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Original Research Article

Characterization of crude protease from *Aeromonas aquariorum* isolated from tofu waste

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Abstract: Food waste has been reported as the potential bioresource for screening microorganisms producing protease since its composition is rich in protein and fat that are good as culture media for microorganisms including bacteria. This study was aimed to characterize crude protease produced by *Aeromonas aquariorum* isolated from tofu waste. Crude enzyme was produced in skim milk with minimum media liquid for 2 days to produce protease specific activity of 0.379 U/mg. Zymographic profile showed that enzyme had broad substrate specificity towards casein, gelatin, and fibrinogen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme consisted of single band with molecular weight of 73 kDa. Enzyme had optimal pH at 12 and optimal temperatures at 37 and 70 °C. Enzyme was inihibited by a serine protease inhibitor, phenylmethylsulfonyl fluoride. The enzyme activity was not affected by ion cofactors. For its enzyme kinetic, the apparent K_m and V_{max} values of *A. aquariorum* crude enzyme toward casein were 4.069 mg/mL and 0.437 U/mg, respectively. Overall, crude protease from *A. aquariorum* had a potential catalytic performance, alkalophilic, thermostable, broad substrate specificity, and maximum catalytic efficiency at low substrate concentration.
Keywords: characterization, protease, *Aeromonas aquariorum*, tofu waste, enzyme activity.

INTRODUCTION

Proteases (EC 3.4) are grouped in degradative enzymes which catalyze the total hydrolysis of proteins. Proteases have been reported to conduct highly specific and selective modification of proteins, such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretary proteins across the membranes. Protease also represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes [1].

Proteases can be naturally found in microorganisms, plants, and animals, however, microbial community is preffered due to their broad biochemical diversity, rapid growth, limited space required for cell cultivation, and simplicity for generation of new recombinant enzymes with desired properties [2]. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent a potential source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Most commercial proteases, mainly neutral and alkaline, are produced by bacteria belonging to the genus Bacillus.

Recently, food waste has been reported as the potential bioresource for bacteria because of the waste generated is composed largely of protein and fat which are good as growing medium for bacteria. Our previous study showed that tofu waste from homemade tofu production is a potential source of microorganisms producing protease, including *Bacillus sp.* and *Aeromonas sp.* (data not shown). *Aeromonas hydrophila* and the related *Aeromonas sp.* belong to opportunistic pathogens of humans and fish. The pathogenicity of the microbe may involve several extracellular enzymes, and it has been suggested that the proteases excreted by *Aeromonas sp.* play an important role in invasiveness and in establishment of the infection, by overcoming the initial host defense, and by providing nutrients for cell proliferation [3]. Two distinct types of extracellular proteases, a temperature-stable metalloprotease (caseinase) and a temperature-labile serine protease (elastase), are found in various strains of *A. hydrophila* and other *Aeromonas sp.* [4]. In this study, crude enzyme produced by *A. aquariorum* isolated from tofu waste was tested for its proteolytic activity and characteristics.

MATERIALS AND METHODS

Aeromonas aquariorum from tofu waste

Thu 2.10 isolate was obtained from Microbiology and Fermentation Technology Laboratory, Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta (Indonesia). This isolate was screened from tofu waste collected from local homemade tofu industry in Bogor and identified as *A. aquariorum* (data not shown). *A. aquariorum* isolate in cryogenic state was refreshed on Luria Agar (LA) media (1% w/v tripton, 0.5%w/v yeast extract, 0.5%w/v NaCl, and 2%w/v bacto agar) and incubated at 37 °C for 24 hours.

Proteolytic activity screening and enzyme production

Proteolytic activity of *A. aquariorum* isolate was detected on skim milk agar (SMA). SMA was composed of minimum media (0.05%w/v NH_4Cl, 0.05%w/v NaCl, 0.03%w/v K_2HPO_4, 0.01%w/v $MgCl_2.6.H_2O$, 0.2%w/v yeast extract, and 2%w/v bacto agar) and 5%w/v skim milk. The inoculated plate was incubated at 37 ºC for 48 hours. Depending upon the zone of clearance, the strain of *A. aquariorum* was selected and enzyme production was performed for further characterization.

