Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 26 (2003) 149–156
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Preparation of enantiomerically pure (*S*)-flurbiprofen by an esterase from *Pseudomonas* sp. KCTC 10122BP

Eun Gyo Lee [a], Hye Soon Won [a], Hyeon-Su Ro [a], Yeon-Woo Ryu [b], Bong Hyun Chung [a,*]

[a] *Laboratory of Integrative Biotechnology, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejeon 305-600, South Korea*
[b] *Department of Molecular Science and Technology, College of Engineering, Ajou University, San 5, Woncheon-dong, Paldal-gu, Suwon 442-749, South Korea*

Received 18 March 2003; received in revised form 26 May 2003; accepted 26 May 2003

Abstract

Esterase PF1-K from *Pseudomonas* sp. KTCC 10122BP was overproduced by the fed-batch culture of *Escherichia coli*. The soluble expression of esterase PF1-K was achieved by shifting the culture temperature from 37 to 25 °C at the time of IPTG induction. The enzyme was partially purified to about 75% purity by a single-step hydrophobic interaction column chromatography. The purified enzyme exhibited a fairly high enantioselectivity towards the hydrolysis of rac-flurbiprofen ethyl ester. The enzymatic chiral resolution was further improved by optimizing the reaction conditions in terms of reaction rate and enantioselectivity. The optimal reaction conditions were found to be 40 °C, pH 10.5 and 600 mM of initial rac-flurbiprofen ethyl ester. After 90 min of batch reaction under the optimal conditions, 50% of the initial rac-flurbiprofen was hydrolyzed with an enantiomeric excess of 99%.

Keywords: Flurbiprofen; Enantioselective hydrolysis; Recombinant esterase; *Pseudomonas*

1. Introduction

Flurbiprofen {(*R,S*)-[2-(3-fluoro-4-phenyl) phenyl] propionic acid} is one of the most prevalent nonsteroidal anti-inflammatory drugs (NSAIDs) [1,2]. It is known that the *S*-enantiomer of flurbiprofen exhibits most of its anti-inflammatory activity, while the presence of the *R*-enantiomer enhances its gastrointestinal toxicity [3]. In the case of NSAIDs, in general, the *S*-isomers have shown significantly greater therapeutic effect than the *R*-isomer (28- and 160-fold for naproxen and ibuprofen, respectively) [2]. In this regard, it is greatly required to resolve the racemic mixture of NSAIDs to avoid the toxicity and potential pharmacokinetical problems.

The enzymes with a high enantioselectivity have been considered as potential catalysts in the chiral-specific resolution of profen drugs. Among a variety of enzymes, lipase and esterase have been most frequently used to prepare optically pure compounds because of its wide substrate specificity and ability to recognize chirality [4–6]. A lipase from *Candida rugosa* exhibited a relatively high hydrolyzing activity towards (*S*)-flurbiprofen ester, but the enantioselec-

* Corresponding author. Tel.: +82-42-860-4442; fax: +82-42-860-4594.
E-mail address: chungbh@kribb.re.kr (B.H. Chung).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.05.004

tivity was unsatisfactory [2,7]. An esterase from *Trichosporon brassicae* CGMCC 0574 was identified to possess a high enantioselectivity towards the ethyl ester form of (*S*)-ketoprofen [8]. Two different esterases from *Citeromyces matriensis* CGMCC 0573 [9] and *Bacillus stearothermophilus* JY144 [10] were identified to be (*R*)-enantiomer-specific. Recently, we have isolated a novel gene from *Pseudomonas* sp. KCTC 10122BP, encoding an esterase PF1-K with enantioselectivity towards (*S*)-ketoprofen ethyl ester, and overexpressed it in *Escherichia coli* [11,12]. To broaden the applications of the enzyme, in this study, we attempted to overproduce the active esterase PF1-K via the fed-batch culture of recombinant *E. coli*, and purified the enzyme by single-step hydrophobic interaction chromatography. In addition, the enantiomercally pure (*S*)-flurbiprofen was produced from rac-flurbiprofen ethyl ester using the purified enzyme, and the chiral resolution conditions were optimized in terms of hydrolysis reaction rate and enantioselectivity.

