months, suggesting that even 15% salt addition could completely inhibit the growth of colifom groups in these *moromi*. This is in agreement with a report of Virulhakul[17] that salt in high concentration limits the growth of spoilage bacteria normally present in fish. This bacteriostatic effect is mainly due to the high concentration of salt inducing the reduction of a_w (water activity) which interferes with the osmotic regulation of most spoilage bacteria.[18]

Changes in the pH of *shishibishio* during fermentation are shown in Fig 2. Initial pH values were not much different among *shishibishio*, ranging from 5.94 to 6.12. These values decreased gradually and had reached to 5.21–5.72 after 3 months, which is within the acceptable range of fermented meat sauces and fish sauces described by Nakamura *et al*[9] and Lopetcharat *et al*.[15] The decrease in pH was caused by acidification in the presence of LAB.[19,20] Several authors[15,20–22] considered that the pH value drop was partly due to the release of peptides and free amino acids during fermentation. This is probably the reason why the pH values in the enzyme treatments were lower ($p < 0.05$) than that in the controls, and why the pH continued to decrease even when LAB dropped to below 300 cfu g^{-1} in the present study (Table 2).

Changes in yield of *shishibishio* from the *moromi* are presented in Fig 3. During the first month, yield increased sharply, thereafter increasing gradually and after 3 months reached 30.2, 49.8 and 50.6%

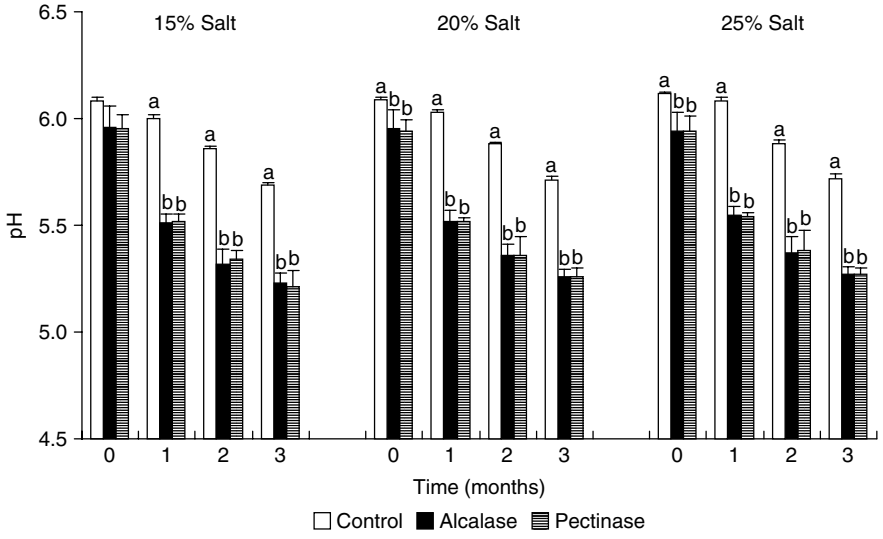

Figure 2. Changes in pH of *shishibishio* during fermentation. Values are expressed as mean ± standard deviation for $n = 3$. [a,b,c]Mean values in a same group with no common superscript differ significantly ($p < 0.05$).

