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Purification and Characterization of a Small Thermostable Protease from *Streptomyces* sp. CNXK100

TAN VIET PHAM[2*], TRUONG CHINH HUA[1], NGOC AN NGUYEN[2], and HANH THI DIEU NGUYEN[2]

[1] Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam
[2] Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam

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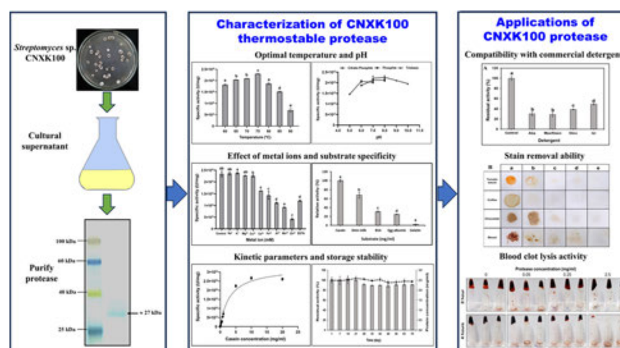
Abstract

Proteases derived from *Streptomyces* demonstrate numerous commendable properties, rendering it extensively applicable in biotechnology and various industrial sectors. This study focused on the purification and characterization of the thermostable protease obtained from *Streptomyces* sp. CNXK100. The purified protease exhibited an estimated molecular weight of 27 kDa, with optimal activity at 75°C and pH 8.0. Notably, the enzyme remained active even without any metal ions and fully active in the presence of Na^+, K^+, Mg^{2+}, and Cu^{2+}metal ions. The kinetic parameters were determined with a K_M value of 3.13 mg/ml and a V_{max} value of 3.28×10^6 U/mg. Furthermore, the protease has demonstrated notable stability when subjected to a treatment temperature of up to 65°C for 60 minutes, and across a broad pH range extending from 5.0 to 10.0. This protease also demonstrated resilience against a spectrum of harsh conditions, including exposure to organic solvents, surfactants, bleaching agents, and proteolytic enzymes. Additionally, the enzyme maintained its activity following treatment with commercial detergents, accomplishing complete thrombus lysis at a concentration of 2.50 mg/ml within 4 hours. Remarkably, the protease exhibited stability in terms of activity and protein concentration for 70 days at 4°C. These findings underscore the potential industrial applications of the thermostable protease from *Streptomyces* sp. CNXK100.



K e y w o r d s: *Actinomycetes*, characterization, *Streptomyces*, purification, thermostable protease

Introduction

Protease is a multifunctional enzyme that breaks down protein through the hydrolysis of peptide bonds, accounting for 60–65% of the world enzyme market (Mostafa et al. 2019; Mechri et al. 2022). It is widely used in many fields, such as detergents, diagnostics, tanning, therapeutics, silver recovery, waste treatment, and peptide synthesis. In recent years, microbial-derived proteases have been increasingly studied due to their specificity and friendly environment (Sharma et al. 2019; Šnajder et al. 2019; Parthasarathy and Gnanadoss 2020). Thermal stability is a crucial property of enzymes for industrial applications. Thermostable proteases are active in the range of 60–70°C without significant changes in their structure or properties. The ability to rapidly convert substrate at high temperatures is a significant factor in improving the efficiency of industrial operations (Zilda et al. 2012; Hussian and Leong 2023). Additionally, implementing high-temperature manufacturing processes offers other benefits, such as reducing the risk of microbial contamination, reducing fluid viscosity, and increasing heat and mass transfer (Synowiecki 2010; Hussian and Leong 2023).

* Corresponding author: T.V. Pham, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam; **e-mail: phamtanviet@iuh.edu.vn**
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Actinomycetes can biosynthesize a variety of efficient extracellular enzymes. In particular, *Streptomyces* species produce more than 80% of active natural products, which are essential in industrial fields (Spasic et al. 2018; Al-Dhabi et al. 2020a). Enzymes, including proteases derived from *Streptomyces* are receiving more and more attention due to their high substrate specificity (Spasic et al. 2018), ability to catalyze at high temperatures, wide pH range, resistance to organic solvents, surfactants, bleaching agents, proteolytic enzymes, and other denaturing agents (Abdullah Al-Dhabi et al. 2020b; Parthasarathy and Gnanadoss). Moreover, *Streptomyces* species such as *S. clavuligerus*, *S. thermoviolaceus*, *S. rimouses* and *S. roseosporus* have been shown to produce large quantities of proteases in different culture media (Abdullah Al-Dhabi et al. 2020b; Al-Dhabi et al. 2020a; Boughachiche et al. 2021). This study thus aimed to isolate and characterize a new thermostable extracellular protease from *Streptomyces* sp. CNXK100, thereby enhancing the database of actinomycete-derived proteases for potential industrial applications.

Experimental

Materials and Methods

Bacterial strain and protease production. *Streptomyces* sp. CNXK100 strain derived from the Laboratory of Microbial Technology, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh city (Vi et al. 2023) was maintained on modified Gause I agar (casein 10.0 g/l, KNO_3 1.0 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, NaCl 0.5 g/l, $FeSO_4$ 0.01 g/l, agar 20.0 g/l, pH 7.2 ± 0.2) at 30°C and stored at –80°C in glycerol 20%. In order to ensure the strain identity and purity, *Streptomyces* sp. CNXK100 was restreaked on the same medium and incubated for 5 days at 30°C prior to use.

The strain was cultured at 30°C shaking at 150 rpm for crude protease production in modified Gause I broth. After 72 hours, 1.0% of inoculum was transferred to three liters of production medium containing skim milk 10.0 g/l, lactose 10.0 g/l, $NaNO_3$ 1.0 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, NaCl 0.5 g/l, $FeSO_4$ 0.01 g/l, pH 7.0 in the same culture conditions. Cell-free crude protease was obtained after 72 hours by centrifugation at 6,000 × *g* at 4°C for 5 minutes.

