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Lipase-catalyzed enantioselective esterifications using different microemulsion-based gels

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Abstract Chiral esters with high optical purity have been synthesized at 298.2 K from racemic 2-octanol and alkanoic acids using the commercial lipases from *Chromobacterium viscosum* (CV) or *Candida* sp. (SP 525) immobilized in microemulsion-based gelatin gels. The microemulsions consisted of water and alkanes stabilized by the anionic surfactant sodium 1,4-bis(2-ethylhexyl) sulfosuccinate (AOT) and the naturally occurring zwitterionic surfactant soybean lecithin, respectively. The enzymes were solubilized both in water-in-oil (W/O) microemulsions and in microemulsions with a bicontinuous structure. Different microstructures of the gels were chosen since the enzyme may undergo conformational changes in different environments resulting in different catalytic efficiencies toward competing substrates. Therefore, it is of great fundamental interest to know the phase behaviour and the microstructures of the used microemulsion systems. Phase diagrams were determined at 298.2 K for the systems water-hexane-AOT and ethanol/water (1:1)-hexadecane-soybean lecithin. The former system exhibited a large one-phase W/O microemulsion region, while in the latter a small one-phase region with bicontinuous structure was present. The kinetic enantiomeric ratios (*E*-values), as determined from enantiomeric excess (e.e.) values at a conversion below 0.5, were higher both in the W/O microemulsion as well as in the bicontinuous microemulsion using the SP 525 lipase, than using the CV lipase. On the other hand, the conversions were higher using gels based on W/O microemulsions (AOT stabilized) than using gels based on microemulsions with a bicontinuous structure (lecithin stabilized).

Key words Phase equilibria – microemulsion-based gels – soybean lecithin – lipase – esterification

Introduction

Thermodynamically stable and optically isotropic solutions of water, oil and surfactant are usually referred to as microemulsions [1]. On a microscopic scale these solutions are structured into aqueous and oil microdomains separated by a monolayer of surfactants [2, 3]. Thus, microemulsions are good solvents for both nonpolar organic molecules and polar inorganic ones. They are therefore involved in many applications, e.g. cleaning, tertiary oil recovery, liquid–liquid extraction, nanoparticles, pharmaceutics, cosmetics or textile processing [4–6]. In recent years microemulsions have widely been used as reaction

media for enzyme catalysis [7–36]. Studies have been performed in systems such as one-phase water droplets-in-oil [7–32] or microemulsions with a bicontinuous structure [13, 33, 34] and in three-phase systems [33–35] with a surfactant-rich bicontinuous middle phase. Especially, the water soluble lipases acting on reactants that are insoluble or poorly soluble in aqueous solutions have been studied intensively [36]. The enzyme is located in the aqueous microdomains, and the reaction is believed to take place at the oil/water interface [36, 37]. However, the different phases in microemulsion systems are very sensitive to additives and problems arise from destabilization effects caused already by moderate substrate and product concentrations leading to phase separation of the microemulsion. Consequently, most of the studies have been done to clarify the physical properties of the microemulsions themselves or the influence of an organized medium on the reaction kinetics.

The enzyme lipase (triacylglycerol acylhydrolase; E.C. 3.1.1.3) usually catalyzes the hydrolysis of ester bonds in aqueous media, but in water-poor media lipases are also able to catalyze the synthesis of various specific esters from acid and alcohol [9, 19, 34]. The water content can be readily controlled in microemulsions and they are well suited to enzymatically catalyze ester syntheses. In recent years Rees and coworkers [38–41] have immobilized lipases in microemulsion-based gelatin gels (MBGs) in order to increase the concentration of the substrates in the reaction medium and to re-use the enzyme. Using these systems, preparative-scale synthesis of a wide variety of esters was possible and repeated usage of the gel showed an enzyme activity of 0.75 times the initial value after 30 days. These improvements have both economical and process technical consequences. The same technique has also been applied by Jenta et al. [42] and by Nascimento et al. [43] to lipase-catalyzed esterifications.

