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Cloning of a Novel Collagenase Gene from the Gram-Negative Bacterium *Grimontia* (*Vibrio*) *hollisae* 1706B and Its Efficient Expression in *Brevibacillus choshinensis*▽

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The collagenase gene was cloned from *Grimontia* (*Vibrio*) *hollisae* 1706B, and its complete nucleotide sequence was determined. Nucleotide sequencing showed that the open reading frame was 2,301 bp in length and encoded an 84-kDa protein of 767 amino acid residues. The deduced amino acid sequence contains a putative signal sequence and a zinc metalloprotease consensus sequence, the HEXXH motif. *G. hollisae* collagenase showed 60 and 59% amino acid sequence identities to *Vibrio parahaemolyticus* and *Vibrio alginolyticus* collagenase, respectively. In contrast, this enzyme showed <20% sequence identity with *Clostridium histolyticum* collagenase. When the recombinant mature collagenase, which consisted of 680 amino acids with a calculated molecular mass of 74 kDa, was produced by the *Brevibacillus* expression system, a major gelatinolytic protein band of ~60 kDa was determined by zymographic analysis. This result suggested that cloned collagenase might undergo processing after secretion. Moreover, the purified recombinant enzyme was shown to possess a specific activity of 5,314 U/mg, an ~4-fold greater activity than that of *C. histolyticum* collagenase.

Bacterial collagenases are metalloproteases containing a consensus motif for zinc proteases, the HEXXH sequence and are capable of digesting both native and denatured collagen. They make multiple cleavages at the Y-Gly bond in repeating X-Y-Gly sequences within triple helical regions, where proline and hydroxyproline residues are most common in the X and Y positions, respectively (17). Because of their characteristics, bacterial collagenases have been widely used in biological experiment as tissue-dispersing enzymes, as well as in medical procedures such as the isolation of pancreatic islet cells for transplantation (14) and the treatment for Dupuytren's disease (6).

Much of our knowledge of bacterial collagenases has come from studies of the enzymes produced by *Clostridium histolyticum* (13, 15–17, 34). Analysis of the primary structure of the gene product from *C. histolyticum* has revealed that clostridial collagenases consist of three domains (catalytic domain, polycystic kidney disease [PKD] domain, and collagen-binding domain [CBD]) in their molecules. Moreover, CBD has utilized for anchoring molecule that growth factors fused to CBD can be functional to bind to collagen fibrils and maintain biological activities (21). On the other hand, one of the other well-investigated bacterial collagenases is *Vibrio alginolyticus* collagenase (7, 10, 11, 28). The collagenase activity of *V. alginolyticus* collagenase is higher than that of any other bacterial collagenase, and it was found highly efficient in debridement of necrotic burns, ulcers and decubitus. To date, bacterial collagenases have been purified from various species, and their genes have been cloned and sequenced (8, 12, 18, 24, 35). However, many collagenases have not yet been both enzymatically and structurally characterized.

Vibrio hollisae is a Gram-negative bacterium first described in 1982 (4) and recently reclassified as the novel genus *Grimontia* (29). *Grimontia* (*Vibrio*) *hollisae* has been reported as a toxic bacterium, whose toxin was clarified as thermostable direct hemolysin (22), and is primarily known to cause moderate to severe cases of gastroenteritis in healthy people (5). *G. hollisae* strain 1706B was isolated from seashore sand collected from the Shin-Kiba coast in Tokyo (27). This organism produces a collagenase with a very high specific activity in the presence of gelatin, and this enzyme even degrades the tanned leather (26). The characteristics of this organism and purified collagenase have been described in a series of papers (25–27). The properties of this collagenase are as follows: (i) it has a molecular mass of 60 kDa; (ii) it degrades insoluble collagen, soluble collagen, Z-GPLGP peptide, and Pz-PLGPR peptide, but not casein; (iii) it has an optimum pH of 7.0 to 8.0 for insoluble collagen hydrolysis; and (iv) it is stable in the range between pH 4.5 and 11 (25). In order to clarify its enzymatic characteristics and to utilize it for biological applications, the primary structure of the collagenase needs to be elucidate.

In the present study, we cloned and sequenced a novel collagenase gene from *G. hollisae* 1706B to elucidate its primary structure and demonstrated the expression and characterization of recombinant mature collagenase using the *Brevibacillus* expression system. Moreover, we discussed the characteristics of the corresponding amino acid sequence of this enzyme and its similarity to those of other bacterial collagenases.

