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ORIGINAL ARTICLE

Purification and Biochemical Characterization of a Novel Thermostable Serine Protease from *Geobacillus* sp. GS53

Seden Güracar Baykara[1] · Yusuf Sürmeli[1,2] · Gülşah Şanlı-Mohamed[1,3]

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Abstract

Proteases account for approximately 60% of the enzyme market in the world, and they are used in various industrial applications including the detergent industry. In this study, production and characterization of a novel serine protease of thermophilic *Geobacillus* sp. GS53 from Balçova geothermal region, İzmir, Turkey, were performed. The thermostable protease was purified through ammonium sulfate precipitation and anion-exchange chromatography. The results showed that the protease had 137.8 U mg^{-1} of specific activity and optimally worked at 55 °C and pH 8. It was also active in a broad pH (4–10) and temperature (25–75 °C) ranges. The protease was highly stable at 85 °C and demonstrated relative stability at pH 4, 7, and 10. Also, the enzyme had high stability against organic solvents and surfactants; enzyme relative activity did not decrease below 81% upon preincubation for 10 min. Ca^{2+}, Cu^{2+}, and Zn^{2+} ions slightly induced protease activity. The protease was highly specific to casein, skim milk, Hammerstein casein, and BSA substrates. These results revealed that the protease might have a potential effect in a variety of industrial fields, especially the detergent industry, because of its high thermostability and stability to surfactants.

Keywords Serine protease · *Geobacillus* · Thermostability · Surfactant

✉ Gülşah Şanlı-Mohamed
gulsahsanli@iyte.edu.tr; gulsahsanli@hotmail.com

[1] Department of Biotechnology and Bioengineering, İzmir Institute of Technology, 35430 İzmir, Turkey

[2] Department of Agricultural Biotechnology, Tekirdağ Namık Kemal University, 59030 Tekirdağ, Turkey

[3] Department of Chemistry, İzmir Institute of Technology, 35430 İzmir, Turkey

Introduction

Proteases are a class of enzymes that break up the peptide bonds in proteins to yield amino acids and short peptides. These enzymes can be categorized as serine protease (EC 3.4.21), cysteine/sulfhydryl protease (EC 3.4.22), aspartate protease (EC 3.4.23), metalloprotease (EC 3.4.24), and threonine protease (EC 3.4.25) according to the role of amino acid residues from the active site [1]. About 33% of proteases can be grouped as serine proteases, and they are named as serine proteases because of its nucleophilic serine amino acid residue found in the active site [2].

Proteases account for about 60% of the enzyme market in the world [3], and they are used in various industrial applications, especially in the food, pharmaceutical, detergent, and leather processing industry [4]. In industrial applications, serine protease is largely utilized in detergents as a supplement for cleaning since they promote the detergent efficiency, facilitating the reduction of the leftover protein stain [5–7]. The serine protease enzyme may attack the substrate uniquely based on the substrate specificity pocket [8].

Proteases are synthesized by different organisms including bacteria, yeast, fungi, animals, and plants [9]. Microorganisms are preferentially used as sources for the production of industrial enzymes, since their growth is low cost and the genes encoding for the enzymes can easily be manipulated to create mutant forms of enzymes having desired characteristics [10, 11]. So far, many studies have been carried out for production and purification of serine protease from many sources [12–16]; however, serine proteases have been purified and characterized in only three members of thermophilic *Geobacillus* genera: *Geobacillus stearothermophilus*, *Geobacillus toebii* strain LBT 77, and *Geobacillus* sp. YMTC 1049 [17–19].

Geobacillus species, gram-positive and endospore-forming bacteria, can survive at extreme temperatures (37 to 75 °C) where plenty of species are not able to grow [20]. Different enzymes synthesized by *Geobacillus* species from different regions possess high stability against extreme conditions such as temperature, pH, chemical denaturants, organic solvents, and detergents [17–26]. Recently, α-L-arabinofuranosidase of thermophilic *Geobacillus vulcani* GS90 and thermostable α-amylase of *Geobacillus* sp. GS33 from Balçova geothermal region, İzmir, Turkey, has been successfully characterized as stable enzymes against various surfactants, pH, and temperature [27–29]. Because proteases are used in the different industrial fields, it has been aimed to investigate a novel thermostable serine protease with convenient biochemical properties from *Geobacillus* source. Here, we reported the purification and biochemical characterization of serine protease of *Geobacillus* sp. GS53 isolated from Balçova geothermal water region, İzmir, Turkey.

