J. Microbiol. Biotechnol. (1998), **8**(3), 245 – 250

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning and Expression of a Collagenase Gene from the Marine Bacterium *Vibrio vulnificus* CYK279H

KIM, BONG JO, HAK-JU KIM, SUN-HEE HWANG, SEOUNG-KWON BAE, SOON-DUCK HA[1], JONG-DEOG KIM[2], AND JAI-YUL KONG*

Department of Biotechnology & Bioengineering, Pukyong University, 599-1 Daeyeon-Dong, Nam-Ku, Pusan 608-737, Korea
[1]Department of Applied Biotechnology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-Ku, Tokyo 113, Japan
[2]Department of Biological Engineering, Yosu National Fisheries University, San 96-1 Doonduk-Dong, Yosu, Chunnam 550-250, Korea

Received: February 6, 1998

Abstract A gene encoding an extracellular collagenase from the marine bacterium *Vibrio vulnificus* CYK279H was cloned into *E. coli* JM83 using the multicopy plasmid vector pUC19. The cloned strain of recombinant *E. coli* showing collagenase activity had an insert fragment of 3.5 kb and was named *E. coli* JM83/pKCL 279H. The cloned strain produced two different collagenase during cultivation. These enzymes, named collagenase-I and -II, were purified from the culture supernatant. SDS-PAGE indicated that collagenase-I had a molecular weight of 41 kDa and collagenase-II had a weight of 37 kDa. The N-terminal amino acid sequence of collagenase-I from the cloned strain, *E. coli* JM83/pKCL279H was determined and was not found to be similar to any other known collagenases. The optimum pH and temperature of the purified collagenase-I were 7.8 and 37°C, respectively.

Key words: *Vibrio vulnificus*, marine bacterium, collagenase, cloning, purification

Collagenases (EC 3.4.23.3), which cleave the structure of collagen with a glycylprolyl sequence in native insoluble collagen, have potential applications to molecular biological, medical, and chemical fields [23]. For example, the enzyme disperses mammalian cells by hydrolyzing collagen, and the enzyme from *Clostridium histolyticum* has been used as a reagent to cleave the fusion protein linked to β-galactosidase via the collagenase recognition sites. Collagenases have been found in several bacteria, including *Clostridium histolyticum* [21], *Streptomyces* sp. [5], *Cytophaga* sp. [20], *Vibrio arginolyticus* [17], and *Vibrio* B-30 [14]. However, only a few reports on the cloning and sequencing of collagenase genes have been published [7, 22] and most of these bacteria have been isolated from a soil environment. In our previous work, a collagen degrading bacterium was newly isolated from the South Sea of Korea and was identified as *Vibrio vulnificus* and named *Vibrio vulnificus* CYK279H [8]. This strain produced an extracellular collagenase which had high degrading activity with gelatin and collagens as substrates. This paper describes the cloning and expression of a collagenase gene from this bacterium into *E. coli* JM83. We also investigated the purification of collagenase produced by the cloned strain.

*Corresponding author
Phone: 82-51-620-6181; Fax: 82-51-620-6181;
E-mail: kongjy@dolphin.pknu.ac.kr

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Vibrio vulnificus CYK279H was grown at 28°C for 18 h with vigorous shaking in a modified marine medium (5 g peptone, 1 g yeast extract, 0.1 g ferric citrate, 0.0016 g ammonium nitrate, 0.008 g disodium phosphate, 23.4 g NaCl, 0.7 g KCl, 10.6 g $MgCl_2 \cdot 12H_2O$, 0.2 g K_2HPO_4, 6.05 g Tris-base, 10 g gelatin in 1 l of distilled water, pH 7.8). *E. coli* JM83 was grown at 37°C for 18 h in Luria Broth medium (5 g yeast extract, 10 g tryptone, 5 g NaCl in 1 l of distilled water, pH 7.4).

Enzymes and Chemicals

Lysozyme, pancreatic RNase A, agarose (type I), collagen (insoluble type I from bovine achielles tendon) and gelatin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All restriction enzymes, DNA modifying enzymes including T4-DNA ligase and calf intestinal

alkaline phosphatase (CIP) were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). A *Hin*dIII-digest λ DNA fragments used as a DNA molecular size marker was purchased from Kosco Biotech Co. (Seoul, Korea). All other chemicals were of analytical grade.

