Mechanism and kinetics of inactivation at 40–70 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F

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Summary. HPLC size exclusion chromatography experiments showed that during inactivation at 40–70 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F small molecular mass fragments were formed, indicating that autoproteolysis was at least one of the major causes of inactivation. The formation of small molecular mass fragments and the reaction order indicated that intermolecular autoproteolysis was more likely than intramolecular autodigestion. This was confirmed by computer simulations. The rate constants and the activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) for the reactions of the intermolecular autoproteolysis model were derived from computer simulations. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the unfolding reaction were 504 kJ mol^{-1} and 1252 J mol K^{-1} respectively. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the refolding reaction were strongly temperature dependent. The estimates for the enthalpy (ΔH^{0}) and entropy (ΔS^{0}) difference between the folded and unfolded state as derived from the reaction rate constants of unfolding and refolding were subject to large deviations, owing to accumulation of errors in the estimation of the kinetic characteristics.

Pseudomonas fluorescens, as well as several other psychrotrophic bacteria, are notorious for their secretion of thermostable proteinases. Even after UHT treatment (e.g. 1 s at 145 °C) part of the proteolytic activity may be retained, so that defects in milk and milk products may occur (Kroll, 1989).

Besides the thermostability of the proteinases from *Ps. fluorescens* at high temperatures, a relatively high susceptibility towards thermoinactivation between 40 and 70 °C has been reported (Barach *et al.* 1976, 1978; Griffiths *et al.* 1981; Stepaniak & Fox, 1983, 1985; Kroll & Klostermeyer, 1984; Christen & Marshall, 1985; Diermayr *et al.* 1987; Patel & Bartlett, 1988; Fortina *et al.* 1989; Stepaniak *et al.* 1991; Kumura *et al.* 1993; Owusu & Doble, 1994). This inactivation is generally referred to as low temperature inactivation, because the mechanism appears to be different from that of inactivation at temperatures > 180 °C.

One of the possible explanations is the occurrence of intermolecular autoproteolysis, which is the hydrolysis of unfolded proteinase molecules by native (not yet unfolded) molecules (Barach *et al.* 1978; Diermayr *et al.* 1987). Other possible inactivation mechanisms include intramolecular autoproteolysis, where one partly unfolded molecule would inactivate itself (Stepaniak & Fox, 1983), and aggregation with milk protein (Barach *et al.* 1978; Stepaniak *et al.* 1991). In this paper the inactivation of the extracellular proteinase from *Ps. fluorescens* strain 22F at 40–70 °C is described.

materials and methods

Organism and enzyme production and purification

Pseudomonas fluorescens strain 22F, originally isolated from raw milk, was obtained from the Netherlands Institute for Dairy Research (NIZO, NL-6710 BA Ede, The Netherlands) and inoculated into sterile skim milk. After incubation at 20 °C for 8 d, the cells were removed by centrifugation (27 000 ***g*** at 4 °C for 30 min). The supernatant, containing the proteinase, was stored at −20 °C, and used as the crude enzyme.

For production of proteinase for purification, the organism was grown in sterile tryptone–lactose growth medium and incubated at 20 °C for 8 d. After removing the cells by centrifugation, the proteinase was purified to electrophoretic homogeneity by ammonium sulphate precipitation, hydrophobic interaction chromatography, ultrafiltration and size exclusion chromatography, as previously described (Schokker & van Boekel, 1997).

Proteinase assay

Proteolytic activity was determined by incubating the enzyme solution with sodium caseinate (DMV, NL-5460 BA Veghel, The Netherlands), followed by quantification of trichloroacetic acid-soluble hydrolysis products using 2,4,6-trinitrobenzenesulphonic acid (Fluka AG, CH-9470 Buchs, Switzerland), as previously described (Schokker & van Boekel, 1997).

Heating experiments

To evaluate the extent of heat inactivation as a function of time and temperature, crude enzyme diluted 10-fold in demineralized water, pH 7·0 ($\sim 1 \times 10^{-7}$ M, $\sim 5\ \mu$g/ml) was heated at various temperatures.

