Kinetic Modeling of Enzyme Inactivation: Kinetics of Heat Inactivation at 90−110 °C of Extracellular Proteinase from *Pseudomonas fluorescens* 22F

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The kinetics of heat inactivation at 90−110 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F was studied. The activation enthalpy $\Delta H^{\ddagger}$ and activation entropy $\Delta S^{\ddagger}$ of the inactivation reaction, when analyzed with a first-order kinetic inactivation model, were found to be 84.5 kJ mol^{-1} and −83.2 J mol^{-1} K^{-1}. Because the fit was not adequate, alternative inactivation models were proposed and modeled to fit the data. The model with the fewest parameters being statistically acceptable consisted of two sequential irreversible first-order reactions and could be used for predictive modeling of the inactivation of the proteinase. A model consisting of two consecutive irreversible reactions, in which the first reaction leads to a partially inactivated enzyme molecule with a relative specific activity of ≈0.6, was statistically better and also appeared to be more in accordance with the mechanism of inactivation.

Keywords: *Kinetic modeling; heat inactivation; (metallo)proteinase; Pseudomonas fluorescens*

INTRODUCTION

The extracellular proteinase from *Pseudomonas fluorescens* 22F, and several extracellular proteinases from other psychrotrophic bacteria, are extremely stable to high temperatures (Barach and Adams, 1977; Alichanidis and Andrews, 1977; Richardson, 1981; Driessen, 1983, 1989; Stepaniak and Fox, 1983; Kroll and Klostermeyer, 1984; Owusu and Doble, 1994); resisting ultrahigh-temperature sterilization, they can reduce the shelf life of food products. To make an estimate of the residual proteolytic activity after heat treatment, a predictive model should be available. Heat inactivation of enzymes is generally shown schematically as

$$\mathrm{N} \underset{k_f}{\overset{k_u}{\rightleftharpoons}} \mathrm{U} \xrightarrow{k_i} \mathrm{I} \qquad (1)$$

In a first unfolding step of the inactivation, the native enzyme molecule (N) is transformed into a denatured, inactive form (U). U can renature back into N. The reversible unfolding and refolding reactions with reaction rate constants k_u and k_f, respectively, can be described by first-order kinetics. This reversible unfolding reaction is followed by an irreversible, often first-order, reaction with a reaction rate constant k_i leading to an irreversibly inactivated enzyme molecule I (Lumry and Eyring, 1954; Ahern and Klibanov, 1988). Typical reactions leading to irreversible inactivation above the denaturation temperature are hydrolysis of peptide bonds, reshuffling of disulfide bonds, destruction of amino acid residues (e.g. deamidation of asparagine and glutamine residues and β-elimination of cysteine residues), and aggregation and formation of incorrect structures (Ahern and Klibanov, 1988). Heat inactivation of many pseudomonal proteinases can be described by this general model.

Kinetic modeling can also be used as tool for the elucidation of the mechanism of enzyme inactivation. Driessen (1983, 1989) found a typical heat inactivation behavior of proteinases from *P. fluorescens* 22F and *Achromobacter* sp. 1−10. In the first few minutes of heat treatment, the rate of inactivation was slower than later on. This behavior can also be seen in the Arrhenius plot shown by Driessen (1983). A relatively slow denaturation reaction has been suggested to explain this behavior (van Boekel and Walstra, 1989), but this appeared not to be the case for this enzyme, as the unfolding of enzyme molecules from *P. fluorescens* 22F takes place between 40 and 60 °C (Schokker and van Boekel, 1997a). In this paper an attempt is made to find an alternative model that can describe the peculiar inactivation behavior.

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) fresh skimmed milk, and after incubation for 8 days at 20 °C, the cells were removed by centrifugation (27000*g*, 30 min at 4 °C). The supernatant, containing the proteinase, was stored until use at −20 °C.

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 trichloroacetic 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 (TNBS; Fluka AG, Buchs, Switzerland), resulting in a yellow complex that was measured spectrophotometrically at 420 nm. The residual activity was defined as the fraction of the initial activity left after heat treatment.

Heating Experiments. Enzyme solutions (2.1 mL), consisting of supernatant diluted 10 times in demineralized water to a final enzyme concentration of ≈1.0×10^{-7} M (≈5 mg/mL), were heated to 90, 100, and 110 °C in stainless steel tubes (7

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× 120 mm), which were rotated in a thermostated glycerol bath. [The enzyme concentration was calculated from the activity of the enzyme solution and molecular weight of the enzyme, as described by Schokker and van Boekel (1997b).] After heating, the tubes were cooled immediately in ice water. The activity after 2 min of heating time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating up.

Reaction Orders. Reaction orders for the inactivation of the proteinase were determined at 100 °C according to the method of Laidler (1987). For the estimation of the reaction order with respect to concentration, the enzyme preparations used were undiluted supernatant from a culture in skimmed milk, and the same supernatant diluted 10 and 100 times in 0.2 M Tris-HCl containing 2 mM $CaCl_2$, pH 7.0. For the estimation of the reaction order with respect to time, results of the experiment with 10 times diluted supernatant were used. Both orders need not be the same: if the order with respect to time is higher than that with respect to concentration, this may indicate autocatalysis; if the order with respect to time is lower than that with respect to concentration, this may indicate inhibition of the reaction under study (Laidler, 1987).

