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PURIFICATION AND CHARACTERIZATION OF THE NEWLY THERMOSTABLE PROTEASE PRODUCED BY *Brevibacillus thermoruber* LII ISOLATED FROM PADANG CERMIN HOTSPRING, INDONESIA

Pemurnian dan Karakterisasi Protease Tahan Panas Baru yang Dihasilkan oleh *Brevibacillus thermoruber* LII yang Diisolasi dari Sumber Air Panas Padang Cermin, Lampung, Indonesia

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ABSTRACT

Thermostability is among of the vital enzyme characteristics for industrial application. *Brevibacillus thermoruber* LII was obtained as a potential isolate from the previous research which screened the potential thermostable protease producing bacteria from Indonesian hotspring. The newly thermostable protease produced by thermophilic *Brevibacillus thermoruber* LII had been purified and characterized. It was predicted that the pure enzyme obtained from *Brevibacillus thermoruber* LII was homo hexameric, having molecular weight of 36 kDa unit protein and its native was 215 kDa. In addition, it was also a neutral metalo serine protease according to biochemical tests that it was totaly inhibited by PMSF (Phenylmethanesulfonyl fluoride) and EDTA (Ethylenediaminetetraacetic acid). It showed optimum activity at pH of 8 and active in acidic buffer (up to pH of 4). All of metal ion in the form of chloride salt (2.5 mM) which were tested on the enzyme enhanced the enzyme activity but Li^{2+}. Ca^{2+} ion increased the activity and the stability of enzyme against thermal. The enzyme also showed the stability against solvent. The protease LII had optimum temperature at 60°C without $CaCl_2$ and 80 – 85°C with addition of 2.5 mM $CaCl_2$. The K_M and V_{max} values for the purified protease LII were 27.2 mg/ml or 0.362 – 0.272 M for substrate HammersteinCasein (MM 75–100 kDa) and 261.1 µg/minute/ml, respectively.

Keywords: thermostable protease, *Brevibacillus thermoruber*, hotspring

ABSTRAK

Ketahanan terhadap panas merupakan salah satu sifat enzim yang penting untuk aplikasi industri. *Brevibacillus themoruber* LII diperoleh dari penelitian sebelumnya pada penapisan isolat potensial penghasil protease tahan panas dari sumber air panas Indonesia. Protease baru tahan panas yang dihasilkan oleh *Brevibacillus thermoruber* LII sudah dimurnikan dan dikarakterisasi. Diprediksi bahwa enzim murni yang dihasilkan oleh mikroorganisma tahan panas, *Brevibacillus thermoruber* LII merupakan heksamer dengan berat molekul unit yang sama yaitu 36 kDa dan *native*nya 215 kDa.Lebih lanjut, protease ini merupakan protease metaloserin netral berdasarkan uji-uji biokimianya yang dihambat sepenuhnya oleh PMSF (Phenylmethanesulfonyl fluoride) and EDTA (Ethylenediaminetetraacetic acid). Enzim menunjukkan aktifitas optimum pada pH 8 dan aktif lebih baik pada pH bufer bersifat asam (sampai pH 4). Semua ion logam (2.5 mM) dalam bentuk garam-garam khlorida yang diujikan dapat meningkatkan aktivitas enzim kecuali Li^{2+} Ion Ca^{2+} paling mempengaruhi aktivitas enzim (meningkatkan sampai 250%) dan kestabilan terhadap panas. Protease LII mempunyai suhu optimum 60°C tanpa penambahan $CaCl_2$ dan 80 - 85°C ketika ditambahkan $CaCl_2$. Enzim juga menunjukkan kestabilan terhadap pelarut organik. Nilai K_M danV_{max}enzim murni masing-masing adalah 27.2 mg/ml atau 0.362 – 0.272 M dengan substrat HammersteinCasein (MM 75–100 kDa) dan 261.1 µg/menit/ ml.

Kata Kunci: protease tahan panas, *Brevibacillus thermoruber*, sumber air panas

1. Introduction

Protease are one of the most important enzyme in industrial and academic fields. It approximately consist of 60% in enzyme market (Rao et al.,1998). The use of protease was predicted up to 250 billon US$ by 2010 (Turk, 2006). They are largely applied in leather, detergent, brewing protein modification, meat, photographic, dairy, waste treatment, and membrane cleansing industries (Kumar, 2002; Chu, 2007). The demand of the enzyme are predicted to increase due to the need of the enzyme, which can resist on rough industrial process.

