STABILITY OF A COMMERCIAL LIPASE FROM *MUCOR JAVANICUS*: KINETIC MODELLING OF pH AND TEMPERATURE DEPENDENCIES

VICTOR M. BALCÃO, TERESA A. OLIVEIRA
and F. XAVIER MALCATA*

*Escola Superior de Biotecnologia, Universidade Católica Portuguesa,
Porto, Portugal*

The present communication reports experimental and modelling work pertaining to the independent roles of pH and temperature on deactivation of a crude lipase from *Mucor javanicus*. Experimental data of lipolytic activities were generated by a classic pH-stat assay on a triolein emulsion following incubation at several pH values for a fixed time, or at several temperatures for various times; postulated models were then fitted by nonlinear fitting to such data. The pH-dependence data were best fit by assumption of three forms of enzyme with increasing states of protonation, with pK_a values of 6.2 and 11.3, respectively, where only the intermediate form is stable within the time frame considered. The thermal-dependence data were best fit by assumption of parallel steps of deactivation and rearrangement, with activation energies of 228.8 and $221.7\,kJ\,mol^{-1}$, respectively.

Keywords: Enzyme; pH; Lipolytic activity; Mathematical modelling; Batch reactor; Activation energy; Stability

INTRODUCTION

Lipases (or acylglycerol acylester hydrolases, EC 3.1.1.3) are enzymes that catalyze cleavage of ester bonds of acylglycerols with concomitant

---

* Corresponding author. Address for correspondence: Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200 Porto, Portugal.

consumption of water molecules (hydrolysis), as well as its reverse reaction under microaqueous conditions (ester synthesis); if such two basic processes are combined in a sequential fashion, a general set of reactions commonly termed interesterifications is obtained (Balcão and Malcata, 1996a; Balcão *et al.*, 1996b).

Application of these enzymes in biotechnological processes is limited frequently by their rates of deactivation. Enzyme deactivation is usually associated with denaturation of the native protein molecule as a result of modifications of the secondary, tertiary, and/or quaternary structure. Several environmental factors promote such changes in the enzyme structure that reduce its catalytic activity, viz. pH, temperature, and physico-chemical nature of the reaction medium.

In most models of deactivation, an active lipase undergoes a reversible or irreversible structural change (i.e. temperature-induced conformational transition) or chemical change (e.g. deamidation or hydrolysis of side groups of amino acid residues, or destruction of disulfide bonds) to produce a catalytically inactive form (Ahern and Klibanov, 1985); such processes are frequently characterized by first-order kinetics (Bailey and Ollis, 1986), and the activation energies associated therewith often range from ca. 75 to 145 kJ mol^{-1}(Malcata *et al.*, 1992b). Although such simple first-order deactivation models have been widely reported in the literature, they are not adequate to describe the behaviour of enzymes in a number of instances (Bailey and Ollis, 1986; Henley and Sadana, 1986); hence, more complex models of enzyme deactivation have been proposed (Malcata *et al.*, 1992b). These models can in general be viewed as multi-step mechanisms which comprise irreversible or reversible rearrangements between various active forms of enzyme coupled with first-order irreversible or reversible deactivation of labile forms (Henley and Sadana, 1986); since, as enzymes, lipases contain basic, neutral, and acidic amino acid residues, such active forms may be accounted for by various prototropic variants in mutual equilibria at any given pH. Such ionizable groups often constitute part of the active site and are frequently involved in general acid–base catalysis (Bailey and Ollis, 1986); hence, the catalytic activity of the lipase is expected to change in a bell-shaped form with pH of incubation (Segel, 1993).

Conformational stability of lipases is an important technological factor, and so attempts to predict the variation in catalytic performance brought about by changes in pH, temperature, and processing time are in order, especially encompassing those enzymes that will most likely be utilized in practice; this research work attempted to meet this goal via mathematically simulating the stability of a commercial lipase.

MATERIALS

Enzyme

The lipase used was a commercial crude powder kindly supplied by Amano Pharmaceutical (Nagoya, Japan) and obtained from the mold *Mucor javanicus* (M10^{TM}). The lipase was used without further purification.

Chemicals

Citric acid monohydrate, *o*-phosphoric acid (85%, v/v), Coomassie brilliant blue G-250, ethanol for spectroscopy (UVASOL®), albumin fraction V (from bovine blood serum), sodium dodecyl sulphate, β-mercaptoethanol, *o*-phthaldialdehyde, tris(hydroxymethyl)-aminomethane, potassium hydroxide, sodium hydroxide, sodium tetraborate, dihydrated calcium chloride, potassium chloride, sodium chloride, and methanol were obtained from Merck (Darmstadt, Germany). Boric acid, trihydrated dipotassium hydrogenophosphate, Sigma Lipase Substrate®, and Wide Molecular Weight-Range SigmaMarker™ protein standards were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid (37%) was obtained from PRONALAB (Lisbon, Portugal). Native PAGE minigels and PhastGel SDS buffer strips were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Prior to use, tap water was subject to successive steps of reverse osmosis, adsorption, deionization, microfiltration, and photooxidation in a Milli-Q Plus 185 water purification system (Molsheim, France) to a final conductivity of $18.2 \, M\Omega \, cm^{-1}$.

Processing Equipment

A 200 mL jacketed glass beaker (Schott, Duran, Germany) was used to carry out the pH-stat assays. The setup comprised a constant temperature bath equipped with a mechanical agitator and a digital temperature controller (model VC) from Julabo Labortechnik (Seelbach, Germany) with external recirculation through the jacketed glass beaker, and a constant temperature bath equipped with a recirculation cooler (model ULTRATEMP FP40-VC, Julabo Labortechnik) for incubation of the lipase solutions.

Analytical Equipment

pH measurements were carried out with a Crison micro pH 2002 unit (Barcelona, Spain). Spectrophotometric measurements were carried out in a UV-VIS spectrophotometer model UV-1203 (Shimadzu, Kyoto, Japan).

Calorimetric studies were carried out in a differential scanning calorimeter (Shimadzu), which comprised a detector (DSC-50) and a thermal analyzer (TA-501). Electrophoretic analyses were carried out in a PhastSystem unit (Pharmacia LKB Biotechnology, Uppsala, Sweden).

EXPERIMENTAL PROCEDURES

Preparation of Stock Solutions

Aqueous Buffers

The tris(hydroxymethyl)aminomethane-HCl (Tris) buffer (pH 8.0) was prepared according to Dawson *et al.* (1969) with modifications: 50 mL of a 5 mmol/L solution of Tris were poured into a glass beaker, and 29.2 mL of a 5 mmol/L solution of HCl were then added, as well as NaCl and $CaCl_2$ up to a final concentration of 40 mmol/L and 20 mmol/L, respectively. The resulting solution was diluted to 100 mL with deionized water, and the resulting buffer was adjusted to pH 8.0 using NaOH. All other buffers were prepared according to the procedures by Dawson *et al.* (1969), viz. McIlvaine buffer (at pH 4, 5, 6, and 7), Clark & Lubs buffer (at pH 9 and 10), and hydroxide–chloride buffer (at pH 12).

