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The Proteinase Distributed in the Intestinal Organs of Fish

1. Purification of the Three Alkaline Proteinases from the Pyloric Caeca of Mackerel, *Scomber japonicus*

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In the previous paper(Kim *et al*, 1986), the alkaline proteinase from the pyloric caeca of mackerel was shown relatively strong activity in the alkaline pH range.

Therefore purification of the enzyme has been undertaken to identify the proteolytic enzyme and three alkaline proteinases were isolated by ammonium sulfate fractionation, DEAE-Sephadex A-50 column chromatography and Sephadex G-100 gel filtration.

One percent sodium chloride solution was the most effective for the extraction of alkaline proteinase from the pyloric caeca of mackerel.

Three alkaline proteinases temporarily designated Enz. A, B and C were isolated from the pyloric caeca of mackerel, and identified to be homogeneous with electrophoresis.

The specific activity of the purified Enz. A, B and C was increased to 34, 53 and 37-fold over the crude enzyme solution, respectively. Yield of them was 1.6, 2.1 and 1.5%, respectively, and a combined yield was 5.2%.

Introduction

The pyloric caeca which develops as blind outgrowths at the anterior end of the mid-gut vary in number between 1 and more than 1,000. These particular organ in fish may be presented in either relatively free and short form or bound together to form a compact gland like mass. The information on digestion and absorption of nutrients at pyloric caeca has been reported intensively(Jansson and Olsson, 1960).

The activities of protease and carbohydrase of the pyloric caeca were stronger than those of stomach or alimentary canal(Kidamikado and Tachino, 1960). Among peptidase in pyloric caeca, a few of them have been classified as a trypsin-like enzyme by several investigators(Yoshinaka *et al*, 1983). The optimum pH of these proteinases was in alkaline pH range. Johnston(1941) reported the pyloric caeca of mackerel had the strongest peptidase activity of all the three species of hake and haddok.

Ooshiro(1971) reported that an alkaline proteinase from the pyloric caeca of mackerel was purified and most active at pH 9.0 and 43°C with casein as a substrate.

As stated above, the pyloric caeca of mackerel contains very strong peptidase. Thus mackerel, an active dark muscle fish, was occured to make allowance for the rapid autolysis, and chosen to be sample in this experiment. Three alkaline proteinases which make allowance for the autolysis of mackerel on distribution in ambient temperature are isolated with the present purification procedure

using ion-exchange column chromatography and gel filtration.

Materials and Methods

1. Materials

Mackerel, *Scomber japonicus*, (body length, 30-35 *cm*; body weight, 220-250 *g*) used in this study were harvested off the Cheju island coast on February, 1985. Lapsed time between capture of mackerel and separation of pyloric caeca was less than 20 hours with ice. The separated pyloric caeca was rinsed with cold distilled water for the elimination of filth. Samples were stored at −20°C for a month until used in this experiment.

All chemicals used in the present study were analytical reagent grade. Solutions were prepared with the distilled and deionized water.

2. Methods

1) Extraction of crude enzyme

Extracting procedure of crude enzyme from the pyloric caeca of mackerel was the same as described previously (Pyeun *et al*, 1986).

2) Preparation of buffer and substrate solution

Buffer and substrate solution were prepared the same as previous paper (Pyeun *et al*, 1986).

3) Enzyme reaction

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

4) Determination of protein concentration

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

Minced sample(Pyloric caeca)
- Extract with 4 vol. of 1% NaCl·1 *mM* EDTA
- Stand at 30°C for 3 hrs and centrifuge(14,000 x *g*, 30 min)

Supernatant — Precipitate(discard)
- Salt-out with powdered ammonium sulfate in the range of 30-70% saturation
- Stand for 3 hrs and centrifuge(14,000 x *g*, 30 min)

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

Supernatant — Precipitate(discard)
- Apply to a Sephadex G-100 column(3 x 100 *cm*) and elute with 0.1 *M* NaCl·0.01 *M* phosphate buffer, pH 6.75
- Pool and concentrate the active fraction in proteolysis