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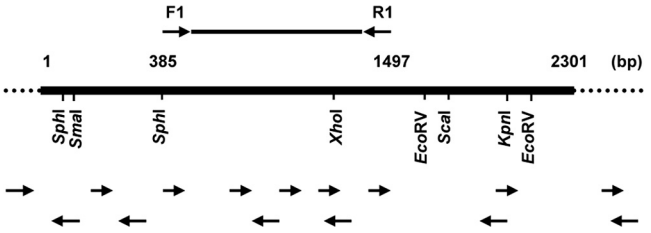

FIG. 1. Sequencing strategy for the collagenase gene inserted into pCC1BAC-2. The thick line represents the collagenase gene inserted into pCC1BAC-2 plasmid. The thin line indicates probe region used for cloning. The lower arrows indicate the direction of sequence determinations, starting from specific primers.

MATERIALS AND METHODS

Bacterial strains and plasmids. *G. hollisae* strain 1706B was obtained around the shore of Shin-Kiba, Tokyo, Japan, and used throughout the present study (27). The plasmid pGEM-T Easy and the *Escherichia coli* competent cells JM109 (Promega, Madison, WI) were used as subcloning vector and host, respectively. The expression plasmid vector pCC1BAC and the host TransforMaxEPI300 *E. coli* (Epicentre Biotechnologies, Madison, WI) were used to make a bacterial artificial chromosome (BAC) clone library of *G. hollisae*. *Bacillus brevis* expression vector pNY326 and *Brevibacillus choshinensis* S5 (Takara Bio, Shiga, Japan) were used for expression of recombinant proteins.

Construction of a genomic library from *G. hollisae* 1706B. The genomic DNA of *G. hollisae* 1706B was purified with a QIA genomic DNA extraction kit (Qiagen, Hilden, Germany). The purified DNA was digested with the restriction enzyme EcoRI and separated on a 0.6% (wt/vol) agarose gel. Only DNA fragments larger than 2 kb were ligated into the *E. coli* expression vector, pCC1BAC (Epicentre Biotechnologies). Then, pCC1BAC was transformed into TransforMaxEPI300 *E. coli* by electroporation, and the transformants were plated onto LB agar plates containing 50 μg of ampicillin/ml.

DNA probe preparation. Degenerate primers were designed based on the internal peptide sequence of original collagenase (see Fig. 2, box 6) for F1 and the consensus sequence of catalytic site from *V. alginolyticus* and *V. parahaemolyticus* collagenase (see Fig. 2, underlined) for R1. The primer sets F1 (5′-GAG GCNATCTTTAGCTCCAATCATATGTAYAAY-3′) and R1 (5′-ATCTAAGT AATGCACGTATTCATGYTCNAGRTT-3′) were used for amplification of probe sequence. Y, R, and N represent C/T, A/G, and A/C/T/G, respectively. Amplification was performed in 50 cycles of 0.5 min at 95°C, 0.5 min at 45°C, and 1 min at 72°C. The amplified 1,080-bp PCR product was electro-eluted from 1% agarose gel, ligated into pGEM-T Easy vector (Promega), and then transformed into *E. coli* strain JM109. The purified plasmid was used as a template to create digoxigenin (DIG)-labeled DNA probes using DIG-high prime labeling reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Screening of the genomic library with DNA probe. The genomic library was screened by colony hybridization according to the manufacturer's instructions (Roche). Briefly, the ampicillin-resistant transformants were blotted onto a nylon membrane (Roche) and lysed with 0.5 M NaOH. The denatured DNA was then immobilized, followed by protease K treatment. The DIG-labeled DNA probe was used for hybridization. Positive clones were picked from cultured LB agar plates and subcultured in LB liquid medium with ampicillin at 37°C. BAC DNA of collagenase-positive colonies was prepared by using the QIA genomic DNA extraction kit (Qiagen).

