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Heat inactivation of lipase from psychrotrophic *Pseudomonas fluorescens* P38: Activation parameters and enzyme stability at low or ultra-high temperatures

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A quantitative model is presented for the heat inactivation of enzymes at low or ultra-high temperatures. As a test of its validity, the heat inactivation of crude or partially purified lipase from *Pseudomonas fluorescens*, strain P38 (P38 lipase) was investigated. The activation energy ($\Delta E^{\#}$), enthalpy ($\Delta H^{\#}$), entropy ($\Delta S^{\#}$) and Gibbs free energy change ($\Delta G^{\#}$) for lipase inactivation at 40–140°C were determined. $\Delta H^{\#}$ for P38 lipase heat inactivation at 40–60°C, 50–80°C and 90–140°C was 170–221 kJ mol^{-1}, -3 – -20 kJ mol^{-1} and 44–78 kJ mol^{-1} respectively. Over these temperature intervals $\Delta S^{\#}$ was 202–380 J mol^{-1} K^{-1}, -318 – -375 J mol^{-1} K^{-1} and -92 – -186 J mol^{-1} K^{-1}. $\Delta G^{\#}$ was 100–115 kJ mol^{-1} for enzyme inactivation at 40–140°C. The results are consistent with different rate-limiting reactions for P38 lipase heat inactivation at low or ultra-high temperatures. Within a narrow range of (intermediate) temperature, a third rate-limiting reaction may lead to 'low-temperature inactivation' phenomena. There was qualitative agreement between experimental results and the current model for enzyme heat inactivation.

INTRODUCTION

Psychrotroph lipases are often implicated in the spoilage of dairy products (Stead, 1986; McKellar, 1989). The enzymes are generally heat resistant at 100–150°C. However, heat inactivation occurs readily at intermediate (40–80°C) temperatures by a 'low-temperature inactivation' (LTI) process. The heat resistance of psychrotroph enzymes does not result from a stable tertiary structure (Owusu *et al.*, 1991). Hence, the relationship between enzyme conformational stability, heat resistance and kinetic stability indices (e.g., inactivation rate constant or activation energy) is uncertain.

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Enzyme stability indices are important for heat process design and control. A combination of the inactivation rate constant (k^*), activation energy ($\Delta E^{\#}$), and an appropriate heating time-course enables the mathematical modelling of enzyme inactivation during heat processing of foods (Naveh *et al.*, 1982; Kessler & Fink, 1986, Togeby *et al.*, 1986).

Recently, the reactions leading to enzyme inactivation were assigned to four classes according to the reaction order with respect to enzyme concentration or time (Laidler & Bunting, 1973). Multiphasic kinetics schemes were also described for the inactivation of isoenzymes (Ling & Lund, 1978) or in the presence of denatured enzyme intermediates with non-zero activity (Henley & Sadana, 1985). The physicochemical changes accompanying the irreversible heat-inactivation of peroxidase (Tomura & Morita, 1975), ribonuclease, lysozyme and

α-amylase (Mozhaev & Martinek, 1982; Zale & Klibanov, 1983; Adhern & Klibanov, 1988; Tamozic & Klibanov, 1988) were identified.

Irreversible heat inactivation of enzymes involves essentially the same heat-induced reactions seen in non-enzyme proteins (Mauron, 1977; Walstra & Jenness, 1984). There is a partial unfolding of the enzymes tertiary structure, followed by one or more irreversible reactions (Ferry, 1948; Mozhaev & Martinek, 1982; Ahern & Klibanov, 1988).

The heat-inactivation of lipase from *Pseudomonas fluorescens*, strain P38, is the subject of this paper. The significance of kinetic stability indices for enzyme inactivation under ultra-high temperature (UHT) or low-temperature conditions is discussed in terms of a two-stage model for enzyme and protein denaturation (Ferry, 1948; Edelhoch, 1960; Zale & Klibanov, 1983; Walstra & Jenness, 1984; Adler-Nielson, 1986; Ahern & Klibanov, 1988; Kroll 1989). The accuracy of this heat-inactivation model will also be examined using literature $\Delta H^{\#}$ values for the unfolding, refolding and irreversible heat-denaturation of α-lactalbumin.

