Biocatalysis and Agricultural Biotechnology 8 (2016) 32–38

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Isolation and characterization of a broad pH- and temperature-active, solvent and surfactant stable protease from a new strain of *Bacillus subtilis*

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ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form
4 July 2016
Accepted 4 August 2016
Available online 5 August 2016

Keywords:
Solvent stable protease
Surfactant stable protease
Alkaline protease
Broad-temperature active protease
Bacillus subtilis

ABSTRACT

Bacillus subtilis BUU1 from marine sediment collected in the Gulf of Thailand produced an extracellular protease. The enzyme was purified by 14.36-fold using 80–90% ammonium sulfate precipitation and Sephadex G-75. Molecular mass of the purified protease was estimated at 32 kDa on SDS-PAGE. The enzyme was highly active in a pH range of 3.0–12.0, the optimum being 11.0. After 6 h of incubation at different pH, the enzyme retained more than 80% of its activity at alkali pHs (7.0–12.0). The enzyme exhibited optimum activity over a broad temperature range (10–80 °C) with the maximum at 50 °C. The enzyme was stable between 60 and 80 °C for 72 h, showed excellent stability towards surfactants and was relatively compatible with oxidizing and bleaching agents. It was partially inhibited by metal salts and displayed remarkable stability towards a range of hydrophobic organic solvents. These promising properties make this enzyme a candidate biocatalyst for future use in biotechnological applications.

1. Introduction

Proteases are versatile enzymes with a long history of catalytic applications in science and industry and contribute about 60% of total worldwide enzyme sales (Banik and Prakash, 2004). Microbial proteases have numerous industrial applications (Rao et al., 1998) due to their high production capacity and catalytic activity (Gupta et al., 1999; Rao et al., 1998). Recently, proteases with high activity over a range of pHs and temperatures have novel applications in a diversity of areas including medicine, detergent and leather products and wastewater treatment (Mesbah and Juergen, 2014; Rao et al., 1998; Raval et al., 2014; Subba Rao et al., 2009).

Microbial proteases, especially from the genus *Bacillus* are the most widely exploited industrial enzymes with major applications as detergent additives (Erikson, 1996; Gupta et al., 1999). Many proteases have been purified and characterized, and have demonstrated significant proteolytic activities, stability, ease of purification, and low cost production (Bhunia et al., 2013; Kumar and Takagi, 1999; Rao et al., 1998). However, there remains a growing industrial need for more potential proteases with respect to pH- and temperature tolerance as well as the stability in the presence of surfactants and organic solvents. In this study, we report the purification and characterization of a protease produced by a strain of *Bacillus subtilis*. We provide also basic information about its potential as an industrial biocatalyst in terms of temperature and pH tolerance and its robustness against solvents, surfactants and oxidants.

2. Materials and methods

2.1. Screening and isolation of protease-producing strain

Bacterium used in this study was isolated from marine sediments collected from Koh Jan, Samaesan, Thailand at depths of 9–24 m. Sediment samples (10 g) was suspended in 100 ml of sterilized distilled water and aliquots of 100 µl were spread on marine agar (BD, Difco, France) and incubated at 30 °C for 24 h. Growing colonies were isolated as single colony by repeated streaking and protease producers detected by plating on skim-milk agar containing 1% (w/v) skim-milk powder, 1.5% (w/v) bacto-tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose and 2.5% (w/v) NaCl (pH 7.2). Plates were incubated at 30 °C for 24 h. Colonies that had formed a clear zone around their margins were chosen as protease producers. Positive isolates were screened further for production

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http://dx.doi.org/10.1016/j.bcab.2016.08.003
1878-8181/

of extracellular protease by assaying protease activity in liquid culture (see below). The strain which showed maximum protease activity was selected for further study.

