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Enhancement of activity of sol–gel immobilized lipase in organic media by pretreatment with substrate analogues

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Abstract

By performing pretreatment with substrate analogues, an activation of lipase immobilized into organic–inorganic hybrid silicate was attempted in esterification of menthol with butyric acid in organic media. With decreasing water content of immobilized lipase preparations, the activity of immobilized lipases by (−)-menthol pretreatment was enhanced, and the highest activity was obtained at water activity, $a_w = 0.11$. However, the pretreatment of immobilized lipases with (−)-menthol could not improve the enantioselectivity. In addition, the activity of pretreated immobilized lipases increased with increasing the chain length of alcohol as a substrate analogue. Consequently, it was found that hydrophobic and bulky alcohol was an effective substrate analogue to activate immobilized lipases. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sol–gel method; Lipase; Esterification; Substrate analogue; Organic–inorganic hybrid silicate

1. Introduction

For the last decade, it has been reported that the sol–gel method is so useful for the immobilization of biomolecules in analytical and commercial applications [1,2]. Recently, many biological materials such as enzymes [3,4], proteins [5], microbes [6], and mammalian and plant cells [7–9] have been immobilized by the sol–gel method. The advantage of the sol–gel method is to form rigid and elastic silica matrices which bring about the high durability against the physical damages [2]. The other advantage is possible insertion of any substituents, which give the efficient environment to the biomolecules entrapped into silica matrices [10].

In previous reports, we demonstrated that the entrapment of lipase into organic–inorganic hybrid silicate formed on Celite 545 resulted in an enhancement of thermal stability [10]. Moreover, we reported that an increase in the chain length or the number of alkyl groups of organic silanes, which were precursors to form a silica matrix, led to an enhancement of the catalytic activity and the enantioselectivity at high temperature of lipase entrapped into the hybrid gel [11]. Consequently, the hybrid gel-entrapped lipase on Celite derived from a mixture of dimethyldimethoxysilane (DMDMOS) and tetramethoxysilane (TMOS) showed the highest activity. Furthermore, it was found that the hybrid gel-immobilized lipase prepared at $r(= [\mathrm{DMDMOS}]/[\mathrm{TMOS}]) = 4$ showed the highest activity and enantioselectivity at 75°C.

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On the other hand, some researchers reported that the pretreatment of enzyme with substrate analogues brought about an improvement of activity and enantioselectivity of enzyme [12,13]. It has been thought that the pretreatment of enzyme with specific molecules such as a substrate changes the local environment in the vicinity of the active site and leads to the improvement of activity and enantioselectivity of enzyme. In addition, it was reported that the pretreatment of support materials with substrate analogues induced the enhancement of the affinity between a gel matrix and a racemic substrate [14,15].

In this study, we aimed at a further activation of the sol–gel immobilized lipase by using this pretreatment. Enhancement of the activity of lipase, as well as the affinity between the hybrid gel matrix and the racemic substrate are expected by the pretreatment of the hybrid gel-immobilized lipase derived from the sol–gel method.

2. Materials and methods

2.1. Chemicals

Candida rugosa lipase (type VII) was obtained from Sigma and used without further purification. Tetramethoxysilane (TMOS), dimethyldimethoxysilane (DMDMOS), and the other chemicals were of reagent grade and were obtained from Tokyo Chemical Industry Co. Celite 545 was obtained from Ishidzu Pharmaceutical Co.

2.2. Immobilization procedures

A typical immobilization procedure of lipase was as follows: at ambient temperature, 2.72 mmol mixture of DMDMOS and TMOS at r (defined as a molar ratio of organic silane to TMOS) = 4, and 5 μl of 40 mM HCl were mixed in a test tube, to obtain a homogeneous solution derived from the hydrolysis of silane precursors. About 1.4 ml of 100 mM phosphate buffer (pH 7.5) was added into this mixture at 35°C, and then 1 ml of enzyme solution (pH 7.5) containing 300 mg lipase was mixed with the buffered sol and 10 μl methanol solution containing a substrate analogue (128 μmol) for pretreatment. These procedures of blending were carried out within a 0.5 min. The resultant mixture was blended well with 1 g powder of Celite 545 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 equilibrated for 1 day in a desiccator containing various saturated salt solutions at 30°C to adjust the water activity of preparations. The salts used were LiCl (water activity, $a_w = 0.11$), K_2CO_3 ($a_w = 0.44$), $CuCl_2$ ($a_w = 0.69$), and KCl ($a_w = 0.84$).

