A Method for Manufacturing of Bonito Spermary Extract by Autolysis

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From the view point of effective utilization of fisheries by-products, a method for manufacturing bonito spermary extracts by autolysis was examined. The optimum lysing temperature was found at 60°C, pH 7.5 with two parts of water. The extract obtained from 20 h autolysates showed favorable characteristic flavor. It was rich in free amino acid and 5'-nucleotide. Total amount of Arg, His, Glu, Ala, and Tau accounted for more than 70% of that of total free amino acid. Thus the products was expected to be used as characteristic and unique seasoning extract.

The total catch of bonito amounts to about 300–350 thousand tons annually in Japan. They have a comparatively large body and are served not only as Sashimi (sliced raw fish) but also as canned products or Katsuwobushi, a traditional dried fish prepared by smoking. Most of the viscera are utilized as Shiokara, a seasoned and fermented food. However, their reproductive organs (the spermary and ovary) are utilized as fish or some livestock feed or organic fertilizer. In the last several years protamine, one of the major spermary proteins, has been reported to have an active anti-bacterial action^{1,2)} and has been examined for use as food preservative.³⁾

As fish reproductive organs, especially spermary, contain some unique and tasty components characteristic of each fish, Japanese people like to eat as local food.⁴⁾ However, bonito spermary is not so eaten though the reason is not obvious. We have tried to get fish extract of superior quality from bonito spermary. This paper deals with a method for preparing the extract by autolysis and describes the chemical nature of its constituents.

Materials and Methods

Materials

Freshly frozen bonito spermary was supplied by a Katsuwobushi manufacturer, Yaizu, Shizuoka Prefecture, in October 1986. It was kept at –25°C until use. A 500 g portion of the frozen block was cut out at each experiment, put into a polyethylene bag and thawed in running water. After thawing, it was washed quickly with tap water and

cleaned to remove blood vessels distributed on its surface. The cleaned raw material was chopped by a hand-operated mincer equipped with a 7-mm diameter hole blade.

Autolysis of Spermary and Extract Preparation

Autolysis was carried out in a cylindrical glass reaction vessel, 14 cm in diameter and 18 cm in depth, equipped with a four blade stirrer and a reflux condenser. The reaction bath was immersed in a water bath of 6 cm in depth. First, a 500 g portion of minced spermary was autolyzed at four different temperatures (40°C, 50°C, 60°C, and 70°C) to determine the optimum temperature. Then, six different ratios of water, (12.5, 25, 100, 200, and 400% of the sample weight) were added to each raw material before the start of the reaction to determine the proper addition of water on the recovery of extractive material. In the last step of this experiment, several different pHs (6.4–8.0) were applied by addition of 6 N and 1 N HCl or 6 N and 1 N NaOH to find out the optimum pH.

Preparation of Press Juice and Extract from Autolysate

The autolysate was pressed in a bag made of Tetoron® yarn to recover press juice. The press cake was stirred again with the additional 200 ml of water for 1 h at the same temperature being used for the autolysis. In some cases instead of compression, centrifugation was also employed to eliminate the adsorption loss of the press juice by the press bag. Centrifugal conditions were

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8,000 rpm ($10,500 \times g$) for 15 min at 4°C.

To prepare the extract, the press juice was deproteinized by an addition of ethanol to a concentration of 80%. Upon separation of the denaturated protein by centrifugation, the supernatant was concentrated by evaporation under reduced pressure to remove ethanol. The concentrate was defatted by washing three times with the same volume of diethyl ether and then made up to 100 ml with water in case the starting raw material was 100 g.

Chemical Analyses of the Extract

1. Total nitrogen. Total nitrogen was determined by macro-Kjeldahl method after AOAC using Quick Digestive and Distillating System (Mitamura Co., Tokyo). All data shown in this paper are expressed as mg-nitrogen per 100 g minced raw material.

2. Approximate amino acid concentration of autolysate. An absorbance of the supernatant of autolysate at 275 nm was measured after being diluted to an appropriate concentration to estimate the increase of free amino acid concentrations with the progress of digestion.

