DEVELOPMENT OF IMMOBILIZED ENZYME ENTRAPPED WITHIN INORGANIC MATRIX AND ITS CATALYTIC ACTIVITY IN ORGANIC MEDIUM

SHIMIO SATO, TADAHIRO MURAKATA,
MOTOHIKO OCHIFUJI, MANABU FUKUSHIMA AND
TOHRU SUZUKI
Department of Materials Science and Engineering,
Yamagata University, Yonezawa, 992

Key Words: Biochemical Engineering, Entrapping Enzyme, Inorganic Matrix, Sol-gel Process, Non-aqueous Medium

A method for entrapment of enzymes within an inorganic matrix was developed using a sol-gel process. Such entrapping immobilization of biocatalysts within inorganic matrices has some benefits but it is rather difficult to apply in contrast to other types of immobilization and therefore has not been commercialized. The method developed was applied to entrapping lipase within silica beads. The entrapped lipase was six times more active for esterification in an organic medium than lipase immobilized over silica glass with a binding method which has conventionally been used. The high activity may come from the characteristics of the present method. It can entrap the enzyme with less denaturation and provide the matrix with physical properties suited to the reaction, e.g. an abumolance of macro pores.

Introduction

Enzymes have several ideal characteristics that enable them to be used as catalysts. They have, however, some shortcomings which prevent them from being efficiently used. Immobilization has thus been studied as a countermeasure. Immobilization is classified into two main categories[9]: binding to a support and entrapping within a matrix. Although the latter is superior to the former in some aspects[3], it has not been applied as often as the former, the reasons being the difficulty[2] in forming polymeric networks and matrices and that immobilization within them must be done simultaneously under mild conditions so that the enzyme does not denature. Thus, matrices applied to the entrapment have been limited to organic ones which can be synthesized under conditions mild enough for the enzyme to retain its activity. In general, inorganic materials have advantages such as stronger mechanical strength and higher resistance to organic solvent. If a method enabling enzymes to be entrapped in inorganic matrices is developed, immobilized enzymes with increased resistance to organic solvent corrosion and mechanical stress can be prepared. Although a method for entrapping yeast cell[10] in silica gel has been developed, it cannot be applied to enzymes because they are considered to be more susceptible to adverse conditions. An enzyme's susceptibility is due to the fact that it lacks ramparts like a cell wall and that it is not a dormant form like yeast which can allow toxic materials to pass through.

Incidentally, a sol-gel process utilizing alkoxide has been noted as a promised material processing technique[8]. It may be useful as a method of biocatalyst entrapment because inorganic polymeric networks can be prepared even under mild ambient conditions. Further, enzymatic reactions in organic media are interesting from various aspects as described in our previous paper[5].

The objectives of the present study are: (1) to show how enzyme can be entrapped within an inorganic matrix using the sol-gel process; (2) to show how the entrapped enzyme alters its catalytic activity to a reaction in organic medium depending on the physical properties of the matrix; and (3) to demonstrate how the entrapped enzyme is active in a reaction when compared with other immobilized enzymes prepared with conventional methods.

* Received April 18, 1994. Correspondence concerning this article should be addressed to S. Sato.

1. Experimental

The present immobilized enzyme is called enzyme entrapped within inorganic matrix (EEIM). For activity comparison among immobilized enzymes (IEZs) prepared with various methods, two other kinds of IEZs were made with conventional methods. One, the enzyme entrapped into organic polymer matrix (EEOM), used a different kind of matrix and a similar immobilization mode. The other, the enzyme bound to inorganic support (EBIS), used a different kind of an immobilization mode and a similar kind of support. IEZs were all prepared at room temperature. The entrapped enzyme was lipase. The reaction catalyzed by the IEZs is esterification of lauryl alcohol with lauric acid in benzene[5] at a constant temperature of 298 K.

