Front. Chem. Eng. China 2008, 2(4): 439–442
DOI 10.1007/s11705-008-0062-8

RESEARCH ARTICLE

Enhancing thermostability of β-mannanase by protective additives

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Abstract The effects of some sugars (glucose, mannose, fructose, sucrose and chitosan) and polyols (glycol, glycerol and sorbitol) as protective additive on the thermostability of β-mannanase were studied. The optimal reaction temperatures of β-mannanase and the thermodynamics and the deactivation kinetics with or without additives were also investigated. The experimental results show that sucrose, chitosan and sorbitol could apparently improve the thermal stability of β-mannanase when their concentration was kept at 2 g/L. The optimal combination additive proportion was sucrose: chitosan : sorbitol = 1 : 2 : 2 (molar ratio) using the orthogonal experimental design. The sucrose, chitosan, glycerol, sorbitol and the combination additive might increase the optimal reaction temperature from 50°C to about 60°C due to their good protection effect. The thermal deactivation curves of β-mannanase accorded with the kinetic rules of first order reaction, and the corresponding kinetic and thermodynamic parameters were calculated. Meanwhile, the protective mechanism of the additives against deactivation of enzyme was also discussed.

Keywords β-mannanase, thermostability, protective additive, thermal inactivation kinetic, protective mechanism

1 Introduction

β-Mannanase (β-1,4-D-mannan mannohydrolase; EC 3.2.1.78) [1] may randomly hydrolyze β-(1 → 4) glucosidic bonds within the main chain of mannan-type polysaccharides, e.g. galactomannan, glucomannan, galactoglucomannan and mannan, which are the important components in hemicellulose. As an important hemicellulase, β-mannanase has been commercially used in food, feed, pulp, paper and oil industries [2,3]. Furthermore, studies on β-mannanase are widely concerned in the food industry since it can be used to refine fruit juice as well as the production of mannan-oligosaccharide, a kind of functional food [4].

Enzyme deactivation is an important factor to limit the industrial production and application of enzymes. Improving enzyme stability is a focus for research in the field of biochemical engineering. In view of the simple processing, low cost as well as easy operation, the additive protection method is usually used to improve enzyme stability. Up to now, there has been no study on the enhancing stability of β-mannanase using protective additives.

In this work, the effects of some additives on the thermal stability of β-mannanase are investigated. The composition of combination additives is optimized. The possible protective mechanism is suggested using kinetic and thermodynamic approaches of thermal deactivation. The systematic research on β-mannanase stability could benefit industrial production and application of enzymes and has an important economic value.

2 Materials and methods

2.1 Materials

Liquid β-mannanase is prepared by tank fermentation from *Bacillus subtilis* TJ-200603 in our laboratory, and purified using the techniques of salt precipitation, ultrafiltration, ion-exchange chromatography and gel exclusion chromatography. All chemical reagents are analytical pure and purchased from the local market.

2.2 Enzyme assay

The activity of β-mannanase is determined by the 3,5-dinitrosalicylic acid (DNS) method [5]. One enzyme activity unit is defined as the amount of β-mannanase liberated

Translated from *Journal of Tianjin University*, 2007, 41(1): 114–118 [译自: 天津大学学报]

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from 1 μmol mannose per minute under the optimal conditions.

2.3 Determination of thermal stability of enzyme

Sugar and polyol solutions of certain concentrations are added into β-mannanase solution, respectively. The enzyme activity of the mixture solution is determined by the DNS method. The enzyme solution without protective additive is the control sample. The relative enzyme activity residue is calculated as follows:

$$A_r = \frac{A_p - A_0}{A_p} \times 100\%, \quad (1)$$

where A_r is the relative enzyme activity, A_p is the enzyme activity with protective additive; A_0 is the enzyme activity without protective additive.

2.4 Orthogonal design of combination additive

Three kinds of protective additives with good performance are combined as the combination additives, and the orthogonal experiments with three factors and three levels, L9 (3^4) are carried out. The results are shown in Table 1. Thus, the protective effect of combination additives is optimized.

