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Hydrolytic properties of crude protease from *Bacillus subtilis* subsp. *subtilis* M13

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Abstract A new strain of *Bacillus subtilis* subsp. *subtilis* M13 which isolated from meat is proved to be the collagenase producing bacteria based on high degradation of denatured form of collagen or gelatin. The molecular weight of the crude enzyme was approximately be 21 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Maximum collagenolytic activity based on gelatin as a substrate was attained at 50°C and pH 6.0 with citrate buffer. Furthermore, this enzyme was preferable to hydrolyze insoluble collagen, and followed by elastin at 37°C and pH 6.0. Maximum hydrolysis for collagen was showed by the highest release of amino acid at 998 μg/ml after incubation for 24 h, and elastin at 656 μg/ml after incubation for 12 h. However, the hydrolytic activity against myofibrillar protein extracted from meat was relatively lower than 3.3 μg/ml at 6 h. There was found either no or slightly hydrolytic effect after prolonged incubation. The results indicated the potential protease for using as meat tenderizing enzyme with high degradation of collagen and elastin with low hydrolysis of meat myofibrillar protein.

Keywords: Bacteria protease, Collagenolytic protease, Meat tenderizing enzyme

Introduction

Proteases are hydrolytic enzymes which efficient for protein hydrolysis into small peptides and amino acids. They are one of the three largest groups of industrial enzymes, accounting for 60% of total global enzyme market share (Ningthoujam *et al.*, 2009). There are several commercially available enzymes originated from plants, animals, and microorganisms. However, microbial origin proteases are preferred rather than other proteases due to economic advantages in terms of easy cultivation and high productivity as well as easy with genetic engineering techniques to improve the catalytic properties (Rao *et al*., 1998; Li *et al*., 2013; Souza *et al*., 2015).

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Amongst microbial proteases, the strains of *Bacillus* sp. present approximately half of commercial production, especially for *B. subtilis* (Beg and Gupta, 2003). The advantage of this strain over than other species is non-pathogenic, lack of endotoxin, easy growth and high rate of protein synthesis (Bhunia *et al*., 2012). It can secrete several types of extracellular proteases to degrade various types of foreign proteins (Bhunia *et al.*, 2012). Recently, Sorapukdee and Tangwatcharin (2019) isolated a new collagenolytic enzyme produced by *B. subtilis* subsp. *Subtili*s M13 with exhibiting a large clear zone in gelatine agar plate, and its crude enzyme had high hydrolytic activity toward gelatine. Generally, gelatine is considered as the denatured form of collagen. Collagen content and also its solubility are one of the important factors affecting meat tenderness. If meat contained with high collagen content and/or low collagen solubility, it decreases meat tenderness. Not only collagen but also elastin is composed of intramuscular connective tissues (Purslow, 2005). The degradation of these cytoskeletal proteins are therefore increase meat tenderness. Moreover, the ideal of meat tenderizing enzyme should be high specificity for collagen and elastin with low cleavage of myofibrillar protein. Therefore, this paper was to characterize the hydrolytic property of crude enzyme produced by *Bacillus subtilis* subsp. *Subtili*s M13. The effect of pH and temperature on gelatinolytic activity was elucidated, and the degradation of this enzyme towards insoluble collagen, elastin, and myofibrillar protein were investigated.

Materials and Methods

Materials

Insoluble collagen from bovine Achilles tendon (Cat No. C9879) and elastin from bovine neck ligament powder (Cat No. E1625), and perfect protein markers 10-225 kDa (Cat No. 69079-3) were bought from Sigma Chemical Co. (St. Louis, MO, USA). Gelatin was brought from Merck (Cat no. 104078, Merck KGaA, Darmstadt, Germany).

