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Purification and properties of lipase from a *Bacillus* strain for catalytic resolution of (*R*)-Naproxen

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Abstract

A *Bacillus* strain was screened for asymmetric resolution of (*R*)-Naproxen. The optical purity (ee (%)) of (*R*)-Naproxen was found to be 86.47% and conversion rate was 40–50% in bacterial cells PBS reaction system. The dissolved lipase was clarified from the *Bacillus* bacterial cells by centrifugation and loaded on a phenyl-Sepharose CL-4B column. After purification by a single hydrophobic chromatography, the activity of lipase was approximately 43 times higher than the crude one. The hydrolytic activity of lipase using Naproxen ethyl ester and *p*-nitrophenyl acetate (*p*-NPA) as substrate remained essentially constant during the purification procedure. A *Bacillus* strain with stereochemical selectivity was obtained.

Keywords: Asymmetric resolution; (*R*)-Naproxen; Lipase; Hydrophobic chromatography; Purification

1. Introduction

2-Arylpropionic acids are an important class of non-steroidal anti-inflammatory drugs. Two of the most commonly prescribed members of this family are *p*-isobutylhydratropic acid (Motrin) and 2-(6-methoxy-2-naphthyl) propionic acid (Naproxen). There is a growing interest in the use of optically pure enantiomers for drugs because they are more efficient target-specific and have less side-effects than racemic mixture [1]. The *S*(+)-enantiomer of Naproxen is 28 times more active than its *R*(−)-enantiomer. (*S*)-Enantiomer of Ketoprofen is 160-fold more active than its (*R*)-enantiomer for anti-inflammation and acesodyne. Chemical resolution methods were studied to obtain the (*S*)-enantiomer of Naproxen. Generally, these methods entail the selective stoichiometric crystallization of a diastereomeric salt by the usage of an expensive amine [2].

In the recent years, the use of biological systems for production of optically enriched compounds has become an alternative to classical methodologies of chemical synthesis. From an industrial point of view the reactions carried out in aqueous media are less expensive than other methods. The preparation of optically active enantiomers has been accomplished by using microorganisms [3] and enzymes. Hydrolytic enzymes such as esterase and lipase have been widely applied for the kinetic resolution of racemic acids. The enzymatically enantioselective resolution of 2-substituted acids by lipases has been the subject of intensitive investigations [1,2,4–7]. For industrial

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applications and research, pure lipases are often desired. The purification of lipase from commercial crude lipase of *Candide cylindradea* has been reported [6]. Gilbert et al. [8] used Tween 80 limited continuous culture to purify lipase of *Pseudomonas aeruginosa* strain EF2.

The aim of the present study is to develop an enzymatic method for the production of the optical enantiomer of 2-arylpropionic acids through stereoselective hydrolysis of their racemic esters. Fourteen seminatural strains were studied for the resolution of Naproxen and Ketoprofen. A *Bacillus* strain with asymmetric resolution of (*R*)-Naproxen was found.

2. Materials and methods

2.1. Materials

Naproxen ethyl ester was synthesized at 160 °C for 2 h by the reaction of racemic Naproxen with ethanol in dichlorosulfoxide and then crystallized as described by Yao [9]. Phenyl-Sepharose CL-4B was purchased from Pharmacia. *p*-Nitrophenyl acetate (*p*-NPA) was synthesized as described by Han et al. [10]. All the other chemicals used were obtained from Tianjin Reagent Company.

2.2. Methods

2.2.1. Screening for Naproxen producing strains

Microorganisms with asymmetric resolution of Naproxen ethyl ester were screened from soil in the vicinity of the Naproxen producing factory. Microorganisms were grown at 37 °C in a solid medium that contained (per liter) 5 g of peptone, 5 g of NaCl, 10 ml of Tween 80, and 15 g of agar (pH 7.0), then grown aerobically at 30 °C for 12–36 h in a liquid medium (pH 7.0) that contained (per liter) 12 g of glucose, 5 g of yeast extract paste, 1 g of peptone, 5 g of NaCl and 5 ml of Tween 80 (pH 7.0). Bacterial growth curves were established and culture conditions were optimized by substituting Tween 20 for Tween 80 in a liquid medium. When Naproxen ethyl ester was used (*S*,*R*)-Naproxen enantioselective products were produced. One strain that was used in the study had stereochemical preference of (*R*)-Naproxen and was identified by Gram staining.

