Kinetic Modelling of Enzyme Inactivation

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of the extracellular proteinase
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E.P. Schokker

Stellingen

1   Het boek van de natuur is in wiskundige taal geschreven.
    Galilei Galileo, Il Saggiatore (Opere, VI, 232)

2   Kinetisch modelleren is een krachtig hulpmiddel bij het ophelderen
    van mechanismen van enzyminactivering.
    Dit proefschrift

3   Lage-temperatuur-inactivering van de extracellulaire proteinase van
    Pseudomonas fluorescens 22F wordt veroorzaakt door
    intermoleculaire autoproteolyse.
    Dit proefschrift

4   Spectroscopische en calorimetrische methoden zijn slechts beperkt
    bruikbaar voor de bepaling van ontvouwing van proteinasen.
    Dit proefschrift

5   Met behulp van autoproteolyse kunnen, in principe, zowel de
    thermodynamische als kinetische parameters van de ontvouwing van
    proteinasen bepaald worden.
    Dit proefschrift

6   Het feit dat de meeste psychrotrofe bacteriën hun extracellulaire
    enzymen vooral produceren in hun laat-exponentiële fase, is voor de
    zuivelindustrie een geluk bij een ongeluk.

7   De optimaliseringscriteria van Schwarz en Akaike zijn
    schaalafhankelijk.
    G. Schwarz, Ann. Statist. 6 (1978) 461-464
    C.M. Hurvich and C.L. Tsai, Biometrika 76 (1989) 297-307

8   De hoge prijs van eco-producten strookt niet met het principe dat de vervuiler betaalt.

9   Nutraceuticals lijken vooralsnog vooral gezond voor de producent.

10   De lage prijs van vliegtickets heeft een negatief effect op het milieu en de veiligheid van de passagiers.

11   Ondanks het feit dat de productie van genetisch gemanipuleerde soya minder milieubelastend is, zijn het vooral de milieu-actiegroepen die weerstand bieden tegen de introductie ervan.

12   De meeste psychrotrofe bacteriën hebben geen baat bij de grote hittestabiliteit van hun extracellulaire enzymen.

voor mijn ouders

Abstract

The kinetics of heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F was studied. It was established, by making use of kinetic modelling, that heat inactivation in the temperature range 35 - 70 °C was most likely caused by intermolecular autoproteolysis, where unfolded proteinase molecules are attacked by still active species. Kinetic modelling also showed that sodium caseinate acted as a competitive inhibitor against autoproteolysis. Autoproteolysis experiments gave indications for the dependence of the conformational stability of the proteinase on metal ions and pH.

Although some mathematical models could describe the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F in the temperature range 80 - 120 °C, the mechanism of inactivation could not be precisely elucidated by making use of kinetic modelling. A model consisting of two consecutive irreversible reactions, possibly involving deamidation, where the first reaction would lead to a partially inactivated enzyme molecule with a specific activity of approximately 0.6, appeared to be in best accordance with the mechanism of inactivation. The inactivation behaviour was dependent on the sodium caseinate concentration and the pH, but not on the calcium ion activity.

Kinetic modelling appeared to be a powerful method to predict enzyme inactivation as function of temperature and time. In combination with analytical methods, kinetic modelling may be a useful tool in the elucidation of the mechanism of enzyme inactivation.

Contents

Chapter 1

Introduction

1.1 Enzymes of psychrotrophic bacteria in milk

Introduction of new methods in production and storage of milk caused new technological problems. In the past, milk was stored in milk cans, which were, at most, water-cooled. After storage at the farm, the cans were collected daily by the dairy, where the milk was heat-treated and stored until further treatment. Because the initial storage temperature was ambient, lactic streptococci and coliforms were the dominant microbes. These bacteria cause an increased acidity of the milk.

Nowadays in many countries, milk is cooled immediately after production and is stored at low temperature in tanks at the farm. The milk is collected by cooled tankers every few days and transported to the dairy. Here the milk is processed immediately or stored in large refrigerated tanks for another few days. Due to the low storage temperature psychrotrophic bacteria become predominant. Eddy (1960) defined psychrotrophs as microorganisms that can grow at 5 °C and below. Food microbiologists define psychrotrophs as microorganisms that can grow relatively fast at 7 °C or less, irrespective of their optimal growth temperature (International Dairy Federation, 1969; Morita, 1975; Vedamuthu et al., 1978; Suhren, 1989; Muir, 1990; Kraft, 1992; Shah, 1994).

Only 10 % of the flora after milking consists of psychrotrophic bacteria, but because they grow relatively fast, they soon dominate the flora during refrigeration. The most common psychrotrophs are *Pseudomonas* spp., particularly *Pseudomonas fluorescens.* Other organisms include *Bacillus, Micrococcus, Flavobacterium, Acinetobacter,* and *Aeromonas* species and Coryneforms (Cousin, 1982; Suhren, 1989; Shah, 1994). Contamination may occur from the environment (water, air, soil, outside of udder) and from inadequately maintained milk equipment (milking machine, bulk tank, road tanker) (Cousin, 1982; Suhren, 1989; Muir, 1990; Bramley and McKinnon, 1990).

Psychrotrophic bacteria as such do not pose a very serious problem to the dairy industry. Pasteurization of the milk will eliminate virtually all of the psychrotrophs (Kraft, 1992; Shah, 1994). In the Netherlands thermization (15 s at 63 °C) is used immediately after the milk has arrived at the dairy. This very mild process is sufficient to kill most of the psychrotrophs (Gilmour et al., 1981; Griffiths et al., 1986).

1

Problems for the dairy industry arise when enzymes, such as proteinases, lipases and phospholipases, are secreted in the milk. These enzymes can be very heat stable; most of them resist pasteurization (15 s at 72 °C) and UHT treatments (1 s at 145°C). Several are even more heat resistant than spores of *Bacillus stearothermophilus* and proteinases can be more heat resistant than thermolysin, the well described thermostable proteinase from *Bacillus thermoproteolyticus* (Barach and Adams, 1977; Driessen, 1983; Kroll, 1989; Sørhaug and Stepaniak, 1991). It is clear that, although psychrotrophs are readily eliminated, their presence is undesired, because production of enzymes must be avoided, since these would cause serious enzymatic deterioration of milk products.

Some proteinases from psychrotrophic bacteria are susceptible to inactivation at relatively low temperature (40 - 60 °C). This process, which is often referred to as low temperature inactivation, appears to be due to autoproteolysis. Other authors believe that this inactivation is caused by aggregation with casein (Barach et al., 1976; Stepaniak et al., 1991). Also lipases from psychrotrophic bacteria can be inactivated at low temperature. Possible mechanisms for this inactivation are proteolytic degradation by associated proteinases, self aggregation or aggregation with milk proteins, or interaction with histidine or Hg^{2+}. Destruction of enzymes at low temperatures may be useful, since heat damage of the product would be avoided (Stead, 1986; Bucky et al., 1988; Kroll, 1989).

Heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F at high temperature (80 - 120 °C) will be discussed in chapter 6 of this thesis, its low temperature inactivation (35 - 70 °C) in chapter 5.

1.2   Physical and biochemical effects on milk components

Milk proteins are mainly composed of casein (80 % of total protein) and whey proteins (19%). Casein consists of four different molecular species, α_{s1}-, α_{s2}-, β- and κ-casein. The structure of caseins is mainly random-coil (Walstra and Jenness, 1984). In milk, most of the caseins are present in large particles, called casein micelles, which also contain calcium and phosphate (Rollema, 1990). Most of the κ-casein is at the outside, giving the micelle a 'hairy' surface, and the α_s- and β-caseins are predominantly located in the interior of the micelle. The κ-casein, being on the outside, is very susceptible to proteolytic attack. At 20 °C and pH 6.7 about 10 % of the casein is located in the serum, presumably in submicelles. When milk is stored at low temperature, this amount increases to 23 %, mainly caused by dissociation of β-casein from the interior of the micelle to the surface and to the serum (Davies and Law, 1983). This causes the casein to be more susceptible to proteolysis (Cousin, 1989).