Thermostability is among of the vital enzyme characteristics for industrial application The benefits of using thermostable enzymes in industry include the capability to use elevated temperatures, thus increasing the solubility of non gaseous reactants/ products, allowing more rapid reactions, and decreasing the occurrence of microbial contamination by mesophilic organisms (Sookkheo et al., 2000; Chen et al., 2006). In a raised temperature range, a hard-to-degrade protein tends to achieve plasticity results in more susceptibleness to enzymatic degradation (Suzuki et al., 2006). Moreover, the use of thermophiles and their derived enzymes is effective for the breaking of hard-to-degrade proteins such as collagen (Okamoto et al., 2001; Watanabe, 2004), keratin (Nam et al., 2012), and prion protein (Tsiroulnikov et al., 2004).

Brevibacillus thermoruber was reported at first as a new species of *Bacillus* by Manachini et al. (1988). This species showed protease producing capacity at optimum temperature of 45°C and optimum pH 9. Wang et al. (2012) reported a *Brevibacillus* species that produced thermostable protease with slightly different characters to the one which was produced by strain LII (optimum temperature 75°C and optimum pH 9) . Another experiment on these bacteria was reported by Lee et al. (2004). Lee's group reported the presence of a gene encoding Lon Protease in *Brevibacillus thermoruber* WR-249. Lon proteases are ATP-dependent *serine peptidases* belonging to the *MEROPS* peptidase family S16 (*Lon protease family, clan SF*). *Brevibacillus thermoruber* had also been reported for its ability to degrade fibroin which is one of the substrates of protease (Suzuki et al., 2009).

Brevibacillus thermoruber LII was obtained as a potential isolate from the previous research which screened the potential thermostable protease producing bacteria from Padang Cermin, an Indonesian hot spring. The intensive screening at elevated temperature not only conducted on solid medium but also whitin liquid medium. The crude enzyme showed the highest activity at 85°C, stable for up to 100 minutes at 75°C with optimum pH of 8 – 9 (Zilda et al., 2012a). The investigation on optimization of enzyme production showed that LII protease was produced optimally at 50-55°C, pH 5-7. The addition of feather keratin in production medium resulted the enzyme with remarkable activity compared to those supplemented with casein, skim milk and collagen. The capability of this isolated on degradation of whole feather keratin also has been investigated (Zilda et al., 2012b). All reports showed that thermostable protease produced by LII had the different characteristics from any thermostable protease produced by the same genus regarding to the optimum temperature and stability against thermal that have been done on crude enzyme.

2. Material and Methods

2.1. Material

Brevibacillus thermoruber LII was isolated from Padang Cermin Hotspring, Indonesia. Feather keratin was prepared by washing chicken feathers with tap water followed by sun drying. Subsequently, the dried feathers were boiled in 0.0125 M NaOH for 20 minutes, neutralized with HCl, and then again sun dried. The above procedure was repeated twice before the samples were ground and used as the substrate (Rahayu, 2010).

The medium for protease production was contained of 0.1% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.05 % yeast extract and 0.5 % feather keratin. The culture was incubated at 50°C for 22 hours as previous reported as optimum condition for protease production (Zilda et al., 2012b). After incubated in respective medium for required time, the culture of *Brevibacillus thermoruber* LII was filtered through Whatman No. 1 filter paper to separate remained feather keratin and then centrifuged at 10,000 rpm for 10 min. The clear cell free culture supernatant was used as enzyme source for further studies.

2.2. Methods

2.2.1. Protease Purification

Two liters of crude protease harvested by centrifugation and the supernatant was concentrated and filtered by continuous ultrafiltration (Sartorius) equipped with a 5000 Da cut-off membrane (Amersham). The sample was loaded onto a column of DEAE Sepharose Fast Flow (1.6 x 10 cm, Amersham) pre-equilibrated with 25 mM Tris-HCl buffer , pH 9.0 (buffer A). Bounded proteins were eluted at a flow rate of 1 ml/min by applying a linear NaCl gradient (0- 1.5 M) in buffer A and fractions were collected at 5

ml. The active fractions were pooled and applied to Sepacryl S200 1cm x 60 cm, (Amersham), equilibrated in 25 mM Tris-HCl buffer, pH 9.0. The elution was performed with the same buffer, at a flow rate of 0.5 ml/min and fractions of 3 ml were collected. The fractions with proteolytic activity were pooled and used for further characterization.