Deposited lipase on Celite (type A) was prepared by replacing the silane mixture with 0.4 ml of distilled water in the procedure described above.

The immobilization procedure of deposited lipase on hybrid gel-covered Celite (type C) was as follows: firstly, the hybrid gel-covered Celite was prepared without lipase. After lyophilization, it was then blended well with lipase solution. The resultant immobilized preparations were treated according to the same procedure as described above.

The non-pretreated immobilized lipase as a control was prepared by the addition of methanol only.

2.3. Measurement

The esterification of (−)- or (+)-menthol with butyric acid was used as a model reaction. The reaction experiments were carried out at 35°C in 100 ml Erlenmeyer flasks containing 0.1 M of both the substrates and the immobilized lipase (*ca.* 1.4 g) in 20 ml *n*-decane. The reaction was initiated by adding the immobilized lipase into the substrate solution magnetically stirred at 180 rpm. To determine the concentrations of the substrates and the products, (−)-/(+)-menthylbutyrate, the organic samples were withdrawn from the reaction mixture at appropriate time intervals, and were analyzed by a gas chromatograph (Shimadzu GC-14 B). No substrate analogues were detected in liquid samples. This means that the substrate analogues were removed by lyophilization. The initial esterification rate measurements were performed over a 0–6 h period during which the rates remained linear. The enantioselectivity was defined as the ratio of the initial esterification rate of (−)-menthol to that of (+)-menthol.

3. Results and discussion

3.1. Water activity dependence of esterification activities of immobilized lipases

Fig. 1 shows the water activity dependence of the initial esterification rates by (a) deposited lipase on Celite (type A) and (b) hybrid gel-entrapped lipase on Celite (type B) pretreated with (−)-menthol, in the esterification of (−)-menthol with butyric acid. At the water activity for hydration, $a_w = 0.11$, both of the pretreated type A and type B showed the highest activities, and which showed 2.3 and 1.5 times higher activities than the non-pretreated ones, respectively. However, the activities decreased gradually with increasing the water activity, and at $a_w = 0.84$, the activities of pretreated and non-pretreated ones became almost the same. On the other hand, the pretreatment with (−)-menthol did not bring about the improvement of enantioselectivity (Table 1).

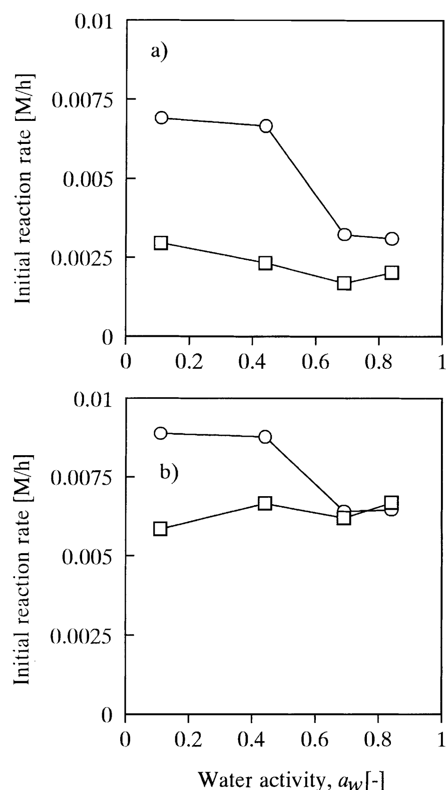

Fig. 1. Water activity dependence of initial reaction rates with immobilized lipases: (a) deposited lipase on Celite (type A) and (b) hybrid gel-entrapped lipase on Celite (type B) pretreated, (○) or non-pretreated (□) with (−)-menthol in esterification of (−)-menthol with butyric acid.

