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Original Article

Contribution of lipid oxidation to bitterness and loss of free amino acids in the autolytic extract from fish wastes: Effective utilization of fish wastes

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SUMMARY: The reason why bitterness of the autolytic extract is enhanced by shaking during autolysis was investigated from the viewpoint of improving the taste of the extract from fish wastes. Two kinds of extract were prepared from the head–viscera mixture of frigate mackerel after autolysis with (S1) and without shaking (S2). A comparison of the two extracts showed significant differences in their organoleptic and chemical properties. More brown in color, S1 tasted much more bitter than S2, while S1 gave lower contents of free amino acids, higher thiobarbiturate (TBA) values and a smaller ratio of polyunsaturated fatty acids to the total ones. This indicates that lipid oxidation occurred more vigorously in S1 than in S2. Furthermore, it was found that the increase in the bitterness and in the loss of free amino acids was correlated with the increase in TBA values of the extract. In conclusion, lipid oxidation accelerated by shaking during autolysis contributed to the enhanced bitterness and brown discoloration and to the loss of free amino acids of the autolytic extract from fish wastes.

KEY WORDS: **autolysis, bitterness, brown discoloration, fish wastes, lipid oxidation.**

INTRODUCTION

In a previous study we reported that the protein in the head and viscera of frigate mackerel *Auxis rochei* could be easily and efficiently recovered as amino acids by autolysis even at low temperature.[1] The amino acids liberated by autolysis can be extracted by hot water. According to sensory evaluation, the hot water extract was rich in umami taste and can be used in seasoning. The autolytic extract, however, would need to be improved, as it had a weak bitterness and unpleasant aftertaste. In addition, the bitterness of the extract was enhanced by shaking during autolysis. However, the reason for this remains unclear and clarification from the viewpoint of improving the taste of the autolytic extract is necessary. Therefore, the objective of the present study was to elucidate what contributes to the enhanced bitterness of the extract after autolysis with shaking.

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MATERIALS AND METHODS

Frigate mackerel *Auxis rochei*, purchased in Kochi central wholesale market, were used as raw materials. The head and viscera of the fish were excised from its trunk and minced with a knife. Its sexual organs and contents of digestive organs were removed from the viscera before mincing. The minced head and viscera were packaged in a small plastic bag, respectively, and kept at –85°C before use. The contents of moisture, protein and lipids in the head were 72.9, 15.5 and 8.9%, and in the viscera were 69.3, 18.6 and 2.4%, respectively.

Autolysis procedure and preparation of the extract

The frozen minced head and viscera were thawed and mixed with water in the ratio of 3 : 1 : 8, then homogenized for 5 min at 10 000 r.p.m. by using Ace Homogenizer (Nihonseiki Co. Ltd, Tokyo, Japan), transferred into a Erlenmeyer flask and incubated with and without shaking in a water bath at 15°C for 24 h. The shaking speed was 120 circles per min (personal Lt-10F low temperature incubator with a shaker; Taitec Co. Ltd, Saitama, Japan). After incubation, 10 g of the autolysate

was homogenized with 10 mL of 10% trichloroacetic acid (TCA) solution to stop autolysis and stood for 30 min at room temperature, then centrifuged for 15 min at 10 000 *g*. The supernatant was collected as TCA-extract for determination of nitrogen content and amino acid composition. For sensory evaluation, autolysis was terminated by heating with an equal volume of water, instead of adding the TCA solution. After boiling for 10 min, the autolysate was cooled and centrifuged as above. When procedures were needed to prevent lipid oxidation, nitrogen gas (N_2) was used to replace the air in head–viscera mixture before incubation. In such a case, a stoppered flask was used to hold the homogenized mixture. After the flask was kept in an ice box, the nitrogen gas was flowed into the mixture for 10 min, then into the head-space for 5 min, at a flow rate of 1 L/min. After that, the flask was stoppered quickly, sealed tightly with a laboratory film and incubated as above.