1. 1 Materials

Tetraethyl orthosilicate (TEOS) was used as the alkoxide to form the inorganic matrix of EEIM and was a reagent grade. Lipase was the same as in our previous

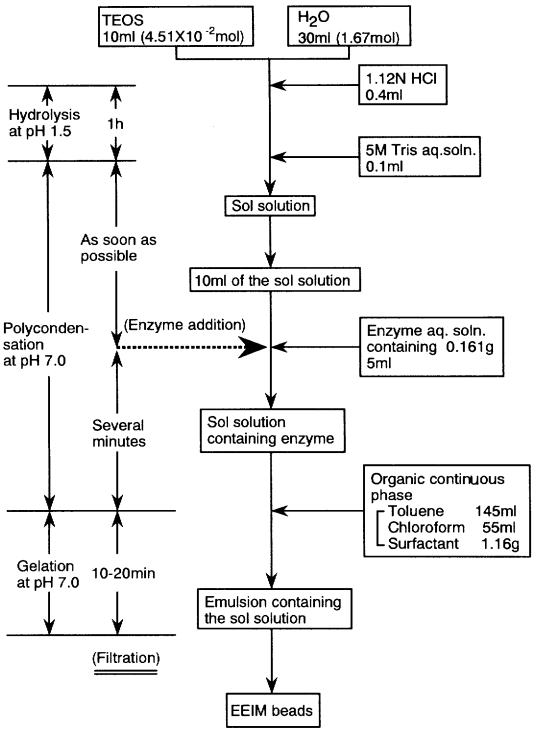

Fig. 1 A flow chart of preparation sequence of EEIM beads

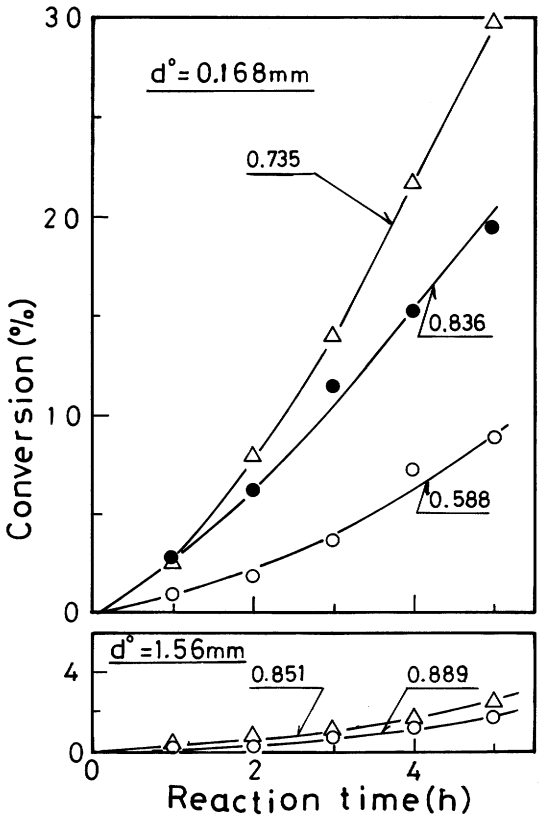

Fig. 2 Effects of diameter(d^0) of EEIM beads in non-dehydrated state and dehydration degrees represented by water content, denoted by the numerals in the figure, on time courses of esterification

work[5]. Deionized water was employed through out preparation of the IEZs. Distilled benzene was used as the organic reaction medium. For EEIM preparation, various bases, *i.e.* aqueous ammonia, pyridine, triethylamine and Tris (hydroxymethyl) aminomethane were used as catalysts for the gelation during a sol-gel process. Studies of which base enabled to prepare EEIM without denaturation of the enzyme were made. Porous silica glass (Vycol Glass, Corning Glass Works) was utilized to prepare EBIS particles. It was purchased as rods and crushed into particles which passed through a 140 mesh screen. The other chemicals were of reagent grade and used as received.

1. 2 Enzymatic reaction

The reaction was studied batchwise with a cylindrical reactor of 5.0×10^{-2} l inner volume of which the interior surface was treated to make it hydrophobic. The experimental procedure and conditions were similar to those in our previous report[5], being as follows. The reaction was started by adding an IEZ of a given amount, to be described later, into 4.0×10^{-2} l of benzene solution containing 0.1 M of the acid and the alcohol, respectively. Several microliters of the solution were taken out periodically during the reaction. The ester concentrations were determined with a thin layer chromatograph equipped with a hydrogen flame ionizing detector. With these concentrations, substrate conversions can be calculated because there is no formation of by-products. The initial esterification rate was estimated using the conversion changes up to two hours of a reaction time.

