Biochemical Properties of Crude Extracellular Proteases from *Chromohalobacter salexigens* BKL5 and *Micrococcus luteus* 11A

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Abstract

In this work, we have reported an enzymatic activity and biochemical properties of extracellular proteases from *Chromohalobacter salexigens* BKL5 and *Micrococcus luteus* 11A. *C. salexigens* BKL5 and *M. luteus* 11A were previously isolated from Bledug Kuwu mud volcano and dietary industry wastewater treatment, respectively. Both bacterial strains were able to produce extracellular proteases, when grown on minimal agar medium supplemented with 1% of skim milk. Proteolytic indexes of *C. salexigens* BKL5 and *M. luteus* 11A were 2.5±0.14 and 2.9±0.42, respectively. Both extracellular proteases exhibited optimum enzymatic activity at pH 7, with specific activity of *C. salexigens* BKL5 was 13.3% higher than that of *M. luteus* 11A. Optimum temperature for enzymatic activity of both proteases was 45°C. Metal cofactor preferences assay showed that extracellular protease from *C. salexigens* BKL5 preferred Zn^{2+}, meanwhile extracellular protease from *M. luteus* 11A mainly preferred Ca^{2+} ion. Metal cofactor preferences assay also suggested that crude extracellular protease from *C. salexigens* BKL5 was categorized as metalloprotease, meanwhile crude extracellular protease of *M. luteus* 11A was common neutral protease. The enzymatic stability assay against various salt concentrations showed that crude extracellular protease from *C. salexigens* BKL5 was more stable than that of *M. luteus* 11A.

Keywords: Extracellular protease, *Chromohalobacter salexigens* BKL5, *Micrococcus luteus* 11A, neutral protease, metalloprotease

Introduction

Protease is an enzyme that specifically cleaves the peptide bond of polypeptide. Protease has been known to be present in all living organism from bacteria to human and also virus. Furthermore the genomic material contains 2–4 % of protease encoding genes (Puente *et al*., 2005). It is not surprisingly that protease is indispensable in every organism, since the proteases have significant role many physiological processes such as those associated with metabolisms, cell signaling, defense response, and cell development (Lopez-Otin and Overall, 2002; Sauer *et al*., 2004; Tripathi and Sowdhamini, 2008). Beside of physiologically important, protease has been widely used in various industrial applications such as foods, pharmacy, detergent, textile, and leather. Recent progress shows that protease has been considered for developing of protease-based drug, which works mostly for drug therapy targeted protease activity (Cudic and Fields, 2009; Drag and Salvesen, 2010).

Generally, proteases are categorized into several groups according to the catalytic mechanism, catalytic environment and also catalytic core residues. Based on the catalytic mechanism, proteases could be categorized into two groups, exo- and endo-proteases (Rao *et al*., 1998). According to the catalytic environment, proteases are separated into acidic, basic, and neutral proteases. This classification is based on the fact that several proteases show the optimum activity either at acidic, basic, or neutral pH (Rao *et al*., 1998). Identification on the catalytic residues

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responsible for the catalytic activity, it makes possible to further discriminate the members of proteases (Hartley, 1960). Serine, sulfhydryl, and metalloenzyme are an example of the member of protease according to the type of important catalytic residue. Among them, serine protease occupied almost one third of total proteases. This class was originally distinguished by the presentation of what was called by "catalytic triads", which consist of Ser-Asp-His residues (Hedstrom, 2002; Polgar, 2005; Tripathi and Sowdhamini, 2008). According to MEROPS database, the serine proteases consist of four clans include chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease (Rawling *et al*., 2016).

Previously we have isolated one moderately halophilic and one non-halophilic bacteria which showed the proteolysis activity when grown in the minimal media containing skim milk as a substrate. Partial identification based on the 16SrRNA gene sequences indicated that the halophilic bacterium was close to *Chromohalobacter salexigens* and therefore we have named as *C. salexigens* BKL5 (Rohman *et al*., 2012). The non-halophilic was close to *Micrococcus luteus* and then we have named as *M. luteus* 11A. In this work we have reported the enzymatic activity of two extracellular proteases produced by moderately halophilic bacterium, *C. salexigens* BKL5 and non-halophilic bacterium, *M. luteus* 11A. Further biochemical properties analyses of those two proteases were also discussed.

