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Purification and Characterization of a Novel Collagenase from *Bacillus pumilus* Col-J

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Abstract The collagenase, produced extracellular by *Bacillus pumilus* Col-J, was purified by ammonium sulfate precipitation followed by two gel filtrations, involving Sephadex G-100 column and Sepharose Fast Flow column. Purified collagenase has a 31.53-fold increase in specific activity of 87.33 U/mg and 7.00% recovery. The collagenase has a relative molecular weight of 58.64 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal temperature for the enzyme reaction was 45°C. More than 50% of the original activity still remained after 5 min of incubation at 70°C or 10 min at 60°C. The maximal enzyme activity of collagenase was obtained at pH7.5, and it was stable over a pH range of 6.5–8.0. The collagenase activity was strongly inhibited by Mn^{2+}, Pb^{2+}, ethylenediamine tetraacetic acid, ethylene glycol tetraacetic acid, and β-mercaptoethanol. However, Ca^{2+} and Mg^{2+} greatly increased its activity. The collagenase from *B. pumilus* Col-J showed highly specific activity towards the native collagen from calf skin. The K_m and V_{max} of the enzyme for collagen were 0.79 mg/mL and 129.5 U, respectively.

Keywords Collagenase · *Bacillus pumilus* · Purification · Characterization

Introduction

China is one of the biggest processing centers of leather products in the world. It manufactures approximately 70 million hides of sheep and other animals. In the meantime, 1.4 million tons of leather scraps are produced and most of them are discarded, causing severe pollution to the environment. An enormous amount of high-quality collagen is also lost during the process. Collagenase is an enzyme that is highly specific for both native and denatured collagen. It is widely used in medical industries, molecular biology experiments,

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as well as in the food industry [1–4]. Since collagenase is mostly extracted from viscera organ of fish or other animals [5, 6], it is not cost-effective.

Collagenases produced by bacteria are well documented for a long time. The diversity of these enzymes is mainly correlated with the phylogenetic relationship of the three bacteria as follows: *Clostridium*, such as *Clostridium histolyticum* [7] and *Clostridium perfringens* [8]; *Vibrio*, such as *Vibrio alginolyticus* [9] and *Vibrio parahaemolyticus* [10]; and *Porphyromonas*, such as *Porphyromonas gingivalis* [11] and *Porphyromonas endodontalis* [12]. These microbes are pathogens or conditional pathogenic bacteria which potentially produce toxin. Thus, the application of collagenases would be restricted. Lately, some microorganisms, which are generally considered safe to produce collagenase, were screened from soil, water, and caviar, such as *Bacillus subtilis* [13, 14], *Bacillus licheniformis* [15], *Streptomyces* sp. [16], and *Thermoactinomyces* [17]. These progresses would make collagenase to have greater applications in the future.

Here, we describe the purification of collagenase, which was isolated from the culture filtrate of the strain *Bacillus pumilus* Col-J, and some of its properties.

Materials and Methods

Microorganism and Culture Conditions

Many soil or sewage samples were collected from leather house, market, and slaughterhouse. Samples were diluted by sterile distilled water into three concentration gradient with 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} g/mL, respectively. Then, 50 μL suspension was spread evenly on the plate and incubated at 37 °C for 24 h. Collagenase activity was tested through elective medium. Elective medium contained (per liter): 20 g gelatin, 0.1 g NaCl, 5 g peptone, 0.5 g KH_2PO_4 and 0.2 g $MgSO_4{\cdot}7H_2O$, pH 7.2–7.5. After 24 h of incubation at 37 °C, the acidic hydrargyrum solution was used to detect the colonies with glutin hydrolyzation activity. The acidic hydrargyrum solution consisted of 15 g $HgCl_2$ and 20 mL dense hydrochloric acid per 100 mL. Then, the positive colonies were selected to be grown in a culture medium as described [18], containing of 20 g glucose, 1.5 g yeast extract, 10 g tryptose, 0.05 g $CaC1_2$, 0.5 g $NaH_2PO_4{\cdot}2H_2O$, and 2.5 g $K_2HPO_4{\cdot}3H_2O$ in 1 L, pH 7.0–7.2. The bacteria with a bigger ratio of gel hydrolysis collar to colony diameter were inoculated into fluid medium. The fresh calf skin 0.5 g was sterilized and incubated together with the culture supernatant. The tubes were set at room temperature and shaken every 12 h. After 48 h, the strain with the top hydrolysis ability to calf skin was screened to be identified following *Bergey's Manual of Systematic Bacteriology* [19].

