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Molecular cloning and expression of a novel mesophilicalkaline protease from *Bacillus* sp. L010 in *Escherichia coli*

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Abstract: [**Objective**] Bacteria producing proteases were isolated and selected from the environment and the alkaline proteases with superior performance for commercial exploitation were screened. [**Methods**] The strain producing extracellular proteases was isolated by a casein plate and was identified by biochemical and morphological tests and by 16S rDNA sequence analysis. To acquire the open reading frame (ORF) of the protease, degenerate primers designing and genome walking method were used. The precursor and mature peptide of the protease were recombinant expressed in BL21 (DE3). After purification of the active protease, the characteristics and the catalytic ability were detected using synthetic peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the substrates. [**Results**] Strain L010 isolated was named as *Bacillus* sp. L010 after identification. The ORF of the protease was 1149-bp long and encoded a protein of 382 amino acids comprised with a 30-residual signal peptide, a 77-residual propeptide, and a 275-residual mature protein, and the encoded protein was one of subtilisins-a member of serine proteases and designated as SprD. The precursor of SprD (pro-SprD) autoprocessed into active SprD mediated by the propeptide when pro-SprD was recombinant expressed in BL21 (DE3). The enzyme exhibited high catalytic efficiency (K_{cat}/K_m) towards synthesis substrates with optimal activity at 70℃ and pH 9 – 10. SprD was stable over a range of pH 7.0 to 10.0 and was thermal stable at 25℃ – 60℃. [**Conclusion**] The high stability of SprD towards alkaline conditions (pH 7 – 10) and under temperature 25℃ – 60℃ and the high catalytic efficiency suggested that the protease would find research value and potential applications.

Keywords: autoprocessing, *Bacillus* sp. L010, BL21 (DE3), expression, PCR, SprD
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Subtilisins, a member of serine proteases, are produced by a diverse range of organisms including achaea, bacteria, fungi, yeasts, higher eukaryotes and even viruses[1]. Recently, subtilisins attract great

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attention based on their ubiquitous presence, non-pathogenicity, easier to isolate and most importantly, their high stability and proteolytic activity under alkaline conditions[2]. Subtilisins were applied in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation[3]. A number of *Bacillus*-*derived* subtilisins such as subtilisin E[4], M-protease[5], DFE[6] had been purified and characterized. According to the difference of the pH value, subtilisins are divided into alkaline enzymes with an optimal pH value of 9 – 10 and high-alkaline enzymes with an optimal pH value of 10 – 11[3]. These *Bacillus*-*derived* alkaline proteases are stable at elevated temperatures and pH, but the majority is incompatible with detergent matrices[2]. Therefore, alkaline proteases with superior performance for commercial exploitations are being sought and further exploration of protease producers and new gene resources were warranted. In addition, vitro expression of subtilisin such as in model organism *E. coli* helps us to better utilize and study about its activity mechanism.

In this study, the strain *Bacillus* sp. L010 producing extracellular alkaline protease was isolated by our labratory. After cloning the novel gene of SprD, we expressed the protease in BL21 (DE3). SprD could only fold into active conformation with the help of propeptide *in vivo* and the propeptide acted as an intramolecular chaperone (IMC) that organizing the correct folding of mature domain. In addition, the polyclonal antibody against mature SprD was prepared and applied, and it was suggested that signal peptide and propeptide of SprD were automatically cleaved before the mature protease was secreted out from L010. Relevant properties of mature SprD were also studied.

1 Materials and Methods

1.1 Bacterial strain, plasmids and media

Bacillus sp. L010 producing extracellular protease was isolated by our labrotary. DH5α (Novagen) was used as clone strain. BL21 (DE3) (Novagen) was used as bacterial host for expression recombinant protein. PMD™ 19-T (Takara) was used as the subcloning plasmid. The plasmid pET28a (Novagen) was used as expression vector. The medium used for expressing protein was LBK containing (1 L) 10 g peptone, 5 g yeast extract, 10 g NaCl, and 50 mg kanamycin.

1.2 Strain L010 isolation and identification

Strain L010 producing extracellular protease was isolated from the soil by a casein plate containing (W/V) KH_2PO_4 0.036%, $MgSO_4 \cdot 7H_2O$ 0.05%, $ZnCl_2$ 0.0014%, $Na_2HPO_4 \cdot 7H_2O$ 0.107%, NaCl 0.016%, $CaCl_2$ 0.0002%, $FeSO_4$ 0.0002%, Casein 0.4%, Peptone 0.005%, Agar 2%. L010 was identified by biochemical and morphological tests and by 16S rDNA gene sequence analysis[7-8]. The bacterial universal primers were given in Supplemental Table 1 and the phylogenetic tree was constructed and given in Supplemental Figure 2. The 16S rDNA sequence has been deposited in GenBank under accession number KC153301.

