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Journal of Molecular Catalysis B: Enzymatic 17 (2002) 23–28

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Effect of imprinting sol–gel immobilized lipase with chiral template substrates in esterification of (*R*)-(+)- and (*S*)-(−)-glycidol

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Received 25 June 2001; accepted 23 November 2001

Abstract

To prepare an immobilized lipase effective for enantioselective esterification of glycidol with *n*-butyric acid in organic media, hybrid gel-entrapped lipase on Celite was prepared by the sol–gel method and pretreated with chiral template substrates. When *n*-butyltrimethoxysilane, (*n*-BuTrMOS) as an organic silane precursor, was mixed with tetramethoxysilane (TMOS) at a molar ratio of 4:1, the hybrid gel-entrapped lipase on Celite showed three times higher activity than the deposited lipase on Celite as a control. The pretreatment of this immobilized lipase with a hydrophobic enantiomer such as (*R*)-(−)-2-octanol brought about a selective enhancement of the activity for the esterification of (*R*)-(+)-glycidol, whereas such pretreatment hardly affected the activity for the esterification of (*S*)-(−)-glycidol. Consequently, the relative initial enantiomeric activity (RIEA) of the hybrid gel-entrapped lipase on Celite, defined as a ratio of the initial esterification rate of (*R*)-(+)-glycidol to that of (*S*)-(−)-glycidol, changed between 0.76 and 2.0. In addition, increasing the concentration of (*R*)-(−)-2-octanol as a template substrate, increased the activity and RIEA, and showed maximum values with the addition of 21 mM of (*R*)-(−)-2-octanol.

Keywords: Sol–gel method; Organic–inorganic hybrid silicate; Lipase; Imprinting; Enantioselective esterification

1. Introduction

Recently, many biological materials such as enzymes [1,2], proteins [3], microbes [4], and mammalian and plant cells [5–7] have been immobilized into silica matrices prepared by the sol–gel method. Because the immobilization of biomolecules into organic–inorganic hybrid silica gels prepared by this method brings about an enhancement of activity, and thermal and operational stabilities, commercial applications of these immobilized biomolecules have been examined by some researchers [8,9]. Previously, we reported that the immobilization of lipase into the organic–inorganic hybrid silica gel derived from a mixture of tetramethoxysilane (TMOS) and dimethyldimethoxysilane (DMDMOS) brought about an enhancement of activity as well as thermal stability in esterification in organic media [10]. It is thought that the activation of lipase by the sol–gel immobilization is due to the opening of the *"lid"*, a loop composed of several

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1381-1177/02/$ – see front matter
PII: S1381-1177(01)00076-5

amino acid residues covering the active site of lipase molecule, which is induced by the hydrophobic interaction between hydrophobic silica matrices and lipase molecules [10].

On the other hand, it has been reported that pretreatment of enzymes with specific template substances such as substrate analogues changes the local environment in the vicinity of the active site, and leads to enantioselective activation in the reactions of racemic substrates. In some early work in this field, Stahl et al. [11] reported that the imprinting pretreatment of α-chymotrypsin with *N*-acetyl-L-tryptophane brought about activation of α-chymotrypsin only for esterification of *N*-acetyl-L-tryptophane, whereas α-chymotrypsin pretreated with *N*-acetyl-D-tryptophane brought about a similar activation only for esterification of *N*-acetyl-D-tryptophane. Pinel et al. [12] attempted imprinting pretreatment with (−)- or (+)-menthol of silica matrices derived by the sol–gel method, and aimed for the preparation of supports which had high affinity to the corresponding substrates. They reported that silica gels pretreated with either enantiomer specifically enhanced the adsorption coefficient of the corresponding substance. Recently, we also attempted to pretreat hybrid gel-entrapped lipase on Celite prepared by the sol–gel method with (−)-menthol as a template substrate, and to improve activity and enantioselectivity of such immobilized lipase in esterification of (−)- and (+)-menthol with *n*-butyric acid [13]. As a result, although the pretreatment of the hybrid gel matrices with (−)-menthol did not affect the adsorption coefficient of (−)-menthol, the pretreatment of lipase molecule with (−)-menthol brought about the enhancement of the activity of sol–gel immobilized lipase not only in esterification of (−)-menthol, but also in esterification of (+)-menthol. Accordingly, this pretreatment did not result in an improvement of enantioselectivity. Thus, it was considered that pretreatment with menthol did not effectively act on the substrate recognition part of the active site of lipase, and did not improve enantioselectivity.

