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Effects of Water and Silica Gel on Enzyme Agglomeration in Organic Solvents

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Abstract It has been observed that water, which is absolutely essential for enzyme activity, can induce the agglomeration of enzyme particles in organic media. Although enzyme agglomeration is significant in that it usually reduces enzyme activity and stability, little attention has been paid to the quantitative analysis of enzyme agglomeration behavior in nonaqueous biocatalytic systems. In this study, the effects of water and silica gel on enzyme agglomeration were investigated using *Candida rugosa* lipase and cyclohexane as a model enzyme and an organic medium. The extent of enzyme agglomeration was quantified by sieve analysis of freeze-dried agglomerates. Increasing the water content of the medium increased the size of the enzyme agglomerates, and it was found that water produced during the esterification reaction could also promote the agglomeration of enzyme particles suspended in organic media. On the other hand, the size of the enzyme agglomerates was remarkably reduced in the presence of silica gel at the same water content. We also show that this increase in the size of enzyme agglomerates results in lower reaction rates in organic solvents.

Keywords: *Candida rugosa* lipase, enzyme agglomeration, sieve analysis, silica gel, water content

INTRODUCTION

In recent years, the use of enzymes in nonaqueous solvents has been extended and the technique has found a variety of applications [1,2], which is due to several advantages, including, increased solubility of a hydrophobic substrate, shift of an equilibrium in a desired direction, and the possibility of conducting reactions that are impossible in water [3]. Although organic solvents in place of water are used as the reaction media, it has been recognized for many years that water is absolutely essential for enzymatic catalysis. Indeed water not only participates in all non-covalent interactions, which maintain protein conformations, but also plays a crucial role in enzyme dynamics. Therefore, it is generally accepted that water is essential for enzymes in organic solvents and that the hydration level of an enzyme significantly affects its catalytic activity. However, an increase in the hydration level of enzymes is not always accompanied by an increased enzymatic activity in microaqueous reaction systems. In general, if there is too little water, the reaction rate will be low or zero, because of a loss of catalytic activity of the enzymes, and if there is too much, then other undesirable effects of the excess water may lower the reaction rates. Consequently, in most cases there exists an optimal water content. It is expected that the rate of enzyme reactions in organic solvents can be raised if adverse effects due to excess water are diminished. It has been suggested that the excess water has unfavorable effects, for example, it may cause hydrolytic reverse reactions, the partitioning of substrates, enzyme inactivation, or the agglomeration of enzyme particles.

In our previous study, we demonstrated that enzyme agglomeration was probably the most significant of the aforementioned factors [4]. It is believed that enzyme agglomeration as induced by water in organic solvents is purely a physical phenomenon, which is also known as spherical agglomeration. When dried barium sulphate is agitated with dry benzene, spherical agglomerates ca. 0.5-1.0 mm in diameter are formed [5]. Moreover, fine particles dispersed in a liquid suspension can be agglomerated by adding a small amount of a second immiscible bridging liquid, which preferentially wets the particles [6-10]. A significant number of studies have been conducted on the spherical agglomeration of a variety of chemical and pharmaceutical powders. Enzyme agglomeration in microaqueous media has also been observed by many workers [11-13], but little attention has been paid to quantifying the effect. Previously, only one research group [14] has reported measuring enzyme particle sizes in organic solvents by direct microscopy using a graduated eyepiece and investigated the effects of hydration on the size of enzyme powder dispersions. However, there has been no report on the effect of additives such as silica gel on enzyme agglomeration. In the

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present work, we investigated the water-induced agglomeration of enzyme particles in organic solvents, particularly with respect to the effect of silica gel on sizes of enzyme agglomerate. Enzyme agglomerates were quantified by sieve analysis. We also examined the effect of enzyme agglomerate size on the rate of enzymatic reactions in organic solvents.

MATERIALS AND METHODS

Enzyme and Reagents

Lipase OF from *Candida rugosa* was purchased from Meito Sangyo (Tokyo, Japan) and used as received. Silica gel (product number: S0507, particle size: 40-63 μm) was from Sigma (St. Louis, MO, USA). Octanoic acid and butanol were also from Sigma (St. Louis, MO, USA). Cyclohexane was obtained from J. T. Baker (Phillipsburg, NJ, USA). All chemicals used in this work were of analytical grade and were used without further purification. Enzyme powders were equilibrated with saturated salt solution (LiCl) prior to use.

