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Purification and Characterization of a Collagenase from the Mackerel, *Scomber japonicus*

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Collagenase from the internal organs of a mackerel was purified using acetone precipitation, ion-exchange chromatography on a DEAE-Sephadex A-50, gel filtration chromatography on a Sephadex G-100, ion-exchange chromatography on DEAE-Sephacel, and gel filtration chromatography on a Sephadex G-75 column. The molecular mass of the purified enzyme was estimated to be 14.8 kDa by gel filtration and SDS-PAGE. The purification and yield were 39.5-fold and 0.1% when compared to those in the starting-crude extract. The optimum pH and temperature for the enzyme activity were around pH 7.5 and 55°C, respectively. The K_m and V_{max} of the enzyme for collagen Type I were approximately 1.1 mM and 2,343 U, respectively. The purified enzyme was strongly inhibited by Hg^{2+}, Zn^{2+}, PMSF, TLCK, and the soybean-trypsin inhibitor.

Keywords: Collagenase, Enzyme characterization, *Scomber japonicus*

Introduction

About 100 million tons of fish are harvested yearly. Of the total catch, 30% is transformed into fishmeal (Receca *et al*., 1991; Kim *et al*., 1997). More than 50% of the remaining fish tissue is considered to be processing waste. This includes offals, fins, skin, internal organs, head, bone, and so on that are not used as food (Nair and Gopakumar, 1982). The mackerel is harvested in Korea exceeds 150,000 tons annually. The intestines are discarded after the fish-processing treatments.

Collagenases are generally defined as enzymes that are capable of degrading the polypeptide backbone. They are divided into two types that play different physiological functions. Serine collagenases are probably involved in the production of hormones and pharmacologically-active peptides, as well as in various cellular functions. These functions include protein digestion, blood-clotting, fibrinolysis, complement activation, and fertilization (Neurath, 1984; Bond and Van Wart, 1987). The collagenolytic-serine proteases have been reported from the following: filefish (Kim *et al*., 2002), fermented fish sauce (Nagano and To, 1999), greenshore crab (Roy *et al*., 1996), Kamchatka crab (Klimova *et al*., 1990; Sakharov and et Litvin, 1992), Atlantic cod (Kristjansson *et al*., 1995), insects (Lecroisey *et al*., 1987), Antarctic krill (Turkiewicz *et al*., 1991), shrimp (Lu *et al*., 1990; Chen *et al*., 1991; Van Wormhoudt *et al*., 1992), and catfish (Yoshinaka *et al*., 1986). The molecular weight range of the enzymes was 24-36 kDa (Roy *et al*., 1996).

On the other hand, metallocollagenases are zinc-containing enzymes that require calcium for stability (Stricklin *et al*., 1977). In addition, these metallocollagenases, extracellular enzymes, are involved in remodeling the extracellular matrix. Their molecular weights vary from 30 to 150 kDa. These enzymes have been widely studied from various mammalian tissues (Sellers and Murphy, 1981; Harris and Vater, 1982), as well as from bacteria (Bond and Van Wart, 1984; Matsuhita *et al*., 1994) and snake venom (Bjarnason and Fox, 1994).

In this paper, we report the purification of a collagenolytic enzyme from the internal organs of mackerel, *Scomber japonicus*, which are discarded after processing treatments, and present the characterization of the purified enzyme.

Materials and Methods

Materials The internal organs of a mackerel (*Scomber japonicus*) were donated from a local market (Busan, Korea), rinsed with deionized water in order to eliminate contaminants, and stored at –20°C until use. DEAE-Sephadex A-50, DEAE-Sephacel, Sephadex G-100, Sephadex G-75, N-P-toluenesulfonyl-L-lysine chloromethyl ketone (TPCK), 1,10-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), chymostatin, trans-epoxysuccinyl-L-leucylamido

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(4-guanidino) butane (E-64), cystatin, pepstatin A, elastatinal, soybean trypsin inhibitor, ethylenediamine tetraacetic acid (EDTA), collagenase (from *Clostridium histolytium*), collagen (type I, II and V from bovine Achilles tendon; type III from calf skin), reagents for electrophoresis, and protein molecular weight markers were purchased from the Sigma Chemical Co. (St. Louis, USA). All of the other reagents were of the highest grade available commercially.