Cloning of *Vibrio vulnificus* CYK279H Collagenase Gene

Genomic DNA was extracted from *Vibrio vulnificus* CYK279H and partially digested with *Hin*dIII. The digested fragments were ligated with T4 DNA ligase into the *Hin*dIII site of plamid pUC19 at 16°C for 18 h. The ligated DNA was transformed into *E. coli* JM83 by the conventional calcium chloride procedure [3, 10, 19]. Transformants were selected by visual inspection from the agar plate containing gelatin (30 mg/ml), ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 200 δ/ml in dimethylformamide) and Isopropyl-β-thiogalactopyranoside (IPTG; 200 μg/ml) after incubation at 37°C for 12 h [9]. The selected transformants were treated with Frazier solution (15 g $HgCl_2$, 20 ml 12 M HCl in 100 ml of distilled water) for 1 min and the extracellular enzyme activity was identified [6]. To determine the intracellular enzyme activity, the selected transformants on the agar plate containing 3 mg gelatin/ml were placed in the desiccator saturated with chloroform for 1 h, and then these were overlaid with 8 ml of soft agar (10 mM Tris · HCl containing 2 mg agarose/ml and 0.5 mg lysozyme/ml, pH 8.0) and were incubated for 16 h at 37°C. The colonies of these trasformants were treated with Frazier solution [23] and the clear zone of gelatin digestion was verified.

Restriction Endonuclease Mapping of Recombinant Plasmid DNA

The following enzymes were used in the construction of plasmid restriction maps: *Hin*dIII, *Dra*I, *Sca*I, *Sac*I, *Pst*I, *Kpn*I, *Sph*I, *Sma*I, *Eco*RI, and *Mlu*I. A combination of single and double digests using appropriate enzymes allowed the unambiguous construction of maps for each of the plasmids [1]. λDNA digested with *Hin*dIII was used as a size marker.

Enzyme Assay

(i) *Insoluble collagen (type I)* as substrate. The reaction mixture contained 10 mg of insoluble collagen, 0.8 ml of 50 mM Tris · HCl (pH 7.5), and 0.2 ml of enzyme solution. The reaction mixture was incubated at 37°C with shaking and the reaction was stopped at various incubation times by the addition of 1.0 ml of 0.1 N acetic acid. The initial rate of increase in the generation of free amino groups was measured by the Ninhydrin method [18]. The reaction mixture was centrifuged at 10,000×g for 15 min and 0.1 ml of the supenatant was taken. The supernatant was added with 1.4 ml of 0.2 M sodium citrate buffer (pH 5.5) and 1.0 ml of 2% ninhydrin in methylcellosolve containing 0.05% $SnCl_2 \cdot H_2O$ and the mixture was heated at 100°C for 15 min.

(ii) *Gelatin as substrate.* Quantitative estimation of the collagenase activity was determined with the initial rate of increase in the generation of free-amino groups by the Ninhydrin method. Gelatin was dissolved in water by boiling. The reaction mixture was composed of 0.3 ml gelatin solution, 0.3 ml of 150 mM Tris · HCl containing 12 mM $CaCl_2$ (pH 7.5), and 0.1 ml enzyme solution. The reaction mixture was incubated at 37°C and the reaction was stopped at various incubation times by addition of 0.6 ml of 0.1 M HCl. After the reaction, these reaction mixtures were cooled with ice water and the absorbance was measured at 570 nm. The specific activity was expressed as mmol of leucine equivalent per min per mg protein.

$$\text{Specific activity (U/mg-protein)} = \frac{\text{reduction amino acid (mmol)}}{\text{reaction time (min)} \times \text{protein amount (mg)}}$$

Protein Measurement

Protein concentration was measured colorimetrically by Lowry's method [12] with bovine serum albumin as the standard.

Zymography of Collagenase

Collagenolytic activity of the crude collagenase from the supernatant of the culture broth was analyzed by zymography on SDS-polyacrylamide gel containing 0.1% gelatin. The crude collagenase was mixed with an equal volume of SDS buffer containing 4% (w/v) sodium dodecyl sulfate, 125 mM Tris · HCl (pH 6.8), 10% (v/v) glycerol, and then electrophoresed without heating. Then, the separated collagenase on the gel was renatured in 2.5% triton X-100 containing 50 mM Tris · HCl (pH 7.5) and 0.1 M NaCl at room temperature for 1 h, followed by incubation in 50 mM Tris · HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 37°C for 20 h. The resultant gel was stained with Coomassie Brilliant Blue R-250.

Purification of Collagenase

E. coli JM83 transformant was grown at 37°C for 18 h in 2 l of LB medium containg 100 μg ampicillin per ml [11, 15]. The cell were removed by centrifugation and the supernatant was precipitated with 70% ammonium sulfate. Dialysis of the pellet was done for desalting and the desalted solution was added to a column (2.5×40 cm) of DEAE Sepharose CL-6B (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The enzyme was eluted with a gradient of 0.0~0.5 M NaCl solution.