2. Materials and Methods

2.1. Chemicals

(*R*,*S*)-Flurbiprofen was purchased from Kolong Co. (Korea). *p*-Nitrophenyl butyrate, isopropyl-β-D-thiogalactopyranoside (IPTG), Triton X-100, Span 80, Tween 80, tetrahydrofuran (THF), dimethylformamide (DMF), and cyclohexane were purchased from Sigma Chemical Co. (USA). Methanol, ethanol and acetonitrile were from Merck Co. (Germany). All other chemicals were of reagent grade.

2.2. Bacterial strain and plasmid

The previously constructed plasmid pEESTa [12] that contains an esterase gene (PF1-K) from *Pseudomonas* sp. KCTC10122BP was introduced into *E. coli* BL21 to overproduce the esterase PF1-K. In brief, an esterase gene (PF1-K) from *Pseudomonas* sp. KCTC10122BP was amplified using polymerase chain reaction (PCR) with N-terminal primer that contains *Nde* I site and C-terminal primer that contains *Xho* I site. Resulting 1.2 kb DNA fragment was digested with *Nde* I and *Xho* I restriction enzymes, and was inserted into the *Nde* I and *Xho* I sites of plasmid pET22b (Novagen).

2.3. Media and culture conditions

The recombinant *E. coli* was grown in Luria–Bertani (LB) medium with an appropriate amount of ampicillin (60 μg/ml). *E. coli* was cultivated in a 5L fermentor (Korea Fermentor, Korea) operated in a fed-batch mode. The initial medium composition for fed-batch culture was 20 g/l glucose, 20 g/l yeast extract, 5 g/l KH_2PO_4, 3 g/l K_2HPO_4, 1.7 g/l $(NH_4)_2SO_4$, 2 g/l $MgSO_4{\cdot}7H_2O$, 0.1 g/l $FeSO_4{\cdot}7H_2O$, 0.02 g/l $CaCl_2{\cdot}7H_2O$ and 0.1 g/l thiamin–HCl in water. The feeding solutions in fed-batch mode composed for cell growth and enzyme production. The medium for the cell growth (Medium A) contained 700 g/l glucose, 7 g/l yeast extract, 20 g/l $MgSO_4{\cdot}7H_2O$, and 20 ml/l trace metal solution water. The trace metal solution contained 2.2 g/l $FeCl_3{\cdot}6H_2O$, 1.05 g/l $ZnSO_4{\cdot}7H_2O$, 0.25 g/l H_3BO_3, 0.19 g/l $CuSO_4{\cdot}7H_2O$, 0.2 g/l $Na_2MoO_4{\cdot}2H_2O$, 0.2 g/l $CoCl_2{\cdot}4H_2O$, and 1.2 g/l $MnCl_2{\cdot}4H_2O$ in water with acidic pH (2.3). The medium for the enzyme production (Medium B) contained 400 g/l glucose, 200 g/l yeast extract, 10 g/l KH_2PO_4, 1.5 g/l $(NH_4)_2SO_4$, and 10 g/l $MgSO_4{\cdot}7H_2O$ in water. The culture pH was adjusted to 6.8 with ammonium hydroxide and hydrochloride solutions. IPTG as an inducer was added to the culture media by 0.03 mM/g cell. The culture temperature was changed from 37 to 25 °C in order to obtain an active soluble esterase when the optical density at 600 nm reached about 0.8 for flask culture and 130 for fermenter culture, respectively.

2.4. Purification of esterase PF1-K

E. coli cells were harvested by centrifugation and then washed twice with 50 mM Tris–HCl buffer (pH 8.5). The cell was diluted to a proper concentration and disrupted using a sonicator (Sonics & Materials, USA) on ice. The soluble fraction, obtained after centrifugation at 12 000 × *g* for 20 min, was clarified with a membrane filter (Millipore, USA) and then loaded onto a Butyl Sepharose (Amersham Bioscience, Sweden) column (1.0 cm × 25 cm, Amicon, USA), that were pre-equilibrated with 0.8 M ammonium sulfate in

50 mM Tris–HCl buffer (pH 8.5). The bound proteins were washed with the equilibration buffer containing 0.8 M of the ammonium sulfate and eluted with a linear gradient of ammonium sulfate from 0.8 to 0 M at a flow rate of 1.5 ml/min. Each of the eluted fractions was subjected to the SDS–PAGE (12%) and activity analysis.

