Changes in Enzyme Activity and Components in the Extract of Oyster during Fermentation

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With (15%) and without NaCl, the pH value of oyster paste dropped to 5 and below 4 respectively, throughout fermentation. The activities of enzymes except amylase in 15% NaCl were completely or partially inhibited during fermentation.

The total amount of free amino acids of oyster was 1,692 mg/100 g, and the major free amino acids were Tau, Glu, Pro, Gly, Ala, and Asp. The six amino acids accounted for 70% of the total free amino acid. About 35 mg/100 g of β -Ala was detected. After fermentation, the maximum amino acid content in the extract with and without sodium chloride reached 2,225 and 3,103 mg/100 g, respectively.

The major nucleosides and their derivatives in oyster were inosine, guanosine, and hypoxanthine. Small quantities of AMP, ADP, and ATP were observed, but IMP was not detected. After four days, nucleotide could not be detected in the presence of salt.

Since the extractive constituents of oyster were first observed in 1929,¹⁾ it has been shown that the components vary according to species,²⁻⁵⁾ season,⁶⁻¹⁰⁾ and region.¹¹⁾ It was also noticed in the last three decades that the taste is closely related to the extractive components. Now, about 60 species of fish and shell-fish have been investigated in terms of extracts and their nitrogenous component profiles.^{9,12)} The relationship between each nitrogenous component and the taste has also been clarified by many workers.¹³⁻¹⁴⁾

Because of its high level of nutrients such as glycogen, sarcoplasmic protein etc., the oyster is called "Sea Milk" and is extensively utilized all over the world. In addition, the oyster, having a high level of taurine, has been recognized as a healthy food for humans.¹⁵⁾ Moreover, it has a special taste and flavor which are different from those of other marine crustaceans and bivalvia, such as lobster, crab, shrimp, scallop, etc. The utilization of the extracts as food additives has been actively developed in Japan.¹⁵⁻¹⁶⁾

Although the profiles of non-nitrogenous and nitrogenous components of oyster have been investigated by some workers,^{1-10,17,18)} the changes of these components and enzyme activities during fermentation have not yet been noticed. In this paper, the relationship between changes in ex-

tractive components and enzyme activities during fermentation is discussed.

Materials and Methods

Materials

Frozen oysters *Crassostrea gigas* were purchased and transported to the laboratory, and then stored at -35°C until use.

Fermentation Conditions

Five kilograms of frozen shucked oyster were homogenized and incubated at 25°C for 0, 1, 2, 4, 7, and 14 days in the presence or absence of 15% NaCl.

Crude Enzyme Extract

From 5 g of each sample (about 500 g), the crude enzyme was extracted with three volumes of cold (5°C) 10 mM Tris-HCl buffer, pH 8, and centrifuged at $10,000 \times g$ for 30 min. The supernatant was treated with solid ammonium sulfate; and then the precipitates (30-70%) were collected and dissolved in a minimum volume of the same buffer solution. Then, the residual ammonium sulfate was removed by dialysis against the same buffer overnight. The crude enzyme solution was centrifuged again at $36,000 \times g$ for 30 min to

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remove undissolved proteins.

Enzyme Activities

The enzyme activities of trypsin, chymotrypsin, carboxypeptidase A, amylases, nucleotidase, glucose 6-phosphate dehydrogenase (Glc-6-P DH), alkaline phosphatase, lactate dehydrogenase (LDH), lipase, and phospholipase were determined using the methods described by Kunitz,¹⁹⁾ Rick,²⁰⁾ Walsh,²¹⁾ Bergmeyer *et al.*,²²⁾ Appel,²³⁾ Yoshinaka *et al.*,²⁴⁾ Bernfeld,²⁵⁾ Heppel and Hilmoe,²⁶⁾ Noltmann *et al.*,²⁷⁾ Bergmeyer *et al.*,²⁸⁾ and Searcy *et al.*,²⁹⁾ respectively.

Preparation of Extract

According to Suyama's method,³⁰⁾ the extract was prepared with 6% perchloric acid. Using the *o*-phthalaldehyde method,³¹⁾ the compositions of free amino acids and dipeptides were determined by high-performance liquid chromatography (HPLC), developed by Jasco Co. Ltd., with Finepak series AAPak Li⁺ column. The nucleotides were also determined by HPLC with Biofine IEC-DEAE column.

