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Expression, Purification, Characterization and Bioinformatics Studies of a Novel Recombinant Alkaline Serine Protease

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Abstract

Background and Objective: Regarding the extensive industrial use of proteases, the discovery and detailed characterization of novel protease enzymes derived from native isolated microbial strains are scientifically and industrially important.

Material and Methods: In this study, a novel recombinant alkaline serine protease was heterologously expressed in *Escherichia coli* BL21. Following the expression, the enzyme was purified and subjected to biochemical characterization and *in silico* analysis.

Results and Conclusion: The purification process yielded an efficiency of 79.5% and the specific activity of the purified enzyme was assessed as 0.12 U mg^{-1}. The enzyme showed significantly thermal stability, preserving approximately 50% of its activity after 3 h of incubation at temperatures ranging 60-70 °C. Furthermore, pH stability assays demonstrated that the enzyme was still functionally stable over a wide pH range, preserving 60-80% of its residual activity after 48 h, particularly under neutral to alkaline conditions. Exposure to sodium dodecyl sulfate, hydrogen peroxide and ethylenediaminetetraacetic acid included partial effects, with almost all enzyme activity was seen. However, the presence of β-mercaptoethanol, phenylmethyl sulphonyl fluoride and NaCl significantly decreased enzymatic activity, with decreases of approximately 50%. *In silico* analysis identified a conserved peptidase S8 and thermitase-like domain spanning amino acid residues of 40 to 270. Catalytically essential residues Asp76, His109 and Ser263 were predicted within the active site. Regarding its robust stability under increased temperatures and alkaline conditions with favorable catalytic characteristics, the recombinant serine protease promises its uses in various industrial processes, compared to the commercial counterpart enzymes.

Keywords: Bioinformatics, Expression, Purification, Recombinant alkaline protease

What is "already known" on this topic:	➢ Alkaline serine proteases are considered important commercial proteases ➢ The subtilisin family is an important industrial biocatalyst widely used in the food industry ➢ Subtilisin demonstrates significant stability under alkaline pH, which is appropriate for protein hydrolysis processes
What this article adds:	➢ Resistance and activity at alkaline pH make the recombinant enzyme appropriate for use in some industries. ➢ The recombinant enzyme has good temperature resistance compared to its commercial counterpart ➢ The gene of the thermostable recombinant enzyme originates from *Bacillus mesophilus* ➢ The calcium ions are not required for activity and stability in the structure of the recombinant enzyme.

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1. Introduction

Proteases are one of the most important groups of industrial enzymes that are used in various industries [1,2]. Based on the Enzyme Commission classification (EC3.4.), proteases belong to Class 3 (hydrolases) and Subgroup 4 (hydrolyzing peptide bonds). The global market is expanding annually, accounting for approximately 60% of the total enzyme industry market [3]. Alkaline protease enzymes include several uses in fermentation, textile, leather, detergent, pharmaceutical, livestock and poultry food supplement production, lens and teeth cleaners, agriculture and food industry [1–3]. Alkaline serine proteases are the most important group of proteases used commercially [3]. Alkaline serine proteases include a molecular weight (MW) of 18–42 kDa and are active in a pH range of 6–11 at a temperature of 50–70 °C [3]. Proteases are used to produce cheese because proteases prevent the coagulation of milk by hydrolyzing casein [4]. In bakeries, proteases are used for faster dough preparation and gluten hydrolysis. The production of protein products with high nutritional value is carried out by adding microbial alkaline proteases. These protein products are vital in preparing baby foods and enriching soft drinks and fruit juices [1,5]. Researchers have reported that the use of protease in poultry feed increases the digestibility of food, improves the absorption of nutrients and digestibility of amino acids in the intestine, improves the digestibility of raw protein and amino acids, improves the growth performance of chickens and increases the food yield [6–8]. In the detergent and food industries, proteases are used, which include high thermal stability and their optimal activity is in alkaline conditions. *Bacillus* species are the major producers of extracellular proteases as microbial sources [1,2]. Since the production and extraction of commercial enzymes from wild-type microbial species include challenges, production of recombinant enzymes uses natural enzyme genes [9] and it seems necessary to identify these enzyme genes to expand the gene database for their recombinant production on an industrial scale. In addition to experimental studies, bioinformatics studies are useful for characterizing the novel enzyme and identifying its industrial use and production [4]. This study continued a previous study; in which, an alkaline serine protease was identified and its gene was cloned into an expression vector. Further investigations were carried out in this study to express and characterize the purified enzyme [9,10]. In 2018, Hadjidj et al. purified and biochemically and molecularly characterized a novel alkaline serine protease from *Bacillus* (*B.*) *licheniformis* K7A [11]. In 2018, Singh et al. isolated and characterized a serine protease enzyme from *B. subtilis* K-1 [12]. In 2013, Joshi et al. investigated the characteristics of an alkaline serine protease isolated from *B. lehensis* Strain MTCC7633 and identified its gene (BLAP), which was then expressed in *Escherichia* (*E.*) *coli* [13]. In 2012, Kaur et al. identified and isolated an alkaline protease gene from *B. circulans* MTCC 7906 and transferred and expressed the gene into *E. coli* DH5-α [14]. In 2010, Sadeghi et al. isolated an alkaline protease gene from *B. subtilis*, cloned it into the pTZ57R cloning vector and transferred it to *E. coli* Hb101 [15]. In 2021, Gurunathan et al. cloned a protease gene from a *B. cereus* strain from shallow marine hydrothermal vents located in the East China Sea and expressed the gene in the host *E. coli* BL21 [16].

