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Purification and Properties of a Collagenolytic Protease Produced by *Bacillus cereus* MBL13 Strain

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Summary

A novel collagenase-producing bacterium has been isolated and identified as *Bacillus cereus* MBL13. From the culture supernatant of *B. cereus* MBL13 grown on bone collagen as the sole carbon and nitrogen source, an extracellular protease with novel property of hydrolyzing waste animal bones was purified. The molecular mass of the purified collagenolytic protease was estimated to be (38.0±1.5) kDa. As determined by amino acid analysis, it had high contents of asparagine, lysine and serine. The optimum temperature and pH for the collagenase activity were 40 °C and pH=8.0, respectively. The results of the effects of some metal ions, inhibitors and protein substrates suggested that the purified collagenolytic protease is a member of the metalloproteases. Type I collagen (the typical collagen in animal bone) was used as the substrate for determination of Michaelis-Menten kinetics. The obtained K_m value was (1.31±0.05) g/L and the corresponding v_{max} value was (12.54±2.5) μmol/min. The study assumes that the collagenolytic protease purified from *B. cereus* MBL13 strain could be applied in the hydrolysis of waste animal bones.

Key words: *Bacillus cereus* MBL13, collagenolytic protease, type I collagen, metalloprotease, purification, waste animal bones

Introduction

Collagens and their peptide fragments are produced in large quantities as by-products in livestock and poultry industries (*1*). Collagen is the predominant constituent of skin, tendons, and cartilage as well as the organic component of bones, teeth and corneas. Type I collagen is the most abundant component of the organic matrix of bone tissue, composing 85–90 % of all bone protein (*2*). Collagen is advertised regularly in the mass media as an ingredient in drugs, drinks, foods, cosmetics, and a variety of healthcare products. It has been reported that collagen peptides, the products of collagen degradation, possess several biological activities of industrial and medical interest. True collagenases are strictly defined as proteases capable of cleaving helical regions of collagen molecules in fibrillar form under physiological conditions of pH and temperature (*3*). The term collagenolytic proteases is used also in a broader sense and refers to enzyme ability to degrade fibrillar or non-fibrillar collagen substrates. Collagenolytic proteases are involved in various physiological and pathological conditions, such as fetal bone development, embryonic development, wound repair, rheumatoid arthritis, malignant tumour invasion, intestinal ulceration and chronic periodontal inflammation (*4*). Collagenolytic proteases are classified into two major groups, metallocollagenases and serine-collagenases. Among bacterial collagenolytic proteases, metalloproteases are the most frequent, whereas serine proteases and other proteases are rarely seen. All metalloproteases share a zinc-containing HEXXH motif in their active sites.

Many collagenolytic proteases have been reported originating from various microorganisms, such as *Vibrio* B-30, *Clostridium histolyticum*, *Clostridium perfringens*, *Can-*

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dida albicans URM 3622, *Bacillus* sp. MO-1 and *Vibrio vulnificus* (*5–10*). Bacterial proteases in various forms have found practical applications worldwide, for example, as additives to laundry detergents and experimental reagents in laboratory work (*4*). Only very little is known about the specificity of collagenases towards animal bones. Reports dealing with bacterial collagenases and collagen-degrading enzymes focus on the potential role of these enzymes in human diseases (*11*).

Bacillus cereus, an aerobic, endospore-forming and mobile Gram-positive rod, is commonly found in soil, air and water. *Bacillus* species are known to secrete several proteases during their transition to the stationary phase (*12*).

Some species of *Bacillus cereus* have previously been reported to produce collagenolytic proteases (*13*). The practical applications of a number of industrial collagenolytic proteases have limitations. Hence, the knowledge of kinetics and catalytic behaviour of the protease secreted from any new strain is a prerequisite for evaluation of its biotechnological potential (*14,15*). Recently, there has been much interest in developing environmentally friendly treatment of waste animal bones in order to obtain collagen through enzymolysis. However, only a limited number of proteases with unique characteristics can trigger bone collagen degradation (*16*). Studies on the isolation of the strains that produce a bone-degrading collagenase, and their application to degrade waste bones have not been reported. In this study, from the culture of a newly discovered *Bacillus cereus* MBL13 strain, isolated from animal bone wastes, a novel collagenolytic protease has been purified. Also, some physicochemical and molecular properties of the collagenase were studied. The results indicate that the purified collagenolytic protease, with its property to degrade bone collagen, could have application(s) in animal bone processing industry.

Materials and Methods

Materials

Bone collagen used in these experiments was purchased from Dongguan Wulong Gelatin Co. Ltd, Guangdong, PR China. Type I collagen (acid-soluble, bovine achilles tendon), type II collagen, type III collagen, gelatin, glucose, casein, yeast extract, tryptone, 1-propanol, phenylazobenzyloxy-carbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-PLGPA), ethylene diamine tetraacetic acid (EDTA), phenylmethanesulphonyl fluoride (PMSF), ethylene glycol tetraacetic acid (EGTA), 2-mercaptoethanol, cysteine, leupeptin and *N*-ethylmaleimide were purchased from Sigma Chemical Co, St. Louis, MO, USA. Furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) was purchased from AppliChem GmbH, Saxony-Anhalt, Germany. Bovine serum albumin (BSA), dye reagent concentrate for protein determination and reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from BioRad Co. (Richmond, CA, USA). For protease purification DEAE-cellulose 52 was purchased from Whatman Biochemicals Ltd, Maidstone, Kent, UK. Sephadex G-100 was obtained from Pharmacia Fine Chemicals Inc, Uppsala, Sweden. Trichloroacetic acid (TCA) was of reagent grade (Wako Pure Chemicals, Osaka, Japan). Animal bones were collected from a local market and washed thoroughly using tap water and then dried. All other reagents used were of the highest grade available.