2.2.2. Molecular Weight Determination

SDS PAGE method in 10% polyacrylamide slab gel described by Laemmli (1970) carried out to analyze the molecular mass. For zymogram analysis, protease was separated in a 10% SDS-polyacrylamide gel containing 0.5% gelatin as substrate. The samples were not heated prior to electrophoresis at a constant current of 8 mA/gel. The gel was washed with 2.5% Triton X-100 for 1 hour and incubated for 30 minute in 25 mM Tris-HCl buffer, pH 9.0 at 75°C. The gel was stained with Coomassie brilliant blue R-250 (0.1% w/v) for 30 min and then destained in distilled water/methanol/acetic acid (50:40:10). The protease band appeared as clear zone surrounded by dark blue color of the gel.

3. Characterization of Protease

3.1. Determination of Active Site by Amino Acid Blocking

Modification of the histidine, cysteine, and serine residue was performed by pre-incubating the protease with 4-bromophenacyl bromide, iodoacetamide (IAA), and phenyl methylsulphonyl fluoride (PMSF), respectively, at a final inhibitor concentration of 1 mM at room temperature (25 °C) for 30 min and then assaying the protease activity against appropriate control (Rai & Mukherjee, 2009). The inhibition effect was also investigated by zymogram analysis.

3.2. Effect of Metal Ions on Protease Activity

Effect of various metal ions on enzyme activity was determined by incubating the enzyme with 2.5 mM metal ions (Co^{2+}, Mg^{2+}, Li^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}; Mn^{2+}, and Ca^{2+}) at 45°C for 30 minutes. The enzyme activity without metal ion served as the control and was considered as 100% activity.

3.3. Effect of Temperature on Protease Activity and Its Stability

To determinate the optimum temperature of enzyme, 250µl of enzyme was mixed with 250 µL of 1 % casein and incubated at different temperatures (50–90°C). After 10 minutes the reaction was terminated by adding 500 µL of 0.4M TCA. Then the protease activity was measured as described earlier. The stability of enzyme was measured by incubating the enzyme at different temperatures (50, 55 and 60 without $CaCl_2$; 60, 65 and 70° C with 2.5 mM $CaCl_2$) up to 3 hours and assayed for its activity. The enzyme without heat treatment was considered as 100% activity.

3.4. Determination of Optimum pH and Its Stability

The effect of pH on protease activity and its stability was determined by incubating the enzyme with different pH (4 – 10) buffer for 2 hours . The enzyme was then loaded on to polyacrylamid-substrate gel and incubated at the same pH buffer. The optimum pH and its stability will determined by the clearest zone appeared on the gel after staining with coomassine blue.

3.5. Effect of Solvents on Protease Activity

Investigation on effect of solvents (ethanol, methanol, acetone, acetonitrile, ethyl acetate and diethyl ether) on protease activity was determined by incubating enzyme with 5% of solvents at 45°C for 30 minutes and assayed for its activity. The enzyme activity without solvents served as the control which was considered as 100% activity.

3.6. Effect of Detergent and Chemical On Pro tease Activity

Investigation on effect of different concentration of surfactant (SDS, Tween-80, Triton X-100), metal ion chelator (ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT) was determined by incubating enzyme with 1% of detergent (1%) and metal 1mM of EDTA and DTT at 45°C for 30 minutes and assayed for enzyme activity. The enzyme activity without chemicals served as the control which was considered as 100% activity

3.7. Determination of Kinetic Parameters

The reaction rate of the purified protease was determined at various concentrations of casein (1 to 15 mg/ml) prepared in 25 mM Tris-HCl buffer. The kinetic parameters were measured using the equation:

$$1/V=1/V_{max}+K_M/V_{max}\,1/[S]$$

The plot of 1/V versus 1/[S] would result a straight line with a y-intercept = $1/V_{max}$ and a slope = K_M/V_{max} which is called a Lineweaver-Burke plot (Voet and Voet, 2004).