Protease assay. The activity of thermostable protease was determined using the modified Anson method with L-tyrosine as the standard. One unit of protease activity (U) was defined as the amount of enzyme required to release 1.0 μmol of tyrosine/ml/min at 75°C. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard (Anson 1938; Bradford 1976; Gohel and Singh 2013).

Thermostable protease purification. The crude protease was obtained from the culture of *Streptomyces* sp. CNXK100 was treated at 70°C for 30 minutes, and denatured thermal sensitive proteins were removed by centrifugation at 11,000 × *g*, 10 minutes, 4°C. The supernatants were subjected to fractional precipitation with $(NH_4)_2SO_4$ at concentrations ranging from 40 to 70% (w/v). Precipitated proteins were collected by centrifugation at 11,000 × *g*, 10 minutes, 4°C then dissolved in buffer A (glycerol 2.0%, Tris 50 mM, 50 μl β-mercaptoethanol, pH 7.5), followed by dialysis with Seamless cellulose tubing (Viskase® Companies, Inc., Japan) in buffer A. Protease activity and total protein content of each fraction were measured. The fraction with highest protease activity was concentrated by Amicon® Pro Purification System (10.0 kDa Amicon® Ultra; Millipore®, Merck KGaA, Germany), and subjected to gel Sephadex G-75 filtration system (GE Healthcare Life Science XK 16/40 Column, Germany). Obtain fractions with high specific activities wer pooled, re-concentrated, and analyzed by SDS-PAGE with a protein ladder (Opti-Protein Express Marker; Smart Science Co., Ltd., Thailand) and the Gel Analyzer software (Touioui et al. 2015; Xin et al. 2015).

Optimal temperature and pH. The influence of temperature on purified protease activity was evaluated in Tris buffer 30 mM, pH 8.0, at 60–90°C. The influence of pH was evaluated at 75°C in different buffers: citrate-phosphate (pH 5.0–7.0), phosphate (pH 6.0–8.0), and Tris (pH 7.0–10.0). Protease activity was determined using the modified Anson method (Mostafa et al. 2019; Parthasarathy and Gnanadoss 2020).

Effect of metal ions. The influence of metal ions on protease activity was studied at optimal temperature and pH with 4.0 mM of individual metal ion solutions or 10.0 mM EDTA (Ethylenediaminetetraacetic acid). A control reaction was done without adding any metal ion solutions or EDTA (Xin et al. 2015).

Enzyme kinetics. Protease solution (0.01 mg/ml) and casein substrate (0.01–20.00 mg/ml) were used to determine of enzyme kinetics in optimal conditions. K_M and V_{max} values were calculated based on Michaelis-Menten plot using GraphPad Prism version 9.0 (GraphPad Software, USA, www.graphpad.com) (Touioui et al. 2015).

Substrate specificity. Substrate specificity was investigated in optimal conditions using a variety of substrates such as casein, BSA, egg albumin, and gelatin at a final concentration of 2.50 mg/ml (Touioui et al. 2015; Xin et al. 2015).

Thermal and pH stability. To assess the thermostability, purified protease was exposed to various temperature points ranging from 60°C to 100°C for 30 or 60 minutes, followed by a rapid cooling to room

temperature. For pH stability evaluation, the protease underwent incubation at room temperature under different pH conditions, including 1.0 N HCl (pH 2.0–4.0), citrate-phosphate buffer (pH 3.0–8.0), phosphate buffer (pH 6.0–8.0), tris buffer (pH 7.0–10.0), and 1.0 N NaOH (pH 9.0–12.0). Following a 120-minute incubation, the protease was readjusted to pH 8.0 using 1.0 N HCl or 1.0 N NaOH. The residual activity of each treatment was calculated in comparison with the non-treated control (100%) (Mostafa et al. 2019; Boughachiche et al. 2021).

Effect of organic solvents, surfactants, bleaching agents, and proteolytic enzymes. The effect of organic solvents (methanol, ethanol, isopropanol, butanol, chloroform), surfactants (SDS, Tween 20, Tween 80, Triton X-100), bleaching agents (NaClO, H_2O_2) on the protease activity was assayed by incubating the protease with 10% organic solvents, 1.0% surfactants, and 0.5–3.0% bleaching agents for 60 minutes in optimal conditions. Each treatment's residual activity was calculated compared to the non-treated control (100%). Additionally, the effect of proteolytic enzymes (proteinase K, trypsin, chymotrypsin, pepsin) was studied by treating CNXK100 protease with each of the proteolytic enzymes at a final concentration of 0.10 mg/ml for 30 minutes. The residual activity of each treatment was calculated in comparison with the non-treated control (100%) (Touioui et al. 2015; Xin et al. 2015).

Enzyme stability and compatibility with commercial detergents. The stability and compatibility of CNXK100 protease was compared with commercial detergents in Vietnam, including ABA, MaxKleen, OMO, and IZI. Proteases, if any, presenting in commercial detergents were inactivated at 70°C for 60 minutes before being used in subsequent experiments. Protease was mixed with different detergents at end concentrations of 0.05 mg/ml and 7.00 mg/ml, respectively, followed by 60-minute incubation. Each treatment's residual activity was calculated compared to the non-treated control (100%). The detergent with the most minor effect on CNXK100 protease is used for the stain removal experiment (Mechri et al. 2022; Tarek et al. 2023).