2.2.2. Isolation and analysis of Naproxen

A reaction system contained 10 ml cultures and 20 μl 20% Naproxen ethyl ester (dissolved by ethyl acetate) was cultured at 30 °C for 24–72 h. After the pH value was adjusted below 3 with 4N HCl, the desired product was extracted with 1 ml ethyl acetate. The extract obtained was assayed by thin-layer chromatography (TLC). The product band was separated from TLC panel, extracted with ethyl acetate. Optical purity (ee (%)) of product (*R*)-Naproxen was assayed by HPLC (American Varian 2010). Chiral column, cellulose-OD column (Japan Daicel Company); mobile phase, petroleum ether/2-propanol/glacial acetic acid = 242/8/0.75 (v/v/v); effluence rate, 0.9 ml/min.

2.2.3. Bacterial reaction system

Substrate–cultures reaction systems were studied in different phases of bacterial growth curve.

After centrifugation of cultures, conversion yield was, respectively, assayed in supernatant and in precipitate (bacterial cells) with 50 mM PBS (pH 7.0).

In bacterial cells PBS reaction and cultures reaction systems, respectively, conversion yields were obtained in different reaction times.

2.2.4. Preparation of crude lipase

Precipitate of bacterial cells was harvested from 500 ml cultures by centrifugation in a high-speed centrifuge at 5000 rpm for 10 min. After weighing the precipitate, it was dissolved by 15 ml 50 mM PBS (pH 7.0) and stored at −30 °C. The bacterial cells PBS was thawed at 20 °C immediately and lysozyme was added (10 mg lysozyme per g bacterial cells), then placed in a water bath at 37 °C for 30 min. The cells were levigated thoroughly by quartz sand at 0 °C, and the supernatant (crude lipase) was obtained by centrifugation in a high-speed centrifuge (12,000 rpm) for 20 min at 4 °C.

2.2.5. Purification of lipase by a single hydrophobic interaction chromatography

A total amount of 40 ml of crude lipase was loaded on a phenyl-Sephrose column (2.0 cm × 25.0 cm), equilibrated with 50 mM piperazine buffer (pH 7.0). Unbound compounds were washed out with 150 ml piperazine buffer and, finally, the lipase was eluted with a linear gradient of isopropanol. Linear gradients

of eluted isopropanol fractions were assayed at 280 nm.

2.2.6. Assays for lipase activity

2.2.6.1. Photometric assay. Photometric assay was performed as described by Bornscheuer et al. [11]. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per min from *p*-NPA.

2.2.6.2. Protein determination. Analysis was performed as described by Bornscheuer et al. [11].

2.2.6.3. Conversion assay. Four fraction tubes (3 ml fraction per tube) containing the highest lipase activities were pooled together and other fraction tubes were pooled together (one reaction flask per four tubes) with 20 μl 20% Naproxen ethyl ester at 30 °C for 20 h. The conversion yields were assayed by TLC.

2.2.7. SDS-PAGE assay

The crude lipase, effluent and eluted fractions were dialyzed against deionized water for 20 h. The dialysates were used for SDS-PAGE. Discontinuous SDS-PAGE and determination of the enzyme were performed with low molecular weight standard mixtures. After electrophoresis for 30 mA the gel were stained with $AgNO_3$ by the method of Heukeshoven and Dernick [12] and scanned by densitometry.

3. Results and discussion

3.1. The screening and determination of bacterial strains for asymmetric resolution of Naproxen ethyl ester

Seventeen bacterial strains were cultured from the soil in the vicinity of Naproxen producing factory, from which 14 strains possessed lipase activity. Table 1 shows the optical activity of product obtained from the asymmetric hydrolysis of Naproxen ethyl ester. From results shown in Table 1 it is evident that there are four strains (nos. 2, 7, 9, 10) which catalyze the conversion of Naproxen ethyl ester to a yield over 10%. (*R*)-Naproxen having an optical purity of 82.3% was obtained from strain #7 which was shown by Gram reaction to be a member of *Bacillus* strain.

Table 1
Comparison of conversion rate and optical purity of Naproxen by different bacterial strains

Bacterial strains	Conversion rate (%)	Optical purity (%)
1	0	–
2	10–20	1.03
3	0	–
4	0	–
5	<5	–
6	0	–
7	10–20	82.32
8	0	–
9	10–20	2.15
10	10	43.32
11	0	–
12	0	–
13	0	–
14	<5	–

Experimental conditions: See Section 2. Tween 80 was contained in the liquid medium.

