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Enantioselective Synthesis of Ibuprofen Esters in AOT/Isooctane Microemulsions by *Candida cylindracea* Lipase

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The enantioselective esterification of racemic ibuprofen, catalyzed by a *Candida cylindracea* lipase, was studied in a water-in-oil microemulsion (AOT/isooctane). By using *n*-propanol as the alcohol, an optimal w_0 ($[H_2O]/[AOT]$ ratio) of 12 was found for the synthesis of *n*-propyl-ibuprofenate at room temperature. The lipase showed high preference for the *S*(+)-enantiomer of ibuprofen, which was esterified to the corresponding *S*(+)-ibuprofen ester. The *R*(−)-ibuprofen remained unesterified in the microemulsion. The calculated enantioselectivity value (*E*) for *S*-ibuprofen ester was greater than 150 (conversion 0.32). The enzyme activities of *n*-alcohols with different chain lengths (3–12) were compared, and it appeared that short- (propanol and butanol) and long-chained (decanol and dodecanol) alcohols were better substrates than the intermediate ones (pentanol, hexanol, and octanol). However, unlike secondary and tertiary alcohols, all of the tested primary alcohols were substrates for the lipase. The reversible reaction (i.e., the hydrolysis of racemic ibuprofen ester in the microemulsion) was also carried out enantioselectively by the enzyme. Only the *S* form of the ester was hydrolyzed to the corresponding *S*-ibuprofen. The reaction yield was, however, only about 4% after 10 days of reaction. The corresponding yield for the esterification of ibuprofen was about 35% (10 days). The high enantioselectivity displayed by the lipase in the microemulsion system was seen neither in a similar esterification reaction in a pure organic solvent system (isooctane) nor in the hydrolysis reaction in an aqueous system (buffer). The *E* value for *S*-ibuprofen ester in the isooctane system was 3.0 (conversion 0.41), and only 1.3 for *S*-ibuprofen in the hydrolysis reaction (conversion 0.32). The differences in enantioselectivity for the lipase in various systems are likely due to interfacial phenomena. In the microemulsion system, the water in which the enzyme is dissolved is separated from the solvent by a layer of surfactant molecules, thus creating an interface with a relatively large area. Such interfaces are not present in the pure organic solvent systems (no surfactant) nor in aqueous systems.
Key words: enantioselectivity • esterification • ibuprofen • lipase • microemulsion • organic solvent

INTRODUCTION

Lipases (EC 3.1.1.3) are hydrolytic enzymes which normally (in aqueous systems) catalyze the hydrolysis of ester bonds. These enzymes have also been shown to catalyze the opposite reaction, e.g., the synthesis of ester bonds, particularly in organic solvents,[6,9,10,15,17,20] and in microemulsions.[3,8,12,23] The interest in lipases as catalysts in organic synthesis reactions has derived from the fact that enzymes are highly selective, in contrast to chemical catalysts. Many fine chemicals in the pharmaceutical and food industries are optical isomers of a chiral molecule but are usually produced only as racemic mixtures of the enantiomers. However, recently it has been shown that many lipases in organic solvent systems act enantioselectively and prefer to catalyze the synthesis or hydrolysis of one of the enantiomers with higher preference.[4,5,16,19,21] To our knowledge, the enantioselectivity of lipases has not been studied in water-in-oil (w/o) microemulsions, although it has been studied to some extent in microemulsion-based organogels.[22] A w/o microemulsion system is a good model system to study lipase-catalyzed reactions, since these enzymes normally act at interfaces (water/lipid) which are abundantly present in a microemulsion (L_2-phase) system.

Ibuprofen, *R*,*S*-2-(4-isobutylphenyl) propionic acid, is known as a nonsteroidal anti-inflammatory agent used in the treatment of arthritis and other similar diseases.[7] It is used as a racemic mixture of the two enantiomers *S*(+) and *R*(−). The chiral center of ibuprofen is in the propionic acid moiety (Fig. 1). The *S*-enantiomer is the biologically active form. It has been shown to be 160 times more potent than the *R*-enantiomer in inhibiting prostaglandin synthesis in vitro.[1] Consequently, there is today an increasing interest in getting pure enantiomeric forms of therapeutically active drugs.

