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Purification and Characterization of Extracellular Collagenase from *Vibrio mimicus*

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Abstract

A collagenase was isolated from the culture filtrate of *Vibrio mimicus* (ATCC 33658). The enzyme was purified to homogeneity by ammonium sulfate precipitation and DEAE-Sephadex A-50 chromatography, which an activity recovery of 22%. The molecular weight of the purified enzyme was estimated to be 42 kDa by SDS-polyacrylamide gel electrophoresis and gel filtration, indicating a monomer structure. The optimum pH and temperature of the enzyme for insoluble collagen (Type I) were around 7.75 and 28℃, respectively. Some chelating agents and serine protease inhibitor inactivated the enzyme, but L-cysteine and histidine did not affect the activity. The amino acid composition indicated that the collagenase contained high amounts of amino acid residues of glycine and alanine. The K_m and k_{cat}/K_m values for the collagenase, using insoluble collagen (Type I) as substrate, were 2.86 mg/ml and 972.28 U/mg-protein, respectively.

Key words : *Collagen, collagenase, protein purification, Vibrio mimicus*

Introduction

Collagenases degrade the collagen molecule in the helical region, predominantly at level of the bond Y-Gly in sequences of the type -Pro-Y-Gly-Pro-, where Y is most frequently a neutral amino acid[1]. Collagenases are metalloproteinase containing Zn^{2+} in the active site region and requiring Ca^{2+} for full activity[2-4]. These enzymes are thought to play important roles in the physiological remodeling of tissues during growth and development as well as in repair and pathologic states[3]. Collagenases have been purified and characterized from a variety of animal tissues[2,5-7] and microorganisms such as *Clostridium histolyticum*[8], *Vibrio arginolyticus*[9-10], *Vibrio B-30*[11], *Pseudomonas marinoglutinosa*[12], *Streptomyces maduraе*[13] and *Streptomyces* sp.[14] Among collagenase of microbial origin, the one produced extracellularly by *Clostridium histolyticum* has been most extensively studied.

In the present study, we have produced and purified collagenase from culture filtrate of *Vibrio mimicus* to apparent homologeneity. Its properties, including the amino acid composition, substrate specificity and proteinase inhibition profiles etc., are described.

Material and Methods

Materials

NZ-amine (Type HD) hydrolyzed casein, insoluble co-

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llagen (Type I, II, V) from bovine Achilles' tendon, acid-soluble collagen (Type III) from calf skin, *p*-phenylazobenzyloxycarbonyl-Pro-Leu-Pro-D-Arg, collagenase (Type I) from *Clostridium histolyticum*, DEAE-Sephadex A-50, L-cysteine, N^{α}-tosyl-L-lysine chloromethyl keton (TLCK), N^{α}-tosyl-L-phenylalanine chloromethyl keton (TPCK), soybean trypsin inhibitor, EDTA, and diisopropylfluorophosphate (DFP) were purchased from Sigma. Sephadex G-150 and L-histidine were obtained from Pharmacia. Other reagents used were of the highest grade available.

Microorganism and cultural conditions

Vibrio mimicus (ATCC 33658) was a gift from the National Health Institute of Korea. Gelatine-containing medium for collagenase production studies had the following composition ; the medium contained 1% NZ-amine (Type HD) hydrolzed casein and 5% gelatine (Fisher Co.) in 1 L of autoclaved natural seawater and adjusted to pH 8.0. Laboratory scale enzyme production was accompolished in 2 L fermentor. The strain was precultured in 5 ml of the gelatine containing medium at 37℃ for 12 hr. The preculture (5 ml) was transferred to 1 L of the same medium and cultured aerobically at 37℃ for 7 days, then the culture was centrifuged at 10,000×g for 20 min at 4℃. The culture filtrate was used for the purification of collagenase.

