The Proteinase Distributed in the Intestinal Organs of Fish

3. Purification and Some Enzymatic Properties of the Alkaline Proteinases from the Pyloric Caeca of Skipjack, *Katsuwonus vagans*

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Purification and some properties of alkaline proteinases in the pyloric caeca of skipjack, *Katsuwonus vagans*, were investigated.

Four alkaline proteinases, temporarily designated proteinases I, II, III and IV, were identified from the tissue extract of the pyloric caeca by ammonium sulfate fractionation, DEAE-Sephadex A-50 chromatography, and Sephadex G−100 and G−200 gel filtration.

Result of disc-polyacrylamide gel electrophoretic analysis showed that the purified proteinases II and III were homogenous with the yields of 1.5% and 1.2%, and those specific activities were increased to 33 to 37 fold over that of the crude enzyme solution, respectively.

Molecular weight of the proteinases II and III determined by sephadex G-100 gel filtration were 28,500 and 24,200, respectively. The optimum conditions for the caseinolytic activity of the two enzymes were pH 9.6 and 48℃.

The reaction rates of the two alkaline proteinases were constant to the reaction time to 80 *min* in the reaction mixture of 3.4 $\mu g/ml$ of enzyme concentration and 2% casein solution. The Km values against casein substrate determined by the method of Lineweaver-Burk were 0.56% for proteinase II and 0.30% for proteinase III.

The proteinases II and III were inactivated under the presence of Ag^{+}, Hg^{2+}, Ni^{2+}, Fe^{2+}, and Cu^{2+}, but activated by Mn^{2+} and Ca^{2+}, and markedly inhibited by the soybean trypsin inhibitor and N-p-toluenesulfonyl-L-lysine chloromethyl ketone.

Therefore, the proteinases II and III were found to be a group of serine proteases and assured to be trypsin-like proteinases.

Introduction

There seems many similarities among the digestive enzymes distributed in the animal digestive tracts. The proteolytic enzymes distributed in the intestinal organs of marine vertebrate of which internal organs are distinct were generally similar to those of mammals, such as pepsin-like enzyme in the stomach and trypsin- or chymotrypsin-like enzyme in the intestine and pancreas.

Among the internal organs of teleostean fish, pyloric caeca is in charge of digestion and abs-

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orption of nutrients, but it's physiological function has not been extensively investigated yet.

Regarding to the pyloric caeca of skipjack, distribution and composition of amino acid(Kakimoto et al 1953 ; Kakimoto et al., 1957), organic acid (Kashiwada et al., 1954) and vitamin B group (Kakimoto et al., 1957 ; Kakimoto, 1957 a ; Kakimoto, 1957b) have been studied, but the function of pyloric caeca especially in chemical phase digestion is still remained in dispute.

To elucidate the function of pyloric caeca, some investigations have been performed – seasonal variation of proteolytic enzyme activity in pyloric caeca of skipjack(Kashiwada, 1952), some enzymatic properties of proteinase of tunny pyloric caeca purified by ion exchange chromatography and crystallization, and distribution of trypsin, chymotrypsin, and carboxypeptidase A and B of chum salmon pyloric caeca in the gland and exocrine juice as a form of zymogens.

In the previous paper(Pyeun and Kim, 1986 ; Kim and Pyeun, 1986), we reported on the purification and properties of the three alkaline proteinases from the mackerel pyloric caeca.

In this study, we purified alkaline proteinases from the pyloric caeca of skipjack and investigated some enzymatic properties in order to compare to that of the alkaline proteinases in the pyloric caeca of other fishes from the view point of phylogenic specificities on the protein digestion.

Materials and Methods

Materials

The skipjack, *Katsuwonus vagans*, used for obtaining pyloric caeca in this study were transported as frozen state at −20℃ from the local fisheries Co.

Throughout the experiment, all reagents used were analytical extra pure grade. Ultrafiltration membrane(Chemlab Industrial Ltd., U.K.) was used to concentrate the enzyme solutions.

Enzyme activity and protein concentration

Buffer and substrate solution were prepared in the same manner that reported in the previous paper(Pyeun et al., 1986).

Proteolytic activity was determined by the modified Anson's method(Anson, 1938). Protein concentration was determined spectrophotometrically according to the Lowry's method(Lowry et al., 1951).

Purification of alkaline proteinases

Unless otherwise indicated, all procedures were carried out at 0~4℃. The purification procedure for the alkaline proteinases from the pyloric caeca of skipjack is shown in Fig. 1. Outline of the procedure from the extraction of crude enzyme to gel filtration with Sephadex G–100 is not much different with the method reported in the previous paper(Pyeun and Kim, 1986). After Sephadex G–100 gel filtration, proteolytic fractions were pooled and concentrated at ultrafiltration membrane. The concentrated Sephadex G–100 fraction(504*mg* of protein) was applied to the first DEAE Sephadex A–50 column(5×50*cm*) equilibrated with 0.01M sodium phosphate buffer, pH 7.0 and eluted with 2,000*ml* of buffer solution by linear gradient ranging from 0 to 0.4M NaCl in 0.01M sodium phosphate buffer, pH 7.0

Four alkaline proteinases(designated proteinases I, II, III and IV) were fractionated in a different concentration of salt solution. Each proteolytic fractions were pooled and concentrated at ultrafiltration membrane and dialyzed against 0.01M sodium phosphate buffer, pH 7.0, for overnight. The dialyzed solutions were centrifuged at 14,000*xg* for 30*min*(first DEAE–Sephadex A–50 fraction).