2.5. Synthesis of (R,S)-flurbiprofen ethyl ester

Flurbiprofen ethyl ester was synthesized according to the previous report [13]. The (*R*,*S*)-flurbiprofen (30 g) was solubilized in 100 ml of ethanol in a round-bottom flask. Sulfuric acid was added as a catalyst for esterification was added to (*R*,*S*)-flurbiprofen solution. The mixture was refluxed for 5 h. The residual ethanol was removed by vacuum evaporation and then washed three times with aqueous 1 M $NaHCO_3$ to eliminate unreacted flurbiprofen, sulfuric acid and ethanol.

2.6. Enzyme activity

Hydrolytic activity of the esterase was measured using both spectrophotometric and HPLC methods. *p*-Nitrophenyl butyrate (5 mM) as a substrate in acetonitrile was prepared in 50 mM Tris–HCl buffer (pH 8.0). The reaction was performed by addition of the enzyme solution (5 ∼ 20 μl) at 30 °C for 10 min and the produced *p*-nitrophenol was measured by a spectrophotometer (Pharmacia, Sweden). One unit of the esterase activity was defined as the amount of enzyme producing 1 μmol of product per minute. With rac-flurbiprofen ethyl ester as a substrate the esterase activity was measured by HPLC (Gilson, France). The reaction was carried out at 30 °C and pH 8.5 for 10 min and the activity was determined by the amount of the produced (*S*)-flurbiprofen.

2.7. Analytical methods

The concentrations of (*R*)- and (*S*)-flurbiprofen were measured by HPLC (Gilson, France). The HPLC conditions were as follows: column, CHIREX (Phase 3005, Phenomenex, CA, USA); detection, UV at 254 nm; mobile phase, methanol containing 10 mM ammonium acetate; flow rate, 0.7 ml/min. The protein concentration was determined by a modified Bradford method using Bio-Rad Protein Assay kit (Bio-Rad, USA).

3. Results and discussion

3.1. Production of active esterase PF1-K

The recombinant *E. coli* cells with the plasmid pEESTa were found to produce the active soluble esterase PF1-K when grown at 20 °C after IPTG induction, while the insoluble inclusion bodies were obtained at 37 °C, as previously reported [12]. To produce the esterase PF1-K on a large scale, we performed a fed-batch culture with an exponential feeding strategy. In the beginning of fed-batch culture, the cells grew up to 130 optical density (OD at 600 nm) by feeding the Medium A (see Section 2.3) at 37 °C. When the cell concentration reached 130 OD, the Medium B (see Section 2.3) was introduced for the production of enzyme. At this production stage, the expression of esterase PF1-K was induced by the immediate feeding of IPTG, and the culture temperature was shifted to 25 °C to reduce the formation of inclusion bodies. In this study, the culture temperature was adjusted to 25 °C instead of 20 °C, because the cell growth at 20 °C was too slow to achieve a high cell-density culture. After 26 h of culture, the cells grew up to 189 OD, and the esterase PF1-K was found to be a major protein judging from SDS–PAGE analysis (data not shown). The cell growth was satisfactory but the soluble fraction of esterase expressed decreased by 30% compared to the flask culture, primarily due to the elevated temperature (20–25 °C).

3.2. Purification of active chiral esterase

In our report [12], the pure esterase PF1-K was obtained by the sequential chromatography of ion exchange and gel filtration. To simplify the purification step, we developed a single-step purification method with hydrophobic column chromatography. The soluble crude extract (225 mg; 4075 units/mg protein) obtained after sonication was subjected to the Butyl-Sepharose FF column chromatography, and the active fractions eluted around 0.3 M $(NH_4)_2SO_4$ were collected (Fig. 1B). The esterase in the active fractions accounted for >75% of the total protein