DNA sequencing and alignment of deduced amino acid sequence. Purified BAC DNA was amplified with cycle sequencing using a thermal cycler (Takara Bio) and sequenced with a DNA autosequencer (ABI Prism 310). Appropriate oligonucleotide primers (Sigma-Aldrich, St. Louis, MO) and Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) were used to walk along the sequence. The sequencing strategy for the pCC1BAC-2 insert was outlined in Fig. 1. Computer analysis of the DNA sequence data was performed using GenBank database and BLAST search programs. The deduced amino acid sequence alignment and homology data were generated by using the CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Recombinant collagenase preparation. pCC1BAC-2 was used as the DNA template. To add an NcoI site to the 5′ region and a HindIII site to the 3′ region of the mature collagenase gene, primers were designed as follows: forward, 5′-AAACCATGGCTTTCGCTGCGGTTGAACAGTGTGATCT-3′; and reverse, 5′-AAAAAGCTTTTACTGACGACACTGGTTAC-3′ (the restriction sites are underlined). The mature domain of 2.1-kb collagenase gene was amplified by using the Expand High Fidelity PCR system (Roche). After treatment with NcoI and HindIII, the double-digested fragment was ligated into the multiple cloning site of the *Brevibacillus* expression vector pNY326, which was located downstream of *Brevibacillus* signal sequence (pNY326-Col2). Plasmid pNY326-Col2, harboring the complete mature collagenase gene, was transformed into *Brevibacillus choshinensis* S5 to express the recombinant enzyme. The *Brevibacillus* transformant was aerobically cultured in 2SLN medium containing neomycin (50 μg/ml). After centrifugation, the supernatant was purified with a DEAE-Sepharose column (26 by 100 mm) with a fast-protein liquid chromatography (FPLC) system under a gradation of sodium concentrations (0.2 to 1.1 M NaCl). The column was eluted isocratically with 0.2 M NaCl–50 mM Tris-HCl (pH 7.5) for 10 min at 5 ml/min, followed by a linear gradient to 1.1 M NaCl for 50 min. The purified recombinant protein was concentrated by ultrafiltration with a 30-kDa cutoff (Pall, Port Washington, NY) and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) at 4°C.

SDS-PAGE. SDS-PAGE was carried out on 7.5 or 10% polyacrylamide gel according to the method of Laemmli (9) unless otherwise stated. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and then destained with 5% methanol and 7.5% acetic acid.

Determination of collagenolytic activity. The collagenolytic activity of recombinant collagenase was measured by using fluorescein isothiocyanate (FITC)-labeled type I collagen as previously described (20). Briefly, the enzyme solution was mixed with 50 mM Tris-HCl (pH 7.5) containing 0.05% FITC-labeled type I collagen, 5 mM $CaCl_2$, and 200 mM NaCl and incubated at 30°C for 30 min. After adding EDTA to stop the enzymatic reaction, the degraded FITC-labeled collagen fragment was extracted with 50 mM Tris-HCl (pH 9.5) containing 70% ethanol. The fluorescence intensity of the supernatant was measured by fluorescence spectrophotometry (530 nm [emission], 485 nm [excitation]). One unit of collagenolytic activity was defined as the amount degrading 1 μg of FITC-labeled collagen at 30°C per min. Protein concentrations were determined by using the Coomassie Plus–The Better Bradford Assay Reagent (Thermo Scientific, Rockford, IL). Collagenase from *Clostridium histolyticum* (Amano Enzyme, Nagoya, Japan) was used as a reaction standard. All assays were carried out in triplicate.

For the measurement of enzyme kinetics, 0.5 μg of enzyme was incubated with various amounts of FITC-labeled type I collagen (10 to 50 μg) at 30°C for 5 min, and the fluorescence intensity of the supernatant was measured. Specific collagenase substrate FALGPA [*N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala; Bachem AG, Bubendorf, Switzerland] was also chosen to determine the enzyme activity. An assay with FALGPA was performed according to the modified method of a previous report (31). Briefly, the enzyme and FALGPA were mixed in 50 mM Tricine buffer (pH 7.5) containing 0.4 M NaCl and 40 mM $CaCl_2$ and incubated at 30°C for 5 min. After incubation, the absorbance change at 345 nm was measured by using a Corona SH-9000 microplate reader (Corona Electric, Ibaraki, Japan). The FALGPA concentrations were varied from 0.5 to 3.0 mM. In the FALGPA assay, one unit of activity was defined as the amount degrading 1 μmol of FALGPA peptide at 30°C per min. The V_{max} and K_m values for hydrolysis of native collagen and FALGPA were estimated from the Lineweaver-Burk plot by using the reaction rates at different substrate concentrations.

Real-time zymography. Real-time zymography was performed as previously described (3). Briefly, recombinant collagenase was subjected to nonreducing SDS-PAGE using a 10% gel containing 0.05% FITC-labeled gelatin. After electrophoresis at 4°C, the gel was washed in 50 mM Tris-HCl (pH 7.5) containing 2.5% TritonX-100 for 30 min and then incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 200 mM NaCl at 37°C for 5 h. The collagenase-digested FITC-labeled gelatin was visualized by using a transilluminator.