Enzyme assay Collagenolytic activity was measured by the method of Moore and Stein (Moore and Stein, 1954) with a slight modification. A reaction mixture, which contained 5 mg of collagen type I, 1 ml of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and 0.1 ml of the enzyme solution, was typically incubated at 37°C for 1 h. The reaction was stopped by adding 0.2 ml of 50% trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at $1,800 \times g$ for 20 min. The supernatant (0.2 ml) was mixed with 1.0 ml of a ninhydrin solution, incubated at 100°C for 20 min, then cooled to room temperature. Subsequently, the mixture was diluted with 5 ml of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as the reference. The concentration of hydrolyzed-amino acids was determined by a standard curve that was based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1mmole of substrate per h.

Purification of collagenolytic enzyme Fresh internal organs were rinsed with chilled-deionized water, and homogenized twice at 12,000 rpm for 2 min with 2 volumes (w/v) of a 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$ (buffer A). The homogenate was centrifuged at $7,000 \times g$ for 20 min. After adjusting the supernatant to a 50% saturation by adding cold acetone, the mixture was precipitated at approximately 4°C for 6 h. The precipitate was centrifuged at $7,000 \times g$ for 10 min, and the precipitated protein was obtained. To remove the insoluble protein from the precipitated protein, the same volume of distilled water was added. The mixture was centrifuged at $9,500 \times g$ for 10 min. The supernatant was used as a crude collagenase-starting material.

The supernatant was put on a column (3×30 cm) of DEAE-Sephadex A-50 that was equilibrated with buffer A. The column was washed, then subsequently eluted with a linear NaCl gradient (0-1.5 M) in buffer A. The collagenolytic protease was collected and concentrated using a Sartorius ultrafiltration membrane system (molecular weight cut off, MWCO: 10 kDa, Goettingen, Germany). The concentrate was dialyzed against buffer A with several changes for 12 h. The dialyzate was put on a column (2×80 cm) of Sephadex G-100 that had been previously equilibrated with buffer A. The enzyme fraction was pooled and concentrated using the same ultrafiltration membrane system. The concentrate was further purified through a DEAE-Sephacel column (1×15 cm) that had been equilibrated with buffer A. The column was washed and subsequently eluted with the linear NaCl gradient (0-1.0 M) in buffer A. The collagenolytic protease was collected, dialyzed, and concentrated using a centrifugal filter (MWCO: 10,000, Millipore Co, Bedford, USA) at $5,000 \times g$ for 5 min. The concentrate was put on a column (2×80 cm) of Sephadex G-75 that had been previously equilibrated with buffer A. The active fraction was pooled. Afterwards, the enzyme solution was concentrated. It was stored at −20°C until use.

Protein determination The amount of protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. In addition, during the enzyme purification with column chromatography, the protein elution profile was monitored spectrometrically at the absorbance of 280 nm.

Molecular mass determination The molecular mass of the enzyme was estimated by gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The standard proteins in the gel filtration were albumin (66.0 kDa), carbonic anhydrogenase (29.0 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). SDS-PAGE was performed by the method of Laemmli (1970). The protein bands were stained with Coomassie Brilliant Blue R-250. The molecular mass standards were bovine ovalbumin (45.0 kDa), pepsin (34.7 kDa), trypsinogen (24.0 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

Effects of pH and temperature The influence of pH on the activity of the collagenase was determined as follows. The reaction mixture was incubated at 37°C for 1 h, as previously described. The buffers used were 50 mM citrate-Na_2HPO_4 (pH 9.0 and 10.0) that contained 5 mM $CaCl_2$. The enzymatic activity was measured by the previously described method (Moore and Stein, 1954). To test the influence of temperature on the activity of the collagenolytic enzyme, the enzyme in the 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$ was incubated in the reaction mixture at various temperatures (30-70°C) for 1 h. The remaining activity was measured as described previously.

Effects of various inhibitors and metal ions The sensitivity of the purified collagenase towards various inhibitors such as PMSF, TPCK, TLCK, elastatinal, E-64, cystatin, pepstatin, chymostatin, EDTA, 1,10-phenanthroline, and soybean trypsin inhibitor was investigated. The concentration of each inhibitor was 1.0 mM (PMSF, EDTA and 1,10-phenanthriline), 0.1 mM (elastatinal, E-64, pepstatin, chymostatin, TPCK and TLCK), and 0.01 mg/ml (cystatin and soybean trypsin inhibitor). Effects of metal ions on the enzyme were investigated by adding the monovalent (Li^+, Na^+ and K^+) and divalent metal ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Sn^{2+}, Hg^{2+} and Pb^{2+}) to the reaction mixture after dialyzing the purified-enzyme solution. The final concentration of each metal ion was 1 mM. The activity was compared with that of the reaction that was free of the corresponding metal ions.