Crude enzyme was produced in liquid minimum media enriched with 5%w/v skim milk [5]. *A. aquariorum* isolate from SMA with proteolytic activity was inoculated in this minimum media, followed by incubation at 37 ºC for 48 hours with agitation (120 rpm) and treatment with 0.2%w/v sodium azide to stop the production. Crude enzyme in supernatant was collected after centrifugation at 13000 ×g for 15 minutes.

Protease activity and protein concentration

The protease activity of crude enzyme was determined according to the modified method of Anson [6] by measuring the release of trichloroacetic acid (TCA) soluble peptides from 1%w/v casein in 50 mM phosphate buffer (pH 8.0) at 37ºC for 10 minutes. Reaction was stopped by the addition of 0.5 mL of 0.1 M TCA, then centrifuged at 16200 ×g for 10 minutes. Absorbances were measured using Folin-ciocalteau reagent (1:8) at 660 nm wavelength with macroplate reader. One unit (U) was defined as the amount of enzyme required that can hydrolyzed casein to produce equivalent colour with 1 µmol (181 µg) tirosin product each minutes under the assay conditions.

Protein concentration of crude enzyme was measured with Bradford method [7]. Absorbances were measured with ELISA reader at 595 nm wavelength. Bovine serum albumine (BSA) at various concentrations was used as the standard.

Substrates specificity and molecular weight

Zymographic assay was used for qualitative enzyme specificity toward various protein substrates (casein, gelatine and fibrinogen). Zymography was performed using 12% polyacrylamide separating gels containing polyacrylamide, 10% SDS, and 0.5% substrates with 6 % polyacrylamide stacking gels. After electrophoresis (100 V), the gels were rinsed with aquadest and soaked for 30 minutes in 1%v/v Triton X-100 at room temperatures to remove the SDS. The gels were rinsed again with aquadest and incubated in 50 mM phosphate buffer (pH 8.0) for denaturation. Staining was done using 0.1 %w/v Coomassie Brilliant Blue (CBB) R-250 in methanol-glacial acetic acid-water (45:10:45), followed by destaining with mehtanol-glacial acetic acid-water (10:10:80) till a clear band appeared.

SDS-PAGE assay was performed to determine molecular weight (MW) of protein by using 12% polyacrylamide separating gels with 5% polyacrylamide stacking gels. After electrophoresis at 100 V for around 120 minutes, the gels were stained with 0.1 %w/v CBB R-250 in methanol-glacial acetic acid-water (45:10:45), followed by destaining with methanol-glacial acetic acid-water (10:10:80) overnight till a blue band appeared with clear backgroud. Molecular weight was calculated using standard curve of low molecular weight (LMW) marker.

Effect of pH and temperature on protease activity

The optimal pH was determined with casein 0.65%w/v as substrate dissolved in various pHs (2-14) using universal buffer. The effect of temperature on the enzyme activity was tested at pH 8.0 within various ranges of temperature (4-70 °C). Relative activity of the enzyme was measured at standard assay according to modified method of Anson [6].

Effect of inhibitors and cofactors on protease activity

The effect of protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and etylenediamine tetraacetic acid (EDTA) was determined by the addition of each inhibitor at various concentrations (0.1-1 mM for PMSF and 1-25 mM for EDTA) to enzyme, followed by the incubation for 60 minutes prior standard assay.

The effect of various metal cofactors with different valence electrons, including K^+, Na^+, Mg^{2+}, Ca^{2+}, Fe^{3+}, and Al^{3+} was determined by the addition of each cofactor with various concentrations (1-10 mM) to enzyme, followed by incubation for 60 minutes prior standard assay.

Enzyme Kinetics

The K_m value was determined by measuring the initial velocity using various substrate concentrations (0.065-2%w/v). The data obtained was fitted to a

hyperbola using the Michaelis-Menten equation. The value was determined by a linear least-squares regression analysis of Lineweaver-Burk plot [3].