Most of the whey proteins are globular, like β-lactoglobulin, α-lactalbumin, and

blood serum albumin. The group of whey proteins also includes immunoglobulins and small peptides. Whey proteins are not or hardly degraded by proteinases from psychrotrophic bacteria, because of their globular conformation. Pseudomonal proteinases, and also proteinases from most other Gram-negative bacteria, in general degrade κ-casein > β-casein > α_s-casein >> whey proteins (Fairbairn and Law, 1986; Cousin, 1989).

The action of proteinases in milk is twofold. Firstly, they hydrolyze κ-casein, causing destabilization of the casein micelle, leading to aggregation. Extensive hydrolysis may cause visible coagulation of the protein in the milk. Secondly, small molecular weight peptides and amino acids are formed, some of which cause bitter off-flavours (Cousin, 1989).

Milk lipids consist of triglycerides (approximately 98.3 %), di- and monoglycerides (0.3 %), free fatty acids (0.1 %), phospholipids (0.8 %), and other lipids (0.5 %). Most milk fat is present in globules, which are surrounded by a thin protective membrane, composed mainly of proteins and phospholipids (Walstra and Jenness, 1984). The intact globular fat in milk is not susceptible to lipolysis. Combined action of phospholipase C and lipase may cause degradation of milk fat (Chrisope and Marshall, 1976). Also mechanical disruption of the globules, for instance by homogenization or foaming, makes the milk fat susceptible to lipolysis, because the globule membrane is replaced by milk proteins and the interfacial tension is increased. Triglycerides are hydrolyzed to 2-monoglycerides and free fatty acids. Free fatty acids cause rancid (C_4 and C_6) and soapy (C_{10} and C_{12}) off-flavours. Fatty acids of chain length C_{14} to C_{18} do not contribute much to flavour (Stead, 1986; Cousin, 1989).

The action of proteinases and lipases in milk is associated with several technological problems occurring during processing and storage of dairy products (Cousin, 1982; Driessen, 1983; Fairbairn and Law, 1986; Stead, 1986; Mottar, 1989; Sørhaug and Stepaniak, 1991; Cromie, 1992; Champagne et al., 1994; Shah, 1994). Defects in pasteurized milk by enzymes of psychrotrophs have not been described; the low storage temperature and the short storage period prevent development of such defects. Ultra-high temperature treated milk is stored for a long time and at intermediate temperatures, and a defect reported in relation to proteinases from psychrotrophs is formation of bitter off-flavours. Also gelation, caused by aggregation of casein micelles has been reported. Lipolysis by psychrotrophs lipase also occurs, but is of less importance than proteolysis (Mottar, 1989; Muir, 1990).

The two most important effects of psychrotroph enzymes in cheese are on yield and flavour (Cromie, 1992). Part of the degradation products of proteolysis, peptides and amino acids, are soluble and may be lost into the whey instead of forming part of the curd, thereby reducing cheese yield. Also fatty acids may be lost in the whey. Formation of bitter and unclean off-flavours caused by proteinases from psychrotrophs

can occur during cheese ripening, although the major part of the proteinases is lost in the whey. Formation of soapy and rancid off-flavours is more important, because lipases are concentrated in the curd (Driessen, 1983).

Lipases that resist pasteurization are concentrated in the cream, and subsequently in the butter, during the butter-making process, causing rancidity. Proteinases of psychrotrophs do not affect the flavour of butter (Stead, 1986; Cousin, 1989).

Proteolytic enzymes, produced by psychrotrophic bacteria, also generate technological problems. Aged milk may cause more fouling in a heat exchanger than fresh milk, because of proteolysis of casein (Jeurnink, 1991).

1.3   Stability and inactivation of enzymes

Enzymes are globular proteins that catalyze chemical reactions in biological systems. They are highly compact proteins of more or less spherical shape. For biological activity a specific conformation of the enzyme is needed. This conformation is called the native state. This three-dimensional structure of globular proteins is stabilized by noncovalent forces including electrostatic interactions, hydrogen bonding, van der Waals forces and hydrophobic interactions, that are in a very subtle balance.

Several agents may distort this balance, like high or low temperature, extreme pH, changed solvent quality, and high pressure. The originally compact structure will unfold, a process that is often called denaturation. The unfolded enzyme molecule is not catalytically active. Denaturation is in principle a reversible process: after removal of the cause of denaturation, the protein molecule may refold to its native conformation. This refolding process is called renaturation.

Generally, the transitions $N \rightleftharpoons U$ are cooperative, because stabilizing interactions are dependent on each other and several bonds are broken simultaneously. This cooperativity leads to a two-state nature of unfolding/folding transitions; the protein is either fully folded or fully unfolded. The temperature at the midpoint of this transition from native to denatured protein is referred to as the thermal denaturation temperature T_d. Different types and extents of denaturating agents may cause different unfolded conformations (Lapanje, 1968; Tombs, 1985; Kristjansson and Kinsella, 1991; Darby and Creighton, 1993).

Denaturation will result in an inactive enzyme molecule, but it will not lead to inactivation, as denaturation is in principle a reversible process. However, full recovery is not always found, because reactions occurring when the protein is unfolded may prevent correct refolding. A general scheme for irreversible inactivation was proposed by Lumry and Eyring (1954):

$$N \overset{K_d}{\rightleftharpoons} U \overset{k_i}{\rightarrow} I \tag{1.1}$$

where N is the native, U the partially unfolded and reversibly denatured and I the irreversibly inactivated enzyme. K_d defines the equilibrium constant between the N and U states of the enzyme, and k_i is the reaction rate constant of the irreversible thermoinactivation processes. In this thesis, the inactivation occurring after denaturation, in the temperature range 80 °C and higher, is referred to as thermal or high temperature inactivation. In chapter 2 the thermodynamics and kinetics of enzyme inactivation are discussed in more detail.

Reactions that cause irreversible inactivation above the denaturation temperature can be subdivided into reactions where covalent bonds are involved and the primary structure of enzyme molecule is affected, and reactions leading to changes in higher orders of structure (Ahern and Klibanov, 1988; Volkin and Middaugh, 1992). Examples of reactions affecting the primary structure include:
- Hydrolysis of peptide bonds at aspartic acid residues in the polypeptide chain. This reaction takes place at high temperature and low pH.
- Reshuffling of disulfide bonds. Enzymes containing cystine may be inactivated by exchange of disulfide bonds. Reshuffling may occur within or among enzyme molecules. This reaction is relatively fast at higher temperature and pH.
- Destruction of amino acids. A typical example is deamidation of asparagine (Asn) and glutamine (Gln), resulting in the formation of aspartic acid (Asp) and glutamic acid (Glu), respectively. At high pH, destruction of cystine residues via β-elimination (formation of dehydroalanine) can cause irreversible thermal inactivation. Also racemization of asparagine and aspartic acid may contribute to thermal inactivation (Zhao et al., 1989).
- Maillard reactions. In milk Maillard reactions may occur between protein-bound lysine and lactose forming protein-bound lactulosyllysine residues.
Examples of reactions affecting higher order structures are:
- Aggregation. Denaturation leads to exposure of hydrophobic amino acid residues, that were buried in the interior of the molecule in the native state, to the aqueous solvent. In order to minimize this unfavourable exposure the protein molecules will associate intermolecularly, primarily via hydrophobic interactions. Also covalent cross-linking may occur.
- Formation of incorrect conformations. The model of Lumry and Eyring (eq. 1.1) suggests that refolding into the native conformation will occur after removal of the cause of unfolding. However, conformations other than the native may be formed, for instance because peptide bonds are still in cis configuration (purely kinetic reasons), resulting in inactive enzymes.

