Journal of Molecular Catalysis B: Enzymatic 55 (2008) 152–156

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enantioselective enzymatic hydrolysis of racemic glycidyl butyrate by lipase from *Bacillus subtilis* with improved catalytic properties

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ARTICLE INFO

Article history:
Received 15 January 2008
Received in revised form 13 March 2008
Accepted 18 March 2008
Available online 25 March 2008

Keywords:
Kinetic resolution
Glycidyl butyrate
Lipase
Enantioselectivity
Hydrolytic activity

ABSTRACT

The lipase from *Bacillus subtillus* (BSL2), a highly active lipase expressed from newly constructed strain of *Bacillus subtilis* BSL2, is used in the kinetic resolution of glycidyl butyrate. A high enantiomeric ratio ($E = 108$) was obtained by using 1,4-dioxane as co-solvent (18%, v/v) and decreasing the reaction temperature to 5 °C. The ratio is about 16-fold more than that ($E = 6.52$) obtained in pure buffer solutions (25 °C, pH 7.8). Under the optimum conditions, the remained (*R*)-glycidyl butyrate with high enantiopure (ee > 98%) was obtained when the conversion was above 52%.

1. Introduction

Optically pure epoxides are important intermediates in organic synthesis. Particularly, optically active 2,3-epoxypropanol (glycidol) and its derivatives have been considered to be versatile chiral synthesis units [1–3]. Both enantiomers of glycidol or its derivatives, *i.e.* glycidyl butyrate, have become widely used as starting materials for the synthesis of many interesting compounds, such as β-blocker drugs, anticancer drugs, protein synthesis inhibitors, as well as a 2-oxazolidinone derivative used against depression. The demand for these valuable intermediates is expected to increase in the near future.

The chemical synthesis of chiral glycidol is usually performed by the Sharpless epoxidation [4–6]. The allylic alcohol is epoxidized in >90% of optical yield and 70% of chemical yield using an oxygen donor [$Ti(O–iPr)_4$, TBHP] and titanium diisopropyl tartrate (DIPT). Due to the interest for the product with high enantiomeric purity, several alternative biocatalytic processes have been developed recently. In most cases, the commercial lipase prepared from *porcine pancreatic* (PPL) has been used in resolution of (±)-glycidol or its esters [7–12]. Several other enzymes have also been used as biocatalysts in resolution of this compound. For example, Iborra and co-workers [13] have used the free and immobilized commercial lipases from *Candida antarctica* (CALA and CALB) and *Mucor miehei* (MML) as catalysts in the synthesis of glycidyl esters from rac-glycidol in non-aqueous conditions. Palomo et al. [14] have described the use of the immobilized lipase from *C. antarctica* (fraction B) (CAL-B) as biocatalyst in the preparation of enantioenriched (*S*)-glycidyl butyrate (ee >90% at 64% of conversion). The lipase from *Rhizopus oryzae* and lipases from *Alcaligenes sp.* (QL) have also been used in resolution of glycidyl butyrate [15–17]. However, all of the selected lipases showed low enantioselectivity against this compound probably due mainly to the small size of the substrates, which reduces the yield of the enantiomerically pure product. The need for these products with high enantiomeric purity requires some efficient techniques to improve the enantioselectivity. The immobilization technique has already been used to improve the enantioselectivity of the used lipase [3,13–18]. Palomo et al. [3] have purified a new lipase (25 kDa) from the pancreatic porcine and immobilized it on DEAE-Sepharose. The immobilized lipase exhibited an excellent enantioselectivity ($E > 100$) in resolution of glycidyl butyrate.

The presence of additives in the reaction system could also improve the activity and enantioselectivity of enzymes [19]. In the present work, we have combined a similar approach with

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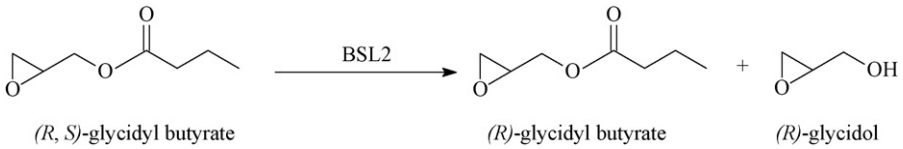
doi:10.1016/j.molcatb.2008.03.007



Scheme 1. Enantioselective hydrolysis of racemic glycidyl butyrate by BSL2.

low temperature method to improve the catalytic properties of BSL2 (prepared from a homely constructed *Bacillus subtilis* strain [20,21]). Moreover, comparison of the hydrolytic activity and enantioselectivity between BSL2 and PPL was made under the optimum conditions (Scheme 1).

