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The Thermodynamic Stability of Lipases and Proteases from Psychrotrophic Bacteria

R. K. Owusu*[a], A. Makhzoum[a] & J. Knapp[b]

[a]Procter Department of Food Science. [b]Department of Microbiology
The University of Leeds, Leeds LS2 9JT, UK

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ABSTRACT

The thermodynamic or conformational stability of psychrotroph lipases and proteases, measured as the Gibbs free energy difference ($\Delta G^{\ominus}$) between the native and denatured enzymes, were estimated from enzyme temperature-activity profile data. $\Delta G^{\ominus}$ estimates of 8–10 kJ/mol and 16–17 kJ/mol were obtained for psychrotroph-derived lipases and proteases, respectively. Pseudomonas fluorescens *strain AR-11 protease was unusually thermolabile ($\Delta G^{\ominus} = 3{\cdot}0$–$7{\cdot}6$ kJ/mol). These values were compared with values for some mesophilic and thermophilic enzymes and the possible relationship of $\Delta G^{\ominus}$ to psychrotrophic enzyme heat-resistance is discussed.*

INTRODUCTION

Extracellular lipases and proteases produced by psychrotrophic micro-organisms are known to withstand ultra-high temperature (UHT) treatment. Thus the development of soapy flavour or hydrolytic rancidity, coagulation and bitter taste in stored UHT treated milk is widely attributed to enzymic (lipase and protease) activity rather than to microbial action *per se* (Cogan, 1977; Law, 1979; Fairbairn & Law, 1986; Stead, 1986; McKellar, 1989).

These enzymes are described as heat-stable or heat-resistant because the *D*-value (time required for a 90% reduction in the initial activity), e.g., for psychrotroph protease, is usually greater than the *D*-value for spores

* To whom correspondence should be addressed.

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isolated from such food spoilage microorganisms as *Clostridium botulinum* and *B. stearothermophilus* (Adams *et al.*, 1975), and comparable to the *D*-value for thermolysin, a protease from the thermophile *B. thermoproteolyticus* (Barach & Adams, 1977).

Past discussions of the stability of psychrotroph enzymes were based solely on kinetic stability using the thermoinactivation first-order rate constant, k (D-value = 2·303/k) and activation energy (E_{act}) as indices. There are no published reports concerning the thermodynamic stability, as measured by the Gibbs free energy difference between the native and the denatured conformation ($\Delta G^{\ominus}$), of lipases and proteases from psychrotrophs.

However, $\Delta G^{\ominus}$, $\Delta H^{\ominus}$ (molar enthalpy change) and $\Delta S^{\ominus}$ (molar entropy change) values are an important basis for unequivocal discussions of structure and stability relations in proteins (Tanford, 1968; Privalov, 1979). In this paper a simple method for the estimation of $\Delta G^{\ominus}$, $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ values for enzymes capable of undergoing reversible thermoinactivation in the presence of substrate, is applied to several lipases and proteases from psychrotrophic bacteria. Thermodynamic quantities were calculated using temperature-activity profile data from the literature (Adams *et al.*, 1975; Alachanidis & Andrews, 1977; Adams & Brawley, 1981*a*; Christen *et al.*, 1986; Mitchell *et al.*, 1986; Omar *et al.*, 1987). $\Delta G^{\ominus}$ estimates and the temperature at which 50% enzyme activity is lost (*Tm*) were compared to values for comparable enzymes from thermophiles. Some implications of the relatively low thermodynamic stability of lipases and proteases from psychrotrophs are discussed.

MATERIALS AND METHODS

For a protein capable of undergoing a reversible conformational change from an active, native (N) conformation to an inactive, denatured (D) form, the thermodynamic stability is directly measurable as the magnitude of the apparent equilibrium constant (K), where $K = [D]/[N]$. This relation is applicable where a protein conformational change is demonstrably reversible, i.e. where removal of the denaturant results in the restoration of the N-form. It is also assumed, particularly in the case of small globular proteins, that denaturation equilibrium involves only two enzyme conformational states, i.e. D and N enzyme forms (Hermans, 1965; Tanford, 1968; Privalov, 1979).

