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Original Article

Purification and characterization of a harsh conditions-resistant protease from a new strain of *Staphylococcus saprophyticus*

Sasithorn Uttatree,[a, b] Jittima Charoenpanich[a, c, *]

[a] *Environmental Science Program and Center of Excellence on Environmental Health and Toxicology (EHT), Faculty of Science, Burapha University, Bangsaen, Chon Buri 20131, Thailand*
[b] *Center of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Burapha University, Bangsaen, Chon Buri 20131, Thailand*
[c] *Department of Biochemistry, Faculty of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand*

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ABSTRACT

A major road block to the industrial usage of known proteases is their limited stability under harsh conditions. Hence, there is always a need for newer enzymes with novel properties that can further satisfy all industrial demands. This study described a benthic marine bacterium, *Staphylococcus saprophyticus* that secretes an alkaliphilic and broad-temperature active protease (10–80 °C). The protease was successfully purified 42.66-fold using 70–80% ammonium sulfate precipitation and gel-permeable column chromatography. It had a relative molecular mass of 28 kDa on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and retained high activity and significant stability at 60–80 °C, over a wide range of pH (3.0–12.0), inhibitors and metal ions. Furthermore, the enzyme was stable in surfactants (such as sodium dodecyl sulfate), oxidizing agents (such as H_2O_2), bleaching agents (such as zeolite) and hydrophobic solvents (such as benzene, hexanes and hexadecane). These properties support the enzyme's potential as a vigorous biocatalyst for industrial applications.

Introduction

Protease is a particularly important group of industrial enzymes that account for 60% of the total world enzyme markets (Rao et al., 1998; Raval et al., 2014). It has diverse applications in a wide range of industrial processes including food, detergent, pharmaceuticals, leather and wastewater treatment (Rao et al., 2009; Mesbah and Juergen, 2014). Among the various industrial protease producers, bacteria with their high production capacity and catalytic activity are the most substantial contributors when compared to animals, plants and fungi (Rao et al., 1998; Kumar and Takagi, 1999; Bhunia et al., 2013). However, a major road block to industrial usage of known proteases is their limited function under harsh conditions (Rao et al., 1998; Kumar and Takagi, 1999). Increasing demand for highly active preparations of proteases with appropriate specificity and stability with regard to pH, temperature, metal ions, surfactants and organic solvent continues to stimulate the search for new enzymes (Sellami-Kamoun et al., 2008; Haddar et al., 2009). Many proteases have been documented and some of them are now commercially available (Rao et al., 1998, 2009; Kumar and Takagi, 1999; Dubin, 2002). *Staphylococcus aureus* V-8 protease is a well-known commercial example. This enzyme shows maximum activity at two optimal pH values (4.0 and 7.8) and at 37 °C. The enzyme is active in the presence of many denaturing agents such as sodium dodecyl sulfate (SDS), urea and guanidine-HCl (Arvidson et al., 1973). However, its stability under multiple extreme conditions is rare. Hence, searching for enzymes that have wider versatility and adaptability to multiple harsh conditions is academically and industrially important.

The marine environment is a source of unique microorganisms with great potential for biotechnological exploitation (Podar and Reysenbach, 2006; Sellami-Kamoun et al., 2008; Haddar et al., 2009). Many studies concerning the isolation and characterization of marine bacteria have been carried out and investigations in this field may lead to many new discoveries. This study described the purification and characterization of a novel extracellular protease produced by the newly isolated marine strain *S. saprophyticus*, with a focus on its biochemical properties under harsh conditions.

* Corresponding author. Department of Biochemistry, Faculty of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand.
E-mail address: jittima@buu.ac.th (J. Charoenpanich).

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Materials and Methods

Isolation of protease-producing strain

Ten marine sediment samples were collected from Koh Jan, Samaesan, Thailand at depths of 9–24 m. Samples (10 g) were mixed with 100 mL of sterile water and the sediment was allowed to settle. The upper phase (100 μL) was spread on marine agar (BD; Difco; Le Pont de Claix, France) and was used for screening. Cultures were maintained at 25 °C for 24 h. Colonies that appeared on the plate were isolated as a single colony by streaking repeatedly and the culture was maintained under the same conditions. Bacteria that secreted protease were screened based on the clear zone surrounding the colonies as shown on skim milk agar (1% (weight per volume; 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). Strains that showed a maximum ratio of clear zone:colony diameters were selected and assayed for protease activity on a substrate of azocasein (see below). The bacterial strain with the highest protease activity after 24 h of cultivation was selected for further experiments.

Bacterial strain identification

Bacterial strain identification was based on the "API skills Bacterial Identification Method" and 16S rRNA gene sequence analysis (Weisburg et al., 1991). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using primers designed from the conserved regions at the base positions 22–41 and 1066–1085 located on the *Escherichia coli* 16S rRNA gene (Precigou et al., 2004). Chromosomal DNA (100 ng) prepared using a GF-1 Nucleic acid extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) was used as a DNA template. PCR was carried out using an initial denaturation step at 95 °C for 10 min which was followed by 30 cycles at 95 °C for 30 s, at 60 °C for 45 s and at 72 °C for 90 s. Final extension occurred at 72 °C for 7 min.