MATERIALS AND METHODS

Organism and cultural conditions

Pseudomonas fluorescens, strain P38, was isolated from raw milk (Bucky *et al.*, 1987) and cultured on half-strength peptone (Oxoid, Basingstoke, UK) water at 10°C with shaking for up to 100 h. Cell-free crude enzyme extract was obtained by centrifugation at 10000 × *g* for 20 min.

Partial purification of P38 lipase

Cell-free enzyme extract (500 ml) was concentrated by ultrafiltration through a 10000 molecular weight cut-off membrane (PM 10, Amicon Ltd). The concentrated enzyme solution (50 ml) was applied to a phenyl Sepharose CL-4B (1·5 cm × 8 cm) column and washed with Tris–HCl buffer (0·05M, pH 8·0). All chromatographic supports were purchased from Sigma Chemical Co (UK). The adsorbed lipase was eluted with a (0–50%) linear gradient of ethylene glycol; 6 ml fractions were collected throughout the chromatography. Lipase-containing fractions were pooled and further concentrated by ultrafiltration. The concentrated fractions were then diluted with Tris–HCl buffer and reconcentrated; the process was repeated four times to render the lipase ethylene glycol-free. Finally, the 'washed' lipase was chromatographed on a (1 cm × 40 cm) Sephadex G75 column equilibrated with Tris–HCl buffer (0·05M, pH 8·0) to remove any remaining ethylene glycol.

Lipase assay

The routine assay of lipase in chromatographic fractions was performed using the agar diffusion assay (Lawrence *et al.*, 1967). Tributyrin (1 ml, 2% emulsion prepared with gum arabic) was added to hot agar (9 ml, 1·2% w/v in Tris–HCl buffer, pH 7·8). The resulting solution was stirred with an overhead stirrer, poured into 9 cm diameter glass Petri dishes and allowed to solidify. Wells (6 mm diameter) were then cut into the gel. Activity assays were conducted by placing 15 μl of lipase solution into each well and incubating at 30°C for 48 h. Thereafter, the radii of zone clearance around the wells were measured. The activity of P38 lipase in chromatography fractions was determined from standard curves of (*Candida cylindracea*) of lipase concentration plotted versus zone clearance diameter.

The residual P38 lipase activity after heat treatment (see below) was assayed by the *p*-nitrophenyl caprylate (*p*NPC) colorimetric method (Winkler & Stuckman, 1979) modified as follows: *p*NPC (1 ml, 0·002% in isopropanol was added to 25 ml of sodium deoxycholate (4 mg ml^{-1} in 0·05M phosphate buffer, pH 7·8) to form a substrate solution. The lipase assay involved adding 20–30 μl of enzyme solution to 2 ml of substrate solution at 37°C. After a 15 min incubation period, 2 ml of acetone was added to clarify the solution and spectrophotometric readings were taken at 410 nm. The activity of P38 lipase was determined from standard curves constructed using solutions with known enzyme activity.

Lipase heat treatment

Samples of crude or partially purified lipase (100 μl) were sealed in capillary glass tubes and then heated in a thermostatically controlled oil bath at 40–140°C. Heating times were corrected for the 'come-up' times after measurement of the latter with a thermocouple sealed within a glass capillary.

Theory

The present model for enzyme heat inactivation is described by eqns (1)–(21) (Tables 1 and 2). Irreversible heat denaturation of enzymes proceeds via a potentially reversible unfolding of the three-dimensional structure ($E \rightleftharpoons D$) followed by an irreversible ($D \dashrightarrow I$) reaction (eqn (1)) (Ferry, 1948; Zale & Klibanov, 1983; Ahern & Klibanov, 1988). In the following discussion E = free and active enzyme, D = reversibly unfolded enzyme and I = the irreversibly inactivated enzyme; k_r, k_f and k_i are the rate constants for the steps shown in eqn (1); K_u is the equilibrium constant for reversible unfolding (eqn (2)).