Coomassie Solution

The stock solution of Coomassie was prepared according to the procedure by Robyt and White (1990) with modifications: 100 mg of Coomassie brilliant blue G-250 were dissolved in 50 mL of 99.9% ethanol, added to 100 mL of 85% *o*-phosphoric acid, and the resulting solution was then diluted to 1 L with deionized water.

o-Phthaldialdehyde Stock Solution

The OPA solution was prepared on a daily basis by mixing 50 mL of 100 mM of sodium tetraborate aqueous solution with 10 mL of 10% (wt/wt) sodium dodecylsulphate aqueous solution, 80 mg of *o*-phthaldialdehyde previously dissolved in 2 mL of methanol, and 0.2 mL of β-mercaptoethanol, and diluting to a final volume of 100 mL with deionized water.

Experimental Methods

Protein Assays

The amount of protein contained in the crude lipase preparation was determined by the Coomassie method (Balcão *et al.*, 1996c). This method

(also known as Bradford method) basically consisted in adding 0.5 mL aliquots of solution of crude lipase in McIlvaine buffer to 4.5 mL of Coomassie stock solution, followed by measurement of absorbance at 595 nm (using disposable cuvettes) after 5 min. Plain McIlvaine buffer was used as blank. The protein content was expressed as equivalent bovine serum albumin via utilization of a calibration curve in the range 0–500 μg/mL.

Electrophoresis Assays

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the crude commercial lipase preparation, as well as on molecular weight standards, using the PhastSystem unit and PAGE minigels (50 mm height × 43 mm width × 0.45 mm thickness) of 12.5% polyacrylamide. The buffer system in the gels was 0.112 M in acetate and 0.112 M in Tris (pH 6.5). The gels were run for 30 min at a constant electrical current of 10 mA using PhastGel SDS buffer strips consisting of 2% Agarose, 0.2 M tricine, 0.2 M Tris, and 0.55% SDS (pH 8.1). SigmaMarker™ protein standards were run in lanes parallel to that of crude lipase, and consisted of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase-b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), and aprotinin (6.5 kDa). After electrical resolution of the sample proteins, the gels were transferred to the development section of the PhastSystem unit and stained with Coomassie Blue.

Lipolytic Activity Assays

The lipolytic activity of the crude lipase powder was determined via a modified version of the classic pH-stat method. For each assay, Sigma Lipase Substrate™ (50% v/v olive oil emulsion stabilized with an emulsifier and further preserved with 0.1% sodium azide) was diluted to 1 : 5 with Tris-HCl buffer. A 40 mL volume of this mixture was transferred to a 200 mL jacketed glass beaker kept at 40°C in which a pH glass electrode was immersed. The emulsion's temperature was allowed to equilibrate for ca. 10 min, and then the pH of the emulsion was brought to ca. 8.0 by adding a few drops of KOH. A volume of 1.0 mL of the sample (ca. 20 mg$_{crude\ lipase\ powder}$/mL$_{Tris\text{-}HCl\ buffer}$) was pipetted into the thermally equilibrated substrate, and the pH was rapidly brought to ca. 8.0 via addition of several droplets of KOH. At this point, the stopwatch was started, the titrating solution of KOH (0.05 mol/L) was added for 10 min from a burette so as to maintain the pH of the reacting

mixture at 8.0, and the titre was calculated from the concentration and total volume of KOH used. For the blank, a similar procedure was applied but 1 mL of Tris-HCl buffer with no enzyme was added instead. The lipolytic activity was expressed in LU (lipolytic units), where 1 LU is defined as the number of micromoles of total free fatty acids released per min and per mg of lipase powder acting for 10 min (time period during which the process reaction curve was reduced to its linear part) on the aforementioned olive oil emulsion at pH 8.0 and 40°C.

pH Stability Assays

To a buffered solution at a desired pH, ca. 100 mg of crude lipase were dissolved in 5 mL of the corresponding buffer, and the resulting lipase solution was incubated at 20°C (temperature at which virtually no thermal deactivation of the commercial lipase preparation could be observed) for ca. 10 min. After thorough mixing, 1 mL of lipase solution was withdrawn and assayed for lipolytic activity using the pH-stat method. Eight pH values were tested, viz. 4, 5, 6, 7, 8, 9, 10, and 12, and for each pH value the experiments were carried out in triplicate.

Thermal Stability Assays

For each experiment at a given temperature, a known amount of crude lipase powder (ca. 400 mg) was poured into a 50 mL glass flask, and dissolved in 20 mL of Tris-HCl buffer (pH 8.0). After complete solubilization, 1 mL samples were taken and assayed for lipolytic activity; these values were taken as the initial activity. The flask containing the remaining solution was immediately placed in a water bath preset at the desired temperature, and samples were taken every 20 min (throughout a total time period of 5 h); the remaining lipolytic activity was determined using the pH-stat method. Five temperatures were tested, viz. 20, 30, 35, 40, and 50°C, and for each temperature the experiments were carried out in triplicate.

Calorimetric Assays

For each differential scanning calorimetry assay, two different patterns were tested. In the first pattern, ca. 10 mg of crude lipase were weighed directly in a high-pressure aluminum cell which was then duly sealed. A blank was also prepared simply by sealing air inside a cell. The sample was then cooled to ca. −10°C using liquid nitrogen, maintained at this temperature for ca. 1 min, and then heated to 120°C at a rate of 2°C/min. In the second pattern, 200 mg of crude lipase were dissolved in 1 mL of McIlvaine buffer (pH 7.0), and 30 μL of this solution were withdrawn with a microsyringe and poured into a

high-pressure aluminum cell which was then sealed. A blank was also prepared simply by sealing 30 μL of plain McIlvaine buffer (pH 7.0) inside another high-pressure cell. The sample was then cooled to ca. $-10°C$ using liquid nitrogen, maintained at this temperature for ca. 1 min, and then heated to 100°C at a rate of 2°C/min.

Proteolytic Activity Assays

The search for (tentative) proteolytic activity in the crude lipase preparation was done following the o-phthaldialdehyde method (Church et $al.$, 1983), modified later by Sousa and Malcata (1996), using bovine serum albumin as substrate. The assay comprised the addition of 300 μL of solution of crude lipase, prepared by dissolving 10 mg of crude preparation in 5 mL of sodium phosphate buffer (50 mmol/L, pH 8.0), to 2.7 mL of a 0.2% (wt/wt) solution of bovine serum albumin in sodium phosphate buffer (50 mmol/L, pH 8.0), and incubation of the resulting mixture at 37°C in a shaking bath for 60 min. Samples (200 μL) were then withdrawn at various time intervals (0, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min), added to 2 mL of the stock solution containing o-phthaldialdehyde (to transform the primary amines of proteins, peptides, and free amino acids into fluorescent adducts), $β$-mercaptoethanol (to reduce disulphide bridges), and sodium dodecyl sulphate (to terminate proteolysis and ensure full exposure of $α$-amino groups), and their absorbance was measured, after 2 min of reaction, at 340 nm in 10 mm quartz cuvettes. Three blanks were used in the calculation of the corrected absorbance (A^*): a blank for the substrate, consisting of 900 μL bovine serum albumin solution and 100 μL sodium phosphate buffer (at the apropriate pH); a blank for the lipase, consisting of 100 μL of solution of crude lipase and 900 μL of sodium phosphate buffer (at the appropriate pH); and a blank for the reagent, consisting of 2000 μL of o-phthaldialdehyde and 1000 μL of sodium phosphate buffer (at the appropriate pH).