Isolation and selection of the strains

Microorganisms were isolated from the chopped animal bone wastes collected at Changsha in Hunan province, PR China. These strains were screened on agar plates (diameter 60 mm, Lianman Industry Co. Ltd, Shanghai, PR China) containing 10 g/L of bone collagen, 20 g/L of agar, pH=7.2–7.5. The plates were incubated at 37 °C for 48 h. Colonies that grew well under such conditions were isolated and retained for subsequent screening. To each individual colony, a drop of mercuric chloride precipitation reagent (15 g of $HgCl_2$, 20 mL of concentrated HCl and distilled water up to the volume of 100 mL) was added. Thirty-two bacteria with larger transparent circles were obtained and then inoculated in the fermentation medium (in g/L): $CaCl_2$ 0.05, $NaH_2PO_4 \cdot 2H_2O$ 0.5, $K_2HPO_4 \cdot 3H_2O$ 2.5, and bone collagen 30, pH=7.0–7.2. The inoculum, 40 g/L, was added into baffled shake flasks (100 mL of liquid medium in 250-mL flasks). The flasks were shaken at 180 rpm on an orbital shaker at 37 °C. After 48 h of incubation, the culture broth was centrifuged (at 4 °C and 4000×*g* for 10 min) and the supernatants were collected for enzyme activity measurement. Among these strains, the strain MBL13 with the highest collagenase activity was isolated and identified according to method described in Bergey's Manual of Determinative Bacteriology (*17*) and 16S rDNA sequence was analysed (*18*).

Growth conditions and collagenase production

For enzyme production, 500-mL liquid medium containing (in g/L): glucose 20, yeast extract 1.5, tryptone 10, $NaH_2PO_4 \cdot 2H_2O$ 0.5, $K_2HPO_4 \cdot 3H_2O$ 2.5, $CaCl_2$ 0.05, pH=7.0–7.2 was poured into the 2-litre baffled flasks for cultivation. The screened strain was inoculated (inoculum concentration of 40 g/L) and cultured at 37 °C for 26 h on a rotary shaker at 180 rpm. Samples were collected at 2-hour intervals in order to determine bacterial growth and collagenase activity. The growth was monitored by measuring $A_{600\ nm}$ values (UV-1800 spectrophotometer Shimadzu Co, Kyoto, Japan).

Purification of collagenolytic protease

All purification steps were carried out at 4 °C. After cultivation, the cells were removed by centrifugation at 10 000×*g* for 20 min. Solid $(NH_4)_2SO_4$ was slowly added into the culture medium supernatant (4 L) until the concentration reached saturation of 30 %, which was calculated using 3.9 M at 0 °C. The volume of the resultant mixture increased and was kept at 4 °C overnight. A volume of 4.1 L of the supernatant was collected by centrifugation at 10 000×*g* for 30 min. Solid $(NH_4)_2SO_4$ was continuously added into the supernatant (30 %) until the concentration reached saturation of 75 %, stirred for 60 min and left overnight at 4 °C. The precipitate was harvested by centrifugation at 10 000×*g* for 30 min, dissolved in Tris-HCl (pH=7.5) and dialyzed against the

same buffer overnight (4 °C), and then concentrated in a freeze dryer (Labconco Corp, Kansas City, MO, USA) (*19,20*). The concentrate was dissolved in 20 mM Tris-HCl buffer (pH=7.5) and the insoluble components were removed by centrifugation at 10 000×*g* for 30 min. The supernatant was dialyzed against the same buffer for 24 h. The resultant dialysate was concentrated by ultrafiltration using an Amicon PM10 membrane (M_r=10 000 Da, Millipore, Billerica, MA, USA). The concentrate was applied to a DEAE-cellulose 52 column (1.6×40.0 cm, Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer, and eluted with a linear gradient of 0–2.0 M NaCl in 20 mM Tris-HCl buffer, pH=7.5, at a flow rate of 3 mL/min. The active fractions were pooled, dialyzed and concentrated. The concentrate was reloaded onto a Sephadex G-100 column (1.6×60 cm, GE Healthcare, Uppsala, Sweden) equilibrated with the same Tris-HCl buffer. The active fractions were pooled and concentrated. Afterwards, the collagenolytic protease solution was concentrated and stored at –20 °C until use. Fractions (1 mL) of purification steps were collected and tested for collagenase activity and analyzed by SDS-PAGE to confirm purity.