These DEAE– Sephadex A–50 fractions were rechromatographed on the second DEAE–Sephadex A–50 column(3×30*cm*) equilibrated with sodium phosphate buffer same as above and eluted with 1,000*ml* of buffer solution by linear gradient ranging from 0.1 to 0.3M NaCl for proteinases III and IV.

Proteolytic fractoins were pooled and concentrated with ultrafiltration membrane. Each suspension was dialyzed against 0.1M NaCl. 0.01M sodium phosphate buffer, pH 7.0(second DEAE–Sephadex A–50 fraction). Each second DEAE–Sephadex A–50 fractions were applied to a Sephadex G–200 column(3×95*cm*) equilibrated with

Minced sample(pyloric caeca)
- Extract with 4 vol of 1% NaCl · 1*mM* EDTA
- Stand at 30℃ for 3*hr*
- Centrifuge(14,000x*g*, 30*min*)

Supernatant / Precipitate

Supernatant:
- Salt-out(ammonium sulfate saturation ; 30~80%)
- Stand for 3*hr*
- Centrifuge(14,000x*g*, 30*min*)

Precipitate / Supernatant

Precipitate:
- Dissolve in 0.1M NaCl · 0.01M phosphate buffer, pH 6.75
- Dialyze against the same buffer for overnight
- Centrifuge(14,000x*g*, 30*min*)

Supernatant / Precipitate

Supernatant:
- Sephadex G-100 column(3×100*cm*)
- Elute with 0.1M NaCl · 0.01M phosphate buffer, pH 6.75
- Pool and concentrate the proteolytic fraction with ultrafiltration membrane

Concentrated effluent
- 1'st DEAE-Sephadex A-50 column(5×50*cm*)
- Elute with 0~0.4M NaCl · 0.01M phosphate buffer, pH 7.0

Proteolytic fraction
- 2'nd DEAE-Sephadex A-50 colum(3×30*cm*)
- Elute with 0.1~0.3M or with 0.2~0.4M NaCl · 0.01M phosphate buffer
- Fractionate into proteolytic fractions.
- Concentrate with ultrafiltration membrane
- Dialyze against 0.1M NaCl · 0.01M phosphate buffer, pH 6.75
- Sephadex G-200 column(3×95*cm*)
- Elute with the above buffer
- Pool the proteolytic fraction
- Concentrate with ultrafiltration membrane
- Dialyze against distilled and deionized water for overnight

Purified alkaline proteinase

Fig. 1. Scheme of purification procedure for the alkaline proteinase from the pyloric caeca of skipjack.

the same buffer as above. Eluted proteolytic fractions were pooled and concentrated with ultrafiltration membrane and dialyzed against distilled and deionized water for overnight(purified alkaline proteinase).

Results and Discussion

Purification of the alkaline proteinases

In the preliminary experiment by using crude enzyme extract from the pyloric caeca, the optimum reaction condition of the crude extract was pH 9.4 and 45℃.

Therefore, the determination of proteolytic activity accompanied by the following purification steps was performed at pH 9.4 and 45℃.

Chromatogram of a Sephadex G-100 gel filtration of the salted-out fraction between 30% to 80% ammonium sulfate saturation is shown in Fig. 2. As shown in the figure, the proteolytic enzyme was eluted from No 32 to No 50 in the tube number. These proteolytic fraction indicated by the horizontal arrow was pooled and concentrated with ultrafiltration membrane. From this gel filtration, the larger and smaller proteins like muscle proteins, the more peptides, and the other constituent except alkaline proteinase was effectively removed. The purity of alkaline proteinase was increased to 9.6 fold over the crude enzyme solution.

The concentrated Sephadex G-100 fraction was applied to the first DEAE-Sephadex A-50 column chromatography(Fig. 3). Four alkaline proteinases designated proteinases I, II, III and IV were fractionated near the ionic strength of 0.13, 0.23, 0.27 and 0.31, respectively. With this first DEAE-Sephadex A-50 chromatography, the purity of the proteinases I, II, III and IV were increased to 15.8, 29.5, 20.6 and 16.0 times, respectively, than that of the crude enzyme. Each alkaline proteinase fractions (tube No. 16~30 for proteinase I, 70~90 for proteinase II, 90~104 for proteinase III and 120~145 for proteinase IV were concentrated and followed the second DEAE-Sephadex A-50 chromatography(Fig. 4). Four alkaline proteinases were eluted at the same salt concentration of first ion exchange chromatography. With these second ion exchange chromatography, the purity of proteinase I, II, III and IV was increased to 21.1, 30.7, 23.1 and 24.6 times than that of the crude enzyme, respectively. The fractions of four alkaline