Concentrated effluent of proteolytic activity
- Apply to the 1'st DEAE-Sephadex A-50 column(3 x 50 *cm*) and elute with 0-0.2*M* NaCl·0.01*M* phosphate buffer, pH 7.0, by linear gradient
- Pool the active fraction in proteolysis and apply to the 2nd DEAE-Sephadex A-50 column and elute with stepwise
- Fractionate into the three proteinase active fractions
- Salt-out with powdered ammonium sulfate
- Centrifuge(14,000 x *g*, 30 min) and dissolve in 0.1 *M* NaCl·0.01 *M* phosphate buffer, pH 6.75
- Apply to the Sephadex G-100 column (2 x 95 *cm*) and elute with 0.1 *M* NaCl·0.01 *M* phosphate buffer, pH 6.75

Purified alkaline proteinases(named Enz. A. B and C)

Fig. 1. Scheme of purification for the three alkaline proteinases from the pyloric caeca of mackerel.

5) Purification of the three alkaline proteinase

Unless otherwise indicated, all operations were done at 4°C. The procedure of separation and purification of the three alkaline proteinases from the pyloric caeca of mackerel is shown in *Fig. 1*. Sixty six gram of powdered ammonium sulfate(A.S.) was gradually added with stirring to 30% saturation to the crude enzyme solution obtained from dialysis. The suspension was allowed to stand for 3 hours to be equilibrated, and then centrifuged at 14,000 x *g* for 30 minutes. The precipitate was discarded and the supernatant was supposed to be salting out, so 110 *gr* of powdered A.S. was added gradually with stirring to 70% saturation. The suspension was standed for overnight to be equilibrated, and then centrifuged at 14,000 x *g* for 30 min. The supernatant is discarded and the precipitate was dissolved in an appropriate volume of 0.1 *M* NaCl·0.01 *M* sodium phosphase buffer, pH 6.75. The fraction was dialyzed for overnight against the same buffer as above(A.S. fraction). The A.S. fraction was applied to a Sephadex G-100 column(3×100 *cm*) equilibrated with o.1 *M* NaCl·0.01 *M* sodium phosphate buffer, pH 6.75, and eluted with the same buffer. After the gel filtration through Sephadex G-100, active fraction in proteolysis was pooled(Sephadex G-100 fraction). The Sephadex G-100 fraction was applied to the 1'st DEAE-Sephadex A-50 column (3×50 *cm*) equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.0, and eluted with a 2,000 *ml* linear gradient ranging from 0 to 0.2 *M* sodium phosphase buffer, pH 7.0. The three alkaline proteinate designted Enz. A, B and C were frationated in a different concentration of salt solution. Each proteolytic fractions were pooled and concentrated by the addition of powdered A.S. and dialyzed against 0.01 *M* sodium phosphate buffer, pH 7.0, for overnight. The dialyzed solution was centrifuged at 14,000 x *g* for 30 min(1st DEAE-Sephadex A-50 fractions). These DEAE-Sephadex A-50 fractions were rechromatographed on the 2nd DEAE-Sephadex A-50 column(3×30 *cm*) equilibrated with sodium phosphate buffer as above and eluted stepwisely with 0.03 *M* and 0.05 *M* against Enz. A fraction, 0.07 *M* and 0.09 *M* against Enz. B, 0.09 *M* and 0.12 *M* against Enz. C in the same buffer. Proteolytic active fractions were pooled and concentrated. The suspensions were dialyzed against 0.1 *M* NaCl·0.01 *M* sodium phosphate buffer, pH 7.0, and centrifuged at 14,000 x *g* for 30 minutes(2'nd DEAE-Sephadex A-50 fractions). The 2'nd DEAE-Sephadex A-50 fractions were applied to a Sephadex G-100 column(2×95 *cm*) equilibrated with the same buffer as above. Proteinase fraction was pooled and concentrated with powdered A.S. and dialyzed against distilled water for overnight (Purified alkaline proteinases).

6) Electrophoresis

Disc-electrophoresis was carried out according to the method of Davis(1964).

