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Separation of enantiopure *m*-substituted 1-phenylethanols in high space-time yield using *Bacillus subtilis* esterase†

Gao-Wei Zheng,[a] Xu-Yun Liu,[a] Zhi-Jun Zhang,[a] Ping Tian,*[b] Guo-Qiang Lin[b] and Jian-He Xu*[a]

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A recombinant *Bacillus subtilis* esterase (BsE) expressed in *E. coli* was found to exhibit excellent enantioselectivity (*E* was always greater than 100) towards *m*-substituted 1-phenylethanol acetates in the enantioselective hydrolysis reaction. An explanation for the high enantioselectivity observed towards these substrates was provided by molecular modeling. Moreover, the BsE also showed strong tolerance towards a high concentration of *m*-substituted 1-phenylethanol acetates (up to 1 M). Based on these excellent catalytic properties of BsE, a kind of *m*-substituted 1-phenylethanols, (*R*)-1-(3-chlorophenyl)ethanol, was efficiently synthesized in space-time yield of 920 g per L per day and 97% ee, indicating that the BsE was considered as a potentially ideal and promising biocatalyst for large-scale production of optically active *m*-substituted 1-phenylethanols.

Introduction

Optically active *m*-substituted 1-phenylethanols are frequently used as important and valuable intermediates for the production of pharmaceuticals and other fine chemicals.[1] For example, enantiopure 1-(3-chlorophenyl)ethanol (**6a**) was recently employed for the synthesis of DP1 (a receptor for prostaglandin D2) antagonists, which has been used for treating the side effects of niacin-induced flushing.[2] In addition, (*R*)-1-(3-methoxyphenyl)ethanol (**16a**) was also used as a precursor for the preparation of (*S*)-rivastigmine, which is the first drug approved by FDA for the treatment of mild to moderate dementia associated with Alzheimer's and Parkinson's disease.[3]

In the past, some physical or chemical methods have been developed for the production of *m*-substituted 1-phenylethanols. For example, a supramolecular chiral host consisting of *N*-(2-naphthoyl)-L-aspartic acid and *meso*-1,2-diphenylethylenediamine was used for enantioseparation of 1-arylethanols, giving 96% ee and 100% inclusion ratio.[4] However, environmental pollution and energy pinch arising from physical or chemical methods are becoming more and more serious. Recently, scientists have shown great interest in biocatalysis because of its lower energy consumption, environmental friendliness, shorter synthetic routes and avoidance of functional group protection. Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) as powerful and promising biocatalysts have been applied in a variety of pharmaceutically useful synthetic reactions because of their excellent activity and robustness. More importantly, they could smoothly catalyze reactions without addition of expensive cofactors and could be reused after immobilization, which makes them commercially available at competitive prices.[5–7] Several lipases and esterases have been utilized for the preparation of optically pure *m*-substituted 1-phenylethanols. For instance, *Candida antarctica* lipase B (CALB) has been used to prepare (*R*)-1-(3-methoxyphenyl)ethanol (**16a**) with excellent enantiomeric excess (>96%).[3] Although a few kinetic resolution processes have been developed for preparing optically active *m*-substituted 1-phenylethanols, most of them provided the low space-time yields, which has limited their practical application. Therefore, efficient synthesis process is still of great interest to achieve economic feasibility and competitiveness for the large-scale preparation of enantiopure *m*-substituted 1-phenylethanols.

We recently cloned and expressed a *p*-nitrobenzyl esterase (BsE) from *Bacillus subtilis* ECU0554.[8,9] The BsE protein exhibited extremely high hydrolytic activity (as high as 45 kU per mg protein) and excellent enantioselectivity towards *l*-menthol. Herein, we found that the BsE also showed excellent enantioselectivity (*E* > 100) towards *m*-substituted 1-phenylethanol acetates in the enantioselective hydrolysis of a series of acetylated *rac*-aryl alcohols. A molecular modelling was performed to