The expected PCR product (~1.1 kb) was purified using a GF-1 Gel DNA recovery kit (Vivantis, Selangor Darul Ehsan, Malaysia) and then was ligated into a pTG19-T vector (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instructions. After transformation into *E. coli* DH5α, plasmids were extracted and purified using a GF-1 Plasmid DNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia). A gene insert was verified using sequence analysis (Sanger et al., 1977). Similarity of nucleotide sequence was determined using BLAST (National Center for Biotechnology Information databases, Bethesda MD, USA) and subsequently analyzed using the Ez-Taxon database (Chun et al., 2007). To generate a 16S rRNA gene-based phylogenetic tree, sequences were aligned using the SILVA aligner (Pruesse et al., 2007). Sequence divergence was calculated using the Kimura 2-parameter model (Kimura, 1980) in the MEGA 6 software (Tamura et al., 2013), which was also used to create the neighbor-joining tree and to perform bootstrap analysis (1000 replicates) (Saitou and Nei, 1987). Sequence data were submitted to the GenBank database under accession no. KM370125.

Measurement of protease activity and protein content

Protease activity was measured using azocasein hydrolysis (Meyers and Ahearn, 1997). The reaction mixture consisted of 1% (w/v) of azocasein (125 μL) and 125 μL of enzyme solution. The reaction was incubated for 15 min at room temperature (28–30 °C). Then, the reaction was stopped by the addition of 250 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 the precipitate. Thereafter, the supernatant was mixed with 0.4 M Na_2CO_3 (625 μL) and Folin-phenol reagent (125 μL). Reaction was allowed at room temperature for 10 min. After that, absorbance was measured at 660 nm wavelength. A blank was set up following the same procedure, except that the enzyme was added after the addition of 0.4 M TCA. One unit (U) of protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine per minute under the assay conditions. The amount of tyrosine was determined from its standard curve. The protein content was determined using the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard protein. All experiments were conducted in triplicate and average values with the standard deviation were reported.

Protease purification

A single colony of the bacterial strain was grown in 3 L of Luria-Bertani broth at 30 °C and 250 rpm for 24 h. The culture supernatant was collected using centrifugation at 10,000 × *g* for 10 min. Solid ammonium sulfate was slowly added to the culture supernatant to 70–80% saturation and continuously stirred for 30 min at 4 °C. The precipitate was harvested using centrifugation at 10,000 × *g* for 30 min and subsequently dissolved in 50 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer overnight (4 °C). The dialyzed sample was assayed for protease activity and protein content and loaded onto a Tris-HCl buffer (pH 8.0) pre-equilibrated Sephadex G-75 gel permeable chromatography column. The same buffer was used for elution of protein with a flow rate of 30 mL/h. The ultraviolet absorbance of each fraction was measured at 280 nm. Fractions were assayed for protease activity. Protease active fractions were pooled and concentrated for further characterization.

Purity and the relative molecular mass of protease were estimated using discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking and 15% separating gel) (Laemmli, 1970), using High-Range Rainbow molecular weight markers (GE Healthcare, Hatfield, England) as the standard protein markers. Gelatin zymography staining was carried out as previously documented (Anbu, 2013). The relative molecular mass of the native enzyme was approximated using gel-permeable column chromatography as mentioned earlier. A gel filtration calibration kit (GE Healthcare, Hatfield, England) containing cytochrome C (MW = 12,400), carbonic anhydrase (MW = 29,000), albumin (MW = 66,000), alcohol dehydrogenase (MW = 150,000) and β-amylase (MW = 200,000) was used for markers.

Effect of pH on protease activity and stability

The effects of pH on the protease activity and stability were studied at 37 °C over a pH range of 3.0–12.0 with azocasein as a substrate. The buffer systems were sodium acetate (pH 3.0–6.0), potassium phosphate (pH 7.0–8.0), Tris-HCl (pH 7.0–9.0) and sodium carbonate (pH 9.0–12.0). For optimal pH determination, the reaction mixture was incubated at 37 °C for 15 min. The effect of pH on protease stability was studied using 50 mM buffer at the specific pH for 6 h at 37 °C. Aliquots were withdrawn and residual activities were determined under standard assay conditions.

Effect of temperature on protease activity and stability

The temperature effect on the purified protease activity was determined after incubation at specific temperatures (10–80 °C) for 15 min in 50 mM Tris-HCl buffer (pH 8.0). The thermo-stability

of the purified enzyme was time-course studied at different temperatures (60–80 °C) in a water bath and subsequently the residual activity was measured using the activity of unheated protease as the control (100% of relative activity).