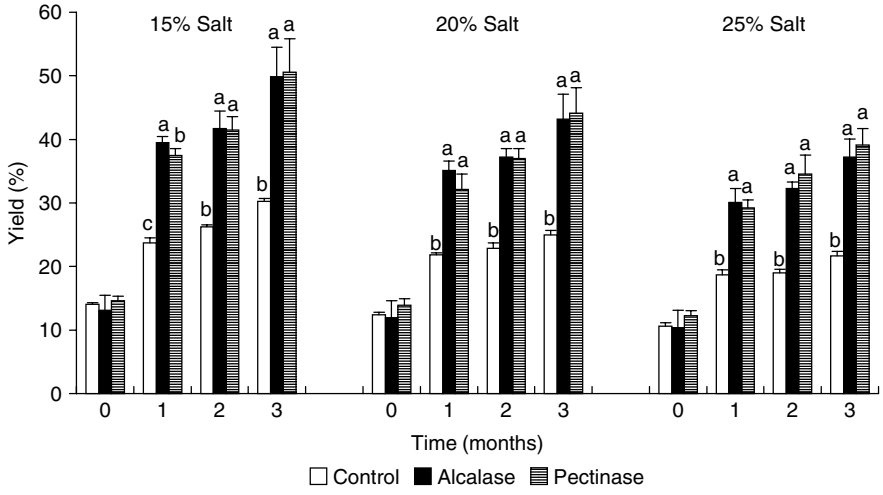

Figure 3. Changes in yield of *shishibishio* from *moromi*. Values are expressed as mean ± standard deviation for $n = 3$. [a,b,c]Mean values in a same group with no common superscript differ significantly ($p < 0.05$).

respectively for the control, Alcalase and Pectinase 3S of *shishibishio* with 15% salt. In case of *shishibishio* with 20 and 25% salt these values were 24.9, 43.2 and 44.2% and 21.6, 37.2 and 39.1%, respectively. The yields from Alcalase treatment and that of Pectinase 3S treatment were not significantly different, but they showed higher yield ($p < 0.05$) than that of the control, suggesting that enzymes might increase the liquefaction of *moromi*. Similar observations were reported when Bae *et al*[23,24] used Complex Enzyme 2000 and Alcalase for rapid processing of modified fish sauce, and when Aquerreta *et al*[6] used exogenous enzymes to elaborate the Roman fish sauce *garum*. Among three levels of salt addition, yield of *shishibishio* with 15% salt was the highest, followed by that with 20% salt and 25% salt. This agrees with results showing that a decrease in the level of salt from 25 to 5% increased the yield of *garum*.[6] This was also attributable to the present study (Table 1) that *moromi* with 15% salt containing more meat than that with 20 and 25% salt, resulting in the higher amount of *shishibishio*.

Changes in protein recovery of *shishibishio* from *moromi* (Fig 4) were paralleled with the changes of yield of *shishibishio* from the *moromi* (Fig 3). Protein recovery of *shishibishio* also increased sharply during the first month, then increased gradually and reached 21.6, 47.4 and 46.0% respectively for the control, Alcalase and Pectinase 3S of *shishibishio* with 15% salt after 3 months. In case of *shishibishio* with 20 and 25% salt, these values were 15.4, 41.3 and 41.0% and 11.9, 35.7 and 36.0%, respectively. There is generally a positive correlation between protein recovery in the fish sauce and proteolytic activities.[25] In the present study, the percentage of protein recovery for the enzyme treatments was around two or three times higher than that for their controls, while there was no significant difference between two enzyme treatments with the same level of salt addition. It is reasonable to conclude that the conversion of insoluble protein to soluble protein during fermentation was faster in the enzyme treatments than in the control. The reason for this was the increased hydrolysis of protein in *moromi* with addition of enzymes. Gildberg and Thongthai[20] reported that both autolytic and microbial activities increased when salt content was reduced to below 20%. In a study that used Complex Enzyme 2000 and Alcalase, Bae *et al*[23,24] considered that the best salt content for accelerating the proteolytic process was 14%. The percentages of protein recovery of *shishibishio* with 15% salt were the highest. These values in enzyme-added *shishibishio* of present study were lower than that in Chinese, Vietnamese, Thai, Korean and Japanese fish sauces (namely 57.8, 61.6, 64.3, 68.5 and 70.4%, respectively) which were reported by Park *et al*,[26] but slightly higher than that in fermented seasonings (44.2%) made from lean beef after 3 months of fermentation which were reported by Nakamura *et al*.[9]

