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Full Length Research Paper

Purification and characterization of collagenase from *Bacillus licheniformis* F11.4

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The extracellular collagenase, produced by *Bacillus licheniformis* F11.4, was purified by ammonium sulfate precipitation followed by DEAE Sephadex A-50. The purified collagenase showed a 26.3-fold increase in specific activity being 1.0 U/mg and 2.6% recovery. The collagenase has an apparent molecular weight of 124 and 26 kD as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymography. The optimal temperature and pH were 50°C and pH 7.0, respectively. The collagenase activity was inhibited by Fe^{2+} (1 mM), Mg^{2+} (1 mM), Mn^{2+} (1 mM), Co^{2+} (1 mM), EDTA (1 mM), and β-mercaptoetanol (1 mM). However, Ca^{2+} (1 mM) and Cu^{2+} (1 mM) increased its activity. The collagenase from *B. licheniformis* F11.4 was capable of hydrolyzing other protein substrates such as casein, gelatin, and fibrin. The K_m and V_{max} of the enzyme for collagen were 0.26 mg/ml and 0.27 U, respectively.

Key words: Collagenase, *Bacillus licheniformis F11.4,* purification, characterization.

INTRODUCTION

Certain bacteria produce collagenases; proteases which cleave native collagen in its triple helical conformation (Takeuchi et al., 1992). Bacterial collagenases possess broad substrate specificities and degrade both native and denatured collagens (Peterkofsky, 1982), while vertebrate collagenases preferentially cleave the native form at a specific site (Birkedal-Hansen, 1987). Collagenase is widely used in medical industries, molecular biology experiments, as well as in the food industry (Beltran et al., 2006). Since collagenase is mostly extracted from viscera organ of fish or other animals (Park et al., 2002; Kim et al., 2002), it is not cost-effective. Collagenases produced by bacteria are well documented for a long time.

Collagenase-producing *Bacillus* reported include: *Bacillus licheniformis* N22 (Asdornnithee et al., 1994), *Bacillus subtilis* FS-2 (Nagano and To, 1999), *B. subtilis* CN2 (Tran and Nagano, 2002), *Bacillus* sp. MO-1 (Okamoto et al*.,* 2001), *B. subtilis* AS1.398 (Rui et al*.,* 2009), *Bacillus pumilus* CoI-J (Wu et al*.,* 2010), *Streptomyces* sp. Strain 3B (Petrova et al*.,* 2006a) and *Streptomyces parvulus* (Sakurai et al*.,* 2009). The present result would make collagenase to have greater applications in the future. Here, we describe the purification and characterization collagenase, which was isolated from the extracellular culture filtrate of the strain *B. licheniformis* F11.4 originated from Palembang, Indonesia.

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MATERIALS AND METHODS

Microorganism

The *B. licheniformis* F11 was screened from a total of 109 isolates originated from Palembang South Sumatera during exploration of chitinase and protease producing indigenous microorganisms.

Growth conditions and media composition

B. licheniformis F11.4 was cultured aerobically at 37°C in 250 ml flask containing 150 ml of growth medium (1% NaCl (Merck), 0.5% triptone (Oxoid), 0.25% yeast extract (Oxoid) and 5% collagen from fish skin) and shaken at 150 rpm.

Enzyme purification

The culture supernatant was precipitated with 50% saturation ammonium sulfate. The precipitate formed was collected by centrifugation, and then dissolved in buffer phosphate, 0.02 M pH 7.0. The enzyme solution was dialyzed (Sigma 12 kD cut off) against buffer phosphate 0.01 M pH 7.0 and stored at 4°C. The sample was next applied to a DEAE Sephadex A-50 column for a separation of the protein mixtures. The column was washed with buffer phosphate 0.02 M pH 7.0 at a flow rate of 0.5 mL/min. Protein fractions were eluted with a step wise of NaCl concentration 0; 0.125; 0.25; 0.5; 0.75 and 1 M in buffer phosphate 0.02 M, pH 8.0 at a rate of 5 ml/min. The fractions with collagenase activity were pooled and concentrated. Finally, the pooled collagenase fractions were stored.