Materials and Methods

Cell and cultures

Two bacterial strains used in this experiment was *C. salexigens* BKL5 and *M. luteus* 11A. *C. salexigens* BKL5 was isolated from Bledug Kuwu mud volcano, which has been characterized as moderate halophilic bacterium, while *M. luteus* 11A was isolated from dietary industry wastewater treatment. Cultivation of both bacteria was carried out in minimal medium containing (g/l) 13 $MgCl_2.6H_2O$, 20 $MgSO_4$, 1.1 $CaCl_2$, 4 KCl, 0.2 $NaHCO_3$, 0.5 NaBr, and 1% skim milk.

Crude extracellular protease production

Extracellular proteases were produced by cultivating of *C. salexigens* BKL5 and *M. luteus* 11A in 100 ml minimal media supplemented with 1% skim milk and incubated at room temperature for 48 hours. Culture supernatants were harvested by centrifugation at 8000 rpm for 10 minutes. Supernatants were collected as crude extract extracellular protease and kept at 4°C for further analysis. Total protein was determined by using BCA Protein Assay Kit (Novagen).

Enzymatic activity assay

A modification of the method of Kunitz was used for enzymatic assay by using skim milk as substrate. The enzymatic activity assay was carried out in 0.5 ml of reaction mixture consisted of 400 µl of 1% skim milk substrate in 50 mM phosphate buffer (pH 7.0) and 100 µl of culture supernatant (OD_{280} ~ 0.1). The enzymatic reaction was started by adding culture supernatant and after incubation the reaction was stopped by adding 500 µl of cold 10% of trichloroacetic acids (TCA) solution. The reaction mixture was incubated on ice for 30 minutes and then centrifuged at 10,000 rpm for 10 minutes. An aliquot was carefully collected and subjected for absorbance measurement at 280 nm (Kunitz, 1946). One unit was defined as the amount of enzyme that increases the absorption at λ= 280 nm by 0.01. The specific activity was defined as the enzymatic activity per milligram of protein.

To examine the effect of temperature, pH, divalent metal ions, and salts on the enzymatic activity, the enzymatic activity assay was employed by similar method as mention previously by some modifications according to factor to be examined. To examine the effect of pH, the phosphate buffer was used to measure the enzymatic activity at pH 5 to 7 and Tris buffer at pH 7–9. Divalent metal ions used in this experiment included $CaCl_2$, $MgCl_2$, and $ZnCl_2$, with the concentration that was varied as follows: 0.5, 1, 1.5, 10, and 50 mM (Hirokoshi, 1971). To test the protein stability against salinity, the crude

extracellular proteases from both bacterial strains were incubated in 0 to 5 molar of NaCl.

Results and Discussion

Crude extracellular protease production from C. salexigens BKL5 and M. luteus 11A

Qualitative assay to test the ability of those bacterial strains to produce extracellular proteases were carried out by examining the index proteolytic of both bacterial strains on minimal media containing 1% skim milk. The assay showed that *C. salexigens* BKL5 and *M. luteus* 11A exhibited similar index proteolytic (IP) with the IP values of 2.5±0.14 and 2.9±0.42, respectively (Table 1). In order to further characterize of extracellular protease from both bacterial strains, both strains were grown in liquid minimal medium supplemented with 1% of skim milk and incubated at room temperature for 48 hours. The harvesting supernatants were used directly for enzymatic assay. By this method of cultivation, the total proteins obtained from the culture supernatants of *C. salexigens* BKL5 and *M. luteus* 11A were 141.67±4.99 mg/ml and 141.06±3.67 mg/ml, respectively. To ensure that the protease enzymes were present in supernatant, we have checked the culture supernatant for the enzymatic activity by using 1% of skim milk as substrate. Enzymatic activity assay showed that both culture supernatants exhibited comparable enzymatic activity. Enzymatic activity assay showed that the culture supernatants from *C. salexigens* BKL5 and *M. luteus* 11A exhibited enzymatic activity which were determined to be 37.3±1.20 U/mg and 32.8±0.91 U/mg, respectively (Figure 1). Both culture supernatants showed enzymatic activity against 1% of skim milk as substrate, therefore, it might be that *C. salexigens* BKL5 and *M. luteus* 11A produced the similar proteases. In order to check whether the two proteases were similar, we have further characterized the biochemical properties of both proteases.