Polymerase Chain Reaction Amplification of the 16S Ribosomal DNA and Sequence Determination

The total DNA of the target strain was extracted by DNA extracting kit Gram-positive bacteria (TianZe Genetic Engineering Co. Ltd., China). Universal polymerase chain reaction (PCR) upstream primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and downstream primer 1492R (5′-TACGG(C/T)TACCTTGTTACGACTT-3′) were used to amplify 16S ribosomal DNA (rDNA) gene. After PCR, fragments were separated by gel electrophoresis, purified from agarose, and cloned into T-vector for sequence analysis. Moreover, 16S rDNA gene sequence data of the isolate were blasted and compared the consensus sequences with GenBank database.

Assay of Collagenase Activity

Collagenase activity was measured according to the method described by Endo [20]. The standard reaction mixture, containing 0.3 mg collagen (Bornstein and Traub Type I; collagen from calf skin; aqueous acid: soluble; C3511) in 500 μL 100 mM Tris–HCl buffer including 50 mM $CaCl_2$ (pH 7.5) was incubated with 100 μL enzyme sample at 37 °C for 30 min. The amount of free amino groups released was measured by the ninhydrin method [21]. One activity unit (U) was defined as the number of 1 μmol L Gly released as a result of the action of 1 mL culture filtrate containing collagenase on collagen for 1 min at 37 °C and pH 7.5.

Collagenase Purification

The culture supernatant was precipitated with 30% and 75% ammonium sulfate saturation level, respectively. The precipitate formed was collected by centrifugation, and then dissolved in a small amount of buffer A (10 mM Tris–HCl, 5 mM $CaCl_2$, pH 7.5). The enzyme solution was dialyzed against buffer A, concentrated by PGE-20,000, and stored at 4 °C. The sample was next applied to a Sephadex G-100 column for a group separation of the protein mixture. The column was washed with buffer A at a flow rate of 0.5 mL/min. The fractions with collagenase activity were pooled and concentrated. The sample was put on anion exchange chromatography Sepharose Fast Flow. The column was pre-equilibrated with a buffer consisting of 20 mM Tris–HCl and 10 mM $CaCl_2$. Protein fractions were eluted with a linear gradient of NaCl concentration from 0 to 1 M in buffer B (1 M NaCl, 10 mM Tris–HCl, 5 mM $CaCl_2$, pH 7.5) at a rate of 2 mL/min. Finally, the pooled collagenase fractions were concentrated and stored.

Molecular Mass Determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% polyacrylamide gels. The standard molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), bovine serum albumin (66.4 kDa), and phosphorylase b (97.2 kDa; Takara Biotechnology (DaLian) Co., Ltd). The protein bands were stained with Coomassie Brilliant Blue R-250.

Effect of Temperature and pH on Collagenase Activity

The optimum temperature for activity was determined at 25, 30, 37, 40, 45, 50, 55, 60, 65, 70, and 80 °C, respectively, and the pH was kept at 7.5. The thermostability of collagenase was investigated by measuring the activity remaining at 45 °C after the purified collagenase was incubated for 5 and 10 min at various temperatures between 45 °C and boiled water in 50 mM Tris–HCl buffer (pH 7.5) in the presence of 50 mM $CaCl_2$, respectively.

For determination of pH dependence on enzyme activity, the following buffers (50 mM) with 50 mM $CaCl_2$ were used: citric acid–sodium citrate (pH 3.0–6.5), Tris–HCl (pH 7.0–9.0), and borax–NaOH (pH 10.0). The reaction temperature was kept at 45 °C. The effects of pH on enzyme stability were analyzed by the photometric assay after preincubation in buffered solutions for 30 min at 45 °C.

Effect of Metal Ions and Various Reagents on Collagenase Activity

The effect of metal ions on the enzyme activity was investigated by adding three monovalent ions (Na^+, K^+, and Li^+) and seven divalent ions (Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Ba^{2+}, and Pb^{2+}) at the final concentration with 10 mM in the reaction mixture, respectively. The residual activities were determined as a percentage of the activity in the control sample without additive metal ions. The influence of chemical reagents on collagenase activity was also tested using ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and β-mercaptoethanol at 10 mM final concentration, as described above for protease inhibitors.