Assay for substrate specificity To measure the digestion of native collagen types I, II, III and V, the reaction mixture that contained 5 mg of collagens of various types, 1 ml of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$, and 0.1 ml of the enzyme solution, was incubated at 55°C for 1 h. The collagenolytic activity was measured as described previously. In addition, after the hydrolysis of the types I, II, III, and V collagen by the purified collagenase, an aliquot of supernatant 20 μl was spotted onto a precoated thin silica gel plate (Merck Co., Darmstadt, Germany). The solvent that was used for the migration was 2-propanol: water (7:3, v/v). NH_2 residues were detected with ninhydrin.

Table 1. Collagenase activity and recoveries in the stages of purification

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Crude extract	57,399.1	855,096.0	16.5	100.0	1.0
Acetone precipitation	1,452.0	614,196.1	42.3	71.8	2.6
DEAE Sephadex A-50	81.1	153,171.0	189.1	17.9	11.5
Sephadex G-100	15.8	6,080.9	384.9	0.7	23.3
DEAE Sephacel	6.4	2,762.1	434.9	0.3	26.4
Sephadex G-75	1.2	776.2	652.1	0.1	39.5

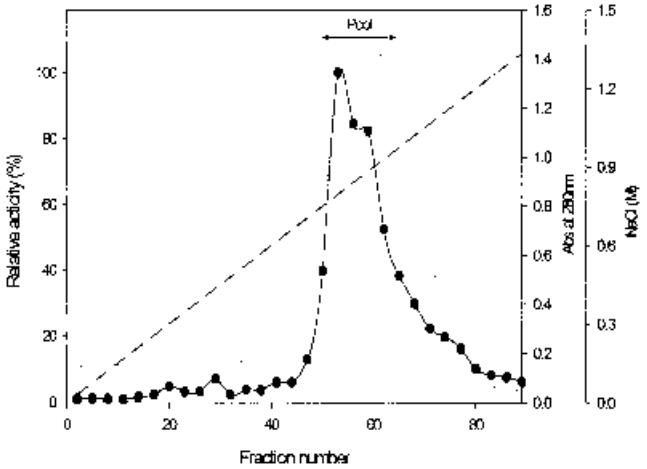

Fig. 1. Chromatogram of DEAE-Sephadex A-50 column (3 × 30 cm) chromatography of the acetone precipitate. The column was eluted with 600 ml of a 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$, then a linear gradient of 0-1.5 M NaCl in the same buffer. Flow rate and fraction volume were 0.5 ml/min and 5 ml, respectively.

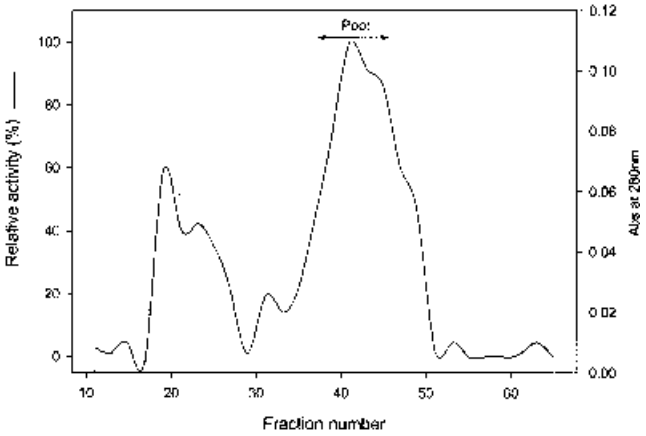

Fig. 2. Gel filtration with Sephadex G-100 from ion-exchange chromatographic fraction. The column (2 × 80 cm) was eluted with a 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$. Flow rate and fraction volume were 0.5 ml/min and 5 ml, respectively.

Assay for kinetic properties The apparent Michaelis-Menten constant (K_m) and the substrate turnover number (V_{max}) were calculated by a least-squares analysis from Lineweaver-Burk plots with 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ at 55°C with collagen type I as the substrate. The range of the substrate concentration that was used in all of the determinations was 1.0-10.0 mg/ml.