For the inactivation experiments above 70 °C, 2·1 ml portions of the enzyme solutions were heated in stainless steel tubes (7 × 120 mm) rotated in a thermostatted glycerol bath. After the desired time the tubes were cooled immediately in ice water, and after 30 min the activity was measured. The residual activity was calculated as a fraction of the activity after 2 min heating, which was considered to be the initial activity, thereby eliminating the effects of heating up.

In the range 40–70 °C, the crude enzyme was diluted 10-fold with water at a temperature just above that desired, in order to overcome the problem of heating up periods. The resulting temperature was recorded and kept constant for the period of the experiment. After the desired time, samples were drawn from the enzyme solution, cooled rapidly in ice water and after 30 min the residual activity was measured. In a parallel experiment the enzyme was preheated at 100 °C for 10 s, subsequently held at the desired temperature and assayed as above. The residual activity was calculated as a fraction of the activity of the unheated enzyme solution.

For the HPLC experiments, 1·0 ml samples of 5×10^{-6} M ($\sim$ 0·25 mg/ml) purified enzyme in 0·02 M-Tris-HCl, pH 7·0 were heated at 55·2 °C in glass tubes. After the desired time, the tubes were cooled rapidly in ice water.

Reaction order

The reaction order (n) for the inactivation of the proteinase was determined using the differential method (Laidler, 1987). Crude enzyme was diluted 10-, 20-, 50- and 100-fold in 0·2 *m*-Tris-HCl–2 mM-$CaCl_2$, pH 7·4, heated at 50 or 52 °C, cooled in ice

water and the residual proteolytic activity determined. The initial rates of inactivation (v) were measured at the various enzyme concentrations (C), then $\log v$ was plotted against $\log C$ and n calculated from the slope.

High performance liquid chromatography

HPLC measurements were performed using a Kontron 414 pump, a Marathon autosampler (fixed loop, 20 μl), a Kratos Spectroflow 757 u.v.–visible variable wavelength detector (all from Separations Analytical Instruments, NL-3341 LL, H.I. Ambacht, The Netherlands) and TSP PC1000 computer software (Thermo Separation Products, San Jose, CA 95161-9031, USA). A 220 × 13 mm Superdex 75 HR 10/30 size exclusion chromatography column (Pharmacia LKB Biotechnology, S-751 82 Uppsala, Sweden) was used. The eluent was 10 mM-phosphate buffer, pH 7·0 at a flow rate of 1·2 ml/min. Detection was at 214 nm.

Differential scanning calorimetry

Differential scanning calorimetry was performed using a Setaram Micro calorimeter (Setaram, F-69300 Caluire, France). Samples of 0·9 ml purified proteinase (1·1 mg/ml 0·02 M-Tris-HCl–10 mM-*o*-phenanthroline, pH 7·0) were heated from 20 to 110 °C at a scanning rate of 0·5 K min^{-1}. Buffer–*o*-phenanthroline was used as a reference. The enthalpy change of unfolding (ΔH^0) and denaturation temperature (T_d) were determined according to Privalov (1979).

Fitting of models to the results

Programs written in TurboPascal 4.0 (Borland International Inc., Scott Valley, CA 95066, USA), run on an IBM-compatible 80486 computer, were used to fit the autoproteolysis models to the experimental results. Ordinary differential equations were derived for the intermolecular and intramolecular autoproteolysis models, and integrated numerically using Gear's algorithm for stiff ordinary differential equations (Stabler & Chesick, 1978; Chesick, 1988). Rate constants were found using Powell's direct search for nonlinear weighted least squares (Lobo & Lobo, 1991). The objective function to be minimized was the χ^2 stochastic.

RESULTS AND DISCUSSION

Mechanism of inactivation

The first target of the work was to establish whether the extracellular proteinase from *Ps. fluorescens* 22F was inactivated in the temperature range 40–70 °C. Therefore, the extent of inactivation after heating the enzyme solution for 30 min at various temperatures was measured (Fig. 1). Above 70 °C the rate of inactivation increased with temperature as for 'normal' reactions. The reactions causing this type of inactivation, such as hydrolysis of the peptide bond, reshuffling of disulphide bonds, destruction of amino acid residues, Maillard reactions, aggregation and formation of incorrect conformations (Ahern & Klibanov, 1988; Volkin & Middaugh, 1992), will be referred to as thermal inactivation. Between 40 and 70 °C, the extent of inactivation was relatively high, indicating that the mechanism was different from the inactivation at high temperature. When the enzyme solution was heated at 70 °C, almost no inactivation occurred. Because at 70 °C no proteolytic activity was observed (Schokker & van Boekel, 1997), it could be concluded that virtually all non-degraded enzyme molecules refolded to an enzymically active conformation following cooling after heat treatment.