Substrate Specificity Determination

To measure the digestion of collagen from calf skin (C3511) and bovine Achilles tendon (C9879) and gelatin, the reaction mixture that contained 300 μL of 1 mg/mL substrate, 200 μL of 100 mM Tris–HCl (pH7.5) that contained 50 mM $CaCl_2$, and 100 μL of the enzyme solution was incubated at 45 °C for 30 min. The collagenase activity was measured as described previously.

Assay for Kinetic Properties

The apparent Michaelis–Menten constant (K_m) and the substrate turnover number (V_{max}) were calculated by a least-squares analysis from Lineweaver–Burk plots with 100 mM Tris–HCl (pH7.5) that contained 50 mM $CaCl_2$ at 45 °C with collagen from calf skin as the substrate. The range of the substrate concentration that was used in all of the determinations was 0.0625–0.5 mg/mL.

Results

Isolation and Screening of the Strains Producing Collagenase

Forty-nine microorganism strains with the activity of gelatin hydrolyzation were screened from water and soil samples. The culture supernatant of five strains showed the strong ability to digest the calf skin, among which strain Col-J showed the highest activity with 35.97 U/mL.

Identification of Bacteria

On the elective medium plate, the colony of strain Col-J was gray in color, dry and crinkly on the surface, and round in shape after 24 h of incubation at 37 °C. Under a light microscope, cells were Gram-positive, 1.3–1.4 μm in length and 0.7–0.9 μm in diameter, and have blunt ends, short rods, and mesogenous spores. Strain Col-J was tentatively identified as *B. pumilus* from the biochemical and physiological characteristics (Table 1). In order to confirm the identity, a full 16S rDNA sequence with 1,513 bp in length was amplified and determined (Accession no. EF197942). The result of 16S rDNA sequence blasting indicated that the strain Col-J had homology of 100% with *B. pumilus* (Accession no. EF528287, and other about ten sequences in GenBank). Then, we concluded that strain Col-J is a strain of *B. pumilus*.

Table 1 Biochemical and physiological characteristics of strain Col-J.

Characteristic	Results
Gram stain	+
Spore	+
Motility at 37°C	+
Catalase	+
Anaerobic growth	+
Voges–Prosakauer	+
Acid from	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Gas from glucose	−
Gelatin liquefaction	+
Hydrolysis of starch	−
Hydrolysis of hippurate	+
Decomposition of casein	+
Use of propionate	−
Phenylalanine deaminase	−
$NO_3^- \rightarrow NO_2^-$	+
Indole	−
Growth at pH6.8 in NB	+
Growth at pH5.7 in NB	+
Growth on NaCl	
2%	+
5%	+
7%	+
Growth at	
5°C	−
10°C	−
30°C	+
40°C	+
50°C	+
55°C	−
65°C	−

Purification of Collagenase

The supernatant of bulk-cultured *B. pumilus* Col-J was concentrated with gradient ammonium sulfate 30% and 75%, and the precipitate was dissolved in buffer A. The solution was then dialyzed against buffer A. Subsequently, the dialyzate was applied to a Sephadex G-100 column. The proteins were eluted in two separate peaks. The collagenolytic activity was detected in the first peak. The active fraction was pooled and concentrated by PEG-20,000. Finally, the enzyme solution was subjected to an anion exchange chromatography on Sepharose Fast Flow column. The fractions containing

Table 2 Purification of collagenase from *Bacillus pumilus* Col-J.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg Pr)	Purification (fold)	Yield (%)
Cultivate superatant	4,265.4	11,800	2.77	1	100
$(NH_4)_2SO_4$ precipitation	836.50	7,837.2	9.40	3.39	66.42
Sephadex G-100 column	101.45	4,578	45.13	16.29	38.80
Sepharose Fast Flow column	9.46	826.14	87.33	31.53	7.00

collagenolytic activity were pooled, concentrated, and stored at −20°C. The results of the collagenase purification were summarized in Table 2. By a three-step procedure, the enzyme was purified 31.53-fold with a yield of 7.00% from crude extract. As judged from SDS-PAGE, collagenase from *B. pumilus* Col-J was purified to homogeneity. The relative molecular mass was estimated to be 58.64 kDa for the enzyme (Fig. 1).

Effect of Temperature on Enzyme Activity and Stability

The optimum activity of the enzyme was observed at 45°C (Fig. 2a). To analyze the stability of the purified collagenase against temperature, collagenase activity was determined as outlined in the "Materials and Methods" section. The residual enzyme activity of heat treatment for 5 min was higher than for 10 min, while their changing curve was similar (Fig. 2b). When the collagenase was stable at 50°C, the enzyme activity dropped sharply about 30% of the full activity. However, the enzyme activity decreased gradually from 60 to 100°C. The results showed that the collagenase kept relatively stable and retained above 50% activity under treatment at 70°C for 5 min or 60°C for 10 min.

