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Enhancement of the thermostability of *β*-1,3-1,4-glucanase by directed evolution*

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Abstract: In order to improve the thermostability of *β*-1,3-1,4-glucanase, evolutionary molecular engineering was used to evolve the *β*-1,3-1,4-glucanase from *Bacillus subtilis* ZJF-1A5. The process involves random mutation by error-prone PCR and DNA shuffling followed by screening on the filter-based assay. Two mutants, EGs1 and EGs2, were found to have four and five amino acid substitutions, respectively. These substitutions resulted in an increase in melting temperature from T_m=62.5 °C for the wild-type enzyme to T_m=65.5 °C for the mutant EGs1 and 67.5 °C for the mutant EGs2. However, the two mutated enzymes had opposite approaches to produce reducing sugar from lichenin with either much higher (28%) for the former or much lower (21.6%) for the latter in comparison with their parental enzymes. The results demonstrate that directed evolution is an effective approach to improve the thermostability of a mesophilic enzyme.

Key words: Directed evolution, Error-prone PCR, DNA shuffling, *β*-1,3-1,4-glucanase, Thermostability
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INTRODUCTION

Enzymes are efficient and specific biocatalysts widely used in food industries, but their application in industrial process often involves special properties not found in natural source enzymes. In order to obtain desirable enzymes, in the past ten years, scientists have developed rational (Kurth *et al*., 1998; Mouratou *et al*., 1999; DeSantis *et al*., 1999) and irrational design methods (Babbitt and Gerlt, 1997; O'Brien and Herschlag, 1999) to improve the enzyme properties. Previous researches indicated that use of the rational design to obtain desirable industrial enzyme had a great challenge when the enzyme 3D structure was not known, so the irrational design-directed evolution was used more and more widely to tailor enzymes for optimal performance in industrial applications. It encompasses a series of experimental techniques that reproduce, on an accelerated timescale in the test tube, the evolution of natural diversity and environmental adaptation.

Many enzymes with enhanced properties have been developed (Arnold and Volkov, 1999; Arnold, 2001; Rohlin and Liao, 2001). Thermostability is often a primary goal for improving the properties of an industrial enzyme. There are three strategies to improve the thermostability of an enzyme: rational design principles, directed evolution, and the consensus concept. Many attempts were made to gain understanding of the principles underlying the stability of proteins. Information accumulated to date indicates that rigidity is a prerequisite for high protein thermostability, that may be correlated with more proline, arginine and tyrosine residues, more salt bridges, disulfide bonds, metal binding sites, side chain-side chain hydrogen bonds and intramolecular interactions by hydrophobic packing, but less asparagine, glutamate, cysteine and serine residues. In

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addition, higher thermostability may be correlated with a large fraction of residues in α-helices.

β-1,3-1,4-glucan is abundant in the endosperm cell walls of barley, rye, sorghum, rice and wheat and is composed of a polymer of glucose monomers linked by β-1,3- and β-1,4-glycosidic bonds in an irregular fashion (Woodward *et al*., 1983; Edney *et al*., 1991). Barley β-glucan can be dissolved in water and often has high solution viscosity due to its unique structure. The released β-glucan during mashing may cause severe problems such as reduced yields of extracts and lower filtration rates, as well as the appearance of gelatinous precipitates in finished beer (Fincher, 1975; Scott, 1972). When barley is used as feedstuff, barley β-glucan can cause high intestinal viscosity, which affects the digestibility of feed and absorption of nutrition (Planas, 2000). Because endogenous β-1,3-1,4-glucanases are heat inactivated during malting, the addition of exogenous enzymes during mashing or malting or feed preparation is an efficient way to reduce the negative effects of barley β-glucan. Bacterial β-1,3-1,4-glucanase, which has similar specificity to the β-1,3-1,4-glucanase found in malted barley, is an important industrial enzyme and often added to reduce viscosity during mashing (Godfrey and Reinchelt, 1983). In animal feedstuff, especially for broiler chickens and piglets, the addition of enzymatic preparations containing bacterial β-1,3-1,4-glucanases can improve digestibility of barley-based diets, and reduce sanitary problems. However, the thermostablility of β-1,3-1,4-glucanase still cannot satisfy the industrial requirements.