Enzyme purification

All procedures of the enzyme purification were done at 4℃ unless otherwise specified. Ammonium sulfate was added to the culture filtrate at 30% saturation and was centrifuged at 10,000×g for 30 min. The supernatant was then brought to 80% saturation by the addition of solid ammonium sulfate. The protein precipitate was collected by centrifugation (10,000×g, 30 min), dissolved in 50 ml of 20 mM Tris-HCl, pH 8.0, containing 0.36 mM $CaCl_2$, and dialyzed at 4℃ for 48 hr against four changes of the same buffer. The enzyme solution was applied to a DEAE-Sephadex A-50 column (3×40 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.36 mM $CaCl_2$. Elution of collagenase was accomplished with a linear gradient of NaCl concentration from 0 to 1 M. The enzyme fraction was concentrated by using ultra-membrane filtrator (Satorius Co.), dialyzed for 24 hr against two changes of 20 mM Tris-HCl, pH 8.0, containing 0.36 mM $CaCl_2$, and centrifugated at 15,000×g for 30 min. The supernatant was applied to a DEAE-Sephadex A-50 column (1.6×25 cm) for rechromatography. As described above, the enzyme was eluted with a linear gradient of NaCl (0~1.0 M) in 20 mM Tris-HCl, pH 8.0, containing 0.36 mM $CaCl_2$. The active fractions were pooled and stored at −80℃.

Assays for collagenolytic activity

1) Hydrolysis of insoluble collagen (Type I) from bovine achilles' tendon : Insoluble collagen was routinely used for assaying collagenase activity in the course of enzyme purification and characterization unless otherwise specified. The collagenase activity was determined essentially based on the method on Peterkofsky[15] with a slight modification. The reaction mixture containing 5 mg insoluble collagen, 50 mM Tris-HCl (pH 7.75), 0.36 mM $CaCl_2$, and the enzyme sample in a total volumn of 1.1 ml was incubated at 28℃ for 24 hr. The reaction was stopped by adding 0.2 ml of 10% trichloroacetic acid. The mixture was allowed to stand at room temperature for 10 min, and was centrifuged at 1,800 ×g for 20 min. Soluble peptide concentration was determined by the method of Moore and Stein[16] using leucine as a standard. A unit of collagenase is defined as the amount of enzyme that liberated 1 mmole of leucine equivalent per hour per mg of protein in the sample.

2) Hydrolysis of synthetic peptide : Hydrolysis of PZ-Pro-Leu-Gly-Pro-D-Arg by collagenase was assayed according to Wüsch and Heidrich[17] with a slight modification. The total reaction volumn was 0.25 ml of 50 mM Tris-HCl, pH 8.4, containing 1 mM $CaCl_2$, 100 mg substrate, and the enzyme. The mixture was incubated at 37℃ for 15 min and the reaction was terminated with 0.5 ml of 0.5% citric acid. The reaction mixture was

diluted with 2.5 ml of ethyl acetate. The cleavage product was measured by spectrophotometer at 320 nm.

Assay for proteolytic activity

Caseinolytic activity was determined according to the method of Pyeun and Kim[18]. One unit of caseinolytic activity was defined as the absorbance equivalent of 1 mmole tyrosine produced per min per 1 mg of protein.

Determination of enzymatic properties

The optimum pH for the activity measured against insoluble collagen (Type I) from bovine achilles' tendon was determined in 50 mM citrate/Na_2HPO_4 (pH 3.0~7.0), 50 mM Tris/HCl (pH 7.0~9.0), and 50 mM Na_2CO_3/$NaHCO_3$ (pH 9.0~10.8) buffers containing 0.36 mM $CaCl_2$. To test the optimum temperature, the enzyme in a 1 ml of 50 mM Tris-HCl (pH 7.75) containing 0.36 mM $CaCl_2$, and 5 mg insoluble collagen (Type I), was incubated at different temperatures for 24 hr, and then the extent of collagen hydrolysis was determined as described above.

To test the effects of inhibitors, the enzyme solution containing each inhibitor was preincubated at pH 7.75 and 37℃ for 30 min then assayed under the standard conditions. The following inhibitors and metals were tested : 1 mM diisopropylfluorophosphate (DFP), 1 mM soybean trypsin inhibitor, 1 mM N^{α}-tosyl-L-lysyl chloromethyl ketone (TLCK), 1 mM N^{α}-tosyl-L-phenylalanyl chlorometyl ketone (TPCK), 1 mM L-cystein, 1 mM histidine, 2 mM EDTA, and 0.15 mM each of NaCl, LiCl, $CaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $HgCl_2$, and $CuSO_4$.

Kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) for insoluble bovine collagen (Type I) were determined according to Lineweaver and Burk[19] as measured in 50 mM Tris-HCl buffer pH 7.75, containing 0.36 mM $CaCl_2$ at 28℃.

Determination of substrate specificity

To examine substrate specificity toward various type of collagen, the reaction mixture consisting of 5 mg of a substrate, 1 ml of 50 mM Tris-HCl, pH 7.75, containing 0.36 mM $CaCl_2$, and 0.1 ml (50 mg protein) of the enzyme was incubated at 28℃ for 24 hr. The peptides and amino acids released were determined by the ninhydrine method[16] using leucine as the standard.