Statistical Analysis. Models were analyzed by unweighted nonlinear regression, using Marquardt's algorithm (Marquardt, 1963) or the derivative-free algorithm DUD (Ralston and Jennrich, 1978; DUD=Doesn't Use Derivatives). These methods minimize the sum of squares (SSE) of the difference between measured (a_{measured}) and predicted residual activity ($a_{\text{predicted}}$):

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

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

Model Comparison. The strategy to discriminate among models was twofold. The fits obtained for the various models were examined for the distribution of the residuals. Residuals of an appropriate fit should represent only the experimental error and should therefore be distributed randomly and not systematically related to the heating time or temperature. The measurement errors were homoscedastic, so there was no necessity to perform transformation or weighting of the errors. Besides assessment of goodness of fit, the models were compared statistically. The various models were tested for lack of fit (Bates and Watts, 1988). The SSE is due to both measuring error and lack of fit. The measuring error can be estimated by the sum of squares of the replication values about their averages. Therefore, the difference between the SSE of a model and the measuring error is an estimate of the lack of fit of the model. If the lack of fit is much smaller than the measuring error, the model may be adequate. If the lack of fit is much larger than the measuring error, the model is not adequate. The comparison between lack of fit and measuring error can be quantified by an *F*-ratio test. The f value is calculated with the equation

$$f = \frac{(\text{lack of fit})/(\nu_1 - \nu_2)}{\text{measuring error}/\nu_2} \tag{3}$$

and is tested against $F_{(\nu 1-\nu 2),\nu 2,0.95}$. Here, ν_1 and ν_2 refer to the number of degrees of freedom (number of data points minus number of parameters) of the proposed model and the measuring error, respectively (Godfrey, 1983; Motulsky and Ransnas, 1987; Bates and Watts, 1988). Formally, the *F*-ratio test may be applied only for models that are linear in their parameters, because only then would the f value be *F*-distributed, but because in our case the sample size is large, the variance ratio is also approximately *F*-distributed for the nonlinear models applied (Godfrey, 1983; Bates and Watts, 1988). For comparison of fits obtained with nonlinear regression also the residual variance s^2, Akaike's optimization criterion AIC (Hurvich and Tsai, 1989), and Schwarz's optimization criterion SC (Schwarz, 1978) were used. These optimization criteria compare models by their SSE, corrected for the number of parameters. The residual variance is defined as

$$s^2 = \text{SSE}/(n - p) \tag{4}$$

Akaike's criterion is defined as

$$\text{AIC} = n \ln\frac{\text{SSE}}{n} + \frac{n(n+p)}{n-p-2} \tag{5}$$

and Schwarz's criterion is

$$\text{SC} = n \ln(\text{SSE}/n) + p \ln n \tag{6}$$

where n is the number of observations and p the number of parameters. The model with the lowest s^2, AIC, or SC, for the residual variance, Akaike's and Schwarz's criteria, respectively, is the best choice from a statistical point of view. The residual variance is independent of scale, whereas Akaike's and Schwarz's criteria are scale dependent.

RESULTS AND DISCUSSION

Modeling Experimental Results with First-Order Inactivation Kinetics. The inactivation of the extracellular proteinase from *P. fluorescens* 22F as a function of heating time at 90, 100, and 110 °C is shown in Figure 1A. Generally, enzyme inactivation is described by first-order kinetics; an inactivation model as given by reaction scheme 1 is used. In general, the rate of inactivation is determined by the rate of unfolding and thermal inactivation reactions (Zale and Klibanov, 1983)

$$k_{\text{obs}} = k_{\text{i}} K_{\text{d}}/(1 + K_{\text{d}}) \tag{7}$$

where k_{obs} is the apparent reaction rate constant for inactivation and K_{d} the equilibrium constant of the unfolding reaction ($k_{\text{u}}/k_{\text{f}}$). However, when the inactivation is examined at temperatures reasonably far above the denaturation temperature, the influence of the folding/refolding equilibrium is negligible, and inactivation is only determined by the secondary reaction leading to irreversible inactivation (Zale and Klibanov, 1983). Most of the inactivation data of pseudomonal proteinases are evaluated this way (Alichanidis and Andrews, 1977; Barach and Adams, 1977; Richardson, 1981; Driessen, 1983; Stepaniak and Fox, 1983, 1985; Kroll and Klostermeyer, 1984). The following equations are used:

$$\text{N} \xrightarrow{k_{\text{i}}} \text{I} \tag{8}$$

$$\text{d[N]}/\text{d}t = -k_{\text{i}}[\text{N}] \tag{9}$$

$$a_t/a_0 = \frac{[\text{N}]_t}{[\text{N}]_{t=0}} = \exp(-k_i t) \tag{10}$$

a_t is the residual activity and a_0 the initial activity. In many cases such a model adequately describes inactivation. Our inactivation data were analyzed with the first-order inactivation model, eq 10. First, the reaction rate constants of the inactivation were determined for each temperature. The estimated reaction rate constants and initial activities (±95% confidence intervals) are given in Table 1.

