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Short Paper

Separation of bitter fraction from the autolytic extract of the wastes from frigate mackerel

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Bitter taste produced during proteolytic hydrolysis of protein is known to be caused by a wide variety of bitter peptides of medium to short chain length with an exceptionally high content of hydrophobic amino acid side chains in protein hydrolyzates.[1,2] Bitter peptides are often accumulated when endopeptidases such as trypsin, pepsin, papain, subtilisin, etc., are used for hydrolysis of proteins without the supplement of exopeptidases, which have shown good effect on debittering of peptides.[3]

In a previous paper,[4] we reported that the autolytic extract from fish wastes had umami taste and can be used in seasoning, although it had weak bitterness and an unpleasant aftertaste. We also found that the bitter taste of autolytic extract was enhanced by shaking treatment because of the accelerated lipid oxidation during autolysis.[5] In these studies, the whole viscera of frigate mackerel was utilized to recover the protein from the fish wastes by autolysis. In the viscera many kinds of proteases including both endopeptidases and exopeptidases were contained. During autolysis, endopeptidases in the fish viscera cleave the polypeptide chain of protein to produce peptides, while exopeptidases hydrolyze the peptides into free amino acids. The bitter taste of autolytic extract might be caused by bitter peptides.

In order to see whether the bitter taste of the autolytic extract is caused by bitter peptides or by other components related to lipid oxidation, separation of bitter fraction from the autolytic extract of fish wastes was carried out by using n-butanol extraction and gel filtration chromatography.

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Minced head and viscera of frigate mackerel *Auxis rochei* were used. Preparation of the minced head and viscera was conducted as previously. Two kinds of extracts were prepared from the fish head and viscera after autolysis with and without shaking (15°C, 24 h), as described previously.[4] Nitrogen content, free and bound amino acid composition were determined as described before.[4] The autolytic extract was homogenized with an equal volume of n-butanol and then centrifuged. After centrifugation, the butanol phase was collected. The water phase was again extracted with the same volume of butanol as above. The two butanol phases were combined. The solvents in both water and butanol phases were removed completely by evaporation under reduced pressure in a rotary evaporator at 50–55°C. Residues from both phases were dissolved in distilled water for sensory evaluation or for a further separation by gel filtration. Sephadex G-15 (fine grade, Pharmacia, Uppsala, Sweden) was used. The column (2.6 × 45 cm) was prepared according to the method described by Fischer[6] and was equilibrated and eluted with distilled water. The eightfold concentrated butanol fraction from autolytic extracts was applied to a Sephadex G-15 column and eluted with distilled water. Every 10 mL of effluent was collected using a fraction collector. Effluent fractions obtained were subjected to the measurement of the absorbance at 280 nm. They were also subjected to ninhydrin reaction as follows; 0.5 mL of an effluent fraction was taken into a tube and mixed with 0.5 mL of 4 M CH_3COONa buffer (pH 5.5) and 0.5 mL of ninhydrin reagent. The outside of the tube was covered with aluminum foil to prevent the effects of light. The mixture was incubated in a boiling water bath for 15 min and then cooled immediately in ice-water. After cooling, the reaction mixture was diluted by adding 5.0 mL of

50% ethanol (v/v) and finally determined the absorbance at 570 nm. Every five fractions near each peak in gel filtration were pooled and lyophilized. The lyophilized powder was dissolved in 8 mL of distilled water and used for sensory evaluation and determination of amino acid composition. The gel filtration chromatography was repeated twice.

Two kinds of extracts from autolysates prepared with and without shaking were treated with n-butanol, respectively. As described above, the extracts were clearly separated into two phases after centrifugation. The butanol phase of both shaken and unshaken extracts had a slight yellow color. On the other hand, the water phase of the shaken extract had a dark brown color and that of unshaken one was slightly brown. The butanol and water phase were collected separately and their solvents were evaporated to dryness under reduced pressure. Residues from the water and butanol phases were redissolved in distilled water and subjected to sensory evaluation.

The water fraction of both shaken and unshaken extracts showed no bitterness. On the other hand, the butanol fraction of both shaken and unshaken extracts showed strong bitterness. This result indicates that bitter components in autolytic extracts were relatively more hydrophobic than other taste components and were fully extracted into the butanol fraction. In a next step therefore the butanol fraction of both shaken and unshaken extracts was subjected to further separation by gel filtration chromatography.

In preliminary experiments, the butanol fraction obtained was subjected to Sephadex column and eluted by 0.01 M sodium phosphate buffer (pH 7.5) and by distilled water, respectively. No difference was found in the gel filtration pattern between two eluents. Therefore, distilled water was selected as the eluent so that the effluents obtained from the gel filtration were allowed to directly conduct sensory evaluation of their bitterness, as reported by Salles *et al.*[7]

Figure 1 shows gel filtration patterns of butanol fractions from the shaken and unshaken extract on a Sephadex G-15 column. No difference was observed in the gel filtration pattern of ninhydrin positive substances (absorbance at 570 nm) between the shaken and unshaken samples. However, remarkable differences were found in the gel filtration pattern of UV absorbance at 280 nm between the two samples. The shaken sample gave a remarkably higher absorbance at 280 nm from 80 to 220 mL of elution volume, while the unshaken one showed almost no absorbance before 220 mL. After the gel filtration, seven fractions were pooled for sensory testing, as given in Fig. 1. Strong bitterness was found in F3 (Fraction No. 18–22) of the