Results and Discussion

A collagenase was isolated from the internal organs of the mackerel, *Scomber japonicus*. The purification of the collagenolytic protease involved a five-step procedure. The procedure involved using acetone precipitation, ion-exchange chromatography on a DEAE-Sephadex A-50, gel filtration on a Sephadex G-100 column, ion-exchange rechromatography on a DEAE-Sephacel, and gel filtration rechromatography on a Sephadex G-75 in order to obtain electrophoretic homogeneity. Table 1 summarizes the results of the purification of the collagenase. The purification factor was about 39.5-fold with a yield of 0.1% from the crude extract. The specific activity of the purified collagenase was higher than those of the collagenase that was reported in the literature.

Acetone was added to the crude extract, and the precipitate was dissolved in a buffer solution. The solution was applied to a DEAE-Sephadex A-50 (Fig. 1). The active fractions were pooled and concentrated by ultrafiltration. The concentrate was put on a Sephadex G-100 column (Fig. 2), and the fractions that contained activity were pooled and concentrated in a similar way. Subsequently, the concentrate was subjected to the DEAE-Sephacel column (Fig. 3). The fractions that contained collagenolytic activity were pooled, concentrated, and dialyzed. Finally, the dialyzate was applied to gel filtration on a Sephadex G-75 column (Fig. 4). The active fraction was pooled, concentrated, and stored at −20°C until use.

The molecular mass of the purified enzyme was about 14.8 kDa by gel filtration and SDS-PAGE (Fig. 5). The molecular mass of the purified collagenase from various sources had been previously measured. The molecular mass of the mackerel collagenase was lower than that of other serine collagenase, such as filefish (*Novoden modestr*us, 27.0 and 42.0 kDa) (Kim and Kim, 1991; Kim *et al*., 2002), Atlantic cod (*cadus morhus*, 24.1 kDa) (Kristjansson *et al*., 1995),

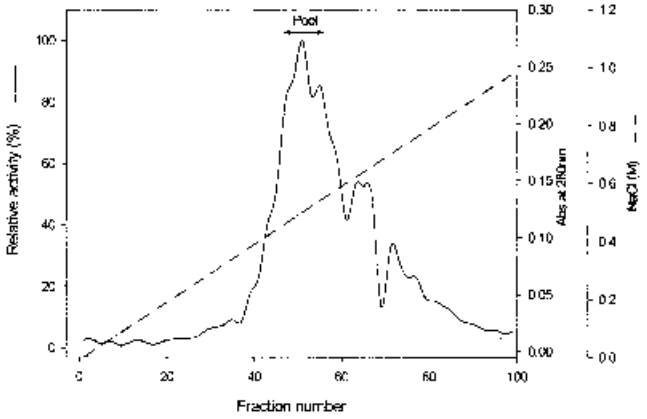

Fig. 3. Chromatogram of DEAE-Sephacel for the collagenolytic fraction pooled from the gel filtration chromatography on a Sephadex G-100 column. The column (1 × 15 cm) was eluted with 300 ml of a 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$, and a linear gradient of 0-1.0 M NaCl in the same buffer. Flow rate and fraction volume were 0.5 ml/min and 3 ml, respectively.

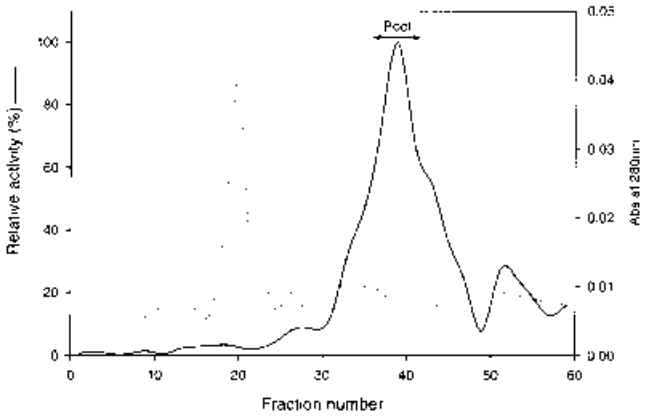

Fig. 4. Gel filtration with sephadex G-75 from ion-exchange rechromatographic fraction. The column (2 × 80 cm) was eluted with a 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM $CaCl_2$. Flow rate and fraction volume were 0.5 ml/min and 5 ml, respectively.