2. Materials and methods

2.1. Materials

Bacillus subtilis lipase (BSL2, 24 kDa) was homely produced from a newly constructed *B. subtilis* strain BSL2 [20,21]. Plasmid pBD64 and two *B. subtilis* strains (A.S.1.1700 and A.S.1.1655) were the gifts from Institute of Microbiology, Chinese Academy of Sciences. Strain IFFI10210 was provided by China Center of Industrial Culture Collection (CICC) Center to obtain lipase gene. PPL was purchased from Shanghai Dongfeng Biochemical Factory (Shanghai, China) and was used without further purification. All the organic solvents were reagent grade from various commercial sources. Other reagents were all of analytical grade or better.

2.2. Construction of *B. subtilis* engineering strain BSL2

The original pBD64 plasmid was obtained from strain A.S.1.1700 as described previously [22]. A novel plasmid, pBSR2, was constructed by incorporating a strong lipase promoter and a terminator into the original pBD64. A mature lipase gene from *B. subtilis* strain IFFI10210, an existing strain for lipase expression, was cloned into the plasmid pBSR2 and transformed into *B. subtilis* A.S.1.1655. Thus, an overexpression strain, BSL2, was obtained.

2.3. Preparation of BSL2

A culture medium (LB), comprising of 1.0% tryptone, 0.5% yeast extract and 1.0% sodium chloride (NaCl) (pH 7.0 before sterilization and pH 6.8 after sterilization) was prepared and dispensed in shake flasks (100 ml and 5 l, respectively). The preculture of *B. subtilis* strain BSL2 was made by shaking the cells in LB supplemented with 30 mg/l kanamycin and incubating the flask at 30 °C on a rotary shaker. The 28 h old preculture thus produced was inoculated (1%, v/v) into 1 l of LB with 30 mg/l kanamycin, and fermentation was carried out at 500 rpm keeping at a constant temperature of 30 °C for 28–30 h. The culture was thereafter centrifuged to collect the cells. The cell pellet was washed twice with 30 mmol/l phosphate buffer (pH 7.0) and the wet cell mass (5.8 g) was thus obtained.

The wet cell mass was crushed by sonication and the supernatant was obtained by centrifugation at 12,000 rpm for 30 min at 4 °C. The crude enzyme was partially purified by ammonium sulfate fractionation procedure. The ammonium sulfate precipitation was dialyzed in 30 mmol/l phosphate buffer (pH 7.0) to remove the excess salt. The enzyme was used after lyophilization.

2.4. Synthesis of racemic glycidyl butyrate

Racemic glycidyl butyrate was prepared according to a procedure previously described in Ref. [23]. The mixture of sodium butyrate and epichlorohydrin with triethyl benzyl ammonium chloride (TEBA) as phase-transfer catalysts was heated to reflux at 85 °C for about 24 h. It was cooled down to room temperature and extracted with 2–3 volumes of water for three times. The organic phase was dried with anhydrous Na_2SO_4 and concentrated *in vacuo* to give the final glycidyl butyrate product (96% of purity by gas chromatography and 70% of yield).

2.5. Hydrolysis of racemic glycidyl butyrate

Racemic glycidyl butyrate (5.0 ml, 36 mmol) was added to 10 mM potassium phosphate buffer (5.0 ml), which consisted of co-solvent (%, v/v) for BSL2 or surfactant CTAB (30 mg/ml) for PPL [24]. The pH of the mixture was adjusted to a given value and the lipase power (100 mg) was added to the mixture. The mixture was stirred vigorously at a given temperature while the pH was kept constant by automatic titration of 2 M NaOH using a pH controller. The activity (μmol/min mg) of the lipases in hydrolysis of (*R*,*S*)-glycidyl butyrate and the conversion (*c*) were determined based on the NaOH consumption. An aliquot of sample was taken and analyzed by chiral high performance liquid chromatography (HPLC) at certain intervals.