Nondenaturing polyacrylamide gel electrophoresis was performed in a 7.5% polyacrylamide gel(0.6×10 *cm*). Separating gel is buffered with 20 *mM* Tris-HCl, pH 8.3, containing 0.1 *mM* dithiothreitol(DTT). Stacking gel(3%) was prepared with an acrylamide to bis(acrylamide) ratio of 4:1 and buffered with 62.5 *mM* Tris-HCl, pH 6.8. Stacking gel was also contained 0.1 *mM* DTT. Electrophoresis was performed at 4 *mA* per column at 4°C in 25 *mM* Tris-glycine buffer, pH 8.3, containing 0.1 *mM* DTT. Electrophoresis was completed in approximately 5 hours. Prior to electrophoresis, samples were dialyzed against 62.5 *mM* Tris-HCl, pH 6.8, at 4°C containing 0.5 *mM* DTT. Proteinase was extracted from 0.4 *cm* slices and homogenized in 1 *ml* of 10 *mM* sodium phosphate buffer, pH 7.0, containing 0.5 *mM* DTT at 4°C, Gel fragments were removed by centrifugation at 2,3000 x *g* and the extracts were assayed for the proteinase activity.

Results and Discussions

1. Preliminary experiments for enzyme purification

1) Effect of extracting solution on enzyme activity

The effect of extracting solution on the specific activity of the crude enzyme was investigated. The

extracting solution used for purpose was NaCl, KCl and sodium phosphate buffer solution containing 1 *mM* 2 Na-EDTA. The concentration of these solution is shown in Table 1. A reference enzyme solution was prepared with distilled water. The proteinase activity of sample solutions obtained with various extracting solution was determined at pH 9.4, 40°C for 30 minutes. Protein concentration was determined by Lowry method. The specific activity shown in Table 1 was calculated by dividing the activity with the protein concentration. The protein concentration and the activity of the crude enzyme were proportional to the increment with the concentration of NaCl, KCl and sodium phosphate. But the specific activity of the crude enzyme did not show any relationship with the concentration of salt. It is the reason of this result that much protein is extracted with high concentration of salt. The specific activity of the crude enzyme extracted with NaCl solution is generally higher than that with KCl and sodium phosphate buffer, especially the crude enzyme extracted with 1% NaCl solution showed the highest specific activity. From the result of this experiment, 1% NaCl solution was the most effective solvent. Therefore 1% NaCl was used as an extracting solution of the crude enzyme in this study.

Table 1. Caseinolytic activity of the extracts obtained by various extracting solution

Extracting solution	Protein conc. (*mg/ml*)	At pH 9.4	
		Activity	Specific activity
Distilled water	5.0	925	185
0.5% NaCl	5.2	936	180
1% NaCl	5.3	1060	200
2% NaCl	5.8	1120	190
0.5% KCl	5.2	939	181
1% KCl	5.3	1010	191
2% KCl	5.8	1044	180
1/60 M phosphate buffer (pH 7.0)	5.3	961	181
1/20 M phosphate buffer (pH 7.0)	6.1	1140	190
1/10 M phosphate buffer (pH 7.0)	6.2	1211	195

Morishita *et al*(1974) investigated caseinolytic activity of the extracts from the posterior salivary gland of octopus prepared with distilled water and various concentration of KCl, NaCl and phosphate buffer, pH 7.0, the crude enzyme prepared with 1% NaCl solution showed the highest specific activity in any other extracting solution. The extraction of proteinase from carp muscle might as well extracted by 2% KCl solution(Iwata *et al*, 1973) or 0.5% NaCl solution(Makinodan *et al*, 1969). Distilled water and various concentration of NaCl, KCl and 0.1 M NaOH-glycine buffer, pH 12.0, were used as an extracting solution of the crude alkaline proteinase from the digestive tracts of echiurid, and distilled water was the most effective extracting solution(Cho *et al*, 1984). These reports are similar to the result of this study. Since most of enzymes are water soluble protein, these enzye were readily extracted with distilled water or low ionic strength of extracting solution. In the extraction of enzyme, salt soluble proteins were increased with the ionic strength of solvent. Therfore specific activity of enzyme was low. In the case of enzyme extraction, low ionic strength of extracting solution might be proper to obtain enzyme solution of higher specific activity.

