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The characteristics of hydrolysis of triolein catalyzed by wheat germ lipase in water-in-oil microemulsions

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Abstract

The enzymatic activities of wheat germ lipase (WGL) (EC.3.1.1.3; triacylglycerol hydrolase) in water-in-oil (w/o) microemulsions of sodium bis-(2-ethylhexyl) sulfosuccinate (AOT)/cyclohexane/water have been investigated by using triolein as the substrate through measurements of absorbency of the product of hydrolysis at the wavelength of 707 nm in the course of reactions. Maximum enzyme activity was obtained at ω_0 (molar ratio of water to surfactant) = 9, pH = 7.4 and $T = 40\,^{\circ}C$. The activation energy of the reaction was calculated from the Arrhenius plot. It was found that the hydrolysis reaction obeyed Michaelis–Menten kinetics in the investigated concentration range (0.01–0.15 mol/l) of the substrate, and the apparent Michaelis constant $K_{m,a}$ and the apparent maximal reaction rate V_m were determined. The stability of wheat germ lipase in w/o microemulsions was also examined and discussed.

Keywords: Wheat germ lipase; Triolein; Hydrolysis; Water-in-oil microemulsion

1. Introduction

In resent years, extensive studies have been carried out on enzyme-containing water-in-oil (w/o) microemulsion systems for their potential in biocatalysis [1–3]. Water-in-oil microemulsion is thermodynamically stable, nanometer-sized water droplets dispersed in an organic phase by means of surfactants. It provides an aqueous phase for hydrophilic enzymes, an interface for surface-active enzymes and an organic phase for hydrophobic substrates or products. The enzyme molecules can be entrapped in the water pools, avoiding direct contact with organic solvent that potentially denatures to enzyme. The w/o microemulsion environment represents a medium where the aqueous/organic interface is very large (approximately 100 m^2/ml) [4]. Increasing the interfacial area is of great technological interest because it leads to an increase in the number of substrate molecules available to react. In this system, the enzymes are active in the conversion of both hydrophilic and hydrophobic compounds.

Wheat germ lipase (WGL) is a plant esterase. There have been a few quantitative studies of the enzymatic reactions in bulk water and w/o microemulsions [5,6], in which only the short-chain substrates were involved. However, planes contain a large amount of long-chain water-insoluble lipids, of which wheat-germ-catalyzed hydrolysis occur to provide the energy source for germination. Therefore, studies of hydrolysis of long-chain water-insoluble lipids catalyzed by wheat germ are of considerable importance in simulating and understanding cell metabolism in plants. These

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studies are also important to practical applications. Muzzarelli et al. [7] reported the depolymerizations of chitosans and substituted chitosans with the aid of wheat germ lipase preparation. Christopher et al. [8] pointed that the deterioration of flour quality over time, which is important in milling and baking industry, was caused by the action of lipase on the storage triacylglycerols. Water-insoluble lipids hydrolyzed by wheat germ lipase can not be processed in aqueous solutions or organic solvents due to hydrophobicity of lipids and insolubility of lipase in organic media. These reactions most likely occur in the interface or in the area of near the interface in the w/o microemulsions, hence have simpler enzyme-catalyzed kinetics. Therefore, it could be a good reaction system to test various theoretical models [1–3]. To the best of our knowledge, hydrolysis of water-insoluble lipids hydrolyzed by wheat germ lipase in w/o microemulsion has not yet been reported. In the present studies, we report the characterization of hydrolysis of triolein catalyzed by wheat germ lipase in w/o microemulsions of sodium bis-(2-ethylhexyl) sulfosuccinate (AOT)/cyclohexane/water. The influences of the characteristic parameters of this system, such as pH, temperature (T), molar ratio (ω_0) of water to AOT, the concentrations of enzyme and substrate, on activity and stability of enzyme are examined. The apparent Michaelis constant $K_{m,a}$ maximum reaction rate V_m and activation energy E_a are also calculated from the initial reaction rates determined by the experiments.