Esterification Reaction in Organic Solvents

Unless otherwise specified, esterification reactions were performed in the following manner. Lipase OF (1.2 g) with or without silica gel (2.4 g) was suspended in cyclohexane solution containing 0.2 M of octanoic acid. This suspension was sonicated to disperse enzyme particles. Another mixture composed of water added and cyclohexane was prepared and also sonicated. The two prepared stocks were mixed together and then incubated with stirring at 30°C and 250 rpm for 10 min. The reactions were carried out in a total volume of 120 mL in a 250 mL spinner flask with an over-head impeller (Bellco Glass Inc., USA) at 30°C and 250 rpm. The reaction was initiated by adding 0.4 M of butanol. At predetermined intervals, 120 μL samples were withdrawn and subjected to GC analysis.

Quantification of Enzyme Agglomeration

Powdered lipase OF weighing 1.2 g with or without 2.4 g of silica gel was put into a 250 mL spinner flask with an over-head impeller and 70 mL of cyclohexane solution containing 0.2 M of octanoic acid was added. In another vessel, water was added to 50 mL of cyclohexane. The above preparations were sonicated separately and then mixed together. This suspension was agitated at 30°C and 250 rpm for 10 min, and then the resulting agglomerates were collected in freeze-drying flasks and were freeze-dried overnight. No significant changes in agglomerate size after freeze-drying were observed. The freeze-dried enzyme agglomerates were first analyzed photographically. The size distribution of the enzyme agglomerates was determined by sieve analysis using standard sieves (Chung Gye Industrial Manufacturing Co., Korea). The size of sieves used were as follows: 0.038, 0.053, 0.063, 0.106, 0.250, 0.355, 0.50, 0.60, 0.71, 0.85, 1.0, 1.18, 1.40, 1.70, 2.0, 2.36, 2.80, 3.35, 4.0, 4.75, and 5.6 mm. Sieve analysis was performed by stacking the sieves in ascending sieve size order and placing the enzyme agglomerates in the top sieve. A closed pan, a receiver, was placed at the bottom of the stack to collect the fines and a lid was placed at the top to prevent powder loss. The stack was shaken at 150 rpm for 10 min and the residual weight of the agglomerates on each sieve was determined. Results are expressed as fractional weight percentage retained. The weight mean sphere diameter for each run was calculated from the size distribution [6].

Analysis of Substrates and Product

The concentrations of octanoic acid and butyl octanoate were analyzed by gas chromatography [Hewlett Packard 5890 Series II (USA)] using a cross-linked polyethylene glycol capillary column (HP-INNOWax, 30 m × 0.32 mm). Helium was supplied as a carrier gas at a rate of 2 mL/min. Hydrogen and air were supplied to the FID at 33.4 mL/min and 330 mL/min, respectively. The injector and the FID temperatures were 250°C and 275°C respectively. The oven temperature was programmed for 2 min at 170°C and was increased to 240°C at a rate of 20°C/min and then maintained at 240°C for 1 min. One μL samples were injected.

RESULTS AND DISCUSSION

Effect of Water Contents on Enzyme Agglomerate Size

Effect of water content on enzyme agglomerate size was investigated by adding different amounts of water to the organic solvent. The amount of water added exceeded the solubility limit (<0.01% v/v) and therefore, because no discrete water phase was observed, the excess water must have associated with the enzyme particles. The images shown in Fig. 1 are of enzyme agglomerates, which were photographed after freeze drying. Fig. 1 shows that the shapes of the enzyme agglomerates are non-uniform and that their sizes increase with increasing amounts of water added. A small amount of water added to the organic solvent plays a role as a bridging liquid. Although the mechanism of spherical agglomeration has not yet been fully elucidated, it has been proposed that the bridging liquid exerts a marked influence on agglomeration [10]. Generally the size of the spherical agglomerates increase with increasing levels of bridging liquid [8]. The size distributions of the enzyme agglomerates obtained from sieve analysis are shown in Fig. 1. The weight mean sphere diameter was 1.4 mm with 0.6 mL of water added (0.5% v/v), and 3.2 mm when 1.2 mL of water was added (1.0% v/v). The weight mean sphere diameter of enzyme agglomerates increased by more than a factor of two. Enzyme agglomeration was also examined on

