A Kinetic Model for the Enzyme-Catalyzed Self-Epoxidation of Oleic Acid

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ABSTRACT: This paper reports a kinetic model for the self-epoxidation of oleic acid with toluene as solvent and Novozym 435 (a commercially available preparation of immobilized *Candida antarctica* lipase) as catalyst at 30°C. The effects of various parameters on the conversion and rates of reaction were studied. Both the initial rate and the progress curve data were used to fit an ordered bi-bi model. At low temperatures, the rate of epoxidation was faster than the rate of deactivation of the enzyme by hydrogen peroxide.

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KEY WORDS: Chemical kinetics, enzyme deactivation, immobilized lipase, oleic acid, self-epoxidation.

Epoxidation of olefinic compounds leads to the formation of commercially important precursors that are employed to manufacture glycols, alcohols, carbonyls, alkanolamines, substituted olefins, polyesters, polyurethanes, and epoxy resins. The unsaturation present in fatty acid molecules and their corresponding esters can be harnessed to prepare a gamut of epoxidized compounds. For instance, epoxides of soybean and other vegetable oils have been employed as plasticizers and stabilizers for the plastics of polyvinyl chloride (PVC) and other related resins to improve the flexibility, elasticity, and toughness of the plastics and also to impart thermal and photo stability. Among the various chemical routes available for the synthesis of epoxidized oils, the most important and widely applied one involves the use of a suitable organic peracid (1–4) with an unsaturated oil. The next important process is the oxidation of unsaturated compounds in the presence of aldehydes (5). However, the disadvantage of the latter process is that the aldehyde should be taken in stoichiometric quantities with the acid, leading to by-product formation. As compared to chemical processes, enzyme-catalyzed epoxidations are advantageous from several viewpoints, such as mild reaction conditions and high selectivity/specificity with little or no by-product formation. Self-epoxidation of unsaturated acids with hydrogen peroxide in the presence of a lipase is a novel application of enzyme catalysis (6) because by-products, such as dihydroxy acids and estolides, that are so common in the epoxidation of fatty acids by chemical routes are not formed in lipase-catalyzed epoxidations; the only by-product is a small amount of epoxy-peroxy acid. The formation of carboxylic acid in lipase catalysis is suppressed as a result of the poor solubility of water in apolar solvents in comparison with hydrogen peroxide. Since the peracid is generated *in situ,* the storage problems associated with it are avoided. Epoxidation of unsaturated acids, including oleic acid with a commercially available immobilized lipase (Novozym 435), involving the addition of hydrogen peroxide in a semibatch mode has been reported (7). However, no kinetic information is available. Therefore, it was thought worthwhile to study kinetic modeling of epoxidation of oleic acid as a model compound in a batch system with Novozym 435 as the catalyst.

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MATERIALS AND METHODS

Enzymes and chemicals. Novozym 435 (*Candida antarctica* lipase immobilized on macroporous polyacrylate resin beads, bead size 0.3–0.9 mm, bulk density 430 kg/m^3, activity of 7,000 PLU/g) was a gift from Novo Nordisk (Bagsvaerd, Denmark). The water content of Novozym 435 was approximately 3% w/w. Oleic acid was a gift from M/s Godrej Soaps Ltd. (Mumbai, India). Toluene and other solvents were of A.R. grade and procured from M/s s.d. Fine Chem Pvt. Ltd. (Mumbai, India). Hydrogen peroxide (50% w/w) was acquired from E. Merck (Darmstadt, Germany).

Experimental setup. The reactions were carried out in a mechanically agitated glass reactor of 4 cm i.d. and 100 mL capacity, equipped with a six-bladed, pitched-turbine impeller. The reactor assembly was immersed in a thermostatic water bath, whose temperature was maintained within 1°C of the desired temperature.