In this study, random mutagenesis and screening were used to search for novel β-1,3-1,4-glucanase with higher thermostablility. In addition, DNA shuffling and screening at higher temperature were applied to accumulate beneficial mutations from the first generation mutants. The preparation of β-1,3-1,4-glucanase variants with high thermostablility is described.

MATERIALS AND METHODS

Enzymes, reagents, bacteria and plasmids

Restriction enzymes, *Taq* DNA polymerase, *Pfu* DNA polymerase, DNase I, T4 DNA ligase and DNA marker were purchased from Takara Shuzo. Lichenin was the product of Sigma (St. Louis, MO, USA). Reagents were purchased from Sangon, Shanghai, China. All other medium components were purchased from Difco. The plasmid vector pET28a (+) and strain *E. coli* BL21 (DE3) were from Invitrogen. *E. coli* BL21 containing plasmids carrying β-1,3-1,4-glucanase gene was stored in −20 °C refrigerator. Cells were grown routinely in Luria-Bertani (LB) liquid or on LB solid medium, as described by Sambrook *et al*.(1989). All media were supplemented, if necessary, with 50 μg/ml kanamycin.

Error-prone PCR

Random mutations were introduced by error-prone PCR. Two oligonucleotides flanked by *Bam*HI and *Hin*dIII restriction sites, 5′-GGGGGATCCATGCCTTATTCTGAAACG-3′ and 5′-GGGAAGCTTATTTACAGAGGGGAGAA-3′, were used as forward and reverse primers, respectively. The conditions of error-prone PCR were optimized to obtain the desired level of mutations (1~2 amino acid substitutions). A 100 μl reaction mixture contains 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 7 mmol/L $MgCl_2$, 0.1~0.5 mmol/L $MnCl_2$, 0.2 mmol/L dATP, 0.2 mmol/L dGTP, 1.0 mmol/L dCTP, 1.0 mmol/L dTTP, 20×10^{-9} mmol/L of each oligonucleotide primer, 2 ng of template DNA, and 5 U of *Taq* polymerase. The mixture was directly subjected to thermal cycling (94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, 30 cycles). The mutagenic PCR products were gel purified, digested with *Bam*HI and *Hin*dIII and ligated with the double-digested pET28a (+) with the same restriction enzymes. Strains *E. coli* BL21 (DE3) were transformed with the ligated DNA and plated onto LB agar plates containing 50 μg/ml of kanamycin.

DNA shuffling

DNA digestion was carried out as described by Stemmer (1994) with minor modification. About 6 μg DNA was dissolved into 90 μl distilled water before 10 μl of 10×DNase I buffer (500 mmol/L Tris-HCl, pH 7.4; 100 mmol/L $MgCl_2$) was added. The mixture was pre-incubated at 15 °C for 15 min. The digestion was initiated by the addition of 0.15 U/μl of DNase I (Takara) and maintained at 15 °C for different time periods. The reaction was stopped by incubating the reaction mixture at 80 °C for 10 min. DNA fragments

of 50~150 bp were purified by inserting a strip of DEAE ion-exchange paper (Whatman) into the gel and running the DNA fragments onto the strip by further electrophoresis. The membrane was removed and the DNA fragments were eluted by incubating the membrane in 20 mmol/L Tris-HCl containing 1.0 mol/L NaCl and 0.1 mmol/L EDTA for 30 min at 65 °C. The DNA fragments were precipitated with ethanol and re-dissolved in water. The reassembly of fragmented DNA and PCR amplification of reassembled products were performed as described by Stemmer (1994).