All chromatographic purification steps were carried out using an ÄKTA™ purifier (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Analytical methods

Collagenolytic protease assay

The activity of collagenolytic protease was assayed by the method of Kang *et al.* (*10*), with modifications. This collagenolytic protease reaction was carried out with the addition of 0.01 mL of the collagenolytic protease solution to 0.3 mL of 1 g/L acid-soluble type I bovine collagen in 20 mM Tris-HCl buffer (pH=7.5) at 37 °C for 30 min, *i.e.* standard assay conditions. The reaction was stopped by the addition of 0.6 mL of 10 % (by mass per volume) trichloroacetic acid (TCA). After that, the solution was centrifuged at 10 000×*g* for 10 min (Avanti J-E, Beckman Coulter Inc, Brea, CA, USA). The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, heated at 100 °C for 15 min, cooled in ice water for 5 min, and the mixture was diluted with 2.5 mL of 60 % (by volume) 1-propanol. After centrifugation at 10 000×*g* for 10 min, the absorbance of the mixture was measured at 570 nm (UV-1800 spectrophotometer Shimadzu Co, Kyoto, Japan). One unit (U) of collagenase activity was expressed as one µmol of glycin equivalents released per mL per minute. The amount of glycin was determined from the glycin standard curve. All experiments were performed in triplicate and average values and corresponding standard deviations (S.D.) were reported.

Protein assay

Protein content was estimated by the method of Bradford (*21*) using BioRad protein assay dye reagent concentrate and BSA as the standard protein.

Molecular mass determination

SDS-PAGE was carried out using the method described by Laemmli (*22*), with 12 % polyacrylamide resolving gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Amino acid analysis

The purified collagenolytic protease was hydrolyzed in sealed vacuum tubes with 6 M HCl at 115 °C for 24 h, using norleucine as internal standard. The liberated amino acids were converted into phenylthiocarbamyl (PTC) derivatives and analyzed by high-performance liquid chromatography (HPLC) on a PicoTag 3.9×150 mm column (Waters, Milford, MA, USA), using citric acid and sodium citrate buffers as the eluents (gradient of pH=3.2, 3.0, 4.0 and 4.9) and ultraviolet-visible detector for detection.

Effect of temperature and pH on collagenase activity and stability

Using a standard reaction mixture (type I collagen) for collagenolytic protease assay, the effect of temperature on the collagenase activity was determined at different temperatures ranging from 10 to 70 °C, followed by assaying the collagenase activity against the control (substrate without enzyme). The thermal stability was determined by preincubating purified collagenolytic protease at the given temperature (10–70 °C) for 1 h and as soon as the sample reached 37 °C, the residual activity was measured by the standard collagenolytic protease assay against enzyme control.

The optimum pH was determined at 37 °C, followed by assaying the enzyme activity against the control (substrate without enzyme). The substrate (20 g/L, type I collagen) was prepared over a range of pH values between 3.0 and 11.0. Protease activity was measured at different pH values under standard assay conditions. pH stability studies were performed by preincubating 5 mL of purified collagenolytic protease in different pH values of the buffer ranging from 3.0–11.0, of the same volume (3.5 mL) at 37 °C for 1 h and then the residual activity was analyzed under standard assay conditions against enzyme control.

The highest collagenase activity was taken as 100 % (purified collagense was kept at 4 °C).

Effect of metal ions and inhibitors on collagenase activity

The effects of metal ions on the collagenase activity were investigated by the addition of monovalent (K^+), divalent (Ba^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Li^{2+}, Mn^{2+} and Fe^{2+}) and trivalent (Al^{3+}) ions. The assay was carried out by preincubating collagenolytic protease with the cations at the final concentration of 2 mM for 1 h at 37 ºC and after that the remaining proteolytic activity was measured using the collagenolytic protease assay as described above.

The effects of inhibitors were also studied using EDTA (metalloprotease inhibitor), PMSF (serine protease inhibitor), EGTA, 2-mercaptoethanol, cysteine, leupeptin, *N*-ethylmaleimide at final concentrations of 2 and 5 mM. After incubation at 37 ºC for 30 min, the remaining activity was assayed as described above.

Collagenase activity measured in the absence of any inhibitor or any ion was taken as 100 %.

Determination of specificity and collagenolytic protease kinetics

The specificity of the collagenolytic protease was tested using various substrates in 20 mM Tris-HCl buffer (pH=7.5). The substrates used were: type I collagen, type II collagen, type III collagen, gelatin, casein, bovine serum albumin (BSA), furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) and phenylazobenzyloxy-carbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-PLGPA) at a final concentration of 3 mM. Reaction was performed at 37 °C for 30 min. One unit of activity was defined as the amount of the enzyme that catalyses the transformation of 1 μmol of substrate per minute. The proteolytic specificity of the purified collagenolytic protease was determined by analyzing peptides, obtained after incubating the protease with type I collagen as the substrate. The hydrolysis products were analyzed by SDS-PAGE.

The kinetic parameters of the purified collagenolytic protease were investigated by using type I collagen at pH and temperature of 7.5 and 40 °C, respectively. The K_m and v_{max} values were determined using Michaelis-Menten equation.