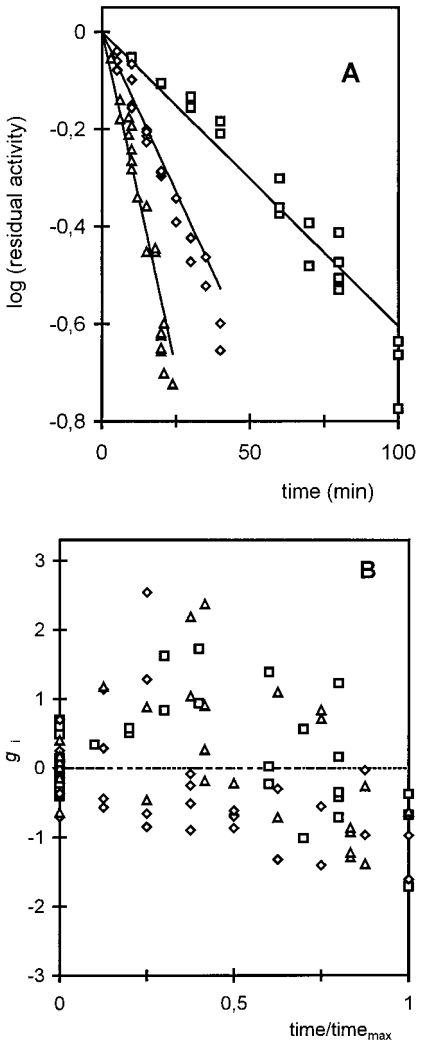

Figure 1. Influence of temperature on inactivation of proteinase from *P. fluorescens* 22F: lines calculated for first-order inactivation (A) and Studentized residuals [$g_i = e_i/s_y$, where $e_i = y_i - y_{model}$ and $s_y^2 = \Sigma(y_i - y_{model})^2/(n - p)$] (B); □, = 90 °C; ◇, 100 °C; △, 110 °C.

Table 1. Inactivation of the Extracellular Proteinase from *P. fluorescens* 22F[a]

T (°C)	k_i (s^{-1})	a_0	n	SSE
90	$2.31 (\pm 0.12) \times 10^{-4}$	1.012 ± 0.020	33	0.398
100	$5.55 (\pm 0.34) \times 10^{-4}$	1.012 ± 0.023	32	0.392
110	$1.06 (\pm 0.07) \times 10^{-3}$	1.015 ± 0.032	29	0.541

[a] Inactivation rate constants (k_i) and initial activities (a_0) (±95% confidence intervals) as estimated with a first-order inactivation model. n is the number of observations, SSE the residual sum of squares.

This study is concerned with the description of thermal inactivation of the proteinase from *P. fluorescens* 22F. Clearly, the rate of inactivation is influenced by temperature. To be able to predict the inactivation at various temperatures, the temperature dependence has to be determined. A consistent temperature dependence is also an additional indication that a model is acceptable. The temperature dependence of reaction rate constants can generally be described by the transition-state theory of Eyring

$$k_i = \frac{k_b T}{h} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(-\frac{\Delta H^{\ddagger}}{RT}\right) \qquad (11)$$

where k_b is Boltzmann's constant (1.38×10^{-23} J K^{-1}), h is Planck's constant (6.62×10^{-34} J s^{-1}), R is the gas constant (8.31 J mol^{-1} K^{-1}), $\Delta S^{\ddagger}$ is the activation entropy, and $\Delta H^{\ddagger}$ the activation enthalpy. The kinetic parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the inactivation reaction can be calculated by linear regression of $\ln(k_i h/k_b T)$ against the reciprocal temperature. Generally, this stepwise procedure results in a relatively large confidence interval of the kinetic parameters due to a large standard deviation and a small number of degrees of freedom (Arabshahi and Lund, 1985; Cohen and Saguy, 1985; Haralampu et al., 1985; van Boekel, 1996). With this method the estimates of the kinetic parameters (±95% confidence interval) were found to be $\Delta H^{\ddagger} = 85.0 \pm 80.2$ kJ mol^{-1} and $\Delta S^{\ddagger} = -81.6 \pm 215.3$ J mol^{-1} K^{-1}, respectively.

$\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the inactivation reactions can also be estimated directly, using the following equation:

$$a_t = a_0 \exp\left[-\frac{k_b T}{h} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(-\frac{\Delta H^{\ddagger}}{RT}\right) t\right] \qquad (12)$$

Direct estimation of the kinetic parameters from this equation is preferable to a stepwise estimation, because in the latter method unnecessary parameters, namely the reaction rate constants, are estimated. Generally, when activation enthalpies and entropies of the inactivation reactions are estimated, a high correlation is found between the parameters, because the experimental range of temperatures studied is narrow compared to the absolute temperature range over which the Eyring equation would apply. Therefore, the temperature was reparametrized:

$$a_t = a_0 \exp[-TX \exp(-Y \Delta H^{\ddagger}) t] \qquad (13)$$

$$X = \frac{k_b}{h} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(-\frac{\Delta H^{\ddagger}}{RT_{av}}\right) \qquad (14)$$

$$T_{av} = \sum T/n \qquad (15)$$

$$Y = \left(\frac{1}{T} - \frac{1}{T_{av}}\right)\frac{1}{R} \qquad (16)$$

When the kinetic parameters are estimated directly by unweighted nonlinear regression (eqs 13−16), the initial activity was set at 1.0, since 1.0 was in the 95% confidence interval of the initial activity at all temperatures. The kinetic parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (±95% confidence interval), estimated with the direct method, were found to be 84.5 ± 5.0 kJ mol^{-1} and -83.1 ± 13.6 J mol^{-1} K^{-1}, respectively. The confidence intervals were much smaller than in the stepwise method. The values of the parameters suggest that the rate-limiting step in the inactivation of the proteinase from *P. fluorescens* 22F most likely is a chemical reaction, and not a protein unfolding reaction, in which case $\Delta S^{\ddagger}$ generally is large and positive because of the unfolding of the molecule. In Figure 1A the calculated inactivation curves are included. In Figure 1B the Studentized residuals of the fit are shown.