Cotton fabric (5 × 4 cm) was stained with either tomato sauce, coffee, chocolate, or blood and then air-dried. The ability of CNXK100 protease to remove stains was evaluated by washing the fabric in shaking condition (150 rpm) at 50°C, for 30 minutes with 100 ml of different treatments: (i) no washing control, (ii) distilled water + buffer A, (iii) CNXK100 protease in buffer A (0.05 mg/ml), (iv) commercial detergent (7.00 mg/ml), (v) CNXK100 protease (0.05 mg/ml) + + commercial detergent (7.00 mg/ml).

Blood clot lysis assay. Purified CNXK100 protease (0.05, 0.25, and 2.50 mg/ml) was incubated with blood clot (100 μg) at 35°C for 4 hours. Tris buffer (30 mM, pH 8.0) was used as a negative control. After incubation, the remaining samples were washed three times with Tris buffer.

Storage stability. Protease was stored in buffer A at 4°C, and enzyme activity and protein concentration were monitored every 7 days.

Statistical analysis. All experiments were repeated thrice, and data were presented as mean ± standard deviation. Charts were drawn using Microsoft Excel or GraphPad Prism version 9.0 (GraphPad Software, USA, www.graphpad.com). Statistical analysis was done using Statgraphics® 19 software (Statgraphics Technologies, Inc., USA). One-way ANOVA was used with a p-value < 0.05, indicating a statistical significance.

Results

Protease purification. A 3-step purification process was used to purify CNXK100 thermostable protease. The initial step involved heat treatment of the crude protease extract at 70°C for 30 minutes, followed by fractional precipitation with $(NH_4)_2SO_4$. Subsequently, gel filtration chromatography was employed for further purification. The results, summarized in Table I, indicate a relatively high purification level of approximately 115 times. Despite a modest activity recovery yield (2.31%), the purified protease exhibited remarkably high specific activity, with a value of 2.40×10^6 U/mg.

A single protein band of approximately 27 kDa appeared on the SDS-PAGE, indicating that the protease was successfully purified (Fig. 1).

Optimal temperature and pH of CNXK100 protease. In Fig. 2A, the CNXK100 protease exhibited high

Table I
Purification of thermostable protease from *Streptomyces* sp. CNXK100.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Activity recovery (%)	Purification level
Crude extract	54.64	1.14×10^6	2.09×10^4	100.00	1.00
Heat-treatment	18.62	4.14×10^5	2.22×10^4	36.32	1.06
Fractional precipitation with 60% $(NH_4)_2SO_4$	0.81	1.95×10^5	2.41×10^5	17.11	11.53
Gel filtration	0.011	2.63×10^4	2.40×10^6	2.31	114.83

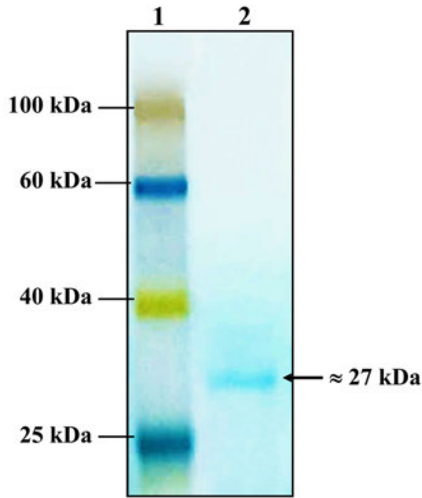
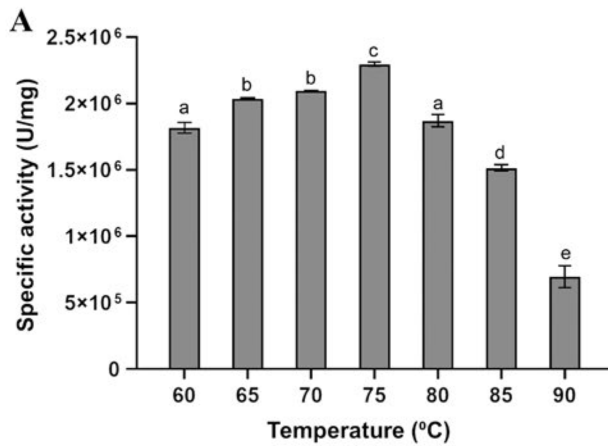


Fig. 1. SDS-PAGE analysis of protease from *Streptomyces* sp. CNXK100. Lane 1 – protein marker, lane 2 – purified protease.

activity within a high-temperature range (60–80°C), with an optimum at 75°C (2.30×10^6 U/mg). Additionally, the CNXK100 protease retained approximately 66% of its maximum activity at 85°C and 30% at 90°C. The CNXK100 protease also demonstrated high activity across a broad pH range of 6.0–10.0 in all three different buffers, reaching optimal activity at pH 7.0–8.0 (2.12×10^6–2.26×10^6 U/mg) in Tris buffer (Fig. 2B). It is noteworthy that even at pH 5.0, the protease retained approximately 65% of its optimum activity.

Effect of metal ions. At a final concentration of 4 mM, Na^+, K^+, Mg^{2+}, and even the heavy metal ion Cu^{2+} had no significant impact on the activity of the CNXK100 protease, with recorded values ranging from 2.25×10^6–2.34×10^6 U/mg. In the presence of Ca^{2+} and Fe^{2+}, the protease retained more than 50% of its catalytic activity. However, the protease activity was diminished by more than 50% in the presence of Al^{3+}, Mn^{2+}, and Zn^{2+}. Upon exposure to the chelating agent EDTA, the protease activity retained 50%, implying the significance of metal ions for the enzymatic function (Fig. 3).

Substrate specificity. The CNXK100 protease demonstrated the ability to catalyze the hydrolysis of four different substrates: casein, skim milk, bovine serum albumin (BSA), and egg albumin. The hydrolysis activity on skim milk by the CNXK100 protease was moderate at 67.92%, while BSA and egg albumin hydrolysis activities were notably lower, ranging from 24.69% to 31.03%. Gelatin was identified as the least specific substrate, with a relative activity of 2.32%. Among these, casein emerged as the most specific substrate for this protease, exhibiting a relative activity value of 100% (Fig. 4).



