Expression and characterization of the *prtV* gene encoding a collagenase from *Vibrio parahaemolyticus* in *Escherichia coli*

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The *prtV* gene, encoding a collagenase of *Vibrio parahaemolyticus*, was expressed in *Escherichia coli* and purified by affinity chromatography. The transformant *E. coli* BL21(DE3)(pPRT2) secreted the recombinant PrtV, and the highest enzyme activity was detected in the culture supernatant after 5 h IPTG induction. The molecular mass of purified PrtV was 62 kDa as determined by gel filtration, which was similar to that obtained by SDS-PAGE (64 kDa). This suggested that PrtV was a monomer protein having no subunit structure. The isoelectric point of PrtV was 8.52. In addition, PrtV contained a 27 amino acid signal peptide, and the amino acid composition of the PrtV showed satisfactory agreement with that predicted from the DNA sequence. The optimum temperature and pH of PrtV were 40 °C and pH 7.5, respectively. The activity of PrtV was inhibited by chelators such as EDTA, EGTA and 1,10-phenanthroline; however, its activity was restored by the addition of various metal ions (Co²⁺, Mn²⁺, Ca²⁺, Cu²⁺, Ni²⁺ and Zn²⁺), indicating that PrtV is a metalloprotease. PrtV degraded both type I collagen and synthetic substrate FALGPA well, showing that PrtV is indeed a collagenase.

Keywords: *prtV* gene, collagenase, *Vibrio parahaemolyticus*

INTRODUCTION

Vibrio parahaemolyticus is an important diarrhoeal agent associated with seafood poisoning in Taiwan, Japan and many coastal areas (Janda *et al.*, 1988). Many proteins are secreted by *V. parahaemolyticus* into the extracellular environment. These include urease (Cai & Ni, 1996), haemolysin (Nishibuchi *et al.*, 1992), chitinase (Zhu *et al.*, 1992), lethal toxins (Sarkar *et al.*, 1987a, b), vascular permeability factors (Honda *et al.*, 1976), protease (Iuchi & Tanaka, 1982) and amylase (Iuchi & Tanaka, 1980).

To date, many proteases from *Vibrio* spp. have been studied in detail. *Vibrio vulnificus*, which causes wound infections and septicemia in humans, produces a neutral metalloprotease with elastolytic activity (Kothary & Kreger, 1987) that has elicited dermonecrosis (Smith & Merkel, 1982). *Vibrio cholerae* haemagglutinin/protease nicks and activates the A subunit of the cholera enterotoxin (Booth *et al.*, 1984), and shows

the ability to cleave several physiologically important substrates, including mucin, fibronectin and lactoferrin (Finkelstein *et al.*, 1983). *Vibrio alginolyticus* produces alkaline serine proteases and a collagenase, which have both been isolated and characterized (Deane *et al.*, 1989; Takeuchi *et al.*, 1992). *Vibrio anguillarum* secretes a 38 kDa metalloprotease, which has elastolytic activity and was strongly implicated in fish invasion (Norqvist *et al.*, 1990). A purified extracellular metalloprotease from *Vibrio mimicus* was reported to enhance vascular permeability in skin and fluid accumulation in rabbit ileal loops (Chowdhury *et al.*, 1991a, b). *Vibrio proteolyticus* secretes a neutral protease which has industrial applications in the synthesis of dipeptides (David *et al.*, 1992). The serine protease gene (*vapT*) derived from *Vibrio metschnikovii* strain RH530 has been cloned in *E. coli*, and the recombinant VapT shows high resistance to SDS and urea (Kwon *et al.*, 1995).

Unlike other *Vibrio* proteases, the proteases of *V. parahaemolyticus* have not been purified and characterized, and their role in pathogenesis is still unclear. Four proteases were analysed by SDS-PAGE (Iuchi & Tanaka, 1982); however, they have not yet been fully purified and characterized. In addition, the protease of

Abbreviations: E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane; FALGPA, 2-furanacryloyl-Leu-Gly-Pro-Ala; CHC, *Clostridium histolyticum* collagenase; VAC, *Vibrio alginolyticus* collagenase.