1.3 Gene cloning and sequence analysis

The genome of strain L010 was extracted as described before[9]. Degenerate primers DP1/DP2 were designed to amplify part of the protease gene (SprD). The touch down PCR programme was : a cycle of 94℃ for 5 min; 5 cycles of 94℃ for 30 seconds, 50℃ for 30 seconds, and 72℃ for 1 min; 5 cycles of 94℃ for 30 seconds, 47℃ for 30 seconds, and 72℃ for 1 min; 20 cycles of 94℃ for 30 seconds, 45℃ for 30 seconds, and 72℃ for 1 min; and a cycle of 72℃ for 5 min. The polymerase was Ex *Taq* (Takara). The product was sequenced when it was ligated to subcloning plasmid pMD™19-T.

To determine the complete sequence of SprD, genome walking method coupled with semi-nested PCR was taken and the long and short adapter were designed (Supplemental Table 1)[10]. *Dra* Ⅰ, *Eco*RⅤ, *Pvu* Ⅱ, and *Sma* Ⅰ were used as restriction enzymes to digest the genomic DNA of L010, and GWL1 (*Dra* Ⅰ genome walking library), GWL2 (*Eco*R Ⅴ genome walking

library), GWL3 (*Pvu* Ⅱ genome walking library), and GWL4 (*Sma* Ⅰ genome walking library) used as the templates for cloning whole sequence SprD were acquired after being ligated to genome walking adapters.

The whole nucleotide sequence of SprD has been deposited in GenBank under accession number KC153302. The deduced amino acid sequence was compared with those of close proteases obtained from the GenBank.

1.4 Protein expression and purification

After subcloning, target fragments from the subcloning plasmids were inserted into pET28a. Primers and restriction sites were given in Supplemental Table 1. Plasmids (including pro-protein and mature peptides, respectively) were designated as pET28-prosprD and pET28-msprD and then transformed into BL21 (DE3). BL21 (DE3) harbouring recombinant plasmids was grown to A_{600} 0.6 - 0.8 in LBK at 37℃, then induced to produce target protein by adding IPTG to a final concentration of 0.3 mmol/L and the incubation was continued overnight at 37℃ or at 21℃. The cells were harvested and washed twice with buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.0 before they were stored at -80℃.

The his-tagged recombinant protein in the soluble fraction was purified by the metal-chelated affinity chromatography according to the Ni-NTA native purification system (Invitrogen). The insoluble fraction was purified by gradient urea elution method and the buffers used were 50 mmol/L $NaH_2PO_4 \cdot 2H_2O$/Urea, pH 7.0. The concentration was measured with BCA™ protein Assay kit (Thermo). The secreted enzymes from L010 were collected as followings. Cell-free supernatant of L010 was collected after 24 h of cultivation. PEG20000 was used in the dialysis to concentrate the enzymes. 100% trichloroacetic acid (TCA) was added to reach a final concentration of 10% to precipitate the protein by centrifugation at 12000 r/min for 15 min. The precipitate was washed with 100% acetone 4 - 5 times, air-dried.

1.5 Preparation for polyclonal antibody against SprD

The fusion protein msprD (>1 mg/ml) as pure as 90% after emulsification mixed with Freund's adjuvant (Sigma) was employed as antigen to immunize healthy male rabbits by three immunizations. The amount of antigen every immunization two weeks separate was about 1 mg.

1.6 SDS-PAGE, Western blot and ELISA

SDS-PAGE was performed on a 5% stacking and a 15% running gel, and protein bands were visualized by staining with Coomassie Brilliant Blue G250. For Western blot analysis, protein was subjected to SDS-PAGE, and then electrically transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% BSA in TBST (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% (V/V) Tween 20) at 37℃ for 2 h, washed 5 × 10 min with TBST, and incubated with the diluted polyclonal antibody serum against msprD in TBST at 37℃ for 1 h. The blot was washed 5 × 10 min with TBST, incubated with the diluted goat anti-rabbit IgG (Millipore) conjugated to HRP (Horseradish Peroxidase) in TBST at 37℃ for 1 h. After the blot washed 5 × 10 min with TBST, it was visualized through X-ray film exposure or DAB kit (CWBIO, China). For ELISA analysis, the steps were according to the indirect Elisa protocol of Abcam. The substrate for HRP was TMB (3, 3′, 5, 5′-teteamethylbenzidine), the process was stopped by adding 2 mol/L H_2SO_4 and the optical density was read at 450 nm.

1.7 Effects of temperature, pH on enzyme activity and stability

For synthetic peptide substrates succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF) (Sigma), assays were performed as described previously[11]. The amount of released *p-nitroaniline* was measured by the absorbance at 410 nm. Activity was calculated as units/mg of protein. One unit is defined as the activity releasing 1 μmol of *p-nitroaniline*/min. For measuring the K_m, the substrate concentrations were varied and the enzymatic activity was estimated by monitoring the release of *p-*

nitroaniline through changes in absorbance at 410 nm. Readings were collected at 10-s time intervals to estimate the reaction velocity.