The irreversibility of enzyme inactivation arises from the $D \rightarrow I$ step (eqn (1)). Consequently, the rate of irre-

Table 1. Equations describing the rate of enzyme irreversible inactivation (see text for explanation of the symbols used)

Equation	No.
$E \underset{k_r}{\overset{k_f}{\rightleftharpoons}} D \quad D \overset{k_i}{\dashrightarrow} I$	(1)
$K_u = [D]/[E] = k_f/k_r$	(2)
$v(i) = k_i\,[D]$	(3)
$e_t = [E] + [ES] + [D]$	(4)
$[D] = K_u\,e_t/\,((1 + [S]/K_s) + K_u)$	(5)
$v(i) = k_i\,K_u\,e_t/\,((1 + [S]/K_s) + K_u)$	(6*a*)
$v(i) = k_i\,k_f\,e_t/\,(k_r\,(1 + [S]/K_s) + k_f)$	(6*b*)

versible inactivation is $V(i)$ (eqn (3)). *A priori*, it may be assumed that an unfolding equilibrium ($E \rightleftharpoons D$) precedes irreversible inactivation (Kroll, 1989). In other words, k_r, $k_f >> k_i$ (eqn (1)). This condition will be uniformly applied to the behaviour of enzymes at low as well as high temperatures. [D] could also reach a constant level as a result of the establishment of a steady state between its rate of formation and removal (Adler-Nielson, 1986). However arbitrary, the rapid equilibrium assumption was adopted, as this leads to results which are readily tested by experiment. From eqn (2), $[E] = K_u/[D]$.

The total concentration of enzyme (e_t), in terms of the mass balance equation (eqn (4)), is the sum of [D], [E] and possible ligand (e.g. substrate, cofactor or metal ion)-bound forms of enzyme [ES]. From the enzyme–ligand binding equilibrium constant (K_s) — K_s = [E] [S]/[ES] and eqn (2), [E] and [ES] may be eliminated from eqn (4), leading to a description of [D] in terms of (e_t), K_s and K_u (eqn (5)). Consequently, the rate of irreversible heat inactivation is given by eqn (6*a*). Also, since $K_u = k_f/k_r$ (eqn(2)) eqn (6*b*) also applies.

It can be seen from eqn (6) that enzyme–ligand binding reduces the rate of irreversible inactivation by the factor $(1 + [S]/K_s)$. In the absence of ligand ([S] = 0) the term $(1 + [S]/K_s)$ becomes unity. In the following discussion, emphasis is placed on enzyme inactivation in the absence of ligands.

Three limiting forms of eqn (6) apply for the heat inactivation of enzymes at ultra-high, intermediate or low temperatures ((i)–(iii)):

(i) Ultra-high temperature (UHT) inactivation

At a temperature exceeding that necessary for the unfolding of the enzyme tertiary structure, $K_u >> 1$ or, in terms of the kinetics of unfolding, $k_f >> k_r$. In the presence of a ligand, the appropriate condition is $K_u >> (1 + [S]/K_s)$ or $k_f >> k_r\,(1 + [S]/K_s)$. For large K_u values, eqn (6) will reduce to eqn (7) (Table 2). Under UHT conditions, the D I reaction (Table 1) will be the rate-limiting step for enzyme inactivation. The apparent rate constant (k^*) will then be k_i (eqn (8)).

(ii) Low-temperature inactivation

At a temperature below that required for the substantial heat unfolding of an enzyme tertiary structure, $K_u << 1$, or $k_f << k_r$. Alternatively, in the presence of ligands, $K_u << (1 + [S]/K_s)$ or $k_f << k_r\,(1 + [S]/K_s)$. For a small value of K_u, eqn (6) will reduce to eqn (15) (Table 2). Therefore, at low temperatures k will be a composite of k_i, k_f and k_r (eqn (16)). The rate of heat inactivation will be determined by the rate of enzyme unfolding, refolding and the irreversible chemical step.

(iii) Intermediate temperature inactivation

At an intermediate temperature, conditions (i) and (ii) will apply simultaneously. Equating eqns (7) and (15) (Table 2) produces the result $K_u = 1$. The (intermediate) temperature at which $K_u = 1$ is the melting temperature (T_m), i.e. the temperature at which 50% of enzyme molecules become unfolded. At the T_m an inflection is expected in the Arrhenius plot for enzyme inactivation.

