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Biocatalysis using microemulsion-based polymer gels containing lipase

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Abstract

Natural gelling agents such as gelatin, agar and κ-carrageenan have been tested for the formation of lecithin microemulsion-based gels as well as hydrogels (without surfactant and oil). The results presented in this work provide information concerning the utility of these solid gels as lipase immobilization matrices. It was found that lipase from *Pseudomonas cepacia* keeps its catalytic function after entrapment in the gels, catalyzing the esterification reaction of propanol with lauric acid in various hydrocarbons at room temperature. Various parameters which affect the lipase catalytic behavior such as the nature and the concentration of the gelling agent, as well as the concentration of the biocatalyst and the mole ratio of the substrates have been examined. High yields (80%) were obtained with agar and κ-carrageenan organogels in isooctane. The remaining lipase activity, in repeated syntheses was found to depend on the nature of the biopolymer used for the formation of the organogels. Gelatin and agar microemulsion-based gels showed the highest operational stability.

Keywords: Biocatalysis; Microemulsion; Polymer gel; Lipase; Esterification

1. Introduction

Biotransformations involving lipases have been established in the last decade. In addition to the hydrolysis of ester bonds of triglycerides, lipase can catalyze esterification or transesterification reactions in low-water-content media [1]. Different approaches have been proposed to facilitate the reversal of the hydrolytic action of lipases, as it has been reviewed by several authors [2–4]. These include various macro-heterogeneous biphasic systems such as liquid–liquid systems composed of a water-immiscible organic solvent and water; nearly anhydrous systems where the lipase is usually suspended as a powder or in an immobilized form adsorbed onto a suitable carrier in organic solvents or gases in supercritical state; various homogeneous media such as mixtures of water and water miscible organic solvent; and micro-heterogeneous systems such as different types of water-in-oil microemulsions or reverse micelles [5].

Microemulsions are spontaneously formed, isotropic thermodynamically stable liquid media

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with a large interfacial area (100 m^2 cm^{-3} solution). Microemulsions provide an aqueous phase where hydrophilic enzymes can be hosted, an interphase where surface-active enzymes can be anchored, and an organic phase where hydrophobic substrates or products are solubilized. In such a system the enzymes are active regarding the conversion of both hydrophilic and hydrophobic compounds [6,7].

The major problem which must be solved for the employment of a microemulsion system in industrial processes is the recovery of the products and the regeneration of the enzyme. Usual techniques such as extraction and distillation lead to poor separation because of the problems of emulsion-forming and foaming caused by the presence of surfactant. One approach to simplifying the recovery of the product and the enzyme reuse from microemulsion based-media has been the use of gelled microemulsion systems [8]. Interestingly, many w/o microemulsions can be 'gelled' by the addition of gelatin yielding a matrix suitable for enzyme immobilization. The preparation of gelled microemulsion systems was first reported in 1986 [9,10], involving the gelation of w/o microemulsions based on AOT or lecithin, into monophasic optically transparent rigid systems by mixing with an hydrogelatin solution above the gelling temperature. By cooling at room temperature a transparent gel is formed which has reproducible physical properties. These enzyme-containing gelatin-based gels are rigid and stable in various non-polar organic solvents and may therefore be used for biotransformations in organic media [8,11–14]. The study of the structural characteristics of these organogels has been the subject of a number of studies [15–18]. Under most conditions the gel matrix fully retains the surfactant, gelatin, water and enzyme components allowing the diffusion of non-polar substrates or products between a contacting non-polar phase and the gel pellets. Using systems based on gelatin and AOT w/o microemulsions preparative-scale synthesis of a wide range of esters was possible in small batch or column reactors even at temperatures as low as -20°C [8].