Assay of collagenase activity and protein determination

Protease activity was measured according to the Bergmeyer method (Bergmeyer et al., 1983) with collagen from fish skin (5%) as the substrate. As much as 50 µl enzyme filtrate was mixed with 250 µl substrate and incubated for 10 minute at 37°C. Trichoracetic acid (TCA) 0.2 M was added and incubated at 37°C for 10 min, followed by centrifuged at 4000 g for 10 min. The supernatant was mixed with Na_2CO_3 0.4 M, followed by addition of Folin Ciocalteau reagent (1:2) and incubation furthur at 37°C for 20 min. The reaction products was measured at λ 578 nm. Substrate solution without enzyme was used as control. One unit (U) of enzyme activity was defined as enzyme which produces 1 µmol of tyrosine per min.

Protein concentration was analysed by Bradford's method (1976) using reagents consisted of 100 mg comassie brilliant blue (CBB) G-250 in 50 ml ethanol 95% and 100 ml phosphate acid 85% in 1 liter. Bovine serum albumin was used as the protein standard. Triplicate experiments were conducted for each measurements.

Molecular weight determination

Molecular weight was estimated by electrophoresis under denaturating polyacrylamide-SDS (SDS-PAGE) with 8% polyacrylamide gels (Laemmli, 1970). The standard moleculer weight markers were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). Enzymes activity *in situ* was determined by zymogram following Choi et al. (2001). SDS-PAGE was performed in 8% acrylamide. For zymogram analysis, collagen at 0.1% was incorporated into the gel.

Effect of pH and temperature on collagenase activity

Collagenase activity of the purified enzyme was measured at buffer universal pH 2.0 to 12.0 containing citrate acid, phosphate acid, borate acid and dietilbarbiturate acid in the absence and presence of 10 mM $CaCl_2$ at a temperature of 50°C, with collagen as the substrate. Plot of enzyme relative activity against pH was constructed to determine the optimum pH for the reaction. The effect of temperature on collagenase activity of the purified enzyme in the absence and presence of 10 mM $CaCl_2$ was measured at 30; 40; 50; 60; 70; 80 and 90°C at pH 7.0, with collagen as the substrate.

Effect of metal ions and various reagents on collagenase activity

The effects of various metal ions were tested on the activity of purified enzyme at 50°C in universal buffer pH 7.0, with collagen as the substrate. The metal ions, such as Co^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+} at the final concentration with 1 mM were applied in the reaction mixture. The residual activity was determined as a percentage of the activity in the control sample without added metal ions. The influence of chemical reagent on collagenase activity was also tested using ethylenediamine tetraacetid acid (EDTA) and β-mercaptoetanol at 1 mM.

Substrate specificity determination

Collagenase activity on various protein substrates: casein, gelatin and fibrin were assayed by mixing 0.2 ml of enzyme and 1 ml of substrate (casein, gelatin and fibrin) and 1 ml 0.05 M buffer phosphate pH 7.0. The collagenase activity was measured as described previously

Assay for kinetic properties

The apparent Michaelis-Menten constant (K_m) and the maximum velocity number (V_{max}) were calculated by a least-squares analysis from Lineweaver-Burk plots with 0.05 M phosphate buffer (pH 7) at 50°C with collagen from fish skin as the substrate. The range of the substrate concentration used in the entire determinant, was 0.409 to 0.440 mg/ml.

RESULTS

Purification of collagenase

The supernatant of bulk-cultured *B. licheniformis* F11.4 was concentrated with 50% ammonium sulfate, and the precipitate was dissolved in phosphate buffer 0.02 M pH 7. The solution was then dialyzed against phosphate buffer 0.02 M pH 7.0. Subsequently, the dialysate was applied to a DEAE Sephadex A-50 column with a step wise NaCl concentration 0; 0.125; 0.25; 0.5; 0.75 and 1 M. The proteins were separated in two separate peaks. The fractions containing collagenolytic activity were pooled, concentrated, and stored at −20°C. The results of the collagenase purification were summarized in Table 1. By a two-step procedure, the enzyme was purified 26.3 - fold with a yield of 2.6% from the crude extract. The specific activity of the purified enzyme was 6.9 U mg^{-1} proteins. Analysis of the purified enzyme revealed two bands with molecular mass of 124 and 26 kDa as determined by SDS-PAGE and zymography (Figure 1).