Materials and Methods

Materials

Casein, L-tyrosine, Folin & Ciocalteu's phenol reagent, and Coomassie Brilliant Blue G-250 were supplied by Merck. DEAE-cellulose and cellulose ion-exchanger resin were purchased from Servacel. Trichloroacetic acid (TCA) and the other chemicals for electrophoresis and characterization studies were also supplied by Sigma Chemical (St. Louis, MO, USA).

Bacterial Strains and Growth Conditions

The environmental *Geobacillus* isolate (V53) [30] obtained from uncontrolled thermal leak of Balçova Geothermal region of İzmir, named *Geobacillus* sp. GS53 in this study, was used as a source of the protease enzyme. Luria-Bertani (LB) agar including 1% (w v^{-1}) tryptone, 0.5% (w v^{-1}) yeast extract, 1% (w v^{-1}) sodium chloride, and 1.5% (w v^{-1}) agar was used to obtain single colony. A complex medium (pH 7) called induction medium containing 1% (w v^{-1}) glucose, 0.5% (w v^{-1}) yeast extract, 0.25% (w v^{-1}) peptone, 0.25% (w v^{-1}) casein, 0.03% (w v^{-1}) $MgSO_4$, 0.002% (w v^{-1}) $FeSO_4$, 0.02% (w v^{-1}) $ZnSO_4$, 0.1% (w v^{-1}) $CaSO_4$, 0.1% (w v^{-1}) KH_2PO_4, and 0.1% (w v^{-1}) K_2HPO_4 was also utilized for induction of extracellular thermostable protease production. Besides, skim milk agar containing 0.8% (w v^{-1}) nutrient broth, 1% (w v^{-1}) skim milk, and 1.5% agar was used for screening of thermostable protease activity. *Geobacillus* sp. GS53 was grown at 55 °C and 200 rpm overnight.

Production and Purification of Extracellular Thermostable Protease

Extracellular protease from *Geobacillus* sp. GS53 was produced using 200 mL of induction medium at 55 °C and 200 rpm for 24 h. For this purpose, overnight culture of *Geobacillus* sp. GS53 was centrifuged at 4 °C and 5000 rpm for 20 min. The supernatant (extracellular soluble protein extract) was kept as a source of extracellular protease. The protease was purified from extracellular soluble protein extract by ammonium sulfate precipitation and anion-exchange chromatography, respectively. Firstly, 80% (w v^{-1}) ammonium sulfate was gradually supplemented to extracellular soluble protein extract, stirring at 4 °C, and then, ammonium sulfate-precipitated soluble protein extract was harvested by centrifugation at 4 °C and 9500 rpm for 15 min. The pellet was resuspended in 10 mL of Tris-HCl buffer (pH 7.2) called a one-step purified sample. For anion-exchange chromatography, one-step purified sample was loaded on equilibrated DEAE-cellulose column (2.5 cm × 10 cm) (Sigma) with 50 mM Tris-HCl buffer (pH 7.2) and then washed using 50 mM Tris HCl buffer (pH 7.2). Protease-active fractions were pooled after gradient elution buffer of 50 mM Tris HCl buffer (pH 7.2) up to 2 M NaCl and stored at – 20 °C. The purified protease was monitored by 12% SDS-PAGE [31] using Coomassie staining. Quantitative protein concentration was determined by Bradford method [32] using standard bovine serum albumin (BSA) solutions.

Biochemical Characterization of Extracellular Protease

Standard Activity Assay

The activity of thermostable protease was determined using non-specific protease activity assay [33, 34]. For this purpose, 100 μL of 0.5% (w v^{-1}) casein in 50 mM Tris-HCl (pH 7.2) was mixed with 100 μL of enzyme solution, and the mixture was incubated at 55 °C for 10 min. The enzymatic reaction was terminated by addition of 100 μL of 15% trichloroacetic acid (TCA). The mixture was incubated on ice for 10 min and centrifuged at 13500 rpm and room temperature for 10 min. Two hundred microliter of the supernatant was mixed with 1000 μL of NaOH and 200 μL of Folin

& Ciocalteu's phenol reagent and then incubated at 37 °C for 1 h. Free tyrosine reacting with the phenol reagent was measured at 660 nm. One unit of enzyme is defined as the amount of 1 μmol of tyrosine equivalent released by the enzyme per minute under the standard activity assay conditions.