2.2. Identification of bacterial strain

Taxonomic characteristics of the protease-producing strain were examined according to API Bacterial Identification Method and 16S rRNA gene sequencing (Weisburg et al., 1991). PCR amplification of 16S rRNA gene was performed using primers designed from the conserved regions at the base positions 22-41 (5′-GCTCAGATTGAACGCTGGCG-3′) and 1066 to 1085 (5′-ACATTTCACAACACGAGCTG-3′) of 16S rRNA gene of *Escherichia coli* (Precigou et al., 2004). Chromosomal DNA (100 ng) prepared by a GF-1 Nucleic acid extraction kit (Vivantis, Malaysia) was used as a DNA template. PCR was carried out by an initial denaturation step at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 90 s. Final extension was done at 72 °C for 10 min. The expected PCR product (~1.1 kb) was purified with a GF-1 Gel DNA recovery kit (Vivantis, Malaysia) and was then ligated into pTG19-T vector (Vivantis, Malaysia) according to the manufacturer's instructions. Ligation product was subsequently transformed into *E. coli* DH 5α and recombinant plasmid was extracted by a GF-1 Plasmid DNA extraction kit (Vivantis, Malaysia) before being used as template for DNA sequencing with dideoxynucleotide method (Sanger et al., 1977). A homology search was performed with Genbank database using BLAST algorithm and EzTaxon database (Chun et al., 2007). Alignment of 16S rRNA sequences of the strain with related bacteria was carried out using the SILVA aligner (Pruesse et al., 2007). The neighbor-joining phylogenetic tree was constructed with MEGA 6 program and 1000 replicates bootstrap analysis (Saitou and Nei, 1987). Sequence divergence was calculated using the Kimura 2-parameter model (Kimura, 1980) of MEGA 6 software (Tamura et al., 2013). Sequence data has been submitted to GenBank database, with the accession number KM370127.

2.3. Protease activity assay

Enzyme assay using azocasein as substrate was carried out. The reaction was performed for 10 min at room temperature in a total reaction volume of 100 µl containing 25 µl of substrate (1% w/v) in Tris-HCl buffer (50 mM, pH 8.0) and 25 µl of enzyme solution (culture supernatant). The reaction was stopped by the addition of 50 µl of 0.4 M trichloroacetic acid (TCA), and the mixture was allowed to stand at room temperature for 15 min. The sample was centrifuged at 10,000 × *g* for 5 min to remove residual azocasein. Then, the supernatant (25 µl) was mixed with 0.4 M Na_2CO_3 (125 µl) and Folin-phenol reagent (25 µl). The reaction was left at room temperature for 10 min and OD660 was measured. A blank was treated in the same manner, except that the enzyme was added after the addition of 0.4 M TCA. A standard curve was generated using solutions of 0–2.0 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the experimental conditions used.

2.4. Protease production and purification

Protease production from the isolated strain was carried out in a Luria-Bertani (LB) medium. Cultivations were conducted in an orbital shaker (250 rpm) for 24 h at 30 °C, in 3-litre Erlenmeyer flasks with a working volume of 1 L. Cultures were subsequently centrifuged for 15 min at 10,000 × g, and the cell-free supernatants used for estimation of enzyme activity. The culture supernatant containing extracellular protease was precipitated at 4 °C using $(NH_4)_2SO_4$. The supernatant was first brought to 0–40% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 12,000 × g for 15 min. The resulting supernatant was then brought to a step-wise 10% saturation $(NH_4)_2SO_4$ and centrifuged. A final concentration of 80–90% $(NH_4)_2SO_4$ saturation was lastly performed with continuous gentle stirring, stirring continued for a further 1 h, and then the suspension was centrifuged at 12,000 × *g* for 15 min at 4 °C. The pellet obtained in this step was suspended in a minimum volume of 50 mM Tris-HCl, pH 8.0 and dialyzed at 4 °C against the same buffer for 12 h. Insoluble materials were discarded by filtration with 0.2 µm pore size of nylon membrane filter. Then, the solution was loaded on Sephadex G-75 chromatography column equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Fractions of 1 ml were collected at a flow rate of 30 ml/h with the same buffer. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. During the course of enzyme purification, absorbance was measured at 280 nm and protease activity was assayed as described before. Fractions showing the highest protease activities were pooled and concentrated for further characterization.

Purity and molecular mass of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to (Laemmli, 1970). Gelatin zymography staining was performed as previously described by (Anbu, 2013). The relative molecular mass of the native enzyme was approximated by gel-permeable column chromatography as mentioned before. Gel filtration calibration kit (GE Healthcare, England) containing aprotinin (MW=6500), ribonuclease (MW=13,700), carbonic anhydrase (MW=29,000), ovalbumin (MW=43,000) and conalbumin (MW=75,000) was used as marker.

2.5. Optimal parameters determination

The pH optimum for purified protease was assayed by analyzing its activity in the pH range of 3.0–12.0 using azocasein as a substrate and buffer systems of 50 mM sodium acetate for pH 3.0–6.0, phosphate for pH 7.0, Tris-HCl for pH 8.0–9.0 and sodium carbonate for pH 10.0–12.0. pH stability studies were performed by pre-incubating the purified protease in selected pH buffer at 37 °C for 6 h and subsequent analysis of residual activities under the used experimental conditions.