Effect of temperature on enzyme activity

In the presence of calcium ions, more than 80% activity

Table 1. Summary of purification of collagenase from *B. licheniformis* F11.4.

Purification steps	Activity (U/ml)	Protein (mg)	Volume (ml)	Total activity (Units)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	0.003	0.082	1000	3.10	82	0.038	1	100
$(NH4)_2SO_4$ 50%	0.032	0.162	10	0.315	1.617	0.195	5	10
DEAE Sephadex A-50	0.016	0.015	5	0.08	0.08	1	26.3	2.6

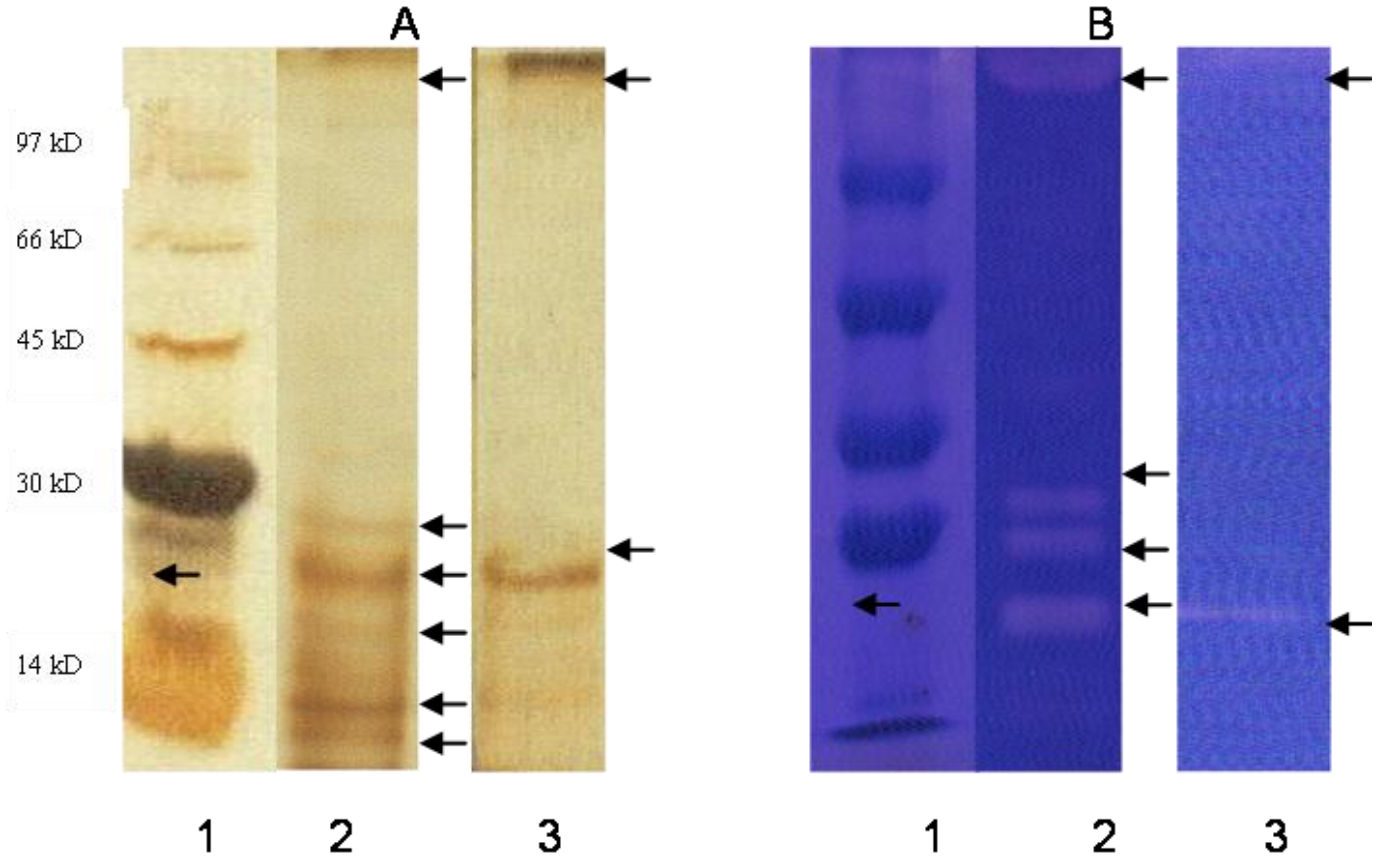