The culture conditions of strain #7 were studied and, in the presence of Tween 80, the cell growth increased with increase in pH value as shown in Fig. 1 which signifies that basic medium was favorable for its growth, therefore, the optimal cell culture was established to be at pH 7–8. Lipase produced from the strain has a highest activity at pH 7–8.

Fig. 2 shows the growth curve of strain #7 at 30 °C, pH 7, and Tween 20, which indicates strain #7 reached

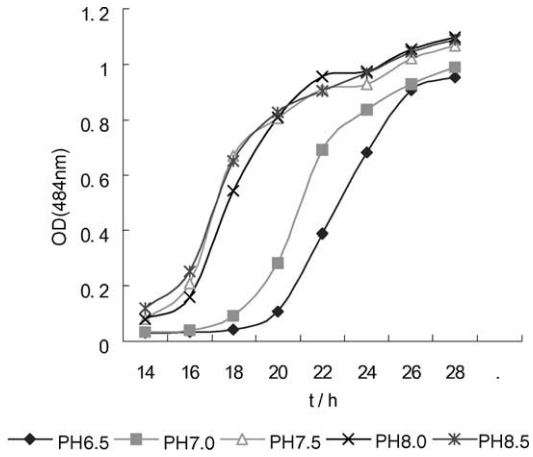

Fig. 1. Growth curve of strain #7 at different pH, 30 °C. Experimental conditions: See Section 2; Tween 80 was contained in the liquid medium.

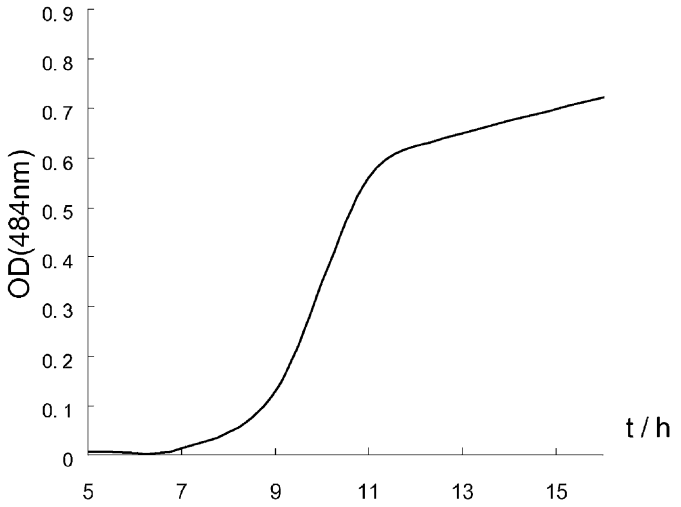

Fig. 2. Growth curve of strain #7 at pH 7.0, 30 °C. Experimental conditions: See Section 2; Tween 20 was contained in the liquid medium.

logarithm growth at 9–12 h of culture. Compared to the logarithm growth using Tween 80 (growth time 20–22 h), the growth time with Tween 20 was shortened by one-fold.

3.2. Optimizing the conversion system of bacterial strain #7

Previous experimental results show the lipase excreted from strain #7 in Tween 80 liquid culture system is an extracellular enzyme [13]. By using Tween 20 instead of Tween 80 it was possible to raise the selective conversion of Naproxen with the enzyme excreted from strain #7. Experimental results show that there was no leakage of enzyme from the bacteria cell when Tween 20 was used and after 11 h of culture the conversion of Naproxen ethyl ester to Naproxen was 10%. Another experiment shows after 11 h culture the bacterial cells were separated by centrifuge and re-suspended in PBS solution adjusted to pH 7, the conversion yield of Naproxen with this system was 30–40%, as shown in Tables 2 and 3. Table 3 also indicates that in culture of strain #7 after 48 h of conversion the yield was 10%, the product purity was 82.32%. If the conversion time was increased to 72 h the conversion rate increased to 15% but the purity of the product decreased to 55.64%. When the bacterial cells were separated by centrifuge and re-suspended in PBS system the Naproxen conversion reached 40–50% and the purity of the product was 86.47%. The above results show PBS conversion system of strain #7 can produce optical pure product and increase the product yield.

