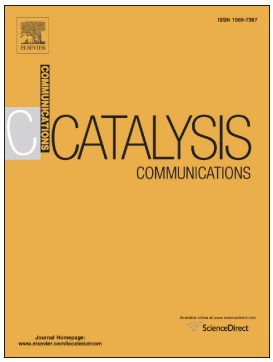

A novel strategy to improve the aromatic alcohols tolerance of enzyme for preparative-scale synthesis of natural glycosides

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A novel strategy to improve the aromatic alcohols tolerance of enzyme for preparative-scale synthesis of natural glycosides

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Abstract

Aromatic alcohols are liable to result in enzyme inactivation. A novel strategy was established for improving the aromatic alcohol tolerance of β-galactosidase (BMG) from *Bacillus megaterium* YZ08. The half-life of BMG in 200 mM vanillyl alcohol solution was dramatically increased by 9–123 times with the addition of hydrophilic solvents. In 30% DMSO, the reaction concentration of aromatic alcohol could reach up to 300–400 mM, and 0.85–2.44 g of natural glycosides were successfully obtained in 100 mL-scale. The simple and effective strategy shows potential applications when dealing with the preparative-scale exploitation of enzymatic reactions.

Keywords: Enzyme inactivation; Substrate tolerance; Hydrophilic solvent; Preparative-scale; Natural glycoside

1. Introduction

Aromatic alcohols are aglycones of a series of important natural glycosides [1]. They have attracted the attention of food and pharmaceutical industries first of all for their well-acquainted antioxidant activity [2]. Not only that, they also possess anti-angiogenic, anti-inflammatory and anti-nociceptive activities [3]. Glycosylation can improve aromatic alcohols' pharmaceutical and pharmacokinetic properties, such as selectivity, bioavailability, water solubility and oxidative stability [4]. More importantly, some important natural glycosides and their derivatives can be easily synthesized by the glycosylation of aromatic alcohols. Salidroside [5] and gastrodin [6] can be efficiently synthesized by using tyrosol and 4-hydroxybenzyl alcohol as substrates, respectively. For the glycosylation of these compounds, conventional chemical glycosylation is difficult to implement due to the insurmountable drawbacks in the multiple steps of protection/deprotection to control regio- and stereoselectivity. Exquisite selectivity is the hallmark of enzymatic glycosylation. However, majority of aromatic alcohols significantly inhibit enzyme activity, and the reactant concentration is generally restricted to relatively low concentrations [7-9].

Several strategies have been reported for reducing the adverse effects of aromatic alcohol and its derivative on enzymes. One such strategy is adsorbing substrates through the use of silica gel or cyclodextrins [10, 11]. Bridiau et al. used silica gel to adsorb aromatic primary alcohols under positive argon pressure away from light; the substrate concentration reached 250 mM [10]. Cyclodextrins successfully limited the substrate inhibition of carboxypeptidase A in catalyzing the hydrolysis of

(*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate) [11]. Both of these methods relieved the effect of the substrate on the enzyme by reducing the concentration of substrate free in solution. This suggests that the catalytic efficiency did not enhance with the increase in the total concentration of the substrate. A potential alternative is the water-organic biphasic system [12, 13], in which the substrate is mainly retained in organic phase. Consequently, the substrate concentration is regulated and minimized around the enzyme [13]. Nevertheless, the mass-transfer limitation in the case of biphasic system is a major challenge for glycosylation reaction, because the hydrophilic sugar donor and the hydrophobic acceptor are in different phases [14]. By contrast, enzymatic glycosylation in hydrophilic organic solvents presents no mass-transfer limitation and provides numerous industrially attractive advantages, such as increased solubility of hydrophobic substrates and reversal of the thermodynamic equilibrium of hydrolysis reactions [15-17].

In this study, a novel strategy was successfully established for reducing the inactivation of β-galactosidase (BMG) from *Bacillus megatherium*YZ08 caused by aromatic alcohols. The addition of hydrophilic solvents remarkably enhanced the stability of BMG in aromatic alcohol solution. To the best of our knowledge, this paper is the first report on improving the substrate tolerance of enzymes by simply adding hydrophilic solvents.