3. Total amino acid in the autolysate. Total amino acid was measured by JEOL JLC-6AS automatic amino acid analyzer after the sample was hydrolyzed with 6 N hydrochloric acid at 110°C for 20 h.

4. Total amino acid in the press juice. Press juice was separated from the autolysate by the centrifugation at 8,000 rpm ($10,500 \times g$) at 2°C for 20 min. Upon hydrolysis of the press juice, amino acids were determined by the same manner as mentioned above.

5. Combined amino acids in the extract. The extract was hydrolyzed to determine combined amino acids which consisted of peptides by the deduction of free amino acids from the analytical data.

6. Free amino acid in the extract. Free amino acids were determined on a Shimadzu amino acid analyzer consisting of LC-6A HPLC modules and a multi-gradient elution system with lithium citrate buffers for physiological fluid analysis. Each amino acids were derivatized with OPA upon elution from a cation exchange resin column (Shim-pack ISCO/S150 L Li, 4×150 mm).

7. Nucleotides and their related compounds in the extract. The extract was pretreated by passing through the Dowex 1-X4 column. Nucleotides were separated with each other by HPLC

using strongly basic anion exchange column Shim-pack WAX-1 (4×50 mm) and stepwise gradient of phosphate buffers (A: 20 mM, pH 7.00, B: 480 mM, pH 6.00) at 45°C at the flow rate of 1 ml/min. Nucleosides and free bases were separated on reversed phase Shim-pack CLC-ODS with linear gradient elution of 0.1 M phosphate buffer (pH 2.0) and 0.2 M sodium perchlorate. Individual peak was detected at 260 nm and calculated automatically using the C-R3A data-processor.

8. Trimethylamine (TMA) and trimethylamine oxide (TMAO) in the extract. TMA in the acidic extract of the autolysate was diluted to the appropriate concentration and determined quantitatively as follows. Two ml of the extract was mixed with 1 ml of diethyl ether and 2 ml of 65% KOH and shaken vigorously for 1 min. One μ l of ether layer was injected into GLC (Shimadzu GC-7A) equipped with a packed glass column ($3 \text{ mm} \times 2 \text{ m}$) of squalane (20%) + glycerine (2.5%) + KOH (2.5%) on Diasolid L (80–100 mesh). Temperatures of injection port and column oven were 80°C and 50°C, respectively. Flow rate of carrier nitrogen gas was 20 ml/min. TMAO was reduced to TMA by the addition of equal volume of 20% TiCl_3 and kept standing at room temperature for 2 h. After an excess reducing reagent was neutralized by the addition of 2–3 drops of saturated KNO_3 , TMA was analyzed by the same GLC manner as mentioned above. The amount of total TMA in the test solution gave that of TMAO by calculation.

9. Organic acids in the extract. The extract was passed continuously through columns of Dowex 50 W-X8 and Amberlite IRA-45. After the columns were washed with water, 1 N NaOH solution was poured into the latter column to elute organic acids. HPLC analysis of organic acids was carried at 50°C by an isocratic elution of acidic pure water (pH 2.10), adjusted with perchloric acid at a flow rate of 1.2 ml/min using cation exchange resin column Shim-pack SCR-101H (7.9×300 mm).

10. Sugars (monosaccharides) in the extract. Sugars were derivatized with TMS reagent in a Reacti-vial (Pearce Co.) and injected into Shimadzu GC-7A equipped with a glass column ($3 \text{ mm} \times 2 \text{ m}$) packed with GP 3% SP-2330 on Supelcoport (100–200 mesh). Temperatures of the injection port and the column oven were 260°C and 225°C, respectively. Carrier nitrogen gas was passed through the column at a flow rate of 40 ml/min.