RESULTS

Proteolytic activity of crude enzyme from *A. aquariorum*

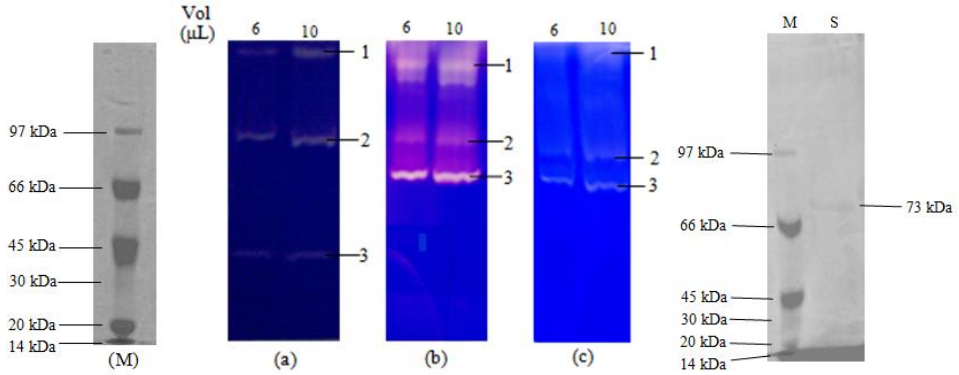
A clear zone around colony of *A. aquariorum* represented proteolytic activity of crude enzyme (Figure 1). Our results showed clear zone diameter (75 mm) from 2-days incubation was more significant compared to that of clear zone diameter (30 mm) from 1-days incubation. Quantification assays demonstrated that unit activity and protein concentration of crude enzyme from *A. aquariorum* were 0.058 U/mL and 0.153 mg/mL respectively. Meanwhile, its specific activity was 0.379 U/mg.

(A) (B)

Fig-1: Clear zone profiles of crude enzyme from *A. aquariorum* on skim milk agar after 1- day (A) and 2-days of incubation periods (B).

Substrate specificity and molecular weight

Crude enzyme from *A. aquariorum* was in situ screened for its substrate specificity against some proteins, including casein, gelatin, and fibrinogen by conducting zymographic assay. Crude enzyme consisted of three clear bands on each protein zymogram profiles (Figure 2A), indicating its broad specificity with caseinolytic, gelatinolytic, and fibrinogenolytic activities. Furthermore, SDS-PAGE analysis was also conducted to measure molecular weight of crude enzyme from *A. aquariorum*. SDS-PAGE profile only revealed single band of crude enzyme from *A. aquariorum* with estimated molecular weight of 73 kDa (Figure 2B).

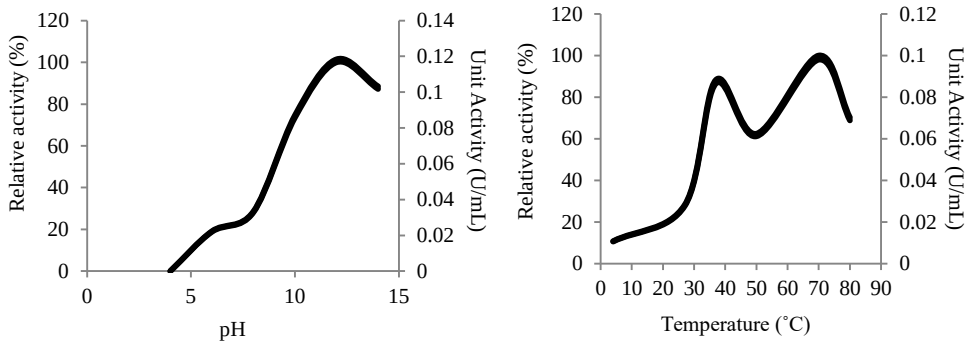


(A) (B)

Fig-2: (A) Zymographic profiles of crude enzyme isolated from *A. aquariorum* in various protein substrates, including casein (a) (1: >97 kDa; 2: 66-97 kDa; 3: 30-45 kDa), gelatin (b) (1: >97 kDa; 2: 66-97 kDa; 3: 66-97 kDa), and fibrinogen (c) (1: >97 kDa; 2: 66-97 kDa; 3: 66-97 kDa) compared with LMW marker (M). (B) SDS-PAGE profiles of crude enzyme (S) from *A. aquariorum* compared with LMW marker (M).