EXPERIMENTAL RESULTS

The protein content of the crude lipase powder was ca. $9.7 ± 1.7%$ (w/w) (as equivalent bovine serum albumin). The results pertaining to stability of crude lipase with respect to pH are depicted in Fig. 1. The results pertaining to stability of crude lipase with respect to time and temperature are plotted in Fig. 2. The thermograms associated with denaturation of crude lipase are presented in Fig. 3. The electrophoretograms of the crude lipase (see Fig. 4) have shown that its molecular weight is ca. $26.1 ± 0.7$ kDa. The evolution of the corrected absorbance (A^*) accounted for by the fluorescent adducts of the
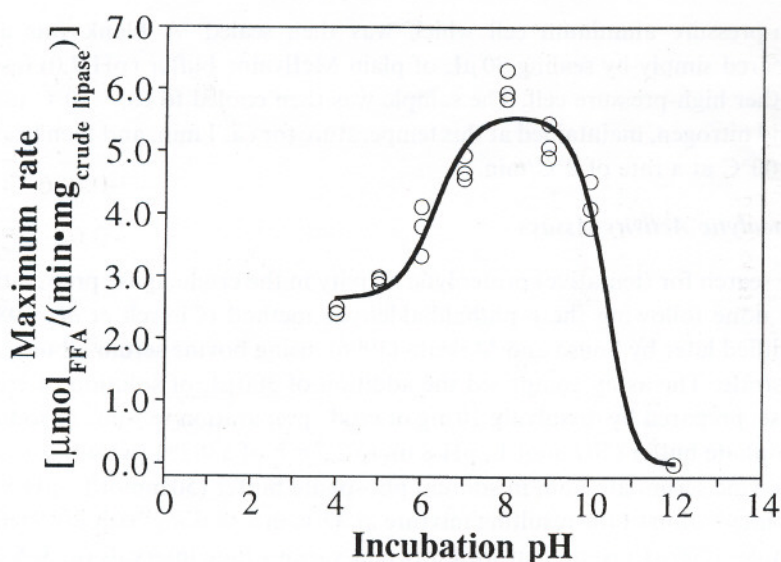
FIGURE 1   Variation of maximum rate with pH of incubation; experimental values (○) and theoretical curve (—).
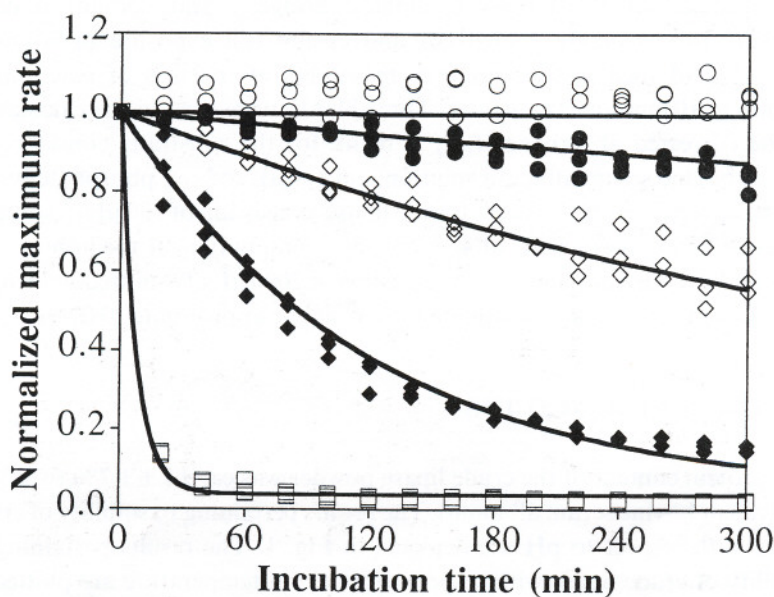

FIGURE 2   Variation of normalized maximum rates with incubation time at the various temperatures tested; experimental values (20°C (○), 30°C (●), 35°C (◇), 40°C (◆), and 50°C (□)) and theoretical curves (—).
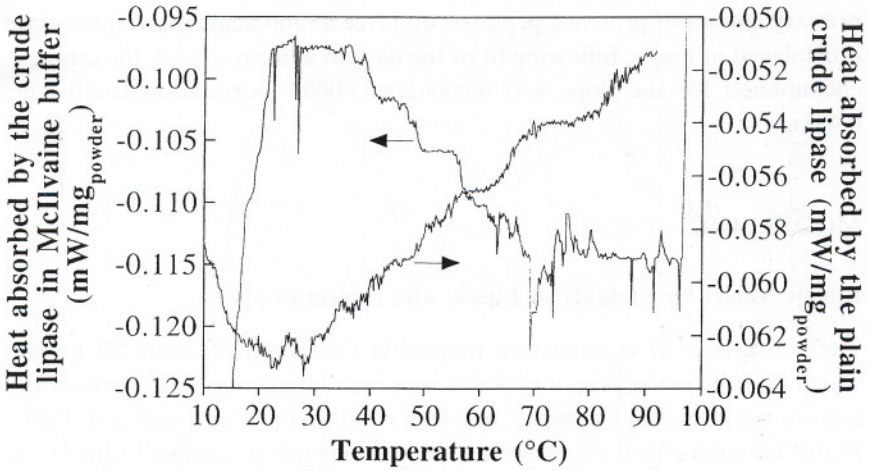
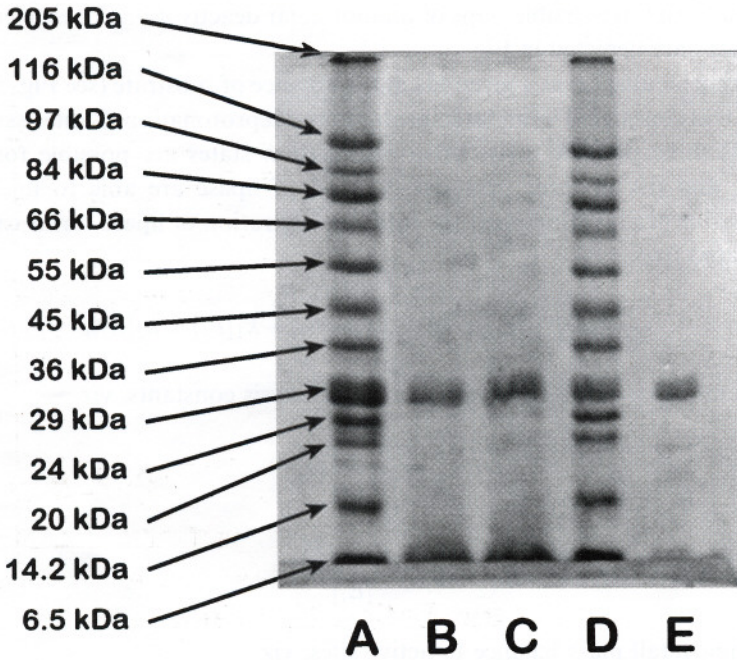FIGURE 3 Thermograms of crude lipase as a plain powder and in buffered aqueous solution.

