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Use of lipases in the resolution of racemic ibuprofen

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Summary. Resolution of (*R,S*)-ibuprofen enantiomers by esterification in different organic solvents was studied using *Candida cylindracea* lipase. This enzyme preparation had high enantiospecificity for *S*(+)-ibuprofen in the esterification reaction of a racemic ibuprofen with primary alcohols. The esterification yields of secondary alcohols were much lower than those of primary alcohols. Esterification with tertiary alcohols was not observed. The synthesis of esters was profoundly affected by the amount of water in the reaction mixture. *C. cylindracea* lipase was active only in very hydrophobic solvents. The esterification activity of the lipase was reduced significantly by addition of water. The *R*- and *S*-enantiomers of ibuprofen were determined without derivatization by HPLC using a chiral column.

Introduction

There is an increasing trend towards the use of optically pure enantiomers for both drugs and agrochemicals, because they are more target-specific and have fewer side-effects than racemic mixtures. Of the 1800 drugs currently available, about half are chiral mixtures (Emsley 1990). Racemic drugs usually have the desired therapeutic activity mainly in only one of the enantiomers. In the case of the anti-inflammatory drug ibuprofen, which is a member of the 2-arylpropionic acid class, only the *S*(+)-form is active. However, it is well documented that a certain proportion of *R*-ibuprofen may undergo metabolic inversion to the active *S*(+)-form (Lee et al. 1984). The inversion apparently proceeds via stereoselective formation of the coenzyme A ester of the *R*(−)-arylpropionic acid, with subsequent racemization and release of the *R*(−)- and *S*(+)-enantiomers. Coenzyme A esters of ibuprofen can replace the natural fatty acids in triacylglycerols to form "hybrid" triglycerides. The hybrid triglycerides may have toxic effects, as they could disrupt normal lipid metabolism and membrane function. The accumulation of ibuprofen via incorporation into hybrid triglycerides can be avoided by use of the active *S*-enantiomer rather than the racemic drug (Williams et al. 1986).

In recent years, the use of enzymes for preparation of optically enriched compounds has become an alternative to chemical synthesis. Lipases have been routinely used for the resolution of racemic alcohols and carboxylic acids through asymmetric hydrolysis of the corresponding esters (Lavayre et al. 1982; Ladner and Whitesides 1984; Fritsche et al. 1989; Barton et al. 1990). In organic media this approach has been extended to stereospecific esterification and transesterification. In particular, the lipase of *Candida cylindracea* has become a versatile catalyst for the resolution of racemic esters and alcohols (Langrand et al. 1985, 1986; Holmberg et al. 1989; Cambou and Klibanov 1984; Kirchner et al. 1985).

The enzymatic enantioselective resolution of 2-substituted propionic acids has been the subject of intense investigation. Much of this effort has centred on the production of *R*-2-chloropropionic acid, due to its high value as an intermediate in the synthesis of herbicides. *C. cylindracea* lipase is also used in this enantioselective hydrolysis (Cambou and Klivanov 1984a, b; Dahod and Siuta-Mangano 1987). Stereoselective ester synthesis and transesterification reactions involving 2-chloropropionic acid and lipase have also been reported (Kirchner et al. 1985).

Of the arylpropionic acids, *S*(+)-naproxen has been produced with *C. cylindracea* lipase via hydrolysis of different esters of a racemic naproxen in aqueous solutions (Sih et al. 1988; Gu et al. 1986). The substrate was introduced as a solid suspension in the reaction mixture. Attempts to disperse the solid substrate using detergents or non-polar organic solvents resulted in a loss of enantiospecificity. The enantiospecificity of *C. cylindracea* lipase was not significantly affected by changes in the ester substituent. High enantiospecificity was also observed for ibuprofen and suprofen, whereas this lipase exhibited low enantiospecificity towards esters of ketoprofen and fluorbiprofen.

A carboxylesterase with high specificity towards *S*-naproxen methyl ester in aqueous solution has been identified from *Bacillus subtilis* Thai 1–8. The methyl

ester of ibuprofen was also hydrolysed with high enatioselectivity by this enzyme (Bertola et al. 1987; Mutsaers 1990). The aim of the present study was to develop an enzymatic resolution process for the production of ibuprofen enantiomers via esterification reaction using lipases in organic solvents.