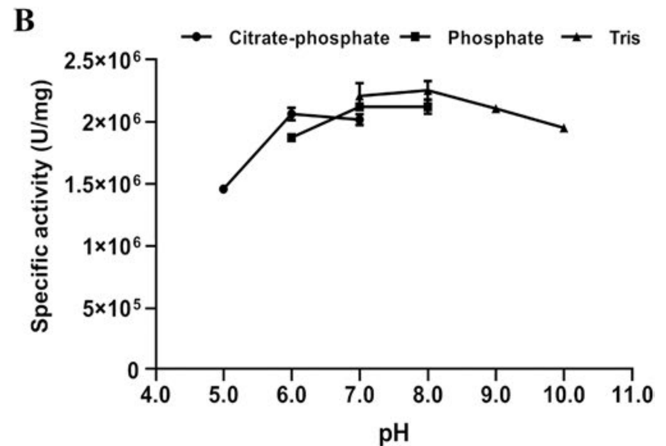


Fig. 2. Effect of temperature (A) and pH (B) on CNXK100 thermostable protease activity.

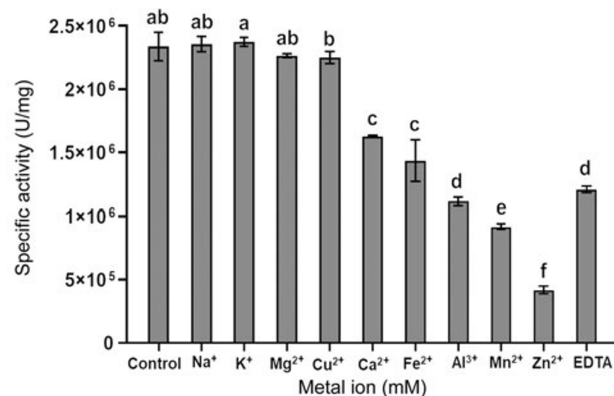


Fig. 3. Effect of metal ions on CNXK100 thermostable protease activity.

Kinetic parameters. The kinetic parameters, K_M and V_{max}, were determined through a Michaelis-Menten plot, yielding values of 3.13 mg/ml and 3.28×10^6 U/mg, respectively, when casein was used as the substrate (Fig. 5).

Thermal and pH stability. The findings depicted in Fig. 6A indicate a marginal variance in protease thermostability between treatments of 30 and 60 minutes. The enzyme exhibited a sustained activity of over 50% following a treatment duration of up to 60 minutes at

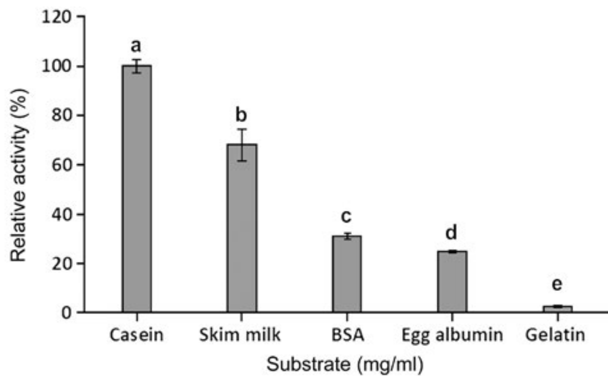


Fig. 4. Substrate specificity of CNXK100 thermostable protease.

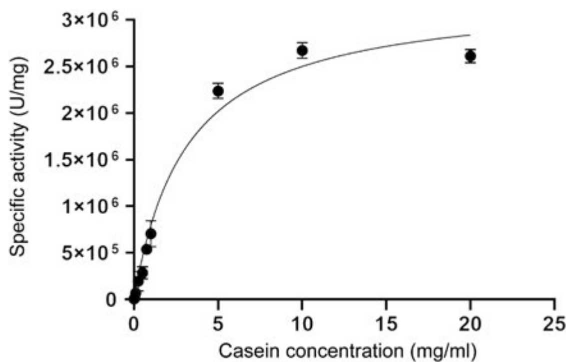


Fig. 5. Michaelis-Menten plot of CNXK100 thermostable protease.

temperatures ranging from 60 to 65°C. However, at 70°C, the protease retained only 14–20% residual activity, and its activity was nearly abolished when subjected to temperatures exceeding 75°C. The pH stability of the protease was further investigated through a 120-minute incubation spanning the pH range of 2.0 to 12.0. The results reveal that over 75% of the enzyme's activity persisted after exposure to the broad pH range of 5.0 to 10.0. Notably, incubation in phosphate buffer at pH 6.0–8.0 resulted in approximately 110% protease activity compared to the control (Fig. 6B).

Effect of organic solvents, surfactants, proteolytic enzymes, and bleaching agents. The CNXK100 protease retained nearly 80% of its activity in the presence of 10% methanol, ethanol, isopropanol, and chloroform. However, butanol had a significant inhibitory effect on the protease, with 44% retention of activity after a 60-minute of treatment. Tween 20, Tween 80, and Triton X-100 were found to have minor effects on the CNXK100 protease, with at least 90% of activity still maintained. Moreover, the activity of the CNXK100 protease exhibited a minor increment when subjected to NaClO concentrations ranging from 0.5% to 3.0%. In contrast, H_2O_2 (0.5% to 3.0%) reduced protease activity by 11% to 33%. While pepsin and chymotrypsin have minor effects on the CNXK100 protease, trypsin caused a 30% reduction, and proteinase K strongly reduced most of its activity (Table II).