1. 3 Preparation of conventional IEZs

EEOM beads were prepared in the same way as described in our previous report[5], corresponding to A-IEB according to the previous definition. Each kilogram of beads contained 0.780 kg-water and 1.5×10^{-2} kg- lipase. Mean diameter was 35.5 μm. 4.5×10^{-3} kg was used for each reaction.

EBIS particles were prepared as follows[3]: The porous glass particles were converted to the alkylamino derivative using 3-aminopropyl triethoxysilane; and lipase was bound covalently to the derivative using glutaraldehyde. The EBIS particles thus prepared contained 0.224 kg-water and 1.92×10^{-2} kg-lipase per unit kilogram. 2.0×10^{-3} kg, being adjusted in their water contents, was used for each reaction.

1.4 Preparation of EEIM

Figure 1 shows the sequence of EEIM preparation. Preparation proceeded via the following reactions: hydrolysis of TEOS to its hydrolysates; polycondensation of the hydrolysates to sol particles; and then gelation by linking the sol particles together. Enzyme was added as these reactions progressed. The overall enzymatic activity of EEIM depends on the physical properties of the gel and on the degree of denaturation occurring with entrapment. The physical properties of the gel are controlled by the extent to which the reactions progress and therefore eventually by the overall conditions of preparation. The degree of denaturation is dominated by the preparation conditions

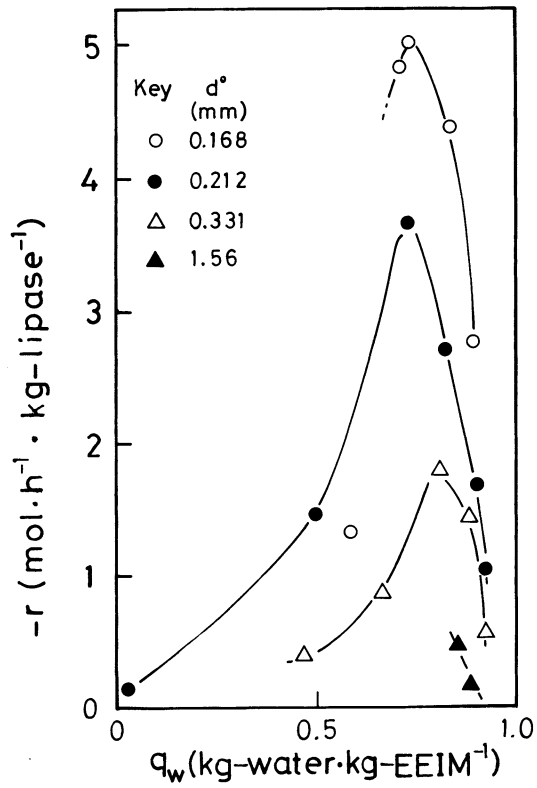

Fig. 3 Effects of bead diameter (d^0) of EEIM in non-dehydrated state and their dehydration degrees represented by water content (q_w) on initial esterification rate ($-r$)

after enzyme addition. Thus, the preparation sequence and conditions were designed and chosen so as to minimize denaturation and allow reactions to progress up to favorable extents.

To avoid denaturation as much as possible, water was used as a solvent for the process. Since TEOS may denature[10] the enzyme added later in the sequence, it was thoroughly hydrolyzed using a large excess of the water. The large excess of water is also advantageous for entrapping the enzyme. Since lower concentration of the hydrolysate retards polycondensation, the enzyme added later can afford to disperse over the sol solution before development of linking networks. To obtain a gel mechanically strong enough for EEIM, the hydrolysis was done at pH 1.5 using hydrogen chloride as catalyst on the basis of a knowledge of the sol-gel process[2, 7]. For making the gel so densely cross-linked that the enzyme was not able to leak out, polycondensation was done using a base catalyst[7]. From tests among the bases mentioned before, Tris(hydroxymethyl)aminomethane was found to be most suitable. The reason is that it worked as a Tris buffer of pH 7.0 associating with the hydrogen chloride of the hydrolysis catalyst and therefore did not make the enzyme denature in contrast to the others. The time when sol solution was neutralized by the base and linking networks were ready to be developed was exactly suited to enzyme addition. Thus, immediately after the base addition, the enzyme was introduced as a solution of concentration 32.2 mg-lipase·l^{-1} to the neutralized solution of volume 1.0×10^{-2} l.