Amino acid composition analysis

The purified enzyme preparation was hydrolyzed with 6N HCl in vacuum-sealed glass tubes at 110℃ for 24, 48, and 72 hr. Amino acids were analyzed with an automated amino acid analyzer (Hitachi Co.).

Other methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[20]. Protein bands were stained with Coomassie Brilliant Blue R-250. Protein concentration was determined by the method of Lowry et al.[21] using bovine serum albumin as a standard.

Results

Purification of collagenase

The results of purification of a collagenase from the culture filtrate of *Vibrio mimicus* are shown in Table 1. Upon 1st DEAE-Sephadex A-50 column chromatography of the ammonium sulfate fraction, the collagenolytic activity was eluted as the major peak (fractions 75-95) (Fig.1). The enzyme was almost completely purified at this stage as examined by SDS-PAGE (data not shown). The enzyme was further purified by DEAE-Sephadex A-50 column rechromatography (data not shown). By these procedures, the collagenase was completely purified from the culture filtrate of *Vibrio mimicus* to apparent homogeneity with a 3.5-fold purification in a yield of 22%. The specific activity of the purified collagenase was 555.5 units/mg of protein (Table 1). The enzyme had only collagenolytic activity, but did not have caseinolytic activity.

Table 1. Purification of *Vibrio mimicus* Collagenase

Purification step	Total protein (mg)	Specific activity[a] (units/mg)	Purification (-fold)	Yield (%)	Caseinolytic activity[b] (units/mg)
Crude	3672	158.7	1.0	100	44.7×10^{-4}
Ammonium sulfate(30−80%)	1541	174.6	1.1	43	26.0×10^{-4}
DEAE-Sephadex A-50(1st)	319	412.7	2.6	23	8.1×10^{-4}
DEAE-Sephadex A-50(2nd)	227	555.5	3.5	22	6.0×10^{-4}

[a]1 unit=activity hydrolyzing 1 μmol of Type I collagen (Bovine achilles' tendon) per hr.

[b]1 unit=activity producing 1 μmol of tyrosine per 1 min.

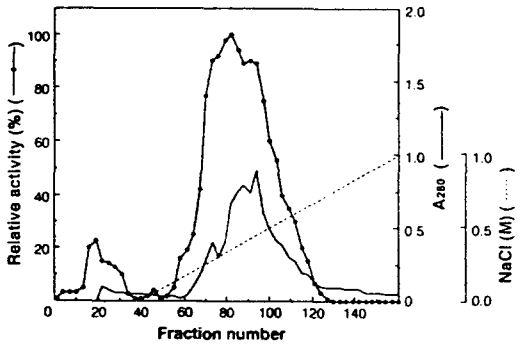

Fig. 1. Chromatography on 1st DEAE-Sephadex A-50. The ammonium sulfate fraction was applied to a column (3×40 cm) of DEAE-Sephadex A-50. The column was washed with 20 mM Tris-HCl, pH 8.0, containing 0.36 mM $CaCl_2$ and then eluted with a linear gradient of 0~1.0 M NaCl in the same buffer. Flow rate was 30 ml/h, and 10 ml fractions were collected.

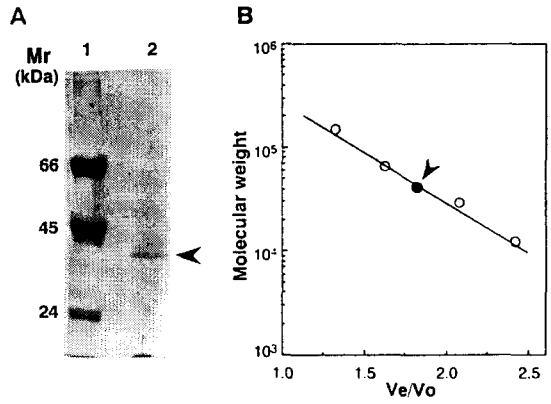

Fig. 2. Determination of the molecular weight of *Vibrio mimicus* collagenase. A : The purified collagenase was electrophoresed on a 10% polyacrylamide gel in the presence of SDS under reducing conditions and stained with Coomassie Brilliant Blue. *Lane* 1, molecular weight standard marker ; *lane* 2, purified collagenase. B : The molecular mass of the enzyme was determined on a calibrated column of Sephadex G-150. The ratio of the elution volumn (Ve) to the void volumn (Vo) determined by using blue dextran was plotted versus the logarithm of the molecular weights of the standard proteins : 150 kDa, alcohol dehydrogenase ; 66 kDa, bovine serium albumin ; 29 kDa, carbonic anhydrase ; 12.4 kDa, cytochrome C. The arrow indicates the position of the purified collagenase, corresponding to a molecular weight of approximately 42,000.