The effect of protease inhibitors on collagenase activity was determined by adding the inhibitors to the incubation buffer. In inhibition studies, protease inhibitors such as EDTA, *o*-phenanthroline, *N*-ethylmaleimide (NEM), or phenylmethylsulfonyl fluoride (PMSF) were used at final concentrations of 20, 2.0, 5.0, and 1.0 mM, respectively.

Amino acid sequence. Amino acid sequence analysis of N-terminal or internal peptide fragments of original collagenase was performed as described previously (19). Briefly, internal sequences were determined by lysyl endoprotease, trypsin, and V8 protease digestion. The enzyme-cleaved fragments and purified collagenase were separated by SDS–10% PAGE and electrophoretically transferred to Immobilon-P (Millipore, Billerica, MA). The membrane was stained with Coomassie brilliant blue R-250, and the protein band was excised from the membrane and then washed extensively with deionized distilled water. The N-terminal sequence was analyzed by using a Procise 491 protein sequencer (Applied Biosystems, Carlsbad, CA). The detected fragments are shown in Fig. 2.

FIG. 2. DNA sequence and deduced amino acid sequence of *Grimontia hollisae* collagenase gene. The N-terminal amino acid sequences of the 74- and 60-kDa collagenases are indicated by box 1. Numbered boxes indicate biochemically identified peptides as follows: boxes 2, 3, and 4, lysyl endoprotease-digested fragments; box 5, trypsin-digested fragment; boxes 6 and 7, V8 protease-digested fragments. The zinc metalloprotease HEXXH consensus motif is underlined. The SD site is indicated by dotted line. The putative transcriptional terminator sequence is indicated by arrows.

Nucleotide sequence accession number. The determined nucleotide sequence was deposited in the DDBJ database under accession number AB600550.

RESULTS

Cloning of collagenase gene from *G. hollisae* 1706B. In order to amplify a fragment of the *G. hollisae* collagenase coding gene, degenerate primers were designed. A primer set yielded a single amplification product, and nucleotide sequencing revealed that the amino acid sequence deduced from this PCR product contained four partial peptide sequences (Fig. 2, boxes 2, 3, 4, and 7). Therefore, the plasmid containing this PCR product was used as a template to create DIG-labeled DNA probes as a hybridization probe for genomic library screening.

A partial genomic library was constructed with EcoRI-digested genomic DNA fragments from *G. hollisae* using a pCC1BAC vector system. The plasmids were transformed into TransforMaxEPI300 *E. coli* using electroporation, yielding about 5,000 colonies on LB-ampicillin plates. By using the colony hybridization technique, 30 positive clones were picked up from the library, and all of the purified BAC contained 2.3-kb inserts encoding the collagenase gene. One of the former clones, designated pCC1BAC-2, was chosen for further study. Restriction analysis revealed that pCC1BAC-2 contained a >50-kb EcoRI insert (data not shown).

Nucleotide sequence of *G. hollisae* collagenase gene. The nucleotide sequence of the pCC1BAC-2 insert was sequenced using the strategy outlined in Fig. 1. The obtained sequences were aligned by their overlaps to form a single contiguous sequence, and the 2.3-kb sequence of *G. hollisae* collagenase was determined (Fig. 2). The entire open reading frame (ORF) of *G. hollisae* collagenase was sequenced on both strands. Analysis of the sequence revealed a complete ORF extending from an ATG codon at nucleotide +1 to a TAA stop codon at position 2301, which encodes a protein of 767 amino acids (aa). A Shine-Dalgarno sequence (AGAAGAA) is observed 5 to 11 bp upstream from the ATG codon. A stem-loop sequence (nucleotide positions 2328 to 2347) with a short run of T's is present downstream of the termination codon.

The N-terminal amino acid sequence (Ala-Val-Glu-Gln-Cys-Asp-Lys-Ser-Gln) of the purified original enzyme corresponded perfectly to the deduced amino acid sequences of our determined sequence in position 262 to 288 downstream of the ATG codon. Moreover, the deduced amino acid sequence of ORF includes a zinc metalloprotease HEXXH consensus motif, which was detected as HEYVH in positions 1474 to 1488. It is known that the amino acid sequence HEXXH is important in facilitating the electron transfer with zinc in enzyme catalysis (30). Moreover, the internal peptide sequences were found to agree completely with the protein sequence deduced from DNA sequencing.