Optimum pH and Temperature

The effect of temperature on protease activity was investigated in a wide range of temperatures, 25 to 85 °C. Besides, the effect of pH on protease activity was analyzed by using varied buffers in diverse pH points, 4 to 10; Na-citrate buffer (pH 4–6); sodium phosphate buffer (pH 7); Tris-HCl buffer (pH 8); and glycine-NaOH buffer (pH 9–10). All experiments were performed using standard activity assay conditions.

pH and Temperature Stability

Thermostability and pH stability of protease enzyme were determined in different temperature and pH values. For thermal stability, the protease enzyme was incubated at two temperature points (55 °C and 85 °C) for 12 h. To study pH stability, the enzyme was incubated at three pH points (pH 4, 7, and 10) throughout 6 h. The residual enzyme activity was then plotted using standard activity assay.

Effect of Various Metal Ions, Organic Solvents, Surfactants, and Inhibitors

The influence of different metal ions, organic solvents, surfactants, and inhibitors on thermostable protease activity was investigated. For this purpose, the enzyme was preincubated in the presence of 10 mM metal ions (NaCl, $CaCl_2$, $MgCl_2$, $CuSO_4$, and $ZnCl_2$), 10% organic solvents (ethanol, methanol, hexane, benzene, dimethyl sulfoxide (DMSO)), 1% surfactants (sodium dodecyl sulfate (SDS), Triton X-100, Tween 20, Tween 80, and H_2O_2), and 1 mM of various inhibitors including phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), iodoacetamide, and protease inhibitor cocktail (AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride)), EDTA, bestatin, pepstatin A, E-64) at 55 °C for 10 min. Then, the reaction with 0.5% casein was initiated, and the residual enzyme activity was determined under standard activity assay conditions.

Substrate Specificity

Substrate specificity of the protease was investigated using various substrates (0.5%) including casein, azocasein, hammers casein, skim milk, and BSA. The relative enzyme activity was determined under standard activity assay conditions.

Data Presentation and Statistical Analysis

All experiments were performed three repetitions. Statistical errors of data were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) (www.graphpad.com).

Results and Discussion

Screening of Protease

Geobacillus sp. GS53 culture was grown on the skim milk agar at 55 °C for 24 h. The screening of protease activity showed that clear zones formed around each colony of *Geobacillus* sp. GS53 strain, indicating that the strain can degrade skim milk (**data not shown**).

Production and Purification of Thermostable Protease

Thermostable protease from *Geobacillus* sp. GS53 was produced by using the induction medium and then purified using ammonium sulfate precipitation and anion-exchange chromatography, respectively. As a result of two-step purification, thermostable protease is purified 1.41-fold with a yield of 33.6%, and specific activity of the purified protease is found as 137.8 ± 7.2 U mg^{-1} (**Table 1**). In literature, serine protease from *Geobacillus toebii* strain LBT 77 had a specific activity of 739.5 U mg^{-1} after three purification steps with a yield of 20% [18]. Also, a specific activity of alkaline serine protease from *Geobacillus stearothermophilus* B-1172 was determined as 97.5 U mg^{-1} with a recovery of 23.6% after ammonium sulfate precipitation and ion-exchange chromatography [17].

Then, the enzyme purity was checked by SDS-PAGE. The protease had three bands with a molecular weight of about 25, 29, and 32 kDa in SDS-PAGE (**Fig. 1**).

Biochemical Characterization of Thermostable Protease

The Influence of pH and Temperature

The purified thermostable protease of *Geobacillus* sp. GS53 from Balçova geothermal water, İzmir, Turkey, is characterized, as shown in Fig. 2, Fig. 3, and Table 2. The protease was investigated in a wide range of pH (4 to 10) and temperature (25 to 75 °C). The maximum relative activity of the enzyme is obtained at 55 °C and pH 8 (**Fig. 2a and b**). In literature, similar optimum characteristics have been determined. In line with this, the protease of *Geobacillus thermoglucosidasius* SKF4 from Sungai Klah Hot Spring Park, Malaysia, optimally worked at 60–65 °C and pH 7–8 [35], whereas protease of *Geobacillus* sp. strain PLS A from undersea fumaroles in Weh Island, Aceh Province, Indonesia, had maximum activity at 60 °C and pH 7 [36]. Also, the protease of *Geobacillus toebii* LBT 77 newly isolated from a Tunisian hot spring owned a maximum activity at 95 °C and pH 13 [18], while a protease *Geobacillus* sp. YMTC 1049 a hot spring in Rehai, Yunnan Province, China, had the highest activity at 85 °C and pH 7.5 [19]. An alkaline protease of *Bacillus lehensis* JO-26 from the

