Kinetic study of esterification by immobilized lipase in *n*-hexane

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The kinetic of the esterification of oleic acid by ethanol catalyzed by immobilized lipase of *Mucor miehei* in *n*-hexane as a solvent has been completely studied. The kinetics of the reaction are suggested to agree with a Ping-Pong Bi Bi mechanism in which only inhibition by excess of ethanol has been identified. Values of all apparent kinetic parameters were computed. No evidence of any significant external diffusional limitation which could account for these values has been detected. Optimization of water content through distribution ratio of water between solvent and support was examined.

Esterification; Immobilized enzyme; Oleic acid; Organic solvent; Kinetic

1. INTRODUCTION

The application of lipase (Triacylglycerol acylhydrolase EC 3.1.1.3) to hydrolyze or synthesize esters has been studied for many years since the work of Iwai [1]. At present there is an increasing interest in the development of the application for this enzyme to the production of esters for flavours, waxes and foods in the oleochemical, the oil and fat industries.

It was demonstrated that esters from a variety of alcohols and acids were synthesized by various lipases, according to their specificity in aqueous medium [2]. However, there are many problems in establishing such a reaction system, the main problem being that an excess of water in the reaction mixture tends to favour hydrolysis rather than esterification.

In order to increase the yield of the reaction, organic solvents are used as suitable reaction media. Zaks and Klibanov have shown that lipase keeps a high activity and stability in organic solvent at 100°C [3]. Numerous authors have reported that the esterification is catalyzed by lipases in many organic solvents that contain only a negligible amount of water [4-8].

Despite all these extensive studies, no reports have been dealing with the kinetics and mechanism of this reaction. Only kinetics of hydrolysis and transesterification catalyzed by lipase were studied and examined [8-10].

The objective of our study is an experimental investigation of the kinetics of esterification catalyzed by immobilized lipase and the suggestion of a mechanism in order to determine kinetic constants. The enzymatic reaction of our present work is a simple esterification of fatty acid and will provide an easy model of study. The reaction catalyzed by a lipase is:

Oleic Acid + Ethanol $\longrightarrow$ Ethyl Oleate + Water

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2. MATERIALS AND METHODS

2.1 *Materials*

Immobilized lipase (EC 3.1.1.3, Lipozyme; 200 U/g) from *Mucor-miehei*, supported on macroporous anionic resin beads, was kindly provided by Novo Industri, Denmark. Oleic acid (*cis*-9-octadecanoic acid (*cis*-9-octadecanoic acid) with an approximate purity of 99% was supplied by Sigma, USA; absolute ethanol and hexane (95%) by Prolabo, France.

2.2 *Enzymatic activities*

Ester synthesis was carried out in glass tubes containing the oleic acid, the ethanol and 10 mg of immobilized lipase. The reaction mixture was incubated at 40°C and agitated with magnetic stirring. Samples were withdrawn at various intervals of time to determine the concentration of substrate and product by GC and HPLC respectively.

2.3 *Water adsorption isotherm measurements*

Dried immobilized enzyme was equilibrated with hexane, ethanol and water during 15 min (equilibration time has been checked) at different temperatures. Then water content of the solvent was determined by the titration method of Karl Fischer. Mass balance provides the equilibrium water content of the immobilized enzyme expressed as gram per gram of dried enzyme ($g \cdot g^{-1}$ dried enzyme).

2.4 *Analytical methods*

The concentration of ethyl oleate was determined by HPLC using a Kontron 420 pump system with a Varian R14 refractive index monitor on a Nucleosil C18 5 μm column (250 $\times$ 4.6 mm) (SFCC, France). Elution was conducted at 40°C with methanol/acetic acid (99.7:0.3, V/V) and a flow rate of 0.7 ml/min. Quantitative data were obtained with an integrator (Hewlett Packard 3396A) after calibration with standards.