Table 1 Orthogonal experimental design for combination additives*

level	ρ_A/g·L^{-1}	ρ_B/g·L^{-1}	ρ_C/g·L^{-1}
1	1	1	1
2	2	2	2
3	3	3	3

*A is sorbitol; B is chitosan; C is sucrose.

2.5 Determination of relative enzyme activity curve

The β-mannanase activities at various temperatures (40°C, 50°C, 60°C, 70°C and 80°C) are determined, respectively. Relative activity curves of enzyme correlated with temperatures are figured based on the experimental data.

2.6 Thermal deactivation kinetic and thermodynamic experiments

The β-mannanase solutions with and without additives are kept at 65°C. The activities of samples obtained from the above enzyme solution were analyzed at 50°C every 30 minutes. Therefore, the deactivation curve is obtained.

For first-order deactivation reaction,

$$\ln A_r = -k_{inact} t, \quad (2)$$

where A_r is relative activity of enzyme, %; k_{inac} is the rate constant of first-order deactivation, 1/min; t is the time maintained at 65°C, min.

The half-life of deactivation is calculated as follows:

$$t_{1/2} = \ln(2/k_{inact}). \quad (3)$$

At the same temperature, the Gibbs free energy is obtained by the following equation [6]:

$$\Delta G = -2.303RT \lg(k_{deact} h / k_B T). \quad (4)$$

In Eq. (4), R is gas constant, 8.314 J/(K·mol); T is absolute temperature, K; k_{deact} is the rate constant for the first order thermal deactivation; h is Planck constant, 6.626×10^{-34} J·S; k_B is Boltzmann constant, 1.381×10^{-23} J/K.

3 Results and discussion

3.1 Effect of single additive on stability of β-mannanase

According to Graber's study [7], the half-life of amylase deactivation added with 4 mol/L sorbitol is 2000 times higher than that without protective additive. Back et al. [8] also found some polyhydroxy substances, such as sorbitol and sucrose, may improve the stability of various proteins. As shown in Figs. 1 and 2, the additives such as sorbitol, sucrose and chitosan could improve the β-mannanase activity.

3.2 Effect of combined additive on stability of β-mannanase

Three good additives (sorbitol, sucrose and chitosan) are combined. The composition of additive combination is optimized using the orthogonal experimental design, as shown in Table 2. It shows that the maximal relative activity 250.5% is obtained. The good performance of combination additive could be explained as the synergistic effect between three additives, which may form more stable structure of β-mannanase.

3.3 Optimal reaction temperature

As is well known, the higher temperature can enhance the biocatalytic reaction rate, and can also affect the stability of active biocatalysts, such as β-mannanase, even resulting in the complete deactivation of biocatalysts. Therefore, it is quite important to understand the effect of temperature on enzyme stability. Figure 3 shows the effect of protective additives on the reaction temperature of β-mannanase. The optimal temperature increases about 10°C after the addition of certain additives, which indicates that the improvement of enzyme stability by