Reaction conditions. Unless otherwise stated, the reaction was carried out by using the standard conditions as follows: A mixture of 0.025 mol oleic acid and 0.0529 mol H_2O_2 was diluted to 65 mL with toluene. The biphasic system consisted of 3.6 mL of aqueous phase and 61.4 mL of organic phase. Oleic acid**,** being soluble in toluene, was preferentially present in the organic phase and thus its amount present in 3.6 mL of the aqueous phase was considered to be negligible. The reaction was carried out at 30°C at a speed of 650 rpm and initiated by adding 300 mg catalyst to the equilibrated reaction mixture. Samples were withdrawn periodically and analyzed by titrimetry.

Analytical methods. The oxirane oxygen content of the epoxide was determined by the tetraethyl ammonium bromide-perchloric acid method of Jay (8). The unsaturation, which was equivalent to the unreacted oleic acid, was deter-

mined by the bromate-bromide method of Lucas and Pressman (9). Peroleic acid was assumed to be in a steady state and no effort was made to analyze it, since excessive sampling might have led to erratic kinetic measurements. It should be noted that an oxirane oxygen content of 5.362% is equivalent to 100% epoxy stearic acid.

Determination of initial rates. Several experiments were performed, and the initial rates of reaction were determined as a function of concentrations of oleic acid, hydrogen peroxide, and the catalyst. The amount of oleic acid varied between 3 and 13 mmol, at different fixed quantities of hydrogen peroxide. The reaction was carried out to 10% conversion of the limiting reactant and the initial rates were calculated from the slopes of the concentration-time curves at zero time.

RESULTS AND DISCUSSION

A variety of immobilized lipases and solvents was employed for the reaction, among which Novozym 435 as catalyst and toluene as solvent were found to give the best results, in consonance with results reported by Björkling *et al.* (10,11). For the measurement of intrinsic kinetics, the effects of various parameters on initial rates and conversion profiles were studied with Novozym 435 as the catalyst and toluene as the solvent.

The effect of speed of agitation on the rate of the reaction was studied in the range of 250 to 850 rpm in order to operate the system in the absence of external mass resistance (Fig. 1). It was observed that the initial rate increased with increasing speeds up to 650 rpm, beyond which there was a decrease in the rate. This could have happened due to one of the following reasons. In a three-phase slurry reaction, containing aqueous and organic phases and solid-bound enzyme, the influence of mass transfer has to be properly assessed. The polymer supported catalyst typically lies at the organic-aqueous interface due to its bulk density, which lies between those of the aqueous and organic phases. Mass transfer between aqueous and organic phases is a function of the interfacial area, which is dependent on the speed of agitation and the aqueous phase–organic phase volume ratio. In the current case an increase in agitation speed led to an increase in the aqueous-organic interfacial area because of the formation of smaller droplets of the aqueous phase (dispersed phase), from whence hydrogen peroxide was transferred to the organic phase, and thus there was a corresponding increase in the rate up to 650 rpm. Thereafter, the rate decreased owing to either abrasion of the enzyme at higher speed or decreased contact between the enzyme and the substrate. Therefore, in further experiments, the speed was maintained at 650 rpm.