Methods of analysis

Moisture was determined according to loss on drying at 105°C. Nitrogen content was determined by the method of Kjeldahl. Free amino acids (FAA) were measured with high-speed amino acids analyzer (Hitachi model L-8500A; Hitachi, Tokyo, Japan). Peptide amino acids (PAA) in extract were analyzed as follows. A portion of the extract was hydrolyzed in 6 N HCl at 110°C for 24 h in a vacuum hydrolysis tube to determine whole amino acids (WAA). The amounts of PAA were calculated by subtracting the amount of FAA in the unhydrolyzed extract from that in the hydrolyzed one. Lipids were extracted with chloroform : methanol (2 : 1, v/v) according to Bligh and Dyer,[2] and lipid content was estimated gravimetrically. After saponification, lipid extracted was esterified with methanolic HCl and fatty acid methyl esters were analyzed by gas chromatography (Shimadzu GC-14A; Kyoto, Japan) equipped with a flame ionization detector. Gas chromatographic conditions were the same as reported previously.[3] The peaks of fatty acid methyl esters were identified by comparison of retention time of fatty acid methyl ester standard, relative retention time and ECL-value in the literature.[4] Thiobarbiturate values (TBA values), expressed as 10^{-5} mM malonaldehyde (MDA)/mL, were determined according to the method of intact sample procedure by Sinnhuber and Yu.[5] At the same time, a standard curve was also plotted by using 1,1,3,3-tetraethoxy propane as a standard.

Sensory evaluation

Sensory evaluation was performed by a panel of 10 members, according to the method of paired comparison by Scheffé[6] with a slight modification. The subjects were presented in small glass cups containing 10 mL each of test solutions. The surface of the cups was heavily covered with a yellow tape so that the color of the extract would not affect the judgment of the observers. The nitrogen content of the test solutions was adjusted to 1 mg/mL and the temperature of them was kept at 40°C by incubating them in a water bath. The observers were asked to calculate the difference between the paired samples and to give their evaluation to the required items on a five-point rating scale (+2, much stronger; +1, stronger; 0, equal; −1, weaker; −2, much weaker). Each observer tested the same paired samples twice but in an reverse order. Results obtained were analyzed statistically based on the method described by Scheffé.[6]

RESULTS AND DISCUSSION

Difference in taste between extracts prepared by autolysis with and without shaking

Processing residues, the head and viscera of frigate mackerel were autolyzed with and without shaking at 15°C for 24 h. Autolysis with shaking was taken to enhance bitterness formation, without shaking as a control. The autolysates were extracted by hot water and the extracts were used for sensory evaluation after their nitrogen content was adjusted to 1 mg/mL. The result of sensory evaluation to their bitterness and other tastes of the extracts are given in Table 1. Significant differences were

Table 1 Bitterness and other tastes of the autolytic extracts from fish head and viscera

Scales (S1, S2)	S1 >> S2 +2	S1 > S2 +1	S1 = S2 0	S1 < S2 −1	S1 << S2 −2
Bitterness*	2	10	7	1	0
Bad aftertaste*	2	16	0	2	0
Odor*	2	14	3	1	0
Umami	3	6	4	6	1
Preference*	0	4	1	13	2

S1, Extract with shaking; S2, extract without shaking.
* $P < 0.01$.

found between the two samples in all the test items except umami taste. Bitterness, bad aftertaste, and odor in the shaken sample were significantly stronger than those in the control ($P < 0.01$). However, in the case of umami taste, no significant difference was found between them. As for preference, the control was better than the shaken one. Besides these, an evident difference in the color of the extracts was also observed between them. The color of the shaken sample was much more brown than the control. In addition, their amino acid compositions were also compared. As shown in Table 2, their major FAA were leucine, lysine, glutamic acid, alanine, aspartic acid, and arginine. In general, the shaken sample gave lower content of all the FAA than the control except arginine and taurine. Such a difference in FAA reached a significant level of $P < 0.01$. However, the content of extractive nitrogen and peptides almost remained constant, regardless of shaking. These results indicated that the bitterness of the extract was enhanced by shaking during autolysis and was directly correlated with its brown color, accompanied by the loss of FAA. Azuma also mentioned such a relationship between bitterness and browning in his review on liquefied protein.[7]