2) Effect of pH on enzyme activity

The effect of pH on the hydrolysis of casein was investigated by incubating proteinase from the pyloric caeca of mackerel at various pH over the range of 1.8 and 11.0 at 40°C for 30 min iscubation. As shown in Fig. 2, the crude enzyme was

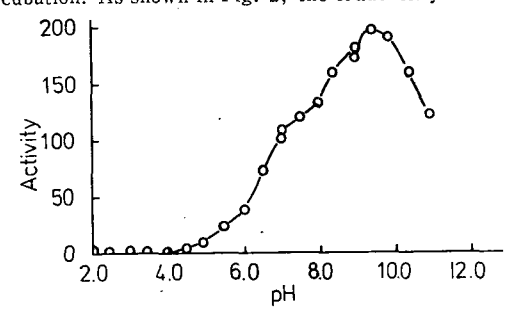

Fig. 2. Effect of pH on the hydrolysis of casein by the tissue extract from the pyloric caeca of mackerel. The reaction mixture was incubated at 40°C for 30 min.

found to be the most active at pH 9.4. The proteolytic activity near the ultimate pH in the post-mortem of fish was demonstrated one third of the maximum activity. Consequently, the optimum activity of the proteinase in the pyloric caeca of mackerel was found to be in the alkaline pH range, the determination of proteolytic activity accompanied with the following experiments of purification was performed at pH 9.4.

This result was similar to the reports of Uchida *et al*(1984), Murakami and Noda(1981), Onishi *et al*(1969) and Ooshiro(1968). The proteolytic activity of the crude enzyme extracted from the digestive tract of marine gastropods(Cho *et al*, 1983) and the optimum condition of tissue extracts from the digestive tract of marine mollusca(Pyeun *et al*, 1983) were reported that the crude enzyme from the digestive tract of marine animals showed the proteolytic activity at acid, neutral and alkaline range. Morishita *et al*(1974) reported the proteolytic enzymes in the posterior salivary gland for casein hydrolysis at 37°C for 20 min incubation showed maximum activity at pH 7.0, and for CGP hydrolysis did maximum activity at pH 8.1. These reports mentioned above were different with present result. The proteolytic enzymes distributed in the internal organs of marine vertebrate, of which internal organs are distinct are generally simillar to those of land vertebrate, such enzymes as pepsin-like in stomach(Noda and Murakami, 1981; Kimoto *et al*, 1981), trypsin- and chymotrypsin-like in intestine, pancreas and pyloric caeca(Uchida *et al*, 1984; Murakami and Noda, 1981; Yoshinaka *et al*, 1983; Perlman and Lorand, 1970).

3) Effect of temperature on enzyme activity

The effect of temperature on the hydrolysis of casein was investigated by incubating the enzyme from the pyloric caeca of mackerel at various temperature over the range of 20°C and 70°C at pH 9.4 for 30 minutes incubation. As shown in Fig. 3, the crude enzyme was found to be the most active from 45°C to 50°C. The activity at 20°C showed 40% of the maximum activity. This result suggests a possibility to the enzyme distributed in the pyloric caeca to make allowance for the autolysis of mackerel

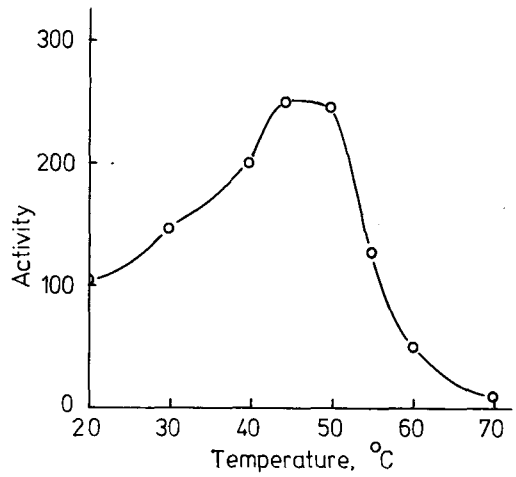

Fig. 3. Effect of temperature on the hydrolysis of casein by the tissue extract from the pyloric caeca of mackerel. Used buffer was 0.1 *M* $NaHCO_3$-Na_2CO_3, pH 9.4.

on an ambient temperature distribution. The activity was rapidly fallen down over 50°C and inactivated at 70°C.