Changes in the total nitrogen contents of *shishibishio* are shown in Fig 5. This soluble nitrogen includes proteins, peptides and free amino acids that derived from the breakdown of meat protein. Total nitrogen content increased rapidly during the initial period of 1 month, and thereafter only a slight increase was observed in any *shishibishio*. This is in good agreement with the findings of Lopetcharat *et al*[15] that the protein conversion rate in *budu* (a fermented fish sauce of north-eastern Malaysia) increased dramatically in the first 60 days of fermentation and then stabilized over a period of 100–200 fermentation days. After 3 months, total nitrogen content was 1.9, 2.6 and 2.5 g 100 ml^{-1} respectively for the control. Alcalase and Pectinase 3S of *shishibishio* with 15% salt. In the cases of *shishibishio* with 20 and 25% salt these values were 1.6, 2.5 and 2.4 g 100 ml^{-1}; and 1.4, 2.5 and 2.4 g 100 ml^{-1}, respectively. Total nitrogen content is the important parameter used for grading the quality of fish sauce

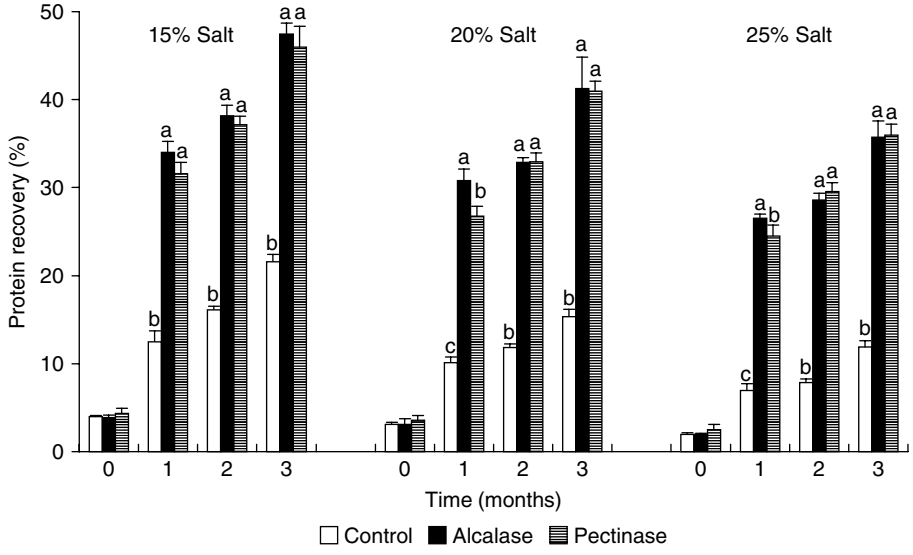

Figure 4. Changes in protein recovery of *shishibishio* during fermentation. Values are expressed as mean ± standard deviation for $n = 3$. [a,b,c]Mean values in a same group with no common superscript differ significantly ($p < 0.05$).

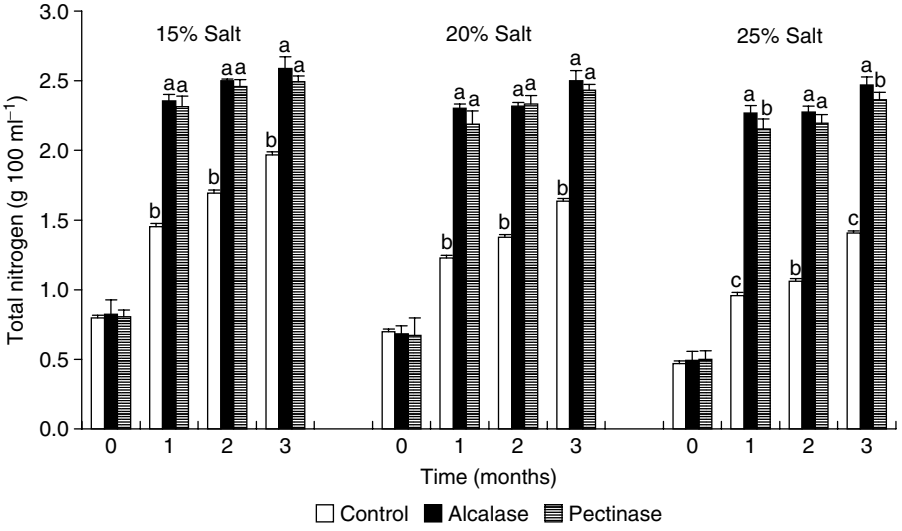

Figure 5. Changes in total nitrogen content of *shishibishio* during fermentation. Values are expressed as mean ± standard deviation for $n = 3$. [a,b,c]Mean values in a same group with no common superscript differ significantly ($p < 0.05$).