As regards the brown discoloration in fish products, many reports have shown that the browning might be derived from autoxidized oil and/or from the Maillard reaction.[8–14] In a previous study we reported that the head of frigate mackerel was fatty and contained about 10% lipids, in which polyunsaturated fatty acids accounted for nearly 50% of the total.[3] This made us consider that lipid oxidation might proceed easily and therefore it might be the main reaction correlated with the bad taste and brown discoloration in such a system rich in FAA and polyunsaturated fatty acids but relatively poor in reducing sugar. In order to reveal this, we carried out the following investigations.

Effect of lipid oxidation on the taste of the extract from autolysate

Thiobarbiturate values of the autolytic extracts and fatty acid compositions of lipids from the residues after autolysis are shown in Fig. 1 and Table 3, respectively. Between both samples, no difference in extractive nitrogen could be observed, but TBA values and fatty acid composition were quite different. Thiobarbiturate values were much higher in the shaken sample during autolysis. In terms of fatty acid composition, before autolysis

Table 2 Amino acid composition and nitrogen content of the autolytic extracts from fish head and viscera

Amino acids[1]	Free amino acids		Peptides	
	S1 (mean ± SD) ($n = 6$)	S2 (mean ± SD) ($n = 6$)	S1 (mean ± SD) ($n = 6$)	S2 (mean ± SD) ($n = 6$)
Taurine	214 ± 19	216 ± 18	—	—
Aspartic acid	216 ± 19	247 ± 4	264 ± 38	250 ± 51
Threonine	122 ± 11	144 ± 10	163 ± 28	156 ± 33
Serine	146 ± 11	156 ± 7	153 ± 27	144 ± 34
Glutamic acid	283 ± 25	326 ± 9	582 ± 62	565 ± 74
Glutamine	89 ± 8	100 ± 7	—	—
Glycine	82 ± 12	107 ± 13	458 ± 52	444 ± 52
Alanine	228 ± 18	253 ± 13	230 ± 33	224 ± 42
Valine	179 ± 15	203 ± 10	157 ± 27	155 ± 35
Cystine	9 ± 2	13 ± 2	38 ± 10	48 ± 11
Methionine	100 ± 16	125 ± 7	81 ± 9	69 ± 24
Isoleucine	157 ± 15	192 ± 7	125 ± 21	115 ± 26
Leucine	299 ± 31	346 ± 20	173 ± 26	165 ± 36
Tyrosine	164 ± 18	184 ± 11	65 ± 17	67 ± 25
Phenylalanine	163 ± 20	189 ± 15	85 ± 15	83 ± 18
Ornithine	35 ± 8	39 ± 12	5 ± 2	5 ± 3
Lysine	289 ± 35	319 ± 22	215 ± 37	215 ± 49
Histidine	134 ± 25	161 ± 19	78 ± 18	66 ± 17
Arginine	222 ± 24	178 ± 57	157 ± 24	148 ± 36
Proline	106 ± 11	131 ± 7	239 ± 30	218 ± 28
Total	3239 ± 274	3627 ± 102*	3266 ± 428	3126 ± 576
Ex-N	13.5 ± 0.2	14.2 ± 0.9	—	—

[1] Amino acids are given as μg/mL. The nitrogen content of the extracts was adjusted to 1 mg N/mL for the sensory evaluation.
Ex-N, extractive nitrogen and is given as mg N/g sample. S1, Extract with shaking; S2, extract without shaking.
* $P < 0.01$.