Today there is an increasing demand for efficient biotechnical methods for synthesis of optically pure compounds. Rees et al. [39] observed a stereoselective esterification of 2-octanol for three different lipases yielding a product with an enantiomeric excess (e.e.) value ranging from 0.8 to 0.92. On the other hand, in the investigation by Uemasu and Hinze [44] the e.e. value was only 0.32, for the esterification of (+/−)-2-methylbutyric acid to ethyl-(+)-2-methylbutyrate by the MBG technique. Recently, Jesus et al. [45] showed that lipases from different sources exhibited high and similar enantioselective efficiencies, differing however in conversions of obtained esters. In a previous work [46] we investigated the catalytic behaviour of the lipase from *Chromobacterium viscosum* (CV) immobilized in MBG on esterification of long-, middle- and short-chain alkanoic acids with racemic 2-octanol according to the following reaction:

$$(R)/(S)\text{-2-octanol} + \text{alkanoic acid} \Leftrightarrow (S)\text{-}(+)\text{-2-octanol} + (R)\text{-}(-)\text{-2-octyl ester} + H_2O \quad (1)$$

The syntheses were carried out with the sectioned gels in various hydrophobic solvents using different initial reactant concentrations. The best conversion was received with hexane as the solvent and the e.e. values were the same or somewhat higher compared with syntheses performed in ordinary organic solvents. It could be concluded that the conversions depended on the distribution of the reactants and the products between the reaction solvent and the gel. We have, therefore, investigated the structural mechanism of the gel in more detail in this work for the enzymatically catalyzed esterifications of nonanoic and hexanoic acids with racemic 2-octanol in hexane. The commercial lipase used was SP 525, originated from a *Candida* sp.

The microemulsion-based gel is prepared by mixing a one-phase microemulsion containing the enzyme with an aqueous gelatin solution. The microemulsions used in this work were stabilized by sodium 1,4-bis(2-ethylhexyl) sulfosuccinate (known as AOT) and soybean lecithin, respectively. The phase behaviour of the system water–hexane–AOT at 293.2 K is known from literature [47]. A large water-in-oil microemulsion is formed at room temperature. However, we were interested in phase equilibria at 298.2 K and re-determined the diagram at some compositions. We also determined the partial phase diagram ethanol/water–hexadecane–soybean lecithin in order to solubilize the lipase in a known one-phase microemulsion before preparing the gel. Since lecithin is slightly too lipophilic to form microemulsions in oil–water systems ethanol was added to the system, where it acts as a cosolvent rather than as a cosurfactant [48–50]. Very few enzyme-catalyzed reactions have been performed in microemulsion systems stabilized by lecithin [33, 50–53] and to our knowledge only one was performed using lecithin-based MBGs [46]. A good reason for studying these kinds of systems might be the use of enzyme-containing lecithin-stabilized microemulsions as models for biomimetic environment for membrane bound proteins.

Experimental

Chemicals

Sodium 1,4-bis(2-ethylhexyl) sulfosuccinate (AOT) of 99% purity was obtained from Sigma Chemicals, USA and soybean lecithin (Epikuron 200) from Lucas Meyer Co.,

FRG. The distribution of fatty acids in the lecithin has been described elsewhere [48, 50]. Hexanoic and nonanoic acids, and (±)-2-octanol (> 98% purity) were from Fluka, Switzerland. Hexane was purchased from Merck, FRG, and hexadecane from Sigma Chemicals. Acetic anhydride of 97% purity was supplied by Merck and ethanol by Oy Alko Ab, Finland. Pyridine with a purity of 99% and the catalyst 4-dimethylaminopyridine were purchased from Sigma Chemicals. The water was distilled and deionized immediately before use. All other chemicals were used without further purification. Racemic 2-octylesters (95% purity) were synthesized using well-known procedures. Gelatin (Bloom 231) was obtained from YA, Finland. The *Candida* sp. lipase, SP 525, was kindly supplied by Novo Nordisk, Denmark and *Chromobacterium viscosum* was purchased from Biocatalysts, UK.