Figure 1. SDS-PAGE (A) and Zymogram (B) of collagenase from *B. licheniformis* F11.4. Line 1, shows relative molecular mass standards: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa); line 2, ammonium sulfate fraction; line 3, purified enzyme.

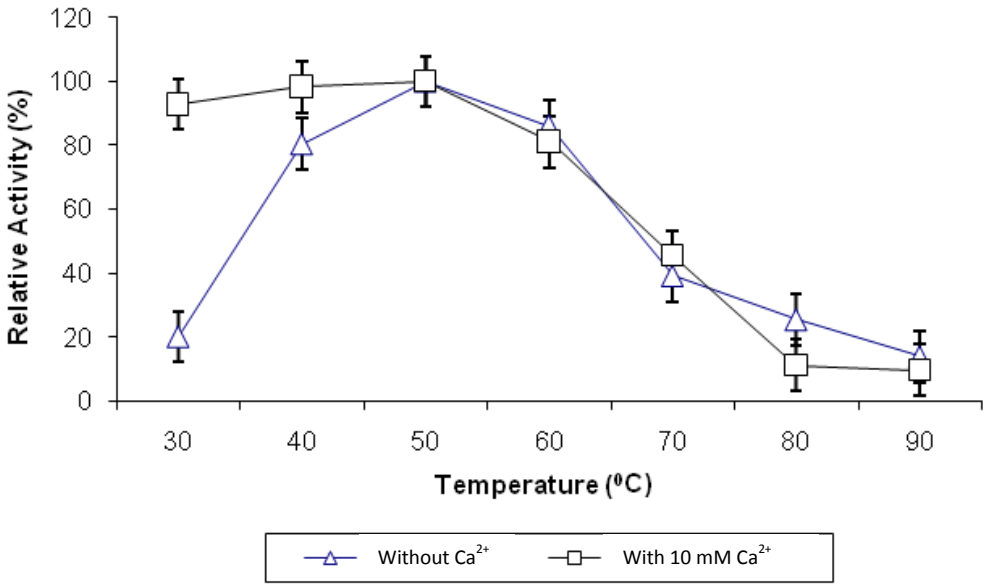

Figure 2. Effects of temperature on purified collagenase activity with 10 mM Ca^{2+} (□) and without 10 mM Ca^{2+} (Δ). Collagenase activity was determined in the buffer phosphate 0.05 M pH 7.0. Each value represents the mean ± SE of three independent experiments.