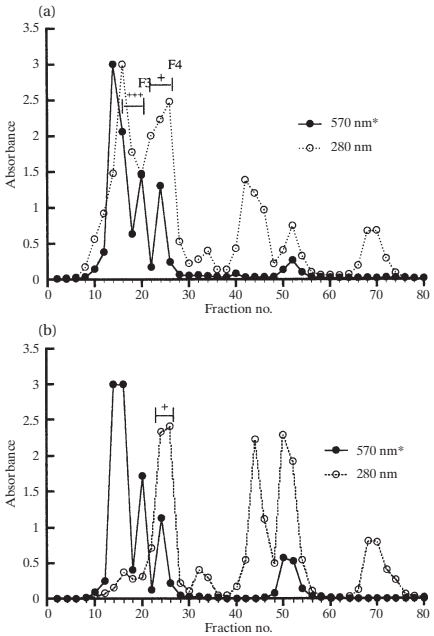

Fig. 1 Gel filtration pattern of butanol fraction from (a) shaken and (b) unshaken extract on a Sephadex G-15 column. The strength of bitterness was rated on a 5-point scale (–, undetectable; +, weak; ++, mild; +++, strong; ++++, very strong).

shaken sample. However, no bitterness was detected in the same fraction of the unshaken sample, though both F4 (Fraction No. 23–27) had weak bitterness. Bad aftertaste in F3 of the shaken sample was also stronger than in the same fraction of the unshaken sample. These results suggest that the bitter taste enhanced by shaking might be different from that without shaking. In addition, the bitterness might not be caused by the ninhydrin positive substances. For confirmation of this assumption, the gel filtration fractions obtained were subjected to estimate their amino acid compositions.

Table 1 exhibits free and bound (peptide) amino acid composition of F3 and F4. It was found that almost all amino acids were free phenylalanine and tyrosine in F3 and F4, respectively. There were very few peptides in F3 and F4. Phenylalanine is known as a bitter amino acid. However, its concentration in the F3 from the shaken extract was much less than that in the same fraction from the unshaken extract. In addition, with an attempt to see if the bitter taste of F3 and F4 obtained from the gel filtration was caused by peptides, leucine aminopeptidase was used to treat these fractions. No change was detected in the bitterness of the fractions after hydrolysis with leucine aminopeptidase.

All results as presented here suggest that the bitter taste of the autolytic extract was not caused by bitter amino acids or peptides produced during

Table 1 Free and bound amino acid composition of gel filtration fractions from shaken and unshaken extract

(μg/mL)	Shaken extract				Unshaken extract			
	F3 (+++)		F4 (+)		F3 (–)		F4 (+)	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Taurine	4	ND	ND	ND	ND	ND	ND	ND
Aspartic acid	ND	2	ND	1	ND	3	ND	2
Threonine	ND	1	ND	ND	ND	2	ND	1
Serine	ND	3	ND	3	ND	3	ND	2
Glutamic acid	ND	4	ND	3	ND	5	ND	3
Glutamine	ND	ND	ND	ND	ND	ND	ND	ND
Alanine	ND	3	ND	ND	ND	4	ND	ND
Valine	3	7	3	3	4	8	3	14
Cystine	1	ND	ND	ND	ND	ND	ND	ND
Methionine	2	16	ND	ND	8	12	ND	ND
Isoleucine	ND	5	ND	ND	ND	6	ND	3
Leucine	ND	6	ND	ND	2	6	ND	3
Tyrosine	ND	9	170	3	1	6	375	3
Phenylalanine	212	6	ND	2	486	4	1	2
Lysine	ND	2	ND	ND	2	ND	ND	ND
Histidine	ND	ND	ND	ND	ND	ND	ND	ND
Arginine	ND	ND	ND	ND	ND	ND	ND	ND
Proline	ND	ND	ND	ND	ND	38	ND	ND
Total	222	65	173	15	503	97	379	34

(–), Bitterless; (+), Weakly bitter; (+++), strongly bitter.
ND, not detected.

autolysis. Substances other than ninhydrin positive substances might contribute to the bitter taste of the extract, although many reports have shown that the bitter taste produced during hydrolysis of protein is caused by bitter peptides with high hydrophobic residues.[1,2] In a previous paper,[5] we reported that TBA values were much higher in the shaken sample than in the unshaken one after autolysis and the polyunsaturated fatty acids such as 22:6n-3, 20:5n-3 etc., decreased about 50% after autolysis with shaking. These results showed that lipid oxidation in the shaken sample occurred more strongly than in the unshaken one. Usuki and Kaneda have pointed out that a dimer isolated from the oxidized oils of soybean had a bitter taste.[8] Miwa has separated pungent components from frozen sea urchin gonad and identified them as many kinds of carbonyl components, decomposition products of unsaturated fatty acids originated from the oxidized lipids and the common constituent of acetal lipids in the urchin gonad.[9] Murata *et al.* have reported that the bitter taste of fresh urchin gonad is not caused by amino acids or peptides.[10]

In summary, lipid oxidation products might be responsible for the bitter taste of the autolytic extract especially for the enhanced bitterness in the shaken extract from the fish processing wastes.

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