Effect of protein content on low temperature inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F

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Previously we have examined the inactivation of unpurified extracellular proteinase from *Pseudomonas fluorescens* 22F diluted in demineralized water (Schokker & van Boekel, 1998) in the range 40–70 °C. It appeared that the inactivation was most probably caused by intermolecular autoproteolysis, which is the hydrolysis of unfolded proteinase molecules by native (not yet unfolded) molecules. It has been reported that purification of proteinases from *Pseudomonas* spp. enhances the susceptibility of the proteinase to autoproteolysis (Barach *et al.* 1976; Griffiths *et al.* 1981; Leinmüller & Christophersen, 1982; Kroll, 1989; Kumura *et al.* 1991). On the other hand, when the proteinase is heated in milk or when proteins are added to the enzyme solution, the rate of inactivation by autoproteolysis diminishes (Barach *et al.* 1978; Kroll & Klostermeyer, 1984; Stepaniak *et al.* 1991). Apparently, proteins stabilize the proteinase against inactivation by autoproteolysis.

Substrate or other ligands stabilize many enzymes against limited proteolysis. Binding of these substances to the enzyme molecule, either to the catalytic centre or to amino acid residues on the enzyme molecule surface, may impose steric difficulties so that the susceptible peptide bonds are protected against proteolysis (Mihalyi, 1978). Such binding may also cause a conformational change of the enzyme molecule, such that susceptible peptide bonds cannot be attacked or that the conformation is stabilized against unfolding (Mihalyi, 1978). In the latter case an increase in the denaturation temperature (T_d) would be expected.

In the case of proteinases, addition of substrate to the enzyme solution may protect the enzyme by a third mechanism. Besides autoproteolysis of the proteinase, the added proteins can be digested. An enzyme molecule digesting a protein is not available at the same time for autoproteolysis, so that the substrate may act as a competitive inhibitor against autoproteolysis.

The aim of this study was to determine the mechanism of protection of the proteinase from *Ps. fluorescens* 22F by sodium caseinate.

EXPERIMENTAL

Production and purification of the extracellular proteinase from *Pseudomonas fluorescens* strain 22F was performed and its proteolytic activity determined using trinitrobenzenesulphonic acid as described previously (Schokker & van Boekel, 1997).

Autoproteolysis curves for the extracellular proteinase from *Ps. fluorescens* 22F

were determined by heating samples of 1×10^{-7} M ($\sim 5\ \mu$g/ml) purified proteinase (0·4 ml) in 0·1 M-Tris-HCl–2 mM-$CaCl_2$, pH 7·0 containing 0, 1, 6 and 15 g sodium caseinate/l for 10 min at various temperatures. After heat treatment the enzyme solution was cooled in ice water and subsequently the activity was measured. Differences in substrate concentration in the proteolytic activity assay due to addition of caseinate to the purified proteinase solutions could be neglected, since residual activity was defined as a fraction of the activity of the unheated enzyme solution.

Another experiment was performed in which enzyme inactivation and product formation were followed as a function of time. Sodium caseinate was added to a solution of 5×10^{-7} M purified proteinase in 0·1 M-Tris-HCl–2 mM-$CaCl_2$, pH 7·0 to final concentrations of 0, 1, 3, 6, 15 and 25 g/l. Samples of enzyme preparations (0·4 ml) were heated at 55·3 °C, cooled in ice water and the residual activity was measured. Product formation was also measured by the trinitrobenzenesulphonic acid method. Directly after heat treatment, trichloroacetic acid to a final concentration of 72 g/l was added to the enzyme solution, precipitating both enzyme and caseinate. After filtration, the trichloroacetic acid-soluble hydrolysis products were reacted with trinitrobenzenesulphonic acid and the absorption at 420 nm was taken as a measure of product formation.

Calculations for fitting the model for stabilization by sodium caseinate to the results were performed in Microsoft Excel 5.0. Ordinary differential equations (eqns 7 and 8) were integrated numerically, using a first order Euler forward algorithm (Press *et al*. 1990). The model was analysed with unweighted nonlinear regression, using Newton's algorithm to minimize the residual sum of squares between the predicted and measured residual activity and product formation (Press *et al*. 1990).

RESULTS

Autoproteolysis curves for preparations of purified extracellular proteinase from *Ps. fluorescens* 22F, containing various amounts of sodium caseinate and heated for 10 min at various temperatures, are given in Fig. 1. The temperature of maximal inactivation shifted a little towards higher temperature when sodium caseinate was added at 1 g/l to the purified enzyme. Addition of more sodium caseinate did not shift the optimum temperature for autoproteolysis any further. Furthermore, a distinct reduction in the extent of inactivation with increasing caseinate content was observed.