Protein

The micro-Kjeldahl method was used to determine the crude protein of each sample. The Lowry method³²⁾ was employed to measure the enzyme protein concentration, using crystalline bovine serum albumin as standard.

Volatile Basic Nitrogen

The volatile basic nitrogen (VB-N) of each sample was determined by Conway's diffusion method, as described by Cobb III *et al.*³³⁾

Glycogen and Its Metabolites

Glycogen and its metabolites, glucose, glucose 1-phosphat (Glc-1-P), Glc-6-P, fructose 6-phosphate (Frc-6-P), fructose 1-phosphate (Frc-1-P), fructose 1, 6-bisphosphate (FBP), and lactic acid, were measured enzymatically with the methods described by Bergmeyer *et al.*²⁸⁾

Inorganic Phosphorus

According to the method described by Allen,³⁴⁾ the inorganic phosphate in the sample solution was measured colorimetrically at 640 nm.

Results and Discussion

Changes in Content of Glycogen and Its Metabolites during Fermentation

The result of analyzing glycogen and its metabolites of oyster during fermentation is shown in Fig. 1. After 14-day fermentation with 15% salt, the glycogen decreased from 24.6 to 0 mg/g, and the quantity of Glc-1-P, Glc-6-P, Frc-6-P, and FBP, were also found to have decreased. The quantity of reducing sugar and inorganic phosphate increased from 8.8 mg/g and 14.9 mmol to 32.9 mg/g and 24.8 mmol, respectively. In addition, the quantity of volatile basic nitrogen also increased from 16.7 to 34.7 mg/100 g and the quantity of lactic acid reached 4.8 mg/g. Consequently, the pH value gradually decreased from 5.9 to 5.1.

On the other hand, the glycogen remained at 9.8 mg/g in the sample without salt. The quantity of hexose phosphate such as Glc-6-P, Glc-1-P, Frc-6-P, and FBP also gradually decreased during fermentation. About 7 days for Glc-6-P and 4 days for the others were required for complete hydrolysis. After 4-day fermentation, lactic acid reached the maximum level, 23.3 mg/g, and VB-N also reached 93 mg/100 g. Consequently, the pH value dropped to 3.5, the minimum value. The VB-N continued to increase and lactic acid showed a decreasing tendency throughout further fermentation. During fermentation, there was no undesirable odor. This phenomenon might come from the reason that generated odorants such as ammonium and amines were neutralized by lactic acid.

There have been many reports to describe the glycogen in fish and shellfish.^{2-3,35-39)} However, all of these have involved themselves in discussing its content or degradation during storage. With (15%) or without NaCl, the change in glycogen showed a rapid decrease, but no significant difference between the two on the first day of fermentation; the former decreased to zero and the latter showed no notable change throughout fermentation. These results will be explained in the next section by the fact that the amylase activity was completely inhibited in the absence of NaCl during further fermentation. Sakaguchi and Murata¹⁰⁾ suggested that the glycogen could be related to the palatable taste of oyster because it accumulates glycogen in winter when the oyster is also at its most delicious taste. On the other hand, Ichikawa¹⁰⁾ reported that oyster

Fig. 1. Changes in the components of oyster during fermentation.

Upper: glycogen ●—●, reducing sugar ■—■, glucose □—□.

Middle: G-6-P ●—●, F-6-P △—△, G-1-P ▲—▲, FBP ○—○, Pi □—□.

Bottom: Volatile basic nitrogen ●—●, lactic acid □—□, pH ○—○.

Fig. 2. Changes in the enzymes activities of oyster during fermentation.
 —, 15% NaCl; ---, 0% NaCl.

sauce of high quality could be prepared by natural fermentation with 15% NaCl. A high level of reducing sugar including glucose was found remaining in the hydrolysate after two weeks' fermentation with 15% NaCl in this study. So, on the basis of these arguments, it was suggested

that sweetness by reducing sugar (glucose) which accumulated in the hydrolysate may contribute to the palatable taste of oyster sauce.