It can be seen from Figure 1B that the distribution of the residuals of the fit with the first-order inactivation model seems reasonable, but they were not optimal. In the beginning of the heating experiment the inactivation seems slower than later on, and the temperature dependence seems not to be consistent in this temperature range. It was concluded that the inactivation could not be described adequately with a single first-

Table 2. Kinetic Parameters of Heat Inactivation of Proteinases from Various *P. fluorescens* Strains, When Analyzed with First-Order Inactivation Model

strain	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol K)	$\Delta G^{\ddagger}$ [a] (kJ/mol)	ref
MC60	77.0	−89.5	112.1	Barach and Adams (1977)
AR11	90.7	−52.8	111.5	Alichanidis and Andrews (1977)
B52	100.5	−33.7	113.7	Richardson (1981)
AFT36	84.5	−66.5	110.7	Stepaniak and Fox (1983)
112	115.1	−2.5	116.0	Kroll and Klostermeyer (1984)
P38	32.8	−201	111.8	Owusu and Doble (1994)
22F	97.8	−48.8	116.0	Driessen (1983)
22F	84.5	−83.1	117.2	this work

[a] At 120 °C.

order reaction. This was confirmed by the orders (±95% confidence interval) with respect to concentration and time of the reaction which were found to be 0.78 (±0.68) and 0.75 (±0.06), respectively. The reaction order with respect to concentration did not differ significantly from first order, because the confidence interval was large due to the small number of observations. However, these reaction orders indicate that the reaction cannot be described with a single first-order reaction but that intermediates must be present in the reaction sequence (Hill, 1977).

Driessen (1983, 1989) also found such inactivation behavior when he investigated inactivation of the same proteinase from *P. fluorescens* 22F, but instead of heating diluted supernatant, he heated the complete culture in skimmed milk medium in which the bacteria had grown. Although non-first-order inactivation was found by Driessen, the kinetic parameters were nevertheless calculated using first-order kinetics. Alternative inactivation models will be discussed below, but for the sake of comparison of our data with those of Driessen and others, we will assume that the first-order inactivation model is correct. Our results are more or less comparable to those of Driessen, who found $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of 97.8 kJ mol^{-1} and −48.8 J mol^{-1} K^{-1}, respectively (Table 2). Although the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are different for both cases, the rate-limiting inactivation reaction most likely is a chemical one, and the activation free energies $\Delta G^{\ddagger}$ at 120 °C were 117.2 and 116.0 kJ mol^{-1} K^{-1} for our and Driessen's results, respectively. Differences may be due to the fact that solutions in which the proteinase was heated were not identical.

We also compared our results to kinetic parameters of inactivation of proteinases from other *P. fluorescens* strains, some of which have been partly recalculated using original data from the publications (Table 2). It must be noted that comparison of the inactivation data is difficult, first, because the experimental conditions were different, second, because in many cases non-first-order inactivation was analyzed with first-order kinetics, leading to misinterpretation of the results, and, finally, because the parameters were estimated with the stepwise method, so that their confidence intervals are large. Nevertheless, this rough comparison shows that the kinetic parameters of the heat inactivation of the various pseudomonal proteinases are more or less similar, because of the sign and value of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$. However, predictive modeling of the heat inactivation of proteinases from *P. fluorescens* strains, as a group, seems not very useful, as the variation in the values of the kinetic parameters is too large, as are their confidence intervals. To deal with this problem, it would be recommendable to study the inactivation behavior of proteinases from many different *P. fluorescens* strains under standard conditions, using the direct method to estimate kinetic parameters, as described above.

Table 3. Models of Inactivation of the Extracellular Proteinase from *P. fluorescens* 22F[a]

1 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U \xrightarrow{k_1} I$

2 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} U_2 \xrightarrow{k_2} I$

3 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U_1 \xrightarrow{k_1} U_2 \xrightarrow{k_2} I$

4 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U_1$, $U_1 \xrightarrow{k_1} N^*$, $N^* \underset{k_f^*}{\overset{k_u^*}{\rightleftarrows}} U_2 \xrightarrow{k_2} I$

5 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} U_2 \xrightarrow{k_2} I$, $U_1 \xrightarrow{k_3} I$

6 $N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U_1 \xrightarrow{k_1} U_2 \xrightarrow{k_2} I$, $U_1 \xrightarrow{k_3} I$

7 $N \underset{k_f}{\overset{k_1}{\rightleftarrows}} U_1$, $U_1 \xrightarrow{k_1} N^*$, $N^* \underset{k_f^*}{\overset{k_u^*}{\rightleftarrows}} U_2$, $U_2 \xrightarrow{k_2} N^{**}$, $N^{**} \underset{k_f^{**}}{\overset{k_u^{**}}{\rightleftarrows}} U_3 \xrightarrow{k_3} I$

[a] Explanation in the text.