In this work, we present results of studies designed to characterize the catalytic behavior of *Pseudomonas cepacia* lipase immobilized on microemulsions-based organogels formulated with biopolymers such as gelatin, agar and κ-carrageenan in non-polar organic solvents. The esterification of 1-propanol with lauric acid was chosen as a model esterification reaction in order to examine aspects of both activity and stability of the immobilized lipase system. We also developed the preparation of hydrogels (without surfactant and oil) which consist of water and gelatin or agar or κ-carrageenan. This type of gels was also tested for the immobilization of *P. cepacia* lipase to catalyze the above esterification in isooctane.

2. Experimental

2.1. Materials

Lipase from *P. cepacia* was supplied by Sigma with a quoted specific activity of 90 U mg^{-1} of powder. Lecithin, containing 18–26% phosphatidylcholine, was purchased from Serva, Heidelberg, Germany. Lecithin was purified by column chromatography using a 35 cm × 3.2 cm column filled with basic alumina (type 5016A, Fluka, Basel, Switzerland). A chloroform/methanol (9/1, v/v) solvent system was used as eluent. The fractions were followed by TLC on silica plates using a solvent mixture of chloroform/methanol/water (60/15/4, v/v). The purified phosphatidylcholine was identified by NMR and an average molecular mass of 800 Da was determined [19]. Gelatin (Bloom 300) and κ-carrageenan were obtained from Sigma, and malt extract agar was obtained from Merck, Germany. 1-Propanol, lauric acid and all organic solvents were of the highest commercially available purity. Millipore highly purified water was used.

2.2. Preparation of microemulsions and microemulsion-based organogels

The lecithin microemulsions were prepared as follows: In 3.8% w/w lecithin in isooctane containing 5% v/v 1-propanol appropriate amounts of lipase in 25 mM Tris/HCl pH 7 were added, and the final water content was adjusted by the addition of the required amount of buffer.

Lipase-containing microemulsions-based organogels were prepared by introducing appropriate amounts of lecithin microemulsion containing lipase to a second solution of a polymer (gelatin, agar or κ-carrageenan) in water. In a typical experiment 2 cm^3 of lecithin microemulsion containing 0.8 mg of lipase, was gelled with 0.8–1.4 g of gelatin and 2.4 cm^3 water at 55°C, 0.45–0.7 g of agar and 5 cm^3 water at 60°C and 0.15–0.3 g of κ-carrageenan and 5 cm^3 water at 60°C. In the case of agar gels agar was first solubilized in water at 100°C. The mixtures were vigorously shaken and stirred until homogeneous (about 5–10 min) and allowed to cool to room temperature to yield organogels. The gels were stored in a freezer until used.

2.3. Preparation of hydrogels

The hydrogels were prepared by mixing aqueous solutions of gelatin, agar or κ-carrageenan with a concentrated lipase solution (9 mg cm^{-3}) in 25 mM Tris/HCl pH 7. In a typical experiment, gelatin gels were prepared from 1.5 g of polymer and 2.4 g water; agar gels from 0.7 g of polymer and 5 g water while κ-carrageenan gels from 0.3 g of polymer and 5 g water. All gels contained 1 mg of *P. cepacia* lipase. The mixtures were vigorously stirred for 5 min at 55–60°C and allowed to cool to room temperature to yield hydrogels. The gels were stored in a freezer until used.

Lipase-catalyzed reactions: apparent lipase activity was based on the measurement of the initial rate of propyl laurate synthesis. The gel (5 cm^3) was cut into approximately 15 pieces and placed into a reaction vial. By adding 15 cm^3 of organic solvent (aliphatic hydrocarbons) which contained various amounts of 1-propanol and lauric acid the reaction was initiated. The vial was stirred at 150 rpm and 25°C. At fixed intervals samples of 50 μl were taken from the vial and analyzed by GC.

2.4. GC analysis

The reactions were monitored by measuring the propyl laureate concentration by gas chromatography, using a Hewlett Packard HP 6890 chromatographer, equipped with a 50 m capillary column, BOX 70 (SEE) and a FID detector. Nitrogen was used as the carrier gas at a flow rate of 10 cm^3 min^{-1}, with detector port temperature at 250°C. The oven temperature was kept constant for 1 min at 120°C, linearly increased (10°C min^{-1}) up to 240°C. The reaction product is quantified on basis of its response relative to the internal standard, i.e., dodecane.