In this study we have used racemic ibuprofen as the substrate in a lipase-catalyzed esterification reaction in sodium bis(2-ethylhexyl) sulfosuccinate (AOT)/isooctane microemulsions. We have also compared the enantioselectivity in this system with the enantioselectivity in a pure organic solvent system in order to elucidate possible effects of the interfaces. Also the reverse reaction, i.e., the hydrolysis of ibuprofen ester, has been studied in both microemulsions and aqueous systems. The lipase used was isolated from *Candida cylindracea*.

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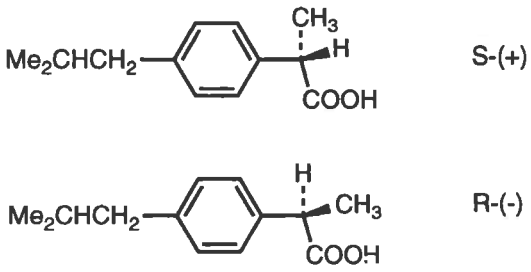

Figure 1. Chemical structure of ibuprofen.

MATERIALS AND METHODS

Chemicals

Aerosol OT (AOT), racemic ibuprofen, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (Hepes), and 2-[*N*-morpholino] ethanesulfonic acid (Mes) were from Sigma Chemicals. The pure enantiomers of ibuprofen were supplied by Ethyl Corporation (Baton Rouge, LA). 2,2,4-Trimethylpentane (isooctane; UV spectroscopy grade) was obtained from Fluka (Switzerland). The water used was deionized and double distilled prior to use. *Candida* lipase B (85,000 units/g, *Candida cylindracea*) was purchased from Biocatalysts (Wales).

Racemic *n*-propyl-ibuprofenate was chemically synthesized by using normal esterification procedures. No remaining ibuprofen could be detected in the product as tested by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Synthesis in Microemulsions

Normally racemic ibuprofen and *n*-propanol were dissolved in 5 mL of 3% (w/w) AOT/isooctane solution to final concentrations of 24 and 48 m*M*, respectively. Immediately before starting the reaction, the lipase was dissolved in 100 m*M* Hepes buffer, pH 7.0. The lipase solution was subjected to mild sonication in a Branson bath sonicator for 1–3 min at ambient temperature. The reaction was started by injecting a certain amount of the lipase solution into the AOT/isooctane substrate solution to give a final lipase concentration of 0.3 mg/mL. The volume of the buffer injection (containing the required amount of the enzyme) was adjusted as needed to give the selected w_0 ($w_0 = \{[H_2O]/[AOT]\}$). The microemulsion was vigorously shaken until clear (less than 30 s). To follow the reaction, 20 μL samples in duplicate were withdrawn, evaporated, and kept at −20°C. The samples were diluted with 1 mL H_2O/methanol (1 : 1) and analyzed by HPLC.

For experiments in which different alcohols were used, the buffer containing the lipase was injected before the second substrate (the alcohol) was added. The addition of the alcohol started the reaction.

Hydrolysis in Microemulsions

The hydrolysis reactions were performed under identical conditions as described for the synthesis reactions, except that ibuprofen and the alcohol were replaced by 24 m*M* racemic *n*-propyl-ibuprofenate.

Synthesis in Isooctane

Ibuprofen and propanol were dissolved in isooctane to final concentrations of 24 and 48 m*M*, respectively. The lipase was added as a dry powder at different amounts into the reaction vials, after which 3 mL of the substrate containing isooctane solution was added to start the reaction. The reaction was performed at 22°C with gentle shaking. At different time intervals, the reaction vials were centrifuged (1500 rpm, 3 min) and 20-μL samples in duplicate were withdrawn, after which the reaction vials were vortexed and returned to the shaking device. The samples were evaporated and kept at −20°C until analyzed by HPLC, before which they were diluted with 1 mL H_2O/methanol (1 : 1).