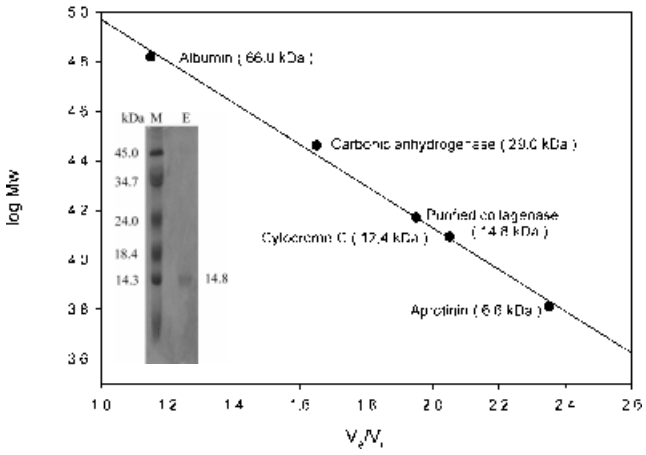

Fig. 5. (A) Molecular mass of purified collagenase from the internal organs of mackerel by gel filtration on a Sephadex G-75 column. Standard proteins in the gel filtration were albumin (66.0 kDa), carbonic anhydrogenase (29.0 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). (B) 15% SDS-polyacrylamide gel electrophoresis of the purified collagenase from the internal organs of mackerel. Lane 1, mark proteins; Lane 2, the purified collagenase. The protein gel bands were stained with Coomassie Brilliant Blue R-250. The standard proteins included bovine ovalbumin (45.0 kDa), pepsin (34.7 kDa), trypsinogen (24.0 kDa), â-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

greenshore crab (*Carcinus maenas*, 23.0 kDa) (Roy *et al*., 1996), shrimp (*Panaeus vannamei*, 25.0 kDa) (Van Wormhoudt *et al*., 1992), insects (*Hypoderma Lineatum*, 25.0 kDa) (Lecroisey *et al*., 1987), and crabs (*Paralithodes camtschatica*, 24.0-36.0 kDa) (Sakharov and et Litvin, 1992). These results suggest that the purified-collagenolytic enzyme shows a character-low molecular weight when compared to the known crustacean collagenase.

The pH dependence of the purified collagenase was investigated. The activity was measured at a pH range of 3.0-10.0. The optimum pH was found at pH 7.5 (data not shown). The results displayed a bell-shaped curve with an apparent maximum that was close to the pH of the greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996). A similar value was reported from the internal organs of filefish (*Novoden modestrus*) (Kim *et al*., 1997), *Paralithodes camtschatica* collagenase A (Klimova *et al*., 1990), and *Parahaliporus sibogae* collagenase (Muramatsu and Kariuchi, 1978). In addition, the effect of temperature was investigated (data not shown). The optimum temperature of the collagenolytic enzyme from mackerel was 55°C, which was the same optimum temperature that was reported for the internal organs of filefish, *Novoden modestrus* (Kim *et al*., 1997). This temperature was higher than that of the 30°C optimum temperature that was reported for Atlantic cod (*Cadus morhua*) (Kristjansson *et al*., 1995) and greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996). It was a lower temperature than that in the tissue of filefish, *Novoden modestrus* (60°C) (Kim *et al*., 1991).

The collagenolytic activity of the purified enzyme was inhibited by various reagents, such as PMSF, soybean trypsin inhibitor, chymostatin, and TLCK (Table 2). However, elastatinal, cystatin, EDTA, and 1,10-phenanthroline were not inhibited. In preliminary experiments, collagenolytic was inhibited by Mn^{2+}, Ni^{2+} ,Cu^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+} (Table 3). Generally, metallo-collagenase specifically requires zinc ion for optimum activity and stability. However, the purified collagenase was inhibited by Zn^{2+}. This suggests that the purified collagenase is a member of the serine protease family. The activity of this purified enzyme was not inhibited by 1,10-

Table 2. Effects of inhibitors on the activity of the purified collagenase

Inhibitor	Class	Concentration (mM)	Activity (%)
None	-	-	100.0
PMSF	Serine	1.0	53.2
Soybean trypsin inhibitor	″	0.01 mg/ml	3.8
Elastatinal	″	0.1	104.0
E-64	Cysteine	0.1	82.2
Cystatin	″	0.01 mg/ml	100.0
Pepstatin	Aspartic	0.1	84.6
Chymostatin	Serine/Cysteine	0.1	26.4
EDTA	Metallo-	1.0	100.0
1,10-Penanthroline	″	1.0	100.0
TPCK	Chymotrypsin-like	0.1	90.3
TLCK	Trypsin-like	0.1	3.7