In the present study, the expression parameters of the recombinant alkaline protease were optimized, followed by purification through affinity chromatography. Then, biochemical characteristics of the purified enzyme were characterized to assess its potential applicability in industrial processes. Based on experimental data and analyses carried out using ExPASy serverwith comparative modelling approaches, the three-dimensional (3-D) structure of the protein and amino acids of its active site were predicted. In this study, a novel recombinant alkaline protease was expressed and purified and its characteristics were described. The innovation of this study included its strong stability at high temperatures and alkaline conditions, as well as favorable catalytic characteristics, which are useful for use in various industrial processes and can be an appropriate alternative to its commercial types.

2. Materials and Methods

2.1. Identifying and isolating the alkaline protease gene

In a previous study, *Bacillus* sp. RAM53 was isolated by Amol Rice Research Institute and identified using biochemical and microbiological assays and molecular analysis [14]. Then, 1194 bp of the alkaline protease gene extracted from *Bacillus* sp. RAM53 was cloned in pET28a $^{+}$ [9] and used in this study for further studies. The gene analysis in Blastn showed 94% similarity with alkaline protease genes of *Bacillus* spp. Phylogenetic tree associated to the 16S rRNA sequence of *Bacillus* sp. RAM53 showed the proximity to *B. cereus* and *B. thuringiensis* [9].

2.2. Expression of the recombinant enzyme and its purification

First, a colony of recombinant bacteria was cultured overnight in 10 ml of Luria Bertani (LB) media containing kanamycin and then inoculated into 500 ml of fresh media [10]. When the bacterial growth at OD600 nm reached 0.4–0.6 in the bacterial culture media, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for induction; then, culture was

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incubated at 20 °C for 20 h. After lysis of the cell wall of bacteria using sonication, the cell extract was precipitated with 80% ammonium sulfate salt. This was centrifuged at 11,000× g for 20 min at 4 °C to separate the precipitate of proteins. After centrifugation, the protein precipitate was dissolved in 20 mM Tris buffer with pH 9. To remove excess salt from the proteins, this was dialyzed in buffer containing 10-mM imidazole using 15-kDa cut-off dialysis bag. To purify the recombinant enzyme due to the presence of a histidine sequence at the carboxyl end of the recombinant protein, cobalt sepharose-resin affinity chromatography (Arg Biotech, Iran) was used. Then, 4 ml of the protein sample were poured onto the column (1.5 × 10 cm) at a flow rate of 1 ml min^{-1}. Imidazole concentration gradient was used to elute the protein samples from the chromatography column. The elution buffer consisted of two buffers of A and B; where, Buffer A contained 50 mM Tris, pH 7 and 1% Triton X-100; and Buffer B contained 150-mM imidazole and the concentration gradient was chosen 50–100% of Buffer B. After purification, the samples containing the recombinant enzyme were filtered to separate the additional proteins from the recombinant protein using 50-kDa cut-off filtration column. Then, 10% SDS-PAGE gel electrophoresis was used to verify the presence of recombinant protein in the samples.

2.3. Western Blot Analysis

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane using transfer buffer containing 39-mM glycine, 48-mM Tris Base, 0.037% SDS and 20% methanol, and Bio-Rad Mini-Protean Tetra Cell System (BioRad, USA). Following the transfer, membranes were blocked with 5% BSA in PBS (pH 7.0) for 2 h at room temperature (RT) to prevent nonspecific binding. The membrane was then incubated with monoclonal anti-6xHis tag antibody (1:3000 dilution in PBS containing 1% Tween 20) for 2 h at RT with gentle agitation. After three washes with PBS/T, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich, USA), diluted 1:5000 in PBS/T containing BSA, at RT for 1 h with gentle shaking. Following another set of three PBS/T washes, protein detection was carried out using HRP chromogenic substrate. The enzymatic reaction was terminated by rinsing the membrane twice with distilled water (DW) [17].

2.4. Enzyme activity assay

For the standard assessment of total enzyme activity, 150 µl of the pure enzyme were mixed with 150 µl of 1% casein as substrate and 300 µl of 50 mM Tris Buffer with pH 9 and then incubated at 40 °C 30 min [10]. The absorbance after enzyme reaction at 280 nm included the absorbance of caseins broken by protease activity, which included a direct relationship with enzyme activity. Based on the definition, a unit is the quantity of enzyme that releases the fragments of peptides produced under standard conditions (0.001 A_{280} nm) within 30 min.

2.5. The effects of temperature and pH on the stability of the purified enzyme

To investigate the effect of temperature on the activity and stability of the recombinant enzyme, the enzyme cocktail was incubated at temperatures of 0, 30, 40, 50, 60 and 70 °C for 3 h and then transferred into ice for 10 min and the quantity of residual enzyme activity was assessed using the standard method [18]. To investigate the effect of various pH levels on enzyme stability, an enzyme cocktail was prepared in various buffers. Sodium citrate buffer for pH 5 and 6, Tris Buffer for pH 7, 8 and 9 and glycine buffer for pH 10, 11, 12 and 13 were used. The enzyme cocktail was incubated for 0 and 48 h at RT and then the rest of enzyme activity was assessed using the standard method [18].

