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Kinetic Characterization of *Penicillium citrinum* Lipase in AOT/Isooctane-Reversed Micelles

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ABSTRACT

A lipase from a wild strain of *Penicillium citrinum* was encapsulated in AOT/isooctane-reversed micelles, and the kinetic parameters were studied relative to triolein hydrolysis. Lipolytic activity was strongly dependent on the water amount in the system (W_o) and presented a bell-shaped curve for this parameter, with a maximum in the range of W_o 10–15. Optimum conditions for enzyme activity were pH 8.0 and 45°C. The influence of substrate concentration was also studied. The enzyme showed a Michaelis-Menten behavior and the apparent kinetics constants were calculated as being $V_{max.app.}$ = 120 U/mg and K_{mapp} = 49.2 m*M*.

Index Entries: *Penicillium citrinum*; lipases; triglyceride hydrolysis; reversed micelles.

INTRODUCTION

Lipases (triacylglycerol hidrolases E.C. 3.1.1.3) are enzymes widely distributed in nature. They catalyze the hydrolysis of glycerol ester bonds and are active almost exclusively at phase interfaces, as well as the reverse reaction in organic solvents at low-water activities. Microbial lipases are diverse in their enzymatic properties and substrate specificities, which

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improve their biotechnological importance and justifies the search for new sources of this type of enzyme *(1)*.

Enzymatic reactions in reversed micelles have attracted considerable interest during the last decade, the most investigated system being the surfactant AOT [sodium di (2-ethylhexyl) sulfosuccinate] in isooctane. The main advantages of this system are the possibility to provide an adequate environment to the enzyme, and therefore protecting it against denaturation by the organic solvent. Additionally, reversed micelles can provide a high superficial area (approx 100 m^2mL^{-1}), which is especially important for the reaction catalyzed by lipases. It also allows a conventional kinetic approach, because the substrate is dissolved in the organic phase and is in a monomeric form *(2–5)*.

A lipase from a strain of *Penicillium citrinum*, isolated from an olive-oil contamination, was purified by chromatograhic methods and characterized in a preliminary work. The main results on the biochemical characterization were the aggregate formation that already occurs in the fermentation broth, the presence of a dimmer with MW 63,000 Dalton containing two subunits with similar molecular weights (31,000–33,000 Dalton) and isoelectric points (4.8–5.0). Additionally, kinetic studies in aqueous media using the crude-extract preparation revealed that the lipase was quite stable at room temperature with an optimal temperature between 34 to 37°C, and a pH value for optimal activity of 8.0 *(6)*. The kinetic constants were determined using p-nitrophenylpalmitate (pNPP) as substrate, and the enzyme showed a Michaelis-Menten behavior in this system. It was also found that this enzyme was capable of hydrolyzing olive oil as well as pNPP.

Reversed-micellar systems have been extensively used for studying the kinetics of lipases *(3–5,17,18)*. This work presents the kinetic characterization and the performance of the *P. citrinum* lipase in an AOT/isooctane reversed-micellar system.

MATERIALS AND METHODS

Chemicals

Sodium di (2-ethylhexyl) sulfosuccinate and p-nitrophenylpalmitate were from Sigma (Munich, Germany); isooctane (analytical grade) was obtained from Riedel de-Häen. Triolein 65%-purity degree was from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and were used without further purification.

Enzyme Production and Purification

Penicillium citrinum isolated as an olive-oil contaminant, as previously described *(7)*, was cultivated in a medium containing 0.5% (w/v) yeast

extract and 1% (v/v) olive oil, in 21 Erlenmeyer flasks containing 500 mL of the medium, with orbital shaking (100 rpm) at 28°C. Fermentation was started with a 10% (v/v) inoculum cultured for 48 h and was finished after 5–7 d of culture *(6)*.

The mycelium was filtered off and the supernatant that contains the enzyme was recovered. Ammonium sulfate was added to the broth supernatant to 80% (w/v) saturation and left for 24 h at 4°C. The precipitate was centrifuged (20,000*g*, 20 min), solubilized in 50 m*M* phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. Further purification was carried out in a gel-filtration Superose 6 preparative-grade column. The enzyme was recovered after this step as a high molecular-weight aggregate that was almost pure, as demonstrated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The fractions with lipolytic activity were collected and concentrated by ultrafiltration (Amicon) using a 10,000 MW cut-off membrane. The resulting concentrated-enzyme solution with a lipolytic activity of 210 U/mg, as determined by pNPP method (*see* below) was used for kinetics experiments.

Protein Determination

The concentration of protein in the aqueous phase was measured using the bicinchoninic-acid method *(7)*.