Table 1. The index proteolytic (IP) of bacterial strains when grown on minimal media agar supplemented with 1% of skim milk.

Bacterial strains	Proteolytic Index*)
C. salexigens BKL5	2.5±0.14a
M. luteus 11A	2.9±0.42a

*) The values followed by the same letter were not statistically significant different at α=0.05.

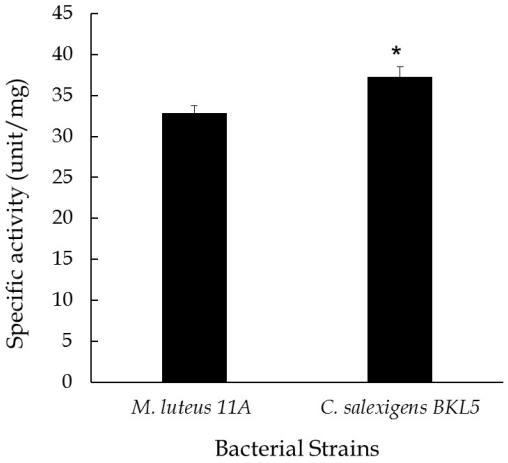

Figure 1. The enzymatic activity of crude extracellular protease from *C. salexigens* BKL5 and *M. luteus* 11A grown in liquid minimal media supplemented with 1% skim milk. Asterisk (*) indicates statistically significant different at α=0.05.

The effect of temperature, pH and metal cations on enzymatic activity of crude extracellular protease

Effect temperature on enzymatic activity of crude extracellular proteases

Crude extracellular proteases were isolated from the two bacterial strains belong to the mesophilic group. We have induced the proteases production from both bacterial strains at temperature conditions that similar to the optimal growth temperature of both bacterial strains. In order to know the effect of temperature on the enzymatic activity of both crude extracellular proteases, we have examined of both proteases at various different temperature conditions. The result showed that crude extracellular protease from *C. salexigens* BKL5 exhibited more broad optimum temperature. In contrast, the crude extracellular protease from *M. luteus* 11A exhibited optimum temperature at 45°C and when the temperature was increased to 55°C,

the enzymatic activity was significantly reduce as much as 55% from the optimum temperature (Figure 2). The results clearly indicated that crude extracellular protease from *C. salexigens* BKL5 was more stable compared to that of *M. luteus* 11A. The result might reflect that both crude extracellular proteases contain several different amino acid residue compositions, which are responsible for protein thermal stability such as proline, arginine, and lysine (Aghajari *et al*., 2003; Nishio *et al*., 2003; Prajapati *et al*., 2007). However, our speculation still needs to be further examined.

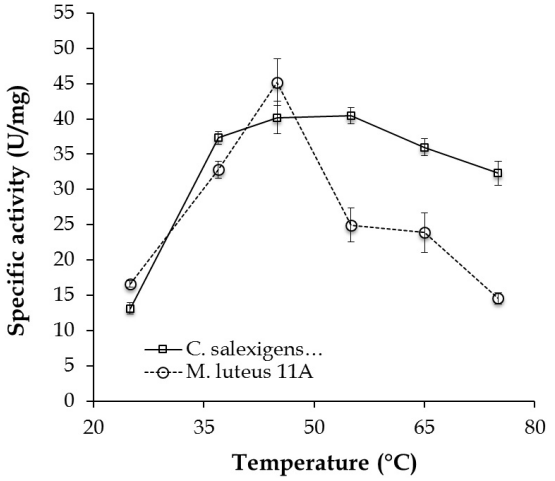

Figure 2. Temperature optimum for enzymatic activity of crude extracellular proteases from *C. salexigens* BKL5 and *M. luteus* 11A. The error bars represent the standard deviations of the corresponding values.

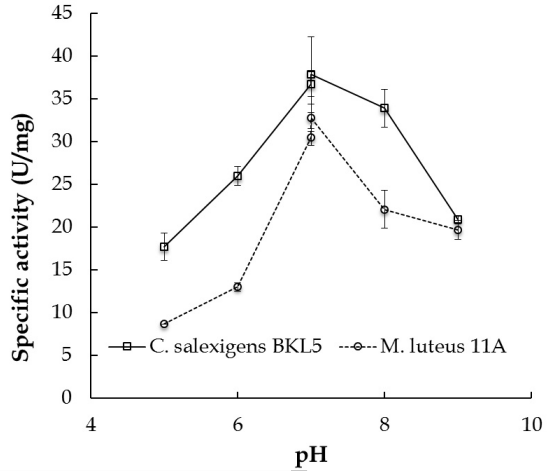

Figure 3. The effect of different pH of assay conditions on the enzymatic activity of crude extracellular proteases from *C. salexigens* BKL5 and *M. luteus* 11A. The enzymatic activity was determined by using the following buffers: phosphate buffer for pH 5-7 and Tris buffer for pH 7-9. The error bars represent the standard deviations of the corresponding values.