2.6. The effects of inhibitors, salt and detergent on the stability of the purified enzyme

First, the pure enzyme was treated with 10-mM PMSF, 10-mM EDTA, 0.5% SDS, 0.5-M NaCl and 5% H_2O_2 for 1 h at RT. Then, residual enzyme activity was assessed using standard method.

2.7. Statistical analysis

Statistical analysis was carried out to compare the differences between the treatments and control using Duncan's method and SPSS software v.22 (IBM, USA) and a significance level was recorded at $p < 0.05$.

2.8. Bioinformatics studies

Briefly, Blastn (NCBI) was used for the gene sequence alignment of the recombinant enzyme. The protein sequence information was used to identify the homologous protein sequences in Blastp (protein-protein BLAST). Protein sequences that were more than 80% identical to the recombinant enzyme sequence and originated from the *Bacillus* genus were used for further analysis. The position and types of amino acids present in the active site of the recombinant enzyme and its active domains were assessed using Merops-the Peptidase Database (https://www.-ebi.ac.uk/merops/), ProScan (http://www.ebi.ac.uk/-interpro/result/InterProScan/) and BLAST (https://blast.-ncbi.nlm.nih.gov/Blast.cgi) databases. The number of partial amino acids, MW and theoretical pI, instability index (II) and aliphatic index were computed using ProtParam and the protein sequence (https://web.expasy.org/protparam/).

The 3D structure of the protein was constructed using Swiss-model and the comparative modelling, corresponding to its amino acid sequences and a template selected from PDB with further protein sequence identity. The amino acids present in the active site of the enzyme were identified in the structure. Structure assessment of the model in Swiss-

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Model was carried out using alignment, Ramachandran plot and QMEAN (qualitative model energy analysis) local score (https://swissmodel.expasy.org/qmean/RzEH8V).

3. Results and Discussion

The recombinant enzyme was purified through cobalt affinity chromatography and the purification efficiency was assessed as 79.5%. The graph associated to purification is seen in Figure 1. The single long peak was associated to the release of the recombinant enzyme, which was eluted from the column at a concentration gradient of 90–100% elution buffer. The purified recombinant enzyme concentration included 130 µg ml^{-1}. The specific activity of the purified recombinant enzyme was 0.12 U ml^{-1} mg$^{-1,}$ and the specific activity of the recombinant enzyme before purification was 0.066 U ml^{-1} mg^{-1}. The enzyme samples extracted from the cell extract after induction, as well as the same sample after concentration with ammonium sulfate salt and the purified recombinant enzyme sample, were electrophoresed on 10% SDS-PAGE gels. Western blot analysis with anti-His tag antibodies revealed a distinct band at approximately 41 kDa, corresponding to the expected MW of the recombinant peptide. The six-His-tag, engineered at the C-terminal end of the recombinant protein for affinity purification, was still intact and was not cleaved from the peptide, indicating successful expression and detection of the full-length His-tagged recombinant protein (Figure 2). The three major elements in the successful production of recombinant protein included host condition, culture media condition and vector type. One of the appropriate microbial strains for exogenous gene expression was the *E. coli* strain [19].

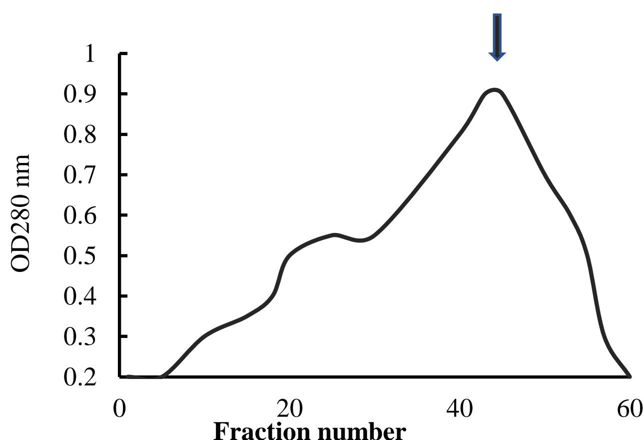

Fig. 1. The graph is relevant to the purification of the recombinant enzyme. The long peak is related to the recombinant enzyme, which is shown by the arrow