Materials and methods

Enzymes. Lipases (E.C. 3.1.1.3) from different organisms were commercial preparations: *Candida cylindracea, Geotrichum candidum, Rhizopus arrhizus, Aspergillus niger* and *Penicillium cyclopium* (Biocatalyst, UK); *A. niger, C. cylindracea* and *Pseudomonas fluorescens* (Amano Pharmaceutical, Japan); *R. arrhizus, C. cylindracea* and porcine pancreas lipase (Sigma, USA); *C. cylindracea* (Meito-Sangyo, Japan); *C. cylindracea* (BDH, UK) and *Rhizomucor miehei* (Lipozyme, Novo Industri, Denmark).

Chemicals. Racemic (*R,S*)-ibuprofen was purchased from Sigma. Pure *R*(−)- and *S*(+)-ibuprofen enantiomers were gifts from The Boots Company (Nottingham, UK). Methyl, ethyl, propyl, butyl and amyl esters of ibuprofen were prepared by chemical synthesis at the VTT Chemical Laboratory.

Analytical methods. Lipase was assayed by the olive oil emulsion method. The substrate was prepared by emulsifying 30 ml olive oil with 70 ml emulsification reagent by homogenizing for 3 min. The emulsification reagent (1 l) contained NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 ml), gum arabic (10 g) and distilled water. The reaction mixture, consisting of 5 ml of the emulsion, 4 ml of 0.2 M sodium phosphate buffer (pH 7) and 1 ml of the enzyme solution, was incubated for 10 min at 37° C. The reaction was stopped by addition of 10 ml of an acetone/ethanol mixture (1 : 1). The liberated fatty acids were titrated with 0.05 M sodium hydroxide solution using an automatic titrator. One unit of lipase activity (nkat) was defined as the amount of enzyme that liberated 1 nmol fatty acid/s under the assay conditions.

Protein determination. The protein content in the enzyme preparations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Esterification. Unless stated otherwise, the reaction mixture was composed of *n*-hexane (40 ml), racemic ibuprofen (12–48 mM) and an amyl alcohol (24–96 mM) without addition of water. The reaction was started by adding 100–500 mg crude lipase to the solution. The reactions were carried out at 30° C by shaking (150 rpm) in 100-ml flasks for a specified time. The enzyme was removed by centrifugation and the liquid phase was analysed by HPLC.

HPLC analysis. The solvent in the samples (10–100 µl) was removed by evaporation at room temperature and the residue was dissolved in 1 ml of a water/acetonitrile mixture (1 : 1). The esterification was monitored by HPLC using a chiral column (Chiral-AGP, Chrom Tech) capable of separating the *R*- and *S*-enantiomers without previous derivatization. The mobile phase was 0.01 M phosphate buffer, pH 7.0 (99%), with added methanol (1%). The column temperature was 40° C and the maximum pressure 400 bar. The compounds were detected spectrophotometrically at 230 nm.

Results and discussion

Selection of enzyme

Several commercial lipases were tested for their ability to catalyse the stereoselective esterification of racemic ibuprofen with methyl, ethyl and amyl alcohols using *n*-hexane as a solvent. Ibuprofen exhibits optical isomerism because of a chiral carbon atom in the propionic acid moiety (Fig. 1). Of the different lipase preparations tested, only *C. cylindracea* lipase and immobilized *R. miehei* lipase were able to catalyse the esterification reaction. These lipases preferentially catalysed the esterification of the *S*-enantiomer of ibuprofen. Other enzymes were almost inactive in the ester formation. Different commercial *C. cylindracea* preparations varied in their esterification activities. Preparations with higher olive-oil-hydrolysing activity were also more active in the esterification reaction (Table 1). The most active *C. cylindracea* preparation (Biocatalysts), which exhibited higher enantiospecificity than *R. miehei* lipase under the test conditions, was selected for further studies.