Optimum temperature of the enzyme was determined by incubating the reaction mixture at different temperatures (10–80 °C) in 50 mM Tris-HCl buffer (pH 8.0) and measuring protease activity. Thermal stability was monitored after enzyme incubation for 72 h at 60, 70 and 80 °C and then measuring residual activity under experimental assay conditions.

2.6. Enzyme stability

2.6.1. Effect of organic solvents

Purified protease stability in relation to an assortment of organic solvents, at 50% (v/v) concentration in enzyme solution, methanol (log $P_{o/w}$ −0.8), ethanol (log $P_{o/w}$ −0.24), isopropanol (log $P_{o/w}$ 0.05), isoamyl alcohol (log $P_{o/w}$ 1.1), benzene (log $P_{o/w}$ 2.0), hexanes (log $P_{o/w}$ 3.6), heptane (log $P_{o/w}$ 4.0), decane (log $P_{o/w}$ 5.6), hexadecane (log $P_{o/w}$ 8.8) was determined after incubation at 37 °C in an orbital shaker at 150 rpm for 72 h. Residual protease activity of each reaction with respect to control (no solvent) was measured under experimental assay conditions.

2.6.2. Effect of metal ions and enzyme inhibitors

The influence of different protease inhibitors (EDTA, DTT and PMSF) was investigated by incubating the enzyme for different times at 37 °C in the selected inhibitor containing reaction mixture

at different concentrations and the residual activity determined. Enzyme activity in the absence of inhibitors was taken as 100%.

The effect of various metal ions (Na^+, Ba^{2+}, Ca^{2+}, $Mg^{2+}$and Hg^{2+} in salt chloride) on protease activity was examined by pre-incubating purified enzyme at 37 °C in 50 mM Tris-HCl buffer (pH 8.0) with specified ions (1 and 5 mM). After 48 h of incubation, residual activity of the enzyme was measured and compared with the control (no chemical added) considered as 100%.

2.6.3. Effect of detergents, oxidizing and bleaching agents

Impacts of detergents (SDS, Tween80 and Triton X-100) and oxidizing agents (H_2O_2 and sodium perborate) on the enzyme stability were examined by pre-incubating the purified protease for 1 h at 37 °C in 50 mM Tris-HCl buffer (pH 8.0) with the selected chemicals after which residual activity was measured. Bleach stability of the enzyme was tested in relation to various concentrations of chemicals. All mixtures were pre-incubated at 37 °C for 1 h and then assayed for protease activity. Bleaching agents used in this study were sodium nitrotriacetate, sodium carbonate, sodium carboxymethyl cellulose, sodium percaborate, sodium pyrophosphate, sodium *p*-toluene sulfonate, sodium silicate, sodium tripolyphosphate, sodium xylenesulfonate, and zeolite. Enzyme activity in the absence of chemicals was assigned as 100%.

2.7. Substrate specificity

Enzyme specificity was tested using azocasien, casein, gelatin, egg albumin, hemoglobin, and BSA as substrate. The Michaelis-Menten's constant (K_m) and the maximum attainable velocity (V_{max}) were determined at different substrate concentrations. K_m and V_{max} values were obtained from a plot Lineweaver and Burk.

3. Results and discussion

3.1. Isolation and identification of the bacterium

Protease producing bacterium was isolated by growing colonies on marine agar plates supplemented with skim milk. Morphological and biochemical characteristics of the isolate indicated the isolate is a gram-positive and rod-shaped bacterium with catalase activity. It fermented glycerol, L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-sorbitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, amidon and glycogen. Negative results were obtained with the assimilation of erythritol, D-arabinose, D- and L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, ducitol, methyl-α-D-mannopyranoside, N-acetylglucosamine, D-lactose, D-melibiose, inulin, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagalose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Biochemical characteristics of the isolate suggested that this bacterium is *Bacillus subtilis* according to API system.

The 16S rRNA sequence was aligned using the NCBI GenBank and Ez-Taxon database to confirm identification. A phylogenetic tree was constructed using MEGA 6.0 software and the neighbor-joining method (Fig. 1). Based on the phylogenetic analyses, the isolate showed 99% homology with the following *B. subtilis* strains: *B. subtilis* JCM 1465 (accession no. **NR 113265.1**), *B. subtilis* NBRC 13,719 (accession no. **NR 112629.1**), *B. subtilis* BCRC 10,255 (accession no. **NR 116017.1**), *B. subtilis* sub sp. OS-105 (accession no. **NR 115001.1**) and *B. subtilis* (accession no. **NR 114996.1**). Thus, through a polyphasic identification approach, the isolate is identified as *B. subtilis* BUU1.