Phase behaviour

The phase diagram by La Mesa et al. [47] for the water–hexane system stabilized by AOT has been re-determined at 298.2 K, and a phase diagram for ethanol/water–hexadecane systems stabilized by soybean lecithin has been determined. The mass ratio between ethanol and water was 1:1. Samples with fixed compositions were homogenized by heating up to 330 K and by the use of a mechanical shaking device. They were then allowed to equilibrate for at least 1 month in a thermostated bath at 298.2 K and analyzed visually between polarizing glasses. The structures of the liquid crystalline phases were identified by a polarizing microscope. Anisotropic liquid crystalline phases are birefringent and the lamellar D phase is identified by the mosaic pattern, while the hexagonal phases E and F usually show a non-geometric texture. The uncertainty in the phase boundaries is about $w_i = \pm 0.005$.

Preparation of gel

The first microemulsion-based gelatin gels were prepared by Haering and Luisi [54] and Quellet and Eicke [55] using microemulsions stabilized by AOT. More recently Robinson and co-workers [39–42, 56] immobilized lipase from different sources in MBGs with retention of catalytic activity. However, the gels used in this work were prepared from isotropic solutions with different microstructures. Usually the gels were prepared at 328 K by adding a water-in-oil (W/O) microemulsion to an aqueous gelatin solution. The AOT-based gel was prepared from a W/O microemulsion consisting of water, hexane and surfactant, at mass fractions of 0.12, 0.71 and 0.17, respectively. The microemulsion, 5.13 g, was gelatinized with 1.4 g gelatin and 1.8 g water. The soybean lecithin-based gels were prepared from microemulsions with a bicontinuous structure at mass fractions of 0.55, 0.25 and 0.20 for water/ethanol (1:1), hexadecane and lecithin, respectively. The same amount of water and gelatin, as for the AOT-based gels, were used to gelatinize 4.53 g of the lecithin-based microemulsion. Two different ways were used to immobilize the enzyme: by injecting an aqueous solution of the enzyme into the oil and surfactant solution or by solubilizing the enzyme in the microemulsion. No significant differences in the progress of the reactions could be detected between the two methods. The enzyme activity was 250 U cm^{-3} when calculated in terms of volume of the gel. The conductivity of the solutions was measured at 298.2 K as a function of added gelatin with a Seibold conductometer and the viscosity of the gel as a function of shear rate (from 1.9×10^{-3} s^{-1} to 1.5×10^{-2} s^{-1}) on a Bohlin VOR rheometer.

Enzymatic reactions

The reactions were mainly performed as previously described [46]. The concentrations were set to 0.33 mol dm^{-3} for both alcohol and acid. The reaction temperature was 298.2 K and the occurrence of produced water (if any) in the reaction medium was determined with Karl Fisher Coulometer 652 (Metrohm). The 2-octanol in the samples withdrawn from the reaction medium was acetylated with acetic anhydride in the presence of pyridine and 4-dimethylaminopyridine prior to the analyzes. The acetylated samples were injected into a Varian 3400 gas chromatograph and analyzed on a Cyclodex-B chiral column (J & W Scientific), 30 m × 0.252 mm, with a film thickness of 0.25 μm. The absolute configuration of the ester was based on the chiral GLC method by using the retention time obtained for the esterification of R-(−)-2-octanol with the alkanoic acid in an enzymatic reaction. The enantiomeric excess (e.e.) values for both product (p) and substrate (s) were calculated from the peak areas by the formula:

$$\text{e.e.} = (A_1 - A_2)/(A_1 + A_2) \quad (2)$$

where A_1 is the area for the dominating enantiomer and A_2 is the area for the other enantiomer. The conversion of the ester formed, c, was determined using a calibration curve with ester standards or by the formula:

$$c = \text{e.e.}(s)/(\text{e.e.}(s) + \text{e.e.}(p)) \, . \quad (3)$$

For the quantitative treatment of biochemical kinetic resolution data Chen et al. [57] have derived the following

equations for irreversible reactions

$$E = \ln[1 - c(1 + \text{e.e.}(p))]/\ln[1 - c(1 - \text{e.e.}(p))] \quad (4)$$

and

$$E = \ln[(1 - c)(1 - \text{e.e.}(s))]/\ln[(1 - c)(1 + \text{e.e.}(s))] \quad (5)$$

where the enantiomeric ratio, E, is a kinetic constant, which describes the enantioselectivity of the enzyme.

