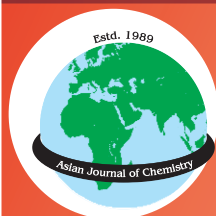

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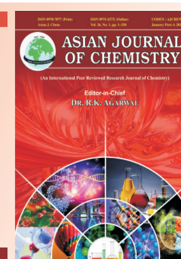

Production and Characterization of Collagenolytic Protease from *Bacillus licheniformis* F11.4 Originated from Indonesia

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Two mutant derivatives of the potent protease producing *Bacillus licheniformis* strain F11, originally isolated from Palembang, Sumatera, were checked for their capacity to secrete proteases: *B. licheniformis* F11.1, with a frame shift mutation in *chiA* and having deleted the genes for polyglutamate synthesis (genotype Δ*chiA*; Δ*pga*) and *B. licheniformis* F11.4 with a deletion spanning both of the chitinase encoding genes (genotype Δ*chiAB*; Δ*pga*). *Bacillus licheniformis* F11.4 was screened and isolated from Palembang, Indonesia. The bacteria produced collagenase enzyme when grown in a collagen media. The purpose of this study was to characterize the collagenase enzyme. Addition of collagen in the media accelerated the collagenase production time and increase the activity. The optimal temperature and pH of the enzyme were 50 °C and pH 9, respectively. At pH 12, the collagenase activity was still high. Collagenase activity was inhibited by Cu^{2+} (I mM) and stimulated by Co^{2+} (1 mM), Mg^{2+} (1 mM) and Ca^{2+} (1 mM). The collagenase from *B. licheniformis* F11.4 was capable of hydrolyzing collagen and other protein substrates such as casein, gelatin and fibrin.

Keywords: Collagenase, Characterization, *Bacillus licheniformis* F11.4.

INTRODUCTION

Collagenase are metalloprotease enzymes which can cleave helical collagen into small peptide fragments. The enzyme plays a major role in connective tissue metabolism and is produced by specific cells involved in repairs and remodeling processes. Previous studies reported that some bacterial collagenase can degrade collagen in both of the denatured and undenatured form. The most commonly used microbial collagenase in medical products was that of *Clostridium histolyticum*[1], *Pseudomonas sp.*[2], *Bacillus licheniformis* N22[3], *Bacillus pumilus* CoI-J[4], (Streptomyces parvulu[5], *Streptomyces* sp. Strain 3B[6] and *Cytophaga* sp. L43-1[7].

In search of the protease producing microorganisms which can be applied in deproteination process for chitin extraction from the shrimp waste, we found isolate *Bacillus licheniformis* F11. *Bacillus licheniformis* F11 was screened from a total of 109 isolates originated from Palembang South Sumatera during exploration of chitinase and protease producing indigenous microorganisms. Study on physiological tests and 16S rRNA gene comparison, as well as microscopic and macroscopic investigations had been conducted[8]. The amino acid alignments revealed 99 to 100 % identity to the known *B. licheniformis* loci, with frameshift mutation detected in F11 strain. This is responsible for lack of producing chitinase but actively hyper secreting protease. The protease from *B. licheniformis* F11 is good for deproteinization of shrimp waste in chitin production[8]. Two mutants of *B. licheniformis* F11 were found: *B. licheniformis* F11.1 which lack of gene encoding *ChiA* and *B. licheniformis* F11.4 which lack of gene encoding *ChiA* and B[9].

In this research, we reported that *B. licheniformis* F11.4 originated from Palembang secreted collagen degrading enzymes. Preliminary characterization of the extracellular collagenase was presented.

EXPERIMENTAL

Effect of collagen on enzyme production: Two types of media were used for bacterial growth and protease production *i.e.*, Luria Broth (LB) and modified Luria Broth plus collagen (MLB + collagen) media. The LB media contained tryptone 1 %, NaCl 1 % dan yeast extract 0.5 % (w/v). The modified LB + collagen media contained tryptone 0.5 %; NaCl 1 %, yeast extract 0.25 % and colllagen 5 % (w/v). The cell growth was monitored turbidimetrically through absorbance at λ = 620 nm. As much as 10 % of seedling culture with optical density of 0.8 (at 620 nm) was sub-cultured into the same

media for enzyme production. Incubation was conducted at 37 °C and samples were taken for analysis of enzyme activity, protein concentration and cell growth.

