Kinetics of Thermal Inactivation of the Extracellular Proteinase from *Pseudomonas fluorescens* 22F: Influence of pH, Calcium, and Protein

Erix P. Schokker[†] and Martinus A. J. S. van Boekel*

Department of Food Technology and Nutritional Sciences, Food Science Group, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

The influence of pH, calcium ion activity, protein, and enzyme purification on the kinetics of heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F was studied in the temperature range 80–120 °C. At pH 5.5–8.6 the rate of inactivation increased slightly with increasing pH values. The pH dependence of inactivation suggests that the inactivation mechanism is mainly through deamidation. Calcium ion activity had no influence on the kinetics of heat inactivation of the proteinase. Addition of 1.8% sodium caseinate to the enzyme solution slightly decreased the heat stability of the proteinase, possibly because part of the inactivation of the proteinase is caused by aggregation to casein. Purification of the proteinase did not change the rate of thermal inactivation.

INTRODUCTION

Due to prolonged cold storage of raw milk at the farm and the dairy, psychrotrophs often become the predominant microbial flora. The most common psychrotrophic bacteria are *Pseudomonas* spp., particularly *Pseudomonas fluorescens* (Cousin, 1982; McKellar, 1989; Shah, 1994). Psychrotrophic bacteria as such do not pose a very serious problem to the dairy industry, as virtually all are eliminated by heat treatment. Problems for the dairy industry arise when enzymes, such as proteinases, lipases, and phospholipases, are secreted in the milk. These extracellular enzymes can be very heat stable and may even resist sterilization (Barach and Adams, 1977; Driessen, 1989; McKellar, 1989; Schokker and van Boekel, 1997a). Residual activity of the enzymes is associated with several technological problems during processing and storage of dairy products, such as loss of cheese yield, formation of bitter or soapy off-flavors, and gelation of milk (Fairnbairn and Law, 1986a; Stead, 1986; McKellar, 1989; Cromie, 1992; Champagne et al., 1994; Shah, 1994; Sørhaug and Stepaniak, 1997).
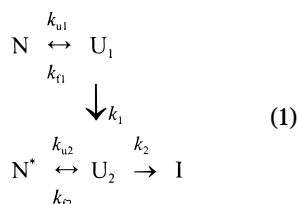
At high temperatures heat inactivation of enzymes is caused by formation of incorrect conformations, due to processes such as hydrolysis of the peptide chain, destruction of amino acids, and aggregation. These reactions have different pH and temperature dependencies. Hydrolysis of the polypeptide backbone, especially at aspartic acid residues, contributes to thermal inactivation of enzymes under acidic conditions (pH 2–4) but to a much lesser extent at higher pH (Ahern

and Manning, 1992). Deamidation of asparagine and glutamine, which results in the formation of aspartic acid and glutamic acid, respectively, is relatively fast at high temperature and pH (Zhang et al., 1993; Riha et al., 1996), and contributes considerably to inactivation of enzymes (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). Exchange of disulfide bonds and destruction of cystine residues by β-elimination contributes considerably to enzyme inactivation in alkaline environment, but the rate constants of these reactions decrease sharply with decreasing pH (Volkin and Klibanov, 1987; Ahern and Klibanov, 1988; Ahern and Manning, 1992). Aggregation is assumed maximal at the isoelectric pH because electrostatic repulsion between molecules would be minimal (Ahern and Klibanov, 1988). Finally, unaltered, unfolded enzyme molecules may refold incorrectly on cooling into conformations that are kinetically stable but that are less or not biologically active. This process is most prevalent near the isoelectric pH of the enzymes, due to reduction of the net charge of the protein that would otherwise hinder the incorrect refolding (Ahern and Klibanov, 1988). Formation of incorrectly refolded molecules contributed largely to the irreversible inactivation of lysozyme at 100 °C (Ahern and Klibanov, 1985) and bovine pancreatic ribonuclease at 90 °C (Zale and Klibanov, 1986), especially at high pH.

Previously we reported a study on the mechanism of heat inactivation of the extracellular proteinase from *P. fluorescens* 22F at 90–110 °C (Schokker and van Boekel, 1997a). The inactivation of the proteinase from *P. fluorescens* 22F could not be described by a single reaction (i.e, simple first-order kinetics inactivation). Therefore, an alternative model, consisting of two consecutive irreversible reactions where the first reaction leads to a partially inactivated enzyme molecule with a relative specific activity of approximately 0.6, was proposed (Schokker and van Boekel, 1997a):

* Author to whom correspondence should be addressed (telephone +31-317-484281; fax +31-317-483669; e-mail tiny.vanboekel@ift.fdsci.wau.nl).
[†] Present address: Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11 222, Palmerston North, New Zealand.

$$N \overset{k_{u1}}{\underset{k_{f1}}{\leftrightarrow}} U_1$$
$$\downarrow k_1 \qquad\qquad (1)$$
$$N^* \overset{k_{u2}}{\underset{k_{f2}}{\leftrightarrow}} U_2 \overset{k_2}{\rightarrow} I$$

In a first unfolding step of the inactivation, the native enzyme molecule (N) would be transformed into a denatured, inactive form (U_1). This reversible unfolding reaction would be followed by an irreversible, first-order reaction with a reaction rate constant k_1 leading to an altered enzyme molecule U_2. A second irreversible, first-order reaction, with reaction rate k_2, would transform U_2 in an irreversibly inactivated enzyme molecule I. After cooling the enzyme solution following exposure to heat, U_1 would refold to the native form N, and U_2 would refold to an active form N^*, with relative specific activity β. The reversible first-order unfolding and refolding reactions have reaction rate constants k_{ux} and k_{fx}, respectively.