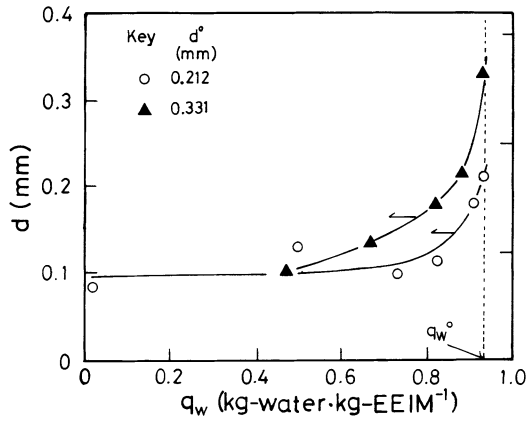

Fig. 4 Change in bead diameter(d) of EEIM with variation in hydration degrees represented by water content (q_w)

The resultant solution gradually became more viscous with time owing to polycondensation. The viscosity then increased exponentially around the phase transition from a sol to a gel. To obtain EEIM as beads, the solution had to be dispersed into the organic continuous phase before the phase transition. The dispersed droplets gelled to form beads within several to about twenty minutes. The beads were separated by filtration and kept in a refrigerator.

The prepared EEIM beads were transparent and almost spherical. Their diameters were controlled to the extent that they were nearly reproducible, being smaller at higher concentrations of the surfactant, and followed a first order algebraic function of concentration. The contents of water and lipase were calculated to be 0.932 kg and 1.02×10^{-2} kg per kilogram of beads, respectively. For each reaction experiment, 1.9×10^{-3} kg of the EEIM beads were taken from the refrigerator. Their water contents were adjusted with evacuation at 2.67 kPa for different periods of time and then loaded into the experimental apparatus. Water content was varied from 0.325 to 174 kg per kg-lipase, an amount far more than the minimum water amount required for enzymatic activity.

1. 5 Determination of physical properties of EEIM

Diameters of EEIM beads were calculated as arithmetic mean values of microscopic observation of one hundred beads. Since the EEIM beads contains water, their pore size distribution had to be measured after drying. Traditional drying caused pore contraction, which interfered with the measurement of inherent pore size distribution of the EEIM beads. Thus, a supercritical fluid drying proces was applied. EEIM beads were immersed in 7.0×10^{-2} l ethanol in an autoclave of 0.1-l inner volume. The autoclave was heated up to the critical temperature of ethanol and the vapor in the autoclave was the released while the autoclave temperature was maintained at the critical temperature. The dried beads had almost the same diameters as the original wet beads and did not appear to shrink. Thus, little pore contraction was considered to occur. Pore size distribution was measured with a porosimeter.

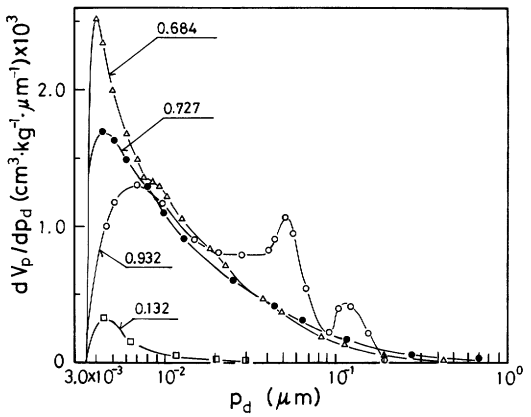

Fig. 5 Change in pore volume distribution (dV_p/dp_d) of EEIM beads having a diameter(d^0) of 0.331 mm with variation in dehydration degrees represented by water content (q_w)

2. Results

2.1 Catalytic activity of EEIM beads

The EEIM beads thus prepared exhibited significantly high activity. They became opaque as the reaction proceeded. They stayed spherical within a few reaction hours but swelled later and finally crushed. The water produced by the reaction was considered to be retained within the EEIM beads because there was no indication of bleeding from the beads into the benzene solution. Consequently, the changes in EEIM appearance were attributable to the water. Such an assumption was confirmed from experimental results showing that the EEIM bead changes accompanied the reaction and occurred in the presence of water without the reaction.

