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The Proteinase Distributed in the Intestinal Organs of Fish

2. Characterization of the Three Alkaline Proteinases from the Pyloric Caeca of Mackerel, *Scomber japonicus*

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The characteristics of the three alkaline proteinases, Enz. A, B and C, from the pyloric caeca of mackerel have been investigated.

The optimum condition for the activity of the Enz. A, B and C was pH 9.4, 9.8 and 9.8 at 45°C for 2% casein solution, and was pH 9.2 10.2 and 9.8 at 45°C for 5% hemoglobin denatured by urea, respectively.

Enz. A, B and C by heat treatment at 50°C for 5 min were inactivated 90, 33 and 37%, respectively, over the original activity.

The reaction rate of the three alkaline proteinases was constant to the reaction time to 40 min in the reaction condition of 2 $\mu g/ml$ of enzyme concentration and 2% casein solution. The reaction rate equation and Km value against casein substrate determined by the method of Lineweaver and Burk were: Enz. A, $Y=3.6X$ and $Km=5.0\times10^{-3}\%$; Enz. B, $Y=6.0X$ and $Km=1.0\times10^{-3}\%$; Enz. C, $Y=4.2X$ and $Km=3.6\times10^{-3}\%$.

The three alkaline proteinases were inactivated by Ag^{+} and Hg^{2+}, but activated by Mn^{2+}, Sn^{2+} and Pb^{2+}. Enz. B and C were remarkably inhibited by the soybean trypsin inhibitor.

Molecular weight of Enz. A, B and C determined by SDS-polyacrylamide gel electrophoresis and Sephadex G-100 gel filtration was in the range of 27,500±2,500, 20,500±1,500 and 15,250±250, respectively.

Introduction

The purified proteinase distributed in the intestinal organs of fish has not been well estabilished to its enzymatic properties which is a role not only in the digestion of feed but also in the activation of zymogens.

On the digestive enzymes of the intestinal organs of fish, Kubota and Ohnuma(1970) reported that two pepsin exist in the stomach of bonito and their optimum condition for activity were found to be pH 2.2, 40-45°C. Kimoto *et al*(1981) reported that molecular weights of the two acid proteinases from antarctic krill were shown to be 45,000 and 64,000. The enzymatic and physicochemical properties of the two acid proteinases from the stomach of sardine were ascertained, and they were similar to mammalian pepsin and microbial acid proteinses in their active site structure having two different carboxyl group(Noda and Murakami, 1981).

The purified carboxylesterases from the pyloric caeca(Kamoi *et al*, 1980) and liver(Suzuki *et al*, 1980) of young yellowtail were studied about optimum reaction condition and some physicochemical

properties. Carboxypeptidase A and B purified from the catfish pancreas appeared their maximum activity at pH 7.0 to 7.5, 40°C and were strongly inhibited by EDTA and o-phenanthroline (Yoshinaka *et al*, 1980 ; 1985).

Ooshiro(1971a, 1971b, 1971c) investigated some properties and substrate specificity of purified proteinase from the pyloric caeca of mackerel. Three alkaline proteinases from the pyloric caeca of sardine belonged to a group of serine protease were distinctly different in their optimum pH, pH stability, net charge and immunological properties (Murakami and Noda, 1981). Uchida *et al*(1984) reported that two anionic trypsin from the pyloric caeca of chum salmon were similer to those of mammalian trypsin.

In the preceding paper(Pyeun and Kim, 1986), we reported the purification precedure of the three alkaline proteinases from the pyloric caeca of mackerel. The present paper describes enzymatic properties of the three purified alkaline proteinases.

Materials and Methods

1. Materials

The three alkaline proteinases(Enz. A, B and C) used in this experiment were isolated from the pyloric caeca of mackerel as described previously (Pyeun and Kim, 1986).

2. Methods

1) Preparation of buffer and casein solution

Preparation of buffer and casein substrate solution were followed to the paper of Pyeun *et al*(1986).