Table 3. Effects of metal ions on the activity of the purified collagenase

Metal ion (1.0 mM)	Relative activity (%)
None	100
Li^{+}	105
Na^{+}	92
K^{+}	107
Mg^{2+}	109
Ca^{2+}	112
Mn^{2+}	21
Fe^{2+}	123
Ni^{2+}	65
Cu^{2+}	30
Zn^{2+}	17
Cd^{2+}	128
Sn^{2+}	142
Ba^{2+}	106
Hg^{2+}	22
Pb^{2+}	24

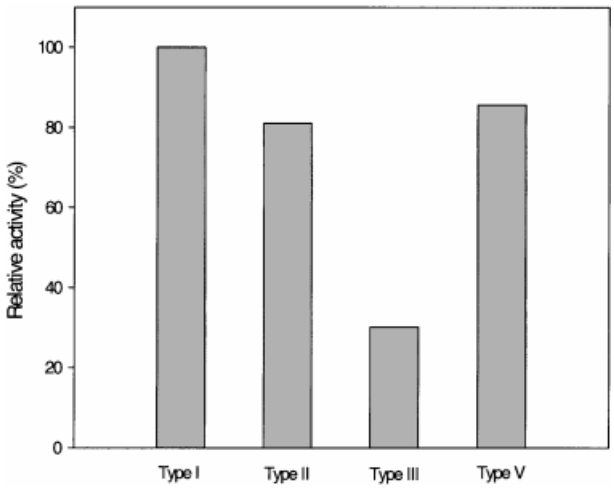

Fig. 6. Substrate specificity of the purified collagenase on various collagens. The collagens were incubated with the purified enzyme: substrate ratio (1 : 200) for 1 h at 55°C.

phenantroline and EDTA, which are inhibitors for metallo-collagenase. The soybean trypsin inhibitor and PMSF had a strong inhibitory activity on this enzyme. Therefore, these results suggest that the collgenolytic enzyme is a member of the serine protease family. It was reported that a serine collagenase family was purified from marine sources, such as greenshore crab (Roy *et al*., 1996; Sakharov and Litvin, 1992), shrimp (Van Wormhoudt *et al*., 1992), and fish (Kristjansson *et al*., 1995; Kim *et al*., 2002).

The mackerel collagenase cleaved the native collagen types I, II, III, and V (Fig. 6). The cleavage rate by the purified collagenase for the substrate was indicated as follows: type I> type V> type II> type III. In addition, Fig. 7 shows the cleavage patterns for various substrates by the purified collagenase. For collagen type I, the cleavage of α2 was observed under conditions when the enzyme-to-substrate ratio was 1 : 200 (w/w), suggesting multiple cleavage sites. In addition, the purified-enzyme catalyzed the hydrolysis of native collagen types II and III; therefore, the release of peptides that contained proline or hydroxyproline residues were stained in yellow on TLC after the ninhydrin vaporization. For the collagen type V, a more distinct cleavage pattern was observed. The β-dimer was fully cleaved; the α1 chain was also partially degraded. The mackerel protease was a collagenase because of its ability to cleave the collagen types I, II, III, and V.

The kinetic parameters of the purified collgenase were measured at pH 7.5 and 55°C using the insoluble collagen type I, as described previously. K_m and V_{max} of the enzyme were determined to be 1.1 mM and 2,343 U, respectively (Fig. 8). Our laboratory recently demonstrated the effectiveness of the fish collagenolytic enzyme as an aid in food processing (Kim *et al*., 1993).