Effect pH on enzymatic activity of crude extracellular proteases

To test whether the crude extracellular protease from both bacterial strains belong to acid, base, or neutral protease, we have examined the enzymatic activity of both crude extracellular proteases in different pH conditions. Our result showed that both proteases exhibited optimum enzymatic activity at pH 7 and when the pH was shifted to more basic then we observed that the enzymatic of both proteases were significantly reduced. Similarly, when we examined at the acidic conditions, both proteases showed less enzymatic activity compared to the basic conditions as well as to the neutral conditions (Figure 3). The results indicated that both proteases belong to neutral protease. Uehara *et al*., (1974) have reported that the optimum pH of the neutral protease was 7.3.

Effect of divalent metal ions on the enzymatic activity of crude extracellular proteases

When the enzymatic activity of both crude extracellular proteases were determined in the presence of various concentrations of $CaCl_2$, $ZnCl_2$ and $MgCl_2$ at pH 7 by using 1% skim milk as substrate, both proteases exhibited different requirement of the divalent metal ions. Crude extracellular protease from *C. salexigens* BKL5 preferred 1 mM of Zn^{2+}, while crude extracellular protease from *M. luteus* 11A required 10 mM of Ca^{2+} ion (Figure 4a and 4b). The result of this study indicated that the crude extracellular protease from *C. salexigens* BKL5 could be categorized as metalloprotease. The common characteristic of metalloproteases was the proteases required Zn as essential metal cofactor (Jiang and Bond, 1992). Furthermore the crystal structure study of psychrophilic metalloprotease of clearly showed that the protease contained Zn, which was located at active site core (Ravaud *et al*., 2003). Meanwhile crude extracellular protease from *M. luteus* 11A could be categorized as common neutral proteases. As shown in Figure 4a, the crude extracellular protease from *C. salexigens* BKL5 also exhibited high enzymatic activity when Ca^{2+} and Mg^{2+} used as divalent metal cofactor (Figure 4a). Unlike

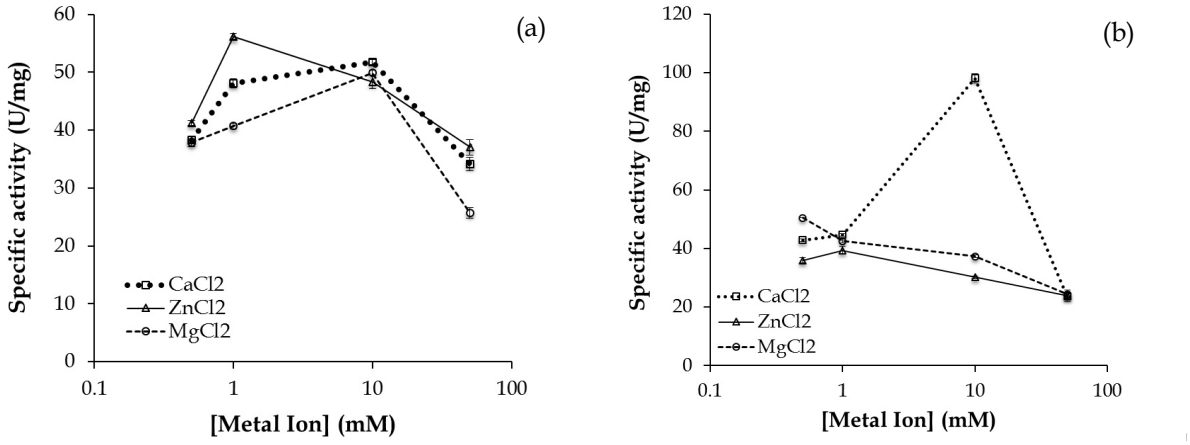

Figure 4. The divalent metal ion preference for enzymatic activity. (a) *C. salexigens* BKL5 and (b) *M. luteus* 11A. Divalent metal ion concentration used in this work was 0.5, 1, 1.5, 10, and 50 mM. The error bars represent the standard deviations of the corresponding values.