[a]*Laboratory of Biocatalysis and Synthetic Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China. E-mail: jianhexu@ecust.edu.cn; Fax: +86-21-6425-0840*

[b]*Key Laboratory of Synthetic Chemistry of Natural Substances, Shanghai Institute of Organic Chemistry, Chinese Academy of Science, 345 Lingling Road, Shanghai 200032, P.R. China. E-mail: tianping@sioc.ac.cn*

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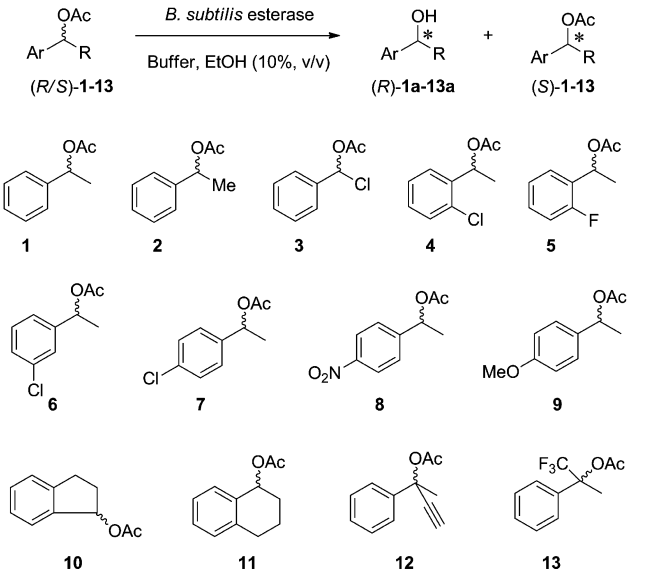
obtain a better understanding of the significant differences observed in enantioselectivity towards different substrates. Furthermore, the study showed that the BsE also exhibited strong tolerance against high substrate concentrations. Finally, a BsE-catalyzed hydrolysis process with high space-time yield for synthesis of optically activity *m*-substituted 1-phenylethanols was developed.

Results and discussion

To learn the substrate specificities of BsE, analytical-scale kinetic resolutions were carried out for a variety of racemic alcohol acetates (**1–13**) using BsE (Scheme 1). The results revealed that the activity and enantioselectivity of BsE differed considerably depending on the structure of substrate (Table 1). An increase in the carbon-chain length (**2**) and the introduction of an electron-withdrawing group (**3**) at the R_2 substituent led to a significant reduction in the enantioselectivity (*E*) from 56 to 21 and 8, respectively. Besides, the introduction of electron-withdrawing and electron-donating groups at the R_1 substituent in the *o*- (**4** and **5**) and *p*-positions (**7–9**) resulted in poor enantioselectivity. However, interestingly, the substrate bearing a chloro-substituent in the *m*-position (**6**) was hydrolyzed with an excellent enantioselectivity (*E* > 100). Based on this preliminary and unexpected result, it was envisaged that the enzyme might also exhibit excellent enantioselectivity towards other *m*-substituted substrates.

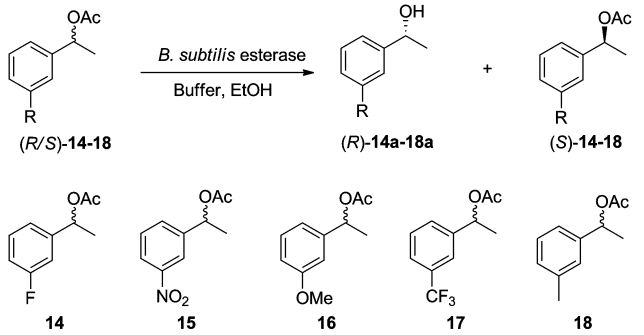


Scheme 1 Enantioselective hydrolysis of racemic alcohol acetates **1–13** by BsE.