Optimal pH and temperature

The effect of pH on enzyme activity was examined at pH value ranging from 2-14, with casein as substrate. The protease was active at alkaline pH (8-12) with maximum activity at pH 12 and specific protease activity of 0.773 U/mg (Figure 3A). Meanwhile, effect of temperature on the protease activity of crude enzyme produced by *A. aquariorum* was shown in Figure 3B. The enzyme was active at temperature ranges of 37-80 ˚C with two optimal temperatures at 37 ˚C and 70 ˚C.



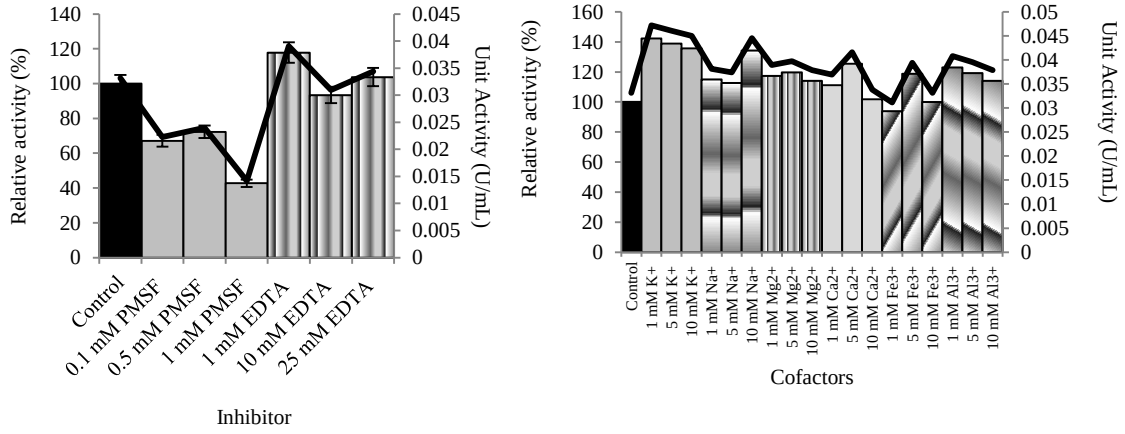
(A) (B)

Figure-3: The effect of pH (A) and temperature (B) on proteolytic activity of crude enzyme from *A. aquariorum*.

Effects of inhibitors and cofactors on protease activity

The effects of serine protease inhibitor (PMSF) and metalloprotease inhibitor (EDTA) on the protease activity of crude enzyme from *A. aquariorum* were tested. Our data showed that enzyme activity was significantly inhibited by PMSF at various concentrations (0.1-0.5-1 mM), indicating that the enzyme was grouped in serine protease (Figure 4A). At highest concentration, PMSF inhibited >50% of enzyme activity.

Furthermore, effect of several cofactors on enzyme activity was also determined. Most ion cofactors at various concentrations (1-5-10 mM) did not affect the increase of enzyme relative activity compared to that of control (Figure 4B). At lowest dose (1 mM), both K^+ and Al^{3+} ions only slightly increased enzyme activities up to ~140%, respectively.



(A) (B)

Fig-4: Effects of inhibitors (a) and cofactors (b) at various concentrations on the protease activity of crude enzyme from *A. aquariorum*.

Enzyme Kinetics

For enzyme kinetics, we measured V_{max} and K_m values of crude enzyme from *A. aquariorum*. The V_{max} is the velocity when all of the enzyme active sites are filled with substrate. The Michaelis constant, K_m, is numerically equal to the substrate concentration that gives half the maximal velocity (V_{max}). Our results showed that the estimated K_m and V_{max} values of crude enzyme were 4.069 mg/mL and 0.437 U/mg (Figure 5).