2.6. Determination of enantiomeric excess and enantioselectivity

The extracted glycidyl butyrate was diluted by the mobile phase and analyzed by chiral HPLC. The column was a Chiralpak AD, the mobile phase was a mixture of hexane and ethanol (90:10, v/v) at a flow rate of 0.4 ml/min and UV detection was performed at 254 nm. The retention time of the enantiomers were 14.32 min for (*R*)-enantiomer and 13.57 min for (*S*)-enantiomer. The enantiomeric excess of the remained glycidyl butyrate (ee_S) was determinated by the peak area of individual enantiomer. The enantioselectivity (*E* value) was calculated according to Eq. (1) as reported by Chen et al. [25].

$$E = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)(1-ee_S)]}. \quad (1)$$

3. Results and discussion

3.1. Effect of pH on the activity and enantioselectivity of BSL2

The influence of pH on both of the activity and the enantioselectivity of BSL2 in resolution of glycidyl butyrate was investigated. The pH was kept constant at a given value by automatic titration of 2 M aqueous sodium hydroxide (NaOH) using a pH controller during the hydrolysis.

As shown in Fig. 1, the *E*-value was increased in the pH range of 6.0–7.8, and then decreased at pH 7.8–9.0. The highest enantioselectivity was achieved at pH 7.8 ($E = 6.52$) and the highest hydrolysis activity (2.9 μmol/min mg) was obtained at pH 7.2. The results above may be explained by that the pH can influence the activity and the enantioselectivity of BSL2 through altering the dimensional structure of the enzyme by breaking weak bonds such as ionic and hydrogen bonds [26].

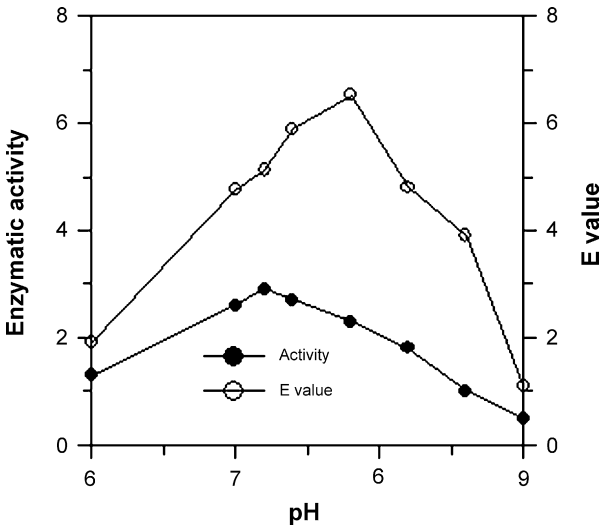


Fig. 1. Effect of pH on the activity (μmol/min mg) and enantioselectivity of BSL2 in the kinetic resolution of glycidyl butyrate. The reaction flask contained (*R*,*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg) and potassium phosphate buffer (5.0 ml, 10 mM). The reaction was performed at 25 °C with pH varying from 6.0 to 9.0.

3.2. Effect of co-solvents on the activity and enantioselectivity of BSL2

Among the methods reported to improve the enzyme-mediated reactions, the additive approach is very attractive because of its simplicity for practical use. Organic co-solvents were often used as additives to enhance the catalytic activity by improving the solubility of hydrophobic ester substrates in aqueous reaction system. In some cases, the addition of organic co-solvents may also enhance the enantioselectivity of the used enzyme [3,14].

3.2.1. Effect of different co-solvents on the activity and enantioselectivity of BSL2

In order to screen out the most effective organic co-solvents to improve the activity and enantioselectivity of BSL2, several hydrophilic organic solvents that are regularly used as co-solvents, such as 1,4-dioxane, acetone, and acetonitrile and so on, were selected and their concentration was controlled at 10% (v/v). As shown in Table 1, the hydrolysis occurred in the presence of all of the co-solvents, but the effects were different. In most cases, the lower enantioselectivity and lower hydrolysis activity were obtained in the presence of the co-solvents, except for 1,4-dioxane, acetone and glycol.