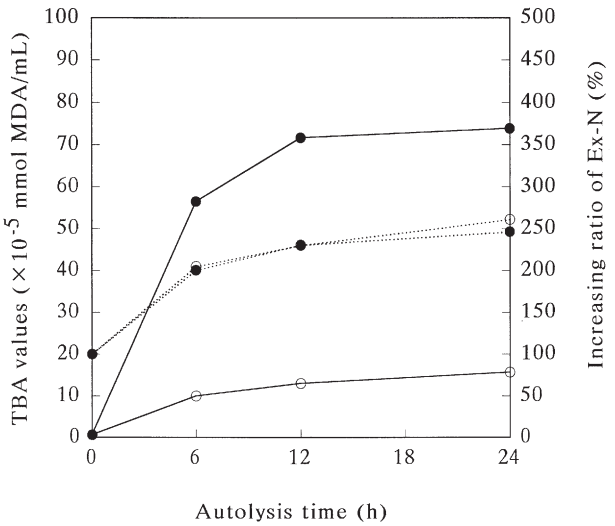

Fig. 1 Variations of thiobarbiturate values and increasing ratio of extractive nitrogen (Ex-N) in the autolysates during autolysis. (—) Thiobarbiturate values; (·····) increasing ratio of Ex-N. (●) The sample with shaking; (○) without shaking.

Table 3 Fatty acid composition of the lipids from fish head and viscera before and after autolysis

	Before autolysis (%)	After autolysis* With shaking (%)	Without shaking (%)
14 : 0	0.8	1.2	0.8
16 : 0	19.2	27.6	18.6
16 : 1n-7	8.4	12.3	8.7
18 : 0	3.8	4.9	3.2
18 : 1n-9	11.2	15.7	11.6
18 : 1n-7	2.5	3.6	2.6
18 : 2n-6	1.2	1.3	1.2
18 : 3n-3	1.0	0.8	0.9
20 : 4n-6	1.8	1.0	1.7
20 : 5n-3	10.8	4.8	10.0
22 : 6n-3	25.4	11.6	25.7
Other	13.8	15.1	15.1
Total	100.0	100.0	100.0

* The lipids were extracted from the residues of the autolysate after centrifugation, according to Bligh and Dyer.[2]

the major fatty acid was 22 : 6n-3 (docosahexaenoic acid, DHA), followed by 16 : 0 (palmitic acid), 18 : 1n-9 (oleic acid), and 20 : 5n-3 (eicosapentaenoic acid, EPA), which accounted for about 66.6% of the total fatty acids. After autolysis with shaking the major fatty acid was 16 : 0, followed by 18 : 1n-9. The polyunsaturated fatty acids such as 22 : 6n-3, 20 : 5n-3 etc. decreased about 50%. After autolysis without shaking, however, the fatty acid composition was just the same as that before autolysis. These

Table 4 Effects of nitrogen on free amino acid composition and thiobarbiturate values of the extracts from fish head and viscera

Amino acids[1]	S1	S2	S3
Taurine	218	200	205
Aspartic acid	228	255	253
Threonine	133	156	165
Serine	154	157	157
Glutamic acid	300	337	356
Glutamine	105	112	113
Glycine	83	113	123
Alanine	251	273	279
Valine	195	220	231
Cystine	11	15	13
Methionine	93	130	131
Isoleucine	168	203	212
Leucine	326	374	389
Tyrosine	181	195	179
Phenylalanine	183	211	209
Ornithine	32	50	49
Lysine	306	307	308
Histidine	124	155	165
Arginine	251	106	79
Proline	106	136	149
Total	3448	3706	3765
TBA values[2]	69.8	13.1	3.1

[1] The unit of amino acid content is the same as in Table 2.
[2] Thiobarbiturate values are expressed as 10^{-5} mM MDA/mL. S1, Extract with shaking; S2, extract without shaking; S3, extract with shaking in the presence of nitrogen gas.

results showed that lipid oxidation in the shaken sample occurred more strongly than in the control. In order to prevent lipid oxidation, we used nitrogen gas (N_2) to replace the air in the head–viscera mixture before autolysis and investigated its influence on tastes and other parameters of the extracts.