The optimal temperature for this protease against AAPF was determined by incubating the reaction mixture (750 μL) containing 50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, 50 μg AAPF, pH 8.0 at different temperature (25℃ - 90℃) for 3 min. Reaction was stopped by adding 250 μL of 2 mol/L Sodium citrate (pH 5.0) to the reaction mixtures, and the amount of released *p-nitroaniline* was measured. The relative enzyme activity was calculated as percentage of the maximal activity. Thermal stability assay was conducted by incubating equal amount of enzyme solution in 50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, pH 8.0 at various temperatures (25℃ - 60℃) for 120 min. The residual activity was calculated as percentage of the starting activity.

The effect of pH 5.5 - 12 on hydrolytic activity against AAPF was measured at 37℃ using (750 μL) 50 mmol/L of Sodium phosphate (pH 5.5 - 7.0)/ Tris-HCl (pH 8.0 - 9.0)/glycine-NaOH (pH 10.0 - 11.0)/Na_2CO_3-NaOH (pH 12.0), 1 mmol/L $CaCl_2$, 50 μg AAPF as the reaction buffers. To assess its pH stability, active SprD was pre-incubated in buffers of different pH values at 25℃ for 6 h prior to determination of the residual activities. Residual activity was calculated as percentage of the enzyme at pH 8.0 and 25℃ in 50 mmol/L Tris-HCl buffer.

2 Results and Discussion

2.1 Microorganism

The strain L010 producing extracellular protease was isolated by a casein plate and the hydrolysis halos of the protease were found after 24 hours at 37℃ (Supplemental Figure 1). L010 was identified by biochemical and morphological tests and by 16S rDNA gene sequence analysis, the results was shown in Supplemental Table 2 and Figure 2. L010 was affiliated with the genus *Bacillus* and closely related to *Bacillus amyloliquefaciens* and *Bacillus subtilis* (99% sequence identity) (Supplemental Figure 2-B) and was named as *Bacillus* sp. L010.

2.2 Cloning and gene structure of SprD from L010

In this study, degenerate primers DP1 and DP2 listed in Supplemental Table 1 were used to amplify part of the protease, which resulted a 470bp DNA identified to be homologous to Apr serine protease (*Bacillus subtilis* FP-133). The full length gene was obtained by genome walking method to amplify the flanking regions using the primers (genome specific primers GSP and adaptor primers AP) listed in Supplemental Table 1, which resulted a 1318 bp DNA fragment including an open reading frame (1149 bp) (Figure 1-A) and its promoting sequences (169 bp) (Figure 1-B).

The DNA open reading frame encoded a protein composed of 382 amino acids (Accession No. KC153302). Nucleotide sequences in GenBank revealed that SprD was a novel gene and had a similarity of 99% with alkaline protease (AFH88394), AprE3 - 17 (ACU32756) and AprE51 (ACA34903), 85% - 86% similarity with the model proteins coupled with propeptides-Subtilisin E (CAA74536), Subtilisin (ZP _ 10165313) and Subtilisin (ACJ07037). The alignments in amino acids showed that the site in 61 in the vicinity of the catalytic triad (Asp32, His64, Ser221) was composed of different amino acids (Arg in SprD, Asn in AprE3 - 17, Asn in AprE51, Gly in other Subtilisins) (Figure 1-C). Strain L010 showed synonymous codon bias in protein expression level. After analyzed with Signal P 3.0, the first 30-residues was a signal peptide, followed by a propeptide with 77-residues, and a mature peptide with 275-residues with molecular mass of 27.5 kDa and isoelectric point (pI) of 8.0. The complete gene sequence started with a GTG codon different from most of other serine proteases and ended with a TAA codon. The potential promoting sequence upstream of the GTG codon was also identified and AGGAGTGG might be the Shine-Dalgarno sequence (Figure 1-B) a little different from other proteases[8].