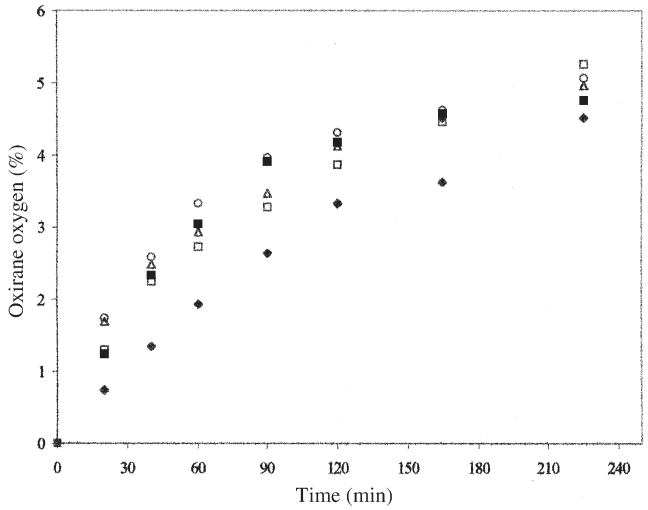

FIG. 1. Effect of speed of agitation for 25 mmol oleic acid (OA) + 52.92 mmol hydrogen peroxide + toluene to make a total volume of 65 mL + 300 mg Novozym 435 (Novo Nordisk, Bagsvaerd, Denmark); and temperature = 30°C (◆ 250 rpm, □ 350 rpm, △ 500 rpm, ○ 650 rpm, ■ 850 rpm).

To verify the effect of intraparticle diffusion limitations, Novozym 435 particles were sieved through sieves of different mesh sizes, and the effect of bead size on the reaction rate was studied. Within experimental error, the rate was found to be the same irrespective of particle size, which suggested the absence of intraparticle diffusion limitations for the particle sizes tested. This was further confirmed by calculating the Thiele's modulus for a spherical particle as 0.2 and the effectiveness factor as 0.99 according to the procedure given by Bailey and Ollis (12). Absence of intraparticle diffusion limitations was further confirmed by invoking the Weisz-Prater criterion (C_{WP}) which is given by the following equation,

$$C_{WP} = R^2 r_{obs} \rho_P / (D_E C) \quad [1]$$

where R = radius of the catalyst particle (cm), r_{obs} = observed rate of reaction (mol/s/g catalyst), ρ_P = bulk density of the enzyme particles (g/cm^3), D_E = effective diffusivity of oleic acid (cm^2/s), and C = concentration of oleic acid (mol/cm^3).

According to the Weisz-Prater criterion, if $C_{WP} \gg 1$, then the reaction is limited by severe internal diffusional resistance, and if $C_{WP} \ll 1$, then the reaction is surface reaction controlled.

In the present case, the typical values are: $\rho_P = 0.43$ g/cm^3, $r_{obs} = 1.15 \times 10^{-5}$ mol·s^{-1}·(g enzyme)$^{-1}$, $C = 4.012$ mol/cm^3, $R = 0.045$ cm, and $D_E = 1.064 \times 10^{-6}$ cm^2/s. Thus, the calculated C_{WP} was found to be equal to 0.00235, indicating that there was no intraparticle diffusional resistance. The effect of catalyst loading was studied under otherwise similar conditions to determine whether the rate of the reaction increased with increasing catalyst loading. The plot of initial rate against catalyst loading was a straight line passing through the origin, which further confirmed that the reaction was kinetically controlled (Fig. 2).

Effect of mole ratio. In one set of experiments, the moles of hydrogen peroxide were kept constant (52.93 mmol). When the mole ratio of oleic acid to hydrogen peroxide was varied from 0.25:1 to 0.5:1, there was no change in the rate of the reaction. However, there was a decrease in the rate when the ratio was further increased to 0.75:1 and 1:1 (Fig. 3), which may be explained as follows: In effective substrate-enzyme complex formation, the substrate will bind to the enzyme in the correct place with correct orientation. But as the amount of the substrate is increased, more substrate mole-

