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Purification and Characterization of a Collagenolytic Protease from the Filefish, *Novoden modestrus*

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A serine collagenolytic protease was purified from the internal organs of filefish, *Novoden modestrus*, by ammonium sulfate, ion-exchange chromatography on a DEAE-Sephadex A-50, ion-exchange rechromatography on a DEAE-Sephadex A-50, and gel filtration on a Sephadex G-150 column. The molecular mass of the filefish serine collagenase was estimated to be 27.0 kDa by gel filtration and SDS-PAGE. The purified collagenase was optimally active at pH 7.0-8.0 and 55°C. The purified enzyme was rich in Ala, Ser, Leu, and Ile, but poor in Trp, Pro, Tyr, and Met. In addition, the purified collagenolytic enzyme was strongly inhibited by N-P-toluenesulfonyl-L-lysine chloromethyl ketone (TLCK), diisopropylfluorophosphate (DFP), and soybean trypsin inhibitor.

Keywords: Collagenase, Purification, *Novoden modesrus*, Characterization

Introduction

Collagenases are generally defined as enzymes that are capable of degrading the polypeptide backbone of native collagen under conditions that do not denature the protein. Two types of proteases with collagenolytic activity have been reported and are thought to play different physiological functions. Metallo-collagenases, first discovered in tadpole tissue explants (Gross and Lapiere, 1962), are zinc-containing enzymes that also generally require calcium for their optimum activity and stability, and cleave the collagen helix at a specific locus under physiological conditions (Gawston and Murphy, 1981; Harris and Vater, 1982; Sellers and Murphy, 1981; Stricklin *et al*., 1977). These enzymes have been widely studied from various mammalian tissues (Harris and Vater, 1982; Sellers and Murphy, 1981) as well as from bacteria, such as *Bacillus cereus* (Makinen and Makinen, 1987), *Clostridium histolyticum* (Bond and Van Wart, 1984a, b; Peterkofsky, 1982), *Achromobacter* (Nguyen *et al*., 1988), *Vibrio alginolyticus* (Takeuchi *et al*., 1992) and *Clostridium perfringens* (Matsuhita *et al*., 1994), and snake venom (Bjarnason and Fox, 1994). These metallo-collagenases, extracellular enzymes, are involved in remodeling the extracellular matrix. Their molecular weights are also found to vary from 30 to 150 kDa.

On the other hand, serine collagenolytic proteases, first isolated from the hepatopancreas of the fiddler crab *Uca pugilator* (Eisen *et al*., 1973), are probably involved in food digestion rather than in morphogenesis. Collagenolytic serine proteases have also been reported from *Bacillus subtilis* FS-2. These were isolated from traditionally fermented fish sauce (Nagano and To, 1999), greenshore crab *Carcinus maenas* (Roy *et al*., 1996), Kamchatka crab *Paralithodes camtschatica* (Klimova *et al*., 1990; Sakharov and et Litvin, 1992), Atlantic cod *Gadus morhua* (Kristjansson *et al*., 1995), insect *Hypoderma lineatum* (Lecroisey *et al*., 1979; Lecroisey and Keil, 1985; Lecroisey *et al*., 1987), Antarctic krill *Euphasia superba* Dana (Turkiewicz *et al*., 1991), midgets of Penaeid shrimps *Penaeus monodon* (Lu *et al*., 1990; Chen *et al*., 1991; Van Wormhoudt *et al*., 1992), and the pancreas of catfish *Parasilarus asotus* (Yoshinaka *et al*., 1986, 1987). These enzymes are involved in the production of hormones and pharmacologically active peptides and in various cellular functions such as protein digestion, blood-clotting, fibrinolysis, complement activation, and fertilization (Neurath, 1984; Bond and Butler, 1987). In addition, these enzymes are widely used in chemical, medical, food industries and molecular biology experiments.

The objectives of this study were to purify a collagenolytic enzyme from the internal organs of filefish *Novoden modestrus*, and to characterize the enzyme with respect to it responses to pH, temperature, and inhibitors.

