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Improvement of cold adaptation of *Bacillus alcalophilus* alkaline protease by directed evolution

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Protein engineering of the *Bacillus alcalophilus* PB92 ATCC 31408 alkaline protease (SBA) was performed to obtain enzymes with improved cold adaptation. The activity of SBA at low temperature was enhanced through direct evolution using error-prone polymerase chain reaction. Two mutation sites, Glu110Ala and Glu134Ala, were obtained in SBA. To identify the mutation of amino acids in E110A/E134A related to its activity at low temperature, single mutants E110A and E134A were obtained via site-directed mutagenesis. The k_{cat}/K_m values of the mutants E110A, E134A and E110A/E134A at 10 °C were 1.5-, 2.2- and 2.7-fold higher, respectively, than that of the wild-type. Through the three-dimensional structure analysis, it was indicated that E110A/E134A showed an improved activity at low-temperature condition as a result of the disrupted hydrogen bond, increased protein hydrophobicity, and decreased calcium affinity. The findings of this study provides the theoretical basis and background data for improvement of the cold adaptation in SBA by protein engineering.

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1. Introduction

Proteases are widely used in different industrial processes such as laundry additive, food processing, leather processing, silk and medicine, especially in the detergent industry because of the high efficiency of removing proteins from textile fibers. Among them, cold-adapted proteases can used as detergent additives, which is mainly due to their excellent cleaning capabilities at low temperature as an environment friendly manner [34]. Cold-adapted enzymes are generally characterized by higher catalytic activity and catalytic efficiency (k_{cat}/K_m) at low temperatures with respect to their warm-adapted counterparts, which was beneficial to environmental application and save energy when used in industrial processes [1,9,10,21,23,24,30,38]. It is assumed that the improvement of the catalytic efficiency in cold-adapted enzymes was achieved by increasing the structure flexibility. Compared with mesophilic and thermophilic counterparts, the cold-adapted enzymes exhibit some characteristics for this structure flexibility, including the better interaction with substrates, higher catalytic rate (k_{cat}), lower thermostability, and lower activation energy (E_a) requirements [17,26–28,38]. Therefore, cold-adapted enzymes are commonly considered to be the result of an enhanced structural flexibility at low temperature through several different structural adaptation strategies, such as reducing number of noncovalent intra- and intermolecular interactions, optimizing the number of hydrophobic side chains exposed to the solvent, lessening compact packing of the hydrophobic core, increasing a polar surface area, decreasing metal ion affinity, lengthening surface loops and reducing the number of salt bridges [2,3,6–8,11,15,19,22,30–32,38–40].

Alkaline detergent is an important detergent which is a formulation designed for use in mechanical cleaning equipment, car washers, instrument washers and disinfectors, and it is effective for removal of protein, blood, fat, grease, and oil. It is stated that alkaline detergent with the addition of cold-adapted proteases could express higher efficiency at low temperature and was beneficial to environmental application and save energy when compared with the detergent without cold-adapted proteases. However, the application of proteases with excellent activity at low temperature is limited as a result of its weak alkali resistance.

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Meanwhile, the mesophilic and thermophilic alkaline proteases exhibit a decreasing activity and catalytic efficiency at low temperature. Therefore, a protease with high catalytic efficiency at low temperature and excellent alkali resistance is desired for alkaline detergent industry.

An alkaline serine protease produced by *Bacillus alcalophilus* PB92 ATCC 31408 (SBA) has an extremely alkaline pH optimum (pH 10.5) and is suitable for use in modern alkaline detergent powders [35]. However, the reaction rates of mesophilic and thermophilic enzymes can be reduced from 30- to 80- folds when the medium temperature is decreased from 37 °C to 10 °C [17]. Therefore, the catalytic efficiency of this protease SBA is greatly influenced by temperature, resulting in the limitation for the application of SBA in the cold environment. Fortunately, the tertiary structure of SBA has been established [36], which was beneficial to improve the properties of SBA by protein engineering. Nevertheless, the study of cold adaptation in SBA so far is still limited, causing little theoretical basis and background data of the structure–function relationship at low temperature on SBA were obtained. Based on these reasons, the cold-adapted mutant SBAs with higher catalytic efficiency at low temperature and excellent alkali resistance were engineered by directed evolution using error-prone PCR in present study. Meanwhile, enzymatic properties combined with three-dimensional structure of the mutant SBAs were analyzed to discuss the molecular mechanism for the cold-adaptation of SBA and improve our understanding on structure–function relationship in enzymes adapted to low temperature.

2. Materials and methods

2.1. Bacterial strains, plasmid, and culture media

Escherichia coli DH5α were purchased from Takara Biotechnology Co. Ltd (Dalian, Liaoning, China), *Bacillus subtilis* WB600 was purchased from Biovector Co. Ltd (Beijing, China), and *B. alcalophilus* PB92 ATCC 31408 was preserved in our laboratory. The plasmid pUAPR was constructed by adding the wild-type (WT) SBA gene *sba* from *B. alcalophilus* PB92 ATCC 31408 to pUC19 (Takara Biotechnology Co. Ltd). The *E. coli*–*B. subtilis* shuttle vector pBE2R, which contained the P43 promoter, the SBA signal peptide and propeptide, and a multiple cloning site that allowed efficient expression of the genes and secretion of the gene products, was constructed in our laboratory. All bacteria were cultured at 37° C in Luria–Bertani (LB) medium containing 5 g l^{-1} of yeast extract, 10 g l^{-1} of peptone and 10 g l^{-1} of NaCl. Ampicillin (30 μg ml^{-1}) or kanamycin (30 μg ml^{-1}) was added to the growth medium when necessary.