In this study we report the influence of pH, calcium ion activity, protein, and purification of the enzyme on the kinetics of thermal inactivation of the proteinase from *P. fluorescens* 22F.

MATERIALS AND METHODS

Production of Enzymes. *P. fluorescens* 22F [obtained from The Netherlands Institute of Dairy Research (NIZO)] was inoculated in sterilized (15 min at 121 °C) growth medium, and after incubation for 8 days at 20 °C the cells were removed by centrifugation (27000*g*, 30 min at 4 °C). Growth media used were 2.5% sodium caseinate in milk ultrafiltrate and tryptone-lactose medium (Schokker and van Boekel, 1997b). The supernatant containing the proteinase was stored at 4 °C after addition of 0.025% NaN$_3$. In some experiments purified enzyme solution was used. The procedure for purification is as described by Schokker and van Boekel (1997b).

Proteinase Assay. Proteolytic activity was determined as previously described (Schokker and van Boekel, 1997b), using 1.0% sodium caseinate (DMV, Veghel, The Netherlands) in 0.1 M Tris-HCl buffer, pH 7.4, as substrate. After incubation for 90 min at 37 °C, the reaction was stopped by adding tri-chloroacetic acid (TCA) to a final concentration of 7.2%, which precipitates the enzyme and the remaining caseinate. After filtration, the TCA-soluble hydrolysis products were allowed to react with 2,4,6-trinitrobenzenesulfonic acid (Fluka AG, Buchs, Switzerland), resulting in a yellow-colored complex that was measured spectrophotometrically at 420 nm. The residual activity was defined as the fraction of the initial activity left after heat treatment.

Calcium Ion Activity. Calcium ion activity was determined using a calcium ion selective electrode (Orion model 93-20; Orion Research Inc., Beverly, MA) (Geerts et al., 1983). The results were expressed as actual activities, not as concentrations, because the activities are important here. (Activities are related to their concentration via the activity coefficient γ: activity = $\gamma \times$ concentration. The activity coefficient depends on conditions such as ionic strength. Note that an ion selective electrode measures the activity of the ions and not their concentration.) Calibration was done with $10^{-2} - 10^{-5}$ M CaCl$_2$ at ionic strength of 0.08 M, in which case the activity coefficient for calcium ions is 0.4 (Geerts et al., 1983).

Heating Experiments. Enzyme solutions (2.1 mL) were heated in stainless steel tubes (7 × 120 mm), which were rotated in a thermostatically controlled glycerol bath. After heating, the tubes were cooled immediately in ice water. The activity after 2 min of heating-up time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating-up.

To study the influence of pH on the inactivation of the proteinase, crude enzyme from a culture of *P. fluorescens* 22F, grown in 2.5% sodium caseinate in milk ultrafiltrate, was diluted 10-fold in 0.2 M Tris-maleate containing 20 mM CaCl$_2$, pH 5.5, 6.5, 7.5, and 8.6, and heated at 90, 100, and 110 °C. The pH during incubation with sodium caseinate deviated slightly from 7.4, due to the influence of the heating buffer, but the activity of the proteinase was not materially changed (Schokker and van Boekel, 1997b).

For preliminary experiments on the influence of calcium ions on the inactivation of the proteinase, crude enzyme from a culture of *P. fluorescens* 22F, grown in tryptone-lactose medium, was diluted in 0.2 M Tris-HCl buffer, pH 7.0, containing either 0.15 mM CaCl$_2$, 20 mM CaCl$_2$, 280 mM CaCl$_2$, or 0.5 mM EDTA, and heated at 110 °C. The calcium ion activities of the enzyme preparations, measured with a calcium ion selective electrode, were 1.1×10^{-4}, 2.2×10^{-3}, 3.8×10^{-2}, and 9.5×10^{-6} M, respectively. To study the influence of the calcium ion activity in more detail, the crude enzyme was diluted in 0.2 M Tris-maleate buffer, pH 7.4, containing either 20 mM CaCl$_2$ or 0.5 mM EDTA, and heated at 90, 100, 110, and 120 °C. The calcium ion activities of these dilutions were 2.2×10^{-3} and 8×10^{-8} M, respectively. Enzyme solutions containing EDTA were kept on ice prior to the heat treatment because addition of EDTA causes a shift in the temperature range in which inactivation of the proteinase from *P. fluorescens* 22F by autoproteolysis takes place, while high concentrations of EDTA make the proteinase susceptible to autoproteolysis even at room temperature (Schokker, 1997).