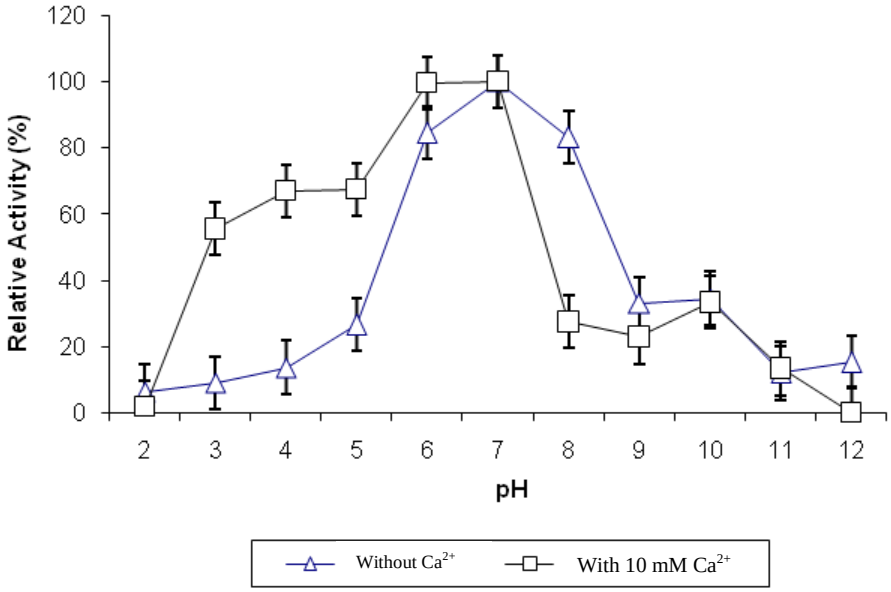

Figure 3. Activity of purified collagenase *B. licheniformis* F11.4 in with (□) and without 10 mM Ca^{2+} (Δ) at different pH values. Collagenase activity was measured at 50°C in the buffer phosphate pH 7. Each value represents the mean ± SE of three independent experiments.

was retained in the 30 to 50°C range with an optimum temperature of 50°C (Figure 2). However, in the absence of calcium, 80% enzyme activity was retained in the 50 to 60°C range with an optimum temperature of 50°C. Thus, the enzyme was moderately thermo-stable (Figure 2).

Effect of pH on enzyme activity

In the absence calcium the enzyme exhibited greatest activity in the pH range of 6.0 to 8.0, with an optimum pH of 7.0 (Figure 3). However, in the presence of calcium,

Table 2. Effects of metal ions and reagents on purified collagenase activity.

Treatment	Concentration (mM)	Relative activity (%)
None	-	100
Ca^{2+}	1	108.36
Cu^{2+}	1	131.56
Fe^{2+}	1	40.75
Co^{2+}	1	6.31
Mg^{2+}	1	13.54
Mn^{2+}	1	48.80
EDTA	1	2.29
β-mercaptoetanol	1	0

the enzyme exhibited greatest activity in the range of 5.0 to 7.0 with an optimum pH of 7.0. Consequently, this collagenase belongs to the group of neutral protease.

Effect of metal ions and reagents on enzyme activity

Among the metal ions tested, only Ca^{2+} and Cu^{2+} stimulated the activity of the enzyme (Table 2). Collagenase activity was inhibited by Co^{2+}, Mn^{2+}, Fe^{2+} and Mg^{2+}. The enzyme was completely inhibited by 1 mM EDTA, a metalloprotease inhibitor and β-mercaptoetanol (Table 2). These results are consistent with the enzyme being a metalloprotease.

Substrate specifity of collagenase

The collagenase was active on variety of other proteins (casein, gelatin, and fibrin). The collagenase exhibited the highest activity on casein. This collagenase could also hydrolyze gelatin and fibrin with 37.03% and 19.96% of the activity toward casein (Figure 4).

Kinetic properties

The kinetic parameters of the purified collagenase were measured at pH 7.0 and 50°C using collagen from fish skin, as described previously. K_m and V_{max} of the enzyme were determined to be 0.26 mg/ml and 0.27 U, respectively (Figure 5).

DISCUSSION

The *B. lichneiformis* F11 showed high protease/ collagenase activity but lacking chitinase activities. The organism strain was identified based on 16S rRNA sequencing, physiological and biochemical characterization. *B. licheniformis* F11.4 displayed a rough colony morphology. Microscopic investigation revealed form motile rods of equal sizes (2.9 by 0.75 µm). According to the physiological and microscopic tests, *B. licheniformis* F11 is suggested to be representatives of the *B. licheniformis* species (Waldeck et al., 2006). These findings agree with the fact that no extra-chromosomal elements (approximately 1 to 20 kb in size) could be detected within F11cells since small plasmids are rarely found in *B. licheniformis* strains (6 to 25%) (Manachini et al., 1998; Yoshimura et al., 1983). However, definite evidence for the affiliation of the isolates with the species *B. licheniformis* was obtained from sequencing the 16S rRNA gene, including the hypervariable regions V1 to V3; 100% identity to *B. licheniformis* DSM13/ATCC 14580, which was totally sequenced only recently, was found (Waldeck et al., 2006). By targeted deletion of the polyglutamate operon (*pga*) in *B. licheniformis* F11, a derivative form, F11.1 (Δ*pga*), was obtained, along with lacking polyglutamate (PGA) formation and, enhanced proteolytic activities. The phenotypic properties were maintained in a strain in which the *chiBA* operon was additionally deleted: F11.4 (Δ*chiBA* Δ*pga*) (Hoffmann et al., 2010).

