Construction of the *Escherichia coli-Bacillus subtilis* Shuttle Vector pBE2R and Identification of the Critical Residues Involved in the Autoprocessing of the Propeptide of the Alkaline Protease

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1 Introduction

The serine alkaline protease, secreted from a wide variety of *Bacillus* species, is an important industrial enzyme and a model system for protein engineering. The alkaline protease can be widely used in washing powders and dehairing hides for its high activity and stability. The alkaline protease generally secreted extracellular for the purpose of scavenging nutrients is specific for aromatic or hydrophobic residues, such as tyrosine, phenylalanine and leucine. However, they are highly sensitive to phenyl methyl sulphonyl fluoride and diisopropyl-fluorophosphate. The *Bacillus*-originated alkaline proteases are mesophilic enzymes with a molecular weight range of 15–30 kDa and an isoelectric point near pI 9. They reflect high activity at 50–70 °C, however, the activity were reduced significantly at low temperature, like 20 °C [1].

Directed evolution has rapidly emerged as a powerful strategy for improving the characteristics of various enzymes in a targeted manner. To generate large gene variant libraries, it is possible to optimize an enzyme for its specific applications through combining error-prone PCR or DNA shuffling with the high-throughput screening which is used to select the specific properies of an enzyme, such as thermostability, catalytic activity and substrate specificity [2]. Therefore, it is available to improve the activity of the alkaline protease at low temperature through

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the directed evolution. The construction of the mutant library was a key step in the process of the alkaline protease directed evolution. In this report, a new *Escherichia coli-Bacillus subtilis* shuttle vector pBE2R was constructed to establish a platform for the high-throughput screening of the alkaline protease in the process of the alkaline protease directed evolution.

The alkaline protease derived from *Bacillus alcalophilus* TCCC11263 can be expressed successfully in *B. subtilis* WB600 by using the pBE2R vector [3]. Apart from this, in the process of construction of the pBE2R vector we found that the correct cleavage of the propeptide after secretion was significant for the formation of the functional protein. As other subtilisins, the *B. alcalophilus* TCCC11263 alkaline protease of 380 residues is synthesized as an inactive precursor that is composed of a 27 residues signal peptide for protein secretion, a 84 residues propeptide for folding and formation of the active protease and a 269 residues mature peptide for the catalytic protein. The signal peptide is cleaved as the protein crosses the inner membrane, and the propeptide remains covalently attached until the protein is secreted from the cell [4]. The propeptides are relatively common in *Bacillus* secretory proteins, and they are divided into two different kinds, long and short [5]. The propeptide region generally functions as a facilitator of folding, stability, and even secretion of the protein. In most cases, the propeptide domain is cleaved from the enzymatic domain autocatalytically to release an active protease. Removal of the propeptide by autocleavage during processing is crucial for the secretion and production of subtilisins [6]. Takahashi et al. [7] showed that the autoprocessing efficiency of a subtilisin E mutant with altered specificity for acid residues was improved by substituting the autoprocessing site Tyr-1 with Asp or Glu. Grande et al. [8] reported that insertion within a 9-amino-acid region in the propeptide caused dramatic reduction in LasA enzymatic activity. All mutant proLasA proteins were still secreted, but extracellular stability was low due to clustered insertions within the propeptide. However, little is known about the critical residues involved in the autoprocessing of the *B. alcalophilus* alkaline protease. Therefore, another primary goal of this study was to identify the critical residues correlated to the autocleavage of the propeptide of the *B. alcalophilus* alkaline protease.

2 Materials and Methods

2.1 Strains and Plasmids

E. coli DH5α, *B. subtilis* WB600, *B. alcalophilu*s TCCC11263 were stored by Tianjin university of science and technology laboratory. The plasmid pWB980 was kindly provided by Sui-Lam Wong (University of Calgary, Canada). The plasmid pBE2 was kindly provided by NanKai University.

2.2 Growth Medium and Conditions

E. coli DH5α, *B. subtilis* WB600 were cultured aerobically in LB medium at 37 °C. For recombinant selection, the kanamycin and ampicillin were applied to the above media. The final concentration of Kanamycin and Ampicillin was 30 and 100 μg/mL respectively. The skim milk plate containing Kanamycin (30 μg/mL) was used for the preliminary screening of the mutants.