To study the influence of protein on the inactivation kinetics, crude enzyme from a culture of *P. fluorescens* 22F in tryptone-lactose medium was diluted 10-fold in 0.2 M Tris-maleate in 2 mM CaCl$_2$, pH 7.4, and 1.8% sodium caseinate or 6.7% skim milk powder was added. Enzyme solutions to which caseinate was added were heated at 80, 95, 105, and 120 °C; solutions to which skim milk powder was added were heated at 80 and 120 °C. To investigate the influence of purification on the heat stability of the proteinase, purified proteinase was diluted in 0.2 M Tris-maleate, pH 7.4, to a final concentration of 1.7×10^{-7} M, and inactivation experiments at 80–120 °C were performed.
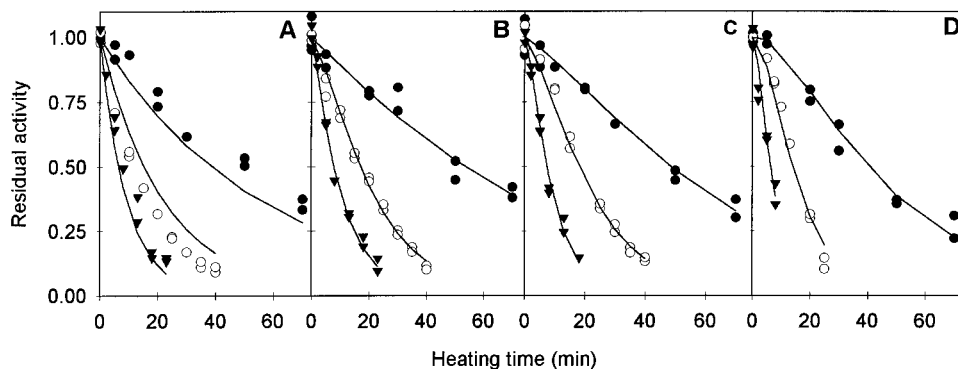
Model Building and Statistical Analysis. The results were analyzed with the above-mentioned inactivation model (eq 1). It was assumed that at room temperature the denaturation equilibrium was shifted completely to the native form N and N^*. Furthermore, it was assumed that the unfolding reaction had proceeded completely after heating the enzyme solution to the temperatures used in this study (i.e., >80 °C), as unfolding occurs between 45 and 65 °C (Schokker and van Boekel, 1998a). These assumptions ruled out any influence of unfolding and refolding reactions on the actual inactivation. When modeling the experimental data, the inactivation during the heating-up time was neglected. The residual activity after 2 min of heating-up time ($t = 0$) was considered to be the initial activity. Consequently, when performing simulations, the fraction of U_1 was considered to be 1 at $t = 0$, the fractions of all other forms zero. The residual activity as function of time is

$$a(t) = a_0\left\{\left(1 + \frac{\beta k_1}{k_2 - k_1}\right)\exp(-k_1 t) - \frac{\beta k_1}{k_2 - k_1}\exp(-k_2 t)\right\} \tag{2}$$

The activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) were estimated with the direct method after reparametrization of the temperature (Schokker and van Boekel, 1997a).

The model was fitted to the experimental data by unweighted nonlinear regression, using the derivative-free algorithm DUD (Ralston and Jennrich, 1978). This algorithm minimizes the sum of squares (SSE) of the difference between measured ($a_{measured}$) and predicted residual activity ($a_{predicted}$)

$$\text{SSE} = \sum(a_{measured} - a_{predicted})^2 \tag{3}$$

Thermal Inactivation of Proteinase

J. Agric. Food Chem., Vol. 47, No. 4, 1999 **1683**

Figure 1. Inactivation of extracellular proteinase from *Pseudomonas fluorescens* 22F at pH 5.5 (A), 6.5 (B), 7.5 (C), and 8.6 (D): ● = 90 °C; ○ = 100 °C; ▼ = 110 °C. Lines were calculated with the inactivation model (eq 1) using kinetic parameters in Table 1.

and calculates the set of parameters with the lowest SSE and their 95% confidence intervals. For estimation of the parameters, the procedure NLIN of the package SAS version 6.09, run on a VMS DEC 3000, was used (SAS, 1985). For estimation of the starting values of the parameters a preliminary grid search was executed.

For comparison of data sets an *F*-ratio test was used (Motulsky and Ransnas, 1987). The sum of residual sums of squares of fits with the separate data sets (SSE$_{sep}$ = ∑SSE) is compared with the residual sum of squares of the fit of the data set in which the separate data sets are pooled and analyzed simultaneously (SSE$_{pool}$). The significance of the improvement of analyzing the sets separately is calculated as

$$f = \frac{(SSE_{pool} - SSE_{sep})/(\nu_{pool} - \nu_{sep})}{SSE_{sep}/\nu_{sep}} \quad (4)$$

and tested against $F_{\nu_{sep},\nu_{pool},0.95}$. Here, ν_{pool} and ν_{sep} are the number of the degrees of freedom of the pooled data set and the separate data sets, respectively, at the 95% confidence level.

