Biocatalysis and Agricultural Biotechnology 19 (2019) 101111

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Cloning, expression, purification and characterization of a thermo- and surfactant-stable protease from *Thermomonospora curvata*

Duangjai Sittipol[a], Pijitra Saelao[a], Tassanee Lohnoo[b], Tassanee Lerksuthirat[b], Yothin Kumsang[b], Wanta Yingyong[b], Pongsak Khunrae[a], Triwit Rattanarojpong[a], Nujarin Jongruja[a,*]

[a] Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, Thailand
[b] Research Center, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Keywords:
Protease
Thermostability
Thermomonospora curvata
Alkaline serine protease
Surfactant

ABSTRACT

Protease is widely used in various industrial applications to hydrolyze the peptide bonds in protein molecules. The appropriate biochemical characteristics of the proteases for the industry, especially thermostability of the enzyme, are the key requirement for the potential applications. Therefore, we identified, expressed, purified and characterized the recombinant protease from thermophilic bacteria, *Thermomonospora curvata* (Tcsp). We identified the putative gene of serine protease from the genome of *T. curvata.* Tcsp which without signal peptide was expressed and purified from *E. coli* as a soluble form. Here, we report that Tcsp worked efficiently at 70 °C and pH 10. The K_m for azocasein was 0.94 mg/ml, and V_{max} was 605.04 unit/mg. We also show the thermal stability of Tcsp after heat treatment at various temperatures indicating that Tcsp is a thermostable protease. To elucidate whether Tcsp is still active after the pre-incubation with surfactants at high temperature, Tcsp was pre-incubated in the presence of nonionic, cationic, anionic and amphionic surfactants at high temperature to give insight into the applications in the detergent industry. Tcsp shows significant residual activity after the pre-incubation with surfactants at high temperature, especially after the pre-incubation with nonionic surfactants. The key point of this study is that the first protease gene from *T. curvata* was intracellularly expressed in *E. coli* and the protease was purified with time-saving strategies. The potential biochemical characteristics of Tcsp are shown for future industrial applications.

1. Introduction

The proteases from microorganisms are widely used for industrial applications, for example, leather, pharmaceuticals, protein processing, foods, diagnostic reagents, and peptide synthesis industries. (Kirk et al., 2002; Ward and Moo-Young, 1988; Zhou et al., 2018; Dayanandana et al., 2003; Cabeza et al., 1988; Samal et al., 1990). Because of the applications in various industries, protease production accounts for a huge proportion of overall industrial enzyme production (Kirk et al., 2002). Among the proteases in industry, serine protease is extensively applied in detergents as a cleaning additive to facilitate the degradation process of remaining protein stains and enhances the efficiency of the detergents. (Banerjee et al., 1999; Saeki et al., 2007; Fuhrmann et al., 2006). The serine protease may attack the substrate differently depending on the substrate specificity pocket. The hydrophobic specificity pocket may efficiently bind to the substrate with hydrophobic amino acids whereas the hydrophilic counterpart may tightly bind with the charged or hydrophilic amino acids (Mukherjee et al., 2008).

Several serine proteases used in industry are produced by *Bacillus* sp., for instance, *B. licheniformis* and *B. subtilis* (Chatterjee, 2015; Abdulrahman, and Yasser, 2004; Sellami et al., 2008; Chouyyok et al., 2005). The mentioned bacteria produce alkaline serine proteases with an optimum pH of 8–10, an optimum temperature at 40 °C - 50 °C and stability in the presence of a chelating agent (EDTA) and surfactant. However, an enzymatic activity of the serine protease from *Bacillus* sp. is significantly lost when the enzyme is treated at high temperatures for a long incubation period. Although the high stability of serine protease produced from *B. pumilus* MCAS8 and *Bacillus sp.* Ak.1 has been reported, the residual activity of the enzymes remains only approximately 50% after incubation at 60 °C - 70 °C. The crystal structure of the serine protease from *Bacillus* sp. Ak.1 suggests that there are specific sites observed which provide substantially enhanced thermostability (Smith et al., 1999). Therefore, the applications at a high temperature of some serine proteases from *Bacillus* sp. may be limited because of their

* Corresponding author. Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, 126 Pracha Uthit Rd, Bang Mod, Thung Khru, Bangkok 10140, Thailand.
E-mail address: nujarin.jon@kmutt.ac.th (N. Jongruja).

https://doi.org/10.1016/j.bcab.2019.101111
Received 30 November 2018; Received in revised form 21 March 2019; Accepted 22 March 2019
Available online 26 March 2019
1878-8181/

Abbreviations

Tcsp	*Thermomonospora curvata* serine protease
Blsp	*Bacillus lichenformis* serine protease
Tk-subtilisin	*Thermococcus kodakarensis* subtilisin
EDTA	Ethylenediaminetetraacetic acid
IPTG	Isopropylthiogalactoside
TE	Tris-Ethylenediaminetetraacetic acid
NaCl	Sodium chloride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Asp	Aspartate
His	Histidine
Ser	Serine
KDa	Kilo Dalton

thermal instability at high temperature after long incubation periods (Uyar and Baysal, 2004).

The thermostable proteases from the hyperthermophilic archaeon, *Thermococcus kodkarensis*, have been reported for their biochemical characteristics and structures (Pulido et al., 2007a, 2007b; Tanaka et al., 2008, 2009). The members of Thermococcus are naturally present in high-temperature environments and are therefore considered to play a major role in the ecology and metabolic activity under the high temperature of an ecosystem (Spaans et al., 2015). From the genome, the thermostable serine protease, Tk-subtilisin, has been extensively studied. The Tk-subtilisin is a highly thermostable protease at an optimum temperature and pH of 80 °C and pH 9.5, respectively. The crystal structure of Tk-subtilisin has shown seven calcium binding sites which the calcium binding site-1, site-2, and site-3 are suggested to have a role for the thermostability of the enzyme, especially the calcium binding site-1 (Pulido et al., 2006).

To apply proteases for detergent formulations, the resistance of proteases to surfactants has been investigated in several studies. The alkaline protease from *Bacillus* sp. HR-08 has been investigated for its stability against Triton X100 and has shown stability against the surfactant at room temperature (Moradian et al., 2009). The protease from *Staphylococcus saprophyticus* BUU1 has been studied for its stability in the presence of SDS at 37 °C for 1 h and has shown resistance to SDS. (Uttatree and Charoenpanich, 2018). The residual enzymatic activity of protease from *Bacillus licheniformis* A10 has been examined after pre-incubation with 1% SDS and 1% Triton X100 at 37 °C for 30 min. The remaining activities of the enzyme are 4% and 41% after the treatment with 15% SDS and 15% Tween 20, respectively (Yilmaz et al., 2016). The study of Tk-subtilisin has shown that the protease is highly stable in 0.1% and 1% of Triton X100 after incubation of the enzyme with the surfactants at 80 °C and 90 °C for 20 min (Hirata et al., 2013).

Thermomonospora curvata is a thermophilic actinomycete bacteria. It is isolated from municipal solid waste and can be grown under a wide range of high temperatures from 45 °C to 65 °C. The complete genome sequence of the microorganism has been reported as a member of the family *Thermomonosporaceae* (Chertkov et al., 2011). Some extracellular enzymes, amylase, cellulose, β-glucosidase, protease, and xylanase, from *T. curvata,* have been reported as secreted enzymes under cultivation of mineral salt minimal medium and induction by various concentrations of protein-extracted lucerne fibers at 53 °C (Janda et al., 2000; Collins et al., 1993). Under cultivation in specified conditions, the extracellular protease from *T. curvata* exhibited the highest protease activity within the growth profile at 2–4 days (Bernier et al., 1988). However, the identification of the putative protease gene and biochemical characteristics of the putative protease from *T. curvata* are yet to be fully understood.