V. parahaemolyticus was also not successfully purified from iron-limited culture (Wong & Shyu, 1994). In our previous study, the protease gene (*prtV*) of *V. parahaemolyticus* was successfully cloned and sequenced (Lee *et al.*, 1995). The *prtV* gene contained a 1761 bp ORF encoding a 587 amino acid protein with a putative signal sequence. This paper reports the expression and characterization of PrtV from *V. parahaemolyticus* in *E. coli* and identifies PrtV as a collagenase.

METHODS

Bacterial strains and plasmids. The protease gene of *V. parahaemolyticus* 93 was cloned and sequenced. A plasmid, pLSD6, carrying a 4.5 kb *Hind*III fragment of the protease gene was constructed in *E. coli* XL-1 Blue (Lee *et al.*, 1995). pET21b(+) and *E. coli* BL21 (DE3)(Novagen) were used as vector and host, respectively, for gene expression. Based on the *prtV* sequence (Lee *et al.*, 1995), two oligonucleotide primers, PRTF1 (5' CCCCACTGAATTCATTGTT 3') and PRTR1 (5' TCCGAAGCTTGCACCTTCAGG 3'), were designed to carry the *Eco*RI and *Hind*III recognition site, respectively. PCRs were performed with pLSD6 as the template, and primers PRTF1 and PRTR1 to amplify the 1.8 kb *Eco*RI–*Hind*III fragment, which carries the *prtV* gene. This fragment was subcloned into the expression vector, pET21b(+), to yield pPRT2 (Lee & Liaw, 1996). The PrtV expressed from pPRT2 was a fusion protein, including the His-Tag oligohistidine domain (6 amino acids) at the C-terminus for convenient purification and the T7-TagTM sequence (11 amino acids) at the N-terminus for immunological detection.

Medium and growth conditions. *E. coli* was grown at 35 °C in LB broth (Difco). LB medium supplemented with 100 µg ampicillin ml⁻¹ (Sigma) was used for plasmid maintenance in *E. coli*.

Enzyme assays. Protein concentrations were determined by using the method of Bradford (1976) with BSA (Bio-Rad) as a standard. For determination of enzyme activity, azocoll was used as a substrate, and the standard conditions were from that used by Chavira *et al.* (1984). Azocoll powder (Calbiochem) was washed once with 50 mM Tris/HCl (pH 8.0) and resuspended in the same buffer to give a concentration of 5 mg ml⁻¹. One millilitre of azocoll was transferred to a glass tube (13 mm diameter) and 10–20 µl enzyme solution was added. The reaction mixture was incubated at 37 °C for 1 h with shaking at 150 r.p.m. The mixture was centrifuged at 11000 g for 10 min, and the A₅₂₀ of the supernatant was noted. One unit of enzyme activity is defined as the amount of enzyme which causes an increase of 1.0 A₅₂₀ unit per hour. For measurement of enzyme kinetics, the specific collagenase substrate FALGPA (2-furanacryloyl-Leu-Gly-Pro-Ala; Sigma) and type I collagen (Sigma) were used. Assays with FALGPA were carried out by monitoring the decrease in absorbance of the substrate after addition of enzyme (Van Wart & Steinbrink, 1981). Ten microlitres enzyme was added to 990 µl FALGPA and the absorbance change was measured using a Hitachi U-2000 spectrophotometer. One unit of enzyme activity was defined as 1 µmol substrate consumed s⁻¹ (1 µkat), which was determined as the ratio of absorbance change corresponding to full hydrolysis multiplied by the substrate concentration. Native collagens were also chosen to determine enzyme activity (Grubb, 1994a). Ten microlitres enzyme was incubated with the collagen at 37 °C for 8 h, and the release of collagen fragments into the supernatant was determined using ninhydrin (Grubb, 1994b). One unit of enzyme activity was

defined as the amount which liberated peptides from collagen equivalent in ninhydrin colour to 1 µmol of L-leucine at 37 °C for 8 h. The kinetic parameters (K_m , V_{max}) for hydrolysis of FALGPA and native collagens were determined from the Lineweaver–Burk double-reciprocal plot. The collagenases of both *Clostridium histolyticum* type A (CHC) and *V. alginolyticus* (VAC) (Boehringer Mannheim) were also compared with the PrtV collagenase in the enzyme kinetic analysis.