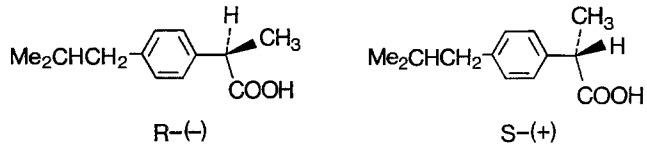


Fig. 1. Stereoisomers of ibuprofen, 2-(4 iso-butylphenyl)propionic acid

Table 1. Esterification of ibuprofen (12 mM) with amyl alcohol (24 mM) by different *Candida cylindracea* lipase preparations (10 g/l) at 30° C using a reaction time of 24 h

Source of lipase	Hydrolytic activity (nkat/mg)	Protein content (% of dry weight)	Ester yield (µmol/g lipase)
Biocatalysts	670	10.0	480
Meito-Sangyo	750	12.9	432
Amano	145	9.0	168
BDH	77	7.3	48
Sigma	85	7.0	24

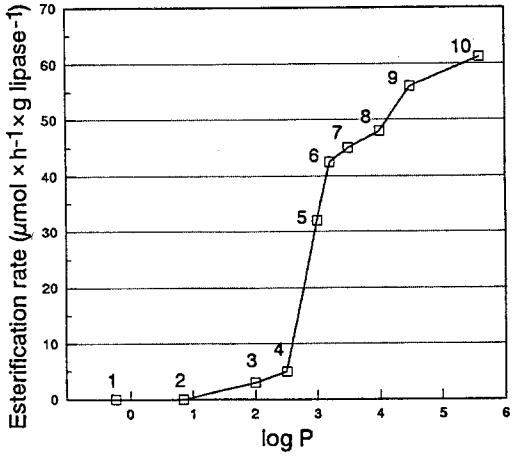
Effect of alcohol moiety

The ester formation catalysed by lipase has been shown to be influenced by the alcohol moiety. *A. niger, G. candidum, Penicillium cyclopium* and *Rhizopus delemar* (Okumura et al. 1979), *Pseudomonas fragi* (Nishio et al. 1988) and *Rhizomucor miehei* (Gatfield 1984) are able to synthesize esters of various primary alcohols, but they are not able to esterify the tertiary alcohols at all. Among these lipases, only those of *G. candidum, P. fragi* and *R. miehei* can use various secondary alcohols as substrates, although the activities are lower than those expressed towards primary alcohols.

In these experiments, a twofold molar excess of alcohol was used because the non-chiral compound should be present in a molar excess over the chiral one in order to ensure complete conversion of the reactive enantiomer of the latter (Cambou and Klibanov 1984a). The alcohol moiety had a great influence on the ester formation

Table 2. Esterification of ibuprofen (48 mM) with different alcohols (96 mM) by *C. cylindracea* lipase (10 g/l) in hexane at 30° C

Alcohol	Rate of esterification (μmol h^{-1} g^{-1} lipase)
Primary	
Methanol	18
Ethanol	20
1-Propanol	53
1-Butanol	51
Amyl alcohol	48
Secondary	
2-Propanol	8
2-Butanol	11
Tertiary	
3-Butanol	0



Solvent	log P
1. Acetone	-0.25
2. Diethyl ether	0.85
3. Chloroform	2.00
4. Toluene	2.50
5. Pentane	3.00
6. Cyclohexane	3.20
7. n-Hexane	3.50
8. Heptane	4.00
9. Iso-octane	4.50
10. Decane	5.60

Fig. 2. Activity of *Candida cylindracea* lipase in the esterification of ibuprofen (48 mM) with amyl alcohol (96 mM) in organic solvents plotted against log P at 30° C. The values of log P are from Laane et al. (1987): P = partition coefficient

by *C. cylindracea* lipase. Primary alcohols, especially the water-immiscible propanol, butanol and amyl alcohols, were good substrates for the esterification of ibuprofen. The esterification yields of the secondary alcohols were much lower than those of the primary alcohols. Esterification with tertiary alcohols was not observed (Table 2). The ester formed was the *S*-isomer in all cases. Only traces of *R*-isomer were detected by HPLC analysis.