2) Preparation of hemoglobin(Hb) substrate

Two point two grams of Hb was placed a 100 *ml* of volumetric flask and the volume was filled with distilled water in a half. Thirty six grams of urea and 8 *ml* of 1 *M* NaOH were added to the flask. The solution was made up to 100 *ml* with distilled water. The alkaline solution was stood at room temperature for 30-60 min to be denatured Hb. The solution was mixed with 10 *ml* of 1 *M* potassium dihydrogen phosphate to bring the pH of solution to be 7.2. Four grams of urea was added to make the final concentration of solution become 8 *M*. After 2 *mg* of sodium azide was added, the solution was kept in a refrigerator for a week.

3) Enzyme reaction

Proteolytic activity was determined by the modified Anson's method(1938).

4) Determination of protein concentration

Protein concentration was determined spectrophotometrically according to the method of Lowry *et al* (1951).

5) Determination of molecular weight

Molecular weight of the three purified alkaline proteinases was determined by Sephadex G-100 gel filtration according to the methed of Andrews (1964) and by SDS-polyacrylamide gel electrophoresis with the method of Weber and Osborn(1969).

Results and Discussions

1. Effect of pH on the hydrolysis of casein and hemoglobin

Effect of pH on the hydrolysis of casein by the three alkaline proteinases from the pyloric caeca of mackerel was experimented in the pH range from 5.0 to 11.0 at 40°C for 30 min incubation. As shown in Fig. 1(A), the optimum pH for Enz. A, B and C was found to be pH 9.4, 9.8 and 9.8, respectively. The activity of the three enzymes at pH 7.0 and 11.0 was considerably decreased on the comparison to the crude enzyme(Pyeun and Kim, 1986). The specific activity of the Enz. B was stronger than that of Enz. A and C.

The three alkaline proteinases from the pyloric caeca of sardine were the most active at pH 10.0 against casein as a substrate(Murakami and Noda, 1981). Tajima *et al*(1984) reported that the purified extracellular protease of *Aeromonas salmonicida*, Ar-4 showed maximum activity at pH 9.4. The reports quoted above are similar to the result of present experiment. The optimum condition for

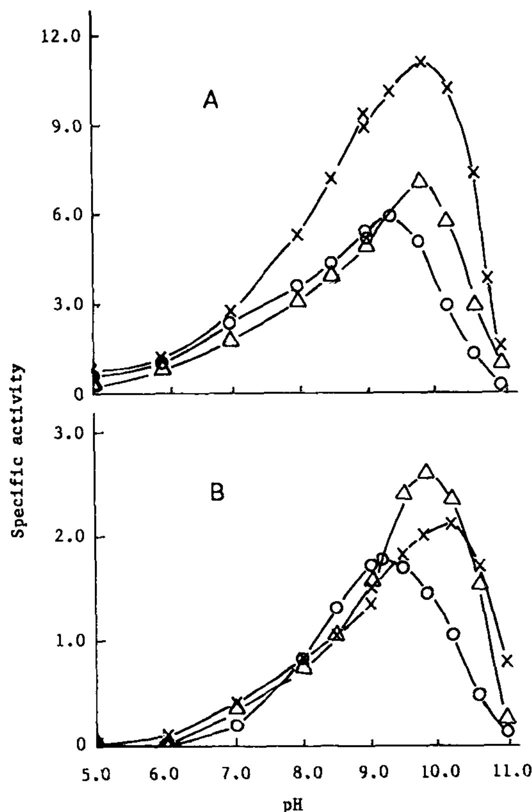

Fig. 1. Effect of pH on the hydrolsis of casein(A) and Hb(B) by the three alkaline proteinases. Buffer used was 01 M citrate-0.2 M Na_2HPO_4 (pH 5.0−7.0), 0.1 M Tris-HCl(pH 7.0−9.0) and 0.1 M Na_2CO_3-$NaHCO_3$ (pH 9.0−11.0). The symbols are as follows : ○, Enz. A; ×, Enz. B; △, Enz. C.