The enantioselectivity was improved when 1,4-dioxane or acetone was used as additive (10%, v/v). The maximum *E*-value (*E* = 27.63) was obtained for 1,4-dioxane and the improvement of the *E*-values was correlated with a significant increase in the activity, which suggests that some conformational changes may take place and this is why the catalytic properties of BSL2 was changed. The improvement of hydrolysis activity could also be observed when glycol was used as co-solvent. For further study, 1,4-dioxane, acetone and glycol were selected as organic co-solvents.

Table 1
Hydrolysis of glycidyl butyrate catalyzed by BSL2 using various co-solvents[a]

Entry	Co-solvents	Enzyme activity (μmol/min mg)	*E* value
1	None[b]	2.3	6.52
2	1,4-Dioxane	4.8	27.63
3	Acetonitrile	2.0	5.21
4	Acetone	2.1	12.69
5	DMSO	0.6	1.27
6	Tetrahydrofuran	0.5	4.78
7	Dimethyl formamide	0.3	2.55
8	Glycol	6.1	6.17

[a] Reaction conditions: 36 mmol substrate (5.0 ml) in a 5.0 ml buffer solution (10 mM, pH 7.8), BSL2 100 mg, temperature 25 °C, co-solvents 10% (v/v).
[b] No co-solvent was added into the reaction system.

3.2.2. Effect of different volume fractions of co-solvents on the activity and enantioselectivity of BSL2

The *E*-values and the relative activity obtained when the amount of selected co-solvents was varied are shown in Fig. 2(a–c), respectively.

It was found in Fig. 2a that the increase of acetone caused an apparent decrease in the activity of BSL2, which may be explained by that the presence of acetone in the reaction mixture may alter and destroy the conformation of BSL2 by interactions with the hydration layer essential for catalysis and proper folding, or alteration of the protein structure by direct interactions with protein salvation sites [27]. With the increase of acetone, the detrimental effect becomes more and more apparent, and then the activity decreases gradually. When the enantioselectivity was considered, the *E* value was increased from 6.52 to 17.43 with the increase of acetone and the maximum *E*-value (*E* = 17.43) was obtained at 15% (v/v) (Fig. 2a). Theses results are in good agreement with those in other literatures [28,29] and its believed that the alternation of the conformation by the addition of acetone may change the binding ability of different enantiomers and then influence the enantioselectivity of BSL2.

Fig. 2b shows that the effect of different volume fractions of glycol on the activity and enantioselectivity of BSL2 in hydrolysis of glycidyl butyrate. The hydrolytic activity was increased with the increase of glycol content in reaction system and the maximum activity was obtained at 25% (v/v). On the other hand, the presence of glycol in reaction system decreased the enantioselectivity of BSL2 in kinetic resolution of glycidyl butyrate. A study of intra- and intermolecular bonds within the protein conformation by Raman methods has revealed that polyols might have direct interactions (specific or non-specific) with the enzyme polypeptide [30]. In this case, glycol may improve the flexibility of BSL2 by interaction with the enzyme polypeptide and then enhance its catalytic activity. However, the increase of the flexibility may reduce the steric hindrance of the two enantiomers of glycidyl butyrate to access the binding site of the enzyme, so the enantioselectivity was decreased in the presence of glycol.

When 1,4-dioxane was used as co-solvent, it was found that addition of 15% 1,4-dioxane (v/v) nearly tripled the hydrolysis activity of BSL2 (Fig. 2c). However, the best value for *E* was observed at 18% (v/v) of 1,4-dioxane, the enantioselectivity (*E* value) was increased by a factor of 8.83 (from 6.52 to 57.6). Further increase of 1,4-dioxane to 30% resulted in a significant decrease in *E* value (*E* = 13.4). It was generally believed that lipases might exist in two different forms [31–33]. When the active center of the lipase was protected from the reaction medium by a polypeptide chain "lid," the enzyme was considered to be inactive (closed form). When the lid was displaced and the active center exposed to the reaction medium, the enzyme was active (open form). In aqueous or homogeneous media, lipase molecules exist in equilibrium between these two forms, with this equilibrium shifting towards the closed form. This dramatic conformational change may make the activity of lipases strongly depend on the experimental conditions. In the present study, the open form of BSL2 might be stabilized by the addition of 1,4-dioxane, resulting in the high enzyme activity. But how can 1,4-dioxane enhance the enantioselectivity? In order to reveal the possible mechanism, the (*R*)-glycidyl butyrate and the (*S*)-one were hydrolyzed by BSL2 with different volume fractions of