2. Experimental

2.1. Materials

AOT was purchased from Fluka was purified according to the method of Martin and Magid [9] and dried over P_2O_5 under vacuum. WGL, triolein (>98%) and cyclohexane (>99.5%) were purchased from Sigma, Shanghai Chemical Factory and Xi'an Chemical Company Inc., respectively, and were used without further purifications.

2.2. Measurement of reaction rate

Reaction rate measurements were principally based on the methods described by Kwon and Rhee [10] and Lowry and Tinsley [11]. The lipase solutions used in experiments were prepared by weighing the desired amount of WGL into the phosphate buffer solutions (PBS, 0.05 mol/l, pH 7.4). The substrate solutions, which contained 0.1 mol/l AOT and desired amount of triolein (S), were prepared by mixing AOT, cyclohexane and triolein. A screw-capped tube was filled with 0.5 ml of substrate solution and a desired amount (2–8 μl) of WGL lipase solution. The desired ratio of water to AOT was adjusted by addition of PBS (1–7 μl) without lipase into the tube. The reaction system then was vortically mixed to initiate the reaction. Usually, after 20 s of mixing, a clear microemulsion formed. The concentrations of AOT, WGL, and triolein in the microemulsion reaction systems were 0.1 mol/l, 1–16 μg/ml, and 0.01–0.15 mol/l, respectively. The tube then was set in a water bath where the temperature was constant within ±0.1 K. After a desired period of time, 0.2 ml cupric acetate-pyridine solution (5% $Cu(Ac)_2$, pH 6.1) was added to the tube and the tube was shaken thoroughly and then heated for 3 min in a boiling-water bath to stop the reaction and to disrupt the structure of w/o microemulsion. After the mixture was separated into two phases, 0.1 ml sample taken from the upper phase was added into a tube containing 1.9 ml of cyclohexane for absorbance measurements at 707 nm by a spectrophotometer (model HP8453). A sample prepared by the same procedure but without the lipase was taken as a reference. The measured absorbance was then conversed to the concentration of oleic acid through a predetermined standard curve of oleic acid concentration versus absorbance. It was found that the initial reaction rate was constant at least in initial 1 h after the reaction started. Therefore, through this work, the reaction time was limited in 1 h and the slopes of straight lines in the plots of oleic acid concentrations versus reaction time were taken as the initial reaction rates. All of the reported data of initial reaction rates were the averages of that measured in triplicate samples and were reproducible within 10%.

2.3. Measurements of lipase stability in w/o microemulsions

The enzyme stability in AOT-based w/o microemulsions may be investigated by measurements of the initial reaction rates for reaction systems after various

pre-incubation periods of time. Each of 0.25 ml solutions, containing 21.6 μg/ml enzyme, and 0.1 mol/l AOT but with various ω_0 and without triolein, was pre-incubated at 40 °C for a period of desired time followed by addition of 0.25 ml substrate solution containing 0.1 mol/l triolein and 0.1 mol/l AOT to start the reaction. After addition of the substrate solution, the value of ω_0 was readjusted to 9 by adding appropriate small amount of PBS. Therefore, each of reactions after pre-incubations was carried out at a standard condition: $[E]_{ov} = 10.8\,\mu g/ml$, $[S] = 0.05\,mol/l$, $[AOT] = 0.1\,mol/l$, $\omega_0 = 9$, $T = 40\,^{\circ}C$ pH = 7.4. Each of initial reaction rates then was measured in a period of 1 h and normalized by the initial reaction rate of the same reaction but without pre-incubation and taken as the relative residual activity.