the tryptic hydrolysis on L-LPA was in the range of pH 9.0-9.5, that of DL-BAPA was at pH 8.0, furthermore the maximum activity for the chymotryptic hydrolysis of GPNA and SPNA appeared at pH 7.5(Erlanger *et al*, 1961 ; Erlanger *et al*, 1966). Eight proteolytic enzymes from the digestive juice of octopus were shown maximum activity for casein to be pH 7.0-8.5, and for BETT and ATEA to be pH 7.5-8.5(Morishita *et al*, 1978). Three proteases from the antarctic krill for casein appeared the most active at pH 7.6, but for TAME showed the most active at pH 8.0(Seki *et al*, 1977). Makinodan *et al*(1969) and Iwata *et al*(1969, 1973) also reported alkaline proteinase existed in the carp muscle had maximum activity at pH 8.0. With the reports adduced above, alkaline proteinases from the muscle of marine vertebrates and that from the intestine of marine mollusca were shown optimum activity near pH 8.0. However alkaline proteinases from the internal organs of the marine vertebrates were shown the optimum activity at pH 9.0-11.0. This difference of optimum pH was resulted from the difference of the species and origin of proteinase separated.

The effect of pH on the hydrolysis of Hb by the three alkaline proteinases from the pyloric caeca of mackerel was examined in the pH range from 5.0 to 11.0 at 40°C for 30 min incubation. As displayed in Fig. 1(B), the optimum pH for Enz. A, B and C was found to be pH 9.2, 10.2 and 9.8, respectively. The three enzymes showed considerably low activity at pH 7.0 and 11.0. The specific activity of Enz. C was stronger than that of Enz. A and B. Generally, proteolytic activity for Hb in this experiment shown one fourth for casein. Some difference appeared to the comparison with activity for casein. Casein is usually used as a sudstrate for the determination of alkaline proteinase activity, because it is more reactive than Hb at neutral and alkaline pH range.

The purified cathepsin D from the porcine spleen for Hb hydrolysis was the most active at pH 3.4 to 3.8, optimum pH was shifted to pH 3.0 when the ionic strength of buffer was increased(Cunningham *et al*, 1976). Ito and Saito(1963) reported that the activity of proteolytic enzyme from the liver of king crab for casein and Hb was the strongest at pH 6.8, and the activity for Hb was 60% of that for casein. Proteolytic activity from the liver of cuttle fish for the hydrolysis of casein or Hb was shown differently, the activity for Hb was 1.7 times stronger than that for casein(Takahashi, 1963). Makinodan *et al*(1982) reported that carp muscle cathepsin D was the most active at pH 2.6-2.8 in 0.6 *M* glycine buffer or at pH 3.2 in 0.12 *M* formate buffer. Makinodan and co-worker ascertained that the hydrolysis of myofibril was the most active at pH 3.0-4.0, furthermore that of casein was at pH 5.0-5.2. With the reports cited above, the optimum pH of proteinases might be also influenced with the kinds of substrates and ionic strength

of buffer solution.

2. Effect of temperature on the hydrolysis of casein and Hb

The effect of temperature on the hydrolysis of casein was investigated by the three proteinases at various temperature over the range of 20°C and 60°C at optimum pH. As figured in Fig. 2(A), three enzymes were the most active at 45°C. The specific activity of Enz. A, B and C at 45°C was 7.1, 12.6 and 9.6 Activity(μmole-Tyr. eq./*mg*-protein/min), respectively. The activities of Enz. A, B and C at