Effect of organic solvents on the stability of protease

Purified protease was mixed with equal volumes of each of the selected organic solvents to prepare the 50% (volume per volume; v/v) organic solution, and mixtures were consequently shaken and incubated at 37 °C for 6 h at 150 rpm. The solvent in the mixture was partially eliminated using evaporation at 37 °C for 5 min before assay. The organic solvents used in this study were 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 $P_{o/w} = 5.6$), and hexadecane (log $P_{o/w} = 8.8$). The residual protease activity was compared to that of the control (without solvent).

Effects of metal ions and inhibitors on protease activity

The impacts of metal ions (Na^+, Ba^{2+}, Ca^{2+}, Mg^{2+} and Hg^{2+}) and inhibitors ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) on the enzyme activity were studied by incubating the purified protease for 48 h at 37 °C in 50 mM Tris-HCl buffer (pH 8.0) with the selected chemicals, after which the residual protease activity was measured. The enzyme activity in the absence of metallic ions or inhibitors was considered as 100%.

Effects of detergents, oxidizing agents and bleaching agents on protease activity

Purified protease was mixed with detergents (SDS, Tween 80 or Triton X-100), oxidizing agents (H_2O_2 and sodium perborate), or bleaching agents (sodium carbonate, sodium carboxymethyl cellulose, sodium pyrophosphate, sodium silicate, zeolite, sodium xylenesulfonate, sodium toluenesulfonate, sodium tripolyphosphate, sodium nitrotriacetate or sodium percarbonate) at 37 °C for 1 h and the residual enzyme activities estimated. The enzyme activity in the absence of chemicals was assigned as 100%.

Substrate specificity

The protease substrate specificity was examined toward substrates of casein, azocasien, egg albumin, gelatin, hemoglobin and BSA. The kinetic parameters were estimated using Lineweaver-Burk plots. Kinetic constants, maximal reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) values were determined at different substrate concentrations.

Results and discussion

Isolation and identification of protease-producing bacteria

Extracellular production of protease was identified in 12 isolates of bacteria from sediment samples. Of these, the one that showed the highest protease production (6.50 ± 0.03 U/mL) was selected as a candidate protease producer. The strain was a cocci-shaped, Gram-positive bacterium that reacted negatively with alkaline phosphatase. Positive results were recorded for catalase

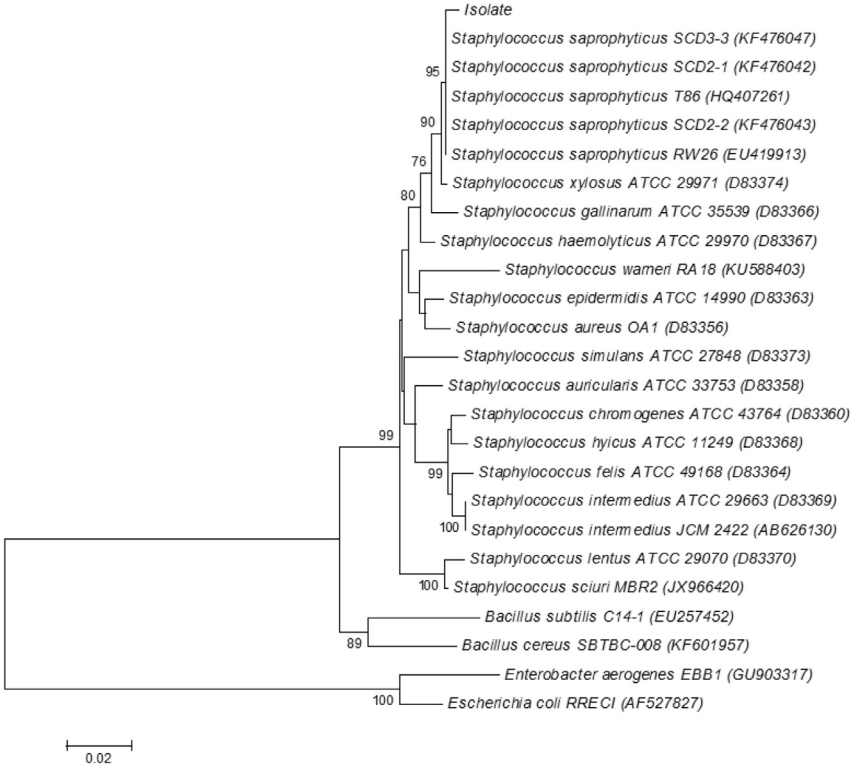

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the isolates and other related reference microorganisms. The value next to the branch is the estimated confidence limit (expressed as a percentage, with only values greater than 70% shown) for the position of the branch as determined by bootstrap analysis (1000 replications). Evolutionary distances were computed using the Kimura 2-parameter method and are presented in the units of the number of base substitutions per site.

Table 1
Summary of the purification of protease from, *Staphylococcus saprophyticus* BUU1.