Hydrolysis of animal bone

A mass of 6.0 g of ground animal bone (0.2×0.2 cm) was added to 30 mL of water. It was heat treated for 20 min at 100 °C, and then cooled to room temperature. A mass of 10 μg of purified collagenase was added into the bone culture. The hydrolysis was performed for 60 min, and stopped by heating the mixture to 90 °C. The inactivated reaction mixture was centrifuged at 4000×*g* for 10 min. Using scanning electron microscopy (JSM-6390, JEOL Ltd, Tokyo, Japan), bone surface was analyzed after the treatment with purified collagenase.

Statistical analysis of the data

Statistical processing of the data was performed using the SPSSX statistical package (*23*). Since the attributes were qualitative in nature, a χ^2 test was applied. To ensure that the test results were sufficiently reliable, rows and columns were either pooled or discarded when the numbers of entries with theoretical frequencies lower than 5 made more than 20 % of the data matrix.

Results and Discussion

Isolation and screening of microorganisms

Collagenase-producing bacteria were isolated from waste animal bones by selective media. Among 32 strains with larger transparent circles, the isolated MBL13 strain showed the highest collagenase activity secreted into the cultivation media. The colony and the transparent circle of MBL13 are shown in Fig. 1. The biochemical and morphological characteristics of MBL13 revealed that the organism belongs to the *Bacillus* genus. Analysis of 16S rDNA sequence showed that the isolated strain was *Bacillus cereus* (MBL13 strain).

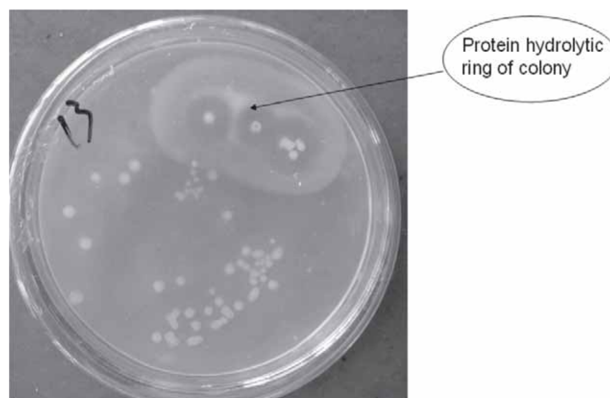

Fig. 1. Colony morphology of *B. cereus* MBL13 strain, and the detection of secreted bacterial proteases into the medium

Change of the growth of B. cereus MBL13 strain and collagenase activity

A time course study was performed in order to determine the growth and collagenase activity of *B. cereus* MBL13 strain. In Fig. 2, bacterial growth (expressed as ln $A_{600\,nm}$) and collagenase activity (U/mL), determined at 2-hour intervals, are presented. Maximal growth of this bacterium was achieved after 12 h of incubation (Fig. 2). *B. cereus* MBL13 grew exponentially from 0–10 h (specific growth rate is 0.47 per h). After 10 h of culturing, the specific growth rate of bacteria decreased, while collagenase activity started to increase. The logarithmic phase commenced at 8 h and continued for another 14 h. Protease production was detected in the late logarithmic phase (after 10 h) and after 22 h of incubation, it reached its optimum production. This situation is very similar to the *B. cereus* cultures described by Adigüzel *et al.* (*24*). The production of enzyme normally occurs in the late logarithmic phase of growth, when the cell density is high. Zaliha *et al.* (*25*) reported that once the cell densities and autoinducers have reached certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and secretion systems is induced. It is believed that the reason why collagenase activity increases with a delay compared to bacterial growth (Fig. 2) is because the culture at time zero has sufficient nutrients and therefore enzyme secretion is repressed. After 10 h, the growth is no longer ex-

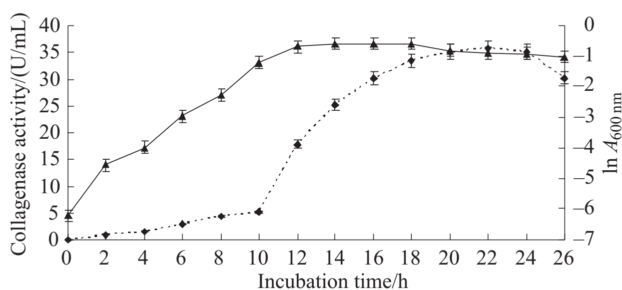

Fig. 2. Changes of the growth rate and collagenase activity of *B. cereus* MBL13 strain during culturing (0–26 h). The growth of *B. cereus* MBL13 (—▲—) is shown as ln $A_{600\,nm}$. Collagenase activity (--◆--) is expressed in U/mL. Results are presented as mean± S.D. based on triplicate data

ponential because the most preferred medium components have been depleted and cells started to secrete collagenase.