Hydrolysis in Buffer Solution

Racemic *n*-propyl-ibuprofenate was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 *M*. Lipase, 25 mg, was dissolved in 1 mL 100 m*M* Hepes buffer, pH 7.0, by sonication for 2–3 min. The reaction was started by adding 1 mL of the lipase-containing buffer solution to 10 μL of the DMSO-dissolved ester, giving a final ester concentration of 10 m*M* (DMSO 1%). The reaction temperature was 22°C, and the reaction vials were subjected to gentle shaking. To 50 μL samples withdrawn from the reaction medium, 500 μL of methanol was added after which the samples were kept at −20°C. Immediately before analysis by HPLC, the samples were further diluted with 450 μL of water.

Analysis by High-Performance Liquid Chromatography

The enantiomers of ibuprofen were separated on a chiral α-1-acid glycoprotein (AGP) column (100 × 4.0 mm; ChromTech AB, Sweden). The mobile phase consisted of 10 m*M* potassium phosphate buffer, pH 6.5, 1 m*M* *N*,*N*-dimethyloctylamine (DMOA), and 3% methanol. The flow rate was set to 0.8 mL/min, and the peaks were detected at 220 nm. Known amounts of the enantiomers of ibuprofen were used for calibration.

For identification of the enantiomers of propylibuprofen ester, the amount of methanol in the effluent was increased to 33%. The chemically synthesized *n*-propyl-ibuprofenate was used as a racemic standard. The column was always washed with the mobile phase containing 40% methanol after about 10–15 analyzed samples to remove the accumulated AOT from the column.

Calculation of Amount of Formed Ester

The decrease in the concentration of unesterified ibuprofen was quantitatively determined. The difference between the amount of ibuprofen at time zero and at different reaction

times was used to quantitate the amount of produced ester. The formation of the *S*- and *R*-enantiomers of the ester was verified in separate HPLC runs (33% methanol in the mobile phase) of the same samples.

RESULTS AND DISCUSSION

The L_2 Phase

The extension of the L_2 phase (thermodynamically stable reversed micelles) for the system AOT/isooctane/water has been well characterized in our previous work.[13] As we included ibuprofen and different alcohols as substrates into the system, we found that the overall system was within the L_2 phase for the $[H_2O]/[AOT]$ ratios used in this study (i.e., <15). We also ruled out the possibility that the system, before and during the reaction, could have generated liquid crystals by examining the reaction mixtures through crossed nicoles.[18]

The Microemulsion System

The overall lipase concentration in all microemulsion reactions was set to 0.3 mg/mL, because we had found in our previous work[14] that higher concentrations did not enhance the reaction rate for the esterification of cholesterol with oleic acid using the same lipase. As we examined the influence of the buffer strength on the esterification activity (Hepes buffer, pH 7.0, $w_0 = 9$), in the range of 10–500 m*M*, we noticed that the lipase showed good catalytic activity over the whole range examined, although slightly higher activity was detected in the range 100–500 m*M* (data not shown). Since ibuprofen is relatively insoluble in water, it was not surprising to find that the buffering capacity of the aqueous phase was enough to cover for the possible influence of the acid on the lipase activity.

To find optimal experimental conditions, we also examined the influence of pH on the lipase activity for the same system using Mes (pH 5.5–7.0) and Hepes buffers (pH 7.0–8.0) at 100 m*M*. The pH optimum for the lipase was known to be quite broad for hydrolytic reactions in aqueous systems (information from the manufacturer), and this was also true for the microemulsion system. The enzyme activity was not much altered by changing the pH in the range of 5.5–8.0 (data not shown), which is in good agreement with results shown by others.[12] However, the Hepes buffer seemed to express slightly higher activity at pH 7.0 than the Mes buffer and hence was chosen for use in further experiments.

