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Purification and Application of a Lipase from *Penicillium expansum* PED-03

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Abstract An extracellular lipase was purified from the fermentation broth of *Penicillium expansum* PED-03 by DEAE-Sepharose chromatography, followed by sephacryl S-200 chromatography. The enzyme was purified 81.8-fold with 19.8% recovery and a specific activity of 85.94 U/mg. The molecular weight of the homogeneous enzyme was about 28 kDa, determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The enzymatic resolution of racemic ibuprofen was carried out by the lipase from *P. expansum* PED-03, and the conversion reached 46% with excellent enantioselectivity ($E>200$), which showed a good application potential in the production of optically pure ibuprofen.

Keywords Lipase · *Penicillium expansum* · Purification · Resolution · Ibuprofen

Introduction

Lipase (triacylglycerol hydrolases; EC3.1.1.3) is one of the most important industrial enzymes that catalyze the hydrolysis or formation of lipids. It has been extensively exploited for catalytic chiral resolution because of the growing demand for enantiopure intermediates and drugs [1, 2]. Lipase is successfully used as chiral catalyst because of its high enantioselectivity and has been widely used for the production of enantiomerically pure compounds, resolving racemic alcohols, and organic acids via hydrolysis, esterification, or transesterification reactions [3].

Ibuprofen is an important member of the nonsteroidal anti-inflammatory drugs. The most common lipases used in the resolution of ibuprofen are from *Candida rugosa* [4–6] and *Rhizomucor miehei* [7, 8]. However, the enantioselectivity of these lipases was relatively low in the case of ibuprofen.

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Recently, our laboratory has isolated and identified a strain of *Penicillium expansum*, *P. expansum* PED-03 [9] from a rap oil manufactory in China and found that it could produce a lipase at a high level with excellent enantioselectivity in the resolution of racemic ibuprofen. This paper describes the purification of the lipase from *P. expansum* PED-03 and its evaluation for the enzymatic resolution of racemic ibuprofen.

Materials and Methods

Microorganism and Culture Conditions

The strain for lipase production was *P. expansum* PED-03, isolated from the waste of a rap oil manufactory in China and deposited in our laboratory. The strain was cultured on potato dextrose agar slants at 26°C for 5 days and stored at 4°C when spores were formed.

The liquid medium for lipase production consisted of 0.5% (*w*/*v*) starch, 4% (*w*/*v*) soybean powder, 0.2% (*w*/*v*) Na_2HPO_4, 0.3% (*w*/*v*) K_2SO_4, 0.3% (*w*/*v*) $MgSO_4$, 0.5% (*w*/*v*) $CaCO_3$, and 0.015% (*w*/*v*) $FeSO_4$. The initial pH of the medium was adjusted to 6.0 with 0.1 M citrate buffer. For inoculum preparation, 1.0 mL of spore suspension (10^8 conidia/mL) of *P. expansum* PED-03 was inoculated into 30 mL of the fermentation medium in a 250-mL Erlenmeyer flask and cultured at 26°C, 300 rpm for 64 h.

Enzyme Assay

The photometric assay substrate was prepared as described by Lianghua and Liming [10] with slight modifications. Solution A contained *p*-nitrophenylpalmitate (pNPP) dissolved in 10 mL of 2-propanol to the concentration of 16.5 mM. Solution B for the pNPP assay consisted of 100 mM Tris–HCl buffer (pH 8) containing 0.4% Triton X-100. The reaction mixture consisting of one part solution A and nine parts solution B was prepared fresh before the assay. A 100-μl volume of an appropriate dilution of the enzyme solution was added to 3,000 μl of the reaction mixture. The kinetics was detected at 410 nm and 37°C with an Ultraspec K photometre (Pharmacia LKB). Under the conditions used, the extinction coefficient (ε_{410}) of *p*-nitrophenol was 1.46×10^5 cm^2/mol.

One unit of lipase activity was defined as the amount of lipase that liberated 1 μmol of *p*-nitrophenol from pNPP per minute.