Modeling Experimental Results with Alternative Inactivation Models. Since the inactivation of the proteinase from *P. fluorescens* 22F was found to show an inactivation behavior deviating from first order, we propose alternative models to fit the experimental data better (Table 3). For all models it is assumed that at room temperature the denaturation equilibrium is shifted completely to the native form N (for models 4 and 7 to forms N* and N**, respectively). Furthermore, it is assumed that the unfolding reaction has proceeded completely after the enzyme solution is heated to the temperatures used in this study (i.e. >80 °C), as Schokker and van Boekel (1997a) found unfolding to occur between 45 and 65 °C. These assumptions rule out any influence of unfolding and refolding reactions on the actual inactivation. When the experimental data are modeled, the inactivation during the heating-up time is neglected. The residual activity after 2 min of heating time ($t = 0$) is considered to be the initial activity. Consequently, in simulations, the fraction of U_1 was considered to be 1 at $t = 0$, the fractions of all other forms zero. By doing so, changes in concentration during the heating-up time are omitted, which may otherwise lead to a distorted representation. The alternative models for the inactivation of the proteinase from *P. fluorescens* 22F can, in principle, account for an initial lag in the inactivation. More complex models than described in Table 3 could be used, but these are considered impractical because of the high number of

Table 4. Estimates of Kinetic Parameters (±95% Confidence Interval; $\Delta H^{\ddagger}$ in kJ/mol; $\Delta S^{\ddagger}$ in J/mol K) for the Alternative Models Describing the Inactivation of the Proteinase from *P. fluorescens* 22F[a]

model	estimates	correlation matrix								SSE
1	$\Delta H_1^{\ddagger} = 84.5$ (±5.0)	1	−0.01							0.143
	$\Delta S_1^{\ddagger} = -83.1$ (±13.6)		1							
2	$\Delta H_1^{\ddagger} = 267.9$ (±829.2)	1	−0.19	0.82	0.48	−0.99	0.06			0.112
	$\Delta S_1^{\ddagger} = 436.8$ (±511.2)		1	−0.70	−0.95	0.18	−0.99			
	$\Delta H_{-1}^{\ddagger} = 55.4$ (±873.2)			1	0.88	−0.80	0.61			
	$\Delta S_{-1}^{\ddagger} = -161.8$ (±1425.3)			1	−0.46	0.91				
	$\Delta H_2^{\ddagger} = 62.5$ (±724.1)					1	−0.04			
	$\Delta S_2^{\ddagger} = -140.8$ (±1951.1)					1				
3	$\Delta H_1^{\ddagger} = 258.5$ (±101.7)	1	0.87	−0.14	−0.43					0.115
	$\Delta S_1^{\ddagger} = 411.6$ (±278.8)		1	0.31	−0.67					
	$\Delta H_2^{\ddagger} = 71.8$ (±7.2)			1	−0.62					
	$\Delta S_2^{\ddagger} = -116.3$ (±20.0)				1					
4	$\Delta H_1^{\ddagger} = 55.8$ (±17.4)	1	−0.91	0.19	0.68	−0.53				0.099
	$\Delta S_1^{\ddagger} = -157.9$ (±48.1)		1	−0.48	−0.84	0.52				
	$\Delta H_2^{\ddagger} = 221.2$ (±44.4)			1	0.69	−0.23				
	$\Delta S_2^{\ddagger} = 298.1$ (±129.0)				1	−0.10				
	$\beta = 0.61$ (±0.17)					1				
5	no convergence									–
6	$\Delta H_1^{\ddagger} = 173.1$ (±278.8)	1	0.91	0.94	−0.96	−0.98	0.61			0.096
	$\Delta S_1^{\ddagger} = 154.9$ (±755.3)		1	0.95	−0.96	−0.86	−0.37			
	$\Delta H_2^{\ddagger} = 5.4$ (±114.0)			1	−0.99	−0.91	0.57			
	$\Delta S_2^{\ddagger} = -288.0$ (±313.5)				1	0.91	−0.60			
	$\Delta H_3^{\ddagger} = 89.5$ (±103.8)					1	−0.57			
	$\Delta S_3^{\ddagger} = -73.1$ (±281.2)						1			
7	$\Delta H_1^{\ddagger} = 453.7$ (±151.3)	1	0.66	−0.78	−0.59	0.81	0.74	0.37	−0.51	0.108
	$\Delta S_1^{\ddagger} = 951.2$ (±411.5)		1	−0.48	−0.67	0.90	0.84	0.48	−0.61	
	$\Delta H_2^{\ddagger} = 56.4$ (±125.0)			1	0.17	−0.77	−0.48	0.19	0.02	
	$\Delta S_2^{\ddagger} = -152.8$ (±349.5)				1	−0.62	−0.92	−0.77	0.97	
	$\Delta H_3^{\ddagger} = 94.7$ (±138.3)					1	0.87	0.23	−0.49	
	$\Delta S_3^{\ddagger} = -51.1$ (±380.4)						1	0.56	−0.83	
	$\beta_1 = 0.88$ (±0.15)							1	−0.89	
	$\beta_2 = 0.72$ (±0.96)								1	

[a] Included are the correlation matrices and the calculated sum of squares of the errors (SSE).

parameters to be estimated. Model 1 is the first-order inactivation model described above (eq 10), in which the inactivation is caused by a single first-order reaction, and is included in Table 3 for comparison. When various models are compared, model 1 is interesting because it has the fewest parameters to be estimated. It is to be expected that models with more parameters can give better fits. In model 2 the unfolded enzyme is subjected to an additional reversible reaction before it can be inactivated. This reaction could be either an additional unfolding or a chemical reaction. Model 3 is similar to model 2, but here it is assumed that k_{-1}, the rate of the reaction from U_2 to U_1, is negligibly small at the temperature of the experiment ($T > 80$ °C). When the enzyme solution is cooled, the rate of refolding is no longer negligible, so that the noninactivated enzyme molecules can return to the native form. In model 4 the secondary reaction is irreversible, even at low temperature. After the enzyme solution is cooled following exposure to heat, U_1 will refold to the native form, U_2 will refold to an active form N*, with relative specific activity β. β can have any positive value and is not restricted to values <1. If the relative specific activity of N* is equal to that of the native molecule ($\beta = 1$), the formula to calculate the residual activity becomes equal to that of model 3. This deterministic unidentifiability between models arises often when only one variable, in our case residual activity, is to be determined (Godfrey, 1983). In models 5 and 6 the proteinase can be inactivated starting from U_1 as well as U_2. In model 5 the secondary reaction is reversible, in model 6 the reaction rate from U_2 to U_1 is negligibly small. Then, in model 7 the proteinase is inactivated by a sequence of three reactions. As in model 4, forms U_2 and U_3 may refold to forms N* and N** with relative specific activities β_1 and β_2, respectively.