Purification step	Total activity(U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	20,600.64	2.49	8273.35	1	100
Ammonium sulfate precipitation (70–80%)	2378.32	0.0043	553,097.67	66.85	11.55
Sephadex G-75	1446.99	0.0041	352,924.39	42.66	7.02

and urea, but results were negative for nitrate. Fermentation or oxidation of some substrates (D-glucose, D-fructose, D-maltose, D-trehalose, D-mannitol, D-saccharose) were detected but did not occur with others (D-mannose, D-lactose, xylitol, D-melibiose,D-raffinose, D-xylose). In addition, the strain could produce L-arginine and produced negative results with methyl-α-D-glucopyranoside and N-acetyl-glucosamine. According to its biochemical characteristics, the strain was classified (97.4% probability) as *Staphylococcus warneri* and confirmed using 16S rRNA gene sequencing.

The 16S rRNA sequence exhibited 99% similarity with the following *Staphylococcus saprophyticus* strains: *S. saprophyticus* ZK-3 (accession no. KM095954.1), *S. saprophyticus* SCD3-3 (accession no. KF476047.1), *S. saprophyticus* T86 (accession no. HQ407261.1), *S. saprophyticus* RW26 (accession no. EU419913.1), *S. saprophyticus* SCD2-1 (accession no. KF476042.1), *S. saprophyticus* ATCC15305 (accession no. NR115607.1) and *S. saprophyticus* SCD2-2 (accession no. KF476043.1). The same confidence level (99% identity) was found in the same genus and subspecies for *Staphylococcus* sp. KJ1-5-94 (accession no. KJ623596.1), *Staphylococcus* sp. WW60 (accession no. JQ687115.1), *S. saprophyticus* subsp. *saprophyticus* ATCC15305 (accession no. AP008934.1) and *Staphylococcus* sp. An35 (accession no. AJ551173.1). Biochemical characteristics suggested the strain was *S. warneri*. However, a phylogenetic tree using the neighbor-joining method (Fig. 1) suggested the strain was closer to *S. saprophyticus*, a common bacterium occurring in urine specimens (Hovelius and Mårdh, 1984). Despite the accuracy of the genotypic identification using the 16S rRNA gene sequence, this bacterium should be identified as *S. saprophyticus* BUU1.

Purification of Staphylococcus saprophyticus BUU1 protease

S. saprophyticus BUU1 protease was 42-fold purified starting from the culture supernatant and achieved apparent homogeneity

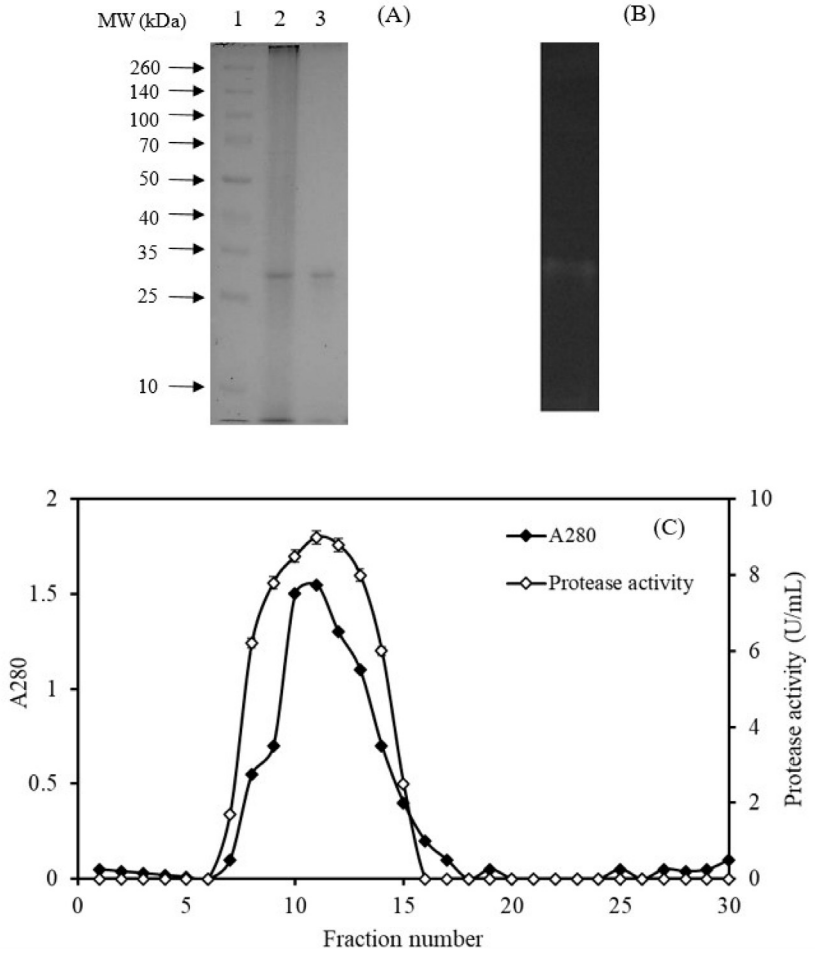

Fig. 2. (A) SDS-PAGE and (B) zymogram activity staining of the purified protease. Lane 1: standard protein markers (expressed in kDa); Lane 2: 70–80% ammonium sulfate precipitated and dialyzed sample; Lane 3: purified protease using Sephadex G-75; (C) Purification profile of the protease from *Staphylococcus saprophyticus* BUU1 using gel filtration on a Sephadex G-75 column. The 70–80% ammonium sulfate precipitates were applied to a 2.0 cm × 10 cm column, equilibrated and eluted with 50 mM Tris-HCl, pH 8.0. Fractions (1 mL each, flow rate = 30 ml/h) collected from the column were assayed for protein content at 280 nm and protease activity as described in the Materials and Methods section. Protease activity shown is the average of three enzyme assays with standard deviation (error bars).