FIGURE 4 Electrophoretogram of crude lipase (lanes B, C, and E) and molecular weight markers (lanes A and D).

primary amines of proteins, peptides, and free amino acids throughout time is displayed in Fig. 7; following fit of the data to a linear model, the estimate encountered for the slope was 0.00005 ± 0.00094 (correlation coefficient: 0.033).

MODELLING

Kinetic Model for Stability of Lipase with respect to pH

The active sites of enzymes are frequently composed of ionizable groups which must be in the proper ionic form to maintain the conformation of the active site able to bind substrate and thus catalyze the reaction (Segel, 1993). In this research effort, assessment of the pH-dependent stability of the crude lipase powder was performed in two consecutive steps, viz. incubation of a lipase buffered solution at a given pH followed by assaying of the residual activity of said soluble lipase. In the development of a suitable kinetic mechanism, the lipase was assumed to undergo quasi-equilibrium steps of rearrangement resulting from proton transfer and complexation with substrate, and irreversible steps of unimolecular deactivation. These elementary steps are depicted in Fig. 5.

During incubation at a given pH in the absence of substrate (see Fig. 5(a)), lipase was considered to undergo protonation/deprotonation. If one assumes that (i) three possible consecutive protonation states are possible for the lipase, and (ii) all three protonated forms of lipase are able to undergo irreversible deactivation, then the total concentration of lipase forms can be calculated from:

$$-\frac{d}{dt}\{[E_0] + [E_1] + [E_2]\} = k_0[E_0] + k_1[E_1] + k_2[E_2], \tag{1}$$

where t denotes time. The definition of protolysis constants, viz.

$$K_1 = \frac{[E_1][H^+]}{[E_2]}, \tag{2}$$

$$K_2 = \frac{[E_0][H^+]}{[E_1]}, \tag{3}$$

and the overall mass balance to active sites, viz.

$$\{[E_0] + [E_1] + [E_2]\}(t = 0) = [E]_{\text{tot},0}, \tag{4}$$

a

$$E_d \xleftarrow{k_0} E_0 + H^+ \underset{\xrightarrow{K_2}}{} E_1 + H^+ \underset{\xrightarrow{K_1}}{} E_2 \xrightarrow{k_2} E_d$$

$$E_1 \xuparrow{k_1} E_d$$

b

$$E_d \xleftarrow{k_2} E_2 + S \underset{\xrightarrow{\alpha K_s}}{} E_2 \cdot S \xrightarrow{\gamma k_p} E_2 + P$$

$$K_{e_1} \updownarrow \qquad \alpha K_{e_1} \updownarrow$$

$$H^+ \qquad H^+$$

$$E_d \xleftarrow{k_1} E_1 + S \underset{\xrightarrow{K_s}}{} E_1 \cdot S \xrightarrow{k_p} E_1 + P$$

$$K_{e_2} \updownarrow \qquad \frac{K_{e_2}}{\beta} \updownarrow$$

$$H^+ \qquad H^+$$

$$E_d \xleftarrow{k_0} E_0 + S \underset{\xrightarrow{\beta K_s}}{} E_0 \cdot S \xrightarrow{\delta k_p} E_0 + P$$
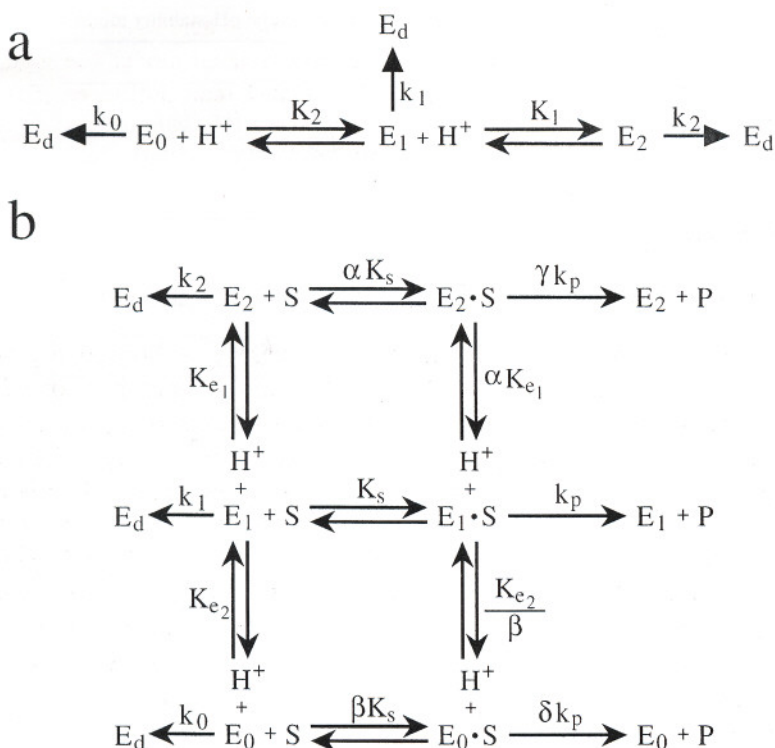
FIGURE 5 Molecular mechanism postulated for lipase incubated in the absence (a) and presence (b) of substrate.

allow Eq. (1) to be rewritten, after integration, as

$$[E]_{\text{tot}(t_{\text{inc}})} = \{[E_0] + [E_1] + [E_2]\}(t = t_{\text{inc}})$$

$$= [E]_{\text{tot},0} \cdot \exp\left\{-\left[\frac{(k_0 K_2/[H^+]_{\text{inc}}) + k_1 + (k_2[H^+]_{\text{inc}}/K_1)}{(K_2/[H^+]_{\text{inc}}) + 1 + ([H^+]_{\text{inc}}/K_1)}\right] \cdot t_{\text{inc}}\right\},$$

$$(5)$$

where subscript 'inc' denotes at the end of incubation and subscript '0' at the beginning thereof.