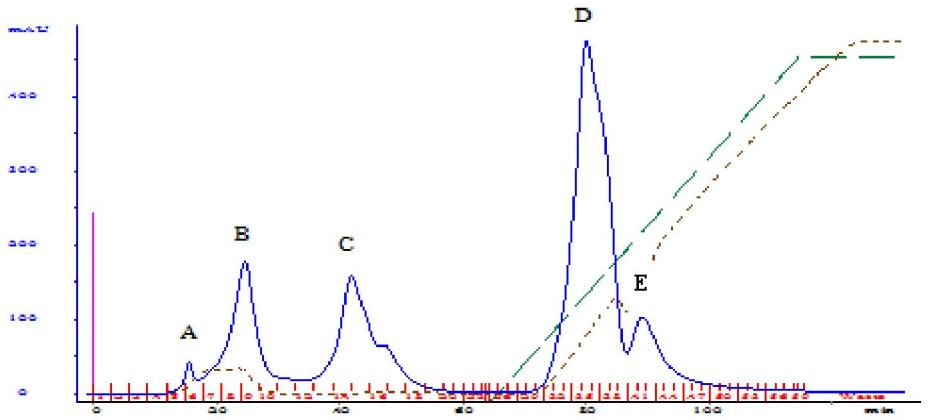

Figure 1. DEAE sepharose profile of concentrated protease LII copied from unicorn software connected to FPLC. The fractionation was carried out with NaCL (0 – 1,5 M) in 25 mM Tris-Cl buffer pH of 9, flow rate 1ml/minute with 5 ml of fraction collection. Arrow indicates the fraction with highest protease activity. Protease activity was measured with 1% casein in 2.5 mM Tris-Cl buffer pH of 9 at temperature of 85°C.

4. Result and Discussion

4.1. Protease Purification

Brevibacillusthemoruber LII was isolated from Indonesian hotspring along with 2 other strain which produced a potential thermostable protease. Previous work showed that crude thermostable protease produced by *Brevibacillus thermoruber* LII optimally active at 85°C, pH 9, and stable at temperature 70°C up to 100 minutes (Zilda, 2012[a]). This characteristics were used as condition for measuring the enzyme activity in this paper.

The fractionation of concentrated enzyme on DEAE sepharose separated the sample into five protease peaks, designated as A – E (Figure 1). Peak B showed the highest activity at 85°C. The peak A showed the protease activity after applied on to sepacryl S200 (Figure 2). The purification summary of this enzyme is displayed in Table 1.

4.2. Molecular Weight Determination

The observed single band in SDS-PAGE suggested that the purified protease from *Brevibacillus thermoruber* LII was homomer with molecular weight of 36 kDa (Figure 3A). Zymogram activity staining revealed one zone of gelatin hydrolysis for the purified sample co-migrating with molecular weight of 215 kDa (Figure 3B). LII protease was confirmed as homomer since the denaturized enzyme showed the single band so that the molecule was predicted consisting about

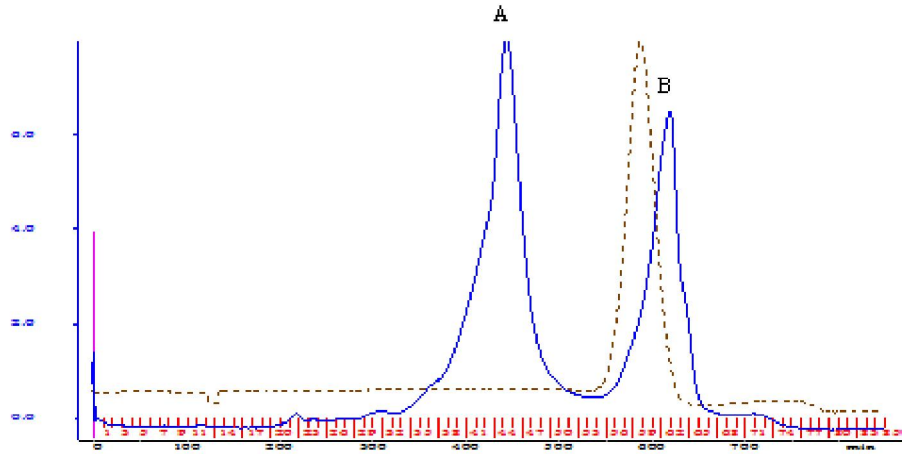

Figure 2. Sepacryl S200 profile of Peak A from DEAE fractionation copied from unicorn software connected to FPLC. The fractionation was carried out using 120 ml matrix equilibrated with 2.5 mM Tris-Cl buffer, flow rate 0.5 ml/ minute and 3 ml fraction collection. Arrow indicates the fraction with protease activity. Protease activity was measured with 1% casein in 2.5 mM Tris-Cl buffer pH of 9 at temperature of 85 °C.