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Materials and Methods

Materials Filefish (*Novoden modestrus*; body length 17.2~19.5 cm; weight 50~78 g), which were used in this study, were obtained from the southern coast of Korea. The lapsed time between the capture and the freezing of both of the internal organs and skin was less than 10 h. The separated internal organs and skin were rinsed with deionized water to eliminate contaminants and stored at −20°C. Reagents for electrophoresis, DEAE-Sephadex A-50, N-P-toluenesulfonyl-L-lysine chloromethyl ketone (TLCK), N-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), diisopropylfluorophosphate (DFP), soybean trypsin inhibitor, L-cysteine, ethylenediamine tetraacetic acid (EDTA), collagen (type I), and collagenase from *Clostridium Histolytium* were acquired from the Sigma Chemical Co. (St. Louis, USA). Sephadex G-50 and L-histidine were obtained from Pharmacia-LKB (Uppsala, Sweden). 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 (1954) with a slight modification. A reaction mixture containing 5 mg of type I collagen, 1 ml of 50 mM Tris-HCl (pH 7.5), and 0.1 ml of the enzyme solution was typically incubated at 55°C for 30 min. 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 × *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 absorption measurement at 570 nm. A buffer (Tris-HCl, pH 8.0) was used instead of an enzyme solution as a reference. The concentration of hydrolyzed amino acids was determined by a standard curve based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme required for the hydrolysis of 1 μmole of substrate per min.

Purification of collagenolytic enzyme Fresh internal organs were washed with chilled water and homogenized with 5 volumes (w/v) of a 100 mM Tris-HCl (pH 8.0) buffer that contained 0.25% Triton X-100 and 10 mM $CaCl_2$. The homogenate was centrifuged at 7,000 × *g* for 20 min. The pellet was re-extracted with 3 volumes (w/v) of the same buffer. The combined supernatants were dialyzed against 20 mM Tris-HCl (pH 8.0) that contained 0.36 mM $CaCl_2$ for 48 h. The dialyzed solution was used as a crude collagenase starting material.

The crude extract was fractionated with ammonium sulfate (30-80%). Briefly, a solid ammonium sulfate was added to the enzyme solution to 30% saturation and centrifuged at 12,000 × *g* for 30 min. The supernatant was then brought to a 80% saturation with a solid ammonium sulfate. After centrifugation at 12,000 × *g* for 30 min, the precipitate was collected and dissolved in a small amount of 20 mM Tris-HCl (pH 8.0) that contained 0.36 mM $CaCl_2$. The solution was dialyzed against the same buffer with several changes.

The dialyzate was put on a column (3 × 40 cm) of DEAE-Sephadex A-50 that was equilibrated with a 20 mM Tris-HCl buffer (pH 8.0) that contained 0.36 mM $CaCl_2$. The column was washed and subsequently eluted with a linear NaCl gradient (0-0.7 M) in the same buffer. The collagenolytic protease was collected and concentrated using a Sartorious ultrafiltration membrane system (molecular weight cut off, MWCO: 10 kDa, Coettingen, Germany). The supernatant was further purified through a DEAE-Sephadex A-50 column (2.5 × 30 cm) that was equilibrated with a 20 mM Tris-HCl buffer (pH 8.0) that contained 0.36 mM $CaCl_2$. The column was washed with the same buffer and eluted with a linear gradient of a NaCl concentration from 0 to 0.7 M. The enzyme fraction was pooled, concentrated with the ultrafiltration membrane system, and dialyzed against the same buffer that contained 0.36 mM $CaCl_2$. The concentrate was put on a column (1.6 × 90 cm) of Sephadex G-150 that was previously equilibrated with a 20 mM Tris-HCl buffer (pH 8.0) that contained 0.36 mM $CaCl_2$. The column was eluted with the same buffer and the enzyme fraction was pooled. The enzyme solution was then concentrated and stored at −20°C until use.