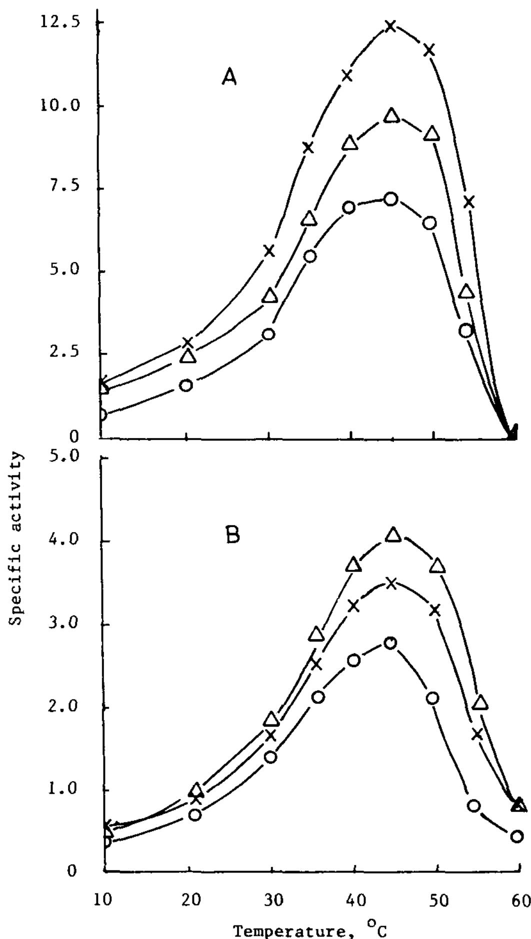

Fig. 2. Effect of temperature on the hydrolysis of casein(A) and Hb(B) by the three alkaline proteinases. Buffer used was 0.1 *M* $NaHCO_3$-Na_2CO_3. The symbols are the same as in Fig. 1.

50°C were decreased to 35, 45 and 55%, respectively, over the maximum activities. These suggest a possibility that these enzymes make allowance for the autolysis of mackerel on ambient temperature distribution.

Three alkaline proteinases from the pyloric caeca of sardine were the most active at 45°C and pH 10.0(Murakami and Noda, 1981), being the same as the result of present study. The extracellular protease produced by *Pseudomonas* 101 isolated from the viscera of sardine showed the maiximum activity at 35°C and pH 7.0(Cho, 1985). Hara *et al* (1984) investigated the effect of temperature of two alkaline protease isolated in the rotifer, *Brachionus plicatilis*, on the hydrolysis of casein. Hara and coworker reported that two enzymes showed maximum activity at 37°C. Makinodan and Ikeda (1977) reported that alkaline proteinase was shifted toward the low temperature side with increment of urea concentration. As the reports described above, optimum temperature of alkaline proteinase from muscle was higher than that from internal organs or that produced by microorganism. The optimum temperature of digestive enzyme was theoretically presumed near the body temperature because that in internal organs is concerned with digestion of diets intaken, but almost all of enzymes have optimum temperature higher than body temperature (Iwata *et al*, 1973; Murakami and Noda, 1981; Ooshiro, 1968; Morishita, 1978). In this experiment, temperature condition for the reaction of purified enzymes was sharper than that of crude enzyme (Pyeun and Kim, 1986). The difference was generated from other proteinases and protein coexisted in crude enzyme solution. Therefore the three alkaline proteinases were protected against thermal denaturation(Dixon and Webb, 1979; Makinodan and Ikeda, 1969).

The effect of temperature on the hydrolysis of hemoglobin was investigated by the three alkaline proteinases at various temperature over the range of 20°C and 60°C at optimum pH for 30 min incubation. As displayed in Fig. 2(B), the three alkaline proteinases were found to be the most active at 45°C, The specific activities of Enz. A, B and C at 45°C

were 2.3, 3.6 and 4.1 Activity(μmole-Tyr. eq./*mg*-protein/min), respectively. The activities of Enz. A, B and C at 60°C were exhibited 13, 19 and 16% of maximum activity, respectively. The specific activity of Enz. B for the hydrolysis of casein was the strongest, while that ef Enz. C for the hydrolysis of hemoglobin was the strongest at optimum reaction condition.

3. Effect of reaction time on enzyme activity

The rate for the hydrolsis of casein was studied with various reaction time by the three alkaline proteinases. The activity(Y) of Enz. A, B and C were direcly increased with reaction time(X)(Fig. 3). The reaction rate of Enz. A, B and C was consistent with the reaction time to 40 min. After the limited reaction time, reaction rate was slowly decreased. The reaction rate equation of Enz. A, B and C was obtained as $Y=3.6X$, $Y=6.0X$ and $Y=4.2X$, respectively, in the reaction condition of 2 μ*g*/*ml* of enzyme concentration and 2% casein sulotion as a substrate.