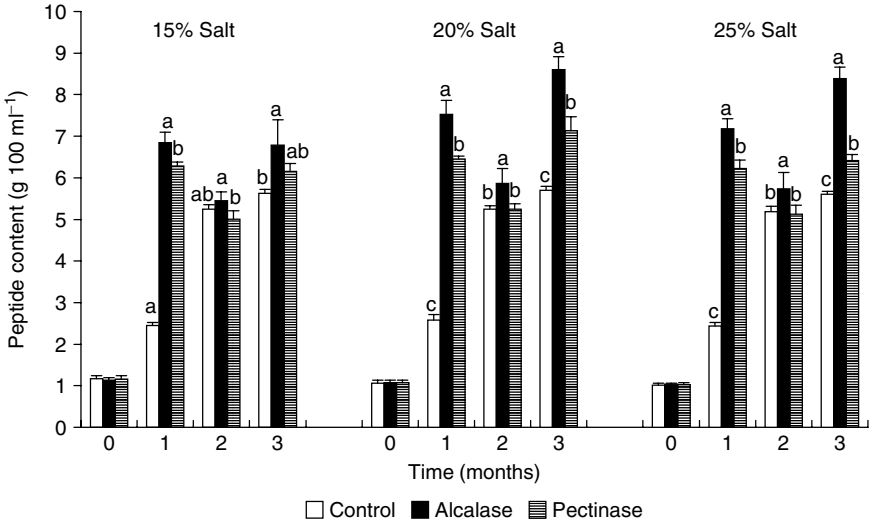

Figure 6. Changes in peptide content of *shishibishio* during fermentation. Values are expressed as mean ± standard deviation for $n = 3$. [a,b,c]Mean values in a same group with no common superscript differ significantly ($p < 0.05$).

and soy sauce. For example, the special grade of soy sauce should contain total nitrogen of more than 1.5% (Japan Agriculture Standard),[3] and total nitrogen in Grade 1 and Grade 2 of fish sauce should be not less than 2.0 and 1.5 g 100 ml^{-1} respectively (Thai Industrial Standard Institute).[17] From both of these points of view, *shishibishio* with enzyme treatment is considered to be rich in total nitrogen, and showed a significantly higher ($p < 0.05$) total nitrogen than the controls.

Figure 6 shows changes in the peptide contents of *shishibishio*. In the controls, peptide content increased gradually, and reached to 5.6, 5.7 and 5.6 g 100 ml^{-1} with 15, 20 and 25% salt, respectively, after 3 months. In the enzyme treatments it rapidly increased during the first month, then slightly decreased during the second month and increased again to reach 6.2–6.8, 7.1–8.6 and 6.4–8.4 g 100 ml^{-1} with 15, 20 and 25% salt, respectively, after 3 months. Overall, the peptide content in *shishibishio* with Alcalase was higher ($p < 0.05$) than that with Pectinase 3S during fermentation. Alcalase is an endoproteinase that was developed to be highly efficient in the hydrolysis of all kinds of proteins. Pectinase 3S has both proteolytic and pectin hydrolytic activities and is often used to accelerate the pressing and filtration of juice from fruits and vegetables; the proteolytic activity of Pectinase 3S is weaker than that of Alcalase.