The eluted active fraction was put in a Sephadex G-100 column (2.0×50 cm, Pharmacia, Uppsala, Sweden) and equilibrated with 50 mM sodium phosphate buffer (pH 7.5). The active fraction was analyzed on 12% SDS-PAGE.

Protein Sequence Analysis

The amino-terminal sequence of purified collagenase-I was determined with an Applied Biosystems protein sequencer. Phenylthiohydantoin(PTH) derivatives were identified by reverse phase HPLC with an Applied Bio-system 120A liquid chromatograph "on-line" with the sequencer.

RESULTS AND DISCUSSION

Cloning and Identification of Collagenase Gene

The cloned *E. coli* JM83 was incubated on LB agar plates containing ampicillin, X-gal, and IPTG at 37°C for 24 h. Each agar plate was flooded with the Frazier solution. In the case of this strain producing extracellular collagenase, a clear zone was formed around the colonies by the treatment of the Frazier solution. The clear zone was due to gelatin or collagen (type I) decomposition into smaller molecules. Among 15,000 transformants screened, only one clone showing the collagenase activity was obtained (Fig. 1). We named this strain *E. coli* JM83/pKCL279H. This cloned strain produced the extracellular collagenase but no intracellular collagenase was found (data not shown). Recombinant plasmid DNA of the cloned strain had a 3.5 kb-fragment

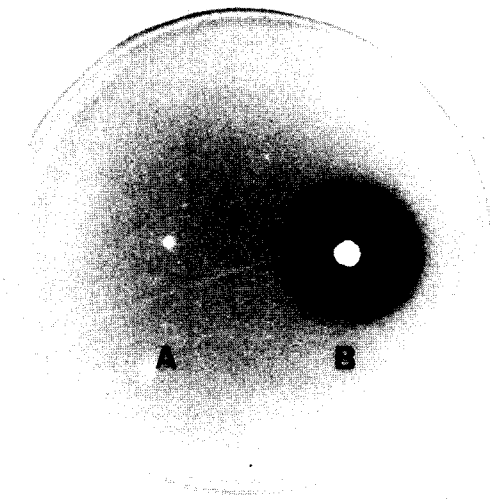

Fig. 1. Photograph of the cloned strain containing the recombinant plasmid DNA carrying the collagenase gene of *Vibrio vulnificus* CYK279H.
The cells were grown on an agar plate containing 3 mg/ml gelatin and the agar plate was treated with Frazier solution. A, *E. coli* JM83/pUC19 (control); B, *E. coli* JM83/ pKCL279H.

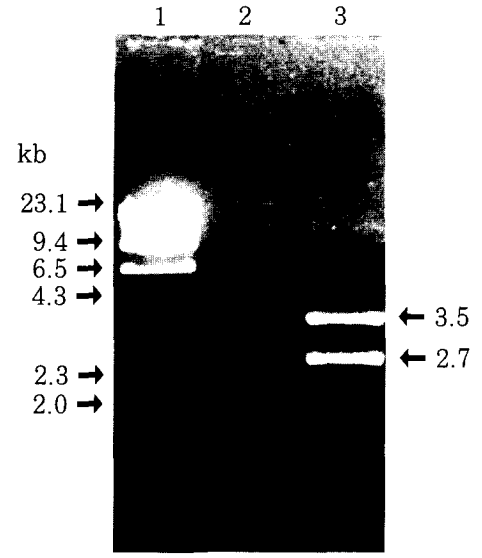

Fig. 2. Agarose gel electrophoresis of the recombinant plasmid DNA (pKCL279H) carrying the collagenase gene of *Vibrio vulnificus* CYK279H.
Lane 1, marker; Lane 2, pUC19 plasmid DNA digested by *Hin*dIII; Lane 3, pKCL279H plasmid DNA digested by *Hin*dIII.

(*E. coli* JM83/pKCL 279H, Lane 3) (Fig. 2). In Lane 2, the fragment of pUC19 plasmid (2.7 kb) is shown.

Gene Mapping of Recombinant Plasmid DNA (pKCL 279H)

The isolated plasmid pKCL279H was digested with several restriction enzymes and analyzed by 0.8% agarose gel electrophoresis. The partial restriction map of plasmid pKCL279H is shown in Fig. 3. Plasmid pKCL279H (A) had *Sac*I and *Kpn*I restriction sites. However, *Dra*I, *Sca*I, *Pst*I, *Sph*I, *Sma*I, *Eco*RI, and *Mlu*I sites were not detected inside the insert plasmid DNA.