3.2. Protease purification and molecular mass determination

Purification of protease was achieved by ammonium sulfate precipitation (80–90% saturation) and Sephadex G-75 gel permeable chromatography. The enzyme was purified to 14-fold with a recovery of ~ 4% and specific activity of 273,250 U/mg protein (Table 1). The low yield might be due to the removal of a significant amount of co-proteinaceous content from the crude enzyme during precipitation. The purified protease migrated as a single band in SDS-PAGE under reducing conditions, suggesting its homogeneity (Fig. 2a). Apparent molecular mass of the purified protease is about 32 kDa which matches with native molecular mass estimated by gel filtration (data not shown). Zymogram activity staining revealed a clear band of gelatinolytic activity for the purified protease with an estimated molecular mass of 32 kDa (Fig. 2b). This suggests that the purified protease is a monomeric protein, similar to those described for other proteases from *Bacillus* sp. (Jain et al., 2012; Rao et al., 2009; Singh et al., 2012). However, the native molecular mass of the enzyme, below 40 kDa, differed from those strains (Anbu, 2013; Bhunia et al., 2013; Jain et al., 2012).

3.3. Effect of pH and temperature

The purified protease had a pH optima at 11.0 and retained more than 80% of its maximum activity over a broad pH range (3.0–12.0) (Fig. 3A). Alkali pHs had little effect on the stability of this enzyme with less than 10% loss of activity when incubated from pH 7.0–11.0 at 37 °C for 6 h (Fig. 3B). These findings are in accordance with several earlier reports on *Bacillus* proteases (Kumar and Takagi, 1999; Margesin et al., 1992), traditionally used for detergents (Annamalai et al., 2014; Joo et al., 2003; Rao et al., 1998).

Purified protease exhibited high activity between 10 and 80 °C, with an optimum at 50 °C (Fig. 4A). Although a similar temperature was reported earlier as optimum for protease activity in *Bacillus* sp. (Kumar and Takagi, 1999), the broad temperature range over which this enzyme functions is a distinguishing feature. Thermal stability profiles of the purified protease found it retained more than 75% of its initial activity after 72 h incubation at 60 °C and 70 °C (Fig. 4B) and lost only 20% and 50% activity after 36 and 72 h at 80 °C, respectively. Together with high pH stability, this protease is probably suited for laundry and tanning industrial (Kumar and Takagi, 1999; Rao et al., 1998).

3.4. Effect of organic solvents

Purified protease was quite stable in the presence of hydrophobic solvents throughout the period of incubation but activity was reduced significantly in the presence of short-chain alcohols like methanol, ethanol and isopropanol (Table 2). Solvents interacted with the essential water molecules surrounding the enzyme reducing its activity (Jain et al., 2012). Hydrophilic solvents are able to absorb the essential water molecule from the enzyme, resulting in the loss of catalytic properties (Sugihara et al., 1991). Moreover, solubility of proteases in azocasein and short-chain alcohols generally is decreased forming a new liquid phase at moderate concentrations leading to enzyme inactivation (Shimada et al., 1999). The stability of this protease in organic solvents with log P values ranging from 2.0 to 8.8 makes its probable applications in peptide synthesis and pharmaceutical industry (Rao et al., 1998; Raval et al., 2014).

3.5. Effect of metal ions and enzyme inhibitors

Metal ions (1 mM) showed neither a positive nor negative effect on enzyme activity after 1 h incubation (Table 3). However,