Table 1 Enzymatic resolution of chiral alcohol acetates **1–13** by BsE at an analytical scale

Substrate	Time (min)	ee_s[a] (%)	ee_p[a] (%)	Conv.[b] (%)	*E*[b]
1	60	91	89 (*R*)	51	56
2	90	81	74 (*R*)	48	21
3	60	60	63 (*R*)	47	8
4	60	50	70 (*R*)	41	9
5	60	79	57 (*R*)	58	8
6	60	95	98 (*R*)	49	>100
7	120	74	71 (*R*)	51	13
8	180	39	37 (*R*)	51	3
9	60	84	75 (*R*)	52	19
10	60	73	59 (*R*)	55	8
11	60	79	85 (*R*)	48	30
12	720	24	21 (*R*)	53	2
13	720	—	—	n.d.[c]	—

[a] Enantiomeric excess (% ee) was determined by chiral HPLC or GC. [b] Conversion and *E* value were calculated from ee_s and ee_p. [c] n.d.: not determined.



Scheme 2 Enantioselective hydrolysis of *m*-substituted 1-phenylethanol acetates with BsE.

Table 2 Enantioselective hydrolysis of *m*-substituted 1-phenylethanol acetates **14–18** using BsE at an analytical scale

Substrate	Time (min)	ee_s[a] (%)	ee_p[a] (%)	Conv.[b] (%)	*E*[b]
14	45	92	96 (*R*)	49	>100
15	90	84	99 (*R*)	46	>100
16	45	97	99 (*R*)	50	>100
17	180	91	97 (*R*)	48	>100
18	45	96	93 (*R*)	51	>100

[a] Determined by chiral GC. [b] Conversion and *E* were calculated from ee_s and ee_p.

Table 3 Results of BsE-catalyzed hydrolysis of *m*-substituent 1-phenylethanol acetates **6** and **14–18** at preparative scale

		Acetate (%)		Alcohol (%)		
Substrate	Time (min)	ee_s[a]	Yield[b]	ee_p[a]	Yield[b]	Conv.[c] (%)
6	360	94	40	98	42	49
14	240	92	45	97	45	49
15	360	93	44	99	40	48
16	200	97	42	99	38	49
17	420	94	42	96	36	49
18	180	94	40	94	41	50

[a] Determined by chiral GC. [b] Isolated yield. [c] Conversion was calculated from ee_s and ee_p.

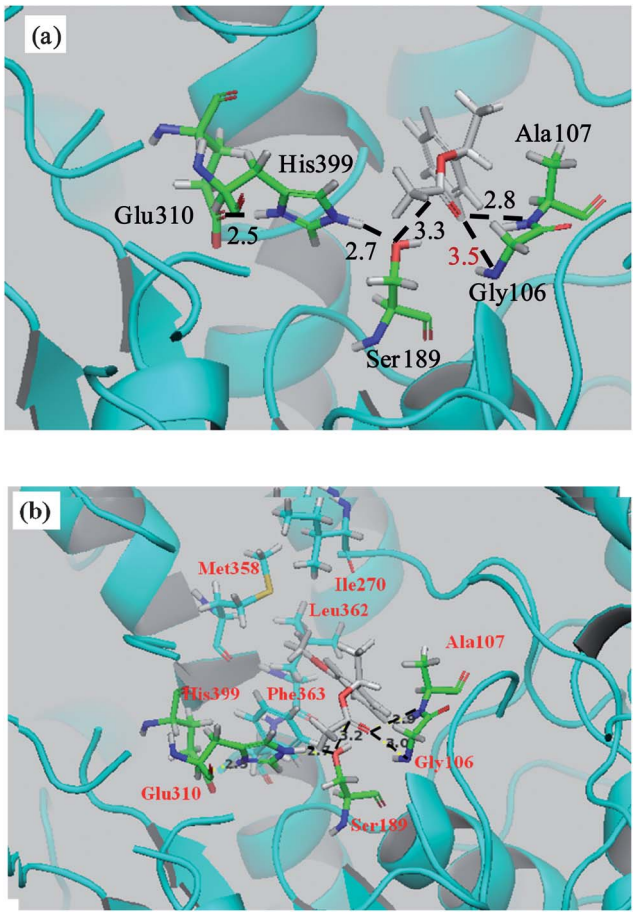


Fig. 1 Comparison of (*R*)-**1** (a) and (*R*)-**16** (b) placed in the active site of BsE. The models suggested that (*R*)-**16** binds more tightly in the active site than (*R*)-**1** likely because of the substituent in the *m*-position, which stretches into a hydrophobic pocket thereby resulting in better binding of the substrate. The greater flexibility of substrates without any substituent at the *m*-position in the active site explains their lower enantioselectivities.