Esterification of Ibuprofen with *n*-Propanol in Microemulsions

The enzyme activity as a function of the water-to-surfactant ratio, w_0, in the microemulsion was examined. The final substrate concentrations were 24 m*M* racemic ibuprofen and 48 m*M* *n*-propanol, since the nonchiral compound should be present in a molar excess over the chiral one.[5] The optimum w_0 was found to be around 12 (Fig. 2), which was slightly higher than the optimum of 9, which was found for the same lipase in the esterification of cholesterol with oleic acid in a similar microemulsion system.[14] The shift of the optimum w_0 toward higher water content was surprising but might be explained by the different solubilities of the substrates in the water and the oil fractions, respectively. Cholesterol and oleic acid are almost insoluble in water, as is ibuprofen, while the propanol used in this study is readily water soluble. Propanol might to some extent be located in the water core of the reverse micelle where the lipase is assumed to be located, and since hydrophilic solvents are likely to withdraw water molecules from the enzyme,[11,24] it might explain why more water is needed to activate the lipase in the present system.

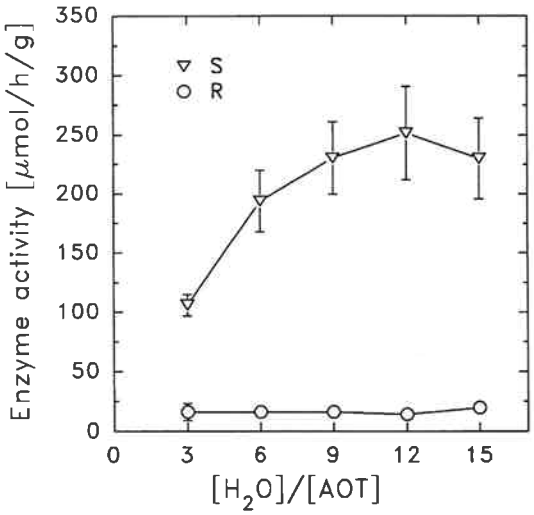

Figure 2. Esterification of ibuprofen by lipase in microemulsions as a function of w_0. The microemulsion contained 3% (w/w) AOT in isooctane, 24 m*M* racemic ibuprofen, and 48 m*M* *n*-propanol. A 100 m*M* Hepes buffer, pH 7.0, including the dissolved lipase was used to adjust the w_0. The final concentration of lipase is 0.3 mg/mL. The average enzyme activity was calculated by using the data from 4 and 7 days of reaction times, from a representative experiment, the error bars giving the range.

As is shown in Figure 2, the lipase from *C. cylindracea* catalyzed only the esterification of the *S*-enantiomer of ibuprofen. In separate HPLC analysis of the esters and in control experiments in which we used only the pure *R*-enantiomer of ibuprofen as the substrate, it was clearly seen that no *R*-ester was produced (data not shown). This total enantioselectivity for the *S*-form can also be seen from Figure 3, where the ester production is plotted as a function of time. The reaction was rather slow, and as can be seen, about 35% of total possible (*S*- and *R*-enantiomer) was formed within 10 days of reaction (Fig. 3).

Hydrolysis of *n*-Propyl-Ibuprofenate in Microemulsions

In our previous work we have shown that the lipase-catalyzed synthesis/hydrolysis reaction of cholesterol ester was reversible in the AOT microemulsion system.[14]

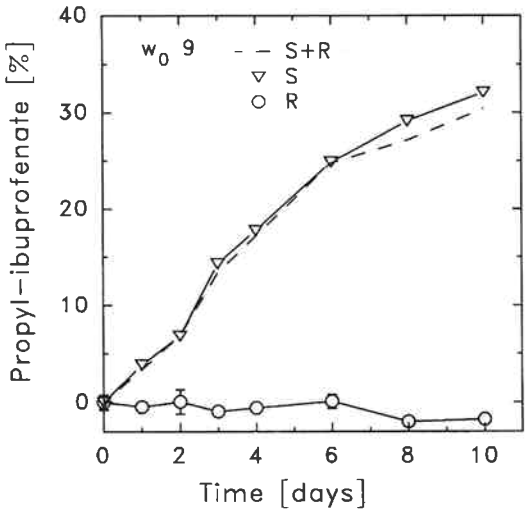

Figure 3. Time curve for the esterification of ibuprofen in microemulsions. The reaction conditions were as in Fig. 2, except that the w_0 was set to 9. The formation of ester is given as percentage of total possible, calculated as the average from samples run in duplicate from a representative experiment, the error bars giving the range.