The incubation mixture which contained 0.4 *mg* of enzyme and 1.8 μmole of substrate in 10 *ml* of 0.1 *M* sodium citrate buffer(pH 2.0) was incubated at 37°C, activiy of human gastricsin and pepsin for the hydrolysis of Z-L-tosyl-L-serine was directly proportional to 6 hour of reaction time(Huang and Tang, 1969). Ooshiro(1971b) reported that alkaline proteinase in the pyloric caeca of mackerel was examined for the hydrolysis of chloroacetyl-DL-methionine, chloroacetyl-DL-leuine and chloroacetyl-L-leucine with reaction time. The release of fluorescent products by lysosomal cathepsin D on fluorescent-hemoglobin as a substrate was linear up to 30 minutes of incubation time(De Lumen and Tapple, 1970). The progress of proteolysis with reaction time was tested by incubating with 5% casein solution and extracts of *Cucumis trigonus roxb* at 4°C, non-protein nitrogen was increased proportionally up 40~45 min(Hujjatullah and A.K.B., 1970). Hippuryl-L-arginine hydrolysis by the carboxy peptidase B of the porcine pancreas was directly proportional to the enzyme concentration within 10 min(Folk *et al*, 1960). I'st reaction rate was obtained from much lower enzyme in comparison with substrate concentration. According to the progress of reaction, substrate concentration in system was decreased. Therefore the reaction rate was decreased with reaction time(Dixon and Webb, 1979; Lin, *et al*, 1969).

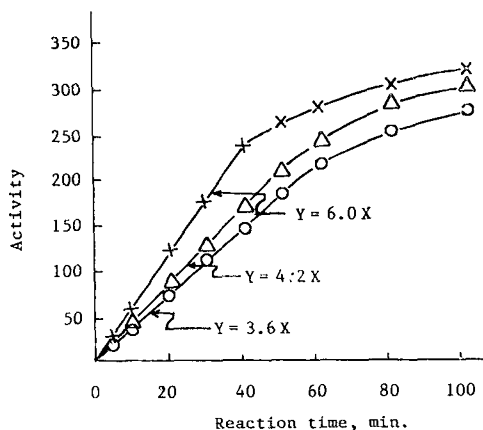

Fig. 3. Effect of reaction time on the hydrolysis of casein by the three alkaline proteinases. The reaction mixture was consisted of 1 μ*g* of enzyme, 0.5 *ml* of 2% casein solution and 2.5 *ml* of 0.1 M $NaHCO_3$-Na_2CO_3, pH 9.4(A) and pH 9.8(B, C). The symbols are the same as in Fig. 1.

4. Thermal stability of the three alkaline proteinases

The three alkaline proteinases were heated in the water bath on the range of 30°C and 60°C for 5 min. The residual activities were determined at optimum reaction condition after heat treatment. The activities of the three enzymes were not changed by treatment until 45°C as displayed in Fig. 4. Enz. A, B and C by heat treatment at 50°C were inactivated 90, 65 and 65%, respectively, over the original activity. The three enzymes were completely inactivated by heat treatment at 60°C for 5 min.

Proteolytic activity of the alkaline proteinase from white croaker and barracuda was lost 95 and 57% of the original activity by heat treatment at 60°C, but that from carp muscle was not influenced(Iwata *et al*, 1974a). Bodwell and Meyer(1981) reported

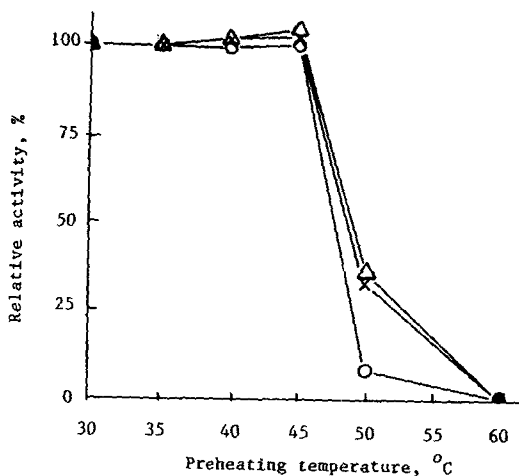

Fig. 4. Thermal stability of the three alkaline proteinases. Enyme solution was preincubated for 5 min at various temperature. After preincubation, the residual activity was determined at the optimum reaction condition.