Screening for thermostable enzymes

The *E. coli* BL21 cells harboring the recombined plasmids were grown on the LB agar plates with IPTG at 37 °C for 15 h. After chilling at 4 °C for about 30 min, the cells were transferred onto nitrocellulose filters. The original plates were incubated continuously at 37 °C to pick the corresponding strains producing thermostable enzymes. The filter-membranes containing the cells were laid on fresh LB agar plates, and incubated at 37 °C for 5 h, then at 4 °C for 2 h. The colonies on the filters were lysed as described by Song and Rhee (2000). The filters were treated at a specific temperature for 2 h, then incubated at 50 °C on screening plates containing 1.0% (w/v) agarose gel, 0.1% (w/v) lichenin, 0.004% (w/v) Congo red and 50 μg/ml kanamycin (sulfate). After 2 h, the filters were lifted up and the screening plates were stained with Congo red (0.1%, w/v). Then the positive *β*-1,3-1,4-glucanase mutants, corresponding to the transparent halos on the screening plates, were picked up from the replica plates as the strains expressing thermostable enzymes.

Purification of the wild-type and mutated *β*-1,3-1,4-glucanase

The culture solution was centrifuged (12 000 r/min, 10 min) to obtain a clear fermentation broth. The resulting supernatant was used as the starting material. Ammonium sulfate was added to the supernatant to achieve 30% saturation. The solution was stored overnight, and centrifuged (10000 r/min) for 30 min. The supernatant was recovered and precipitated by 70% saturated ammonium sulfate. The resulting precipitate was recovered by centrifugation (10000 r/min, 30 min), dissolved in phosphate buffer solution (50 mmol/L, pH 6.0), and dialyzed against the same buffer solution to remove the ammonium sulfate at 4 °C. The dialyzate was collected, concentrated and placed on a DEAE-Cellulose column (2 cm ×60 cm) which had been equilibrated previously with phosphate buffer (20 mmol/L, pH 6.0) containing 30 mmol/L NaCl. The column was washed with the same buffer, followed by elution with a linear concentration gradient from 30~300 mmol/L NaCl in the same buffer. Fractions (2.0 ml each) were collected at a flow rate of 15 ml/h. Each fraction was monitored by the absorbance at 280 nm. The active fractions were dialyzed, concentrated and stored at 4 °C.

Assay of *β*-1,3-1,4-glucanase activity

The activity of *β*-1,3-1,4-glucanase was measured by a modified method described by Cantwell and McConnell (1983). The enzyme was appropriately diluted with PBS (0.2 mol/L, pH 6.0). An aliquot of prepared 0.1 ml enzyme solution was added to 0.9 ml substrate solution (2 mg/ml lichenin) which was pre-incubated at 50 °C for 10 min, and incubated at 50 °C exactly for 10 min. The reaction was stopped by adding 1.5 ml dinitrosalicylic acid solution and boiling for 5 min. Then, the mixture was cooled immediately with cold water and added to 25 ml with distilled water. The amount of reducing sugar was calculated by the absorption value at 540 nm. One unit of activity was defined as the amount of enzyme capable of producing 1 μmol reducing sugar per minute (using maltose as reference) under the above conditions.

Thermostability of native and evolved *β*-1,3-1,4-glucanase

For determination of enzyme thermostability, 0.1 mg of the purified enzyme was diluted in PBS (0.2 mol/L, pH 6.0) and incubated at different temperatures for 20 min. The sample solution was then cooled immediately in an ice bath. The remaining *β*-1,3-1,4-glucanase activity was measured as described in "enzyme assay".