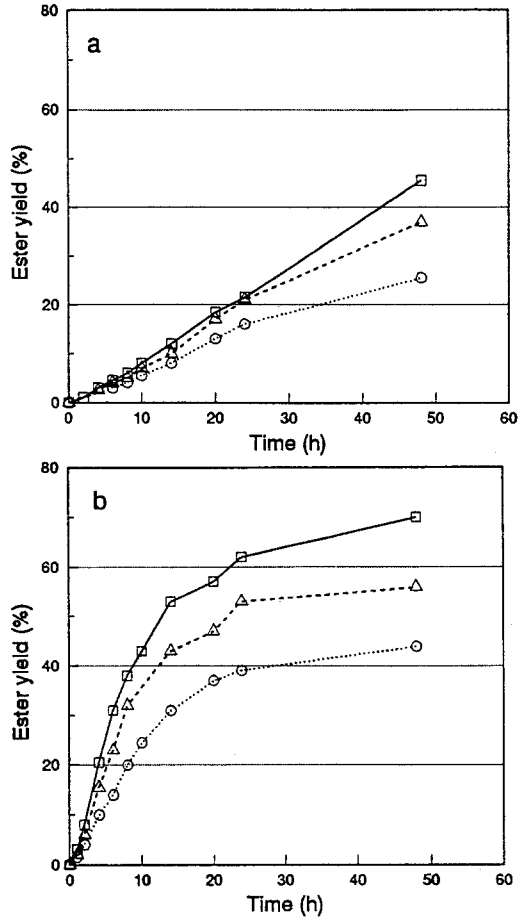


Fig. 3a, b. Effect of substrate and enzyme concentration on the esterification of ibuprofen (12, 24 and 48 mM) with amyl alcohol (24, 48 and 96 mM) in hexane by *C. cylindracea* lipase at 30° C: □, 12 mM ibuprofen + 24 mM amyl alcohol; △, 24 mM ibuprofen + 48 mM amyl alcohol; ○, 48 mM ibuprofen + 96 mM amyl alcohol. **a** Lipase concentration, 2.5 g/l. **b** Lipase concentration, 12.5 g/l

Effect of solvent on the lipase activity

From the literature data of biocatalysis in organic media by microbial lipases it seems clear that biocatalytic activity is high in non-polar solvents, whereas the activity is low in more hydrophilic and water-miscible solvents (Laane et al. 1987). Log P (the partition coefficient of a given compound in the octanol/water two-phase system) was found to correlate well with the activity of *C. cylindracea* lipase in the esterification of ibuprofen with amyl alcohol. The enzyme was active only in very hydrophobic solvents (Fig. 2). Although the esterification rates in heptane, iso-octane and decane were somewhat higher than in hexane, hexane was chosen as a solvent in further studies, because this solvent is already widely used in industrial processes.

Effect of enzyme and substrate concentrations

The production of amyl ester of ibuprofen was almost linear over a period of 2 days with a substrate concen-