Concentration of ethanol was determined by an Intersmat IGC 120

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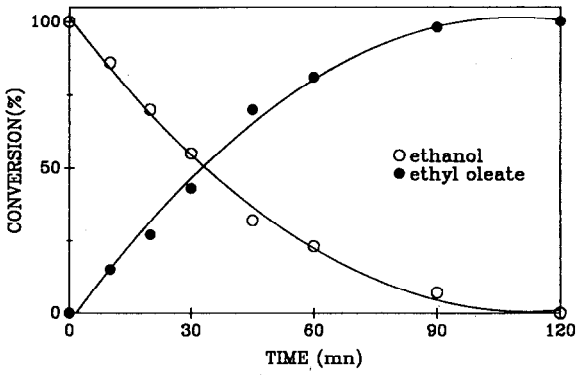


Fig. 1. Kinetics of esterification of oleic acid and ethanol into ethyl oleate. The reaction was carried out in 6 ml hexane containing 60 mM oleic acid, 60 mM ethanol and 10 mg of immobilized lipase. The initial water content was 0.067 $g \cdot l^{-1}$ in hexane and 0.08 $g \cdot g^{-1}$ of enzyme support.

DFL gas chromatograph equipped with a flame ionization detector. Separation took place in a column length of 2 m and a diameter of 3.2 mm packed with Porapak QS 80–100 mesh. Nitrogen was used as carrier gas. Injector, detector and column temperature were 250, 250, 150°C respectively. Quantitative data were obtained by peak integration (Intersmat ICR 1B) after calibration with an internal standard.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of ethyl oleate*

The reaction of esterification was investigated from a reaction volume of 6 ml hexane with 60 mM oleic acid, 60 mM ethanol and 10 mg of lipase. In this condition, the initial water content of hexane and enzyme support were determined by the method of Karl Fischer to be 0.067 $g \cdot l^{-1}$ and 0.08 $g \cdot g^{-1}$ respectively. The kinetics of ethyl oleate and ethanol are shown in Fig. 1. After 2 h, almost complete conversion of oleic acid was obtained. Otherwise, the measurement of initial velocities demonstrated the proportionality between reaction rate and immobilized enzyme quantity (data not shown).

3.2 *Mass transfer limitation study*

In order to study the influence of mass transfer phenomena on our kinetic results, we have performed experiments in a specific reactor where external mass transfer may be controlled. That specific reactor is a 'spinning basket' type allowing to use 40 mg of immobilized lipase in 100 ml solvent. The reaction medium consisted of 30 mM oleic acid and 30 mM ethanol. The rotating speed may be varied from 100 rpm to 500 rpm and the plateau obtained for curves of initial velocity against rotating speed shows that external mass transfer limitations are not significant when the rotating speed is greater than 200 rpm. Because the value of initial velocity obtained on the plateau (where mass transfer is not the limiting factor) is the same as in agitated tubes (under the same concentration conditions, and at the agitation speed chosen) we may assert that external mass transfer is not significant in our kinetic results. However, the internal diffusion effect has not been tested because of the unavailability of the different catalyst sizes, but it is suspected to have an influence on the kinetic constants.

3.3 *Effect of water content*

One of the most important parameters affecting the enzyme activity in organic solvent is the amount of water. We have studied the influence of the quantity of water upon the evolution of the catalytic activity of immobilized lipase in *n*-hexane at 3 different temperatures (27, 40, 55°C). The reaction medium consisted of 8 mM oleic acid, 150 mM ethanol and 10 mg of enzyme in 6 ml of hexane. Before use, the immobilized enzyme was dehydrated under vacuum. Then a known quantity of distilled water was added via ethanol solution. The initial velocity of reaction in different amounts of water was studied. The initial velocity increased with respect to the increasing amount of water and temperature. The catalytic activity reached a maximum and then progressively decreased to zero in every case but with different amounts of water depending on the temperature (5, 6, 7 μl of water at 27, 40, 55°C respectively). It is known that enzymes need a small amount of water to maintain their active conformations. Our results show that the optimal water content was shifted with increasing temperature (0.5, 1.0, 1.5 μl of water added at 27, 40, 55°C respectively).