Often a thermal inactivation behaviour deviating from the model of Lumry and Eyring is found, and more complex models to describe this inactivation have been proposed (Sadana, 1991). In chapter 6 the thermal inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F will be evaluated with the classical inactivation model (eq. 1.1) and with adjusted inactivation schemes. Some proteolytic enzymes will hydrolyze partially unfolded molecules of their own type, either intramolecularly or intermolecularly (Barach et al., 1978; Kawamura et al., 1981; Adler-Nissen, 1986; Owusu and Doble, 1994). This type of inactivation will occur near the denaturation temperature of the proteinase, for the extracellular proteinase from *Pseudomonas fluorescens* 22F in the temperature range 35 - 70 °C, and will be referred to in this thesis as low temperature inactivation. This inactivation is absolutely not related to cold denaturation of proteins, occurring at sub-zero temperatures (Privalov, 1990). In chapter 5 the low temperature inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F is discussed

1.4   Outline of this thesis

As described in this chapter, proteinases secreted by psychrotrophic bacteria can be very heat stable and therefore can cause deterioration of milk and milk products if insufficiently inactivated. In order to make an estimate of the residual proteolytic activity after heat treatment a predictive model should be available. Besides predicting residual activity, kinetic modelling can also be used as tool for elucidation of the mechanism of enzyme inactivation. The aim of this study is to gain more fundamental knowledge about the kinetics of the thermal inactivation of the extracellular proteinase of a psychrotrophic bacterium.

As kinetics is the tool to study the inactivation quantitatively, some basics of thermodynamics and kinetics of enzyme inactivation will be discussed in chapter 2. *Pseudomonas fluorescens* 22F, a psychrotroph isolated from raw milk, was chosen to produce the proteinase. In chapter 3 the production, purification and characterization of its extracellular proteinase is discussed. For kinetic study of enzyme inactivation a reliable method to measure the activity is essential. In chapter 4 two assays to determine the proteolytic activity are evaluated.

In chapter 5 the inactivation of the proteinase in the temperature range 35 - 70 °C, and the factors influencing this inactivation, are discussed. The inactivation at temperatures above 80 °C is discussed in chapter 6. In chapter 7, an attempt to elucidate the mechanism of thermal inactivation by means of urea is described. Finally, in chapter 8 the consequences of the heat stability of the proteinases, and the possibilities and limitations of kinetic modelling are evaluated.

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Chapter 2

Fundamentals of Enzyme Inactivation Kinetics

2.1   Introduction

Knowledge of enzyme inactivation kinetics is of great importance. It can provide biochemists insights into the structure, function and composition of enzymes (Sadana, 1991). Also, it is essential for food technologists to estimate the residual activity of quality-related enzymes. In this chapter some fundamentals of enzyme inactivation kinetics are described. Whether a reaction takes place and in what direction is determined by the thermodynamics of a system. Some thermodynamics of reactions is described in the second section of this chapter. Kinetics is the study of the rate and mechanism of reactions. In section 2.3 some basic concepts of reaction rates and their dependence on temperature are explained. In section 2.4 these concepts are applied to enzyme inactivation. Finally, in section 2.5 the use of kinetic models as predictive tools is discussed.

2.2   Thermodynamics of chemical reactions

The driving force of chemical reactions is the difference in free energy of reactants and products. Reactions will proceed in the direction that minimizes free energy until thermodynamical equilibrium is reached. Also in enzyme inactivation equilibria are involved, for instance:

$$\text{Native} \rightleftharpoons \text{Unfolded} \tag{2.1}$$

Under constant pressure the equilibrium between the folded and unfolded state of an enzyme is determined by their respective Gibbs energies:

$$K_d = \frac{[\text{Unfolded}]}{[\text{Native}]} \tag{2.2}$$

$$\Delta G_{N \to U} = \Delta G^\circ = G_N^\circ - G_U^\circ = -RT \ln K_d \tag{2.3}$$

reaction coordinate
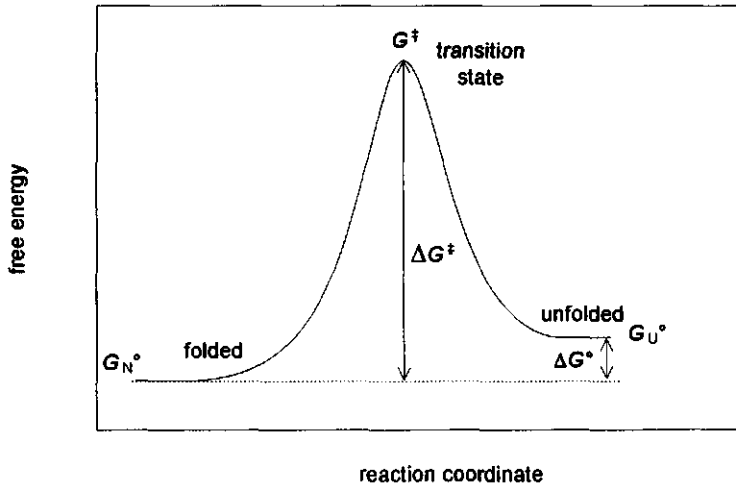
Figure 2.1:   Schematic energy profile for the unfolding/refolding transition. $\Delta G°$ = Gibbs energy difference between the folded and unfolded state, $\Delta G^{\ddagger}$ = the activation free energy.

in which K_d is the equilibrium constant and $\Delta G°$ the difference in Gibbs energy between the native and unfolded state (figure 2.1). The temperature where $\Delta G° = 0$ ($K_d = 1$), is defined as the denaturation temperature of the enzyme T_d.

Because $\Delta G° = \Delta H° - T\Delta S°$, equation 2.3 can be rewritten as:

$$\ln K_d = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} \qquad (2.4)$$

This equation is known as van 't Hoff 's equation. The enthalpy change $\Delta H°$ and the entropy change $\Delta S°$ may be obtained from a van 't Hoff plot, assuming that $\Delta H°$ and $\Delta S°$ are independent of temperature. For small temperature ranges, this is a fairly good approximation.

Thermodynamics can not describe the rate of the reactions, it can only predict the concentrations of products when equilibrium is reached. An energy barrier may prevent a thermodynamically favourable reaction to proceed, or it may determine the rate of the reaction (Hill, 1977; Chang, 1981; Laidler, 1987; Van Boekel and Walstra, 1995).

2.3   Some basic concepts of chemical kinetics

Chemical kinetics is the study of the rate of a reaction, its mechanism by which one chemical species is converted into another, and its dependence on concentration

of reactants, temperature, and other possible factors influencing the rate, such as pH and ionic strength.

The rate of reaction is defined as the change in moles of a component in time, per unit of volume of reaction mixture. In principle, the rate of a reaction is directly related to its stoichiometric equation. Suppose an elementary reaction:

$$aA + bB \rightarrow pP + qQ \tag{2.5}$$

then the rate of the reaction v can be described as :

$$v = k \ [A]^{\alpha} \ [B]^{\beta} \tag{2.6}$$

the reaction has an order α with respect to A, an order β with respect to B and an overall reaction order $\alpha + \beta$. The reaction rate constant k is dependent on conditions such as temperature, pressure, presence of catalysts, and so on.

The way a reaction proceeds may be different than the stoichiometric equation of that reaction indicates, for example when intermediates exist that do not appear in the final equation. That means that the order of a reaction has to be distinguished from the molecularity of that reaction. The molecularity indicates the number reactants participating in an elementary reaction, while the order indicates the empirical dependence of reaction rate on concentration. Also if the complete stoichiometry of a reaction is not known, the order can be determined from experimental data. The rate at which a concentration c of a single reactant in batch systems changes with time can be written as a differential equation:

$$v = -\frac{d c}{d t} = k c^{\tilde{n}} \tag{2.7}$$

in which $\tilde{n}$ is the order with respect to c. The order is an empirical quantity, and it may be non-integer or zero.

The order of a reaction can be determined using the differential method. Initial reaction rates are measured at various concentrations, and ln v is plotted versus ln c, resulting in a straight line with slope $\tilde{n}$ and intercept ln k. The order determined in this way is the order with respect to concentration $\tilde{n}_c$, or true order. When the reaction rates are measured at various times during a single run, corresponding to a number of values of reactant concentration, the order found is the order with respect to time $\tilde{n}_t$.

A method to determine the order $\tilde{n}_t$ is by integration. Equation (2.7) can be integrated for concentration of a single reactant as function of time:

$$c_t^{1-n} = c_0^{1-n} - (1-n) k t \qquad\qquad (n \neq 1) \qquad\qquad\qquad (2.8)$$

$$c_t = c_0 e^{-kt} \qquad\qquad\qquad (n = 1) \qquad\qquad\qquad (2.9)$$

in which c_0 is the initial concentration. Units of c and k are M and M^{1-n} s^{-1}, respectively.