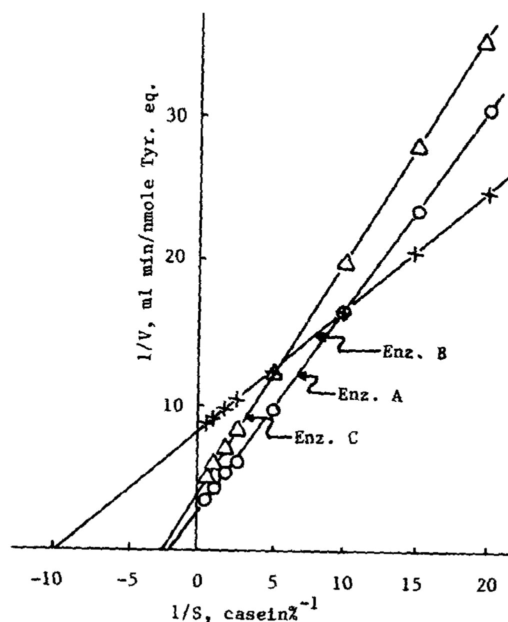

Fig. 5. Lineweaver-Burk plots for the hydrolysis of casein by the three alkaline proteinases. Km value : Enz. A, 5.0×10^{-3}%; Enz. B, 1.0×10^{-3}%; Enz. C, 3.6×10^{-3}%.

that carboxypeptidase A from rat skeletal muscle was substantially more heat sensitive than its pancreatic counterpart. Su and Lanier(1981) investigated the thermal stability of the alkaline proteinase from the various tissue such as skin, muscle, alimentary canal and kidney in chum salmon, and reported that proteinase from skin and muscle remained fairly stable by heat treatment at 60°C for 20 min, but that from alimentary canal and kidney showed unstable. Uchida *et al*(1984a) reported that two anionic tryptins isolated from the pyloric caeca of chum salmon were stable by treatment at 35°C and activities were decreased the with rise of temperature of heat treatment. With the reports cited above, most of the alkaline proteinase in fish muscle was stable by heat treatment up to 60°C, whereas digestive enzyme in internal organs was stable up to 35~45°C.

5. Affinity against casein substrate

The Km value of the three alkaline proteinases for casein as a substrate is determined by the method of Lineweaver and Burk. As shown in Fig. 5, the Km value of Enz. A, B and C was found to be 5.0×10^{-3}, 1.0×10^{-3} and 3.6×10^{-3}%, respectively. These result was coincided with Fig. 3.

Hara *et al*(1984) reported that the Km values using casein and BAPNA as substrate were determined with alkaline proteinases of rotifer, the Km values of the two proteinases for casein and BAPNA were considerably different. The kinetic constant obtained with the carboxypeptidase A_1 of porcine pancreas for several peptide substrates and ester substrate was reported(Fork and Schirmer, 1963).

6. Effect of metal ions on enzyme activity

The effect of metal ions on the enzyme activity has been investigated with the three alkaline proteinases. As shown in Table 1, the activities of the three enzymes were significantly decreased with the addition of Ag^{+}, Hg^{2+} and Ni^{2+}. The activities of Enz. B and C decreased with the addition of Cu^{2+}, while the activity of Enz. A was increased. and Enz. C was moderately inactivated. The three enzymes were activated with the addition of Mn^{2+} and slightly activated with the addition of Sn^{2+} and Pb^{2+}. The

Table 1. Effect of metal ions on the activity of Enz. A, B and C

Metal ion	Conc. (*mM*)	Relative activity(%)		
		Enz. A	Enz. B	Enz. C
None(control)		100	100	100
K^+	3	132	100	93
Na^+	3	124	95	151
Ag^+	3	23	33	22
Ba^{2+}	3	116	101	92
Li^+	3	130	93	97
Cd^{2+}	3	92	96	98
Mn^{2+}	3	190	260	270
Ca^{2+}	3	114	100	87
Co^{2+}	3	92	96	106
Mg^{2+}	3	126	95	93
Hg^{2+}	3	7	6	5
Zn^{2+}	3	87	91	22
Sn^{2+}	3	127	153	169
Ni^{2+}	3	86	50	47
Cu^{2+}	3	137	43	22
Pb^{2+}	3	122	155	131
Fe^{3+}	3	94	77	44

activity of Enz. A was inereased with K^+, Li^+ and Mg^{2+} and that of Enz. C was also increased with Na^+ and Co^{2+}. Na^+ activated Enz. A and C. This result is coincided with the result that NaCl solution was effective extracting solution shown in previous paper (Pyeun and Kim, 1986).