Curves for inactivation of the proteinase and product formation in enzyme preparations containing various amounts of sodium caseinate as a function of time at 55·3 °C are given in Fig. 2. Fig. 2(*a*) clearly shows the protective function of the sodium caseinate. Addition of caseinate at > 15 g/l did not give further protection. Fig. 2(*b*) shows that at 55·3 °C the proteinase was still active, but that the activity, approximately proportional to d[product]/dt, decreased with time. Product formation in the absence of sodium caseinate was negligibly small, so that the increase in A_{420} must be ascribed virtually completely to proteolysis of the substrate, and not to autoproteolysis. The rate of product formation did not increase further when the substrate concentration was increased to > 15 g/l, indicating that the proteolytic reaction was inhibited by substrate. Inhibition of enzymic activity of the extracellular proteinase from *Ps. fluorescens* 22F by sodium caseinate at concentrations > 10 g/l was also found at 37 °C (Schokker & van Boekel, 1998).

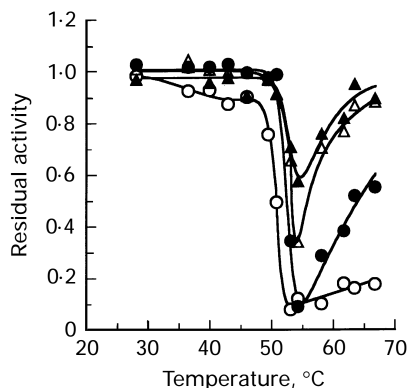


Fig. 1. Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F after 10 min heating with ○, 0; ●, 1; △, 6 and ▲, 15 g sodium caseinate/l at the temperatures indicated.

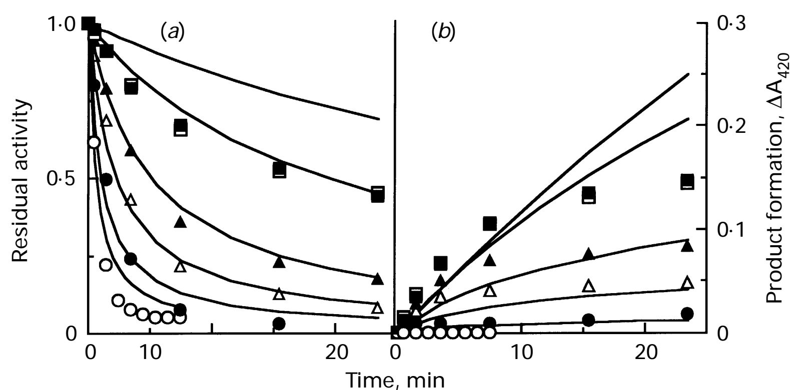


Fig. 2. (*a*) Residual activity and (*b*) product formation of the extracellular proteinase from *Pseudomonas fluorescens* 22F as a function of heating time at 55·3 °C with ○, 0; ●, 1; △, 3; ▲, 6; □, 15 and ■, 25 g sodium caseinate/l. ——, Fitted lines for model describing inhibition of autoproteolysis by 0, 1, 3, 6 and 15 g sodium caseinate/l (eqns 4–8).

DISCUSSION

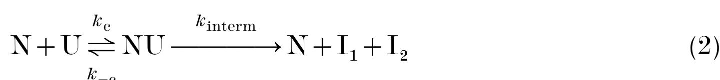
An initial observation when comparing the autoproteolysis and inactivation curves of the purified proteinase from *Ps. fluorescens* 22F to which no sodium caseinate had been added with those of the unpurified proteinase (Schokker & van Boekel, 1998) was that the purified proteinase was inactivated much faster, as has been observed for other pseudomonal proteinases (Barach *et al.* 1976; Griffiths *et al.* 1981; Leinmüller & Christophersen, 1982; Kroll, 1989; Kumura *et al.* 1991). Clearly, substances in the crude supernatant exerted a protective effect against autoproteolysis. Probably these were concomitant or associated proteins or peptides, but other substances are also possible candidates.

The mechanism of inactivation of the proteinase is by intermolecular autoproteolysis (Schokker & van Boekel, 1998). Therefore, the extent of inactivation is determined by the reaction rates of the unfolding, refolding and autoproteolysis reactions. According to the intermolecular autoproteolysis model, the temperature at which inactivation is maximal roughly corresponds with T_d, where the relative fractions of native and unfolded enzyme molecules are equal, and the product of the fractions maximal. The temperature shift of maximal inactivation towards higher temperature when sodium caseinate was added to the purified enzyme at 1 g/l

indicated that small amounts of sodium caseinate could stabilize the native proteinase molecule against unfolding, resulting in an increased T_d. The reduction in the extent of inactivation with increasing caseinate content may be caused by association of proteins, making it sterically impossible to break susceptible peptide bonds, or by competitive inhibition of the autoproteolysis reaction by substrate molecules. The relatively large fraction of the proteinase that was inactivated at temperatures above T_d was mainly due to inactivation during heating up, as was shown by computer simulations (Schokker, 1997).

