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

Mei-Shiuan Yu and Chia-Yin Lee

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 diarrheal 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), chitobiase (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 septicaemia 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 dipptides (David et al., 1992). The serine protease gene (*vapT*) derived from *Vibrio metchnikovii* 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...
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 4.5kb HindIII 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’ CCCCCACTGAACTCTATTGTT 3’) and PRTR1 (5’ TCCGAAGCCTTGCATTG 3’), were designed to carry the EcoRI and HindIII recognition site, respectively. PCRs were performed with pLSD6 as the template, and primers PRTF1 and PRTR1 to amplify the 1.8 kb EcoRI-HindIII 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 signal sequence. This paper reports the expression and characterization of PrtV from V. parahaemolyticus in E. coli.

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 gel containing carrier ampholytes that generate a linear pH gradient from pH 3.5-9.5 during electrophoresis. The proteins were separated on a 7.5% SDS-polyacrylamide gel as described by Laemmli (1970). 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-leucylamid-(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⁺, Fe³⁺, Fe²⁺, 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 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 x His) at the carboxyl terminus, which was used for purifying the

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

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Cell density (A₆₀₀)</th>
<th>Specific activity (U mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inducer absent</td>
<td>Inducer present</td>
</tr>
<tr>
<td>0</td>
<td>0.646</td>
<td>0.662</td>
</tr>
<tr>
<td>1</td>
<td>0.966</td>
<td>0.873</td>
</tr>
<tr>
<td>2</td>
<td>1.116</td>
<td>0.867</td>
</tr>
<tr>
<td>3</td>
<td>1.214</td>
<td>0.853</td>
</tr>
<tr>
<td>4</td>
<td>1.318</td>
<td>0.834</td>
</tr>
<tr>
<td>5</td>
<td>1.369</td>
<td>0.839</td>
</tr>
<tr>
<td>6</td>
<td>1.369</td>
<td>0.841</td>
</tr>
<tr>
<td>7</td>
<td>1.318</td>
<td>0.849</td>
</tr>
</tbody>
</table>

* 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.
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 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.
Terminal portion of PrtV. In addition, comparison of the point, PrtV was rich in basic residues (histidine, arginine predicted from the DNA sequence shows satisfactory triplicate determinations.

20% loss. Preincubation at temperatures above hydrolysate.

amino acid composition of purified PrtV with that agreement (Table 2). As suggested by the high isoelectric acid hydrolysis also releases NH, from the side chain of Asn and Gln; these residues are then detected as Asp and Glu. ND, Not determined.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Composition derived from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotide sequence*</td>
</tr>
<tr>
<td>Asp</td>
<td>39</td>
</tr>
<tr>
<td>Asn</td>
<td>33</td>
</tr>
<tr>
<td>Asp + Asn</td>
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</tr>
<tr>
<td>Thr</td>
<td>25</td>
</tr>
<tr>
<td>Ser</td>
<td>36</td>
</tr>
<tr>
<td>Glu</td>
<td>37</td>
</tr>
<tr>
<td>Gln</td>
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<tr>
<td>Glu + Gln</td>
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</tr>
<tr>
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<td>19</td>
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<tr>
<td>Gly</td>
<td>29</td>
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<td>Met</td>
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<tr>
<td>Ile</td>
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</tr>
<tr>
<td>Leu</td>
<td>54</td>
</tr>
<tr>
<td>Tyr</td>
<td>27</td>
</tr>
<tr>
<td>Phe</td>
<td>24</td>
</tr>
<tr>
<td>His</td>
<td>16</td>
</tr>
<tr>
<td>Lys</td>
<td>14</td>
</tr>
<tr>
<td>Arg</td>
<td>31</td>
</tr>
<tr>
<td>Cys</td>
<td>5</td>
</tr>
<tr>
<td>Trp</td>
<td>9</td>
</tr>
</tbody>
</table>

* 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 hydrophathy 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 decreased the activity (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 Kₘ (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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FALGPA</th>
<th>Type I collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mM)</td>
<td>(V_{max}) (μkat kg⁻¹)</td>
</tr>
<tr>
<td>PrtV</td>
<td>1.06</td>
<td>(4.41 \times 10^4)</td>
</tr>
<tr>
<td>VAC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CHC</td>
<td>14.82</td>
<td>(5.82 \times 10^9)</td>
</tr>
</tbody>
</table>

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., 1993). 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 pET2lb(+), 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\(\downarrow\)-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 (Birkedal-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 \((\alpha, \beta, \gamma, \delta, \xi, \zeta, \eta)\) 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 their activity in 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.

ACKNOWLEDGEMENTS

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