on SDS-PAGE using 70–80% ammonium sulfate precipitation and gel filtration using Sephadex G-75 (Table 1). The specific activity of the final purified protease was approximately 353,000 U/mg proteins indicating almost 7% recovery. The lower yield might have been due to the removal of a substantial amount of the co-proteinaceous content from the protease mixture during precipitation. However, low yields (below 15%) have been previously documented (Arvidson et al., 1973). The decrease in the specific activity after Sephadex G-75 column chromatography may have been the result of either a technical problem or the loss of protease activity during purification. The molecular mass of *S. saprophyticus* BUU1 protease was estimated as 28 kDa using SDS-PAGE (Fig. 2A), corresponding to that obtained from zymogram activity (Fig. 2B) and gel filtration (Fig. 2C). This was similar to the proteases of *Staphylococcus* spp. For which the molecular masses generally were in the range 15–36 kDa, being a monomeric protease (Arvidson, 1973; Arvidson et al., 1973; Rydbén et al., 1974; Saheb, 1976; Takeuchi et al., 1999).

Effects of pH and temperature on protease activity and stability

S. saprophyticus BUU1 protease was highly active (>70% relative activity) over a wide pH range of 3.0–12.0 and exhibited maximum hydrolytic activity toward azocasein at pH 11.0 (Fig. 3A). Surprisingly, protease stability occurred also over the same pH range and maintained almost 90% of its initial activity even after 6 h incubation (Fig. 3B). It should be noted the enzyme's broad-pH activity differed from that of other bacterial proteases (Arvidson, 1973; Arvidson et al., 1973; Saheb, 1976; Takeuchi et al., 1999).

Purified protease of *S. saprophyticus* BUU1 could hydrolyze azocasein at all temperatures (10–80 °C; Fig. 4A). The optimal enzyme temperature was 30–60 °C and no substantial change in protease activity occurred to 80 °C. The enzyme retained more than 80% of its initial activity at 60 °C for 72 h, while more than 70% residual activity was detected at 48 h incubation at 70–80 °C (Fig. 4B). Previously, proteases from *Staphylococcus* sp. were reported to be stable below 40 °C and generally to have their optimal