Protein Determination

Protein was assayed by the method of Lowry et al. [11].

Purification of the Lipase

Ion-exchange Chromatography

Cell-free supernatant (100 mL) was adjusted to pH 8.0 with 0.5 M NaOH.

An IEC was used with a glass column (16×200 mm) filled with 30 mL DEAE-Sepharose. After the column was equilibrated with a buffer consisting of 20 mM Tris–HCl buffer (pH 8), the supernatant was loaded onto the column. Then, the column was washed with 120 mL of starting buffer at a flow rate of 1 mL/min.The lipase was finally eluted at a flow rate of 1 mL/min with 45 mL of 20 mM Tris–HCl buffer (pH 8) containing 150 mM NaCl.

Table 1 Purification of a lipase from *Penicillium expansum* PED-03.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Cell-free supernatant	74,600	78,000	1.05	1	100
DEAE-Sepharose	1,063	29,484	27.74	26.4	37.8
Sephacryl S-200	180	15,470	85.94	81.8	19.8

Gel filtration

The combined active fractions from the DEAE-Sepharose chromatography were freeze-dried and resuspended in 1 mL of 0.2 M Tris–HCl buffer (pH 8). The precipitate was centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant was loaded onto a Sephacryl S-200 column (16×650 mm). Then, the column was washed with 240 mL of 0.2 M Tris–HCl buffer (pH 8) at a flow rate of 1 mL/min. The fractions were assayed by the pNPP method.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 15% polyacrylamide) was performed according to Laemmli [12].

Enzymatic Resolution

The resolution was performed in a reactor containing 20 mg of (*R*,*S*)-ibuprofen, 15 mL of isooctane, 45 μL 1-propanol, and 15 mg of purified lipase. The suspension was incubated at 55°C under continuous shaking (200 rpm). Samples (0.5 mL) were collected at predetermined time intervals for analysis by HPLC. The enantiomeric excess of ibuprofen was

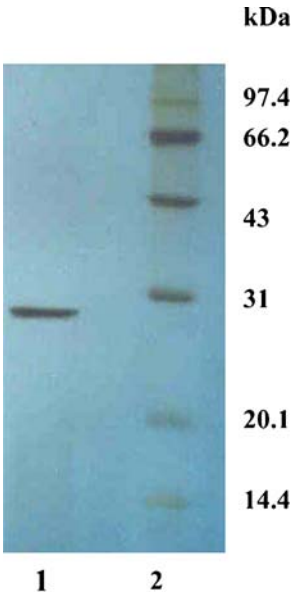

Fig. 1 Separation of lipase containing fractions by SDS–PAGE *1* Sephacryl 200 eluate fraction, *2* low molecular weight marker proteins; *from top to bottom* rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa)

Table 2 Resolution of ibuprofen catalyzed by the lipase from *P. expansum* PED-03[a].

Reaction time (h)	ee_s^b(%)	ee_p^b(%)	Conversion (%)	Enantioselectivity (*E*)
24	38	99	33	323
48	90	99	46	536

Solution contained 20 mg of (*R*,*S*)-ibuprofen, 15 mL of isooctane, 45 μl 1-propanol, and 15 mg of purified lipase at 55°C, and the results were averages of three replicates.

[a] The reactions were carried out in 15 mL of isooctane

[b] Enantiomeric excesses for the substrate (ee_s) and for the product (ee_p) were determined by chiral HPLC.

determined by HPLC with a Chiracel OD column (4.6×250 mm). Samples (20 μl) were eluted by a mixture of *n*-hexane/2-propanol/trifluoroacetic acid (98:2:0.1, by volume) at 0.5 mL/min and detected at 254 nm, and the conversion (*C*) and enantioselectivity (*E*) were calculated as described by Straathof and Jongejan [13].