Assay of collagenase activity and protein determination: Collagenase activity was measured according to the Bergmeyer method[10] 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 min at 37 °C. 500 µL Trichoracetic acid (TCA) 0.2 M was added and incubated at 37 °C for 10 min, followed by centrifuged at 4000 g 10 min. The supernatant was mixed with 1.25 µL Na_2CO_3 0.4 M, followed by addition 250 µL 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 produce 1 µmol of tyrosine per min.

Protein concentration was analyzed by Bradford's method[11] using reagents consisted of 100 mg Coomassie Brilliant Blue (CBB) G-250 in 50 mL ethanol 95 % and 100 mL phosphate acid 85 % in 1 L. Bovine serum albumin was used as the protein standard. Triplicate experiments were conducted for each measurements.

Effect of pH and temperature on collagenase activity: Collagenase activity of the enzyme was measured using buffer universal pH 2-12 containing 0.029 M of A solution (citrate acid, phosphate acid, borate acid and dietilbarbiturate acid) and B solution (NaOH 0.2 N) in the absence and presence of 10 mM $CaCl_2$ at a temperature of 50 °C, with collagen (0.5 %) 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 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, with collagen (0.5 %) as the substrate.

The thermal stability of enzyme was measured from the residual collagenase activities after incubation the enzyme solution at various temperature (50 and 70 °C). Sample were taken and measured their activities every 20 min.

Effect of metal ions on collagenase activity: The effects of various metal ions were tested on the activity of enzyme at 50 °C in universal buffer pH 7, with collagen 5 % (w/v) as the substrate. The metal ions, such as $CaCl_2$ and $ZnCl_2$ at the final concentration with 1, 5 and 10 mM and $CoCl_2$, $MgCl_2$, $FeCl_2$ and $CuCl_2$ 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.

Determination of molecular weight: Molecular weight was estimated by electrophoresis under denaturating polyacrylamide-SDS (SDS-PAGE) with 8 % polyacrylamide gels[12]. 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.*[13]. Acrylamide gel was copolymerized with the 1 % protein (collagen) substrates. Following electrophoresis, the gel was soaked in Triton X-100 2.5 % for 1 h and furthur incubated for enzyme substrate reaction in buffer *Tris*-Cl 10 mM, pH 8, 37 °C for 24 h. The activity bands was visualized after incubation in staining solution (50 % methanol + 10 % acetic acid + 0.06 % coomassie brilliant blue R-250) for 30 min followed by incubation in destaining solution (5 % methanol + 7.5 % acetic acid). Positive result was seen as clear bands.

Substrate specificity determination

Collagenase activity on various protein substrates: casein (0.5 %) gelatin (0.5 %) and fibrin (0.5 %) was assayed by mixing 0.2 mL of enzyme and 1 mL of substrate (collagen, casein, gelatin and fibrin) and 1 mL 0.05 M buffer phosphate pH 7. The collagenase activity was measured as described previously.

RESULTS AND DISCUSSION

Effect of collagen on enzyme production: Fig. 1 showed collagenase production of *B. licheniformis* 11.4 in LB media and addition of collagen into the media (modified LB media). The optimum production time of *B. licheniformis* F11.4 was between 20-35 h of fermentation, with specific enzyme activity of 0.157 U/mg protein. Addition of collagen into the media, resulted in different responses (Fig. 1). In this case, the concentration of tryptone and yeast extract in the original LB media was reduced by half and collagen was added at 5 %. The activity of *Bacillus licheniformis* F11.4 was increased from 0.157 U/mg protein to 0.546 U/mg protein with optimum production being 10 h incubation which is within the logarithmic