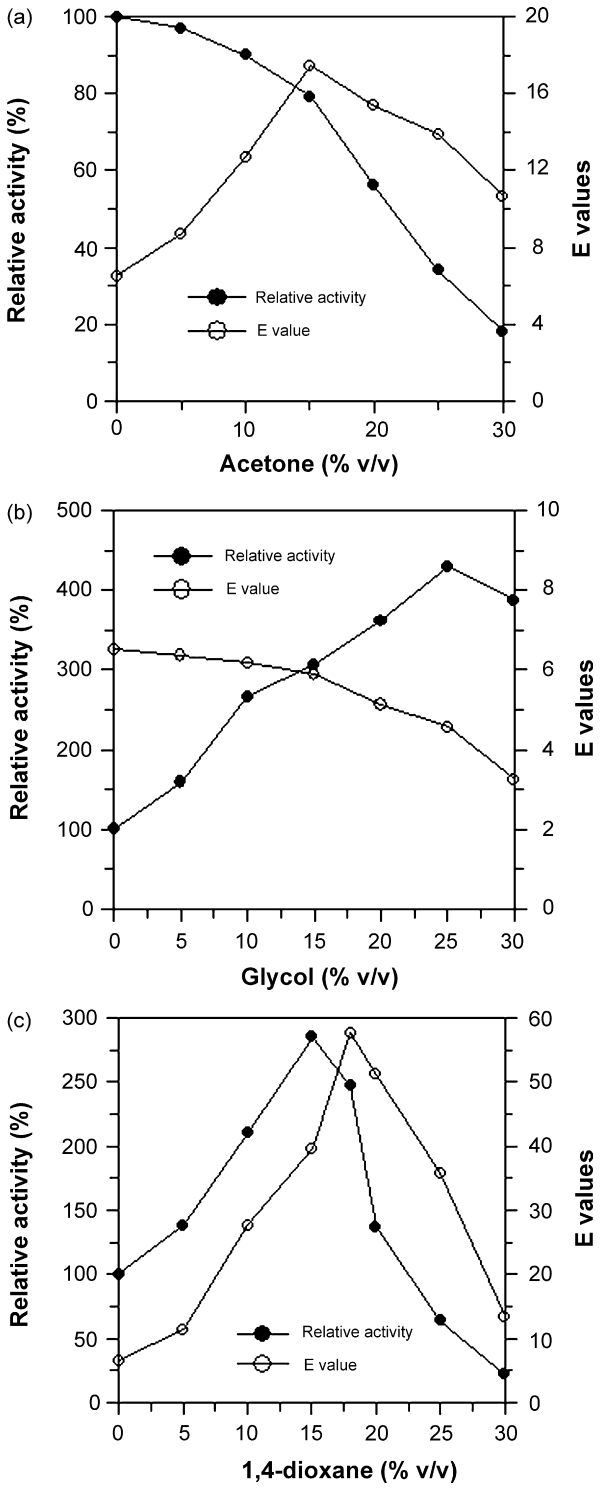


Fig. 2. (a) Effect of different volume fractions of acetone on the activity and enantioselectivity of BSL2 in hydrolysis glycidyl butyrate. The reaction flask contained (*R*,*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg), potassium phosphate buffer (pH 7.8, 5.0 ml, 10 mM) with different volume fractions of acetone as additive. The reaction was performed at 25 °C. (b) Effect of different volume fractions of glycol on the activity and enantioselectivity of BSL2 in hydrolysis glycidyl butyrate. The reaction flask contained (*R*,*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg), potassium phosphate buffer (pH 7.8, 5.0 ml, 10 mM) with different volume fractions of glycol as additive. The reaction was performed at 25 °C. Fig. 2c. Effect of different volume fractions of 1,4-dioxane on the activity and enantioselectivity of BSL2 in hydrolysis glycidyl butyrate. The reaction flask contained (*R*,*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg), potassium phosphate buffer (pH 7.8, 5.0 ml, 10 mM) with different volume fractions of 1,4-dioxane as additive. The reaction was performed at 25 °C.

