Engineering better thermostability into a GH16 β-13-1,4-glucanase using a directed evolution strategy

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β-glucan is a non-starch polysaccharide widely found in plant cell walls as well as in some fungi, algae and bacteria. Barley has the highest content, which can reach 2-20g/100g dry weight (65% soluble). β-glucan in nature is mostly formed by β-glucose residues linked by (1,3)/(1,4) or (1,3)/(1,6) bonds. Therefore according to the type of glycosidic bond in a β-glucan, It can be divided into: β-1,3-1,4-glucan (lichen chitosan), β-1,4-glucan (cellulose), β-1,3-glucan (seaweed polysaccharide) and β-1,3 (4)-glucan [1].

β-1,3-1,4-glucanase can specifically hydrolyze β-1,4-glycosidic bonds in 3-O-substituted pyranose. Because of the specificity of its hydrolysis, β-1,3-1,4-glucanase is in high demand in industries that rely on cereals such as oats or barley as feedstock [2]. For example, as a feed additive, β-1,3-1,4-glucanase can hydrolyze β-glucan to oligosaccharides of low molecular weight, eliminating the anti-nutritional effect; In beer brewing, β-1,3-1,4-glucanase can reduce the viscosity of liquor and increase the extraction rate of malt. In addition, β-1,3-1,4-glucanase is also used to produce functional oligosaccharides with probiotics, which are commonly used in the food processing industry. Therefore, the development of β-glucanase with excellent enzymatic properties (high activity and thermostability) is of great significance for industrial production [3].

BisGlu16B_ΔC, the GH16 glucanase from *Bispora* sp. MEY-1, has excellent catalytic performance. The specific activity of BisGlu16B_ΔC was up to 77000U/mg when barley glucan was used as substrate. However, the poor thermostability of BisGlu16B_ΔC limited its application in the feed industry [4]. In this study, BisGlu16B_ΔC was used as the starting material, and its thermostability was rapidly artificially evolved and modified by a directional evolution strategy. Through thermostability evaluation and comparison of mutants, dominant mutants with improved thermostability were finally obtained. This study provides a reference for the industrial production of glucanase and its application in feed.

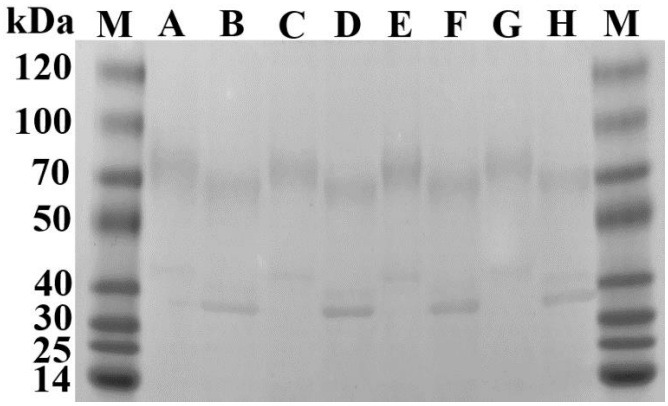

Fig. 1 SDS–PAGE analysis of the purified BisGlu16B_ΔN and its three mutants. Lanes: M, the standard protein molecular weight markers; A, C, E, and G: the purified wild-type BisGlu16B_ΔC, and three mutants A43P, T59I, G165K; B, D, F, and H: deglycosylated the wild type and three mutants.

The yeast expression mutant library was constructed by error-prone PCR, and the thermostability of the three mutants A43P, T59I and G165K was confirmed to be significantly higher than that of the wild type by high-throughput screening under high temperature conditions. After purifying the wild type and three mutants, the purity of glucanase was more than 95%, and the molecular weight of glucanase decreased significantly after the removal of N-glycogroup (Fig. 1). The enzymatic properties of wild type and mutant were determined by using lichen polysaccharide as substrate. As shown in Fig. 2A, the optimal pH of wild-type and mutant was not significantly different from each other, and both ranged from 3.5 to 4.0. The pH-stability of the three mutants between pH7.0 and 10.0 was higher than that of the wild type. Among them, mutant T59I increased the relative enzyme activity by 11%-11.3 times between pH1.0-10.0, especially under an alkaline environment (pH7.0-10.0), the increased range was more than 2 times (Fig. 2B). The optimum temperature of the enzyme was determined at pH4.0, 0.1mol/L McIlvaine buffer and different temperatures (37-95℃). As shown in Fig. 2C, the optimal temperatures of the wild type and the three mutants A43P, T59I and G165K were 55℃, 60℃, 60℃ and 55℃, respectively. The relative enzyme activity of the three mutants (5%-94%) was significantly higher than that of the wild type (0.2%-

16%) at high temperatures (65-75℃). The half-lives ($t_{1/2}$s) of glucanases were determined by incubation at 60℃ for 0-120 min. As shown in Fig. 2D, the $t_{1/2}$s of A43P, T59I, and G165K at 60℃ were 34, 49, and 24 min longer than that of the wild type (16 min), respectively.

