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Heat-stability of a proteinase from psychrotrophic *Pseudomonas fluorescens* P38, chymotrypsin and thermolysin

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The heat-stabilities of proteinases from a psychrotrophic *Psuedomonas fluorescens* strain P38 (P38 proteinase), chymotypsin, succinyl–chymotrypsin and thermolysin are compared on the basis of their respective thermoinactivation-rate constants and Arrhenius plots (40–145°C). The Arrhenius plots for P38 proteinase and succinyl-chymotrypsin showed an inversion at 80–90°C characteristic of enzymes showing low-temperature inactivation (LTI). Thermolysin showed a biphasic log k versus $1/T$ plot consistent with the two-state model for protein and enzyme denaturation. The results are discussed in terms of the kinetics of proteinase autolysis and possible physico-chemical features necessary for LTI.

NOTATION

BTEE	benzoyl tyrosine ethyl ester
D	denatured/unfolded protein(ase)
ϵ	native enzyme concentration
ϵ_o	total enzyme concentration
$\Delta E^{\#}$	activation energy
fu	fraction of unfolded protein(ase)
$\Delta G^{\#}$	activation free energy for irreversible inactivation
h	Planck's constant
$\Delta H^{\#}$	activation enthalpy for irreversible inactivation
I	irreversibly inactivated protein(ase)
k'	observed rate constant for irreversible inactivation
k_A	rate constant for an autolytic D → I step
k_c	enzyme turnover number
k_f	folding-rate constant
k_i	rate constant for a non-autolytic D → I step
k_p	rate constant for a proteolytic D → I step
k_u	unfolding-rate constant
K	unfolding equilibrium constant
K_m	Michaelis constant
N	native state
$\Delta S^{\#}$	activation entropy for irreversible inactivation
T	temperature
v	observed rate of proteolysis or proteinase inactivation

INTRODUCTION

Proteinases from psychrotrophic *Pseudomonas fluorescens* are of interest owing to their detrimental effects on dairy-product quality (Fairbairn & Law, 1986; Mitchell *et al.*, 1986; McKellar, 1989). The temperature-dependence of the thermoinactivation-rate constant for psychrotroph proteinases does not usually conform to the Arrhenius equation over a wide temperature range. The rate of heat-inactivation is usually disproportionately high at moderate temperatures when compared with the rate at ultra-high temperatures (UHT). The phenomenon is termed low-temperature inactivation (LTI). There exists a patent considering the use of LTI as an alternative to UHT processing of milk and dairy products (Bucky *et al.*, 1986). Though LTI has been extensively studied in the last decade (Barrach *et al.*, 1978; Stepeniak & Fox, 1983; Diermayr *et al.*, 1987), the underlying processes remain uncertain. LTI seems to be confined to psychrotroph proteinases and lipases (McKellar, 1989).

A useful approach to the study of LTI, adopted in this paper, is to compare the irreversible thermoinactivation reactions for proteinases from psychrotrophic, mesophilic and thermophilic sources, that is, P38 proteinase, chymotrypsin and thermolysin, respectively. The thermoinactivations of a proteinase from psychrotrophic pseudomonas MC60 and thermolysin have been compared but only under UHT conditions (Barrach & Adams, 1977). The results of the current study are discussed with reference to the kinetics of autolysis and to the general two-stage model for protein and enzyme thermoinactivation. We also comment on some probable mechanisms for LTI of psychrotroph proteinases based on results obtained for succinyl–chymotrypsin.

MATERIALS AND METHODS

Enzyme preparation

Fluorescent pseudomonas strain P38 was isolated from raw milk (Bucky *et al.*, 1987) and cultured in half-strength peptone (Oxoid, Basingstoke, UK) water at 25°C in a shaking water bath (Gallenkamp) at 100 r/min. Cell-free crude-enzyme extract, obtained by centrifugation at 10000 × *g* for 20 min, was concentrated ten-fold by ultrafiltration through a 10-kDa-molecular-weight cut-off PM-10 membrane (Amicon Ltd). The concentrated enzyme was used as crude P38 proteinase.