In this report, we have accomplished the identification of the putative serine protease gene from *T. curvata* (Tcsp). The results of amino acid sequence alignment show the amino acid sequence of Tcsp in relation to the proteases from other organisms. The first report of identification, cloning, expression, purification and biochemical characterization of the protease from *T. curvata* is provided in this study. We also discuss the possible applications of Tcsp in industries, particularly the detergent industry.

2. Material and methods

2.1. Strains, culture conditions and genomic DNA extraction

Thermomonospora curvata Henssen ATCC 19,995 was obtained from the American Type Culture Collection. The bacteria were grown in a static condition in the recommended medium containing 2 mg of cobalt chloride, 1 g of yeast extract, 1 g of beef extract, 2 g of tryptone and 10 g of dextrin in 1 L of distilled water, pH 7.2 at 50 °C. The growth of bacterial cells was monitored by absorbance at 660 nm. The bacteria were separated from the media by filtration through Whatman qualitative filter paper No. 1. The bacteria were ground to a fine powder in the presence of liquid nitrogen. The ground powder of bacteria was transferred to a conical tube. 1–5 ml of 100 mM Tris-EDTA buffer pH 7.0 (TE buffer pH 7.0) and 2.5 mg of lysozyme was added to the bacteria. The incubation process was carried out at 30 °C for 60 min. Then, 40 µl of proteinase K and 10% SDS were added to the mixture. The incubation temperature was shifted to 56 °C. A gentle inverted mixing was performed every 30 min for 4 times before overnight incubation at 56 °C. DNA was extracted with phenol:chloroform (1:1). The clear mixture was then transferred to a fresh tube, and an equal volume of ice-cold isopropanol was used to precipitate the DNA. The DNA precipitant was added to 1 ml of 70% ethanol. The sample was centrifuged at 5000 g for 15 min, and the precipitant was added to 1 ml of 90% ethanol before being centrifuged again at 5000 g for 15 min. The DNA pellet was separated from the supernatant, and the pellet was air dried. The DNA was dissolved in 100 µl of TE buffer pH 7.0.

2.2. Bioinformatic analysis

The complete genome sequence of *Thermomonospora curvata* (**CP001738.1**) was investigated for genes encoding putative proteases. The protein encoded by the gene with the accession number of **ACY97035.1** was selected for further analysis. The coding sequence of the gene was from 1,657,273 to 1,658,493 in the genomic sequence of *Thermomonospora curvata*. Basic Local Alignment Search Tool (BLAST) was used to find the homolog of the gene (Altschul et al., 1990). The multiple sequence alignment was analyzed by Clustal Omega. The amino acid sequence of Tcsp was used for protein homology modeling and secondary structure prediction by SWISS-MODEL (Waterhouse et al., 2018).

2.3. Cloning and expression of Tcsp

The extracted genomic DNA of *T. curvata* was used as a template for protease gene amplification. The nucleotide sequence of the gene with the accession number of ACY97035.1 was used for primer design. The primer designing strategy was to use the gene ACY97035.1 from the whole genome sequence of *T. cuvata* and analyze the signal peptide by SignalP. The primers were designed for gene amplification without signal peptide. The primers for gene amplification by PCR were 5′-GGAATTCCATATGGCCCCGTCCGCCTCGGCC-3′ and 5′- GCGGATCC TCAGAAGG TGGAGACGTTGAGGAGCTTCTC -3′, in which the *Nde*I and *Bam*HI (Biorad) sites are underlined, respectively. The PCR mixture was performed under the following conditions: denaturation at 94 °C for

5 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The resultant DNA fragment was digested with *Nde*I and *Bam*HI and ligated into the *Nde*I-*Bam*HI sites of pET25b(+) (Novogen) to generate pET-Tcsp. All DNA oligomers for PCR were synthesized by Bioneer, Korea. The DNA sequences were confirmed by Bioneer, Korea. The *E. coli* BL21 (DE3) strain (ThermoFisher) was transformed with pET-Tcsp and grown at 37 °C. When the absorbance at 600 nm reached approximately 0.6, 1 mM isopropyl thio-β-D-galactoside (IPTG) was added to the culture medium. The cultivation continued at 37 °C for an additional 3 h. Cells were harvested by centrifugation at 5000*g* for 15 min and resuspended in 100 mM Tris HCl buffer pH 7.0. Cells were disrupted by sonication lysis and centrifuged at 11,000 *g* for 30 min.

2.4. Protein purification of Tcsp

Protein purification was performed using Bio-Rad NGC™ Chromatography Systems. The soluble Tcsp was collected, dialyzed against 100 mM Tris HCl pH 6.0 containing 20 mM NaCl, and applied to a pre-packed column (5 ml) of DEAE (Bio-Rad) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of NaCl from 0 to 1 M. The fractions containing the protein were collected, dialyzed using 3.5-KDa molecular cut-off dialysis bag against 100 mM Tris HCl pH 6.0 containing 20 mM NaCl, and loaded onto the Superdex 200 column equilibrated with the same buffer. The fractions containing the protein were collected and dialyzed against 10 mM Tris-HCl (pH 6.0). The purity of the protein was analyzed by SDS/PAGE on a 15% polyacrylamide gel (Smith, 1984) followed by staining with Coomassie Brilliant Blue. The protein concentration was determined by the method of Bradford (1976).

2.5. Enzymatic activity of Tcsp

The protease activity was determined using azocasein (Sigma) as a substrate. 30 µl of a suitable dilution of enzyme solution was added to 270 µl of azocasein (2%, wt/vol) in a reaction buffer (100 mM Glycine-NaOH pH 10) and the mixture was incubated for 30 min. The reaction was terminated by adding 150 µl of 15% (vol/wt) trichloroacetic acid and was left for 30 min on ice, followed by centrifugation at 15,000 × *g*, at 4 °C for 10 min. 160 µl of the supernatant was neutralized by adding 40 µl of 2 N NaOH, and the absorbance at 440 nm (A440) was measured using a spectrophotometer. One unit of protease activity was defined as the amount of enzyme that increased the A440 value of the assay reaction mixture by 0.1 in 1 min (Park et al., 2013). The specific activity was defined as the enzymatic activity per milligram of protein.

The effect of temperature on the proteolytic activity of the enzyme was determined at various temperatures ranging from 30 °C to 100 °C using azocasein as a substrate. The reaction mixture containing 30 µl of the purified enzyme was added to 270 µl of 100 mM Glycine-NaOH buffer (pH 10), 20 mM NaCl and 2% azocasein, and then incubated at various temperatures for 30 min. To experimentally compare the optimum temperature of Tcsp with that of protease from *B. licheniformis* (Sigma) (Abdulrahman and Yasser, 2004). The enzymatic activity of the protease from *B. licheniformis* was examined under its optimum condition as recommended in the product instruction at various temperatures ranging from 30 °C to 100 °C using azocasein as a substrate.

The effect of pH on the proteolytic activity of the enzyme was determined at various pHs ranging from pH 3.5 to pH 11 using azocasein as a substrate. The reaction mixture containing 30 µl of the purified enzyme was added to 270 µl of 100 mM buffer at the specified pH, 20 mM NaCl and 2% azocasein, and then incubated at 70 °C for 30 min.

The effect of NaCl on the proteolytic activity of the enzyme was determined at various concentrations of NaCl ranging from 0 M to 2M using azocasein as a substrate. The reaction mixture containing 30 µl of the purified enzyme was added to 270 µl of 100 mM Glycine-NaOH buffer (pH 10) in the presence of various concentration of NaCl and 2% azocasein and then incubated at 70 °C for 30 min.

2.6. Stability of Tcsp against high temperature and surfactants

The Tcsp was pre-incubated at specific high temperatures ranging from of 60 °C - 100 °C for 1 h. After treatment at the specified temperatures for 0, 10, 20, 30, 40, 50 and 60 min, the residual enzymatic activity of Tcsp was examined at the optimum condition of 100 mM Glycine-NaOH buffer (pH 10) at 70 °C for 30 min.