The temperature dependence of enzyme heat inactivation (eqns (9) and (17), Table 2) is in accordance

Table 2. Equations describing the irreversible thermoinactivation of an enzyme at ultra-high temperature (UHT) or low temperatures (see the text for explanation of symbols)

UHT conditions		Low Temperature conditions	
$v(i) = k_i\,e_t$	(7)	$v(i) = k_i\,k_f\,e_t/\,k_r = k_i\,K_u\,e_t$	(15)
$k^* = k_i$	(8)	$k^* = k_i\,k_f/\,k_r$	(16)
$d\,(\ln k^*)/dT = d\,(\ln k_i)/dT$	(9)	$d\,(\ln k)/dT = d\,(\ln k_i)/dT + d\,(\ln k_f)/dT - d\,(\ln k_r)/dT$	(17)
$d\,(\ln k^*)/dT = E^{\#}/RT^2$	(10)	$\Delta E^{\#} = \Delta E^{\#}\,(i) + \Delta E^{\#}\,(f) - \Delta E^{\#}\,(r)$	(18)
$\Delta E^{\#} = \Delta E^{\#}\,(i)$	(11)	$\Delta H^{\#} = \Delta H^{\#}\,(i) + \Delta H^{\#}\,(f) - \Delta H^{\#}\,(r)$	(19)
$\Delta H^{\#} = \Delta H^{\#}\,(i)$	(12)	$\Delta G^{\#} = \Delta G^{\#}\,(i) + \Delta G^{\#}\,(f) - \Delta G^{\#}\,(r)$	(20)
$\Delta G^{\#} = \Delta G^{\#}\,(i)$	(13)	$\Delta S^{\#} = \Delta S^{\#}\,(i) + \Delta S^{\#}\,(f) - \Delta S^{\#}\,(f)$	(21)
$\Delta S^{\#} = \Delta S^{\#}\,(i)$	(14)		

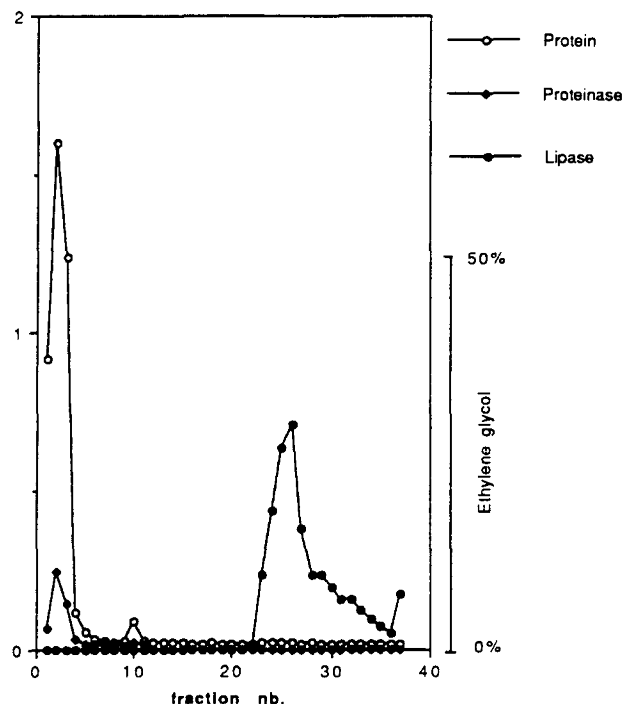

Fig. 1. Hydrophobic interaction chromatography of P38 lipase on phenyl-Sepharose CL 4B with Tris-HCl buffer (0·05M, pH 8·0) solvent. Lipase was eluted with a 0–50% ethylene glycol gradient.

with the Arrhenius formula (eqn (10)). Consequently, the observed activation energy ($\Delta E^{\#}$) for UHT or low-temperature enzyme inactivation will be described by eqns (11) and (18), respectively. From the relationship between $\Delta E^{\#}$, $\Delta H^{\#}$ and $\Delta G^{\#}$ it is readily shown that eqns (12)–(14) and (19)–(21) follow from eqns (11) and (18), respectively (Moore, 1985).