2. Experimental

2.1. Materials

2-Nitrophenyl-β-D-galactopyranoside, vanillyl alcohol and other aromatic

alcohols were purchased from Aladdin (Shanghai, China). One Step Cloning Kit was purchased from Novoprotein (Shanghai, China). Organic solvents were purchased from Sinopharm (Shanghai, China).

2.2. Enzyme preparation and activity assay

The preparation of purified BMG and the determination of BMG activity were conducted as described previously [18].

2.3. Effect of vanillyl alcohol on BMG stability in different systems

The effect of different concentrations of vanillyl alcohol on the activity and stability of purified BMG was investigated in Na_2HPO_4/KH_2PO_4 (50 mM, pH 7.4) buffer containing 5 mM Mg^{2+}. The stabilizing effect of hydrophilic solvents in 200 mM vanillyl alcohol solution was then detected. The samples were incubated for appropriate periods and the residual activities were determined as described above.

2.4. Kinetic analysis of galactosylation of vanillyl alcohol catalyzed by purified BMG

The kinetic parameters were determined in the reaction mixture containing vanillyl alcohol as the substrate and 500 mM lactose in Na_2HPO_4/KH_2PO_4 buffer (50 mM, pH 7.4) containing 5 mM $MgCl_2$. The substrate concentration was in the range of 0.5–5 mM. Purified BMG (final concentration 12.15 μg/mL) was added, and the reaction was incubated at 35 °C for 10 min, or 20 min. The reaction was stopped by adding 900 μL methanol to 100 μL reaction solution. The reaction products were analyzed by HPLC.

2.5. HPLC analysis

The mixture of enzymatic galactosylation was analyzed by RPHPLC on an

Agilent TC-C18 column (4.6 mm×250 mm, 5 mm) with a flow rate of 0.9 mL/min. The elution is detailed in supplementary material.

2.6. Preparative-scale synthesis, purification and structure determination of the products

The reaction mixture (70 mL Na_2PO_4/KH_2PO_4 buffer (50 mM, pH 7.4) + 30 mL DMSO) containing $MgCl_2$ (5 mM), lactose (50 mmol), aromatic alcohols (30–40 mmol), crude enzyme solution (100 U) was prepared in a 250 mL Erlenmeyer flask. The reaction was conducted at 35 °C with shaking at 200 rpm for 12 h. AB-8 macroporous resin column and flash column chromatography were used to purify the products. The procedure is detailed in supplementary material. The product structure was determined by NMR (BrukerAV-400 spectrometer, Switzerland).

3. Results and Discussion

3.1. Effect of aromatic alcohols on BMG

Many aromatic alcohols severely affect the activity of enzymes, including BMG. The effect of aromatic alcohol on the BMG was analyzed using vanillyl alcohol as the model substrate. The half-lives of BMG in vanillyl alcohol solution added at different concentrations were measured (Table 1). As the concentration of vanillyl alcohol was increased, the half-life of BMG was increasingly shortened. When the concentration of vanillyl alcohol was only 25 mM, the half-life of BMG was less than 1 hour, which was only 2.31% of that without addition of vanillyl alcohol. These results indicated that BMG was unstable in vanillyl alcohol aqueous solution presumably because of the powerful capability of vanillyl alcohol to seize essential water molecules from

BMG. Moreover, after 1 h of incubation, a small amount of white precipitate was observed in the mixture of BMG and 50–200 mM vanillyl alcohol, further reinforcing BMG inactivation.

3.2. Hydrophilic solvent for improving aromatic alcohol tolerance of BMG

To date, hydrophilic solvents have been rarely used to reduce the effect of substrates on enzymes. The reason is that hydrophilic solvents are generally considered toxic to most enzymes because of the powerful capability of such solvents to seize essential water molecules from protein [19, 20]. Fortunately, BMG is stable in various 10–30% (v/v) hydrophilic solvents [21]. In addition, BMG is more stable in aqueous hydrophilic solvent than in aqueous solution [18]. On this basis, the half-lives of BMG in hydrophilic solvents with different log *P* values at 10% (v/v) concentration containing 200 mM vanillyl alcohol were tested (Table 2). There is no apparent association between stabilizing effect and the log *P* values of solvents. The half-lives were markedly improved in all tested aqueous hydrophilic solvents except for 10% acetonitrile. The ideal results were obtained in 10% DMSO and methyl alcohol, and the half-life of BMG was increased by 98 and 123 times in the two solvent systems, respectively. These may be because that the addition of DMSO and methyl alcohol could significantly enhance the rigidity of BMG, but BMG was unstable in 10% acetonitrile.