As a preliminary step, temperature-activity profile data were replotted in the form of Arrhenius plots (Fig. 1). Enzyme thermoinactivation equilibrium constants were then determined over a range of assay temperatures (T) as described below.

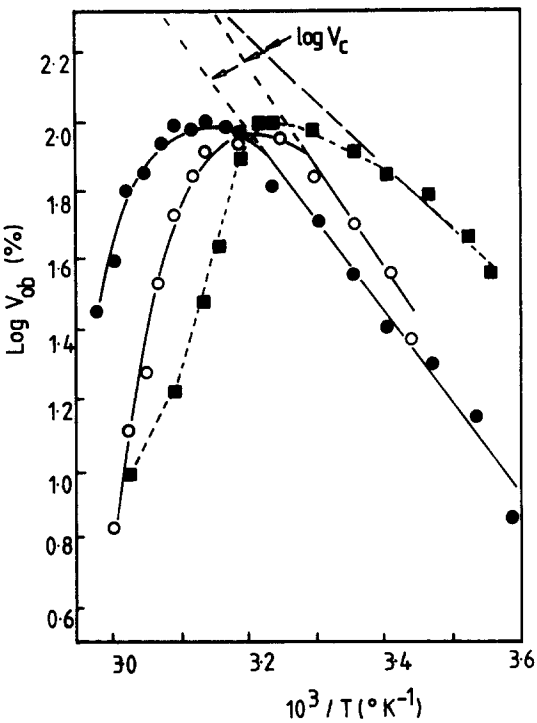

Fig. 1. Arrhenius plot of log observed enzyme activity (log V) versus $1/T$ for A32 lipase (○), *P. fluorescens* OM82 (●) and strain AR-11 (■) proteases. Equation of the least-squares line is $\log V_c = 12 - 3250\ (1/T)$ for A32 lipase and $\log V_c = 11 - 2797\ (1/T)$ for OM82 protease; $r > 0{\cdot}98$.

It was first necessary to consider the activity response of an enzyme capable of undergoing a reversible transition with increasing assay temperature, in the presence of a saturating concentration of substrate.

At a low assay temperature (e.g. $T \ll Tm$), the observed rate of catalysis (V_{ob}) will be a maximum, determined by the total enzyme concentration and the enzyme turnover number. At these temperatures, V_{ob} may be approximated by a theoretical value (V_c) calculated using a least-squares straight line equation fitted to experimental points, i.e. $\log(V_{ob}) = \log(V_c) = m(1/T) + C$; where *m and C* are the slope and intercept of the linear phase of the Arrhenius plot (Fig. 1).

At a high assay temperature (e.g. as T approaches Tm), due to a significant conversion of the *N*-form to the *D*-form of enzyme, values of V_{ob} would deviate from rates of enzyme reaction extrapolated from the ideal Arrhenius-type response at low temperature (V_c). The fraction of active enzyme (F_N) at any temperature and the equilibrium constant for reversible thermoinactivation are then given by $F_N = V_{ob}/V_c$ (Fig. 2) and $K = F_N - 1$ (Herman & Scheraga, 1961).