In conclusion, it is expected for UHT inactivation of enzymes, that activation parameters ($\Delta E^{\#}$, ΔH, $\Delta S^{\#}$ and $\Delta G^{\#}$) will be characteristic of the irreversible D --> I reaction. However, during low-temperature inactivation, $\Delta E^{\#}$ (and related parameters) would correspond to enzyme unfolding, folding and one or more irreversible reaction(s).

RESULTS AND DISCUSSION

Hydrophobic interaction chromatography (HIC) on phenyl-sepharose CL-4B resulted in the effective separation of P38 proteinase from lipase (Fig. 1); proteinases were assayed using the azo-casein method. Thus it was possible to study the heat inactivation of P38 lipase, apparently with or without proteinases present.

The heat-inactivation of crude or partially purified *Ps. fluorescens* P38 lipase (P38 lipase) followed first-order kinetics with respect to time at 100–140°C and 40–140°C, respectively. For the partially purified lipase the apparent first-order rate constant for heat inactivation (k^*) was determined from a plot of log (residual activity) versus time (Fig. 2). Inactivation of crude lipase at a temperature <100°C produced a biphasic semilog plot (results not shown). Therefore, the initial rate of enzyme inactivation was used as an estimate for k^*.

Activation parameters were determined using the Arrhenius plot, $\log k^* = -E^{\#}/2{\cdot}303RT + C$ (Figs 3 and 4), and the Eyring relation $\Delta G^{\#} = RT \ln (k^*h/kT)$, where k, h and R are the Boltzmann, Planck and gas constants respectively. $\Delta G^{\#}$ is the observed or apparent Gibbs free energy of activation for irreversible enzyme inactivation. The enthalpy ($\Delta H^{\#}$) and entropy ($\Delta S^{\#}$) of activation (Table 3) were determined from $\Delta H^{\#} = \Delta E^{\#} - RT$ and $\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T$ (Walstra & Jenness, 1984; Moore, 1985).

Activation parameters for the heat inactivation of P38 lipase at 40–60°C are summarised in Table 3. The $\Delta H^{\#}$ values are near to the estimates of 200–300 kJ mol^{-1} expected for the unfolding of the proteins tertiary structure (Walstra & Jenness, 1984). Therefore, the unfolding of the P38 lipase tertiary structure is probably the rate-limiting step for low-temperature inactivation.

$\Delta H^{\#}$ values for P38 lipase inactivation at low temperature (Table 3) compare with 550 kJ mol^{-1} and 424 kJ mol^{-1} for *Ps. fluorescens* 112 proteinase and *Ps.*

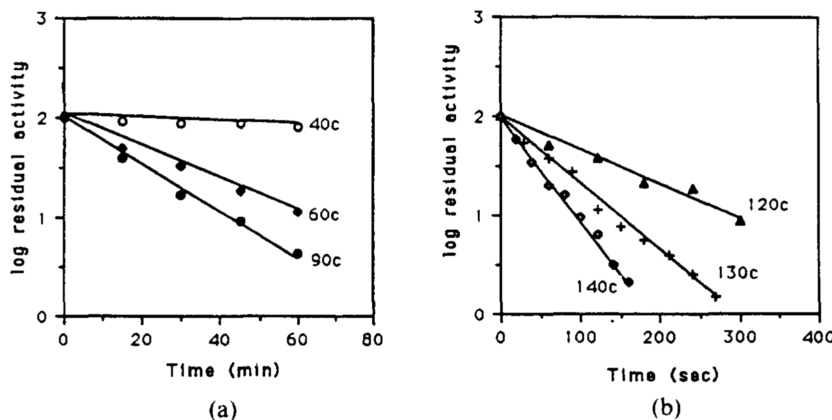

Fig. 2. Semilog plots for heat inactivation of partially purified P38 lipase at selected temperatures: (*a*) 40–90°C, (*b*) 120–140°C.