Total free amino acid contents of *shishibishio* obtained after 3 months are shown in Table 3. Values were highest in the Pectinase 3S treatments (6213–7934 mg 100 ml^{-1}) followed by the Alcalase treatments (5792–7427 mg 100 ml^{-1}) and then the controls (4099–5662 mg 100 ml^{-1}) at any salt level. The higher ($p < 0.05$) total free amino acid content in the enzyme treatments than that in their control could have resulted from the more proteolytic activity contributed by enzyme addition. Between the two

Table 3. Free amino acids composition of *shishibishio* obtained after 3 months (mg 100 ml^{-1})

Amino acids	15% salt			20% salt			25% salt		
	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase	Control	Alcalase	Pectinase
Asp	365.6 ± 12.3^{c}	476.1 ± 22.4^{b}	564.4 ± 10.8^{a}	298.5 ± 19.0^{c}	395.9 ± 8.0^{b}	468.1 ± 19.6^{a}	258.7 ± 26.9^{b}	326.6 ± 31.8^{a}	330.9 ± 19.1^{a}
Thr	303.8 ± 15.6^{b}	386.7 ± 31.6^{a}	421.4 ± 33.3^{a}	200.4 ± 21.0^{c}	299.6 ± 12.7^{b}	404.4 ± 38.3^{a}	164.4 ± 14.5^{c}	260.1 ± 24.2^{b}	373.8 ± 41.2^{a}
Ser	317.5 ± 35.1^{b}	384.2 ± 51.3^{ab}	459.2 ± 31.0^{a}	245.3 ± 14.0^{c}	343.2 ± 21.1^{b}	403.0 ± 33.8^{a}	216.2 ± 11.5^{b}	298.1 ± 30.0^{a}	339.8 ± 22.6^{a}
Asn	147.7 ± 7.0^{c}	216.1 ± 8.0^{b}	257.8 ± 12.0^{a}	139.3 ± 12.1^{c}	231.9 ± 26.4^{b}	297.3 ± 10.7^{a}	98.1 ± 7.6^{b}	198.3 ± 27.6^{a}	236.0 ± 22.9^{a}
Glu	741.3 ± 72.2^{c}	1029.0 ± 36.4^{b}	1159.4 ± 44.8^{a}	651.1 ± 15.7^{c}	990.6 ± 13.0^{a}	960.3 ± 15.3^{b}	566.9 ± 29.4^{c}	926.5 ± 19.9^{a}	703.6 ± 12.5^{b}
Gln	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pro	143.3 ± 11.1^{b}	186.3 ± 14.5^{a}	214.6 ± 27.2^{a}	94.4 ± 5.3^{b}	141.7 ± 23.0^{a}	170.2 ± 8.8^{a}	85.6 ± 7.4^{c}	132.8 ± 11.4^{b}	165.6 ± 8.4^{a}
Gly	151.7 ± 5.1^{b}	197.8 ± 21.0^{a}	227.2 ± 27.5^{a}	100.8 ± 5.1^{b}	119.3 ± 26.7^{b}	191.4 ± 5.6^{a}	95.3 ± 9.2^{b}	101.1 ± 24.3^{b}	189.0 ± 9.7^{a}
Ala	421.2 ± 15.7^{b}	643.9 ± 11.8^{a}	624.7 ± 39.8^{a}	402.8 ± 13.3^{c}	521.4 ± 57.0^{b}	652.8 ± 31.0^{a}	352.5 ± 31.5^{c}	463.5 ± 38.2^{b}	583.6 ± 13.8^{a}
Val	343.9 ± 31.9^{b}	560.9 ± 34.1^{a}	590.8 ± 27.5^{a}	259.5 ± 16.6^{c}	427.5 ± 13.0^{b}	588.1 ± 26.2^{a}	231.4 ± 21.6^{c}	366.0 ± 36.3^{b}	532.2 ± 45.4^{a}
Cys	ND	ND	ND	ND	ND	ND	ND	ND	ND
Met	224.4 ± 19.2	220.6 ± 19.7	200.4 ± 16.8	167.4 ± 10.3^{b}	202.3 ± 16.7^{ab}	207.9 ± 24.4^{a}	152.7 ± 13.5	177.4 ± 10.8	181.3 ± 19.9
Ile	299.5 ± 24.6^{b}	370.4 ± 3.7^{a}	377.9 ± 23.0^{a}	211.1 ± 18.8^{b}	329.8 ± 25.4^{a}	361.0 ± 9.4^{a}	208.6 ± 5.5^{c}	245.6 ± 13.6^{b}	329.3 ± 18.4^{a}
Leu	496.8 ± 21.0^{a}	494.6 ± 29.8^{a}	432.3 ± 26.4^{b}	516.6 ± 11.7^{a}	475.4 ± 35.0^{ab}	428.6 ± 19.3^{b}	427.1 ± 15.5	407.7 ± 27.6	393.9 ± 26.3
Tyr	145.7 ± 13.2	155.0 ± 17.5	165.3 ± 21.5	186.8 ± 12.4^{a}	161.2 ± 10.4^{b}	155.5 ± 12.5^{b}	139.3 ± 14.8	136.4 ± 7.7	126.5 ± 19.8
Phe	244.1 ± 31.0^{b}	340.9 ± 29.3^{a}	372.2 ± 19.7^{a}	180.3 ± 21.9^{b}	319.9 ± 18.9^{a}	348.8 ± 15.2^{a}	163.1 ± 33.4^{b}	287.7 ± 38.6^{a}	313.7 ± 23.4^{a}
Lys	757.6 ± 33.0^{c}	911.8 ± 38.5^{b}	1065.3 ± 21.2^{a}	624.5 ± 18.3^{c}	889.8 ± 78.6^{b}	1119.7 ± 11.3^{a}	534.4 ± 30.2^{c}	771.3 ± 30.4^{b}	934.0 ± 3.6^{a}
His	112.2 ± 11.1^{b}	137.8 ± 19.7^{b}	175.2 ± 14.3^{a}	77.3 ± 5.9^{c}	114.2 ± 23.4^{b}	165.1 ± 14.6^{a}	85.4 ± 21.1^{b}	103.2 ± 28.2^{ab}	145.1 ± 10.6^{a}
Arg	445.8 ± 29.7^{c}	715.4 ± 5.8^{a}	625.8 ± 8.8^{b}	326.6 ± 18.8^{c}	649.6 ± 13.2^{a}	476.0 ± 26.4^{b}	319.4 ± 12.4^{b}	589.3 ± 12.5^{a}	334.3 ± 19.2^{b}
Total	5662.0 ± 69.9^{c}	7427.4 ± 105.3^{b}	7933.9 ± 101.0^{a}	4682.5 ± 59.4^{c}	6613.2 ± 118.7^{b}	7398.2 ± 67.2^{a}	4099.1 ± 86.5^{c}	5791.5 ± 67.1^{b}	6212.6 ± 37.0^{a}