Results and discussion

Phase behaviour

The one-phase region, the L_2 phase in Fig. 1, of the system water–hexane–AOT was much larger at 298.2 K than at 293.2 K as determined by La Mesa et al. [47]. The phase consisted of inverse swollen micelles and water droplets-in-oil microemulsion. As these closed structures grow with increased water contents the conductivity decreased all the way up to $w_{H_2O} = 0.62$ (figure not shown), as a result of a decreased droplet concentration and mobility. Jada et al. [58] have shown that only W/O microemulsions are present at 298.2 K (by conductivity measurements). The gels have been prepared from the L_2 phase in Fig. 1, for the composition **a**, described in the experimental section.

When the natural, biological component soybean lecithin was mixed with hexadecane and an ethanol/water solution, one-, two-, and three-phase regions were observed. The partial phase diagram of the water/ethanol (1:1)-hexadecane–lecithin system in Fig. 2 resembled that of a water–alkane-nonionic surfactant system at a certain temperature [59]. The bottom phase of the three phases in equilibrium at low lecithin contents was a dilute ethanol/water solution of lecithin, the top phase was a dilute solution of lecithin and ethanol/water in hexadecane and the middle phase contained most of the lecithin. Depending on the nature of the surfactant this phase may be an isotropic solution or a liquid crystalline phase. It has previously been shown that these three phases form a Winsor III system, i.e., the middle phase is also a solution [50]. Outside the three-phase area the microemulsions were in equilibrium with almost pure hexadecane (Winsor I) and an aqueous/ethanol solution (Winsor II), respectively. The one-phase area in the middle of the triangle diagram was a microemulsion with bicontinuous structure (Winsor IV), as concluded previously [46]. The enzyme has been solubilized in this phase (point **a** in Fig. 2) before preparing the gel. At higher lecithin contents a lyotropic crystalline phase with indefinitely long, mutually parallel inversed rods in hexagonal array i.e., phase F, was found. At still higher lecithin contents a lamellar phase with bilayers of planar topology was present. This phase D extended over a very large dilution range, from the binary ethanol/water–lecithin system up to 0.40 hexadecane mass fractions.

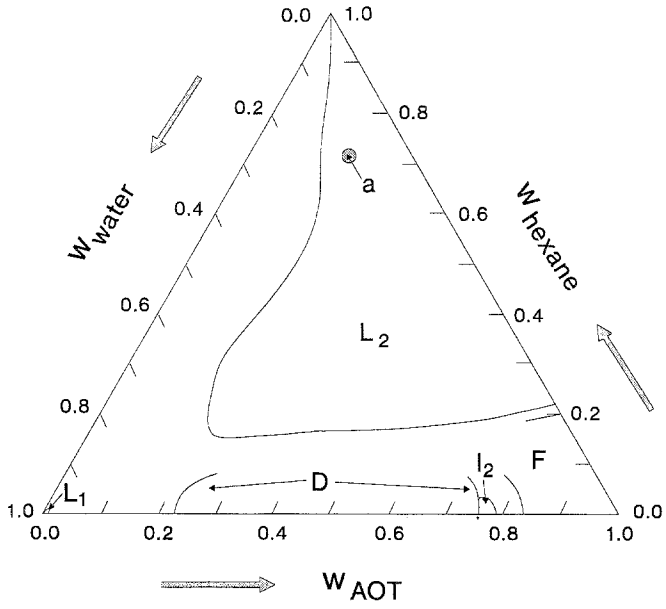

Fig. 1 Extensions of the isotropic L_2 and L_1 phases at 298.2 K for the system water–hexane–sodium 1,4-bis(2-ethylhexyl) sulfosuccinate. Only the phase boundaries of D and F (liquid crystalline phases) and I_2 (isotropic cubic phase) on the binary axes were determined. Point **a** represents the composition of the microemulsion used in the preparation of the MBG