For simple reactions, the order may be zero, first or second. A reaction is zero-order if the rate of the reaction is independent of reactant concentration, for example for such an excess of a reactant so that its concentration is virtually constant during reaction. Typical for a first-order reaction is that the halving-time $t_{\frac{1}{2}}$ (time needed for 50 % of the reaction to proceed) does not depend on the initial concentration. Examples of first-order reactions are radioactive decay or protein denaturation. Pseudo-first-order reactions may occur when in bimolecular reactions one of the reactants is present in large excess.

For second-order reactions two possibilities are of interest. The first possibility is that the rate is proportional to the product of two unequal concentrations of two reactants. The reaction is second-order for the overall reaction, but first-order with respect to the individual reactants. The second possibility is that the rate is proportional to the product of equal concentrations. Besides these fairly simple reactions also more complex reaction schemes may occur, including reversible and chain reactions (Hill, 1977; Chang, 1981; Laidler, 1987; Van Boekel and Walstra, 1995).

Reaction rate constants strongly depend on temperature for most reactions. Most food scientists apply the empirical Arrhenius model for the effect of temperature on reaction rates:

$$k = k_0 \exp\left(\frac{-\Delta E_a}{RT} \right) \qquad\qquad\qquad (2.10)$$

where k_0 is a constant, known as the pre-exponential factor or frequency factor, and ΔE_a is the activation energy. ΔE_a is usually determined experimentally from a plot of ln k versus $1 / T$.

A theoretically better justified approach to describe the relation between reaction rate constants and temperature was developed by Eyring and others, and is called the activated complex or transition state theory. In this theory kinetics are interpreted in terms of thermodynamic properties. It is assumed that an activated complex $X^{\ddagger}$ (or transition state) is formed from reactants, which is in thermodynamic equilibrium with the reactants, and which subsequently decomposes to products:

$$A + B \rightleftharpoons X^\ddagger \rightarrow \text{products} \tag{2.11}$$
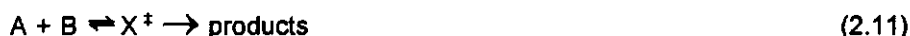
Since equilibrium is assumed for the first step, the concentration of $X^\ddagger$ is determined by the equilibrium constant $K^\ddagger$:

$$[X^\ddagger] = K^\ddagger [A] [B] \tag{2.12}$$

The rate v of the overall reaction is determined by the frequency of decomposition of the complex and its equilibrium concentration $[X^\ddagger]$. It can be shown that the decomposition frequency $\upsilon = k_b T / h$, where h is Planck's constant ($6.62 \cdot 10^{-34}$ J s), and k_b is Boltzmann's constant ($1.38 \cdot 10^{-23}$ J K^{-1}), so that:

$$k = \frac{k_b T}{h} K^\ddagger \tag{2.13}$$

The equilibrium constant can now be related to thermodynamic quantities:

$$\Delta G^\ddagger = -RT \ln K^\ddagger \tag{2.14}$$

where $\Delta G^\ddagger = G^\circ_{\text{activated complex}} - G^\circ_{\text{reactants}}$. Furthermore,

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

so that the reaction rate constant becomes:

$$k = \frac{k_b T}{h} \exp\left(\frac{\Delta S^\ddagger}{R}\right) \exp\left(\frac{-\Delta H^\ddagger}{RT}\right) \tag{2.15}$$

(Hill, 1977; Chang, 1981; Laidler, 1987; Van Boekel and Walstra, 1995).

   Food technologists often use other kinetic parameters than those discussed in this section. The temperature dependence parameter Q_{10} is defined as the factor by which the reaction rate is increased if the temperature is raised by 10 °C:

$$Q_{10} = \frac{k_{T+5}}{k_{T-5}} \approx \exp\left(\frac{10 E_a}{RT^2}\right) \tag{2.16}$$

Q_{10} itself is strongly temperature dependent. At about 100 °C, Q_{10} is 2 - 3 for many chemical reactions, and 6 - 175 for protein denaturation (Walstra and Jenness, 1984).

The z-value is the temperature rise (K) needed to increase the reaction rate by a factor 10:

$$z = \frac{10}{\log Q_{10}} \approx 2.303 \frac{RT^2}{E_a} \tag{2.17}$$

Also the Z value strongly depends on temperature. The decimal reduction time D is defined as time needed to reduce c to $0.1\ c_0$. For first-order reactions it is related to k by:

$$D = \frac{2.303}{k} \tag{2.18}$$

2.4   Kinetics of enzyme inactivation

In chapter 1 the general scheme for enzyme inactivation as proposed by Lumry and Eyring was already mentioned:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \overset{k_i}{\rightarrow} I \tag{2.19}$$

where N is the native, U is the partially unfolded and reversibly denatured and I the irreversibly inactivated enzyme. k_u, k_f, and k_i are the reaction rate constants for denaturation, renaturation and subsequent irreversible thermoinactivation processes, respectively. If we consider this simple inactivation scheme, the rate of inactivation is given by:

$$v = k_i\ [U] \tag{2.20}$$

Since measurement of residual activity involves [N] + [U] (U will renature upon cooling), the observed rate of inactivation (k_{obs}) is:

$$k_i\ [U] = k_{obs}\ ([N] + [U]) \tag{2.21}$$

Because $K_d = [U]\ /\ [N]$ (eq. 2.2), assuming rapid equilibrium, equation 2.21 can be transformed into:

$$k_{obs} = \frac{k_i\ K_d}{1 + K_d} \tag{2.22}$$

Consequently, at high temperatures, where $K_d \gg 1$, the observed rate constant of inactivation k_{obs} equals k_i, and does not depend on the reversible denaturation. At temperatures near the thermal denaturation temperature T_d, where $K_d \approx 1$, the observed rate constant of inactivation is a combination of k_i and K_d. In the latter case the inactivation will depend on factors influencing the equilibrium constant K_d, such as pH and ionic strength (Zale and Klibanov, 1983). Using eq. 2.22, different types of thermostable enzymes must be distinguished. Some enzymes unfold at relatively high temperature, but the rate of thermal inactivation may be high, for instance many enzymes from thermophilic microorganisms. Other enzymes unfold at relatively low temperatures, but their resistance against thermal inactivation at high temperatures (where $K_d \gg 1$ and $k_{obs} = k_i$) is much higher. Proteinases and lipases from many psychrotrophic bacteria are typical examples of this type of heat stable enzymes.

The model for inactivation of enzymes, according to eq. 2.19, may be applied in many cases. However, often more complex inactivation kinetics are found, because inactivation may occur by consecutive or parallel processes or by some other mechanism. Many alternative mechanisms are described by Sadana (1991). Some of these alternative mechanisms may result in apparent first-order inactivation kinetics (Sadana, 1991; Lencki et al., 1992). In this thesis the inactivation kinetics of the proteinase from *Pseudomonas fluorescens* 22F is described. The autodigestion reaction, mentioned in section 1.1, responsible for inactivation at relatively low temperatures (35 - 70 °C), is a complicated bimolecular process, showing non-first-order kinetics. The kinetics of low temperature inactivation of the proteinase is described in chapter 5. Also at high temperatures (80 - 120 °C), the proteinase shows an inactivation behaviour deviating from eq. 2.19. This is described in chapter 6 of this thesis.

2.5   Modelling enzyme inactivation during heat processing of foods

Study of kinetics of enzyme inactivation can provide insight into the mechanism of inactivation. This would involve precise determination of molecular processes involved in the inactivation. The determination of the reaction mechanism is a process of inductive and deductive thinking, although general guidelines can be given for finding the mechanism (Hill, 1977). Additional methods and techniques may be necessary to check the validity of the proposed mechanism.

Often it is not possible to find a complete scheme of elementary equations describing all molecular processes. For those interested in the estimation of residual activity of enzymes, for instance subsequent to heat treatment, knowledge of the reaction mechanism may be useful, but it is not essential. For analysis and interpretation of kinetic data mathematical models can be used. These simplified

schemes may help to model the enzyme inactivation. These mathematical models are no longer mirrors of the actual mechanism, but they describe the inactivation as a black-box. Still, speculations can be made about what is happening in this black-box. Food technologists often use simple first-order models to describe transformation and inactivation processes. In order to justify the use of these simple models instead of more complex ones, statistical methods must be used (van Boekel, 1996).