Purification of collagenolytic protease

In the presence of bone collagen as the major carbon and nitrogen source, *B. cereus* MBL13 secreted collagenolytic protease into the culture medium. The secreted collagenase was purified to electrophoretic homogeneity by the combination of three purification steps. Using ammonium sulphate as the first purification step (Table 1), 80 % of proteins secreted into the medium were removed. As shown in Table 1, the enzyme was purified 20.4-fold with a 25.2 % yield. The specific activity of the final enzyme preparation was estimated to be 2443 U/mg. Collagenolytic protease samples extracted in different purification steps were tested by SDS-PAGE (Fig. 3). After Sephadex G-100 chromatography, collagenase activity was detected in a single peak. The corresponding SDS-PAGE showed the homogeneity of the purified collagenolytic protease with a molecular mass of (38.0±1.5) kDa (S.D., *N*=3) (Fig. 3). This molecular mass is in agreement with the data from the literature which indicate that the molecular masses of proteases are rarely higher than 50 kDa (*26*). The molecular mass of the purified collagenolytic enzyme was much smaller than the molecular mass of the collagenases isolated from *C. histolyticum*, *C. perfringens*, *Acinetobacter* sp. and *Vibrio alginolyticus* (molecular masses of 120, 66-125, 102 and 82 kDa) (*6,7,27,28*). Also, the molecular mass of the protease isolated in the present paper was lower than the molecular mass of collagenolytic proteases purified from *Bacillus cereus* by Mäkinen and Mäkinen (*29*) and Lund and Granum (*30*), which had molecular masses of 87 and 105 kDa, respectively. The molecular mass of our preparation was close to the molecular mass of proteases from *Bacillus circulans* and *Bacillus cereus* (39.5 and 45.6 kDa, respectively) (*14,28*).

Determination of amino acids

Amino acid composition, expressed as residues per 1000 total residues, is shown in Table 2.

Regarding the amino acid composition, asparagine is the major amino acid in the purified collagenolytic protease from *B. cereus* MBL13. High contents of lysine, serine, threonine and glutamine, very little methionine,

Table 1. The summary of the purification of *B. cereus* MBL13 collagenolytic protease

Purification step	Total activity/U	*m*(total protein)/mg	Specific activity/(U/mg)	Purification/fold	Yield/%
Crude enzyme	77570	643	120	1.0	100.0
Ammonium sulphate	51563	117	441	3.7	66.5
DEAE-cellulose 52	30210	21	1439	12.0	38.9
Sephadex G-100	19542	8	2443	20.4	25.2

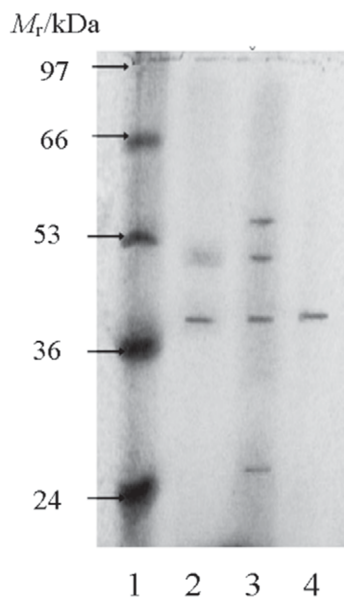

Fig. 3. SDS-PAGE of the protein fractions obtained from various purification steps. Electrophoresis was performed on 12 % gels. Lane 1: molecular mass markers (97, 66, 53, 36 and 24 kDa), lane 2: fraction after the second DEAE-cellulose 52 column chromatography, lane 3: fraction after the ammounium sulphate precipitation step, lane 4: purified collagenase. Protein was visualized by Commassie Brilliant Blue R 250 staining of the gel. The picture is a representative of three similar experiments

Table 2. Amino acid composition of *B. cereus* MBL13 collagenolytic protease

Amino acid	Nearest integer	*n*/%
Glutamine	65	8.6
Glycine	45	6.0
Asparagine	88	11.7
Threonine	67	8.9
Serine	72	9.6
Arginine	32	4.3
Isoleucine	40	5.3
Methionine	8	1.1
Alanine	44	5.9
Valine	53	7.0
Tyrosine	35	4.7
Leucine	51	6.8
Phenyalanine	31	4.1
Histidine	15	2.0
Lysine	74	9.8
Proline	25	3.3
Cystine	0	0
Tryptophan	7	0.9

The values shown are medians from three separate analyses except for the value for methionine, which is the nearest integer of the mean of two determinations

tryptophan and no cysteine in the amino acid composition were found. These results show that the amino acid composition of the collagenase from *B. cereus* MBL13 is similar to that of the collagenase purified from *Bacillus cereus* (*29*).

Enzymological features of collagenolytic protease

Optimum temperature and thermal stability

The optimum temperature for collagenase activity was measured at various temperatures, ranging between 10 and 70 °C. As shown in Fig. 4, the maximum collagenase activity was observed at 40 °C. The increase of temperature above 40 °C drastically reduced the collagenase activity. The temperature maximum obtained in this study is similar to the temperature maximum for *Bacillus* sp. MO-1, which is between 40 and 50 °C (*9*). The temperature maximum of the protease isolated in this study was higher than the temperature maxima of collagenases from the greenshore crab (*Carcinus maenas*) and marine bacterium *Vibrio vulnificus* CYK279H (*10,31*). From the temperature profile, it can be seen that the collagenolytic protease maintained over 60 % of its hydrolitic activity between 10–50 °C. At 60 °C, the relative collagenase activity was only 20 % (Fig. 4).