We next compared the deduced primary sequence of *G. hollisae* collagenase gene with other known protein sequences using the BLASTP programs of the National Center for Biotechnology Information. The predicted amino acid sequence of *G. hollisae* collagenase showed 59 and 60% identities with collagenase from *V. alginolyticus* (28) and *V. parahaemolyticus* (8), respectively (Fig. 3A). Moreover, the alignment predicted that *G. hollisae* collagenase consists of a pre-pro region (aa 1 to 87), a catalytic domain (aa 88 to 615), and a bacterial prepeptidase C-terminal (PPC) domain (aa 688 to 749) (33) (Fig. 3B). Furthermore, a database search revealed that *G. hollisae* collagenase showed <35% similarity to any other reported *Vibrio* metalloproteases (data not shown). On the other hand, *G. hollisae* collagenase showed 12 and 11% identities to ColG and ColH, respectively, from *Clostridium histolyticum* (data not shown).

Expression and characterization of recombinant collagenase in *Brevibacillus*. To examine whether the product of the *G. hollisae* collagenase gene possesses similar proteolytic activity as the original enzyme, we first made a construct for expressing the recombinant mature collagenase (aa 88 to 767) and produced the recombinant enzyme using the *Brevibacillus* expression system. When the collagenolytic activity of the culture supernatant of transformants (carrying pNY326-Col2) was measured using FITC-labeled collagen, the activity was found to be higher than that of mock transformants (data not shown). This result indicated that recombinant collagenase was successively produced.

To characterize certain biochemical properties, recombinant *G. hollisae* collagenase was purified from a *Brevibacillus* culture medium using a DEAE-Sepharose column with an FPLC system. The expression typically yielded 0.2 g of pure collagenase from 1 liter of culture. When the *Brevibacillus* culture medium and purified recombinant enzyme were confirmed by SDS-PAGE, three bands (74, 60, and 40 kDa) derived from this enzyme were detected (Fig. 4A, lane 3). The deduced molecular mass of mature protein, without the possible pre-pro peptide of 87 aa, was 74 kDa, and matched with the molecular mass determined by SDS-PAGE. The major 60-kDa form was the same molecular mass as the purified form from the original bacterial collagenase. Zymography showed that all three forms of collagenase possessed gelatinase activity; however, the activity of the 40-kDa form was weaker than that of the other forms (Fig. 4B). Moreover, the gelatinolytic band disappeared in the presence of the metal ion chelators, EDTA and *o*-phenanthroline, but not cysteine and serine protease inhibitors, such as NEM and PMSF (Fig. 4C). We also found that the three forms of collagenase have the same N-terminal amino acid sequence (data not shown), suggesting that the C-terminal region of the mature enzyme would be autodegraded and then become 60- and 40-kDa enzymes. Furthermore, the 60-kDa enzyme seems to be the most stable form. The purified recombinant enzyme could digest insoluble and soluble type I collagen, and a collagenolytic activity assay showed a specific activity of 5,841 U/mg using FITC-collagen, which was elevated by ~5.8-fold compared to the culture medium (data not shown). In contrast, this recombinant enzyme could not degrade casein (data not shown).

Kinetic parameters for the hydrolysis of type I collagen by collagenase. The kinetic parameters of collagenase were determined using native type I collagen and the synthetic peptide substrate, FALGPA (Table 1). *G. hollisae* collagenase showed a 4.2-fold lower K_m value and a slightly higher V_{max} value against FITC-collagen than *C. histolyticum* collagenase, resulting in a higher specific constant (~6.7-fold higher). On the other hand, the two enzymes have comparable substrate affinity to FALGPA, whereas the V_{max} values increased 25-fold in *G. hollisae* compared to *C. histolyticum* collagenase. As a result, *G. hollisae* collagenase showed a high specific constant