3. Results and discussion

3.1. Effect of the concentration of biopolymers on lipase activity

In this work, we have investigated the ability of some natural polymers such as gelatin, agar and κ-carrageenan to form stable microemulsion-based gels (MG) in non polar organic solvents. The goal was to find gels that are suitable for enzyme immobilization and their application in enzyme-catalyzed reactions. For this purpose, lecithin w/o microemulsions containing enzyme (lipase from *P. cepacia*) were gelled to pseudosolid gels after mixing with aqueous solutions of natural polymers. The lipase-containing gel was cut into suitable pieces and added to a hydrocarbon which contained lipase substrates (1-propanol and lauric acid).

The formation of MGs with various natural polymers depends on the nature of polymers

and on their molar fraction. Namely, gelatin forms lecithin MGs with mass fraction of gelatin in the gels ($w_{gelatin}$) from 0.16 to 0.25, agar forms MGs with w_{agar} from 0.060 to 0.095 while κ-carrageenan forms MGs with $w_{carrageenan}$ from 0.02 to 0.045, respectively. MGs formed with gelatin and agar at the above concentration range of biopolymers, were stable and maintained their structural integrity for several days in contact with the external organic solvent (i.e., isooctane). However, κ-carrageenan MGs were unable to maintain their pellet form since they became glutinous.

The effect of the mass fraction of biopolymers on the lipase activity was determined by comparing the rates of esterification obtained using MGs prepared with different polymer concentrations. Table 1, shows the effect of the mass fraction (w) of polymers in the gels on the rate of esterification of 1-propanol with lauric acid catalyzed by immobilized *P. cepacia* lipase in isooctane. Fig. 1 shows typical reaction profiles for the enzymatic synthesis of propyl laurate. As can be seen, the esterification rate depends on the nature of biopolymer used. The higher esterification rate was observed with gels formulated with κ-carrageenan while the lower rate observed with gelatin gels. As can be seen from Table 1, an increase of the mass fraction of the biopolymers increases the esterification rate for the all three biopolymers used.

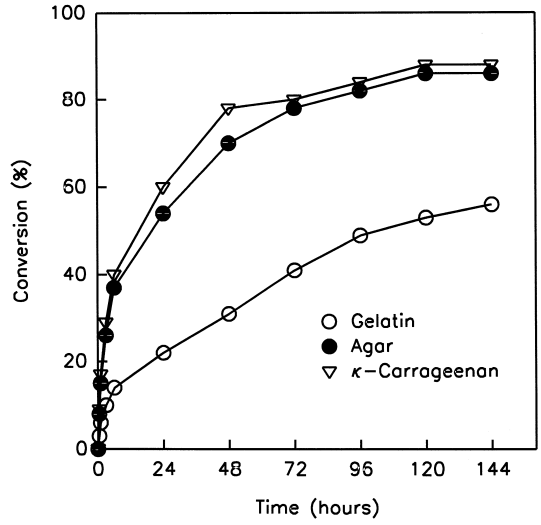

Fig. 1. Typical reaction profiles for the synthesis of propyl laurate catalyzed by *P. cepacia* lipase (0.8 mg) in lecithin microemulsion-based gels formulated with gelatin, agar and κ-carrageenan. Organogels were prepared as in the experimental section; the mass fraction of biopolymers was 0.20 for gelatin, 0.075 for agar and 0.035 for κ-carrageenan.

