

Study on Thermostability of *Bacillus Subtilis* Lipase by Site-Directed Mutagenesis

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Abstract. BSLA is an extracellular hydrolase secreted by *Bacillus subtilis*, which is an enzyme at room temperature and its optimum temperature is only 35 °C. Thermal stability is an important indicator for the evaluation of industrial biocatalysts. The industrial increase of reaction temperature can improve the conversion rate, the solubility of the substrate and the possibility of reducing the microbial contamination. Therefore, by improving the heat resistance of BSLA through molecular modification, Adapt to the requirements of industrial production process, has important practical value. The mutant BSLAN174E was constructed by whole plasmid PCR and the thermal stability of the mutant strain was verified by experiments. After the purified BSLA and BSLAN174E enzyme solutions were stored at 55 °C for 5, 20 and 60 min respectively, the relative residual activity of BSLAN174E Respectively 1.6, 2.0, 2.3 times of the original strain BSLA.

1. Introduction

BSLA is an extracellular hydrolase secreted by *Bacillus subtilis*. The lipA gene encoding BSLA is located at 291,800 bp of the *B. subtilis* genome. The full length of the structural gene is 636 bp, encoding 212 amino acids. These amino acids are assembled into lipase precursors. The BSLA precursor removed the 31 amino acid signal peptide during post-processing and the resulting 181 amino acids were assembled and folded into BSLA with a molecular weight of only 19 kDa.

BSLA is room temperature enzyme, its optimum temperature is only 35 °C. Thermal stability is an important indicator for the evaluation of industrial biocatalysts. To raise the reaction temperature industrially, the conversion rate, the solubility of the substrate and the possibility of reducing the microbial contamination can be improved. Therefore, increasing the temperature resistance of the enzyme is an urgent problem to be solved in the industrialization of the enzyme one. Finally, mutants X and XI were obtained, whose T5015 reached 89 °C and 93 °C respectively. Thus, the iterative saturation mutation in the enzyme to improve the thermal stability than the traditional methods showed great superiority.



2. Experimental materials and methods

2.1. Experimental Materials

Table 1. Experimental strains, plasmids and primers

	Strain, plasmid or primer	Feature, genotype or sequence	source
Strain	B. subtilis 168	Wild type	The laboratory preservation
	E. coli DH5 α	supE44 Δ lacU169 (ϕ 80 lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Vazyme company
	E. coli BL21 (DE3)	F - ompT hsdS (rB- mB-) gal dcm (DE3)	Vazyme company
	DH5 α -BSLA	Amp ^r , Recombinant bacteria	constructed by this paper
Plasmid	pET22b	Amp ^r , pBR322 origin, Plac, 5493 bp	TaKaRa company
	pET22b-BSLAN174E	Mutant gene N174E	constructed by this paper
	pET22b-BSLAN95E	Mutant gene N95E	constructed by this paper
	pET22b-BSLAQ61D	Mutant gene Q61D	constructed by this paper
Primer	P1	5'-CCGCATATGGCTGAACACAATC-3'	constructed by this paper
	P2	5'-CCCAAGCTTATTCGTATTCTGGC-3'	constructed by this paper
	P3	5'-CAAGAAGGGCTGGAGGGCGGGGGC-3'	constructed by this paper
	P4	5'-CCCCGCCCTCCAGCCCTTCTTTAAT-3'	constructed by this paper

2.2. Experimental method

2.2.1. Molecular simulation method.

a). Predict BSA amino acid mutations in hot spots

The HotSpot Wizard online server was used to analyze and compare the primary sequence of more than 50 Bacillus subtilis lipase family proteins to obtain the mutation rate and mutation of each residue of BSLA. The amino acid residues with high mutation rate were screened. The active amino acids around the catalytic activity center and the substrate channel are excluded to predict the relevant amino acid sites in the hot spot of the amino acid mutation.

b). Mutation site selection.

The RMSF (Root Mean Square Fluctuation) of BSLA was obtained by using molecular dynamics method to determine the more flexible area of BSLA. Pymol visualization software and the B-factor were used to determine the amino acid sites in the protein surface and in more flexible regions.

c). Analyze the mechanism by which ionic bonds introduce a stabilizing BSLA protein structure.