During incubation at the reference pH in the presence of substrate (see Fig. 5(b)), all three aforementioned forms of lipase were assumed to be able to bind substrate and yield product according to a general Dixon–Webb methodology. The corresponding rate expression has been derived elsewhere (Segel, 1993) for intact enzyme; if the enzyme has been already subject to

TABLE I Definition of lumped parameters in the pH-stability model

Parameter	Definition
Ψ	$\vartheta_{max,0} \cdot \dfrac{(\gamma[H^+]/\alpha K_{e1}) + 1 + (\delta K_{e2}/\beta[H^+])}{(K_{e2}/\beta[H^+]) + 1 + ([H^+]/\alpha K_{e1})}$
$\vartheta_{max,0}$	$k_p \cdot [E]_{tot,0}$

deactivation as depicted in Eq. (5), then the resulting expression is

$$\vartheta_{max} = \Psi \cdot \exp\left\{ -\left[\frac{(k_0 K_2/10^{-pH}) + k_1 + (k_2 10^{-pH}/K_1)}{(K_2/10^{-pH}) + 1 + (10^{-pH}/K_1)} \right] \cdot t_{inc} \right\}, \quad (6)$$

where Ψ is a lumped parameter defined in Table I (where α, β, γ, and δ are dimensionless constants depicted in Fig. 5(b), and k_p is the catalytic constant associated with reaction catalyzed by the intermediate form of lipase); k_0, k_1, and k_2 are elementary kinetic constants associated with deactivation of all protonated states of (free) lipase, K_1 and K_2 are dissociation constants associated with consecutive states of protonation of lipase, pH refers to the pH prevailing during incubation, and t_{inc} is the incubation time at that pH (viz. 10 min). In the derivation of Eq. (6) advantage was taken from the fact that the pH-stat assays were carried out using such a high concentration of substrate and for such a little time that the rate expression could reduce to pseudo-zero-order (i.e., $K_S \ll [S]$).

Kinetic Model for Stability of Lipase with respect to Temperature

In its most general form, the substrate-independent thermal deactivation of enzymes can be described by a multi-step mechanism which involves only unimolecular steps (Henley and Sadana, 1986; Malcata, 1991; Van der Padt *et al.*, 1992; Malcata *et al.*, 1993) to yield the following general rate expression:

$$\tilde{a}\{t, n\} = \alpha_{n,1}e^{-\beta_{n,1}t} + \alpha_{n,2}e^{-\beta_{n,2}t} + \cdots + \alpha_{n,n-1}e^{-\beta_{n,n-1}t} + \{1 - \alpha_{n,1} - \alpha_{n,2} - \cdots - \alpha_{n,n-1}\}e^{-\beta_{n,n}t}, \quad (7)$$

where $\tilde{a}$ is the activity of the lipase normalized by its initial value, t is time elapsed, and the $\alpha_{n,i}$'s and the $\beta_{n,i}$'s are adjustable parameters functionally dependent on the intrinsic activities of each enzyme form and the first-order kinetic constants of rearrangement between, and deactivation of, said enzyme forms.
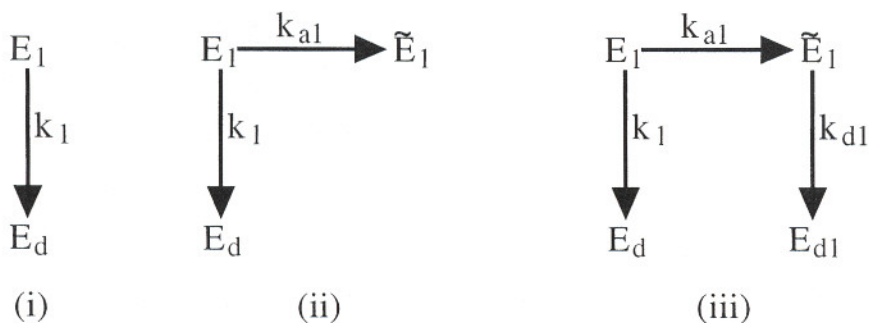
FIGURE 6 Schematic representation of unimolecular models considered for lipase deactivation.

In attempts to model data taken at various times but at a constant (optimal) pH, three levels of nesting of the aforementioned mechanism for enzymatic thermal deactivation were considered (and are depicted in Fig. 6): Mechanism I, which assumes that the native enzyme form (E_1) may be deactivated to an inactive form (E_d) with rate constant k_1; Mechanism II, which assumes that the native enzyme form may be rearranged to another active (but stable) species ($\tilde{E}_1$) with rate constant k_{a1}, but may be deactivated in parallel to an inactive form (E_d) with rate constant k_1; and Mechanism III, which assumes that the native enzyme form may be rearranged to another active (but stable) species ($\tilde{E}_1$) with rate constant k_{a1}, and both these forms may be deactivated to inactive forms (E_d, and E_{d1}, respectively) with rate constants k_1 and k_{d1}, respectively. The lumped rate expressions associated with these three postulated mechanisms are mathematically depicted below.

Mechanism I

$$\tilde{a}\{t\} = e^{-\beta_{1,1}t},$$
$$\beta_{1,1} = k_1. \tag{8}$$

Mechanism II

$$\tilde{a}\{t\} = \alpha_{2,1}e^{-\beta_{2,1}t} + \{1 - \alpha_{2,1}\},$$
$$\beta_{2,1} = k_{a1} + k_1. \tag{9}$$

Mechanism III

$$\tilde{a}\{t\} = \alpha_{3,1}e^{-\beta_{3,1}t} + \{1 - \alpha_{3,1}\}e^{-\beta_{3,2}t},$$
$$\beta_{3,1} = k_{a1} + k_1,$$
$$\beta_{3,2} = k_{d1}. \tag{10}$$

Postulating Arrhenius temperature dependencies for the elementary first-order kinetic constants depicted in Eqs. (8)–(10), and centering the reciprocal temperatures with their median value (T_m) in order to improve convergence and decrease interparameter correlation in the numerical procedure required by nonlinear regression fitting, one will obtain the following form of the rate expressions:

Model I

$$\tilde{a} = \exp\left\{-\left[\omega \cdot \exp\left(-\varphi\left\{\frac{1}{T} - \frac{1}{T_m}\right\}\right)\right] \cdot t\right\}. \tag{11}$$

Model II

$$\tilde{a} = \alpha_{2,1} \cdot \exp\left\{-\left[\omega \cdot \exp\left\{-\varphi\left(\frac{1}{T} - \frac{1}{T_m}\right)\right\}\right.\right.$$
$$\left.\left. + \chi \cdot \exp\left\{-\gamma\left(\frac{1}{T} - \frac{1}{T_m}\right)\right\}\right] \cdot t\right\} + \{1 - \alpha_{2,1}\}. \tag{12}$$

Model III

$$\tilde{a} = \alpha_{3,1} \cdot \exp\left\{-\left[\omega \cdot \exp\left\{-\varphi\left(\frac{1}{T} - \frac{1}{T_m}\right)\right\} + \chi \cdot \exp\left\{-\gamma\left(\frac{1}{T} - \frac{1}{T_m}\right)\right\}\right] \cdot t\right\}$$
$$+ \{1 - \alpha_{3,1}\} \cdot \exp\left\{-\left[\delta \cdot \exp\left\{-\Psi\left(\frac{1}{T} - \frac{1}{T_m}\right)\right\}\right] \cdot t\right\}, \tag{13}$$

where the definitions of the lumped parameters (α, ω, φ, χ, γ, δ, and Ψ) in the three nested models can be found in Table II. It is apparent from comparison