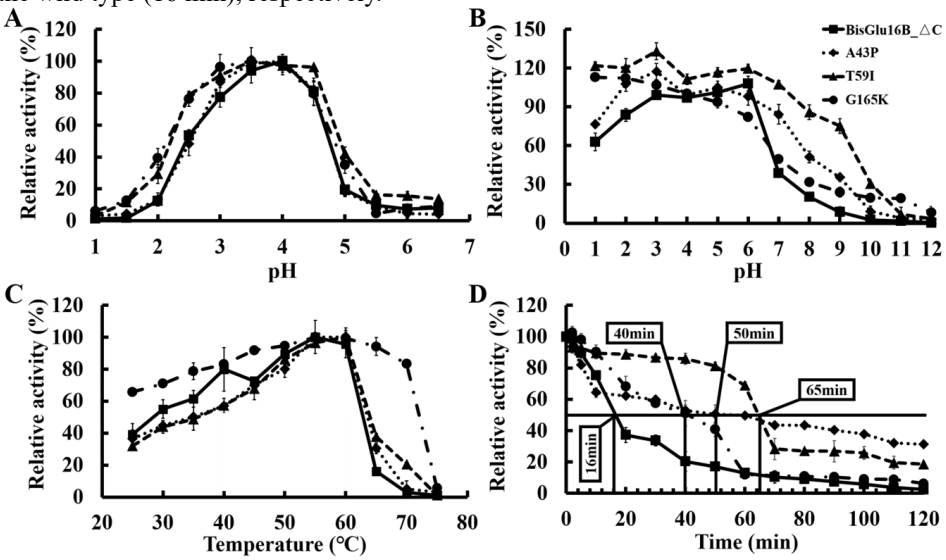

Fig. 2 Properties of the purified recombinant wild-type BisGlu16B_ΔC and its mutants. A pH–activity profiles of each enzyme tested at the respective optimal temperatures; B pH–stability profiles. After the enzymes were incubated for 1 h at 37 °C in buffers with pH ranging from 1.0 to 12.0, the residual activities were determined in 100 mM McIlvaine buffer at the optimal temperature and pH of each enzyme; C temperature–activity profiles tested at pH4.0 of each enzyme; D half-lives of wild-type BisGlu16B_ΔC and its mutants at 60 °C.

The kinetic parameters and specific activity of glucanase were determined by using lichen polysaccharide as substrate at pH4.0 and 55℃. As shown in Table 1, the specific activity (V_{max}) and catalytic efficiency of the mutant G165K were 60% and 62% higher than those of the wild type, respectively. The enzyme activities of the other two mutants A43P and T59I were not significantly different from those of the wild type.

Table 1 Kinetic parameters and specific activity of glucanases against lichenin at 55 °C.

Enzymes	K_m (mg/mL)	V_{max} (μmol/min·mg)	k_{cat}/K_m (mL/s·mg)	Specific activity (U/mg)
BisGlu16B_ΔC	3.14 ± 0.29	64600 ± 4400	11900 ± 260	62000 ± 1300
A43P	2.56 ± 0.04	54300 ± 890	12300 ± 140	55900 ± 4600
T59I	2.67 ± 0.09	64900 ± 1490	14100 ± 170	65400 ± 2600
G165K	4.16 ± 0.65	103200 ± 11500	14400 ± 611	100600 ± 1500

In this study, the thermostability of GH16 β-1,3-1,4-glucanase was improved by directed evolution for the first time. Three mutants with improved thermostability were obtained by high-throughput screening. Enzymatic properties showed that the thermostability and catalytic activity of mutant G165K were improved simultaneously. The dominant mutants obtained in this study show great application potential in the feed industry, and provide a strong reference for the molecular improvement of enzymes.

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