Zymography of Collagenase in the Culture Supernatant

The presence of collagenase in the culture supernatant of *E. coli* JM83/ pKCL279H was analyzed by zymography. As shown in Fig. 4, two clear active bands were separated on the gel. Therefore, it was confirmed that *E. coli* JM83/pKCL279H produced two different sizes of

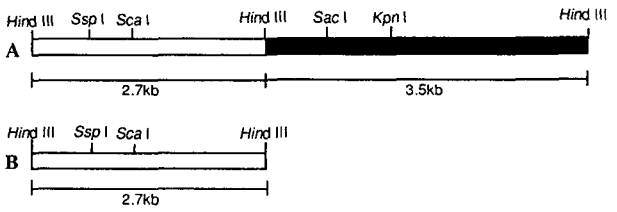

Fig. 3. Restriction enzyme map.
A, *E. coli* JM83/pKCL279H; B, *E. coli* JM83/ pUC19.

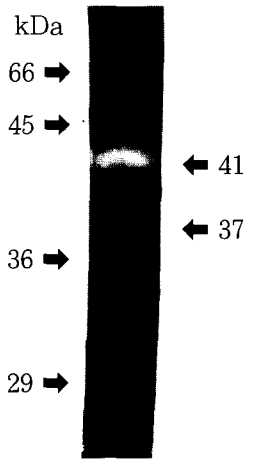

Fig. 4. Zymogram of collagenases produced by recombinant *E. coli* JM83/ pKCL279H.
Positions of markers (actual bands not shown in this figure): albumin bovine serum, 66 kDa; albumin egg, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa.

collagenases. These enzymes were named collagenase-I and collagenase-II. The M_r of collagenase-I was 41 kDa and that of collagenase-II was 37 kDa.

Purification of Collagenase-I, -II

The extracellular collagenase released into the culture broth of *E. coli* JM83/ pKCL279H was purified by the procedure summarized in Table 1. The supernatant obtained by centrifugation of the culture broth was treated with 70% ammonium sulfate. After centrifugation at 6,000×g for 20 min, the precipitate was dialyzed and the dialysate was put in a column of DEAE Sepharose CL-6B. The eluted collagenases were pooled and each elution was treated again with ammonium sulfate. The precipitated collagenases were put in a column of Sephadex G-100. All of the purification steps were carried out at 4°C. Finally, the specific activity of a major collagenase-I was 100.1 U/mg and that of -II was 135.4 U/mg. The purities of collagenase-I, -II were indentified as a single band of protein on SDS-PAGE (Fig. 5).

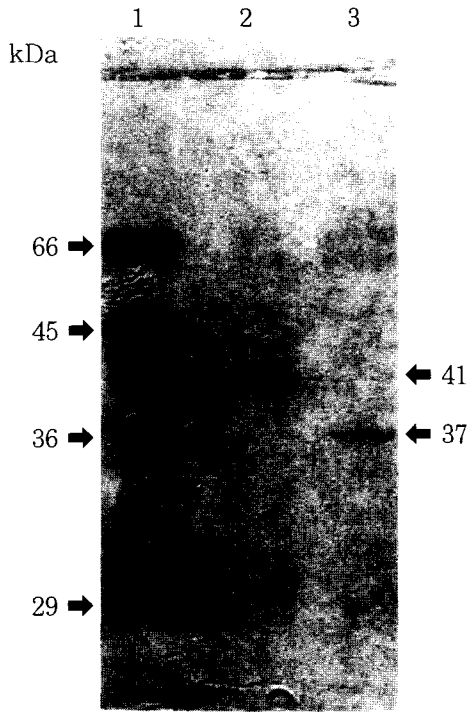

Fig. 5. SDS-polyacrylamide gel electrophoresis of the purified collagenase-I, -II from *E. coli* JM83/pKCL279H.
The purified enzymes were subjected to electrophoresis on a 12% polyacrylamide gel in a Tris-glycine buffer (pH 8.0) and the protein was stained with Coomassie Brilliant Blue R-250. Lane 1, markers (albumin bovine serum, 66 kDa; albumin egg, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa); Lane 2, Purified collagenase-I (41 kDa), Lane 3, purified collagenase-II (37 kDa).

N-terminal Amino Acid Sequence

The N-terminal amino acid sequence of collagenase-I as a main product from *E. coli* JM83/pKCL279H was analyzed and was compared with those of other previously reported collagenases (Table 2). The N-terminal amino acid sequence of collagenase-I was determined as Thr-Ala-Ile-His-Glu-Leu-Phe-His-Asp-Glu. The results showed a difference from those of other known collagenases and the sequence homologies were relatively low.