3. Results and discussion

3.1. Effect of "water pool" size in w/o microemulsion on enzyme activity

The activity of lipase in the w/o microemulsion is related to the microstructure of the w/o microemulsion. The diameter of water pools in w/o microemulsion appears to be the most important factor that determines the reaction rate and it has been studied most extensively in recent years. The diameter of water pools in w/o microemulsion strongly dependents on ω_0. It was reported that altering ω_0, from the minimal value necessary to stabilize the microemulsion to higher value, results in three different activity curves of the enzymes [2]: the saturation cure, the bell-shaped curve, and a curve where enzyme activity decreases continuously as the value of ω_0 increases.

At a constant total AOT concentration of 0.1 mol/l and a constant overall enzyme concentration $[E]_{ov}$ (referring to whole volume of system) of 16.2 μg/ml, the effect of ω_0 on the WGL activity in the w/o microemulsion was determined under the conditions of pH 7.4 and 40 °C. The experimental results showed that the activity of wheat germ lipase in the AOT microemulsion system had a typical bell-shaped dependence on ω_0 with a maximum value of initial reaction rate V at $\omega_0 = 9$ as shown in Fig. 1a.

Keeping $[E]_{ov}$ and the concentration of AOT constant yields the change of the enzyme concentration in the water pool ($[E]_{wp}$) with ω_0. As pointed by Han et al. [12], it is not clear whether the overall enzyme

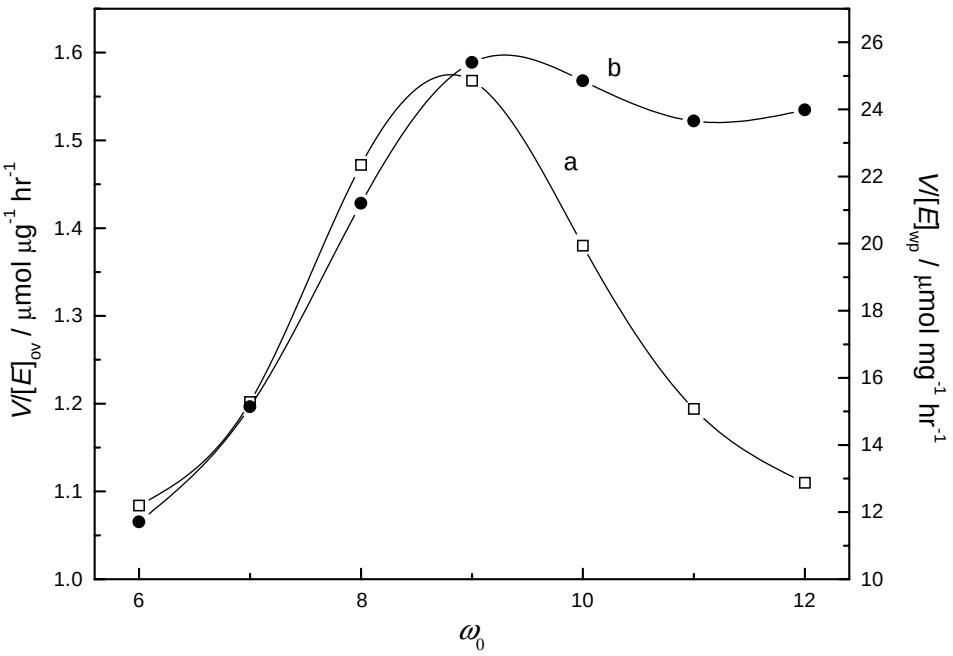

Fig. 1. Dependence of $V/[E]_{ov}$ (a) and $V/[E]_{wp}$ (b) on ω_0 at 40 °C, pH = 7.4, [S] = 0.05 mol/l, [AOT] = 0.1 mol/l, and $[E]_{ov} = 16.2\,\mu g/ml$.

concentration in the system ($[E]_{ov}$) or the enzyme concentration in the water pool ($[E]_{wp}$) should remain constant. A wrong choice of a concentration to be constant will yield the distortion of the profiles of activity versus ω_0. For the system we studied, the lipase contacts with the water pool interface and the substrate triolein is partitioned between the organic phase and interface. Thus, the reaction most likely takes place at the interface or near the interface and the enzyme concentration in water pool may affect the initial reaction rate. Fig. 1b shows a plot of $V/[E]_{wp}$, which is similar to that with $[E]_{wp}$ being constant reported by Piera-Velazquez et al. [13]. The existence of optimal ω_0 for lipase activity was probably related to conformational changes of the protein, to the changes in the water structure in the hydration shell, and to the activity of water in the microemulsion.