Microorganism and culture condition

The microorganism with collagenolytic activity used in this study was isolated from meat. It was identified as *B. subtilis* subsp. *Subtilis* M13 by 16S rRNA gene sequencing (Sorapukdee and Tangwatcharin, 2019). A bacterium was kept frozen at -20 ℃ in nutrient broth (NB) supplemented with 20 % (v/v) glycerol. The strain was sub-cultured in NA plate. The cultures were grown at 37 ℃ for 24 h. A loopfull of strain in sub-culture medium was transferred to

10 ml of NB in sterile 50-ml centrifuge tubes and incubated at 37 ℃, 180 rpm for 24 h in an incubator shaker (NB-205, N-Biotek, N-Biotek Co., Ltd., Gyeonggi-Do, Korea). To produce collagenase, culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of production medium according to the method of Suphatharaprateep, Cheirsilp, and Jongjareonrak (2000) with some modification. The medium contained gelatin (10 g/l), glucose (5 g/l), yeast extract (1 g/l), K_2HPO_4 (1 g/l), KH_2PO_4 (0.5 g/l), $MgSO_4.7H_2O$ (0.2 g/l), and $CaCl_2.2H_2O$ (0.2 g/l) at pH 7.0. After 24 h of cultivation at 37 ℃, the cells were removed by centrifuged at 13,000 ×g, 4 ℃ for 5 min (Sigma 2-16 KL, Sigma, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The cell-free supernatant was designed as crude enzyme.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight estimation

The protein pattern of crude enzyme was evaluated by SDS-PAGE using the method described by Laemmli (1970), with 10% polyacrylamide resolving gels. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. After running, the relative migration distance (R_f) of the protein standards and the protein in crude enzyme were determined using the equation (1):

$$R_f = \frac{\text{migration distance of the protein}}{\text{migration distance of the dye front}} \quad (1)$$

Based on the values obtained for the protein bands, the logarithm of the molecular weight (log MW) of denatured protein and its relative migration distance (R_f) is plotted. Then, the MW of the unknown protein (protein from crude enzyme) was calculated from generating the equation (2):

$$Y = mX + C \quad (2)$$

Where Y is the MW of the unknown protein, m is the gradient of the line, X is relative migration distance (R_f), and C is the Y-intercept, respectively.

Assay for collagenolytic activity

Collagenolytic activity was defined by using a gelatin as substrate according to procedure by Tran and Nagano (2000) with a slight modification. The reaction mixture composed of 0.6 ml of 0.2% (w/v) gelatin in water, 0.4 ml of 150 mM Tris-HCl, pH 7.5 containing 12 mM $CaCl_2$ and 0.2 ml of cell-free supernatant. The reaction was incubated at 37 ℃ for 30 min and then stopped by the addition of 1.2 ml of 0.1 N HCl. The content of liberated free amino acid was estimated by the ninhydrin method using glycine as the standard amino acid. One unit (U) of collagenolytic activity was defined as the amount of

enzyme which released 1 μg of glycine per minute. Specific activity was shown as U/mg protein.

Effect of pH and temperature

Collagenolytic activity was assayed over a pH range of 4.0-6.0 (50 mM sodium citrate buffer), pH 6.0-7.5 (50 mM potassium phosphate buffer), and pH 7.5-9.0 (50 mM Tris-HCl buffer) at 37℃ for 30 min. For effect of temperature, the enzyme activity was evaluated at temperature between 30 and 70℃ for 30 min at the obtained optimum pH. The collagenolytic activity was determined by the method as explain above.

Hydrolysis of insoluble collagen and elastin

The hydrolysis insoluble collagen and elastin were performed according to the reported of Takagi *et al*. (1992) with some adjustment. Twenty milligrams of insoluble collagen or elastin was incubated with 0.4 ml of crude enzyme in 2 ml of 50 mM citrate buffer, pH 6.0. The enzymatic hydrolysis was incubated at 37℃ for 3, 6, 12, 18, 24, 30, 36, 42, and 48 h. Thereafter, the reaction was stopped by adding 2.4 ml of 10% w/v trichloroacetic acid, followed by centrifugation at 10,000×g for 5 min. The amounts of relased amino acids (μg/ml) during hydrolysis were determined by ninhydrin reagent. Additionally, the digested peptides in supernatant were also assayed by SDS-PAGE with 10% acrylamide.

Hydrolysis of myofibrillar protein

Myofibrillar protein components were fractionated from beef according to the method of Ha *et al*. (2013) and then freeze-dry prior to use as a substrate for enzyme hydrolysis. The hydrolysis of myofibrillar protein was also performed as same as the method for hydrolysis of collagen and elasin. Additionally, the digested peptides in supernatant were also assayed by SDS-PAGE with 10% acrylamide.