Thermolysin (Type X from *Bacillus thermoproteolyticus*) and chymotrypsin (Type II from bovine pancreas) were supplied by Sigma, UK. The enzymes (0·05–0·5 mg/ml) were each dissolved in Tris–HCl buffer. The thermoinactivation of thermolysin was also examined in Tris-HCl buffer containing 8 mM calcium chloride (Ca^{2+} ions). Chymotrypsin was succinylated as described by Maneepun and Klibanov (1981) and Habeeb *et al.*, 1958). Enzyme solutions were heat-treated by using the sealed-capillary method of Davies *et al.* (1977) as described previously (Owusu *et al.*, 1992).

Proteinase assay

Proteinase residual activity was assayed using azocasein (1% w/v in 0·1 M Tris–HCl buffer, pH 7·0) as substrate at 37°C (P38 proteinases and chymotrypsin) and 50°C (thermolysin) (Sarath *et al.*, 1989). The activity of succinyl–chymotrypsin was determined using bezoyl tyrosine ethyl ester (BTEE) as substrate (Rao & Lombardy, 1975). The kinetics of enzyme thermoinactivation were analysed using relations for first- or second-order kinetics. Changes in inactivation-rate constants (k') with temperature were examined according to the Arrhenius equation,

$$\log k' = (\Delta E^{\#}/2{\cdot}303RT) + C,$$

where $\Delta E^{\#}$ is the experimental activation energy for enzyme thermoinactivation. The parameter log k' was plotted against $1/T$ to give a slope equal to $\Delta E^{\#}/2{\cdot}303\ RT$. The free energy of activation ($\Delta G^{\#}$) was calculated using

$$\Delta G^{\#} = RT \ln (k'h/kT),$$

where k, h, and R are the Boltzman, Planck and gas constants respectively. Other activation parameters were determined from

$$\Delta H^{\#} = \Delta E^{\#} - RT \text{ and } \Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T$$

(Moore, 1985).

Theory of proteinase thermoinactivation

According to Linderstrøm-Lang (1938), the hydrolysis of a protein substrate by a proteinase involves an enzymic attack on the denatured substrate molecule (D);

$$N \Leftrightarrow D \Rightarrow I \qquad (1)$$

N and I are the native and hydrolysed protein substrates, respectively. The rate of the proteinase-catalysed D ⇒ I reaction (v) can be described using Michaelis–Menten kinetics. In detail, the D ⇒ I reaction involves the steps, $\epsilon + D \Leftrightarrow \epsilon D \Rightarrow \epsilon + I$; where ϵD is an enzyme–substrate complex. The Michaelis–Menten equation, when [D] is lower than the Michaelis constant (K_m) is,

$$v = k_c\ \epsilon_o\ [D]/K_m = k_c\ \epsilon_o\ fu\ [S]/K_m = K_p\ [D] \qquad (2)$$

where v = overall rate of proteolysis, k_c = the enzyme turnover number, ϵ_o = total enzyme concentration, S = total substrate concentration ([N] + [D]), fu = fraction of denatured substrate and k_p = microscopic first-order rate constant for the proteolytic D ⇒ I reaction.

Autolytic inactivation of a proteinase involves denatured proteinase molecules serving as substrate and by analogy with eqns (1) and (2) then eqns (3) and (4) apply:

$$\epsilon \Leftrightarrow D \Rightarrow I \qquad (3)$$

$$v = k_c\ [\epsilon]\ [D]/K_m = k_c\ [\epsilon_o](1 - fu)[D]/K_m = k_A\ [D] \qquad (4)$$

Here, k_A is the autolysis-rate constant for the D ⇒ I step. From the relations, $[\epsilon_o] = [\epsilon] + (D)$, $fu = [D]/[\epsilon_o]$ and $[\epsilon] = [\epsilon_o]\ (1 - fu)$, substitution for [D] in eqn (4) leads to eqn (5) below:

$$v = k_c\ \epsilon_o^2\ fu\ (1 - fu)/K_m \qquad (5)$$

Equation (5) shows that autolysis is second-order with respect to enzyme concentration (ϵ_o). Furthermore, autolysis will become extinguished under denaturing conditions when all proteinase molecules are unfolded ($fu \rightarrow 1$). In fact, the kinetics of autolysis can be first or second order depending on the rate of proteinase (un)folding and the magnitude of k_A (see below).

