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Esterification with Solid Enzymes

Water and Solvent Influence

Scientific Note

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ABSTRACT

Water activation of the enzyme (an esterase of Mucor miehei) is studied during oleic acid/1-decanol esterification with or without solvent. The activation is rapid, but not instantaneous. Reaction water and water added before the beginning of the reaction do not have the same influence. The activation of the enzyme is effected by its swelling with water. When the initial quantity of water is sufficient, the reaction order is zero. But from a certain conversion, the rate decreases very suddenly or very slowly. This observation is attributed to the partitioning of an aqueous phase around the enzyme.

Index Entries: Esterification; solid enzyme; water influence; solvent; phase separation; swelling.

INTRODUCTION

Enzymes are generally used in aqueous or biphasic medium (water-organic solvent). More recently, studies have demonstrated the possibility of using enzymes in organic medium without the need for an aqueous phase (*1–4*). However, A. R. Macrae with *Aspergillus Niger* lipase (*5,6*), M. D. Lilly et al. with *Nocardia Rhodocrus* cells (*1*), and T. Funuda et al. (*7*) have demonstrated the necessity of activation of the biocatalyst by low quantities of water. The enzymatic catalysis for esterification reactions

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has already been surveyed by S. Okumura et al. (*8*), I. Gatfield et al. (*9,10*), A. R. Macrae (*11*), T. Funuda et al. (*7*), J. L. Boyer et al. (*12–14*), A. M. Klibanov et al. (*15*), and more recently by D. F. Tai et al. (*16*).

We have studied the esterification reaction of oleic acid by 1-decanol with or without a solvent by means of a Mucor mieihi esterase. Water is very important in the control of the rate in the reaction where the enzyme is not soluble. The correlation between the catalytic activity and the swelling of the enzyme has been shown. Yet, a large number of solvents have been proposed to perform this type of reaction (*17–19*). They permit to operate at an ambient temperature when reactants are solid.

Our study is focused on the relation between the reaction rate and the distribution of water between the solid enzyme and liquid phase. Our objective is to understand the role of water and to identify the optimum operating conditions.

MATERIALS AND METHODS

Reactants and Solvents

The acid used was oleic acid (Prolabo RP) and the alcohol was 1-decanol (Sidobre-Sinnova Chemical Products Company Boussens, Haute-Garonne, France or Fluka, Buchs, Switzerland). All these nonvolatile reagents facilitate the elimination of water by displacement with an inert gas (in our case, nitrogen). Four solvents were used: tetrahydrofuran (THF), toluene, *n*-Heptane, and *n*-Dodecane. Reactants and solvents are reagent grade.

Enzyme

The enzyme used was an esterase (EC 3.1.1.3) obtained by the fermentation of Mucor miehei, supplied by the Gist Brocades Company (Séclin, Nord, France). It's a crude industrial enzyme. The batch, E 34000, titrated 34000 BGEU (1 BGE unit: the amount of enzyme that catalyzes the formation of 1 μmol butyric acid/g min during the hydrolysis of tributyrine) and contained 2.2% (w/w) water. In all cases, the enzyme was used in powder form.

Reaction Conditions and Kinetic Studies

The reaction was carried out in a glass reactor maintained at constant temperature (35°C) and at atmospheric pressure. In all cases, acid and alcohol were in stoichiometric proportions.

When no solvent is used, the following reaction medium is generally used: oleic acid, 0.125 mol; 1-Decanol, 0.125 mol; ([oleic acid]=[1-decanol]=1.92 *M*); Enzyme, 1 g; ([enzyme]=15.6 g/L) with or without water and in the presence or absence of nitrogen. When a solvent is used, the

concentrations of oleic acid and alcohol in 50 mL of reaction medium are 1*M*. The concentration of the enzyme with or without water is the same as without solvent (15.6 g/L). With a solvent, nitrogen is never used. Experimental conditions are given under each figure or table.

For the kinetic studies, the product detected analytically is oleic acid. It was detected by means of a titration with NaOH. Whether or not the enzyme has been hydrated, alcohol is added, and the reaction medium is stirred vigorously. The first sample (time zero) from the assay previously described is taken approximately 1.5 min after the beginning of the operation. All the samples are weighed and titrated with NaOH 0.1*N*. The conversion ($0 \leq X \leq 1$) is then determined vs time. To represent the water percentage in the organic phase, the enzyme is separated from the latter by centrifugation and water titrated vs time.