Foods are complex systems with many ingredients, and many reactions that complicate enzyme inactivation may take place. Impurities may inhibit the enzyme or act as catalysts for inactivation, and substrate may stabilize the enzyme. Using purified enzymes may lead to a more precise determination of kinetic parameters, but translation of the results to food systems is often not possible. Other factors complicating accurate prediction of heat inactivation of enzymes in foods are the finite rate of heat transfer and variation in temperature. During heating and cooling of the reaction mixture, some inactivation will take place.

Despite the limitations, mathematical modelling of enzyme inactivation can be very useful. Even without knowing the molecular processes underlying the inactivation, kinetic models can be used for product improvement, new product development, and shelf-life testing.

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Chapter 3

Production, purification and partial characterization of the extracellular proteinase from *Pseudomonas fluorescens* 22F

Abstract

Pseudomonas fluorescens 22F was inoculated into tryptone-lactose medium, and incubated at 20 °C for 8 days. Proteinases were produced during the exponential and stationary phase. Cells were removed by centrifugation, and from the supernatant an extracellular proteinase from the bacteria was purified to electrophoretic homogeneity by ammonium sulfate precipitation, hydrophobic interaction chromatography, ultrafiltration and gel filtration. The increase in specific activity was 57-fold, the yield was 40%. The purified enzyme had an apparent molar mass of 52 kDa as determined by SDS-PAGE, 47 kDa as determined by gel filtration. The proteinase was characterized as an alkaline metalloproteinase with an optimum pH for activity on sodium caseinate of 9.5-10. The optimum temperature of the enzyme was 50 °C; activity decreased rapidly with increasing temperatures. With sodium caseinate concentrations up to 1%, Michaelis-Menten-type reaction kinetics was observed, whereas at higher concentrations substrate inhibition occurred. V_{max} and K_m were estimated at 1560 TNBS units/ml and 0.24 % sodium caseinate, respectively.

3.1   Introduction

The heat stability of extracellular proteinases from psychrotrophic bacteria has been studied by several authors. Various bacteria were used for the production of the proteinases, and although most of these bacteria were *Pseudomonas fluorescens* strains, the proteinases investigated were all slightly different from each other (Fairbairn and Law, 1986a; Fox et al., 1989), making it difficult to compare heat inactivation results (Kroll, 1989).

In our study on the heat stability of proteinases from psychrotrophs we used the proteinase produced by *Pseudomonas fluorescens* 22F. The proteinase of this bacterium had been found to be very heat stable (Driessen, 1983). In this chapter production, purification and partial characterization of the extracellular proteinase from *Pseudomonas fluorescens* 22F are described.

3.2   Materials and methods

Culture

Pseudomonas fluorescens strain 22F, originally isolated from raw milk (Driessen and Stadhouders, 1974), was obtained from the Netherlands Institute for Dairy Research (NIZO). *Pseudomonas fluorescens* 22F was inoculated into conical flasks containing sterile growth medium, consisting of 2.0 % tryptone, 1.0 % lactose, 0.2 % $(NH_4)_2SO_4$, 0.1 % KH_2PO_4, 0.05 % $MgSO_4$, 0.02 % yeast extract, 0.01 % NaCl, 0.01 % $CaCl_2$, and 0.01 % $ZnSO_4$ in demineralized water, and incubated for 8 days at 20 °C. Bacterial growth was followed by measuring the optical density at 580 nm. Cells were removed by centrifugation (27,000 *g* for 30 min at 4 °C), resulting in a clear supernatant containing the proteinase.

Enzyme purification

All experiments were performed on ice water or at 4 °C. Ammonium sulfate was added to the supernatant up to 40 % saturation and this solution was centrifuged (27,000 *g* for 10 min at 4 °C). To the supernatant ammonium sulfate was added to 80 % saturation. After centrifugation (27,000 *g* for 10 min at 4 °C) the pellet was dissolved in a small volume of 0.02 *M* TrisHCl, pH 7.0, containing 2 m*M* $CaCl_2$, 2 m*M* $ZnSO_4$, and ammonium sulfate up to 20 % saturation. The material not dissolving was removed by centrifugation.

The clear supernatant was applied to a 29 x 1.6 cm Phenyl Sepharose 6 FF column (Pharmacia), which is a hydrophobic interaction chromatography column. The column was washed thoroughly with 0.02 *M* TrisHCl, pH 7.0, containing 2 m*M* $CaCl_2$, 2 m*M* $ZnSO_4$, and ammonium sulfate up to 20 % saturation. The proteinase was

eluted with the same buffer containing ammonium sulfate up to 10 % saturation. The linear flow rate was 100 cm/h. Fractions with proteolytic activity were pooled and concentrated by ultrafiltration in stirred cells with a cut-off of 3 kDa (Filtron). The concentrate was purified further by gel filtration using a 92 x 2.6 cm Sephadex G75 (superfine, Pharmacia) column, equilibrated with 0.02 M TrisHCl, pH 7.0, containing 2 mM CaCl$_2$, 2 mM ZnSO$_4$, and eluted with the same buffer. The linear flow rate was 4.9 cm/h.

To check whether only one or more extracellular proteinases were produced by *Pseudomonas fluorescens* 22F, the unpurified supernatant was applied to the Sephadex G75 column, and fractions were examined for proteolytic activity.

Enzyme assays

Proteolytic activity was determined using the TNBS and azocasein methods, as described in chapter 4 of this thesis. The TNBS method, being quantitatively more accurate than the azocasein method, was used for determination of the yield. The azocasein method was applied to identify proteolytic activity in the fractions obtained by liquid chromatography.

Protein content

Fractions eluting from the chromatography columns during purification were tested for protein content by ultraviolet absorption at 280 nm (Stevens, 1992). Determination of the protein content of the pooled fractions after dialysis (4 °C, Spectrapor membrane, Spectrum Medical Industries, 6 - 8 kDa) was done with the Bichinchoninic Acid method (Smith et al., 1985) using bovine serum albumin as a standard.

Molar mass

Relative molar mass of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) according to the method of Laemmli and Favre (1973), performed on a Pharmacia Phastsystem apparatus on precast gradient gels (Pharmacia Phastgel gradient 8-25). Visualization of the bands was accomplished by Coomassie staining. Proteins used for calibration were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.1 kDa), and bovine α-lactalbumin (14.4 kDa).

Relative molar mass was also estimated by gel filtration chromatography on a 220 x 13 mm Superdex 75 HR 10/30 column (Pharmacia), calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine β-lactoglobulin (36 kDa) and bovine α-lactalbumin (14.4 kDa) as standards. The column was equilibrated with 0.06 M phosphate buffer, pH 7.0 at a flow rate of 1.2 ml/min, 20 °C.

Isoelectric point (pI)

Isoelectric focusing was performed on a Phastsystem apparatus (Pharmacia) on PhastGel IEF 3-9 gel using trypsinogen (p*I* 9.3), basic lentil lectin (p*I* 8.65), neutral lentil lectin (p*I* 8.45), acidic lentil lectin (p*I* 8.15), basic horse myoglobin (p*I* 7.35), acidic horse myoglobin (p*I* 6.85), human carbonic anhydrase (p*I* 6.55), bovine carbonic anhydrase (p*I* 5.85), bovine β-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55) and amyloglucosidase (p*I* 3.50) as standard proteins. Visualization was done by silver staining. Prior to the electrophoresis run *o*-phenanthroline, a specific Zn^{2+} chelator, was added to the enzyme solution in order to prevent autoproteolysis.