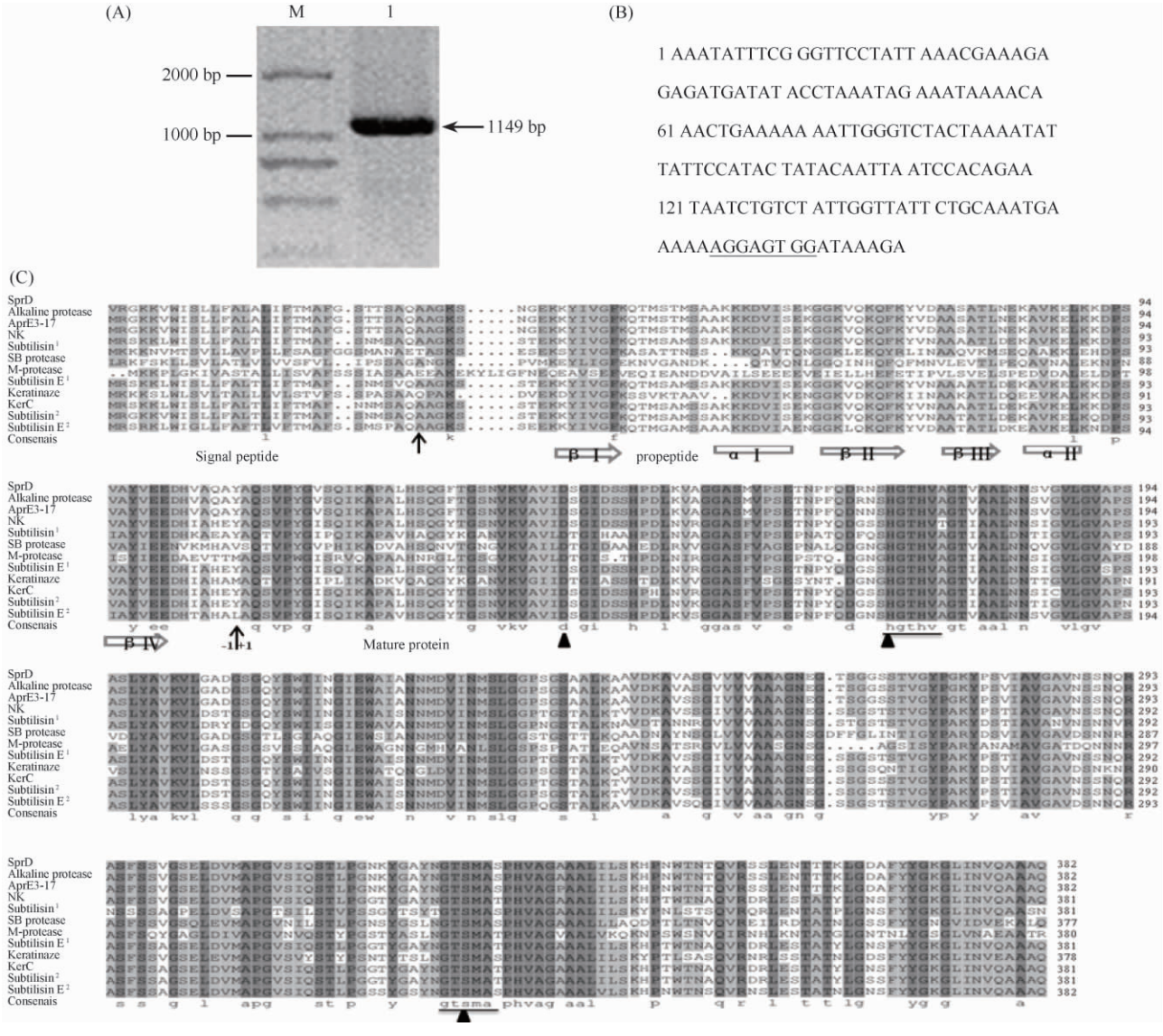

Figure 1. PCR amplification of the whole sequence of SprD and sequence analysis. A: The whole sequence of SprD. Lane M, Trans 2K marker; Lane 1, the whole gene of SprD (1149 bp). B: The promoting sequences of SprD: the sequence unlined may be its SD sequence. C: Amino acid sequence alignment of SprD and other *Bacillus* serine proteases. The active sites were indicated as "▲". The amino acides were composed of signal peptide, propeptide and mature protein (from +1), separated by arrows. Degenerate primers were designed according to the conserved regions in underlines. The sequences used for analysis were: Alkaline protease (AFH88394), AprE3 – 17 (ACU32756), NK (AEV91244), Subtilisin[1] (ZP_10165313), SB protease (BAD21128), M-protease (Q99405), Subtilisin E[1] (CAA74536), Keratinaze (BAE92942), KerC (ABY65903), Subtilisin[2] (ACJ07037), Subtilisin E[2] (YP_003972439).

Similar molecular mass and pre-pro-peptide regions had been reported in other extracellular proteases (Figure 1-C). The signal peptide functioned as a signal for protein secretion across the membrane[12]. The propeptide did not form part of the mature domain, however, it acted as an intramolecular chaperone (IMC) that facilitating folding by acting as a template for its mature domain[13]. There were studies reported about the three-dimensional structural model of the propeptide-subtilisin complex[14], and they showed that the propeptide was composed of two α-helices and four β-strands playing different roles in the refolding of the mature protein (Figure 1-C). The catalytic triads of SprD are determined to be Asp32, His64 and Ser221 included in the mature peptide, which is a common property in the serine proteases of

subtilase superfamily[1].