Table 1 Two-step purification of thermostable protease from *Geobacillus* sp. GS53

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Soluble protein extract	894 ± 5.83	9.33	95.85 ± 0.62	100 ± 0.65	1
Ammonium sulfate precipitation	723 ± 21.3	6.43	112.4 ± 3.3	81.5 ± 3.3	1.17
Ion-exchange chromatography	300 ± 15.8	2.182	137.8 ± 7.2	33.6 ± 1.8	1.44

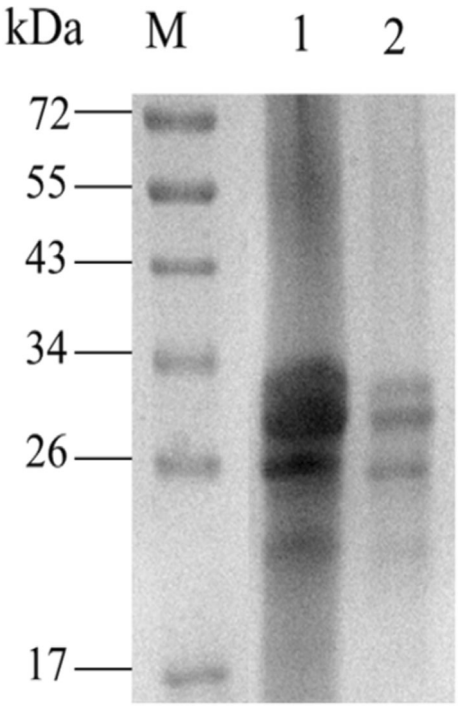

Fig. 1 SDS-PAGE analysis of the extracellular serine protease from *Geobacillus* sp. GS53. **M** protein ladder. **1** soluble protein extract. **2** the purified protease

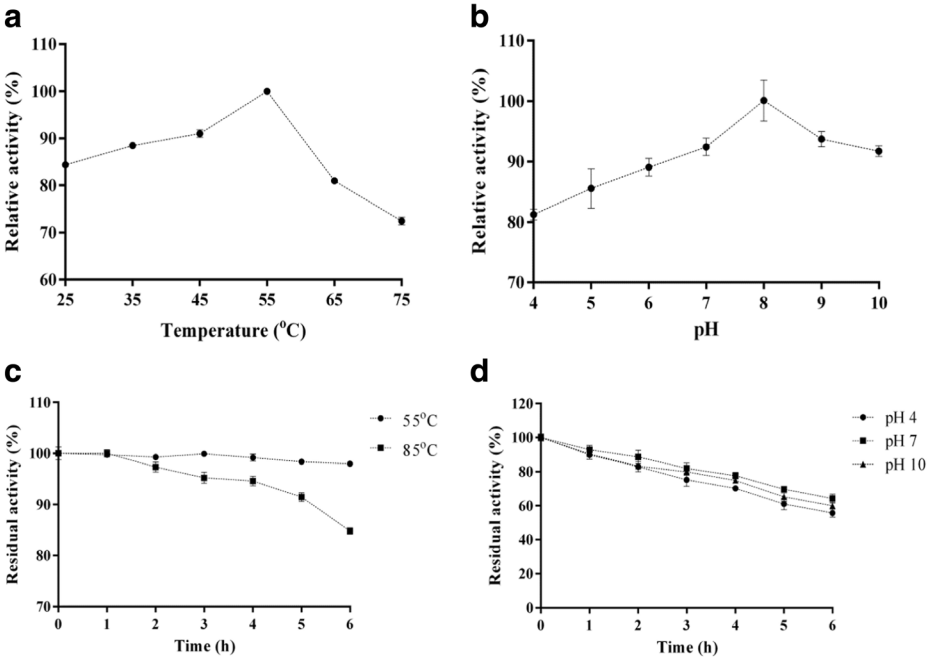

Fig. 2 Thermal and pH effects on the activity and stability of the extracellular serine protease from *Geobacillus* sp. GS53. (**a**) Temperature effect, (**b**) pH effect, (**c**) thermostability, (**d**) pH stability