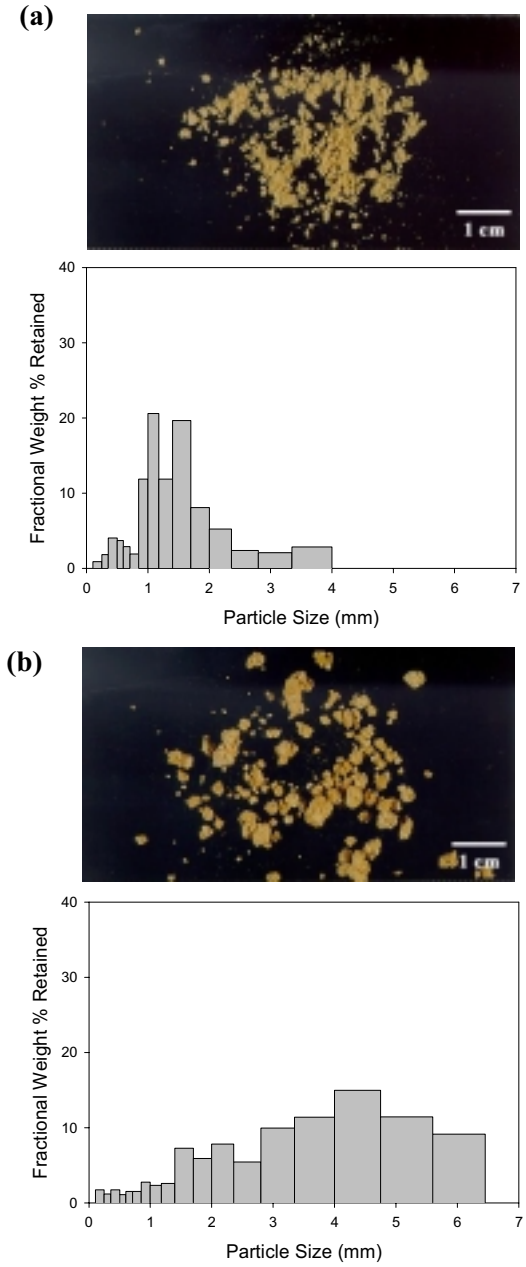

Fig. 1. Photographs and size distribution of the enzyme agglomerates induced by adding water in the absence of silica gel. The amount of water added: (a) 0.6 mL; (b) 1.2 mL. The enzyme agglomeration occurred under the conditions used for the esterification reaction i.e., 30°C and 250 rpm for 10 min and then the resultant enzyme agglomerates were freeze-dried overnight.