Temperature optimum

The optimum temperature was determined by measuring the increase of free amino groups during 10 and 90 min, using the TNBS method. The concentration of the purified enzyme used in the 10 minutes experiment was 12 µg/ml, in the 90 minutes experiment 2.8 µg/ml. Experiments were performed in triplicate.

pH optimum

The optimum pH was determined by measuring the increase of free amino groups with the TNBS method, after incubation of 1.5 µg/ml proteinase in 1.1 % sodium caseinate in 0.1 *M* Universal buffer with various pH values (James and Lord, 1992).

pH stability

Purified enzyme was brought into 25 m*M* Universal buffer with various pH values (James and Lord, 1992) to a final concentration of 1.5 µg/ml and kept at 25 °C for 1 h or at 4 °C for 24 h. After incubation 0.5 ml of the enzyme solution was transferred to a sodium caseinate solution in 0.1 *M* TrisHCl, pH 7.15 at a final concentration of 1.0 % and the residual activity was measured using the TNBS method. The actual pH during the incubation with sodium caseinate varied from 6.9 to 7.3.

Effect of inhibitors

Equal volumes (0.25 ml) of proteinase (3 µg/ml) and inhibitor in 0.2 *M* TrisHCl, pH 7.0, were mixed and incubated for 30 minutes at 30 °C. The remaining activity was assayed with the TNBS method, with the modification that the TrisHCl solution did not contain calcium chloride. The activity was expressed as a percentage of the control, to which only buffer was added. Proteinase inhibitors used were EDTA (Janssen Chimica), ethylene glycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA, Sigma), *o*-phenanthroline (*o*PA, Sigma), *p*-hydroxymercuribenzoic acid (PCMB, Sigma), phenylmethylsulfonylfluoride (PMSF, Sigma), bestatin (Sigma), soybean-trypsin-inhibitor(Serva),trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane(E64,

Determination of V_{max} and K_m

Volumes of 0.5 ml of a 3.3 µg/ml enzyme solution were incubated with sodium caseinate solution in 0.2 *M* TrisHCl, pH 7.0 at a final concentration of 0.5 - 50 mg/ml. Except for the caseinate concentration the proteolytic activity was measured according to the standard procedure.

3.3   Results and discussion

Growth of the culture and production of proteinase

Biomass production and production of proteinases were followed in time. Results are shown in figure 3.1. Proteinases were produced during the exponential and early stationary phase, paralleling growth. This was also seen for a few other strains of *Pseudomonas fluorescens* (Adams et al., 1975; Gebre-Egziabher et al., 1980; Birkeland et al., 1985; Rowe et al., 1990; Hellio et al., 1993), although the majority of strains have a maximal production during late exponential and early stationary phase (McKellar, 1982; Stead, 1987; Kohlmann et al., 1991a). Driessen (1983) investigated growth and proteinase production of *Pseudomonas fluorescens* 22F in fresh skimmed milk at 7 °C and found maximal production towards the end of the exponential growth phase. Differences are possibly due to different growth media or temperature. Although further investigation of the relation between growth and enzyme production is beyond the scope of this thesis, it is important to realize that it determines whether or not proteinases are secreted in the milk under normal storage conditions.
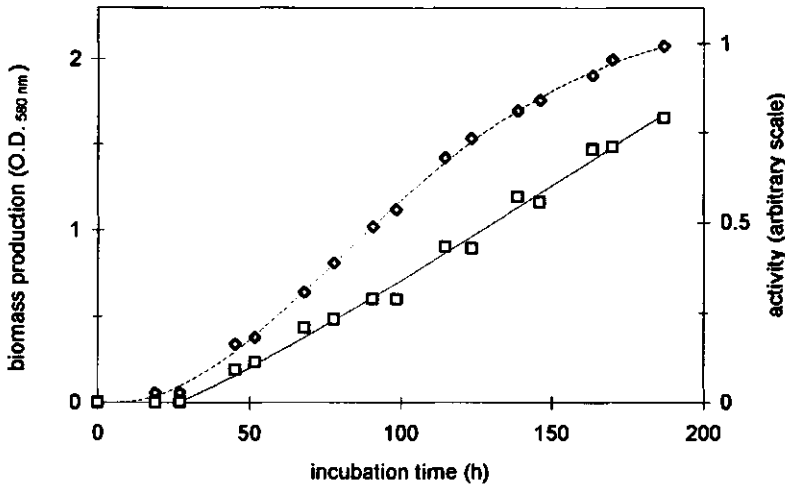
Figure 3.1:   Biomass production (◊) and proteinase production (□) by *Pseudomonas fluorescens* 22F.
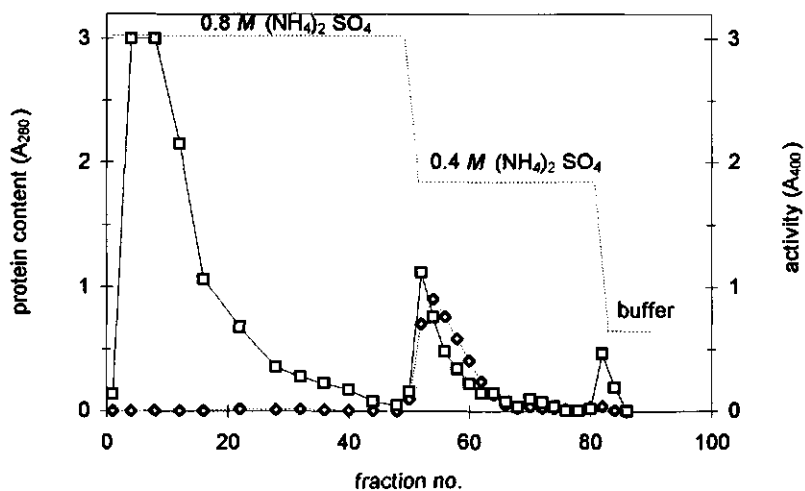
Figure 3.2: Protein content (□) and proteolytic activity (◊) on azocasein of samples eluting from a Phenyl Sepharose 6 FF column.
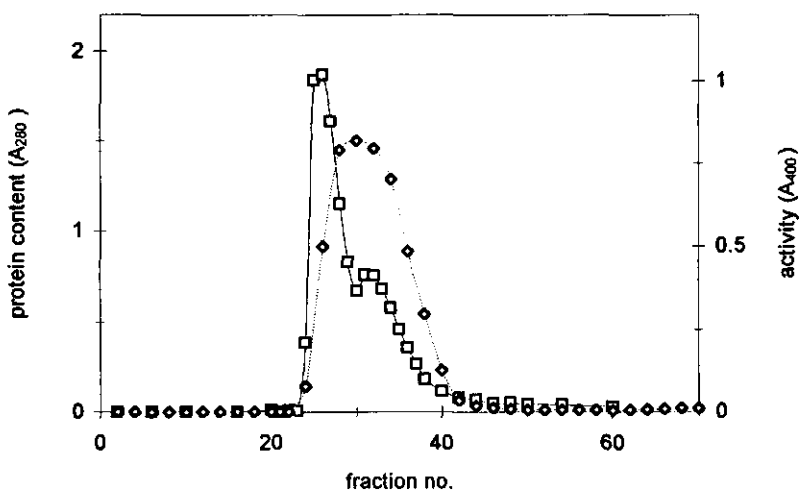
Figure 3.3: Protein content (□) and proteolytic activity (◊) on azocasein of samples eluting from a Sephadex 75 column.

Only one peak with proteolytic activity was detected when the unpurified supernatant was separated on the Superdex G75 gel filtration column, suggesting that probably one single proteinase is produced by *Pseudomonas fluorescens* 22F. This was found for most strains of *Pseudomonas fluorescens*, although some strains were found to produce more than one proteinase (Fairbairn and Law, 1986b; McKellar, 1989).

Purification

The results of a typical purification procedure of the proteinase from *Pseudomonas fluorescens* 22F are summarized in table 3.1. Determination of proteolytic activity and protein content of pooled fractions after each purification step were performed after dialysis (6 - 8 kDa) against 0.02 *M* TrisHCl containing 2 m*M* CaCl$_2$, pH 7.0.

Ammonium sulfate precipitation led to a thirteenfold increase in specific activity, removing, amongst other materials, the greatest part of peptides and amino acids from the culture broth. Another threefold purification was achieved with hydrophobic interaction chromatography (figure 3.2). In this purification step also most of the green pigment was removed from the enzyme solution. Gel filtration (figure 3.3) led to purity of the proteinase, as SDS-PAGE of the pooled fractions 34 to 40 showed a single band. The complete purification procedure resulted in 57-fold purification and 40 % recovery.