Protein determination The amount of protein was determined by the method of Lowry (1951) using bovine serum albumin as the standard. In addition, during the enzyme purification with column chromatography, the protein elution profile was monitored spectrometrically-the absorbance at 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 done by the method of Laemmli (1970). Protein bands were stained with Coomassie Brilliant Blue R-250. The molecular mass standards were bovine serum albumin (66.0 kDa), carbonic anhydrase (29.0 kDa), cytochrome C (12.4 kDa), and insulin (5.7 kDa).

Effects of pH and temperature To determine the effect of pH on the enzyme reaction, the reaction mixture was incubated at 55°C for 20 min. The buffers used were 50 mM citrate-Na_2HPO_4 (pH 3.0-7.0), 50 mM Tris-HCl (pH 7.0-9.0), and 50 mM Na_2CO_3-$NaHCO_3$ (pH 9.0-11.0) that contained 0.36 mM $CaCl_2$. The enzymatic activity was measured by the previously described method (Moore and Stein, 1954). In order to test the thermal stability, the enzyme in the 50 mM Tris-HCl buffer (pH 7.75) that contained 0.36 mM $CaCl_2$ was incubated at different temperatures for 20 min. The remaining activity was measured as described previously.

Amino acid composition analysis The purified enzyme was hydrolyzed with 6 N HCl at 110°C for 24 h in vacuum-sealed ampoules. The result was subsequently analyzed with an amino acid analyzer (Biochrom 20, Biochrom Ltd., Cambridge, UK). Tryptophan residues were measured by the method of Hugli and Moore (1972).

Effects of metal ions and various reagents on collagenolytic activity The effect of metal ions on the enzyme activity was investigated by adding the monovalent (K^+, Na^+, and Li^+) and divalent metal ions (Ba^{++}, Ca^{++}, Hg^{++}, Cu^{++}, Fe^{++}, Zn^{++}, Cd^{++}, Sn^{++}, Mn^{++}, Pb^{++}, Ni^{++}, Co^{++}, and Mg^{++}) to the reaction mixture. The final concentration of each metal ion was 60 mM. The activity was compared with the reaction that is free of the corresponding metal

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

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude extract	792.23	90436.05	114.15	100.00	1.00
Ammonium sulfate	400.15	58158.90	145.34	64.30	1.27
1st DEAE-Sephadex A-50	35.35	34514.02	976.31	38.16	8.60
2nd DEAE-Sephadex A-50	2.55	31138.84	12,204.61	28.77	89.39
Sephadex G-150	0.93	9825.11	10,553.28	10.90	92.40

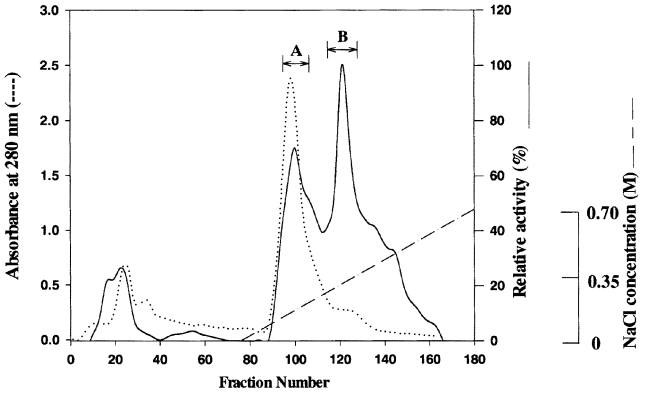

Fig. 1. Chromatogram of DEAE-Sephadex A-50 column (3 × 40 cm) chromatography of the ammonium sulfate saturation in the range of 30 to 80%. The column was eluted with 700 ml 20 mM Tris-HCl buffer, pH 8.0, containing 0.36 mM $CaCl_2$, and then a linear gradient of 0~0.7 M NaCl in the same buffer. The flow rate and the fraction volume were 0.5 ml/min and 10 ml, respectively.