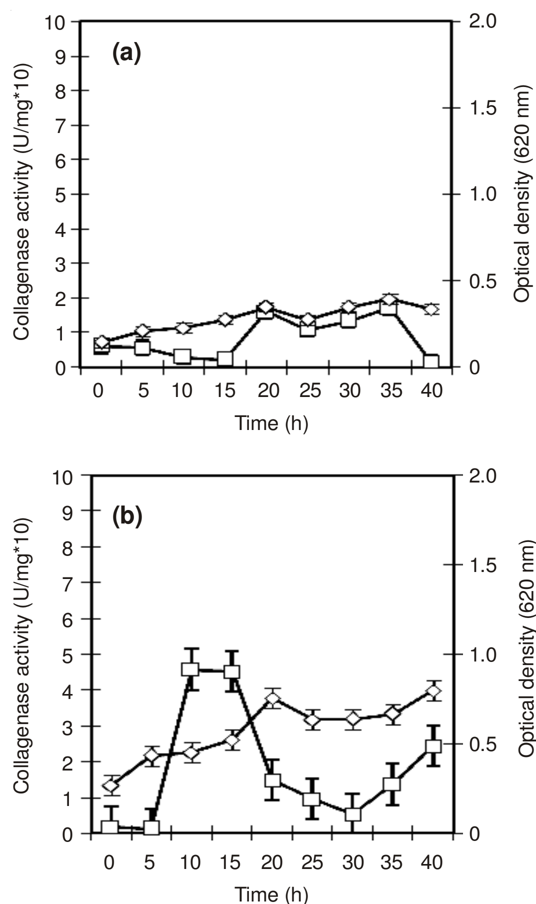

Fig. 1. Collagenase production (□) and Growth (◇) as a function of cultivation time by *Bacillus licheniformis* F11.4 grown on LB media (a) and modified LB supplemented with collagen (5 %) (b) in shake flasks at initial pH 7 and at 50 °C

growth phase. The optimum enzyme production of *B. licheniformis* F11.4 in LB media was achieved at the stationary growth phase, while that of *Bacillus licheniformis* F11.4 in modified LB media was achieved during logarithmic growth phase. Induction of collagenase enzyme was clearly observed in *B. licheniformis* F11.4 which responded to collagen addition by increasing the collagenase enzyme activity rapidly. The high enzyme activity was short lived and rapidly reduced following its optimum activity at 10 h of incubation. This may relate to the possible auto degradation of the protease.

B. licheniformis F11.4 of Palembang Indonesia was found to produce extracellular alkaline collagenase enzyme. Induction of collagenase enzyme was clearly observed in *B. licheniformis* F11.4 which responded to collagen addition by increasing the collagenase enzyme activity rapidly. The high enzyme activity was short lived and rapidly reduced following its optimum activity at 10 h of incubation. The optimum fermentation time for synthesis of a particular enzyme is affected not only by different bacterial strain but also by varieties of fermentation conditions. In general, the *Bacillus subtilis* CN2 was reported to synthesize protease optimally by 14 h of fermentation time[14]. Shorter optimum fermentation time of protease, *i.e.* 9 h was reported when *Bacillus* SMIA-2 was grown in media containing tri sodium citrate 1 %[15]. *Bacillus subtilis* PE-11 produced protease optimally at longer incubation time, that is by 48 h when grown in nutrient broth[16].

Effect of pH on collagenase activity: A pH range between 2 and 12 was used to study the effect of pH on collagenase activity (Fig. 2). Optimum pH was found to be 9. At pH 5 only 8.3 % of the maximum enzyme activity was obtained, increasing to 38.6 and 91% at pH 6 and 7, respectively. The collagenase displayed a broad pH activity profile in the neutral to basic range, at pH 12 the activity of enzyme was still at 80 % of its optimum level.