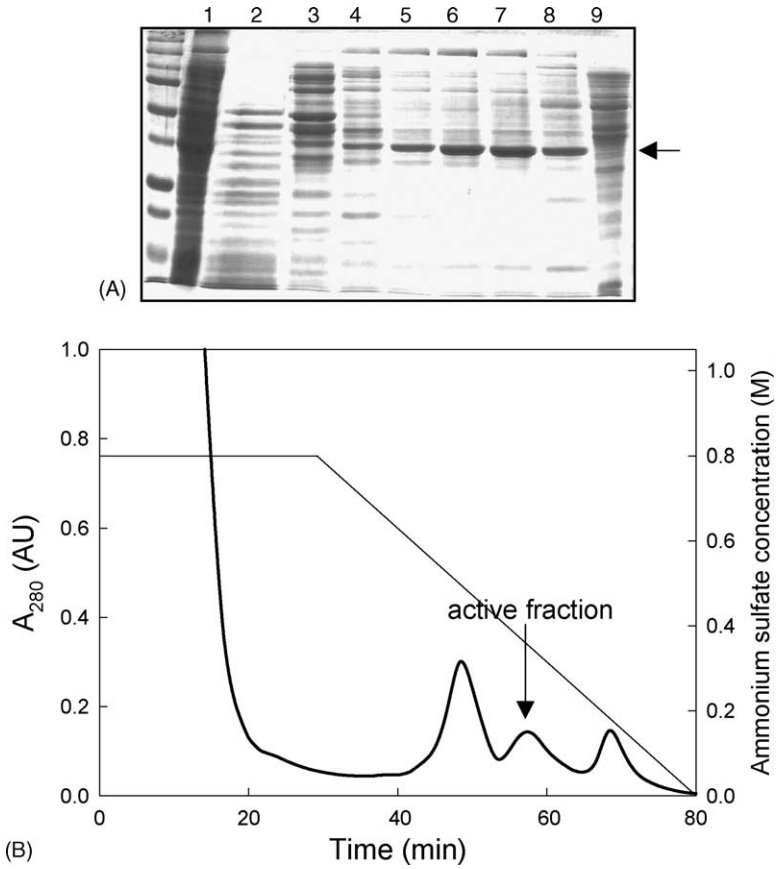

Fig. 1. Hydrophobic interaction chromatography of the esterase PF1-K. (A) SDS–PAGE analysis of the fractions. Lanes 1–8: (1) loading sample; (2) flow-through; (3–4) the first peak in chromatogram; (5–8) the second peak (active fraction); (9) the third peak. (B) Elution profile of the esterase PF1-K. The arrow indicates the peak that contains the active esterase PF1-K.

(Fig. 1A). Finally, the total protein (27 mg) with a specific hydrolysis activity of 28 875 units/mg protein was obtained with a recovery yield of 85%.

3.3. Effects of pH and temperature

The enzymatic hydrolysis of rac-flurbiprofen ethyl ester was examined in the range of pH 6–11 (phosphate buffer for pH 6–7.5, Tris–HCl buffer for pH 7.5–9, glycine–NaOH buffer for pH 9–11). The esterase activity increased with increasing pH in the range of pH 6–10 (Fig. 2). The activity was not affected by a further increase in pH. The highest reaction rate was obtained at pH 10.5, whereas the enantioselectivity remained satisfactory, producing (*S*)-flurbiprofen over 99% e.e. in the overall pH range tested (data not shown). The pH optimum of the esterase PK1-F observed here was 1 pH unit higher than our previous result on the chiral resolution of rac-ketoprofen ethyl ester (optimum pH 9.5) [12]. While the pH-activity profile for the chiral resolution of rac-ketoprofen ethyl ester with the partial-purified enzyme was the same with that for the rac-flurbiprofen ethyl ester (data not shown), it was conceivable that the crude enzyme in [12] co-precipitated with the contaminated cellular proteins at above pH 9.5 thereby yielding lowered activity.