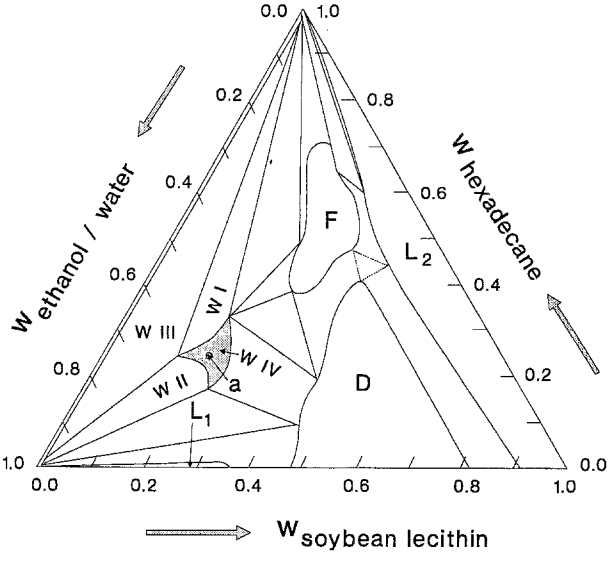

Fig. 2 Partial phase diagram at 298.2 K for the system ethanol/water–hexadecane–soybean lecithin. The mass ratio between ethanol and water was 1. W I-W IV denote Winsor systems of different types. L_1 and L_2 are isotropic solution phases. D and F are liquid crystalline phases. Point **a** in the W IV system (shadowed) represents the used microemulsion

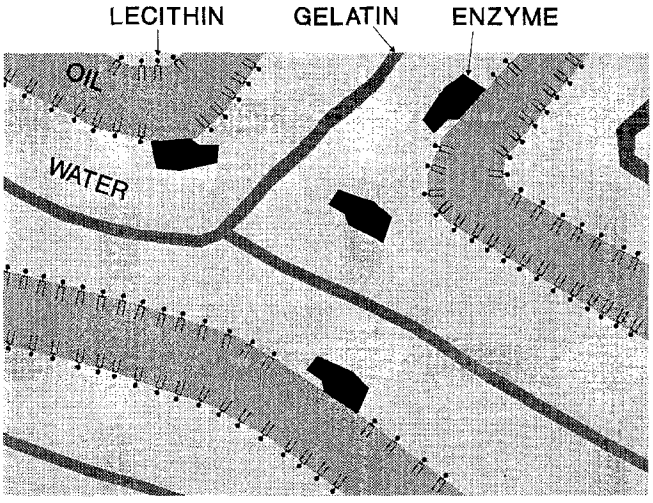

Fig. 3 A proposed model for the microstructure of a microemulsion-based gel [61]. Rigid gelatin network surrounded by water channels stabilized by soybean lecithin. No water droplets are present

The structure of the gel

The conductivity of the AOT-based microemulsion increased remarkably upon addition of gelatin, while the change for the lecithin system was small [46]. In the former microemulsion bicontinuous structures were formed during the gelation process, while the latter already exhibited this structure before addition of gelatin. Furthermore, there were no free ions in the lecithin system. Circular dichroism (CD) measurements gave a positive signal for the AOT-based gel; i.e., the same as for a gel based only on gelatin and water, while the lecithin-based gel gave a negative signal [60]. The interpretation was that lecithin does not participate to its whole extension in the gel structure. That the used gels have different structures could also be seen from atomic force microscopy (AFM) images [60].

The viscosity can be taken as a measure on the rigidity of the gel. The MBGs used in this work exhibited a very high viscosity i.e., the mean value was $20\,kPa\,s^{-1}$ for the shear rates studied and the components of the gel do not leach out even when stored for a long time in the reaction solvent. In spite of this high rigidity, structural characterizations of MBGs using a wide variety of techniques suggested the existence of extensive ion-conducting channels and a high mobility of the organic solvent molecules [39].

Petit et al. [61] have proposed three different models for the microstructure of the MBG: 1) Aggregated gelatin molecules in a rigid network surrounded by water channels intertwined with oil channels separated by the surfactant film. This is shown in Fig. 3 for the lecithin-based gelatin gel. 2) A gelatin network as in the first model coexisting with water droplets in oil stabilized by the surfactant, i.e., AOT, and finally 3) reverse micelles in oil containing water and a part of the gelatin. The micelles are connected by the rest of the gelatin, arranged in helical strands.