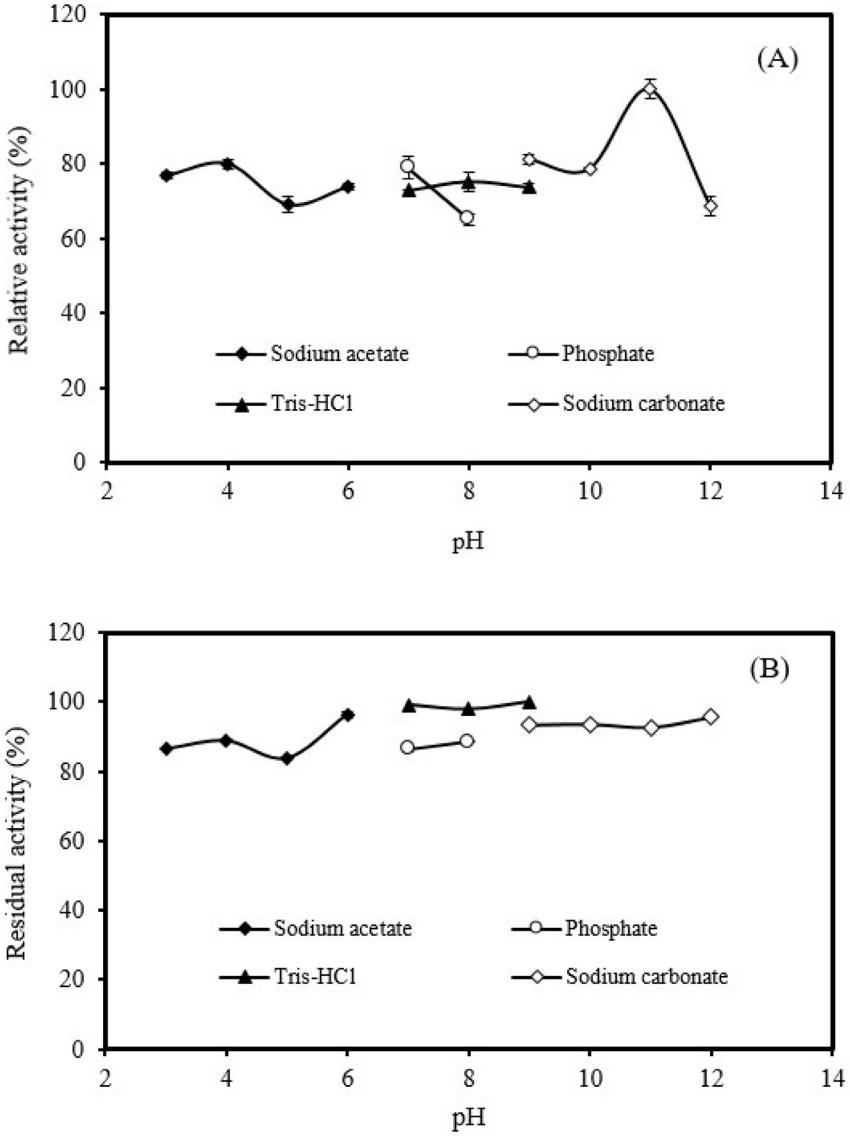

Fig. 3. Effect of pH on activity (A) and stability (B) of the purified *Staphylococcus saprophyticus* BUU1 protease. Activity was determined by incubating the reaction mixture in 50 mM buffer of a specific pH. Buffer systems used were sodium acetate (pH 3.0–6.0; closed diamonds), phosphate (pH 7.0–8.0; open circles), Tris-HCl (pH 7.0–9.0; closed triangles) and sodium carbonate (pH 9.0–12.0; open diamonds). pH stability was estimated from residual activities after 6 h incubation relative to the control (100% relative activity) at pH 9.0. Results are expressed as the mean of three determinations with standard deviation (error bars).

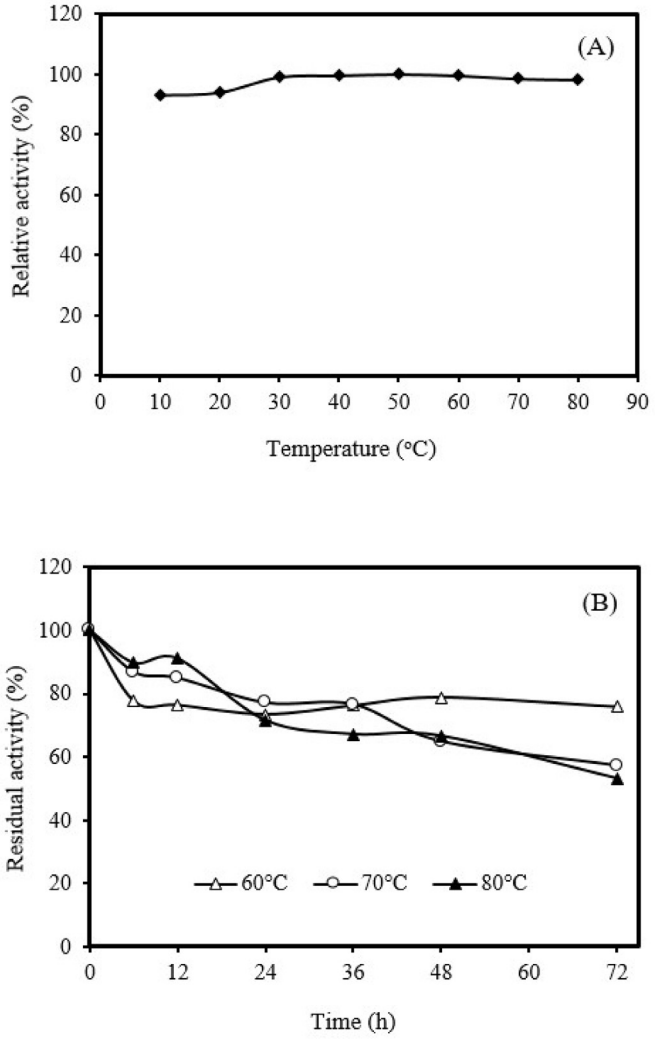

Fig. 4. Temperature effect on activity (A) and stability (B) of the purified *Staphylococcus saprophyticus* BUU1 protease. The temperature profile was determined by assaying the protease activity between 10 °C and 80 °C. Enzyme activity at 50 °C was taken as 100%. Protease thermal stability was measured by pre-incubating enzyme solution at different temperatures for 72 h at pH 8.0. Residual enzyme activities were estimated at 6 h, 12 h, 24 h, 36 h, 48 h and 72 h under standard conditions. The unheated enzyme was taken as 100%.

temperature in the range 30–50 °C (Drapeau et al., 1972; Shaw et al., 2005; Akram et al., 2014). Until now, there has been no report of fully stable *Staphylococcus* proteases over the pH and temperature ranges described in the present study (Arvidson, 1973; Arvidson et al., 1973; Shaw et al., 2005). Thus, the ability to work over broad pH and temperature ranges as well as the enzyme's stability make it particularly suitable for industrial use (Iyer and Ananthanarayan, 2008; Bhunia et al., 2013).

