IMMOBILIZATION OF LIPASE WITH POLYION COMPLEX AND ITS ESTERIFICATION ACTIVITY IN ORGANIC MEDIUM

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A novel method to immobilize lipase on inorganic particles or on an inorganic porous plate is proposed. The method consists of entrapment of lipase into polyion complex, followed by fixation of the complex on the inorganic supports. It was simpler and offered much saving in time, comparing with conventional entrapping and covalently binding methods. Both the lipases immobilized on the particles and the plate showed high catalytic activity for esterification in organic medium, and the activity depended on kinds of polyion complex and water contents in the immobilized-lipases. The immobilized-lipases sustained their activity up to a significantly high temperature of 60°C, which suggests that lipase and polyion complex interact to enhance thermal stability of lipase. Activity holding with repeated uses was also studied.

Introduction

Recently, development of methods to use enzymes in organic solvents has attracted considerable attention. An enzymatic system surviving in organic solvents has some advantages such as extensive application of enzyme to reactions involving substrates poor in solubility and stability in water, and equilibrium shift of hydrolytic reactions to synthetic ones (Klibanov, 1986; Dordick, 1989; Fitzpatrick and Klibanov, 1991). Thus, several systems have been developed to apply enzymes to reactions in organic solvents, *e.g.*, reverse micellar systems (Menger and Yamada, 1979; Levashov *et al.*, 1982; Fletcher *et al.*, 1985; Luisi and Laane, 1986), aqueous-organic two phase systems (Chen *et al.*, 1987; Eggers, 1989) and immobilized enzyme systems (Yokozeki *et al.*, 1982; Nakatsuka, 1987; Tanaka and Sonomoto, 1990; Murakata *et al.*, 1993; Sato *et al.*, 1994). Of these systems, the last ones have advantages that enzyme can be recovered easily and used repeatedly. To be catalytically active in organic solvents, however, immobilized enzymes should contain enough enzyme to be active and enough water to sustain enzyme active. In addition, industrial application requires preparation as easy as possible.

From the above viewpoint, we considered a method of immobilization accompanying polyion complex (PIC) formation as effective, because PIC entraps various compounds together with water, insoluble in water and usual organic solvents, and to

be formed easily in such a way of mixing solutions of cationic and anionic polymer. Enzymatic reactions using PIC containing an enzyme and microorganisms have actually been reported by a few workers (Osada *et al.*, 1982; Kokufuta *et al.*, 1982). However, these were carried out in aqueous media, and those done in organic media are nonexistent as far as we know. PIC is usually formed as suspensions or lumps of gel which are so sticky and lumpy as to be difficult to handle. Thus, it is not practical that enzyme contained in PIC is used as such. Further, PIC as a support to immobilize enzyme is likely more expensive than others having conventionally been used, *e.g.*, celite. The usefulness of an immobilized enzyme, however, should be evaluated from various aspects in addition to such an economic one.

In the present work, for convenience of use, we fixed PIC containing enzymes on inorganic supports as follows: fixation on inorganic particles (lipase immobilized on inorganic particles, LIP) and on an inorganic plate as a film (lipase immobilized as a film, LIF). Esterification was carried out in organic media and effects of factors that were considered as controlling the enzymatic activity, such as the kind of PIC and its water content, were studied. In addition, activity sustainability against elevated temperature and repeated use were investigated.

1. Experimental

1.1 Chemicals

Lipase (300×10⁴ unit/g) was purchased from Nagase Biochemical Ltd. It was the same as that used in our previous work (Murakata *et al.*, 1993).

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Fig. 1 Preparation procedure of LIP and LIF

Polyacrylic acid (PAA), poly(methacrylic acid) (PMAA) and potassium polyvinylsulfate (PVSK) were used as anionic polymers, and polyethylenimine (PEI) and poly(4-vinylpyridine hydrochloride salt) (PVPy⁺) as cationic polymers. PAA, PVSK and PEI were purchased from Wako Pure Chemical Ind., and used as received. PMAA was synthesized from a corresponding monomer by a conventional radical polymerization. PVPy⁺ was obtained by a reaction of poly(4-vinylpyridine), synthesized in a way similar to that of PMMA synthesis, with hydrochloric acid. The polymers were dissolved in 0.067 M phosphate buffer solution (pH=7.2) to obtain the starting polymer solutions. The polymer solutions contained 0.1 M of their corresponding monomers and 4 wt% of NaCl, which was added to promote PIC formation. Lauric acid and lauryl alcohol used as substrates were purchased from Kanto Chemical Co. All the organic solvents utilized were distilled before use.