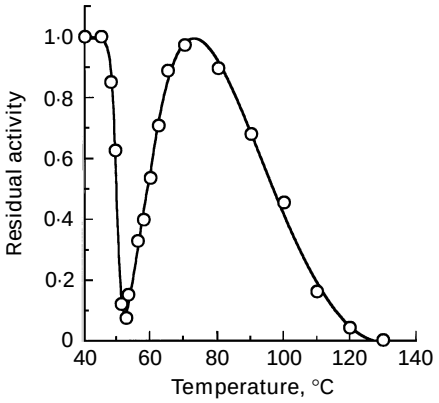

Fig. 1. Residual activity of the crude extracellular proteinase from *Pseudomonas fluorescens* 22F after 30 min heating at the temperatures indicated.

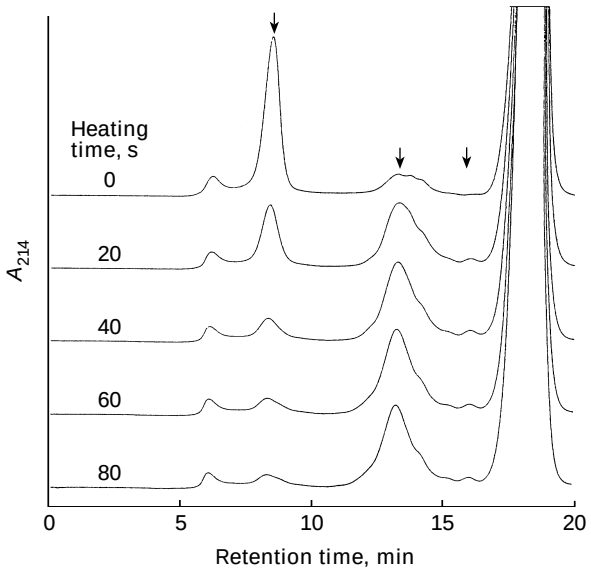

Fig. 2. HPLC chromatograms of 5×10^{-6} M purified extracellular proteinase from *Pseudomonas fluorescens* 22F in 0·02 M-Tris-HCl, pH 7·0 heated at 55·2 °C. The proteinase eluted at 8·3 min and the autoproteolysis products at 13·2 and 16·0 min (as indicated by arrows). The large peak at 18 min was caused by elution of Tris.

In order to investigate the relatively high loss of activity between 40 and 70 °C, this was followed by HPLC. Samples of purified enzyme in 0·02 M-Tris-HCl, pH 7·0 were heated at 55·2 °C for 0, 20, 40, 60 and 80 s, cooled in ice water and run on a size exclusion chromatography column (Fig. 2). The proteinase eluted at 8·3 min, corresponding to an apparent molecular mass of ~ 45 kDa. During the heat treatment the amount of enzyme was rapidly reduced, in proportion to the loss in proteolytic activity. At the same time new peaks eluted at 13·2 and 16·0 min, roughly corresponding to molecular masses of 2 and 1 kDa respectively, indicating that the proteinase molecules were digested into small fragments. From these results it could be concluded that autoproteolysis caused at least part of the inactivation at

40–70 °C. The formation of small fragments suggested that inactivation was caused by intermolecular autoproteolysis, rather than by intramolecular autoproteolysis, for which the last proteolytically active species in the breakdown sequence of the enzyme were expected to be much larger (Diermayr *et al.* 1987).