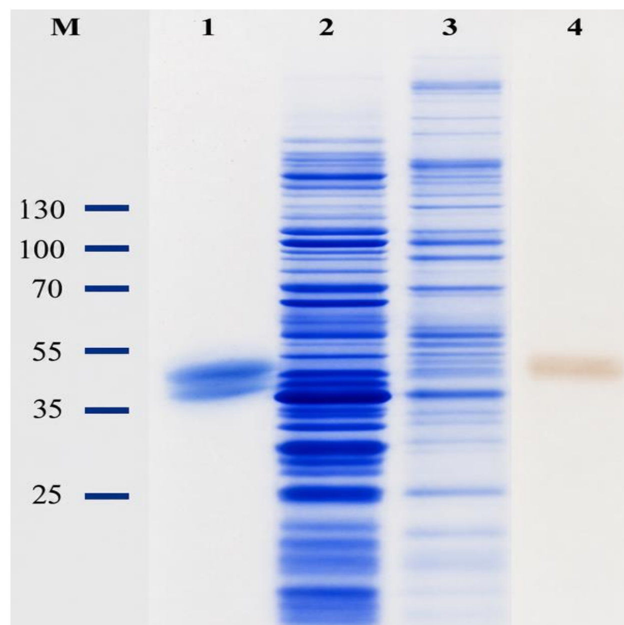

Fig. 2. SDS-PAGE gel before and after purification of the recombinant enzyme and Western blot analysis. M, Protein Molecular Weight Marker (MyBioSource); Line 1, the band corresponding to the purified enzyme at about 40 kDa; Line 2, sample of cell extract extracted after induction; Line 3, sample of cell extract extracted after concentration with 80% ammonium sulfate salt; Line 4, distinct band at approximately 41 kDa, corresponding to the expected size of the full-length His-tagged recombinant enzyme after Western blot analysis.

The activity of the recombinant enzyme was assessed before and after purification. The quantity of absorbance after enzyme reaction at 280 nm included the absorbance of caseins broken by protease activity, which included a direct relationship with enzyme activity. Based on the definition, a unit is the quantity of enzyme that releases the fragments of peptides produced under standard conditions (0.001 A_{280} nm) within 30 min. In the present study, a novel recombinant serine alkaline protease derived from the native strain of *Bacillus* sp. RAM53 was expressed in a laboratory environment. The culture conditions were optimized for optimal production of this recombinant enzyme. The recombinant enzyme gene and protein with the scientific name of subtilase-like protease (MARF) were deposited in GenBank with accession numbers of OP922123.1 (https://www.ncbi.nlm.nih.gov/nuccore/OP922123.1) and WCO70948.1, respectively. The pure enzyme did not decrease its activity; instead, it increased its specific activity.

The specific activity of the purified recombinant enzyme was 0.12 U ml^{-1} mg$^{-1,}$ and the specific activity of the recombinant enzyme before purification was 0.066 U ml^{-1} mg^{-1}. The concentration of the recombinant enzyme was assessed based on Bradford's standard method using a standard curve. The concentration of the recombinant enzyme was 0.3 mg ml^{-1} before purification and 0.13 mg ml^{-1} after purification.

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Results of the thermal stability of the pure enzyme at various temperatures showed that the pure recombinant enzyme included relatively good stability for 3 h at 30 and 40 °C; hence, more than 70% of its activity were included. At 70 °C, 46% of the enzyme activity remained (Fig. 3). At all temperatures, a significant difference from the control was observed ($p<0.05$). The recombinant enzyme demonstrated relatively good stability at high temperatures such as 60 and 70 °C without addition of stabilizing ions, compared to commercial enzymes. The subtilisin family of serine proteases represent an important class of industrial biocatalysts widely used in the food industry [3]. Their extensive use is largely attributed to their favorable biochemical and catalytic characteristics. In addition, their high thermostability allows them to preserve activity under increased temperatures associated to industrial heat treatments [3,5,8,20]. The recombinant enzyme of the present study included good temperature resistance, compared to commercial enzymes.

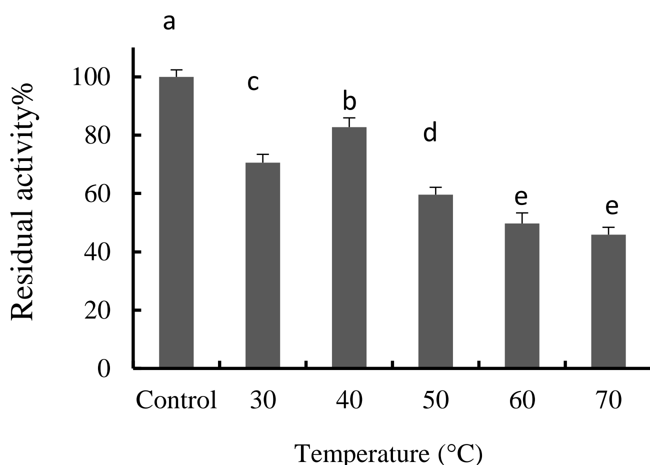

Fig. 3. Graph of enzyme stability at different temperatures after 3 hours of incubation. The common letters illustrate no statistically significant difference (P<0.05)

Results of the stability to various pHs showed that the enzyme was stable at various pHs after 48 h of treatment, and its residual activity included 60–80% at neutral to alkaline pHs (Figure 4).

At all pHs, a significant difference with the control was observed ($p < 0.05$), and it showed similar stability at neutral to alkaline pHs, except for pH 9, and the highest stability was reported at pH 9. Subtilisin typically demonstrated significant stability under alkaline pH conditions, making it particularly appropriate for protein hydrolysis processes commonly used in food manufacturing [3,21].

Aryaei et al. identified and isolated an alkaline protease gene from a native strain of *Bacillus* sp. RAM and cloned the gene into pET28a+. The enzyme showed maximum activity at pH 9 and 40 °C [10]. Moradian et al. purified an alkaline serine protease from *Bacillus* sp. HR-08 and the protease activity was at pH 10 and 60 °C [18].