Values are expressed as mean ± standard deviation for $n = 3$, ND: not detected.
[a,b,c] Mean values in a row with no common superscript differ significantly ($p < 0.05$).

Table 4. Sensory evaluation of *shishibishio* obtained after 3 months[a]

	15% salt			20% salt			25% salt		
	Control	Acalase	Pectinase	Control	Acalase	Pectinase	Control	Alcalase	Pectinase
Colour									
Very good	3	12	11	5	11	12	4	12	12
Good	10	4	5	10	5	4	10	4	4
Bad	3	0	0	1	0	0	2	0	0
Aroma									
Very good	5	11	9	6	10	7	4	8	7
Good	11	5	7	10	6	9	12	8	9
Bad	0	0	0	0	0	0	0	0	0
Flavour									
Very good	4	10	9	3	9	7	3	6	5
Good	5	6	7	6	5	6	5	6	6
Bad	7	0	0	7	2	3	8	4	5
Overall									
Very good	4	10	8	3	7	5	3	6	5
Good	6	6	8	7	7	9	4	5	6
Bad	6	0	0	6	2	2	9	5	5

[a] Total of panelists = 16.

enzyme treatments, the higher ($p < 0.05$) total free amino acid content for Pectinase 3S than for Alcalase suggested that Pectinase 3S has more aminopeptidase activity than Alcalase. Comparing the results obtained from samples with and without enzymes at the three levels of salt, it can be seen that a decrease in the level of salt increased the proteolytic activity, resulting in the higher total free amino acid content, especially when enzymes were used. Aquerreta *et al*[6] reported a similar tendency when they used exogenous enzymes to elaborate the Roman fish sauce *garum*. Individually, glutamic acid, alanine, valine, leucine, lysine and arginine were the predominant free amino acids in any *shishibishio*. Similar observation in commercial fish sauces have been reported.[15] Nearly all these free amino acids could contribute to the final taste of the product and, among them, glutamic acid makes an important contribution.[27] In the present study, glutamic acid contents in *shishibishio* ranged from 567 to 1159 mg 100 ml^{-1}. The differences in glutamic acid content among *shishibishio* might contribute an important role to the difference of sensory evaluation of products (Table 4).