Table 1
Effect of mass fraction of bipolymers in the gels on the esterification rate of 1-propanol with lauric acid catalyzed by lipase from *P. cepacia* in lecithin microemulsion-based gels in isooctane. Organogels were prepared as described in the experimental section with 2 cm^3 of lecithin microemulsion. The amount of lipase was 0.8 mg

Mass fraction (w)	Esterification rate (mM h^{-1})
Gelatin	
0.16	2.9
0.20	3.0
0.25	3.3
Agar	
0.060	7.5
0.075	8.6
0.095	8.6
κ-*Carrageenan*	
0.020	9.4
0.035	9.6
0.045	9.5

3.2. Effect of lipase concentration on esterification rates

The effect of the lipase concentration on the reaction rate in various organogels has been tested. Fig. 2 shows the effect of varying the concentration of *P. cepacia* lipase on the esterification rate of 1-propanol with lauric acid in lecithin MGs formulated with gelatin, agar and κ-carrageenan. As can be seen, the esterification rate increases as the amount of lipase in the organogels increases. However, at high amounts of immobilized lipase the dependence of the reaction rate upon enzyme concentration gave a nonlinear profile over the measured range due to a probable mass-transfer limitation. It must be noted that transport phenomena are rate limiting when the surface area of the gels is rela-

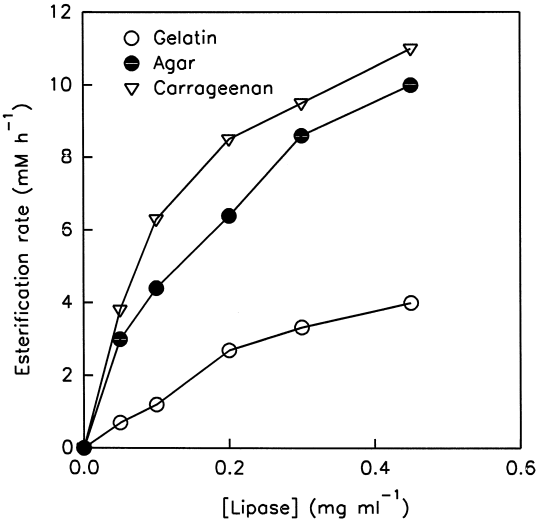

Fig. 2. Effect of *P. cepacia* lipase concentration (calculated in terms of volume of the microemulsion), on esterification rate of 1-propanol with lauric acid in various lecithin microemulsion-based organogels in isooctane. Organogels were prepared as described in the experimental section with 2 cm^3 of lecithin microemulsion. The mass fraction of biopolymers as described in Fig. 1.

tively small. In our work, the catalytic activity of immobilized lipase depends on the surface area per unit volume of organogels since the lipase activity in fragmented gels (especially in gelatin and agar gels) was 30–50% higher than in whole ones (data not shown). This has been also observed when using AOT microemulsions-based gelatin organogels by other researchers [14].

3.3. Effect of alcohol concentration

In order to study the effect of the 1-propanol concentration on the lipase activity in the MGs, different concentrations of alcohol were used in the range 50–400 mM at constant concentration of lauric acid (150 mM). The reaction was carried out in isooctane while *P. cepacia* lipase was immobilized in lecithin MGs formulated with gelatin and agar, respectively. The results of this study are summarized in Fig. 3. As can be seen, the reaction rate in both organogels increases as the mole ratio of propanol to lauric acid increases. It must be noted that in all MG cases studied here no inhibitory effect of alcohol was observed, in contrast to the one observed in various w/o microemulsions with the same lipase [6].

3.4. Effect of the oil nature

One of the most important criteria for solvent selection in biocatalytic processes seems to be the ability of solvent to strip the essential water bound to the enzyme. Therefore, the polarity of the organic solvent is a fundamental factor that determines the behavior of enzymes [20].