RESULTS AND DISCUSSION

Influence of pH on Thermal Inactivation. Since inactivation of enzymes can be caused by various reactions, each having its own pH and temperature dependency, the influence of pH on the kinetics of thermal inactivation must be studied at several temperatures. The inactivation at 90–110 °C of the proteinase from *P. fluorescens* 22F was studied at pH values 5.5, 6.5, 7.5, and 8.6 (Figure 1A–D). The results show a tendency of an increasing inactivation rate with increasing pH. At 100 °C, the inactivation showed the typical lagged inactivation behavior as described by Schokker and van Boekel (1997a) in the case of pH 7.5 and 8.6. At pH values 5.5 and 6.5 the typical inactivation was not found, and presumably first-order inactivation kinetics would apply equally well.

We analyzed the inactivation with a model of two sequential reactions of which the first leads to an enzyme molecule with a lower relative specific activity β (eq 1), as it was found previously that this model described the inactivation of the proteinase the best (Schokker and van Boekel, 1997a). In the cases where first-order kinetics would give better results, the model in eq 1 may still hold, although β will have a small value or the second reaction proceeds very fast, so that the inactivation can be solely described by the first reaction. The kinetic parameters of the inactivation are given in Table 1. The difference in inactivation at different pH values was statistically significant. However, since the

Table 1. Kinetic Parameters (±95% Confidence Interval) for the Inactivation of the Extracellular Proteinase from *Pseudomonas fluorescens* 22F at Various pH Values

pH	$\Delta H_1^{\ddagger}$ (kJ mol^{-1})	$\Delta S_1^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H_2^{\ddagger}$ (kJ mol^{-1})	$\Delta S_2^{\ddagger}$ (J mol^{-1} K^{-1})	β
5.5	43 ± 13	−188 ± 36	623 ± 621	1402 ± 1678	0.74 ± 0.12
6.5	51 ± 24	−166 ± 66	244 ± 49	359 ± 138	0.71 ± 0.15
7.5	78 ± 31	−94 ± 87	155 ± 51	116 ± 147	0.76 ± 0.15
8.6	36 ± 50	−202 ± 147	170 ± 52	157 ± 152	1.04 ± 0.16

inactivation model was not always the best description of the inactivation, the confidence intervals of the kinetic parameters were found to be large. Although pH had some influence on the inactivation at high temperatures, the influence was not as dramatic as found for enzymes such as hen egg white lysozyme (Ahern and Klibanov, 1985) or bovine pancreatic ribonuclease A (Zale and Klibanov, 1986). The inactivation of these enzymes were analyzed with first-order kinetics, and the rate constants of inactivation were found to be different by orders of magnitude between pH 4 and 8. This difference was considered to be a result of the fact that at different pH values different reactions caused the inactivation of the enzymes (Ahern and Klibanov, 1985, 1988; Zale and Klibanov, 1986).

The isoelectric pH of the extracellular proteinase from *P. fluorescens* 22F was estimated to be pH 7.4 (Schokker and van Boekel, 1997b). At this pH, inactivation was not faster than at other pH values, which suggests that aggregation or incorrect refolding of the molecules does not play an important role in the inactivation of the proteinase. The fact that activity was largely recovered after prolonged heating of the enzyme at 70 °C, which is above the denaturation temperature (Schokker and van Boekel, 1998a), confirmed this conclusion. Because most proteinases from *P. fluorescens* do not contain any cysteine (Mayerhofer et al., 1973; Barach and Adams, 1977; Richardson, 1981; Diermayr and Klostermeyer, 1984; Mitchell et al., 1986), exchange of disulfide bonds and destruction of cystine residues by β-elimination are probably not very important in our case, and it is thus not strange that pH has only a small influence on inactivation. Therefore, an inactivation involving deamidation of one or more asparagine or glutamine residues appeared the most probable mechanism. Deamidation was also suggested to be the mechanism for thermal inactivation of the proteinase from *P. fluorescens* 112, because heating caused a decrease in the isoelectric pH of the proteinase (Diermayr et al., 1987). For other enzymes it has been found that deamidation of a single asparagine or glutamine residue would not
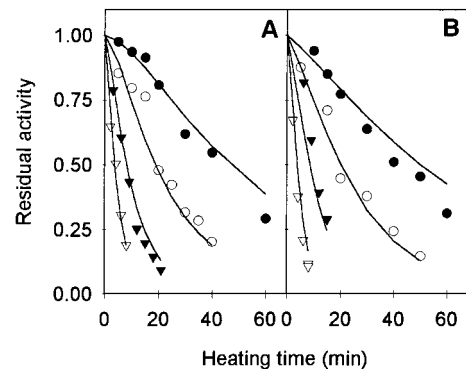
1684 *J. Agric. Food Chem.,* Vol. 47, No. 4, 1999