Effect of pH on Enzyme Activity and Stability

The effect of pH on collagenase activity was measured in a range from pH3 to 10. As shown in Fig. 3a, the purified enzyme had the pH optimum at 7.5. Consequently, this

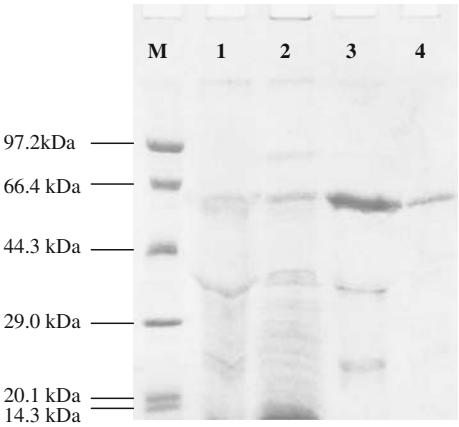

Fig. 1 SDS-PAGE and molecular mass determination of collagenase from *Bacillus pumilus* Col-J. *M* Standard molecular weight, *1* sample from initial culture supernatant, *2* sample after $(NH_4)_2SO_4$ precipitation, *3* sample after Sephadex G-100 column, *4* sample after Sepharose Fast Flow column

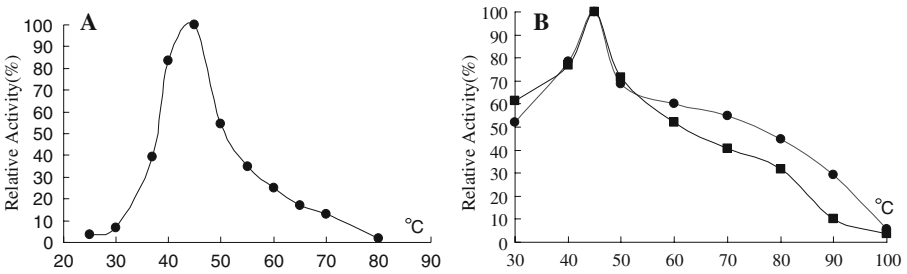

Fig. 2 **a** Effect of temperature on enzyme activity; **b** effect of temperature on enzyme stability (*filled circle*, treatment for 5 min; *filled square*, treatment for 10 min)

collagenase belongs to the group of neutral protease. After incubation between pH6.5 and 8.0 at 45 °C for 30 min, the collagenase was stable and retained above 84% of full activity (Fig. 3b). However, the stability was lower at pH<5 or >9, and the activity lost dramatically over 40%.

Effect of Metal Ion and Reagents on Enzyme Activity

Most of the metal ions tested exhibited either no effect or a slight stimulatory effect (Table 3). Some of the metal ions such as Li^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Ba^{2+} increased and stabilized the collagenase activity, and especially the Ca^{2+} ion displayed the strongest activation ability with 127%. Further, Mn^{2+} and Pb^{2+} ions showed obvious inhibition of the enzyme by 37.8% and 55.2%. Additionally, the collagenase was drastically inhibited by EDTA, EGTA, and β-mercaptoethanol. In particular, EGTA had maximum inhibition of the enzyme activity by about 90%.

Substrate Specificity

Among the protein substrates, the native collagen from calf skin was found to be the most suitable substrate for the enzyme from *B. pumilus* Col-J. Meantime, this collagenase could also hydrolyze the native collagen from bovine Achilles tendon and gelatin with relative cleavage rate of 50.72% and 62.56%, respectively (Fig. 4). The substrate specificity data were consistent with the high hydrolyzation ability to calf skin mentioned above.

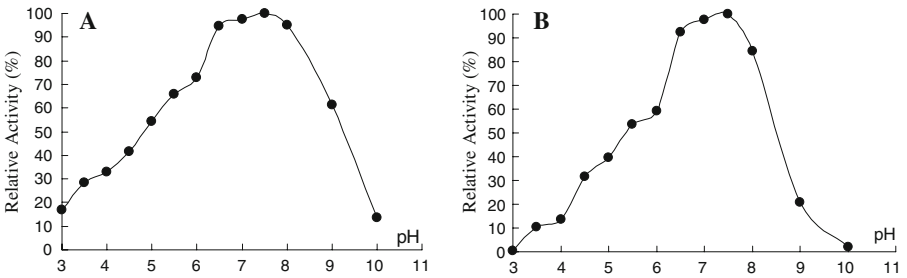

Fig. 3 **a** Effect of pH on enzyme activity; **b** effect of pH on enzyme stability (The pH activity profiles were determined at 45 °C; pH stability was determined at pH7.5 after incubation of enzymes for 30 min at various pH buffers)

Table 3 Effect of metal ions and inhibitors on activity of collagenase from *Bacillus pumilus* Col-J.