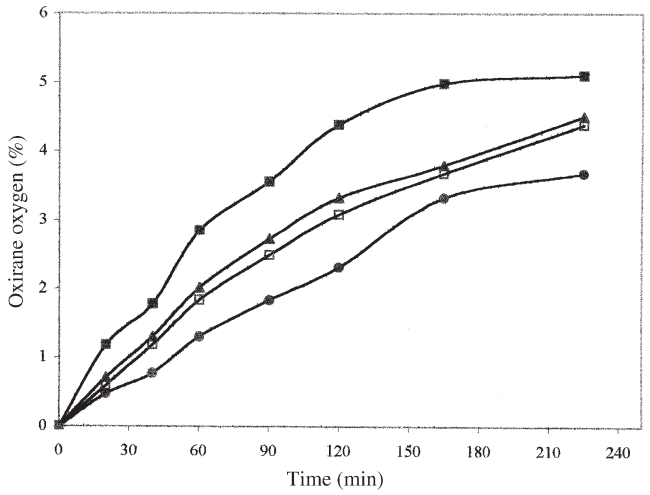

FIG. 2. Effect of catalyst loading for 25 mmol OA + 52.96 mmol hydrogen peroxide + toluene to make a total volume of 65 mL + required quantity of Novozym 435; temperature = 30°C and speed = 650 rpm (● 200 mg, □ 300 mg, ▲ 400 mg, ■ 500 mg). See Figure 1 for abbreviation and manufacturer.

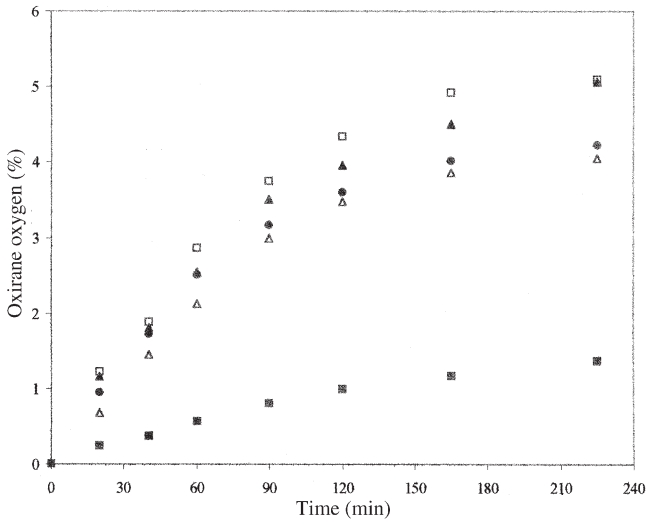

FIG. 4. Effect of mole ratio (oleic acid constant) on 25 mmol OA + required quantity of hydrogen peroxide + toluene to make a total volume of 65 mL + 300 mg Novozym 435; temperature = 30°C; and speed = 650 rpm (■ 1:1 H_2O_2/OA, □ 1.6:1 H_2O_2/OA, ▲ 2:1 H_2O_2/OA, ● 2.6:1 H_2O_2/OA, △ 3.7:1 H_2O_2/OA). See Figure 1 for abbreviation and manufacturer.

cules will try to bind to the enzyme molecule at the same sites. The absence of any free sites will make the substrate-enzyme complex ineffective, thus retarding the reaction.

In another set of experiments, the mole ratio was changed, keeping oleic acid constant at 25 mmol. When the mole ratio of hydrogen peroxide to oleic acid was increased from 1:1 to 1.6:1, the conversion rate increased, reaching a maximum at 1.6:1 (Fig. 4). When the mole ratio was increased further from 2:1 to 3.7:1, there was a decrease in the rate as well as the extent of conversion. At higher quantities of hydrogen peroxide, this decrease may be due to the inactivation of the enzyme in the presence of hydrogen peroxide, which was studied independently (14).

From the above experiments it can be inferred that higher concentrations of oleic acid caused inhibition of the reaction by forming an ineffective substrate complex. However, this inhibition did not lead to any irreversible deactivation. It was observed by Warvel and gen Klaas (7) that the enzyme could be used 15 times without any significant loss in activity when an excess quantity of oleic acid was used and hydrogen peroxide added dropwise. Our results are in conformity with these observations.