The contents of Glc-6-P were higher than those of any other hexose phosphate in the oyster (Fig. 1). It was reported that the amount of Glc-6-P

Table 1. The enzyme activity of oyster before fermentation

Enzymes	Activity
Protease (unit/ml-10 min)	0.09
Trypsin-like (unit/ml-10 min)	0.07
Chymotrypsin (unit/ml-10 min)	0.03* ¹
Carboxypeptidase-A (unit/ml-10 min)	0.22
α -Amylase (G mg/ml-30 min)	8.5
β -Amylase (G mg/ml-30 min)	6.6
Amyloglucosidase (G mg/ml-30 min)	9.2
Alkaline Phosphatase (Pi μ mol/ml-30 min)	19.9
Glu-6-P Dehydrogenase (μ mol/min)	132.0
Lactate Dehydrogenase (μ mol/min)	0.9
Nucleotidase (Pi μ mol/ml-10 min)	49.0
Lipase (μ mol)* ²	0.5
Phospholipase (μ mol)* ²	+

*¹ The chymotrypsin activity was detected after 1-day fermentation.

*² One unit of enzyme activity was defined as 1 μ mole of released fatty acid.

was highest in all glycolytic metabolites in kuruma prawn,³⁸⁾ scallop adductor,³⁹⁾ and seabass³⁷⁾ muscles. Glc-6-P plays an important role in Maillard reaction, as is well known,³⁸⁾ and Nagayama⁴⁰⁾ suggested that most acids inhibit browning by their pH-lowering action. Therefore, although the remaining high level of Glc-6-P in hydrolysates could induce a brown reaction, the extremely high level of lactic acid might prevent it from occurring.

Changes of Enzyme Activities during Fermentation

The activities of enzymes existing in the oyster are determined during fermentation. The results are shown in Table 1. The activities of proteolytic enzymes, protease, trypsin-like, and carboxypeptidase A were 0.09, 0.07, and 0.22 units/ml-

10 min, respectively; the activities of amylolytic enzymes, α - and β -amylase, and amyloglucosidase were 8.5, 6.6, and 9.2 G mg/ml-30 min, respectively. The activity of Glc-6-P DH was 132 μ mol/min. Alkaline phosphatase and nucleotidase activity were 19.9 Pi μ mol/ml-30 min and 49 Pi μ mol/ml-10 min, respectively. LDH activity was 0.9 μ mol/min. Lipase activity was 0.5 μ mole of released fatty acid. Chymotrypsin and phospholipase showed very weak activity before fermentation.

During incubation at 25°C with (15%) and without NaCl, the changes of enzyme activities were traced (Fig. 2). The protease was found to lose 70–80% of its initial hydrolytic activity within 2 days, but it kept this level during further fermentation. Trypsin-like protease was found to lose about 90% of its activity and then kept its level throughout fermentation. The activity of carboxypeptidase A gradually decreased to 75% of its initial value after 14 days' fermentation in the presence of NaCl. In the absence of NaCl, however, the activity decreased to about 60% during the initial 2 days' fermentation and showed almost no change after that. The activity of chymotrypsin could hardly be detected throughout fermentation with 15% NaCl. On the other hand, it was detected after one day's fermentation without salt, and was taken as 100%, but then decreased during further fermentation. Therefore, it was considered that this enzyme might exist in the form of apoenzyme in the oyster.

In addition, the amylolytic enzymes α - and β -amylase and amyloglucosidase were found to lose their activities quickly during fermentation without salt. The enzyme protein was probably denatured and lost its hydrolytic ability due to a

Table 2. Changes in content of nucleotide and its derivatives of oyster during fermentation with 15% and without NaCl (mg/100 g meat)

Nucleotide	Incubation time (Day)										
	15% NaCl						0% NaCl				
	0	1	2	4	7	14	1	2	4	7	14
Inosine	55	58	56	61	57	71	71	33	32	31	39
Guanosine	25	30	35	54	35	66	91	29	66	69	77
Hypoxathine	57	44	52	47	68	30	0	18	47	0	30
IMP	0	4	4	0	0	0	2	0	0	0	0
AMP	3	0	0	1	0	1	3	2	0	0	0
ADP	7	5	5	6	5	2	5	1	0	0	0
ATP	1	0	0	0	0	0	0	0	0	0	0
Total amount	148	141	152	169	165	170	172	83	145	100	146

low pH condition over a long period. In contrast, with 15% salt, the activities of α -amylase and amyloglucosidase did not show any significant change. However, the activity of α -amylase increased sharply within 4 days' fermentation. This phenomenon might be explained by the fact that the halophilic microorganism secreted the same as this enzyme.