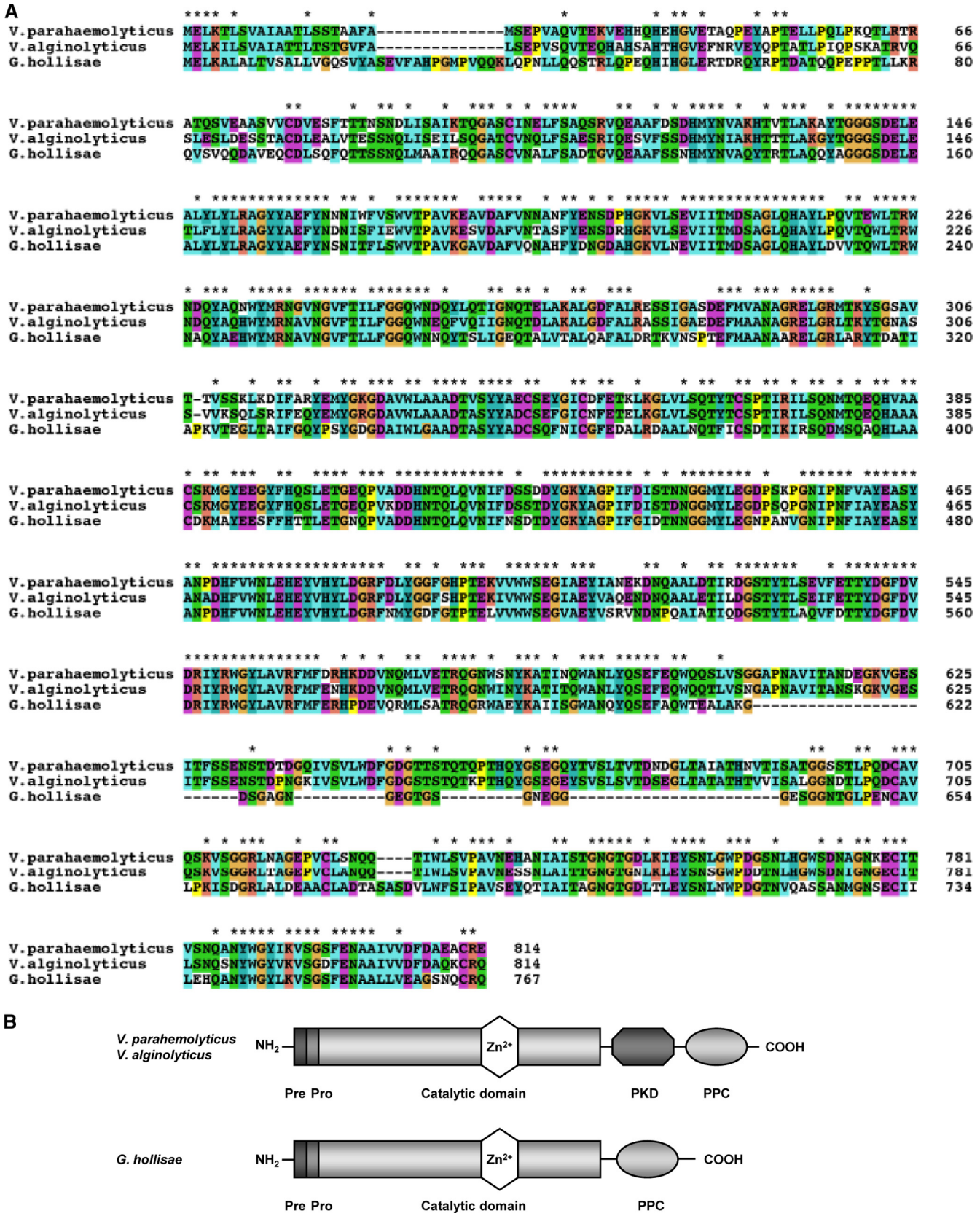

FIG. 3. Amino acid sequence comparison of *Grimontia hollisae* collagenase with homologous collagenase. (A) The amino acid sequences from *G. hollisae* (the present study), *Vibrio parahaemolyticus* (NP_797719), and *Vibrio alginolyticus* (CAA44501) were aligned by using the CLUSTAL W2 program. Identical residues among the three sequences are indicated by asterisks. (B) Schematic representation of the domain architecture of *G. hollisae* (the present study), *V. parahaemolyticus* (NP_797719) and *V. alginolyticus* (CAA44501). Pre, signal peptide; pro, putative pro-domain; PKD, polycystic kidney disease-like domain; PPC, pre-peptidase C-terminal domain.