The Tcsp was pre-incubated with a variety of surfactants (Sigma) — three nonionic surfactants (Emulgen 147, Tween 20 and Triton X100), one anionic surfactant (SDS), and two cationic surfactants (Quartamin

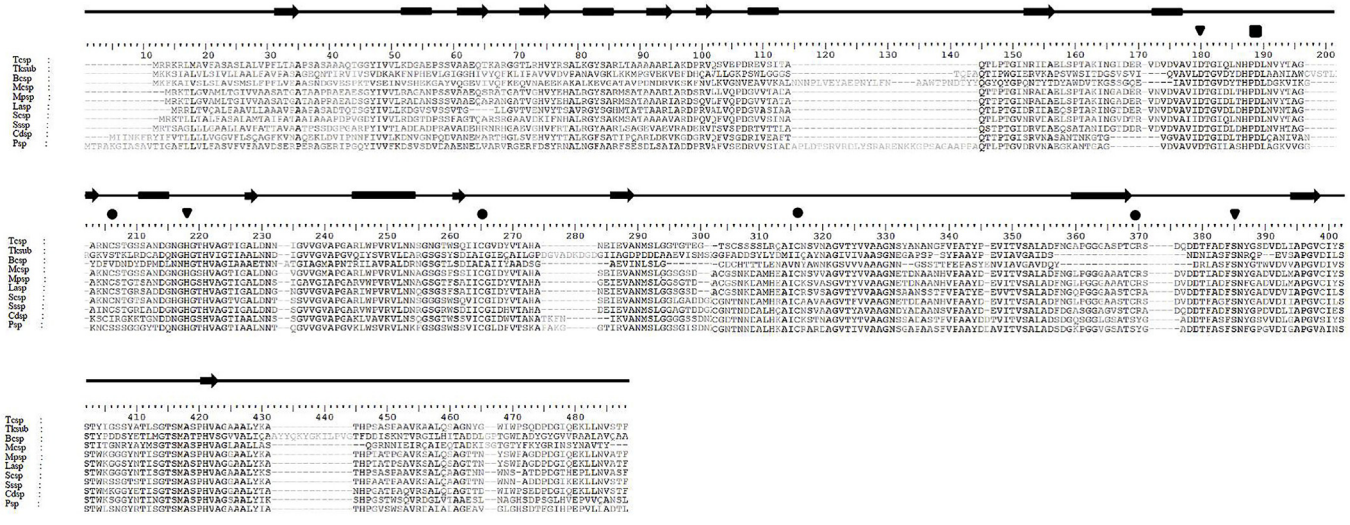

Fig. 1. Alignment of the amino acid sequences. The amino acid sequence of Tcsp is compared with those of representative proteases, Tksub: *Thermococcus kodakarensis* protease (**WP_011,250,640.1**), Bcsp: *Bacillus* sp. AK1 (**Q45670.1**), Mcsp: *Micromonospora chersina* protease (**WP_091,310,642.1**), Mpsp: *Micromonospora purpureochromogenes* peptidase (**WP_088,962,026.1**), Lasp: *Lechevalieria aerocolonigenes* protease (**WP_030,472,647.1**), Scsp: *Streptosporangium canum* protease (**WP_093,890,752.1**), Sssp: *Saccharomonospora saliphila* protease (**WP_019,816,301.1**), Cdsp: *Candidatus daviesbacteria* protease (**KKQ66970.1**) and Psp: Parcubacteria group protease (**KKW19509.1**). The ranges of secondary structures of the structural model of Tcsp are shown above the sequence of Tcsp. The filled rectangles and arrows represent alpha-helices and beta-sheets of the structural model of Tcsp, respectively. Gaps are denoted by dashes. The numbers represent the positions of amino acid residues relative to the initiator methionine of protease from Parcubacteria.

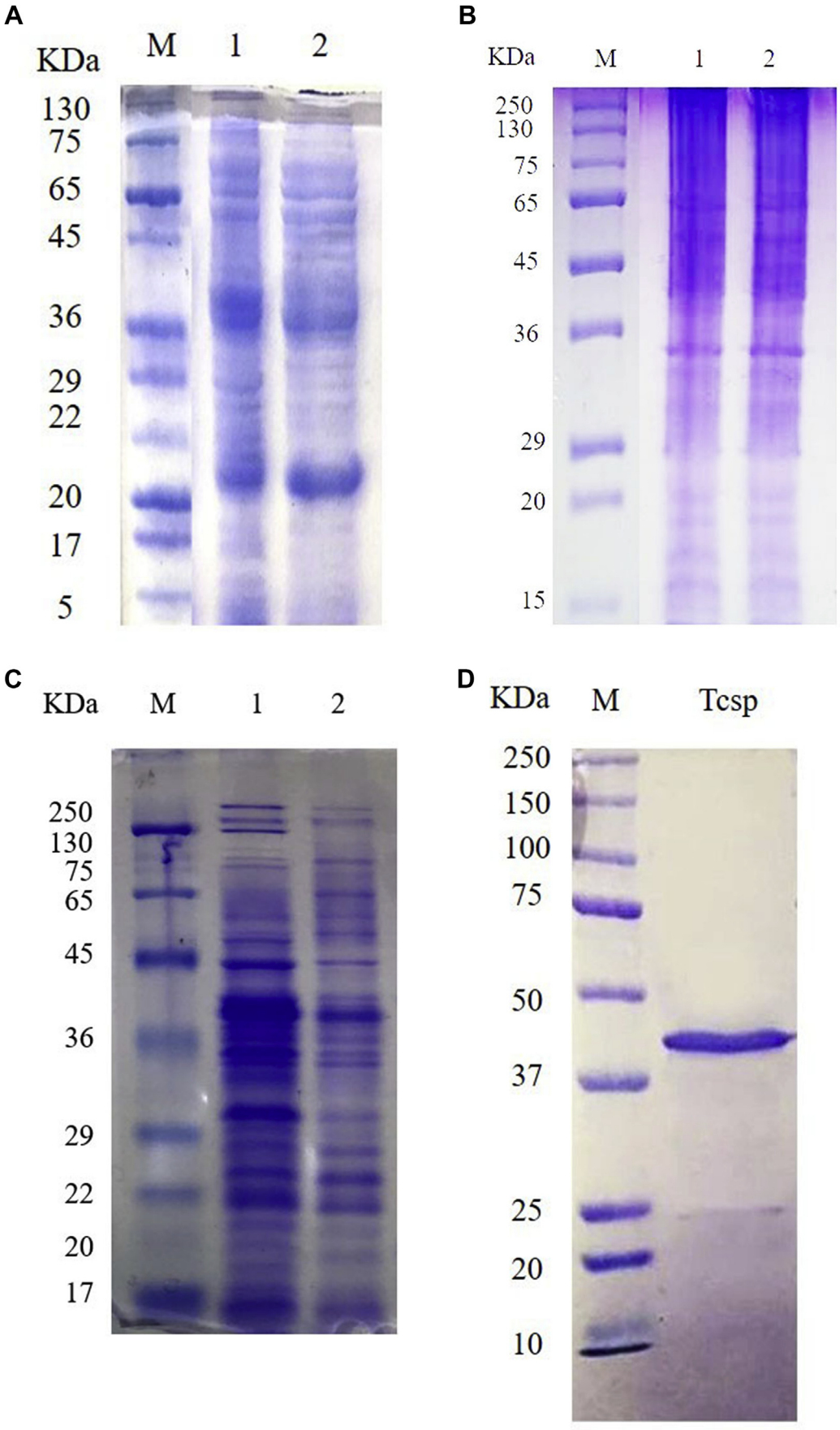

Fig. 2. SDS/PAGE of Tcsp. The Tcsp was subjected to electrophoresis as described in Materials and Methods and shown in Fig. 2A, B, 2C, and 2D. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. The proteins from a whole cell expressing Tcsp are shown in Fig. 2A. The protein samples after IPTG induction at 3 and 0 h are subjected in lane 1 and 2, respectively. The proteins from the cell pellet after 3 and 0 h of IPTG induction and sonication are shown in lane 1 and 2 of Fig. 2B, respectively. The proteins from the supernatant after 3 and 0 h of IPTG induction and sonication are shown in lane 1 and 2 of Fig. 2C, respectively. The purified Tcsp is shown in Fig. 2D. Lane M, a protein marker kit (Bio-Rad). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

60 W and Sensitol) — at various concentrations. The pre-incubation of the enzyme with the surfactants was performed at 75 °C for 0, 10, 20, 30, 40, 50 and 60 min. The residual enzymatic activity of Tcsp was examined at the optimum condition of 100 mM Glycine-NaOH buffer (pH 10) at 70 °C for 30 min.