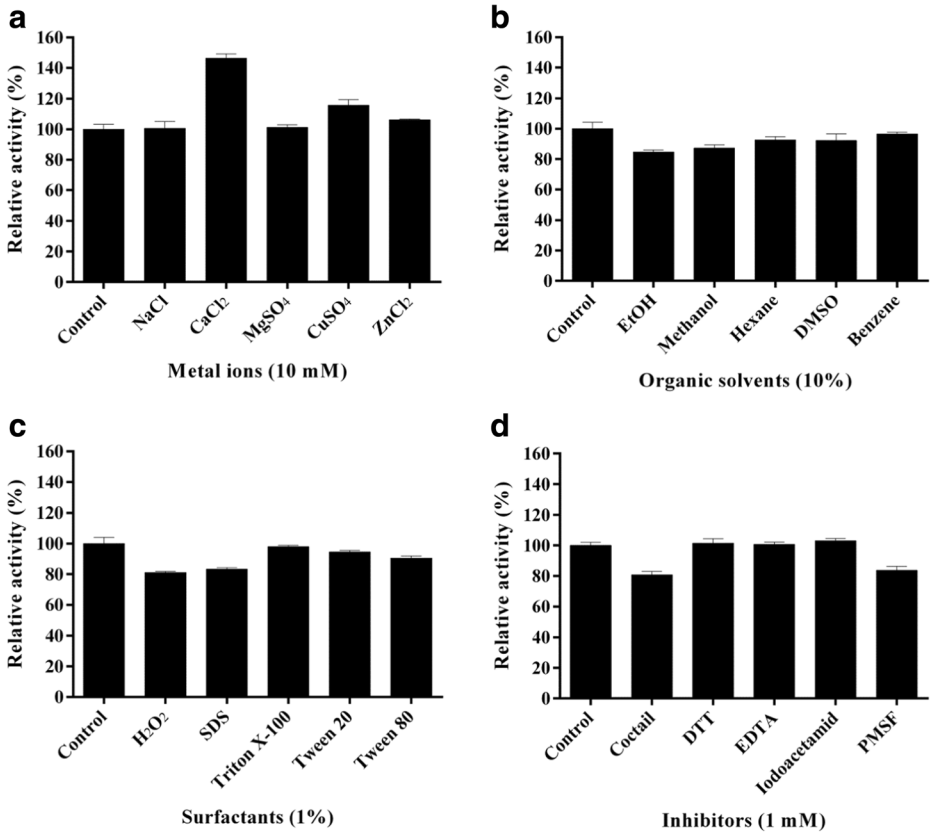

Fig. 3 Effect of various chemicals on the activity of the extracellular serine protease from *Geobacillus* sp. GS53. (**a**) Metal ion effect, (**b**) organic solvent effect, (**c**) surfactant effect, (**d**) inhibitor effect

saline desert, Little Rann of Kutch, India, had a maximum activity at pH 10 and 50 °C [15]. Optimum temperature and pH of a protease of *Virgibacillus* sp. strain CD6 from salted fish were 60 °C, pH 7, and pH 10, respectively [37]. This indicated that the optimum characteristics of the present protease were comparable with literature findings.

Various temperature and pH effects on the thermostable protease stability were investigated. Thermostability analysis showed that the enzyme kept almost all of its activity at 55 °C upon incubation of 6 h. Also, the enzyme retains 85% of residual activity at 85 °C even after 6 h (**Fig. 2c**). In literature, an alkaline serine protease from *Geobacillus stearothermophilus* lost more than half of its residual activity at 80 °C and 90 °C [38]. A thermostable and

Table 2 Substrate specificity of thermostable protease from *Geobacillus* sp. GS53