Effects of N_2 on amino acid composition and TBA values of the extracts are shown in Table 4. In the absence of N_2, the shaken sample (S1) gave an evidently lower content of FAA and higher TBA values than the control (S2), as described before. However, in the presence of N_2, even though the autolysis was proceeded with shaking (S3), the content of FAA in S3 showed the same level as in S2. Effects of N_2 on bitterness and bad aftertaste of the extract are given in Table 5. In the absence of N_2, S1 was significantly more bitter than S2; in the presence of N_2, the differences disappeared. The same trend was found in bad aftertaste. In addition, effect of N_2 on their color also showed the same trend (Fig. 2). In the absence of N_2, the color of the shaken sample (S1) was much more brown than that of the unshaken one (S2). But in the presence of N_2 (S3), no difference in color was observed between the samples with (S3) and

Table 5 Effects of nitrogen on bitterness and bad aftertaste of the autolytic extracts from fish head and viscera

Scales (A, B)[1]	A >> B +2	A > B +1	A = B 0	A < B −1	A << B −2
Bitterness					
S1, S2*	4	12	2	2	0
S1, S3**	7	10	1	2	0
S2, S3	2	7	6	4	1
Bad aftertaste					
S1, S2**	5	9	2	4	0
S1, S3**	4	9	5	2	0
S2, S3	1	4	9	5	1

[1] A represents one sample and B the other one of the paired samples.
* $P < 0.05$; ** $P < 0.01$.
S1, Extract with shaking; S2, extract without shaking; S3, extract with shaking in the presence of nitrogen gas.

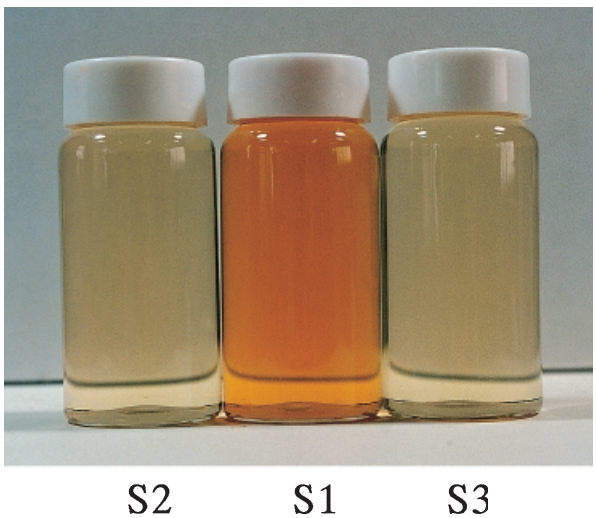

Fig. 2 Effect of nitrogen on the color of the autolytic extracts from fish head and viscera. S1, Extract with shaking; S2, extract without shaking; S3, extract with shaking in the presence of nitrogen gas.

without shaking (S2). This indicates that the development of bitter taste and brownish pigments could be effectively inhibited by N_2, due to prevention of lipid oxidation during autolysis.

From the results of the present study, we could preliminarily see that lipid oxidation contributed to the development of bitterness, brown discoloration and the loss of FAA. In order to confirm this, we used a lean material, the dorsal meat of the fish (lipid content, 0.8%) to replace its head (lipid content, 8.9%) to do a proven experiment as follows. To prepare the extract, the dorsal meat was mixed with viscera and water in the ratio of 3 : 1 : 8. After homogenization, the mixture was divided into four parts. Two parts, as controls, were autolyzed

Table 6 Effects of lipid addition on bitterness and bad aftertaste of the autolytic extracts from fish dorsal meat and viscera

Scales (A, B)[1]	A >> B +2	A > B +1	A = B 0	A < B −1	A << B −2
Bitterness					
M1, M2	0	4	10	6	0
M1, M3*	3	1	2	11	3
M3, M4*	7	12	1	0	0
Bad aftertaste					
M1, M2	0	4	11	5	0
M1, M3*	0	2	2	11	5
M3, M4*	5	10	0	4	1

[1] A and B represent the same as in Table 5.
M1, extract from meat-viscera mixture after autolysis with shaking; M2, extract from meat-viscera mixture after autolysis without shaking; M3, extract from meat-viscera-lipids mixture after autolysis with shaking in the absence of nitrogen gas; M4, extract from meat-viscera-lipids mixture after autolysis with shaking in the presence of nitrogen gas.
* $P < 0.01$.

directly with (M1) and without shaking (M2). The other two parts were mixed with lipids extracted from the head. The final lipid content was adjusted to 7.3%, the same as in the head–viscera mixture, and then autolyzed with shaking in the absence (M3) and presence of N_2 (M4).