RESULTS AND DISCUSSION

Random mutagenesis of *β*-1,3-1,4-glucanase gene

Error-prone PCR was used to introduce random mutations into the *β*-1,3-1,4-glucanase gene due to

the reduced fidelity of *Taq* polymerase. Error-prone PCR was performed by increasing the concentration of Mg^{2+}, adding Mn^{2+}, increasing and unbalancing the concentrations of the four dNTP's, and increasing the concentration of *Taq* polymerase. Wan *et al*.(1998) found that the Mn^{2+} concentration in the reaction solution was a decisive factor for random mutagenesis; the mutagenesis rate could be finely tuned by controlling the concentration of Mn^{2+}. Additionally, the Mn^{2+} concentration affected the amount of PCR product, so the amount of mutated product was firstly determined at different Mn^{2+} concentrations (Fig.1). The results showed that the amount of PCR product was noticeably less than that of the non-mutagenized reaction when the Mn^{2+} concentration was higher than 0.3 mmol/L. This might be due to the fact that minimal amount of Mn^{3+} oxidized from Mn^{2+} inhibits the *Taq* polymerase activity.

To probe the effect of Mn^{2+} concentration on the mutation rate of bgls gene, five mutant pools were constructed by ligating the mutated gene, amplified at 0.0, 0.1, 0.2, 0.3 and 0.5 mmol/L Mn^{2+}, with plasmid pET28a (+), and transforming the recombined plasmid into *E. coil* BL21. Four or five colonies from each mutant pool were picked randomly and subjected to DNA sequencing. The relations between Mn^{2+} concentration and the mutagenic rate are shown in Table 1.

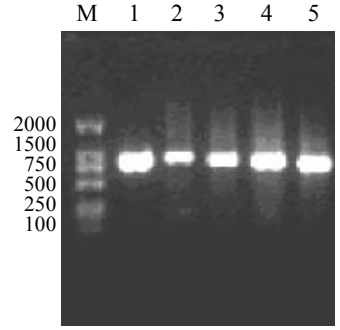

Fig.1 Effect of Mn^{2+} concentration on the error-prone PCR product
M: DL2000; Lane 1: Standard PCR control; Lanes 2~5: Error-prone PCR with 0.5, 0.3, 0.2, 0.1 mmol/L Mn^{2+}, respectively

The results indicated that the error-prone PCR system had a null mutation rate (about 0.05%) which increased with increasing Mn^{2+} concentration. 0.3 mmol/L Mn^{2+} should be the proper concentration for the error-prone PCR system. Under the mutagenic conditions, the PCR reaction gave a mutation rate of 2~3 base substitutions per β-1,3-1,4-glucanase gene corresponding to about 1~2 amino acid substitutions per β-1,3-1,4-glucanase. However, all possible mutations might not be represented equally in the thermostable variants (Table 2). Of the 32 mutations in the sense strand of bgls gene, 26 resulted from the conversion of A and T, and transitions outnumbered transversions 20 to 12, with 16 transitions of A→G and T→C.

Furthermore, the annealing temperature of the error-prone PCR should be lower than that of normal PCR, and the mutagenized PCR products tend to polymerize to high molecular polymers. Although the causative reason for polymerizing is not clear, the polymers can be unpolymerized by incubation at 94 °C for 5 min (Fig.2).

Under the above error-prone PCR conditions, β-1,3-1,4-glucanase gene was used as the template. A

Table 1 The mutation rate at different Mn^{2+} concentrations

Mn^{2+} concentration (mmol/L/30 cycles)	Nucleotide substitutions (%)	Amino acid substitutions (%)
0.00	0.05	0.030
0.10	0.07	0.045
0.20	0.12	0.080
0.30	0.23	0.150
0.50	0.35	0.240

Table 2 Mutations found in the sense strand of all sequenced mutants of the bgls gene after PCR random mutagenesis

