Purification and Properties of a Collagenolytic Protease Produced by Marine Bacterium *Vibrio vulnificus* CYK279H

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Abstract A collagenolytic enzyme, produced by *Vibrio vulnificus* CYK279H, was purified by ultrafiltration, dialysis, Q-Sepharose ion exchange and Superdex-200 gel chromatography. The enzyme from the supernatant was purified 13.2 fold, with a yield of 11.4%. The molecular weight of the purified enzyme was estimated by SDS-PAGE to be approximately 35.0 kDa. The N-terminal sequence of the enzyme was determined as Gly-Asp-Pro-Cys-Met-Pro-Ile-Ile-Ser-Asn. The optimum temperature and pH for the enzyme activity were 35°C and 7.5, respectively. The enzyme activity was stable within the pH and temperature ranges 6.8~8.0 and 20~35°C, respectively. The purified enzyme was strongly activated by Zn^{2+}, Li^{2+}, and Ca^{2+}, but inhibited by Cu^{2+}. In addition, the enzyme was strongly inhibited by 1, 10-phenanthroline and EDTA. The purified enzyme was suggested to be a neutral metalloprotease.

Keywords: collagenase, gelatin, metalloprotease, purification, *Vibrio vulnificus*

INTRODUCTION

Collagen is an insoluble structural protein found in animals, which is produced in large quantities as a by-product in livestock industrials; moreover, it is contained in fish skin at levels exceeding 80%.

Collagenolytic proteases are defined as proteases capable of degrading the native triple helix of collagen, and are involved in various physiological and pathological situations, such as fetal bone development, embryonal development, wound repair, rheumatoid arthritis, malignant tumor invasion, intestinal ulceration and chronic periodontal inflammation [1]. The collagenolytic proteases are classified into two major groups; metallocollagenases and serinecollagenases. Hydrolysis by metalloproteases takes place mostly between the peptide bond of residue X and Gly-Pro [2,3]. Metallocollagenases, first discovered in tadpole tissue explants, are zinc-containing enzymes, but also generally require calcium for their optimum activity and stability [4,5], and are involved in re-modeling the extracellular matrix. Conversely, serinecollagenases were first isolated from the hepatopancreas of the fiddler crab (*Uca pugilator*), and are probably involved in food digestion rather than morphogenesis [6], as well as the production of hormones and pharmacologically active peptides, and in various cellular functions, such as protein digestion, blood-clotting, fibrinolysis and fertilization [7]. The proteolytic products of collagen re-

cently been widely used in the chemical, medical, cosmetic and food industries, as well as molecular biology experimental applications [8]. They can serve as a seasoning, a non-allergic preservative for medicines, ingredients for dietary materials and parentally-fed products [9]. Collagenolytic proteases have been widely studied from a variety of microorganisms, such as *Vibrio* B-30, *Clostridium perfringens*, *Bacillus* sp. MO-1 and *Vibrio harveyi* [10-13]. In particular, the collagenase produced by *Clostridium histolyticum* has been extensively studied from both pathological and enzymological viewpoints [14]. However, only a few reports have been published on the biochemical and molecular biological techniques of microbial collagenolytic proteases [15,16]. In addition, previous reports dealing with bacterial collagenases and collagen-degrading enzymes have focused on the potential role of those enzymes in human diseases [2,5]. In this paper, the purification and properties of the collagenolytic enzyme from the marine bacterium, *Vibrio vulnificus* CYK279H, are described. Some physicochemical and molecular properties of the enzyme were studied, and subsequently compared with those of other known collagenases. Also, attempts were made to screen new collagenase inhibitors from other marine bacteria with pharmacological activity.