We tried to discriminate between intramolecular and intermolecular autoproteolysis by kinetic modelling. For intermolecular autoproteolysis the following reaction schemes were used.

$$\mathrm{N} \underset{k_\mathrm{f}}{\overset{k_\mathrm{u}}{\rightleftharpoons}} \mathrm{U} \tag{1}$$

$$\mathrm{N} + \mathrm{U} \xrightarrow{k_\mathrm{interm}} \mathrm{N} + \mathrm{I}_1 + \mathrm{I}_2 \tag{2}$$

In the first reaction sequence, the unfolding–refolding transition is described, where N and U represent the native and unfolded enzyme molecule respectively, and k_u and k_f the rate constants for the unfolding and refolding reactions respectively. In the second reaction sequence, which is the actual autoproteolysis reaction, the digestion of the unfolded molecule by the native proteinase takes place. Here, k_interm is the rate constant of the digestion, and I_1 and I_2 the products of autoproteolysis, i.e. the fragments of the enzyme molecule. The autoproteolysis reaction might continue, resulting in even more fragments. The fragments would be catalytically inactive. The intermolecular autoproteolysis model could be simulated and fitted to the experimental results, using the following system of differential equations.

$$\frac{\mathrm{d}[\mathrm{N}]}{\mathrm{d}t} = -k_\mathrm{u}[\mathrm{N}] + k_\mathrm{f}[\mathrm{U}] \tag{3}$$

$$\frac{\mathrm{d}[\mathrm{U}]}{\mathrm{d}t} = k_\mathrm{u}[\mathrm{N}] - k_\mathrm{f}[\mathrm{U}] - k_\mathrm{interm}[\mathrm{N}][\mathrm{U}] \tag{4}$$

$$-\frac{\mathrm{d}\,(\text{residual activity})}{\mathrm{d}t} = k_\mathrm{interm}[\mathrm{N}][\mathrm{U}], \tag{5}$$

with $(\text{residual activity})_t = [\mathrm{N}]_t + [\mathrm{U}]_t$, as U would refold to a catalytically active molecule upon cooling.

In the case of intramolecular autoproteolysis the following reaction schemes were used.

$$\mathrm{N} \underset{k_{\mathrm{f},1}}{\overset{k_{\mathrm{u},1}}{\rightleftharpoons}} \mathrm{N}^* \underset{k_{\mathrm{f},2}}{\overset{k_{\mathrm{u},2}}{\rightleftharpoons}} \mathrm{U} \tag{6}$$

$$\mathrm{N}^* \xrightarrow{k_\mathrm{intram}} \mathrm{I}_1 + \mathrm{I}_2 \tag{7}$$

In the first reaction sequence the unfolding–refolding transition is described, but the enzyme is assumed to unfold in more than one step (eqn 6). A first unfolding step would result in a proteinase molecule that is partly unfolded but still catalytically active (N*). A second unfolding step would make the proteinase inactive (U). Here, $k_{\mathrm{u,x}}$ and $k_{\mathrm{f,x}}$ represent the rate constants for the unfolding and refolding reactions respectively. The partly unfolded proteinase molecule is supposed to inactivate itself, for instance, if a flexible loop, which is unfolded, is attacked by the catalytic site of the same molecule, causing inactivation of the proteinase. Here, k_intram represents the

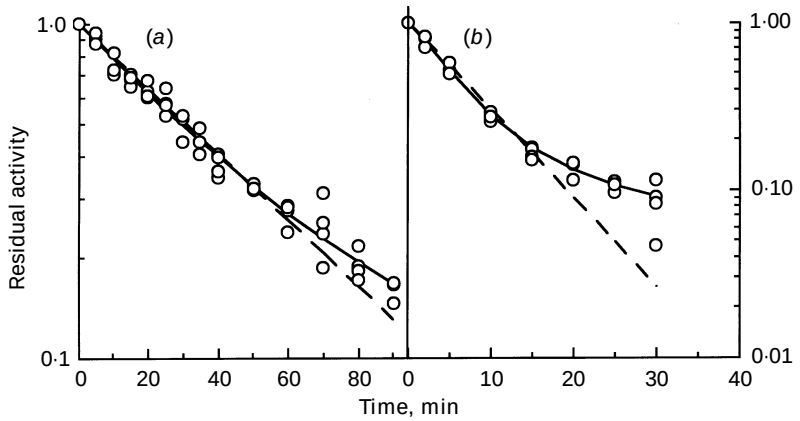

Fig. 3. Inactivation of the crude extracellular proteinase from *Pseudomonas fluorescens* 22F at (*a*) 50 and (*b*) 52 °C. Fits of ——, the intermolecular and – – –, the intramolecular autoproteolysis model are shown.