3.2. Effects of pH and temperature on enzyme activity

The activity and stability of lipase in w/o microemulsion are sensitive to pH, as the charge density of enzyme surface changes with the variation of pH, which results in change of interaction between enzyme and surfactant head groups. The effect of pH on the activity of the wheat germ lipase was investigated at 40 °C for various PBS of pH 6.8–8.0. The results showed a maximum activity around pH 7.4.

The effect of temperature on the activity of the wheat germ lipase in the temperature range of 20–50 °C was investigated at pH 7.4, which indicated that the optimum reaction temperature was around 40 °C. When the temperature is below 40 °C, the reaction rate increased with the temperature. However, beyond this temperature, the reaction rate decreased rapidly due to the enzyme denaturation.

Activation energy E_a of this lipase reaction in the microemulsion system was calculated from the Arrhenius plot (see Fig. 2), which was found to be (19.7 ± 0.2) kJ/mol.

3.3. Effects of enzyme concentration and substrate concentration on enzyme activity

The enzyme-catalyzed kinetics has been investigated in details in AOT-based w/o microemulsions and

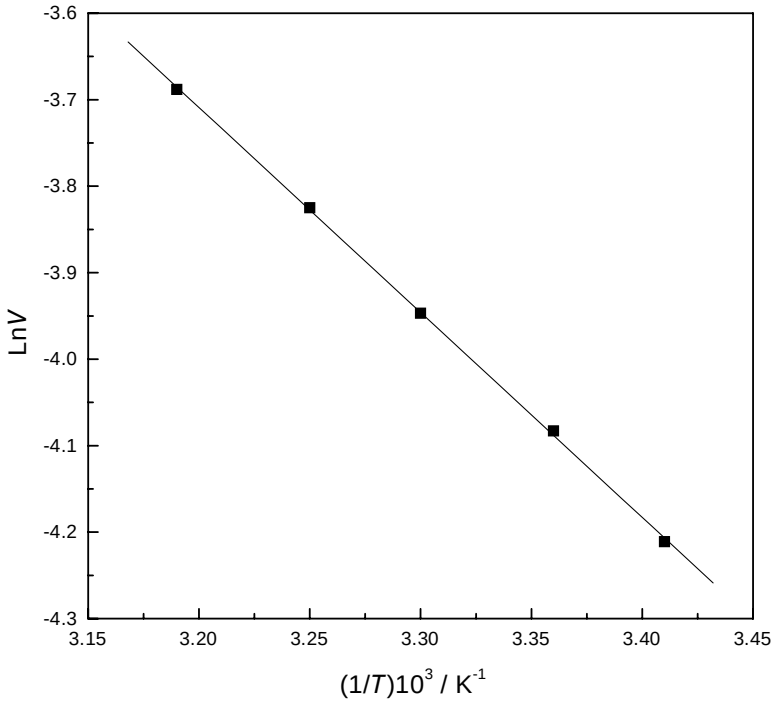

Fig. 2. Arrhenius plot of initial reaction rate of triolein hydrolysis at pH = 7.4, $\omega_0 = 9$, [S] = 0.05 mol/l.

