Heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F: inactivation during heating up and cooling periods

By ERIX P. SCHOKKER and MARTINUS A. J. S. VAN BOEKEL

Wageningen Agricultural University, Department of Food Technology and Nutritional Sciences, Division of Food Science, PO Box 8129, NL-6700 EV Wageningen, The Netherlands

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We have reported previously on the kinetics of thermal inactivation at 80–120 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F (Schokker & van Boekel, 1997, 1999*b*). During these studies, we noted some inactivation during the heating up and cooling periods, but allowed for this by calculating the residual activity as a fraction of the activity after the heating up period of 2 min followed by cooling to 0 °C. However, it may be of interest to evaluate the extent of inactivation during these heating up and cooling periods. If the temperature dependence of the reaction rate behaves according to Eyring's theory, inactivation would, of course, be slower than at the final heating temperature. However, during the heating and cooling of the enzyme solution, the temperature also passes the region in which autoproteolysis occurs (Schokker & van Boekel, 1998*a*). Prolonged residence time in the critical zone for autoproteolysis may cause increased inactivation, as has been demonstrated in electrophoresis experiments for proteinases from other *Ps. fluorescens* strains (Barach & Adams, 1977; Richardson, 1981; Diermayr *et al*. 1987). Consequently, the inactivation during the first few minutes would be dependent on factors influencing both autoproteolytic and thermal inactivation.

In most of our heating experiments (Schokker & van Boekel, 1997, 1999*b*), inactivation during heating up was relatively rapid compared with inactivation at the final heating temperature, leading to a biphasic inactivation curve. This was also found for proteinases from many other *Ps. fluorescens* strains. In some studies the inactivation during heating up was not taken into account when analysing the kinetics of thermal inactivation (Patel *et al*. 1983; Yan *et al*. 1985; Fairbairn & Law, 1986), which led to misinterpretation of the mechanism or the kinetic values. Others explained the biphasic inactivation curve by autoproteolysis (Barach & Adams, 1977; Richardson, 1981; Stepaniak & Fox, 1983; Kroll & Klostermeyer, 1984; Diermayr *et al*. 1987), or stabilization by Ca^{2+} of a small portion of the proteinase to heat inactivation (Stepaniak & Fox, 1983; Azcona *et al*. 1988).

In this paper we discuss the influence of protein, enzyme purification and Ca^{2+} activity on inactivation during the heating up and cooling periods. The aim of this study was to determine, using kinetic modelling, whether the inactivation during heating up and cooling periods could be explained by autoproteolysis and thermal inactivation, or whether other mechanisms are involved in the strong initial inactivation.

experimental

Inactivation measurements were taken from the experiments described in Schokker & van Boekel (1999*b*). In these experiments the relative activity of unheated samples was compared with that of samples heated for 2 min (i.e. the heating up time). These values provide information about the amount of proteinase inactivated during the heating up and cooling periods. In the experiments the enzyme solutions (2·1 ml) were heated in stainless steel tubes (7×120 mm), which were rotated in a thermostatted glycerol bath. The tubes were then immediately cooled in ice–water. Assuming that the temperature was uniform throughout the tubes, the following equation can be used to describe the profile of temperature increase and decrease (Hiddink, 1975).

$$V\rho c_p \frac{dT}{dt} = \Phi A(T_w - T), \tag{1}$$

where V is the volume of the liquid to be heated ($2{\cdot}1 \times 10^{-6}$ m^3), ρ the density of the liquid (1025 kg m^{-3}), c_p the heat capacity of the liquid (3950 J kg^{-1} K^{-1}), Φ the overall heat transfer coefficient (W m^{-2} K^{-1}), A the heating surface area ($2{\cdot}6 \times 10^{-3}$ m^2) and T_w the temperature of the tube wall (K). Integration gives

$$\frac{T_w - T}{T_w - T_0} = \exp\left(-\frac{\Phi A t}{V\rho c_p}\right), \tag{2}$$

where

$$\frac{1}{\Phi} = \frac{1}{\alpha_{\text{glycerol}}} + \frac{d_w}{\lambda_w} + \frac{1}{\alpha_{\text{enzyme solution}}}, \tag{3}$$

and where α is the heat transfer coefficient (W m^{-2} K^{-1}), d_w the thickness of the tube wall ($1{\cdot}0 \times 10^{-3}$ m), λ_w the heat conductivity of the tube wall (50 W m^{-1} K^{-1}) and t the heating time. Because α_{glycerol} and $\alpha_{\text{enzyme solution}}$ were unknown, Φ had to be estimated, such that the calculated temperatures fitted the measured temperature profiles. When the tubes were not rotating, Φ was estimated at 120 W m^{-2} K^{-1}. When the tubes were rotating, Φ would have been larger and the temperature would change faster.

results and discussion

The extent of inactivation during heating up and cooling the enzyme solution, taken from experiments described in Schokker & van Boekel (1999*b*), is given in Table 1. In most cases this inactivation was relatively rapid compared with that at the final heating temperature.