During fermentation without salt, the activities

of Glc-6-P DH, alkaline phosphatase, LDH, and nucleotidase decreased quickly to zero after 1 day or 4 days, and the lipase activity gradually fell to 40%. In contrast, during fermentation with 15% NaCl, the activities of Glc-6-P decreased sharply to zero within 2 days, but activities of other enzymes keeping approximately 60, 70, or 50%, and gradually fell to 15% throughout it. The activity of phospholipase showed no changes in

Table 3. Changes in free amino acids contents of oyster during fermentation at 25°C with 15% NaCl (mg/100 g meat)

Amino acid	Incubation time (Day)					
	0	1	2	4	7	14
Nitrogen	271	280	295	344	346	356
P-Ser	8	8	10	11	11	13
Tau	614	607	574	586	565	534
<i>o</i> -PEA	8	5	6	6	6	9
Asp	72	82	91	122	117	141
Hyp	57	81	86	134	120	127
Thr	51	59	68	83	86	92
Ser	28	34	43	56	60	66
Asn	0	0	0	0	0	0
Glu	168	168	187	221	223	233
Gln	0	0	0	0	0	0
Sar	0	0	0	0	0	0
α -AAA	19	21	20	22	19	18
Pro	137	26	98	109	58	34
Gly	78	73	80	91	92	99
Ala	149	158	174	198	192	211
Cit	0	0	0	0	0	0
α -ABA	9	0	9	7	10	5
Val	34	49	68	86	99	108
Cys	0	0	0	0	0	0
Met	0	0	0	0	0	0
Cyt	0	0	0	0	0	0
Ile	16	21	27	34	38	42
Leu	23	34	42	51	55	60
Tyr	16	22	26	31	34	35
Phe	15	24	30	37	40	43
β -Ala	35	35	34	36	34	36
β -ABA	0	0	0	0	0	0
γ -ABA	0	0	0	0	0	0
EA	27	36	21	26	38	26
NH ₃	24	29	20	23	27	26
Orn	9	15	14	11	10	10
Lys	36	82	40	88	107	122
1-MeHis	0	0	0	0	0	0
His	15	19	17	20	20	21
3-MeHis	0	10	0	10	0	0
Ans	0	0	11	0	25	34
Car	0	0	0	0	0	0
Arg	44	52	50	51	76	80
Total amounts	1,692	1,750	1,846	2,150	2,162	2,225

this study.

Changes in Nucleotides during Fermentation

Nucleotides and their derivatives in oyster were analyzed during fermentation with (15%) and without NaCl. The results are shown in Table 2. The total nucleotides were 148 mg/100 g and no significant change in content was observed during fermentation. The major nucleotides and

their derivatives of oyster are inosine, guanosine, and hypoxanthine, while small quantities of AMP, ADP, and ATP were also observed, though IMP could not be detected. Although adenine nucleotides were found in large quantity in living crustaceans and mollusks, the taste enhancers AMP and IMP were easily degraded in postharvest storage²⁾ and struggling fish.³⁰⁾ Therefore, after 4-day fermentation without salt, all nucleotides

Table 4. Changes in free amino acids contents of oyster during fermentation at 25°C without sodium chloride (mg/100 g meat)