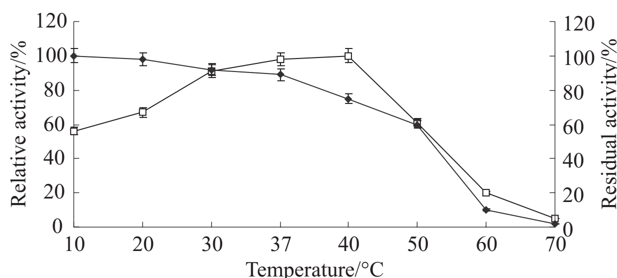

Fig. 4. Effect of temperature on the activity and stability of purified collagenolytic protease. The optimum temperature for the collagenase activity (—□—) was determined at different temperatures (10–70 °C). The enzyme was preincubated at 10–70 °C for 1 h to determine its stability. The stability of the enyme (--◆--) is expressed as residual activity. Results are expressed as mean±S.D. based on triplicate data

Analysis of the temperature-dependent collagenase activity revealed that the enzyme catalytic behaviour was similar in the temperature range of 35–45 °C. The collagenase was thermostable in the temperature range of 10–40 °C (more than 75 % of the residual activity). However, further increase of temperature influenced the collagenase activity. The residual activity was 60 % at 50 °C, and only 10 % at 60 °C (Fig. 4).

Effect of pH on the activity and stability

The effect of pH on the collagenase activity was estimated in three different buffer systems, 50 mM sodium citrate (pH=3.0–6.6), 50 mM Tris-HCl (pH=7.0–9.0), and 50 mM Na_2CO_3/$NaHCO_3$ (pH=9.16–11.0). The optimum pH of the collagenolytic protease was estimated to be 8.0 at 37 °C, which was the same as for the collagenolytic protease from *Treponema denticola* ATCC 35405-A (*32*). Over 70 % of protease activity was maintained within the pH range of 7.0–8.0 (Fig. 5). The relative activity of the purified collagenolytic protease, which was highly

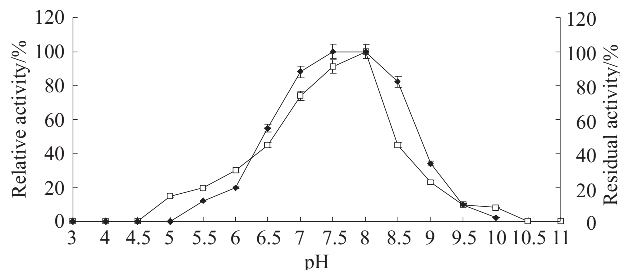

Fig. 5. Effect of pH on the activity and stability of purified collagenolytic protease. The optimum pH for the activity (—□—) and pH stability (--◆--) of the enzyme were determined at various pH values (pH=3.0–11.0). Before assaying, enzymes were preincubated at the given pH values for 1 h. The buffers used were 50 mM sodium citrate (pH=3.0–6.6), 50 mM Tris-HCl (pH=7.0–9.0), and 50 mM Na_2CO_3/$NaHCO_3$ (pH=9.16–11.0). The results are expressed as mean±S.D. based on triplicate data

stable in the pH=7.0–8.5 range, was 45 and 23 % at pH=8.5 and pH=9.0, respectively. This pH range is lower than pH ranges determined for *Bacillus circulans* and *Bacillus licheniformis* NH1 proteases (*14,33*). With the decrease of pH (pH=3.0–7.0), collagenase activity also sharply decreased. Collagenase activity rapidly decreased from 34 to 2 % with the increase of pH above pH=9.0 (pH=9.0–11.0) (Fig. 5).

Bacillus genus secretes mostly two types of extracellular proteases, neutral or metalloprotease, and an alkaline protease, which is functionally a serine endopeptidase and referred to as subtilisin. The first exhibits optimal pH at 7.0, whereas the latter has pH optima between 9 and 11. Therefore, the collagenolytic protease produced by *B. cereus* MBL13 is a neutral protease as indicated by its pH activity and stability. However, compared to the other proteases produced by *B. cereus*, this collagenolytic protease has new characteristics.

Effect of metal ions on the activity

The effect of metal ions on the collagenase activity is presented in Table 3. The collagenase activity of the

Table 3. Effect of different metal ions on the purified protease activity

c=2.0 mM	Residual activity/%
None	100
K^+	(89±2.23)*
Ca^{2+}	(130±2.20)*
Cu^{2+}	(2±0.51)*
Li^{2+}	96±2.43
Zn^{2+}	(110±1.92)*
Mn^{2+}	92±3.05
Mg^{2+}	(107±2.05)*
Ba^{2+}	98±2.11
Fe^{2+}	(86±2.30)*
Al^{3+}	96±2.29

Mean±S.D., calculated on the basis of peak areas; the results represent the mean value of at least three independent experiments performed in duplicate

*significantly different from 100 % activity (Mann-Whitney *U* test, $p<0.05$)

purified collagenolytic protease was increased in the presence of Ca^{2+}, Zn^{2+} and Mg^{2+}, but strongly inhibited by Cu^{2+}. These results indicate that the collagenolytic protease requires Ca^{2+} for its optimal activity. Calcium cations are known to be stabilizers of many enzymes, protecting them from conformational changes. The effect of metal ions on the activity of *B. cereus* MBL13-secreted collagenolytic protease was similar to those of *Bacillus* sp. MO-1 and *Bacillus* sp. JH108 (*9,34*). Generally, bacterial collagenases specifically require zinc and calcium for their optimum activity and stability. Metallocollagenases, first discovered in tadpole tissue explants, are zinc-containing enzymes, but also generally require calcium for their optimum activity and stability (*35,36*).