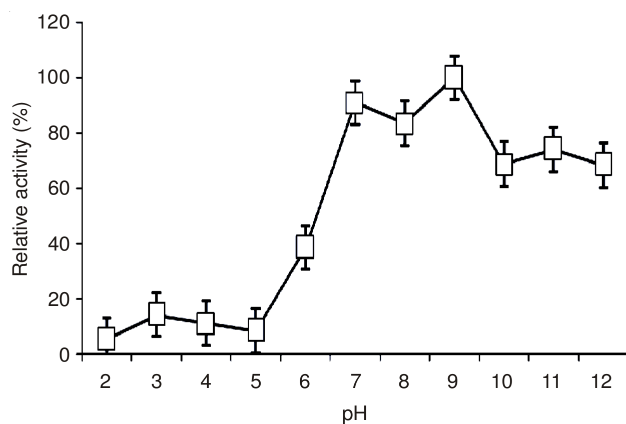

Fig. 2. Activity of collagenase of *B. licheniformis* F11.4 at different pH values. Collagenase activity was measured at 50 °C in the buffer universal. Each value represents the mean ± SE of three independent experiments

The pH optimum of the enzyme was 9 (Fig. 2), which was also observed in collagenase from *Bacillus sp*. MO-1[17]. and *Streptomyces parvulus*[5]. Interestingly we found that the collagenase of *B. licheniformis* F11.4 was still significantly active at pH 12. Consequently, this collagenase belongs to the group of alkaline protease.

Effect of temperature on collagenase activity: The collagenase activity was assayed at different temperature ranging from 30 to 90 °C at a constant pH of 9, the hydrolysis of collagen was also influenced by temperature (Fig. 3). Enzyme activity increased with temperature within the range of 30 to 50 °C. A reduction in enzyme activity was observed above 60 °C.

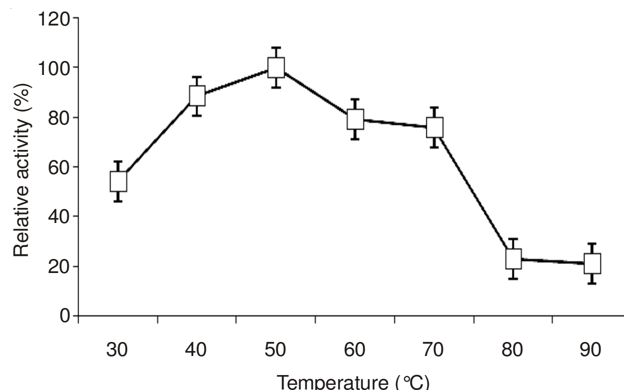

Fig. 3. Effects of temperature on collagenase activity. Collagenase activity was determined in the buffer phosphate 0.05 M pH 7. Each value represents the mean ± SE of three independent experiments

The pH optimum of the enzyme was 9, which was also observed in collagenase from *Bacillus sp*. MO-1[17] and *Streptomyces parvulus*[5]. Interestingly we found that the collagenase of *B. licheniformis* F11.4 was still significantly active at pH 12. Consequently, this collagenase belongs to the group of alkaline protease. The optimum temperature of this collagenase was 50 °C which is similar to *Bacillus subtilis* FS-2[18].

Thermostability: Temperature stability was studied at 50 and 70 °C (Fig. 4). The residual enzyme activity of heat treatment at 50 °C was higher than at 70 °C, while their changing curve was similar. The result showed that the collagenase kept relatively stable and retained a bove 50 % activity under 20 min incubation at 50 and 70 °C. However, the enzyme activity decreased gradually after 20 min incubation.

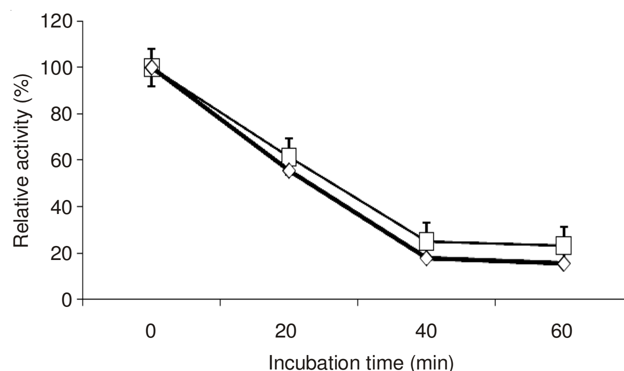

Fig. 4. Thermostability of collagenase from *Bacillus licheniformis* F11.4 at 50 °C (□) and 70 °C (◇) Collagenase activity was determined in the buffer phosphate 0.05 M pH 7.0. Residual activity monitored at various time interval after incubation at various temperature. Each value represents the mean ± SE of three independent experiments