MATERIALS AND METHODS

Bacterial Strain and Culture Condition

The *V. vulnificus* CYK279H used in these experiments

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was obtained from the seawater from southern Korea [17]. The composition of culture broth consisted of 0.3% (w/v) galactose, 0.6% (w/v) yeast extract, 4.0% (w/v) gelatin, 0.2% (w/v) $(NH_4)_2SO_4$, 0.2 mM ferric citrate, 2.0% (w/v) NaCl, 0.2% (w/v) $(NH_4)_2SO_4$ and artificial sea water. The organism was cultured at 25°C for 18 h on a rotary shaker at 180 rpm.

Enzyme Assay

The activity of the collagenolytic enzyme was assayed by the method of Moore and Stein, with slight modification [18]. This enzyme reaction was carried out with the addition of 0.01 mL of the enzyme solution to 1.0 mL of 2 mg gelatin in 50 mM Tris-HCl buffer (pH 7.5) at 37°C for 18 h. The reaction was stopped by the addition of 0.1 mL 10% trichloroacetic acid. The reaction was centrifuged at 10,000 × g for 10 min. The supernatant (0.2 mL) was mixed with 0.5 mL ninhydrin solution, heated at 100°C for 10 min, cooled on ice water for 5 min, and the mixture then diluted with 2.5 mL 50% 1-propanol. After centrifugation at 12,000 × g for 10min, the absorbance of the mixture was measured at 570 nm. One unit (U) of enzyme activity was expressed as μmol of leucine equivalents released per minute. All experiments were performed in triplicate.

Enzyme Purification

All purification steps were carried out at 4°C. After cultivation, the cell was removed by centrifugation at 9,000 × g for 20 min, and the supernatant concentrated using a freeze dryer. The concentrate was dissolved in 20 mM Tris-HCl (pH 7.5, buffer A) and dialyzed against the same buffer for 24 h. The dialysate was concentrated by ultrafiltration using an Amicon PM10 membrane (M.W 10,000), with the concentrate then applied to a Q-Sepharose column (2.6 × 10.0 cm, Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer, and eluted with a linear gradient of 0~2.0 M NaCl at a flow rate of 1 mL/min. The active fractions were pooled, dialyzed and concentrated. The concentrate was reloaded onto a Q-Sepharose column (2.6 × 10.0 cm, Pharmacia Biotech) equilibrated with buffer A. The elution was performed with a linear gradient of 0~1.0 M NaCl at a flow rate of 1 mL/min. The active fractions were pooled and dialyzed and concentrated. The concentrate was applied to a Superdex-200 column (1.6 × 60.0 cm, Pharmacia Biotech) equilibrated with the same buffer. The active fractions were pooled and concentrated. The concentrate was reloaded onto a Superdex-200 column (1.6 × 60.0 cm, Pharmacia Biotech) equilibrated with the same buffer. Afterwards, the enzyme solution was concentrated and stored at -20°C until used.

Protein Assay

The protein level was determined by the method of Bradford [19], using bovine serum albumin as a standard protein, or by measuring the absorbance at 280 nm.

Molecular Weight Determination

The molecular weight of the purified enzyme was determined by SDS-PAGE according to the method of Laemmli [20], using a 12% polyacrylamide gel stained with Coomassie brilliant blue R-250.

N-terminal Sequence Analysis

The N-terminal amino acid sequence analysis was performed by automated Edman degradation [21], using a model 491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

Effect of pH and Temperature on Activity and Stability

The effect of temperature on the enzyme activity was investigated between 10 and 60°C. To determine enzyme stability, the enzyme was preincubated at the given temperatures for 1 h. The optimal pH for the enzyme activity was investigated at various pHs ranging from 3 to 10.4. To determine the pH stability of the enzyme activity, the reactions were preincubated at the various pHs for 1 h.

Effect of Metal ions and Inhibitors

The effects of metal ions on the enzyme activity were investigated by the addition of monovalent (K^+) and divalent cations (Zn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Li^{2+}, Mn^{2+}, and Fe^{2+}) at a metal ioin concentration of 1 mM. The effects of inhibitors were also studied using 1, 10-phenanthroline, EDTA, PMSF, L-cysteine, N-Ethylmaleimide, and 2-mercaptoethanol at final concentrations of 0.1 and 1.0 mM.