Wild-type	Mutations				Types of mutations		
	T	C	A	G	Transitions	Transversions	Total
T	–	4	10	0	4	10	14 (44%)
C	4	–	0	0	4	0	4 (12%)
A	0	0	–	12	12	0	12 (38%)
G	2	0	0	–	0	2	2 (6%)
Total	32				20 (62.5%)	12 (37.5%)	32 (100%)

mutant library comprising about 8000 colonies was established. The library was screened by the filter-based visual screening method after being heat-treated at 100 °C for 2 h, which was found to be sufficient to inactivate the wild-type β-1,3-1,4-glucanase. Eight positive variants with enhanced thermostability were isolated and named as EGe1, EGe2, EGe3, EGe4, EGe5, EGe6, EGe7, and EGe8. Sequence of these mutants revealed the following amino acid changes: EGe1 (F45L), EGe2 (S43T, P204L), EGe3 (N66A), EGe4 (M69I, N198V), EGe5 (N66A, D163A), EGe6 (M57V, N157I), EGe7 (H233E), EGe8 (Q47E).

Shuffling positive variants with enhanced thermostability

In order to combine the beneficial mutations generated by error-prone PCR into one gene clone, the genes of the eight improved mutants were subjected to DNA shuffling, in the process of which the time of DNase I digestion was crucial for the success of DNA fragmentation. The DNA mixture was digested at 15 °C for different time periods and agarose electrophoresis (2%) was used to estimate the amount and the molecular size of the fragments (Fig.3). The results showed that the proper digestion time was 1~3 min when approximately 6 μg DNA was digested with 0.15 unit of DNase I at 15 °C. If the reaction time lasted over 3 min, the amount of fragments (50~150 bp) tended to decrease and the molecular size of the fragments tended to become shorter.

Fig.4 shows the flowchart and the results of DNA shuffling of β-1,3-1,4-glucanase gene. It includes PCR amplification of the eight mutated genes;

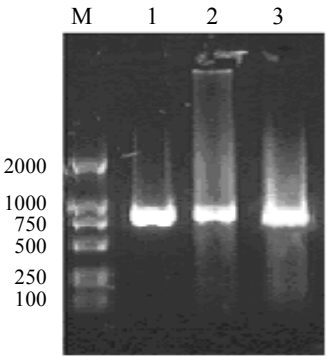

Fig.2 High molecular polymers of error-prone PCR
M: DL2000; Lane 1: Standard PCR control; Lane 2: High molecular polymer in the mutagenized PCR products; lane; Lane 3: Heat-treatment of high molecular polymer

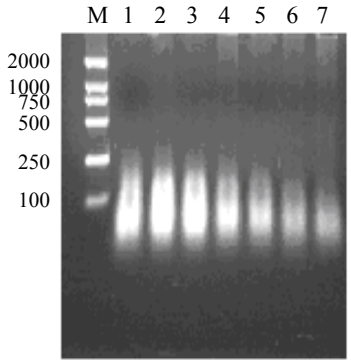

Fig.3 The molecular size of DNA fragments digested by DNase I for different time
M: DL2000; Lane 1: 1 min; Lane 2: 2 min; Lane 3: 3 min; Lane 4: 4 min; Lane 5: 5 min; Lane 6: 6 min; Lane 7: 7 min