1.2 Preparation of LIP and LIF

1.2.1 Preparation of LIP Vycor glass rods (mean pore diameter < 10 nm) were used for the preparation of inorganic particles. The glass rods were ground to particles to be washed first with 30% H₂O₂ aqueous solution and then three times with deionized water, and dried at 40°C for 6 h. They were sieved and those having diameters ranging from 105 to 149 μm were used, unless otherwise stated. **Figure 1(a)** shows the preparation procedure of LIP. Glass particles weighing 2 g and 15 ml of anionic-polymer solution containing 100 mg of lipase were put in a glass vessel of 50 ml inner volume. Then, 15 ml of cationic-polymer solution was added dropwise to

the solution while the mixture was stirred vigorously with a magnet driven stirrer. By addition of the cationic-polymer solution, PIC formed immediately and adhered on the glass particles. The stirring was continued for a further 30 min so that adhesion proceeded sufficiently. After that, the mixture was filtrated with suction by using an aspirator to collect LIP. The suction was continued for a constant time of 30 min to remove excess water from LIP and to keep LIP water content constant at every preparation.

1.2.2 Preparation of LIF A filter glass of which pore diameter ranged from 20 to 30 μm was used as the inorganic support. **Figure 1(b)** shows the vessel used and the procedure for the preparation. The filter glass was 4 cm square in a size and settled at the bottom of the vessel. The vessel was charged first with 10 ml of cationic-polymer solution containing 100 mg of lipase and then quickly with 10 ml of anionic-polymer solution. After standing for 30 min, the solution was sucked from the vessel outlet located below the filter glass. The suction was continued for a constant time of 30 min in a way similar to LIP preparation. The filter glass covered with PIC was taken out from the vessel and used as LIF. It was observed with a scanning electron microscope (SEM, Jeol T-330) how LIF was covered by PIC. For both LIP and LIF preparation, the filtrate was collected and its absorbance at 280 nm was measured to determine the protein concentration in it. Consumed rate of fed protein was calculated from the protein concentration to be used as an indication of the immobilized rate of lipase.

1.3 Esterification activity measurements

Fig. 2 Reactors employed for esterification catalysed with LIP (a and c) and LIF (b)

1.3.1 Control of water content in LIP and LIF

LIP and LIF prepared as described above contained significant amounts of water and their enzymatic activity was considered as being affected by its amount. Thus to discuss the effect on their enzymatic activity, they were controlled in water content with vacuum-dehydrating and employed in the reaction.

Controlling and determination of the water content were carried out as below. LIP, after dehydration for an arbitrary time from 0 to 12 h, was sampled and weighed (W_T) and the rest was applied to the reaction. The sampled part was vacuum-dehydrated again until reaching a constant weight (W_D), which was defined as its dry weight. In case of LIF, two pieces of LIF were prepared and vacuum-dehydrated as LIP was treated. One was weighed (W_T) and vacuum-dehydrated again to measure its dry weight (W_D). The other was used for the esterification. The water content of LIP and LIF employed in the esterification was determined to be $[(W_T - W_D) / W_T] \times 100$.

1.3.2 Esterification experiments

For all the esterification experiments, a benzene solution containing 0.1 M of lauric acid and 0.1 M of lauryl alcohol was used as a substrate solution. **Figure 2(a, b, c)** shows the reactors employed in esterification. The reactors were immersed in a water bath to control reaction temperature. The reaction temperature was kept constant at $25 \pm 0.1^\circ\text{C}$, except for the case that the activity sustainability against elevated temperature was studied. Conversion was determined by measuring the amount of the ester formed in the solution with a thin layer chromatograph equipped

with a hydrogen flame ionizing detector (Iyatron, TH-10). The esterification experiments with LIP were carried out by using reactor **a**. The reactor was a cylindrical glass vessel of 50 ml inner volume. LIP and 30 ml of the substrate solution were placed in the reactor. During the esterification, the reaction mixture was stirred with a magnet-driven stirrer. Since the activity should be compared on the basis using the same amount of the enzyme, LIP of a weight corresponding to 1.5 g of the dry weight was used in the experiments for all cases.

For the esterification with LIF, reactor **b** was used. It consisted of a glass vessel of 200 ml inner volume and a shaft connected to a geared motor. LIF adjusted in water content was attached to the shaft with a tilt angle of 10 deg. against the horizontal direction. The substrate solution of 100 ml was put in the reactor and esterification was done by stirring the solution using the LIF as a stirrer at a rotation rate of 98 rpm.

1.3.3 Esterification activity sustainability

Activity sustainability against elevated temperatures was studied for LIP with the same procedure as stated above. To compare the activity change with that of native lipase, additional reaction experiments were carried out in an aqueous organic two-phase system: 2 ml of the buffer solution containing 45 mg of lipase and 30 ml of the substrate solution were fed into the reactor and the mixture was stirred vigorously during esterification. The amount of the lipase used was almost the same as that for the esterification with LIP.

