Structural gene and complete amino acid sequence of *Vibrio alginolyticus* collagenase

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The DNA encoding the collagenase of *Vibrio alginolyticus* was cloned, and its complete nucleotide sequence was determined. When the cloned gene was ligated to pUC18, the *Escherichia coli* expression vector, bacteria carrying the gene exhibited both collagenase antigen and collagenase activity. The open reading frame from the ATG initiation codon was 2442 bp in length for the collagenase structural gene. The amino acid sequence, deduced from the nucleotide sequence, revealed that the mature collagenase consists of 739 amino acids with an M_r of 81 875. The amino acid sequences of 20 polypeptide fragments were completely identical with the deduced amino acid sequences of the collagenase gene. The amino acid composition predicted from the DNA sequence was similar to the chemically determined composition of purified collagenase reported previously. The analyses of both the DNA and amino acid sequences of the collagenase gene were rigorously performed, but we could not detect any significant sequence similarity to other collagenases.

INTRODUCTION

In 1973, Welton & Woods isolated and described the Gram-negative aerobic bacterium *Achromobacter iophagus* [1]. In the presence of collagen or macromolecular fragments of collagen [2], a subculture of this organism (strain I029) produces a collagenase having a very high specific activity [3,4]. This strain was later classified as *Vibrio alginolyticus* chemovar. *iophagus* [5]. The structure and functional properties of this *Vibrio* collagenase (EC 3.4.24.8) have been described in a series of papers by Keil and his colleagues [2,3,6–9]. The collagenase activity of *Vibrio alginolyticus* collagenase is higher than that of any other bacterial collagenase, and it has very low neurotoxic activity. Thus this enzyme is of practical importance, because it is very effective in promoting healing of abnormal skin scars [10,11] and can tenderize meat by cleaving collagen.

The *Vibrio* collagenase is a zinc proteinase which cleaves native collagen and synthetic substrates at the Xaa–Gly bond in the Xaa–Gly–Pro–Xaa sequence [12]. Three active forms of the collagenase can be isolated from the supernatant of the culture of the organism; their molecular masses are 110, 90, and 70 kDa respectively. The different active forms of the collagenase are assumed to be cleavage products derived from the same simple polypeptide chain [9]. There are other reports of the DNA sequence of the collagenase gene, namely that of vertebrates [13–15]. Owing to its large size, it has been difficult to clone the bacterial collagenase gene. The molecular size of the native collagenase is still unknown, owing to the ease of auto-degradation. To clarify its molecular size and enzymic characteristics, the primary structure of the collagenase needs to be clarified. Recently we cloned the collagenase gene from *Vibrio alginolyticus* [16]. In the present study we completely sequenced the collagenase gene and clarified these major issues. In addition, the characteristics of the corresponding amino acid sequence of

this protein and its similarity to that of other proteinases are also discussed.

MATERIALS AND METHODS

Bacteria and antibody

Both *Vibrio alginolyticus* chemovar. *iophagus* (*V. alginolyticus*) and purified collagenase were described previously [4,12]. Anti-collagenase antibody was produced in rabbits by three 250 µg injections of collagenase/animal. It was further purified through a TSK-DEAE35W h.p.l.c. column [9]. The anti-collagenase antibody was detectable up to 16-fold dilution by Ouchterlony assay.

Construction and screening of the gene library

Chromosomal DNA was collected from *V. alginolyticus* by conventional methods and partially digested with restriction endonuclease *Sau3A*. Only DNA fragments over 7 kb were ligated to pUC18, the *Escherichia coli* expression vector, and a gene library was constructed. From this gene library, several clones were selected with the conventional rabbit anti-collagenase antibody, followed by ¹²⁵I-labelled anti-(rabbit Ig) antibody treatment.

Collagenase assays

Enzyme activity was measured colorimetrically using 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine hydrochloride dihydrate (Fluka) according to the method of Wunsch & Heidrich [17]. Protein concentration was determined by the method of Lowry *et al.* [18]; 5 µl (about 20 µg of protein) of each sonicated sample were used for assays of collagenase activity of each gene product.