Substrate	Wavelength (nm)	Relative activity (%)
Casein	660	100 ± 1.92
Skim milk	660	100 ± 1.02
Hammersten casein	660	97.2 ± 3.02
BSA	660	90.4 ± 3.63
Azocasein	440	5.4 ± 0.01

haloalkaline protease from *Geobacillus toebii* strain LBT 77 retained its residual activity of 80% and 40% at 80 °C and 90 °C after 3 h, respectively [18], whereas haloalkaliphilic protease from *Bacillus lehensis* JO-26 lost all of its activity at 80 °C after 3 h [15]. Beside this, a protease from halophilic *Virgibacillus* sp. CD6 lost about half of its residual activity at 70 °C upon incubation of 1 h [37]. Also, thermostable serine alkaline protease from *Caldicoprobacter guelmensis* lost its activity at 80 °C and 90 °C for 3 h and 1 h, respectively [39]. A thermostable protease of *Streptomyces* sp. M30 retained active at 70 °C for only 30 min [40]. A protease from the thermophilic *Anoxybacillus kamchatkensis* M1V kept 75% of its activity at 80 °C after 6 h [41]. It was concluded that thermostable protease from *Geobacillus* sp. GS53 exhibited better thermal stability than those of the above-mentioned proteases. Also, pH stability analysis results show that the protease exhibited a similar pattern at pH 4, 7, and 10, retaining residual activity between 60% and 65% upon incubation of 6 h (**Fig. 2d**). In literature, there have been many studies having comparable results with the present work [15, 18, 37–39, 41].

Effect of Metal Ions, Organic Solvents, and Surfactants/Inhibitors

Thermostable protease activity was investigated in the presence of 10 mM various metal ions (Ca^{+2}, Cu^{+2}, Mg^{+2}, Na^{+1}, and Zn^{+2}), 10% organic solvents (ethanol, methanol, hexane, DMSO, and benzene), 1% surfactants (SDS, Triton X-100, Tween 20, Tween 80, and H_2O_2), and 1 mM inhibitors (PMSF, DTT, EDTA, iodoacetamide, and inhibitor cocktail), using casein as the substrate. Among all tested metal ions, Ca^{2+}, Cu^{2+}, and Zn^{2+} increase the protease activity by approximately 46%, 15%, and 6%, respectively (**Fig. 3a**). Most serine proteases are recognized by stimulating the activity in the presence of Ca^{2+}, as reviewed by Gurumallesh and colleagues [12]; thus, the present enzyme might belong to a group of a serine protease. On the other hand, Na^{+} and Mg^{2+} did not change the protease activity (**Fig. 3a**). In literature, there have been similar Ca^{2+} effect results in various *Geobacillus* sp., but not the other metal ions. Regarding these studies, 5 mM Ca^{2+} induced the activity of thermostable serine protease from *Geobacillus toebii* by about 50%, whereas Cu^{2+} and Zn^{2+}, to a different extent, reduced its activity [18]. Similarly, the activity of protease RH-1 from a thermophilic *Geobacillus* sp. YMTC 1049 was activated by 10 mM Ca^{2+} by 55%; however, Cu^{2+} and especially Zn^{2+} strongly inhibited its activity by 11% and 78%, respectively [19]. Besides, the activity of serine protease from *Geobacillus stearothermophilus* did not changed in the presence of 1 mM, 5 mM, and 10 mM Ca^{2+} [38].

The results of organic solvent effect showed that the protease activity was not considerably reduced in the presence of 10% organic solvents (ethanol, methanol, hexane, DMSO, and benzene), exhibiting a relative activity of above 84%. Accordingly, ethanol and methanol repress the protease activity by 13% and 15%, respectively, whereas benzene slightly decreases its activity (**Fig. 3b**). Given that many reports about organic solvent effects on protease activity, the activity of every protease is differently affected. Regarding this, ethanol and methanol considerably reduced alkaline thermostable serine protease from *Bacillus lehensis* by 46% and 24%, respectively, while benzene considerably increased its activity [15]. In addition, ethanol decreased the activity of protease from *Streptomyces* sp. M30 by 40% although methanol did not change its activity [40].