Effect of inhibitors on the activity

To investigate the nature of the alkaline protease produced by *B. cereus* MBL13, the collagenase activity in the presence of 2.0 and 5.0 mM of different protease inhibitors was analyzed. By comparing collagenase acivity without the added inhibitors to the collagenase activity in the presence of inhibitors in the assay mixture, it can be seen that the collagenase activity changed in regard to two different concentrations of inhibitors. The results revealed that EDTA and EGTA (metalloprotease inhibitors) completely inhibited the collagenase activity even at very low concentration (2 mM). Leupeptin, 2-mercaptoethanol and *N*-ethylmaleimide inhibited collagenase activity only at a higher concentration (5 mM), while PMSF (serine protease inhibitor) and cysteine showed no or very little effect on protease activity (Table 4).

Table 4. Effects of inhibitors on enzyme activity

Inhibitors	*c*=2 mM	*c*=5 mM
	Residual activity/%	
None	100	100
EDTA	(7±2.15)*	(3±0.23)*
EGTA	(10±1.54)*	(6±0.35)*
PMSF	98±2.21	93±3.05
Cysteine	100±1.43	99±2.91
2-mercaptoethanol	(78±1.86)*	(62±2.25)*
Leupeptin	93±3.01	(87±1.99)*
N-ethylmaleimide	(89±2.42)*	(81±2.16)*

Mean±S.D., calculated on the basis of peak areas; the results represent the mean value of at least three independent experiments performed in duplicate

*significantly different from 100 % activity (Mann-Whitney *U* test, $p<0.05$)

Metalloproteases require a divalent metal ion at their reaction centre, and are usually inhibited by divalent metal ion chelators, such as EDTA or EGTA. North (*37*) classified proteases based on their sensitivity to various inhibitors. Therefore, it could be concluded that the purified collagenolytic protease produced by *B. cereus* MBL13 is a member of metalloproteases.

Specificity of substrate and enzyme kinetics

The specificity of the purified collagenolytic protease was tested using various substrates at 37 °C for 30 min. Collagenase displays a great deal of specificity in hydrolyzing a single polypeptide bond on each chain of the native triple-stranded collagen helix. Of the protein substrates, FALGPA, gelatin and PZ-PLGPA were found to be suitable substrates for the purified collagenolytic protease from *B. cereus* MBL13 strain. The purified protease showed high activity towards the above mentioned substrates, but no activity towards BSA was observed (Table 5). In this study, no other proteins or synthetic peptide substrates were investigated. The cleavage rate for the substrates due to the purified collagenolytic protease was as follows: FALGPA=gelatin>PZ-PLGPA>type I collagen>type III collagen>casein>type II collagen.

Table 5. Substrate specificity of collagenolytic protease

Substrate	Relative activity/%
Type I collagen*	85±2.13
Type II collagen	67±2.05
Type III collagen	76±2.19
Gelatin	100
Casein	67±1.71
BSA	n.d.
FALGPA	100
PZ-PLGPA	98±2.01

Mean±S.D., calculated on the basis of peak areas; the results represent the mean value of at least three independent experiments performed in duplicate

*acid-soluble, from bovine achilles tendon; n.d. – not detected

Using type I collagen, the kinetic parameters of the purified collagenolytic protease were investigated at pH=7.5 and 37 °C. The hydrolysate was analyzed by SDS-PAGE (Fig. 6). By comparing lanes 2 and 3, it can be seen that undigested type I collagen (lane 2), became a smear of small uncontinuous molecular mass polypeptides after hydrolysis (lane 3). These results also indicate that enzymatic hydrolysis is specific for type I collagen.

The kinetic parameters v_{max} and K_m of the purified protease were determined by measuring the collagenase activity at different substrate concentrations (0.2–2.0 g/L). For collagen I as a substrate, it was determined that this collagenolytic protease had K_m of (1.31±0.05) g/L (S.D., N=3) and v_{max} of (12.54±2.5) μmol/min (S.D., N=3) (Fig. 7). The K_m value of the protease described in this research is in the range of K_m value reported for *Bacillus* sp. No. 8-16 (1.3 mg/mL) (*38*). The K_m value of our collagenolytic protease was lower than the K_m values of proteases isolated from *Bacillus clausii* GMBAE 42 (1.8 mg/mL), haloalkaliphilic *Bacillus* sp. (2 mg/mL) and *Pseudomonas aeruginosa* PseA (2.69 mg/mL) (*39–41*).