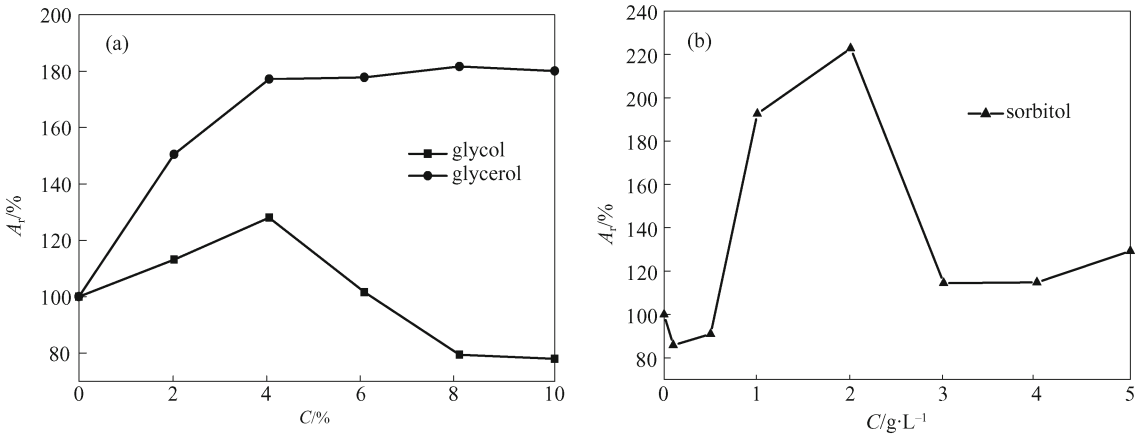

Fig. 1 Relative activity of β-mannanase in presence of different polyols
(a) Liquid polyol protective agent; (b) solid polyol protective agent

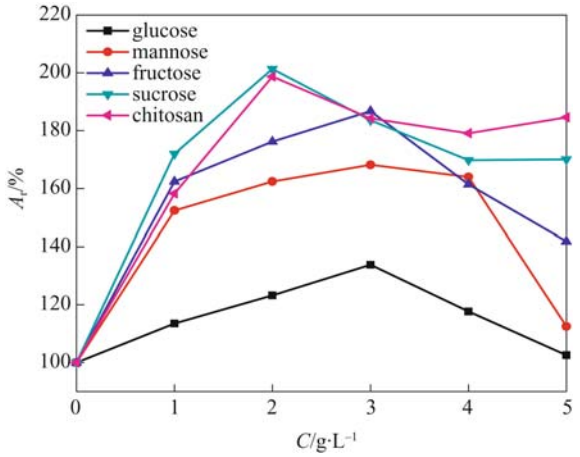

Fig. 2 Relative activity of β-mannanase in presence of different sugars

protective additives may increase the optimal reaction temperature.

3.4 Kinetics and thermodynamics of thermal deactivation and protective mechanism

As was shown in Fig. 4, the deactivation reaction of β-mannanase followed first-order reaction. Various rate constants of thermal deactivation were calculated by Eq. (2), half-life by Eq. (3) as well as Gibbs free energy by Eq. (4). The computed results are listed in Table 3.

Table 2 Effect of additive combinations on relative activity of enzyme

trial	ρ_A/g·L^{-1}	ρ_B/g·L^{-1}	ρ_C/g·L^{-1}	A_r/%
1	1	1	1	207.7
2	1	2	2	173.6
3	1	3	3	170.4
4	2	1	2	215.1
5	2	2	1	250.5
6	2	3	3	200.5
7	3	1	3	219.1
8	3	2	1	212.0
9	3	3	2	230.1

From the data in Table 3, it was revealed that protective agents, especially additive combination, may largely enhance the stability of β-mannanase and decrease enzyme deactivation. According to Samborska's dynamic deactivation analysis [9], some polyhydroxy agents can prolong the half period of amylase deactivation at high temperature, and thus also improve the stability of enzyme.

It was shown in Table 3 that the Gibbs free energy of β-mannanase deactivation was not largely changed with the additives, which was similar to Amjum's result [10]. That is, the protective additives would not significantly affect the internal conformation of enzyme molecule.

The mechanism of enhancing enzyme stability by polyhydroxy agents was studied in many previous papers [8,11–13]. Based on the previous works, the polyhydroxy sugars and polyols had better modification performance than the single hydroxy agent, because the more hydrogen bonds supplied from polyhydroxy sugars and polyols could stabilize the enzyme structure

4 Conclusions

1) For the single additive, three additives (sucrose, chitosan and sorbitol) show good protective performance to improve the activity of β-mannanase. When their concentration was kept at 2 g/L, the relative activities were 201.3%, 198.7% and 222.8%, respectively.

2) From the orthogonal experiment of combined additives, the optimized additive combination was obtained (sucrose : chitosan : sorbitol was 1 : 2 : 2 in molar ratio). Under the optimized conditions, β-mannanase relative activity was 250.5%.