RESULTS AND DISCUSSION

Purification of Collagenolytic Enzyme from *Vibrio vulnificus* CYK279H

A collagenolytic enzyme was obtained from the culture supernatant of *V. vulnificus* CYK279H. A summary of the purification procedure is shown in Table 1. After the final purification step, the purified enzyme showed a specific activity of 12,638.8 units/mg protein, indicating a purity and yield of 13.2 fold and 11.4%, respectively (Table 1).

The molecular weight of the purified collagenolytic enzyme was estimated by SDS-PAGE to be approximately 35 kDa (Fig. 1).

This molecular weight was higher than those of the collagenases from *Aspergillus niger* and the greenshore crab (*Carcinus maenas*) [22,23], but lower than those from *C. perfringens*, *Bacillus* sp. MO-1, *Streptococcus gordnii*, and *Cytophage* sp. L43-1 [11,12,24,25]. The N-terminal sequence of 10 residues was determined to be Gly-Asp-Pro-Cys-Met-Pro-Ile-Ile-Ser-Asn. No significant homology was obtained from the current database analysis using the BLAST Program of the NCBI [26]. This protease was named *V. vulnificus* CYK279H, and com-

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Table 1. Summary of the purification steps for the collagenolytic protease from the culture supernatant of *Vibrio vulnificus* CYK279H

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	55,377.0	58.0	954.7	100.0	1.0
Q-Sepharose[1st]	32,121.9	12.0	2,676.8	58.0	2.8
Q-Sepharose[2nd]	10,983.7	3.5	3,138.2	19.8	3.3
Superdex-200[1st]	7,970.2	1.2	6,641.8	14.4	7.0
Superdex-200[2nd]	6,319.4	0.5	12,638.8	11.4	13.2

Table 2. Comparison of the N-terminal amino acid sequence of the collagenolytic protease from *Vibrio vulnificus* CYK279H
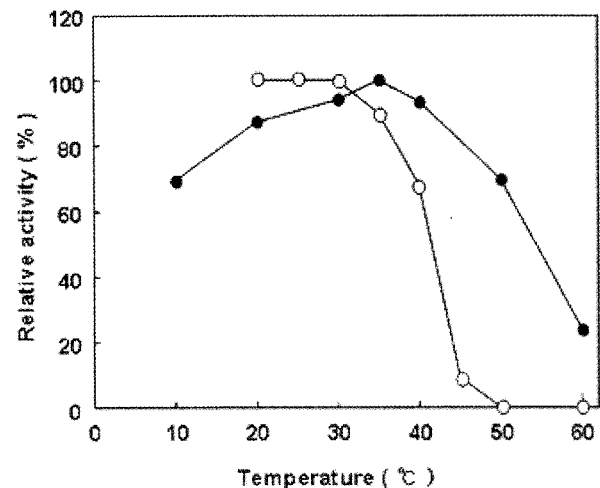
Strains	N-terminal sequence	Reference
V. vulnificus CYK279H	Gly-Asp-Pro-Cys-Met-Pro-Ile-Ile-Ser-Asn	This work
Clostridium perfringens	Ala-Arg-Asn-Asn-Lys-Ile-Tyr-Thr-Phe-Asp	[11]
Bacillus sp.	Met-Lys-Tyr-Ser-Lys-Glu-Leu-Val-Glu-Ala	[12]
V. harveyi AP6	Leu-Glu-Ala-Glu-Gly-Pro-Gly-Gly-Asn-Gln	[13]
V. Parabaemolyticus prtV	Gln-Asn-Gln-Cys-Ala-Val-Ala-Asp-Leu-Gln	[40]
V. alginolyticus	Met-Glu-Leu-Lys-Ile-Leu-Ser-Val-Ala-Ile-Ala	[41]
V. mimicus	Val-Tyr-Ser-Gln-Pro-Leu-Phe-Arg-Arg-His	[42]
E. coli JM83/pKCL279H	Thr-Ala-Ile-His-Glu-Leu-Phe-His-Asp-Glu	[43]
Cytophaga sp.	Thr-Leu-Thr-His-Glu-Phe-Gly-His-Phe-Leu	[44]
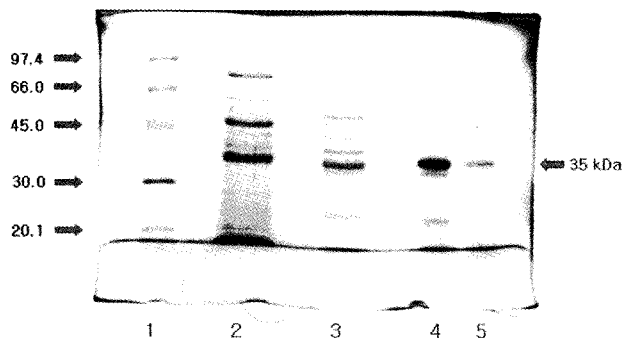
Fig. 1. 12% SDS-PAGE of the purified collagenolytic protease. Lane 1, protein molecular weight markers; phosphorylase b (97.4 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa); Lane 2, supernatant; Lane 3, dialysis; Lane 4, Q-Sepharose[2nd]; Lane 5, Superdex-200[2nd].