Table 3. Activation parameters for the irreversible heat inactivation of P38 lipase.[a]

	T^b(°C)	$\Delta E^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (kJ mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
c	40–50	224	221	380	100
pp	40–60	172	170	202	104
c	50–70	0·0	-3·0	-318	103
pp	60–80	-17	-20	-375	105
c	90–140	47	44	-186	115
pp	90–140	84	78	-92	115

[a] c and pp = crude and partially purified P38 lipase.
[b] T = inactivation temperature (°C).

fluorescens 22f lipase, respectively (Kroll, 1989). The lipase $\Delta H^{\#}$ values seem unexpectedly high for psychrotroph enzymes with molecular weights of about 40000–50000. By comparison, $\Delta H^{\#}$ was 375 kJ mol^{-1}, 450 kJ mol^{-1} and 775 kJ mol^{-1} for the heat inactivation of chymosin (MW = 31000), alkaline phosphatase (MW = 40000) and lactoperoxidase (MW = 82000), respectively (Walstra & Jenness, 1984).

For the high-temperature (90–140°C) inactivation of P38 lipase, the observed activation parameters (Table 3) compare favourably with literature values of $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ of 64–80 kJ mol^{-1}, -79 – -114 J mol^{-1} K^{-1} and 112–121 kJ mol^{-1}, respectively (Adams & Brawley, 1981; Dring & Fox, 1983; Fox & Stepaniak, 1983). A $\Delta H^{\#}$ value of about 60 kJ mol^{-1} is usual for 'ordinary' chemical reactions, e.g. the heat-inactivation of vitamins and Maillard browning (Laidler & Bunting, 1973, Walstra & Jenness, 1984; Kessler & Fink, 1986; Dannenberg & Kessler, 1988). The rate-limiting step for UHT inactivation of P38 lipase is therefore likely to be a simple chemical reaction ($\Delta H^{\#}$ = 44–78 kJ mol^{-1}), in contrast to protein unfolding as the rate-limiting reaction for low-temperature inactivation.

$\Delta S^{\#}$ for high-temperature inactivation was negative (Table 3). The activated complex for protein unfolding is probably more disordered compared with the native conformation; hence, $\Delta S^{\#}$ would be positive if the rate-limiting reaction was protein unfolding. A negative $\Delta S^{\#}$ for the irreversible inactivation of P38 lipase indicates that the rate-limiting (D $\dashrightarrow$ I) reaction probably involves the aggregation of (partially unfolded) enzyme molecules (Dannenberg & Kessler, 1988). It is feasible that other heat-induced chemical changes could also become important under UHT conditions.

The above results seem in good agreement with the model of enzyme heat inactivation presented above (Tables 1 and 2). According to eqn (19) (Table 2) there are three contributions to $\Delta H^{\#}$ for low-temperature inactivation. The $\Delta H^{\#}$(i) term in eqn (19) can be expected to have a small value, as discussed above. $\Delta H^{\#}(r)$ will also be small because of the fast rate of (re)folding for proteins at low temperatures (Kuwajima & Sugai, 1978; Creighton, 1984). By contrast, $\Delta H^{\#}(f)$ is expected to be large since unfolding occurs at a relatively slow rate at physiological temperatures (Creighton, 1984). Therefore, the condition $\Delta H^{\#}(f) > \Delta H^{\#}$(i), $\Delta H^{\#}(r)$ is likely to apply in relation to eqn (19).

This view is supported by literature $\Delta H^{\#}$ values for α-lactalbumin which, like psychrotroph lipases (Owusu *et al.*, 1992), has a moderately stable conformation and a high resistance to irreversible heat-inactivation (Ruegg *et al.*, 1977). $\Delta H^{\#}$ for the irreversible heat inactivation of α-lactalbumin in whole milk, was 265·6 kJ mol^{-1} at temperatures below 70°C (Dannenberg & Kessler, 1988). $\Delta H^{\#}(f)$ and $\Delta H^{*}(r)$, for the unfolding of α-lactalbumin in water at 57°C, were 215 kJ mol^{-1} and 14 kJ mol^{-1} respectively (Kuwajima & Sugai, 1978). Based on eqn (19) and the literature data cited, $\Delta H(i)$ (= 265·6 + 14 – 215 kJ mol^{-1}) is 64·6 kJ mol^{-1}. By comparison, the experimental $\Delta H^{\#}(i)$ value was 65 kJ mol^{-1}. This was the $\Delta H^{\#}$ value for the irreversible inactivation of α-lactalbumin at 90–150°C (Dannenberg & Kessler, 1988), i.e. under conditions when $\Delta H^{\#}$ would be expected to equal $\Delta H^{\#}(i)$ (Table 2).