various kinetic models also have been proposed to explain the mechanism of the reactions [1–3,14]. It was well known that enzyme-catalyzed reactions usually obey Michaelis–Menten kinetics, and the reaction rate may be expressed as:

$$V = \frac{V_m[S]}{K_{m,a} + [S]} \quad (1)$$

where

$$V_m = k_{cat}[E] \quad (2)$$

$K_{m,a}$, V_m, [S], and [E] represent the apparent Michaelis constant, maximum activity, the concentration of substrate, and the concentration of enzyme, respectively. The initial reaction rate is proportional to the enzyme concentration when the substrate concentration constant. With AOT concentration, substrate concentration, ω_0, pH, and temperature being fixed at 0.1 and 0.05 mol/l, 9 and 7.4, and 40 °C respectively; the initial reaction rates were measured for various concentrations of wheat germ lipase and are illustrated in Fig. 3. The above conditions ensured that the concentrations [E] in our experiment for both "overall" and "in water pool" were changed simultaneously. A good linear relationship between V and [E] indicates the validity of Eq. (1) to hydrolysis of long-chain water-insoluble lipids catalyzed by wheat germ lipase.

When the overall enzyme concentration $[E]_{ov}$ was kept constant (24.4 U/ml), a series of initial reaction rates for various substrate concentrations were measured while the other above variables being fixed at the same values. Here, 1 U of enzyme was defined as the amount of lipase liberating 1 μmol of triolein/h under the constant assay conditions: [AOT] = 0.1 mol/l, [S] = 0.05 mol/l, pH = 7.4, $\omega_0 = 9$, and $T = 40$ °C. A profile of V versus [S] is shown in Fig. 4. A plot (Fig. 5) of $1/V$ versus 1/[S] (Lineweaver–Burk plot) yielded a good straight line. The values of apparent $K_{m,a}$ and V_m were obtained from the slope and the intercept of the straight line, which were (76 ± 4) mmol/l and (59 ± 2) μmol/(ml h), respectively.

3.4. Lipase stability

An important problem for application of the lipase to hydrolysis in w/o microemulsion is the denaturation or deactivation of the enzyme in the micro-phase,

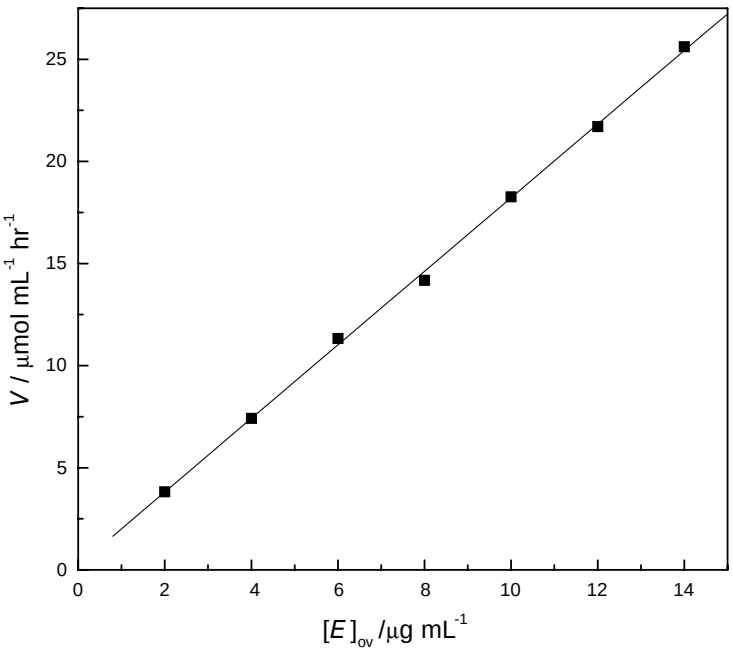

Fig. 3. Dependence of initial reaction rate on enzyme concentration at 40 °C, pH = 7.4, $\omega_0 = 9$, [S] = 0.05 mol/l, [AOT] = 0.1 mol/l.