In order to examine whether the ibuprofen reaction also was reversible, we used chemically synthesized *n*-propyl-ibuprofenate (24 m*M*) as the substrate for a hydrolysis reaction. We can see from Figure 4 that the reversible reaction occurred to some extent in a microemulsion system, where the w_0 was set to 9. The yield was, however, much lower than the yield for the synthesis reaction; after 10 days of reaction no more than about 4% of the ester had been hydrolyzed. The interesting thing, however, was that the hydrolysis reaction also was carried out enantioselectively by the *C. cylindracea* lipase. Only the *S* form of the ester was hydrolyzed to the corresponding *S*-ibuprofen. No *R*-ibuprofen was detected in the reaction samples.

Comparison of Enantioselectivity of Lipase in Microemulsions, Isooctane, and Buffer Solutions

In order to compare the enantioselectivity of the *C. cylindracea* lipase in the microemulsion medium with other systems, we chose to test the same reactions in a pure organic solvent and in an aqueous buffer solution. As isooctane and Hepes buffer was used to build up the microemulsion systems, we selected isooctane as the organic solvent for the esterification of ibuprofen and Hepes buffer (100 m*M*, pH 7.0) as the aqueous system for the hydrolysis of propyl-ibuprofenate.

The esterification reaction was performed with the lipase suspended as a powder in the solvent with gentle shaking. The reaction yield increased linearly as a function of the amount of lipase added to the solvent (data not shown). The ester yield in the pure isooctane system was negligible with the same amount of enzyme (0.3 mg/mL solvent) that was used in the microemulsion study. However, when we drastically increased the lipase concentration, we obtained a high yield of propyl-ibuprofenate. Figure 5 shows a yield-versus-time function for lipase concentration of 30 mg/mL. After 3 days of reaction, about 90% of total possible propyl-ibuprofenate had been produced. Esterification (100%) was achieved after 3 days of reaction, when the lipase concentration was increased to 50 mg/mL (data not shown).

As we compared the enantioselectivity of the lipase in this system with that in the microemulsion system, we could see that it was much less pronounced in isooctane

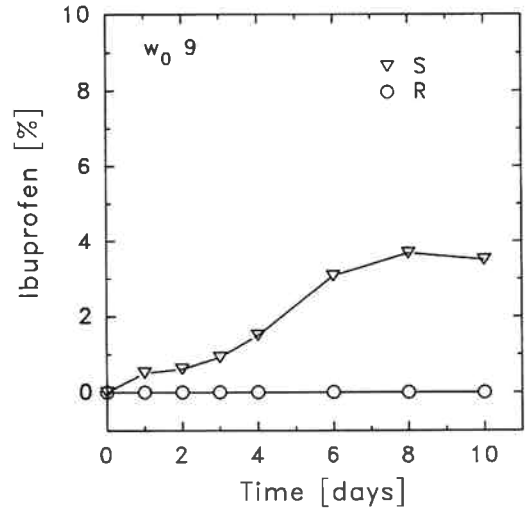

Figure 4. Time curve for the hydrolysis of *n*-propyl-ibuprofenate in microemulsions. *N*-propyl-ibuprofenate 24 m*M*, was used as the substrate in the AOT/isooctane microemulsion at $w_0 = 9$. Samples run in duplicate were used to calculate the formation of ibuprofen as percentage of total possible. The error bars which show the range from the average values are within the symbols.