The preceding example confirms that $\Delta H^{\#}(f) > \Delta H^{\#}(r)$, $\Delta H^{\#}(i)$. Thus, in spite of the three activation enthalpy terms in eqn (19) (Table 2), protein unfolding is likely to be the rate-limiting step for low-temperature inactivation of heat-resistant enzymes and proteins.

A valid inactivation model, should be a useful device in the discussion of the properties of psychrotroph enzymes, *vis-à-vis* low temperature inactivation phenomena, the high stability under UHT conditions and the usefulness of $\Delta G^{\#}$ as an index of enzyme structural stability. Such topics are now briefly examined in the light of present results.

The rate of P38 lipase heat inactivation at 40–80°C was greater than calculated from the rate of inactivation under UHT conditions (cf. extrapolation of line A at 90–140°C to low temperatures, Fig. 3). The maximum difference, at 50°C for crude lipase and 60°C for partially purified lipase, was 11-fold and 9-fold, respectively. The high rate of inactivation of psychrotroph enzymes at low temperatures compared to UHT condi-

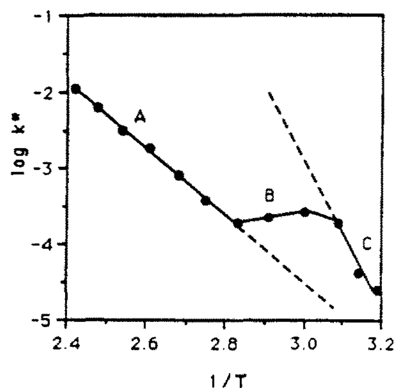

Fig. 3. Arrhenius plot for the heat inactivation of partially purified P38 lipase. A, B and C indicate three straight-line segments; (-----) shows extrapolated rate constants (see the text).

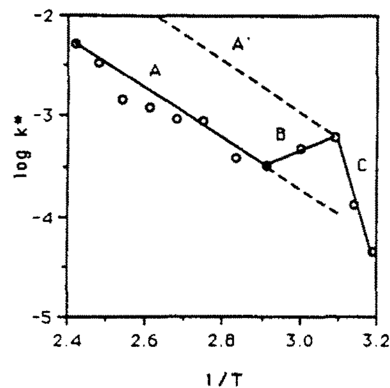

Fig. 4. Arrhenius plot of the heat inactivation of crude P38 lipase. Line A'-----C is the expected biphasic plot in the absence of LTI or UHT stabilisation (see the text).

tions is termed, LTI (Kroll, 1989). There is a patent protecting an LTI procedure for milk enzymes (Bucky *et al.*, 1986).

Conversely, the rate of UHT inactivation of P38 lipase was lower than calculated from the LTI rate (cf. extrapolation of line C at 40–50°C to high temperatures, Fig. 3). At 120°C, the rate of UHT inactivation of crude or partially purified P38 lipase was, respectively, $3{\cdot}4 \times 10^4$ -fold and $4{\cdot}2 \times 10^3$-fold lower than expected. The topography of the Arrhenius plot for P38 lipase is typical of those obtained for other psychrotroph enzymes (Dring & Fox, 1983). In general, such enzymes appear to show a high level of stabilisation against UHT inactivation.