The effect of temperature on the rate of reaction was studied at 30, 37, 45, and 52°C (Fig. 5). There was an increase in

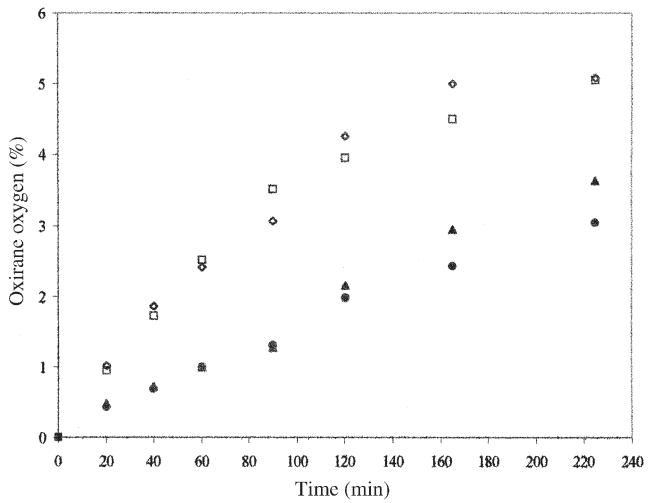

FIG. 3. Effect of mole ratio (hydrogen peroxide constant) on required quantity of OA + 52.92 mmol hydrogen peroxide + toluene to make a total volume of 65 mL + 300 mg Novozym 435; temperature = 30°C; and speed = 650 rpm (◇ 0.25:1 OA/H_2O_2, □ 0.5:1 OA/H_2O_2, ▲ 0.75:1 OA/H_2O_2, ● 1:1 OA/H_2O_2). See Figure 1 for abbreviation and manufacturer.

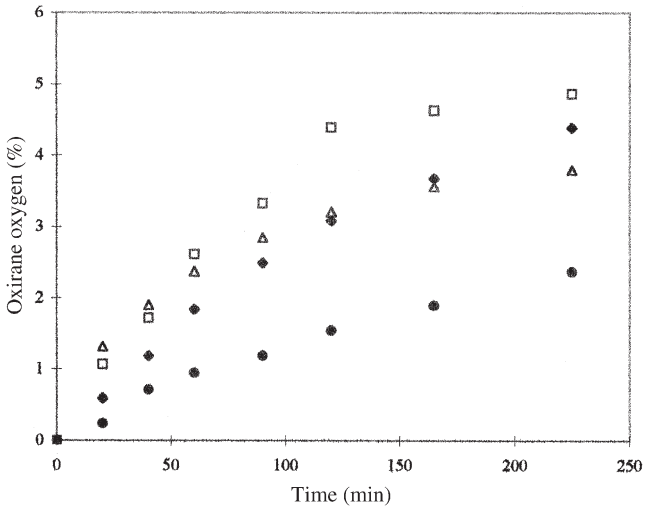

FIG. 5. Effect of temperature on 25 mmol OA + 52.92 mmol hydrogen peroxide + toluene to make a total volume of 65 mL + 300 mg Novozym 435; speed = 650 rpm (◆ 30°C, ■ 37°C, ▲ 45°C, ● 52°C). See Figure 1 for abbreviation and manufacturer.

the initial rate with a temperature increase from 30 to 45°C. However, the enzyme was found to be deactivated as the reaction progressed at 45°C, and it showed little activity at 52°C. Novozym 435 is reported to be stable for several thousand hours between 60 and 80°C (15). We have studied the deactivation of Novozym 435 at different temperatures in the preparation of perlauric acid, wherein it was found that the thermal instability was due to the presence of hydrogen peroxide (14).

Reusability of catalyst. During the first use, the fresh enzyme lost about 50% of its activity. After the second use, i.e., first reuse, the activity decreased to 12% of the original activity (Fig. 6). Our earlier studies in the preparation of perlauric acid showed that the enzyme is stable in the presence of toluene and lauric acid, and is deactivated by hydrogen peroxide. In the current studies too, it is likely that the deactivation may be due to hydrogen peroxide. Warwel and gen Klaas (7) reused the enzyme 15 times with almost full retention of activity by using excess amounts of oleic acid, which suggests that oleic acid does not cause any irreversible deactivation of the enzyme. It is assumed that the rate of peroleic acid conversion to oleic acid *via* oxygen release immediately after its formation is faster than the formation of peroleic acid and hence there is no accumulation of peroleic acid to cause any deactivation of the enzyme. Thus no term for the concentration of peroleic acid appears in the kinetic model.