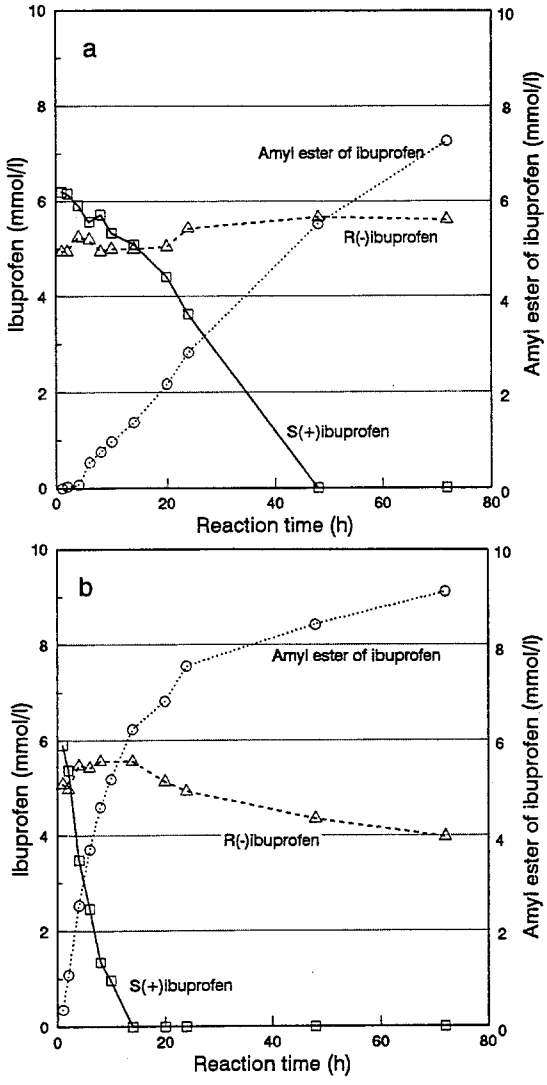


Fig. 4a, b. Stereoselective esterification of racemic ibuprofen (12 mM) with amyl alcohol (24 mM) in hexane at 30° C using *C. cylindracea* lipase. □, *S*(+)-ibuprofen; △, *R*(−)-ibuprofen; ○, amyl ester of ibuprofen. **a** Lipase concentration, 2.5 g/l. **b** Lipase concentration, 12.5 g/l

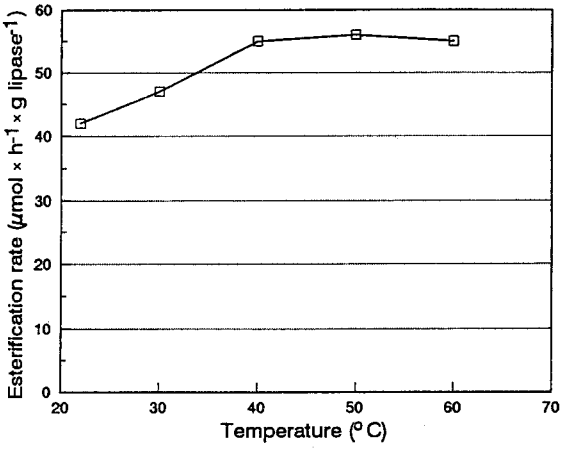


Fig. 5. Effect of temperature on the activity of *C. cylindracea* lipase (2.5 g/l) in the esterification of ibuprofen (12 mM) with amyl alcohol (24 mM)

tration of 12 mM ibuprofen/24 mM amyl alcohol and a lipase concentration of 2.5 g/l (Fig. 3a). With higher substrate concentrations the reaction was inhibited and the yield of amyl ester was decreased (Figs. 3a and b). In the esterification reaction the *S*(+)-enantiomer of ibuprofen disappeared entirely from the reaction mixture before esterification of the other enantiomer started. The *S*(+)-isomer was consumed within 48 h using a lipase concentration of 2.5 g/l and within 17 h with a lipase concentration of 12.5 g/l (Fig. 4a and b). The ester formed was the *S*-isomer, without any detectable *R*-isomer when less than 50% of the ibuprofen was esterified.

Effect of temperature

The effect of temperature on the esterification of ibuprofen with amyl alcohol was tested at temperatures ranging from 22° C to 60° C. In aqueous solutions the lipase lost its activity at temperatures above 45° C (results are not shown), whereas the esterification of ibuprofen in hexane proceeded at 60° C as well as at lower temperatures (Fig. 5).

Effect of water content on the ester synthesis

The effect of water content in the reaction mixture on ester synthesis by *C. cylindracea* was studied using ibuprofen (12 mM) and amyl alcohol (24 mM) as substrates. It was found that most of the water involved in the reaction mixture came from the enzyme powder. The water content of *C. cylindracea* lipase was 10%. Therefore, a reaction mixture containing a lower amount of water was prepared by adding enzyme powder that had been dried under vacuum for specified times (0–80 min). By changing the drying time of the enzyme powder, reaction mixtures with different moisture contents were obtained. On the other hand, to prepare a reaction mixture with a higher amount of water, different amounts of water (1, 2.5 and 5 mmol) were added to the hexane. The final water contents of these reaction mixtures were 0.1, 0.2 and 0.4%.