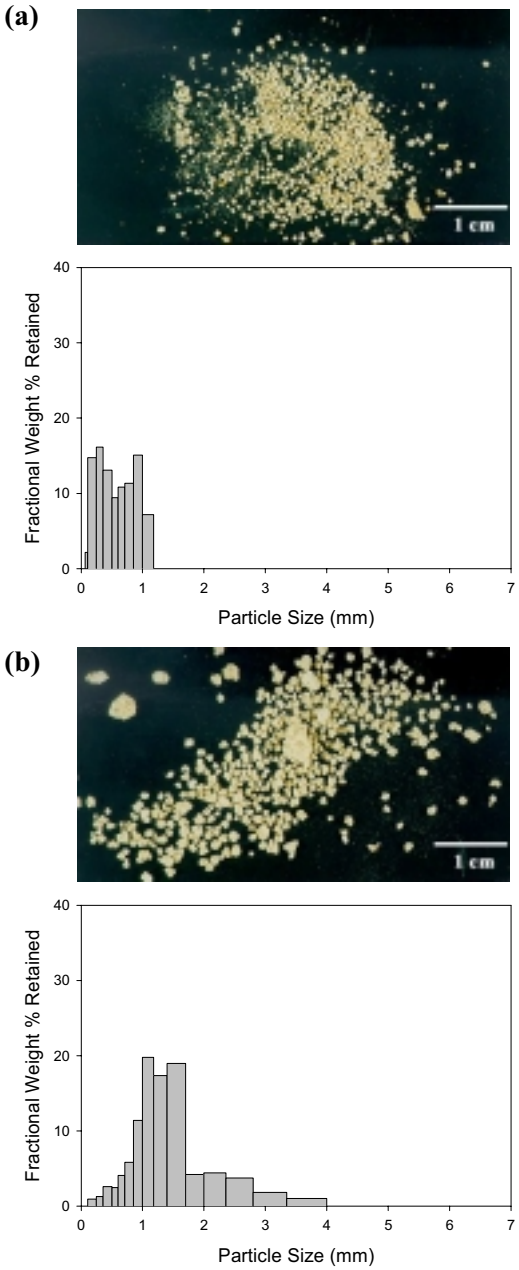

Fig. 2. Photographs and size distribution of the enzyme agglomerates induced by esterification without silica gel. (a) Before the reaction and (b) after 5 h reaction (conversion = 100%). The esterification reaction was carried out at 30°C and 250 rpm without silica gel.

adding more water. However, water additions of greater than 1.8 mL, to the medium, resulted in the solid materials adhering to the flask walls and bottom, and therefore, this beyond the scope of our experiment. Further water addition lead to a separate water which contained dissolved enzyme.

For further investigations, enzymatic esterification in organic solvents was carried out without further water addition. During esterification water is produced as a by-product. Much of the water produced during the reaction is adsorbed by the enzymes. Because water can cause enzyme particles dispersed in organic medium to agglomerate, it might be expected that the enzyme particles would be larger after reaction. The photographs of enzyme agglomerates before and after reaction are shown in Fig. 2. When the reaction time was 5 h, conversion was 100 % and 0.2 M of water (0.432 mL) was formed. Fig. 2 shows that the weight mean sphere di-