To determine the optimal reaction temperature for the enantioselective hydrolysis of rac-flurbiprofen ethyl ester, the effect of reaction temperature was investigated in the range of 30–80 °C at pH 8.5. As shown in Fig. 3, the activity profiles against temper-

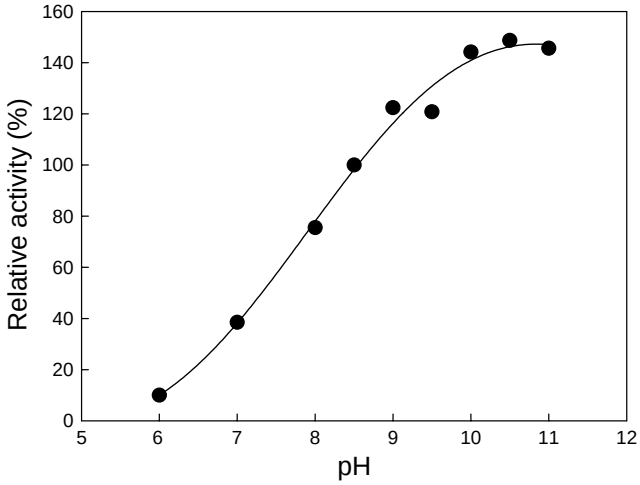

Fig. 2. Effect of pH on the conversion of rac-flurbiprofen ethyl ester. The activity was measured with rac-flurbiprofen ethyl ester (5 mM) as substrate at 30 °C. The buffer used were sodium phosphate buffer for pH 6–7.5, Tris–HCl buffer for pH 7.5–9, and glycine–NaOH buffer for 9–11. Relative activity was calculated on the basis of the activity at pH 8.5 as 100%.

ature were bell-shaped with the optimal temperature of 40 °C. The optimal temperature for the chiral resolution of rac-flurbiprofen ethyl ester was up-shifted 5 °C when as compared with that for the chiral resolution of rac-ketoprofen ethyl ester (35 °C) [12]. It is probable that the presence of fluoro group at rac-flurbiprofen ethyl ester may decrease the solubility of the substrate, requiring higher temperature to dissolve the substrate. In addition, the thermal stability was examined by measuring the residual activity after the pre-incubation of enzyme for 1 h at a given temperature. The esterase PF1-K was stable up to 40 °C but rapidly lost its activity above this temperature (Data not shown). The half-life of esterase PF1-K was found to be 1 h at 45 °C.

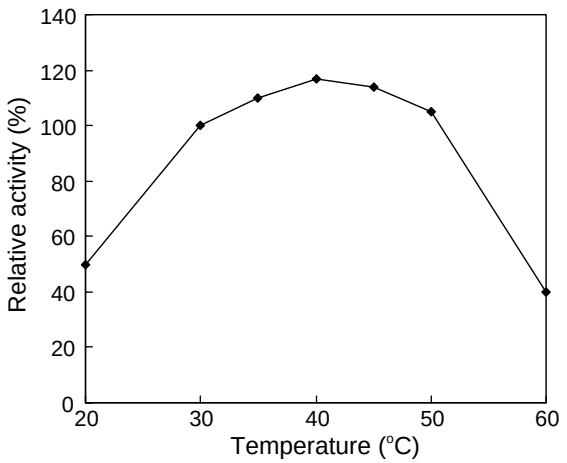

Fig. 3. Effect of temperature on conversion of rac-flurbiprofen ethyl ester. Relative activity was calculated on the basis of the activity at 30 °C as 100%.

3.4. Effects of surfactants and solvents

Additives and solvents have been known to affect the esterase activity [14–16]. Our previous results have also shown that the activity of the esterase PF1-K in the enantioselective resolution of (*S*)-ketoprofen ethyl ester was significantly affected by the presence of the surfactants and solvents [11,12]. To examine the effects of solvents and surfactants on the enantioselective resolution of (*S*)-flurbiprofen ethyl ester, we chose a few solvents and surfactants based on our previous report [11]. As shown in Table 1, the addition of solvents decreased the activity and the enantioselectivity. On the other hand, the presence of surfactants showed a slight increase in the reaction rate while retaining the enantioselectivity. The addition of surfactants in the case of (*S*)-flurbiprofen ethyl ester was less effective than that of (*S*)-ketoprofen ethyl ester [11]. It appears that this is due to the difference in sol-