2.2. Generation of a random mutant library by error-prone PCR

For random mutagenesis, the PCR reaction mixture was prepared by adding 10 ng of template DNA (pUAPR), 100 pmol of upstream primer (Primers P1 5′-CGGGATCCGGCG-CAATCAGTGCCATGGGGA-3′, the underlined sequences is *Bam*HI restrict digestion site), 100 pmol l^{-1} of downstream primer(P2 5'-CCCAAGCTTCTATTAGCGTGTTGCCGCTTCTGC-3′, the underlined sequences is *Hin*dIII restrict digestion site), 1× PCR buffer, 0.2 mmol l^{-1} dATP, 0.2 mmol l^{-1} dGTP, 0.2 mmol l^{-1} dTTP, 0.2 mmol l^{-1} dCTP, 7 mmol l^{-1} $MgCl_2$, 0.25 mmol l^{-1} $MnCl_2$, and 5 U *Taq* DNA polymerase (Promega) in water to 100 μl. PCR was performed for 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The PCR fragments digested by *Bam*HI–*Hin*dIII was cloned into *Bam*HI–*Hin*dIII site of pBE2R and transformed into competent *E. coli* DH5α by electroporation. Subsequently the plasmids were extracted from the colonies of *E. coli* DH5α and transformed into competent *B. subtilis* WB600 as described by Liu et al. [16].

2.3. Screening of mutant library

Colonies of *B. subtilis* WB600 on the plates were picked with sterile toothpicks and inoculated in 96-well plates containing LB medium supplemented with 30 μg l^{-1} kanamycin individually. The plates were incubated at 300 rev min^{-1} and 37 °C for 24 h and then centrifuged at 3800 × *g* for 20 min. Subsequently, 200 μl of the supernatant were transferred into replica 96-well plates. After cooling the replica plates at 10 °C for 20 min, 100 μl of 25 mmol l^{-1} borax–NaOH buffer solution (pH 10.5) containing 10 mmol l^{-1} $CaCl_2$, 100 mmol l^{-1} NaCl, and 1 mmol l^{-1} *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF) was added to the wells at 10 °C to measure the activities of the mutant SBAs. Product formation was assessed by reading the absorbance at 410 nm in a 96-well plate reader.

2.4. Site-directed mutagenesis

The *sba* was mutated by site-directed mutagenesis using a standard overlap extension PCR technique [14]. Oligonucleotide primers were synthesized by Sangon (Shanghai, China). The forward primer P1 and reverse primer P2 were used to amplify the full length mutant SBA genes. Mutation E110A was created using sense primer P3 5′-AAGGATTGGC(A→C)ATGGGCAGGGAACAA-3′ and anti-sense primer P4 5′-TTGTTCCCTGCCCATG(T→G)CCAATCCTT-3′. Mutation E134A was created using sense primer P5 5′-CCACACTTGC(A → C)GCAAGCTGTTAATAG-3′ and anti-sense primer P6 5′-CTATTAACAGCTTGCG(T → G)CAAGTGTGG-3′. Mismatched sites for site-directed mutagenesis were underlined. The mutant genes were confirmed by DNA sequencing, digested by *Bam*HI–*Hin*dIII, and cloned into pBE2R. *E. coli* DH5α strain was used as the host for propagation of plasmids containing the mutant SBA genes. The recombinant plasmids were transformed into competent *B. subtilis* WB600, individually.

2.5. Expression and purification of WT and mutant SBAs

The recombinant *B. subtilis* strains harboring WT and mutant SBA genes were cultivated in 100 ml of LB medium supplemented with 30 μg ml^{-1} kanamycin at 200 rev min^{-1} and 37 °C for 24 h. The centrifugal supernatant of the culture broth was salted out with ammonium sulfate at 70% saturation. The precipitate formed was dialyzed against MES buffer (20 mmol l^{-1}, pH 7.0; buffer A), and the retentate was subjected to ion-exchange chromatography on a CM-Sephadex fast flow column (2.5 × 20 cm) of pre-equilibrated with buffer A. The column was washed with buffer A and proteins were eluted with the buffer A using a linear gradient from 0 to 1 mol l^{-1} NaCl. The fractions containing protease activity was applied to a Superdex G-75 gel column (1.6 × 80 cm) which was pre-equilibrated with buffer A. Buffer A (0.5 ml min^{-1}) was used to elute the purified proteins. The protein concentrations of the purified enzyme samples were measured by the Bradford method [4]. The purity of the recovered samples was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. Activity assay and kinetic properties of WT and mutant SBAs

Protease activity was measured by monitoring the release of *p*-nitroaniline as a result of enzymatic hydrolysis of AAPF. The activity assay with AAPF as substrate was defined as standard assay. The reaction was initiated by adding 10 μl of enzyme sample (containing 0.05 mg purified enzyme) to 990 μl of 25 mmol l^{-1} borax-NaOH buffer (pH 10.5) containing 1 mmol l^{-1} of AAPE, 10 mmol l^{-1} of

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