Results

SDS-PAGE and molecular weight estimation

Crude protease from *Bacillus subtilis* subsp. *Subtilis* M13 exhibited a collagenolytic acivity as 1.68 U/ml, and its specific activity was approximately 0.68 U/mg protein (data not shown). The single protein band of the crude protease was mainly observed in SDS-PAGE, where was displayed in

Figure 1a. The molecular mass of this crude enzyme was evaluated to be about 21 kDa (Figure 1b), suggesting low molecular weight of this enzyme.

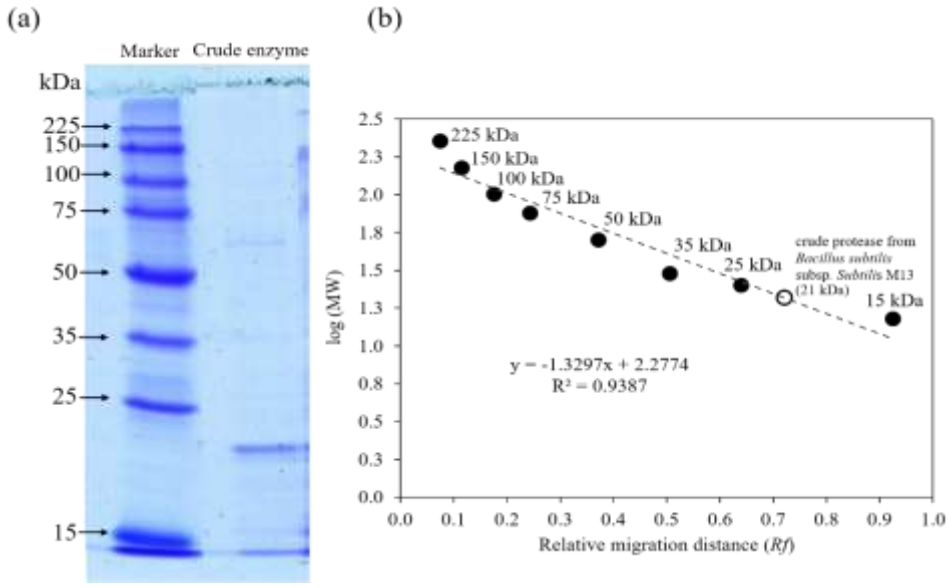

Figure 1. Protein pattern observed in SDS-PAGE for crude protease from *Bacillus subtilis* subsp. *Subtili*s M13 (a), and the estimated molecular weight from generated curve between the log (MW) versus R_f (b)

Effect of pH

The effect of pH on the collagenolytic activity was analyzed in three different buffer systems to defind the optimum buffer and pH. The results showed that the maximum activity was observed at pH 6.0 in citrate buffer, followed by pH 6.0 in phosphate buffer (Figure 2).

Effect of temperature

Collagenolytic enzyme from *Bacillus subtilis* subsp. *Subtili*s M13 had an optimal temperature at 50 ℃ as presented in Figure 3. The activity could maintain over 90% of maximum hydrolytic activity, if it incubated between 30 and 45 ℃. However, a decrease in activity was observed, especially when incubated enzyme at 70 ℃, the relative gelatinolytic activity was only 68% of maximum hydrolytic activity.

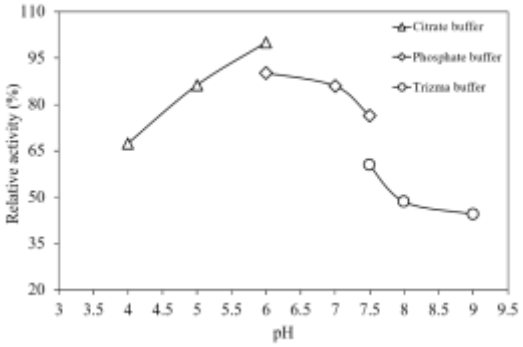

Figure 2. Effect of pH on activity of crude protease from *Bacillus subtilis* subsp. *Subtili*s M13