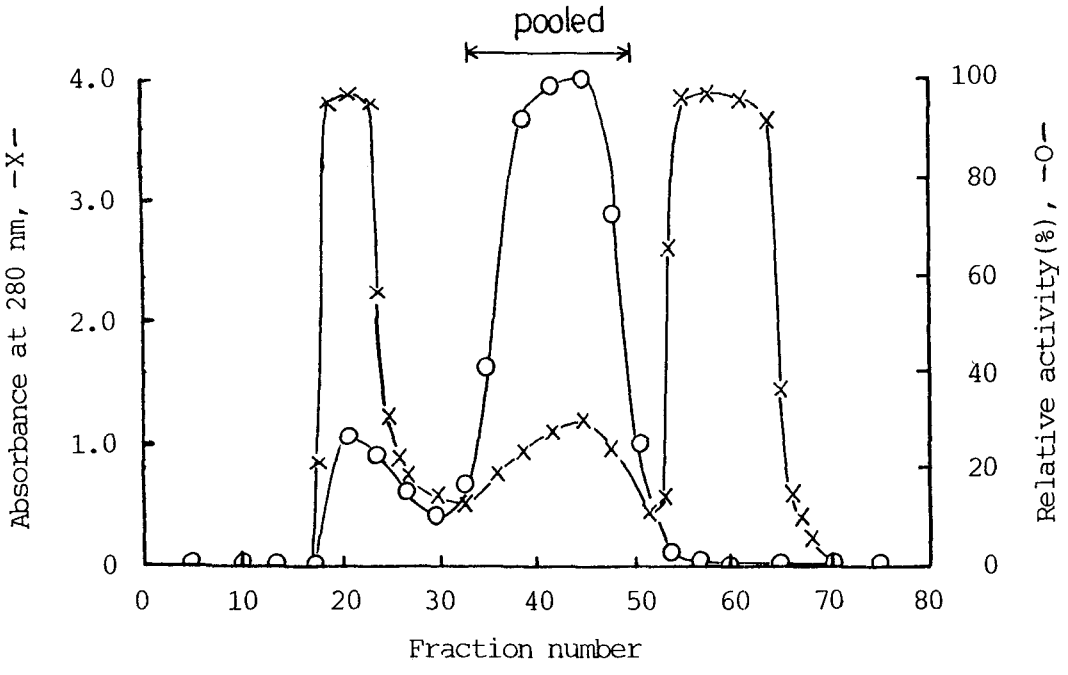

Fig. 2. Gel filtration of 30~80% ammonium sulfate saturated fraction on Sephadex G-100 column(3×95*cm*). *The column was eluted with* 0.1M NaCl · 0.01M phosphate buffer, pH 6.75. Contents in tube number 33~50 were pooled and concentrated for applying DEAE-Sephadex A-50 chromatography. Flow rate, 20*ml/hr* ; Fraction volumn, 10*ml*.

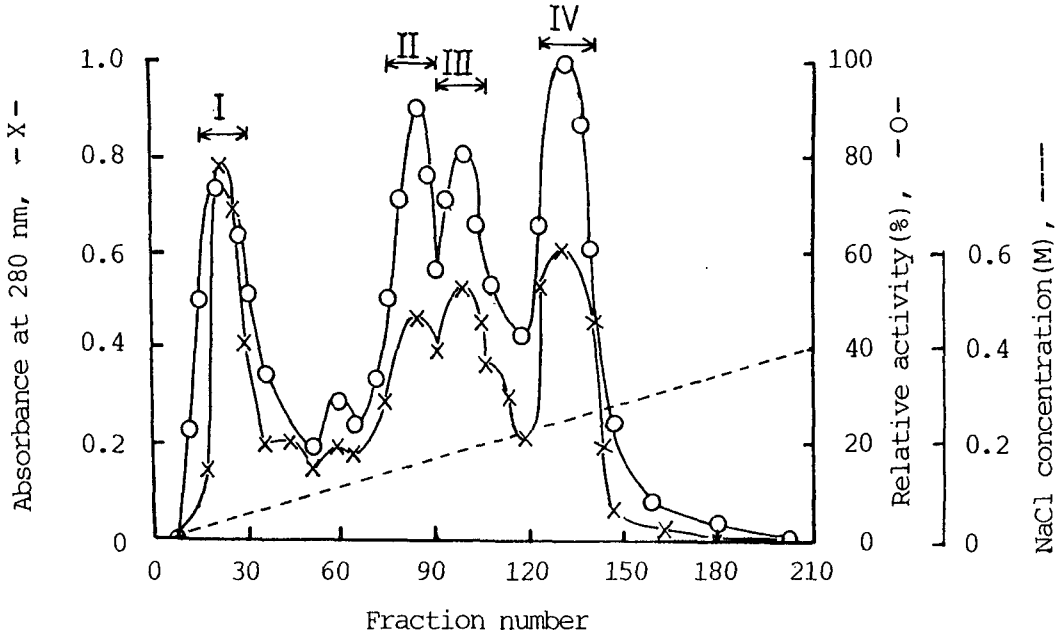

Fig. 3. 1'st DEAE-Sephadex A-50 chromatography of the Sephadex G-100 proteolytic fraction. The column(5×50*cm*) was eluted with 0.01M phosphate buffer, pH 7.0, and further eluted using a 2,000*ml* linear gradient of 0~0.4M NaCl in the same buffer. Flow rate, 40*ml/hr* ; Fraction volume, 10*ml*.