In this study, we examined the esterification of (*R*)-(+)- and (*S*)-(−)-glycidol with *n*-butyric acid as a model reaction, and investigated improvements of activity and enantioselectivity of the hybrid gel-entrapped lipase on Celite pretreated with several template substrates.

2. Materials and methods

2.1. Chemicals

Lipase originated from *Rhizopus javanicus* (Lipase F-AP), kindly supplied by Amano Pharmaceutical Co. Ltd., was used without further purification. Ethyltrimethoxysilane (ETrMOS), propyltrimethoxysilane (PrTrMOS), *n*-butyltrimethoxysilane (*n*-BuTrMOS), *i*-butyltrimethoxysilane (*i*-BuTrMOS), *n*-hexyltrimethoxysilane (*n*-HxlTrMOS), *n*-hexadecyltrimethoxysilane (*n*-HxdlTrMOS), and *n*-octadecyltrimethoxysilane (*n*-OctdlTrMOS) were kindly donated by Dow Corning Toray Silicone Co. Celite 545 was obtained from Ishidzu Pharmaceutical Co. (*R*)-(−)-2-, (*S*)-(+)-2-octanol and (*S*)-(−)-β-citronellol were purchased from Merck, and (*R*)-(+)- and (*S*)-(−)-glycidol were purchased from Aldrich. TMOS, methyltrimethoxysilane (MTrMOS), DMDMOS, trimethylmethoxysilane (TrMMOS) and other chemicals were of reagent grade and obtained from Tokyo Chemical Industry Co.

2.2. Immobilization procedures

A typical immobilization procedure of lipase is follows: at ambient temperature, 1.36 mmol mixture containing organic silane and TMOS at a molar ratio of 4:1, 0.1 ml distilled water, and 2.5 μl of 40 mM HCl were mixed in a test tube to obtain a homogeneous solution by the hydrolysis of silane precursors. Then, 0.7 ml of 100 mM phosphate buffer (pH 7.5), 0.5 ml of 100 mM phosphate buffer (pH 7.5) containing 150 mg lipase, and 5 μl methanol solution containing a template substrate (64 μmol) at a final concentration of 21 mM for pretreatment were added into this homogeneous sol solution and mixed well. These procedures were carried out within 30 s. The resultant mixture was then blended well with 0.5 g Celite 545 powder in a petri dish, and left for 1 day at room temperature. The solid mass formed was lyophilized for 1 day, crushed in a mortar, and hydrated for 1 day in a desiccator containing a saturated aqueous LiCl solution (water activity, 0.11) at 30 °C.

Deposited lipase on Celite was prepared by replacing the silane mixture with 0.2 ml distilled water in the same procedure as described above.

Non-pretreated immobilized lipase as a control was prepared by the addition of methanol solution not containing a template substrate.

2.3. Measurement

The esterification of (*R*)-(+)- and (*S*)-(−)-glycidol with *n*-butyric acid was used as a model reaction. All the experiments were carried out at 35 °C in 20 ml vials with screw caps, with the reaction mixture containing 0.02 M of both the substrates and the immobilized lipase (ca. 0.7 g) in 10 ml isooctane. The reaction was initiated by adding the immobilized lipase into the substrate solution and was performed in a water bath shaker at 160 min^{-1}. To determine the concentrations of the substrates and the product, (*R*)-(+)- or (*S*)-(−)-glycidol *n*-butyrate, the organic samples were withdrawn from the reaction mixture at appropriate time intervals and analyzed by a gas chromatograph (Shimadzu GC-14 B) equipped with a FSS ULBON HR-20 M capillary column. Analyses were typically carried out at an injection and a detector temperature of 250 °C. The column temperature was elevated from 90 to 220 °C over 15 min. In order to check whether the template substrates remained in the esterification medium or not after immobilization and imprinting, the pretreated immobilized lipase was added into isooctane, and the liquid samples were analyzed. No template substrate was detected in the liquid samples. This result indicated that the template molecules were removed by vaporization during lyophilization. The initial esterification rate measurements were performed over a 0–20 min period during which the rates remained constant. The relative initial enantiomeric activity (RIEA), defined as a ratio of the initial esterification rate of (*R*)-(+)-glycidol to that of (*S*)-(−)-glycidol, was used as a measure for enantioselectivity.