Kinetic modeling from initial rate measurements. From the initial rate measurements it was observed that the rate increased with increasing quantity of oleic acid. The Lineweaver-Burk plot (Fig. 7) demonstrates that the slope increases whereas the intercept decreases with increasing concentrations of hydrogen peroxide. The reverse is the case when the concentration of hydrogen peroxide is low, which implies that hydrogen peroxide acts as an inhibitor. The progress curve analysis, enzyme reusability studies, and deactivation studies taken together confirm that hydrogen peroxide causes irreversible deactivation of the enzyme. However, for initial rate analysis it is assumed that hydrogen peroxide acts only as a dead-end inhibitor and the inhibition step is shown to be reversible.

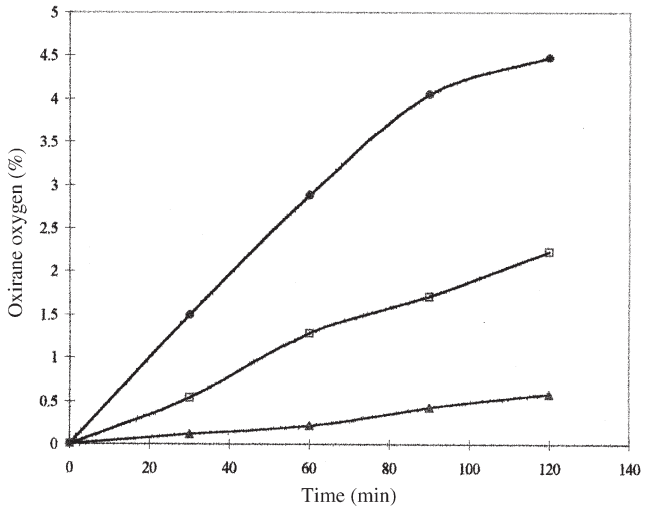

FIG. 6. Reusability of catalyst for 25 mmol OA + 52.92 mmol hydrogen peroxide + toluene to make a total volume of 65 mL + 300 mg Novozym 435; temperature = 30°C; and speed = 650 rpm (● fresh use, □ first reuse, ▲ second reuse). See Figure 1 for abbreviation.

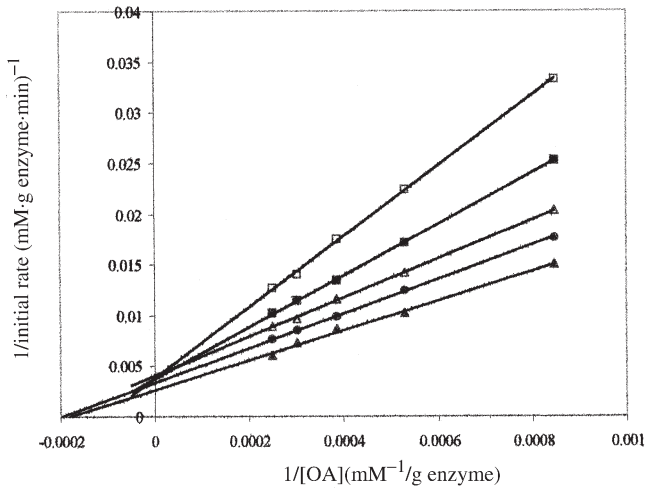

FIG. 7. 1/Initial rate vs. 1/concentrated OA. Quantity of OA varied between 1150 and 4020 mM/g catalyst at different constant quantities of hydrogen peroxide in total volume of 65 mL with toluene as solvent + 300 mg Novozym 435; temperature = 30°C; and speed = 650 rpm (● 3,430.37 mM H_2O_2/g catalyst, ▲ 4,901.87 mM H_2O_2/g catalyst, △ 6,370.67 mM H_2O_2/g catalyst, ■ 7,840.83 mM H_2O_2/g catalyst, □ 9,311 mM H_2O_2/g catalyst). See Figure 1 for abbreviation.