2.3 Recombinant expression of SprD in BL21 (DE3)

At high temperature (37℃), BL21 (DE3)/pET28-prosprD expressed its target protein in its precursor-prosprD (lane 3), while at low temperature (<30℃), target protein was expressed in its mature (active) form-SprD and hydrolyzed most of the essential proteins in BL21 (DE3)/pET28-prosprD (lane 4), therefore, active SprD protease was only expressed at a lower level (lane 4) (Figure 2). Whereas, BL21 (DE3)/pET28-msprD expressed its target protein only in inclusion bodies (not active form) (lane 7), whatever the temperature was. The reason was that temperature affected the folding rate of pro-protein into mature protein, and the results was also consistent with other studies that only mature sequence without propeptide could not fold into an active conformation and was only found in inclusion bodies[13]. An autoprocessing procedure of pro-SprD (38 kDa) (lane 1) into mature SprD (29 kDa) (lane 2) *in vivo* after purification was shown when the induced temperature was altered in BL21 (DE3)/pET28-prosprD (Figure 4-A). When BL21 (DE3)/pET28-prosprD was induced at 21℃ for 15 h, the final yield of active SprD was about 0.25 – 0.3 mg/g (wet weight of strains). The expression level was not high. Through research and exploration, the time that pro-protein autoprocessing into mature protein may be shortened to improve the expression level, and the results will be shown in another paper (being underwritten).

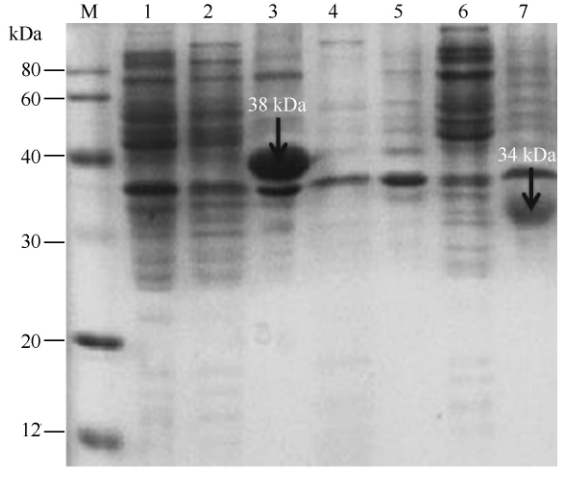

Figure 2. SDS-PAGE analysis of the expressed products. Lane M, protein MW markers; Lane 1, BL21 (DE3)/pET28a-prosprD uninduced; Lane 2, supernatant of BL21 (DE3)/pET28a-prosprD induced at 37℃; Lane 3, precipitation of BL21 (DE3)/pET28a-prosprD induced at 37℃; Lane 4, supernatant of BL21 (DE3)/pET28a-prosprD induced at 21℃; Lane 5, precipitation of BL21 (DE3)/pET28a-prosprD induced at 21℃; Lane 6, supernatant of BL21 (DE3)/pET28a-msprD induced at 37℃; Lane 7, precipitation of BL21 (DE3)/pET28a-msprD induced at 37℃. The recombinant protein prosprD (38 kDa) and msprD (34 kDa) were shown in arrows.

Fusion protein msprD (34 kDa, 3 mg/ml) as pure as 90% was employed as antigen to immunize rabbits (lane 2) (Figure 3-A). Several kinds of enzyme were secreted by L010 (lane 1) (Figure 4-A), and this phenomenon showed that L010 had relatively perfect secretion mechanism.

2.4 Detection and application of the polyclonal antibody against SprD

After the generation of polyclonal antibody against msprD, the serum titer was validated by ELISA (the titer, 1:640000) (Figure 3-B) and Western blot (the titer, 1:32000) (Figure 3-C).

The polyclonal antibody against msprD was applied to confirm the expression products in BL21 (DE3)/pET28-prosprD and the products secreted by L010. The results showed that the molecular weight of active SprD protease by L010 was about 27.5 kDa (lane 3), a little smaller than that of active SprD protease (29 kDa) (lane 2) with a 6×His-tag in its C terminal by BL21 (DE3)/pET28-prosprD at lower temperature (Figure 4-B). Auto-processing procedure of SprD *in vivo* was totally shown in Fig 4. Western blot analysis demonstrated that the protein with 27.5 kDa was indeed the protease SprD. It was also suggested that the signal peptide and the propeptide of SprD were automatically cleaved before the active SprD was secreted out of cells (lane 3 in Figure 4-B), and that the conserved regions designed for degenerate primers in SprD were different from the sequences of other secreted enzymes by L010. In addition, the results from lane 3 in Fig 4-A also showed that the secretion mechanism in *Bacillus* was relatively perfect

and a variety of enzymes were secreted outside besides SprD.