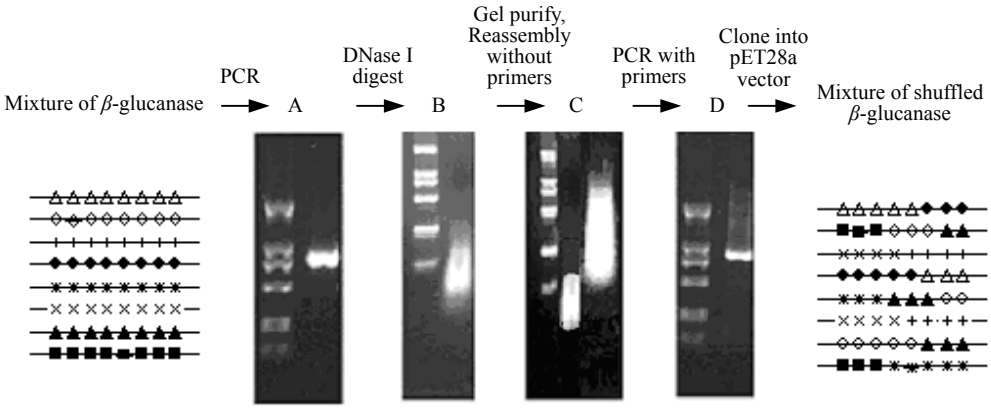

Fig.4 The flowchart and the results of DNA shuffling of β-1,3-1,4-glucanase gene
A: 849 bp DNA fragment encoding β-glucanase was amplified by PCR; B: The bgls gene was digested with DNase I into random small fragments; C: 50~100 bp fragments were purified and reassembled by PCR in the absence of primers; D: Additional cycles of PCR in the presence of primers

digestion of the PCR products into random small DNA fragments with DNase I; purification of 50~150 bp DNA fragments from 2% agarose gel; reassembly of the DNA fragments in a PCR-like reaction without primers; PCR amplification of the reassembled products by standard PCR; followed by cloning into the vector and selection by filter-based visual screening method.

Under the above DNA shuffling conditions, a mutant library comprising about 16000 colonies was established. The library was screened by the filter-based visual screening method after being heat-treated at 110 °C for 2 h, which was found to be sufficient to inactivate the first generation of mutants. Finally, two thermostable *β*-1,3-1,4-glucanases were isolated, designated as EGs1 and EGs2. DNA sequences of the thermostable *β*-1,3-1,4-glucanase genes were determined. They had the following amino acid substitutions: EGs1 (S43T, N66A, M69I, N198V), EGs2 (F45L, Q47E, M57V, D163A, H233E). Also the effects of these mutations on the thermostability of *β*-1,3-1,4-glucanase were evaluated using a variety of proposed mechanisms for the enhanced thermostability (Vieille and Zeikus, 2001). Amino acid residues with polar side chains were substituted for those with nonpolar hydrophobic side chains except the S43T substitution in all of the mutations of EGs1. The alanine, isoleucine and valine substitutions may allow for tighter packing in hydrophobic cores and S43T might be a silent mutation. In general, the thermostability of EGs1 could be ascribed to the changes during which the hydrophobic amino acids increase the tightness of the hydrophobic core packing. By contrast, EGs2 had five amino acids substitutions. The two glutamic acid substitutions might form ionic pairs with nearby amino acids, and the valine, alanine and the leucine substitutions might increase the tightness of the hydrophobic core packing. Similarly the increased thermostability of EGs2 might be due to a combination of increased ionic pairing and hydrophobic strength.

Characterization of the thermostable *β*-1,3-1,4-glucanase

Wild-type and mutated *β*-1,3-1,4-glucanase were purified from culture solution by two-step precipitation with ammonium sulfate, and ion-exchange DEAE-Cellulose chromatogram. The final products gave a single band on SDS-PAGE with a molecular mass of about 27 kDa.

The kinetic parameters of these enzymes in the hydrolysis reaction of lichenin were measured (Table 3). The Michaelis constants (K_m) of the mutant EGs1 and EGs2 enzymes were 6.56 mg/ml and 6.89 mg/ml, which was almost the same as the wild-type value of 6.35 mg/ml. The maximal velocities (V_{max}) of the mutant EGs1 and EGs2 enzymes were 356 μmol/(mg·min) and 238 μmol/(mg·min), respectively, which was much higher (by 28%) or lower (by 21.6%) than the wild-type value of 278 μmol/(mg·min). The results indicated that the affinity for lichenin of the two mutated enzymes was almost unchanged, but the catalytic activity on lichenin was changed. The catalytic activity of EGs1 was increased and the catalytic activity of EGs2 was decreased.