Perlmann and Lorand(1970) reported that heavy metal ions such as Cu^{2+}, Hg^{2+} and Ag^+ inhibit chymotrypsin activity. Yoshinaka *et al*(1981) studied *in vitro* activation of trypsinogen and chymotrypsinogen in the pancreas of catfish. Yoshinaka and co-workers reported that zymogens were activated in the presence of Ca^{2+} and Mn^{2+}, but inhibited with Zn^{2+}. Therfore Enz. B and C were presumed to be a serine protease. Kanai and Wakabayashi(1984) reported that the proteolytic activity of the protease produced by *Aeromonas hydrophila* was considerably inhibited by Ni^{2+}, Cu^{2+} and Cd^{2+}, whereas it was inhibited about 60% of its original activity by Hg^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+}.

7. Effect of chemical reagents on enzyme activity

The effect of inhibitors and activators on the enzyme activity was examined with the three alkaline proteinases. As displayed in Table. 2, those were neither inhibited nor activated by EDTA, monoiodoacetate, 1,4-dithiothreitol, mercaptoethanol and o-phenanthlorine. Only Enz. A was inhibited by cysteine and p-chloromercuribenzoate. Soybean trypsin inhibitor inhibited Enz. B and C. These result indicates that Enz. B and C are classified as a serine protease.

Murakami and Noda(1981) reported that two enzymes of three alkaline proteinases from the pyloric caeca of sardine were classified serine protease. The purified alkaline proteinase in carp muscle was found to be a serine protease(Iwata *et al*, 1974b). The purified extracellular protease of *Aeromonas salmonicida*(Tajima *et al*, 1984) and that of *Aeromonas hydrophila*(Kanai and Wakabayashi, 1984) were also found to be a serine protease.

8. Determination of molecular weight

SDS-polyacrylamide gel electrophoresis of the three alkaline proteinases showed a single band which indicated the homogeneity and the enzymes were

Table 2. Effect of various reagents on the activity of Enz A, B and C

Chemical reagents	Conc. (*mM*)	Relative activity(%)		
		Enz. A	Enz. B	Enz. C
None		100	100	100
Ethylendiamine tetraacetate	2	84	107	109
Iodoacetate	2	107	93	107
Cysteine	2	60	100	98
1,4-Dithiothreitol	2	103	96	101
o-Phenanthlorine	2	98	92	102
p-Chloromercuribenzoate	2	54	107	100
Soybean trypsin inhibitor	2	42	21	13

consisted of single polypeptide. Enzymes and marker protein(10 μg per column) were subjected to electrophoresis in 7.5% polyacrylamide gel in the persence of 0.1% SDS. Molecular weights of Enz. A, B and C were estimated to be 30,000, 22,000 and 15,500,

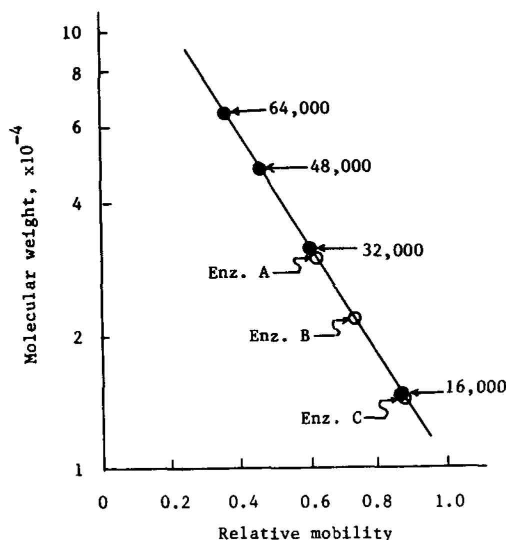

Fig. 6. Estimation of molecular weight of the three purified alkaline proteinases with SDS-polyacrylamide gel electrophoresis. Reference porotein was cross-linked hemoglobin,