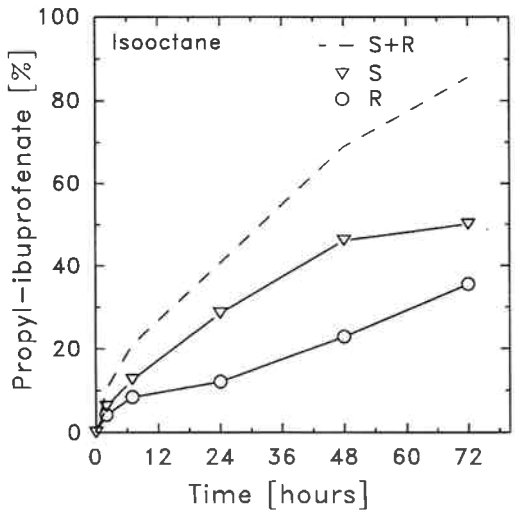

Figure 5. Time curve for the esterification of ibuprofen in isooctane. The concentrations of racemic ibuprofen and propanol were 24 and 48 m*M*, respectively. Lipase, 30 mg/mL, was suspended as a dry powder in the substrate containing isooctane. Samples in duplicates from a representative experiment were taken, as described in detail in Materials and Methods, and analyzed to calculate the average amount of formed ester as percentage of total.

than in the microemulsion. Both *S*- and *R*-ibuprofen were esterified in isooctane; however, the *S* form was still the faster reacting enantiomer. The results are in agreement with those found by Mustranta,[19] who has used the same lipase to esterify ibuprofen in *n*-hexane, although in those experiments the lipase started to use the *R*-enantiomer only after the *S* form had been depleted.

The hydrolysis in an aqueous system was performed by making a concentrated solution of the ester in DMSO, before adding the buffer, including the lipase to start the reaction. The reaction was accomplished under gentle shaking at room temperature. The reaction products *S*- and *R*-ibuprofen appeared in the reaction vials at about the same rate until a yield of 55% was reached, after which no net increase in the product was detected (Fig. 6). The conclusion drawn was that the enantioselectivity was completely lost in this aqueous system and that the ester was hydrolyzed to give a racemic mixture of ibuprofen.

In order to compare the enantioselectivities between the different media, we performed the calculations used by Allenmark and Ohlsson.[2] These calculations are described for irreversible reactions only, but since the reversibility in our microemulsion system was fairly minimal and no reversibility was detected in isooctane or buffer, we assume that the possible error in this case is small and negligible. As can be seen from the enantioselectivity values (E) in Table I, the microemulsion system was completely superior compared to the other systems.

Because ee_p values close to 1.00 have dramatic effects on the enantioselectivity values, we have chosen to follow the principle used by Allenmark and Ohlsson,[2] to set an upper limit for E at 150. The great difference in enantioselectivity for the lipase between a microemulsion consisting of reversed micelles in isooctane, and a pure isooctane system is not easy to explain. In the first case, however, the lipase was dissolved in water, whereas in the second case it was suspended as a dry powder in the organic solvent. This will most likely affect the conformation of the enzyme. But the water environment in the micelle alone cannot be the only reason for the difference, because the lipase was even more unselective in a totally aqueous environment (Fig. 6).

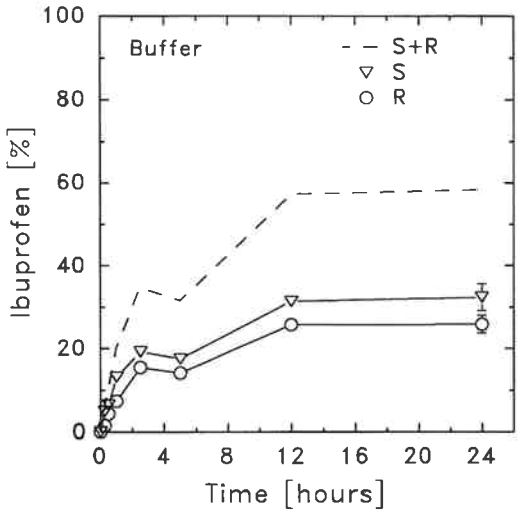

Figure 6. Time curve for the hydrolysis of *n*-propyl-ibuprofenate in buffer solution. The reaction medium consisted of 100 m*M* Hepes buffer, pH 7.0, 25 mg/mL lipase, and 10 m*M* *n*-propyl-ibuprofenate (added from a concentrated DMSO solution). From samples (duplicate) withdrawn from the reaction medium, the average formation of ibuprofen as percentage of total was calculated. The error bars give the range.