Enzyme Hydration

To modify the hydration of the enzyme, the selected quantity of water is added to the required oleic acid with or without solvent. The medium is emulsified with a vigorous magnetic agitation. The enzyme, in powder form, is then poured into the emulsion, and the magnetic agitation is kept at room temperature and during the require time, generally a night (15–17 h).

Water Measurement

The water concentration (P or % H_2O) is expressed in mass percentage vs the total mass of the reaction medium (reactants + enzyme + water) at the beginning of the reaction. The water concentration in the organic phase (% H_2O w/w) was determined by the Karl Fischer method after enzyme separation.

Maximal Water Solubility

Samples of synthetic media corresponding to different conversions are prepared. A large excess of water is added to each of them (two liquid phases). These samples are kept for 24 h at 35°C under vigorous magnetic agitation. The organic phase is separated from the aqueous phase by centrifugation, and its water content is titrated.

RESULTS AND DISCUSSION

Study of the Initial Rate

Water is absolutely necessary for the enzyme to operate normally, but its action is not instantaneous. In order to obtain significant initial kinetic data, the influence of the time of contact between the water and enzyme was studied (Table 1). Ten minutes after it has been placed in oleic acid,

alcohol can be added since the initial rate hardly varies any longer. For convenience reasons, 17 h of contact (one night) were chosen.

By adding increasing quantities of water to the system, the initial rate increases up to a maximum value. This phenomenon can be observed with the reactants alone, as well as with various solvents (Tables 2 and 3). The graph $X=f(t)$, which permits us to obtain the initial rate, is a straight line of variable length showing an apparent zero order up to values of X that can reach 0.4 (Fig. 1). If the reaction is studied without adding any water to the enzyme, the graph $X=f(t)$ shows an inflection point (Fig. 2) and the rate goes through a maximum. The initial increase of the rate is owing to the action of the produced water. In this part of the graph, the rates nevertheless are lower than the initial rates obtained with the same water quantities. Graph (a) in Fig. 3 represents the initial rates for different water contents of the reaction medium, graph (b) in the same figure, the rates during the reaction for the same water contents without addition of water at the beginning of the reaction. Here as well, the action of water is not instantaneous. Together with the increase of the rate obtained by addition of water, a swelling of the solid enzyme is observed; since the enzyme swells, the active sites are released. The swelling of the enzyme can be seen through a microscope. This swelling is not immediate.

Results concerning the existence of the relationship between the catalytic activity and swelling of the solid enzyme have already been presented (*20*). Table 4 shows the influence of hydration (and consequently the influence of swelling) in THF and *n*-Heptane.

Rate During the Reaction

With variable water quantities, the initial zero order that leads to very high reaction rates is maintained for a variable length of time; depending on the operating conditions, the rate may decrease very suddenly or very slowly (Figs. 1, 4, and 5). To discuss this part of the graph, the apparent zero order must be justified. If the reactants are dilute in toluene, it is possible to demonstrate that the rate follows a Michaelis-Menten law of the type

$$r = r_{max}\,[\text{oleic acid}] / K_M + [\text{oleic acid}]$$

up to about $X=0.85$. The K_M determination in this way, at a temperature of 35°C, equals 0.115 *M* (*14*). So the zero order thus obtained with a very high concentration in substrate ([oleic acid]=1 to 1.92 *M*) corresponds to a simplification of this equation. Indeed $K_M << 1$ or 1.92 *M* and is negligible compared to the substrate concentration. The rate given by the Michaelis-Menten law is constant: $r=r_{max}$ (zero order). Consequently, if water is eliminated during the reaction, the experimental results up to $X=0.8$ verify this law.

Table 1
Influence of the Hydration Time of the Enzyme[a]

Hydration time	r_i ($10^2 M$/min)
1.5 min	2.04
10 min	3.27
30 min	3.25
1 h	3.56
17 h	3.61

[a]Oleic acid: 0.125 mol, 1-Decanol: 0.125 mol, Enzyme (E34000): 1 g; temperature: 35°C, atmospheric pressure; P (time zero): 1.38%; nitrogen flow rate: 73 L/h; r_i: initial rate.

Table 2
Influence of Water of the Esterification Reaction[a]

P % (W/W)	r_i ($10^2 M$/min)
0.97	3.04
1.39	3.53
2.28	4.72
3.55	4.90

[a]Oleic acid: 0.125 mol, 1-Decanol: 0.125 mol, Enzyme (E34000): 1 g [oleic acid] = [1-decanol] = 1.92 *M*, [enzyme] = 15.6 g/L, temperature: 35°C, atmospheric pressure; hydration time: 17 h; r_i: initial rate.