Esterification reactions

In our previous work [46] it was concluded that the conversion of esters depended on the distribution of the reactants and products between the MBG and the solvent. Therefore we have examined in more detail the working mechanism of the gels as reaction media. For this purpose the resolution of racemic (*R*)/(*S*)-(±)-2-octanol by selective esterification of the (*R*)-(−)-enantiomer with alkanoic acids was carried out using MBGs containing the lipase SP 525. Using this technique both the enzymatic enantioselectivity and the reaction rate are independent of the initial reactant concentrations [46]. Therefore, the substrate concentrations were kept at the same level, i.e., $0.33\,mol\,dm^{-3}$, in all performed experiments.

For the AOT-based gel in Fig. 4 both acid and alcohol go together into the gel and the produced ester comes simultaneously out of the gel. The chemical conversion of (*R*)-(−)-2-octyl hexanoate was practically stopped after 3 days of reaction time and closely corresponded to the theoretical conversion of 0.5 according to Eq. (1). The conversion could be kept at this high level for at least 13 days and no (*S*)-(+)-ester could be detected in these samples and no (*S*)-(+)-2-octanol was consumed.

Lipase-containing MBGs maintain their integrity for temperatures below the gel melting point at 303 K and therefore the gels were stored in a freezer when not in use. The effect of long time storage at this low temperature and of the accumulated water in the gel, produced in former esterifications, was tested by using a 1 year old AOT-based gel containing *Chromobacterium viscosum*-lipase. The conversion of (*R*)-(−)-2-octyl nonanoate in Fig. 5 was still high after the storage. After five days the e.e.(*p*) value was > 0.99 corresponding to a chemical conversion of 0.24. From Fig. 5 it can be seen that the e.e.(*p*) value decreased with time and after 24 days it was 0.86 corresponding to a conversion of 0.49. These values can be compared with the values of 0.87 (e.e.(*p*)) and 0.50 (conversion), respectively [46], after 3 days using a fresh AOT-based gel containing the same CV-lipase. Thus, it can be concluded that the storage of the gel has no apparent effect on the conversion of ester nor to any greater extent on the enantioselectivity of the enzyme. Only the reaction rate was slowed down.

The lecithin-based gel containing SP 525 lipase also catalyzed the esterification of racemic 2-octanol with high enantioselectivity. The progress of the reaction can be seen

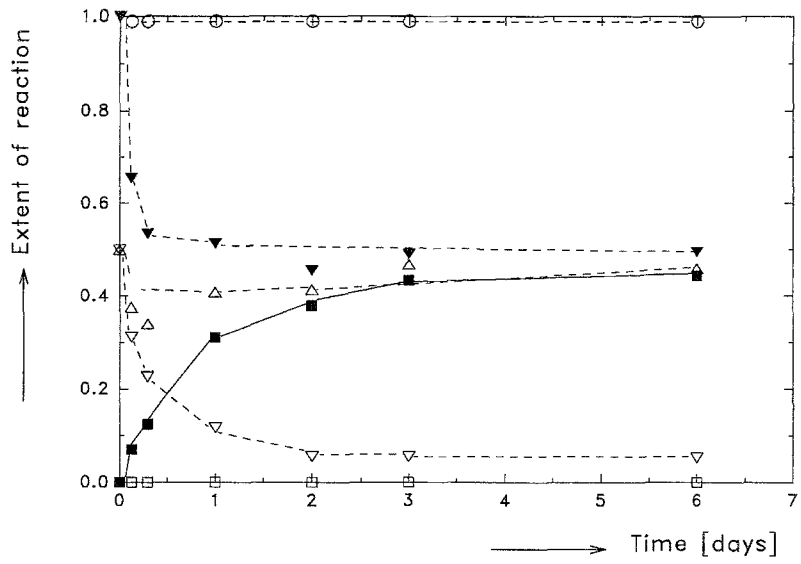

Fig. 4 Extent of reaction using the AOT-gel with SP 525 lipase. The initial concentrations were 0.33 mol dm^{-3} for both alcohol and acid. The symbols represent: (*R*)-2-octanol (▽), (*S*)-2-octanol (△), hexanoic acid (▼), (*R*)-2-octyl hexanoate (■), (*S*) -2-octyl hexanoate (□), and e.e.(*p*) (○)