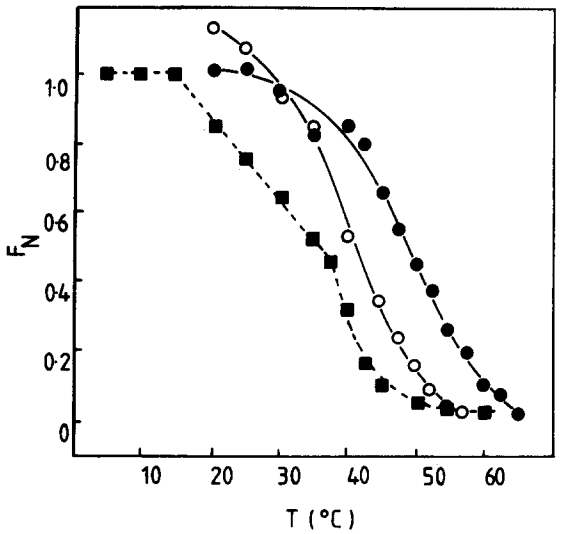

Fig. 2. A plot of the fraction of native enzyme (F_N) versus assay temperature T(°C) for A32 lipase (○) and *P. fluorescens* OM82 (●) or strain AR-11 proteases (■).

The molar enthalpy for denaturation was estimated from the slope ($-\Delta H^{\ominus}/2{\cdot}303RT$) of a van't Hoff plot of log K versus $1/T$ (Fig. 3). At an assay temperature equal to Tm, $\log K = 0$, $\Delta G^{\ominus} = 0$ and $\Delta S^{\ominus} = \Delta H^{\ominus}/Tm$ (Herman & Sheraga, 1961). Finally, the standard Gibbs free energy change $\Delta G^{\ominus}$ was then computed using $\Delta G^{\ominus} = \Delta H^{\ominus} - 298\,\Delta S^{\ominus}$.

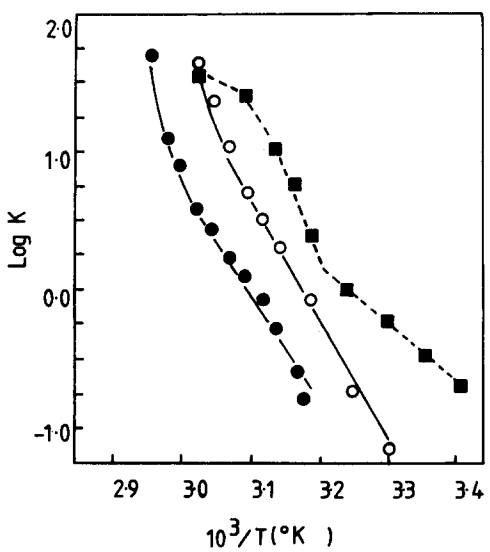

Fig. 3. Van't Hoff plot of log K versus inverse temperature ($1/T$) for A32 lipase (○) and *P. fluorescens* OM82 (●) or strain AR-11 (■) proteases.

RESULTS AND DISCUSSION

The Arrhenius plot (Fig. 1) based on temperature-activity data for *Acinetobacter* 32 (A32) lipase (Christen *et al.*, 1986), *P. fluorescens* OM82 protease (Mitchell *et al.*, 1986) and *P. fluorescens* strain AR-11 protease (Alichanidis & Andrews, 1977) showed the anticipated deviation of V_{ob} from V_c at high assay temperatures ($Tr \rightarrow Tm$). Furthermore, V_{ob} and V_c were approximately coincident at assay temperatures below *Tm*, as expected. Similar results were observed for *P. fluorescens* MC50 lipase (Adams & Brawley, 1981*a*), *Huminocola lanuginosa* No. 3 lipase (Omar *et al.*, 1987) and *P. fluorescens* MC60 protease (Adams *et al.*, 1975).