Metal ion/chemical reagent (10 mM)	Relative activity (%)
Control	100
Li^{+}	109.1
Na^{+}	96.7
K^{+}	110.3
Mg^{2+}	119.5
Ca^{2+}	127
Zn^{2+}	85.1
Fe^{2+}	98.3
Mn^{2+}	62.2
Ba^{2+}	105.4
Pb^{2+}	44.8
EDTA	15.4
EGTA	10.1
β-Mercaptoethanol	20.7

Kinetic Properties

The kinetic parameters of the purified collagenase were measured at pH 7.5 and 45 °C using the soluble acidic collagen from calf skin, as described previously. K_m and V_{max} of the enzyme were determined to be 0.792 mg/mL and 129.5 U, respectively (Fig. 5).

Discussion

As the denaturation collagen, the amino acid composition of gelatin is similar to collagen. Collagenase has the ability to hydrolyze gelatin; then, gelatin is used as the substrate to originally collect bacteria producing collagenase. Bacteria on the raw skins with collagenase activity were considered as an important reason for leather decomposing. In this study, we chose samples from tannery, butcher shop, abattoir, and so on. Most of the other bacteria were removed by hydrolysis assay of the gelatin plate and fresh calf skin. Then, the strain Col-J producing collagenase was screened by quantitative assay of collagenase in broth using the acid-soluble collagen from calf skin as the substrate.

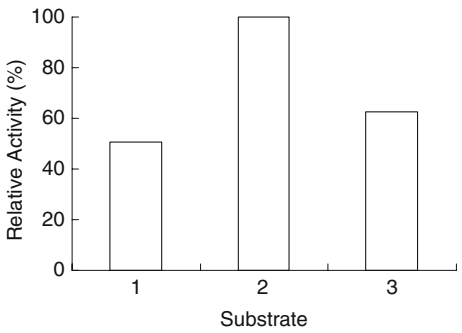

Fig. 4 Substrate specificity of collagenase from *Bacillus pumilus* Col-J. *1* Collagen from bovine Achilles tendon, *2* collagen from calf skin, *3* gelatin

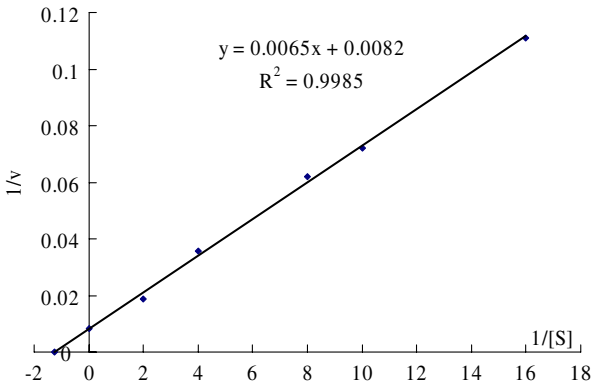

Fig. 5 Determination of kinetic parameters of collagenase from *Bacillus pumilus* Col-J by Lineweaver–Burk plot

The strain Col-J coincides with the property of genus *Bacillus* according to colony morphology and cultural characteristic. Most of the biochemical and physiological data showed that it was a *B. pumilus* according to *Bergey's Manual of Systematic Bacteriology* except the positive anaerobic growth and nitrate deoxidization. Moreover, the blasting of 16S rDNA gene of strain Col-J in GenBank also confirmed the conclusion.

The collagenase was easily purified from the culture supernatant by separation with ammonium sulfate (30% and 75% saturation level), but this fraction was highly contaminated with brown pigment. After the sample was filtered through to a Sephadex G-100 column, it was divided in two groups. The first group had high A_{280} value and protein concentration, as well as collagenase activity. By comparison, the other group showed lower protein concentration and enzyme activity, but higher A_{280} value and deep brown pigment. This phenomenon proved that the brown pigment had strong absorbency at 280 nm like protein, which was similar to the report by Paul et al. [22]. Then, the first group was pooled and purified by Sepharose Fast Flow column.