A thermostable serine-protease enzyme from *Aeribacillus pallidus* c10 strain was purified by Yildirim et al. and the optimal activity of this enzyme was at 60 °C and pH 9 [22]. *Bervibacillus brostelensis* isolated from a hot spring in Qeinerjeh produced an alkaline protease and showed maximum activity at 60 °C and pH 9 [23]. Alkaline proteases produced by *Bacillus* species are greatly important due to their thermal stability and stability at various pH levels, which are important in industries [1,24].

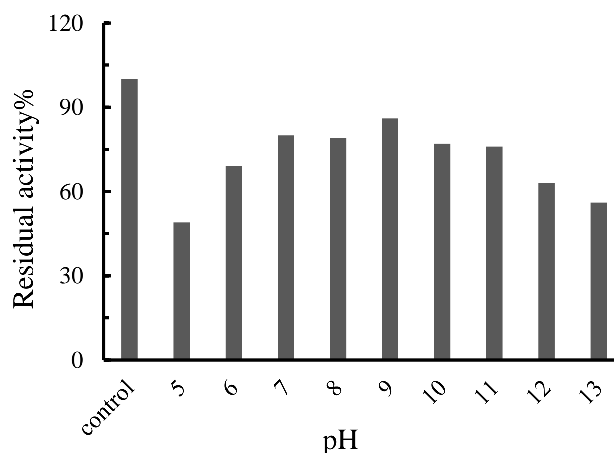

Fig. 4. Graph of enzyme stability at different pH levels after 48 hours of incubation. The common letters illustrate no statistically significant difference (P<0.05)

Results of pure enzyme treatment with 10-mM PMSF, 10-mM EDTA, 0.5% SDS, 0.5-M NaCl and 5% H_2O_2 after 1 h are reported in Figure 5. The EDTA did not affect the activity, and beta-mercaptoethanol decreased the activity of the enzyme a little with no significance ($p < 0.05$). The PMSF and NaCl decreased the enzyme activity by nearly 50% with significance ($p < 0.05$). In the presence of SDS and H_2O_2, almost all enzyme activity was preserved.

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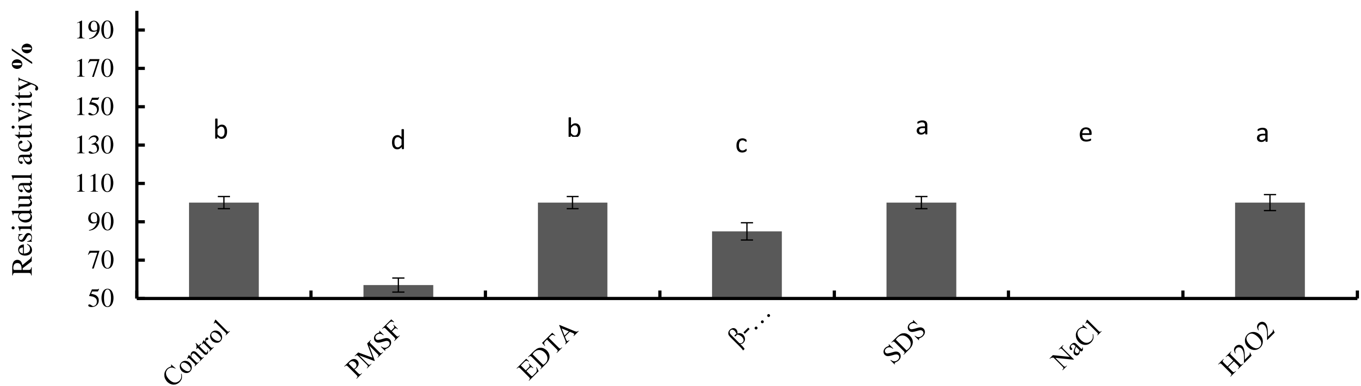

Fig. 5. Graph of enzyme stability in different treatments after 1 hour of incubation. The common letters illustrate no statistically significant difference ($P<0.05$)

Several members of the S8 family (e.g. subtilisin) bind calcium for stability; inhibition can be seen with EDTA and EGTA, which are often reported as specific inhibitors of metallopeptidases. Calcium binding stabilizes these proteins in natural extracellular environments but poses a problem in industrial environments containing high concentrations of metal chelators. Subtilisin *Carlsberg* possesses three Ca^{2+} binding sites that stabilize its 3-D structure. The important industrial enzymes, subtilisin Carlsberg and subtilisin BPN, respectively included thermostability of 3.4 and 2.4 min at 50 °C and their thermal stability enhanced in presence of calcium [25,18]. These proteases are addressed as important commercial proteases and, therefore, the recombinant enzyme of the present study includes good temperature resistance, compared to commercial enzymes. In addition, the gene of this enzyme originates from *B. mesophilus*. A serine protease gene of *B. megaterium*, ispK, was cloned into *E. coli*. The thermostability increased 32.9-fold from 3.3 to 108.5 min at 60 °C in the presence of 2 mM of Ca^{2+} [26]. In the present study, the recombinant enzyme activity did not decrease in the presence of a chelating agent, resulting in calcium ions not neccessary for activity and stability in the structure. This characteristic is important for use in industry. *Bacillus* proteases have been shown stable in a wide range of pH and temperature [27]. A serine alkaline protease purified from *Bacillus* sp. HR08 was stable to hydrogen peroxide and its activity was complete [18].