In conclusion, we isolated and purified a new serine

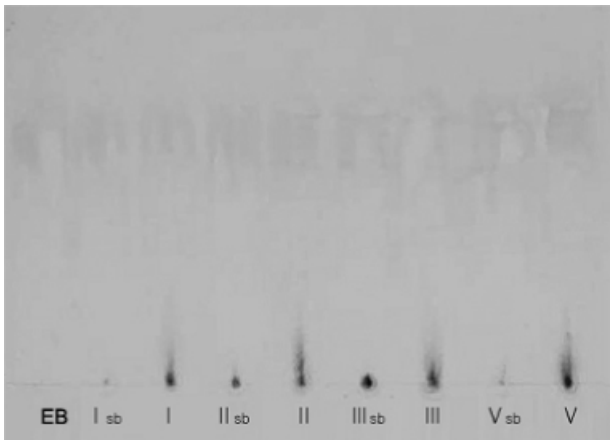

Fig. 7. Digestion of the native collagen types I, II, III, and V by mackerel collagenolytic proteinase. After hydrolysis of the types I, II, III, and V collagen by the purified collagenase, an aliquot of supernatant 20 µl was spotted onto a precoated thin silica gel plate. The solvent that was used for the migration was 2-propanol: water (7 : 3 v/v); NH_2 residues were detected with ninhydrin. EB, enzyme blank; I_{SB}, substrate blank of collagen type I; I, digestion pattern of collagen type I; II_{SB}, substrate bank of collagen type II; II, digestion pattern of collagen type II; III_{SB}, substrate blank of collagen type III; III, digestion pattern of collagen type III; V_{SB}, substrate blank of collagen type V; V, digestion pattern of collagen type.

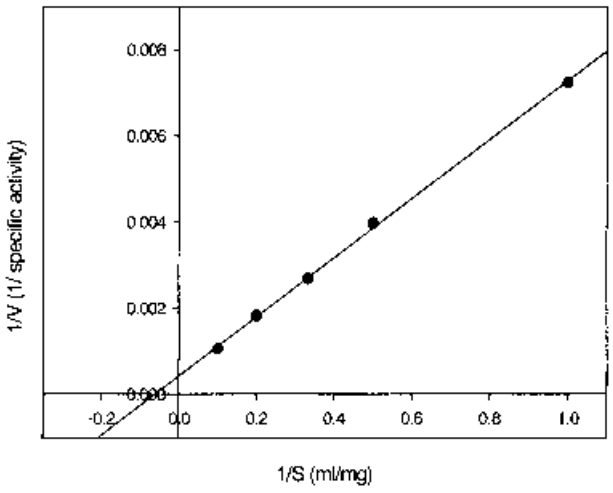

Fig. 8. Determination of kinetic parameters of the mackerel collagenase by Lineweaver-Burk plot. The reaction was carried out at 55°C for 1 h in 50 mM Tris-HCl, pH 7.5 that contained 5 mM $CaCl_2$ using collagen type I as the substrate. K_m and V_{max} of the purified mackerel collagenase were found to be 1.1 mM and 2,343 U.

collagenase with a low molecular weight from the internal organs of mackerel, *Scomber japonicus.* However, the identification of the partial amino acid sequence was not carried out, because the N-terminal chain of the purified collagenase was blocked. Therefore, we are presently investigating the information for the gene of the mackerel collagenase.

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References

Bjarnason, J. B. and Fox, J. W. (1994) Hemorrhagic methalloproteinases from snake venom. *Pharmacol. Ther.* **62**, 325-372.

Bond, M. D. and Van Wart, H. E. (1984) Characterization of the individual collagenases from *Clostridium histolyticum. Biochemistry* **23**, 3085-3091.

Bond, M. D. and Van Wart H. E. (1987) Purification and separation of individual collagenases of Clostridium histolyticum using red dye chromatography. *Biochemistry* **23**, 3077-3085.

Chen, Y. L., Lu, P. J. and Tsai, I. H. (1991) Collagenolytic activity of crustacean midgut serine proteases: comparison with the bacterial and mammalian enzymes. *Comp. Biochem. Physiol.* **100B**, 763-768.

Harris, E. D. and Vater, C. A. (1982) Vertebrate collagenases. *Methods Enzymol*. **82**, 423-452.

Hurion, N., Fromentin, H. and Keil, B. (1979) Specificity of the collagenolytic enzyme from the fungus *Entomophthora coronata*: comparison with the bacterial collagenase from *Achromobacter iophagus. Archs. Biochem. Biophys.* **192**, 438-445.

Kim, S. K., Byun, H. G., Choi, K. D., Roh, H. S., Lee, W. H. and Lee, E. H. (1993) Removal of skin from filefish using enzymes. *Bull. Korean Fish. Soc.* **26**, 159-172.

Kim, S. K., Jeon, Y. J., Byun, H. G., Kim, Y. T. and Lee, C. K. (1997) Enzymatic recovery of cod frame proteins with crude proteinase from tuna pyloric caeca. *Fisheries Science* **63**, 421-427.