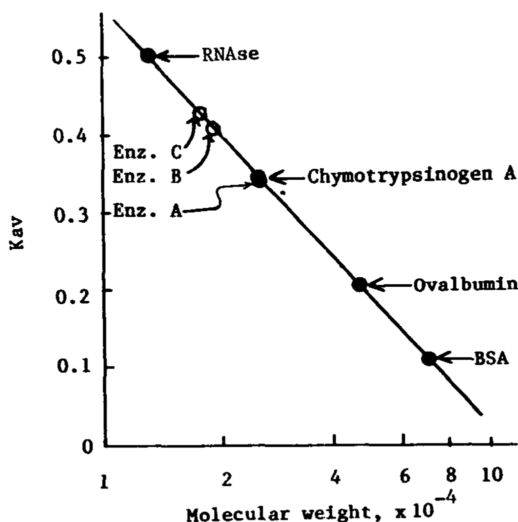

Fig. 7. Estimation of molecular weight of the three purified alkaline proteinases with Sephadex G-100 gel filtration.

respectively(Fig. 6). Relative mobility was defined as a ratio of mobility of protein to Bromophenol blue. The used marker protein was crosslinked hemoglobin(M. w. ; 64,000, 48,000, 32,000 and 16,000).

The molecular weights of the three alkaline proteinases were also determined by gel filtration on Sephadex G-100 column(2 x 95 *cm*). As shown in Fig. 7, molecular weights of Enz. A, B and C were estimated to be 25,000, 19,000 and 15,000, respectively. The used standard proteins were ribonuclease A(Mw. ; 13,700), α-chymotrypsinogen A(Mw. ; 25,000), ovalbumin(Mw. ; 43,000) and bovine serum albumin(Mw. ; 67,000).

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魚類의 臟器組織에 分布하는 蛋白質分解酵素에 관한 硏究

2. 고등어 幽門垂組織中에 分布하는 3種 알칼리性 蛋白質分解酵素의 特性

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前報(Pyeun and Kim, 1986)에서와 같이 고등어 幽門垂組織에서 精製한 3種의 알칼리性蛋白質分解酵素에 對하여 活性最適條件, 熱安定性·基質親和度, 化學藥劑에 대한 影響 및 分子量 등을 究明하였다. 各 精製酵素의 反應最適條件을 基質別로 檢討한 結果, casein에 대하여 Enz. A는 pH 9.4, Enz. B와 Enz. C는 pH 9.8이었으며, Hb에 대하여 Enz. A는 pH 9.2, Enz. B는 pH 10.2, 그리고 Enz. C는 pH 9.8이었고, 最適反應溫度는 共히 45°C였다.

酵素濃度 2 $\mu g/ml$, 2% casein 基質의 反應條件에서 反應時間(x)에 대한 活性度 (y)의 關係를 分析한 結果, Enz. A는 60分, Enz. B는 40分, 그리고 Enz. C는 50分까지 1次 反應의 關係가 成立하였으며, 이때의 反應速度式은 Enz. A는 $y=3.6x$, Enz. B는 $y=6.0x$, 그리고 Enz. C는 $y=4.2x$였다.

熱安定性을 檢討하기 위하여 50°C에서 5分間 加熱했을때, Enz. A는 90%, Enz. B는 33%, 그리고 Enz. C는 37%가 各各 不活性化하였다.

Lineweaver-Burk의 圖式에 의한 酵素의 基質親和度를 測定한 結果, casein基質에 대하여 Enz. A는 Km이 5.0×10^{-3}%, Enz. B는 Km이 1.0×10^{-3}%, 그리고 Enz. C는 Km이 3.6×10^{-3}%였다.
金屬 ion에 의한 影響을 檢討한 結果, Ag^{+}, Hg^{2+}는 酵素活性을 低下시켰으나, Mn^{2+}, Sn^{2+} 및 Pb^{2+} 이온은 活性을 增加시켰다.

Enz. B와 Enz. C는 soybean trypsin inhibitor에 의해 상당히 저해되었다. 따라서 酵素 B와 C는 serine 系 蛋白質分解酵素로 判斷되었다.

SDS-PAG 電氣泳動과 Sephadex G-100 겔 濾過法에 의하여 各 精製酵素의 分子量을 測定한 結果, Enz. A는 27,500±2.500, Enz. B는 20,500±1,500, 그리고 Enz. C는 15,250±250이었다.