To confirm this hypothesis, a series of racemic 1-phenylethanol acetates that were substituted in the *m*-position with different electron-withdrawing or electron-donating groups, were subjected to biocatalytic hydrolysis (Scheme 2, compounds **6** and **14–18**). As expected, the BsE displayed excellent enantioselectivities (E > 100) towards all of the *m*-substituted substrates tested (Table 2). The subsequent preparative-scale hydrolysis of high concentration of **6** and **14–18** (up to 1 M) was also smoothly catalyzed by BsE (Table 3). The results showed that the BsE exhibited not only excellent enantioselectivity, but also strong tolerance towards all of the *m*-substituted substrates tested.

To obtain a better understanding of the significant differences observed in enantioselectivities of BsE towards substrates with and without substituent in the *m*-position, protein modeling was performed using faster reacting enantiomers (*R*)-1 and (*R*)-16 as examples. The modeling showed that the substituent in the *m*-position of the substrate molecule stretched into a hydrophobic pocket consisting of Ile-270, Met-358, Leu-362 and Phe-363, and the hydrophobic interaction between the substituent and the hydrophobic pocket could result in better binding of the substrate in the active site. This suggestion was confirmed by the distances between the nitrogen atom in the oxyanion hole and the carbonyl oxygen atom of the substrates. In the case of (*R*)-16, the distance was found to be 3.0 Å, whereas, the distance in the case of (*R*)-1 was 3.5 Å, (Fig. 1), suggesting that BsE bound (*R*)-16 more tightly than (*R*)-1. The enhanced flexibility of the substrates without any substituent at the *m*-position in the active site could provide an explanation for the lower enantioselectivities observed towards these substrates.

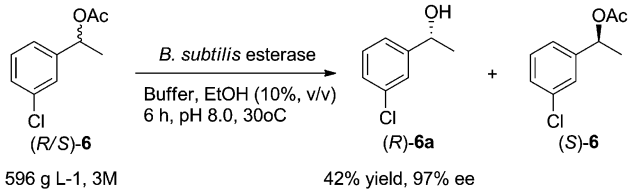
To investigate the feasibility of using BsE for the large-scale preparation of *m*-substituted 1-phenylethanols, substrate **6** was selected for further investigation. Pleasingly, a relatively high space-time yield (307 g per L per day) was achieved in conjunction with a nearly complete conversion (49%) within 6 h (Table 4, entry 1) for the hydrolysis of 199 g L^{-1} of substrate **6** (1.0 M). Further increases in the substrate loading to 397 g L^{-1} (Table 4, entry 2) and 596 g L^{-1} (Table 4, entry 3) resulted in excellent conversion (50%) and extremely high space-time yields of 1-(3-chlorophenyl)ethanol (**6a**) (as high as 940 g per L per day), which markedly exceeded the threshold of industrially feasible space-time yield (500 g per L per day).[10] The BsE derived from *B. subtilis* ECU0554 was further demonstrated to be a robust biocatalyst with excellent tolerance towards high substrate loading; enabling the efficient preparation of optically active *m*-substituted 1-phenylethanols as well as their esters.