Purification of PrtV. *E. coli* BL21(DE3)(pPRT2) was grown in 400 ml LB until the OD₆₀₀ reached 0.4–0.6, when 1 mM IPTG was added. After IPTG induction, cells were pelleted by centrifugation at 6510 g for 15 min at 4 °C. The supernatant fraction was collected and the proteins were precipitated on ice by the addition of solid ammonium sulphate to 70% saturation. The precipitate was pelleted by centrifugation at 8050 g for 20 min at 4 °C. The pellet was dissolved in 3 ml binding buffer (20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 8 M urea) and dialysed at 4 °C overnight, changing the buffer at least three times. A His-bind affinity column was prepared as described by the supplier (Novagen), with slight modifications. His-bind resin was charged with 8 bed vols 50 mM NiSO₄ and equilibrated with 2 bed vols binding buffer. The dialysed protein solution was applied to a His-bind affinity column (bed vol, 2.5 ml). After unbound proteins had been washed off, PrtV was eluted with buffer containing 20 mM imidazole. The pooled active fractions were dialysed against 50 mM Tris/HCl (pH 8.0) and concentrated by ultrafiltration with Macrosep (Filtron) to approximately 1 ml. This protein solution was used for further characterization.

Preparation of the intracellular fractions. To compare the enzyme activity of intracellular with extracellular fractions, intracellular fractions were obtained as follows. Cells were harvested and suspended in 50 mM Tris/HCl (pH 8.0). The cell suspension was sonicated in an ice-bath and centrifuged at 8050 g for 20 min. After this procedure, the intracellular fractions were divided into supernatant (soluble form) and pellet (insoluble form or inclusion body). Finally, the insoluble form was resuspended in 50 mM Tris/HCl, pH 8.0 containing 8 M urea.

SDS-PAGE and isoelectric focusing. The proteins were separated on a 7.5% SDS-polyacrylamide gel as described by Laemmli (1970). Isoelectric focusing was performed with an Ampholine PAGplate (Pharmacia), a precast polyacrylamide gel containing carrier ampholytes that generate a linear pH gradient from pH 3.5–9.5 during electrophoresis. The purified enzyme was applied to the Ampholine PAGplate, and electrophoresed for 1.5 h at 1500 V. Bands were detected by Coomassie staining. The pI of PrtV was estimated by determining its relative mobility in the pH gradient.

Determination of molecular mass. Estimation of the molecular mass of the PrtV collagenase was made by gel filtration chromatography using a Sephadex G-100 column (Pharmacia). The molecular mass of the PrtV was also estimated by 10% SDS-PAGE.

Determination of N-terminal amino acid sequence. Electroblotting of proteins to PVDF Immobilon membrane (Millipore) for protein sequence determination was performed according to the method of Matsudaira (1987). The N-terminal sequence of PrtV was determined by Edman degradation, using a model 477A pulse-liquid protein sequencer (Applied Biosystems).

Amino acid analyses. Protein samples (10–30 µg) were hydrolysed with 6 M HCl/trifluoroacetic acid (4:1, v/v) for 3 h at 130 °C and analysed for amino acid composition on a Hitachi 835 amino acid analyser.

Effects of temperature and pH on enzyme activity. The effect of temperature was determined with azocoll at various temperatures (25–90 °C), and the enzyme activity was measured as described above. For determination of thermostability, the enzyme was preincubated at the indicated temperature for 15 min and the residual enzyme activity was measured. To determine the effect of pH on enzyme activity, the enzyme was assayed with azocoll in the following buffers: 50 mM acetate buffer (pH 3·6–5·6), phosphate buffer (pH 5·7–8), Tris/HCl (pH 7·2–8·8) and glycine/NaOH (pH 8·6–10·4). For the determination of pH stability, the enzyme was preincubated at 4 °C in the indicated buffer for 1 h and the remaining activity was measured.