Table 1
Effects of organic solvents and surfactants on hydrolysis of rac-flurbiprofen ethyl ester by esterase PF1-K

	rac-Flurbiprofen ethyl ester		rac-Ketoprofen ethyl ester[a]	
	Relative activity[b] (%)	e.e.$_p$ (%)	Relative activity (%)	e.e.$_p$ (%)
Control	100	99	100	82
DMF	78	99	56	100
Cyclohexane	74	99	79	97
THF	25	95	59	94
Triton X-100	133	100	410	95
Tween 80	122	100	140	84
Span 80	118	100		

[a] Data from our previous report [11].
[b] Relative activity was determined in the presence of solvent (10%) and surfactant (1%) under standard assay condition.

ubilities of these two compounds. The lower solubility of flurbiprofen ethyl ester might require higher concentrations of surfactants to be solubilized. This interpretation was further supported by (1) the poor effect of Triton X-100 on the resolution of (*S*)-flurbiprofen ethyl ester when compared to that of the resolution of (*S*)-ketoprofen ethyl ester (Fig. 4), and (2) the fact that ketoprofen is 60 times more soluble than flurbiprofen [17]. It seems that much higher concentration of surfactants would be required to achieve higher reaction rate by the addition of surfactants in the enzymatic resolution of (*S*)-flurbiprofen ethyl ester.

3.5. Effect of substrate concentration for the enantioselective resolution of (S)-flurbiprofen ethyl ester

To further optimize the reaction conditions, we examined the effect of the initial concentration of flurbiprofen ethyl ester on the enzyme activity. Fig. 5 showed that the enzyme activity rapidly increased with an increase in the substrate concentration up to 600 mM. But, above this concentration, the activity gradually decreased with increase in the substrate concentration, suggesting the presence of substrate inhibition effect. Since the solubility of the ethyl

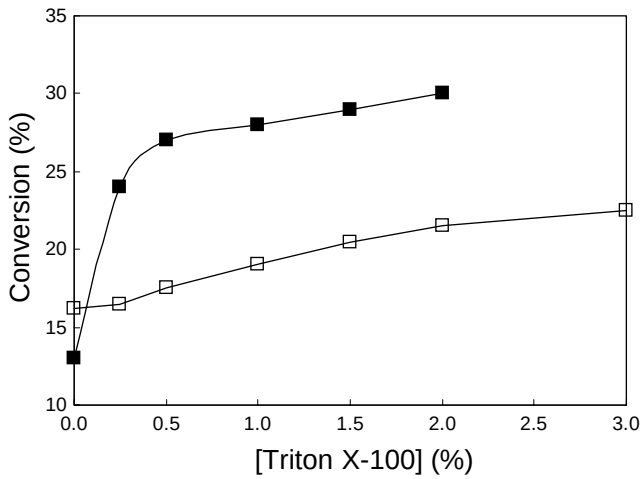

Fig. 4. Effect of Triton X-100 concentration on the conversion of rac-flurbiprofen ethyl ester. The data for rac-ketoprofen ethyl ester were reproduced from our previous report [11]. (□), rac-Flurbiprofen ethyl ester; (■), rac-ketoprofen ethyl ester.

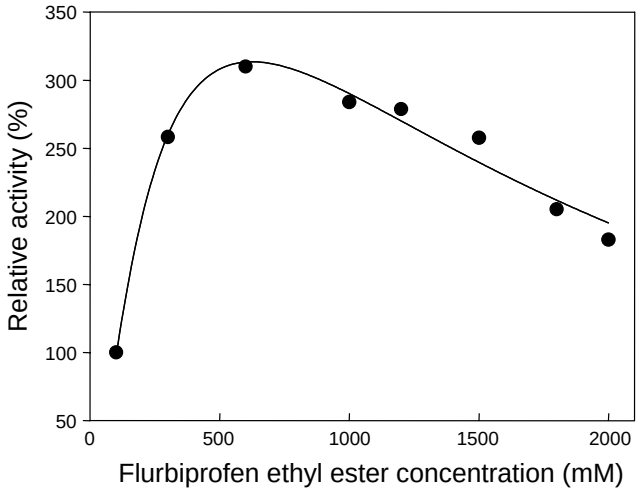

Fig. 5. Effect of the initial concentration of rac-flurbiprofen ethyl ester on the activity. Relative activity was calculated on the basis of the activity at 100 mM rac-flurbiprofen ethyl ester as 100%.

ester form of flurbiprofen was expected to be less than that of flurbiprofen (34 μM, see [17]), the optimal concentration of the substrate for the enzymatic conversion in this case was far beyond its' solubility limit. The gradual decrease in the enzyme activity after 600 mM substrate concentration might reflect the increase of inactivation rate of the enzyme at the interphase between water(enzyme) and oil(substrate) phases.