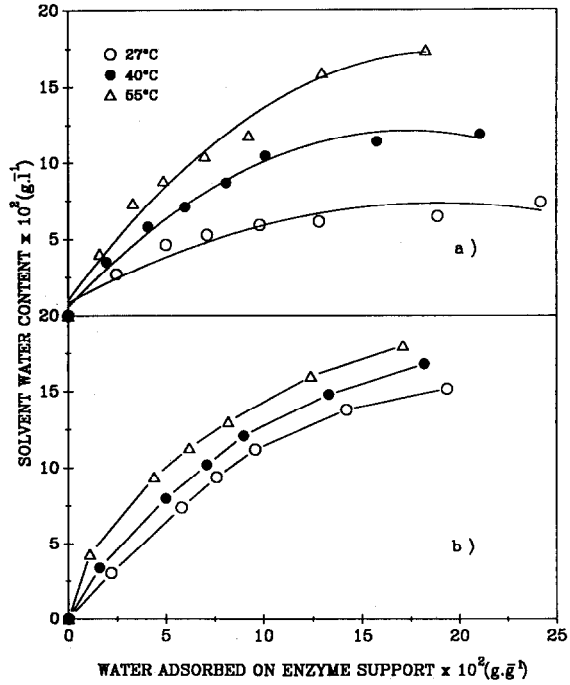


Fig. 2. (a) Water adsorption isotherms between immobilized enzyme and hexane. (b) Water adsorption isotherms between immobilized enzyme and hexane with 150 mM ethanol. 80 mg of dried immobilized enzyme was equilibrated for 15 min with 50 ml hexane, different amounts of water (for a and b) and ethanol mixture corresponding to 150 mM (for b only).

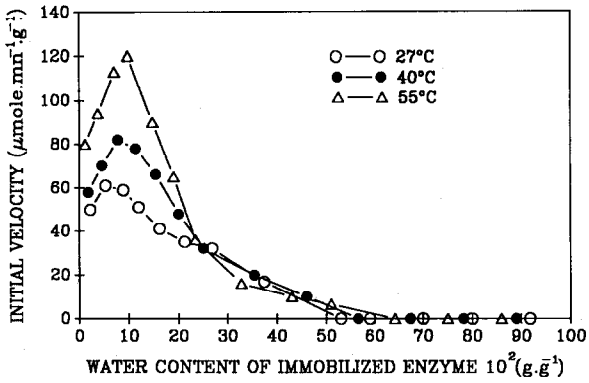


Fig. 3. Effect of water content of immobilized enzyme on initial reaction velocity. The reaction was carried out in 6 ml hexane containing 8 mM oleic acid, 150 mM ethanol and 10 mg of dried immobilized enzyme. The initial water content of hexane was 0.067 $g \cdot l^{-1}$.

In hexane, thermodynamic partition of water between the organic phase and the hydrophilic support is dependent upon temperature and other solutes in the solvent, i.e. ethanol or oleic acid in our case. We have determined water adsorption isotherms at different temperatures for hexane alone and for hexane and ethanol at the concentration of 150 mM used in the kinetic experiments. It has been checked that oleic acid has no influence on adsorption. Results are shown in Fig. 2. The actual quantity of water present on the support during kinetic experiments can then be derived and curves may be drawn using water content of the solid phase as a coordinate. It can be concluded from Fig. 3 that initial velocity maxima are obtained at the roughly identical water content of 0.1 $g \cdot g^{-1}$ of dried enzyme for all tested temperatures.

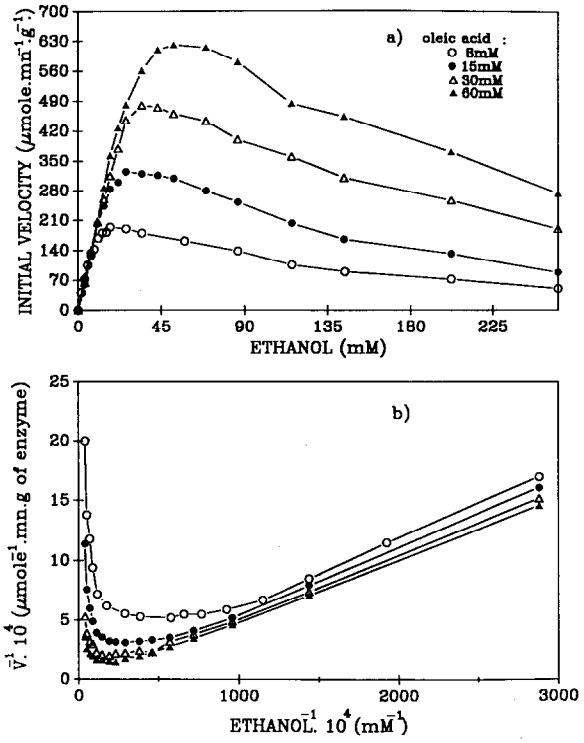


Fig. 4. (a) Effect of the ethanol concentration on the initial velocity of the reaction. (b) Reciprocal initial velocity of the reaction versus reciprocal ethanol concentration. The reaction was carried out in 6 ml hexane containing different concentrations of substrates with 10 mg of immobilized lipase. The initial water content was 0.067 $g \cdot l^{-1}$ in hexane and 0.08 $g \cdot g^{-1}$ of enzyme support.