Next, the biohydrolytic reaction with the highest space-time yield (Table 4, entry 3) was scaled up to 100 mL to evaluate the feasibility of this bioprocess. As a result, the hydrolysis of substrate **6** (59.6 g) over a 6 h period provided the desired product with a conversion of 49% and an enantiomeric excess of 97% (Scheme 3). Finally, following an extraction and standard work-up, 19.6 g of (*R*)-1-(3-chlorophenyl)ethanol was isolated

Table 4 Enantioselective hydrolysis of substrate **6** at high loads with BsE[a]

Entry	Substrate ($g\ L^{-1}$)	Substrate (M)	Enzyme (kU)	Time (h)	ee_s[b] (%)	ee_p[b] (%)	Conv.[c] (%)	Space-time yield (g per L per day)
1	199	1.0	47	6	94	98	49	307
2	397	2.0	94	6	94	97	50	626
3	596	3.0	141	6	95	97	50	940

[a] *Reaction conditions*: substrate (2.0–6.0 g, 10–30 mmol), enzyme (47–141 kU), EtOH (10%, 1 mL), phosphate buffer (100 mM, pH 8.0, 9 mL), 30 °C. [b] Determined by chiral GC analysis. [c] Conversion was calculated from ee_s and ee_p.



Scheme 3 BsE-catalyzed enantioselective hydrolysis of 1-(3-chlorophenyl)ethyl acetate with BsE at 59.6 g/100 mL scale.

(42% yield), representing an outstanding space-time yield of 920 g per L per day. The results suggest that the reaction could be potentially used as a competitive process for the large-scale production of enantiopure *m*-substituted 1-phenylethanols.

Materials and methods

Molecular modeling

Molecular modeling was performed with Maestro (version 9.2, Schrodinger, New York, NY, USA) using OPLS-2005 force field[11] starting with the X-ray crystal structure of *Bacillus subtilis* esterase (PDB file ID: 1QE3).[12] The dielectric constant was set to 1 to mimic the solvation effects of water. Hydrogen atoms were added and His-399 was protonated. The geometry of structure was optimized in a stepwise manner. Initially, the geometries of the hydrogen atoms on water were optimized followed by the hydrogen atoms on the protein. The whole protein with water molecules was then optimized followed by water, whole protein and the bound substrate. Each step in the optimization of the geometry was conducted using the PolaRibiere conjugated gradient (PRCG) method with a maximum of 5000 iterations and a convergence threshold of 0.005 kcal mol^{-1} $Å^{-1}$.

Enantioselective preparation of (*R*)-6a

BsE esterase (1.24 g) was added to a mixture of substrate **6** (59.6 g, 0.3 mol), ethanol (10 mL) and phosphate buffer (90 mL, pH 8.0, 100 mM). The reaction mixture was magnetically agitated at 30 °C for 6 h. The pH was automatically adjusted to 8.0 by titrating 2 M NaOH. Following the removal of the enzyme from the reaction mixture by filtration, the filtrate was extracted three times with ethyl acetate. The organic layers were combined, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the crude residue, which was purified by column chromatography on silica gel using a mixture of petroleum ether and ethyl acetate (10 : 1, v/v) as the eluent. The separated substrate and product were recovered by removal of the solvent under reduced pressure and dried under vacuum. The product was characterized by ^{1}H NMR and its optical purity was determined by chiral gas chromatography and polarimetry.

(*R*)-1-(3-Chlorophenyl)ethanol (6a)[13]

Yield: 42% (19.6 g). $[\alpha]_D^{30}$: +43.2 (*c* 1.00, $CHCl_3$), 97% ee; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.36 (s, 1H), 7.29–7.21 (m, 3H), 4.88–4.82 (m, 1H), 2.10 (d, $J = 3.2$ Hz, 1H), 1.47 (d, $J = 6.0$ Hz, 3H).

Conclusion

Enantioselective hydrolysis of racemic alcohol acetates by the *p*-nitrobenzyl esterase (BsE) from *Bacillus subtilis* ECU0554 is noteworthy for its excellent enantioselectivity ($E > 100$) and strong tolerance against high substrate loading for all of the *m*-substituted 1-phenylethanol acetates tested. This enantioselective hydrolysis using the BsE also provided an easy access to *m*-substituted 1-phenylethanols with high enantio-purity and the extremely high space-time yield.

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