Zn^{2+}, Ca^{2+} was required for protein stability, instead of enzymatic activity (Tajima *et al*., 1976; Feder *et al*., 1971).

Furthermore the molar concentration of Ca^{2+} or Mg^{2+} was higher than molar concentration of Zn^{2+}. In the case of Mg^{2+}, it might be that the divalent metal ion could replace the role of Ca^{2+}, since both divalent metal ions have similar preference on ligand binding although both divalent metal ions differs ionic radii (Glusker *et al*., 1999). In the case of thermolysin, a kind of metalloprotease, it stoichiometrically contained 1g-atom of Zn and 4 g-atom of Ca per enzyme molecule (Tsuru *et al*., 1966). Previously, we have reported that the extracellular cellulase preferred Mg^{2+} and Zn^{2+} for enzymatic activity, since both have similar ionic radii (Rohman *et al*., 2015). It is apparently that the preference of ligand binding formation is more important for structure stability of the proteins, instead for enzymatic reaction. Regarding to the divalent metal ion concentrations, the results clearly indicated that the divalent metal ions required for enzymatic activity of both proteases was 1 to 10 mM. Increasing concentration of all the divalent metal ions examined exceeding than 10 mM greatly reduced the enzymatic activity of both proteases. Therefore concentration of divalent metal ions higher than 10 mM was inhibitory for those two crude extracellular proteases (Figure 4).

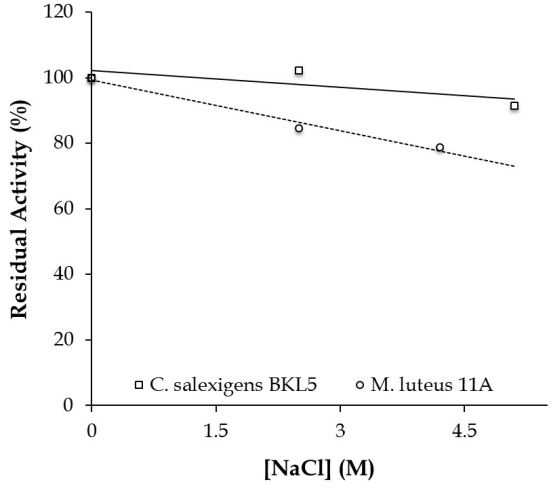

Figure 5. Effect of salt concentration on the enzymatic activity. NaCl concentrations were varied from 0 to 5 M. The error bars represent the standard deviations of the corresponding values.

Effect of salinity on enzymatic activity of crude extracellular proteases

In this work, we have tested the protein stability against the various concentrations of NaCl. The enzymatic activity was determined in the presence of NaCl with concentration from 0 to 5 M (Figure 5). The result indicated that crude extracellular protease from *C. salexigens* BKL5 showed enzymatic activity that relatively stable with the decreasing rate of enzymatic activity was as much as 1.71% per molar of NaCl. In contrast, crude extracellular protease from *M. luteus* 11A exhibited faster decreasing rate of enzymatic activity. The enzymatic activity of crude extracellular protease from *M. luteus*

11A was decrease by the rate of 5.14% per molar of NaCl or 3 times faster than that of *C. salexigens* BKL5. As expected that the crude extracellular protease from *C. salexigens* BKL5 was more stable than from *M. luteus* 11A, since *C. salexigens* BKL5 was moderate halophilic bacteria that could grow in the presence of NaCl up to 20% (Rohman *et al*., 2012).

Conclusions

We have characterized the crude extracellular protease from *C. salexigens* BKL5 and *M. luteus* 11A. Both proteases were belong to the group neutral protease, however both proteases showed different divalent metal ion requirement. The crude extracellular protease from *C. salexigens* BKL5 preferred Zn^{2+} as metal cofactor meanwhile crude extracellular protease from *M. luteus* 11A preferred Ca^{2+}, and therefore, crude extracellular protease from *C. salexigens* BKL5 belongs to the zinc metalloprotease. Crude extracellular protease from *M. luteus* 11A was less stable than that of *C. salexigens* BKL5 in the presence of high concentration of salt.

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