When the quantity of oleic acid was low, the rate decreased with increasing amounts of hydrogen peroxide. At higher quantities of oleic acid, the rate increased with increasing quantities of hydrogen peroxide, which is in agreement with the observations made by the progress curve analysis.

From the Lineweaver-Burk plots, it was observed that there were no parallel lines, ruling out the ping-pong bi-bi mechanism. In fact, the lines were intersecting at a point suggesting the formation of ternary complex. To confirm the application of the ternary complex mechanism, the same data were analyzed by two methods, namely, (i) ENCORA, which uses a modified Simplex optimization method (Hennipman, J.W., B. Romein, and A.J.J. Straathof, Delft University of Technology, Delft, The Netherlands), and (ii) nonlinear regression analysis using the package MathCad.

By both methods, the sum of squared errors (SSE) was found to be minimum for the ternary complex mechanism. The kinetic parameters determined by the two methods are given in Table 1 along with the sum of squared errors. Formation of the ternary complex can be depicted as follows:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AE$$

$$AE + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ABE$$

$$ABE \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EQ + P$$

TABLE 1
Kinetic Parameters Obtained for the Epoxidation of Oleic Acid[a]

Parameter	ENCORA analysis	SSE	MathCad analysis	SSE
V_{max} (mmol/L/min/g enzyme)	2.229×10^5	0.0623	3.614×10^5	0.013
K_{mA} (mmol/L/g enzyme)	1.054×10^5		3.852×10^5	
K_{mB} (mmol/L/g enzyme)	3.789×10^6		5.617×10^6	
K_{iA} (mmol/L/g enzyme)	59.986		65.618	
K_i^1 (mmol/L/g enzyme)	9.892×10^2		2.55×10^2	

[a] V_{max} = maximum velocity; K_{mA} = Michaelis constant for oleic acid; K_{mB} = Michaelis constant for hydrogen peroxide; K_{iA} = dissociation constant for enzyme-oleic acid complex; ENCORA analysis, a modified Simplex optimization method developed by J.W. Hennipman, B. Romein, and A.J.J. Straathoff (Delft University of Technology, Delft, The Netherlands); MathCad, Version 6.0, MathSoft Inc., Cambridge, MA, 1996; SSE, sum of squared errors.

$$EQ \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + Q$$

$$E + B \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EB$$

where A = oleic acid, E = free enzyme, B = hydrogen peroxide, AE = enzyme-oleic acid complex, ABE = ternary complex of the enzyme + oleic acid + hydrogen peroxide, EQ = enzyme-peroleic acid complex, P = water, and Q = peroleic acid.

The rate or velocity (v) equation obtained with the above mechanism is as follows:

$$v = \frac{V_{max}[A]\cdot[B]}{K_{iA}\cdot K_{mB}\cdot\left(1+\frac{[B]}{K_i^1\cdot[B]}\right)+K_{mB}\cdot[A]+K_{mA}\cdot[B]\cdot\left(1+\frac{[B]}{K_i^1}\right)+[A][B]} \quad [2]$$

where V_{max} = maximum velocity, K_{iA} = dissociation constant for enzyme-oleic acid complex, K_{mA} = Michaelis constant for oleic acid, K_{mB} = Michaelis constant for hydrogen peroxide, and K_i^1 = inhibition constant due to hydrogen peroxide.

Based on crystallographic and molecular modeling studies of Lipase B from *C. antarctica,* it is postulated that the active site pocket can be partitioned into two sides, an acyl side and an alcohol side, where the corresponding parts of the substrate will be located during catalysis (16). In the current case, it is assumed that oleic acid fits into the acyl side and hydrogen peroxide fits the alcohol side to form the ternary complex.

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