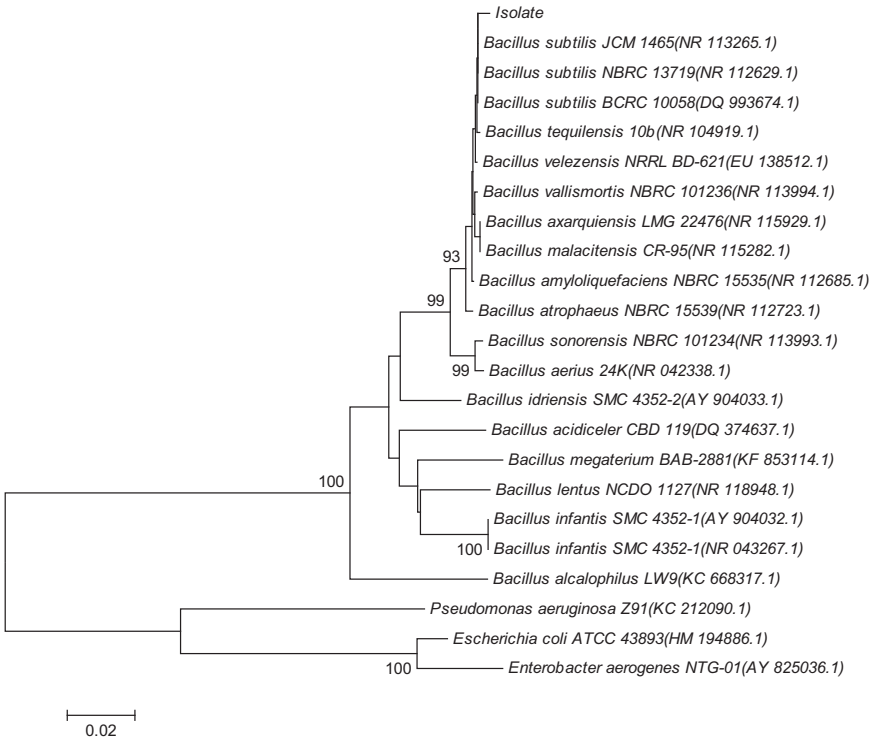

Fig. 1. Phylogenetic neighbor-joining tree based on 16S rRNA gene sequence of the isolate and other *Bacillus* sp. The digit at each branch point represents percentage bootstrap support calculated from 1000 replicates. Evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

Table 1
Purification of the purified protease from *Bacillus subtilis* BUU1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	26,455	1.39	19,032	1	100
Ammonium sulfate precipitation (80–90%)	143	0.001	143,000	7.51	0.54
Sephadex G-75 chromatography	1093	0.004	273,250	14.36	4.13

with longer incubation, enzyme activities were marginally affected (~20% inhibition). Half activities still retained when incubation the enzyme in high ion concentration for 48 h. Magnesium and sodium typically act as a salt or ion bridge between two adjacent amino acid residues in the active site and maintain the enzyme molecule's rigid conformation (Kumar and Takagi, 1999). Calcium is known to prevent unfolding of the protease and plays a vital role in maintaining its native conformation or is involved in the catalytic reaction (Annamalai et al., 2014; Haddar et al., 2009; Rao et al., 2009). The interaction between protease and Ca^{2+} likely improves both proteolytic activity and stability (Deng et al., 2010).

Protease activity of *B. subtilis* BUU1 was small affected by DTT and EDTA. Serine inhibitor PMSF caused a 40% reduction of protease activity after 48 h incubation. The weak impact of EDTA on enzyme activity suggests protease activity does not rely on any divalent cation, consistent with the fact that proteases are generally independent of cofactors (Najafi et al., 2006). PMSF blocks serine residue at the active site of proteases, resulting in a loss of enzyme activity (Kumar, 2002).

3.6. Effect of detergents, oxidizing and bleaching agents

Remarkably, the addition of strong anionic (SDS) and non-ionic detergents (Tween 80 and Triton X-100) had little effect on enzyme activity (Table 4). This is in contrast to some earlier reports on *Bacillus* proteases and notable (Annamalai et al., 2014; Doddapaneni et al., 2009). *B. subtilis* BUU1 protease showed improved stability in the presence of H_2O_2 and sodium perborate at 1% concentration. The activities were slightly reduced with high concentration (5% and 10%). Increased activity in the presence of oxidants may result from oxidization of methionine, cysteine, tyrosine or tryptophan residue present next to catalytic serine and prevent a critical step in the formation of tetrahedral intermediates during proteolysis (Siezen and Leunissen, 1997; Singh et al., 2012).

The protease from *B. subtilis* BUU1 was highly stable in all bleaching agents (1% concentration) tested except sodium carboxymethyl cellulose after 1 h incubation at 37 °C (Table 5). It retained > 70% of its initial activity at a 5% concentration. Sodium carbonate and sodium silicate did not affect protease activity at this concentration. Furthermore, at a 10% concentration of bleaching agent, protease retained residual activity in the range of 51–92%. A good detergent protease is expected to be compatible and stable with all commonly used detergent formulations (Gupta et al., 2002; Rao et al., 1998). The remarkable stability of this enzyme in the presence of high detergent concentrations underscores its usefulness as a detergent additive.