Fig. 2. Effect of temperature on the activity and stability of the purified collagenolytic protease. The optimal temperature for the enzyme activity (●) was determined at different temperatures (10~60°C). The enzyme was preincubated at 20~60 °C for 1 h to determine the stability (○) of the enzyme activity.

pared with those from other microorganisms (Table 2).

Effect of pH and Temperature on Activity and Stability

The optimal temperature for the enzyme activity was measured at various temperatures, ranging between 10 and 60°C. As shown in Fig. 2, the enzyme exhibited maximum activity at 35 °C. This is lower than the 40~60 ℃ of *Bacillus* sp. MO-1, *Cytophage* sp. L43-1, and the northern shrimp (*Pandalus eous*) [12,25,27], but higher than that of the collagenase from the greenshore crab (*C.*

maenas) [23]. However, the enzyme maintained over 60% of its activity at around 10~50 °C. The thermal stability of the enzyme was stable up to 40 °C, and at 45 °C only 10% of the activity remained (Fig. 2).

The effect of pH on the enzyme activity was estimated in three different buffer systems; 50 mM sodium citrate (pH 3.0~6.2), 50 mM Tris-HCl (pH 6.8~9.0), and 50
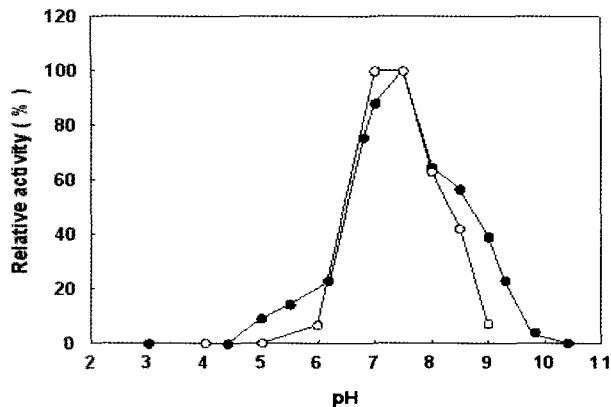
Fig. 3. Effect of pH on the activity and stability of the purified collagenolytic protease. The optimal pH for the activity (●) and pH stability (○) of the enzyme were determined at various pHs (3.0~11.0), with the reactions preincubated at the given pHs for 1 h. The buffers used were 50 mM sodium citrate (pH 3.0~6.2), 50 mM Tris-HCl (pH 6.8~9.0), and 50 mM Na₂CO₃-NaHCO₃ (pH 9.3~10.4). To determine the stability, the enzyme was preincubated at 37°C for 1 h in each of the specified buffer.