3. Result

3.1. Amino acid sequence analysis

The gene of Tcsp consists of 406 amino acids. The amino acid sequence of Tcsp is compared with those of representative proteases. The amino acid sequence of Tcsp shows the amino acid sequence identities of 69.87% to Mcsp, 68.88% to Mpsp, 45.46% to Cdsp, 42.36% to Psp, 43.34% to Lasp, 42.91% to Sssp, 41.02% to Scsp, 23.44% to Bcsp and 23.15% to Tk-subtilisin. The ranges of secondary structures of the Tcsp model are shown above the sequence in the amino acid sequence alignment in Fig. 1. All active sites (Asp, His and Ser) are fully conserved in Tcsp and indicated by inverted triangles. The calcium-binding Asp is also conserved in Tcsp and other proteases shown by the closed square in Fig. 1. The novelty of Tcsp was assessed on the basis of the protease sequence showing relatively low amino acid sequence identities to the extensively characterized proteases.

3.2. Recombinant Tcsp production and purification

To characterize the enzymatic activities of Tcsp, the protease gene lacking a signal peptide (residues 24–406) was constructed. Tcsp was overproduced in *E. coli* BL21 (DE3) with 1 mM IPTG induction. The proteins from the whole cell of Tcsp were analyzed after 3-h induction as shown in Fig. 2A. After cell lysis by sonication, the cell pellet (Fig. 2B) and supernatant (Fig. 2C) were analyzed by SDS/PAGE. The Tcsp was overproduced in soluble form as shown in Fig. 2C. The protein band at approximately 40 kDa after 3-h IPTG induction in the whole cell and supernatant proteins has been more intensely observed than that observed after 0-h induction suggesting that the status of the expression is the overexpression with 1 mM IPTG induction. The distribution of the expressed gene product is also in the soluble fraction. The overproduced Tcsp was then purified to give a single band on SDS/PAGE as shown in Fig. 2D. The concentration of NaCl to elute out the recombinant Tcsp was approximately 650 mM. The molecular mass of the protein was estimated to be 40 kDa by gel filtration column chromatography. The estimated molecular weight from the experimental result corresponded with the theoretical molecular weight based on the amino acid sequence which is 38,994 suggesting that Tcsp exists as a monomer.

3.3. Enzymatic activity assay and thermal stability analysis

The dependencies of Tcsp activities on pH and temperature were analyzed. When the enzymatic activity was determined over the range pH 3.5–11, Tcsp exhibited the highest activity at around pH 10. It exhibited approximately 60% and 80% of maximum activity at pH 3–5.5 and pH 7–9, respectively, as indicated in Fig. 3.

The enzymatic activity of Tcsp was determined at various temperatures ranging from 30 °C–100 °C. The activity of commercial protease from *B. licheniformis* (Blsp) was examined at the same range of temperatures and compared with that of Tcsp as shown in Fig. 4. Tcsp and Blsp exhibited the highest activity at 70 °C and 60 °C, respectively. The enzymatic activity of Tcsp at 30 °C and 100 °C retained 78% of the highest activity of Tcsp at the optimum temperature, whereas the enzymatic activity of Blsp critically halted at 100 °C. These results indicate that the optimum temperature for enzymatic activity of Tcsp is higher than that of Blsp, and Tcsp is significantly more stable than Blsp at high temperatures.

The kinetic parameters of Tcsp were determined at 70 °C in the presence of 100 mM Glycine-NaOH buffer (pH 10) and 20 mM NaCl using azocasein as a substrate. The K_m was 0.94 mg/ml, and V_{max} was 605.04 unit/mg.

The stability of Tcsp was analyzed against heat inactivation by incubation of Tcsp in 100 mM Glycine-NaOH buffer (pH 10) and 20 mM NaCl at 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C. An aliquot was withdrawn after incubation at the specified temperature for 0, 10, 20, 30, 40, 50 and 60 min to analyze the residual activity at 70 °C using azocasein as a substrate. As indicated by the result in Fig. 5, Tcsp was stable for at least 1 h at 60 °C and 70 °C. The enzymatic activity was diminished approximately 17% by incubation at 80 °C for 1 h whereas the incubation at 90 °C and 100 °C for 1 h caused cessation of enzymatic activity of Tcsp by 27% and 32%, respectively. Therefore, Tcsp is a thermostable protease as suggested by the significant enzymatic activity remaining after heat incubation for 1 h.

The enzymatic activity of Tcsp in the presence of NaCl was analyzed by incubation of Tcsp in 100 mM Glycine-NaOH buffer (pH 10) in the presence of 0, 0.5, 1, 1.5, and 2 M NaCl at 70 °C. The enzymatic activity was analyzed at 70 °C using azocasein as a substrate. The result in Fig. 6 shows that Tcsp lost approximately 20% of enzymatic activity in the presence of 2 M NaCl, whereas significant loss of enzymatic activity of Tcsp in the presence of 0.1, 0.5, and 1 M NaCl was not observed. Therefore, Tcsp is a high-salt resistant protease enzyme.

To demonstrate the effectiveness of Tcsp for applications in the

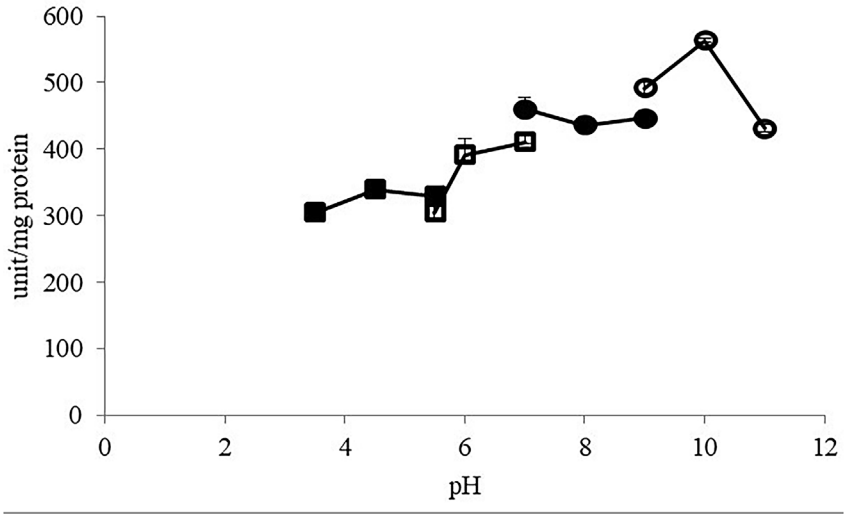

Fig. 3. pH dependency of Tcsp. The optimum pH analysis with a wide range of pH buffers; pH 3.5–5.5: sodium acetate buffer (closed square), 5.5–7: sodium phosphate buffer (open square), 7.0–9.0: Tris HCl buffer (closed circle) and pH 9.0–11.0: glycine-sodium hydroxide buffer (open circle). Each experiment was carried out three times, and the average values are shown together with the error bars.