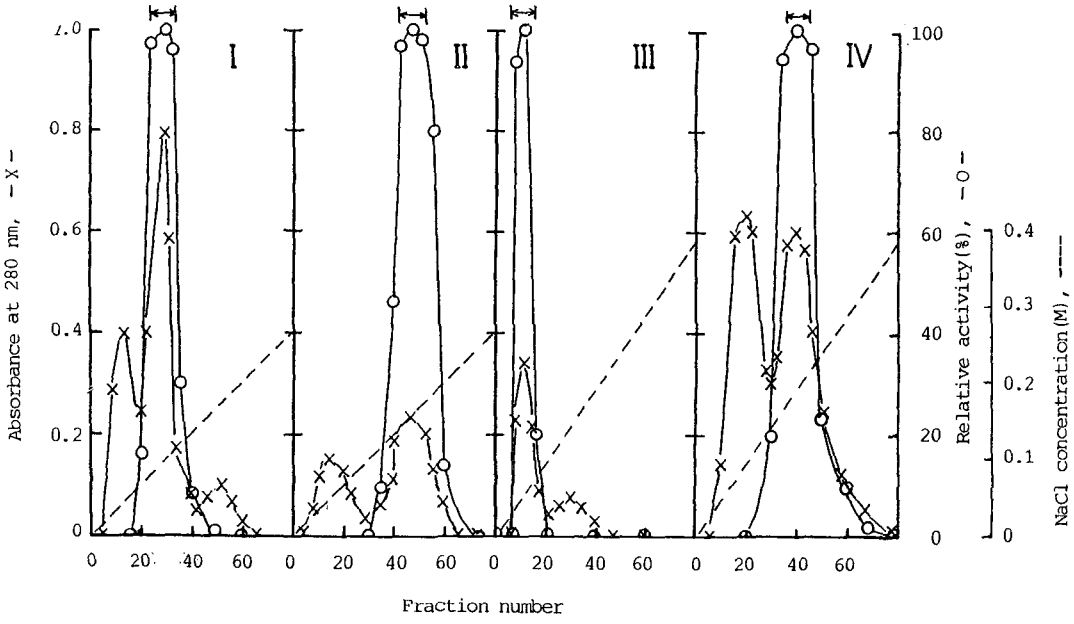

Fig. 4. 2'nd DEAE-Sephadex A-50 chromatography of the proteolytic fraction I, II, III and IV obtained from the 1'st DEAE-Sephadex A-50 chromatography. The column was eluted with 0.01M phosphate buffer, pH 7.0, and further eluted using a 1,000*ml* linear gradient of 0.1~0.3M NaCl in the same buffer for Proteinases I and II, and 0.2~0.4M NaCl for Proteinases III and IV. Flow rate, 40*ml/hr*; Fraction volume, 10*ml*.

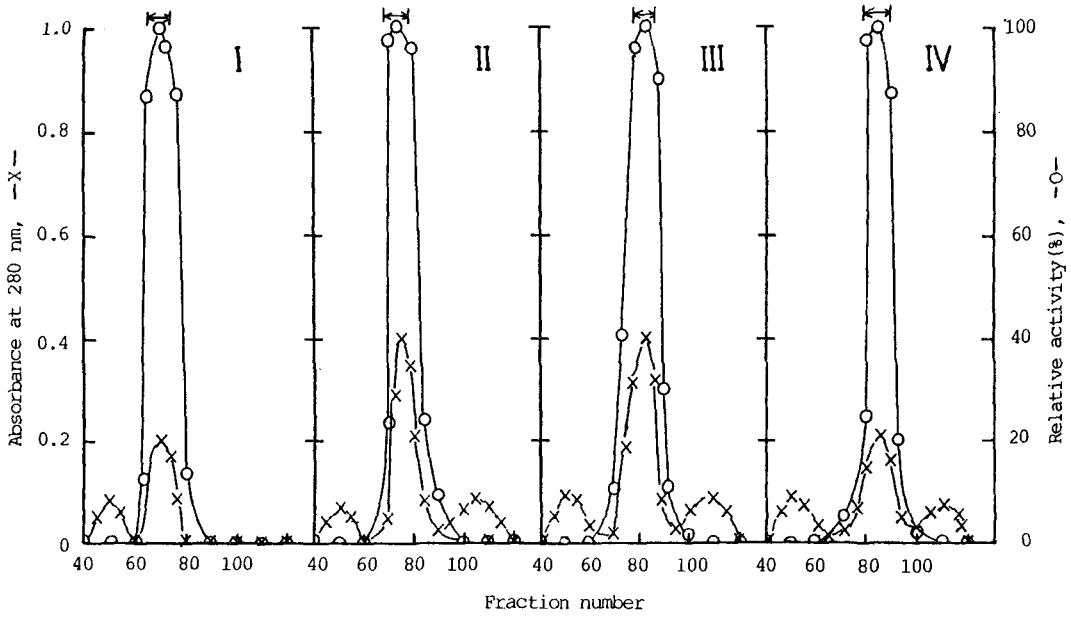

Fig. 5. Gel filtration of 2'nd DEAE-Sephadex A-50 fraction on a Sephadex G-200 column(3×95*cm*). The column was eluted with 0.1M NaCl · 0.01M phosphate buffer, pH 6.75. Flow rate, 20*ml/hr*; Fraction volume, 5*ml*.