After 3 months, NaCl contents were around 22.3, 25.0 and 24.5% in the control, 20.7, 22.4 and 22.5% in the Alcalase treatment, and 21.1, 22.9 and 22.7% in the Pectinase 3S treatment with 15%, 20%, and 25% initial salt in *moromi*, respectively. The NaCl contents of our *shishibishio* samples were slightly lower than the average values of commercial fish sauces (26 (±3.7)%), but higher than that of commercial soy sauce (16–18%) as reported by Lopetcharat *et al*.[15]

The sensory evaluation of *shishibishio* obtained after 3 months is shown in Table 4. The most important factors for consumer acceptability of the products are flavour and aroma, although the colour can also be significant.[27] The enzyme treatments had a clear brown colour similar to commercial fish sauce, but the control had a lighter colour so that several panelists assessed that the colour was 'bad'. No unpleasant smell was found in any *shishibishio*. The panelists commented that all *shishibishio*, especially those with added enzyme, had a nice aroma. In enzyme treatments, *shishibishio* with 15% salt were graded 'very good' and 'good' in flavour, while some panelists gave a 'bad' evaluation for that with 20 and 25% initial salt, because of to their saltier taste. Overall enzyme treatments were judged to be 'very good' and 'good' by most panelists. It is important to note that the 'bad' evaluation for that with 20 and 25% salt was due to their saltier taste. The 'bad' evaluation for the controls was probably attributed to their low content of free amino acids.

CONCLUSIONS

Shishibishio obtained after 3 months was an acceptable seasoning with high nitrogen content and hygienic quality. In particular, the products had an attractive colour and a good aroma and flavour, with no unpleasant smell and taste. The addition of commercial enzymes markedly increased yield of *shishibishio* and protein recovery from *moromi* by accelerating the liquefaction and the proteolysis, especially in *shishibishio* with 15% salt, resulting in the improvement of sensory quality of product. *Shishibishio* with Alcalase had a higher peptide content but lower total free amino acid content than that with Pectinase 3S. However, there was not much difference in sensory evaluation between the two enzyme treatments.

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REFERENCES

1 Beddows CG, Fermented fish and fish products, in *Microbiology of fermented Foods*, ed by Wood BJB. Vol II. Elsevier Applied Science, London, pp 1–39 (1985).

2 Michihata T, Yano T and Enomoto T, Volatile compounds of headspace gas in the Japanese fish sauce *Ishiru*. *Biosci Biotechnol Biochem* **66**:2251–2255 (2002).

3 Yokotsuka T, Fermented protein foods in the Orient, with emphasis on shoyu and miso in Japan, in *Microbiology of fermented foods*, ed by Wood BJB. Vol I. Elsevier Applied Science, London, pp 197–247 (1985).

4 Park JH, Kung SN, Yun US and Shim WM, Study on the fermentation of fish sauce by koji. *Chungang Uihak* **37**:93–101 (1979).

5 Funatsu Y, Kawasaki K, Yuan C, Uchida M, Satomi M and Fukuda Y, A comparison of volatile compound in fish sauces prepared from Silver Carp by use of soy sauce Koji and lactic acid bacteria with those in Chinese commercial fish sauce. *Nippon shokuhin kagaku kogaku kaishi* **49**:106–118 (2002).

6 Aquerreta Y, Astiasaran I and Bello J, Use of exogenous enzymes to elaborate the Roma fish sauce 'garum'. *J Sci Food Agric* **82**:107–112 (2002).

7 Santos AC, Hernandez VS, Reyes AR and Strength DR, The preparation and use of papain for the production of fish hydrolysates. *Philippine Agric* **11**:91–100 (1968).