Effect of inhibitors and metal ions on enzyme activity. The inhibitors EDTA, EGTA, 1,10 phenanthroline, PMSF, E-64 [L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane] and pepstatin A (Sigma) were added to the enzyme and incubated at 4 °C for 1 h before the assay. The residual enzyme activity was then measured with azocoll. To test the reactivation effects of metal ions, the enzyme was inactivated with 5 mM EDTA and the metal-chelator complex was removed using Sephadex G-25 (Pharmacia). Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Mg²⁺, Mn²⁺, Ni²⁺ or Zn²⁺ was added at various concentrations to activate the enzyme, and the mixtures were then incubated at 4 °C for 1 h. The restored activities against azocoll were measured.

Effects of denaturants, detergents and reducing agents on enzyme activity. The reagents were added to the enzyme and incubated at room temperature for 1 h before the activity assay. The reagents used were urea and guanidine-HCl as denaturants, SDS and Triton X-100 as detergents, and β -mercaptoethanol and DTT as reducing agents.

RESULTS

Expression of the *prtV* gene in *E. coli*

The 1·8 kb *EcoRI*–*HindIII* fragment containing the *prtV* gene was ligated into pET21b(+) in the same orientation as the T7 promoter, and the recombinant plasmid, pPRT2, was transformed into *E. coli* BL21(DE3) (Lee & Liaw, 1996). The resulting transformant was grown in LB medium until the OD₆₀₀ reached 0·4–0·6 and then

Fig. 1. SDS-PAGE analysis of PrtV. Lane 1, protein molecular mass markers; lane 2, the crude enzyme before loading onto His-bind column (5 µg); lane 3, PrtV purified by His-bind column (5 µg).

induced by adding 1 mM IPTG. Although IPTG induction slowed the growth of *E. coli* BL21(DE3), enzyme activity dramatically increased after the IPTG was added (Table 1). After 5 h induction, the highest activity (38·14 U mg⁻¹) was detected in the culture supernatant. Enzyme activity in intracellular fractions was also detected. The intracellular fractions were divided into soluble and insoluble (inclusion body) form. The culture supernatant was the main fraction containing enzyme activity; its activity was about 16 and 90 times higher than that of the soluble and insoluble form, respectively. This result also indicates that the PrtV can be secreted by *E. coli* BL21(DE3).

Purification of PrtV by affinity column

The *prtV* gene on pPRT2 encodes PrtV tagged with an extension of six histidyl residues (6×His) at the carboxyl terminus, which was used for purifying the

Table 1. Effect of inducer on the growth stage and production of PrtV by *E. coli* BL21(DE3)(pPRT2)

Incubation time (h)	Cell density (<i>A</i> ₆₀₀)*		Specific activity (U mg ⁻¹)†	
	Inducer absent	Inducer present	Inducer absent	Inducer present
0	0·646	0·662	0·48	0·31
1	0·966	0·873	0·25	4·30
2	1·116	0·867	1·27	26·74
3	1·214	0·853	4·50	30·13
4	1·318	0·834	11·43	34·08
5	1·369	0·839	20·68	38·14
6	1·369	0·841	23·29	34·23
7	1·318	0·849	24·98	32·79

* Cells were grown at 37 °C, shaking at 150 r.p.m. in LB.

† The supernatant was collected by centrifugation, then azocoll was used to determine enzyme activity.

Fig. 2. Isoelectric focusing of PrtV. The cathode was at the top of the gel. Lane 1, pI standards; lane 2, purified PrtV (10 µg). The gel was stained with Coomassie brilliant blue R-250.

protein from a His-bind affinity column. From 400 ml culture supernatant, 2.5 mg of purified protein was obtained. The PrtV (with a molecular mass of 64 kDa) was visible on 7.5% SDS-polyacrylamide gels (Fig. 1). The specific activity against azocoll and the recovery of PrtV are 113.07 U mg^{-1} and 77%, respectively.