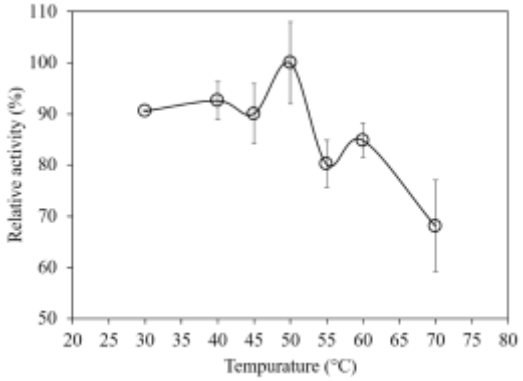

Figure 3. Effect of temperature on activity of crude protease from *Bacillus subtilis* subsp. *Subtili*s M13

Hydrolysis of insoluble collagen, elastin, and myofibrillar protein

The hydrolytic properties of crude enzyme assay towards collagen, elastin, and myofibrillar protein both activity and degraded peptides are presented in Figure 4 to Figure 6. This crude protease had the highest increase in amino acids released from insoluble collagen during the first 24 h of hydrolysis (998 μg/ml) and then decreased in 48 h (Figure 4a). The pattern of insoluble collagen was also largely degraded after prolonged incubation (Figure 4b).

The maximum of hydrolysis of elastin was observed during the first 12 h (656 μg/ml) and then gradually decrease until 48 h of hydrolysis (Figure 5a). The low molecular weight peptides were more produced with increasing incubation time (Figure 5b).

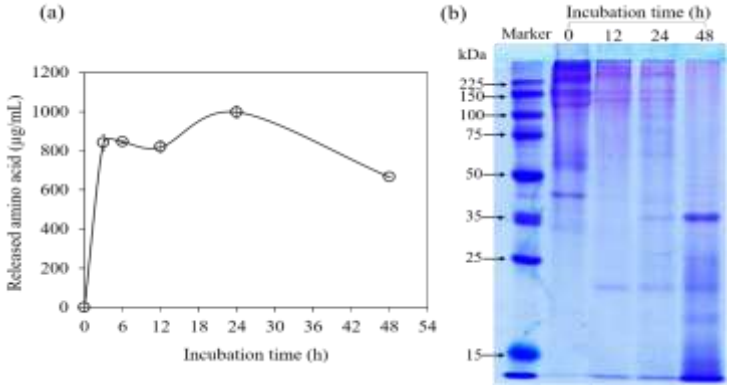

Figure 4. The amount of released amino acids during hydrolysis of collagen substrate (a), and SDS-PAGE analysis of the digestion pattern of collagen substrate treated with protease from *Bacillus subtilis* subsp. *Subtili*s M13 at 37 ℃ for 0, 12, 24, and 48 h

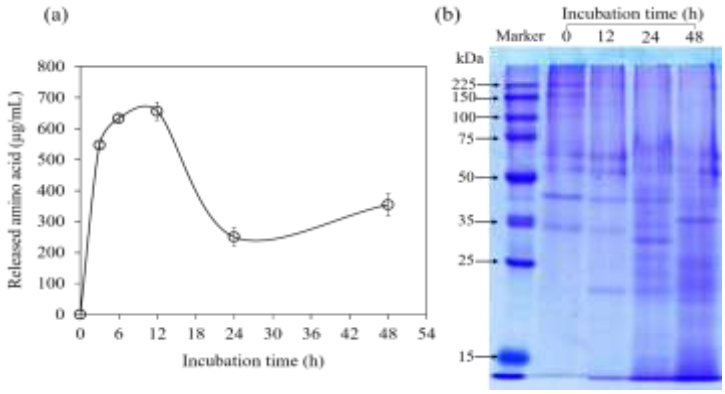

Figure 5. The amount of released amino acids during hydrolysis of elastin substrate (a), and SDS-PAGE analysis of the digestion pattern of elastin substrate treated with protease from *Bacillus subtilis* subsp. *Subtili*s M13 at 37 ℃ for 0, 12, 24, and 48 h

Regarding myofibrillar protein, the little degradation was found during first 6 h of hydrolysis (3.3 μg/ml) and there was no further hydrolysis after prolonged incubation (Figure 6a). These results are in agreement with myofibrillar protein patterin on SDS-PAGE, which there is no observable degradation of protein (Figure 6b). The results revealed that this enzyme was preferable to hydrolyze insoluble collagen, followed by elastin, with minimal effect on degradation of myofibrillar protein.