Purity and molecular weight

The purified collagenase was judged to be homogeneous by the SDS-PAGE under reducing condition and Sephadex G-150 gel filtration column, as all of the results gave a single band (Fig. 2A) or a single peak (data not shown). The apparent molecular weight of the purified collagenase was calculated to be about 42,000 Da by SDS-PAGE under reducing condition and Sephadex G-150 gel filtration (Fig. 2). These indicate that the purified collagenase is a monomeric enzyme.

Amino acid composition

The amino acid composition of the purified collagenase is listed in Table 2 in forms of residues per molecule of enzyme. The enzyme was composed of 366 amino acid residues characterized by high contents of glycine and alanine, and low contents of phenyalanine, methionine, isoleucine, and lysine. This results indicated that the amino acid composition of *Vibrio mimicus* collagenase is different from those of *C. histolyicum* collagenase[22] *Streptomyces C-51* collagenase[14].

Table 2. Amino acid compositions of *Vibrio mimicus* collagenase

Amino acid	Collagenase		
	V. mimicus[a]	*C. histolyticum*[b]	*Streptomyces sp*[c]
Asx	35	92	100
Thr	15	34	101
Ser	21	44	71
Glx	38	58	73
Gly	91	47	108
Ala	43	27	104
Half-Cys	20	2	Nd[d]
Val	14	39	54
Met	7	11	18
Ile	10	37	22
Leu	16	46	69
Tyr	11	39	53
Phe	7	23	31
Lys	10	54	23
His	ND	12	16
Arg	14	8	57
Pro	14	23	32
Trp	ND	5	ND
Molecular weight	42,000	70,000	100,000

[a]*Vibrio mimicus* collagenase in this work. Values are the nearest integer expressed as amino acid residues/mole based on molecular weight by SDS-PAGE and gel filtration.
[b]Quoted from paper by Emöd *et al.*, (1981).
[c]Quoted from paper by Endo *et al.*, (1987).
[d]ND, not determined.

Effects of pH and temperature on the enzyme activity

The enzyme activities were measured at various pHs from 2.0 to 11.0 in several buffer. As shown in Fig. 3 A, the enzyme was active in weakly alkaline pH and the optimum pH was about pH 7.75. The enzyme activity at pH 7.0 was approximately 76% of the maximal activity. Fig. 3B shows the temperature dependence profile at various temperatures. The optimum temperature for insoluble collagen (Type I) hydrolysis was at 28℃.

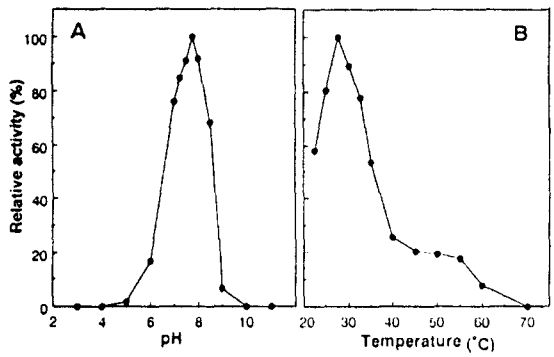

Fig. 3. Effects of pH and temperature on the activity of *Vibrio mimicus* collagenase. A : Enzyme activity was measured in the appropriate buffer containing 0.36 mM $CaCl_2$ at 28℃ for 24 h. Buffers : 50 mM citrate/Na_2HPO_4, pH 3.0-7.0 ; 50 mM Tris/HCl, pH 7.0-9.0 ; 50 mM Na_2CO_3/$NaHCO_3$, pH 9.0-10.8. B : Enzyme activity was measured in 50 mM Tris-HCl (pH 7.75) containing 0.36 mM $CaCl_2$ at the various temperatures for 24 h.

Effects of various compounds on the enzyme activity

The effects of various proteinase inhibitors on the activity of the purified collagenase are shown in Table 3. The enzyme was completely by EDTA and strongly inhibited by DFP, TLCK and TPCK. The activity was partially inhibited by soybean trypsin inhibitor and L-cysteine, and slightly activated by histidine. The enzyme activity was increased 1.2-fold by 1 mM $CaCl_2$, $HgCl_2$ and $CoCl_2$, while the activity was inhibited about 40% by 1 mM $MnCl_2$ and $CuSO_4$. These results suggest that the enzyme is a metalloproteinase.