In the purification of the collagenase produced by *B. licheniformis* F11.4, precipitation by ammonium sulfate was important in the removal of contaminating proteins and exopolysaccharides. The collagenase was easily purified from the culture supernatant by ammonium sulfate precipitation (50% saturation level), Then, it was pooled and purified by DEAE sephadex A-50 column. After ammonium sulphate precipitation, the enzyme was purified 5-fold with a yield of 10.1% from the crude extract (Table 1). Other work with *B. pumilus* COI-J collagenase, when ammonium sulphate was used precipite the enzyme was purified 3.39-fold with a 66.4% yield (Wu et al., 2010). Collagenase from *Streptomyces exfoliates* was purified by ammonium sulphate precipitation to 3.29-fold with a 67.21% yield (Jain and Jain, 2009). In our study purification with DEAE Sephadex A-50 column, resulted in 26.3-fold increase in the specific activity with a 2.6% yield (Table 1). Petrova et al. (2006a) used DEAE Sephadex A-25 column, for purification of collagenase from *Streptomyces* sp strain 3B which increased the enzyme purity by 18.9-fold with a yield of 38% from the crude extract.

Both of SDS-PAGE and zymography indicated that the molecular mass of the purified collagenase were 124 and 26 kDa. Different molecular masses for different collagenases have been reported: 125 kDa for *B. subtilis* FS-2 collagenase (Hiroko and Kim, 1999); 120 and 29 kDa for *B. licheniformis* N22 (Asdornnithee et al., 1994); 42.8 kD for *Bacillus cereus* (Sela et al., 1998); 58.64 kD for *B. pumilus* Col-J (Wu et al., 2010); 50 kDa for *Thermoactinomyces* sp. 21E (Petrova et al., 2006b); and 33 and 19.8 kDa for *Pseudomonas* sp. (Hisano et al., 1989).

The optimum temperature of 50°C was recorded for the

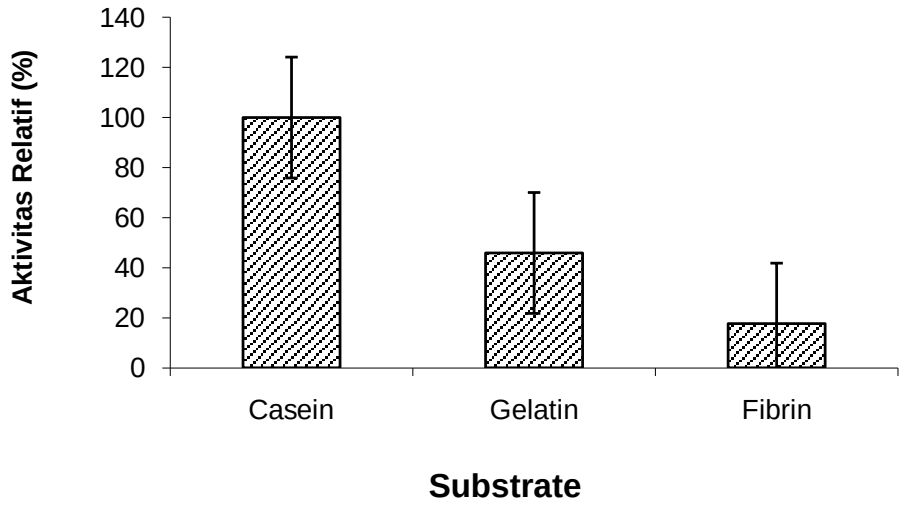

Figure 4. Substrate specificity of purified protease. Specific activity values are means of triplicate determinations ± SD.