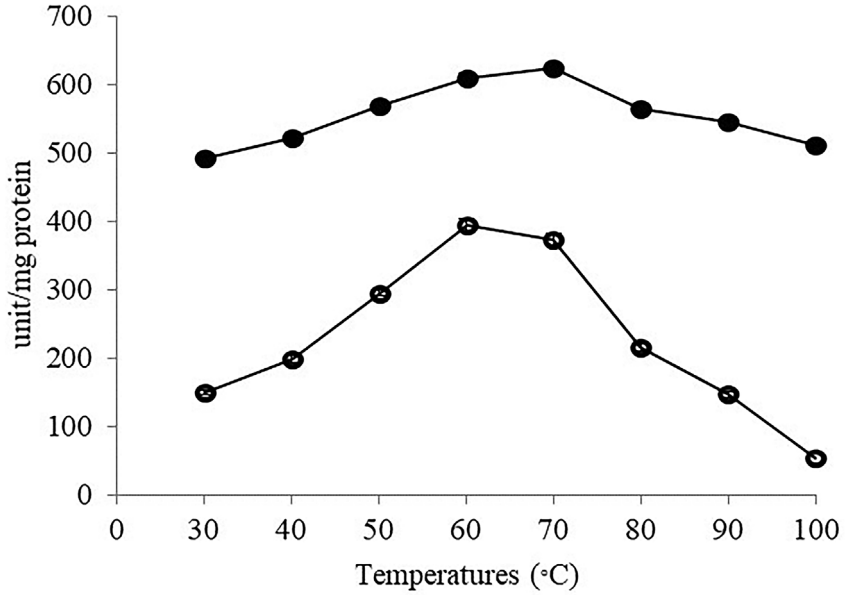

Fig. 4. Temperature dependencies of Tcsp (closed circle) and Blsp (open circle). The optimum temperature was obtained through a process of analysis using a wide range of temperatures. Each experiment was carried out three times, and the average values are shown together with the error bars.

detergent industry, the residual enzymatic activity of Tcsp was examined after pre-incubation of the enzyme in the presence of nonionic, cationic, anionic and amphionic surfactants at 75 °C for 1 h. The residual enzymatic activity of Tcsp after the pre-incubation of 0.1% surfactants for 1 h is shown in Fig. 7A. Tcsp retained at least 83% of enzymatic activity after 1 h pre-incubation with 0.1% nonionic surfactants, Tween20, Emulgen 147 and Triton X100 whereas Tcsp, after the pre-incubation of 0.1% cationic, anionic and amphionic surfactants, showed significant loss of its enzymatic activity as the residual activity retained only 31%–63%. The higher concentration of nonionic, cationic, anionic and amphionic surfactants, 1% surfactants, was applied and shown in Fig. 7B. The residual activity of Tcsp after the pre-incubation of 1% nonionic surfactants was retained at 76%, 76% and 43% for Tween 20, Emulgen 147 and Triton X100, respectively. The pre-incubation of 1% cationic, anionic and amphionic surfactants with Tcsp caused significant loss of the enzymatic activity. The remaining enzymatic activity of Tcsp after the incubation with 1% of SDS, Quatamin, Sensitol and Amphitol 24N for 1 h was 21%, 21%, 19%, and 1%, respectively. The residual enzymatic results after the surfactant and high-temperature treatments indicate that Tcsp is more stable against nonionic surfactants than cationic, anionic and amphionic surfactants. The residual enzymatic activity of Tcsp was, therefore, further examined after the pre-incubation of 10% nonionic surfactants with Tcsp at 75 °C for 1 h (Fig. 7C). After the incubation, Tcsp pre-incubated with Tween 20 showed 70% of the residual activity whereas it showed 58% and 17% of the residual activity after the incubation with 10% of Emulgen 147 and Triton X100, respectively, suggesting that among nonionic surfactants, Tcsp is more resistant to the presence of Tween 20 than Emulgen 147 and Triton X-100.

4. Discussion

Protease has been the focus of extensive studies due to its significant physiological functions and applications. The alkaline serine protease from *Bacillus mojavensis* A21 has been reported for its optimum temperature and pH at 60 °C and 8.5, respectively. The stability of the enzyme against Triton X100 has been shown as 73% after the treatment of maximal concentration of surfactants of 5% at 30 °C for 1 h (Haddar

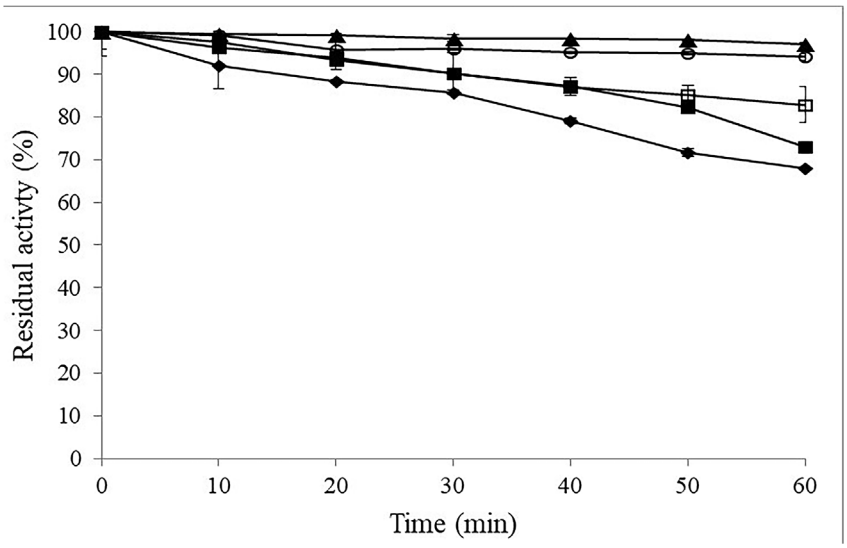

Fig. 5. Thermal stability against heat inactivation. Plots of residual activity versus incubation time are shown. Tcsp was incubated at 60 °C (closed triangle), 70 °C (open circle), 80 °C (open square), 90 °C (closed square) and 100 °C (closed diamond) in 100 mM Glycine-NaOH buffer (pH 10) and 20 mM NaCl. The aliquot of the solution was withdrawn after 10 min interval, and the residual activity was determined at 70 °C by using azocasein as a substrate. Each experiment was carried out three times, and the average values are shown together with the error bars.

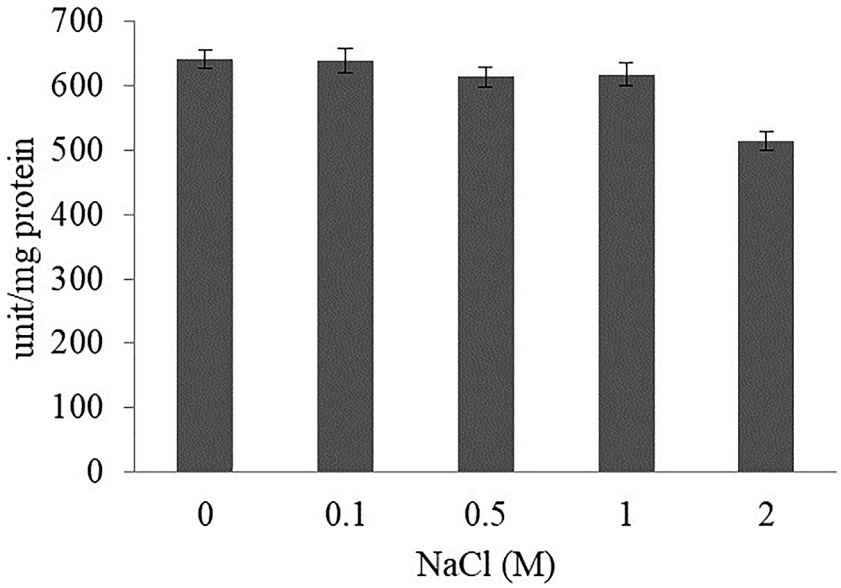

Fig. 6. Effect of NaCl on the enzymatic activity of Tcsp. The enzymatic activity of Tcsp was analyzed in the presence of 0, 0.1, 0.5, 1 and 2 M NaCl. Each experiment was carried out three times, and the average values are shown together with the error bars.