rate constant for intramolecular autoproteolysis. The intramolecular autoproteolysis model could be simulated and fitted to the experimental results, using the following system of nonlinear differential equations.

$$\frac{d[N]}{dt} = -k_{u,1}[N] + k_{f,1}[N^*] \tag{8}$$

$$\frac{d[N^*]}{dt} = k_{u,1}[N] - (k_{f,1} + k_{u,2} + k_{intram})[N^*] + k_{f,2}[U] \tag{9}$$

$$\frac{d[U]}{dt} = k_{u,2}[N^*] - k_{f,2}[U] \tag{10}$$

$$-\frac{d\,(\text{residual activity})}{dt} = k_{intram}[N^*], \tag{11}$$

with $(\text{residual activity})_t = [N]_t + [N^*]_t + [U]_t$, as N* and U will refold to catalytically active molecules upon cooling.

In the above reaction schemes, the reactions causing thermal inactivation are omitted, because their rates were negligibly small at 70 °C (Fig. 1), and the rate of thermal inactivation appeared to be virtually zero at temperatures of < 70 °C.

At several temperatures the inactivation of the proteinase was followed with time, and both autoproteolysis models were fitted to the results. At temperatures up to 50 °C both models could adequately describe the results (Fig. 3*a*). The unfolding reaction appeared to be rate limiting at these temperatures, resulting in a pseudo first order inactivation for both models, in which unfolded enzyme molecules were digested as soon as they were formed. At higher temperatures the intermolecular autoproteolysis model could fit the results, while the intramolecular autoproteolysis model could not (see Fig. 3*b* for an example at 52 °C). Here, the autoproteolysis reaction would be more important. Apparently, an autoproteolysis model including a second order reaction was needed to fit the results, indicating that the proteinase from *Ps. fluorescens* 22F was most likely inactivated by intermolecular autoproteolysis, confirming the results of the HPLC experiments.

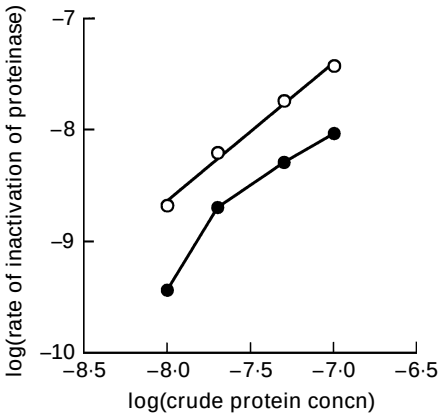

Fig. 4. Plots of log(rate of inactivation of proteinase from *Pseudomonas fluorescens* 22F) *v*. log(crude proteinase concentration) at ●, 50; ○, 52 °C.

The reaction order for the inactivation was determined, as it may illuminate the mechanism of inactivation. When $\log v$ was plotted against $\log C$ (Fig. 4), a straight line with slope $n = 1{\cdot}23$, was found at 52 °C. A reaction order $\geqslant 1$ may suggest that inactivation is a combination of a first and a second order reaction, as described in the intermolecular autoproteolysis model, while in the case of intramolecular autoproteolysis, which is a combination of two first order reactions, a reaction order $\leqslant 1$ would be expected. At 50 °C the plot was not linear. The inactivation rate was almost proportional to the enzyme concentration at relatively high concentrations, which may indicate that the unfolding reaction was rate limiting at this temperature. The inactivation of the most diluted enzyme solution clearly deviated, possibly because the rate of autodigestion became increasingly important when the enzyme concentration was lower.