The caseinolytic activity of the crude extracts from krill, shrimp and mysis was shown maximum activity at 50°C, 55°C and 45°C, respectively(Konagaya, 1980). This report is similar to the present study. Noguchi et al(1976) also reported the purified protease of antarctic krill displayed the optimum temperature at 40°C. Iwata *et al*(1974) investigated that the optimum caseinolytic activity of the enzymes distributed in the white muscle of 4 species of fresh water fish, 21 species of marine fish, and 2 species of mammalian, and the internal organs of carp was found to be 60~65°C and that in internal organs was found to be 45~55°C. The activity of the alkaline proteinase from the muscle and internal organs of file fish, hag fish and cat shark was examined, the maximum activity of the alkaline proteinase in muscle of the 3 species of fish was found to be 60~65°C and that in liver, spleen, alimentary canal, kidney and pancreas was found to be 45~55°C(Nam *et al*, 1983). As the reports quoted above, maximum proteolytic activity in internal organs of the fish is found to be appeared at lower temperature than that in muscle. Therefore the determination of proteolytic

activity accompanied in the following experiments of purification was performed at pH 9.4 and 40°C.

2. Purification of three alkaline proteinases

A chromatogram of Sephadex G-100 gel filtration for salted out fraction with 30-70% saturation of $(NH_4)_2SO_4$ is shown in Fig. 4.

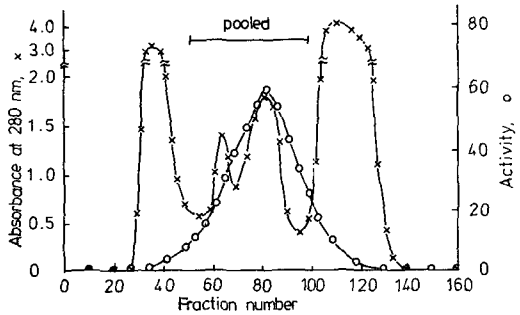

Fig. 4. Gel filtration of fraction with 30-70% saturation of $(NH_4)_2SO_4$ on Sephadex G-100 column(3×95*cm*). Contents in tube No. 52-100 were pooled and concentrated for applying DEAE-Sephadex A-50 chromatography.

The alkaline proteinase was eluted in tube number 52 to 100. The Sephadex G-100 fraction was applied to the 1'st DEAE-Sephadex A-50 column chromatography. Three alkaline proteinases, designated Enz. A, B and C, were isolated near the ionic strength of 0.053, 0.093 and 0.123, respectively(Fg. 5). With

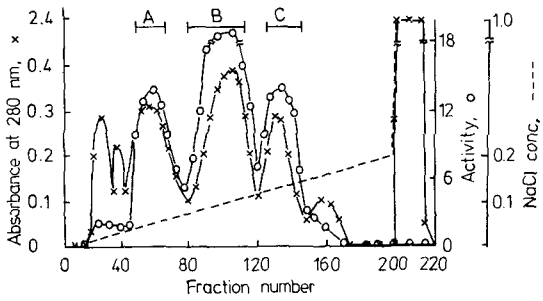

Fig. 5. 1'st DEAE-Sephadex A-50 chromatography of the Sephadex G-100 proteolytic fraction. The column(3×50 *cm*) was eluted with 0.01 *M* phosphate buffer, pH 7.0, and then with a 2000 *ml* linear gradient from 0 to 0.2 *M* NaCl in the same buffer. The flow rate was 40 *ml*/hr. and fraction volume was 10 *ml*.