3.7. Substrate specificity

B. subtilis BUU1 protease exhibited the highest activity towards casein (Table 6). This protease showed K_m of 0.2 mg ml^{-1} and V_{max} of 220.76 μmol min^{-1} (data not shown) indicating its high affinity

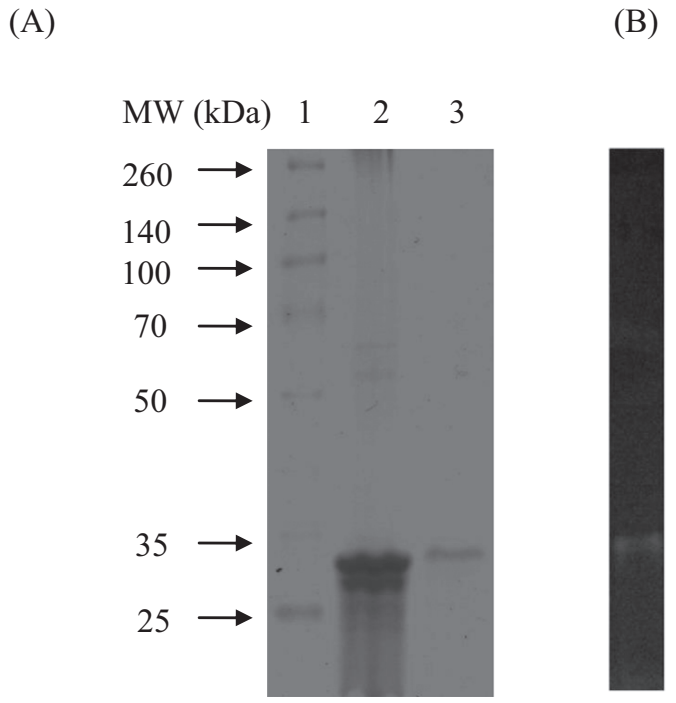

Fig. 2. (A) SDS-PAGE analysis of the purified protease from *Bacillus subtilis* BUU1. Lane 1: protein markers, Lane 2: 80–90% ammonium sulfate precipitated and dialyzed sample, Lane 3: purified protease by Sephadex G-75. (B) Zymogram activity staining of the purified protease with gelatin as a substrate.

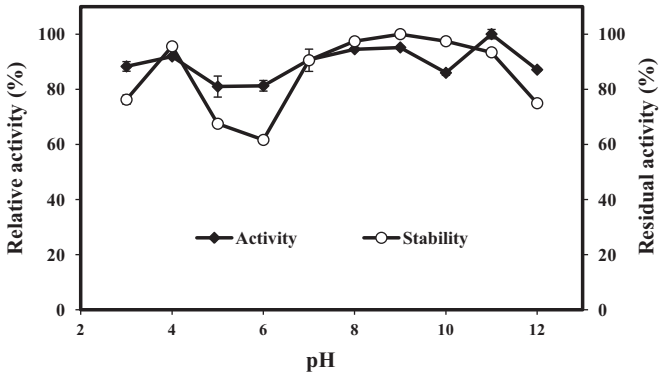

Fig. 3. Effect of pH on activity and stability of the purified *Bacillus subtilis* BUU1 protease. Protease activities were evaluated in the pH range of 3.0–12.0 using buffers of different pH values at 37 °C. The pH stability of the enzyme was determined by incubating enzyme in different buffers for 6 h at 37 °C and the residual activity was measured. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard deviations (mean ± SD). Buffer solution used for pH activity and stability are presented in materials and methods.

compared to literature documented proteases from *B. circulans* (K_m of 0.597 mg ml^{-1}) (Rao et al., 2009) and efficient catalytic role compared to protease from *B. clausii* GMBAE 42 (K_m of 1.8 mg ml^{-1} and V_{max} of 11.50 μM) (Kazan et al., 2005) and *Bacillus* sp. (K_m of 2 mg ml^{-1} and V_{max} of 289 μg min^{-1}) (Gupta et al., 2005). The enzyme scored 40% relative activity with azocasein as substrate whereas it was approximate 20% on gelatin, hemoglobin, BSA and egg albumin. Similarly, proteases from other *Bacillus* species exhibited their highest activity towards casein (Annamalai et al., 2014; Arulmani et al., 2007).