proteinases by second DEAE-Sephadex A-50 chromatography were applied to a Sephadex G-200 column chromatography(3×95*cm*) and the chromatogram is shown in Fig. 5. With these gel filtration, proteins except alkaline proteinases were effectively removed. The purity of proteinases I, II, III and IV was increased to 26.0, 33.0, 37.0 and 31.1 times than that of the crude enzyme, respectively. The outline and result of the purification of four alkaline proteinases are summarized in Table 1. With 300*g* of the tissues, 6.0*mg* of proteinase I, 8.3 *mg* of proteinase II, 5.4*mg* of proteinase III and 10.1 *mg* of proteinase IV were isolated with a combined yield of 6.4% from the present purification procedure. These enzymes were purified to a 26 to 37 fold with this purification procedure. The final specimens after Sephadex G-200 gel filtration were stored at −20℃ and used for subsequent experiments.

Murakami and Noda(1981) reported that the specific activities of three alkaline proteinases from the pyloric caeca of sardine were increased to 50~120 fold than that of the crude enzyme and combined yields were 17%. Uchida et al.,(1984)

Table 1. Purification of skipjack pyloric caeca alkaline proteinases I, II, III and IV

Proteinase	I		II		III		IV	
Procedure	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)
Crude extract	0.13	100	0.13	100	0.13	100	0.13	100
Ammonium sulfate fractionation(30~80%)	1.06	45.3	1.06	45.3	1.06	45.3	1.06	45.3
Sephadex G-100 gel filtration	1.26	22.3	1.26	22.3	1.26	22.3	1.26	22.3
First DEAE-Sephadex A-50 chromatography	2.06	4.4	3.85	5.1	2.70	6.1	2.10	3.7
Second DEAE-Sephadex A-50 chromatography	2.76	1.8	4.02	2.2	3.03	2.1	3.22	3.2
Sephadex G-200 gel filtration	3.41	1.0	4.32	1.5	4.86	1.2	4.07	2.7

Specific activity : U/*mg* - protein

reported that six anionic trypsins having isoelectric point of pH 4.1~4.3 and one cationic trypsin having isoelectric point of pH 11.1 were isolated from chum salmon pyloric caeca by ammonium sulfate salting-out, batch mathod of P-and DEAE-cellulose, and gel-filtration chromatography on Sephadex G-100.

Comparing the specific activities of the purified alkaline proteinases isolated from the pyloric caeca of mackerel(Pyeun and Kim, 1986) and sardine (Murakami and Noda, 1981) to the results of the present study, the proteinases isolated from the pyloric caeca of skipjack has lower specific activities and less yields than those from mackerel and sardine.

Purity and molecular weight

It was presumed that proteinases I, II, III and IV were purified, since Sephadex G-200 gel chromatogram (Fig. 5) showed matched curves between protein concentration and enzyme activity. In order to check the purity of four alkaline proteinases obtained from Sephadex G-200 gel filtration, disc-electrophoresis was performed at pH 8.3. As shown in Fig. 6, proteinases II and III were displayed a single

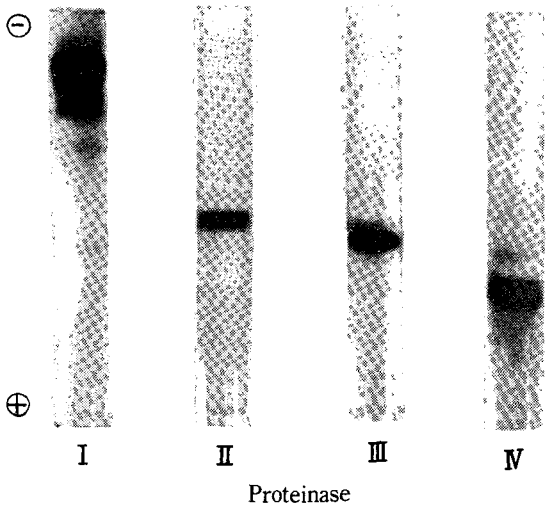

Fig. 6. Disc-gel electrophoresis of the four alkaline proteinases I, II, III and IV from the pyloric caeca of skipjack. The enzyme protein(50*μg*) was placed on 7.5% polyacrylamide gel column(0.6×10*cm*) at pH 8.3 and permitted to migrate in an electric field of 4*mA* current per tube for 2.5 hours. Protein bands were stained by Coomasie brilliant blue R-250.