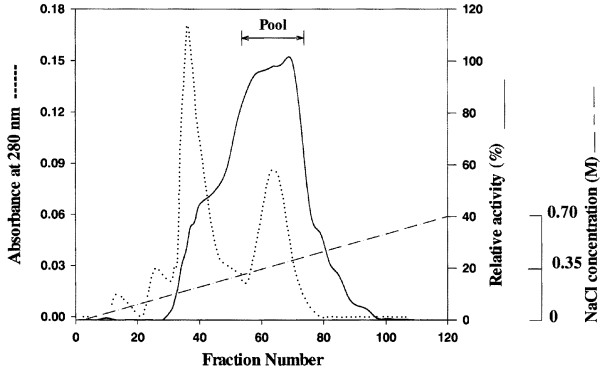

Fig. 2. Rechromatogram of the collagenolytic fraction B pooled from the DEAE-Sephadex A-50 chromatography. The column (2.5 × 30 cm) was eluted with 5 ml Tris-HCl buffer, pH 8.0, containing 0.36 mM $CaCl_2$, and a linear gradient of 0~0.7 M NaCl in the same buffer. The flow rate and the fraction volume were 0.4 ml/min and 10 ml, respectively.

ions. The effect of inhibitors and activators were examined by adding chemical reagents (TPCK, TLCK, L-cysteine, histidine, and the soybean trypsin inhibitor) to the reaction mixture before the activity measurement. The final concentration of each reagent was 1.0, 0.5, and 0.2 mM.

Results

Purification of collagenase The collagenolytic enzyme from the internal organs of the filefish, *Novoden modestrus*, was purified by ammonium sulfate (30-80%) and consecutive column chromatography using DEAE-Sephadex A-50 and Sephadex G-150. The results of the purification of the collagenolytic protease are summarized in Table 1. Using a four-step procedure, the enzyme was purified 92-fold with a yield of 10.9% from crude extract.

Ammonium sulfate was added to the crude extract and the precipitate was dissolved in a buffer solution. The solution was then dialyzed against the same buffer that contained 0.36 mM $CaCl_2$. Subsequently, the dialyzate was applied to a DEAE-Sephadex A-50 that was separated into two fractions (Fig. 1). The active fractions were pooled, concentrated by ultrafiltration, and applied to the second DEAE-Sephadex A-50 column, which was different in column size and flow rate when compared to the first DEAE-Sephadex A-50 column (Fig. 2). The fractions containing activity were pooled, concentrated in a similar manner, and dialyzed. Finally, the dialyzate was subjected to gel filtration chromatography on the Sephadex G-150 column (Fig. 3). The fractions containing

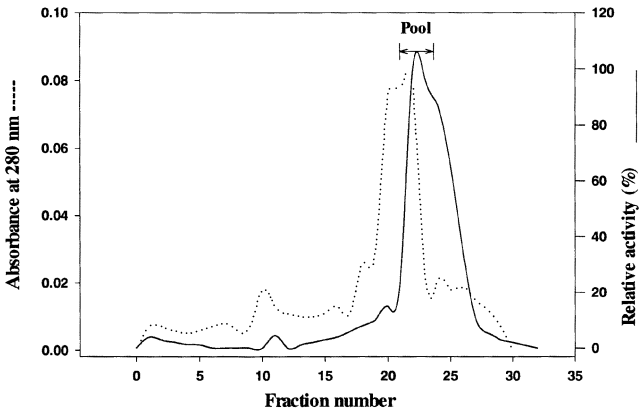

Fig. 3. Gel filtration with Sephadex G-150 from rechromatographic fraction. The column (1.6 × 90 cm) was eluted with 300 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 0.36 mM $CaCl_2$. The flow rate and the fraction volume were 0.5 ml/min and 10 ml, respectively.