The EEIM changes indicated that mechanical strength was not as sufficient as would be expected and that improvement was necessary in the future. The crushed EEIM beads, however, were able to be recovered easily and found to be as active as the original beads. Thus they can be reused a number of times without any improvement, one of the benefits of immobilized enzymes.

Figure 2 shows time courses under the influence of two EEIM beads of which diameters were the smallest and largest among the beads prepared in the present work. The figure shows that the catalytic activity of EEIM depended heavily on the diameter of the beads. It also indicates that water contents, i.e. dehydration degrees, affected considerably the catalytic activity of EEIM.

The catalytic properties of EEIM stated above were discussed in more detail. Initial rates were calculated from data points on the time courses within two hours where no deformation and crushing of EEIM beads were observed. They were plotted against water content q_w using beads diameter as a parameter. **Figure 3** shows the results. From the figure, EEIM was found to be more active with small beads and maximum catalytic activity was observed at a dehydration degree of about $q_w = 0.7$ kg·kg-EEIM^{-1}.

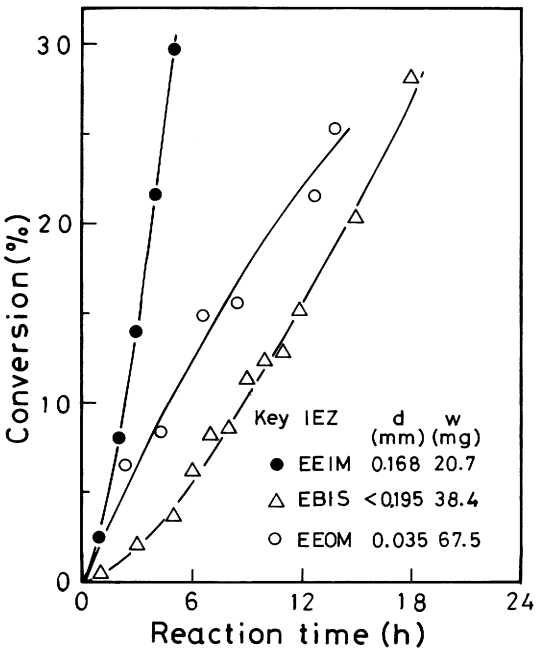

Fig. 6 Comparison of esterification activity of different immobilized lipases

2. 2 Change in EEIM's physical properties with dehydration

EEIM shrunk with dehydration while they roughly maintained their spherical shape as long as dehydration was not extreme. **Figure 4** shows the relationship of diameter of EEIM beads with their water contents. The diameter decrease exponentially as dehydration progresses.

Figure 5 shows pore volume distributions of EEIM beads non-dehydrated and dehydrated to various degrees. The figure indicates that the EEIM beads had numerous macro pores and the distribution changed considerably along with dehydration. On the whole, relative populations of smaller pores increased with greater dehydration which suggests that the pores contracted further as dehydration became severer.

2. 3 Activity comparison among IEZs

Since IEZ's activity depends on various experimental conditions such as pH at which they are prepared, rigorous activity comparison needs a rather complicated discussion which is beyond the present objectives. For straightforward comparison, the IEZs were prepared individually so as to attain their own highest activity and were tested. EEOM and EBIS were made under conditions where they had maximum catalytic activity with adjustment of diameter[4] and of water content[1], respectively. EEIM was represented by the most active beads prepared in the present work.

Figure 6 shows the time courses observed for the IEZs. Their initial reaction rates were evaluated, being converted to the rate, $-r$, per unit weight of lipase loaded and compared. As a result, EEIM had a rate of 5.01, EEOM 1.28 and EBIS 0.736 in units of mol·h^{-1}·kg-lipase^{-1}. EEIM is smaller in size than EEOM, being advantageous for less intra mass transfer against EEOM. Such an advantage,

however, is not considered to be so great as to change the activity order observed above owing to little size difference. Thus EEIM is most active under the present conditions.