Lipase Activity Measurement

Aqueous System

In the aqueous phase, enzyme activity for p-NPP was assayed as described *(6,8)*. Release of p-nitrophenol at 37°C, and pH 8.0 (50 m*M* phosphate buffer) was detected at 410 nm (Shimadzu UV-265 FW spectrophotometer). One enzyme-activity unit was defined as 1 nmol of p-nitrophenol released per min., at pH 8.0 and 37°C. The molar-absorption coefficient of pNPP ($1.32 \times 10^4\ M^{-1} \cdot cm^{-1}$) *(9)* was used to calculate the enzyme activity.

Micellar System

Reversed micelles with the required water content ($W_o = [H_2O]/[AOT]$), were prepared with 100 m*M* AOT in isooctane, adding the appropriated amount of the enzyme solution. The activity assays were carried out in a stirred batch reactor with a water jacket, using 4 mL of the micellar medium and 1 mL of triolein, and samples were taken at given time intervals. For the optimization of the physicochemical parameters (W_o, pH, and temperature) of the reversed-micellar system, triolein, was added as substrate to the reactor immediately after the reversed-micellar solution became clear. The optimization studies of W_o and pH were carried out at 37°C and in 50 m*M* buffer solution. The pH values (pH of the stock

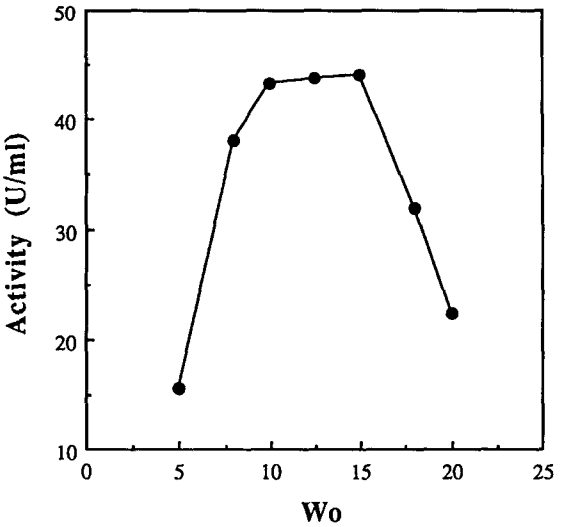

Fig. 1. Effect of the water content on triolein hydrolysis by *Penicillium citrinum* lipase in reversed micelles (100 m*M* AOT in isooctane, pH 7.0, T = 37°C; [enzyme] = 12 µg/mL micellar solution).

solution) are those of the aqueous buffer, from which the micellar solutions were prepared.

The spectrophotometric method of Lowry and Tinsley *(10)* was used to quantify the formation of the fatty acid during the enzymatic reaction in reversed micelles.

RESULTS AND DISCUSSION

Effect of the Water Content

The effect of water amount in the reversed-micellar system on the lipolytic activity was determined in the range of W_0 5–20, at pH 7.0, using triolein as substrate. To prepare the microencapsulated lipase, the enzyme solution was added to the reversed-micellar solution and the water amount was corrected by adding 50 m*M* pH 7.0 phosphate buffer, to achieve the required W_0. Reversed-micellar solutions were prepared so as to maintain the overall enzyme concentration constancy throughout the whole range of W_0 values.

The results are shown in Fig. 1. The microencapsulated lipase from *P. citrinum* showed a typical bell-shaped profile of activity, which is characteristic of monomeric enzymes *(11)*. The maximum activity (44 U/mg) was obtained in the range of 10–15 of W_0. This result is in agreement with other data reported in the literature for lipases and also for other

enzymes *(3,4,12)*. Several factors seem to contribute to the existence of a W_o optimal for enzyme activity, one of the most important being the size of the inner cavity of the micelle correspondent to the size of the protein *(4,12)*. *P. citrinum* lipolytic preparation used in this experiments exists as a high molecular-weight aggregate, as determined by gel filtration in a preliminary work, and it is formed by a dimmer of 63,000 Dalton, as determined by SDS-PAGE; the dimmer consists of two subunits of similar molecular weights, in the range of 31,000–33,000 Dalton. From the W_o values, it is possible to calculate the micelle radii, according Luisi et al. *(13)*, using the equation: $r_{micelles}$ (Å) = 1.64 W_o.

The radius of the monomeric form of the enzyme, considering a MW of 33,000 Dalton is also obtainable *(14)* by the equation: $r_{protein}$ (Å) = 0.7 $(MW)^{1/3}$.

The comparison of the radii of the inner cavity of the reversed micelles in the range of W_o 10–15, 16.4 and 24.6 Å respectively, with the radius of the monomeric form of the lipase, 22.4 Å leads to the conclusion that the enzyme is probably encapsulated as the monomeric form. This conclusion is in accordance to that presented by Kabanov et al. *(14)*. These authors reported the possibility of utilization of AOT/isooctane reversed micelles at low W_o values to disrupt enzyme aggregates in nondenaturating conditions, and found different W_o values for the encapsulation of more than one species of the same enzyme.