In Figs 3 and 4, the segment labelled B seems unique to the Arrhenius plot for psychrotroph enzymes, i.e. systems demonstrating LTI phenomena. In the absence of segment B, the expected Arrhenius plot would be biphasic, consisting of the lines A'–C (Fig. 4). This type of Arrhenius plot does not indicate LTI phenomena or exceptional enzyme stability under UHT conditions (as defined in the preceding two paragraphs). Biphasic Arrhenius plots are common for mesophile enzymes and proteins (Walstra & Jennes, 1984; Kessler & Fink, 1986; Dannenberg & Kessler, 1988) and can be described in terms of the present inactivation model (Tables 1 and 2). The displacement of line A' to position A in Fig. 4 was not anticipated in Tables 1 and 2.

Three possible LTI mechanisms have been suggested: (a) proteolysis of partially unfolded lipase by associated proteolytic enzymes; (b) interaction of lipase histidine residues with magnesium ions in whole milk; and (c) aggregation of unfolded lipase molecules via hydrophobic bonding. It is envisaged that with increasing temperature, there is a switch from an LTI reaction to a less efficient inactivation process (Kroll, 1989).

The three straight-line segments in Figs 3 and 4 are an indication of the presence of two (more possibly three) distinct rate-limiting reactions for lipase inactivation. Because LTI is a kinetic phenomenon, prospective mechanisms for this process should meet the requirement of being rate-limiting for enzyme inactivation at about 60–80°C (i.e. corresponding to line B in Figs 3 and 4). A probable LTI reaction would probably show a temperature optimum corresponding to the beginning of segment B (Figs 3 and 4). For instance, proteolysis of P38 lipase could become rate-limiting for enzyme heat-inactivation as a result of the heat deactivation of psychrotroph proteinases. Although the partially purified lipase was proteinase-free, it is possible that a very low concentration of proteinase contaminant was present. Aggregation could also become rate-limiting as the strength of hydrophobic bonding (mechanism (b)) decreased at 70–80°C.

The exposure of nonpolar residues at the onset of enzyme unfolding would facilitate aggregation via hydrophobic interactions. Proteolysis would also increase as unfolded lipase molecules would be better substrates for proteinases. In this regard, it seems important that unfolding of the P38 lipase tertiary structure began at 45°C and was completed at 60°C; the T_m was 49·1°C (Makhzoum *et al.*, 1991). This temperature range corresponds closely to the LTI region in the the Arrhenius plot for P38 lipase inactivation (Figs 3 and 4).

The heat resistance of P38 lipase is not due to a stable conformation (Owusu *et al.*, 1991) and probably has to do with the type of ($D \dashrightarrow I$) rate-limiting reaction for UHT inactivation. It has been suggested that the absence of cysteine residues in the primary structure of many psychrotroph enzymes may lead to protection against disulphide-mediated heat aggregation (Mitchell *et al.*, 1986). Similarly, the presence of a low number of other heat-sensitive amino acids could reduce the likelihood of irreversible inactivation in psychrotroph enzymes. For example, glutamine/asparagine, aspartate, proline and lysine are associated with heat-induced deamination peptide-bond hydrolysis, peptide-bond isomerisation and Maillard reactions (in milk), respec-

tively (Mauron, 1977; Walstra & Jenness, 1984; Ahern & Klibanov, 1988). A possible mechanism for enhancing enzyme heat resistance might involve the use of protein engineering methods to substitute for amino acids that render proteins susceptible to heat-induced irreversible reactions (Suzuki *et al.*, 1989).

$\Delta G^{\#}$ values for irreversible inactivation are often a poor measure of enzyme tertiary structure stability (Owusu *et al.*, 1991). To optimise the heat inactivation of enzymes for technological purposes (e.g. blanching and high-temperature short-time processing), foods may be heated to temperatures above those required for enzyme unfolding (Svensson, 1977). Under such conditions, kinetic stability indices (e.g. k^*, $E^{\#}$, $\Delta H^{\#}$, *D*-values and *Z*-values) are likely to be a function of the type of rate-limiting $D \dashrightarrow I$ reaction. As discussed, those enzymes that possess the least numbers of heat-sensitive amino acids should be the most heat-resistant under UHT conditions. Kinetic stability indices determined at low temperature will be expected to be an accurate reflection of enzyme conformational stability with respect to irreversible inactivation processes.

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