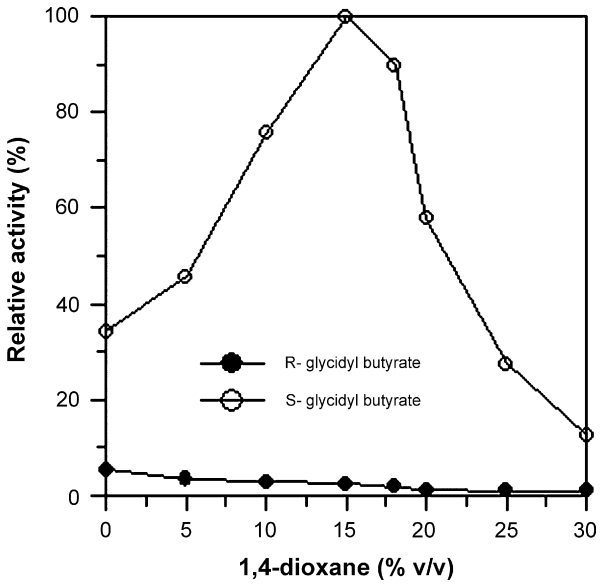


Fig. 3. Effect of different volume fractions of 1,4-dioxane on the activity of BSL2 in hydrolysis of (*R*)-glycidyl butyrate and (*S*)-glycidyl butyrate. The reaction flask contained (*R*)- or (*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg), potassium phosphate buffer (pH 7.8, 5.0 ml, 10 mM) with different volume fractions of 1,4-dioxane as additive. The reaction was performed at 25 °C.

1,4-dioxane used as co-solvent, respectively. The results obtained from Fig. 3 may give a possible explanation.

Fig. 3 showed that the hydrolytic activity of BSL2 for (*S*)-glycidyl butyrate was increased in the volume fraction range of 0–15%, and the maximum activity was obtained at 15% (v/v), and then the catalytic activity was decreased. However, the (*R*)-activity was decreased with the increase of 1,4-dioxane content. So it means that a suitable volume fraction of 1,4-dioxane may act as activator to (*S*)-glycidyl butyrate, but inhibitor to the (*R*)-enantiomer. As a result, the enantioselectivity is improved largely. It is well known that the enhanced catalytic activity and the enantioselectivity of lipases are often associated with structural changes. The research to clarify the effect of 1,4-dioxane on the conformation of BSL2 is currently in process and will be reported in due course.

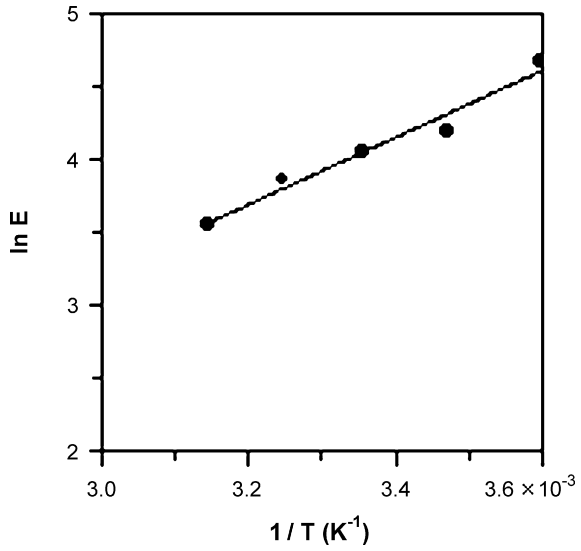


Fig. 4. Effect of temperature on the enantioselectivity in hydrolysis of glycidyl butyrate. The reaction flask contained (*R*,*S*)-glycidyl butyrate (5.0 ml, 36 mmol), BSL2 (100 mg), potassium phosphate buffer (pH 7.8, 5.0 ml, 10 mM) with 1,4-dioxane (18%, v/v) as additive. The reaction was performed at varied temperature from 45 to 5 °C.