Adler-Nielson (1986) emphasised that the concentration of denatured proteinase [D] reaches a steady state during autolysis. From eqn (3), we have:

$$k_u[\epsilon] = [D]\ (kf + k_A) \qquad (6)$$

where k_u = the unfolding-rate constant, k_f = folding-rate constant. Combining eqns (4) and (6) results in eqn (7):

$$v = k_A\ k_u\ [\epsilon]/(k_f + k_A) \qquad (7)$$

From the rates of autolytic D → I reaction and proteinase conformational change, when $k_A >> k_f$ then eqn (7) reduces to an expression for first-order kinetics with respect to enzyme concentration:

$$v \cong k_u\ [\epsilon] = k_u\ \epsilon_o\ (1 - f_u) \qquad (8)$$

Consequently, the observed rate constant for inactivation k (s^{-1}) will reflect the rate of proteinase unfolding; k' (s^{-1}) ≅ ku. Note that, k_A in eqn (7) increases with enzyme concentration (cf. eqn (4)), so a first-order autolytic reaction is more likely at a high initial proteinase concentration or under conditions where the autolytic rate exceeds the rate of enzyme (un)folding. However, when $k_A << kf$, then eqn (7) reduces to eqn

(9), which describes second-order kinetics with respect to the total enzyme concentration:

$$v = k_A\, k_u\, \epsilon/kf = k_c\, \epsilon_o^2\, (1 - fu)\, k_u/(K_m\, k_f) \qquad (9)$$

The observed second-order rate constant for inactivation, $k(M^{-1}\ s^{-1}) \equiv k_c k_u(1-fu)/k_m\, k_f$. The condition $k_A << k_f$ is most likely at a low initial proteinase concentration. Incidentally, both eqns (3) and (9) describe second-order kinetics; the more general eqn (9) reduces to eqn (3) where it is assumed that $ku \rightarrow 0$, $k_u k_f \rightarrow fu$ or $K << 1.0$.

At an elevated temperature, the degree autolysis will decrease owing to enzyme unfolding (i.e. as $fu \rightarrow 1$; cf eqns 4, 8, and 9). Autodigestion will therefore be only one of a range of possible D $\Rightarrow$ I reactions, that is

$$v = (k_A + k_i + k_i + 1 + \ldots\ k_n)\ [D]$$

(Ahern & Kilanov, 1988). At least two D $\Rightarrow$ I reactions appear necessary to explain the low-temperature inactivation of psychrotroph proteinases.

Irreversible thermoinactivation is preceded by a two-state equilibrium between the ϵ and D states. An equilibrium constant (K) may be assumed, where $K = [D]/\epsilon$. The corresponding rate equation for autolysis is

$$v = (k_A + k_i)\epsilon_o K/(1 + K) \qquad (10)$$

where, k_i is the rate constant for a non-enzymatic D $\Rightarrow$ I reaction (Kroll, 1989; Owusu *et al.*, 1992; Sanchez-Ruiz, 1992). Consequently, at a temperature below the proteinase unfolding temperature (T_m, $K << 1$ and, from eqn (10), we have:

$$v \equiv (k_A + k_i)\epsilon_o K = (k_A + k_i)\epsilon_o(k_u/k_f) \qquad (11)$$