Activity sustainability against repeated uses was studied for both LIP and LIF. As for LIP, when reactor **a** was employed, it was pulverized to finer

particles along with an increase in times of repeated uses. To avoid effects of the pulverization, esterification was also carried out using reactor **c** which was a glass column of 8 mm in inside diameter and 10 cm in length. In this experiment, vycor glass particles with rather large diameters ranging from 149 to 250 μm were used to prepare LIP so that the liquid flow pressure drop through the column was low and the substrate solution in the column was able to flow at a high rate. 1.37 g of LIP corresponding to 1 g of the dry weight was loaded in the column and 30 ml of the substrate solution was cycled at a rate of 32 ml/h. In any case for LIP and LIF, after esterification for 24 h, they were recovered and washed with benzene to remove the remaining substrates and products. The water formed was removed with methods used depending on the material: the LIP was washed with benzene and placed on filter paper and filtered for 30 min by an aspirator; LIF was washed with benzene and vacuum-dehydrated for 1 h under a pressure of 200 mmHg. The LIP and LIF were applied repeatedly to esterification in this way.

2. Results and Discussion

2.1 Characterization of LIP and LIF

2.1.1 LIP characterization Results of experiments to form PIC showed that three kinds of PIC enabled the enzyme to be entrapped well and were formed using the following polymer pairs: PAA and PEI; PMAA and PEI; PVSK and PVPy⁺. Whether or not the PICs mentioned above adhere to supporting particles was investigated further. As a result, the PICs were found to adhere successfully when supporting particles were hydrophilic and to be formed separately from the particles as lumps of gel-like material without adhering when the particle surface was hydrophobic. Thus, the PICs adhered to the Vycor glass which was hydrophilic and other commercially available inorganic supports such as Al₂O₃ and TiO₂ can be used as well. Under the present conditions, on average about 70 and 50% of the enzyme added were immobilized to PICs made from pairs of PAA and PEI, and PMAA and PEI, respectively. The pair of PVSK and PVPy⁺, however, did not allow a determination of the amount of lipase immobilized. It was because the UV absorption measurement could not be applied to the filtrate owing to its turbidity. The turbidity was responsible for the relevant PIC which did not have adhesive force great enough to fix completely on the supporting particles, and passed through a filter. Similar turbidity was observed in the preparation of LIF using the same PIC. The LIP obtained were white particles and easy to handle.

2.1.2 LIF characterization LIF was prepared employing PIC which was found to have strong

adhesive force by the LIP characterization described above. Thus, a pair of PMMA and PEI was employed for the preparation. The appearance of the prepared LIF showed that the PIC covered the whole surface of the filter glass though nonuniform in thickness. From SEM observation, the PIC was found to deposit as a layer on the surface of the filter glass but not grow its layer into the inside of the filter glass. In spite of the SEM image that the PIC layer did not invade adequately into the filter glass, the PIC was deposited so strongly that no separation was observed for the esterification runs, except swelling and limited separation for the use in aqueous media within several hours. Thus, its application to reactions in aqueous media requires some treatments for waterproofing. By this method, about 75% of the lipase added was immobilized. The value was a little greater than that for LIP.

2.2 Esterification with the immobilized-lipases

2.2.1 Esterification with LIP **Figure 3** shows the reaction-time courses obtained using LIP, prepared with the PIC formed by a pair of PMMA and PEI, containing various water contents. From the figure the courses are found to have depended greatly on the water content. Similarly, the other reaction-time courses by LIPs prepared with different PICs depended on the water contents. Further they depended on the kind of PIC. It is interesting to know how LIP was controlled by these experimental factors in their activity. To make clear the point in question, the conversion at 24 h after the reaction start was employed as an indicator of LIPs' activity to be discussed as below. Although the initial rate can also be the typical activity indication, it was difficult owing to an induction period accompanying many of the courses. The conversion at 24 h was employed, because the time was far beyond the induction period and was prior to time of the reaction reaching to its equilibrium. **Figure 4** shows conversions for 24 h esterification at various water contents. All the LIPs had the maximum conversion against change in the water content. Similar behavior has been reported for other enzymatic reaction systems in organic media such as reverse micellar systems (Kavanov *et al.*, 1988; Martinek *et al.*, 1989) and aqueous organic two-phase systems (Shield *et al.*, 1986; Kise *et al.*, 1987; Eggers *et al.*, 1989). It has been explained generally in terms of equilibrium shift favoring reaction progress to the synthetic side and denaturation of enzyme. Such an explanation may also be applied to the behavior of LIP, but additional effects should be taken into account because PIC changed its state from swelling to shrinking as the water content decreased. PIC has been thought to be composed of an intricate networks formed by electrostatic attraction between the charged polymers (Michaels, 1965). The networks have pores, which shrink or enlarge according to removal