Table 2
Comparison of hydrolytic activity and enantioselectivity between BSL2 and PPL

Lipases	Enzyme activity (μmol/min mg)	Conversion (%)	ee (%)	*E* values	Stereoselectivity
PPL[a]	2.4	60	>98	21	*S*
BSL2[b]	1.6	52	>98	108	*S*

[a] Reaction conditions: 36 mmol substrate (5.0 ml) in a 5.0 ml buffer solution (5 mM, pH 7.4), PPL 100 mg, 30 mg/ml CTAB, at 30 °C.
[b] Reaction conditions: 36 mmol substrate (5.0 ml) in a 5.0 ml buffer solution (10mM, pH 7.8), BSL2 100 mg, 1,4-dioxane 18% (v/v), at 5 °C.

In subsequent experiments we used 1,4-dioxane (18%, v/v) as the co-solvent for the hydrolysis of glycidyl butyrate.

3.3. Effect of temperature on the activity and enantioselectivity of BSL2

Among a variety of methods for increasing the enantioselectivity in the lipase-catalyzed resolution of alcohols, the temperature control is now also accepted as a simple and theoretically reliable method [34]. The temperature effect on the enantioselectivity in hydrolysis was examined at the range from 45 to 5 °C in this work. As shown in Fig. 4, the plot of $\ln E$ versus 1/T showed a linear correlation, which indicated that the enantioselectivity of this BSL2-catalyzed kinetic resolution is temperature-dependent. When the reaction mixture was cooled, the *E* values were markedly increased and the maximum *E*-value ($E = 108$) was obtained at 5 °C. However, low temperature would also cause low reaction velocity. Therefore, the optimum reaction temperature was selected to be 5 °C because the enantioselectivity of the enzyme is sufficient for further practical use.

3.4. Comparison of hydrolytic activity and enantioselectivity between BSL2 and PPL

Lipase catalytic resolutions depended mainly on the type and origin of the enzyme. It is well known that the commercial lipase preparation from PPL was often used in resolution of glycidyl esters. The optimum conditions for PPL-catalyzed the kinetic resolution of glycidyl butyrate had been screened out in our previous work [24]. Then the hydrolytic activity and enantioselectivity of BSL2 were compared with those of PPL under the optimum conditions, respectively (Table 2).

In BSL2-catalyzed resolution, the remained (*R*)-glycidyl butyrate with high enantiopurity (ee > 98%) was achieved when the conversion was above 52%. As for PPL [24], the high enantiopurity (ee > 98%) of (*R*)-glycidyl butyrate can only be obtained when the conversion was more than 60%. These data indicate that both BSL2 and PPL can be used to give (*R*)-glycidyl butyrate with high enantiopurity. Nevertheless, high conversion was requested for PPL to achieve an appropriate enantiomeric excess of the remained glycidyl butyrate because of its low enantioselectivity, which decreased the yield of the enantiomerically pure (*R*)-enantiomer.

4. Conclusion

A kinetic resolution process for producing (*R*)-glycidyl butyrate from its racemate using BSL2-catalyzed hydrolysis was investigated. The results showed that use of 1,4-dioxane as co-solvent resulted in a higher enantioselectivity and low temperature can also improve the enantioselectivity. The highest enantioselectivity ($E = 108$) was achieved when the reaction system contains 18% (v/v) of 1,4-dioxane and the reaction was carried out at 5 °C. The enantioselectivity is about 16-fold more e than that in pure buffer solutions (pH 7.8, $E = 6.52$). Under the optimum conditions, the remained (*R*)-glycidyl butyrate with high enantiopurity (ee > 98%) was obtained when the conversion was above 52%.

Acknowledgements

The authors are grateful for the financial support from National Natural Science Foundation of China (Nos. 20072011 and 20272017) and the Foundation of Research Program of Jilin University, China.

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