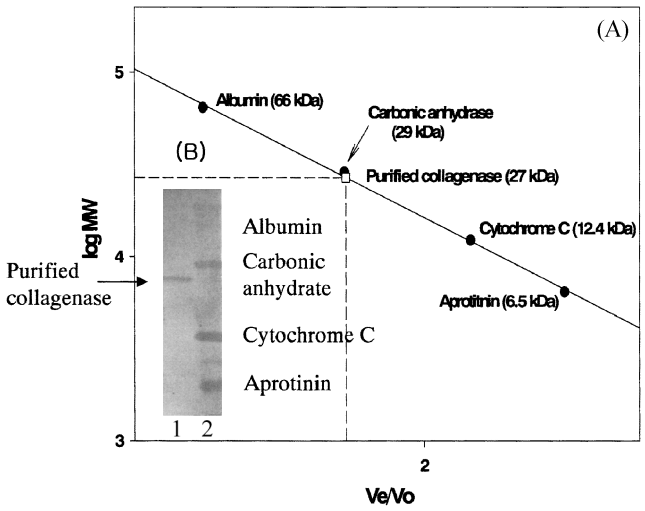

Fig. 4. (A) Molecular weight of the collagenase purified from the internal organs of filefish by gel filtration on the Sephadex G-100 column. The standard proteins in the gel filtration were albumin (66.0 kDa), carbonic anhydrogenase (29.0 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). (B) A 15% SDS-polyacrylamide gel electrophoresis of the purified collagenase from the internal organs of filefish. Lane 1, the purified collagenase; Lane 2, marker proteins. The protein bands of the gel were stained with Coomassie Brilliant Blue. The standard proteins included bovine serum albumin (66.0 kDa), carbonic anhydrase (29.0 kDa), cytochrome C (12.4 kDa), and insulin (5.7 kDa).

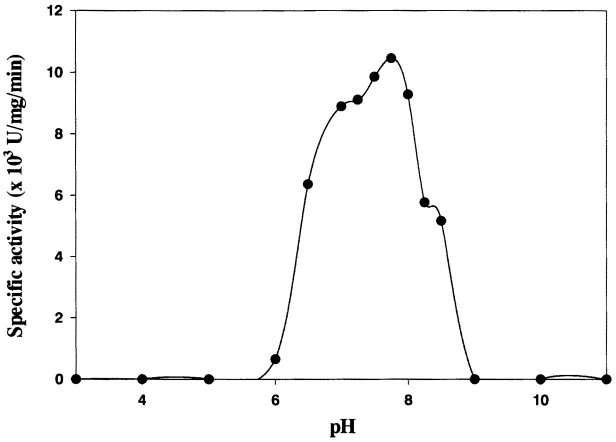

Fig. 5. Effect of pH on the hydrolysis of the type I collagen by the collagenase from the internal organs of filefish. The buffers used were 50 mM citrate-Na_2HPO_4 (pH 3.0~7.0), 50 mM Tris-HCl (pH 7.0~9.0), and 50 mM Ca_2CO_3-$NaHCO_3$ (pH 9.0~10.8). All of the buffers contained 0.36 mM $CaCl_2$. The reaction was conducted at 55°C for 20 min.

collagenolytic activity were pooled, concentrated, and stored at −20°C until use.

Native PAGE of the purified collagenase showed a single band, indicating the homogeneity of the enzyme (data not shown). A gel filtration profile and SDS-PAGE indicated a molecular mass of 27 kDa for the enzyme (Fig. 4).

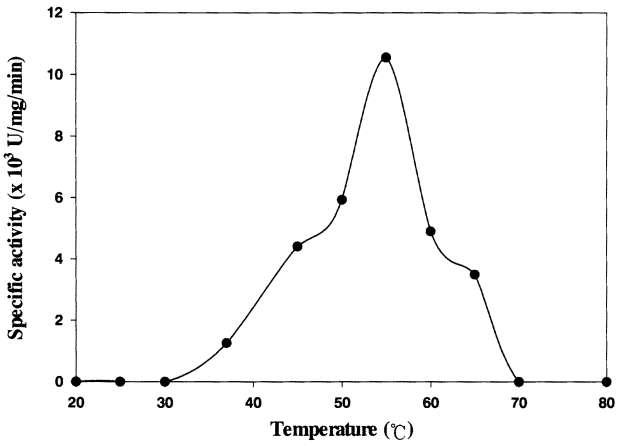

Fig. 6. The Effect of temperature on the hydrolysis of Type I collagen by the collagenase from filefish internal organs. The used buffer was 50 mM Tris-HCl, pH 7.75, containing 0.36 mM $CaCl_2$. The mixture was incubated at various temperatures for 20 min.