That is, the rate of proteinase thermoinactivation at moderate temperatures will depend on the rates of unfolding and folding, a non-autolytic D $\Rightarrow$ I reaction, and, k_A (Owusu *et al.*, 1992). As discussed above, the autolytic reaction may conform to first- or second-order kinetics depending on the ratio of k_A:k_f. Finally, at thermoinactivation temperatures exceeding T_m, the rate of proteinase inactivation will involve D $\Rightarrow$ I reaction(s) not featuring autolysis, i.e. in eqn (10), where $K >> 1$ (or as $fu \rightarrow 1{\cdot}0$ then $k_A \rightarrow 0$, cf. eqn (4) then:

$$v = k_i\, \epsilon_o \qquad (12)$$

Computer simulations by Sanchez-Ruiz (1992) give graphic representations of how the concentrations of ϵ, D and I species may change as functions of temperature.

RESULTS AND DISCUSSION

The kinetics of proteinase thermoinactivation were determined as first- or second-order according to whether a plot of log residual activity (percentage initial activity remaining after heat treatment, RA) or 1/RA versus time (t) produced a straight-line graph. According to such criteria, the heat-inactivation of P38 proteinase conformed to first-order kinetics (Fig. 1(a)). The non-

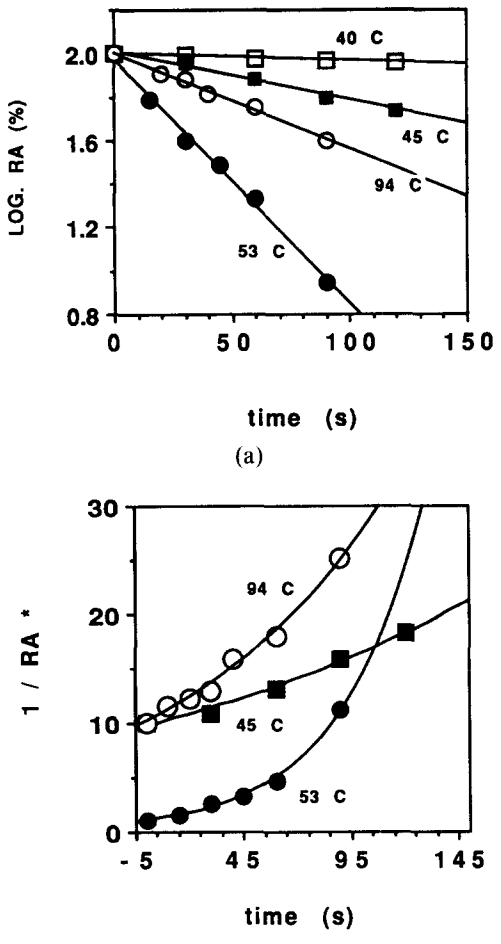

Fig. 1. A comparison of first-order (a) and second-order (b) kinetic plots for *Psuedomonas fluorescens* P38 proteinase heat-inactivation at selected temperatures. * 1/RA values at 45°C and 94°C have been multiplied by 100 or 1000, respectively.

linearity of graphs in Fig. 1(b) shows that second-order kinetics do not apply. Figure 2 shows a plot of log k' versus $1/T$ for P38 proteinase thermoinactivation. These results agreed with 'initial-rate' results where k' was estimated $k' = (2{\cdot}303/t)\ \log\ (A/A_o)$; A_o = initial

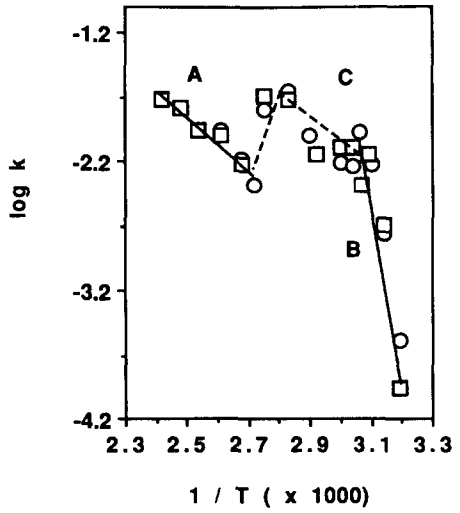

Fig. 2. Arrhenius plots for *Pseudomonas fluorescens* P38 proteinase heat-inactivation; (○) first-order constants or (□) initial-rate data plotted versus $1/T$ (see text for details).