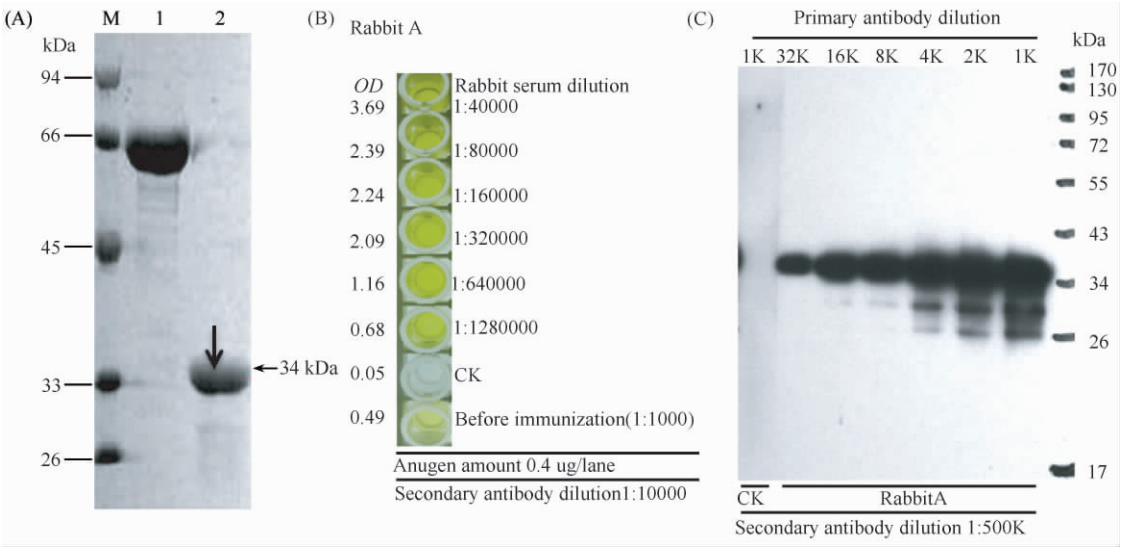

Figure 3. Preparation for polyclonal antibody against SprD. A: Purification of recombinant protein msprD. Lane M, protein MW markers; Lane 1, Bovine serum albumin served as the control; Lane 2, recombinant protein msprD. B: Polyclonal antibody against msprD detected by ELISA. C: Polyclonal antibody against msprD detected by Western blot.

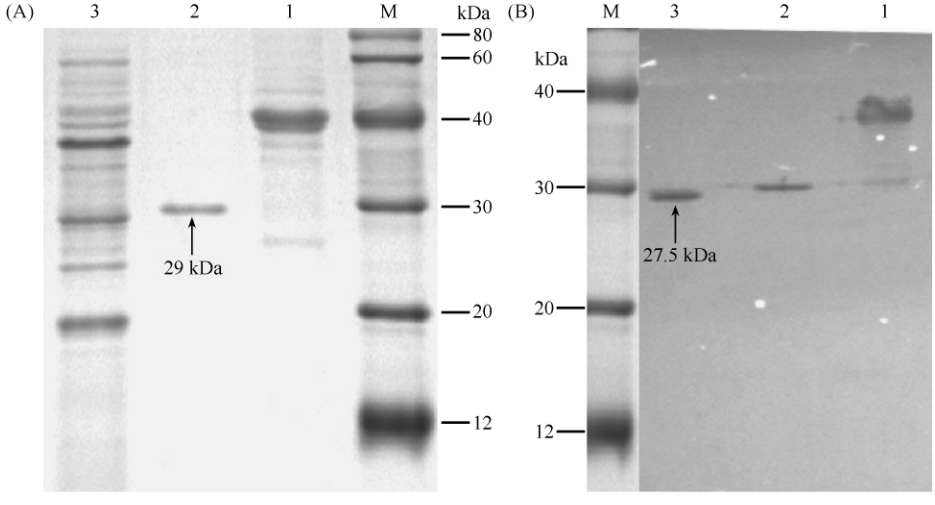

Figure 4. Application of the polyclonal antibody against msprD. A: Active sprD/pro-SprD detected by SDS-PAGE. Lane M, protein MW markers; Lane 1, pro-SprD purified from BL21 (DE3)/pET28a-prosprD induced at 37℃; Lane 2, active SprD with His-tag purified from BL21 (DE3)/pET28a-prosprD induced at 21℃; Lane 3, extracellular proteases purified from *Bacillus* sp. L010. B: Active SprD/prosprD detected by Western Blot. Lane M, protein MW markers; Lane 1, pro-SprD purified from BL21 (DE3)/pET28a-prosprD induced at 37℃; Lane 2, active SprD with His-tag purified from BL21 (DE3)/pET28a-prosprD induced at 21℃; Lane 3, extracellular proteases purified from *Bacillus* sp. L010.