Schokker and van Boekel
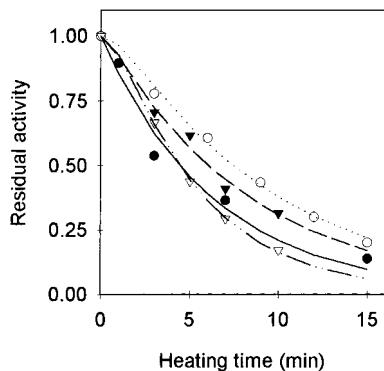
Figure 2. Inactivation of proteinase from *Pseudomonas fluorescens* 22F at 110 °C: $-\bullet- = 0.15$ mM $CaCl_2$; $\cdot\cdot\bigcirc\cdot\cdot = 20$ mM $CaCl_2$; $--\blacktriangledown-- = 280$ mM $CaCl_2$; $\cdot-\triangledown-\cdot = 0.5$ mM EDTA.

Figure 3. Inactivation of proteinase from *Pseudomonas fluorescens* 22F in 0.2 M Tris-maleate, pH 7.4, containing 20 mM $CaCl_2$ (A) or 0.5 mM EDTA (B): $\bullet = 90$ °C; $\bigcirc = 100$ °C; $\blacktriangledown = 110$ °C; $\triangledown = 120$ °C. Lines were calculated with the inactivation model (eq 1) using kinetic parameters in Table 2.

Table 2. Kinetic Parameters (±95% Confidence Interval) for the Inactivation of the Extracellular Proteinase from *Pseudomonas fluorescens* 22F at Various Calcium Ion Activities

aCa^{2+} (M)	$\Delta H_1^{\ddagger}$ (kJ mol^{-1})	$\Delta S_1^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H_2^{\ddagger}$ (kJ mol^{-1})	$\Delta S_2^{\ddagger}$ (J mol^{-1} K^{-1})	β
2.2×10^{-3}	92 ± 17	-60 ± 47	124 ± 631	41 ± 177	0.93 ± 0.78
8×10^{-8}	89 ± 74	-61 ± 211	108 ± 57	-17 ± 158	0.82 ± 1.04

always lead to a completely inactivated molecule but that a molecule with a lower relative specific activity was found (Ahern and Klibanov, 1985; Zale and Klibanov, 1986; Tomizawa et al., 1994). This would be in agreement with previous results (Schokker and van Boekel, 1997a), in which a model describing a partial inactivation by a first reaction followed by complete inactivation by another was found to be one of the two best models.

Influence of Calcium Ion Activity on Thermal Inactivation. The influence of calcium ion activity on the inactivation varies among extracellular proteinases of various *P. fluorescens* strains, although it should be noted that comparison of inactivation kinetics from various literature sources is difficult because of variation in experimental conditions. The calcium content of the heating menstrua has been reported to have a positive effect on the heat stability at high temperatures of several pseudomonal proteinases (Barach et al., 1976; Barach and Adams, 1977; Stepaniak and Fox, 1983; Kroll and Klostermeyer, 1984; Yan et al., 1985; Patel et al., 1986; Stepaniak et al., 1987; Roussis et al., 1990). Lowering the calcium ion activity by use of phosphate buffer or by addition of EDTA caused reduced heat stability, indicating that divalent cation bridges may be involved in the stability of the proteinases. The native calcium content of milk appeared to be sufficient to stabilize the enzymes, since addition of extra calcium gave no further protection (Fairbairn and Law, 1986b). However, in other studies a protective influence of calcium was not observed (Christen and Marshall, 1985; Stepaniak and Fox, 1985).

In a preliminary experiment to study the influence of calcium ions, the proteinase from *P. fluorescens* was heated at 110 °C in the presence of 0.15, 20, and 280 mM $CaCl_2$, or 0.5 mM EDTA (Figure 2). Increasing the calcium ion activity by addition of calcium slightly stabilized the proteinase against thermal inactivation. However, at high calcium ion activity this effect appeared to be less pronounced. This may have been caused by displacement of Zn^{2+} by Ca^{2+}, causing a decreased activity, as was suggested by Barach et al. (1976), but it may also be caused by the increased ionic strength. Lowering the calcium ion activity with EDTA caused some destabilization.

To study the influence of the calcium ion activity in more detail, enzyme was heated at 90, 100, 110, and 120 °C in the presence of 20 mM $CaCl_2$ or 0.5 mM EDTA (Figure 3). The results were analyzed with the inactivation model given in eq 1 (Table 2). Calcium ion activity

had no significant influence on the heat stability of the proteinase at high temperatures. The reason that no significant difference in heat stability between both experiments was found may partly be the result of large variation in duplicate values, causing large confidence intervals. On the other hand, considering the processes that may cause thermal inactivation, influence of calcium is not very likely. Although calcium ion activity did not affect heat stability at high temperature, the extent of inactivation during heating-up varied considerably (Schokker, 1997).