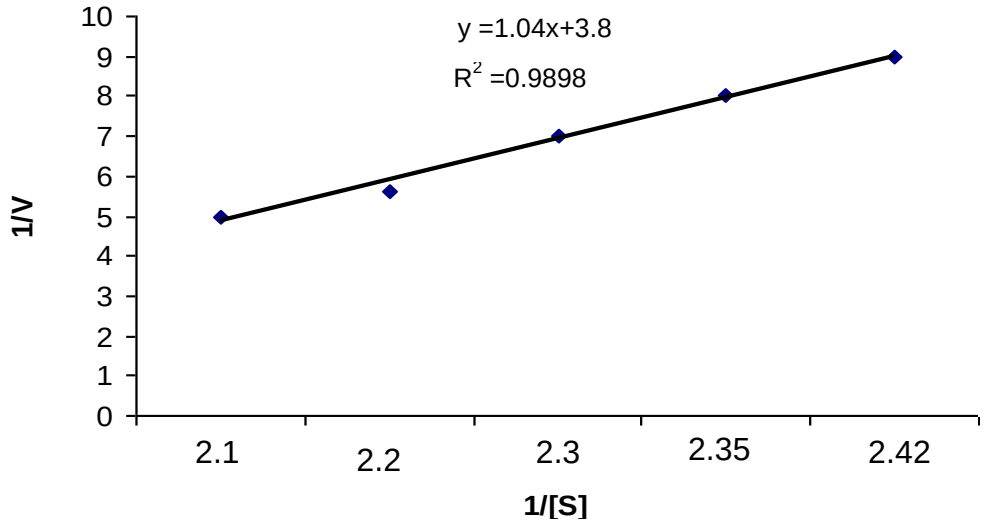

Figure 5. Determination of kinetic parameters of collagenase from *Bacillus licheniformis* F11.4 by ineweaver-Burk plot.

collagenase purified in this study. This is similar to *B. subtilis* FS-2 (Hiroko and Kim, 1999), and higher than with the 30 to 42°C optimum temperature reported for *Cytophaga* sp. L43-1 (Sasagawa et al., 1993), *Cytophaga perfringens* (Matsushita et al., 1994), but lower than 70 to 75°C of *Bacillus* sp. MO-1 (Okamoto et al., 2001) and *Thermoactinomyces* sp. 21E (Petrova et al., 2006b).

In the presence of Ca^{2+} ion (1 mM), more than 80% activity was retained in the 30 to 50 °C range but at temperature above 50°C, the activity decreased drastic-cally. While in the absence of Ca^{2+} ion, 80% enzyme activity was retained in the 50 to 60°C range. The presence of Ca^{2+} ion appeared stabilizes enzyme activity in the 30 to 50°C range. These temperatures, the enzyme conformation might be more responsive toward the presence of Ca^{2+}. Temperature of 30 to 50°C range has not yet altered the Ca^{2+} binding domain of the enzyme. At temperature above 50°C, the enzyme conformation (hydrogen bond, van der walls bond, hydrophobic bond and electrostatic interaction) was probably altered such that addition of Ca^{2+} did not have positive effect.

Maximum collagenolytic activity was observed at pH 7.0. The result was similar to most reports which were pH 7.0 to 7.5, but lower than pH 9.0 of *B. subtilis* FS-2 (Hiroko and Kim, 1999) and *Bacillus* sp. MO-1 (Okamoto et al., 2001), and pH 9.0 to 9.5 of *Thermoactinomyces* sp.

21E (Petrova et al., 2006b).

Most metal ions affect the enzyme activity. Fe^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} showed inhibitory effect on collagenase in our case, which was similar to the results claimed that the enzymes from *B. pumilus* CoI-J (Wu et al., 2010) which was inhibited by Fe^{2+} and Mn^{2+}. However, Ca^{2+} and Cu^{2+} enhanced its activity. Besides, these cations were also reported to enhance the thermal stability of another alkaline protease from *Bacillus* sp. by protecting the enzyme against thermal denaturation and maintaining the active conformation of the enzyme at high temperature (Donaghy and Mckay, 1993).

EDTA, a chelating agent for calcium ions strongly inhibited the enzyme consistent with the finding that, activity of enzyme was increased in the presence of Ca^{2+} (Figure 2). The collagenase was inhibited by β-mercaptoethanol completely, indicating that the structure of this collagenase contained disulfide bonds. The results indicated that the collagenase was a Ca^{2+}-dependent protease, and it is an enzyme with disulfide bonds.

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