8 Beddows CG, Ismail M and Steinkraus KH, The use of bromelain in the hydrolysis of mackerel and the investigation of fermented fish aroma. *J Food Technol* **11**:379–388 (1976).

9 Nakamura T, Yano Y and Hada T, Studies on fermented seasoning production from meat and meat by-products. *Jpn J Zootech Sci* **56**:851–859 (1985).

10 Yano Y, Hada T and Nakamura T, Studies on accelerated fermentation and improvement in taste and aroma of fermented meat by-products seasonings. *Jpn J Zootech Sci* **58**:639–647 (1987).

11 AOAC, Crude protein in meat, in *Official Methods of Analysis of AOAC International*, 16th edn, ed by Patricia C. Vol II. *AOAC International*, Arlington. Virginia, Chapter 39, Section 981.10, pp 7–8 (1995).

12 Mikami M, Nagao M, Sekikawa M, Miura H and Hongo Y, Effects of electrical stimulation in the peptide and free amino acid contents of beef homogenate and sarcoplasma during storage. *Anim Sci Technol (Jpn)* **65**:1034–1043 (1994).

13 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275 (1951).

14 Statistical Analysis Systems Institute, SAS/STAT® Software: Changes and Enhancements through Release 6.12, Cary NC, USA (1997).

15 Lopetcharat K, Choi YJ, Park JW and Daeschel MA, Fish sauce products and manufacturing: a review. *Food Rev Int* **17**:65–88 (2001).

16 Paludan-Muller C, Madsen M, Sophanodora P, Gram L and Moller PL, Fermentation and microflora of plaa-som, a Thai fermented fish product prepared with different salt concentrations. *Int J Food Microbiol* **73**:61–70 (2002).

17 Virulhakul P, The processing of Thai fish sauce. *Infofish Int*, **5**: 49–52 (2000).

18 Hayes PR, *Food microbiology and hygiene*. Elsevier Applied Science Publishers Ltd, UK, pp 1–23 (1985).

19 Ijong FG and Ohta Y, Microflora and chemical assessment of an Indonesian traditional fermented fish sauce 'Bakasang'. *J Fac Appl Biol Sci Hiroshima Univ* **34**:95–100 (1995).

20 Gildberg A and Thongthai C, The effect of reduced salt content and addition of halophilic lactic acid bacteria on quality and composition of fish sauce made from Sprat. *J Aqua Food Produ Technol* **10**:77–88 (2001).

21 Lopetcharat K and Park JK, Characteristics of fish sauce made from Pacific whiting and surimi by-products during fermentation stage. *J Food Sci* **67**:511–516 (2002).

22 Chou CC and Ling MY, Biochemical changes in soy sauce prepared with extruded and traditional raw materials. *Food Res Int* **31**:487–492 (1998).

23 Bae TJ, Han BH, Cho HD, Kim JC, Kim BS and Choi SI, Condition for rapid processing of modified fish sauce using enzymic hydrolysis and improvement of product quality. 2. Fish sauce from sardine waste and its quality. *Han'guk Susan Hakhoechi* **23**:125–136 (1990).

24 Bae TJ, Han BH, Cho HD, Kim BS and Choi SI, Condition for rapid processing of modified fish sauce using enzymic hydrolysis and improvement of product quality. 3. Fish sauce from whole sardines and its quality. *Han'guk Susan Hakhoechi* **23**:361–372 (1990).

25 Gildberg A, Utilisation of male Arctic capelin and Atlantic cod intestines for fish sauce production—evaluation of fermentation conditions. *J Biores Technol* **76**:119–123 (2001).

26 Park JN, Fukumoto Y, Fujita E, Tanaka T, Washio T, Otsuka S, Shimizu T, Watanabe K and Abe H, Chemical composition of fish sauce produced in Southeast and East Asian countries. *J Food Comp Anal* **14**:113–125 (2001).

27 Sanceda NG, Kurata T and Arakawa N, Overall quality and sensory acceptance of a lysine-fortified fish sauce. *J Food Sci* **55**:983–988 (1990).