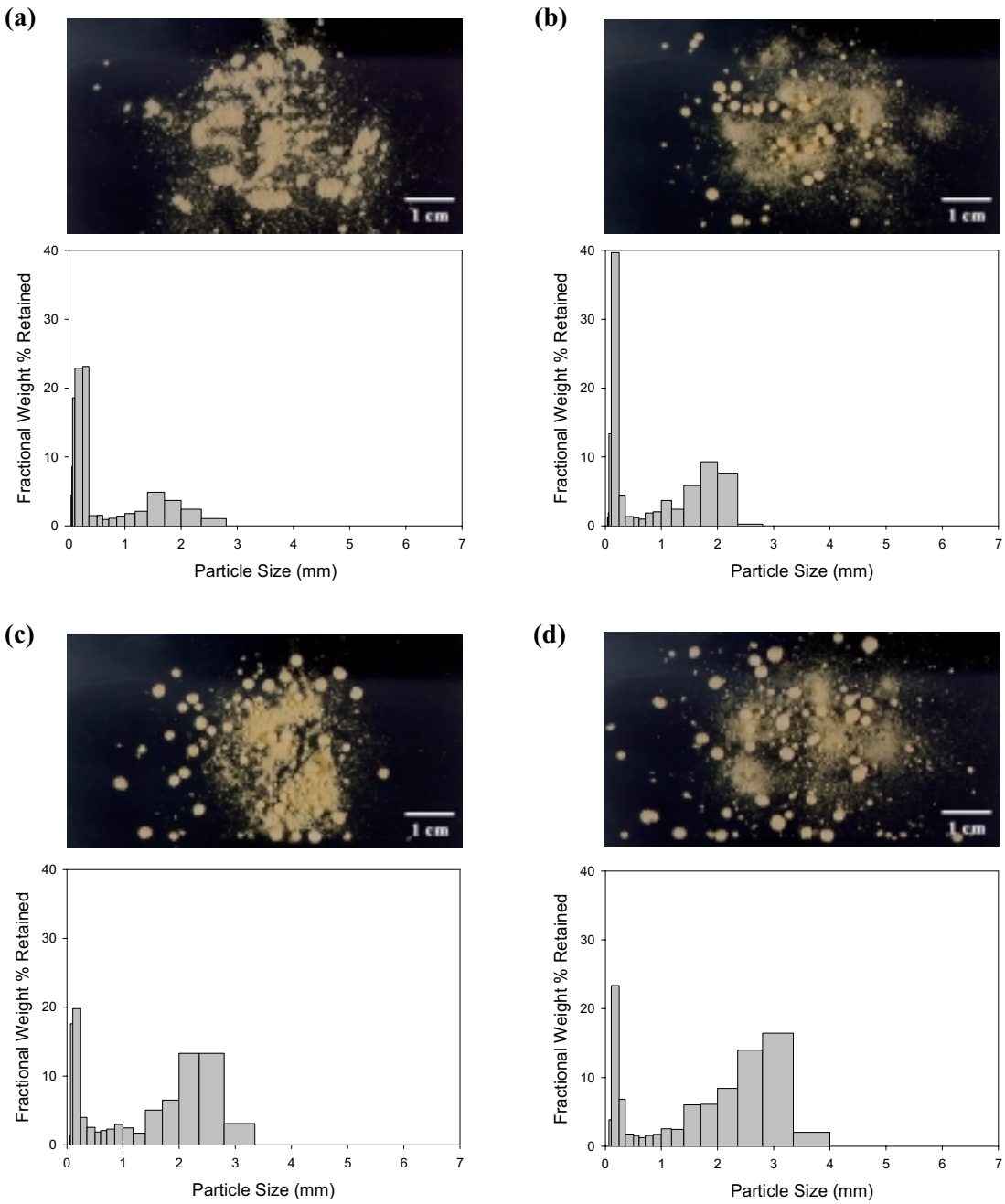

Fig. 3. Photographs and size distribution of the enzyme-silica gel agglomerates induced by adding water in the presence of silica gel. The amounts of water added were: (a) 0.6 mL; (b) 1.2 mL; (c) 1.8 mL; (d) 2.4 mL. Enzyme agglomeration occurred at 30°C and 250 rpm for 10 min and the resultant enzyme agglomerates were freeze-dried overnight.

ameter of the enzyme before the reaction was 0.56 mm, and after the reaction was 1.3 mm. This value is consistent with a weight mean sphere diameter of 1.4 mm at 0.6 mL of added water. This result, when viewed in conjunction with the previous result, suggests that the water produced by the reaction within the medium can also agglomerate suspended enzyme particles.

Effect of Silica Gel on Enzyme Agglomerate Size

The effect of silica gel on enzyme agglomerate size was analyzed by adding various amounts of water to the organic medium. Silica gel was simply added to the medium before agglomeration took place. The pictures shown in Fig. 3 are of enzyme-silica gel agglomerates, which were freeze-dried after agglomeration. As shown earlier, the agglomerate size was found to increase gradually as the amount of added water increased. The size distributions of these agglomerates are also shown in Fig. 3. The size distribution curves show two different peaks. The first sharp peak that appeared at about 100 μm was mainly due to the presence of silica gel, and