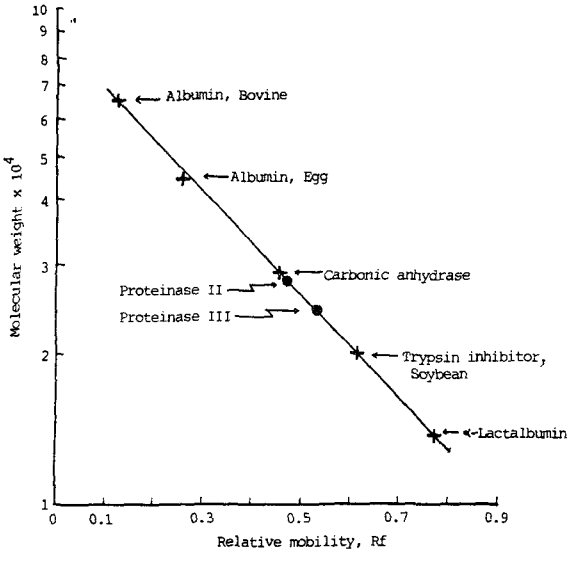

Fig. 7. Estimation of molecular weight of the two alkaline proteinases with SDS-polyacrylamide gel electrophoresis.

band which indicates homogeneity. But proteinases I and IV were not completely isolated to a homogeneity by the present purification procedure.

The alkaline proteinases II and III were corresponded to the relative mobilities of the molecular weight of 28,500 and 24,200, respectively on the electrophoretograms of marker proteins(Fig. 7). The molecular weights of the two alkaline proteinases were also determined by gel filtration on Sephadex G-100

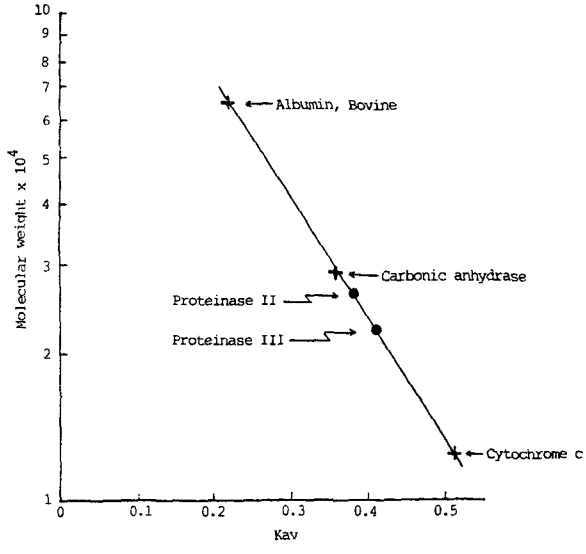

Fig. 8. Estimation of molecular weight of the two alkaline proteinases with Sephadex G—100 gel filtration.

column(2 x 95*cm*). As shown in Fig. 8, molecular weights of the proteinases II and III were estimated to be 27,000 and 22,700, respectively. In regard to the determination of molecular weight by two methods, the results were in agreement each other.

Effect of pH and temperature on the hydrolysis of casein

Effect of pH on the hydrolysis of casein by the two proteinases was experimented in the pH range from 5.0 to 11.0 at 40℃ for 30 minutes incubation. The optimum pH for the proteinases II and III were found to be pH 9.6(Fig. 9). The pH condition of the two enzymes was coincided with the condition of

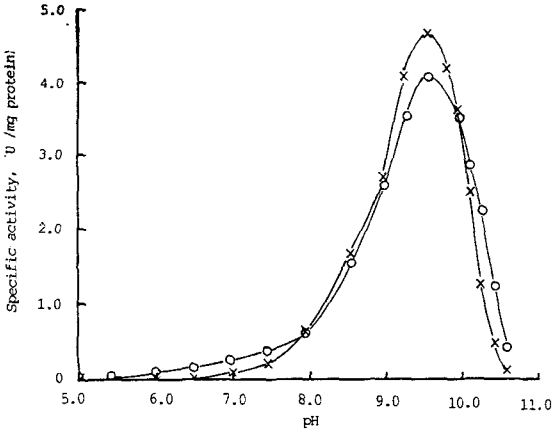

Fig. 9. Effect of pH on the hydrolysis of casein by the two alkaline proteinases. The used buffer were 0.1M Citrate-Na_2HPO_4 (pH 5.0-7.0), 0.1M Tris-HCl (pH 7.0-9.0) and 0.1M Na_2CO_3-$NaHCO_3$ (pH 9.0-11.0). Proteinase II, o ; Proteinase III, x.

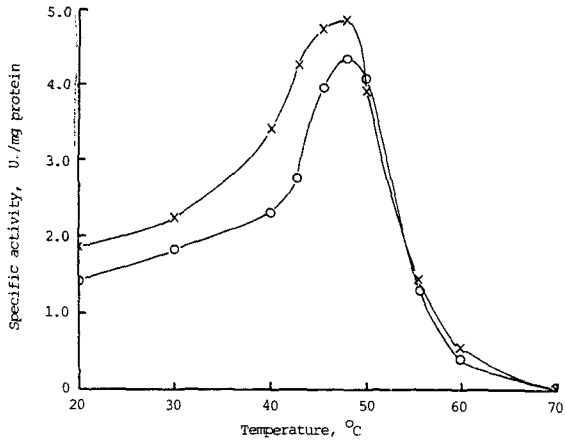

Fig. 10. Effect of temperature on the hydrolysis of casein by the two alkaline proteinases. The used buffer was 0.1M Na_2CO_3-$NaHCO_3$, pH 9. 6. Proteinase II, o ; Proteinase III, x.