Table 1
Enantioselectivities of deposited lipase on Celite (type A) and hybrid gel-entrapped lipase on Celite (type B) at various water activities, a_w

a_w	0.11	0.44	0.69	0.84
Pretreated type A	10	12	7	7
Non-pretreated type A	14	17	11	10
Pretreated type B	9	6	6	7
Non-pretreated type B	6	5	6	6

It is thought that the enzymes have rigid structures at low water activity, but they become flexible ones with increasing the water content in the vicinity of lipase molecule. Stahl et al. [12] reported that the pretreatment with a substrate analogue induced the activated structure of enzyme. Therefore, it was thought that the immobilized lipase at a low water activity could keep its activated structure, and with increasing the water content, lipase structure became more flexible, which resulted in the deformation of the activated structure. In addition, from the result that the enantioselectivities were not improved by the pretreatment, it was thought that imprinting effect by the incorporation of

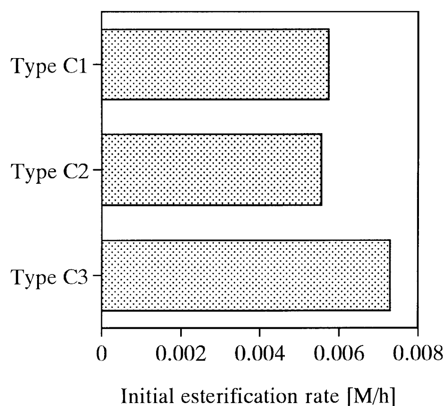

Fig. 2. Comparison of the initial esterification rates by three subtypes of deposited lipase on hybrid gel-covered Celite (type C) hydrated at $a_w = 0.11$ in esterification of (−)-menthol with butyric acid: type C1; non-pretreated (control), type C2; pretreated with (−)-menthol during preparing the hybrid gel-covered Celite, and type C3; pretreated with (−)-menthol during depositing lipase on the hybrid gel-covered Celite.

(−)-menthol into the active site of the lipase molecule was too rough to distinguish between (+)-menthol and (−)-menthol. Thereafter, we prepared all the immobilized lipases at $a_w = 0.11$.

3.2. Comparison of the esterifiacation activity by deposited lipases on hybrid gel-covered Celite

Fig. 2 shows the comparison of initial esterification rates by the three subtypes of the deposited lipases on hybrid gel-covered Celite (type C) in esterificaiton of (−)-menthol with butyric acid. We prepared the three subtypes of type C to investigate the reason of this activation induced by the pretreatment with (−)-menthol. As described above, the preparation procedure of type C composed of two steps; to prepare the hybrid gel-covered Celite in the first step and to deposit lipase on the resultant hybrid gel-covered Celite in the second step. Type C1 was prepared as a control without any additives. Type C2 was prepared by mixing (−)-menthol with precursor sol solution in the first step. Type C3 was prepared by mixing (−)-menthol with lipase solution in the second step. Consequently, type C3 showed the highest