The recombinant enzyme protein with the scientific name of subtilase-like protease was deposited in GenBank with the accession no. of WCO70948.1 (https://www.ncbi.nlm.nih.gov/protein/WCO70948.1). Based on the sequence alignment of S8 family peptidase (Bacillus) using Blast, NCBI, a sequence with reference number WP_000790938.1 was selected, which had nearly 86% identity to the recombinant enzyme sequence (Table 1). Then, the information on the amino acids of its active site was used to find amino acids in the active site of the recombinant alkaline serine protease. Based on the alignment and sequence homology, which included 86% identity with the subtilase family and available information on the active amino acids in the active site of the enzymes of this family, aspartic acid, histidine and serine (MEROPS - Peptidase Database) were assessed for the active site of the recombinant enzyme. The S8 termitase-like peptidases region is located at amino acids 40 to 270 in the recombinant enzyme.

Table 1. Sequences producing significant alignments of alkaline serine proteases represent different *Bacillus* species retrieved from BLASTp-NCBI.

Accession	Description	Query Cover	Per. Identity	Length
WCO70948.1 (Query)	alkaline serine protease *Bacillus sp*. (in: *firmicutes*)	100%	100%	296
MDA2070431.1	S8 family peptidase *Bacillus cereus*	97%	86.55%	397
WP_000790938.1	S8 family peptidase *Bacillus*	97%	86.21%	397
WP_086395645.1	S8 family peptidase *Bacillus thuringiensis*	97%	86.21%	397
WP_200458572.1	S8 family peptidase *Bacillus* (in: *firmicutes*)	97%	86.21%	397

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In sequence analysis using ProScan (http://www.ebi.ac.uk/interpro/result/InterProScan/), the type of protease from the S8 subtilase family and the presence of a domain similar to thermitase were verified (Figure 6a). In addition, presence of Asp76, His109 and Ser263 in the active site of the enzyme was verified using ProScan and PROSITE (Figures 6b and 6c). Based on ProtParam prediction, the instability index (II) was computed as 18.38. This classified the recombinant protein as stable for more than 10 h in *E.coli* expression system. The aliphatic index of the recombinant enzyme was assessed as 71.52 as a thermostable protein. Several important subtilisins have been produced using various bacilli, including subtilisin Carlsberg from *B. licheniformis*; subtilisin BPN and Dj-4 from *B. amyloliquefaciens*; subtilisin E, NAT and J from *B. subtilis*; and subtilisin amylosacchariticus from *B. amylosacchariticus* [27]. The alkaline protease gene (Apr) from *B. licheniformis* 2709 was cloned into an expression vector of pET28b (+) and expressed in a high-expression strain of *E. coli* BL21 [29]. The recombinant enzyme included identities with S8 family peptidase from *B. cereus*, S8 family peptidase from *Bacillus sp*., S8 family peptidase from *B. firmicutes* and S8 family serine peptidase from *B. thuringiensis* as 86, 86, 86 and 85%, respectively. The subtilisin family is one of the largest serine peptidase families characterized to date. Structures have been assessed for several members of the subtilisin family that contain similar catalytic triad residues in an order of DHS (Asp, His, Ser) (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR034202/). Subtilisin possess broad substrate specificity, enabling efficient degradation of diverse protein substrates and demonstrates strong catalytic efficiency, which contributes to reduce processing times and improved production yields. Collectively, these characteristics highlight the significant potential of S8/thermitase-like proteases in various food processing uses [3,21]. The tertiary structure of the present alkaline serine protease was made based on a template selected by the Swiss model building. The template included thermitase with PDB code 1thm.1A, as well as sequence identity of 85% (Figure 7a). In Ramachandran plot, distribution of the backbone dihedral angles (ϕ and ψ) showed that a majority of residues were located in the most favored regions (dark green areas), primarily corresponding to α-helical and β-sheet conformations. A smaller fraction of residues was set within additionally allowed regions (light green areas), which was acceptable for certain amino acids in specific conformations. Overall, the plot suggests that the protein model includes good stereochemical quality, with most residues adopting energetically favorable conformations and a minimal number of outliers that are unlikely to affect the structural integrity significantly (Figure 7b). The local quality assessment in QMEAN, expressed as the predicted local similarity to the target, showed that the majority of residues included high similarity scores (> 0.8), indicating reliable modelling in most structural regions. Peaks close to 0.9–1.0 suggested well-defined secondary structures with high confidence in backbone conformation. However, a few distinct regions particularly near residues of 25–30 and 235–245 showed significantly lower quality scores (< 0.6), with the lowest approaching 0.3. These decreases often corresponded to loop regions, flexible termini or poorly conserved areas in the template alignment. Overall, the QMEAN profile supported a generally reliable global structure, with local weaknesses restricted to specific flexible or unresolved segments (Figure 7c). Using experimental data and ExPASY analysis as well as comparative modelling, this study predicted the protein 3-D structure and active site amino acids. Assessmentn of the predicted 3-D structure using Ramachandran plot revealed that a majority of residues were located in the most favored regions, with only a few residues in additionally allowed areas and a minimal number in disallowed regions. This distribution indicated that the backbone dihedral angles were largely in stereochemically favorable conformations, supporting the overall correctness of the model [29]. Collectively, these results suggest that the predicted protein model possesses good overall stereochemical quality and global reliability.