Results and Discussion

When fish solubles are manufactured commercially from viscera,²⁾ minced raw materials are autolyzed near 50°C for 2 h in some Japanese factories. In the first step of this experiment, similar experimental conditions were employed to establish the optimum temperature in bonito spermary autolysis. The autolysis at 40°C was excluded from further experimentation because of its foul smell suggesting the presence of propagating microorganisms. At 50°C, 60°C, or 70°C, the minced spermary became an aromatic viscous paste. The press juice obtained was clear, bright-yellow and smelled of tasty boiled fish.

However, the amount of the press juice obtained by one squeeze was much lower than expected probably in some part due to its absorption by the press bag. A considerable portion of the extractive compounds seemed to remain in the press cake. Thus 200 ml of water were added to the press cake and stirred at the same temperature. After 1 h of stirring, a liquid fraction was separated by the same manner as used for the first separation.

In the next step of this experiment, an appropriate amount of water was added to the minced raw material at the start of the process expecting the hydrolysis of the protein to improve the recovery of juice. The data were compared with that for autolysis without addition of water. At first, 200 g of water (being equivalent to 40% weight of the starting raw material) was added to the reaction system and the increase of press juice was estimated on the weight bases. The

data shown in Table 1 indicate the considerable increase of the press juice by addition of water to the reaction system. However, it should be obvious that as more water is added, more press juice would be obtained.

The amount of water soluble nitrogen in the press juice obtained by digestion with or without water at each temperature tested was summarized in Table 2. From these observations the addition of water increased 3 to 4 times the total water soluble nitrogen. The maximum recovery was obtained at 50°C and it diminished along with the increase of temperature for autolysis. Water soluble nitrogen thus obtained at 60°C with water showed about 7 times higher recovery than that at the same temperature without water. In the case of 50°C and 70°C with water, the recovered water soluble nitrogen was about 4.7 and 2.5 times higher than those without water. From the results of this experiment, it was confirmed that the addition of water to the minced bonito spermary increased not only the volume of press juice but also increased the recovery of water soluble nitrogenous compounds.

Absorbance at 275 nm on the same sample was measured and converted into the weight of free tyrosine to estimate the release of free amino acid as indicated in Table 3. In this estimation, the converted tyrosine in an autolysate with water also revealed a higher amount than that of no water addition. When autolyzed at 60°C, the converted tyrosine in the press juice gave the highest value and it was more than 7.5 times the value of unautolyzed bonito spermary itself.

Table 1. Weight of press juice obtained from 500 g of the autolysate incubated at 50, 60, and 70°C with or without water and that of unautolyzed materials (g)

Temp. incubated (°C)	without water			with water* ¹			unautolyzed
	50	60	70	50	60	70	
First press	19.0	13.0	16.5	202.5	265.0	122.5	55.0
Second press	90.5	28.5	67.0	200.5	71.0	82.0	124.0
Total	109.5	41.5	83.5	403.0	336.0	204.5	179.0

*¹ Water was added at a level of 40%.

Table 2. Extractive nitrogen in the press juice autolyzed at 50, 60, and 70°C with or without water and that of unautolyzed materials (mg nitrogen/100 g of minced material)

Temp. incubated (°C)	without water			with water* ¹			unautolyzed
	50	60	70	50	60	70	
First press	28	17	15	247	161	151	18
Second press	49	22	80	122	80	87	35
Total	77	39	95	369	241	238	53

*¹ See the footnote of Table 1.

Table 3. Tyrosine equivalent concentration liberated during the autolysis at 50, 60, and 70°C with or without water and that of unautolyzed minced materials (mg tyrosine/100 g of minced material)

Temp. incubated (°C)	without water			with water* ¹			unautolyzed
	50	60	70	50	60	70	
First press	51.0	100.0	97.2	519.7	958.8	454.4	44.4
Second press	121.6	118.1	227.3	284.6	266.0	319.1	92.3
Total	172.6	218.1	324.5	804.3	1124.8	773.5	136.7

*¹ See the footnote of Table 1.**Table 4.** Weight of the juice squeezed by centrifuging 500 g of autolysates incubated at 60°C with 6 different portions of water (g)

Water added (%)	12.5	25	50	100	200	400
First centrifugation	28.6	95.2	57.7	96.6	173.6	316.2
Second centrifugation	66.4	35.2	36.1	37.4	35.0	76.5
Total	95.0	130.4	93.8	134.0	208.6	392.7

Fig. 1. Ratio of amino acid nitrogen to total extractive nitrogen in the press juice autolyzed at 50 and 60°C with (40%) or without water. Columns filled with vertical lines and painted out with black indicate total extractive nitrogen and amino acid nitrogen, respectively. Arabic numerals given on the columns show ratios of two nitrogens. (%).