To further elucidate how hydrophilic solvents can protect BMG from destruction by vanillyl alcohol molecules, the kinetic parameters were determined by using purified BMG in different solvent systems (Table 3). The vanillyl alcohol

concentration used to measure the kinetic parameters was kept within 5 mM to minimize BMG denaturation. Compared with that in the aqueous solution, the K_m values of purified BMG to vanillyl alcohol in aqueous hydrophilic solvents obviously increased, demonstrating the decreased affinity of BMG for vanillyl alcohol in aqueous hydrophilic solvents. The changes in affinity to the substrate suggested that the addition of hydrophilic solvents reduced the interaction between BMG and vanillyl alcohol. The κ_{cat} values in 10% methanol, ethanol, DMF and acetone were slightly lower than that in aqueous solution, although BMG was stable in these solvent systems, which may be because the enhanced rigidity of BMG resulted in the loss of activity. Surprisingly, κ_{cat} value in 10% DMSO was significantly enhanced, indicating that the addition of DMSO enhanced the activity of BMG. The values of kinetic parameters were different from solvent to solvent, presumably because the addition of different solvents induced different conformational changes in the catalytic active center of BMG. Therefore, the addition of hydrophilic solvents not only enhanced the rigidity of BMG but also reduced the interaction between BMG and vanillyl alcohol, and only in 10% DMSO, the enhanced stability of BMG was not at the cost of enzyme activity.

3.3. Preparative-scale synthesis of natural glycosides at high concentrations

With simple and convenient addition of hydrophilic solvents, the inactivation of BMG due to vanillyl alcohol could be relieved effectively. Fig. 1 showed the effects of DMSO and methanol on the conversion at different concentrations of vanillyl alcohol. As expected, in 10% DMSO and methanol, with the increase of vanillyl

alcohol concentration, the decreasing trend of conversion became slower compared with that in aqueous solution. When the concentration of vanillyl alcohol was increased to 300 mM, the conversion in 10% DMSO and methanol only decreased by 22% and 37%, respectively, while the conversion in aqueous solution dropped by more than 90%. It is worth noting that the maximum conversion in 10% methanol was about half of that in aqueous solution and 10% DMSO, which probably caused by the slight conformational changes in the catalytic active center of BMG in 10% methanol. Pioneering studies in this field have proven that organic solvents could alter conformation of enzyme active site [22]. There are several cases in which various types of enzyme selectivity have been profoundly changed by switching from one solvent to another, including substrate, enantiomeric, prochiral, regio- and chemoselectivities [23, 24].

With the further increase of DMSO concentration, the maximum conversion increased slightly. More importantly, the decreasing trend of conversion became slower further. In 30% DMSO, about 40% conversion was successfully obtained at vanillyl alcohol concentration of 400 mM, while the reaction almost could not be carried out at the same concentration of vanillyl alcohol in aqueous solution.

BMG successfully synthesized a series of important natural glycosides from vanillyl alcohol, 4-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 2-hydroxybenzyl alcohol, 3-phenyl-2-propene-1-ol, 4-methoxybenzyl alcohol and 4-methoxyphenethyl alcohol, except phenol (Table 4), indicating that BMG is selective of the alcohol hydroxyl groups. In aqueous solution, when the substrate

concentration was 10 mM, BMG could effectively catalyze the galactosylation of most substrates. Under the same substrate concentration, slightly higher conversions were obtained in 30% DMSO because of the change in the thermodynamic equilibrium of hydrolysis reaction. As the concentration of aromatic alcohols increased, the BMG inactivation in aqueous solution intensified. When the substrate concentration was increased by 30–40 times, all galactosylation reactions in the aqueous solution became inefficient, although most substrates were completely dissolved in aqueous solution. By contrast, BMG still effectively catalyzed galactosylation in 30% DMSO under the same concentration, and 0.85–2.44 g of natural glycosides were successfully obtained. Bridiau et al. [10] used silica gel to adsorb aromatic primary alcohols, and the substrate concentration reached 250 mM. However, adsorption was conducted under positive argon pressure and away from light. The proposed strategy for improving the aromatic alcohol tolerance of enzymes by adding hydrophilic solvent is a simple yet effective approach that is feasible practical applications.