Compatibility of CNXK100 thermostable protease with commercial detergents. The stability of the CNXK100 protease showed variability, retaining 30–50% residual activity after treatment with various commercial detergents (Fig. 7A). However, the individual treatment of different types of stains, such as tomato sauce, coffee, chocolate, and blood, using either the CNXK100 protease or the IZI commercial detergent demonstrated equally effective removal capabilities. Interestingly, the combination of the CNXK100 protease with IZI exhibited enhanced stain removal activity, particularly in the case of blood stains (Fig. 7B).

Blood clot lysis activity. The CNXK100 protease has demonstrated varying levels of blood clot lysis capability. The protease partially lysed blood clots at a concentration of 0.25 mg/ml, while at a concentration of 2.50 mg/ml, it completely lysed blood clots after 4 hours of incubation (Fig. 8).

Storage stability. Throughout the initial 21-day storage period at 4°C, the CNXK100 protease maintained 100% activity. Remarkably, even after 70 days

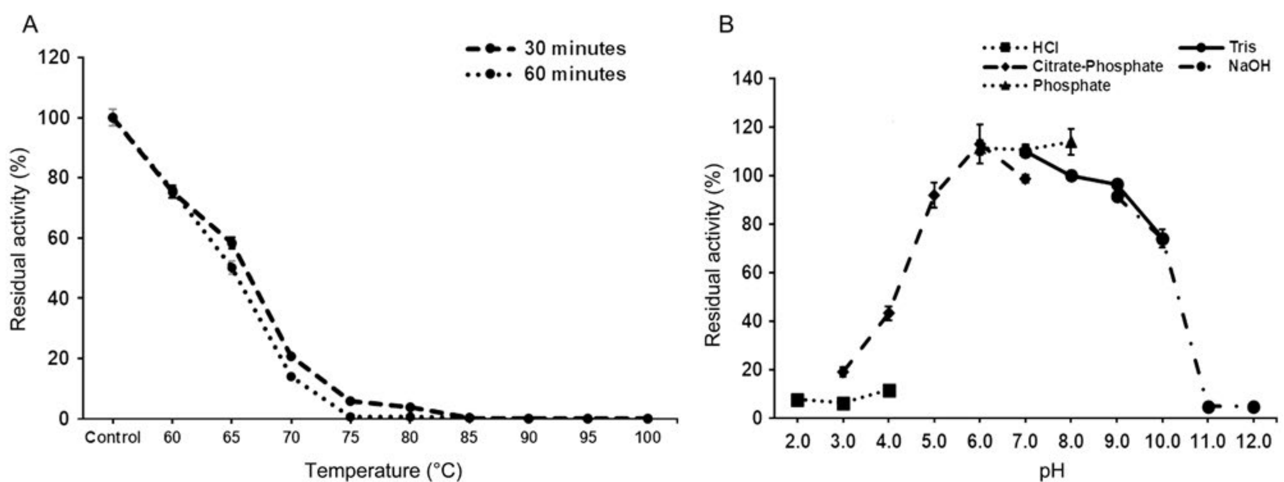


Fig. 6. Stability of CNXK100 thermostable protease; thermostability (A); pH stability (B).

Table II
Effect of various chemicals on CNXK100 thermostable protease activity.

Group	Chemical	Concentration	Residual activity (%)
Control	–	–	100.00
Organic solvents	Methanol	10.0%	89.28 ± 3.63
	Ethanol		86.54 ± 2.36
	Isopropanol		79.47 ± 1.85
	Butanol		43.93 ± 2.32
	Chloroform		86.54 ± 2.34
Surfactants	Triton X-100	1.0%	101.27 ± 2.58
	Tween 20		88.13 ± 1.93
	Tween 80		92.06 ± 3.21
	SDS		53.42 ± 3.05
Bleaching agents	NaClO	0.5%	105.86 ± 0.57
		1.0%	111.36 ± 0.86
		1.5%	113.17 ± 1.62
		2.0%	114.11 ± 0.53
		2.5%	121.47 ± 2.28
		3.0%	120.75 ± 1.34
	H_2O_2	0.5%	89.21 ± 2.03
		1.0%	77.85 ± 9.51
		1.5%	74.12 ± 10.34
		2.0%	71.68 ± 12.29
		2.5%	75.51 ± 2.12
		3.0%	66.96 ± 13.07
Proteolytic enzymes	Pepsin	0.1 mg/ml	101.54 ± 1.43
	Trypsin		68.97 ± 0.51
	Chymotrypsin		91.34 ± 0.13
	Proteinase K		10.80 ± 0.44

of storage, the activity remained consistent at approximately 90%. Furthermore, the protease concentration showed minimal variation during storage (Fig. 9).

Discussion

In the past few decades, there has been a growing interest in proteases that can function effectively at high temperatures and under harsh conditions, making them suitable for diverse applications. The CNXK100 thermostable protease was successfully purified in the present study, revealing a high specific activity. The purified CNXK100 protease, with a molecular weight of approximately 27 kDa, is similar to the protease from *Streptomyces* sp. D1 (28 kDa) (Mane et al. 2013), and these are among the smallest proteases in *Streptomyces* in comparison to those previously purified from *S. griseorubens* E44G (35 kDa) (Al-Askar et al. 2015) and *S. koyangensis* TN650 (45 kDa) (Ben Elhoul et al. 2015). The specific activity of this enzyme surpasses that of *Streptomyces* sp. AH.4 by 36 times (6.60×10^4 U/mg) (Touioui et al. 2015) and exceeds that of *S. clavuligerus* Mit-1 by an impressive 350 times (6.80×10^2 U/mg) (Thumar and Singh 2007).