Enz. A isolated from the mackerel pyloric caeca(Kim and Pyeun, 1986).

Murakami and Noda(1981) reported that the purified alkaline proteinase from the pyloric caeca of sardine showed the optimum pH at 10.0 toward milk casein.

The optimum temperature of the two enzymes with casein substrate were found to be 48℃ at the optimum pH(Fig. 10). Consequently, the reaction conditions of the two proteolytic enzymes against casein substrate were slightly differed to that of the other proteolytic enzymes from the pyloric caeca of mackerel (Kim and Pyeun, 1986) and sardine(Murakami and Noda, 1981).

Effect of reaction time on the hydrolysis of casein

The reaction rate for the casein substrate was studied with the two alkaline proteinases. A linear correlation was observed between the enzyme activity (y) and the reaction time(x) under 80 minutes of reaction time, 1.36 *μg/ml* of enzyme concentration and 0.4% of casein solution. The reaction rate equations of the proteinases II and III were found to be y=5.63x and y=4.13x, respectively(Fig. 11).

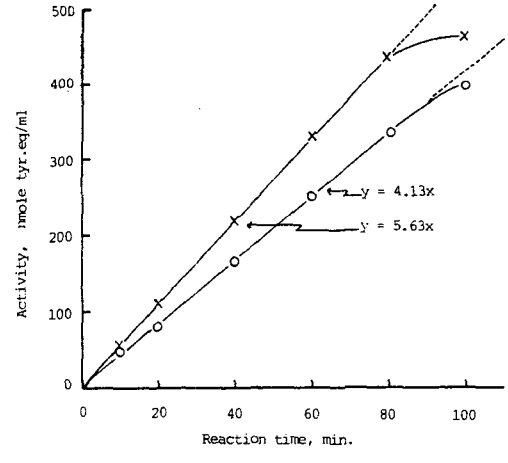

Fig. 11. Effect of reaction time on the hydrolysis of casein by the two alkaline proteinases. The reaction mixture was consisted of 3.4*μg* of enzyme, 0.5ml of 2% casein solution and 2.0*ml* of 0.1M Na_2CO_3-$NaHCO_3$, pH 9.6. Proteinase II, o ; Proteinase III, x.

Thermal stability of the two alkaline proteinases

The two alkaline proteinases were preincubated for 5 minutes in 6.8*μg/ml* of enzyme concentration to distilled deionized water at 30~60℃. The residual activities were determined under the optimum reaction conditions after preincubating.

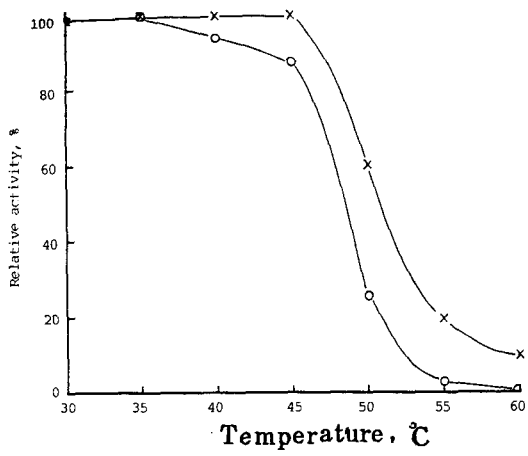

Fig. 12. Thermal stability of the two alkaline proteinases. Enzyme solution were preincubated for 5*min* at various temperature. After preincubation, residual activities were determined at the optimum reaction condition. Proteinase II, o; Proteinase III x.

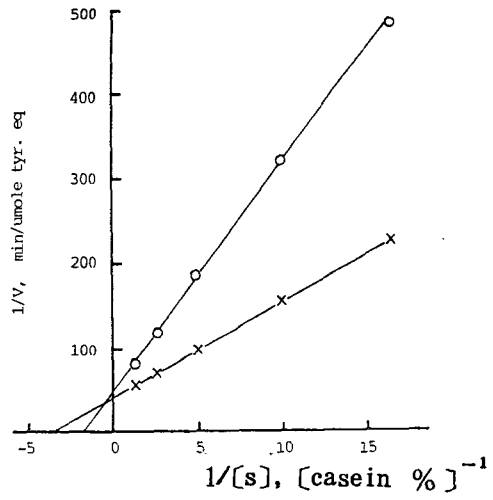

Fig. 13. Lineweaver - Burk plots for the hydrolysis of casein by the two alkaline proteinases. Km value : Proteinase II, 0.56%; Proteinase III, 0.30%. Proteinase II, o; Proteinase III, x.