Equations that describe residual activity as a function of time were derived for each model (Schokker, 1997), and these equations were fitted to the data to find the parameters of interest, using unweighted nonlinear regression. The results of the simulations are given in Table 4 and are shown in Figure 2. Problems occurred when statistical analysis of the larger models was performed. When the inactivation data were analyzed with model 5, no convergence was obtained, because of the high correlation between the parameters. For model 7 a solution was found, but obviously it was not the best possible: considering that model 7 is a special case of model 4, a much lower SSE was expected. Also, high correlations between parameters were found for other models (Table 4). Most estimates of the parameters in these models were not significant, as zero was in the confidence interval. As was stated above, there is always a strong correlation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ that determine a single reaction rate. This problem was circumvented by reparametrization. However, high correlation was also found between the kinetic parameters determining different reactions rates, especially when models consisting of many parameters were estimated. A high $|\rho|$ value (correlation coefficient) in the correlation matrix means that the correlation between the parameters is strong. In general, it is recommended to avoid correlation coefficients higher than |0.99| (Bates and Watts, 1988), because these may cause problems: parameters that are strongly correlated are difficult to estimate, because a change in one

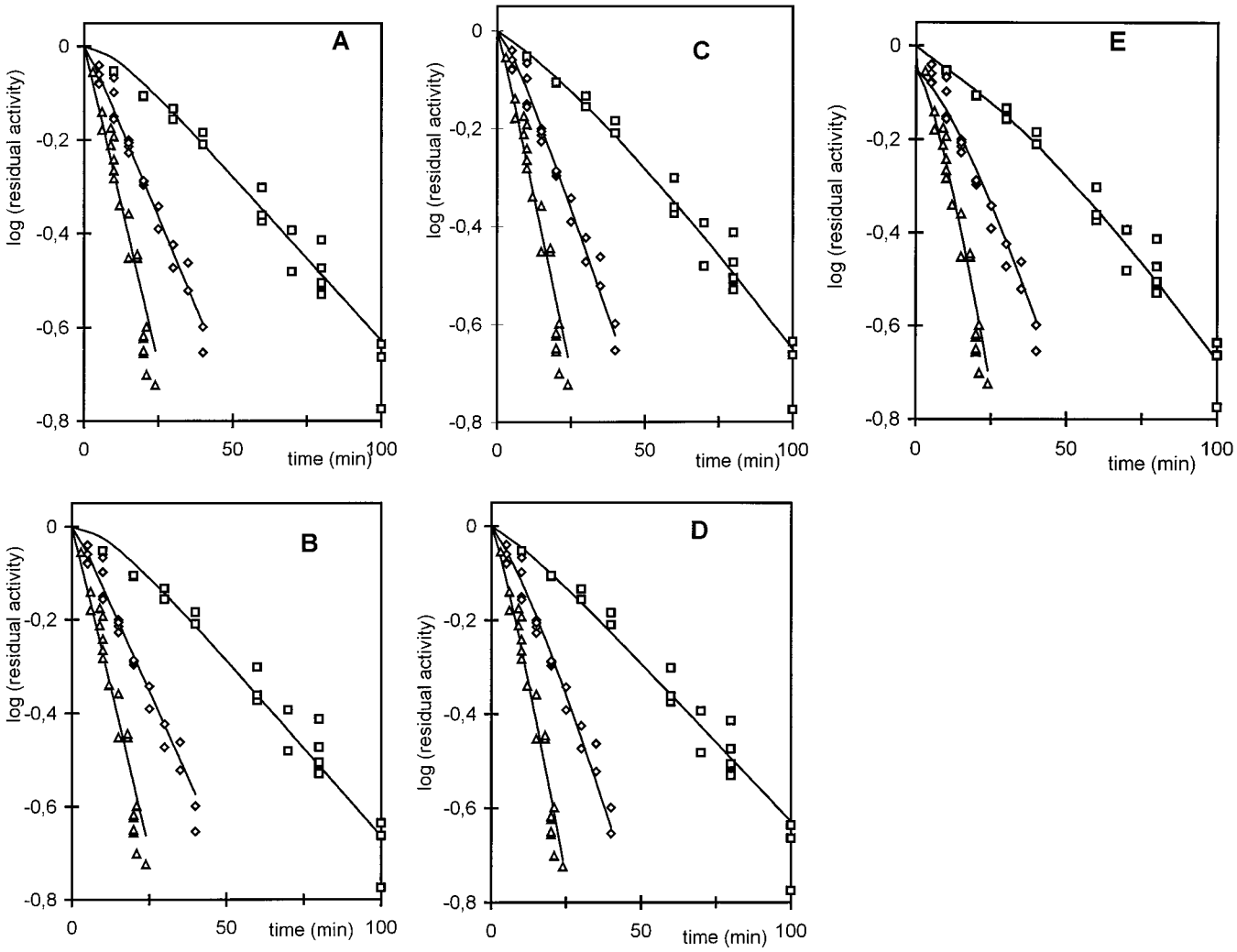

Figure 2. Inactivation of the extracellular proteinase from *P. fluorescens* 22F, analyzed with model 2 (A), model 3 (B), model 4 (C), model 6 (D), and model 7 (E): □ = 90 °C; ◇, 100 °C; △, 110 °C.

parameter will be compensated for by a change in a correlated parameter, and numerous iterations will be necessary.