Significantly, the CNXK100 protease exhibited activity within a high-temperature range of up to 90°C, with an optimal temperature (75°C) surpassing those of specific proteases from *Streptomyces* sp. Al-Dhabi-82 (40°C) (Abdullah Al-Dhabi et al. 2020b), *S. flavogriseus* HS1 (50°C) (Ghorbel et al. 2014), and *Streptomyces* sp. AH4 (70°C) (Touioui et al. 2015).

The high-temperature activity of this protease may be attributed to the presence of numerous proline residues, intermolecular hydrogen bonds, disulfide, salt bridges, and hydrophobic interactions within its molecular structure (Zhou and Pang 2018; Xu et al. 2020). In addition, the CNXK100 protease demonstrated high activity in different buffers within a wide pH range of 5.0–10.0. This enzyme's optimum pH range of 7.0–8.0 was lower than that of proteases from other strains, such as *Streptomyces* sp. GS-1 (pH 8.5) (Sarkar

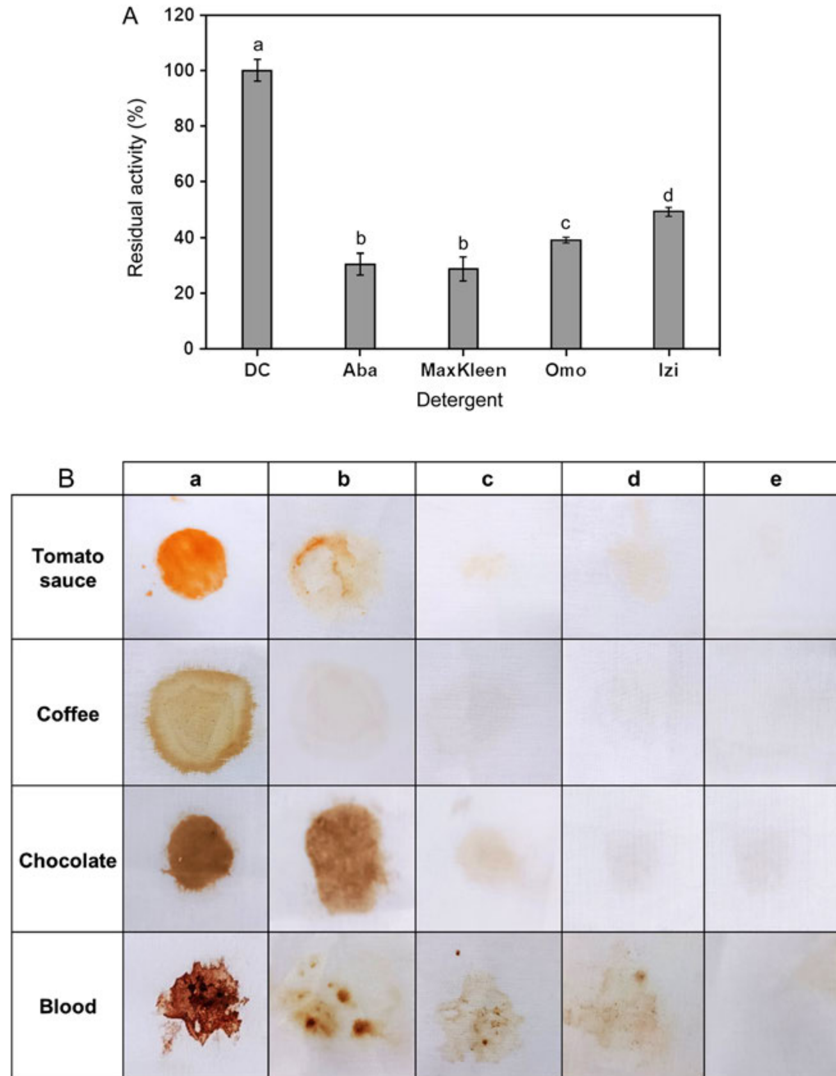


Fig. 7. Compatibility of CNXK100 thermostable protease with commercial detergent (A) and (B) stain removal ability (a – unwashed control; b – distilled water + buffer A; c – CNXK100 protease + buffer A; d – IZI detergent; e – CNXK100 protease + IZI).

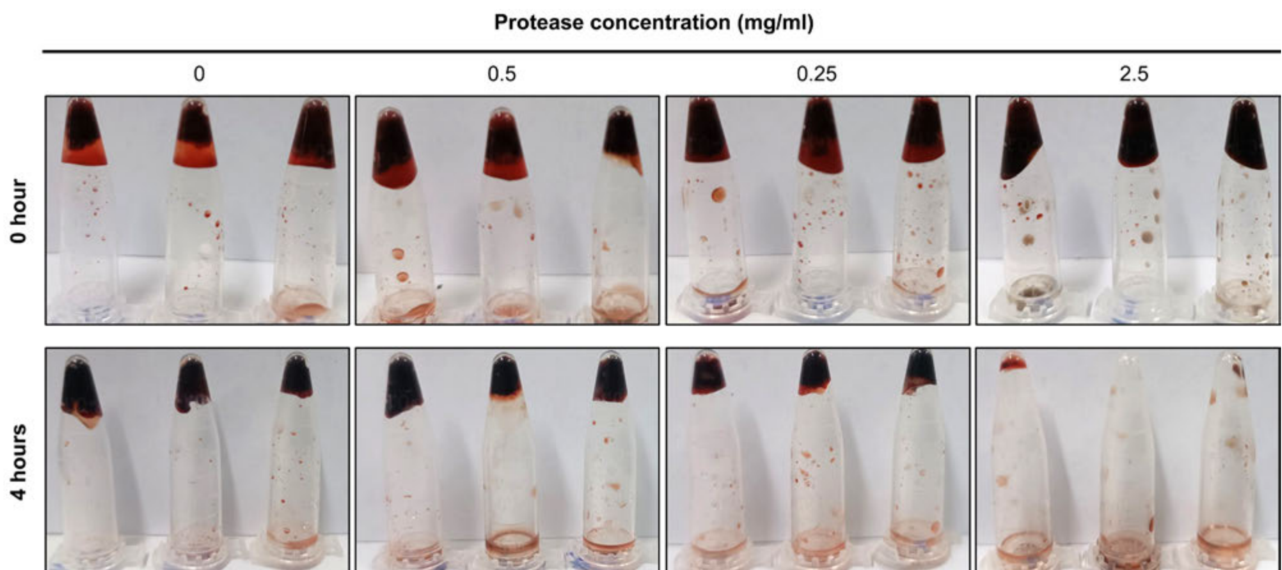


Fig. 8. Blood clot lysis activity of CNXK100 thermostable protease.