Influence of Protein and Enzyme Purification on Thermal Inactivation. Previously we found that protein had a strong protective influence on the inactivation of proteinase from *P. fluorescens* 22F in the temperature range 40–60 °C in which the inactivation is caused by autoproteolysis (Schokker and van Boekel, 1998a,b). The influence of accompanying proteins on inactivation at high temperatures as described in the literature appears to vary among extracellular proteinases from various *P. fluorescens* strains. Some proteinases are protected against thermal inactivation by companion proteins (Mayerhofer et al., 1973; Richardson, 1981; Kroll and Klostermeyer, 1984; Uplacksh et al., 1994), while others were not affected (Barach et al., 1976; Christen and Marshall, 1985). However, it is not always clear whether the protective action at high temperature was actually due to less inactivation by autoproteolysis during the heating-up of the enzyme solution (Schokker, 1997).

Enzyme solutions containing sodium caseinate or skimmed milk were heated at 80, 95, 105, and 120 °C and 80 and 120 °C, respectively (Figure 4). Addition of sodium caseinate (1.8%, an arbitrarily chosen level) to the enzyme solution resulted in a slight, but statistically significant increase of the inactivation rate. This may be caused by aggregation of enzyme molecules with caseinate molecules. Addition of skimmed milk powder (containing 25–28% casein and 6–7% whey protein) did not influence the heat stability of the proteinase. The

Thermal Inactivation of Proteinase

J. Agric. Food Chem., Vol. 47, No. 4, 1999 **1685**
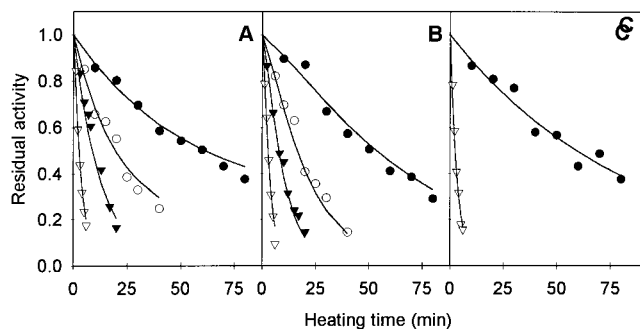
Figure 4. Inactivation of proteinase from *Pseudomonas fluorescens* 22F in 0.2 M Tris-maleate in 2 mM CaCl$_2$, pH 7.4, containing no added protein (A), 1.8% sodium caseinate (B), or 6.7% skimmed milk powder (C): ● = 80 °C; ○ = 95 °C; ▼ = 105 °C; ▽ = 120 °C. Lines were calculated with the inactivation model (eq 1) using kinetic parameters in Table 3.
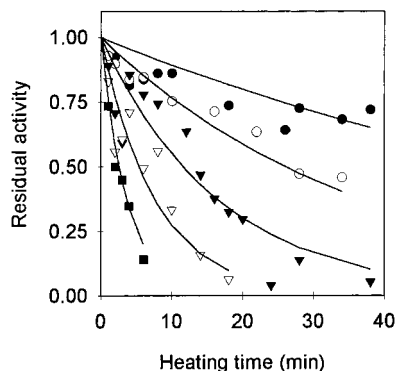
Figure 5. Inactivation of purified proteinase from *Pseudomonas fluorescens* 22F: ● = 80 °C; ○ = 90 °C; ▼ = 100 °C; ▽ = 110 °C; ■ = 120 °C. Lines were calculated for first-order inactivation with $\Delta H^{\ddagger}$ = 89.8 kJ mol^{-1} and $\Delta S^{\ddagger}$ = −63.4 J mol^{-1} K^{-1}.

Table 3. Kinetic Parameters (±95% Confidence Interval) for the Inactivation of the Extracellular Proteinase from *Pseudomonas fluorescens* 22F in the Presence and Absence of 1.8% Sodium Caseinate

[caseinate] (%)	$\Delta H_1^{\ddagger}$ (kJ mol^{-1})	$\Delta S_1^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H_2^{\ddagger}$ (kJ mol^{-1})	$\Delta S_2^{\ddagger}$ (J mol^{-1} K^{-1})	β
	74 ± 9	−104 ± 27	229 ± 104	303 ± 284	0.28 ± 0.21
1.8%	78 ± 19	−83 ± 58	85 ± 11	-73 ± 32	0.85 ± 0.31

level added was 6.7% so that the casein level was about the same as in the above experiment in which only sodium caseinate was added. The results of the inactivation experiments in the absence and presence of sodium caseinate were analyzed with the inactivation model described in eq 1 (Table 3). Besides the effect of caseinate on thermal inactivation, a particular strong influence of proteins on the inactivation during heating-up has been found (Schokker, 1997).