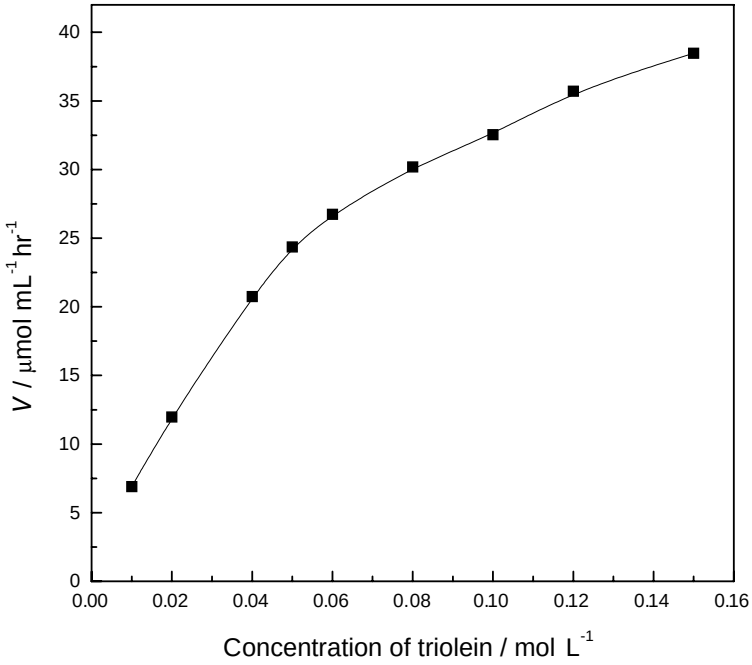

Fig. 4. Dependence of initial reaction rate on substrate concentration at 40 °C, pH = 7.4, $\omega_0 = 9$, [AOT] = 0.1 mol/l, and $[E]_{ov} = 24.4$ U/ml.

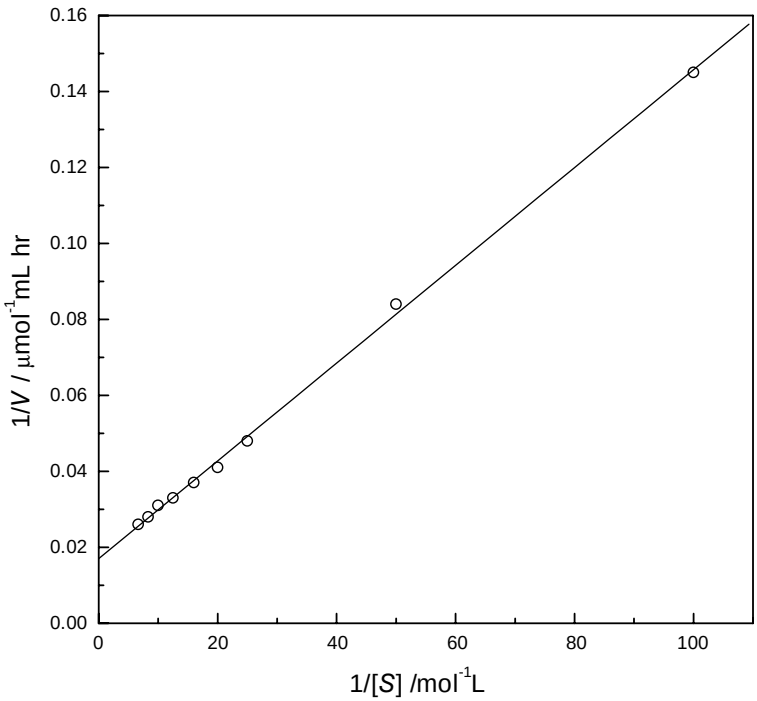

Fig. 5. Lineweaver–Burk plot of initial reaction rate of triolein hydrolysis at 40 °C, pH = 7.4, $\omega_0 = 9$, [AOT] = 0.1 mol/l, $[E]_{ov} = 24.4$ U/ml.