Using molecular simulation and analysis software GROMACS4.5.4 simulation software, the force field GROMOS9643a1, temperature 500K, pH = 7.0; system using a periodic cubic box, BSLA

surrounding water molecules filled with 1.0 nm, adding CL ions Neutralization charge and solvent model adopt explicit model SPC. Simulate the steepest descent in 5000 steps first, and then optimize constrained BSLA, using Parrinello-Rahman and V- rescale. The PME (ParticleMeshEwald) method was used for the long-range electrostatic interaction. The threshold of short-range force was 1.0 nm and the cut-off value of vdW interaction was 1.0 nm. Coulomb interaction cut-off (rcoulomb) is 1.0 nm; Finally, the molecular dynamics simulation without constraint, the simulation time is 7 ns, the time step is 2 fs; the coordinate of each atom in the system is collected once every 1 ps; Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of Gyration (Rg) Solvent Accessible Surface, The content of secondary structure, hydrogen bond, and salt bond parameters were used to resolve the contribution of the mutation sites to the thermal stability of BSLA Important amino acid sites for the thermal stability of BSLA.

2.2.2. Construction of Recombinant Plasmid.

a). Obtaining and PCR amplification of BSLA gene: *Bacillus subtilis* 168 genome was extracted (using Takara's extraction genome kit), and a pair of PCR primers was designed and synthesized according to the sequence of *Bacillus subtilis* genome in Genebank (accession number AL009126.3) Gene amplification and recovery of PCR products: P1: 5'-CCGCATATGGCTGAACACAATC-3', P2: 5'-CCCAAGCTTATTCGTATTCTGGC-3' NdeI and HindIII sites were designed for ligation to the expression vector pET22b was amplified by PCR using the DNA of *Bacillus subtilis* 168 as a template. The PCR conditions were as follows: pre-denaturation at 94 °C for 2 min, 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, and 35 cycles after 72 °C for 10 min.

b). Agarose gel electrophoresis.

c). Plasmid extraction and gum recovery.

d). Cleavage of BSLA Gene and Plasmid: A plasmid-containing strain was amplified in an LB liquid medium containing 50 µg mL⁻¹ of ampicillin and a plasmid (using Takara's Extraction Plasmid Kit) was extracted. NdeI and HindIII were selected as restriction sites to double-digest *Bacillus subtilis* 168 and pET-22b plasmids. The double-digested products were dephosphorylated and ligated with BSLA gene to verify the results of nucleic acid electrophoresis.

e). Transformation of recombinant plasmids.

f). Whole plasmid PCR amplification.

g). Whole plasmid amplification of the product conversion.

2.2.3. *Inducible expression of BSLA*. The DH5α-pET22b-BSLA and mutant plasmids were extracted and transformed into the expression vector *E. coli* BL21 (DE3) respectively. The BL21-pET22b-BSLA strain was constructed and plated on LB plates containing ampicillin overnight. The transformants were picked on LB liquid medium containing 50 µg mL⁻¹ ampicillin and cultured at 37 °C for about 2 h. When the OD₆₀₀ reached 0.6-0.8, IPTG was added to a final concentration of 0.5 mmol L⁻¹, Placed in 30 °C shaker continue to train 6-8 h induction expression.

2.2.4. *Isolation and purification of BSLA*. The bacterial cells were collected by centrifugation in the fermentation broth and the cells were resuspended in an equal volume of Tirs-HCl buffer (pH 7.4) in the fermentation broth. The cells were then disrupted by sonication and centrifuged at 12,000 rpm min⁻¹ for 15 min the supernatant was collected and the supernatant collected was crude enzyme solution of *Bacillus subtilis* lipase. The crude enzyme solution was purified by Ni column affinity chromatography, and the purification effect of the effluent in the collection tube was verified by SDS-PAGE electrophoresis. After purification, the crude enzyme solution was stored at 4 °C.

2.2.5. Determination of related enzymatic properties

a). P-NPP assay lipase activity, Bradford assay protein concentration,

b). Thermal Stability Assay: The purified wild-type BSLA and mutant enzyme solutions were stored at 55 °C for 5, 10, 20, 30, 40, 50 and 60 min, respectively. SpectraMax M3 microplate reader

Determination of residual activity, absorbance was read at 405 nm wavelength every 30 s and samples were taken to determine their protein content. The residual enzyme specific activity after incubation at different times is plotted against time.

3. Results and discussion

3.1. Predict BSLA mutation hot spot area

The HotSpot Wizard online server was used to screen mutation hot spots to obtain the mutation rate and mutation of each residue of BSLA. The amino acid residues with high mutation rate were screened out, and the functional amino acids around the catalytic activity center and substrate channel were excluded. The round of screening for heat stable spots was performed at a mutation rate of 9 residues. If the first round of mutations at a rate of 9 residues can not be selected reasonable mutation point, then the second round of the mutation rate of 7 or 8 residues. Finally, the results of this round of screening are: Ala20, Tyr25, Val27, Ser28, Ser32, Tyr49, Asn50, Val50, Val54, Phe58, Gln60, Ala97, Gly111, Pro118, Gln150, Asn174, Gly175, Gly176.