SWISS-MODEL is a fully automated protein structure homology-modelling server, accessible via the Expasy web server or DeepView program (Swiss Pdb-Viewer). Homology modelling allows the protein engineer to design modifications of a protein before the determination of the 3-D structure using X-ray technique. Of all current theoretical approaches, comparative modelling is the best method that can reliably generate a 3-D model of a protein from its amino acid sequence [30,31]. The alkaline protease from thermophilic bacteria included an aliphatic index of 69.4 [32], while that of the recombinant enzyme was assessed as 71.52, showing that the recombinant enzyme included higher aliphatic index than the thermophilic type and thermal stability despite its mesophilic origin, compared to other thermophilic enzymes. This index should be addressed as a positive factor for increasing the thermal stability of globular proteins [33].

More than one million proteolytic enzyme sequences have been recorded in the Merops Database [34]. Therefore, identifying novel strains with the ability to produce enzymes appropriate for industrial uses and genes of these enzymes for the production of recombinant enzymes has always been useful. Especially in countries that buy industrial enzymes, this decreases the purchase costs. In addition to laboratory studies, bioinformatics studies for characterizing alkaline proteases help identify their industrial use and large-scale production. Therefore, protein-structure prediction tools are appropriate for the study of protein structures and functions [35].

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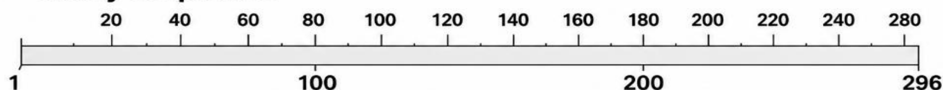
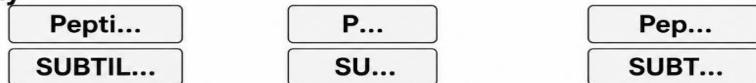
A)

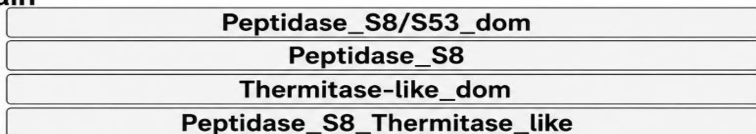
A Query sequence



B Family



C Domain

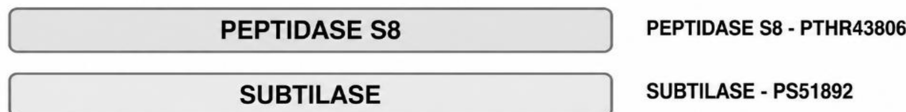


D Homologous Superfamily

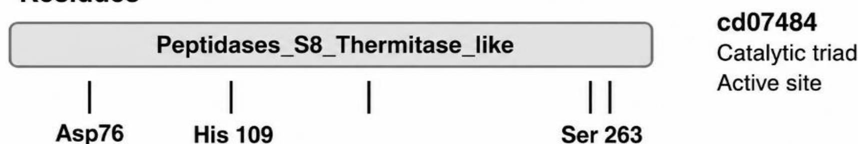
B)

A Active Site

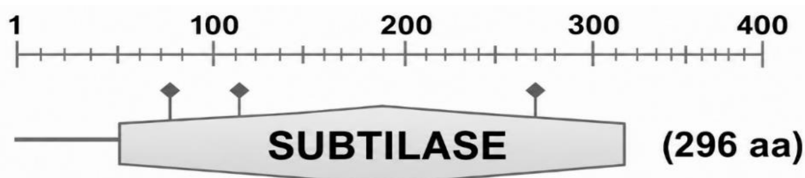
B Unintegrated



C Residues



C)



PS51892 | SUBTILASE *Serine proteases, subtilase domain profile*

Predicted features:

FEATURE	FROM	TO	DESCRIPTION	CONDITION
DOMAIN	49	296	Peptidase S8	—
ACT_SITE	76	—	Charge relay system	[condition: D]
ACT_SITE	109	—	Charge relay system	[condition: H]
ACT_SITE	263	—	Charge relay system	[condition: S]

Fig. 6. Domains and the presence of amino acids in the active site of the recombinant enzyme were determined by ProScan. A) Peptidase S8 and Thermitase domains, B) the amino acids Asp76, His109, and Ser263 in the active site of the enzyme, C) the conserved domains in the structure of the recombinant enzyme in the Prosite database

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Fig. 7. The tertiary structure of the alkaline serine protease was generated using the Swiss Model building software. A) The amino acids Asp76, His109, and Ser263 in the active site of the enzyme were determined from the structure. B) Ramachandran Plot, the distribution of backbone dihedral angles (ϕ and ψ) for amino acids, indicating stereochemical correctness. C) QMEAN Local Quality Estimate**,** the local quality assessment in QMEAN, expressed as the predicted local similarity to the target, shows that the majority of residues have high similarity scores (>0.8), indicating reliable modelling in most structural regions.