Physical characteristics of PrtV

Analysis of the purified PrtV by SDS-PAGE yielded a band with a molecular mass of 64 kDa. However, the molecular mass of PrtV, as determined by the Sephadex G-100 column, was 62 kDa. Therefore, the similarity of the estimated molecular mass by SDS-PAGE and gel filtration suggested that PrtV is a single polypeptide chain with no subunit structure. In addition, isoelectric focusing of the purified PrtV in a native gel revealed an isoelectric point of pH 8.52 (Fig. 2).

N-terminal sequence and amino acid composition of PrtV

The nucleotide sequence of the *prtV* gene was reported in our previous study (Lee *et al.*, 1995). The cloned DNA fragment contained a 1761 bp ORF encoding a predicted 587 amino acid protein. The predicted amino acid

Fig. 3. Partial deduced amino acid sequence of PrtV from *Vibrio parahaemolyticus* (Lee *et al.*, 1995). The N-terminal sequence determined by Edman degradation is underlined. The arrow pointing downward indicates the signal peptide cleavage site. For characterization of the signal peptide, the symbols + and • represent basic charged and polar residues, respectively.

Fig. 4. The hydropathy profile of PrtV. The profile starts from the initiating methionine residue. The portions below the midpoint line indicate hydrophilic regions.

sequence from position 28 to 42 corresponded to the N-terminus of purified PrtV, as determined by Edman degradation (Fig. 3). This indicated that 27 amino acid residues of signal peptide were excised during protein translocation, instead of 25 amino acids residues predicted in our previous paper (Lee *et al.*, 1995). Bacterial signal peptides usually contain up to three domains (N, H and C) (Pugsley, 1993). The N domain (2–15 residues or more) is polar and carries a net positive charge. The H domain (7–15 residues) is composed of predominantly hydrophobic residues and is required to initiate translocation across the cytoplasmic membrane. The C domain precedes the cleavage site. Characterization of the PrtV signal peptide is shown in Fig. 3; it resembles a typical signal peptide sequence for secreted proteins of prokaryotic origin. Moreover, the signal peptidase cleavage site (Ala²⁷–Gln²⁸) conforms to the $-3, -1$ rules (von Heijne, 1986). The hydropathy of the PrtV along the amino acid sequence was calculated by the method of Kyte & Doolittle (1982), and is shown in Fig. 4. The mean hydropathy of the first 27 amino acids and the remainder of the protein (residues 28–587) is 0.69

Table 2. Amino acid composition of PrtV (residues mol⁻¹ PrtV)

Two amino acid residues, Cys and Trp, were not detected under acid hydrolysis conditions. Since the indole structure was completely destroyed, Trp was detected as Gly. In addition, acid hydrolysis also releases NH₃ from the side chain of Asn and Gln; these residues are then detected as Asp and Glu. ND, Not determined.

Amino acid	Composition derived from:	
	Nucleotide sequence*	Analysis of purified PrtV†
Asp	39	
Asn	33	
Asp + Asn	72	73 ± 1.4
Thr	25	25 ± 0.4
Ser	36	41 ± 5.7
Glu	37	
Gln	32	
Glu + Gln	69	76 ± 2.2
Pro	19	26 ± 0.6
Gly	29	39 ± 1.8
Ala	70	67 ± 2.5
Val	32	35 ± 1.1
Met	14	10 ± 0.8
Ile	14	12 ± 1.7
Leu	54	52 ± 0.9
Tyr	27	22 ± 2.1
Phe	24	21 ± 2.2
His	16	14 ± 0.6
Lys	14	15 ± 0.9
Arg	31	34 ± 0.8
Cys	5	ND
Trp	9	ND

* Based on a molecular mass of 62 947 (Lee *et al.*, 1995).