3. Results and discussion

3.1. Effect of organic silanes mixed with TMOS for preparing hybrid gel-entrapped lipase on Celite

Fig. 1 shows a comparison of initial esterification rates of (*R*)-(+)-glycidol at 35 °C by hybrid gel-entrapped lipase prepared from a mixture of various organic silanes and TMOS at a molar ratio of 4:1. The hybrid gel-entrapped lipase derived from TMOS alone showed a rather low esterification activity, probably due to a closed gel structure [14]. However, by increasing the number of the methyl group (MTrMOS to TrMMOS) and the chain length of the alkyl group (MTrMOS to OctdlTrMOS), the esterification activity increased, and reached a maximum with *n*-BuTrMOS.

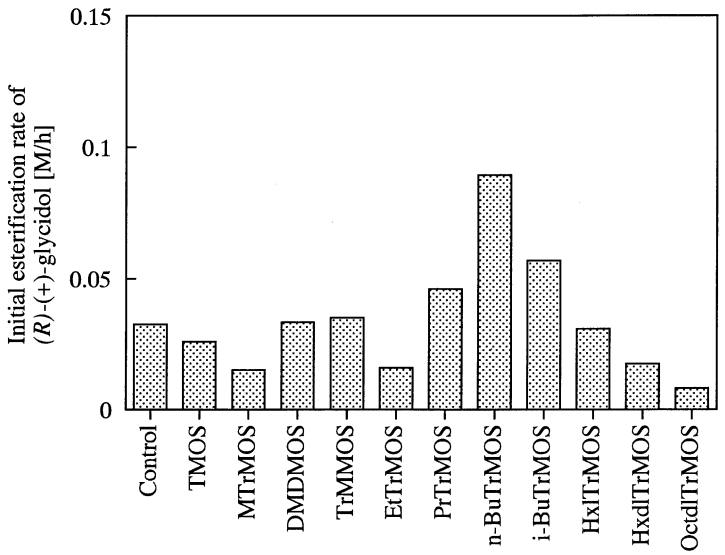

Fig. 1. Effect of substituent groups in organic silanes mixed with TMOS on activity of hybrid gel-entrapped lipase on Celite for esterification of (*R*)-(+)-glycidol with *n*-butyric acid. Molar ratio of organic silane to TMOS, 4:1. Control means deposited lipase on Celite.

This hybrid gel-entrapped lipase on Celite showed three times higher activity than the deposited lipase on Celite. In the esterification of menthol with *n*-butyric acid, the hybrid gel-entrapped lipase on Celite derived from a mixture of DMDMOS and TMOS showed the highest activity [10]. Therefore, the optimum organic silane for preparing hybrid gel-entrapped lipase seems to be dependent upon the substrates and the origin of the lipase used. In subsequent experiments, we used *n*-BuTrMOS as the organic silane precursor, and investigated the effect of pretreatment with several template substrates for further enhancement of esterification activity and enantioselectivity.

3.2. Effect of various alcohols as template substrates

In our first attempt to bioimprint the sol–gel immobilized lipase, we used (*R*)-(+)- or (*S*)-(−)-glycidol as a template substrate, the substrate in this reaction. A comparison of initial activities in esterifications of (*R*)-(+)- and (*S*)-(−)-glycidol is shown in Fig. 2. Pretreatment of hybrid gel-entrapped lipase on Celite with (*R*)-(+)- or (*S*)-(−)-glycidol did not induce a significant activation in the esterification of the corresponding substrate. In a previous study, we reported that bulky and hydrophobic molecules were effective for enhancement of the activity, and that this might be due to the hydrophobic interaction between those molecules and lipase molecules [13]. Therefore, it was thought that as the hydrophobicity of (*R*)-(+)- and (*S*)-(−)-glycidol was not so high, accordingly the hydrophobic interaction between them did not effectively function. From this hypothesis, we adopted more hydrophobic substrate templates such as (*R*)-(−)-2-octanol, (*S*)-(+)-2-octanol and (*S*)-(−)-β-citronellol.