3.3. Purification and analysis of lipase from strain #7

Fig. 3 shows the purification elution pattern of crude lipase by phenyl-Sepharose CL-4B chromatography column. Results indicate the chromatogram has three peaks at 280 nm using piperazine–isopropanol as a continuos gradient eluent. The concentration of isopropanol was <1, 9–14 and 30%, respectively. Among

Table 2
Effect of different growth time on conversion of Naproxen

Growth time (h)	Conversion rate in culture (%)	Conversion rate in bacterial cells PBS (%)
9	0	<5
10	<5	10
11	10	30–40
12	<5	10
13	<5	10

Experimental conditions: Substrate–cultures reaction and substrate–bacterial cells PBS reaction systems were studied in different phases of bacterial growth curve. In substrate–cultures reaction systems, strain #7 were grown aerobically at 30 °C in a liquid medium (pH 7.0) containing Tween 20 as shown in Section 2. After centrifugation of cultures in different growth phases, conversion yield was assayed in precipitate (bacterial cells) with 50 mM PBS (pH 7.0) as shown in Section 2.

Table 3
Comparison of enantiospecific hydrolysis of Naproxen ethyl ester by different reaction systems

Conversion time (h)	Conversion rate (C); optical purity (ee, %)	Cultures (under Tween 20 limitation)	Bacterial cells PBS
24	C	<10	20
	ee	83.21	88.25
48	C	10	40–50
	ee	82.32	86.47
72	C	15	40–50
	ee	55.64	85.50

Experimental conditions: In bacterial cells PBS reaction and cultures reaction systems, respectively, conversion yields were obtained in different reaction times. After centrifugation of cultures, conversion yield was, respectively, assayed in supernatant and in precipitate (bacterial cells) with 50 mM PBS (pH 7.0) as described in Section 2. C, conversion rate; ee, optical purity.

the three protein peaks only the one eluted by 9–14% isopropanol showed lipase activity for the conversion of Naproxen.

Fig. 4 and Table 4 show the purity before and after SDS-PAGE separation of lipase. The crude enzyme was loaded on phenyl-Sepharose CL-4B column and the eluting fraction tube 12, 13 which have the

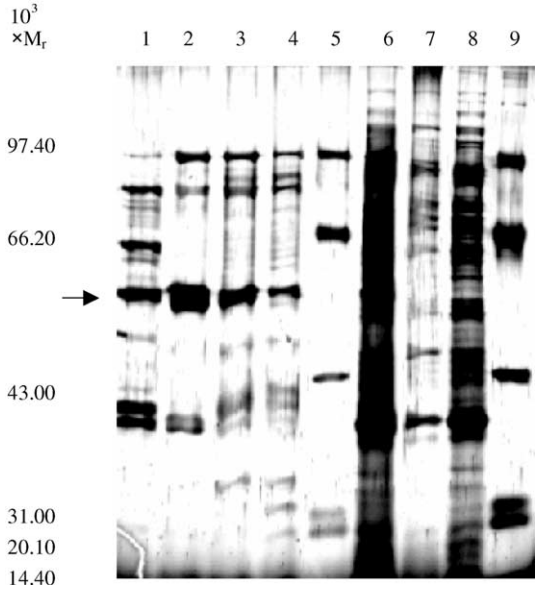

Fig. 4. Electrophoretic purification of lipase from the *Bacillus* strain. SDS-PAGE lanes: 1, 2, 3, 4 correspond to fraction tube 11, 12, 13, 14, respectively; 5, 9, molecular weight standards; 6, crude lipase; 7, effluent; 8, eluted fractions by piperazine buffer. Experimental conditions: See Section 2.