Since the optimum autolyzing temperature was not clearly established from the above results, the ratio of free amino acid nitrogen to total extractable nitrogen in each sample autolyzed at 50°C and 60°C was calculated and shown in Fig. 1. The results clearly indicate a remarkable recovery

of amino acid and perhaps an acceleration of the digestion process of the bonito spermary. The ratio of amino acid nitrogen to total nitrogen in the autolysate incubated at 60°C showed 2.3–2.8 times higher than those incubated at 50°C, so it was concluded that the optimum autolyzing temperature was 60°C as long as the amount of digested nitrogenous products was thought to be one of the efficiency index.

Six different volumes of water, which represented 12.5, 25, 50, 100, 200, and 400% of raw material weight, were added to the minced spermary in order to determine an optimum volume of water. In this study, centrifugation at 8,000 rpm (10,500 × g) for 20 min was adopted instead of hand press to reduce the loss of extract in the course of separation. Table 4 shows the total volume of the extract obtained from the first and second centrifugations. The amount of extractive nitrogen in the press juice separated from each autolysate incubated with 6 different volumes of water was given in Table 5. Out of 6 autolysates thus obtained, 50% addition of water gave the least extractive nitrogen and 12.5, 25, and 100% addition showed only a little increase. Both 200 and 400% addition showed better nitrogen extractability than other 4 experimental conditions. As shown in Table 4, the amount of extract separated by centrifugation increased in proportion to the volume of water added. Between these two amounts, the 200% addition of water was found to be the optimum when the energy saving in concentration process was taken into consideration.

The minced raw material with 200% water was

Table 5. Extractive nitrogen in the press juice squeezed from autolysate incubated at 60°C with 6 different portions of water (mg-nitrogen/100 g minced material)

Water added (%)	12.5	25	50	100	200	400
First centrifugation	135.2	247.2	182.0	216.9	325.7	329.5
Second centrifugation	138.8	47.3	38.3	39.5	33.8	32.5
Total	274.0	294.5	220.3	256.4	359.5	362.0

Fig. 2. Ratios of water-soluble proteinous, combined, and free amino acids in the extracts autolyzed at different pHs to total amino acid in the starting minced materials. Columns filled with vertical lines indicate, total amino acids in the starting materials; with horizontal line, water soluble proteinous amino acid; and with oblique line, combined amino acid; painted out with black, free amino acid.

adjusted to four different pHs of 6.5, 7.0, 7.5, and 8.0. All these test samples were incubated at 60°C, the optimum autolyzing temperature. Proportion of total, combined, and free amino acids in the extracts, which were prepared from the autolysates adjusting to the four different pH and taken out at 45 h after the reaction started, were compared to the total amino acid of minced raw material with twice amount of water just before the start of autolysis. An increase of total weight of each amino acid specimen samples during the 45-hr incubation is shown in Fig. 2. The highest recovery of free amino acids by the autolysis at pH 7.5 accounted for 60% of the total

amino acid and was more than twice as much as that in the raw material. The highest free amino acid recovery indicates the optimum pH 7.5 for the bonito spermary extract preparation.

In the last step of this study, the chemical composition of the final products were analyzed in detail. The products were prepared by the autolysis under the optimum and two other conditions as described above. Analytical results are summarized in Table 6.