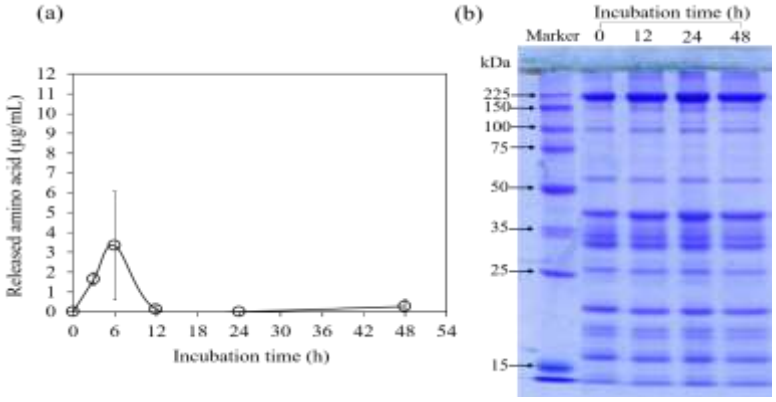

Figure 6. The amount of released amino acids during hydrolysis of myofibrillar protein (a), and SDS-PAGE analysis of the digestion pattern of myofibrillar protein treated with protease from *Bacillus subtilis* subsp. *Subtilis* M13 at 37 ℃ for 0, 12, 24, and 48 h

Discussion

The crude protease obtained from *Bacillus subtilis* subsp. *Subtilis* M13 had a molecular mass around 21 kDa. As reviewed by Pal and Suresh (2016), most of microbial collagenases have a high apparent molecular mass between 50 to 120 kDa. However, there are numbers of reported are in agreement with present study. Kumar and Takagi (1999) reported that the molecular weight (MW) of proteases secreted by microorganism were between 15 and 45 kDa. Previous studies for proteases from *Bacillus* sp. including *B. subtilis* AP-MUS 6 with MW of 18.3 kDa, *B. mojavensis* A21 with MW of 20 kDa, *B. cereus* SIU1 with MW of 22 kDa, *B. cereus* with MW of 28 kDa, and *B. subtilis megatherium* with MW of 45 kDa were found (Gerze *et al.*, 2005; Doddapaneni *et al.*, 2009; Haddar *et al.*, 2009; Singh *et al*., 2012; Maruthiah *et al.*, 2013).

The pH and temperature profiles of microbial collagenases were varied depending on the microbial species and strains. The present study found that the crude collagenase from *B. subtilis* subsp. *Subtilis* M13 had an optimum pH at pH 6.0 with optimum temperature at 50 ℃. A close to result was stated by Baehaki *et al.* (2011), who obtained that collagenase produced by *B. licheniformis* F11.4 had on optimal pH at 7.0, and optimum temperature at 50 ℃. Suphatharaprateep *et al*. (2011) demonstrated that *B. cereus* CNA1 had maximum activity at pH 7.0 and 45 ℃. While, Wu *et al.* (2010) reported that collagenase from *B. pumilus* Col-J was more active at pH 7.5 for 45 ℃ and Okamoto *et al*. (2001) described that collagenase from *B. subtilis* FS-2 was the highest acivity at pH 8 at the temperature of 60 ℃.

The crude proteases obtained from our isolated strain exhibited the efficacy to hydrolyze insoluble collagen and elastin, which could be observed in both hydrolyzing activity and SDS-PAGE. In contrast, the hydrolysis of myofibrillar protein to small peptides was very slight, eventhough it was incubated for 48 h. The result revealed that this enzyme had higher specific toward collagen and elastin than myofibrillar protein. Protease from *Bacillus* sp. YaB (Yeh *et al.*, 2002) also showed the similar specific degradation as same as our report. However, enzymes from those reported are active at the alkaline pH around 8-11 (Yeh *et al.*, 2002), while enzyme from our report had an optimum pH nearly the ultimate pH of meat (pH~ 5.6-5.8 for normal meat). Takagi *et al.* (1992) discovered elastase from alkalophilic *Bacillus* sp. that showed the optimum pH at 10.5 and favor to hydrolysis of elastin, followed by collagen, with no proof for myofibrillar protein degration. Therefore, crude enzyme from present study could be a candidate for meat tenderizing enzyme, where the toughness of meat was caused by high connective tissue protein mainly collagen.

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