TABLE II Definition of lumped parameters in temperature stability models; $k_{a,0}$, $k_{d,0}$, and $k_{d,1}$ are pre-exponential factors: $E_{a,d}$, $E_{a,1}$, and $E_{ad,1}$ are activation energies; and R is the ideal gas constant

Model	Parameter	Definition
I	ω	$k_{d,0}\exp\{-E_{a,d}/RT_m\}$
	φ	$E_{a,d}/R$
II	α	$\alpha_{2,1}$
	ω	$k_{a,0}\exp\{-E_{a,a1}/RT_m\}$
	φ	$E_{a,a1}/R$
	χ	$k_{d,0}\exp\{-E_{a,d}/RT_m\}$
	γ	$E_{a,d}/R$
III	α	$\alpha_{3,1}$
	ω	$k_{a,0}\exp\{-E_{a,a1}/RT_m\}$
	φ	$E_{a,a1}/R$
	χ	$k_{d,0}\exp\{-E_{a,d}/RT_m\}$
	γ	$E_{a,d}/R$
	δ	$k_{d1,0}\exp\{-E_{a,d1}/RT_m\}$
	Ψ	$E_{a,d1}/R$

between Figs. 5 and 6 that Model I corresponds to the middle portion of the mechanism depicted in Fig. 5(b) (as expected since only the optimum pH was considered), which implies that most enzyme is in the form possessing the intermediate state of protonation. Conversely, although Models II and III are also consistent with Fig. 5(b), they encompass long-term conformational rearrangement of lipase (to form $\tilde{E}_1$) which was not postulated for the pH-dependent stability mechanism because it could not be experimentally detected in the short time frame considered.

STATISTICAL ANALYSES

Model parameters were estimated by nonlinear regression using a General REGression package, GREG (Stewart *et al.*, 1992), at level 10. At this level, the program performs nonlinear, uniresponse regression analyses of the data using finite differences as approximants of the derivatives of the objective function with respect to each parameter, and using as objective function minimization of the sum of squares of the residuals between model and experimental data. Given starting estimates, this advanced regression software expands the objective function as a local quadratic, finds a solution for the feasible minimum of this quadratic expansion in terms of parameter values, and implements a weak line search for a smaller value of the objective function. The results of the regression analysis (as provided by the post-convergence report generated by GREG) for the model postulated for the pH-dependent deactivation are tabulated in Table III, whereas the results of the regression analysis for each one of the three nested models postulated for temperature-dependent deactivation are tabulated in Table IV.

In order to decide whether a simpler nested model (i.e., Models I or II) rather than the full model (i.e., Model III) fits the data set adequately, one

TABLE III Parameter estimates and associated marginal inference intervals for the parameters of the model fitted to the data pertaining to the pH-dependent deactivation of the crude commercial lipase

Parameter	Estimate $\pm 2\sigma$
Ψ	$5.60 \pm 3.26 \times 10^{-1}$
k_0	$6.88 \times 10^{-1} \pm 3.35$
k_1	$2.72 \times 10^{-15} \pm \infty$
k_2	$7.48 \times 10^{-2} \pm 1.31 \times 10^{-2}$
K_1	$6.72 \times 10^{-7} \pm 5.19 \times 10^{-7}$
K_2	$4.73 \times 10^{-12} \pm 2.43 \times 10^{-11}$

TABLE IV Parameter estimates and associated marginal infer-
ence intervals for the parameters of the three nested models fitted
to the data pertaining to temperature-dependent deactivation of
the crude commercial lipase

Model	Parameter	Estimate $\pm 2\sigma$
I	ω	$1.95 \times 10^{-3} \pm 1.16 \times 10^{-4}$
	φ	$2.67 \times 10^{4} \pm 1.22 \times 10^{3}$
II	α	$9.63 \times 10^{-1} \pm 1.65 \times 10^{-2}$
	ω	$5.70 \times 10^{-11} \pm \infty$
	φ	$2.67 \times 10^{4} \pm \infty$
	χ	$2.05 \times 10^{-3} \pm 1.30 \times 10^{-4}$
	γ	$2.75 \times 10^{4} \pm 1.34 \times 10^{3}$
III	α	$9.90 \times 10^{-2} \pm 4.27 \times 10^{-2}$
	ω	$2.26 \times 10^{-11} \pm \infty$
	φ	$6.55 \times 10^{4} \pm \infty$
	χ	$1.86 \times 10^{-6} \pm \infty$
	γ	$5.55 \times 10^{4} \pm 4.67 \times 10^{3}$
	δ	$2.24 \times 10^{-3} \pm 2.00 \times 10^{-4}$
	Ψ	$2.89 \times 10^{4} \pm 1.76 \times 10^{3}$

TABLE V Extra sum of squares analyses for simpler and full model

Nested models	Source	Sum of squares	Number of degrees of freedom	Mean square	F_{ratio}	Standard F_{ratio} [1% significance level]
I to II	Extra parameters	0.05858	3	0.019527	6.407	3.948
	Model II	0.71616	235	0.0030475		
	Model I	0.77474	238			
II to III	Extra parameters	0.02382	2	0.01191	4.008	4.788
	Model III	0.69234	233	0.0029714		
	Model II	0.71616	235			

has proceeded as in the linear case and used a likelihood ratio test (Draper
and Smith, 1981). Because of the spherical normal assumption, this test leads
to an assessment of the extra sum of squares due to the extra parameters
involved in going from a partial to the full model (Bates and Watts, 1988); the
results of the extra sum of squares analyses involved in going from Model I
(2-parameter model) to Model II (5-parameter model), and in going from
Model II (5-parameter model) to Model III (7-parameter model) are
summarized in Table V.

Since the data generated in this research effort include replications, it was
possible to perform a test of lack of fit for the best (nested) model found, i.e.
that associated with Eq. (12). Such analysis (which is based on the fact that
the replication subspace is always orthogonal to the subspace containing the
averages of the replicated data and the model postulated) (Bates and Watts,
1988) proceeded via comparison of the ratio of the lack of fit mean square to

TABLE VI Lack of fit analysis for the statistically selected model proposed for thermal deactivation (Model II)

Source	Number of degrees of freedom	Sum of squares	Mean square	F_{ratio}	Standard F_{ratio} [1% significance level]
Lack of fit	75	0.260074	0.00346765	1.216	1.562
Replications	160	0.456086	0.00285054		
Residuals	235	0.716159			

the replication mean square with the appropriate value of the F distribution. This analysis is depicted in Table VI.

DISCUSSION

Although several methods are available, the Coomassie method was selected for determination of the protein content of the crude lipase assayed because it is easy, reproducible, and much less susceptible to interfering substances than, e.g. the Lowry or the Folin–Lowry methods (Robyt and White, 1990). On the other hand, although glycerol trioleate is the most universal substrate for lipase (Brockerhoff and Jensen, 1974), a less expensive substitute (viz. olive oil) was used because the former is unduly expensive, even though only a minimum of 70% oleic acid residues can be guaranteed. Furthermore, for monitoring the rate of lipolysis via continuous titration of free fatty acids released, selection of pH 8 to carry out the reaction was a consequence of the pK_a of aliphatic acids (ca. 5), which results in all of the fatty acids being dissolved in the aqueous phase in dissociated form, as required by quantitative assaying.