2.3 Related Enzymes and Reagents

GeneRuler, restriction endonuclease and T4 DNA ligase were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. *Taq* DNA polymerases and Unstained Protein Molecular Weight Marker were purchased from Fermentas. PCR primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. All other reagents were analytically pure.

2.4 Construction of the pBE2R

P43 promoter is a strong promoter containing two overlapping promoters which are recognized in vitro by σ55- and σ37-containing RNA polymerase holoenzymes from *B. subtilis* [9]. The 317 base pair length fragment P43 promoter was released from the plasmid pWB980 by digestion with *Eco*R I and *Kpn*I. The fragment was isolated by gel purification and inserted into the MCS of the pBE2 vector directionally. Cohesive ends ligation of both fragments resulted in the plasmid pBE2a. Then the DNA fragment containing signal peptide and pro-peptide of alkaline protease derived from *B. alcalophilus* TCCC11263 was amplified by the Polymerase Chain Reaction (PCR). Based on the DNA sequence of the alkaline protease (*aprE*) of *B. alcalophilus* reported on NCBI, a primer pair (Psp1 and Psp2) (Table 1) with indicated engineered restriction sites were designed to amplify the signal peptide and pro-peptide gene (*sp*).

Table 1 Primers used for PCR experiments

Targert gene	Primer sequence
Signal peptide and pro-peptide gene	
Forward	5′-CGGGGTACCATTATAGGTAAGAGAGGAATGTACA CATGAAGAAACCGTTGGGGAAAATTG-3′ (*Kpn*I)
Reverse	5′-CGCGGATCCCGCCATTGTCGTTACTTCTGCATCC-3(*Bam*H I)
Mature peptide encoding gene	
Forward	5′-CGCGGATCCCAATCAGTGCCATGGGGAATTAGCCG-3′ (*Bam*H I)
Reverse	5′-ACGCGTCGACTTAGCGTGTTGCCGCTTCTGCATTGAC-3′ (*Sal*I)

The *Kpn*I-*Bam*H I fragment of the PCR product was inserted into pBE2a directionaly, forming plasmid pBE2R. Then the pBE2R was transformed into *E. coli* DH5α. The pBE2R was sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd for DNA sequencing.

The mature peptide encoding gene of alkaline protease was introduced into the pBE2R to detect the function of the new shuttle vector. A primer pair (Pmp1and Pmp2) (Table 1) were designed to amplify the mature peptide encoding gene. The *Bam*H I-*Sal*I fragment of the PCR product of the mature peptide (*mp*) was introduced into pBE2R directionaly to generate pBE2R-*mp*. Then the recombinant plasmid was transformed into *B. subtilis* WB600 to express the alkaline protease.

2.5 Construction of the Mutants with Different Insertional Positions of the Restriction Sites Around the Region Between Propeptide and Mature Peptide

Four pairs primers were designed to amplify the fragment of signal and pro-sequences of alkaline protease with the different insertional positions of restriction site around the region between propeptide and mature peptide. The *Bam*H I-*Sal*I fragments of PCR products were inserted into the pBE2a to construct plasmids containing different insertional positions. According to the insertional position, The recombinant plasmids were named pBE2a∷Rs109, pBE2a∷Rs110, pBE2a∷Rs111, pBE2a∷Rs112, respectively. The mature peptide encoding gene was amplified by PCR. Then the PCR products were introduced into the recombinant plasmids described above, generating plasmids pBE2a∷Rs109-*mp′*, pBE2a∷Rs110-*mp′*, pBE2a∷Rs111-*mp′* and pBE2a∷Rs112-*mp′*. Meanwhile, a recombinant plasmid pBE2a-*apr* containing the signal and pro-sequences, as well as the mature sequence of the alkaline protease was constructed without the insertion of restriction site at the junction between propeptide and mature peptide. Then these recombinant plasmids were transformed into the *B. subtilis* WB600 to express the alkaline protease. The skim milk plate containing Kanamycin (30 μg/mL) was used to screen the mutants preliminarily.

2.6 Assay of the AprE Activity

The *B. subtilis* WB600 carrying recombinant plasmids were cultured in 100 mL LB medium containing Kanamycin (30 μg/mL) at 37 °C for 48 h. The culture supernatants were collected by centrifugation at 4 °C and 12,000 r/min for 10 min. The supernatants were used as crude enzyme solutions for the activity determination of the AprE protease. The general methods of determination for industrial enzymes was employed to assay the activity of the alkaline protease quantitatively.