2.5 Effects of temperature, pH on enzyme activity and stability

The activity of the subtilisins was a Ca^{2+}-dependent process. The activity of SprD was operated with 1 mmol/L Ca^{2+} and decreased sharply without Ca^{2+}, probably due to formation of a stabilized active conformation in the presence of Ca^{2+} [15]. The activity of purified active SprD against AAPF at 37℃ in 50 mmol/L Tris-HCl with 1 mmol/L $CaCl_2$, pH 8.0 was about 7531 U/mg and the affinity for its substrate (K_m) and the catalytic constant (K_{cat}) were detected (Table 1). The results showed that the catalytic

efficiency (K_{cat}/K_m) of SprD was higher than NK[14], Subtilisin E[11] and M-protease[5]. The affinity of SprD with AAPF was greater than that of NK, Subtilisin E and M-protease.

Table 1. Comparison of enzyme activity from different sources. Assays were carried out in 50 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L Ca^{2+} at 37℃ using AAPF

Enzymes	Reference	K_m/(μmol/L)	$K_{cat}/(s^{-1})$	$(K_{cat}/K_m)/(s^{-1}\cdot mmol^{-1}\cdot L)$
SprD	This study	75.69±12	47.02±7	621
NK	Jia et al. 2010	161±7.9	9.15±0.6	56.8
Subtilisin E	Takagi et al. 1988	1900±200	21±4	11
M-protease	Kobayashi et al. 1995	800	139.5	174.4

The activity of protease SprD was detected at temperature between 25℃-90℃ and pH 5.5-12. It showed the maximum enzyme activity with 1 mmol/L Ca^{2+} at 70℃, and retained 20% of its maximum activity at 90℃ (Figure 5-A). The enzyme showed the maximum activity at pH 9.5, retained about 98% of its maximum activity at pH 9.0 and pH 10.0 (Figure 5-B). However, it almost lost all its activity at pH 12.0 (Figure 5-B). Thermal stability of SprD was evaluated by incubation at different temperatures for 120 min, and the results showed that the enzyme retained about 100%, 80%-90%, 50%-60% of

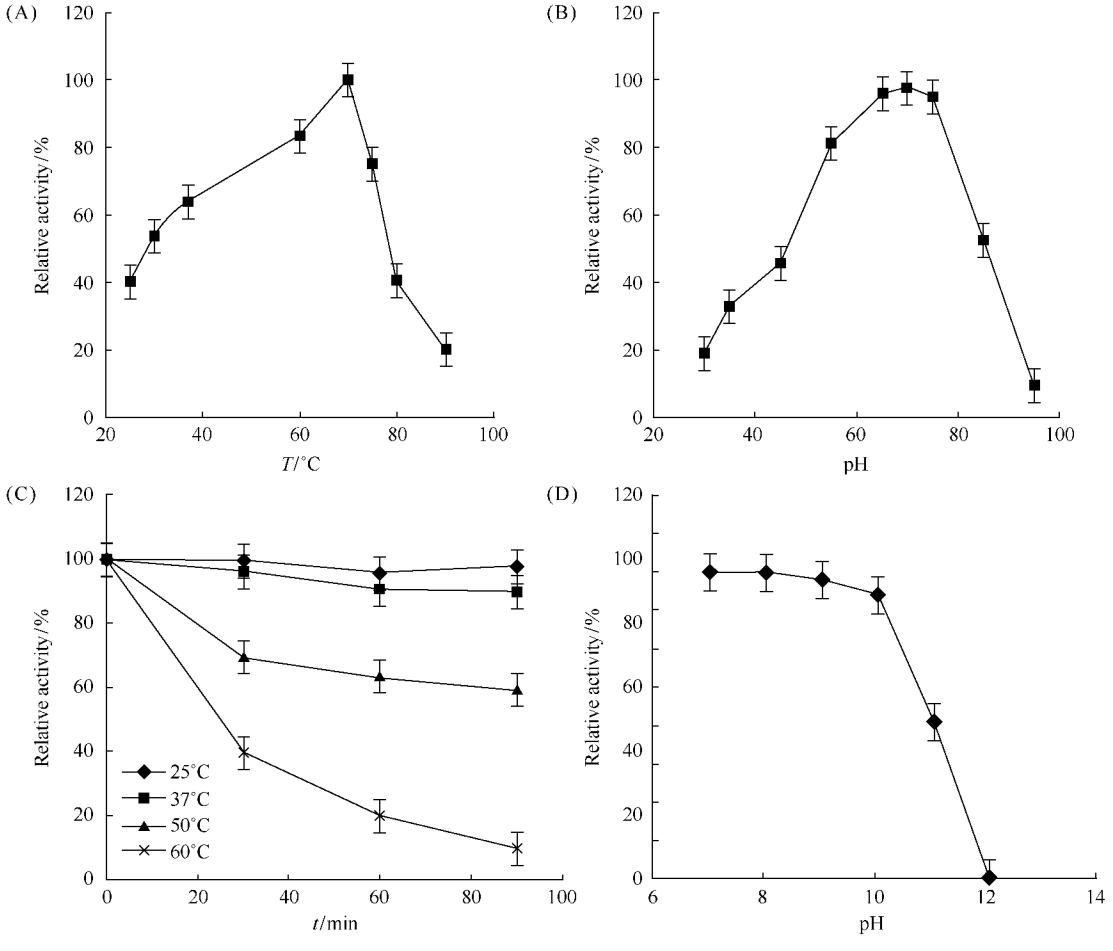

Figure 5. Effects of temperature (℃) and pH on the protease activity and stability. A. Effect of temperature on the protease activity. B. Effect of pH on the protease activity. C. Residual activities after incubation for various times at different temperatures. D. Stability at different pH values. The values presented correspond to the mean values of at least three replicates.