Table 1. Purification summary of thermostable protease produced by *Brevibacillus thermoruber* LII

Step	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	88242,11	210,50	419,21	1	100
DEAE	23648,42	6,53	3618,98	8,63	26,79
sepacryl	2817,68	0,60	4696,98	11,20	3,19

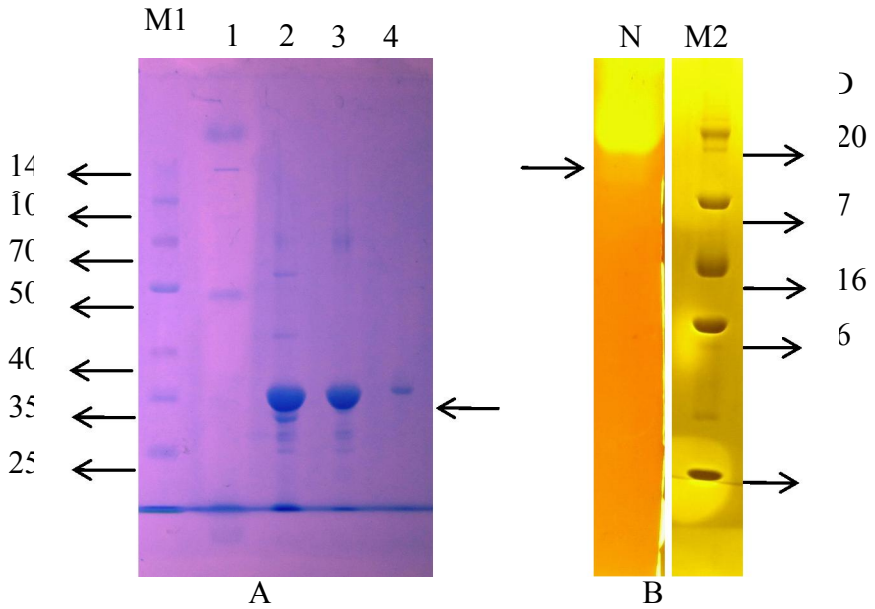

Figure 3. SDS-PAGE in 12% (w/v) polyacrylamide slab gel of thermostable protease LII purification (A) Lane M1: Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific), lane 1: Crude enzyme, Lane 2: Ultra filtration, Lane 3: DEAE fraction, Lane 4: Sepacryl S200 fraction. (B) Lane N:LII (native), M2: Native Molecular Weight Marker (Amersham).

six subunits or hexameric. The observation to determine the native molecular weight of protease using zymogram analysis also described by Bhargavi & Prakasham, (2013) and Jaouadi et al., (2010) who determine the molecular weight of protease produced by *Seratia* sp. RSPB 11 and *Streptomyces* sp. strain AB1 respectively.

Zymography is an electrophoresis technique, based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and a substrate co-polymerized with the polyacrylamide matrix. Proteins are prepared by the standard SDS-PAGE buffer under non-reducing conditions (no boiling and no reducing agent), and are separated by molecular weight in the standard denaturing SDSPAGE. The active enzymes will hydrolyze the embedded substrate, and then proteolytic activity can be visualized as cleared bands on a coomassie stained background (Heussen & Dowdle, 1980).

Some of serine proteases were reported as monomeric protease (Zhu et al., 2007; Rachadech et al., 2010; Mazar et al., 2012). *Bacillus* sp. strain MO-1 produced protease consisted of two large sub units (105 kDa) or dimeric (Okamoto et al., 2001). Dimeric protease also produced by *Aeromonas veronii* PG01 with molecular weight of 33 kDa as denaturized protein and 66 kDa as non-denaturized one (Divakar et al., 2010). There were no reports found about hexameric protease as be produced by *Brevibacillus thermoruber* LII.

Varieties of molecular weight for denaturized proteases from other *Bacillus* species had been reported: 30.9 kDa from thermophilic *Bacillus* strain HS08 (Huang et al., 2006); 27.0 kDa from *Bacillus megaterium* (Reungsang et al., 2006); 75.0 kDa from *Bacillus sp.* S17110 (Jung et al., 2007); 34.0 kDa from *Bacillus thuringiemis* (Kunitate et al., 1989); 38.0 kDa from *Bacillus cereus* KCTC 3674 (Kim et al., 2001);

15.0 kDa from *Bacillus subtilis* PE-11 (Adinarayana *et al*., 2003) and 34.0 kDa from *Bacillus cereus* BG1 (Ghorbel-Frikha *et al*., 2005).

4.3. *Determination of Active Site by Amino Acid Blocking*

The active site of protease can be determined by modification of the histidine, cysteine, and serine residue which was performed by pre-incubating the protease with 4-bromophenacyl bromide (BPB), iodoacetamide (IAA) and phenyl methylsulphonyl fluoride (PMSF). After incubation LII protease with PMSF for 30 minutes at 45°C, the zymogram analysis showed no clear zone (Figure 4). The measurement of enzyme activity after incubation showed that PMSF completely inhibited enzyme activity and there was no effect on the enzyme activity with addition of BPB but with IAA the activity was retained 91% (Table 2) . Based on the results, the LII protease could be classified as a serine protease.