Effects of pH and temperature on the collagenolytic activity The pH dependence of the purified collagenolytic enzyme is shown in Fig. 5. The activity was measured on a pH range of 3.0-11.0. The optimum pH was found at pH 7.0-8.0. Fig. 6 shows the thermal stability profile at various temperatures. The optimum temperature of collagenolytic enzyme from filefish was 55°C.

Amino acid composition The amino acid composition of the purified collagenolytic enzyme from filefish is summarized in Table 2. Tryptophan residues were measured by the method of Hugli and Moore, because of the sensitivity of this amino acid to acid hydrolysis. The enzyme was composed of Ala, Ser, Leu, and Ile as its major amino acids; whereas Trp, Pro, Tyr, and Met were present in minor quantities.

Effects of metal ions and various reagents on the collagenolytic enzyme The enzyme was strongly inhibited by TLCK, the soybean trypsin inhibitor, and DFP. However, only a slight inhibition was detected with TPCK, EDTA, and histidine. On the other hand, L-cysteine was totally ineffective (Table 3). In preliminary experiments, the effect of metal ions (such as K^+, Na^+, Li^+, Ba^{++}, Ca^{++}, Hg^{++}, Cu^{++}, Fe^{++}, Zn^{++}, Cd^{++}, Sn^{++}, Mn^{++}, Pb^{++}, Ni^{++}, Co^{++}, and Mg^{++}) on the collagenolytic activity was estimated. The collagenolytic activity was inhibited by Zn^{++}, Cd^{++}, Cu^{++}, and Ni^{++}, but activated by K^+, Li^+, Ba^{++}, Ca^{++}, and Mg^{++} (Table 4).

Discussion

In this work, a serine collagenase was isolated from the internal organs of the filefish *Novoden modestrus*. The purification of the serine collagenolytic protease involved a four-step procedure using ammonium sulfate, ion-exchange

Table 2. Amino acid composition of the collagenase from the internal organs of filefish

Amino acid	Residue/mol-protein
Lys	14
His	13
Arg	8
Asp	9
Thr	8
Ser	30
Glu	7
Pro	1
Gly	13
Ala	32
Half-Cys	4
Val	5
Met	2
Ile	21
Leu	28
Tyr	2
Phe	9
Trp	ND[a]
Ser	10
Total residues	216

[a]ND: not detected.

Table 3. Effects of various inhibitors on the collagenolytic activity

Reagents	Concentration in reaction mixture (mM)	Collagenolytic activity (%)
None*		100
TLCK	1.00	0
	0.50	0
	0.20	0
TPCK	1.00	46
	0.50	75
	0.20	85
L-cysteine	1.00	115
	0.50	104
	0.20	101
EDTA	1.00	0
	0.50	5
	0.35	18
	0.20	54
Histidine	1.00	0
	0.70	7
	0.50	20
	0.20	49
Soybean trypsin Inhibitor	1.00	0
	0.50	0
	0.20	0
DFP	1.00	0
	0.50	0
	0.20	0

*0.05 M Tris-HCl buffer containing 0.36 mM $CaCl_2$

Table 4. Effects of metal ion on the collagenolytic activity

Metal ion (0.06 mM)	Collagenolytic activity (%)
None	100.0
Hg^{2+}	81.3
Cu^{2+}	74.1
Fe^{2+}	96.0
Cd^{2+}	71.0
Sn^{2+}	88.5
Zn^{2+}	63.7
Mn^{2+}	84.5
Na^{+}	92.1
K^{+}	123.0
Pb^{2+}	92.5
Ni^{2+}	70.5
Li^{+}	110.8
Co^{2+}	91.7
Ba^{2+}	119.1
Mg^{2+}	110.8
Ca^{2+}	135.3

chromatography on a DEAE-Sephadex A-50, ion-exchange rechromatography on a DEAE-Sephadex A-50, and gel filtration on a Sephadex G-150 column in order to obtain electrophoretic homogeneity. The purification factor was about 92-fold with a yield of 10.9% from the crude extract. The specific activity of the purified collagenase was also higher than those reported in the literature.