Table 3
Enzymatic Esterification with Solvents[a]

THF		Toluene		*n*-Heptane		*n*-Dodecane	
P	$r_i 10^2$ *M*/min	P	$r_i 10^2$ *M*/min	P	$r_i 10^2$ *M*/min	P	$r_i 10^2$ *M*/min
0.09	0.14	0.98	2.40	0.67	3.26	0.81	3.50
0.88	0.53	1.28	2.82	0.83	3.50	1.28	3.74
1.67	0.52	1.72	2.67	1.32	4.27	1.75	2.95
3.18	0.34			1.81	4.09		

[a][oleic acid] = [1-decanol] = 1 M; (enzyme] = 15.6 g/L; temperature: 35°C, atmospheric pressure; hydration time: 17 h; r_i: initial rate.

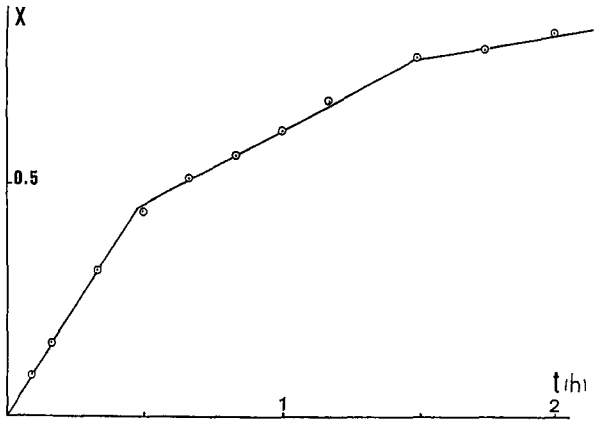

Fig. 1. Esterification reaction with the addition of water: [oleic acid] = [1-decanol] = 1.92 *M*; [enzyme] = 15.6 g/L; temperature: 35°C, atmospheric pressure; P (time zero): 0.97%; hydration time = 17 h.

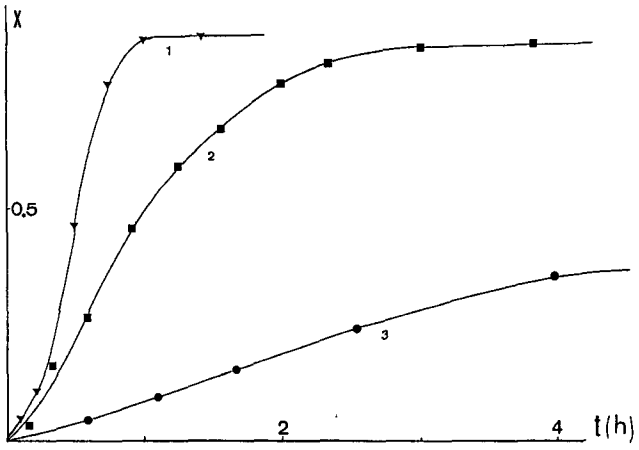

Fig. 2. Esterification reaction without the addition of water: temperature: 35°C, atmospheric pressure; [enzyme] = 15.6 g/L; curve (1) solvent: toluene, [oleic acid] = [1-decanol] = 1 *M*; curve (2) without solvent: [oleic acid] = [1-decanol] = 1.92 *M*; curve (3) solvent: THF, [oleic acid] = [1-decanol] = 1 *M*.

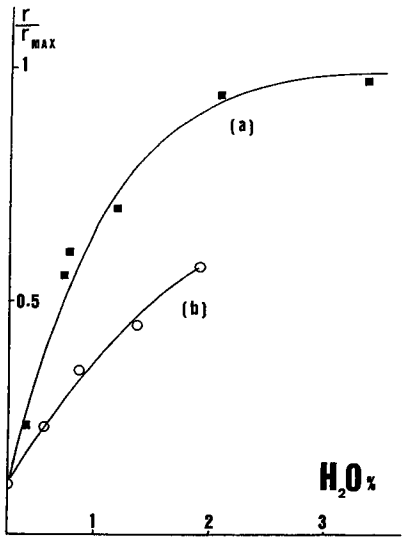

Fig. 3. Relative rate vs percentage water: [oleic acid] = [1-decanol] = 1.92 *M*; [enzyme] = 15.6 g/L; temperature: 35°C, atmospheric pressure; curve (a): relative initial rate, hydration time: 17 h; curve (b) relative rate determined as the ratio of the slope of the tangent at the S-shaped curve $X = f(t)$ for different X values vs r_{max} obtained in the case of curve (a).