Another great difference between the microemulsion system and the pure organic solvent system is the interfacial region, which consists of a layer of surfactant molecules, in the microemulsions. The substrate and/or the lipase molecules probably penetrate the micelle surface to some extent, since contact between the enzyme located in the water pool and the substrates located mostly in the hydrophobic solvent is necessary for the reaction to take place. These types of interfacial regions are not present in the organic solvent system, where the enzyme is suspended as a dry powder in the solvent. This might be an explanation for the difference in enantioselectivity between microemulsions and organic solvent systems.

Table I. Enantioselectivity values for the lipase-catalyzed synthesis or hydrolysis of propyl-ibuprofenate in different media.

Medium	ee_p	c	E
Synthesis			
Microemulsion	1.00	0.32	>150
Isooctane	0.40	0.41	3.0
Hydrolysis			
Microemulsion	[a]	[a]	[a]
Buffer	0.11	0.32	1.3

Abbreviations: ee_p = enantiometric excess of the product = $(x_1 - x_2)/x$, where x_1 and x_2 are the concentrations of the product enantiomers and $x = x_1 + x_2$; c = conversion factor = x/a, where a is the initial concentration of the racemate; E = enantioselectivity = $\ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.

[a]Reaction rate too slow to be determined.

Influence of Alcohol on Esterification Reaction in Microemulsions

To test the influence of different alcohols on the *C. cylindracea* lipase, we determined the enzyme activities as a function of the chain lengths of the alcohol. The conditions used were slightly different compared to the experiments described above; the concentrations of both ibuprofen and alcohol were set to 12 m*M*, and Hepes buffer, in which the lipase was dissolved, was used at a concentration of 50 m*M*. The enzyme activities for the alcohols with carbon chain lengths between 3 and 12 were about the same (Fig. 7); the short chained (3 and 4 carbon atoms) and the long chained (10 and 12 carbon atoms) showing slightly higher activities than the intermediate ones. Although the water solubilities of the alcohols differ (propanol and butanol are water soluble, whereas the others are insoluble),

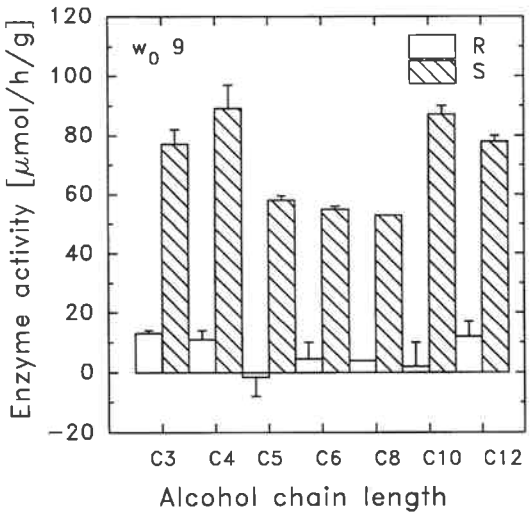

Figure 7. Lipase activity as a function of the chain length of the alcohols used in the esterification of ibuprofen in microemulsions. The microemulsion contained 3% (w/w) AOT in isooctane, 12 m*M* racemic ibuprofen, and 12 m*M* alcohol. A 50 m*M* Hepes buffer, pH 7.0, including the dissolved lipase was used to adjust the w_0 to 9. The final concentration of lipase was 0.3 mg/mL. The average enzyme activity was calculated by using the data from 3 and 6 days of reaction, the error bars giving the range. Abbreviations: C3, 1-propanol; C4, 1-butanol; C5, 1-pentanol; C6, 1-hexanol; C8, 1-octanol; C10, 1-decanol; and C12, 1-undecanol.