Effect of organic solvents on the stability of protease

The protease in this study retained almost 60% of its original activity in the presence of short-chain alcohols (methanol, ethanol and isopropanol) while activity was reduced by approximately 15% in isoamyl alcohol (Table 2). Hydrophobic solvents (benzene, hexanes, heptane, decane and hexadecane) did not significantly affect the protease activity. Perhaps the operational mechanism of these solvents is to hinder efficient interaction between enzymes and substrates (Laane et al., 1987). It is possible that hydrophilic solvents are able to dissolve proteases increasing inactivation (Sugihara et al., 1991). The solubility of proteases in azocasein and short-chain alcohols likely reduced the formation of a new liquid phase at moderate concentrations leading to enzyme inactivation (Shimada et al., 1999). Proteases are increasingly being used for peptide synthesis and pharmaceutical applications (Rao et al., 1998; Raval et al., 2014) that require reactions containing water-immiscible organic solvents. Thus, the stability of this protease in hydrophobic solvents makes the enzyme suitable for such applications as well.

Table 2
Effect of organic solvents on the activity of *Staphylococcus saprophyticus* BUU1 protease.

Organic solvent	log $P_{o/w}$	Residual activity (%)[a]
Control	–	100 ± 1.29
Methanol	−0.8	58 ± 1.06
Ethanol	−0.24	60 ± 1.15
Isopropanol	0.05	56 ± 0.64
Isoamyl alcohol	1.1	85 ± 1.62
Benzene	2.0	94 ± 1.10
Hexanes	3.6	93 ± 0.90
Heptane	4.0	95 ± 1.09
Decane	5.6	93 ± 1.19
Hexadecane	8.8	97 ± 0.48

[a] The remaining protease activities were measured after pre-incubation of enzymes with an equal volume of each organic solvent at 37 °C for 6 h. Enzyme activities measured in the absence of any solvent were taken as 100%. Results are expressed as average of three determinations ± standard deviation (SD).

Effect of metal ions and inhibitors on protease activity

Many bacterial proteases normally require a divalent cation or a combination of such cations for activity and stability. The protease activity of *S. saprophyticus* BUU1 was stimulated nearly 44% after 48 h incubation with Ca^{2+} (Table 3). Appreciable changes did not occur in the presence of Na^{+}, Ba^{2+} and Mg^{2+}. About 20% inhibition of protease activity occurred in the presence of Hg^{2+} after 48 h incubation. Calcium is well known to induce both proteolytic activity and thermal stability (Haddar et al., 2009; Deng et al., 2010) possibly by protecting enzymes against thermal denaturation and playing a vital role in maintaining their active conformation (Rao et al., 2009). Magnesium and sodium normally act as a salt or ion bridge conserving histidine in active sites and maintaining the enzyme molecule's rigid conformation (Brockerhoff and Jensen, 1974; Dharmsthiti et al., 1998; Kumar and Takagi, 1999; Dzubiella, 2008).

Table 3
Effect of different metal ions and inhibitors on purified protease activity.

Metal ions and inhibitors	Relative activity (%)[a]		
	6 h	24 h	48 h
Control	100 ± 4.01	100 ± 2.01	100 ± 0.02
Na^{+}	104 ± 0.70	100 ± 0.61	100 ± 0.08
Ba^{2+}	100 ± 2.27	95 ± 0.89	96 ± 0.65
Ca^{2+}	100 ± 0.93	114 ± 0.32	144 ± 0.17
Mg^{2+}	97 ± 1.19	93 ± 0.68	96 ± 1.36
Hg^{2+}	90 ± 0.03	86 ± 0.01	81 ± 0.83
EDTA	107 ± 0.14	94 ± 0.31	94 ± 0.42
DTT	94 ± 1.91	95 ± 0.43	98 ± 0.25
PMSF	100 ± 2.42	99 ± 0.81	92 ± 0.12

[a] Enzyme activity was determined by incubating the enzyme in the presence of various metal ions and inhibitors at 37 °C for different times. The activity is expressed as a percentage of the activity level in the absence of metal ions and inhibitors. Results are shown as the mean of triplicate determination ± SD.

The reducing, DTT, and chelating, EDTA, agents, respectively, had practically no influence on protease activity and stability. These indicate *S. saprophyticus* BUU1 protease was neither a cysteine-like protease nor a metalloprotease. No activity change was found after the addition of PMSF for 48 h. PMSF is known to sulfonate the essential serine residue in the active protease site resulting in activity loss (Kumar, 2002). Protease stability after the addition of PMSF may reflect resistance imparted by the serine hydrolase inhibitor (Dharmsthiti et al., 1998). It should be noted that protease is configured in such a way that it does not allow inhibitors to reach active site residues and affect its activity or that protease does not respond to these inhibitors (Najafi et al., 2006). Almost full protease activity in the presence of EDTA is particularly valuable for a detergent supplement especially for water softeners and stain removal formulae. This study suggested that *S. saprophyticus* BUU1 protease seems to be the only one reported to retain its stability in the presence of DTT, EDTA and PMSF.