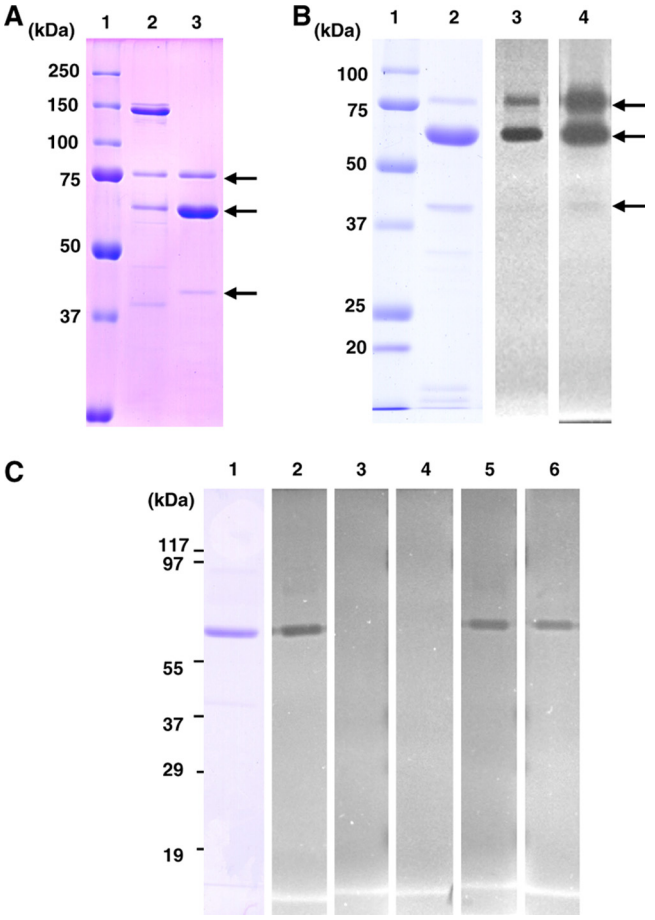

FIG. 4. Analysis of recombinant collagenase purified from *Brevibacillus* culture media. (A) Purified recombinant collagenase was analyzed by SDS-PAGE using a reducing 7.5% gel. Lane 1, molecular weight marker; lane 2, culture medium from *Brevibacillus* (carrying pNY326-Col2); lane 3, purified collagenase. (B) Real-time gelatin zymography using a nonreducing 10% gel. Lane 1, molecular weight marker; lane 2, SDS-PAGE of purified collagenase; lanes 3 and 4, gelatin zymogram of purified collagenase after 2 h (lane 3) or 19 h (lane 4) of incubation. (C) Inhibition assay using real-time gelatin zymography. Lane 1, SDS-PAGE of purified collagenase; lanes 2 to 6, gelatin zymogram of purified collagenase in the absence (lane 2) or presence of the inhibitors EDTA (lane 3), *o*-phenanthroline (lane 4), NEM (lane 5), and PMSF (lane 6).

also against FALGPA (~24-fold higher). These results help to explain the specific activity of the two enzymes. Previously, *V. parahaemolyticus* collagenase has been reported to have a K_m value of 1.06 mM toward FALGPA, at pH 8.0 and 25°C (35), and this K_m value was comparable to that of the present collagenase. Based on the specific constant, *G. hollisae* collagenase had a higher specificity for type I collagen and FALGPA than did *C. histolyticum* collagenase.

DISCUSSION

In the present study, we isolated the *G. hollisae* collagenase gene by a cloning and sequencing method. The isolated gene consisted of 2,301 nucleotides, and the 767-amino-acid protein deduced from the ORF revealed high homology to several *Vibrio* collagenases. Moreover, we succeeded in the effective expression of its recombinant enzyme in *Brevibacillus*. It hydrolyzed type I collagen, gelatin, and FALGPA peptide more efficiently than *C. histolyticum* collagenase, and the inhibition study showed that the enzyme was inhibited by metal ion chelators, such as EDTA, indicating that the cloned collagenase was a metalloprotease.

A BLASTP search indicated that the deduced amino acid sequences of *G. hollisae* collagenase show extensive homology with *V. alginolyticus* and *V. parahaemolyticus* metalloproteases. On the other hand, *G. hollisae* collagenase shared <20% identity with ColG and ColH from *C. histolyticum* and ColA from *C. perfringens* (data not shown). This result indicates that *G. hollisae* collagenase can be classified into the M9A subfamily in the MEROPS database (23). In the previous study, *Vibrio* metalloproteases were classified into three subgroups (class I, II, and III) based on the HEXXH sequence and substrate specificity (8). According to this classification, *G. hollisae* collagenase belongs to the class III group that includes *V. alginolyticus* and *V. parahaemolyticus*. However, *G. hollisae* collagenase has little caseinase activity (data not shown), while *V. alginolyticus* and *V. parahaemolyticus* collagenase reportedly possess apparent caseinase activity. One of the possible reasons for this difference in substrate specificity may be the difference in domain structures among these enzymes. Given that the PKD domain was absent from *G. hollisae* collagenase (Fig. 3), this domain might participate in caseinase activity when bound to substrate. These results indicate that *G. hollisae* collagenase should be classified into a new group among the