Abbreviations used: KLH, keyhole-limpet haemocyanin; SD, Shine–Dalgarno.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X62635.

Fig. 1. Restriction map and sequencing strategy for the collagenase gene

The thick line represents the DNA insert containing the collagenase gene. The top arrow indicates the span of the collagenase gene and the direction of transcription. Broken lines represent the span of pLCO-4 and pLCO-1 plasmid genes. The lower arrows indicate the direction and length of sequence determinations, starting from the universal primer (●) or specific primers (○) (see the Materials and methods section).

Fig. 2. Detection of collagenase by Coomassie Blue staining and Western blotting with peroxidase-labelled rabbit anti-collagenase antibody

(a) Coomassie Blue staining; (b) Western blotting. Lane 1, *E. coli* HB101 (pLCO-1); lane 2, *E. coli* HB101 (pLCO-4); lane 3, purified collagenase. Molecular-mass (*M*) markers in (b) are Prestained SDS-PAGE Standards (Bio-Rad).

Amino acid sequence

The methods for amino acid sequence analyses of internal or *N*-terminal peptide fragments were described previously [19,20]. Hydropathy was analysed as described previously [21].

Peptide synthesis

Peptides deduced from the collagenase DNA sequence were synthesized on a 430A peptide synthesizer (Applied Biosystems,

Table 1. Measurement of the collagenase activity (A_{320}) of *E. coli* pLCO-4, pLCO-1, pUC18 and *V. alginolyticus*

Each sonicated and solubilized bacterial sample was assayed.

Bacterial sample	A_{320}
<i>E. coli</i> pLCO-1	0.002
<i>E. coli</i> pLCO-4	0.521
<i>E. coli</i> pUC18	0.003
<i>V. alginolyticus</i>	0.433

Foster City, CA, U.S.A.) using chemicals and program cycles supplied by the manufacturers. All synthesized peptides were purified through h.p.l.c. columns (TSK-ODS 120T). For immunization, each peptide was conjugated with keyhole-limpet haemocyanin (KLH; Sigma).

Detection of proteins

The supernatant from the sonicated bacterial suspension was loaded on to SDS/10%-(w/v)-polyacrylamide gels. Proteins were revealed by staining with Coomassie Blue. To identify proteins as collagenase, both Western blotting [22] and an e.l.i.s.a. [23] were employed. Both anti-collagenase antibody and also anti-peptide antibodies were employed. Sonicated bacterial samples were used, and the antibodies were obtained by injection of 300 μ g of peptide-conjugated KLH into rabbits on three occasions. Each solubilized bacterial sample was fixed on a plate and each anti-peptide antibody was allowed to react with it. Peroxidase-labelled anti-rabbit antibody was then allowed to

Fig. 3. DNA sequence and deduced amino acid sequence of collagenase

A putative ribosome-binding site (SD-like sequence) is underlined. ORF indicates an open reading frame of an undefined protein. The *N*-terminus of the mature protein was identified on the basis of its sequence similarity to the chemically determined *N*-terminal sequence of the collagenase. The letters and numbers beneath the underlined regions indicate chemically identified peptides. Numbers written on both sides of the lines indicate the positions of both nucleotides and amino acids from the initiation codon of the *N*-terminal amino acid. Abbreviation: SR, stacking region.

react, and the bound antibodies were stained with H_2O_2 and benzidine. We evaluated the titre of antibodies by observing the colour as it was disappearing. After the 16th dilution, no more colour was detectable.

Subcloning

The plasmids of collagenase-positive bacteria were collected, and the appropriate DNA fragments were prepared with various restriction enzymes (from Takara Shuzo Co., Kyoto, Japan). The cleaved samples were applied to agarose gels for electrophoresis, and a restriction-enzyme map was drawn (Fig. 1). For nucleotide-sequence analysis, staggered deletions of about 300 bp each were prepared by unidirectional digestion with exonuclease III according to the method of Henikoff [24]. To determine the span of the collagenase structural gene, the reactivity to the antibody of each *E. coli* clone transformed with deleted DNA was measured.