its original activity after incubation at 25℃, 37℃ and 50℃ for 120 min, lost almost all its activity after incubation at 60℃ for 120 min (Figure 5-C). The enzyme was unstable at 70℃ and almost lost all its activity after incubation for 30 min. The enzyme was stable between pH 7.0 and 10.0 and retained about 90% – 100% of its activity after incubation at 25℃ for 6 h (Figure 5-D). The enzyme retained about 60% and 20% – 30% of its activity after incubation at pH 11.0 and pH 12.0 for 6 h (Figure 5-D).

Although the high similarity with alkaline protease (AFH88394), AprE3-17 (ACU32756) and AprE51 (ACA34903), the characteristics of the alkaline protease (AFH88394) and AprE3-17 (ACU32756) were not reported, the research about AprE51 (ACA34903)[16] showed that the optimal pH and temperature for partially purified AprE51 activity were 6.0 and 45℃, in a large difference with the results of other highly similarity proteins-AprFP-133 (BAJ41479)[17], NK[18] and SprD. The kinetic parameters about AprE51 were not measured in the study[16]. The properties about AprFP-133 were also not clear[17]. The Arg61 site of SprD in the vicinity of the catalytic triad (Asp32, His64, Ser221) (Asn61 in AprE51, Asn61 in AprFP-133, Gly in other Subtilisins) might contribute to the alkaline adaption and thermal stability of SprD. Similar results have been seen in a comparison between crystal structures of an alkaline protease-Subtilisin Carlsberg and a high alkaline protease-M-protease and the comparison showed that Arg and His residues increased in the high alkaline protease, whereas Asp, Glu, Lys residues decreased[3,19]. The extra Arg residues contributed to an increase of hydrogen bonds or ion pairs in the protease[3,19].

For most applications in industry, alkaline proteases with an optimal temperature of 50℃ – 70℃ and an optimal pH of 9 – 12 are desirable[2-3], and SprD has these characteristics. Our results revealed that SprD was stable between pH 7.0 and 10.0, more thermal stable than DFE[6,20], NK[14,18], as comparable as M-protease in thermal stability[5,19] and had a greater catalytic efficiency than NK[14], Subtilisin E[11] and M-protease[5]. M-protease and many other subtilisins had been used in industry applications as detergent additives[3]. All these characteristics suggest that the novel SprD identified in new strain L010 will find the research value and potential applications.

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一新中温碱性蛋白酶基因的克隆及原核表达

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摘要:【目的】从环境中分离筛选产蛋白酶、降解蛋白质的菌株,寻找使用价值较高的碱性蛋白酶。【方法】通过酪蛋白平板法分离筛选产蛋白酶菌株,经生理生化方法及 16S rDNA 基因序列鉴定菌株;利用简并引物及基因组步移克隆蛋白酶完整开放阅读框;蛋白酶前体蛋白及成熟肽序列在大肠杆菌(*Escherichia coli*) BL21 (DE3) 中进行重组表达;纯化活性蛋白酶后,利用化学合成多肽底物(succinyl-Ala-Ala-Pro-Phe-p-nitroanilide)检测酶活性质及其催化活力。【结果】分离到的菌株 L010 被鉴定命名为芽胞杆菌(*Bacillus* sp.) L010;蛋白酶开放阅读框包含了 1149 个碱基,编码 382 个氨基酸,氨基酸序列按其功能分为 N 端的 30 个氨基酸残基组成的信号肽,77 个氨基酸残基构成的前导肽,C 端 275 个氨基酸残基组成的成熟肽;此蛋白属于丝氨酸蛋白酶家族中枯草杆菌蛋白酶类(Subtilisins)成员,并命名为 SprD;SprD 的前体蛋白在大肠杆菌(*Escherichia coli*) BL21 (DE3) 中重组表达时,在前导肽辅助下自加工为活性蛋白酶;SprD 呈现出较高的催化活力,其反应最适条件为温度 70℃,pH 9 – 10。【结论】SprD 在碱性(pH 7.0 – 10.0)、中高温(25℃ – 60℃)条件下的稳定性及较高的催化能力使其具有一定的研究和潜在利用价值。

关键词:自加工,芽胞杆菌(*Bacillus* sp.) L010,大肠杆菌(*Escherichia coli*) BL21 (DE3),表达,聚合酶链式反应,蛋白酶 SprD

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