TABLE 1. Kinetic constants of *Grimontia hollisae* and *Clostridium histolyticum* collagenases[a]

Substrate	Enzyme strain	Sp act (U/mg)	Mean ± SD		
			K_m (mM)	V_{max} (mM/min)	Specific constant (V_{max}/K_m)
FITC-collagen	*G. hollisae*	5,314	$(2.83 \pm 0.76) \times 10^{-3}$*	8,889 ± 924†	$(3.18 \pm 0.27) \times 10^{6}$*
	C. histolyticum	1,289	$(11.8 \pm 1.95) \times 10^{-3}$	5,556 ± 962	$(0.48 \pm 0.04) \times 10^{6}$
FALGPA	*G. hollisae*	7.40	2.41 ± 0.19	33.2 ± 1.11*	13.8 ± 0.81*
	C. histolyticum	0.39	2.40 ± 0.52	1.35 ± 0.12	0.57 ± 0.07

[a] The activities of *G. hollisae* and *C. hystolyticum* collagenase were determined by using FITC-labeled collagen (FITC-collagen) or the synthetic peptide substrate FALGPA. Assays were carried out in 50 mM Tris-HCl–0.3 M NaCl–10 mM $CaCl_2$ (pH 7.5) at 30°C for the FITC-labeled collagen or 50 mM Tricine–0.4 M NaCl–40 mM $CaCl_2$ (pH 7.5) at 30°C for FALGPA. Each collagenase was used at the amount of 0.5 μg for FITC-collagen. When used for FALGPA, the amounts of *G. hollisae* and *C. hystolyticum* collagenase were 1.0 and 20 μg, respectively. The data represent the means three separate experiments. *, $P < 0.01$; †, $P < 0.05$.

Vibrio collagenases and suggest that the three enzymes, which differ in function and origin, are evolutionary related.

Surprisingly, we found that *G. hollisae* collagenase possessed markedly greater activity compared to *C. histolyticum* collagenase (Table 1). Since *G. hollisae* collagenase showed high affinity to native collagen and high catalytic activity to FALGPA compared to *C. histolyticum* collagenase, these results suggested that the degradation mechanism of the two collagenases appear to be different against collagen or gelatin. However, the *C. histolyticum* collagenase used in the present study should be considered a commercially available enzyme, which is a purified native protein. Since we found that CBD and PKD domains were absent from this purchased enzyme by SDS-PAGE (data not shown), this result raises the possibility that the loss of CBD and PKD domains may lead to a decrease in activity of this purchased enzyme compared to the intact form.

Analysis of kinetic parameters led to our considerable interest in why and how *G. hollisae* collagenase degrades collagen effectively. Because collagen is highly resistant to most proteases, collagenase seems to possess an effective degradation mechanism against collagen. For example, a recent study indicated that mammalian collagenases locally unwind the triple-helical structure through the coordinated action of the catalytic domain and collagen-binding domain (called the hemopexin domain) and then hydrolyze the peptide bonds (1). Moreover, the PKD domain of deseasin MCP-01, which is a bacterial collagenolytic serine protease, is reported to bind collagen and to swell collagen fascicles, suggesting that the PKD domain may improve the collagenolytic efficiency of the catalytic domain (32, 36). However, *G. hollisae* collagenase possesses neither a CBD nor a PKD domain. Since CBD has been reported to be necessary for the collagenolytic activity of mammalian and bacterial collagenase (2, 16), *G. hollisae* collagenase may contain an unidentified CBD in the 60-kDa form and/or possess a novel degradation mechanism against collagen. The domain structure-function relationship remains to be clarified in order to elucidate the mechanism of action of this enzyme.

It is noteworthy that the recombinant collagenase of *G. hollisae* was produced with stable activity using the *Brevibacillus* expression system. Since *Brevibacillus* is a Gram-positive bacterium, this system leads to the expression of recombinant proteins with low endotoxin contamination, which has been known to enhance the immunological response of higher animals. In addition, this recombinant enzyme can be used for dispersion of human fibroblasts in collagen gel and appears to have no obvious cytotoxicity (data not shown). Therefore, it can be utilized for biological applications, specifically for medical applications.

In conclusion, we cloned a novel collagenase gene from *G. hollisae* 1706B and produced a high yield of recombinant enzyme by using the *Brevibacillus* expression system. Moreover, we provided evidence that this enzyme showed higher collagenolytic activity than *C. histolyticum* collagenase, indicating that *G. hollisae* collagenase is suitable for both basic and applied research.

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