Among free amino acids, Tau a nonproteinous amino acid was the most abundant (350 mg in 100 g of starting material). Basic amino acids, such as Arg, His, and Lys, were the second richest (50–110 mg,) followed by Glu, Ala, and Leu at a little lower level. The sum amount of these 7 amino acids comprised about three-fourth of the total amino acids. In general, fish spermary is composed of the nucleic protein protamine which consists of Arg as a major amino acid. Although Arg accounts for the most part of the total amino acids which are obtained after complete acid hydrolysis, our data on the autolysate shown in Table 6 did not confirm this. Since the autolytic enzyme in the spermary has not been separated or characterized yet, the reason for the above mentioned phenomenon is not known. Presumably, the major enzyme responsible for the autolysis is endopeptidases and not exopeptidases.

Among compounds related to nucleic acids, about 20 mg of IMP and UMP each was detected as 5'-nucleotide and only a trace amount of nucleosides was found to exist. Besides these compounds, three free nucleobases were determined for 10 mg order. Both sugars and TMAO were found to be below the detection limit. In organic acid, only a few mg of propionic acid was detected together with a trace of acetic acid.

An autolytic extract of bonito spermary had strong sweetness and Umami (glutamate-like meaty taste), with weak saltiness, but had no bitterness and sourness at all. The product was clear and light bright yellow in color. It had almost no specific spermine like odor which was characteristic of raw fish spermary and its products.

Table 6. Extractive nitrogenous components in the autolysate produced with 3 different portions of water (mg/100 g)

Water (%)	25	100	200	Water (%)	25	100	200
Tau	347	384	354	AMP	—	—	5
Asp	32	34	30	CMP	9	—	—
Thr	28	25	24	IMP	34	36	6
Ser	37	37	38	UMP	49	33	25
Glu	95	102	77	Total	92	69	36
Pro	16	30	+	Ado ^a	3	—	4
Gly	49	55	44	Guo ^b	1	—	—
Ala	73	70	67	Uri ^c	—	8	—
Val	39	34	37	Ade ^d	24	7	5
Met	21	21	15	Cyt ^e	21	18	20
Ile	28	21	20	Ura ^f	30	7	5
Leu	62	46	55	Total	79	40	34
Tyr	42	30	45	PA ^g	86	29	6
Phe	33	26	34	AA ^h	—	1	1
His	107	77	106	Total	86	30	7
Lys	56	42	54				
Arg	115	86	144				
Total	1175	1120	1146				

(a) Ado=adenosine, (b) Guo=guanosine, (c) Uri=uridine, (d) Ade=adenine, (e) Cyt=cytosine, (f) Ura=uracil, (g)=PA propionic acid, (h) AA=acetic acid.

Its flavor was weak but similar to dried bonito. The viscosity of the final product was quite low.

In conclusion, the manufactured autolytic extract of bonito spermary was rich in free amino acids whose profile was somewhat different from that of fish meat extract.⁶⁾ It was also abundant in taste-active 5'-nucleotide whose level was a little higher than that of ordinary fish muscle extract.⁷⁾ Our product could be used as a manufacturing material to blend with other types of fish, meat or vegetable extract in order to produce a characteristic and unique seasoning extract.

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References

- 1) F. Miller, R. Abrams, A. Dorfman, and M. Klein: *Science*, **96**, 428-429 (1942).
- 2) N. M. Islam, T. Motohiro, and T. Itakura: *Nippon Suisan Gakkaishi*, **51**, 811-815 (1985).
- 3) N. M. Islam, T. Motohiro, and T. Itakura: *Nippon Suisan Gakkaishi*, **52**, 913-918 (1986).
- 4) E. Tanikawa: in "Marine products in Japan" (ed. by E. Tanikawa), Koseisha-Koseikaku, Tokyo, 1971, pp. 295-339.
- 5) S. Lassen: in "Fish as food III" (ed. G. Borgstrom), Academic Press, New York, 1965, pp. 281-299.
- 6) S. Konosu: in "Food taste chemistry" (ed. by J. C. Boudreau), ACS, Washington, D. C., 1979 pp. 185-203.
- 7) M. Murata and M. Sakaguchi: *J. Food Sci.*, **51**, 321-326 (1986).