DNA sequencing

Various restriction fragments of the collagenase gene were subcloned into M13mp18 [25]. Sequencing reactions were carried out by dideoxy chain termination [26] with ^{32}P -labelled nucleotide. The region of the structural gene was sequenced by using universal or synthetic oligonucleotide primers. The oligonucleotides were synthesized with an automatic DNA synthesizer (381A DNA synthesizer; Applied Biosystems).

RESULTS

Cloning the collagenase gene

As a first step, we constructed *Sau*3AI-digested genomic DNA libraries from *V. alginolyticus*, using pUC18 as a cloning vector [16,19]. The libraries were screened with rabbit anti-collagenase antibody. Several positive clones were obtained out of about 5000 colonies. However, when the antibody assays were redone, only three clones of *E. coli* HB101, namely pLCO-1, pLCO-2 and

pLCO-3, reacted repeatedly. These pLCO-1, pLCO-2 and pLCO-3 plasmids contained fragments of *V. alginolyticus* of approx. 7.2, 6.2 and 5.5 kb respectively. From these clones we constructed several subclones for DNA-sequencing studies. In addition, a smaller plasmid (pLCO-4), containing a 2.8 kb *Hinc*II-*Eco*RV fragment, was also constructed from the pLCO-1 plasmid for studying the function of these gene products and the span of the collagenase structural gene.

Detection of collagenase

We detected the antigenicity of the collagenase protein of newly constructed collagenase genes. The sonicated bacterial samples were analysed. Fig. 2 shows the results of both protein staining (a) and Western blotting (b) of pLCO-1 and pLCO-4, as well as chemically purified collagenase. A collagenase band of about 90 kDa and several collagenase bands of lower molecular mass were observable in *E. coli* HB101 (pLCO-4). Some of these lower-molecular-mass bands (80 kDa and lower) were also observed with the purified collagenase. Several of the lower-molecular-mass bands seemed to be cleaved products of the collagenase. It is possible that the *Vibrio* collagenase may be synthesized as a larger proenzyme form, possibly containing a signal peptide. The collagenase activity of these cloned gene products were assayed using sonicated bacteria. As shown in Table 1, a strong collagenase activity was observed in *E. coli* HB101 (pLCO-4). This plasmid gene includes only 2.8 kb length, but its product has a strong collagenase activity.

DNA sequencing

To sequence this cloned gene, a detailed restriction map and sequencing strategies were prepared (Fig. 1). Several smaller DNA fragments about 500 bp in length were made from the pLCO-1 plasmid. The nucleotide sequence of the collagenase gene and the deduced amino acid sequence of the protein encoded by this gene are shown in Fig. 3. The entire *V. alginolyticus* collagenase reading frame was sequenced on both strands. The *N*-terminal amino acid sequence of the purified collagenase (Thr-Ala-Cys-Asp-Leu-Glu-Ala-Leu-Val) corresponded perfectly to the deduced amino acid sequences of our determined DNA sequence. The G + C content of this mature collagenase gene was 46.6%, which is typical of the *V. alginolyticus* genome [27]. After the ATG, the initiation methionine codon, we observed some hydrophobic amino acids. A Shine-Dalgarno (SD)-like sequence was also observed 6–12 bp upstream from this ATG codon. The codon of the *N*-terminal amino acid of mature protein was observed from 225 to 227 bp downstream of the ATG codon. From the initiation of *N*-terminal peptide to the stop codon TAG (739 amino acids) we were able to detect the chemically determined amino acid sequences of 20 peptide fragments in the deduced amino acid sequence of mature collagenase. The amino acid sequence His-Glu-Xaa-Xaa-His, which was suggested to participate in the formation of a ligand with Zn, has been suggested to be the important catalytic site of the Zn-metalloproteinase [1,9,17,19,20], was also observed. After the stop codon we were able to observe a stacking region, which also supported the existence of a termination codon at this region.