The synthesis of amyl ester of ibuprofen was markedly affected by the amount of water in the reaction mixture. The initial esterification rate was first increased but later decreased with increasing water content, reaching its maximum rate at a water content of 0.05%. The yield of the ester reflected the effect of the moisture content on reaction rate (Fig. 6). At higher moisture contents, the rate of ester synthesis was lowered due to the reversibility of the reaction. The reverse reaction (hydrolysis of the ester) becomes significant as the moisture content increases.

The water content of the enzyme also influenced the enantioselectivity of the lipase of *C. cylindracea*. The enantioselectivity decreased with increasing water content (Table 3). With decrease in water content the conformation of the enzyme becomes more rigid. The substrate specificity is limited, but the stereo/enantioselec-

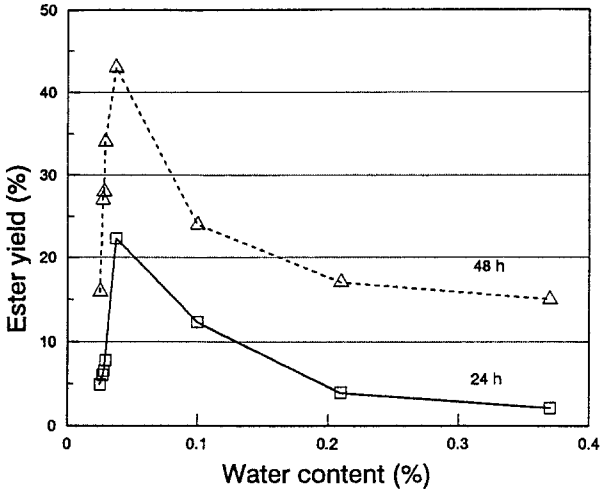


Fig. 6. Effect of water content in the reaction mixture on the esterification of ibuprofen (12 mM) with amyl alcohol (24 mM) by *C. cylindracea* lipase (2.5 g/l) using reaction times of 24 (□) and 48 h (△)

Table 3. Esterification of (*R,S*)-ibuprofen (12 mM) with amyl alcohol (24 mM) by *C. cylindracea* lipase (2.5 g/l) in hexane at 30° C using a reaction time of 24 h: effect of the addition of water to the reaction system on ester formation and enantiomeric excess (ee) of the ester

Addition of water (mmol)	Conversion (%)	Ester (% ee [S])
0.0	42	99
1.0	24	98
2.5	17	90
5.0	13	88

tivity is considerably enhanced (Langrand et al. 1986; Zaks and Klibanov 1984). Water affords a high conformation flexibility on the enzyme molecules (Klibanov 1986).

Conclusions

A convenient method was developed for the resolution of a racemic ibuprofen via an esterification reaction using *C. cylindracea* lipase in anhydrous organic solvent. In the esterification reaction of ibuprofen with amyl alcohol the *S*(+)-enantiomer of ibuprofen disappeared completely from the reaction mixture before esterification of the other enantiomer started. The *S*(+)-isomer was consumed in 48 h at 30° C with an ibuprofen concentration of 12 mM (2.5 g/l), an amyl alcohol concentration of 24 mM and a lipase concentration of 2.5 g/l. The ester formed was the *S*-isomer (5.5 mmol/l) without any detectable *R*-isomer when less than 50% of the ibuprofen was esterified. In practice, this means that when the esterification reaction is monitored continuously, it can be stopped before the *R*(−)-isomer has started to react. The residual *R*(−)-ibuprofen could be recovered easily from the *S*(+)-ester produced on the basis of the different solubility properties of esters and acids. Among several solvents evaluated, only very hydrophobic solvents (e.g. hexane) were found to be suitable. Primary alcohols were the best alcohol moieties for the esterification of ibuprofen by *C. cylindracea* lipase. Compared with aqueous systems, the lipases can easily be separated from the organic solvents and reused. However, drying of the enzyme is necessary because the water that is produced in the reaction is adsorbed by the catalyst. The method developed in this work appears promising for the preparation of optically pure ibuprofen enantiomers.

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