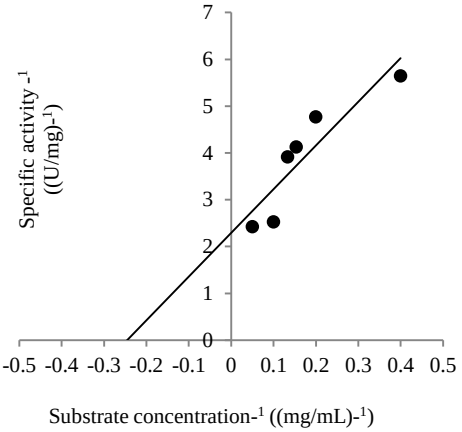


Fig-5: Lineweaver-Burk plot for enzyme kinetics of crude enzyme from A. aquariorum.

DISCUSSION AND CONCLUSION

In this study, we employed Thu.2 isolate, identified as *A. aquariorum*, from tofu waste for screening its potential proteolytic activity. After 2-days incubation of *A. aquariorum* in SMA agar, our data showed a significant clear zone with diameter of 75 mm (Figure 1). This result is in line with previous study by Usharani and Muthuraj [5]. Proteolytic activity of crude enzyme from *Bacillus laterosporus* had the highest activity after 48 hours of incubation. Specific enzyme activity is used to determine the potential enzyme unit and purity. Specific activity of crude enzyme produced by *A. aquariorum* isolated from tofu waste (0.379 U/mg) was similar with that of another species of Aeromonas, *A. hydrophila* (0.340 U/mg) [3]. However, its specific activity was quite low compared to those of other proteolytic producing-bacteria, such as *Pseudomonas sp.*, *Bacillus sp.*, *Proteus sp.*, and *Enterobacter sp*.

Enzyme had a unique characteristic in term of its substrate specificity. Aspartase only has absolute substrate specificity; meanwhile other enzymes exert relative broad substrate specificity. In this study, screening for substrate specificity of protease from A. aquariourum was done using in situ zymogram assay. Zymographic profiles demonstrated that crude enzyme from *A. aquariorum* had broad substrate specificity against various protein substrates, including casein, gelatin, and fibrinogen (Figure 2A). Based on zymogram results, one caseinolytic band with MW of 30-45 kDa is in accordance with casein zymographic profiles of purified protease from *B. circulans* (39.5-43 kDa) and *A. hydrophila* (45 kDa) [2, 8].

Furthermore, SDS-PAGE analysis was done for determining the molecular weight of protein. Sodium dodecyl sulfate (SDS), known as an anionic detergent, binds to protein and covers the protein with negative charges. The SDS and β-mercaptoethanol also denature proteins and break up any polymers into their subunits. The β-mercaptoethanol reduces disulfide bridges that lead to the similar shape (random coil) and charge/mass ratio of protein subunits. Then, proteins are separated based on their mass. SDS-PAGE profile showed that crude enzyme from *A. aquariorum* only revealed one single band with MW of 73 kDa (Figure 2B). This data is quite simillar with purified protease from A. hydropila with MWs of 65.9 and 68 kDa [3, 4]. According to Esteve and Birkbeck, purified proteases from A. hydropila demonstrated both serine and metalloprotease properties with MWs of 31, 44, and 60 kDa [4].

Enzymes are affected by changes in pH. Enzyme has a typical optimal pH that correlated with its maximum activity. The pH value of enzyme represents pH when carboxyl and amino groups receive or donor a proton at the enzyme catalytic site to be in the desired degree of ionization. Our data showed that crude enzyme from *A. aquariorum* was tolerable and active in alkaline condition, with optimal pH value at 12 (Figure 3A). This data is quite similar with *A. hydrophila* protease that had optimal alkaline pH at 9 [3]. Interestingly, other study by Kannan et al. showed that *A. hydrophila* protease exerted optimal pH at pH 5 for its growth [9]. In contrast with our results, *A. aquariorum* crude protease did not work at acidic condition.