Table 4
Influence of the Hydration Time with Solvent[a]

Hydration time	r_i-THF, 10^2 *M*/min	r_i-*n*-Heptane, 10^2 *M*/min
1.5 min	0.37	2.68
17 h	0.53	4.27

[a] [Oleic acid] = [1-Decanol] = 1 M; (Enzyme] = 15.6 g/L; temperature: 35°C, atmospheric pressure; P (time zero): 0.90% in THF; P (time zero): 1.32% in *n*-Heptane; r_i: initial rate.

A more precise comparison of the kinetic model and the experimental results was carried out under the following conditions: [oleic acid] = (1-decanol] = 1 *M*; P (time zero) = 1.28%. Figure 5 shows the resulting curve which, for a conversion superior to 0.55, is much above the experimental curve. In our opinion, this difference is not owing to an erroneous choice of the model, but to another cause that is going to be discussed. We attrib-

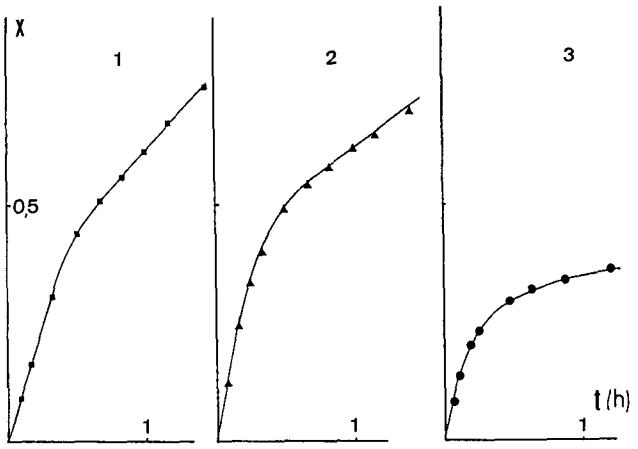

Fig. 4. Influence of the water content. Reaction conditions, *see* Table 2. Curve (1): P (time zero) = 0.97%; curve (2): P (time zero) = 2.28%; and curve (3): P (time zero) = 3.53%.

ute this difference to the phase separation phenomenon characterized by the partitioning of an aqueous phase around the enzyme. This aspect is going to be discussed more precisely in relation to the reaction medium (with or without any solvent).

The solubility of the organic phase in water has been the subject of numerous discussions (*17–19*). Our study demonstrates the importance of the solubility of water in the organic phase. What causes a decrease of activity when a second liquid phase (an aqueous phase) appears will not be studied in detail, but the correlation between phase separation and the decrease of activity will be demonstrated experimentally. The distribution of water between the enzymatic solid and solution depends very much on the nature of this medium. The swelling of the enzyme, as well as the reaction rate, depend closely on the ratio of the water content of the reaction medium to its maximum water content.

For fixed experimental conditions (concentration of the reactants and enzyme, initial concentration in water, presence or absence of water...) corresponding to the highest initial rate, the conversion and water percentage in the liquid phase are determined vs time. The water percentage is compared to the maximum solubility of water in mixtures corresponding to different compositions of the reaction medium without enzyme.

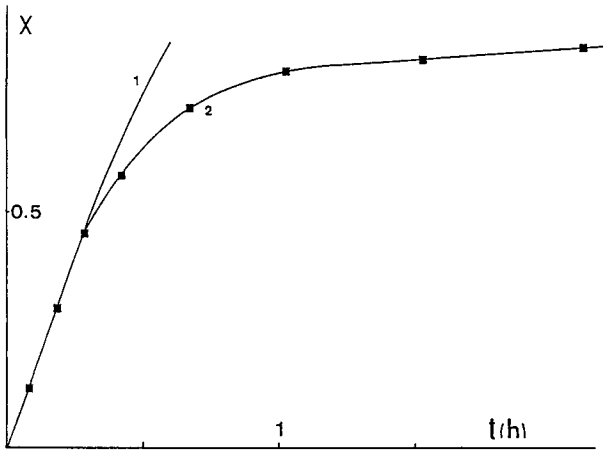

Fig. 5. Esterification reaction in Toluene. Reaction conditions, *see* Table 3, except P (time zero) = 1.28%. Curve (1): curve calculated with the model; Curve (2): experimental curve.