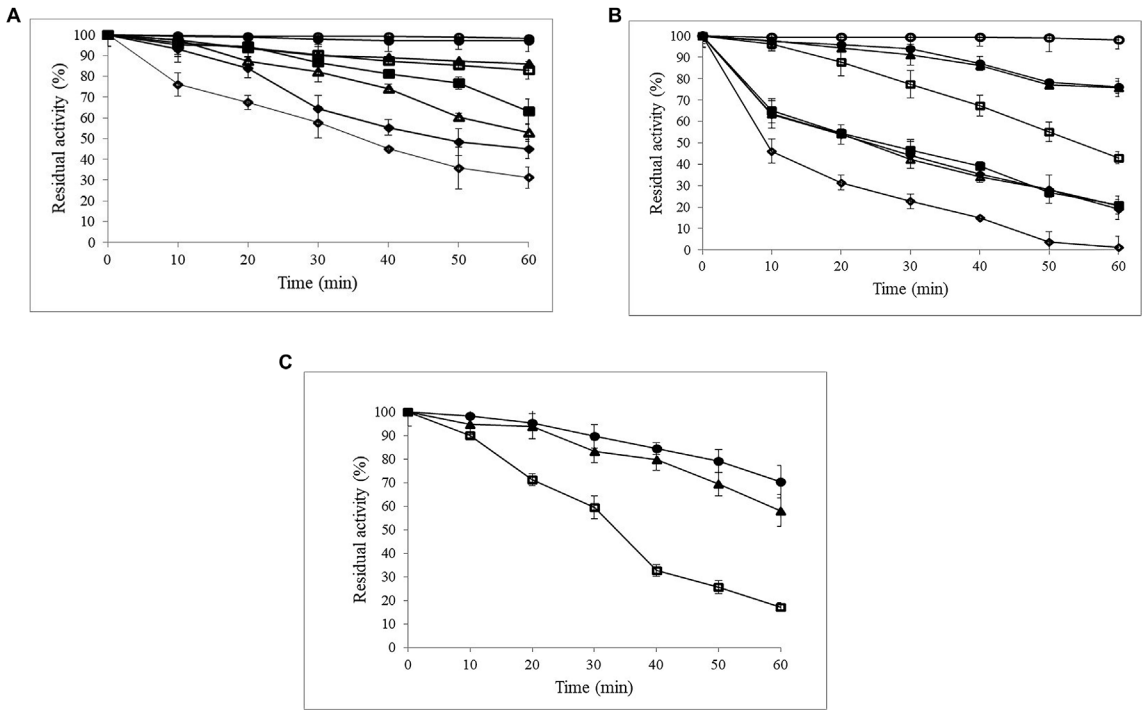

Fig. 7. Stability against surfactants. Plots of residual activity versus pre-incubation time are shown. (A) Tcsp was pre-incubated at 75 °C with 0.1% of Tween20 (closed circle), Emulgen 147 (closed triangle), Triton X100 (open square), SDS (filled square), Quartamine 60 W (open triangle), Sensitol (closed diamond) and Amphitol 24N (open diamond). Tcsp incubated with the absence of surfactant is shown (open circle). The residual enzymatic activity of Tcsp was examined at its optimum condition. (B) Tcsp was incubated at 75 °C with 1% of Tween20 (closed circle), Emulgen 147 (closed triangle), Triton X100 (open square), SDS (filled square), Quartamine 60 W (open triangle), Sensitol (closed diamond) and Amphitol 24N (open diamond). Tcsp incubated in the absence of surfactant is shown (open circle). (C) Tcsp was incubated at 75 °C with 10% of Tween20 (closed circle), Emulgen 147 (closed triangle), Triton X100 (open square).

et al., 2009). Biochemical characterization of an alkaline protease from *Bacillus psudofirmus* SVB1 shows that after 1% of SDS pre-treatment, the remaining activity of the enzyme is 71% (Sen et al., 2011). After pre-incubation with 20% Tween 80 at 40 °C for 1 h, the alkaline serine protease from *Serratia* sp. SYBC H has retained 76%–82% of its activity (Li et al., 2011). A further report for a serine protease from *Salimicrobium halophilum* has indicated residual activity of 78%–99% after 1-hr. pre-incubation of the enzyme with 20 mM SDS, Tween 80, and Triton X100 (Li and Yu, 2012). Although the studies of those enzymes demonstrate that the enzymes substantially retained their enzymatic activity after specified incubation periods with surfactants, the continuously examined residual activity of the enzyme during the pre-incubation with surfactants was first reported in this study. The retained enzymatic activity as a time-course analysis may be informative for further applications as the period of pre-incubation to maintain the expected enzymatic activity may be simply decided on the basis of

experimental data.

The presence of salt in the protein solution has been reported to have various effects on the protein stability depending on the protein surface charges (Lindman et al., 2006; Mao et al., 2007). The industrial applications of enzymes in the conditions containing high salt concentrations are possible when the enzymes reveal high activity under the previously discussed conditions. The enzymatic activity of Tcsp is relatively high especially at the concentrations of 0.5 M–1 M NaCl which resembles a similar range of salt concentration to sea water. The results from this study suggest that Tcsp could be a potential protease for applications within the aquaculture industry or the salt-fermenting process.

To control microorganisms and infections, especially in hospitals, it is commonly recommended by Centers for Disease Control and Prevention as the standard guideline for environmental infection control in health-care facilities (2017) to apply at least 71 °C heat for a minimum of 25 min for hot washing. The detergents comprising any enzymes should be stable in the mentioned condition. That means the enzymes added into the detergents should be thermostable enzymes exhibiting enzymatic activity in the presence of surfactants. Protease is one of the enzymes added to detergents to diminish protein stain or residuals (Yildirim et al., 2017; Thebti et al., 2016). The characterization of Tcsp against various surfactants indicates that the nonionic surfactants, unlike cationic, anionic and amphionic surfactants, have minimal effects on the enzymatic activity of Tcsp (Fig. 7). The minimized enzymatic activity of Tcsp after the pre-incubation of cationic, anionic and amphionic surfactants may be due to the interference of the ionic interactions of the ionic surfactants with the charged surface of the enzyme molecule by strongly interacting with the charged amino acids usually located at the surface of the protein. When the protein molecules interact with the molecules of ionic surfactants, the molecule of the surfactant electrostatically binds to the oppositely charged residues of the protein molecules and causes the denaturation of the enzyme due to the instability of the protein structure (Schor et al., 2016). The addition of nonionic surfactants to the protein solution causes hydrophobic interactions or hydrogen bonds between the molecules of surfactants and uncharged amino acids. The hydrophobic interactions and hydrogen bonds may not strongly influence the conformational change of the protein. The structure of the protein is, therefore, more likely to be retained in the presence of nonionic surfactants than in the presence of ionic surfactants (Jelinska et al., 2017). According to the results shown in Fig. 7, the presence of 0.1% of nonionic and ionic surfactants does not completely destroy the enzymatic activity of Tcsp suggesting that the low concentration of surfactants could feasibly be applied to the enzymatic reaction or detergents containing the enzyme. However, only nonionic surfactants should be used when high concentrations of surfactants are required as an additive in the detergents or protein solution as the enzymatic activity of Tcsp after pre-incubation of 1% or 10% ionic surfactant is dramatically lost.