Kinetics of intermolecular autodigestion

In order to establish the kinetics of intermolecular autodigestion, two types of inactivation experiments were planned, one without pretreatment, and one with a preheating step of 10 s at 100 °C, as described in Materials and Methods. We assumed that at the beginning of the first experiment all enzyme molecules were in their native form (N). After bringing the enzyme solution to the desired temperature, the native enzyme N would start to unfold to form U at an initial reaction rate $v = k_u[N]$. Then, U could be digested by the native N (if still present), to form the inactive fragments I_1 and I_2 at a reaction rate $v = k_{interm}[N][U]$. In the second experiment a complete transformation of the proteinase to its unfolded form U would be induced by a heat treatment of 10 s at 100 °C. At the desired temperature, U would refold to N at an initial reaction rate of $v = k_f[U]$, and subsequently be inactivated with a reaction rate $v = k_{interm}[N][U]$ (eqns 3–5).

The extent of inactivation of the proteinase is determined by the reaction rates of the unfolding, refolding and autoproteolysis reactions. According to the intermolecular autoproteolysis model, the temperature of maximal inactivation would roughly correspond with the denaturation temperature (T_d), where the relative fractions of native and unfolded enzyme molecules would be equal, so the product of the fractions would be maximal. Generally, T_d would coincide with the temperature of the midpoint of a cooperative transition leading to a completely unfolded and inactive enzyme molecule. However, it may also mark a local transition, leading to

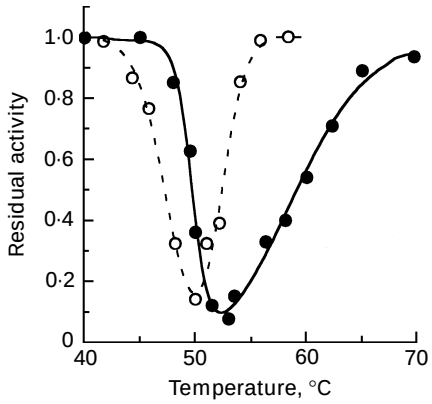

Fig. 5. Residual activity of the crude proteinase from *Pseudomonas fluorescens* 22F after holding the proteinase solution for 30 min at pH 7·0 and the temperature indicated: ○, enzyme solution preheated at 100 °C for 10 s; ●, enzyme solution not preheated.

disruption of the catalytic site of the enzyme. In Fig. 5, the residual activity after 30 min heating is given in an autoproteolysis curve. The temperatures of maximal inactivation were 52 and 50 °C for the not preheated and preheated enzyme respectively. These were similar to denaturation temperatures found for other pseudomonal proteinases (Barach *et al.* 1976; Leinmüller & Christophersen, 1982; Stepaniak *et al.* 1982; Stepaniak & Fox, 1983, 1985; Kroll & Klostermeyer, 1984; Diermayr *et al.* 1987; Patel & Bartlett, 1988; Owusu *et al.* 1991; Kumura *et al.* 1993; Owusu & Doble, 1994). The shape and position of the autoproteolysis curve depended on the conditions that determine the conformational stability of the enzyme, such as pH, ionic strength and ion composition (Schokker, 1997).

There was a clear difference in inactivation between treatments (Fig. 5). This was mainly caused by the reaction rates for unfolding and refolding having finite values, as can be confirmed by computer simulations (Schokker, 1997). Furthermore, the enzyme solutions could not be brought instantaneously to the desired temperature. Although precautions were taken to shorten the heating up period as much as possible, the inactivation was overestimated where the temperature of maximal inactivation had to be passed. This was especially true for experiments in the higher temperature range for the experiment without preheating. Finally, it should be considered that the unfolded state as induced by preheating the enzyme at 100 °C for 10 s need not be equal to the unfolded state induced by heating to 40–70 °C, and therefore the reaction rate for refolding to an active form, and consequently the autoproteolysis curve, might be different. A few proteinases from *Ps. fluorescens* strains have been reported to be less sensitive or even insensitive to autoproteolysis when cooled after being preheated to high temperatures. Susceptibility to autoproteolysis recurred after holding the enzyme at temperatures sufficiently below T_d (Griffiths *et al.* 1981; Stepaniak & Fox, 1983, 1985; Diermayr *et al.* 1987).