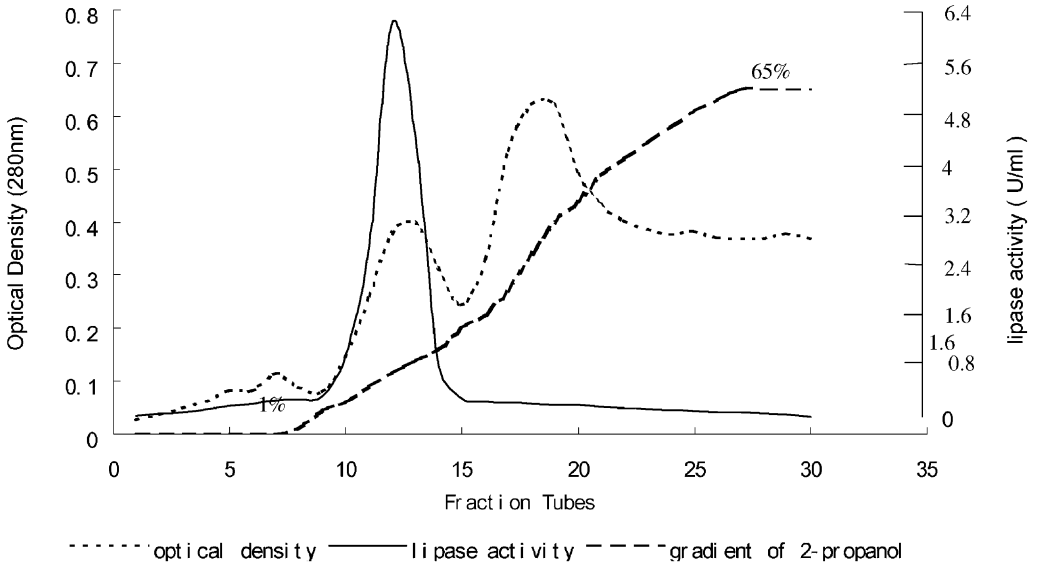

Fig. 3. Elution pattern of hydrophobic interaction chromatography of lipase of *Bacillus* from a phenyl-Sephrose column eluting gradient from 0 to 65% isopropanol. Experimental conditions: See Section 2 and experimental conditions in Table 4.

Table 4
Purification result of lipase from *Bacillus* strain

Fraction	Protein concentration (mg/ml)	Total protein (mg)	Lipase activity (U/ml)	Specific activity (U/mg)	Lipase purity[a] (%)
Crude lipase	5.075	230	3.2	0.61	0.2
Fraction tube 11	0.330	1.32	2.86	8.58	19.01
Fraction tube 12	0.235	0.940	6.2	26.4	66.84
Fraction tube 13	0.225	0.900	4.44	20.0	61.27

Experimental conditions: Photometric assay was performed as described by Bornscheuer et al. [11]. Photometric assay using *p*-nitrophenyl acetate (*p*-NPA) was used for a quick analysis of crude lipase and chromatographic fractions. Solution A contained 90 mg *p*-NPA and was dissolved in 30 ml 2-propanol with a sonicator for 6 min at room temperature. Solution B contained 0.4% Triton-X 100 and 0.1% gum arabic. Both solutions are stable for about 2 week in a refrigerator. The reaction mixture consisted of one part of solution A and nine parts of solution B and was freshly prepared before each assay. A 100 μl volume of an appropriate dilution of the enzyme solution was added to 900 μl of the reaction mixture. Finally, the lipase reactions were analyzed at 410 nm and 30 °C with an Ultrospec-K photometer. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per min from *p*-NPA. Analysis of protein determination was performed as described by Bornscheuer et al. [11] with 0.1% Coomassie brilliant blue in 1% acetic acid, 40% methanol.
[a] The data of lipase purity is derived from Fig. 4.

highest enzyme activity contained 66 and 61% of the total protein. The molecular weight of lipase in these two fractions was determined by SDS-PAGE to be 55–56 kDa.

Table 4 also shows the lipase activity before and after hydrophobic chromatography purification. Results signifies that crude enzyme from strain #7 had a concentration of 5.075 mg/ml, of which lipase only contained 0.2%. After loading on phenyl-Sepharose CL-4B column the lipase amount in eluting tube no. 12 was 66% of the total protein which indicates an increase in purity of 330-fold and increase of specific activity of 43-fold.

The above results clearly indicate that hydrophobic chromatography (phenyl-Sepharose CL-4B) is a powerful purification method for low content lipase containing enzyme.

The lipase which we purified has a molecular weight of 55–56 kDa, very similar to *Ps. fluorescens* MC 50 (molecular weight 55 kDa having only one sub unit) reported by Bozoglu et al. [14]. But it is quite different from *Ps. aeruginosa* EF2 (29 kDa), *Ps. fragi* AFO 3458 (14.6 kDa) and *Staphylococcus hyicus* (86 kDa) reported by Gilbert et al. [8], Kugimiya et al. [15] and Van Oort et al. [16].

Acknowledgements

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