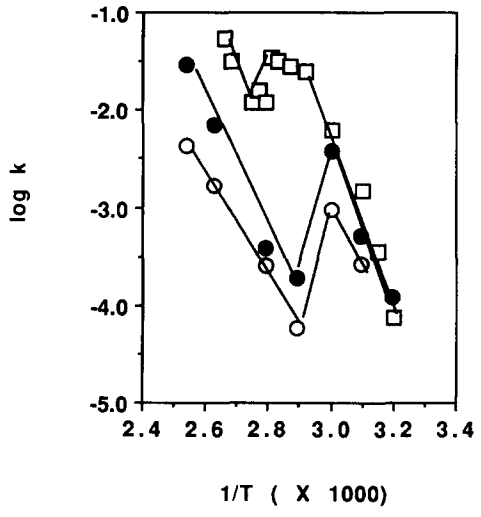

Fig. 3. A comparison of Arrhenius plots for chymotrypsin (□) and succinyl–chymotrypsin (○,●), showing first-order (○) and second-order (●) inactivation kinetics.

activity, A = activity remaining after 15 seconds of heating. The non-linear Arrhenius plot for P38 proteinase is typical of results obtained for other psychrotrophic proteinases (Barrach *et al.*, 1978; Diermayr *et al.*, 1987; Fairbairn & Law, 1986; McKellar, 1989).

The heat-inactivation of chymotrypsin (Fig. 3) and thermolysin (Fig. 4) also conformed to first order kinetics. Bimolecular-inactivation kinetics were often observed for succinyl–chymotrypsin (results not shown). The Arrhenius plot for succinyl-chymotrypsin shows results based on first- and second-order kinetics (Fig. 3). Bimolecular rate constants (k, M^{-1} s^{-1}) were transformed according to, $k'(s^{-1}) = k(M^{-1}\ s^{-1})\epsilon_o(M)$.

Proteinase inactivation occurs by autolysis at low temperatures (Barrach *et al.*, 1978; Stepeniak & Fox 1983; Diermayr *et al.*, 1987). The present results therefore demonstrate that apparent second-order kinetics are not a reliable indicator for autolysis. From the earlier theoretical discussions, first-order thermoinactivation kinetics may arise from a rate-limiting (un)folding of the proteinase conformation ($k_f << k_A$). This is likely where k_A is large owing to a high initial concentration of proteinase, a high k_c value, and/or a low value for K_m. An important point is that, though the molecularity of proteinase thermoinactivation could change as a function of temperature or *fu*, this cannot account for the non-linear Arrhenius plots (Figs. 2–4) obtained.

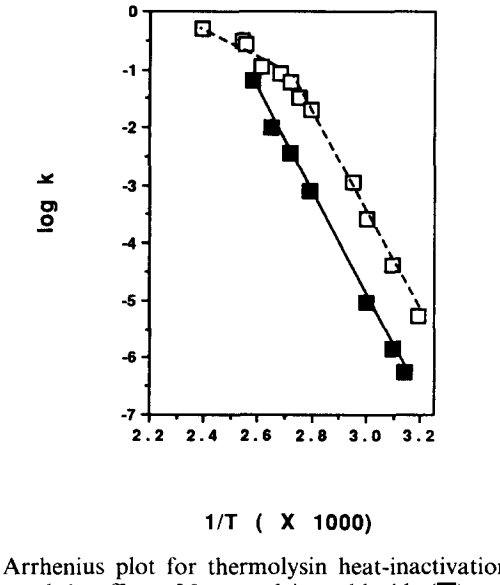

Fig. 4. Arrhenius plot for thermolysin heat-inactivation (□) and the effect of 8 mM calcium chloride (■).