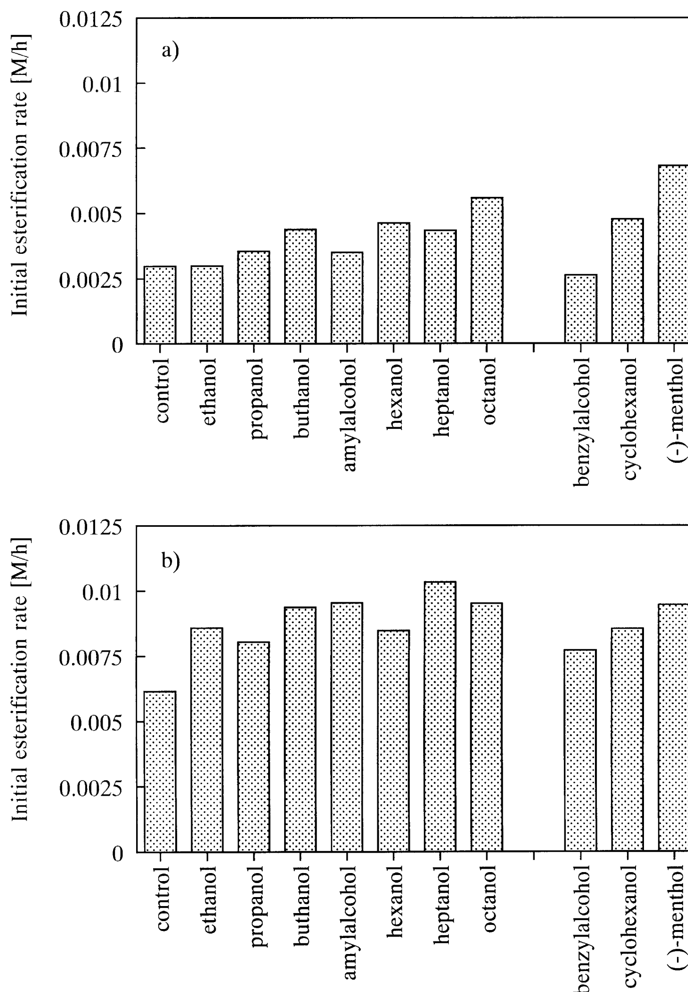

Fig. 3. Effect of hydrophobic groups of alcohol used for the pretreatment of: (a) deposited lipase on Celite (type A) and (b) hybrid gel-entrapped lipase on Celite (type B) hydrated at $a_w = 0.11$ on initial rate of esterification of (−)-menthol with butyric acid.

activity, whereas type C1 and type C2 showed almost the same activities. From this result, it was confirmed that the activation of immobilized lipases was not owing to the enhancement of affinity between substrate and hybrid gel but the activation of lipase molecule itself by the (−)-menthol pretreatment.

3.3. Effect of various alcohols as substrate analogues

Fig. 3 shows the initial esterification rates by (a) type A and (b) type B preparations pretreated with various alcohol molecules as substrate analogues in esterification of (−)-menthol with butyric acid. The activities of both the immobilized lipases were enhanced with increasing chain length of alcohol, and type A pretreated with octanol and type B pretreated with heptanol resulted in the highest activity. In addition, the pretreatment with alcohol molecules having cyclic structure such as (−)-menthol and cyclohexanol also induced the activation of immobilized lipase. From these results, the pretreatment with hydrophobic and bulky alcohol, which showed high $\log P$ values and had circular structures, facilitated the activation of lipase. We propose two possible reasons in terms with this activation. One is that the pretreatment with the hydrophobic molecule brought about the hydrophobic atmosphere in the vicinity of lipase molecules and this made the lipase structure the activated one by the hydrophobic interaction. Another is that these hydrophobic and bulky substrate analogues are incorporated into the active site of lipase molecule locally and this contact also enhances the affinity between the active site of lipase molecule and the substrate molecule.

3.4. Effect of butyric acid as a substrate analogue

Table 2 shows the initial esterification rates by the deposited lipase on Celite (type A) and the hybrid gel-entrapped lipase on Celite (type B) pretreated with butyric acid in esterification of (−)-menthol with butyric acid. The pretreatment of type A with butyric acid brought about a remarkable enhancement of esterification activity, whereas that of type B did not. It was thought that this activation resulted from the hydrophobic environment in the vicinity of lipase molecule induced by the pretreatment with butyric acid. From this reason, pretreatment of type B with butyric acid did not bring about such activation because type B was hydrophobic enough by the organic–inorganic hybrid silica matrix. From this result, the use of butyric acid did not bring about the activation induced by the incorporation of a substrate analogue into the active site.

Table 2
Comparison of the initial esterification rates by deposited lipase on Celite (type A) and hybrid gel-entrapped lipase on Celite (type B) pretreated with butyric acid and hydrated at $a_w = 0.11$ in esterification of (−)-menthol with butyric acid

	Pretreated (M/h)	Non-pretreated (M/h)
Type A	0.00547	0.00296
Type B	0.00756	0.00726

4. Conclusions

We applied the pretreatment method with a substrate analogue to the hybrid gel-immobilized lipases derived from the sol–gel method. The pretreatment of the hybrid-gel-immobilized lipase with a substrate analogue brought about a further enhancement of esterification activity only at a low water activity, but the enhancement of enantioselectivity was not shown by this pretreatment. The hydrophobic and bulky molecules as a substrate analogue were considerably effective for the activation of immobilized lipases. Now, a more detailed study on this activation mechanism is under investigation.

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