Calorimetric assays for the crude lipase (see Fig. 3) exhibit several peaks of heat absorption which are probably associated with unfolding of various proteins present therein. The electrophoretogram obtained for the crude lipase (see Fig. 4) shows only a major band for the crude lipase; as separation was effected on the basis of molecular weight, this implies that the proteins in the crude lipase powder would probably be a family of proteins with similar molecular weights.

As discussed by Lee et al. (1989), lipases are apparently not sensitive to shear stress when acting in free form in a batch stirred tank reactor, and so it is not expected that our deactivation data reflected mechanical, rather than thermal or other forms of, deactivation. Furthermore, if one assumes that the increase of the inner area of the vortex caused by magnetic stirring is negligible and no extensive foam forms, then denaturation of lipase caused by adsorption onto the high surface tension air/water interface throughout our experiments can be assumed to be negligible. On the other hand, it can be

seen from Fig. 2 that the activity of the crude lipase decays faster and faster as temperature increases as would be expected from plain application of the Arrhenius law to the deactivation constants. The decay in lipase activity can safely be attributed solely to thermally driven changes in the three-dimensional conformation of the enzyme molecule because, in addition to the foregoing arguments, virtually no proteolytic activity could be found in the crude preparation assayed (see Fig. 7, where the null hypothesis that the concentration of products of proteolytic breakdown does not change with time was accepted at the 1% level of significance).

The most common method of determining the temperature dependence of kinetic parameters is to independently fit data taken at each temperature, and then fit the resultant kinetic parameters to the Arrhenius relationship using a logarithmic/reciprocal plot. However, a better method is to fit the global model to the entire set of data points (as done in this research effort) because fitted parameters do not behave statistically as actual experimental data. On the other hand, centering the reciprocal absolute temperatures allowed mutual correlation between parameters be kept to a minimum, with off-diagonal elements of the normalized covariance matrix lying between -0.65 and $+0.73$ (results not shown).

Although convergence to best parameter estimates occurred in all fits, the confidence intervals for some parameters overlapped the null hypothesis
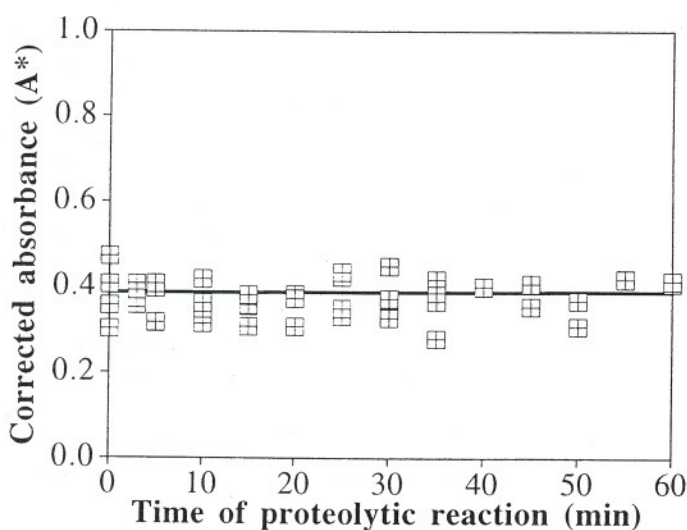

FIGURE 7 Corrected absorbance produced by fluorescent adducts of primary amines in proteins, peptides, and free amino acids as a function of time of incubation of crude lipase with bovine serum albumin.

probably because of the limited range of the data set generated. Nonlinear fitting of the general model for thermal deactivation to experimental data at various temperatures proceeded through consideration of increasing levels of nesting. Inspection of Table V indicates that Model II should be selected to describe the experimental data because any further refinement of the fit resulting from a lower sum of squares of residuals is not statistically justified in view of the increased number of parameters. Therefore, postulation of an alternative active and stable form of lipase in the rate expression for deactivation is relevant, but assumption that this alternate form also deactivates irreversibly does not significantly improve the fit of the data at all temperatures. These conclusions are in agreement with results reported elsewhere for a lipase produced by *Aspergillus niger* (Malcata *et al.*, 1992a). Since Model II was selected to describe the data, further confirmation of the statistical adequacy of the fit was based upon a *posteriori* lack of fit analysis; inspection of Table VI does not raise any statistical queries as of the form of Model II, on the 1% level of significance.

Inspection of Table IV indicates that the pre-exponential factor associated with k_{a1} is nonnegative (as required for physical significance) and that the activation energy associated therewith is $221.7\,\text{kJ mol}^{-1}$; this value, which is much larger than that found for a similar rearrangement by Malcata *et al.* (1992a) for a lipase from *Aspergillus niger*, clearly suggests that the process of rearrangement of the native active lipase to a more stable, active form involves rearrangement of several (at least 10) hydrogen bonds. Inspection of Table IV also indicates that the activation energy associated with the deactivation of the native lipase, k_d, is $228.8\,\text{kJ mol}^{-1}$; this value is of the order of magnitude of the activation energies associated with deactivation of enzymes (Malcata *et al.*, 1992a). Experimental studies with a pure lipase from *Mucor javanicus* performed by Ogiso *et al.* (1972) have resulted in activation energies for deactivation of this enzyme of ca. $26.6\,\text{kJ mol}^{-1}$, which are rather low when compared with our case; however, our values for the activation energy associated with deactivation of the native lipase are of the same order of magnitude of those for a lipase from *Chromobacterium viscosum* in an aqueous system at pH 8.0, viz. $161.5\,\text{kJ mol}^{-1}$ (Prazeres *et al.*, 1993), for pancreatic lipase, viz. $192.5\,\text{kJ mol}^{-1}$ (Laidler and Bunting, 1973), and for *Candida rugosa* lipase, viz. $113.0–142.3\,\text{kJ mol}^{-1}$ (Shaw *et al.*, 1990).

Departing from the values of parameters ω and φ, and χ and γ, for Model II in Table II, the values for the elementary kinetic constants k_{a1} and k_1, respectively, were calculated to range in the intervals $6.79 \times 10^{-13}–3.00 \times 10^{-9}$ and $2.13 \times 10^{-5}–1.30 \times 10^{-1}\,\text{min}^{-1}$, respectively, for the temperature range 20–50°C. Therefore, deactivation of the native active form of lipase is apparently much faster than rearrangement of this form to

another active species. The range of k_1 encompasses the values reported by Ogiso *et al.* (1972) for the kinetic constant describing heat denaturation at 40, 50, and 60°C.