Figure 1 also shows that the activity strongly decreases when the W_o decreases at values lower than 8, and at W_o 5.0, the lipolytic activity was only approx 33% of that presented at W_o 15. This could be explained by assuming that there is a minimum amount of water required for enzyme hydration and to take part in the hydrolytic reaction. The decrease of the enzyme activity at W_o over 15 could be explained by the correlated increase of water amounts inside the micelle, with a consequent increase of enzyme mobility, resulting in catalytic conformation disruption, as proposed by several authors *(2–4)*. It was also noticed that the micelle formation became difficult with the increase of the water amount in the system, and for W_o 20 almost 3 min were necessary to encapsulate the enzyme. Therefore, W_o 10 was chosen to proceed with further experiments.

Effect of pH

The effect of the pH of the aqueous phase on the lipolytic activity was carried out in the range of 4.0–9.0, using diffirent buffer solutions (acetate, pH 4.0–5.5, phosphate, pH 6.0–8.0, and Tris-HCl, pH 8.0–9.0). The enzyme preparation was obtained after gel filtration with a pH 7.0 phosphate buffer. To obtain the required pH value, a volume of the lipolytic solution was dialyzed overnight against a 50 m*M* buffer. The pH values were checked after the dialysis.

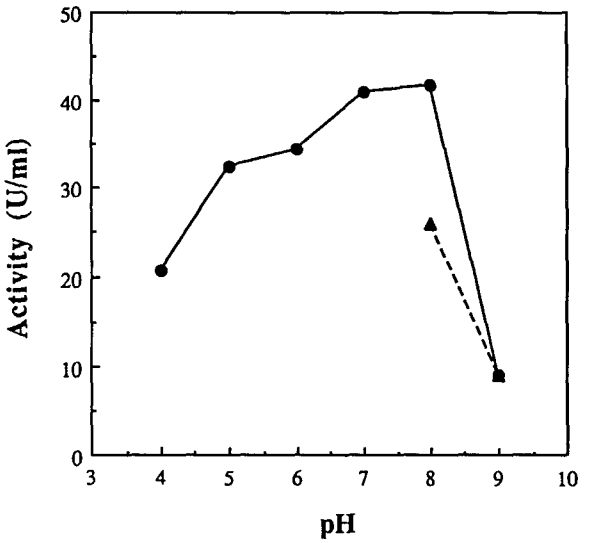

Fig. 2. Effect of pH on triolein hydrolysis by *Penicillium citrinum* lipase in reversed micelles. Conditions: 100 m*M* AOT in isooctane, T = 37°C; [enzyme] = 9 μg/mL micellar solution. Buffers (50 m*M*): acetate, pH 4.0–5.5, phosphate, 6.0–8.0, and Tris-HCl 8.0–9.0).

From Fig. 2 it can be seen that the maximum activity was found at pH 8.0. This value is similar to that reported in previous work for the crude extract of the *P. citrinum* lipase in aqueous medium *(6)*. The type of the buffer is also important for the determination of the lipolytic activity. The low activity found for pH 9.0 was not only caused by the pH value itself, but also by the Tris-HCl buffer. This can be seen from the comparison of the values found at pH 8.0 with phosphate and Tris-HCl buffers, being the activity with phosphate approx 38% higher than that with the other buffer. A similar effect was also found during the characterization of the crude extract of *P. citrinum* in aqueous medium *(6)*.

Effect of Temperature

The effect of temperature on lipolytic activity was investigated in the range of 20–60°C, using 50 m*M* phosphate buffer at pH 8.0 and W_0 10 (Fig. 3). An optimum temperature of 45°C was observed, and even at 50°C the residual activity of the enzyme was very high (96.4%). Beyond this temperature the activity falls sharply, and at 55°C the residual activity was only 60% of the maximum activity, probably owing to protein denaturation. Higher temperatures for maximum enzymatic activity in reversed micelles have been also reported by other authors and have been attributed to a lower degree of freedom of the enzyme into the reversed micelles, leading to a higher stability against temperature *(4,12)*.

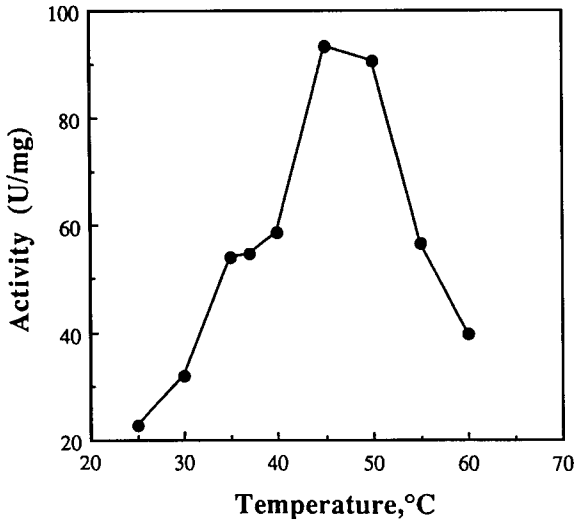

Fig. 3. Effect of temperature on on triolein hydrolysis by *Penicillium citrinum* lipase in reversed micelles. Conditions: 100 m*M* AOT in isooctane, [enzyme] = 7.5 μg/mL micellar solution, 50 m*M* phosphate buffer pH 8.0.