† Based on a molecular mass of 62 000. The values are the mean of triplicate determinations (± SD).

and -0.41, respectively. The hydropathy pattern also suggests the presence of a signal peptide in the N-terminal portion of PrtV. In addition, comparison of the amino acid composition of purified PrtV with that predicted from the DNA sequence shows satisfactory agreement (Table 2). As suggested by the high isoelectric point, PrtV was rich in basic residues (histidine, arginine and lysine), which represented 11% of the protein hydrolysate.

Effect of temperature and pH on enzyme activity

The optimal temperature for PrtV was 40 °C. Preincubation of PrtV at 25–45 °C did not cause significant loss of enzyme activity, while at 50 °C, it resulted in a 20% loss. Preincubation at temperatures above 55 °C led to complete loss of activity. The optimal pH for enzyme activity was pH 7.5. PrtV retained at least 80%

of its original activity during preincubation at pH values ranging from pH 3.6–10.4, and retained 90% during preincubation at above pH 5. This indicates that PrtV is much more stable in alkaline than acidic conditions.

Effect of inhibitors, metal ions and chemical interference on enzyme activity

The enzyme activity of PrtV was inhibited by the metal chelators EDTA, EGTA and 1,10-phenanthroline at concentrations of 1 and 10 mM. In contrast, PrtV was resistant to serine and cysteine protease inhibitors such as PMSF and E-64. It was also resistant to pepstatin A, an inhibitor of aspartate proteases. The sensitivity of PrtV to metal chelators indicates that PrtV is a metalloprotease. The inhibition of PrtV activity brought about by 5 mM EDTA could be restored by the addition of various metal ions. The metal ions most effective in restoring activity were Co²⁺ and Mn²⁺ (100 µM), with restoring activities of 62 and 77%, respectively. However, significant reactivation was also observed with Ca²⁺, Cu²⁺, Ni²⁺ and Zn²⁺. The activity of PrtV was also inhibited by protein denaturants, such as urea and guanidine-HCl. PrtV retained 86% of its original activity in the presence of 2 M urea, but only 6% with 4 M urea. However, guanidine-HCl completely inhibited the activity (2–8 M). It therefore appears that guanidine-HCl inhibits PrtV activity more effectively than urea. SDS is an anionic detergent that thoroughly inhibited the activity of PrtV (0.2–10%, w/v). In contrast, Triton X-100 (a nonionic detergent) had no inhibitory effect on enzyme activity even at 10%, and it showed the property of stimulation of enzyme activity. Reducing agents, such as β-mercaptoethanol and DTT, decreased the activity of PrtV as the concentration increased. With β-mercaptoethanol and DTT at a concentration of 20 mM, the enzyme retained 37 and 74% of its original activity, respectively. These results also indicate that β-mercaptoethanol is more effective than DTT in inhibiting PrtV activity.

Kinetic parameters for the hydrolysis of FALGPA and type I collagen by collagenase

A comparative study of the kinetic analysis of collagenase was undertaken with the synthetic substrate FALGPA and native type I collagen. FALGPA is hydrolysed more rapidly by collagenases than any other commonly used synthetic substrate but is not cleaved by the well-known proteases such as trypsin, elastase or thermolysin (Van Wart & Steinbrink, 1981). In addition to PrtV, VAC and CHC were also used in this study. Compared with CHC, PrtV showed a higher binding affinity (1.06 mM) with FALGPA, while VAC did not bind and degrade FALGPA (Table 3). In addition, the specific constant (reaction velocity of each collagenase at the same substrate concentration) showed no significant differences between PrtV and CHC. In contrast, for the hydrolysis of type I collagen (Table 3) CHC showed a lower K_m (13.86 mg ml⁻¹) and a higher specific constant (7.14 U ml⁻¹) than those for both PrtV and

Table 3. Substrate specificity and kinetic constants of the PrtV collagenase

Assays were carried out in 50 mM Tricine, 0.2 M NaCl, 10 mM CaCl₂, pH 8.0 at 25 °C for FALGPA and 37 °C for type I collagen. The collagenase concentration was 10 µg ml⁻¹. –, Undetectable.