Kim, S. K., Park, P. J., Kim, J. B. and Shahidi, F. (2002) Purification and characterization of a collagenolytic protease from filefish, *Novoden modestrus*. *J. Biochem. Mol. Biol.* **35**, 165-171.

Kim, Y. T. and Kim S. K. (1991) Purification and characterization of the collagenase from the tissue of filefish, *Novoden modestrus. Korean Biochem. J.* **24**, 401-409.

Klimova, O. A., Borukhov, S. I., Solovyeva, N. I., Balaevskaya, T. O. and Strongin, A. Y. (1990) The isolation and properties of collagenolytic proteases from crab hepatopancreas. *Biochem. Biophys. Res. Commun.* **166**, 1411-1420.

Kristjansson, M. M., Cudmundsdottir, S., Fox, J. W. and Bjarmason, J. B. (1995) Characterization of a collagenolytic serine proteinase from the Atlantic cod (*Gadus morhua*). *Comp. Biochem. Physiol*. **110B**, 707-717.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lecroisey, A., Gilles, A. M., De Wolf, A. and Keil, B. (1987) Complete amino acid sequence of the collagenase from the insect *Hypoderma lineatum. J. Biol. Chem.* **262**, 7546-7551.

Lowry, O. H., Rosebrough, A. L., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin-phenol reagent. *J.*

Biol. Chem. **193**, 165-175.

Lu, P. J., Liu, H. C. and Tsai, I. H. (1990) The midgut trypsins of shrimp (*Penaeus monodon*). High efficiency toward native protein substrates including collagens. *Biol. Chem. Hoppe-Seyler* **371**, 851-859.

Matsuhita, O., Yoshihara, K., Katayama, S. I., Minami, J. and Okabe, A. (1994) Purification and characterization of a *Clostridium perfringes* 120 kilo Dalton collagenase and nucleotide sequence of the corresponding gene. *J. Bacteriol.* **176**, 149-156.

Moore, S. and Stein, W. (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**, 907-913.

Muramatsu, T. and Kariuchi, H. (1978) Proteolytic activity of extracts from the hepatopancreas of shrimp, *Parahaliporus sibogae* (De Man). *Bull. Jap. Soc. Sci. Fish.* **44**, 171-174.

Nagano, H. and To, K. A. (1999) Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. *Biosci. Biotechnol. Biochem.* **63**, 181-183.

Nair, A. L. and Gopakumar, K. (1982) Soluble protein isolates from low cost fish and fish wastes. *Fishery Technol.* **19**, 101-103.

Neurath, H. (1984) Evolution of proteolytic enzymes. *Science* **224**, 350-357.

Receca, B. D., Pena-Vera, M. T. and Deaz-Castaneda, M. (1991) Production of fish protein hydrolysates with bacterial proteases; Yield and nutritional value. *J. Food Sci.* **56**, 309-314.

Roy, P., Colas, B. and Durand, P. (1996) Purification, Kinetical and molecular characterizations of a serine collagenolytic protease from greenshore crab (*Carcinus maenas*) digestive gland. *Comp. Biochem. Physiol.* **115B**, 87-95.

Sakharov, I. Y. and et Litvin, F. E. (1992) Substrate specificity of collagenolytic proteases from the hepatopancreas of the Kamchatka crab. *Biochem.* (*USSR*) **57**, 44-49.

Sellers, A. and Murphy, G. (1981) Collagenolytic enzymes and their naturally-occurring inhibitors. *Int. Rev. Connect. Tissue Res.* **9**, 15-190.

Stricklin, G. P., Bauer, E. A., Jeffrey, J. J. and Eisen, A. Z. (1977) Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. *Biochemistry* **16**, 1607-1615.

Turkiewicz, M., Galas, E. and Kalinowska, H. (1991) Collagenolytic serine proteinase from *Euphasia superba* Dana (Antarctic krill). *Comp. Biochem. Physiol.* **99B**, 359-371.

Van Wormhoudt, A., Le Chevalier, P. and Sellos, D. (1992) Purification, biochemical characterization and N-terminal sequence of a serine-protease with chymotrypsic and collagenolytic activities in a tropical shrimp, *Penaeus vannamei (Crustacea, Decapoda). Comp. Biochem. Physiol.* **103B,** 675-680.

Yoshinaka, R., Sato, M., Itoko, M., Yamashita, M. and Ikeda, S. (1986) Purification and characterization of a collagenolytic serine proteinase from the catfish pancreas. *J. Biochem.* **99**, 459-467.