2.7 SDS-PAGE Analysis

The SDS-PAGE was employed to detect the relative content of the extracellular proteins. The hosts were cultured in 100 mL LB medium containing Kanamycin (30 µg/mL) at 37 °C for 48 h. Supernatants and the cells were harvested by centrifugation at 4 °C and $5000 \times g$ for 10 min, respectively. Supernatants were precipitated with 100% TCA at −20 °C for 5–10 min and then 4 °C for 12 h. Centrifugating at 4 °C and $5000 \times g$ for 10 min to collect the precipitate. The precipitate was washed by acetone for three times and volatiled thoroughly. Then the precipitates were dissolved with the 1× loading buffer before they were boiled. The cells collected by centrifugation described above were suspended with 40 µL of distilled water and 10 µL of 1× loading buffer. Then the solutions were boiled at 100 °C for 20 min before loading. SDS-PAGE was performed in a 30% polyacrylamide gel.

# 3 Results

3.1 Expression of the Alkaline Protease (AprE)

The *Escherichia coli-Bacillus subtilis* shuttle vector pBE2R was constructed by fusing P43 promoter and signal as well as pro-sequence of the *aprE* gene to the pBE2 vector (Figs. 1 and 2). The plasmid was verified by double digestion (*Kpn*I/*Bam*H I). The results demonstrated that the sequences inserted into pBE2.

The mature peptide encoding gene of alkaline protease was introduced into pBE2R to generate pBE2R-*mp*, which was transformed into *B. subtilis* WB600 to detect the function of the new shuttle vector. The transformants were cultured on the skim milk plate containing Kanamycin (30 µg/mL) at 37 °C for 16 h. It was observed from the production of the proteolytic ring that the shuttle vector pBE2R can express the alkaline protease successfully in *B. subtilis* WB600 (Fig. 3).
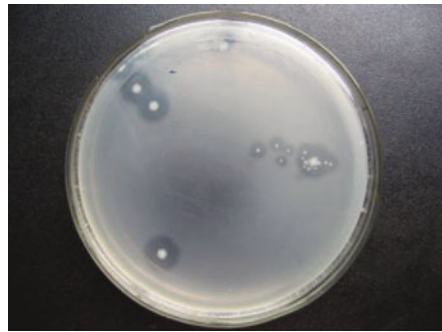
Fig. 1 Verification of the recombinant plasmid pBE2a
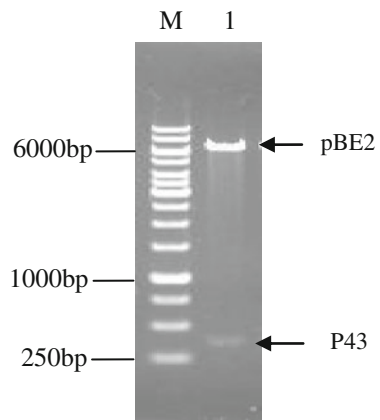
Fig. 2 Verification of the recombinant plasmid pBE2R
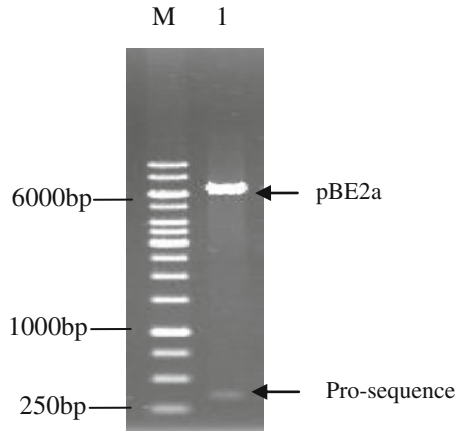
Fig. 3 Expression of the pBE2R-*mp* in the *B. subtilis* WB600

3.2   Deduction of the Critical Residues Involved in the Autoprocessing of the Propeptide

The new constructed shuttle vector pBE2R can be used in the directed evolution of the alkaline protease. The mutational gene encoding mature peptide of the alkaline protease produced by error-prone PCR and DNA shuffling can be inserted into the MCS of pBE2R with the connection of *Bam*H I. Therefore, the pBE2R provided a convenient tool for the high-throughput screening of the forward mutation in the directed evolution of the alkaline protease.