The residuals for model 1 showed a nonrandom behavior with respect to heating time and temperature (Figure 1B). In models 2 and 3 this nonrandomness was still observed, although to a lesser extent. Models 4 and 6 showed random behavior. Finally, the residuals in model 7 showed that the residuals for the 100 °C experiment were not randomly distributed, obviously because the calculated residual activity dropped rapidly to 0.88 as result of a very fast first reaction.

Besides examination of the distribution of the residuals, the models were compared statistically by using the lack of fit test (see Materials and Methods). In addition, the residual variance and Akaike's and Schwarz's criteria were calculated for the different models (Table 5). It was concluded from the lack of fit test that models 1, 2, and 7 were not acceptable, because the *f* value was larger than the tabulated *F* value. Therefore, these models were rejected for these data set. Models 3, 4, and 6 were statistically acceptable, because the *f* value was lower than the *F* value. From a statistical point of view these models may be used to describe the inactivation. From the residual variance and Akaike's and Schwarz's criteria it was concluded that models 4 and 6 were the best models to describe the inactivation.

Table 5. Evaluation of Models Describing the Inactivation of Proteinase from *P. fluorescens* 22F with *F*-Ratio Test, Residual Variance (s^2), Akaike's Criterion (AIC) and Schwarz's Criterion (SC)

model	*n*	*p*	SSE	*f*	F_{tab}	$10^3\ s^2$	AIC	SC
measurement error	67	26	0.058			1.42	−312	−363
1	67	2	0.143	2.49	1.79	2.20	−339	−404
2	67	6	0.112	1.91	1.83	1.88	−344	−402
3	67	4	0.115	1.80	1.81	1.81	−349	−410
4	67	5	0.099	1.38	1.82	1.60	−356	−415
5	67	8						
6	67	6	0.096	1.34	1.83	1.57	−356	−413
7	67	8	0.108	1.95	1.86	2.27	−334	−395

Model Selection. The selection of a model depends on the purpose of the study. Mathematical modeling of the kinetics of heat inactivation of enzymes can be used for predicting the residual activity after a heat treatment and for elucidating the mechanism of the inactivation. When the purpose is predictive modeling, it is recommendable to choose the model in which the fewest parameters are estimated, because it is the easiest model to use. Moreover, this model would be the most stable, because the parameters are the least correlated. Furthermore, the model with the fewest parameters has the largest number of degrees of freedom, which can be important when the number of measurements is small. In our case, the statistically

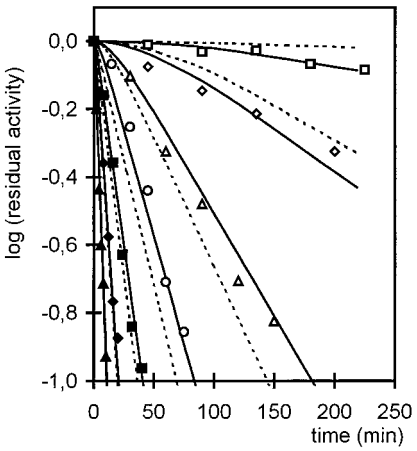

Figure 3. Calculated inactivation of the extracellular proteinase from *P. fluorescens 22F*, using kinetic parameters as in Table 4 (- - -; $\Delta H_1^{\ddagger} = 258.5$ kJ mol^{-1}, $\Delta S_1^{\ddagger} = 411.6$ J mol^{-1} K^{-1}, $\Delta H_2^{\ddagger} = 71.8$ kJ mol^{-1}, $\Delta S_2^{\ddagger} = -116.3$ J mol^{-1} K^{-1}), newly fit parameters (—; $\Delta H_1^{\ddagger} = 139.3$ kJ mol^{-1}, $\Delta S_1^{\ddagger} = 81.2$ J mol^{-1} K^{-1}, $\Delta H_2^{\ddagger} = 79.8$ kJ mol^{-1}, and $\Delta S_2^{\ddagger} = -96.2$ J mol^{-1} K^{-1}). Inactivation data are from Driessen (1983): □, 70 °C; ◇, 80 °C; △, 90 °C; ○, 100 °C; ■, 110 °C; ◆, 120 °C; ▲, 130 °C.

acceptable model with the fewest parameters is model 3.

When the purpose of modeling is to elucidate the mechanism of the inactivation, all models that are statistically acceptable may be used. In fact, it is profitable to perform many measurements to be able to distinguish also between very complex models. Of the models studied in this paper, three were acceptable. Models 4 and 6 were found to be the best models according to the residual variance and Akaike's and Schwarz's criteria. Both models described the data adequately, as the distribution of the residuals showed random behavior. The drawback of model 6 was the high correlation between the parameters and, consequently, the large confidence intervals.