3.6. Production of pure (S)-flurbiprofen

The batch production of pure (*S*)-flurbiprofen was performed in a glass reactor. We used the crude esterase PF1-K, which was obtained after sonication of the harvested cells. The enzymatic reaction was designed to be over in 1 h. Since the reaction rate gradually reduced, a three-fold excess amount of the enzyme was introduced. The conversion experiment was performed under the optimized conditions (50 mM glycine–NaOH, pH 10.5, 40 °C, 3% Triton X-100). A successful enzymatic chiral resolution was achieved with around 50% yield in 90 min under the optimized conditions. The enantiomeric excess was over 99% for overall period of reaction. In conclusion, the data shown in this work indicate that the recombinant esterase PF1-K is highly effective in enantio-specific production of (*S*)-flurbiprofen and the reaction conditions that established in this work are effectively applicable in the production of (*S*)-flurbiprofen.

Acknowledgements

This work was supported by the Korean Ministry of Science and Technology and 21C Frontier R&D program (Microbial Genomics and Application).

References

[1] P.J. Hayball, Drugs 52 (1996) 47.
[2] A.R. Alcantara, J.M. Sanchez-Montero, J.V. Sinisterra, in: R.N. Patel (Ed.), Stereoselective Biocatalysis, Marcel Dekker, New York, 2000, p. 659 (Chapter 22).
[3] S.V. Bhandarkar, S.H. Neau, J. Biotechnol. 3 (2000) 195.
[4] V.T. John, G. Abraham, in: J.S. Dordick (Ed.), Biocatalysts for Industry, Plenum Press, New York, 1991, p. 193 (Chapter. 10).
[5] R. Verger, TIBTECH 15 (1997) 32.
[6] S. Benjamin, A. Pandey, Yeast 14 (1998) 1069.
[7] S.H. Wu, Z.W. Guo, C.J. Sih, J. Am. Chem. Soc. 112 (1990) 1990.
[8] D. Shen, J.H. Xu, P.F. Gong, H.Y. Wu, Y.Y. Liu, Can. J. Microbiol. 47 (2001) 1101.
[9] P.F. Gong, H.Y. Wu, J.H. Xu, D. Shen, Y.Y. Liu, Appl. Microbiol. Biotechnol. 58 (2002) 728.
[10] J.Y. Kim, G.S. Choi, Y.J. Kim, Y.W. Ryu, G.J. Kim, J. Mol. Catal. B: Enzymat. 18 (2002) 133.

[11] G.J. Kim, G.S. Choi, J.Y. Kim, J.B. Lee, D.H. Jo, Y.W. Ryu, J. Mol. Catal. B: Enzymat. 17 (2002) 29.
[12] G.J. Kim, E.G. Lee, B. Gokul, M.S. Hahm, D. Prerna, G.S. Choi, Y.W. Ryu, H.S. Ro, B.H. Chung, J. Mol. Catal. B: Enzymat. 22 (2003) 29.
[13] M.G. Kim, E.G. Lee, B.H. Chung, Proc. Biochem. 35 (2000) 977.
[14] Y.Y. Liu, J.H. Xu, Y. Hu, J. Mol. Catal. B: Enzymat. 10 (2000) 523.
[15] K. Veeraragavan, Anal. Biochem. 186 (1990) 301.
[16] I.J. Colton, S.N. Ahmed, R.J. Kazlauskas, J. Org. Chem. 60 (1995) 212.
[17] Y. Sugaya, T. Yoshiba, T. Kajima, Y. Ishihama, Yakugaku Zasshi 122 (2002) 237.