The effect of heating up could also partly explain the asymmetrical curve in Fig. 5. This asymmetry was even more pronounced if no precautions were taken to shorten the heating up period. Other reasons for asymmetry of the autoproteolysis curve might be the increase in the rate of autoproteolysis with increasing temperature, and the finite values of the reaction rates for unfolding and refolding, as can be shown by computer simulation (Schokker, 1997). Similar asymmetrical curves have been found by others (Barach *et al.* 1976; Leinmüller & Christophersen,

Table 1. *Values of the reaction rate constants of the intermolecular autoproteolysis model for inactivation of extracellular proteinase from* Pseudomonas fluorescens *22F*

(Values derived from kinetic modelling, ±SD from the covariance matrix)

	Enzyme not preheated			Enzyme preheated at 100 °C for 10 s		
Temperature, °C	$k_u \times 10^5$, s^{-1}	$k_f \times 10^5$, s^{-1}	$k_{interm} \times 10^{-4}$, $M^{-1}\ s^{-1}$	$k_u \times 10^5$, s^{-1}	$k_f \times 10^5$, s^{-1}	$k_{interm} \times 10^{-4}$, $M^{-1}\ s^{-1}$
48·0	12±57	1833±50	25	8±2	233±18	25
49·5	37±2	233±266	28			
50·0	52±8	433±1100	29	17±17	38±5	28
50·1				17±7	25±2	29
51·5	162±6	233±76	32			
52·0	283±41	32±90	33	200±208	6±2	33
52·9	200±16	17±73	35			
53·0	308±100	33±366	35			
54·0				500±217	1±15	38
56·3	2300±683	38±13	44	?	< 2	44
58·0	10500±1480	142±2	50			
60·0	14000±2333	102±17	57			
62·0	28100±833	58±5	66			

k_u, k_f, and k_{interm}, rate constants for unfolding, refolding and intermolecular autodigestion reaction respectively (eqns 1 and 2); k_{interm} was varied with $Q_{10} = 2$.

?, Indeterminate.

1982; Stepaniak *et al.* 1982; Kroll & Klostermeyer, 1984; Christen & Marshall, 1985; Patel & Bartlett, 1988).

From the inactivation experiments at several temperatures we then tried to derive the three reaction rate constants of the intermolecular autoproteolysis model, namely k_u, k_f, and k_{interm}. It may be expected that several combinations of these reaction rate constants could fit the model to the results, since we were able to determine only the residual activity. However, preliminary calculations showed that the rate constant k_u was quite critical for experiments with not preheated enzyme, while k_f was critical for experiments with preheated enzyme. Comparison of preliminary results indicated that k_{interm} usually gave about the same value for all the experiments, without much effect of the temperature. This is to be expected, as k_{interm} reflects the proteolytic activity. Therefore the value of k_{interm} was allowed to vary only within the range found in preliminary experiments, while forcing a Q_{10} of 2, as this was found for the digestion of sodium caseinate by this enzyme (Schokker & van Boekel, 1997). This fixed k_{interm} within a narrow range, so that the possibility of finding unique values for k_u and k_f was increased.

The results of the fitting procedure for the experiments on not preheated and preheated enzyme are given in Table 1. The approximate SD were taken from the covariance matrix, assuming that the errors were normally distributed (Press *et al.* 1990). In Fig. 6 the results of the experiments and simulations at 52 °C are presented as an example, showing that the fits were adequate. The fits were also acceptable from a statistical point of view: the reduced χ^2 (χ^2 divided by the number of degrees of freedom) was in most cases ~ 1.

The values for the rate of the unfolding reaction were found to be of the same order of magnitude for the preheated and not preheated enzyme (Table 1). The value of k_u increased sharply with temperature, as is to be expected for an unfolding reaction. The temperature dependence was analysed using the Eyring equation (Laidler, 1987). The activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$), means ±95%