The curve that gives the percentage of water in the liquid phase vs conversion meets the maximum solubility graph for a composition that corresponds either to the break (absence of solvent) or the end of the linearity (presence of solvent). In order to illustrate this observation, the variation of the reaction rate vs conversion was plotted in the case of Toluene (Fig. 6). On this figure, the conversion for which the two water solubility curves meet is the beginning of the reaction rate decrease (this correspondence is shown by an arrow). Similar results were obtained with *n*-Heptane, *n*-Dodecane, and without any solvent. During the reaction, the formed water dissolves in the liquid phase and is adsorbed on the enzyme. When the water concentration in the liquid phase reaches the maximum solubility, the water remains on the enzyme, forms an aqueous phase, and inhibits the enzyme gradually.

The results obtained with Tetrahydrofuran are very different. The initial rate is much lower in this solvent than in any of the others. The zero order is maintained only up to $X = 0.16$, and the rate decreases very quickly (Fig. 7). In addition to this behavior, the water distribution is very different. This water remains mainly in the liquid phase; phase separation is far from being achieved. In tetrahydrofuran, we could not verify that this low rate corresponded to a limited swelling of the enzyme. Indeed, it

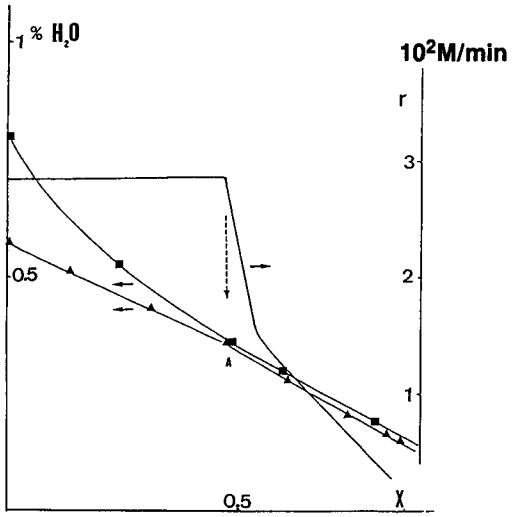

Fig. 6. Phase separation effect: rate vs conversion in relation with water solubility in the organic phase: [oleic acid]=[1-decanol]=1 *M*; [enzyme]=15.6 g/L; temperature: 35°C, atmospheric pressure; P (time zero): 1.28%, hydration time=17 h; solvent: toluene (▲)% H_2O in the organic phase vs conversion and (■) Maximal water solubility in the organic phase. A: phase separation.

is possible that tetrahydrofuran have an inhibiting effect. The lack of interest of this solvent was already mentioned by C. Laane et al. (*19*).

CONCLUSION

For the studied reactions, the use of solvents does not seem relevant in the production of esters unless this production is required to dissolve the reactants. The control of water during the reaction is necessary if short reaction times are desirable; the formation of an aqueous phase must be avoided by all means. By controlling the water content in these conditions, a conversion of $X=0.94$ was obtained in 1.5 h. Therefore, it is possible to think of developing processes for an industrial production in the future if the separation of the ester from the reaction medium can be effected at the end of the reaction. This is the object of our current study.

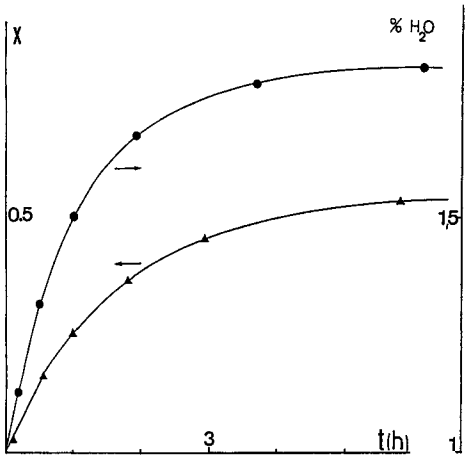

Fig. 7. Esterification reaction in tetrahydrofuran: [oleic acid]=[1-decanol]=1 *M*; [enzyme]=15.6 g/L; temperature: 35°C, atmospheric pressure; P (time zero): 0.90%, hydration time=17 h; (▲) Curve, $X=f(t)$; (●) Curve, % $H_2O=f(t)$.

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