The influence of the chain length of the hydrocarbon oil used on the esterification activity of *P. cepacia* lipase immobilized in MGs formulated with gelatin and agar has been studied. The results are summarized in Table 2. As can be seen, the higher esterification rate was observed in *n*-octane followed by cyclohexane and isooctane for both organogels tested, while the reaction rate slightly drops in longer hydrocarbons. For all solvents studied, the final conversion yield at equilibrium was estimated to be

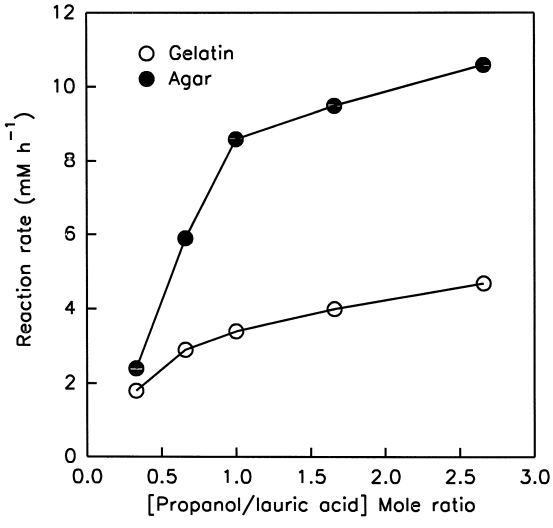

Fig. 3. Effect of propanol to lauric acid mole ratio on reaction rate of synthesis of propyl laurate catalyzed by *P. cepacia* (0.8 mg) in microemulsion-based organogels in isooctane formulated with gelatin and agar. The mass fraction of biopolymers was 0.20 for gelatin and 0.075 for agar.

Table 2
Influence of the oil chain length on the esterification rate of 1-propanol with lauric acid catalyzed by *P. cepacia* lipase (0.8 mg) in lecithin microemulsion-based organogels formulated with agar and gelatin. Organogels were prepared as described in Section 2; the mass fraction of biopolymers was 0.20 for gelatin and 0.075 for agar

Solvent	Esterification rate (mM h^{-1})	
	Gelatin organogel	Agar organogel
n-Hexane	3.3	7.9
Cyclohexane	3.4	8.5
n-Heptane	3.2	8.1
n-Octane	3.6	8.6
Isooctane	3.3	8.5
n-Decane	2.9	7.9
n-Dodecane	2.8	8.0

about 80% for agar gels and about 60% for gelatin gels. It must be noted, than in other studies concerning the activity of immobilized *Chromobacterium viscosum* lipase in AOT microemulsion-based organogels formulated with gelatin a decrease in the esterification activity was observed as the alkane chain length increased from 6 to 10 carbon atoms [14], while the conversion yield of the esterification of pentanol with oleic acid catalyzed by the above lipase varied with the solvent polarity showing a maximum in *n*-octane.

3.5. Effect of enzyme reuse

In this study, the ability of lipase from *P. cepacia* to be reused, was investigated. Immobilized lipase in various MGs formulated with gelatin, agar and κ-carrageenan were reused 5 times for the esterification of 1-propanol with lauric acid in isooctane. Reaction was followed during a 24-h incubation at 25°C as previously described with esterification rates measured by GC. After each run, organogels containing lipase were washed with isooctane and filled with fresh substrate solution.

Reaction rates of propyl laurate formation with repeated use of immobilized lipase are shown in Fig. 4. As can be seen, the remaining lipase activity, after each run, depends on the nature of the biopolymer used for the formation of organogels. Gelatin gels showed the higher operational stability while the lipase stability in κ-carrageenan gels decreased rapidly even after the first run. It must be noted that the physical appearance of the gelatin and agar gels were unchanged while the integrity of κ-carrageenan gel was modified during the 5-day incubation period since it became glutinous.

The batch lipase stability presented in this work, seems to be lower than that observed by Rees et al. [8], for the *C. viscosum* lipase immobilized in gelatin organogels, where 75% of initial activity remained after 15 successive runs performed over a 30-day period in *n*-heptane. This difference can be attributed to the fact that in the case of *C. viscosum* lipase, the operational stability was measured to only 10% substrate conversion while in our work the conversion after 24 h of incubation was significantly higher (30–60% depending on the nature of biopolymer). The large quantities of water formed as a coproduct in the latter case, accumulate in the gel matrix and therefore on the lipase microenvironment affecting the lipase catalytic behavior [14].