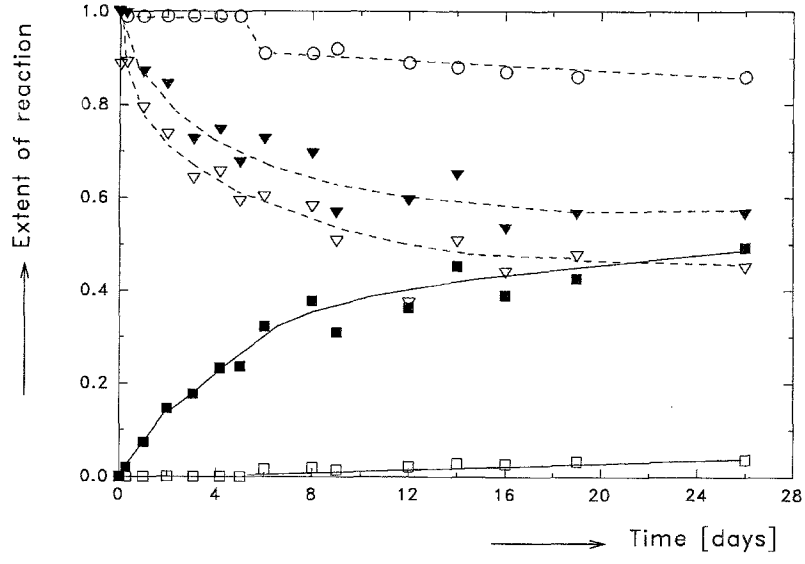

Fig. 5 Extent of reaction using a 1-year old and many times used AOT-gel with *Chromobacterium viscosum* lipase. The initial concentrations were the same as in Fig. 4. The symbols represent: (*R*)/(*S*)-2-octanol (▽), nonanoic acid (▼), (*R*)-2-octyl nonanoate (■), (*S*)-2-octyl nonanoate (□), and e.e.(*p*) (○)

in Fig. 6 for hexanoic acid. However, the reaction stopped at a conversion of about 0.24 for both hexanoic and nonanoic acid as the acylating agent. As the AOT- (Fig. 4) and lecithin-based (Fig. 6) gels are compared, a difference in the acid behaviour appears. For the lecithin gel the acid rapidly decreased in the reaction solution, i.e., it penetrated the gel. For the AOT gel the amount of acid in the reaction solution decreased at the same rate as the alcohol. The interpretation is that the two used gels have different microstructures as pointed out previously (see section **Structure of the gel**).

The enantiomeric excess values (e.e.(*p*)), taken as a measure on the optical purity are close to 1 for the esterifications in Figs. 4 and 6 (only the (*R*)-enantiomers of the esters could be detected by the chiral GLC and only the (*R*)-octanol was consumed). Both the AOT- and the lecithin-based gels were highly stereoselective for the substrates tested, when the enzyme used was SP 525. Our results are compared with literature data in Table 1. Calculated *E*-values using the Eqs. (4) or (5) for irreversible reactions are also included in Table 1. Lipase-catalyzed esterifications at the oil/water interface are irreversible reactions since the surface active properties of enantiomers are identical, i.e., the net penetration rate of the enzyme into the interface is not affected by changes in the concentrations of the enantiomers [62]. The very high