this 1'st ion exchange chromatography, purity of Enz. A, B and C was increased to 16, 29 and 19 fold, respectively, over the crude enzyme solution. Each alkaline proteinase fractions(Enz. A, tube No. 50-70; Enz. B, tube No. 80-116; Enz. C, tube No. 127-147) were

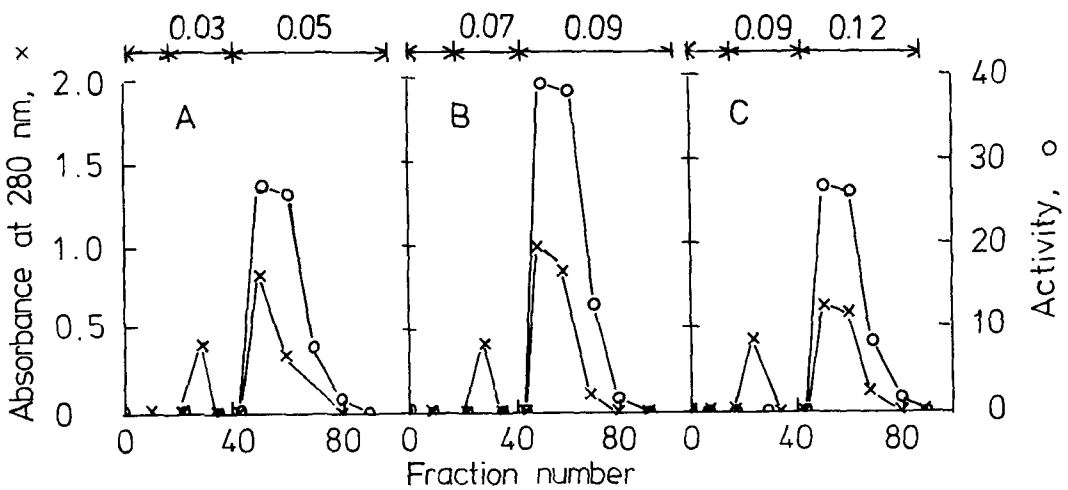

Fig. 6. 2'nd DEAE-Sephadex A-50 chromatography of the pooled fraction A, B and C obtained from 1'st DEAE-Sephadex A-50 chromatography. Elution was stepwise with 0.03, 0.05, 0.07, 0.09 and 0.12 *M* NaCl in the 0.01 *M* phosphate buffer, pH7.0. The flow rate was 40 *ml*/hr and each fraction volumn was 5 *ml*.

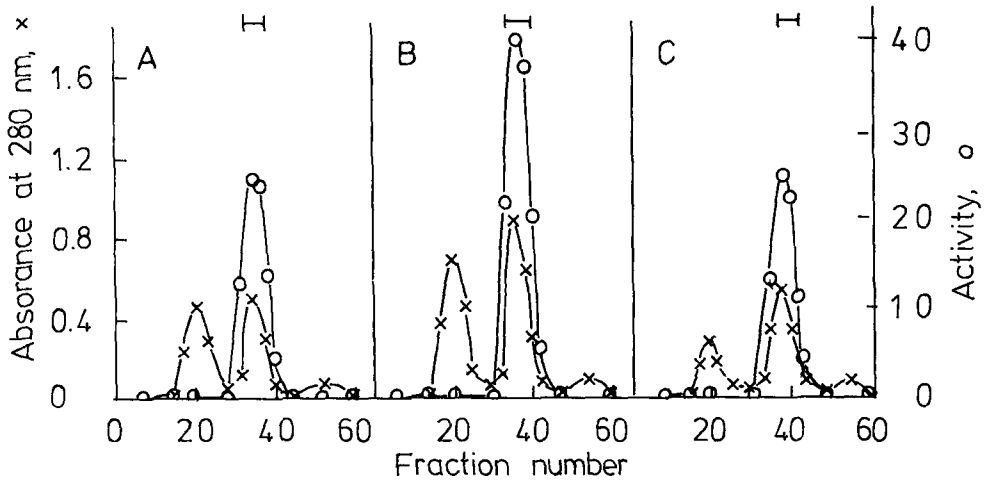

Fig. 7. Gel filtration of 2'nd DEAE-Sephadex A-50 fraction on a Sephadex G-100 column (2×95 *cm*). The column was eluted with 0.01 *M* phosphate buffer, pH 6.75, containing 0.1 *M* NaCl. The flow rate was 20 *ml*/hr. and fraction volume was 5 *ml*.