Table 1. Purification of collagenases-I, -II from the culture supernatant of *E. coli* JM83/pKCL279H.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Supernatant	1276.4	60.0	21.3	1.0	100.0
Ammonium sulfate precipitation	420.3	15.3	27.5	1.3	32.9
DEAE Sepharose CL-6B	276.5	8.9	31.0	1.5	21.6
Sephadex G-100 (Collagenase-I)	30.0	0.3	100.1	4.7	2.4
Sephadex G-100 (Collagenase-II)	10.8	0.1	135.4	6.4	0.8

Table 2. Comparison of N-terminal amino acid sequence of the collagenase-I produced by *E. coli* JM83/pKCL279H and others.

Strains	N-terminal sequence	Reference
E. coli JM83/pKCL279H	Thr-Ala-Ile-His-Glu-Leu-Phe-His-Asp-Glu	This work
Astacus fluviatilis	Thr-Ile-Ile-His-Glu-Leu-Met-His-Ala-Ile	[2]
Pseudomonas aeruginosa	Thr-Leu-Thr-His-Gl-Leu-Thr-His-Ala-Val	[4]
Vibrio arginolyticus	Asp-Leu-Glu-His-Glu-Tyr-Val-His-Tyr-Leu	[13]
Serratia sp.	Thr-Phe-Thr-His-Glu-Ile-Gly-His-Ala-Leu	[16]
Cytophaga sp.	Thr-Leu-Thr-His-Glu-Phe-Gly-His-Phe-Leu	[20]

Properties of Collagenase-I

Effect of pH on collagenase activity. The optimal pH of collagenase-I was determined with 50 mM sodium acetate (pH 3.6 to 5.6), 50 mM sodium phosphate (pH 5.8 to 8.0) and 50 mM Tris-HCl (pH 7.8 to 10.1) buffers in the enzyme assay. The activity of collagenase-I was stable from pH 5.0 to 8.0 and had maximum values at pH 7.8 (Fig. 6).

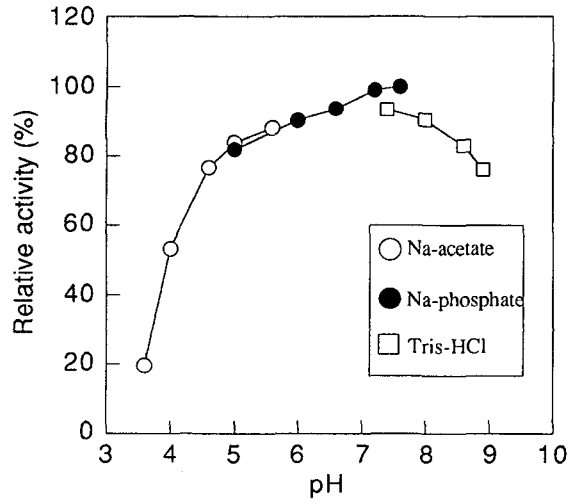

Fig. 6. Effect of pH on collagenase-I activity.
50 mM sodium acetate, pH 3.6 to 5.6; 50 mM sodium phosphate, pH 5.8 to 8.0; 50 mM Tris-HCl, pH 7.8 to 10.1.

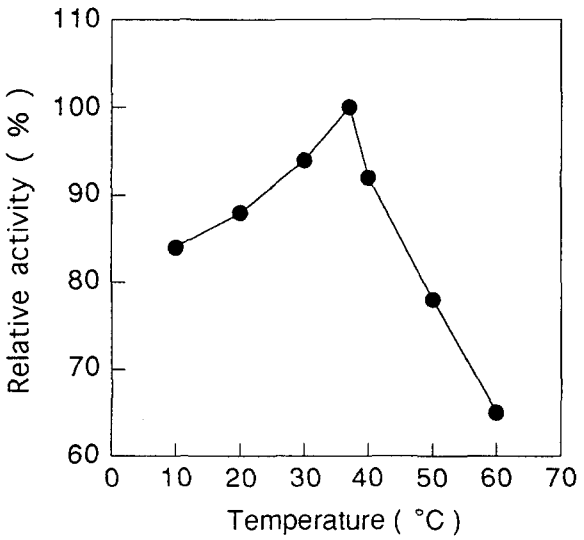

Fig. 7. Effect of temperature on collagenase-I activity.

Effect of temperature on collagenase activity. Collagenase-I showed maximum enzyme activity at 37°C (Fig. 7). This enzyme was stable at the temperature range of 20°C~40°C and the stability was maintained for 12 months at 4°C (data not shown).

Acknowledgments

This study was supported by the academic research fund of Ministry of Education, Republic of Korea. Project No. BSRI-97-4410.

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