The purity of the enzyme is also reported to have a strong effect on the inactivation kinetics of pseudomonal proteinases (Barach et al., 1976; Richardson, 1981; Kumura et al., 1991). Possibly, the thermostability of purified enzymes may be reduced due to partial denaturation during the purification process. Again, it is not always clear whether the thermostability is really reduced at high temperature or whether the increased inactivation is caused by autoproteolysis during the heating-up period. If the heating-up period is not taken into account, inactivation will be considerable in the absence of concomitant proteins (Schokker, 1997).

Purified proteinase from *P. fluorescens* 22F was heated at 80−120 °C. The results were poorly reproduc-

ible, probably due to variation in the extent of inactivation during heating-up (Figure 5). Because of the absence of accompanying protein, small differences in the temperature−time profile may cause relatively large differences in the extent of inactivation (Schokker, 1997). As a consequence of the large errors, it appeared impossible to analyze the results with the inactivation model given in eq 1; therefore, the results were analyzed with first-order inactivation kinetics. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (±95% confidence interval) of the reaction were 89.8 ± 7.4 kJ mol^{-1} and −63.4 ± 21.6 J mol^{-1} K^{-1}, respectively. There is no statistically significant difference between these kinetic parameters and those found for the inactivation of unpurified proteinase (Schokker and van Boekel, 1997a).

LITERATURE CITED

Ahern, T. J.; Klibanov, A. M. The mechanism of irreversible enzyme inactivation at 100 °C. *Science* **1985**, *228*, 1280−1284.

Ahern, T. J.; Klibanov, A. M. Analysis of processes causing thermal inactivation of enzymes. *Methods Biochem. Anal.* **1988**, *33*, 91−127.

Ahern, T. J.; Manning, M. C. *Stability of Protein Pharmaceuticals, part A: Chemical and Physical Pathways of Protein Degradation*; Plenum Press: New York, 1992.

Barach, J. T.; Adams, D. M.; Speck, M. L. Stabilisation of a psychrotrophic *Pseudomonas* protease by calcium against thermal inactivation in milk at ultrahigh temperature. *Appl. Environ. Microbiol.* **1976**, *31*, 875−879.

Barach, J. T.; Adams, D. M. Thermostability at ultrahigh temperatures of thermolysin and a protease from a psychrotrophic *Pseudomonas*. *Biochim. Biophys. Acta* **1977**, *485*, 417−423.

Champagne, C. P.; Laing, R. R.; Roy, D.; Mafu, A. A. Psychrotrophs in dairy products: their effects and their control. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 1−30.

Christen, G. L.; Marshall, R. T. Effect of histidine on thermostability of lipase and protease of *Pseudomonas fluorescens* 27. *J. Dairy Sci.* **1985**, *68*, 594−604.

Cousin, M. A. Presence and activity of psychrotrophic microorganisms in milk and dairy products. *J. Food Prot.* **1982**, *45*, 172−207.

Cromie, S. Psychrotrophs and their enzyme residues in cheese milk. *Aust. J. Dairy Technol.* **1992**, *47*, 96−100.

Diermayr, P.; Klostermeyer, H. A new metalloproteinase from *Pseudomonas fluorescens* biotype I. *Hoppe-Seyler's Z. Physiol. Chem.* **1984**, *365*, 1345−1350.

Diermayr, P.; Kroll, S.; Klostermeyer, H. Mechanisms of heat inactivation of a proteinase from *Pseudomonas fluorescens* biotype I. *J. Dairy Res.* **1987**, *54*, 51−60.

Driessen, F. M. Inactivation of lipases and proteinases (indigenous and bacterial). *Bull. Int. Dairy Fed.* **1989**, *238*, 71−92.

Fairbairn, D. J.; Law, B. A. Proteinases of psychrotrophic bacteria: their production, properties, effects and control. *J. Dairy Res.* **1986a**, *53*, 139−177.

Fairbairn, D. J.; Law, B. A. Purification and characterization of an extracellular proteinase from *Pseudomonas fluorescens* NCDO 2085. *J. Dairy Res.* **1986b**, *53*, 457−466.

Geerts, J. P.; Berkhof, J. J.; Scherjon, J. W. Determination of calcium ion activities in milk with an ion-selective electrode. A linear relationship between the logarithm of time and the recovery of the calcium ion activity after heat treatment. *Neth. Milk Dairy J.* **1983**, *37*, 197−211.

Kroll, S.; Klostermeyer, H. Heat inactivation of exogenous proteinases from *Pseudomonas fluorescens* I. Possibility of inactivation in milk. *Z. Lebensm. Unters. Forsch.* **1984**, *179*, 288–295.

Kumura, H.; Mikawa, K.; Saito, Z. Effect of protease on concomitant lipase produced by *Pseudomonas* sp. No. 33. *Milchwissenschaft* **1991**, *46*, 215–218.