Table 2. Purification of the three alkaline proteinases, Enz. A, B and C

Fraction	Enz. A		Enz. B		Enz. C	
	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)
Crude extract	200	100	200	100	200	100
Ammonium sulfate fractionation (30-70%)	590	65	590	65	590	65
Sephadex G-100 gel filtration	1650	38.6	1650	38.6	1650	38.6
1'st DEAE-Sephadex A-50 chromatography	3240	5.2	5820	7.2	3580	4.8
2'nd DEAE-Sephadex A-50 chromatography	4850	3.8	8720	4.9	5360	3.6
Sephadex G-100 gel filtration	6720	1.6	10600	2.1	7400	1.5

applied to the 2'nd DEAE-Sephadex A-50 chromatography(Fig. 6). Enz. A, B and C were eluted with NaCl concentration of 0.05, 0.09 and 0.12 M, respectively. With these 2'nd ion-exchange chromatography, purity of Enz. A, B and C was increased to 24, 44 and 27, fold, respectively, over the crude enzyme solution. The fractions of Enz. A, B and C by 2'nd DEAE-Sephadex A-50 chromatography were applied to a Sehpadex G-100 column. The chromatograms were shown in Fig. 7. The proteins except the alkaline proteinases were removed through these gel filtration. With these gel filtration, purity of Enz A, B and C was increased to 34, 53 and 37 fold, respectively, over the crude enzyme solution. The outline and results of purification of the three alkaline proteinases are summarized in Table 2. With 100 *gr* of the pyloric caeca, 4.6 *mg* of Enz. A, 8.6 *mg* of Enz. B and 6.6 *mg* of Enz. C were isolated at a combined yield of 5.2% by the present purification procedure. These enzymes were purified to a 34-53 fold with this purification procedures. The final specimens after the Sephadex G-100 gel filtration were stored at −20°C and used for subsequent experiments.

3. Purity of the three alkaline proteinases

The purity of the three alkaline proteinases from the pyloric caeca of mackerel is illusterated visually at each purification steps, from the crude enzyme solution to the 2'nd DEAE-Sephadex A-50, with Disc-polyacrylamide gel clectrophoresis(Fig. 8). As shown in Fig. 8, the purity of the three alkaline proteinases was increased with the purification procedures.

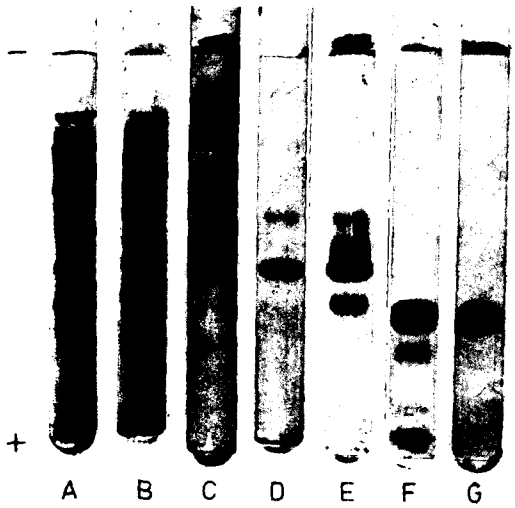

Fig. 8. Polyacrylamide gel electrophoresis patterns at various steps of purification. A, crude enzyme; B, Sephadex G-100 fraction; C, Enz. A of 1'st DEAE-Sephadex A-50 fraction; D, Enz. B of 1'st DEAE-Sephadex A-50 fraction; E, Enz. B of 2'nd DEAE-Sephadex A-50 fraction; F, Enz. C of 1'st DEAE-Sephadex A-50 fraction; G, Enz. C of 2'nd DEAE-Sephadex A-50 fraction.