Results and Discussion

Enzyme Production

Penicillium expansum PED-03 produced an extracellular lipase in a medium(pH 6.0) consisting of 0.5% (*w*/*v*) starch, 4% (*w*/*v*) soybean powder, 0.2% (*w*/*v*) Na_2HPO_4, 0.3% (*w*/*v*) K_2SO_4, 0.3% (*w*/*v*)$MgSO_4$, 0.5% (*w*/*v*) $CaCO_3$, and 0.015% (*w*/*v*) $FeSO_4$. Maximum enzyme activity (780 U/ml) was found after 64 h under shaking conditions (300 rpm) at 26°C.

Enzyme Purification

When the cell-free supernatant was loaded on a DEAE-Sepharose column, most of the protein bound to DEAE-Sepharose, and 37.8% of the total lipase was eluted with the Tris–HCl buffer containing 150 mM NaCl. A 26.4-fold increase in specific activity was obtained by the DEAE-Sepharose chromatography.

When the concentrated active fractions from DEAE-Sepharose chromatography passed through a Sephacryl S-200 column, 19.8% of the total lipase activity was obtained. The enzyme, in the form of homogeneous lipase, exhibited an 81.8-fold increase in specific activity as compared with the cell-free supernatant.

The extracellular lipase from *P. expansum* PED-03 was purified employing a two-step procedure. A summary of the purification procedure was shown in Table 1. The lipase was purified 81.8-fold with 19.8% recovery and a specific activity of about 85.9 U/mg. The yield in lipase purification procedure was relatively low, which was in agreement to previous studies [10, 14].

Table 3 Comparison of enzyme enantioselectivity for ibuprofen.

Lipase producer	ee (%)	Conversion (%)	Enantioselectivity (*E*)
Candida rugosa	–	30	24.1
Rhizomucor miehei	93.8	49.9	113
Aspergillus niger	79.1	48%	32
Penicillium expansum PED-03	99	46%	536

Enzyme Characterization

A molecular mass of approximately 28 kDa was determined for the purified lipase by SDS–PAGE (Fig. 1). *P. expansum* PED-03 lipase is smaller than those from *P. cyclopium* [15] and *P. simplicissimum* [16] but similar to those from *P. candidum* [17], *Penicillium* sp. [18], and *P. expansum* DSM1994 [19].

Application in the Enzymatic Resolution of Racemic Ibuprofen

The course of the enzymatic resolution of ibuprofen through esterification reaction catalyzed by the purified lipase from *P. expansum* PED-03 was monitored by HPLC. The enantiomeric excess ee and enantiomeric ratio *E* were calculated as described by Straathof and Jongejan [13]. For the substrate (*R*,*S*)-ibuprofen only (*S*)-ibuprofen reacted, and therefore, in the HPLC chromatogram, the concentration of (*R*)-ibuprofen remained constant. The conversion of the racemate reached 46% after 48 h, and the product of (*S*)-ibuprofen *n*-propyl ester was the pure *S*-enantiomer (Table 2). The enantiomeric excess was 99% for the product, resulting in enantiomeric ratio much larger than 200 ($E \approx 536$). The enantioselectivity of *P. expansum* PED-03 lipase for ibuprofen was excellent. The selectivity with an enantiomeric ratio *E* of 536 was much higher than that of those lipases from *C. rugosa* [5], *R. miehei* [8], *Aspergillus niger* [20] (Table 3).

Ibuprofen is an important member of the nonsteroidal anti-inflammatory drugs. Its racemic mixture of the two enantiomers *R*-(−) and *S*-(+) is being used, but the biological activity is mainly from the *S*-ibuprofen. Therefore, the resolution of racemic ibuprofen is of great benefit in preventing the side effects from the use of racemic mixture. The result of our research work demonstrates that the lipase from *P. expansum* PED-03 is a good biocatalyst with excellent enantioselectivity in the enzymatic resolution of racemic ibuprofen.

Conclusion

The results obtained in this study show that *P. expansum* PED-03 lipase has a high enantioselectivity for the esterification of the *S*-ibuprofen. This result renders the lipase attractive for kinetic resolution of racemic ibuprofen.

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