4. Conclusions

BMG inactivation caused by aromatic alcohols was successfully relieved by adding hydrophilic solvents. The stability of BMG in aromatic alcohol solution was remarkably enhanced by the simple addition of hydrophilic solvents. The conspicuous stabilization of BMG in aromatic alcohol solution is beneficial to preparative-scale synthesis of natural glycoside from aromatic alcohol. In 30% DMSO, the concentration of aromatic alcohol reached up to 300–400 mM, and 0.85–2.44 g of

natural glycosides were successfully obtained in 100 mL-scale. Therefore, this method provides a new idea for relieving the inactivation of enzymes caused by aromatic alcohols.

Acknowledgements

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Figure legends

Fig. 1. Effect of hydrophilic solvent on galactosylation of vanillyl alcohol catalyzed by BMG. ■, aqueous system; ●, 10% methanol; ▲, 10% DMSO; □, 20% DMSO; ○, 30% DMSO.

Table 1 Stability of BMG in the presence of vanillyl alcohol in aqueous solution

Vanillyl alcohol concentration (mM)	Half-life (h)
0	35.9 ± 2.2
10	6.2 ± 0.3
25	0.80 ± 0.05
50	0.43 ± 0.03
75	0.33 ± 0.03
100	0.30 ± 0.03
150	0.22 ± 0.03
200	0.18 ± 0.02

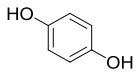
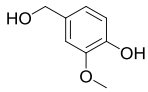
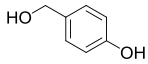
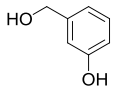
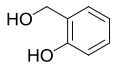
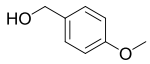
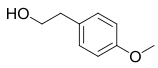
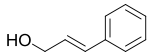
Table 2 Stability of BMG with addition of 200 mM vanillyl alcohol in different solvent systems

System	log *P*	Half-life (h)	
		Without vanillyl alcohol	With 200 mM vanillyl alcohol
Aqueous solution	-	35.9 ± 2.2	0.18 ± 0.02
10% DMSO	-1.49	148.9 ± 8.7	18.7 ± 1.5
10% DMF	-0.6	66.2 ± 2.8	2.9 ± 0.2
10% Methyl alcohol	-0.27	168.3 ± 11.7	23.4 ± 1.7
10% Ethyl alcohol	0.07	152.4 ± 9.1	5.6 ± 0.4
10% Acetone	0.2	14 ± 1.1	1.8 ± 0.1
10% Acetonitrile	0.17	0.93 ± 0.08	0.33 ± 0.03

Table 3 Effects of media on kinetic parameters for galactosylation of vanillyl alcohol catalyzed by BMG

Solvent	K_m (mM)	κ_{cat} (s^{-1})	κ_{cat}/K_m (s^{-1} mM^{-1})
Aqueous solution	38.1 ± 1.9	11.1 ± 0.5	0.29 ± 0.03
10% DMSO	87.9 ± 4.2	23.2 ± 0.8	0.26 ± 0.02
10% DMF	78.6 ± 6.9	10.2 ± 0.8	0.13 ± 0.01
10% Acetone	66.7 ± 5.2	8.3 ± 0.8	0.12 ± 0.01
10% Methyl alcohol	72.1 ± 6.3	9.2 ± 0.8	0.13 ± 0.01
10% Ethyl alcohol	79.4 ± 7.2	8.6 ± 0.5	0.11 ± 0.01

Table 4 Enzymatic galactosylation of aromatic compounds in aqueous solution and 30% DMSO[a]