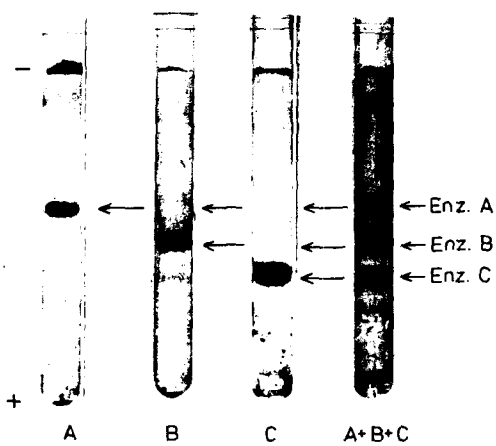

Fig. 9. Polyacrylamide gel electrophoresis of the three purified alkaline proteinases. The electrophoresis was performed at pH 8.3, 4 *mA* per tube, 25°C for 3hr in 0.6×10 *cm* columns of 7.5% polyacrylamide gel. A, B and C represented purified alkaline proteinases Enz. A, B and C, respectively. Protein bands were stained by Coomassie-brilliant blue R-250.

In order to check the purity of the three alkaline proteinases obtained from Sephadex G-100 gel filtration, Disc-polyacrylamide gel electrophoresis was performed at pH 8.3. As shown in Fig. 9, only a single band was appeared on the gel, accordingly, it indicates the homogeneity of the Enz. A, B and C.

Polyacrylamide gels developed Enz. C were sectioned with 4 *mm* in width. Alkaline proteinase was extracted and its proteolytic activity was determined. As shown in Fig. 10, the activity peak was only one and coincided with a protein band from other gels. So it is concluded that any other proteinases were not coexisted in the alkaline proteinase C.

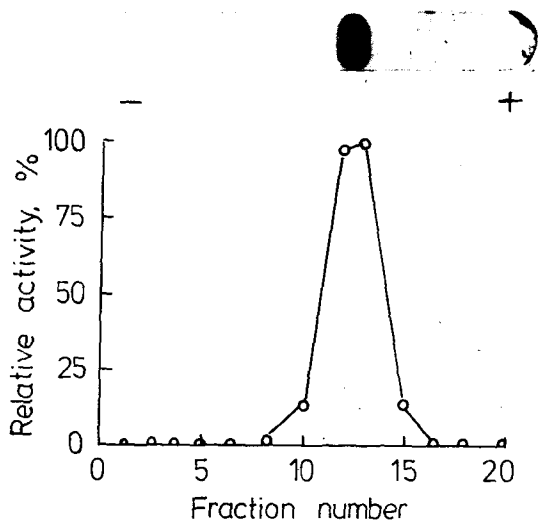

Fig. 10. Disc electrophoresis of the purified alkaline proteinase C. Graph demonstrated relative activity of the extracted enzyme from the unstained gels. Section number; number of sliced gel 4 *mm*.

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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

1. 고등어 幽門垂組織으로 부터 3種의 알칼리性 蛋白質分解酵素의 分離·精製

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고등어 幽門垂組織中에 分布하는 알칼리性蛋白質分解酵素의 精製條件을 檢討하고 鹽析, DEAE-Sephadex A-50 칼럼 크로마토그래피 및 Sephadex G-100 겔 濾過에 의해 3種類의 알칼리性蛋白質分解酵素의 分離·精製方法을 確立하였다.

幽門垂組織中에 分布하는 알칼리性蛋白質分解酵素의 抽出溶媒는 1% NaCl 溶液이 가장 效果的이었다.

精製된 假稱 Enz, A, B 및 C의 各 알칼리性蛋白質分解酵素는 電氣泳動的으로 均質함이 證明되었으며, 粗酵素에 比해 固有活性이 Enz. A는 34배, B는 53배 그리고 C는 37배로 各各 增加하였다. 各 酵素의 收率은 粗酵素에 對해 1.6%, 2.1% 및 1.5%였고, 總收率은 5.2%였다.