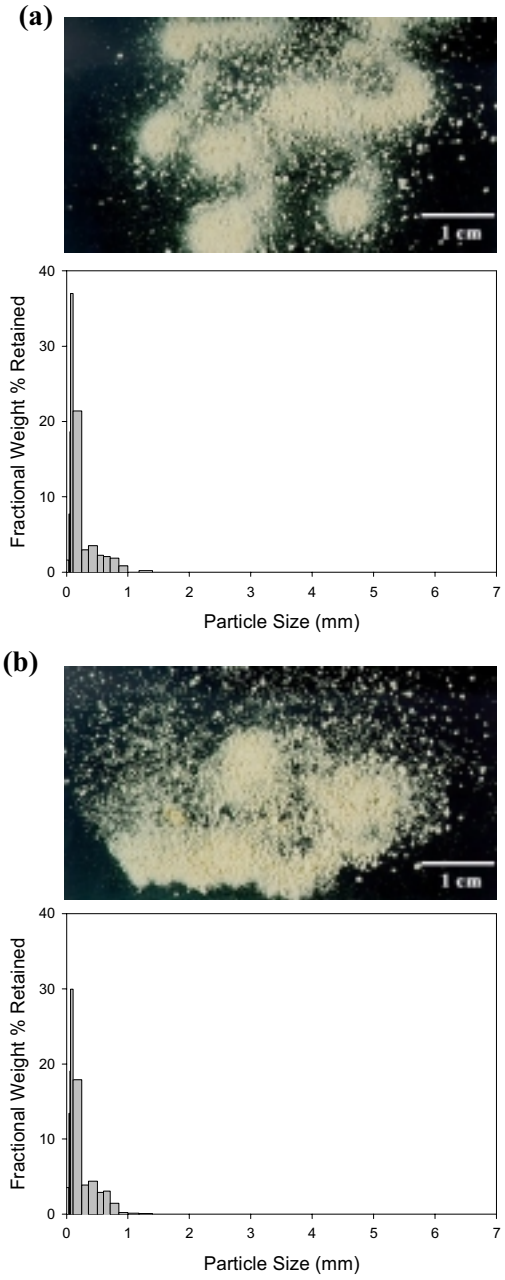

Fig. 4. Photographs and size distributions of the enzyme agglomerates induced by the esterification with silica gel. (a) Before the reaction and (b) after 5 h reaction (conversion = 100%). The esterification reaction was carried out at 30°C and 250 rpm with silica gel.

hence, excluded from the calculation of weight mean sphere diameter of the enzyme agglomerates. The weight mean sphere diameters calculated in this manner were 0.84, 1.4, 1.7, and 2.0 mm at 0.6, 1.2, 1.8, and 2.4 mL of added water, respectively.

In the presence of silica gel, the size of the enzyme agglomerates also increased upon adding water to the medium. However, as Fig. 3(a) and (b) show, the size of the enzyme agglomerates was much smaller than that

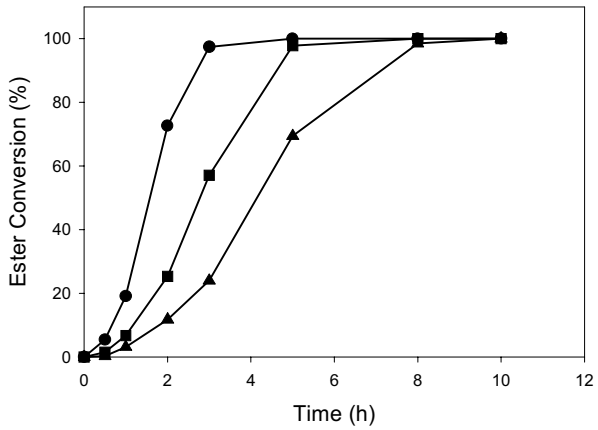

Fig. 5. Effect of the size of enzyme agglomerates on reaction rate. The size of used enzyme agglomerates: (●) < 1.0 mm; (■) 1.0-2.0 mm; (▲) 2.0-3.35 mm.

observed without silica gel at the same water content (Fig. 1(a) and (b)), which is explained by the partitioning of water to the silica gel. Hydrophilic silica gel as well as enzyme particles in organic medium can adsorb water [15]. Hence, in the presence of silica gel, the amount of water available to the enzyme particles is reduced, and this results in a reduction of enzyme agglomerate size.

In addition, enzyme agglomeration was observed during the esterification reaction in the presence of silica gel. The reaction conditions used were the same as those described in Fig. 2. In common with the results obtained without silica gel, conversion reacting for 5 h was 100%, and 0.2 M of water (0.432 mL) was produced. Photographs and size distributions of agglomerates before and after this reaction are shown in Fig. 4. In contrast to the results obtained in the absence of silica gel, the weight mean sphere diameter varied only slightly in the presence of silica gel (from 0.55 mm to 0.51 mm). The reason for this may be that silica gel added to the reaction medium adsorbs much of the water produced during the course of the reaction and therefore, the amount of water around enzyme particles remains almost constant. Other investigators have also used silica particles to buffer the water content during a reaction [15-17].

Effect of Enzyme Agglomerate Size on the Reaction Rates

In our previous study, it was suggested that enzyme agglomeration should be considered to be the most significant cause of reduced enzymatic activity at high water [4], and it has been shown in this study that enzyme particles suspended in organic media are agglomerated by water and that the agglomerate size increases with increasing amounts of water. Lyophilized enzyme agglomerates were divided into three groups according to their sizes, and enzymatic esterification was performed using three enzyme preparations in cyclohexane.

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