3.2. Selection of amino acid sites related to thermal stability

Due to the dissociation of acidic amino acid side chains into negative ions, the basic amino acids dissociate into positive ions. These groups are distributed on the surface of the protein molecule to form an ordered hydration layer with the water molecules. Occasionally, a few oppositely charged side chains are located in the molecule Hydrophobic internal salt-forming, can play a role in stabilizing the protein structure. According to the results obtained in the previous section, the amino acid mutation sites related to thermal stability were determined. If the mutation was mutated to Asp, Glu, Lys and Arg, Any one of the amino acid residues can increase the thermostability of the enzyme. Pymol visualization software was used to search for the oppositely charged amino acid residues in the 4 amino acids in the 4 Å range centered on the selected amino acids:

PyMOL> select60, resi60 (selected amino acids that can be mutated);

PyMOL> select near60, 60expand4 (Residues in the range of 4 are selected to determine whether residues oppositely charged thereto).

Ultimately, the following high-mutation rate residues (Table 2) and their results were confirmed and may play an important role in the thermal stability of BSLA.

Table 2. High mutation rate of BSLA residues and mutation results

Mutation site	Gln60	Gly111	Ser167	Asn174	Gly175
Mutation rate	9	9	9	9	9
Mutation results	6×D, 1×E, 1×N	1×D	1×D	1×E	1×E

3.3. The effect of introduction of the predicted mutation site on the stability of BSLA protein was simulated

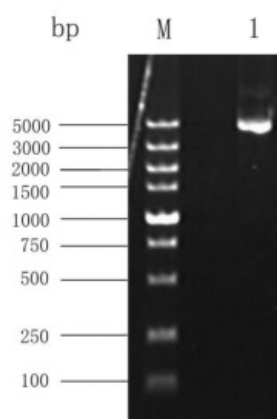
According to the results in Table 2, Gln60Lys, Gln60Asp, Gln60Glu, Gly111Asp, Ser167Asp, Asn174Glu and Gly175Lys mutants were constructed by on-line server SWISS-Model homology modeling. The mutants were evaluated and optimized with Verify_3D, Space conformation. The results of molecular modeling showed that the conformation of the protein after 5500 ps tended to be higher than that of the mutant BSLAN174E by analyzing RMSD and percentage of secondary structure (α , β) of BSLA with mutant BSLAN174E. The average RMSD of wild-type BSLA at the final 2 ns was 1.21 ± 0.02 nm. The average RMSD of the mutant BSLAN174E was 0.69 ± 0.01 nm, which was lower than that of wild-type BSLA. The lower the RMSD was, the more stable the molecular conformation was. It was confirmed that Asn174Glu mutation may play a role in the thermal stability of BSLA.

Table 3. BSLA and BSLAN174E secondary structure percentage content

	BSLA	BSLAN174E
α -helix content	3.67%	3.58%
β -helix content	16.68%	21.41%

3.4. Construction, Expression and Purification of Mutants

3.4.1. Construction of recombinant strains. First, double digestion of the DNA fragment and the expression vector pET-22b were performed using restriction enzymes NdeI and HindIII, respectively. In accordance with the ratio of the components added to 0.5 mL of sterile centrifuge tube, mix well placed in a constant temperature reactor, 30 °C for 3 h or more. After the reaction, the PCR reaction products were detected by electrophoresis. The digestion results of the target gene and pET-22b were consistent with the expected results, and then two double digestion products were recovered using TaKaRa's "mini-gel recovery kit". Then, the target gene recovered from the gel and the double-stranded fragment of pET-22b were mixed with 1 μ L of the double-digested product of pET-22b (0.03 pM), 4 μ L of the double-digested product of the target gene the ligation reaction, 16 °C overnight connection. After the reaction was completed electrophoresis test, the results shown in Figure 1. Finally, the ligation solution was transformed into *E. coli* DH5 α competent cells to construct the recombinant DH5 α -pET22b-BSLA strain.



M: DNA Marker DL5000, Lane 1: Recombinant plasmid

Figure 1. Recombinant plasmids

3.4.2. Whole-plasmid PCR site-directed mutagenesis. The plasmid was extracted by TaKaRa's "plasmid mini-extraction kit". For details, see the TaKaRa Operation Manual. The whole plasmid was amplified using primSTAR. Primers P3, P4, primers containing Asn174Glu:

P3: 5'-CAAGAAGGGCTGGAGGGCGGGGGC-3 '

P4: 5'-CCCCGCCCTCCAGCCCTTCTTAAAT-3 '

The mutated product was ligated into TAKARA's pMD20-T vector and sent to sequencing company for sequencing. The results showed that the mutant BSLAN174E was successfully constructed.