3.4 *Kinetic and mechanism of reaction*

The effect of the concentration of both substrates on the rate of reaction was investigated. Fig. 4 shows that when the concentration of ethanol was increased, the initial velocity was proportionally increased but reached a maximum at a critical concentration value. A subsequent increase in ethanol concentration ultimately led to a decrease in the initial velocity for all oleic acid concentrations tested. The stability of the enzyme was excellent in the reaction mixture under the highest concentration tested for both substrates (oleic acid 60 mM and

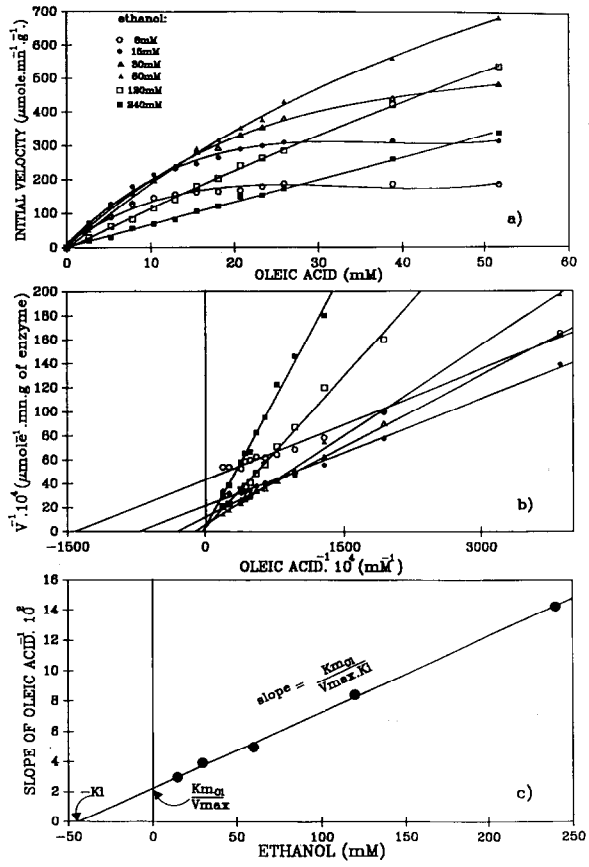
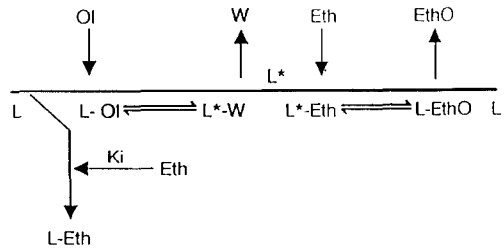


Fig.5. (a) Effect of the oleic acid concentration on the initial velocity of the reaction. (b) Reciprocal initial velocity of the reaction versus reciprocal oleic acid concentration. (c) Slope of 1/oleic acid versus ethanol concentration. The reaction was carried out in 6 ml hexane containing different concentrations of substrates with 10 mg of immobized lipase. The initial water content was 0.067 $g \cdot l^{-1}$ in hexane and 0.08 $g \cdot g^{-1}$ of enzyme support.



Ol : oleic acid, Eth : ethanol, L : lipase, W : water, L* modified lipase, EthO : ethyl oleate, L-Eth : dead end enzyme-ethanol complex

Fig. 6. Scheme of the mechanism of esterification catalyzed by lipase.

ethanol 240 mM) in hexane (data not shown) within the time range of our experiment (60 min) and therefore, it may be deduced that ethanol is a substrate inhibitor for lipase catalyzed esterification. The decrease in initial velocity could have been attributed to an 'entrainer effect' by ethanol that 'dries' the support and lowers the activity (left side of the bell shape in the curve of Fig. 3) but experiments with 15% water content of the solid phase present the same decreasing activity with increasing ethanol concentration and so validate the hypothesis of a mechanistic inhibition effect (data not shown). Fig. 5 shows that no evidence of inhibition by oleic acid was found at all concentrations of ethanol tested.