P38 proteinase inactivation at <50°C resulted in large $\Delta H^{\#}$ (345·5 kJ mol^{-1}) and $\Delta S^{\#}$ (784 J mol^{-1} K^{-1}) values. However, at temperatures exceeding 90°C, the values of $\Delta H^{\#}$ and $\Delta S^{\#}$ decreased to 32·8 kJ mol^{-1} and −201 J mol^{-1} K^{-1}, respectively. The results are comparable with those reported for proteinases from other pseudomonas strains (Barrach & Adams, 1977; Stepeniak & Fox, 1983; Diermayr *et al.*, 1987). Similar trends were observed for the temperature-dependence of activation parameters for chymotrypsin and thermolysin (without added Ca^{2+} (Table 1).

The parameter $\Delta G^{\#}$ was 106·4 (kJ mol^{-1}), 96·8 (kJ mol^{-1}) 91·8 (kJ mol^{-1}) for thermolysin, chymotrypsin or P38 proteinase thermoinactivation, respectively, at 50°C. From this, the ratio of thermoinactivation-rate constants for thermolysin : chymotrypsin : P38 proteinase was 1 : 36 : 230. Moreover, in the presence of Ca^{2+}, thermolysin was 1000-fold and 6400-fold more heat-resistant than chymotypsin or P38 proteinase. Interestingly, under UHT conditions (120°C), the ratio of P38 proteinase : thermolysin heat-resistance was 25 : 1.

At low temperatures, the values of $\Delta H^{\#}$ and $\Delta S^{\#}$ were highest for the thermoinactivation of P38 proteinase (Table 1). That is, the number of bonds broken and the increase in conformational entropy were greater for the formation of a transition state ($N^{\#}$) for the psychrotroph enzyme. In the reaction, $N \Leftrightarrow N^{\#} \Leftrightarrow D$, there is resemblance between the $N^{\#}$ and N states. The observed changes in activation parameters (Table 1) are therefore consistent with the following order of conformational rigidity for these enzymes: thermolysin > chymotrypsin > P38 proteinases. Inflexions occurred in

Table 1. Activation parameters for proteinase thermoinactivation

Temperature (°C)	$\Delta G^{\#}$ (kJ/mol)[a]	$\Delta H^{\#}$ (kJ/mol)[b]	$\Delta S^{\#}$ (J/mol K)[b]
P38 Proteinase			
40–50	99·8–91·8	345·4	784·0
100–145	180–117	32·8 (± 0·3)	−201 (± 0·3)
Chymotrypsin			
40–50	102–96·8	249·6	473 (± 0·2)
90–103	103–102	121·5	50·8 (± 0·6)
Thermolysin			
40–95	108–99·2	161 (± 0·2)	169 (1·3)
95–145	99·2–101	71·2	−77 (± 1·8)
60–120[c]	114–104	167	158 (± 1·8)

[a] Range of values corresponds to temperature range.
[b] Mean value for temperature range shown (S.D ≥ 0·4% are given in parentheses)
[c] Effect of 8 mM calcium chloride.

the Arrhenius plot at 50°C for P38 proteinase and at 92°C for thermolysin. For chymotrypsin, there was a more gradual deviation from linearity at $T > 50$–60°C, culminating in the inversion of the Arrhenius plot at 80–90°C. These temperatures agree closely with the apparent T_m values of 45–50°C, 55–60°C and 90°C for psychrotroph proteinases, chymotrypsin and thermolysin (Owusu *et al.*, 1991; Owusu & Berthalon, 1993) and confirm the order of proteinase conformational rigidity discussed above.