In the process of pBE2R construction, we found that the insertional position of *Bam*H I which was located at the junction between the propeptide and mature peptide had a significant effect on the activity of the alkaline protease. It was indicated that the inactive protease was due to the aborting autoprocessing of the propeptide after secretion. Five recombinant plasmids pBE2a::Rs109 ∼ pBE2a::Rs112 and pBE2a-*apr* were constructed to detect the critical residues involved in the autoprocessing of the propeptide. Then the mature peptide encoding gene amplified by PCR was ligated to the pBE2a::Rs109 ∼ pBE2a::Rs112 to generate pBE2a::Rs-*mp'* respectively.

Finally, the plasmids pBE2a::Rs-*mp′* and pBE2a-*apr* were transformed into *B. subtilis* WB600 to express the alkaline protease.

It was observed that the proteolytic ring could be produced by the hosts carrying pBE2a-*apr* and pBE2a::Rs112 but could not by the others carrying pBE2a::Rs109 ∼ pBE2a::Rs111. The results showed that the activity of the alkaline protease produced by the hosts carrying pBE2a-*apr* and pBE2a::Rs112-*mp′* could be detected and were parallel. While the activity of the protease produced by the hosts carrying pBE2a::Rs109-*mp′* ∼ pBE2a::Rs111-*mp′* could not be detected (Table 2). The determination of the activity of AprE was consistent with the results of the proteolytic ring.

To determine if there are mutations in the coding domain of the alkaline protease which couldn't produce the proteolytic ring. DNA sequencing was used to detect the mutation in the mature peptide encoding gene which was probably produced by PCR. However, the results of the DNA sequencing revealed that there was no mutation in the coding region of the mature peptide. The protein electrophoresis was employed to determine if the insertions adversely affected the secretion of the alkaline protease. The electrophoresis result of the intracellular protein showed that no increased accumulation of 41-kDa proAprE protein was observed in cell (Fig. 4a). The results of the extracellular protein electrophoresis showed that there was no obvious difference in the secretion level between the two active proteases which were produced by the hosts carrying pBE2a-*apr* and pBE2a::Rs112-*mp′*, respectively, while there was a great difference between the active protease and the inactive protease. The active proteases, a 38-kDa protein bond could be detected in the hosts producing inactive proteases, while the mature peptide which is 29-kDa in molecular mass was not detected (Fig. 4b). The results described above implied that the mutant proAprE protein resulted from the different insertions of *Bam*H I appeared to be secreted normally but abnormally in autoprocessing of the propeptide outside of the cell.

In our experiments, the insertion occured at the residues 109, 110, 111 and 112, respectively (Table 3). The insertion of the restriction sites between propeptide and mature peptide might interfere with the recognition of the autoprocessing site so that the pro-peptide couldn't be cleaved from preproenzyme correctly. It was deduced from the experimental results described above that the amino acids

Table 2 Determine result of the enzyme activity of the samples

Plasmid designation	Average value of OD600	Activity of AprE (U/mL)	Standard deviation
pBE2a (control)	0.000	0	±0.1
pBE2a-*apr*	0.478	18	±0.9
pBE2a::Rs109-*mp′*	0.000	0	±0.2
pBE2a::Rs110-*mp′*	0.000	0	±0.3
pBE2a::Rs111-*mp′*	0.000	0	±0.2
pBE2a::Rs112-*mp′*	0.484	18	±2.0

(a)