Table 3. Effects on Inhibitiors and metal ions on the activity of *Vibrio mimicus* collagenase

Reagent	Concentration (m.)	Relative activity (%)
None		100
EDTA	1	0
DFP	1	10
TLCK	1	19
TPCK	1	28
Soybean trypsin inhibitor	1	40
L-cysteine	1	49
Histidine	1	175
Ca^{2+}	0.15	124
Hg^{2+}	0.15	116
Co^{2+}	0.15	115
Na^{2+}	0.15	93
Zn^{2+}	0.15	85
Li^{2+}	0.15	84
Mg^{2+}	0.15	79
Mn^{2+}	0.15	68
Cu^{2+}	0.15	62

Substrate specificity and kinetic properties

The substrate specificity of the purified collagenase was examined by using various substrates such as Type I, Type II and Type V collagen from achilles' tendon and Type III collagen from calf skin. The results are shown in Fig. 4. The enzyme hydrolyzed various type collagen, and the enzymatic activity on acid-soluble collagen (Type III) was the highest among the substrates tested. The rate of cleavage by the enzyme decreased in the order : Type III>Type I>Type II>Type V. On the other hand, there are reported that most microbial collagenase cleaved a synthetic peptide, PZ-Pro-Leu-Gly-Pro-D-Arg, at a single position[15,17,23]. We confirmed in the present study using the purified collagenase that the enzyme cleaved the synthetic peptide between Leu-Gly residues.

The kinetic parameters of the collagenase were measured at pH 7.75 and 28℃ using insoluble collagen (Type I) described above. K_m and k_{cat}/K_m values of the enzyme were determined to be 2.86 mg/ml and 972.28 U/mg-proein, respectively.

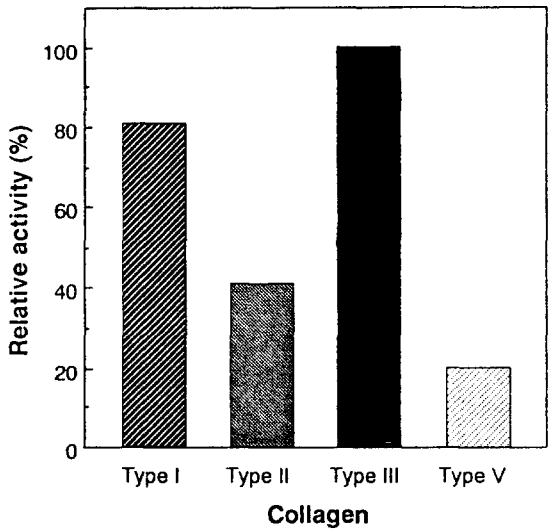

Fig. 4. Substrate specificity of *Vibrio mimicus* collagenase on various collagen. Reaction mixture contained 5 μg collagen and 50 mg collagenase in 1 ml of 50 mM Tris-HCl, pH 7.75, containing $CaCl_2$. The reaction mixture was incubated at 28 ℃ for 24 h. The hydrolyzed peptide was determined as described under "Material and Methods". Collagen Type I ; insoluble collagen from bovine Achilles', Type II ; insoluble collagen from bovine Achilles', Type III ; acid-soluble collagen from calf skin, Type V ; insoluble collagen from bovine Achilles'.

Comparison of enzymatic activities of the purified collagenase and various proteinases

To investigate the collagenolytic activity of the purified collagenase and various proteinases (*Clostridium histolyticum* collagenase, papain, α-chymotrypsin, trypsin, and pepsin), the collagenolytic activities of the enzymes were measured as described above at 28℃ and 37℃,

respectively. The results of the activities of the enzymes on insoluble collagen (Type I) are shown in Table 4. The purified collagenase showed the highest collagenolytic activity (555 U/mg-protein) at 28℃. On the other hand, the activity of *Clostridium histolyticum* collagenase was 225 U/mg-protein, and the activities of papain, α-chymotrypsin, trypsin, and pepsin were much lower than that of *Clostridium histolyticum* collagenase. At 37 ℃, the collagenolytic activities of the purified collagenase and *Clostridium histolyticum* collagenase were 290 U/mg-protein and 255 U/mg-protein, respectively, but the other enzymes did not have a little collagenolytic activity.