It should be noted that the selection of the model is, in principle, only valid for this data set. It is not unlikely that the mechanism of inactivation is different under other conditions, so that a different model would have to be chosen. Selection of a different model may also be a consequence of the measurement error in the data set. If the measurement error is relatively large or the number of measurements is small, simpler models may not be rejected, while it is more difficult to find solutions for more complicated models. Therefore, it is recommended that the procedure to select a model is carried out with a number of data sets to find the best model to describe the specific sets of data.

Model Validation. According to our results, model 3 could describe the inactivation of the proteinase from *P. fluorescens* 22F in the temperature range 90−110 °C. To check whether the model can also predict inactivation of the proteinase outside this temperature range, we used our results to model inactivation between 70 and 130 °C and compared these with the results of Driessen (1983). First, the kinetic parameters found in our experiments (Table 4) were used for modeling the inactivation data from Driessen (Figure 3). The results of this fit were very reasonable, leaving, however, some discrepancy between the modeled inactivation and the actual inactivation data, especially at lower temperatures. This is probably due to different experimental conditions, such as difference in heating menstrua. New values for kinetic parameters were estimated to describe the results of Driessen better. The new values were found to be $\Delta H_1^{\ddagger} = 139.3$ kJ mol^{-1}, $\Delta S_1^{\ddagger} = 81.2$ J mol^{-1} K^{-1}, $\Delta H_2^{\ddagger} = 79.8$ kJ mol^{-1}, and $\Delta S_2^{\ddagger} = -96.2$ J mol^{-1} K^{-1} (Figure 3). At 80 and 90 °C, and to a lesser extent at 70 and 100 °C, the logarithmic curves were not linear, indicating that non-first-order inactivation occurred. At the other temperatures, more or less straight lines were found. Here, the inactivation could be described with pseudo-first-order kinetics, presumably because one of the reactions then is rate limiting. We could not establish whether the difference between Driessen's and our results is statistically significant, because we did not have the disposal of Driessen's raw data.

Mechanism of Heat Inactivation. From the kinetic data presented above it is not yet possible to draw conclusions about the mechanism of thermal inactivation of the extracellular proteinase from *P. fluorescens* 22F, as various models could describe the inactivation. Also, the large confidence intervals of the estimated parameters and their intercorrelation made it difficult to relate the values of the parameters directly to processes that cause thermal inactivation. The only rigorous conclusion that could be drawn is that the inactivation is not caused by a single reaction but by a sequence of at least two reactions. We may speculate about the mechanism of inactivation on the basis of circumstantial evidence.

Inactivation studies at different pH values (pH 5.5−8.6) showed that the rate of inactivation of the proteinase slightly but significantly increased with pH of the enzyme solution (Schokker, 1997). This finding ruled out that the inactivation is caused by reactions such as reshuffling or β-elimination of cysteine residues, which are extremely pH dependent. In general, pseudomonal proteinases contain no or few cysteine residues, so that it was not very likely anyway that these reactions were a main cause of inactivation. The pH range studied also ruled out inactivation caused by hydrolysis of the peptide chain. It would appear that inactivation caused by deamidation of asparagine or glutamine is the most likely reaction to cause the inactivation of this particular proteinase. This would confirm results of Diermayr et al. (1987), who found lowered isoelectric p*I* after heating the proteinase from *P. fluorescens* Biotype I at 90 °C. Assuming that deamidation of residues is the main cause of inactivation, a model consisting of two consecutive reactions, in which the first reaction leads to an enzyme molecule with a lower specific activity (model 4), seems to be reasonable to describe the inactivation, confirming the indications given by the residual variance and Akaike's and Schwarz's criterion. For other enzymes it has also been found that deamidation of a single asparagine or glutamine residue does not lead to a completely inactivated molecule (Ahern and Klibanov, 1988; Tomizawa et al., 1994). The calcium ion activity of the heating menstrua had no significant influence on the heat inactivation of the proteinase from *P. fluorescens* 22F (Schokker, 1997). Assuming that inactivation is caused by deamidation, no influence of the calcium ion activity was expected. Purification of the proteinase did not alter the inactivation behavior, while addition of 1.8% sodium caseinate to the heating menstrua increased the rate of inactivation slightly, possibly because of aggregation of enzyme molecules with casein (Schokker, 1997).

Conclusion. In this paper we have selected a model that could predict the inactivation of the extracellular

proteinase from *P. fluorescens* 22F. This model applies as yet only for this particular proteinase. We do not know whether or not other pseudomonal proteinases also show inactivation behavior deviating from first-order kinetics. It was until now only clear from the work of Driessen (1983, 1989) for *P. fluorescens* 22F. Other authors who studied pseudomonal proteinases usually did not apply such a rigorous statistical treatment as we did, so the phenomenon may have escaped notice. Even if the inactivation of the proteinase from *P. fluorescens* 22F was to be described with a first-order inactivation model, comparison with inactivation results from the literature is difficult. To make predictions on the inactivation of pseudomonal proteinases as a group, it is necessary to standardize heat inactivation experiments, both for experimental setup and for statistical analysis.

Kinetic modeling appeared to be a useful tool in elucidating the mechanism of enzyme inactivation, but the method has its limitations. We were able to find mathematical models that could describe the thermal inactivation in a statistically acceptable way, but other techniques will be needed to precisely elucidate the mechanism of inactivation. Furthermore, it appears that without complete insight into primary and higher structures of the proteinase it is not possible to explain the causes of inactivation. Therefore, further research into elucidation of the processes inactivating pseudomonal proteinases should start with determination of the enzyme structure.

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