Effects of lipid addition on their tastes, amino acid composition and TBA values are compared in Tables 6 and 7, respectively. Before adding lipids, no difference could be found in organoleptic and chemical properties between the lean samples with (M1) and without shaking (M2). Both M1 and M2 were rich in umami taste. Almost no bitter taste nor brown color could be detected. In addition, their TBA values and composition of FAA showed the same. After adding lipids, some differences appeared. In comparison with M1, the lipid-added shaken sample (M3) was much more bitter and showed a remarkable decrease in FAA with an increase in TBA values. The differences almost disappeared after N_2 was used to prevent lipid oxidation. In the presence of N_2, the lipid-added shaken sample (M4) had a less bitter taste and less brown coloration than without N_2 (M3). In addition, FAA in M4 increased sharply with an evident decrease in TBA values. In other words, the higher the TBA values the extracts gave, the more bitter the taste they had and the more FAA they lost. As for peptides, no such trend could be found. This further demonstrated that the increase in bitterness and the loss of FAA were correlated with the increase in TBA values of the extracts.

As regards the loss of FAA in the shaken extract, it might be caused by an amino-carbonyl reaction (*i.e.* some FAA in the fish muscles reacted with carbonyl

Table 7 Effects of lipid addition on free amino acid composition and thiobarbiturate values of the extracts from fish dorsal meat and viscera

Amino acids[1]	M1[2]	M2[2]	M3[2]	M4[2]
Taurine	59	58	59	56
Aspartic acid	163	153	112	144
Threonine	89	91	56	78
Serine	91	92	58	84
Glutamic acid	278	264	180	216
Glutamine	78	80	50	71
Glycine	51	54	32	47
Alanine	180	186	124	161
Valine	137	143	94	126
Cystine	23	23	14	19
Methionine	117	118	78	113
Isoleucine	134	142	83	127
Leucine	282	291	208	269
Tyrosine	151	146	120	151
Phenylalanine	169	172	134	166
Ornithine	14	18	16	33
Lysine	254	267	212	265
Histidine	276	296	251	278
Arginine	212	158	192	199
Proline	88	90	54	84
Total	2847	2845	2126	2688
TBA values[3]	0.6	0.5	63.2	15.9

[1] The unit of amino acid content is the same as in Table 2.
[2] M1, M2, M3 and M4 represent the samples as shown in Table 6.
[3] The unit of thiobarbiturate values is the same as in Table 4.

compounds produced during lipid oxidation)[15] and/or by a decrease in the activity of protease due to interactions between enzymes and oxidized lipids during autolysis.[16–18] A further investigation is in progress in order to confirm the reason why FAA decreased remarkably in the shaken sample.

We conclude that the lipid oxidation accelerated by shaking during autolysis contributed to the enhancement of the bitterness, brown discoloration and to the loss of FAA of the autolytic extract. It is well known that occurrence of lipid oxidation is often a serious problem during the process and storage of fisheries products because it is usually associated with a deterioration of the organoleptic, chemical and nutritive properties of the products. As discussed, the head and viscera of frigate mackerel might be easily oxidized because of its high content of lipids abundant in polyunsaturated fatty acids. In the present study, it was found that lipid oxidation occurred during autolysis even without shaking and more vigorously during autolysis with shaking. Thus, the first step to improve the taste of the autolytic extract is to delay lipid oxidation during autolysis so that the development of the bitter taste can be effectively inhibited.

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