Effect of metal ions on collagenase activity: The effect of different metal ions on collagenase is shown in Table-1. A significant inhibitory effect was observed in presence of Cu^{2+}.

TABLE-1
EFFECTS OF METAL IONS ON COLLAGENASE ACTIVITY

Metal ions	Concentration (mM)	Relative activity (%)
Control	-	100
$CaCl_2$	1	112.11
$CaCl_2$	5	105.19
$CaCl_2$	10	93.28
$ZnCl_2$	1	98.31
$ZnCl_2$	5	107.93
$ZnCl_2$	10	94.40
$CuCl_2$	1	60.61
$FeCl_2$	1	71.41
$CoCl_2$	1	159.76
$MgCl_2$	1	123.75

Collagenase activity was stimulated by Co^{2+} (159.8 %), Mg^{2+} (123.7 %) and Ca^{2+} (112.1 %)

Collagenase activity was inhibited by Cu^{2+} (1 mM) and stimulated by Co^{2+} (1 mM), Mg^{2+} (1 mM) and Ca^{2+} (1 mM). Similar effect of Ca^{2+} and Mg^{2+} on the activity of collagenase were also observed[4,19]. These result suggest that these metal ions apparently played a vital role in maintaining the active conformation of the enzyme[20].

Apparent molecular weight of collagenase: SDS analysis of the crude extract revealed several protein bands and zymography analysis indicated that the molecular mass of the collagenase fraction were approximately 124, 35, 31 and 26 kDa (Fig. 5), which appeared as clear bands.

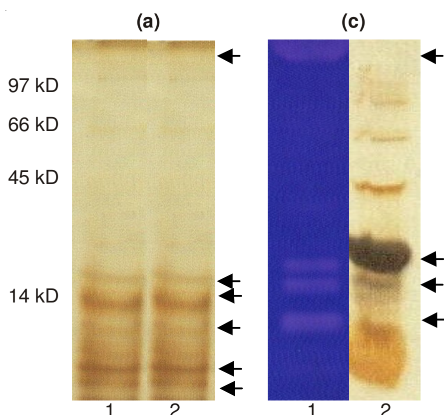

Fig. 5. SDS-PAGE (a) and Zymogram (b) of collagenase from *B. licheniformis* F11.4. Lane 1, molecular mass standards: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa); lane 2, crude collagenase; SDS-PAGE was done under reduced conditions

Zymography analysis indicated that the apparent molecular mass of the collagenase fractions were approximately 124, 35, 31 and 26 kDa (Fig. 5). Multiple collagenase in the range 14.5 -210 kDa produced by bacteria have been reported previously. In the case of *Bacillus licheniformis* N22, two collagenase corresponding to 29 and 120 kDa were found[3].

Substrate specifity of collagenase: The activity of collagenase on various protein substrates (collagen, casein, gelatin and fibrin) is shown in Table-2. All experiment for substrate were done under the same conditions. This collagenase were highly active for casein and collagen, more than gelatin and fibrin.

TABLE-2
SUBSTRATE SPECIFICITY OF THE COLLAGENASE

Substrate	Relative activity (%)
Collagen	100.00
Casein	217.88
Gelatin	71.43
Fibrin	38.49

The bacteria produced several collagenases, probably to hydrolyze to diverse protein substrates found in nature. The capability of diverse substrates degradation was confirmed. In this study, the enzyme was found capable of hydrolyze not only collagen but also casein, gelatin and fibrin. Even though the best substrate is still casein, the enzyme activity to collagen was higher that the other 2 substrates (gelatin and fibrin).

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