The reaction mechanism has been elucidated by the plot of $1/V$ versus $1/S$ (oleic acid) presented in Fig. 5b, which shows that at low fixed ethanol concentrations (8 and 15 mM), the plots appear parallel. As the fixed ethanol is increased (30, 60, 120, 240 mM), the slope increases and the $1/V$ axis intercept decreases as usual to a limit of $1/V_{max}$. These results agree with an assumed Ping-Pong Bi Bi mechanism with dead-end inhibition by one substrate as described in Fig. 6 [11]. In this typical reaction sequence, the lipase may react with ethanol to yield a dead-end enzyme-ethanol complex or with oleic acid to yield the lipase-oleic acid complex. Then the lipase-oleic acid complex transforms to an carboxylic-lipase intermediate and water is released. This is followed by interaction of carboxylic-lipase with ethanol to form another binary complex which then yields the ethyl oleate and free lipase.

The equation for the immobilized lipase catalyzed esterification is expressed as:

$$\frac{v}{V_{max}} = \frac{[Ol][Eth]}{K_{m(Ol)}\,[Eth]\,\{1 + [Eth]/K_i\} + K_{m(Eth)}\,[Ol] + [Ol]\,[Eth]}$$

where V_{max} is the maximum rate of reaction, [Ol] is the initial oleic acid concentration, [Eth] is the initial ethanol concentration, $K_{m(Ol)}$ and $K_{m(Eth)}$ are the Michaelis constant of oleic acid and ethanol respectively, K_i is the inhibitor constant of ethanol.

Our proof for the mechanism of esterification is the occurrence of alcohol inhibition (ethanol) in Ping-Pong systems. The study of transesterification previously reported by Zaks and Klibanov [8] did not show alcohol inhibition but also agreed with Ping-Pong mechanism. So, alcohol substrate molecule can act as a dead-end inhibitor in the esterification reaction (by preventing enzyme-acid complex formation) but not in transesterification (enzyme-ester complex is first formed).

The V_{max} and $K_{m(Eth)}$ values could be determined from Fig. 5b as described above. Slopes from Fig. 5b may be replotted versus ethanol concentrations; the straight line obtained (Fig. 5c) gives the value for K_i at the intercept with the abscisse axis. The values of $K_{m(Ol)}$ may also be obtainable from this replot as described in Fig. 5c. However, to ensure better accuracy, the values of $K_{m(Eth)}$, $K_{m(Ol)}$, K_i and V_{max} were computed from the equation for reaction velocity by numerical parametric identification using the Gauss-Newton algorithm of error minimization with 6.2% of mean deviation on 72 experimental points. These values are 190 mM, 120 mM, 40 mM and 5700 $\mu mol \cdot min^{-1} \cdot g^{-1}$ respectively. It is reminded that K_m values are apparent values and are suspected to be affected by internal diffusion. This sound knowledge of the immobilized-lipase mechanism and the kinetic behaviour will be applied to the optimisation of esterification in bioreactors.

REFERENCES

[1] Iwai, M., Tsujisaka, M. and Fukumoto, J. (1964) J. Gen. Appl. Microbiol. 10, 13-22.
[2] Iwai, M., Okumura, S. and Tsujisaka, Y. (1980) Agric. Biol. Chem. 44, 2731-2732.
[3] Zaks, A. and Klibanov, A. (1984) Science 224, 1249-1251.
[4] Bell, G., Blain, J.A., Patterson, J.D.E., Shaw, C.E.L. and Todd, R. (1978) FEMS Microbiol. Lett. 3, 223-225.
[5] Seo, C., Yamada, Y. and Okada, H. (1982) Agric. Biol. Chem. 46, 405-409.
[6] Marlot, C., Langrand, G., Triantaphylides, C. and Baratti, J. (1985) Biotechnol. Lett. 7, 647-650.
[7] Langrand, G., Triantaphylides, C. and Baratti, J. (1988) Biotechnol. Lett. 10, 549-554.
[8] Zaks, A. and Klibanov, A. (1985) Proc. Natl. Acad. Sci. USA 82, 3192-3196.
[9] Bell, G., Blain, J.A., Patterson, J.D.E. and Todd, J.R. (1976) J. Appl. Chem. Biotechnol. 26, 582.
[10] Bell, G. and Todd, J.R. (1981) Biotechnol. Bioeng. 23, 1703-1719.
[11] Segel, I.H. (1975) Enzyme Kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley-Interscience, USA.