A possible interpretation for the non-linear Arrhenius plot (Figs 2–4) is that breaks coincide with temperatures where changes occur in the rate-limiting step for heat-inactivation. At low temperatures, proteinase heat-inactivation is likely to proceed via a rate-limiting unfolding step giving rise to large $\Delta H^{\#}$ and $\Delta S^{\#}$ values (cf. Fig. 2; Table 1) associated with protein denaturation (Stearn, 1949). At temperatures perhaps only slightly exceeding the T_m autodigestion, associated with relatively low $\Delta H^{\#}$ and $\Delta S^{\#}$ values, this would become rate-limiting. Whether there occurs an inversion (i.e. change to a negative slope) in the Arrhenius plot at such temperatures (Fig. 2) may depend on the value for T_m. A precipitous fall in the thermoinactivation rate is likely if proteinase unfolding occurs (and autolysis is therefore extinguished) at a temperature that is too low to support an alternative, non-autolytic D $\Rightarrow$ I reaction.

At temperatures greater than T_m (when $fu \rightarrow 1{\cdot}0$ or the concentration of native enzyme is insignificant) proteinase inactivation would require a non-autolytic irreversibility reaction. A detailed discussion of specific mechanisms and possible values for associated activation parameters for enzyme thermoinactivation under low-temperature and UHT conditions has been presented previously (Owusu *et al.*, 1992; Owusu & Berthalon, 1993). A rate-limiting D $\Rightarrow$ I reaction for heat-inactivation would reflect characteristics of proteinase primary rather than tertiary structure. The heat-resistance of psychrotrophic enzymes has been attributed to the presence of a low level of heat-sensitive amino acids, e.g. cysteine, in the primary structure of these enzymes (Barrach & Adams, 1977; Mitchell *et al.*, 1986).

The Arrhenius plot for P38 proteinase shows that this enzyme is susceptible to LTI whereby psychrotroph proteinases are more heat-resistant at UHT than at moderate temperature. For example, extrapolation of Section B of Fig. 2 to high temperatures reveals that the rate of thermoinactivation of P38 proteinases at 50°C is disproportionately high compared with the rate of thermoinactivation at 140°C. Succinyl–chymotrypsin also showed an inverted Arrhenius plot (Fig. 3) resembling, at least superficially, the profiles obtained with psychrotroph proteinases and lipases. Indeed, modification of chymotrypsin or trypsin with various agents thought to increase their hydrophilicity, produces 'zigzag' Arrhenius plots (Siksnis *et al.*, 1990; Mozhaev, 1992), which are indistinguishable from those associated with psychrotroph proteinases. The same feature was also present, to a lower degree, for the thermal inactivation of non-modified chymotrypsin (Fig. 3). On the other hand, the simple, two-phase Arrhenius plot for thermolysin conforms to the two-stage mechanism for enzyme and protein denaturation discussed previously (Owusu *et al.*, 1992). In the presence of calcium ions, heat-inactivation of thermolysin produced a linear Arrhenius graph (Fig. 4). This is consistent with the stabilisation of thermolysin by calcium ions (Feder, 1971).

The thermal-inactivation characteristics of succinyl–chymotrypsin suggest that an increase in the net negative charge and hydrophilisation (Habeeb *et al.*, 1958; Maneepun & Klibanov, 1981; Siksnis *et al.*, 1990) is associated with the characteristic inversion of Arrhenius plots, which is the basis of the LTI of psychrotroph proteinases. A low p*I* (McKellar, 1989) or a naturally high level of glycosylation in psychrotroph proteinases could produce physico-chemical properties analogous to those observed for succinylated proteins, e.g. increased solubility and resistance to aggregation, increased resistance towards proteolysis and reduced conformational stability. Succinylation of RNAse does not result in a zigzag Arrhenius plot (Leach & Boyd, 1973). Thus far, it would appear that the LTI phenomenon is mainly associated with proteinases or enzyme preparation probably contaminated with proteinases. To undergo LTI, it may be necessary that a proteinase unfolds at low temperatures, where the rates of non-autolytic (D $\Rightarrow$ I) irreversibility reactions are low. Hence P38 proteinase with a relatively low unfolding temperature showed LTI, whereas thermolysin with a higher T_m did not show the LTI phenomenon.

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