Enzyme	FALGPA			Type I collagen		
	K_m (mM)	V_{max} (µkat kg ⁻¹)	Specific constant ($V_{max} K_m^{-1}$)	K_m (mg ml ⁻¹)	V_{max} (U ml ⁻¹)	Specific constant ($V_{max} K_m^{-1}$)
PrtV	1.06	4.41×10^8	4.16×10^8	68.68	27.32	0.40
VAC	–	–	–	60.00	14.37	0.24
CHC	14.82	5.82×10^9	3.9×10^8	13.86	99.01	7.14

VAC. In general, based on the specific constant, PrtV and CHC showed a similar specificity for FALGPA, but CHC had a higher specificity for type I collagen than PrtV (18-fold) and VAC (30-fold).

DISCUSSION

The *prtV* gene, encoding an extracellular protease of *V. parahaemolyticus*, has been cloned and sequenced as previously reported (Lee *et al.*, 1995). The deduced amino acid sequence of PrtV shows 32% identity with that of the collagenase of *V. alginolyticus*. In this study, the *prtV* gene has been subcloned into expression vector pET21b(+), and the PrtV protein was purified in a single step using a His-bind affinity column and further characterized. The molecular mass of the mature PrtV, as determined by gel filtration, is 62 kDa, which correlates well with the value analysed by SDS-PAGE. The first 12 N-terminal residues, Gln-Asn-Gln-Cys-Ala-Val-Ala-Asp-Leu-Gln-Gln-Ser, completely matched those deduced from the nucleotide sequence. This reveals that PrtV is synthesized as a precursor with a 27 amino acid signal peptide and a 560 amino acid extracellular mature form. The 27 residue signal peptide derived from *V. parahaemolyticus* can be recognized by the peptidase in *E. coli*. Therefore, further investigations will be done to assess the potential of the PrtV signal peptide for the secretion of heterologous proteins from *E. coli*.

Since PrtV degrades native collagens, it can be classified as a collagenase. Collagenases specifically cleave the typical collagenous sequence, -Pro-X↓-Gly-Pro-, whether the molecule is in the native or denatured form (Keil, 1979) and where X can be almost any amino acid. Comparing the collagenases of bacteria and vertebrates, the former show broad substrate specificity and degrade both native and denatured collagens (Peterkofsky, 1982), while the latter preferentially cleave the native form at a specific site and other proteases are necessary for complete degradation (Birkeday-Hansen, 1987). The destructive power of bacterial collagenase has been widely used for the disintegration of connective tissue, separation of tissue culture cells (Wolters *et al.*, 1995), selective cleavage of gene products in biotechnology

(Sassenfeld, 1990), improving the tenderness of meat (Miller *et al.*, 1989) and also for clinical therapy to eliminate necrotic tissue from burns, ulcers and decubitus (Ohmi *et al.*, 1989; Sank *et al.*, 1989).