The molecular mass of the purified enzyme was about 27.0 kDa by SDS-PAGE using the method of Laemmli *et al.* (1970) on PAGE 15%. The molecular weight of the purified collagenase from various sources had previously been measured. The molecular mass of the filefish collagenase was close to that of other serine collagenase. It was slightly higher than that of Atlantic cod (*Cadus morhua,* 24.1 kDa) (Kristjansson *et al*., 1995), greenshore crab (*Carcinus maenas*, 23.0 kDa) (Roy *et al*., 1996), shrimp (*Panaeus vannamei*, 25.0 kDa) (Van Wormhoudt *et al*., 1992), insect (*Hypoderma lineatum*, 25.0 kDa) (Lecroisey *et al*., 1987), and *Aspregillus niger* (21.0 kDa) (Barthomoeuf *et al*., 1992). It was somewhat lower than collagenases from crab (*Paralithodes camtschatica*, 24.0-36.0 kDa) (Sakharov *et al*., 1992), *Vibrio mimicus* (42.0 kDa) (Kim and Kim, 1996), and the tissue of filefish (*Novoden modestrus*, 42.0 kDa) (Kim and Kim, 1991).

The optimum pH for the collagenolytic activity of the filefish against type I collagen was between pH 7.0 and 8.0. The results displayed a bell-shaped curve with an apparent maximum close to the pH of greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996). A similar value was reported for the *Paralithodes camtschatica* collagenase A (Klimova *et al*., 1990) and *Parahaliporus sibogae* collagenase (Muramatsu and Kariuchi, 1978). The optimum temperature for the collagenase was 55°C. This is higher than 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 lower than that in the tissue of filefish *Novoden modestrus* (60°C) (Kim and Kim, 1991).

The purified collagenase was rich in Ala, Ser, Leu, and Ile. However, it was poor in Trp, Pro, Tyr, and Met by an amino acid composition analysis. However, the identification of the N-terminus amino acid sequence was not determined, because the N-terminal chain of the purified collagenase was blocked.

The collagenase activity of the purified enzyme was inhibited by metal ion (Zn^{++}) and various reagents, such as the soybean trypsin inhibitor, DFP, and TLCK. Generally, metallo-collagenase specifically requires zinc ion for optimum activity and stability. However, the purified collagenase in the study was inhibited by zinc ion. In addition, the soybean trypsin inhibitor and DFP have a strong inhibiting effect on collagenase activity, suggesting the possibility of having a serine collagenase in the internal organs of filefish *Novoden modestrus*. These results for the purified collagenase showed the effect of specific serine protease inhibitors and the inhibitory effect of Zn^{2+}, which could bind SH groups in proteins. This suggests that the collagenolytic enzyme is a member of the serine protease family. Many serine protease have been isolated from various sources, such as fungus (Hurion *et al*., 1979), insect (Lecroisey *et al*., 1979; Lecroisey and Keil, 1985; Lecroisey *et al*., 1987), bacteria (Nagano and To, 1999), crab (Eisen *et al*., 1973; Klimova *et al*., 1990; Sakharov and et Litvin, 1992; Roy *et al*., 1996), and fish (Yoshinaka *et al*., 1986; 1987; Kristjansson *et al*., 1995) among others.

In conclusion, we isolated and purified a new serine collagenolytic protease, which possesses high specific activity from organs of filefish by using consecutive chromatographic procedures. However, the identification of the partial amino acid sequence was not carried out, because the N-terminal chain of the purified collagenase was blocked. Future work will investigate the industrial application of the collagenase from the internal organs of filefish according to its properties.

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