5. Biochemical Characterization

5.1. Effect of Metal Ions on Protease Activity

Metal ions such as Mn^{2+}, Zn^{2+},Ca^{2+} and Mg^{2+} had a stimulatory effect on protease activity. This effect was also reported to increase the thermostability of other *Bacillus* proteases (Paliwal et al.,1994; Rahman et al., 1994). The remarkable activity was observed after the incubation of LII protease with metal ions (2.5 mM). This experiment showed that the protease activity increased more than 200% in the presence of Mg^{2+} , Mn^{2+} , Ca^{2+} and Cu^{2+}. There was no metal ion used in this research inhibiting the activity of the protease (Figure 5). Some thermostable serine proteases were inhibited by ion Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+} such as protease produced by *Pseudomonas* strain which was inhibited by ion Zn^{2+} and Mn^{2+} (Patil & Chaudhari 2009; Gupta et al., 2005) and thermostable serine protease produced *Bacillus pumilus* which was inhibited by Zn^{2+} and Fe^{2+} (Jayakumar et al., 2012) . Thermostable serine protease generated by *Bacillus* sp. strain MO-1 was completely inhibited by Fe^{2+} (Okamoto et al., 2001). *Brevibacillus* sp. strain PLI-1 produced thermostable protease which was completely inhibited by Zn^{2+} and Fe^{3+} (Wang et al., 2012).

5.2. Effect of Temperature and Its Stability

Since thermostable protease produced by *Brevibacillus thermoruber* LII was serine metaloprotease, which was stimulated by Ca^{2+} ion, the effect of temperature on enzyme activity and its stability was investigated through the addition of 2.5 mM of $CaCl_2$ at temperature of 55 – 95°C as shown in Figure 6.

The result showed that optimum temperature of purified protease produced by *Brevibacillus thermoruber* LII was 60°C without addition of $CaCl_2$ and 80-85°C with addition of 2.5 mM of $CaCl_2$. The activity of the enzyme with the presence of $CaCl_2$ was seen to be lower at temperature 55 – 60°C compared to enzyme activity without $CaCl_2$ (Figure 6). The

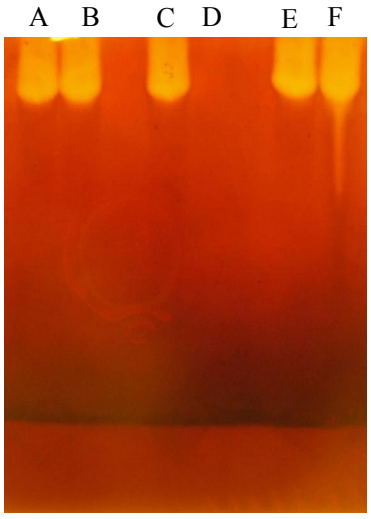

Figure: 4. Zymogram analysis of amino acid blocking. The purified protease LII was loaded after 30 minutes incubation at 45°C with chemical blocker. The experiment was carried out using 12 % polyacrylamyde and incubated at 75°C with Tris-Cl buffer pH of 9 for 30 minutes. (Lane A: Control IAA, B. Enzyme with IAA, C. Control PMSF, D. Enzyme with PMSF, E. Control DBP andF. Enzyme with BPB).

Table 2. Effect of various inhibitors and chemicals on the activity of thermostable protease produced by *Brevibacillus thermoruber* LII

Characteristics / *Inhibitors/Chemicals*	Relative Activity (%)
PMSF	0
IAA	92,75
BPB	99,22
EDTA (1mM)	0
DTT (1mM)	0
Tween 80 (1%)	98,25
SDS (1%)	22
Triton x100 (1%)	97.68
Metal ions	
Li^{2+}	99,88
Mg^{2+}	203,39
Mn^{2+}	206,15
Ca^{2+}	258,57
Zn^{2+}	166,18
Cu^{2+}	235,71
Fe^{3+}	126,69
Co^{2+}	199,21
Organic solvents	
Ethanol	100
Methanol	93,5301
Acetone	100
Acetonitrile	69,69
Ethyl acetate	97,15
Diethyl ether	97,6