The activation energy of the hydrolysis reaction calculated from an Arrhenius plot was 13.4 kcal · mol^{-1}. This value is somewhat higher than that reported by Han and Rhee *(3)* for the hydrolysis of olive oil by *Candida rugosa* (8.0 kcal · mol^{-1}), and quite higher than those reported by Prazeres *et al. (4)* for the hydrolysis of triolein in the same micellar system (4.7 kcal · mol^{-1}). The calculated value for the aqueous medium using the crude extract and pNPP as substrate (11.4 kcal · mol^{-1}) is slightly lower than that obtained for the enzyme encapsulated in reversed micelles, suggesting that the enthalpy of the transition complex for the latter system is higher than that of the aqueous medium. This result is in agreement with those of a higher temperature for maximum activity and was found in the reversed-micellar system. This means that a larger amount of distortion, or even breaking of chemical reaction, is necessary for the formation of the transition state in reversed micelles *(4)*, and is in accordance with the fact that entropy and interfacial area of reversed-micellar systems are much higher than in aqueous-organic systems.

Effect of Substrate Concentration

The effect of substrate concentration in 100 m*M* AOT/isooctane-reversed micelles was studied using triolein as substrate, 50 m*M* phosphate buffer, pH 8.0, temperature of 37°C and W_0 = 10. The results are shown in Fig. 4. The Lineweaver-Burk plot was applied to the experimental values and the kinetics parameters were calculated: $K_{m\text{app.}}$ of 49.2 m*M* and $V_{max.\text{app.}}$ of 0.9 μmol/min · mL (120 U/mg) were obtained.

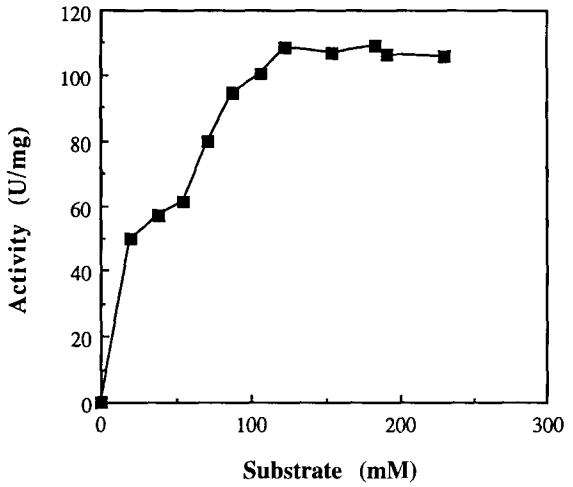

Fig. 4. Effect of substrate on the reaction rate of the hydrolysis of triolein by the *Penicillium citrinum* lipase in AOT (100 m*M*) reversed-micellar system. Conditions: 50 m*M* phosphate buffer pH 8.0, W_0 10, and temperature, 37°C. [enzyme] = 7.5 mg/mL micelles.

Michaelis-Menten behavior for lipases in reversed-micellar media has been frequently reported *(3–5)*, and the apparent kinetic constants were calculated. For other fungal lipases, the apparent kinetic constants obtained in AOT/isooctane-reversed micelles were: for *Candida rugosa (3)* (W_0 = 10.5 and olive oil as substrate) a $K_{m\ app.}$ = 10 m*M* and $V_{max\ app.}$ = 1020 U/mg; for *Rhizopus arrizus (17)* (W_0 = 13 and palm oil as substrate) a $K_{m\ app.}$ = 136 m*M* and $V_{max\ app.}$ = 5714 U/mg; and for *Rhizopus delemar (18)* (W_0 = 11 and tricaprylin as substrate) a $K_{m\ app}$ = 32 m*M* and $V_{max\ app.}$ = 87 U/mg.

CONCLUSIONS

The lipase from *P. citrinum* was encapsulated in AOT/isooctane-reversed micelles. The enzyme showed an optimum pH of 8.0 and temperature of 45°C. Lipase activity was strongly dependent of the water amount in the system (W_0), and presented a bell-shaped curve for this parameter, with a maximum in the range of 10–15 of W_0. The enzyme showed a Michaelis-Menten behavior for triolein as substrate, and the kinetics constants could be calculated for this system.

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