In surfactant and inhibitor effects, the results show that thermostable protease activity was, to a different extent, inhibited in the presence of 1% surfactants (SDS, Triton X-100, Tween 20, Tween 80, and H_2O_2) and 1 mM inhibitors (PMSF, DTT, EDTA, iodoacetamide, and cocktail), but none of them could not reduce the protease activity below 81% (**Fig. 3c and d**). The enzyme is more stable in the presence of non-ionic surfactants including Triton X-100 (98%), Tween 20 (95%), Tween 80

(91%) than ionic surfactant SDS (83%), and detergent constituent H_2O_2 (81%) (**Fig. 3c**). In literature, there have been some recent reports of surfactant effects on the activity of protease, which is suitable for detergents. Accordingly, one report has shown that Triton X-100 and Tween 80 dramatically reduced the activity of *Bacillus lehensis* JO-26 serine protease, which is effective as a detergent additive, to 37% and 45%, respectively, whereas SDS enhanced its activity to 275% [15]. Also, Triton X-100 did not change, and Tween 80 increased the activity of *Geobacillus stearothermophilus* serine protease, which could be a promising candidate for the detergent industry, whereas SDS and H_2O_2 substantially decreased its activity to 46% and 61%, respectively [38]. Tween 20 increased the activity of detergent-compatible protease from *Virgibacillus* sp. strain CD6 to 106%; however, it was reduced to 85%, 87%, 78%, and 97%, in the presence of Triton X-100, Tween 80 SDS, and H_2O_2, respectively [37]. Thus, it was suggested that the present protease might be used as a good candidate in the detergent industry. In addition, inhibitor effect analysis results show that PMSF and inhibitor cocktail (AEBSF, EDTA, bestatin, pepstatin A, and E-64) partially inhibited thermostable protease, whereas DTT, iodoacetamide, and EDTA did not change its activity (**Fig. 3d**). It is well known that PMSF and AEBSF are serine protease inhibitors [42, 43], confirming that the present protease in this study belongs to a class of serine protease. Serine protease is classified by existence of serine amino acid residue in catalytic region [44], and its catalytic binding region possesses three amino acid residues as aspartate, histidine, and serine. Most of the serine protease conserves a main concept of catalytic mechanism, where lysine or histidine is bound with the catalytic residue serine, involving a couple of residues. On the other hand, remaining serine proteases induce catalytic action via novel triple residues, e.g., including a couple of histidine residue associated with the nucleophile serine residue [45].

Substrate Specificity

The substrate specificity of thermostable protease was investigated using various substrates including 5% casein, 5% azocasein, 5% hamersten casein, 5% skim milk, and 5% BSA under the standard activity assay conditions. The substrate specificity analysis showed that the highest relative activity of the protease enzyme was found in the presence of casein (100%) and skim milk (100%), whereas it was obtained 97.2% and 90.4% of relative activity in the presence of Hammersten casein and BSA, respectively. However, the lowest relative activity is determined as 5.4% using azocasein as a substrate (**Table 2**). In literature, the alkaline protease of *B. licheniformis* A10 exhibited the highest relative activity toward casein (100%) and azocasein (97%); however, it showed low specificity using BSA (18%) [46]. In addition, protease from *B. iranensis* exhibited the highest specificity with casein [47]. Alkaline protease enzyme purified from *A. pallidus* C10 showed the highest substrate specificity to casein, whereas it showed a relative activity of 15% and 5% to azocasein and BSA, respectively [48]. Besides, protease from wild-type *Bacillus* sp. had the highest activity to casein (100%), but it exhibited 0.04% of relative activity to BSA [49].

Conclusions

The present study revealed that novel thermostable protease from Geobacillus sp. GS53 was produced, purified, and characterized for the first time. Thermostable protease could emerge as an effective candidate in various industries, especially the detergent industry since the present study has demonstrated it was stable to metal ions, organic solvents, and surfactants, and also temperature. Regarding this, the protease was very stable in the presence of surfactants and

organic solvents, exhibiting at least 81% of relative activity after 10 min of preincubation. Additionally, the protease was activated by Ca^{2+}, Cu^{2+}, and Zn^{2+}, and no metal ion could not inhibit its activity. Thermostable protease also had high thermal stability, retaining its residual activity above 85% at 85 °C even after 6 h. The enzyme had a high substrate specificity to casein, skim milk, Hammersten casein, and BSA.

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Authors' Contributions Seden Güracar Baykara: Research, methodology, data collection, and writing
Yusuf Sürmeli: Writing—review and editing
Gülşah Şanlı-Mohamed: Research conceptualization, investigation and methodology, supervision, writing—review, and editing

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Availability of data and materials Not applicable

Ethical Approval Not applicable.

Consent to Participate All authors are consent to participate in the manuscript.

Consent to Publish All authors are consent to publish the manuscript.

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