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4. Conclusion

The current study focused on the biochemical characteristics and tertiary structure, physicochemical characteristics and catalytic potential of a novel recombinant alkaline serine protease. Despite lack of sufficient funding to sequence the N-terminal region of the purified protein that was a limitation of the present study, structural engineering methods can be used to improve enzyme production for the production of a novel high-quality recombinant enzyme on an industrial scale by predicting the tertiary structure of this enzyme using bioinformatics. Given its favorable biochemical characteristics, including pH stability over the range of 6–13, thermal stability at 60 °C, independent of Ca^{2+} and resistance to SDS and oxidizing agent of H_2O_2 with its classification as an alkaline protease, the enzyme demonstrates strong potential for use in various industrial sectors such as detergent, food and animal and poultry feed productions.

5. Declaration

5.1. Acknowledgements

This study was the result of a research project associated with the student core project of Sari University of Agricultural Sciences and Natural Resources. The authors of the article thank the Research Vice-Chancellor of Sari University of Agricultural Sciences and Natural Resources for providing financial help.

5.2. Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

5.3. Funding

This study was funded by Sari Agricultural Sciences and Natural Resources University.

5.4. Financial interests

The authors declare no financial interests.

5.5. Declaration of competing interest

The authors have no competing interest to declare that are relevant to the content of this article.

5.6. Authors' Contributions

F. Moradian contributed to the study's conception and design. Material preparation, data collection and analysis were carried out by O. Rezaie, Sh. Shafieyan and F. Norani. The first draft of the manuscript was written by F. Moradian, O. Rezaie, Sh. Shafieyan and F. Norani. F. Moradian commented on previous versions of the manuscript and edited the final version. All authors read and approved the final manuscript.

5.7. Consent to Participate

Informed consents were collected from all participants of this study.

5.8. Using Artificial Intelligent Chatbots

In this article, artificial intelligence was used to improve the abstract and some sentences. Artificial intelligence was also used to improve the quality of some images.

5.9. Ethical Consideration

In this study, a native strain isolated from the Mazandaran region was used, and all ethical considerations related to studying this strain in the laboratory were taken into account.

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بیان، خالص‌سازی، تعیین ویژگی و مطالعات بیوانفورماتیک سرین پروتئاز قلیایی نوترکیب جدید

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چکیده

سابقه و هدف: با توجه به کاربرد گسترده صنعتی پروتئازها، کشف و تعیین ویژگی دقیق آنزیم‌های پروتئاز جدید مشتق‌شده از سویه‌های میکروبی بومی، از اهمیت تحقیقاتی و صنعتی قابل توجهی برخوردار است.

مواد و روش ها: در این مطالعه، یک سرین پروتئاز قلیایی نوترکیب جدید به صورت هترولوگ در *اشرشیا کلی* BL21 بیان شد. پس از بیان، آنزیم خالص‌سازی شده و تحت بررسی‌های بیوشیمیایی و آزمون این سیلیکو قرار گرفت.

یافته‌ها و نتیجه‌گیری: فرآیند خالص‌سازی، راندمان ۷۹/۵٪ را به همراه داشت و فعالیت ویژه آنزیم خالص‌سازی شده ۰/۱۲ واحد بر میلی‌گرم تعیین شد. آنزیم پایداری حرارتی قابل توجهی از خود نشان داد و تقریباً ۵۰٪ از فعالیت خود را پس از ۳ ساعت گرمخانه گذاری در دماهای ۶۰ تا ۷۰ درجه سانتی‌گراد حفظ کرد. علاوه بر این، سنجش‌های پایداری pH نشان داد که آنزیم در طیف وسیعی از pH از نظر عملکردی پایدار باقی می‌ماند و ۶۰ تا ۸۰ درصد از فعالیت باقیمانده خود را پس از ۴۸ ساعت، به‌ویژه در شرایط خنثی تا قلیایی، حفظ می‌کند. قرار گرفتن در معرض SDS، پراکسید هیدروژن و EDTA اثرات ناچیزی داشت و تقریباً تمام فعالیت آنزیم در حضور آن‌ها باقی ماند. با این حال، حضور β-مرکاپتواتانول، PMSF و NaCl فعالیت آنزیمی را به طور قابل توجهی کاهش داد و تقریباً ۵۰٪ کاهش یافت. آزمون و تحلیل این سیلیکو یک پپتیداز S8 حفاظت‌شده و دومین شبه‌ترمیتاز را شناسایی کرد که شامل واحدهای اسید آمینه ۴۰ تا ۲۷۰ بود. واحدهای اصلی کاتالیزوری Asp76، His109 و Ser263 نیز در جایگاه فعال پیش‌بینی شدند. با توجه به پایداری قوی آن در دماهای بالا و شرایط قلیایی، همراه با خواص کاتالیزوری مطلوب، در مقایسه با آنزیم‌های تجاری مشابه، سرین پروتئاز نوترکیب نویدبخش استفاده در فرآیندهای صنعتی مختلف است.

واژگان کلیدی: بیوانفورماتیک ▯ بیان ▯ خالص‌سازی ▯ پروتئاز قلیایی نوترکیب

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