In order to check the possibility of reduced inactivation of the proteinase by competitive inhibition of autoproteolysis by the substrate, we modelled the inactivation by intermolecular autoproteolysis simultaneously with substrate breakdown according to Michaelis–Menten kinetics.

$$\mathrm{N} \underset{k_f}{\overset{k_u}{\rightleftharpoons}} \mathrm{U} \tag{1}$$



$$\mathrm{N}+\mathrm{U} \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} \mathrm{NU} \xrightarrow{k_{\mathrm{interm}}} \mathrm{N}+\mathrm{I}_1+\mathrm{I}_2 \tag{2}$$

$$\mathrm{N}+\mathrm{S} \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} \mathrm{NS} \xrightarrow{k_p} \mathrm{N}+\mathrm{P} \tag{3}$$

In the first reaction scheme the unfolding–refolding transition is described, where N and U represent the native and unfolded enzyme molecules respectively, and k_u and k_f the rate constants for the unfolding and refolding reactions respectively. The second reaction scheme is the actual autoproteolysis reaction, where NU is the native enzyme–unfolded enzyme complex and k_c, k_{-c} and k_{interm} are the rate constants for the formation of NU complex, its reverse reaction and the reaction forming the autoproteolysis products respectively (Schokker & van Boekel, 1998). The proteolytic breakdown of substrate (eqn 3) was assumed to follow unireactant Michaelis–Menten kinetics (Segel, 1975; Whitaker, 1994). Here, S represents the substrate (in our case sodium caseinate), NS the enzyme–substrate complex, and P the products formed; k_s, k_{-s}, and k_p are the reaction rate constants of the formation of the enzyme–substrate complex, its reverse reaction and the product formation reaction respectively. Compared with the situation in which no substrate was available, the amount of enzyme available for autoproteolysis was reduced, because it was complexed in the enzyme–substrate complex, and the rate of inactivation by autoproteolysis was decreased. The proportion of substrate-complexed enzyme increased as the substrate concentration increased.

For the calculations we assumed the proportion of enzyme molecules in the complex NU to be negligible. Furthermore, we assumed an equilibrium between N, U and NS (steady state), so that

$$[\mathrm{N}]_t = \frac{[\mathrm{N}]_{\mathrm{tot},t}}{1+K_d+([\mathrm{S}]/K_m)} \tag{4}$$

$$[\mathrm{U}]_t = \frac{[\mathrm{N}]_{\mathrm{tot},t}K_d}{1+K_d+([\mathrm{S}]/K_m)} \tag{5}$$

$$[\mathrm{NS}]_t = \frac{[\mathrm{N}]_{\mathrm{tot},t}([\mathrm{S}]/K_m)}{1+K_d+([\mathrm{S}]/K_m)}, \tag{6}$$

where $[\mathrm{N}]_{\mathrm{tot}}$ is the total amount of active enzyme ($[\mathrm{N}]_{\mathrm{tot}} = [\mathrm{N}]+[\mathrm{NS}]$), K_d the

equilibrium constant for the unfolding transition ($K_d = [U]/[N]$), and K_m the Michaelis–Menten constant ($K_m = (k_{-s}+k_p)/k_s$). Ordinary differential equations derived for enzyme inactivation and product formation are respectively

$$-\frac{d\,(\text{residual activity})}{dt} = k_{\text{interm}}\,[N]\,[U] \tag{7}$$

$$\frac{d\,[P]}{dt} = k_p\,[NS]. \tag{8}$$

The model described above was fitted to the results. The results with 25 g sodium caseinate/l were excluded from the calculation, because of the inhibition of proteolytic activity discussed earlier. The values for K_d, K_m, k_{interm} and k_p were estimated as 1·13, $6{\cdot}7\times10^{-5}$ M, $1{\cdot}9\times10^{5}$ M^{-1} s^{-1} and $2{\cdot}6\times10^{3}$ s^{-1} respectively. The fitted lines are included in Fig. 2. The fit for the inactivation of the proteinase (Fig. 2*a*) appeared reasonable. The calculated inactivation of the proteinase in the absence of sodium caseinate deviated from the measured results, but it should be noted that the mechanism of stabilization by small amounts of substrate would be different, as indicated above. The estimated values agreed reasonably well with those found in other experiments. K_m was found to be 7×10^{-5} M at 37 °C (Schokker & van Boekel, 1997); k_{interm} was estimated at 4×10^{5} M^{-1} s^{-1} (Schokker & van Boekel, 1998). The temperature of maximal inactivation, an estimate for T_d, was $\sim$ 54 °C (Fig. 1), so K_d should be slightly > 1 at 55·3 °C.

The value of k_p was more difficult to evaluate, because this rate constant indicated the proportionality between the amount of enzyme–substrate complex and change in A_{420}. The calculated product formation, expressed as change in A_{420}, did not agree well with the experimental results. From the tangents in Fig. 2(*a*) the activity could be estimated. The activities at 55·3 °C deviated from the modelled activities. This may indicate that the assumption of [NU] being negligibly small was not correct. It may also indicate that competitive inhibition of autoproteolysis by substrate was not the only mechanism of protection, but that formation of a reversible complex between enzyme molecules and caseinate also helped to prevent autoproteolysis.

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