The fraction of active enzyme (F_N) showed a sharp transition with temperature, consistent with a cooperative unfolding of protein structure (Fig. 2). Also, van't Hoff plots were linear in the region of $\log K = 0{\cdot}5$ to $-0{\cdot}5$ indicating reversible two (N & D) state enzyme thermoinactivation (Fig. 3); following the suggestions of other workers, $\Delta H^{\ominus}$ values were determined from the slope of the van't Hoff plots in the above linear region (Herman & Scheraga, 1961). Notably, a $\log K$ versus $1/T$ plot for *Ps. fluorescens* AR-11 protease possessed a discontinuity within the $\log K = 0{\cdot}05$ to $-0{\cdot}5$ region (Fig. 3). Thus two estimates of thermodynamic parameters were obtained (Table 1). It is uncertain whether or not this was due to the presence of two enzymes differing in thermodynamic stability. It may also be significant that AR-11 protease, alone of the proteases considered in this paper, was originally assayed in potassium phosphate buffer (Alachinidis & Andrews, 1977) which chelates calcium. The role of calcium in the stabilization of these microbial proteases is now widely recognised (Fairbairn & Law, 1986). Pending further studies of such biphasic van't Hoff plots, and the role of buffer choice, it is assumed that AR-11 protease thermostability is unrepresentative of psychrotroph protease stability, e.g. in the presence of calcium ions (see below).

The $\Delta G^{\ominus}$ values for *Ps. fluorescens* MC50 lipase and A32 lipase were 8 kJ/mol and 10·2 kJ/mol, respectively. Whilst $\Delta G^{\ominus}$ was 16 kJ/mol for *P. fluorescens* MC60 protease and 17 kJ/mol for *P. fluorescens* OM82 protease (Table 1). By comparison, $\Delta G^{\ominus}$ for the lipase from the thermophilic fungus *H. lanuginosa* was 13·0 kJ/mol. The thermodynamic stability of thermolysin was 82·2 kJ/mol and 118·0 kJ/mol for the reversible thermoinactivation of calcium ion-stabilized apo- and holothermolysin, respectively, based on $\Delta H^{\ominus}$ and *Tm* values reported previously (Dahlquist *et al.*, 1976). Present ΔG estimates compare with 60 kJ/mol, 35–51 kJ/mol and 40–54 kJ/mol reported for lysozyme, chymotrypsin and ribonuclease (Privalov, 1979).

The estimation of thermodynamic parameters as described above is valid only if it may be assumed that the activity decreases, which were observed

TABLE 1
Thermodynamic Parameters for the Reversible Thermoinactivation of some Psychrotroph Enzymes

Enzymes	$\Delta H^{\ominus}$ (*kJ/mol*)	$\Delta S^{\ominus}$ (*J/mol°K*)	$\Delta G^{\ominus}$ (*kJ/mol*)	*Tm* (°C)
Acinetobacter 32 lipase	192·0	610	10·2	41·7
P. fluorescens MC50 lipase	198·3	639	8·0	37·5
*H. lanuginosa** lipase	128·8	388·6	13·0	58·3
P. fluorescens OM82 protease	165·0	497	17·0	49·4
P. fluorescens MC60 protease	245·1	770	16·0	45·6
P. fluorescens AR11 protease	82·8 202·0	268 652	3·0 7·6	35·8 34·0
Thermolysin[a]**	545 545	1 555 1 437	82·4 118·0	78 92

* Enzymes from thermophilic microorganisms.
[a] $\Delta G°$ estimated from $\Delta H°$ and *Tm* values from Dalquist *et al.* (1976).

in the temperature-activity profiles, were the result of reversible and non-irreversible thermoinactivation. However, it may be supposed that significant irreversible thermoinactivation over the low temperature ranges considered is not consistent with the well-documented heat resistance of psychrotroph derived lipases and proteases (Fairbairn & Law, 1986; Stead, 1986; McKellar, 1989). It is worth noting that the present thermodynamic methods are not expected to be sensitive to kinetic instability phenomena, e.g. low temperature-inactivation.

The thermodynamic parameters reported above may well incorporate substrate effects. Thus proteases may be stabilized by substrates. The adsorption of lipase at the water/oil interface may involve partial unfolding (O'Connor & Bailey, 1989), in which case $\Delta G^{\ominus}$ would be different in the absence of substrate. It is known for instance that the nature of the oil phase and emulsifier present may affect the kinetic stability of lipase in the presence of emulsified lipase substrate (Adams & Brawley, 1981*b*). There are also differences in the assay conditions for various enzymes; the use of phosphate buffer, in the assay of P. fluorescence strain AR-11 protease and *H. lanuginosa* lipase, may be rather important.