Both of PAGE and SDS-PAGE indicated that the collagenase was in monomeric form, and its molecular mass is 58.64 kDa and different from all of the microbial collagenases reported. Unlike the collagenases from *Pseudomonas* sp. [23], and *Bacillus* sp. strain MO-1 [24], which consist of two subunits, most of collagenases known are monoenzymes with various molecular weights as follows: 125 kDa of *B. subtilis* FS-2 [13]; 97 and 116 kDa of *Streptomyces* sp. 3B [16]; 50 kDa of *Thermoactinomyces* sp. 21E [17]; 50, 110, 150, and 200 kDa of *Pseudomonas aeruginosa* [18]; 120 kDa of *Cytophaga* sp. L43-1 strain [25] and *C. perfringens* [26]; 30 and 40 kDa of *Photobacterium logei* [27]; 33 and 19.8 kDa of *Pseudomonas* sp. [28]; 120 and 29 kDa of *B. licheniformis* N22 [29]; and 30–40 kDa of *Streptomyces* sp. 1349 [30]. Moreover, isoenzyme PAGE indicated that there was at least three gelatinase isoenzymes produced by *B. pumilus* Col-J (data not shown). However, only one collagenase was purified and identified, while the other two were neglected because there was no collagen hydrolyzation activity or low expression.

The optimum temperature of 45 °C was recorded for the collagenase purified in this study. This is higher than with the 30–42 °C optimum temperature reported for *Cytophaga* sp. L43-1 [25], *C. perfringens* [26], *P. aeruginosa* [18], and *P. logei* [27], and similar to *Pseudomonas* sp. [28], but lower than 50 °C of *B. subtilis* FS-2 [13], 70–75 °C of *Bacillus* sp. MO-1 [24] and *Thermoactinomyces* sp. 21E [17]. On heating, the collagenase in this study was relatively stable and retained above 50% activity under treatment at 70 °C for 5 min or 60 °C for 10 min. It is more thermostable than most known collagenases, but is

lower than the others from *Bacillus* strains, and *Thermoactinomyces* sp. 21E, which is the most thermostable reported so far.

Maximum collagenolytic activity was observed at pH7.5. The result was similar to most documents with pH7.0–7.5, but lower than pH8.5 of *P. logei* [27], pH9.0 of *B. subtilis* FS-2 [13] and *Bacillus* sp. MO-1 [24], and pH9.0–9.5 of *Thermoactinomyces* sp. 21E [17]. While the pH stability of the collagenase is in the range of pH6.5–8 at 45 °C for 30 min, the enzyme retained above 84% of full activity. It is consistent with the characteristic of the neutral proteinases.

Most metal ions did not affect the enzyme activity too much. Fe^{2+} and Zn^{2+} showed lower inhibiting function on collagenase in our case, which differed from the result claiming that the enzymes from *Streptomyces* sp. 3B [16] and *Thermoactinomyces* sp. 21E [17] were inhibited almost completely by Fe^{2+} and Zn^{2+}. However, Ca^{2+} and Mg^{2+} (strongly) and Li^{+}, K^{+}, and Ba^{2+} (slightly) enhanced its activity. Besides, these cations were also reported to enhance the thermal stability of other alkaline protease from *Bacillus* sp. by protecting the enzyme against thermal denaturation and maintaining the activity conformation of the enzyme at high temperature [31, 32]. The metal chelators (EDTA and EGTA) could strongly inhibit the enzyme. Particularly, Ca^{2+} had the greatest activating activity while EGTA had the strongest inhibition. EGTA as the specific Ca^{2+} chelator could inhibit the enzyme more acutely than EDTA, which suggested that the enzyme was a Ca^{2+}-bound enzyme. However, Kim et al. [6] reported that EDTA inhibited the collagenase partially and Yang et al. [18] reported that EDTA inhibited the enzyme more intensely than EGTA. Otherwise, the collagenase was inhibited by β-mercaptoethanol dramatically, which indicated that this contracture of collagenase contained disulfide bond in this study. The results indicated that the collagenase was a Ca^{2+}-dependent protease, and it is an enzyme with disulfide bonds.

Conclusion

The results obtained from our study show that the collagenase purified from *B. pumilus* Col-J is a novel neutral collagenase with a medium molecular weight. This collagenase would be very useful for medicine, food industry, and leather scrap recycle.

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