Each enzyme has a temperature that it works optimally in. Our data demonstrated that *A. aquariorum* crude protease had two optimal temperatures at 37 ˚C and 70 ˚C (Figure 3B). It seems that crude enzyme consisted of two different enzymes and/or had isomers with different forms. In linier with study from Cho et al., *A. hydrophila* protease also showed optimal pH at thermostable temperature (60 ˚C) [3].

Enzyme works because its substrate fits into the active site on the protein molecule. Active site has main function that is associated with the way protein is folded into its tertiary structure. Enzyme treatment with heating gives protein chains extra energy and makes them move more. If proteins move enough, then the bonds holding the tertiary structure in place will come

under increasing strain. As soon as these bonds holding the tertiary structure together are broken, then the shape of the active site is likely to be lost and broken permanently if given enough time and high enough temperature.

Crude protease of *A. aquariorum* was inhibited (>50%) by a serine protease inhibitor, PMSF at 1 mM, but not by a metalloprotease inhibitor of EDTA (Figure 4A). This result is in accordance with Cho et al. that reported *A. hydrophila* protease was also grouped in serine protease since its activity was strongly blocked (>85%) by 2.5 mM PMSF [3]. Other protease inhibitors, such as E64, EDTA, pepstatin, and 1, 10-phenanthroline had no influence on its activity. PMSF is a protease inhibitor that reacts with serine residues to inhibit trypsin, chymotrypsin, thrombin, and papain. Proteolytic inhibition occurs when a concentration between 0.1-1 mM PMSF is used.

We also tested the effect of several ion cofactors on increasing protease activity from *A. aquariorum*. Our data showed that most ions did not show significant inhibition against protease activity (Figure 4B). The addition of 1 mM K^+ and Al^{3+} slightly enhanced protease activity up to 140% compared to that of control. Interestingly, protease from A. hydropila was slightly inhibited by the addition of ion cofactors, such as Ca^{2+}, Mg^{2+}, and Zn^{2+} [3]. Protease activity from *B. stearothermophilus* had no inhibitory effect on Zn^{2+} ion [10]. Meanwhile, the addition of Ca^{2+}, Mg^{2+} and Na^+ ions enhanced alkaline serine protease from *B. pumilus* [11]. Metal ion as a cofactor affects enzyme activity by binding to active site of enzyme. The effect of metal ions on protease activity may differ depending on the source of microorganisms and enzyme production.

The mechanism of enzyme catalyzed reactions is also studied by conducting kinetic measurements on enzyme-substrate reaction systems, including measuring rates of the enzyme-catalyzed reactions at different substrate and enzyme concentrations. To characterize an enzyme-catalyzed reaction, K_m and V_{max} values need to be determined by determining the rate of catalysis (reaction velocity) for different substrate concentrations. Our study demonstrated that Lineweaver-Burk plot presented the apparent K_m and V_{max} values of crude protease from *A. aquariorum* using casein was estimated to be 4.069 mg/mL and 0.437 U/mg (Figure 5). Enzyme achieved its maximum catalytic efficiency at 0.4%w/v casein concentration. However, its catalytic is much lower compared to that of protease from A. hydropila with K_m value of 0.32 mg/mL [3].

K_m is equal to the substrate concentration at which the reaction rate is half its maximum value. An enzyme with a small value of K_m achieves its maximum catalytic efficiency at low substrate concentrations. The value of K_m for an enzyme depends on substrate, pH, and temperature at which the reaction is carried out. Therefore, crude protease from *A. aquariorum* is needed to be further purified for determining its exact catalytic and kinetic reactions.

In summary, crude protease of A. aquarioum isolated from tofu waste has been characterized biochemically. Enzyme had broad substrate specificity towards casein, gelatin, and fibrinogen with MW of 73 kDa. Optimal pH enzyme was reached at pH 12, while the optimal temperature of enzyme was 37 and 70 °C. Enzyme was grouped in serine protease and did not need ion cofactors for its activity. K_m and V_{max} values of the enzyme were 4.069 mg/mL and 0.437 U/mg. According to these characteristics, crude enzyme produced by *A. aquariorum* was classified as an endopeptidase serine protease of hydrolase class of enzyme (EC 3.4.21).

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