In spite of such limitations, $\Delta G^{\ominus}$ and *Tm* values for lipases and proteases for psychrotrophs were of comparable magnitude, whereas $\Delta G^{\ominus}$ and *Tm* values for *H. lanuginosa* No. 3 lipase and thermolysin were significantly higher. The ratio of thermodynamic stability for a pair of enzymes (A & B), e.g. K_A/K_B is $10^{(1/5706)[\Delta G_A - \Delta G_B]}$. Thus from present estimates of thermodynamic parameters, the thermophile lipase was five-fold more stable than either psychrotroph lipase examined. More impressive conformational stability differences are observed for thermolysin. Thus holothermolysin may be as much as 6×10^{17}-fold more thermodynamically stable than either *P. fluorescens* MC60 or OM82 protease.

Therefore, employing the thermodynamic criterion, $\Delta G^{\ominus}$, appears to result in a clear distinction between conformationally stable enzymes (i.e., *H. lanuginosa* lipase and thermolysin) which remain active at high temperatures, and conformationally less stable enzymes (e.g. psychrotroph lipases and proteases) which generally have a low T_{opt}. The former class of enzyme can always be expected to show high kinetic stability against irreversible thermoinactivation. However, as illustrated by the psychrotroph enzymes examined, high kinetic stability need not be associated with a high thermodynamic or conformational stability.

A clear distinction between kinetic and thermodynamic stability is also important because experiments designed to investigate kinetic stability will not in general provide information about the degree of thermodynamic stability (Bozoglu *et al.*, 1984). As present results show, the thermodynamic or conformational stability of the psychrotrophic enzymes studied is significantly lower than the conformational stability of corresponding thermophilic enzymes (or a miscellaneous group of eukaryotic enzymes) and does not account for the high resistance to heat-inactivation observed with psychrotroph enzymes. Therefore the great heat resistance of psychrotroph lipases and proteases may be the result of efficient renaturation after high temperature treatment. Indeed, the propensity towards reversible unfolding may be a general characteristic of lipases and proteases from psychrotrophs owing to the inherent flexibility of such enzymes (Mitchell *et al.*, 1986). This might explain how such enzymes survive exposure to high temperatures but exhibit relatively low activity-temperature optima.

Discussions of enzyme thermostability based entirely on kinetic data such as the Gibbs free energy of activation (ΔG^{++}) for irreversible thermoinactivation, can lead to ambiguous results. For instance, ΔG^{++} for enzymes with greatly varying degrees of conformational thermostability, e.g. *P. fluorescens* MC60 protease, subtilisin Carlsberg, subtilisin BPN', thermomycolase and thermolysin ΔG^{++}, was only within a range of 92–113 kJ/mol (Voordouw *et al.*, 1976; Barrach & Adams, 1977). Also ΔG^{++} for caldolysin, a zinc metalloprotease from *Thermus aquaticus* T-351, arguably one of the

most conformationally thermostable enzymes known (T_{opt} = 95–97°C), was only 40·3 kJ/mol (Cowan & Daniel, 1982).

Finally, microbial protein thermostability generally shows a positive correlation with the optimal growth temperature of the source microorganism (Owusu & Cowan, 1989). For life in a low temperature niche, interspersed with short periods at high temperature, it might be that psychrotrophic microorganisms would possess proteins with a low thermodynamic thermostability and high flexibility, enabling efficient renaturation after heat-denaturation. By contrast it would appear necessary for thermophile enzymes to maintain conformational and functional integrity in order that thermophilic microorganisms can live at high temperatures. If, as suggested by the present data, thermophile and psychrotroph-derived enzymes are fundamentally different in their response to high temperature, there is no reason to suppose that the two classes of enzymes are especially related structurally (Barach & Adams, 1977).

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