3.4.3. Mutant enzyme expression and purification. The recombinant plasmid DH5 α -pET22b-BSLA was transformed into *E. coli* BL21 (DE3) competent cells by chemical transformation. The plates were plated to construct BL21-pET22b-BSLA. The transformant was picked up in LB liquid medium containing 50 μ g/mL ampicillin and cultured in a shaker at 37 °C. When the OD₆₀₀ reached about 0.8, IPTG was added to a final concentration of 0.5 mmol/L and the culture was continued Hour for induction of expression.

After the crude enzyme solution was purified by Ni column, the protein band of each collected solution was verified by SDS-PAGE electrophoresis, as shown in FIG. 2. In the figure, the protein contained in the effluent collected in each stage of the purification process is finally a single band with a size of 20.1 kDa, which achieves an ideal purification effect.

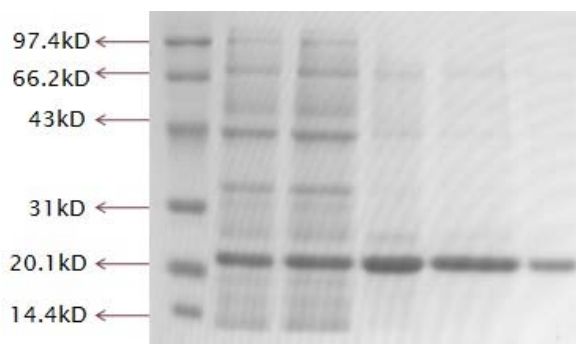


Figure 2. Wild type BSLA purification process SDS-PAGE electrophoresis

3.5. Thermal stability determination

The purified BSLA and BSLAN174E enzyme solutions were separately stored at 55 °C for 5, 10, 20, 30, 40, 50 and 60 min, and the residual activity was measured using a SpectraMax M3 multi-plate reader (Molecular Devices). According to the above standard curve, the activity of the original enzyme and the mutant enzyme, the protein content and the specific activity of the enzyme were obtained at each time period. Since the activity of the enzyme was relatively low, the specific activity was used as a reference to plot the time, as shown in FIG 3.

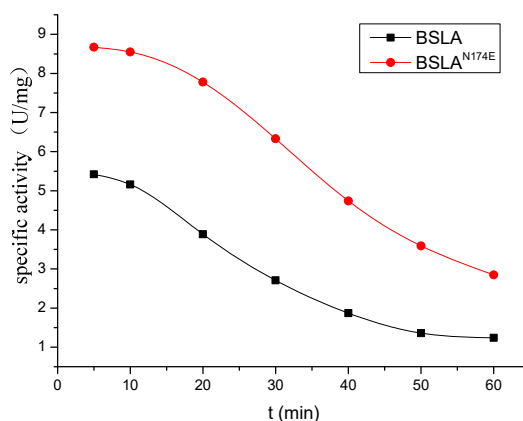


Figure 3. Specific activity curves of BSLA and BSLAN174E

Based on this, the relative residual viability of mutant BSLAN174E after incubation for 5, 20 and 60 min was calculated by multiplying the original strain BSLA as shown in Table 4. Can be seen that the thermal stability of mutant BSLAN174E compared with the original enzyme does have a certain increase, but the increase is not obvious.

Table 4. The relative residual activity of the mutant BSLAN174E is a multiple of the original enzyme BSLA

Time/min	5	20	60
Fold(BSLAN174E/BSLA)	1.6	2	2.3

4. Summary

(A). By HotSpot Wizard on-line server virtual screening mutant hot spots, combined with Pymol visualization software and B factor, the mutation sites and mutation residues were identified to obtain amino acid sites of the protein surface and more flexible regions. The effect of mutation on the thermostability of the enzyme was analyzed by molecular simulation. Finally, the Asn174Glu mutation site was found to have a positive effect on the thermal stability of BSLA.

(B). The BSLA encoding gene was cloned from the genome of *B. subtilis* 168 and sequenced. The whole plasmid PCR method was used to carry out site-directed mutagenesis of the plasmid to construct the mutant BSLAN174E, which was successfully sequenced.

(C). The expression and purification of BSLA and BSLAN174E were achieved. The Ni column purification of enzyme, the determination of lipase activity by p-NPP method and the thermal stability of BSLA174E were established, which laid the foundation for further molecular transformation and molecular biology experiments.

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