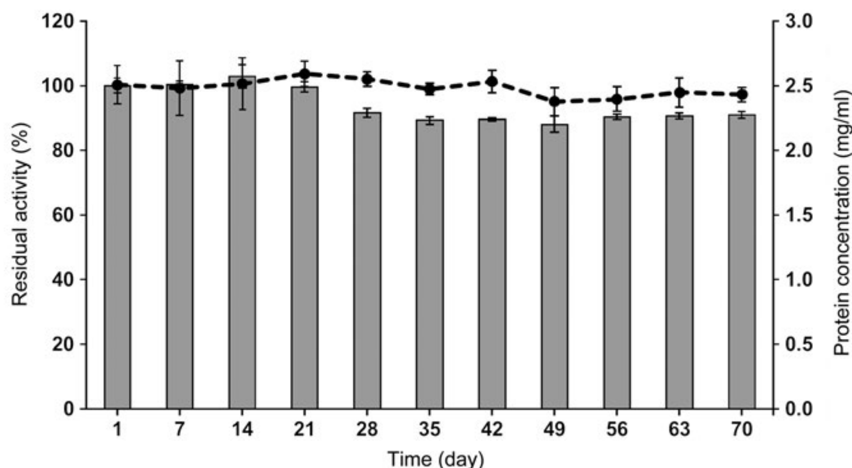


Fig. 9. Residual activity (column) and protein concentration (line) of CNXK100 thermostable protease in storage condition.

and Suthindhiran 2020), *Streptomyces* sp. M30 (pH 9.0) (Xin et al. 2015), and *S. mutabilis* (pH 10.0) (Mechri et al. 2022). This disparity in optimal pH values suggests that this protease can be characterized as a neutral enzyme (Singh and Bajaj 2017).

Metal ions can exhibit varying effects on different thermostable proteases from *Streptomyces* strains. In the presence of the heavy metal Cu^{2+}, the activity of the studied enzyme remained unaffected, whereas Cu^{2+} induced a loss of protease activity from *Brachystreptospora xinjiangensis* OM-6 (Gohel and Singh 2013) and *Streptomyces* sp. P3 (Cheng et al. 2015). Besides, Mn^{2+} and Fe^{2+} induced a partial reduction in the activity of the CNXK100 protease whereas both were observed to completely abolish the protease activity from *S. olivochromogenes* (Simkhada et al. 2010). This observation underscores the potential applicability of the enzyme under conditions characterized by the presence of these heavy metals.

Among the five tested substrates, the CNXK100 protease displayed the highest hydrolysis activity toward casein. It exhibited similarities to proteases from other *Streptomyces* strains, including *S. koyangensis* TN650 (Ben Elhoul et al. 2015), *Streptomyces* sp. LCJ12A (Parthasarathy and Gnanadoss 2020), and *S. speibonae* (Tran et al. 2022). Consequently, this protease could be employed to produce peptones and peptides from casein (Rao et al. 2020).

The K_M value serves as an indicator of the enzyme's affinity for its substrate. The K_M of the CNXK100 protease was 1.3–4.2 times higher than that of the metalloprotease from *S. olivochromogenes* (0.74 mg/ml) (Simkhada et al. 2010) and the alkaline serine protease from *S. griseus* (2.50 mg/ml) (El-Khonezy et al. 2015). However, the V_{max} value of the CNXK100 protease, representing the maximum reaction rate of substrate conversion, was remarkably higher (846–17200 times) than those of the protease from *S. olivochromogenes* (3876 U/mg) (Simkhada et al. 2010) and *S. griseus* (190 U/mg) (El-Khonezy et al. 2015).

The CNXK100 protease has been assessed for its thermal stability, exhibiting resilience up to 65°C for 60 minutes, with its activity surpassing that of the protease derived from *Streptomyces* sp. P3 (Cheng et al. 2015). However, it is imperative to note that this protease underwent heat treatment at 70°C for 30 minutes during the purification process. Consequently, this protease is considered a relatively thermostable enzyme, and its activity is anticipated to remain stable for at least 30 minutes at 70°C.

In addition, the CNXK100 protease demonstrated notable substrate hydrolysis activity across a wide range of pH levels following incubation in three distinct buffers: citrate-phosphate, phosphate, and Tris. Mainly, slightly enhanced activity was observed when the enzyme was subjected to processing at pH 6.0 in citrate-phosphate buffer or within the pH range of 6.0–8.0 in phosphate buffer, surpassing the corresponding control. Possessing inherent pH robustness and compatibility with diverse buffer systems, the CNXK100 protease holds promise for applications in the medical field (pH 5.0–6.0) (Singh et al. 2016), as well as in the domains of bakery and brewing (pH 6.0–8.0) (Gurumallesh et al. 2019), and various formulations within the industrial area of detergent production (pH 8.0–10.0) (Ben Elhoul et al. 2015). The pH stability of CNXK100 protease aligns with that of proteases from *S. flavogriseus* (pH 4.5–9.5) (Mostafa et al. 2019), *Streptomyces* sp. XE-1 (pH 5.0–9.0) (El-Khonezy et al. 2015), and *S. koyangensis* TN650 (pH 5.0–11.0) (Ben Elhoul et al. 2015).