The proteolytic activity of the purified collagenase on casein was compared to the activities of other enzymes. The activity of pronase E was 0.226 U/mg-protein, which is the highest proteolytic activity. Those of a-chymotrypsin and papain were 0.204 U/mg-protein and 0.201 U/mg-protein, respectively, while the proteolytic activities of the purified collagenase and *Clostridium histolyticum* collagenase were 0.0006 U/mg-protein and 0.160 U/mg-protein, respectively. The proteolytic activity of the purified enzyme was approximately 267-fold lower than that of *Clostridium histolticum* collagenase (Table 4).

Discussion

In the present study, we purified an collagenase from the culture filtrate of *Vibrio mimicus* (ATCC 33658), gram negative marine bacterium, using ammonium sulfate fractionation and two times DEAE-Sephadex column chromatographics. The collagenase obtained after 3.5-fold purification showed apparent homogeneity on SDS-PAGE and Sephdex G-150 gel filtration.

The molecular mass of the purified collagenase from *Vibrio mimicus* was 42 kDa by SDS-PAGE and gel filtration (Fig. 2). These results suggest that the enzyme molecule is present as a monomer. Over the years, molecular weight of collagenases from other species of microorganism have been reported. *Clostridium histolyticum* collagenases have been found to have a molecular weight ranging from 68~125 kDa[24]. Also, the molecular weights of collagenase from *Vibrio* B-30 (24 & 28 kDa) and *Streptomyces* sp. C-51 (100 kDa) were determined [11,14]. Thus, the molecular weight of *Vibrio mimicus* collagenase is lower than general bacteria collagenases.

Many other properties of *Vibrio mimicus* collagenase are similar to those reported for *Clostridium histolyticum* [8], *Streptomyces* sp. C-51[14], and *Vibrio* B-30 collagenases [11] : e.g., optimum pH (7.75) ; the peptide specificities

Table 4. Comparison of enzymatic activities of *Vibrio mimicus* collagenase and various proteinase

Enzyme	Collagenolytic activity (units/mg)		Caseinolytic activity (units/mg)
	28℃[a]	37℃[b]	
Collagenase (*V. mimicus*)[c]	555	290	0.001
Collagenase, Type I (*C. histolyticum*)	225	255	0.160
α-chymotrypsin	20	60	0.204
Papain	35	65	0.201
Pepsin	5	20	0.008
Pronase E	100	180	0.226
Trypsin	35	70	0.187

[a]In collagenase assay, the reaction mixture was incubated at 28℃ for 24 h.

[b]In collagenase assay, the reaction mixture was incubated at 37℃ for 24 h.

[c]*Vibrio mimicus* collagenase in this work.

are apparently identical (extensive degradation of native collagen and cleavage pattern of a oligopeptide) ; EDTA inhibit all the enzyme completely. The optimum temperature (28℃) of *Vibrio mimicus* collagenase is slightly lower than that for *Streptomyces* sp. C-51[14] and *Vibrio* B-30 collagenases[11].

Vibrio mimicus has several advantages over other bacteria for the production and isolation of collagenase. The fact that this strain does not produce the toxins often encountered in *Clostridium* cultures makes its large-scale production attractive for clinical and indutrial viewpoints[25]. In addition, *Vibrio mimicus* collagenase can be purified by simple procedures as described above.

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초록 : *Vibrio mimicus*가 생산하는 collagenase의 정제 및 특성

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Vibrio mimicus(ATCC 33658)의 최적 배양조건하에서 배양액으로부터 collagenase를 황산암모늄 염석과 DEAE-Sephadex A-50 이온교환크로마토그래피에 의해 분리·정제하였다. SDS-PAGE 전기영동분석법 및 겔여과법으로 정제된 collagenase의 분자량은 42 kDa이였다. 기질인 불용성 콜라겐(Type Ⅰ)에 대한 collagenase의 최적 pH 및 온도는 각각 7.75 및 28℃였다. 금속착물제와 serine protease 저해제는 collagenase의 활성을 저해하였지만 L-cysteine과 histidine은 효소의 활성을 저해하지 않았다. Collagenase의 아미노산 조성은 glycine 및 alanine의 아미노산 잔기가 많이 함유되어 있었다. 불용성(Type Ⅰ) 콜라겐에 대한 collagenase의 속도상수인 K_m값 및 k_{cat}/K_m값은 각각 2.86 mg/ml 및 972.28 U/mg-protein이였다.