Mayerhofer, H. J.; Marshall, R. T.; White, C. H.; Lu, M. Characterization of a heat-stable protease of *Pseudomonas fluorescens* P26. *Appl. Microbiol.* **1973**, *25*, 44–48.

McKellar, R. C. *Enzymes of Psychrotrophs in Raw Food*; CRC Press: Boca Raton, FL, 1989.

Mitchell, G. E.; Ewings, K. N.; Bartley, J. P. Physicochemical properties of proteases from selected psychrotrophic bacteria. *J. Dairy Res.* **1986**, *53*, 97–115.

Motulsky, H. J.; Ransnas, L. A. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* **1987**, *1*, 365–374.

Patel, T. R.; Jackman, D. M.; Williams, G. J.; Bartlett, F. M. Physicochemical properties of heat-stable proteases from psychrotrophic Pseudomonads. *J. Food Prot.* **1986**, *49*, 183–188.

Ralston, M. L.; Jennrich, R. I. DUD, a derivative-free algorithm for nonlinear least squares. *Technometrics* **1978**, *20*, 7–14.

Richardson, B. C. The purification and characterization of a heat-stable protease from *Pseudomonas fluorescens* B52. *N. Z. J. Dairy Sci. Technol.* **1981**, *16*, 195–207.

Riha, W. E.; Izzo, H. V.; Zhang, J.; Ho, C. T. Nonenzymatic deamidation of food proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 225–255.

Roussis, I. G.; Drainas, C.; Alichanidis, E. Some properties of extracellular protease from a *Pseudomonas fluorescens* strain. *Lebensm.–Wiss. Technol.* **1990**, *23*, 106–111.

SAS Institute Inc. *SAS User's Guide: Statistics*, version 5 ed.; SAS Institute Inc.: Cary, NC, 1985.

Schokker, E. P. Kinetic modelling of enzyme inactivation. Kinetics of heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands, 1997.

Schokker, E. P.; van Boekel, M. A. J. S. Kinetic modelling of enzyme inactivation: kinetics of heat inactivation at 90–110 °C of extracellular proteinase from *Pseudomonas fluorescens* 22F. *J. Agric. Food Chem.* **1997a**, *45*, 4740–4747.

Schokker, E. P.; van Boekel, M. A. J. S. Production, purification and partial characterization of the extracellular proteinase from *Pseudomonas fluorescens* 22F. *Int. Dairy J.* **1997b**, *7*, 265–271.

Schokker, E. P.; van Boekel, M. A. J. S. Mechanism and kinetics of inactivation at 40–70 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F. *J. Dairy Res.* **1998a**, *65*, 261–272.

Schokker, E. P.; Van Boekel, M. A. J. S. Effect of protein content on low-temperature inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F. *J. Dairy Res.* **1998b**, *65*, 347–352.

Shah, N. P. Psychrotrophs in milk. *Milchwissenschaft* **1994**, *49*, 432–437.

Sørhaug, T.; Stepaniak, L. Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends Food Sci. Technol.* **1997**, *8*, 35–41.

Stead, D. Microbial lipases: their characteristics, role in food spoilage and their industrial uses. *J. Dairy Res.* **1986**, *53*, 481–505.

Stepaniak, L.; Fox, P. F. Thermal stability of an extracellular proteinase from *Pseudomonas fluorescens* AFT 36. *J. Dairy Res.* **1983**, *50*, 171–184.

Stepaniak, L.; Fox, P. F. Isolation and characterization of heat stable proteinases from *Pseudomonas* isolate AFT 21. *J. Dairy Res.* **1985**, *52*, 77–89.

Stepaniak, L.; Birkeland, S. E.; Sørhaug, T.; Vagias, G. Isolation and partial characterization of heat stable proteinase, lipase and phospholipase C from *Pseudomonas fluorescens* P1. *Milchwissenschaft* **1987**, *42*, 75–79.

Tomizawa, H.; Yamada, H.; Imoto, T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 °C. *Biochemistry* **1994**, *33*, 13032–13037.

Uplasksh, V. K.; Mathur, D. K.; Malik, R. K. Thermal resistance of partially purified proteinase of *Pseudomonas fluorescens* P-26. *J. Appl. Bacteriol.* **1994**, *76*, 356–360.

Volkin, D. B.; Klibanov, A. M. Thermal destruction processes in proteins involving cystine residues. *J. Biol. Chem.* **1987**, *262*, 2945–2950.

Yan, L.; Langlois, B. E.; O'Leary, J.; Hicks, C. L. Purification and characterization of four extracellular proteases isolated from raw milk psychrotrophs. *J. Dairy Sci.* **1985**, *68*, 1323–1336.

Zale, S. E.; Klibanov, A. M. Why does Ribonuclease Inactivate at High Temperature? *Biochemistry* **1986**, *25*, 5432–5444.

Zhang, J.; Lee, T. C.; Ho, C. T. Comparative study on kinetics of nonenzymatic deamidation of soy protein and egg white lysozyme. *J. Agric. Food Chem.* **1993a**, *41*, 2286–2290.