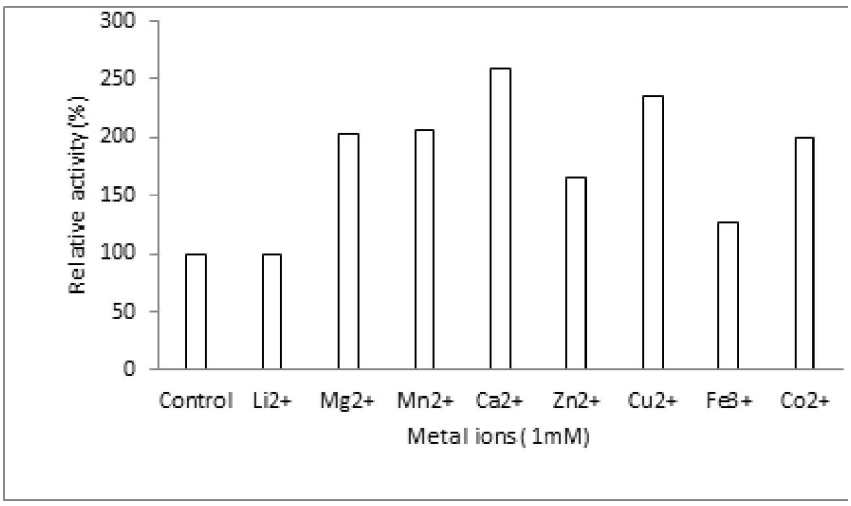

Figure 5. The activity of purified protease LII after incubation with various of 2.5 mM metal ions at 45°C for 30 minutes. The activity was measured with 1% casein in 2.5 mM Tris-Cl pH of 8 and temperature of 85°C.

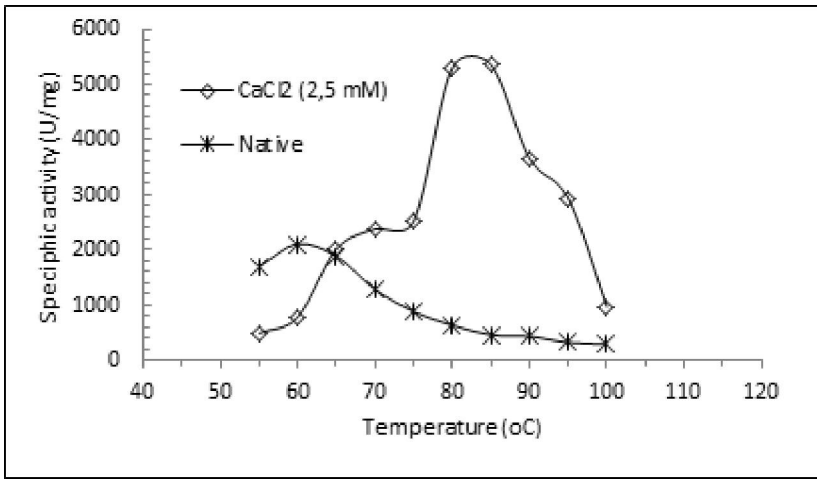

Figure 6. Activity of purified protease LII (native and with 2.5 mM $CaCl_2$) at different of temperature (55 – 100°C). The activity was measured with 1% casein in 25mM Tris-Cl buffer pH of 8.

enzyme with $CaCl_2$ showed maximum stability at temperature of 65°C (not presented). The thermostability of thermostable protease is mostly influenced by Ca^{2+} ion as reported by Asoodeh and Musaabadi, (2012), Jayakumar et al. (2012), Ghorbel et al. (2003) and Johnvesly & Naik, (2001). Two extracellular enzymes produced by thermophilic microorganisms required Ca^{2+} ion for their stabilities (Peek et al., 1992; Arulmani et al., 2007).

5.3. Effect of pH and It's Stability

The previous result showed that LII preferred acidic to basic medium to produce the protease (Zilda et al., 2012[a]). The zymogram analysis which was conducted by incubating the acrylamid-substrate gel in various of pH (4-10) buffer showed that the enzyme was active in all of pH range with optimum pH of 8 (data not shown).

The same characteristic showed by *Brevibacillus* sp. PLI-1 thermostable protease (Wang et al., 2012). The protease produced by *Bacillus thermantarcticus,* strain M1 was active over pH range 4.0-10.0 with an optimum of pH 7.0 (Dipasquele et al., 2008). *Pseudomonas*sp. DR89 produced neutral protease which was active in range pH from 5 to 11 with an optimum pH of 8.0 (Assodeh & Musaabadi, 2012).

5.4. Effect of Solvent on Protease Activity

It was reported that the proteases are unstable in organic solvents (Jayakumar et al.,2012). Hence, the need for organic solvent stable and thermostable protease is of much concerned as it could withstand at harsh industrial process conditions. Purified enzyme of *Bervibacillus thermoruber* LII inhibited about 2.4, 2.9 and 6.5 and 30,3%, by diethyl ether, ethyl acetate, methanol and acetonitrile repectively after preincubated for 30 minutes. No inhibition detected with addition of ethanol and acetone (Table 2).