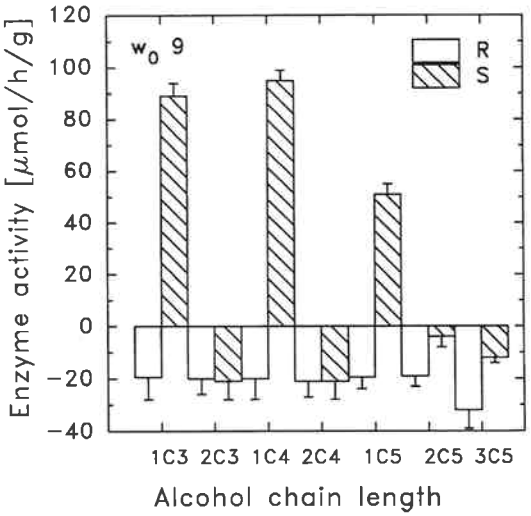

Figure 8. Lipase activity as a function of different alcohols in the esterification of ibuprofen in microemulsions. The microemulsion contained 3% (w/w) AOT in isooctane, 24 m*M* racemic ibuprofen, and 48 m*M* alcohol. A 100 m*M* Hepes buffer, pH 7.0, including the dissolved lipase was used to adjust the w_0 to 9. The final concentration of lipase was 0.3 mg/mL. The average enzyme activity was calculated using the data from 7 and 11 days of reaction, the error bars giving the range. Abbreviations: 1C3, 1-propanol; 2C3, 2-propanol; 1C4, 1-butanol; 2C4, 2-butanol; 1C5, 1-pentanol; 2C5, 2-pentanol; 3C5, 3-pentanol.

there was no correlation between enzyme activity and water solubility.

Other investigators[15] have also shown that *n*-propanol and *n*-butanol were esterified most rapidly in a lipase-catalyzed reaction in hexane when alcohols with chain lengths between 1 and 16 were compared. On the other hand, it has been demonstrated that the stereoselective esterification of halogen-containing carboxylic acids by lipase in benzene was more stereoselective with longer chain lengths, although the overall reaction rate decreased with increasing chain length.[20]

The greatest influence from the alcohol substrate was seen as we compared alcohols of the same chain length but with the hydroxy group at different positions. The primary alcohols were good substrates of the lipase, whereas the secondary and tertiary alcohols were not used by the lipase in these microemulsion systems (Fig. 8). In the study by Mustranta,[19] it was shown that secondary alcohols were substrates of the lipase, although not as good substrates as the primary alcohols. The different substrate properties of the secondary alcohols seen in the microemulsion system, on the one hand, and with the neat organic solvent system, on the other, can probably be related to effects of the ordered interface on the interaction between enzyme and substrate compounds. The hydroxy groups in the secondary alcohol molecules might not be available for the active site of the enzyme in the microemulsions, although they are available for the enzyme in the organic solvent system. Tertiary alcohols have also previously been shown not to be substrates for lipase-catalyzed reactions in organic solvents.[16,19]

SUMMARY AND CONCLUSIONS

The AOT/isooctane microemulsion was shown to be a good medium for enantioselective catalysis by *C. cylindracea* lipase. The enantioselectivity was total for the *S*-enantiomer of ibuprofen used in this work. The *R* form of ibuprofen did not become a substrate, even when no *S* form was available to the enzyme. The ibuprofen esters formed by this method can be separated from the microemulsion and chemically hydrolyzed to give pure *S*-ibuprofen.

The drawback for the microemulsion system was, however, the slow reaction rates. Another difficult task can be the separation of the substrates and products from the system. These problems might have been solved by using an organic solvent system, which indeed showed higher reaction rates in our experiments than did the microemulsion system. However, the lipase did not display the same total enantioselectivity in isooctane, although it showed preference for the *S*-enantiomer over the *R* form. The microemulsions seem to be better enantioselective media for the lipases than pure organic solvents, but the problems with reaction rates, product recovery, and enzyme reuse have not been solved. A possible solution could be the use of a combination of these two systems—microemulsion-based organo gels used in organic solvents—which has recently been described.[22]

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