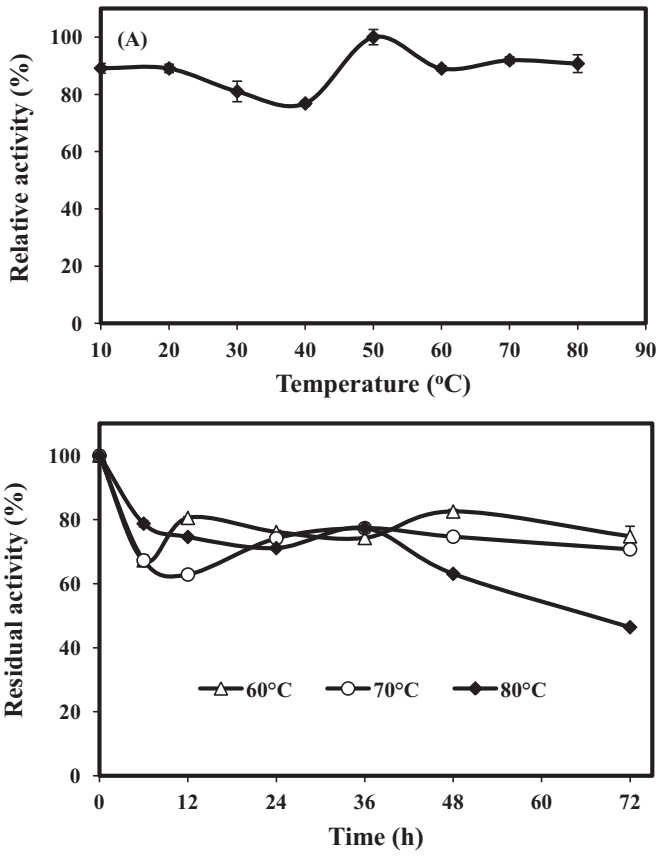

Fig. 4. Effect of temperature on activity (A) and stability (B) of the purified protease from *Bacillus subtilis* BUU1. Activity was measured by incubating the purified protease in the temperature ranges of 10–80 °C in a thermostatically controlled water bath. Heat stabilities of the enzyme were determined by pre-incubating protease solution at different temperatures for 72 h, after which residual activities were calculated relative to enzyme activity before incubation (100% of relative activity). All experiments were done in triplicate and expressed as the mean of three determinations with the standard deviations.

Table 2
Effect of organic solvents on protease stability.

Organic solvents	log $P_{o/w}$	Residual activity (%)[a]			
		12 h	24 h	48 h	72 h
Control	–	100 ± 1.54	100 ± 3.68	100 ± 0.41	100 ± 0.38
Methanol	−0.8	59 ± 0.79	56 ± 0.74	50 ± 0.61	50 ± 1.03
Ethanol	−0.24	68 ± 0.63	60 ± 1.17	57 ± 0.04	53 ± 0.95
Isopropanol	0.05	54 ± 0.15	54 ± 0.98	53 ± 0.22	52 ± 0.31
Isoamyl alcohol	1.1	93 ± 0.06	92 ± 0.09	87 ± 0.20	84 ± 0.01
Benzene	2.0	98 ± 0.46	97 ± 1.48	89 ± 0.42	89 ± 1.48
Hexanes	3.6	100 ± 0.85	98 ± 1.83	97 ± 1.09	92 ± 1.46
Heptane	4.0	98 ± 0.32	97 ± 0.14	96 ± 0.90	90 ± 0.09
Decane	5.6	99 ± 0.55	95 ± 1.22	95 ± 0.17	94 ± 0.04
Hexadecane	8.8	101 ± 0.37	100 ± 0.50	99 ± 1.32	95 ± 0.24

[a] The purified protease was incubated in the presence of organic solvents (50% v/v in enzyme solution) at 37 °C for several hours. Residual activity was determined and the enzyme activities measured in the absence of any solvent were taken as 100%.

4. Conclusions

This study describes the functional activity of *Bacillus subtilis* BUU1 protease over a broad range of pH (3.0–12.0), temperatures (10–80 °C) and solvent stability. The stability of the enzyme in the presence of various surfactants, bleach and oxidizing agents is particularly useful for its application in detergent, food, pharmaceutical, and leather industries. Most of the reported protease-

Table 3
Effect of various metal ions and some inhibitors on the activity of purified protease.