3) Sucrose, chitosan, glycerol, sorbitol and the combination additive may increase the optimal reaction tem-

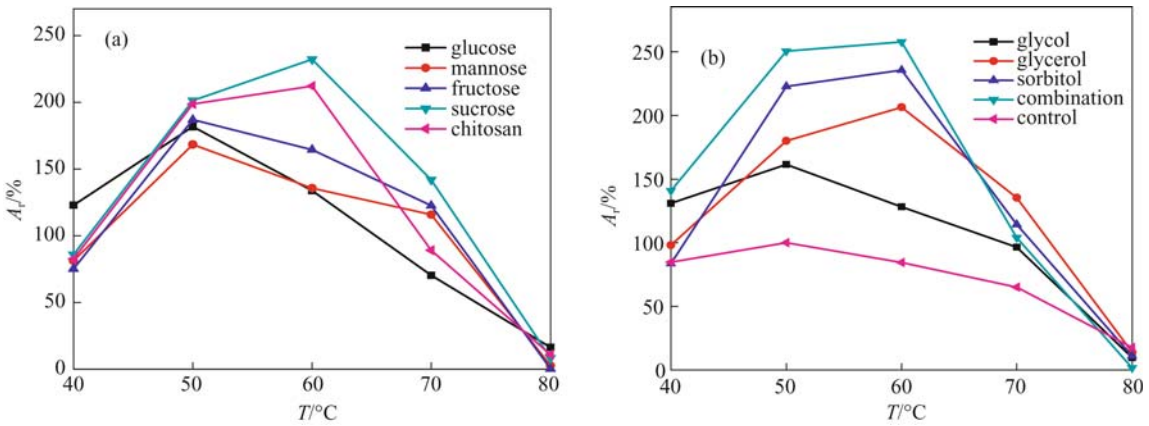

Fig. 3 Effect of protective additives on reaction temperature of β-mannanase
(a) Sugar protective agents; (b) polyols and combination protective agents

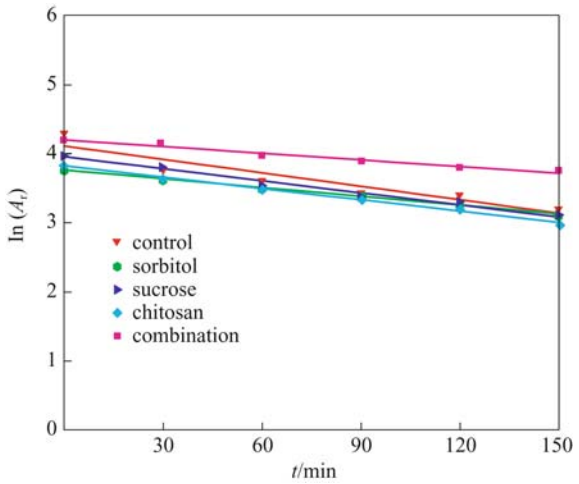

Fig. 4 Thermal inactivation curves of β-mannanase

Table 3 Kinetic and thermodynamic parameters for thermal deactivation of β-mannanase

agent	k_{inact}/min^{-1}	ΔG/kJ·mol^{-1}	$t_{1/2}$/min
none	0.0065	108.87	106.64
sorbitol	0.0042	110.10	165.04
sucrose	0.0058	109.19	119.51
chitosan	0.0055	109.34	126.03
combination	0.0032	110.86	216.61

perature from normal 50°C to about 60°C due to their good protective effect.

4) Based on the research on the deactivation kinetics and protection mechanism, the reaction parameters were calculated. Using thermal deactivation kinetic and thermodynamic methods, the mechanism of enhancing enzyme stability using polyhydroxy agents was analyzed.

Acknowledgements This work was supported by the Tianjin Science & Technology Commission of China (No. 05YFGZGX04600), the National Key Technology R&D Program (No. 2007BAD42B02) and the International Science and Technology Cooperation Program (No. 2006DA62400).

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