Table 3.1: Purification of proteinase from *Pseudomonas fluorescens* 22F. AS = ammonium sulfate, HIC = hydrophobic interaction chromatography.

step	protein conc. µg / ml	activity TNBS- units / ml	specific activity TNBS- units / µg	purification factor	yield %
supernatant	520	434	0.83	1	100
AS precipitation	1506	16216	10.7	13	96
HIC	203	5685	28.0	34	73
Ultrafiltration	1232	48425	39.3	47	66
Gel filtration	190	9072	47.7	57	40

Molar mass

The denatured proteinase in disintegrating buffer, as estimated by gel electrophoresis, had a molar mass of 52 kDa. Estimation with gel filtration gave a lower value of 45 kDa, suggesting that the enzyme occurs as a monomer. A lower estimate from gel filtration was also found for other enzymes (Richardson, 1981; Baral et al., 1995). The molar mass is in accordance with other proteinases from *Pseudomonas fluorescens* reported (Fairbairn and Law, 1986a; Fox et al., 1989).

Isoelectric focusing

The IEF gel with the proteinase showed a single band, focused at pH 7.4. Isoelectric pH values of other proteinases from *Pseudomonas fluorescens* strains vary widely from 5.1 to 8.8 (Fox et al., 1989).

Optimum temperature

The optimum temperature of an enzyme is dependent on the length of the experiment, because denaturation and inactivation may occur during the experiment. Also other factors, such as pH and ionic strength, may influence the optimum temperature. The optimum temperature under standard conditions, found in the experiment lasting 90 minutes was around 47 °C, in the 10 minutes experiment 50 °C (figure 3.4). This is somewhat higher than optimum temperatures of most other pseudomonal proteinases, which were between 30 and 45 °C (Fairbairn and Law, 1986a; Fox et al., 1989), although Richardson (1981) also found a temperature optimum between 45 and 50 °C.
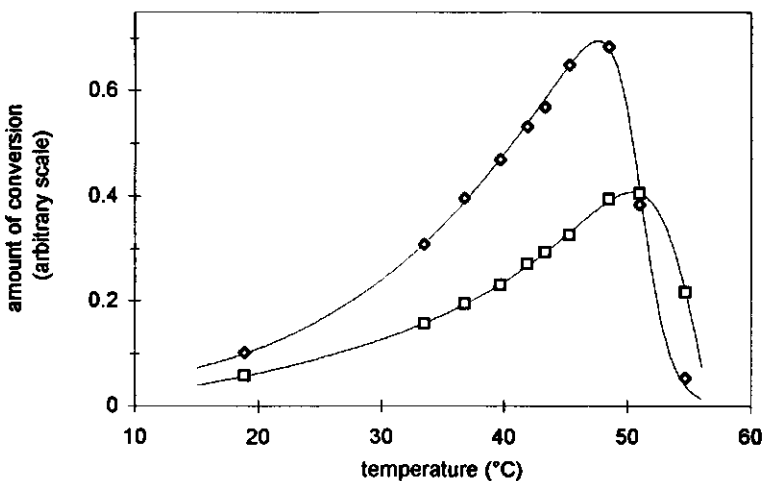
Figure 3.4:   Effect of incubation temperature on activity of proteinase from *Pseudomonas fluorescens* 22F. ◊ = experiment of 90 min; □ = experiment of 10 min.
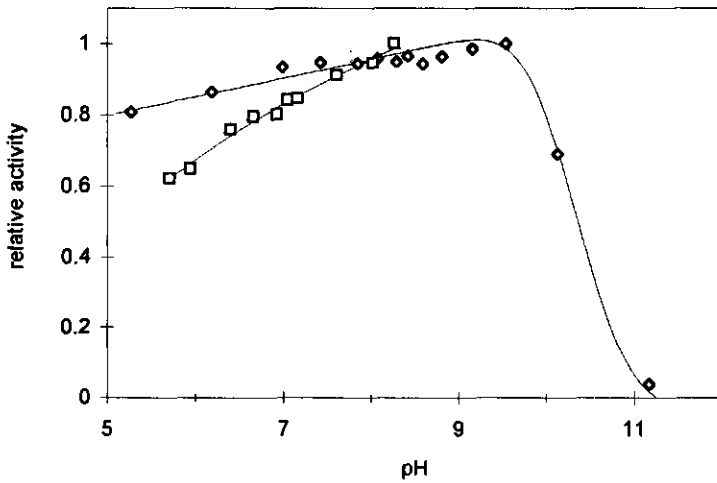
Figure 3.5: Effect of pH on proteolytic activity, using 1.2 % sodium caseinate in 20 m*M* TrisHCl (□) or in 25 m*M* Universal buffer (◊) as a substrate.
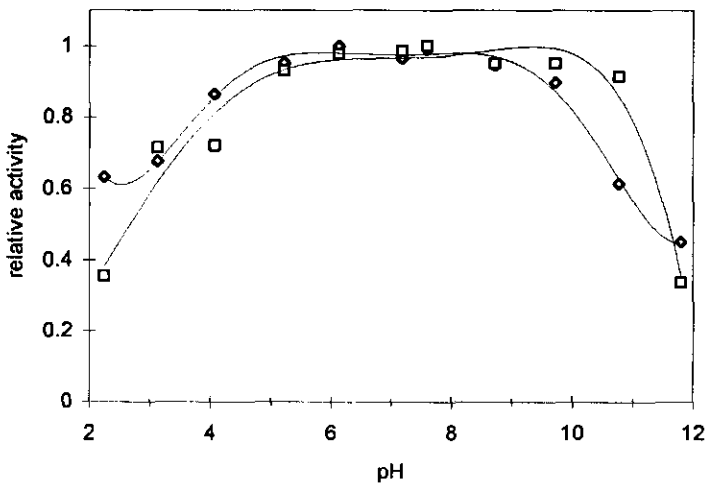
Figure 3.6: Effect of pH on the stability of proteinase from *Pseudomonas fluorescens* 22F, using 25 m*M* Universal buffer. Incubation 1 h at 25 °C (□) and 24 h at 4 °C (◊).

pH optimum and stability

The isolated proteinase from *Pseudomonas fluorescens* 22F had a broad pH range of activity towards caseinate as substrate (figure 3.5). The optimum was near pH 9.5-10, which means that it was an alkaline proteinase. At the pH of bovine milk (6.6) the proteinase had an activity in this buffer of 90 % of its maximum. The proteinase was stable between pH 5 and 10 at 4 and 25 °C (figure 3.6).

Inhibitors of the proteinase

The effects of various inhibitors on the activity of the proteinase are summarized in table 3.2. EDTA, a chelator of divalent cations, was a strong inhibitor, indicating that the proteinase was a metalloproteinase, like most proteinases from *Pseudomonas fluorescens* (Fairbairn and Law, 1986a; Fox et al., 1989). EGTA, which especially chelates Ca^{2+}, had only little effect on the activity, whereas o-phenanthroline (oPA), a Zn^{2+}-chelator, could inactivate the proteinase. Inactivation curves of EDTA and oPA are shown in figure 3.7. Because oPA has a much higher association constant for zinc ($2.5 \cdot 10^6$ M^{-1}) than for calcium (3.2 M^{-1}), inhibition by oPA is a diagnostic for a Zn^{2+}-metalloproteinase (Salvesen and Nagase, 1990). Calcium is assumed to stabilize the structure of proteinases from psychrotrophs, zinc is assumed to be essential in the active site. Phosphoramidon, which is a strong inhibitor of some metalloproteinases like thermolysin, did not strongly inhibit the proteinase.