Experimental evidence (Neklyudov *et al.*, 1982) has indicated that serine and histidine residues form the active site of lipases, and that histidine must be in the proper ionic form to be able to abstract a proton from the hydroxyl group of serine (a preliminary step necessary to make a pair of electrons available for subsequent nucleophilic attack on the acyl moiety of the ester bond of the glyceride substrate); an amino acid residue containing a carboxylic acid as side group (i.e. aspartic acid or glutamic acid) in the close vicinity of the active site has also been implicated in the formation of a three-dimensional pocket where the substrate must bind before its ester bond can be attacked by lipase. If the aforementioned His and Asp/Glu residues are not in the proper ionic form, physical binding and subsequent chemical transformation of substrates will be prevented. It is thus expected that the effect of pH upon the stability of the lipase (Bailey and Ollis, 1986) displays a behaviour at least similar to that depicted in Fig. 1, although the values for pK_1 and pK_2 (viz. 6.2 and 11.3, as obtained from Table III) are somewhat higher than those usually associated with such residues (Segel, 1993). Inspection of the data generated by nonlinear fitting of the stability model (viz. Eq. (6)) to the experimental data (see Fig. 1) shows that the optimum pH in terms of stability occurs at ca. 8.2, which is in agreement with the optimum pH for a similar lipase reported by Ogiso *et al.* (1972). (Since such fit possesses a correlation coefficient above 0.98, it is expected that it should not be considerably affected by the absence of a datum at pH 11, which was due to an unavoidable experimental problem.)

Inspection of Table III shows that the value obtained for k_1 is very small, virtually zero, which implies that the intermediate enzyme form in the model depicted in Fig. 5(a) is very stable; conversely, k_0 and k_2 are much higher than k_1, which implies that the two putative enzyme forms E_0 and E_2 are quite prone to inactivation. The rate of deactivation of form E_1, at pH 8, will only be accurately estimated at longer reaction times, as apparent from inspection of the values of χ and γ in Table IV, which yield the range 2.13×10^{-5} $-1.30 \times 10^{-1} \, min^{-1}$ for k_1 in the temperature range 20–50°C.

CONCLUSIONS

From this research it has been possible to simulate the kinetic stability of a lipase of industrial importance at various temperatures and pH values; such

simulation is important in attempts to design biochemical processes for which the aforementioned lipase is a potential candidate. It was concluded that (i) only thermal deactivation played a relevant role in terms of the performance of such crude enzyme, which could be described via parallel steps of deactivation and rearrangement (with activation energies of ca. 230 and $220 \, kJ \, mol^{-1}$, respectively), and that (ii) the observed stability behaviour was consistent with an assumption of three forms of active enzyme with increasing states of protonation (with pK values of ca. 6 and 11), where the intermediate form is particularly stable.

Acknowledgements

References

Ahern, T.J. and Klibanov, A.M. (1985) The mechanism of irreversible enzyme inactivation at 100°C. *Science*, **228**, 1280–1284.

Bailey, J.E. and Ollis, D.F. (1986) *Biochemical Engineering Fundamentals*. McGraw-Hill, New York, USA, pp. 135–144, 148–152.

Balcão, V.M. and Malcata, F.X. (1996a) Reactors with immobilized lipase. Mathematical modelling. In: *Engineering Of/With Lipases*, Ed., F.X. Malcata. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 435–454.

Balcão, V.M., Paiva, A.L. and Malcata, F.X. (1996b) Bioreactors with immobilized lipases: state-of-the-art. *Enzyme Microb. Technol.*, **18**, 392–416.

Balcão, V.M., Vieira, M.C. and Malcata, F.X. (1996c) Adsorption of protein from several commercial lipase preparations onto a hollow-fiber membrane module. *Biotechnol. Progr.*, **12**, 164–172.

Bates, D.M. and Watts, D.G. (1988) *Nonlinear Regression Analysis and its Applications*. Wiley Interscience, New York, USA.

Brockerhoff, H. and Jensen, R.G. (1974) *Lipolytic Enzymes*. Academic Press, New York, USA, p. 20.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. (1983) Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J. Dairy Sci.*, **66**, 1219–1227.

Dawson, R.M.C., Elliott, W.H., Elliott, D.C. and Jones, K.M. (1969) *Data for Biochemical Research*. Oxford Science Publications, Oxford, UK, p. 427.

Draper, N.R. and Smith, H. (1981) *Applied Regression Analysis*. Wiley, New York, USA.

Henley, J.P. and Sadana, A. (1986) Deactivation theory. *Biotechnol. Bioeng.*, **28**, 1277–1285.

Laidler, K.L. and Bunting, P.S. (1973) *The Chemical Kinetics of Enzyme Action.* Oxford University Press, London, UK.

Lee, Y.-K. and Choo, C.-L. (1989) The kinetics and mechanism of shear inactivation of lipase from *Candida cylindracea. Biotechnol. Bioeng.*, **33**, 183–190.

Malcata, F.X. (1991) Hydrolysis of butterfat by immobilized lipase using three-phase membrane reactors, Ph.D. thesis, University of Wisconsin – Madison, USA.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1992a) Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: IV. Effects of temperature. *Biotechnol. Bioeng.*, **39**, 1097–1111.

Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, C.G. and Amundson, C.H. (1992b) Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme Microb. Technol.*, **14**, 426–446.

Malcata, F.X., Hill, C.G. and Amundson, C.H. (1993) Hydrolysis of butteroil by immobilized lipase using a hollow fiber reactor: Part V. Effects of pH. *Biocatalysis*, **7**, 177–219.

Neklyudov, A.D., Shvedov, B.D. and Tsibanov, V.V. (1982) Properties of a preparation of immobilized *Rhizopus oryzae* 14–14 lipase. *Appl. Biochem. Microbiol.*, **17**, 378–382.

Ogiso, T., Sugiura, M. and Kato, Y. (1972) Studies on bile-sensitive lipase. X. Preparation and properties of carrier-bound *Mucor* lipase. *Chem. Pharm. Bull.*, **20**, 2542–2550.

Prazeres, D.M.F., Garcia, F.A.P. and Cabral, J.M.S. (1993) Temperature, pH and media influence on lipase stability. In: *Stability and Stabilization of Enzymes*, Eds., W.J.J. van den Tweel, A. Harder and R.M. Buitelaar. Elsevier, Amsterdam, The Netherlands, pp. 445–450.

Robyt, J.F. and White, B.J. (1990) *Biochemical Techniques – Theory and Practice.* Waveland Press, Chicago, USA.

Segel, I.H. (1993) *Enzyme Kinetics – Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.* Wiley, New York, USA.

Shaw, J.-F., Chang, R.-C., Wang, F.F. and Wang, Y.J. (1990) Lipolytic activities of a lipase immobilized on six selected supporting materials. *Biotechnol. Bioeng.*, **35**, 132–137.

Sousa, M.J. and Malcata, F.X. (1996) Effects of processing conditions on the caseinolytic activity of crude extracts of *Cynara cardunculus* L. *Food Sci. Tech. Int.*, **2**, 255–263.

Stewart, W.E., Caracotsios, M. and Sørensen, J.P. (1992) *GREG Software Package Documentation.* University of Wisconsin, Madison, USA.

Van der Padt, A., Sewalt, J.J.W., Ágoston, S.M.I. and Van't Riet, K. (1992) *Candida rugosa* lipase stability during acylglycerol synthesis. *Enzyme Microb. Technol.*, **14**, 805–812.