3. Discussion

The higher activity of EEIM at smaller bead diameter can be expressed in terms of the decrease in intra particle diffusion resistance of the components affecting the reaction rate. The components may include the benzene of the reaction medium because benzene can alter the reaction rate with change in its concentration within EEIM pores through effects such as the variation in partition equilibrium of the substrates between inner and outer pores.

The intra particle diffusion resistances may also be responsible for an occurrence of an activity induction period, as shown in Fig. 2, where conversion time courses exhibit a concave response. As a matter of fact, no induction period was found when crushed EEIM beads recovered after the reaction were reused in further reactions.

The occurrence of maximum rate against change in the dehydration degree may be explained as follows, when a fact that the water around the lipase was over the minimum requirement for its activity, even at the largest degree of dehydration, is considered. As dehydration progresses, the water in the pores is squeezed out and the pores contract. The water squeezing and pore contraction may change the following three factors in the pores: substrate transfer rate; esterification equilibrium; and enzymatic activity. Water squeezing favors the first and second factor because it makes the interior of the pores hydrophobic. It affects the third factor on either side because lipase tends to favor hydrophobic environments but is affected negatively by such environments owing to denaturation. Pore contraction negatively affects the first factor, having no effect on the third one unless it is so great as to deform the lipase structure and also having no effect on the second one. Thus, at first the favorable effects prevail but then the negative effects prevail as dehydration proceeds. As a result, EEIM bead attained their maximum activity at a certain degree of the dehydration.

Considerably great activity of EEIM may imply that the present immobilization denatured the enzyme a little and was able to give the matrix the physical properties favorable to the enzymatic reaction. The favorable physical properties were regarded as mainly being responsible for the significantly large populations of macro pores. Macro pore formation may be attributable to the effect of lipase advantageous to the growth of silica sol particles[6].

Conclusion

Immobilization of enzymes by entrapment within an inorganic matrix was investigated. It was applied to lipase entrapment in silica gel. Esterification activity of the entrapped lipase was studied in an organic medium, being compared with that of other immobilized lipases prepared with conventional methods.

When beads of the present entrapped lipase were dehydrated to different degrees, their catalytic activity changed and reached a maximum at a certain degree of dehydration. They were more active at smaller diameters

Among the immobilized lipases, the present entrapped lipase was most active, which implies the superiority of the present entrapment method.

Nomenclature

d	=	diameter of dehydrated EEIM beads and IEZ particles	[mm]
d^0	=	diameter of non-dehydrated EEIM beads	[mm]
EBIM	=	enzyme bound to inorganic matrix	
EEIS	=	enzyme entrapped within inorganic support	
EEOM	=	enzyme entrapped within organic matrix	
IEZ	=	immobilized enzyme	
q_w	=	water content of dehydrated EEIM beads	[kg·kg-EEIM^{-1}]
q_w^0	=	water content of non-dehydrated EEIM beads	[kg·kg-EEIM^{-1}]
p_d	=	pore diameter	[μm]
V_p	=	pore volume	[cm^3]
$-r$	=	esterification rate	[mmol·h^{-1}·kg-lipase^{-1}]
w	=	total lipase weight in IEZ loaded to reaction experiments	[mg]

Literature Cited

1) Chiba, M.: Master thesis, Yamagata univ., p.63, (1993)
2) Chibata, I: "Koteika Kouso", p.74, Kodansya, Japan (1981)
3) Ibid, p.36, p.49
4) Liu,T.-X.: Master thesis, Yamagata univ. ,p.46, (1992)
5) Murakata.T.-X. Liu and S. Sato: *J. Chem. Eng. Japan*, **26**, 681-685(1993)
6) Sato. S.,T. Murakata, T. Suzuki and T.Ohgawara: *J. Mater. Sci.*, **25**, 4880-4885(1990)
7) Scherer, G. W.: *Yogyo Kyoukaishi*, **95**, 21-24(1987)
8) Segal,D.L.: *J. Non-Cryst. Solids*, **63**, 183-191 (1984)
9) Tanaka, A. and K. Sonomoto: *CHEMTECH*, 112-117 (1990)
10) Uo, M., K. Yamashita, M. Suzuki, E. Yamaya, I.Karube and A. Makishita: *Seramikku Ronbunshi*, **100**, 426-429(1992)