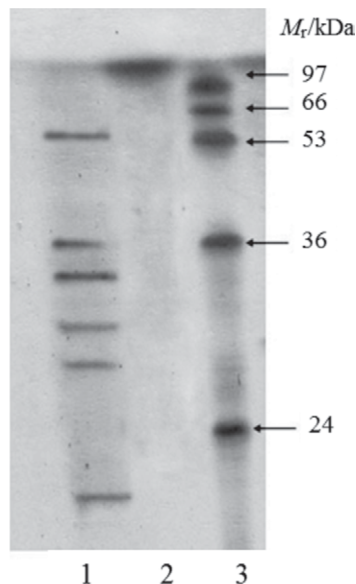

Fig. 6. SDS-PAGE of type I collagen and its hydrolysis products obtained by the collagenolytic protease of *B. cereus* MBL13. Proteins were separated on 18 % gel. Lane 1: type I collagen hydrolysates, lane 2: undigested type I collagen, lane 3: protein molecular mass markers (97, 66, 53, 36 and 24 kDa)

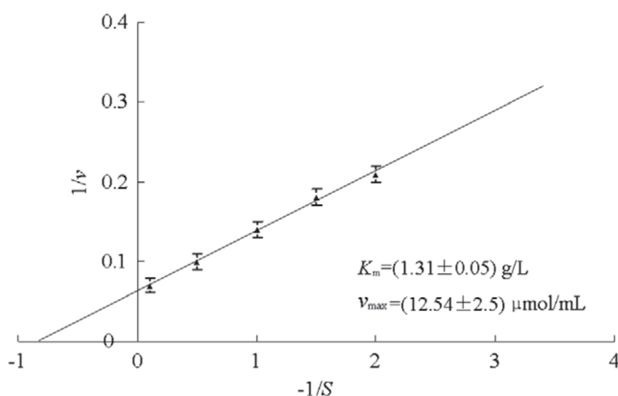

Fig. 7. Lineweaver-Burk plot for the hydrolysis of type I collagen by the *B. cereus* MBL13 collagenolytic protease. The collagenase activity was measured at different type I collagen concentrations (0.2–2.0 g/L) in 50 mM Tris-HCl buffer (pH=7.5). Each point is the mean of three determinations. Standard deviation is based on triplicate data

Hydrolysis of animal bone

Animal bone is composed of a well organized extracellular matrix that contains embedded crystals of hydroxyapatite (HA). The major part, 90 % of the organic matrix, is collagen. In mature collagens, the amino acid contents of both glycine and proline exceed 20 %. Proline is a peculiar amino acid due to its pyrrolidine ring structure, and the proline peptide bond is one of the hardest to hydrolyze. Thus, because of the bone's tight structure, it is hardly hydrolyzed. Scanning electron microscopy (SEM) analysis (Fig. 8) showed that *B. cereus* MBL13 collagenolytic protease hydrolyzed bone surface. An interesting effect is that the collagenolytic protease 'cracked' the bone surface.

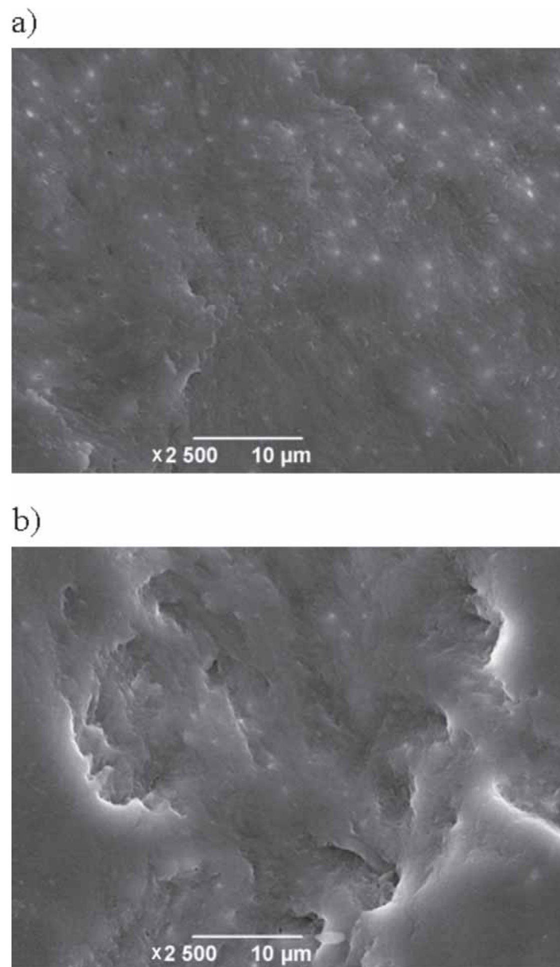

Fig. 8. High resolution scanning electron microscopy image of animal bone surface (2500× magnification): a) untreated, b) treated by *B. cereus* MBL13 collagenolytic protease

Conclusions

In this work, *B. cereus* MBL13 was selected based on the level of production of collagenolytic protease as well as in regard to characteristical growth of these bacteria on bone collagen. *B. cereus* MBL13 collagenolytic protease was purified to electrophoretic homogeneity using a combination of three purification steps. For the purified collagenolytic protease thermostability and activity over a certain range of pH values were determined. The protease inhibition pattern, a pattern typical for metalloproteases, suggested that purified collagenolytic protease is a metalloprotease. Furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) and phenylazobenzyloxy-carbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-PLGPA) were found to be suitable protein substrates for the protease. With respect to substrate specificity, enzymatic hydrolysis was specific for type I collagen. The result of the action of the purified enzyme towards animal bone showed that the protease is a collagenase-type and is able to degrade animal bones. Therefore, the presented results indicate that *B. cereus* MBL13 collagenolytic protease will most probably play an important role in the process of efficient waste animal bones hydrolysis.

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