Substrate	Substrate concentration (mM)	Conversions (%)[b]	
		Aqueous solution	30 % DMSO system
 Phenol	10	0	0
 Vanillyl alcohol	10	44.7 ± 3.9	47.2 ± 2.1
	400	2.2 ± 0.2	39.6 ± 1.7
 4-Hydroxybenzyl alcohol	10	44.2 ± 1.9	46.3 ± 2.7
	400	3.9 ± 0.6	38.8 ± 1.1
 3-Hydroxybenzyl alcohol	10	43.2 ± 3.9	45.3 ± 2.9
	400	3.8 ± 0.3	37.1 ± 2.4
 2-Hydroxybenzyl alcohol	10	45.3 ± 1.9	48.3 ± 3.6
	400	4.7 ± 0.3	42.8 ± 2.9
 4-Methoxybenzyl alcohol	10	43.9 ± 3.3	45.5 ± 2.8
	400	3.3 ± 0.4	37.7 ± 2.9
 4-Methoxyphenethyl alcohol	10	38.1 ± 2.1	40.8 ± 3.2
	300	2.2 ± 0.3	32.2 ± 2.1
 3-Phenyl-2-propene-1-ol	10	1.3 ± 0.1	30.1 ± 2.1
	300	0	22.1 ± 1.8

[a]Cinnamyl alcohol was insoluble in aqueous solution, but soluble in 30% DMSO, and the other aromatic alcohols were soluble in aqueous solution and 30% DMSO.

[b]Conversions were calculated by the ratio of the galactosylated aromatic alcohol

concentration to the initial aromatic alcohol concentration.

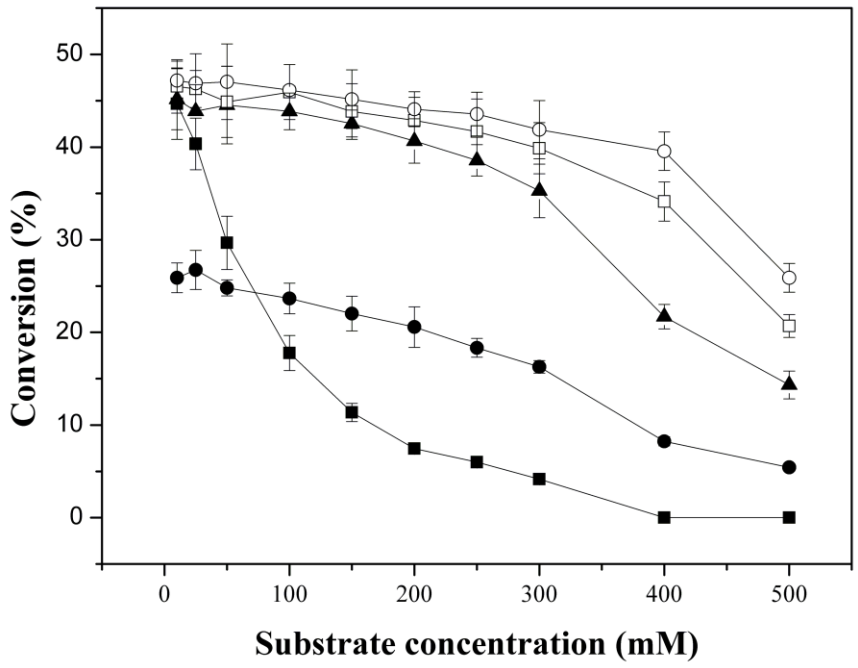
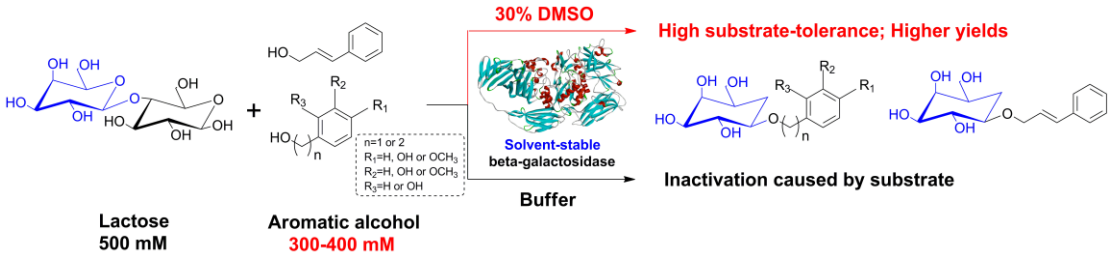


Figure 1



Graphical abstract

Highlight

- A novel strategy was established to improve substrate tolerance of β-galactosidase.
- Hydrophilic solvent was employed to increase the stability of BMG in vanillyl alcohol solution.
- 0.85–2.44 g of natural glycosides were successfully obtained in 100 mL-scale.