This is the first report that specifically discusses the identification, cloning, expression, purification and characterization of a recombinant protease from *T. curvata*. Although there is another report on the production and biochemical characterization of recombinant serine protease from *Thermomonospora fusca*, the recombinant serine protease from the previous study extracellularly produced in *Pichia pastoris* required a six-day period of protein overproduction (Kim and Lei., 2005). For the reasons mentioned above, this study set out specifically to demonstrate the intracellular expression of serine protease from *T. curvata* in an *E. coli* system and shows the biochemical characteristics of the soluble active protease including its possible industrial applications. The recombinant protease from *T. curvata* in this study has been successfully expressed in an *E. coli* system and purified with time-saving protocols. The thermostability and surfactant resistance of this alkaline protease indicate the potential of this enzyme for detergent formulation.

5. Conclusions

This is the first report to demonstrate that the putative protease gene of protease from *T. curvata* could be intracellularly expressed in a soluble and active form as a recombinant protease in an *E. coli* system. The purified protease exhibited thermal stability and retained high enzymatic activity after heat treatment. The high enzymatic activity in alkaline pH and the presence of a high concentration of NaCl is also the biochemical characteristics of the recombinant protease identified within this study. The kinetic parameters of the enzyme have been reported. The residual enzymatic activity of the purified enzyme after incubation with surfactants at high temperature has also been determined. The protease has been particularly resistant to nonionic surfactants. This report demonstrates a newly identified thermostable protease with highly promising characteristics for industrial applications.

Declaration of interest

- Thai patent application (pending)
- Research funding from Faculty of Science, King Mongkut's University of Technology Thonburi, KMUTT Research Fund and Petch Pra Jom Klao-Ph.D. scholarship.

Acknowledgments

This work was supported by a research fund from the Faculty of Science, King Mongkut's University of Technology Thonburi, KMUTT Research Fund and Petch Pra Jom Klao-Ph.D. scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101111.

References

Abdulrahman, A.S.M., Yasser, M.S., 2004. Production and some properties of protease produced by *Bacillus licheniformis* isolated from Tihamet Aseer Saudi Arabic Pakistan. J. Biol. Sci. 7, 1631–1635. http://doi.org/10.3923/pjbs.2004.1631.1635.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Banerjee, U.C., Rajesh, K.S., Sani, R.K., Azmi, W., Sani, R., 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochem. 35, 213–219. https://doi.org/10.1016/S0032-9592(99)00053-9.

Bernier, R., Kopp, M., Trakas, B., Stutzenberger, F., 1988. Production of extracellular enzymes by *Thermomonospora curvata* during growth on protein-extracted Lucerne fiber. J. Appl. Bacteriol. 65, 411–418. https://doi.org/10.1111/j.1365-2672.1988.tb01910.x.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding (PDF). Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Cabeza, L.F., Taylor, M.M., DiMaio, G.L., Brown, E.M., Mammer, W.N., Carrio, R., Celma, P.J., Cot, J., 1988. Processing of leather waste: pilot scale studies on chrome shavings. Isolation of potentially valuable protein products and chromium. Waste Manag. 18, 211–218. https://doi.org/10.1016/S0956-053X(98)00032-4.

Centers for disease control and prevention (CDC) Atlanta, 2017. Guidelines for Environmental Infection Control in Health-Care Facilities. https://www.cdc.gov/infectioncontrol/pdf/guidelines/environmental-guidelines.pdf, Accessed date: 7 March 2019.

Chatterjee, S., 2015. Production and estimation of alkaline protease by immobilized *Bacillus licheniformis* isolated from poultry farm soil of 24 Parganas and its reusability. Adv Pharm Technol Res 6, 2–6. https://doi.org/10.4103/2231-4040.150361.

Chertkov, O., Sikorski, J., Nolan, M., Lapidus, A., Lucas, S., Del Rio, T.G., Tice, H., Cheng, J.F., Goodwin, L., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Mikhailova, N., Ovchinnikova, G., Pati, A., Chen, A., Palaniappan, K., Olivier, D., Djao, N., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Brettin, T., Han, C., Detter, J.C., Rohde, M., Goker, M., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Hans-Peter Klenk, H.P., Kyrpides, N.C., 2011. Complete genome sequence of *Thermomonospora curvata* type strain (B9T). Std. Gen Sci. 4, 13–22. http://doi.org/10.4056/sigs.1453580.

Chouyyok, W., Wongmongkol, N., Siwarungson, N., Prichnont, S., 2005. Extraction of alkaline protease using an aqueous two-phase system from cell-free *Bacillus subtilis*