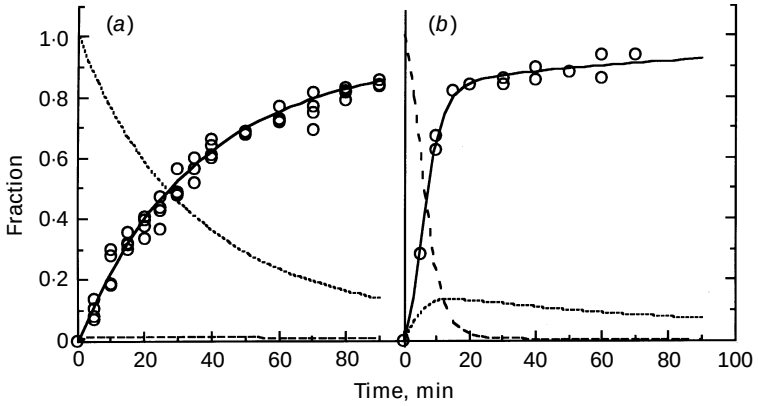

Fig. 6. Results of heating experiments and simulations, using the intermolecular autoproteolysis model for the inactivation of the proteinase from *Pseudomonas fluorescens* 22F at 52·0 °C (*a*) without preheating and (*b*) after preheating at 100 °C for 10 s: ○, fraction of inactivated enzyme (measured); ·····, fraction of native proteinase (simulation); – – –, fraction of unfolded proteinase (simulation); ——, fraction of inactive (hydrolysed) proteinase (simulation).

confidence intervals, were estimated as 504 ± 51 kJ mol^{-1} and 1252 ± 165 J mol^{-1} K^{-1} respectively for the experiment without preheating, and 665 ± 291 kJ mol^{-1} and 1743 ± 900 J mol^{-1} K^{-1} respectively for the experiment with preheating. These values are quite normal for the unfolding of proteins. The difference between the values for not preheated and preheated enzyme was not statistically significant. Large differences were found for the values of k_f between not preheated and preheated enzyme. This implies that the temperature shift in the autoproteolysis curves of not preheated and preheated enzyme, as shown in Fig. 5, should be ascribed to this difference in k_f. In the case of not preheated enzyme, k_f was of not much significance, as indicated by its very large SD values.

From the values of the reaction rate constants for unfolding and refolding (Table 1), a rough estimation could be made of the thermodynamic equilibrium constant $K_d = k_f/k_u$ at various temperatures. Furthermore, the denaturation temperature T_d (where $k_d = 1$) could be estimated. From the temperature dependence of K_d, the enthalpy and entropy difference ΔH^0 and ΔS^0 between the folded and unfolded state could be estimated using van't Hoff's equation (Laidler, 1987). The estimated denaturation temperatures were 50·8 °C and 50·2 °C for not preheated and preheated enzyme respectively. These values corresponded rather well with the estimates of T_d from Fig. 5. ΔH^0 and ΔS^0 for the unfolding reaction were estimated as 1128 kJ mol^{-1} and 3481 J mol^{-1} K^{-1} respectively for the experiment without preheating, and 1453 kJ mol^{-1} and 4493 J mol^{-1} K^{-1} respectively for the experiment with preheating. The values for the thermodynamic factors were very large compared with those for other pseudomonal proteinases or enzymes in general (Privalov, 1979; Owusu *et al.* 1991). Presumably, the present indirect method of determining the thermodynamic characteristics by using the reaction rates of unfolding and refolding was not very useful, owing to inaccuracy in the determination of the kinetic values.

We also tried to determine the enthalpy change of unfolding by differential scanning calorimetry. In order to prevent autoproteolysis during the run, 10 mM-*o*-phenanthroline, an inhibitor of the proteinase (Schokker & van Boekel, 1997), was added to the enzyme preparation. An enthalpy change of 435 kJ mol^{-1} was found, which is a more reasonable value than the estimates from the indirect method described above. However, owing to the addition of *o*-phenanthroline the

denaturation temperature shifted from 51 °C to ~ 44 °C, indicating a decreased conformational stability of the proteinase by removal of Zn^{2+}, as was also observed in the autoproteolysis curves in the presence of *o*-phenanthroline (Schokker, 1997). Measurement of the stability of proteinases, especially metalloproteinases where the metal ion is involved in both catalysis and conformational stability, seems to be impossible with differential scanning calorimetry. Optical methods such as circular dichroism or fluorescence spectrometry will face the same problem. Therefore, determination of thermostability of proteinases under physiological conditions by autoproteolysis may be useful to circumvent this problem.

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