E.I.s.a. with anti-peptide antibodies

To detect the initiation codon of the collagenase gene we synthesized four peptides (Fig. 4). Three were synthesized in the 5' region of the proposed initiation codon (from -223 to -225). The other one is the C-terminal-region peptide. The binding ability of these antisera, obtained from immunized rabbits, was assayed by e.i.s.a. As shown in Table 2, we were not able to detect antibodies which bound to the sonicated fraction of *V. alginolyticus*.

DNA sequence	-412	GATTTCGTGCAAGCCCGTCAACGCACTCGAGTACCAAAACT	-369
Peptide 1		AspPheTrpGlnAlaArgAlaThrGlnLeuGluTyrGlnLys	
Peptide 2		IleSerGlyLysProValGlnArgAsnSerSerThrLysAsn	
Peptide 3		PheLeuAlaSerProCysAsnAlaThrArgValProLysThr	
DNA sequence	2173	AATGCCGCCATCGTGTGATTTTGTATGCTCAGAAGTGTGCTCAG	2217
Peptide 4		AsnAlaAlaIleValValAspPheAspAlaGlnLysCysArgGln	

Fig. 4. Synthetic peptides for antibody production deduced from the DNA sequence

Table 2. Detection of antigens by various anti-peptide antibodies

Sonicated bacterial sample or peptide	Anti-peptide...	Binding activities of anti-peptide antibody [as final detectable dilution (fold)]			
		1	2	3	4
pLCO-4		< 100	< 100	< 100	25600
pLCO-1		< 100	< 100	< 100	6400
<i>V. alginolyticus</i>		< 100	< 100	< 100	51200
<i>E. coli</i> (pUC18)		< 100	< 100	< 100	< 100
Peptide 1		25600	< 100	< 100	< 100
Peptide 2		< 100	12800	< 100	< 100
Peptide 3		< 100	< 100	12800	< 100
Peptide 4		< 100	< 100	< 100	25600

Table 3. Amino acid composition of collagenase from *V. alginolyticus*

Amino acid	No. of residues	
	Determined from purified enzyme*	Predicted from DNA sequence
Asp } Asn }	91.4	{ 43 48
Thr	54.1	57
Ser	63.2	65
Glu } Gln }	99.9	{ 51 42
Pro	16.7	16
Gly	66.3	61
Ala	56.9	59
Val	47.4	45
Met	5.9	10
Ile	32.8	37
Leu	51.4	52
Tyr	34.8	38
Lys	26.8	22
His	17.7	16
Arg	21.3	18
Cys	8.2	10
Trp	13.7	18
Phe	30.8	31

* The amino acid composition was calculated using the M_r of the collagenase reported by Trocheris *et al.* [28].

Fig. 5. Hydropathic character of collagenase

The profile shown is of the hydropathic index of the amino acid sequence as a function of the residue number, which starts from the initiating methionine residue. Each value is a mean hydropathy index value for 11 successive residues.

Comparison of amino acid composition

In a previous study, Trocheris *et al.* [28] reported the amino acid composition of *V. alginolyticus* collagenase from a chemically purified protein sample. The comparison of the two amino acid compositions is shown in Table 3. The amino acid composition deduced from our DNA sequence was similar to that determined on the purified enzyme.

Hydropathy profile

The hydropathy profile is shown in Fig. 5. Hydrophobic amino acids were observed in the initial sequence. The amino acid sequence of the collagenase had a hydrophilic trend, particularly in the C-terminal half of the collagenase.

DISCUSSION

We have cloned the *Vibrio* collagenase gene and determined the entire DNA and amino acid sequences. The amino acid composition of the *Vibrio* collagenase predicted from the nucleotide sequence is in close agreement with that determined by amino acid analysis of the purified enzyme. In addition, the

amino acid sequences of 20 experimentally sequenced peptide fragments of the enzyme were completely identical with those of deduced amino acid sequence. These results strongly indicate that the gene that we cloned is the *Vibrio* collagenase gene.