The extent of inactivation of the proteinase during heating up was strongly affected by the composition of the solution. The presence of 15 g sodium caseinate/l reduced the inactivation. This stabilizing effect of proteins against inactivation during heating up was also found by Barach *et al.* (1976), Richardson (1981) and Yan *et al.* (1985). Assuming that the rapid inactivation during heating up is caused by autoproteolysis, the presence of other proteins would be expected to have an influence, as they would reduce the rate of autoproteolysis (Barach *et al.* 1978; Stepaniak & Fox, 1983; Schokker & van Boekel, 1998*b*). Other explanations for the protective action of proteins could be that they stabilize a critical conformation near the active site by an enzyme–substrate complex or facilitate refolding (Mihalyi, 1972).

Table 1. *Relative activity of the extracellular proteinase from* Pseudomonas fluorescens *22F after 2 min heating up time compared with that of the unheated enzyme solution*†

(Values are means ± SD for $n = 3$)

Temperature, °C	Unpurified enzyme in				Purified enzyme in solution E
	Solution A	Solution B	Solution C	Solution D	
80	0·62 ± 0·09	0·98 ± 0·03	0·66 ± 0·02	—	0·75 ± 0·14
90	—	—	0·80 ± 0·11	0·52 ± 0·06	0·74 ± 0·09
95	0·74 ± 0·10	0·92 ± 0·08	—	—	—
100	—	—	0·77 ± 0·09	0·36 ± 0·03	0·66 ± 0·32
105	0·73 ± 0·02	0·90 ± 0·06	—	—	—
110	—	—	0·79 ± 0·06	0·57 ± 0·01	0·63 ± 0·18
120	0·67 ± 0·03	0·72 ± 0·07	0·68 ± 0·08	0·60 ± 0·08	0·51 ± 0·09

† Solutions were A, 0·2 M-Tris–maleate buffer with 2 mM-$CaCl_2$; B, 0·2 M-Tris–maleate buffer with 2 mM-$CaCl_2$ and 15 g sodium caseinate/l; C, 0·2 M-Tris–maleate buffer with 20 mM-$CaCl_2$; D, 0·2 M-Tris–maleate buffer with 0·5 mM-EDTA; E, 0·2 M-Tris–maleate buffer alone (pH 7·4 in all cases). For details of these solutions, see Schokker (1997).

The Ca^{2+} activity also appeared to have a strong influence on the inactivation during heating up. Reduction of the Ca^{2+} activity by adding EDTA increased the initial inactivation of the proteinase (Table 1). This difference was unexpected, because the rates of autoproteolysis and thermal inactivation are not affected by Ca^{2+} activity (Schokker & van Boekel, 1999*a*,*b*). A mechanism for the protective effect of calcium was proposed by Barach & Adams (1977). Most of the extracellular proteinases from *Ps. fluorescens* strains are metalloproteinases, containing a zinc ion at the active site. In addition to this catalytic metal ion, Ca^{2+} ions are present as structural elements, forming salt bridges. These salt bridges are thought to be important for regeneration of the native conformation of the proteinase, rather than maintenance of native structure during heating. Ca^{2+}, as well as other divalent metal ions, would stabilize part of the native structure near the active site of the proteinase, allowing rapid and accurate enzyme renaturation. Possibly partial unfolding by heat is necessary for removal of the divalent metal ions by EDTA. Another mechanism for initial inactivation in the presence of EDTA was found for thermolysin, the extracellular metalloproteinase from *Bacillus thermoproteolyticus* (Vita *et al.* 1985; Fassina *et al.* 1986; Fontana, 1988). In the presence of EDTA, susceptibility to autoproteolysis of the native thermolysin molecule is increased, and the molecule can be hydrolysed into three peptide chains. These parts do not separate, but form a complex with a relative specific activity lower than that of the native molecule. Upon heating, the complex falls apart, resulting in irreversible inactivation.

The increased inactivation during heating up and cooling was more pronounced when the heating temperature was relatively low (80–90 °C), except when sodium caseinate was present in the solution. Assuming that the increased inactivation was caused by autoproteolysis, this would be expected, since the time necessary to pass through the critical temperature range for autoproteolysis was relatively long (eqns 1–3).