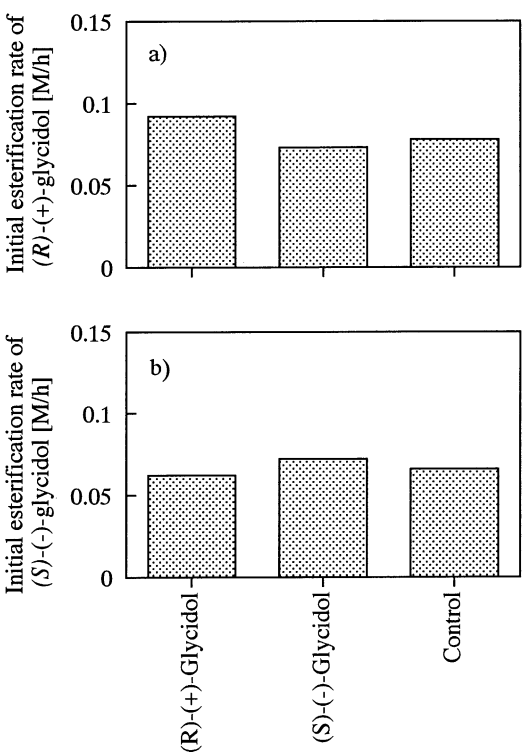

Fig. 2. Effect of template substrates used for pretreatment of hybrid gel-entrapped lipase on Celite on initial rates of esterification of (a) (*R*)-(+)-glycidol and (b) (*S*)-(−)-glycidol with *n*-butyric acid. Concentration of template substrates, 21 mM. Control means unpretreated hybrid gel-entrapped lipase on Celite.

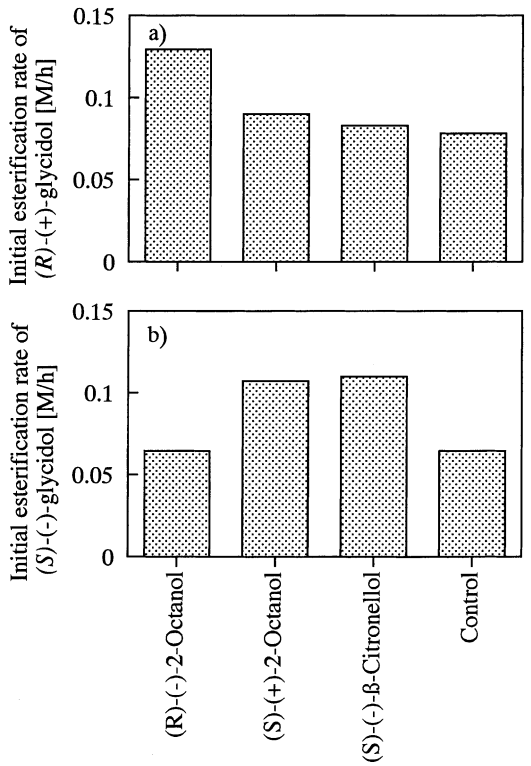

Fig. 3. Effect of hydrophobic enantiomers used for pretreatment of hybrid gel-entrapped lipase on Celite on initial rates of esterification of (a) (*R*)-(+)-glycidol and (b) (*S*)-(−)-glycidol with *n*-butyric acid. Concentration of template substrates, 21 mM. Control means unpretreated hybrid gel-entrapped lipase on Celite.

Fig. 3 shows comparisons of the activities of the hybrid gel-entrapped lipase pretreated with the above templates in esterifications of (*R*)-(+)- and (*S*)-(−)-glycidol. These pretreatments brought about selective enhancements of the activities of the hybrid gel-entrapped lipase on Celite. Interestingly, pretreatment with the template substrate having a (*R*)-structure induced selective activation only for the esterification of (*R*)-(+)-glycidol, whereas that with the template substrate having a (*S*)-structure selectively activated only the esterification of (*S*)-(−)-glycidol. Accordingly, RIEA of the hybrid gel-entrapped lipase on Celite pretreated with (*R*)-template substrate increased from 1.2 to 2.0, and that pretreated with (*S*)-template decreased from 1.2 to 0.76, as shown in Table 1. Thus, it was shown that pretreatment with hydrophobic molecules brought about the effective bioimprinting of these immobilized lipases.