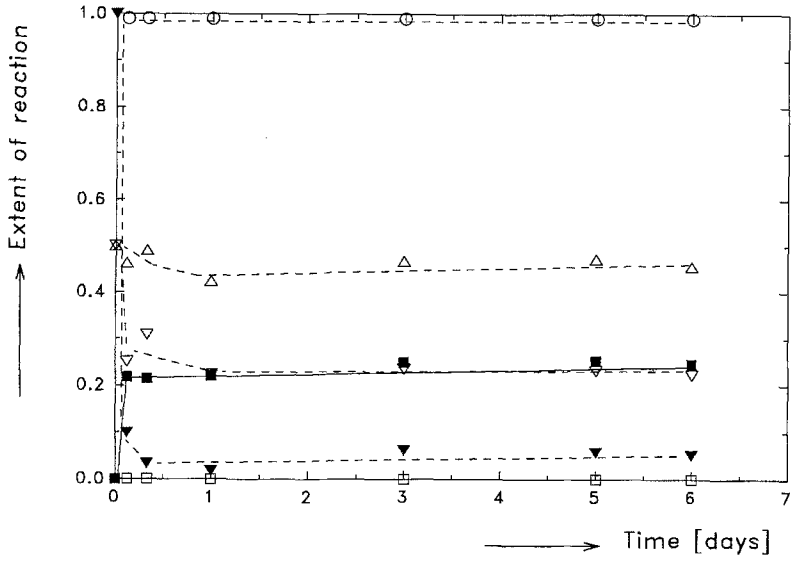

Fig. 6 Extent of reaction using the lecithin-gel with SP 525 lipase. The initial concentrations were the same as in Fig. 4. The symbols represent: (*R*)-2-octanol (▽), (*S*)-2-octanol (△), hexanoic acid (▼), (*R*)-2-octyl hexanoate (■), (*S*)-2-octyl hexanoate (□), and e.e.(*p*) (○)

Table 1 Resolution of racemic (*R*)/(*S*)-2-octanol by stereoselective esterification with different alkanoic acids in this work and current literature using microemulsion-based gels. Incubation temperature, 298.2 K

Source of lipase	Gel	Acylating agent	Conversion	e.e.(*p*)	E^a	Ref.
SP 525	AOT-MBG	Hexanoic acid	0.45	> 0.99	> 150	this work
SP 525	AOT-MBG	Nonanoic acid	0.42	> 0.99	> 150	this work
SP 525	Lecithin-MBG	Hexanoic acid	0.24	> 0.99	> 150	this work
SP 525	Lecithin-MBG	Nonanoic acid	0.23	> 0.99	> 150	this work
CV	AOT-MBG	Decanoic acid	0.45	0.92		39
Pseudomonas sp.	AOT-MBG	Decanoic acid		0.88		39
Microbial	AOT-MBG	Decanoic acid		0.80		39
CV	AOT-MBG	Hexanoic acid	0.25	0.90	25	46
CV	AOT-MBG	Nonanoic acid	0.52	0.87	50	46
CV	Lecithin-MBG	Nonanoic acid	0.40	0.86	25	46
CV	AOT-MBG	Hexanoic acid	0.18			45
Pseudomonas sp.	AOT-MBG	Hexanoic acid	0.15			45
Microbial	AOT-MBG	Hexanoic acid	0.16			45

[a] The *E*-values are calculated from Eq. [4] and [5].

stereoselectivity of the enzyme is also reflected by the high *E*-values for SP 525 lipase.

Concluding remarks

Lipase-containing microemulsion-based gelatin gels provide a unique medium for performing stereoselective esterifications of racemic alcohols with alkanoic acids in hydrophobic solvents. Due to the relatively concentrated reactant solutions and the high conversions these gels are of practical use in preparative scale-synthesis. The conversions were higher using gels based on water droplets-in-oil microemulsions than using gels based on microemulsions with bicontinuous structures. Careful selections of aqueous medium, oil and surfactant as well as familiarity with their phase behaviours are therefore of fundamental importance in preparing the gels. The same gel may be used several times to catalyze reactions involving different substrates, since remaining products and reactants may be washed out from the gel by using fresh hydrophobic solvents. The efficiency depends on the hydrophobicity of the solvents. However, long time storage of the gel decreases the reaction rate. The optical purities of the esters, especially those with SP 525-lipase, are the highest ever reached with the gel technique.

The gel method used in this work is thus characterized by high conversions and high optical purities, relatively

concentrated reactant solutions, room temperature, no stirring, no additional buffer, re-use of enzyme, and reasonable reaction times. All these improvements, compared with pure microemulsions, have economical as well as process technical consequences.

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