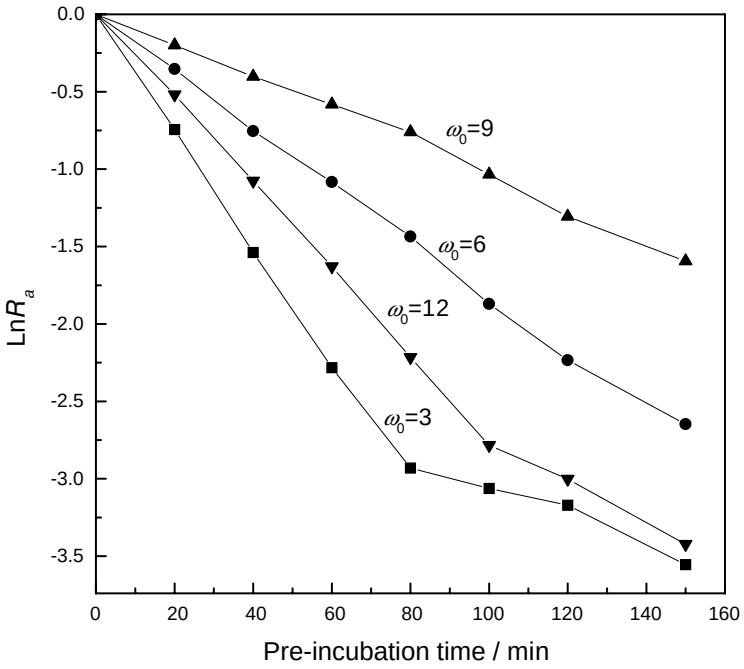

Fig. 6. Stability of enzyme in w/o microemulsions at different ω_0 at 40 °C, pH = 7.4 and $[E]_{ov}$ = 10.8 μg/ml, (▲) ω_0 = 9, (●) ω_0 = 6, (▼) ω_0 = 12, (■) ω_0 = 3.

which has received great attention recently [2,3,15–18]. In this work, we investigated the stability of wheat germ lipase in AOT-based w/o microemulsions with different ω_0 values by measurements of relative residual activities (R_a).

Fig. 6 shows a plot of ln(R_a) versus pre-incubation time for various ω_0, from which one can see that the enzyme has its best stability at $\omega_0 = 9$ in w/o microemulsions. Denaturation of enzyme in w/o microemulsions proceeds faster at lager or smaller value of ω_0 than 9. This ω_0-dependent stability may be explained by changes of the interactions among the enzyme molecules and the heads of surfactant molecules and the organic phase.

Losing nearly 45% of lipase activity within a period of pre-incubation time of 60 min at $\omega_0 = 9$ seems contradictory to the constant initial reaction rate in the same period. This "contradictory" also has been found by Han et al. [15]. However, it may be explained by the contributions of substrate (triolein) and products (diglyceride, monoglyceride, oleic acid) to enzyme stabilization [15–18].

4. Conclusion

We have studied the triolein hydrolysis catalyzed by wheat germ lipase in w/o microemulsions, which is unable to be proceeded in aqueous solutions or organic solvents. We found the optimum values for this bioconversion to be 7.4, 9 and 40 °C for the pH, the molar ratio of water to AOT, and the reaction temperature, respectively. The initial reaction rate has been found to be proportional to the enzyme concentration. The Lineweaver–Burk plot has shown as a straight line with the values of apparent $K_{m,a}$ and V_m being (76 ± 4) mmol/l and (59 ± 2) μmol/(ml h), respectively. This phenomenon confirmed the validity of Michaelis–Menten kinetics. The profile of enzyme activity versus size of the water pool was found to be dependent on what concentration of enzyme, the overall concentration in the w/o microemulsion or the concentration in the water pool, being kept constant. When the overall concentration was constant, the profile showed a bell-shaped curve. It was also found that the optimum value of the molar ratio of water to

surfactant is not only for activity but also for the stability of the wheat germ lipase in w/o microemulsions.

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