Table 1
Comparison of relative initial enantiomeric activities (RIEA) of hybrid gel-entrapped lipase on Celite pretreated with various template substrates

Template substrate	RIEA
Not pretreated	1.2
Pretreated with	
(*R*)-(+)-glycidol	1.5
(*R*)-(−)-2-octanol	2.0
(*S*)-(−)-glycidol	1.0
(*S*)-(+)-2-octanol	0.84
(*S*)-(−)-β-citronellol	0.76

3.3. Effect of concentration of the template substrate during pretreatment of hybrid gel-entrapped lipase on Celite

Finally, we investigated the effect of the concentration of template substrates used for pretreatment of hybrid gel-entrapped lipase on Celite. As above, (*R*)-(−)-2-octanol was used as the template substrate. As shown in Fig. 4, with increasing concentration of the template substrate, the initial esterification activity for (*R*)-(+)-glycidol of the hybrid gel-entrapped lipase pretreated with (*R*)-(−)-2-octanol increased, whereas the activity for (*S*)-(−)-glycidol was almost the same. Consequently, RIEA was enhanced up to 2.0 by the addition of 21 mM of the template substrate to the hybrid gel-entrapped lipase on Celite, although further addition of the template substrate did not induce a further improvement of RIEA.

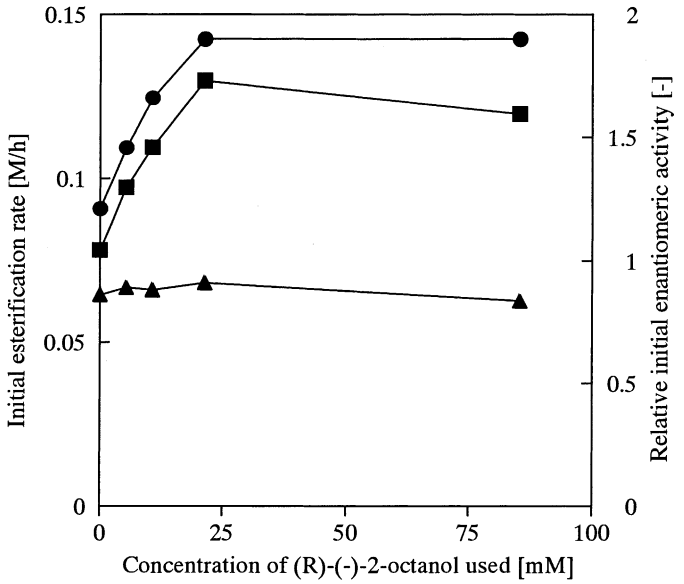

Fig. 4. Effect of concentration of (*R*)-(−)-2-octanol used for pretreatment of hybrid gel-entrapped lipase on Celite on initial esterification rates of (*R*)-(+)-glycidol (closed square), (*S*)-(−)-glycidol (closed triangle) and relative initial enantiomeric activity (RIEA) (closed circle).

4. Conclusions

We attempted to pretreat hybrid gel-immobilized lipase prepared by the sol–gel method with a template substrate. The hybrid gel-entrapped lipase on Celite derived from a mixture of *n*-BuTrMOS and TMOS showed three times higher activity than the deposited lipase on Celite. Further, pretreatment of hybrid gel-entrapped lipase on Celite with chiral template substrates resulted in a further enhancement of esterification activity and RIEA. Especially, pretreatment of hybrid gel-entrapped lipase on Celite with (*R*)-(−)-2-octanol brought about a selective enhancement of activity for esterification of (*R*)-(+)-glycidol, whereas this hardly affected activity for esterification of (*S*)-(−)-glycidol. Additionally, the pretreatment with (*S*)-(+)-2-octanol or (*S*)-(−)-β-citronellol also brought about the selective enhancement of activity for esterification of (*S*)-(−)-glycidol. Therefore, RIEA of pretreated hybrid gel-entrapped lipase on Celite varied from 0.76 to 2.0. Increasing the concentration of (*R*)-(−)-2-octanol as the template substrate, increased the activity and RIEA, and showed maximum values with the addition of 21 mM of (*R*)-(−)-2-octanol.

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