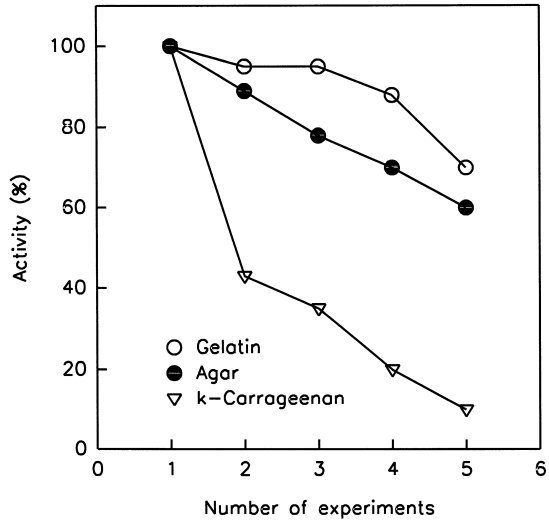

Fig. 4. Operational stability of microemulsion-based organogels containing *P. cepacia* (0.8 mg) lipase in isooctane. The mass fraction of biopolymers as described in Fig. 1.

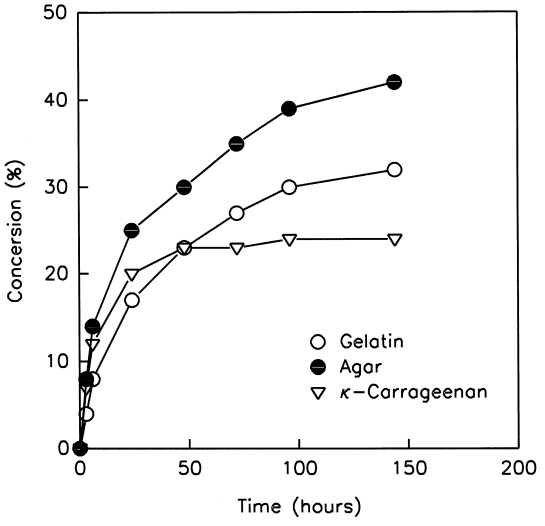

Fig. 5. Typical reaction profiles for the synthesis of propyl laurate catalyzed by *P. cepacia* lipase (1 mg) in aqueous gels formulated with gelatin, agar and κ-carrageenan. The mass fraction of biopolymers was 0.38 for gelatin, 0.12 for agar and 0.057 for κ-carrageenan.

3.6. Esterification with immobilized lipase in hydrogels

We have also investigated the potential of natural biopolymers, such as gelatin, agar and κ-carrageenan, to form gels containing lipase without the addition of microemulsion phase, for the catalysis of esterification reactions in organic solvents. Fig. 5, shows a typical reaction progress for the esterification of 1-propanol with lauric acid in isooctane catalyzed by *P. cepacia* lipase immobilized on gelatin, agar and κ-carrageenan hydrogels. Conversion rates observed for the three hydrogels used, are 4–5 times lower than in the case of microemulsion-based organogels as described in the previous sections. In this case the mass fraction of polymers was 0.38 for gelatin, 0.12 for agar and 0.057 for κ-carrageenan, respectively. It must be noted that the operational stability of the hydrogels containing lipase depends on the biopolymer content. Gels formulated with gelatin and agar are rigid when the mass fractions of polymers are higher than 0.35 and 0.10, respectively while gels formulated with κ-carrageenan became glutinous and totally crashed after 24 h storage in isooctane. Gelatin and agar hydrogels may be repeatedly used since they retain their integrity for several days in isooctane.

4. Conclusions

Lipase-containing microemulsions-based organogels formulated with various biopolymers such as gelatin, agar and κ-carrageenan have considerable potential for their application in biotranformations. Lipase immobilized in gelatin and agar organogels showed good stability in catalyzing esterification reactions under mild conditions with high conversion yields. Moreover, aqueous gelatin and agar gels containing only lipase, water and biopolymer retain their integrity in organic solvents and can also be used for the synthesis of esters.

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