Chemicals	Relative activity (%)[a]									
	1h		6h		12h		24h		48h	
	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM
Control	100 ± 1.67	100 ± 1.67	100 ± 1.15	100 ± 0.33	100 ± 0.58	100 ± 1.06	100 ± 0.28	100 ± 0.65	100 ± 0.28	100 ± 0.42
Na^{+}	99 ± 1.19	66 ± 2.76	92 ± 0.61	66 ± 0.53	91 ± 3.79	62 ± 0.09	85 ± 0.09	58 ± 0.19	85 ± 0.49	52 ± 0.16
Ba^{2+}	97 ± 1.59	66 ± 2.11	93 ± 1.50	63 ± 0.40	93 ± 0.17	63 ± 0.20	87 ± 1.05	56 ± 0.27	83 ± 0.62	54 ± 0.94
Ca^{2+}	98 ± 0.21	63 ± 1.62	92 ± 1.66	62 ± 0.30	92 ± 0.24	60 ± 0.15	92 ± 0.55	54 ± 1.35	87 ± 0.16	51 ± 0.25
Mg^{2+}	103 ± 6.08	65 ± 1.30	101 ± 1.01	63 ± 0.52	93 ± 0.12	60 ± 0.70	93 ± 0.65	57 ± 0.28	90 ± 1.26	47 ± 0.95
Hg^{2+}	105 ± 2.80	57 ± 0.24	97 ± 0.30	56 ± 0.47	87 ± 0.58	49 ± 0.49	85 ± 1.16	49 ± 0.11	77 ± 0.58	43 ± 0.40
EDTA	95 ± 0.27	59 ± 0.38	91 ± 0.59	57 ± 0.24	85 ± 0.73	57 ± 1.11	82 ± 0.79	54 ± 0.12	76 ± 0.73	46 ± 0.48
DTT	105 ± 1.47	73 ± 0.93	99 ± 0.24	70 ± 1.44	93 ± 1.49	66 ± 0.64	93 ± 1.01	63 ± 0.07	88 ± 0.55	56 ± 0.60
PMSF	104 ± 3.45	67 ± 1.50	93 ± 1.19	64 ± 0.09	86 ± 0.93	53 ± 0.70	75 ± 0.30	42 ± 0.23	58 ± 1.14	36 ± 0.04

[a] Purified protease was pre-incubated with various chemicals for different hours at 37 °C and the residual activity was measured under standard conditions. Activity of the enzyme pre-incubated in the absence of any additive was taken as 100%.

Table 4
Effect of detergents and oxidizing agents on protease activity.

Detergents and oxidizing agents	Relative activity (%)[a]		
	1%	5%	10%
Control	100 ± 1.67	100 ± 1.08	100 ± 0.39
H_2O_2	120 ± 3.25	103 ± 0.20	77 ± 1.23
Sodium perborate	116 ± 1.57	91 ± 0.11	74 ± 0.90
SDS	100 ± 2.91	100 ± 0.67	90 ± 0.69
Triton X-100	102 ± 1.02	86 ± 0.05	79 ± 0.18
Tween 80	97 ± 4.22	96 ± 0.59	81 ± 3.45

[a] The purified protease was pre-incubated with various detergents and oxidizing agents at 37 °C for 1 h and then the remaining activity was measured. Enzyme activity determined in the absence of any additives was taken as 100%.

Table 5
Protease stability in different concentration of bleaching agents after 1 h.

Bleaching agents	Relative activity (%)[a]		
	1%	5%	10%
Control	100 ± 4.41	100 ± 0.43	100 ± 1.70
Sodium nitrotriacetate	107 ± 1.10	92 ± 0.14	82 ± 0.12
Sodium carbonate	117 ± 0.53	101 ± 0.36	90 ± 1.67
Sodium carboxymethyl cellulose	72 ± 0.95	70 ± 0.52	70 ± 0.11
Sodium percaborate	95 ± 0.49	86 ± 0.14	68 ± 0.21
Sodium pyrophosphate	110 ± 0.68	80 ± 0.12	59 ± 1.84
Sodium *p*-toluene sulfonate	102 ± 0.15	71 ± 0.22	53 ± 0.15
Sodium silicate	103 ± 0.15	102 ± 1.11	92 ± 0.27
Sodium tripolyphosphate	94 ± 0.63	73 ± 0.01	51 ± 0.09
Sodium xylenesulfonate	96 ± 0.41	77 ± 0.27	53 ± 0.31
Zeolite	108 ± 0.80	77 ± 0.02	56 ± 0.16

[a] The purified protease was incubated with different concentration of bleaching agents for 1 h at 37 °C. The remaining activity was measured and expressed as a percentage comparing with control (no additives).

producing bacteria are from *Bacillus* genera. *B. subtilis* BUU1 is a novel strain that produces a broad pH- and temperature -tolerant protease and contribute to current knowledge on the biodiversity of protease producers.

Acknowledgments

Research grant was provided to J.C. from National Research Council of Thailand through Burapha University (Grant no. 37/2558). We are grateful for financial support to S.U. from Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education. We appreciate F.W.H. Beamish's proofreading assistance.

Table 6
Substrate specificity of *B. subtilis* BUU1 protease.

Substrates	Relative activity (%)[a]
Casein	100 ± 1.20
Azocasein	40 ± 0.58
Egg albumin	21 ± 0.07
Gelatin	22 ± 0.43
BSA	22 ± 0.25
Hemoglobin	22 ± 0.07

[a] Percentages shown are relative to maximum activity of casein substrate and expressed as the mean of three determinations with the standard deviation.

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