(b)
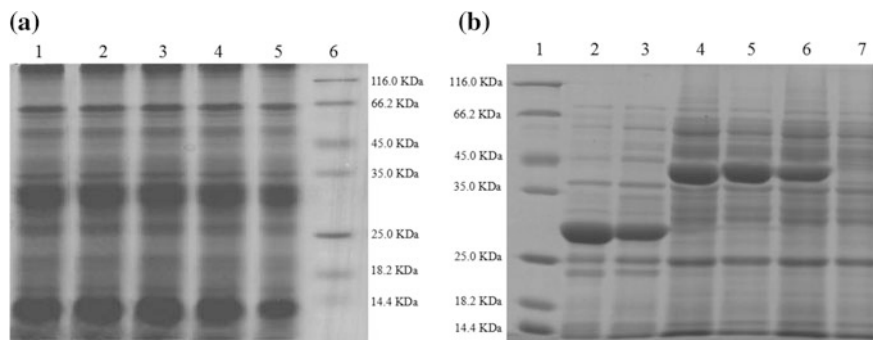
Fig. 4 The SDS-PAGE results of the intracellular and extracellular protein from different Samples. **a** The SDS-PAGE result of the intracellular protein. The strains examined were as follows *1–5* hosts carrying pBE2a::Rs109-*mp′* ∼ pBE2a::Rs112-*mp′* and pBE2a-*apr*, respectively; *6* protein marker. **b** The SDS-PAGE result of the extracellular protein. *1* Protein marker; the strains examined were as follows: *2–3* hosts carrying pBE2a-*apr* and pBE2a::Rs112-*mp′*; *4–6* hosts carrying pBE2a::Rs109-*mp′* ∼ pBE2a::Rs111-*mp′*; *7* host of carrying pBE2 (negative control)

Table 3 The insertional position of the *Bam*H I

Plasmid designation	Insertion position	Amino acids sequence
pBE2a-*apr*	–	TTMAQS
pBE2a::Rs109-*mp′*	109	**TG**STMAQS
pBE2a::Rs110-*mp′*	110	TT**GS**MAQS
pBE2a::Rs111-*mp′*	111	TTM**GS**AQS
pBE2a::Rs112-*mp′*(pBE2R-*mp*)	**112**	TTMA**GS**QS

The pBE2a-*apr* is the plasmid carrying the intact peptide of alkaline protease without the insertion of the *Bam*H I. pBE2a::Rs109 ∼ pBE2a::Rs112 are plasmids containing different insertional positions of *Bam*H I between propeptide and mature peptide. The black body "GS" represents two amino acids introduced by insertion of *Bam*H I

"TTMA" which located at the junction between propeptide and mature peptide, as well as its arranging sequence were critical in autoprocessing of the propeptide. The autoprocessing would be disturbed when this amino acid sequence was destroyed.

4 Discussion

This report described the construction of the *Escherichia coli-Bacillus subtilis* shuttle vector pBE2R and the identification of the critical residues involved in the autoprocessing of the propeptide in alkaline protease. The new shuttle vector contained P43 promoter and the signal as well as pro-peptide encoding gene of alkaline protease. It was demonstrated that the signal peptide of the alkaline protease could guide the foreign protein to secrete effectively. The propeptide acting as

an intramolecular chaperone had a significant effects on the folding and stability of the protease, as well as the formation of the active protease [10–15]. In our experiment, It was shown that the deletion of the propeptide resulted in the acute instability of the alkaline protease. The alkaline protease could not fold itself into a suitable state without the assistance of the propeptide so that could be recognized and digested by the proteases produced by the host (not data shown). On the other hand, the correct cleavage of the propeptide after the secretion of the protein was also indispensable to an active protease. It was revealed from the mutant experimental results that several crucial amino acids "TTMA" locating at the 3′ terminal of the propeptide, as well as the maintenance of its integrated sequence played a key role in the autoprocessing of the propeptide. The autoprocessing might be disturbed when this amino acid sequence was destroyed. In addition, the inactive protease caused by the aborted autoprocessing of the propeptide suggested that the propeptide was a potent inhibitor of the mature domain although it was needed in the folding of the protein before secreting.

The pBE2R could be used to express the alkaline protease in *B. subtilis* WB600 successfully. The shuttle vector pBE2R not only provided a convenient tool for the expression of the foreign protein in the *B. subtilis*, but also established a platform for the high-throughput screening of the alkaline protease in the process of the directed evolution of the alkaline protease. The mutated encoding genes of the mature peptide which were produced by error-prone PCR and DNA shuffling were introduced into the pBE2R to express the alkaline protease with the different mutation in *B. subtilis* WB600. The desired mutant containing different positive characters was obtained after multiple rounds of error-prone PCR and DNA shuffling as well as the high-throughput screening.

The test for the expression of mesophilic alpha-amylase gene in *B. subtilis* WB600 by using pBE2R revealed that the long propeptide of the alkaline protease could not guide the alpha-amylase which belongs to the short propeptide protein to produce the functional protein.

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