Fig. 4. Thermoinactivation of the purified collagenolytic protease in the presence of 1 mM $CaCl_2$. To determine the enzyme activity, the reactions were preincubated from 0 to 90 min at 30, 40, and 50°C.

-●- In the absence of $CaCl_2$ at 30 °C -■- In the presence of $CaCl_2$ at 30 °C
-○- In the absence of $CaCl_2$ at 40 °C -□- In the presence of $CaCl_2$ at 40 °C
-●- In the absence of $CaCl_2$ at 50 °C -■- In the presence of $CaCl_2$ at 50 °C

Table 3. Effect of metal ions on the activity of the purified collagenolytic protease

Metal ions (1.0 mM)	Relative activity (%)
None	100.0
K^+	89.2
Zn^{2+}	160.3
Cu^{2+}	3.8
Co^{2+}	77.2
Li^{2+}	110.3
Ca^{2+}	131.6
Mg^{2+}	90.0
Fe^{2+}	58.3
Mn^{2+}	81.5

The enzyme was preincubated with various metal ions in 50 mM Tris-HCl buffer for 30 min at 37°C. All experiments were performed in triplicate.

mM Na₂CO₃-NaHCO₃ (pH 9.3~10.4). The optimum pH of the enzyme was estimated to be 7.5, which was the same as for the collagenolytic enzyme from *Cytophage* sp. L43-1, the northern shrimp (*P. eous*) and the marine crab (*Scylla serrata*) [25,27,28], with over 70% of the activity maintained within the pH range 6.8~8.0 (Fig. 3). Most enzymes are stable at the pH where the maximum activity is shown. The purified enzyme was highly stable at neutral pH (pH 7.0~7.5); therefore, the purified enzyme was stable over a very narrow pH range.

In general, the pH range of the enzyme stability is wider than that of the enzyme activity, due to the conformational structure of an active or quaternary structure of the whole protein.
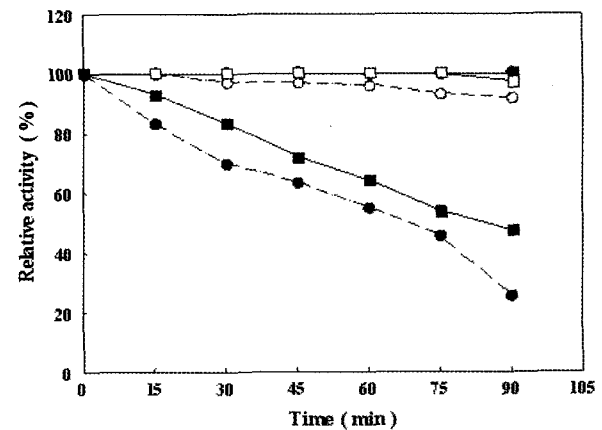
Effect of Metal Ions and Inhibitors

The effects of metal ions on the collagenolytic activity are presented in Table 3. The collagenolytic activity of the purified enzyme was increased in the presence of Zn^{2+}, Li^{2+}, and Ca^{2+}, but strongly inhibited by Cu^{2+} ions (Table 3).

Interestingly, the activity of the collagenolytic protease from *V. vulnificus* CYK279H was similar to those of *Bacillus* sp. MO-1, marine crab (*S. serrata*), *Bacillus* sp. JH108, *Empedobacter collagenolyticum*, and *Aneurinibacillus* sp. AM-1 [12,28-31]. Generally, bacterial collagenases specifically require zinc and calcium for their optimum activity and stability. Above all, Ca^{2+} often participates in the stabilization of the protein structure, which is required for protection against heat inactivation [32].

In these experiments, during the thermoinactivation of the purified enzyme in the presence of 1 mM $CaCl_2$ over 50% of the activity was retained at 50°C (Fig. 4); while during thermoinactivation of the purified enzyme in the absence of $CaCl_2$, only 20% of the activity was retained.