To investigate whether or not autoproteolysis was the sole cause of the relatively rapid inactivation during heating up and cooling, we estimated the loss of proteolytic activity by autoproteolysis and thermal inactivation during heating up and cooling, using reaction rate constants determined previously. To estimate inactivation in the

Table 2. *Loss of activity, expressed as a fraction of the initial activity, of the extracellular proteinase from* Pseudomonas fluorescens *22F during heating up and cooling periods in the presence and absence of sodium caseinate*

	In the presence of 15 g sodium caseinate/l			In the absence of 15 g sodium caseinate/l		
Temperature, °C	Owing to autoproteolysis	Owing to thermal inactivation	Total	Owing to autoproteolysis	Owing to thermal inactivation	Total
80	0·007	0·006	0·013	0·007	0·047	0·054
100	0·031	0·004	0·035	0·030	0·031	0·061
120	0·118	0·003	0·121	0·116	0·023	0·139

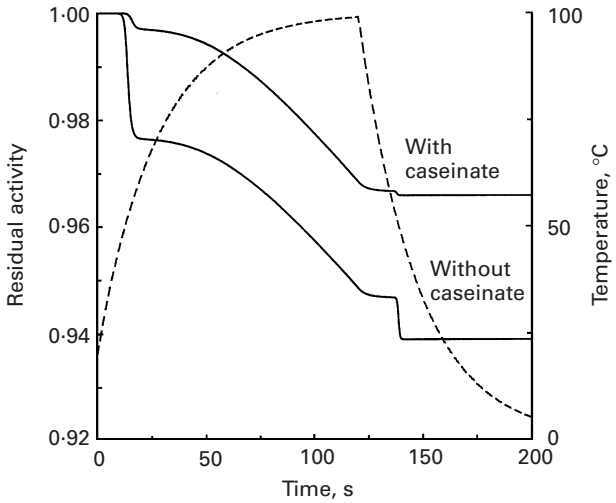

Fig. 1. Calculated inactivation of the proteinase from *Pseudomonas fluorescens* 22F during heating up from 20 to 100 °C and then cooling to 0 °C in the presence and absence of 15 g sodium caseinate/l as a function of time: ——, residual activity; – – – –, temperature. Residual activity is expressed as a proportion of the original activity.

temperature range 40–70 °C, reaction rate constants for unfolding, refolding and autoproteolysis at various temperatures were estimated by interpolation from results of Schokker & van Boekel (1998*a*). The influence of sodium caseinate on the rate of inactivation by autoproteolysis was calculated as described in Schokker & van Boekel (1998*b*), with a Michaelis–Menten constant, K_m, of $6{\cdot}7 \times 10^{-5}$ M. For temperatures > 70 °C, we assumed thermal inactivation by a single first order reaction with activation enthalpy, $\Delta H^{\ddagger}$, of 84·5 kJ mol^{-1} and activation entropy, $\Delta S^{\ddagger}$, of $-83{\cdot}2$ J mol^{-1} K^{-1} (Schokker & van Boekel, 1997). Inactivation as a function of time was calculated numerically, using a first order Euler forward algorithm with steps of 0·1 s (Press *et al.* 1990). The results of the calculations are given in Table 2. As an example, the calculated inactivation during heating to 100 °C and cooling in the presence and absence of 15 g sodium caseinate/l is shown graphically in Fig. 1. It is clear that the contribution of autoproteolysis to the inactivation during the heating up and cooling periods was greater at relatively low heating temperatures and in the absence of sodium caseinate.

Of course, this calculation is a rough approximation of the initial inactivation. Temperature–time profiles of the solution in the tubes were measured at rest, while in the actual experiments the tubes were rotated in the glycerol bath, so that heat

transfer would have been faster and thermal inactivation more effective. Furthermore, while the reaction rate constants for unfolding, refolding and autoproteolysis from Schokker & van Boekel (1998*a*) may be useful to describe their inactivation experiments, they could not accurately describe the unfolding equilibrium. The temperature dependence of the unfolding equilibrium would be expected to be less than calculated (Schokker & van Boekel, 1998*a*), so that inactivation by autoproteolysis was underestimated in these calculations. Finally, the reaction rate constants used were derived for the inactivation of an unpurified enzyme preparation of supernatant from a skim milk culture diluted in demineralized water (Schokker & van Boekel, 1997, 1998*a*). However, the experiments described in Schokker & van Boekel (1999*b*) used supernatant from a culture in tryptone–lactose diluted in Tris–maleate, or a purified enzyme preparation. Besides having a different Ca^{2+} activity and ionic strength, the enzyme preparation used in Schokker and van Boekel (1997, 1999*b*) also had a slightly higher protein content than the enzyme preparations used in these experiments. As the presence of other proteins reduces the rate of autoproteolysis, the extent of inactivation is underestimated in our calculations, especially for the purified enzyme. Nonetheless, this approximation indicates that autoproteolysis and thermal inactivation cannot completely explain the loss of proteolytic activity during the first 2 min, especially when purified proteinase is used or EDTA is added to the enzyme solution. Hence other, as yet unknown, mechanisms must also play a role in the strong initial inactivation.

Kinetic modelling can be a powerful tool to explain enzyme inactivation results. In this communication we have shown with computer simulations that inactivation of the proteinase from *Ps. fluorescens* 22F during heating up and cooling could not be ascribed completely to autoproteolysis. Moreover, kinetic modelling allows rigorous checking of proposed reaction schemes. Similar simulations (Schokker, 1997) have been used to explain the shape and position of autoproteolysis curves of the proteinase (Schokker & van Boekel, 1998*a*, *b*).

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