TISTR 25 fermentation broth. Process Biochem. 40, 3514–3518. https://doi.org/10.1016/j.procbio.2005.03.052.
Collins, B.S., Kelly, C.T., Fogarty, W.M., Doyle, E.M., 1993. The high maltose-producing alpha-amylase of the thermophilic actinomycete, *Thermomonospora curvata*. Appl. Micro.Biot. 39, 31–35. http://doi.org/10.1007/BF00166844.
Dayanandana, A., Kanagarajb, J., Sounderraja, L., Govindarajua, R., Suseela Rajkumar, G., 2003. Application of an alkaline protease in leather processing: an ecofriendly approach. J. Clean. Prod. 11, 533–536. https://doi.org/10.1016/S0959-6526(02)00056-2.
Fuhrmann, C.N., Daugherty, M.D., Agard, D.A., 2006. Subangstrom crystallography reveals that short ionic hydrogen bonds, and not a His-Asp low-barrier hydrogen bond, stabilize the transition state in serine protease catalysis. J. Am. Chem. Soc. 128, 9086–9102. http://doi.org/10.1021/ja057721o.
Haddar, A., Bougatef, A., Agrebi, R., Sellami-Kamoun, A., Nasri, M., 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. Process Biochem. 44, 29–35. https://doi.org/10.1016/j.procbio.2008.09.003.
Hirata, A., Hori, Y., Koga, Y., Okada, J., Sakudo, A., Ikuta, K., Kanaya, S., Takano, K., 2013. Enzymatic activity of a subtilisin homolog, Tk-SP, from *Thermococcus kodakarensis* in detergents and its ability to degrade the abnormal prion protein. BMC Biotechnol. 28, 13–19. https://doi.org/10.1186/1472-6750-13-19.
Janda, L., Damborsky, J., Petricek, M., Spizek, J., Tichy, P.J., 2000. Molecular characterization of the *Thermomonospora curvata* aglA gene encoding a thermotolerant alpha-1, 4-glucosidase. Appl. Microbiol. 88, 773–783. https://doi.org/10.1046/j.1365-2672.2000.01019.x.
Jelinska, A., Zagozdzon, A., Gorecki, M., Wisniewska, A., Frelek, J., Holyst1, R., 2017. Denaturation of proteins by surfactants studied by the Taylor dispersion analysis. PLoS One 12, e.0175838. http://doi.org/10.1371/journal.pone.0175838.
Kim, T., Lei, X.G., 2005. Expression and characterization of a thermostable serine protease (TfpA) from *Thermomonospora fusca* YX in *Pichia pastoris*. Appl. Micro.Biot. 68, 355–359. http://doi.org/10.1007/s00253-005-1911-8.
Kirk, O., Borchert, T.V., Fuglsang, C.C., 2002. Industrial enzyme applications. Curr. Opin. Biotechnol. 13, 345–351. https://doi.org/10.1016/S0958-1669(02)00328-2.
Li, G., Cai, G., Liao, X., Yin, J., 2011. A novel nonionic surfactant- and solvent-stable alkaline serine protease from *Serratia* sp. SYBC H with duckweed as nitrogen source production, purification, characteristics, and application. J. Ind. Microbiol. Biotechnol. 38, 845–853. http://doi.org/10.1007/s10295-010-0855-x.
Li, X., Yu, H.Y., 2012. Purification and characterization of novel organic-solvent tolerant b-amylase and serine protease from a newly isolated *Salimicrobium halophilum* strain LY20. FEMS Microbiol. Lett. 329, 204–211. https://doi.org/10.1111/j.1574-6968.2012.02522.x.
Lindman, S., Xue, W.F., Szczepankiewicz, O., Bauer, M.C., Nilsson, H., Linse, S., 2006. Salting the charged surface: pH and salt dependence of protein G B1 stability. Biophys. J. 90, 2911–2921. http://doi.org/10.1529/biophysj.105.071050.
Mao, Y.J., Sheng, X.R., Pan, X.M., 2007. The effects of NaCl concentration and pH on the stability of hyperthermophilic protein Ssh10b. BMC Biochem. 8, 28–35. https://doi.org/10.1186/1471-2091-8-28.
Moradian, F., Khajeh, K., Naderi-Manesh, H., Sadeghizadeh, M., 2009. Isolation, purification, and characterization of a surfactants-, laundry detergents- and organic solvents-resistant alkaline protease from *Bacillus* sp. HR-08. Appl. Biochem. Biotechnol. 159, 33–45. http://doi.org/10.1007%2Fs12010-008-8402-1.
Mukherjee, A.K., Adhikari, H., Rai, S.K., 2008. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using Imperata cylindrical grass and potato peel as a low-cost medium: characterization and application of enzyme in detergent formulation. Biochem. Eng. J. 39, 353–361. https://doi.org/10.1016/j.bej.2007.09.017.
Park, M.H., Walpola, B.C., Ho Yoon, M., 2013. Purification and characterization of protease enzyme from *Burkholderia stabilis*. Afr. J. Biotechnol. 12, 1408–1418. https://doi.org/10.5897/AJB12.1574.
Pulido, M.A., Koga, Y., Takano, K., Kanaya, S., 2007a. Directed evolution of Tk-subtilisin from a hyperthermophilic archaeon: identification of a single amino acid substitution responsible for low-temperature adaptation. Protein Eng. Des. Sel. 20, 143–153. https://doi.org/10.1093/protein/gzm006.
Pulido, M., Saito, K., Tanaka, S., Koga, Y., Morikawa, M., Takano, K., Kanaya, S., 2006. Ca^{2+}-dependent maturation of subtilisin from a hyperthermophilic archaeon, *Thermococcus kodakaraensis*: the propeptide is a potent inhibitor of the mature domain but is not required for its folding. Appl. Environ. Microbiol. 72, 4154–4162. http://doi.org/10.1128/AEM.02696-05.
Pulido, M.A., Tanaka, S., Sringiew, C., You, D.J., Matsumura, H., Koga, Y., Takano, K., Kanaya, S., 2007b. Requirement of left-handed Glycine residue for high stability of the Tk-subtilisin propeptide as revealed by mutational and crystallographic analyses. J. Mol. Biol. 374, 1359–1373. https://doi.org/10.1016/j.jmb.2007.10.030.
Saeki, K., Ozaki, K., Kobayashi, T., 2007. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J. Biosci. Bioeng. 103, 501–508. https://doi.org/10.1263/jbb.103.501.
Samal, B.B., Karan, B., Stabinsky, Y., 1990. Stability of two novel serine proteinases in commercial laundry detergent formulations. Biotechnol. Bioeng. 35, 650–652. https://doi.org/10.1002/bit.260350611.
Schor, M., Reid, J.L., MacPhee, C.E., Stanley-Wall, N.R., 2016. The diverse structures and functions of surfactant proteins. Trends Biochem. Sci. 41, 610–620. http://doi.org/10.1016/j.tibs.2016.04.009.
Sellami, K.A., Haddar, A., Ali, N.E., Ghorbel-Frikha, B., Kanoun, S., Nasri, M., 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiol. Res. 163, 299–306. https://doi.org/10.1016/j.micres.2006.06.001.
Sen, S., Venkata Dasu, V., Dutta, K., Mandal, B., 2011. Characterization of a novel surfactant and organic solvent stable high-alkaline protease from new *Bacillus pseudofirmus* SVB1. Res. J. Microbiol. 6, 769–783. http://doi.org/10.3923/jm.2011.769.783.
Smith, B.J., 1984. SDS polyacrylamide gel electrophoresis of proteins. Nucleic Acids Res. 1, 41–56. http://doi.org/10.1385/0-89603-062-8:41.
Smith, C.A., Toogood, H.S., Baker, H.M., Daniel, R.M., Baker, E.N., 1999. Calcium-mediated thermostability in the subtilisin superfamily: the crystal structure of *Bacillus* Ak.1 protease at 1.8 Å resolution. J. Mol. Biol. 294, 1027–1040. https://doi.org/10.1006/jmbi.1999.3291.
Spaans, S.K., Oost, J., Kengen, S., 2015. The chromosome copy number of the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1. Extremophiles 19, 741–750. http://doi.org/10.1007/s00792-015-0750-5.
Tanaka, S., Matsumura, H., Koga, Y., Takano, K., Kanaya, S., 2009. Identification of the interactions critical for propeptide-catalyzed folding of Tk-Subtilisin. J. Mol. Biol. 394, 306–319. https://doi.org/10.1016/j.jmb.2009.09.028.
Tanaka, S., Takeuchi, Y., Matsumura, H., Koga, Y., Takano, K., Kanaya, S., 2008. Crystal structure of Tk-subtilisin folded without propeptide: requirement of propeptide for acceleration of folding. FEBS Lett. 582, 3875–3878. https://doi.org/10.1016/j.febslet.2008.10.025.
Thebti, W., Riahi, Y., Belhadj, O., 2016. Purification and characterization of a new thermostable, haloalkaline, solvent stable, and detergent compatible serine protease from *Geobacillus toebii* strain LBT 77. Biom. Res. Int. 9, 9178–9262. http://doi.org/10.1155/2016/9178962.
Uttatree, S., Charoenpanich, J., 2018. Purification and characterization of a harsh conditions-resistant protease from a new strain of *Staphylococcus saprophyticus*. Agri. and Natural Res. 52, 16–23. https://doi.org/10.1016/j.anres.2018.05.001.
Uyar, F., Baysal, Z., 2004. Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Process Biochem. 39, 1893–1898. https://doi.org/10.1016/j.procbio.2003.09.016.
Ward, O.P., Moo-Young, M., 1988. Thermostable enzymes. Biotechnol. Adv. 6, 39–69. https://doi.org/10.1016/0734-9750(88)90573-3.
Waterhouse, A., Bertoni, A., Bienert, M., Studer, S., Tauriello, G., Gumienny, G., Heer, De Beer, R.F.T., Rempfer, T.A.P., Bordoli, C., Lepore, L., Schwede, R., 2018. SWISS-MODEL: homology modeling of protein structures and complexes. Nucleic Acids Res. 46, 296–303. https://doi.org/10.1093/nar/gky427.
Yildirim, V., Baltaci, M., Ozgencli, I., Sisecioglu, M., Adiguzel, A., Adiguzel, G., 2017. Purification and biochemical characterization of a novel thermostable serine alkaline protease from *Aeribacillus pallidus* C10: a potential additive for detergents. J. Enzym. Inhib. Med. Chem. 32, 468–477. HYPERLINK. https://doi.org/10.1080/14756366.2016.1261131.
Yilmaz, B., Baltaci, O., Sisecioglu, M., Adiguzel, A., 2016. Thermotolerant alkaline protease enzyme from *Bacillus licheniformis* A10: purification, characterization, effects of surfactants and organic solvents. J. Enzym. Inhib. Med. Chem. 31, 1241–1247. https://doi.org/10.3109/14756366.2015.1118687.
Zhou, C., Qin, H., Chen, X., Zhang, Y., Xue, Y., Ma, Y., 2018. A novel alkaline protease from *alkaliphilic Idiomarina* sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry. Sci. Rep. 8, 16467–16493. https://doi.org/10.1038/s41598-018-34416-5.