Fig. 3 Reaction-time course with LIP (PMMA-PEI) at various water content

Fig. 5 Effect of water content of LIF on conversion

Fig. 4 Effect of water content of LIP on conversion PICs: (○) PMMA-PEI, (△) PAA-PEI, (△) PVSK-PVPy⁺

or filling of water. Thus, when the reaction proceeds in PIC, its rate, *i.e.* the conversion must become greater or less depending on PIC's water content because the substrates diffuse fast in enlarged pores and slow in shrunk ones. Under conditions where LIP contained water over 10 or 15%, although pores were enlarged to allow the substrates to diffuse fast, an excess of water might interfere with contact of the substrates to the enzyme owing to hydrophobicity of the substrates and eventually decrease the conversion against its increase. Under the counter-conditions, the substrates diffused slow because of pore shrinking, but the substrates might contact easily the enzyme to react faster as the water content increased. The conversion depended also on the kind of PIC: The maximum conversion and the conversion dependence upon the water content were different among the PICs. The activity difference was considered as arising from differences in PIC network structure such

Fig. 6 Effect of reaction temperature on conversion

as pore size distribution. Further, interaction between lipase and PIC may be responsible for the activity difference. In the present work, no additional experiment to make clear the network structure and the interaction was carried out. As for the interaction, however, it was confirmed indirectly by experiments on activity sustainability at elevated temperature which will be mentioned below.

2.2.2 Esterification with LIF Change in the esterification activity of LIF was studied also in connection with the water content. **Figure 5** shows the conversion change after esterification for 24 h. The conversion decreased linearly with water content, which is different from the LIP properties shown in Fig. 4. Such a property can be explained as follows. The filter glass used for LIF held far larger amounts of water than LIP, even at the lowest water content of LIF. Therefore, further increase in the water content affected the activity of LIF to decrease the conversion.

2.3 Activity sustainability at elevated temperature

Activity sustainability at elevated temperature was studied for LIP. **Figure 6** shows the conversion versus the temperature in the esterification for 24 h. At 25°C, conversions for two kinds of LIP and for the

aqueous organic two-phase system lay in a range from 60 to 70%. All the systems decreased the conversion at higher temperatures and the conversion decrease was the most significant for the two-phase system for which conversion dropped to zero at 60°C. In contrast to such a great activity decrease in the aqueous organic two-phase system, the LIPs sustained their activity at significantly high temperatures. For example, the pair of PMMA and PEI gave considerably high conversions of 72% at 25°C and 64% at 60°C. This indicates that about 90% of the activity at 25°C was sustained even at 60°C. Thus, it is evident that lipase was thermally stabilized by entrapment into PIC. The thermal stability is probably due to an interaction between lipase and PIC. Electrostatic interaction and hydrogen bonding between lipase and PIC can be speculated from the structure of the polymers, although details of the interaction are not clear at the present stage.

2.4 Activity sustainability during repeated use

Activity sustainability with repeated use was studied for both LIP and LIF, which were made from a typical PIC prepared with a pair of PMAA and PEI. Figure 7 shows the conversion against repeated times of runs which were duplications of the reaction continuing for 24 h.

In the case of LIP, the conversion, when reactor c was employed, remained almost constant against the number of times repeated, while it decreased with an increase of the repeated times when reactor a was used. For employment of reactor a, pulverization of LIP was observed and became more significant with an increase of the number of times repeated, whereas no pulverization was observed for employment of reactor c. These results indicate that LIP can sustain its activity with repeated use as long as it was not mechanically broken down.

In the case of LIF, the conversion increased initially and decreased gradually after three repetitions. Since any mechanical damage of LIF, including peeling of PIC from the filter glass, was not observed during the repetitions, it was not a cause of the conversion change. The conversion change may mainly be caused from change in water content of LIF with repeated use and may be explained as below. LIF was vacuum-dehydrated under definite conditions after each repetition as stated previously. LIF was possibly dehydrated at every vacuum-dehydration more than the water amount formed in LIF and LIF's water contents were regarded as decreasing upon every repetition of LIF. Thus every dehydration after first few repetitions allowed LIF to raise its activity, because LIF before application to repetition contained significant amounts of water as described before, and its water content decreased after every repetition towards a value to attain its maximum activity. Further dehydration along with

Fig. 7 Conversion change against number of repeated use of LIP with reactor a (●) and reactor c (○) and LIF with reactor b (△), where the conversions were for repeated times of esterification continuing for 24 h

the successive use of LIF decreased LIF activity. The above results show that both LIP and LIF can be used for several repetitions.

In conclusion, besides the ease of preparation, the present method was found to have the following advantages: 1) PIC can hold enough water to sustain lipase active and can be used repeatedly, 2) PIC stabilized thermally lipase with the entrapment and can be used at significantly higher temperatures. 3) The present method may be applied to immobilization of other biocatalysts through enabling them to be entrapped with less degree of denaturation. 4) The present method is also applicable to a membrane reactor.

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