Table 3 Kinetic parameters of the wild-type enzyme and the mutated enzymes

Strain	K_m (mg/ml)	V_{max} (μmol/(mg·min))
Wild-type	6.35	278
EGS1 variant	6.56	356
EGS2 variant	6.89	238

The thermostability of these enzymes was assessed by measuring their remaining enzymatic activities after incubating them at various temperatures for 20 min. To evaluate the effect of different temperatures on the enzyme activity, enzyme reactions were initiated at 30 °C, 40 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C by using the purified enzyme. The results showed that the thermostability of EGs1 and EGs2 enzymes was shifted upward by 3 °C and 5 °C compared with that of the wild-type enzyme (Fig.5a). The specific activity of the mutants approached to their maximum value at 60 °C (Fig.5b). This indicated that the optimal temperatures (T_{opt}) of EGs1 and EGs2 enzymes were both at 60 °C, which was increased by 5 °C compared with that of the wild-type enzyme.

CONCLUSION

The purpose of this study is to create a thermostable *β*-1,3-1,4-glucanase through directed evolution method involving random mutation by error-prone

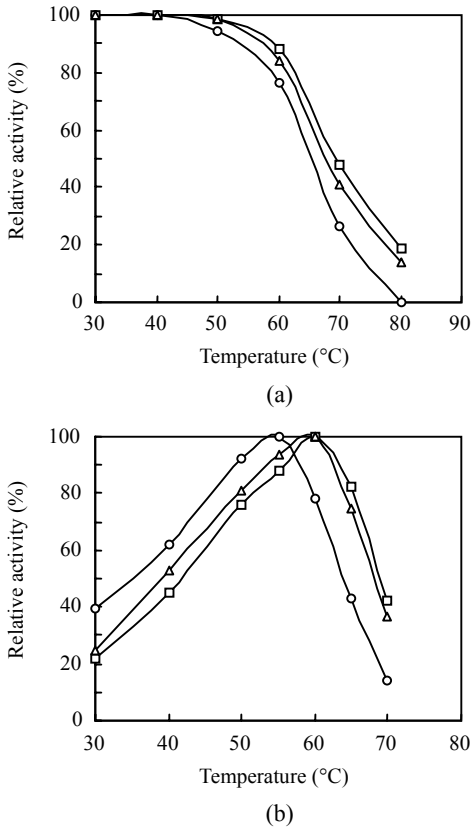

Fig.5 The thermostability (a) and the optimum temperature (b) of natural (○) and evolved *β*-1,3-1,4-glucanase (△: EGs1; □: EGs2)

PCR and DNA shuffling followed by screening for increased thermostable mutants on the screening plates. In the random mutation reaction system, Mn^{2+} concentration is a decisive factor for the mutation rate. Error-prone PCR reaction system containing 0.3 mmol/L Mn^{2+} produced 2~3 base substitutions per *β*-1,3-1,4-glucanase gene corresponding to about 1~2 amino acid substitutions per *β*-1,3-1,4-glucanase. The beneficial mutations generated by error-prone PCR were combined into one gene through DNA shuffling, during which the DNase I digestion time is crucial to the DNA fragmentation. One to three minutes is proper when 0.15 U/μl DNase I is used for about 6 μg DNA. Under these conditions, the DNA is digested into small fragments (<200 bp), and the amount of about 100 bp fragments is the largest.

Through random mutation, DNA shuffling and filter-based screening, two thermostable mutants were obtained. Analysis of the amino acid substitutions indicates that there is no single mechanism that is responsible for the remarkable stability of the thermostable enzymes. They may be stabilized by tight packing of the hydrophobic core, or formation of ionic pairs, hydrogen bonds, disulfide bonds, etc. Different enzymes possess particular thermostability from different evolutionary pathways.

The results of the characterization of the thermostable *β*-1,3-1,4-glucanases indicated that the ability of the thermostable EGs1 enzyme to produce reducing sugar from lichenin was enhanced in comparison with the parental enzyme. The results also demonstrated that enhancing the thermostability does not necessarily result in the loss of the low-temperature activity of the enzyme; and that directed evolution is an effective approach to improve the thermostability of a mesophilic enzyme.

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