Although three molecular masses for collagenase have been proposed, it has been difficult to determine the exact value because of enzymic autodegradation. Our DNA sequencing data clearly demonstrate that the molecular mass of the mature protein is 81 704. About 225 bp downstream of the ATG codon we observed the N-terminal peptide sequence, which was reported by Tong *et al.* [9,29]. In addition to this peptide sequence they also proposed two other N-terminal amino acid sequences (Val-Xaa-Leu-Lys-Ile-Xaa-Leu-Trp-Leu and Ala-Val-Xaa-Xaa-Tyr-Val-Leu-Ile), which were attributed to the N-terminal sequences of 110 kDa and 90 kDa molecules respectively [9]. According to that assumption, the N-terminal sequences of the 90 kDa and 110 kDa collagenase-coding sequence should exist at the region around 300 bp and 850 bp upstream of the ATG initiation codon. However, we were not able to find these amino acid sequences in the reading frames of these regions. In addition, using synthetic peptides, we tried to find some peptides which may be encoded in this region in *Vibrio* bacteria. However, we were not able to detect any antigenicity in these solubilized bacterial samples (Table 2 and Fig. 4). Additionally, four to six stop codons were observed in these regions (between -1150 bp and -280 bp in Fig. 3). Although there were some ATG codons, which are possible initiation codons for protein, we did not detect any SD-like sequences upstream of these ATG codons. Furthermore, adenine and thymidine are abundant in these regions; this is a typical characteristic of promoter regions. So far we have not been able to detect any DNA sequence which would code for the N-terminal region of the 110 kDa collagenase molecule as proposed previously [9,29]. We also observed both 90 kDa and lower-molecular-mass molecules by Western blotting. The slightly larger size of the collagenase from *E. coli* HB101 (pLCO-4) might be result of the premature collagenase containing a signal peptide. Perhaps the smaller molecules are those of cleaved collagenase. A long open reading frame is situated from -1552 bp to -1198 bp (Fig. 3) of our cloned collagenase gene, but it is premature to formulate any hypothesis on the nature of this protein.

A comparison of amino acid composition of five metalloproteinases was done. The collagenase and other proteinases showed some sequence similarities in some small regions of the protein. We could not find any extensive sequence identity such as that observed between thermolysin [30] or *Bacillus subtilis* neutral proteinase [31] and *Pseudomonas* elastase or between *Serratia* proteinase [32] and *Pseudomonas* alkaline proteinase [20]. There was no sequence similarity between the *Vibrio* collagenase and the eukaryotic collagenases [13-15,33]. Collagenase can be regarded as a Zn-metalloproteinase, although its enzymic properties are unlike to those of other bacterial proteinases (*B. subtilis* thermolysin [30], *B. subtilis* neutral proteinase [31], *Serratia* proteinase [32], *Pseudomonas* elastase [19] and *Pseudomonas* alkaline proteinase [20]). In discussing the mechanism of the action of metalloproteinase, Matthews *et al.* [34,35] proposed that, in the process of catalysis, the amino acid structure of His-Glu-Xaa-Xaa-His is important in facilitating the electron transfer with Zn. As shown in Fig. 3, the proposed catalytic amino acids His-402, Glu-403 and His-406 are also found in the *Vibrio* collagenase sequence. These observations confirm that the collagenase gene we cloned possesses one of the structures typical of a metalloproteinase.

We detected 20 peptide fragments in the entire amino acid sequence of mature collagenase. As shown in Fig. 3, no peptide fragments, determined by amino acid sequencing, were found in

the deduced amino acid sequence in the region downstream from Lys-508. One possibility is that, during the process of chemical purification, the collagenase was cleaved and became bereft of its C-terminal region. In addition, the results of e.l.i.s.a. (Table 2) also suggests that the amino acid sequence Asn-Ala-Ala-Ile-Val-Val-Asp-Phe-Asp-Ala-Gln-Lys-Cys-Arg-Gln, deduced from the nucleotide sequence of the C-terminal region, is involved in the collagenase.

We were not able to detect any significant similarity between the DNA and amino acid sequences of *Vibrio* collagenase and other collagenases [23] so far determined, such as insect collagenase [33], synovial-cell collagenase [36] and others [13–15]. This result suggests that *Vibrio* collagenase may be one of a new family of collagenases, but further work is necessary to prove this hypothesis.

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