The purified enzyme activity was inhibited by both 1, 10-phanthroline and EDTA, but not by PMSF (Table 4). These results suggest the purified enzyme to be a member of the metalloprotease family.

The collagenolytic enzyme is a common feature of the neutral metallo-proteases of the marine crab (*S. serrata*), *Streptomycete* sp. A8, *Streptococcus mutans*, *Xenorhabdus nematophilus*, and *paracoccus* sp. WJ-98 [28,33-36]. Metalloproteases require a divalent metal ion at their reaction center, and are usually inhibited by divalent metal ion chelators, such EDTA or 1, 10-phenanthroline.

Substrate Specificity and Enzyme Kinetics

The specificity of the purified enzyme was tested using

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Table 4. Effect of inhibitors on the activity of the purified collagenolytic protease

Inhibitors	Collagenolytic activity (%)	
	0.1 mM	1.0 mM
None	100.0	100.0
1,10-phenanthroline	48.6	7.5
EDTA[a]	25.4	12.4
PMSF[b]	90.5	83.2
Cysteine	95.4	107.8
N-ethylmaleimide	86.7	75.4
2-mercaptoethanol	45.7	51.9

The enzyme, with various metal ions, was preincubated in 50 mM Tris-HCl buffer for 30 min at 37°C. All experiments were performed in triplicate. [a] EDTA: ethylene diamine tetra-acetic acid, [b] PMSF: phenyl methyl sulfonyl fluoride.

Table 5. Substrate specificity of collagenolytic protease

Substrate	Relative activity (%)
Collagen type I [a]	30.5
Collagen type II [a]	75.3
Collagen type V [a]	56.3
Gelatin	100.0
Casein	53.8
Elastin	8.9
BSA	ND[b]

[a] insoluble collagen from bovine Achilles.
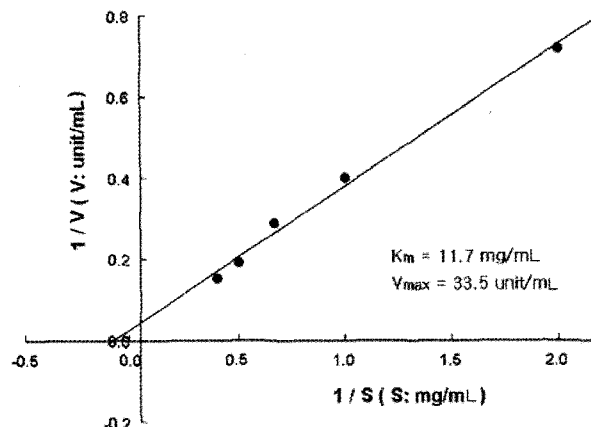[b] not detected.

Fig. 5. Lineweaver-Burk plot for the hydrolysis of gelatin by the collagenolytic protease of *Vibrio vulnificus* CYK279H. The collagenolytic protease activity was measured at different gelatin concentrations (0.5~2.0 mg/mL) in 50 mM Tris-HCl buffer (pH 7.5).

various substrates. Collagenases display a great deal of specificity in hydrolyzing a single polypeptide bond on each chain of the native triple-stranded collagen helix [37]. Of the protein substrates, collagen type II and gelatin were found to be suitable substrate for the purified enzyme from strain CYK279H (Table 5), showing high activity toward gelatin, but no activity toward BSA. The cleavage rate for the substrate due to the purified enzyme was as follows: gelatin > type II collagen > type V collagen > casein> type I collagen > elastin. No other proteins or synthetic peptide substrate were investigated in this study.

The Kinetic parameters of the purified collagenolytic enzyme were investigated at a pH and temperature of 7.5 and 37°C, respectively, using gelatin. The K_m and V_{max} of the enzyme were calculated to be 11.7 mg and 33.5 units, respectively (Fig. 5).

The K_m value of the protease was higher than that reported for *Bacillus* sp. No. 8-16 (1.3 mg/mL) and No. AH-101 (8.2 mg/mL) [38,39].

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