Amino acid	Incubation time (Day)					
	0	1	2	4	7	14
Nitrogen	271	337	394	427	496	457
P-Ser	8	11	12	13	15	14
Tau	614	550	557	536	619	607
<i>o</i> -PEA	8	8	6	10	11	10
Asp	72	27	99	135	179	168
Hyp	57	114	156	138	156	150
Thr	51	81	99	113	144	131
Ser	28	36	40	60	84	53
Asn	0	0	0	0	0	0
Glu	168	221	256	299	355	270
Gln	0	0	0	0	0	0
Sar	0	0	0	0	0	0
α -AAA	19	22	20	15	16	16
Pro	137	113	111	125	77	75
Gly	78	109	106	105	141	128
Ala	149	264	270	283	341	306
Cit	0	0	0	0	0	0
α -ABA	9	6	8	3	3	9
Val	34	85	123	151	192	175
Cys	0	0	0	0	0	0
Met	0	0	6	11	12	10
Cyt	0	0	0	0	0	0
Ile	16	34	56	64	75	64
Leu	23	55	86	97	111	94
Tyr	16	34	42	48	43	33
Phe	15	43	67	73	84	74
β -Ala	35	36	35	34	37	36
β -ABA	0	0	0	0	0	0
γ -ABA	0	7	11	9	16	16
EA	27	39	14	12	17	26
NH ₃	24	29	29	29	37	51
Orn	9	33	25	16	26	40
Lys	36	53	109	118	172	189
1-MeHis	0	0	0	0	0	0
His	15	19	20	21	27	31
3-MeHis	0	0	9	10	6	9
Ans	0	43	20	33	0	11
Car	0	0	0	0	0	0
Arg	44	36	71	105	107	59
Total amounts	1,692	2,108	2,463	2,666	3,103	2,855

entirely degraded into their derivatives.

Changes in Free Amino Acids and Dipeptides during Fermentation

The changes in the contents of free amino acids and dipeptides of oyster during fermentation were investigated. As shown in Tables 3 and 4, the total amount of free amino acids of the initial stage was 1,672 mg/100 g and the major free amino acids were Tau, Asp, Glu, Pro, Gly, and Ala. The six amino acids accounted for 70% of total free amino acids. About 35 mg/100 g of β -Ala was observed. Cysteine was not found in initial or further fermentation.

After fermentation, total free amino acids showed no remarkable increase in the first two days and then slowly increased to 2,225 mg/100 g in the group with 15% NaCl on the 14th day. To explain this phenomenon, the high salt content was considered to be able to inhibit the growth of microorganisms; but it could not completely inhibit the activity of proteolytic enzymes.

On the contrary, total free amino acids rapidly increased and reached their maximum level, 3,103 mg/100 g, in the group without NaCl on the 7th day, and then decreased during further one-week fermentation. In general, the free amino acids and small peptides were the carbon source and nutrients of microorganisms. So, this result could be explained by the fact that the resulting free amino acids and small peptides offered good growth conditions for microorganisms, and that the protein for decomposition was exhausted during fermentation.

Since Tau and β -Ala were not the constituent amino acid of protein, there were no remarkable changes to be observed during fermentation either with (15%) or without salt. However, Orn was observed to increase to 445% during fermentation without salt. According to Tokunaga *et al.*,⁽¹⁾ Arg could be degraded by arginase to form Orn. Therefore, a part of the increasing Arg might be transferred into ornithine by arginase existing in oyster paste during fermentation. As for the constituent amino acids of protein, the quantity of each amino acid increased to about 130–340% after two-week fermentation with 15% NaCl (Table 3) and 150–800% after one-week fermentation without salt (Table 4). Moreover, Murata and Sakaguchi⁽²⁾ also reported that the Pro of oyster had a decreasing tendency in the whole body and an increasing tendency in the adductor

muscle during ice storage. The Pro was also found to have a declining tendency (Table 3) and fluctuation (Table 4) during fermentation with (15%) and without salt, respectively.

Konosu and Yamaguchi⁽²⁾ reported that Glu, Gly, Ala, Arg, and AMP were the taste components in fish and shellfish. Although free amino acids which have a palatable taste, such as Glu, Gly, and Ala, increased during fermentation, Arg and Val, amino acids which give a bitter taste, also increased in content. However, the total content of the former was higher than that of the latter (Tables 3 and 4).

The dipeptides, glutathionine, balenine, Car, and Ans existing in fish and shellfish muscle have been identified. All of them have been observed in abundance in tuna, skipjack, eel, and shark. Small amounts of Car, Ans, and balenine were also found in crustaceans and mollusks.⁽²⁾ The oyster was shown to have Ans by Lukton and Olcott⁽⁴³⁾ (1958). Although not all dipeptides were detected at the initial state, nevertheless a small quantity of anserine was found in further fermentation.

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