Effects of detergents, oxidizing agents and bleaching agents on protease activity

The purified protease was highly stable in all detergents tested. Enzyme activity was not affected by a strong anionic surfactant (SDS) or by a non-ionic surfactant (Triton X-100) (Table 4). Stability towards SDS is considered rare among proteases (Choi et al., 2009). Tween 80 had little effect on the enzyme activity perhaps by changing the protease conformation or its interfacial properties. The enzyme was stable in the presence of sodium perborate, while hydrogen peroxide induced enzyme activity by 37%. Many available proteases have low activity and stability toward oxidants which are common ingredients in modern bleach-based detergents. This effect is related to the oxidization of the methionine residue present next to catalytic serine, preventing a critical step in the formation of tetrahedral intermediates during proteolysis (Siezen and Leunissen, 1997). Enhancement of the protease activity by H_2O_2 may result from further oxidization of cysteine, tyrosine or tryptophan residue next after methionine which tends to undergo oxidative inactivation (Siezen and Leunissen, 1997; Singh et al., 2012). Stability of *S. saprophyticus* BUU1 protease regarding SDS and oxidizing agents is an important feature for its eventual use in detergent formulations.

In the present study, the stability of the protease enzyme was reduced only slightly in the presence of bleach. Traces of sodium carbonate and sodium carboxymethyl cellulose only reduced the protease activity to 93% and 60%, respectively (Table 5). The enzyme retained half of its original activity in the presence of sodium carboxymethyl cellulose at 5–10% concentration. At slightly higher concentrations, little antagonistic effect on the protease activity was found in the presence of 10% (v/v) concentration of the bleaching agents sodium carbonate (93% relative protease activity), sodium percarbonate (91% relative protease activity), zeolite (87% relative protease activity), sodium silicate (84% relative protease activity), sodium pyrophosphate (83% relative protease activity), sodium tripolyphosphate (80% relative protease activity), sodium xylenesulfonate (77% relative protease activity) and sodium p-toluene sulfonate (75% relative protease activity), while sodium nitrotriacetate did not inhibit the enzyme activity. Surfactant stability makes *S. saprophyticus* BUU1 protease a good candidate biocatalyst for laundry applications (Rao et al., 1998; Raval et al., 2014).

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

Detergents and oxidizing agents	Relative activity (%)[a]
Control	100 ± 0.34
H_2O_2	137 ± 2.21
Sodium perborate	100 ± 0.07
SDS	100 ± 2.66
Triton X-100	102 ± 0.70
Tween 80	97 ± 0.30

[a] The purified protease was pre-incubated with various detergents and oxidizing agents (1% concentration) for 1 h at 37 °C and then the residual activity was triplicate determined and average ± SD reported. Enzyme activity measured in the absence of any additive was taken as 100%.

Table 5
Relative activity of purified protease at different concentrations of bleaching agents.

Bleaching agent	Relative activity (%)[a]		
	1%	5%	10%
Control	100 ± 0.34	100 ± 4.41	100 ± 0.21
Sodium nitrotriacetate	103 ± 0.27	107 ± 1.10	100 ± 0.24
Sodium carbonate	93 ± 0.09	90 ± 0.53	93 ± 1.48
Sodium carboxymethyl cellulose	60 ± 0.11	55 ± 0.03	52 ± 0.07
Sodium percarbonate	106 ± 0.07	105 ± 0.49	91 ± 0.17
Sodium pyrophosphate	109 ± 1.05	110 ± 0.68	83 ± 0.57
Sodium *p*-toluene sulfonate	107 ± 0.83	102 ± 0.15	75 ± 0.12
Sodium silicate	103 ± 1.67	92 ± 0.15	84 ± 0.58
Sodium tripolyphosphate	103 ± 0.64	94 ± 0.63	80 ± 0.37
Sodium xylenesulfonate	99 ± 1.57	96 ± 0.41	77 ± 0.02
Zeolite	108 ± 0.09	100 ± 0.80	87 ± 0.72

[a] The purified protease was incubated with different concentrations of bleaching agents for 1 h at 37 °C and the remaining activity was measured under standard conditions. The activity is expressed as a percentage of the activity level in the absence of additives. Results are indicated as the mean of three determinations with SD values.

Substrate specificity

The substrate specificity of *S. saprophyticus* BUU1 protease indicated it was most active towards casein. The protease had a K_m value of 0.83 mg/mL and a V_{max} value of 592.86 μmol/min (data not shown). In contrast, proteases of *Staphylococcus* spp. were reported to have no activity on casein (Choi et al., 2009; Jadhav et al., 2010; Park et al., 2011). The enzyme scored 40% relative activity with azocasein as substrate and approximately 20% on hemoglobin, BSA, gelatin and egg albumin.

Although proteases from *Staphylococcus* spp. are well-known, previous studies have not reported their activity or stability under multiple extreme conditions. Thus, the discovery of a new protease from *S. saprophyticus* BUU1 which shows chelator resistance, surfactant stability and broad ranges of pH- and temperature-active characteristics described in this study fills a missing part of this versatile *Staphylococcus* protease. Its unique properties make the enzyme especially attractive for a number of biotechnological applications.

Conflict of interest

The authors declare that there are no conflict of interests regarding the publication of this article.

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