Furthermore, ethanol, isopropanol, chloroform, and methanol were found to only marginally decrease the enzymatic activity of CNXK100 protease, with a maximum reduction of 20%. In contrast, ethanol resulted

in a 40% reduction in the activity of protease derived from *S. koyangensis* TN650 (Ben Elhoul et al. 2015) and *Streptomyces* sp. M30 (Xin et al. 2015). On the other hand, chloroform resulted in 40% and 90% reductions in the activity of *Streptomyces* sp. GS-1 (Sarkar and Suthindhiran 2020) and *S. koyangensis* TN650 protease, respectively (Ben Elhoul et al. 2015), while isopropanol caused reductions of 50% and 70% in the activity of *S. koyangensis* TN650 protease (Ben Elhoul et al. 2015) and *Streptomyces* sp. M30 protease, respectively (Xin et al. 2015). The stability of the CNXK100 protease was further exemplified through its resistance to Triton X-100, Tween, and SDS. On the contrary, Triton X-100 induced a 40% reduction in the activity of a new *S. flavogriseus* HS1 protease (Ghorbel et al. 2014). In comparison, Tween caused a reduction exceeding 40% in the activity of serine proteases from *Thermoactinomyces* spp. (Aksoy et al. 2012). Additionally, a 0.5% SDS solution resulted in approximately 75% reduction in the activity of antifungal protease from *Streptomyces* sp. A6 (Singh and Chhatpar 2011) and even completely abolishes the activity of *S. flavogriseus* HS1 protease (Ghorbel et al. 2014).

The CNXK100 protease also demonstrated higher stability than other microbial proteases in the presence of two oxidative agents, NaClO and H_2O_2. While 1.0% NaClO has been reported to reduce the activity of *Bacillus siamensis* protease by 20% (Tarek et al. 2023), 0.5–3.0% NaClO concentrations resulted in an enhancement of 5.0–20% in the activity of CNXK100 protease. Additionally, 0.5% H_2O_2 reduced the activity of *S. flavogriseus* HS1 protease by 40% (Ghorbel et al. 2014), whereas this effect on CNXK100 protease was only observed at a 3.0% H_2O_2 concentration.

Limited research exists on the tolerance of proteases from the genus *Streptomyces* towards common proteolytic enzymes. Pepsin primarily cleaves peptide bonds at the N-terminal positions of aromatic amino acids, including tyrosine, tryptophan, and phenylalanine (Ahn et al. 2013), while chymotrypsin cleaves peptide bonds at the carboxyl positions of tyrosine and phenylalanine, as well as at the N-terminal position of tryptophan (Zhou et al. 2011). Neither of these enzymes exhibited a significant effect on the CNXK100 protease, suggesting that these amino acids do not play a crucial role in the protease's active site. Trypsin, known for its ability to hydrolyze peptide bonds on the carboxyl side of arginine or lysine at the C-terminal, excluding those followed by proline, only causes a marginal reduction of approximately 30% in the activity of the CNXK100 protease (Vorob'ev 2019). Meanwhile, proteinase K, a non-specific serine endopeptidase, can cleave peptide bonds of aromatic, aliphatic, and hydrophobic amino acids at the C-terminal (Mótyán et al. 2013), thereby exerting a substantial influence on CNXK100 protease structure and its catalytic function. The CNXK100 protease has been found to possess fibrinolytic activity, a characteristic documented in a few other studies involving proteases derived from *S. radiopugnans* VITSD8 (Verma et al. 2018), *S. rubiginosus* VITPSS1 (Dhamodharan et al. 2019), and *Streptomyces* sp. 214L–11 (He et al. 2023). Due to its stability towards pepsin, trypsin, chymotrypsin, and fibrinolytic activity, the CNXK100 protease holds potential applications in medications (Bose et al. 2019).

The enduring characteristics of CNXK100 protease with surfactants, oxidizing agents, and diverse proteases were also demonstrated via its sustained functionality and even enhanced blood stain removal capability when used in conjunction with commercial detergents. This indicates its promising potential for utilization in the detergent industry.

Last but not least, to our knowledge, limited research has been conducted on the storage stability of purified proteases from *Streptomyces*. Nevertheless, the CNXK100 protease storage stability mirrors a few proteases derived from *Bacillus*, which retained 80–90% activity after 2 months at 4°C (Kotb et al. 2023; Wang et al. 2018).

Conclusions

In this study, one of the smallest thermostable proteases from *Streptomyces* sp. CNXK100 was successfully purified and characterized. The protease exhibited optimal activity at high temperatures and demonstrated activity in diverse buffers across a wide pH range. Additionally, metal ions were found to be important for enzyme activity and exhibited significant fibrinolytic activity. Notably, the protease displayed a preference for casein, with a V_{max} value thousands of times higher than other *Streptomyces* proteases. The enzyme demonstrated its stability at high temperatures in a wide pH range and resistance to polar and non-polar organic solvents, surfactants, bleaching agents, and proteolytic enzymes. Although the protease exhibited partially decreased activity in commercial detergents, their combination increased the destaining effect, particularly on blood stains. Moreover, the enzyme remained stable for at least 70 days at 4°C. In conclusion, the thermostable protease originating from *Streptomyces* sp. CNXK100 is a versatile candidate for various applications in different fields.

ORCID

Truong Chinh Hua https://orcid.org/0000-0001-8228-1077
Ngoc An Nguyen https://orcid.org/0000-0002-7574-2952
Hanh Thi Dieu Nguyen https://orcid.org/0000-0002-3735-9127
Tan Viet Pham https://orcid.org/0000-0002-1061-1257

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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