Many bacterial collagenases have been widely reported. The first bacterial collagenases to be studied in detail were those produced by *Clostridium histolyticum* (EC 3.4.24.3) (Mookhtiar & Van Wart, 1992). Seven collagenases (α , β , γ , δ , ϵ , ζ , η) were purified to homogeneity from the culture supernatant and all were fully characterized. Recently, the *colH* gene, encoding a collagenase and a gelatinase, was cloned from *C. histolyticum* (Yoshihara *et al.*, 1994), and recombinant *colH* was successfully expressed in *Bacillus subtilis* (Jung *et al.*, 1996). *colA*, encoding a 120 kDa collagenase from *Clostridium perfringens*, was cloned in *E. coli*. This collagenase showed significant homology with VAC and *Achromobacter lyticus* protease I, suggesting that these three enzymes are evolutionarily related (Matsushita *et al.*, 1994). Purification of hemorrhagic toxin from *Clostridium sporogenes* suggested that hemorrhaging caused by the toxin depends on its collagenase activity (Hara-Kudo *et al.*, 1996). Two high-molecular-mass forms (90 and 110 kDa) of *V. alginolyticus* collagenases (formerly called *Achromobacter* collagenase, EC 3.4.24.8) have been purified and well characterized (Tong *et al.*, 1986). The DNA encoding the collagenase of *V. alginolyticus* has been cloned and its complete nucleotide sequence determined (Takeuchi *et al.*, 1992). Another *Vibrio* species, *Vibrio harveyi* (formerly called *Vibrio* B-30), produced two collagenases; some of the properties of the enzymes are similar to CHC, such as molecular mass, specificity and mode of collagen hydrolysis (Merkel & Dreisenbach, 1978). The *prtC* gene, encoding a collagenase, was isolated from *Porphyromonas gingivalis*, which is implicated as a periodontal pathogen (Kato *et al.*, 1992). Enzyme activity was enhanced by Ca²⁺ and inhibited by EDTA and thiol-blocking agents.

Because the activity of the collagenases described above was inhibited by EDTA and restored by adding metal ions, collagenases can be regarded as metalloproteases. The activity of PrtV was inhibited by EDTA, EGTA and 1,10-phenanthroline, but the activity was restored by

Co²⁺, Ni²⁺, Ca²⁺, Cu²⁺, Ni²⁺ and Zn²⁺. In addition, the primary sequence motif HEXXH was found in PrtV, suggesting that PrtV is a zinc metalloprotease (Lee *et al.*, 1995). Since six different metal ions could restore the activity of EDTA-inhibited PrtV, this implies that they function in a catalytic role. Participation of metal ions in CHC catalysis has been reported (Mookhtiar & Van Wart, 1992), with Zn²⁺, Co²⁺, Ni²⁺ and Cu²⁺ reconstituting apo-CHC, which contains no metal ions. However, all of the CHC contained a single zinc atom and a variable amount of calcium (Bond & Van Wart, 1984). For the hydrolysis of FALGPA by γ -CHC, Co²⁺ and Ni²⁺ decreased the K_m approximately twofold in comparison with Zn²⁺, while Cu²⁺ lowered the K_m 30-fold. In terms of K_{cat}/K_m^{-1} , the activity of γ -CHC followed the order Cu²⁺ > Co²⁺ > Zn²⁺ > Ni²⁺. Because of the variation in kinetic parameters of γ -CHC on metal substitution, the metal ions may play an important role in the binding and catalysis of the substrate. To resolve the content of metal ions and their role in catalysis in PrtV, further study is needed.

The proportion of hydrophobic amino acids in PrtV was approximately 47%, whereas in CHC and VAC (Keil-Dlouha, 1976) it was about 47 and 44%, respectively. In addition, it was found that PrtV bound very strongly to phenyl-Sepharose gels even at low ion concentrations (data not shown). Therefore, we suggest that PrtV is a highly hydrophobic protein. The hydrophobicity of the active domain is supported by the ability of the enzyme to hydrolyse insoluble hydrophobic substrates, such as collagen.

Comparative studies of the specificity of CHC and VAC were undertaken with native collagen and the synthetic substrate, Pz-Pro-Leu-Gly-Pro-D-Arg (Lecroisey & Keil, 1979; Keil, 1992). The stages of degradation of native collagen were observed by electron microscopy and automatic Edman degradation. CHC was shown to cleave native collagen at several sites, but not progressively from the N-terminus. VAC, however, cleaved native collagen preferentially at two sites corresponding to interbands 33–34 and 41–42. For the synthetic substrate, both collagenases cleaved the same bond at Gly-Pro. If the sequence Gly-Pro is changed to Gly-Ala, it is cleaved only by VAC. In fact, the specificity of the two collagenases to the collagen substrate differed greatly. To compare PrtV with CHC and VAC, further investigations will study the pattern for the digestion of native collagen by PrtV and, by substituting different amino acid residues, the specificity of PrtV with synthetic substrates.

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