5.5. Effect of Chemicals and Detergent on Protease Activity

Thermostable protease produced by *Brevibacillus thermoruber* LII completely was inhibited by EDTA and DTT. SDS also found inhibited this enzyme. Enzyme activity was not influenced by Incubation of the enzyme with 1 % of Triton-X 100 and Tween 80 . The inhibition produced by DTT might be due to reduction of intramolecular disulphide bonds required to maintain the activity and stability of the enzyme (Rai et al., 2010).

The summary of biochemical characteristics of thermostable protease produced by *Brevibacilus thermoruber* LII is presented in Table 2.

5.6. Determination of Kinetic Parameters

The kinetic parameters, including Km and Vmax values towards casein as a substrate, were determined. The K_M and V_{max} values for purified protease LII were 27.2 mg/ml or 0.362 – 0.272 M for its substrate HammersteinCasein (MM 75–100 kDa) and 261.1 µg/minute/ml respectively (Figure 7).

Thermostable protease produced by *Pseudomonas* sp. DR89 (Assodeh & Musaabadi, 2012), *Bacillus thermantarcticus* M1 (Dipasquale et al., 2008), *Paenibacillus tezpurensis* sp. nov. AS-S24-II (Rai et al., 2010) have Km value of 0.76, 1 and 0,2 mg/ml

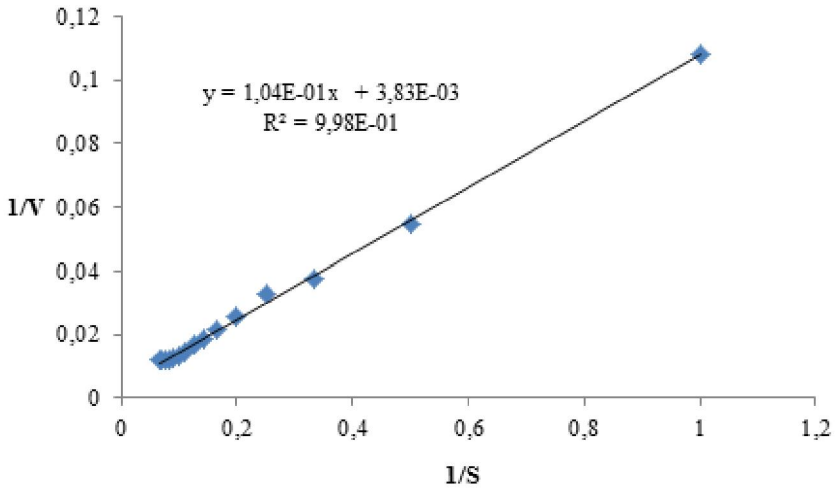

Figure 7. Lineweaver-Burk curve of the purified protease LII activity.

respectively which are lower than that produced by LII.

Dramatic loss of enzymatic activity, after observation of purified protease post-modification of serine residues by PMSF, suggested the involvement of this amino acid in the catalytic activity of the enzyme. Bacteria of the *Bacillus* genus secrete mostly two types of extracellular proteases, a neutral or metallo protease, and an alkaline protease which is functionally a serine endopeptidase, referred to as subtilisin. The first exhibited optimal pH at 7.0, whereas the latter had optimum pH between 9 and 11 (Ghorbel et al., 2003). LII protease belongs to the first group since the optimum pH was 8.0 and active more at acid pH than basic one. Subsequently, the addition of EDTA (1 mM), a potent inhibitor of metalloprotease, totally inhibited enzyme activity as a result of metal ion chelation. These biochmical characterization and inhibition pattern provides a clear results that the protease produced by LII was neutral metalo serine protease.

The unique about this enzyme was all of metal ions tested had no inhibition effect and increased the enzyme activity. The increasing of protease activity indicated that there were protection of the protease activity by metal ions against thermal denaturation there by playing a vital role in maintaining the active confirmation of the protease at higher temperatures (Kumar & Takagi, 1999). The kinetics parameter showed that LII protease has high K_M value. It may means that LII protease have capability to work efficiently at a broad range of substrate.

6. Conclusion

LII protease may be a good choice for application in food industries and peptide synthesis. Nevertheless, intensive investigations were needed more since this result obtained from native enzyme. The activator such as Ca^{2+} and other metal ions can be used to improve its stability against organic solvent and other environment parameters.

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