The residual activity of proteinase III was not decreased till the preincubation temperature reached to 45℃, but that of proteinase II was decreased to 87 % over the original activity by the preincubation at 45℃ (Fig. 12).

Consequently, it may be concluded that proteinase III is more stable than proteinase II under the preincubating thermal condition.

The activities of proteinases II and III were decreased to 75% and 40% over that of original purified enzymes when they were preincubated at 50℃, respectively.

Affinity against casein substrate

The Km values of the two alkaline proteinases were determined using casein as substrate by the method of Lineweaver - Burk. As shown in Fig. 13, the Km values of the proteinases II and III were found to be 0.56% and 0.30%, respectively.

Effect of metal ions and chemical reagents on the enzyme activity

Effect of metal ions and chemical reagents on the proteolytic activity was investigated with the two alkaline proteinases. Activities of the two proteinases were significantly decreased with the addition of Hg^{2+} Ag^{+}, Ni^{2+}, Cu^{2+}, and Fe^{3+}, as shown in Table 2. The activity of proteinase II was also decreased by Pb^{2+}. On the other hand, the activity of two proteinases were increased with the addition of Mn^{2+}, and Ca^{2+}, and only proteinase II was increased its activity in the presence of Ba^{2+}, and Co^{2+}. Therefore, the two proteinases were presumed to be a serine proteinase.

The reactivity of the two proteinases against the metal ions was seemed to be an analogous to that of the three alkaline proteinases from the mackerel pyloric caeca(Kim and Pyeun, 1986).

Effect of various reagents on the proteolytic activity was examined with the two alkaline proteinases. As shown in Table 3, the activities of the proteinases II and III were significantly inhibited in the presence of the soybean trypsin inhibitor and TLCK, and those were slightly inhibited by the presence of mono - iodoacetate, *o* - phenanthlorine and *p*-chloromercuribenzoate. Those were neither inhibited nor activated by EDTA, cysteine, 1,4 - dithiothreitol and TPCK.

In regard to these results, we conclude that two proteinases from the skipjack pyloric caeca might be trypsin - like proteinases since they are remarkably

Table 2. Effect of metal ions on the proteolytic activity

(Relative activity,%)

Metal ion(2×10^{-3}M)	Skipjack proteinase		Mackerel Enz.*		
	Ⅱ	Ⅲ	A	B	C
None(control)	100	100	100	100	100
K^+	98	90	132	100	93
Na^+	107	103	124	95	151
Ag^+	12	7	23	33	22
Li^+	100	114	130	93	97
Ba^{2+}	140	103	116	101	92
Cd^{2+}	94	92	92	96	98
Mn^{2+}	245	225	190	260	270
Ca^{2+}	154	120	114	100	87
Co^{2+}	118	99	92	96	106
Mg^{2+}	73	90	126	95	93
Hg^{2+}	10	1	7	6	5
Zn^{2+}	73	84	87	91	22
Ni^{2+}	28	32	86	50	47
Cu^{2+}	40	20	137	43	22
Pb^{2+}	69	100	122	155	131
Fe^{3+}	54	53	94	77	44

* Kim and Pyeun, 1986.

Table 3. Effect of various reagents on the proteolytic activity

(Relative activity,%)

Reagents(2×10^{-3}M)	Skipjack proteinase		Mackerel Enz.[1]		
	Ⅱ	Ⅲ	A	B	C
None (control)	100	100	100	100	100
EDTA[2]	105	106	84	107	109
Iodoacetate	61	82	107	93	107
Cysteine	123	110	60	100	98
1,4-Dithiothreitol	114	114	103	96	101
o-Phenanthlorine	87	72	98	92	102
p-Chloromercuribenzoate	67	50	54	107	100
Soybean trypsin inhibitor	14	9	42	21	13
TLCK(0.2mM)[3]	14	28	86	75	0
TPCK(0.2mM)[4]	92	96	19	55	107

1) Kim and Pyeun, 1986.

2) EDTA ; ethylenediamine tetaacetate,

3) TLCK ; N-p-toluenesulfonyl-L-lysine chloromethylketone,

4) TPCK ; N-p-toluenesulfonyl-L-phenylalanine chloromethylketone.

inhibited by soybean trypsin inhibitor and TLCK.

Enzymatic properties of two alkaline proteinases are summarized in Table 4.

Table 4. Properties of the two skipjack alkaline proteinases

Property	Proteinase II	Proteinase III
Specific activity for casein[1]	4.32	4.86
Optimum pH	9.6	9.6
Optimum temperature, ℃	48	48
Inactivation by metal ion	Ag^+, Hg^{2+}, Ni^{2+}	Ag^+, Hg^{2+}, Ni^{2+}, Cu^{2+}
TLCK(0.2mM)[2]	14	28
TLCK(0.2mM)[2]	92	96
Thermal stability at 50℃[2]	25	60
Km for casein, %	0.56	0.30
Molecular weight[3]	27,000	22,700
Molecular weight[4]	28,500	24,200

1) U/*mg*-protein
2) Residual activity, %
3) Molecular weight by Sephadex G-100 gel filtration
4) Molecular weight by SDS-polyacrylamide gel electrophoresis

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