Inhibitors for serine proteinases (PMSF), cysteine proteinases (PCMB, E_{64}), aspartic proteinases (Pepstatin A), and aminopeptidases (Bestatin) had no significant influence on the activity of the isolated proteinase, and neither had soybean trypsin inhibitor. Sulfhydryl blocking agents such as cysteine and β-mercaptoethanol caused inhibition, which may indicate that cysteine residues are essential for the tertiary structure, although β-mercaptoethanol is also used for inhibition of metalloenzymes (Whithaker, 1994). Inhibition by cysteine and β-mercaptoethanol was also found for a few other proteinases from *Pseudomonas fluorescens* (Alichanidis and Andrews, 1977; Fairbairn and Law, 1986b; Kohlmann et al., 1991b), although for several proteinases from *Pseudomonas fluorescens* strains it was found that they did not contain any cysteine (Mayerhofer et al., 1973; Barach and Adams, 1977; Richardson, 1981; Diermayr and Klostermeyer, 1984; Mitchell et al., 1986).

Kinetics of sodium caseinate degradation

The Michaelis-Menten constants were estimated with nonlinear regression. V_{max} was found to be 1560 TNBS units/ml, and K_m = 2.44 mg / ml (0.11 mM); in figure 3.8 a Lineweaver-Burke plot is given. At caseinate concentrations of 10 mg/ml and higher substrate inhibition was observed. Other proteinases from *Pseudomonas fluorescens* strains have similar K_m values on caseinate (Alichanidis and Andrews, 1977; Stepaniak et al., 1982; Patel et al., 1983).

Table 3.2: Influence of various inhibitors on the activity of proteinase from *Pseudomonas fluorescens* 22F. Enzyme concentration 3 µg/ml.

Inhibitor	concentration	activity
none	-	1
EDTA	0.1 mM	1.03
EDTA	1.0 mM	0.03
EDTA	10 mM	0.01
EGTA	0.1 mM	1.03
EGTA	1.0 mM	1.04
EGTA	10 mM	0.97
oPA	0.1 mM	1.00
oPA	1.0 mM	0.50
oPA	10 mM	0.01
phosphoramidon	2 mM	0.80
PCMB	1.0 mM	0.91
PCMB	10 mM	0.87
β-mercaptoethanol	5 mM	0.79
E_{64}	3 mM	1.01
cysteine	10 mM	0.23
bestatin	3 mM	0.99
pepstatin A	1 mM	0.93
trypsin inhibitor	0.2 %	1.11
PSMF (+isopropanol)	10 mM	0.97
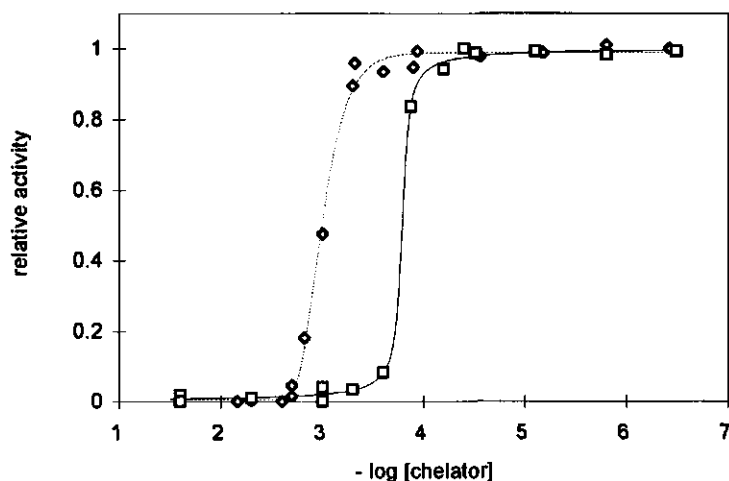
Figure 3.7: Influence of o-phenanthroline (□) and EDTA (◊) on the activity of proteinase from *Pseudomonas fluorescens* 22F.
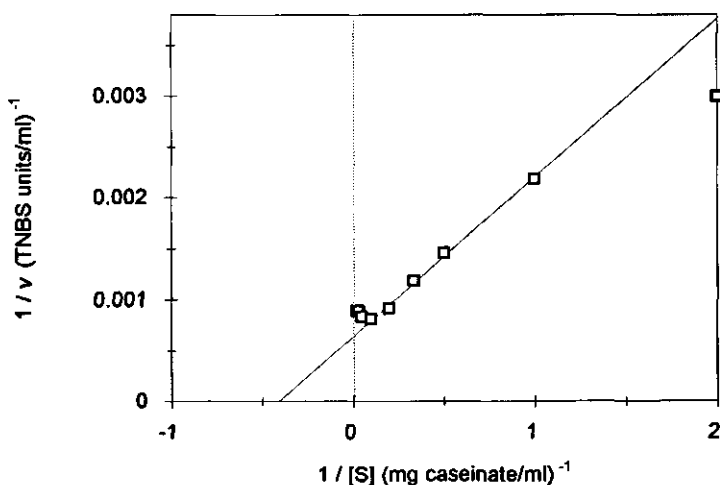
Figure 3.8:   Lineweaver-Burk plot for the proteolytic activity of proteinase from *Pseudomonas fluorescens* 22F on sodium caseinate in 0.2 *M* TrisHCl, pH 7.0.

Substrate specificity

In order to complete the characterization of the proteinase from *Pseudomonas fluorescens* 22F, we like to mention results on substrate specificity, as found earlier by Driessen (1983). When milk is incubated with the proteinase at 37 °C, κ-casein and β-casein were notably digested to form para-κ-casein and γ- casein, respectively. Para-κ-casein and γ- casein were digested further to form peptides and amino acids. $α_s$-Casein and whey proteins were not hydrolyzed.

Summarizing the results, it can be concluded that the extracellular proteinase from *Pseudomonas fluorescens* 22F is quite similar to other *Pseudomonas fluorescens* proteinases for all characteristics studied.

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Chapter 4

Evaluation of two proteolytic activity assays: azocasein and TNBS method

Abstract

The TNBS method and the azocasein method were investigated for their proportionality of response to the enzyme concentration/activity and for their precision. The TNBS method showed a linear correlation between the enzyme concentration and the response. The coefficient of variation was acceptable (1.4 %). With the azocasein method, however, the linear correlation was not found for the proteinase from *Pseudomonas fluorescens* 22F. The coefficient of variation was 1.6 %. The TNBS method can be used for characterizing the heat stability of the proteinase; the azocasein method, being faster and less sensitive for interfering substances, can be used for identifying fractions containing activity during the purification of the proteinase.

4.1   Introduction

Various assays to measure the proteolytic activity of proteinases from psychrotrophic bacteria are used by various workers, as reviewed, among others, by Fairbairn (1989) and Rollema et al. (1989). We used two assays: the azocasein method and the TNBS (trinitrobenzene sulfonic acid) method. In the first method azocasein is hydrolyzed by the proteinase, releasing red-coloured azopeptides, in the latter method sodium caseinate is digested and the newly formed amino groups are complexed with TNBS, resulting in a yellow colour.

When investigating the inactivation of proteinases, an essential property of the proteolytic activity assay should be that the activity measured is proportional to the actual proteinase concentration, or shows at least a simple constant relation. In this chapter the azocasein and TNBS methods will be evaluated with respect to our work on the heat inactivation of the proteinase from *Pseudomonas fluorescens* 22F.

4.2   Materials and methods

Enzyme production and purification

For the experiments the extracellular proteinase from *Pseudomonas fluorescens* 22F was used. Production and purification of the enzymes were performed as described in chapter 3. *Pseudomonas fluorescens* 22F was grown in a tryptone-lactose medium. After incubation of 8 days at 20 °C the cells were removed by centrifugation. Purification was done by ammonium sulfate precipitation, hydrophobic interaction chromatography, ultrafiltration and gel filtration. Purity was checked by SDS-PAGE.

TNBS method

The TNBS method for measuring the proteinase activity was performed according to McKellar (1982), with minor modifications. Samples of 2.5 ml, containing 1 ml of 2.5 % sodium caseinate in demineralized water, 1 ml of 0.2 M TrisHCl buffer containing 2 mM CaCl$_2$ (pH 7.0), and 0.5 ml of enzyme solution, were incubated for 90 min at 37 °C. The reaction was stopped by adding 4.0 ml of 0.72 N trichloroacetic acid (TCA) to a final concentration of 7.2 %. The precipitated casein was filtered using Schleicher & Schüll 589[5] red ribbon filter paper. The TCA-soluble amino groups in the filtrate were determined with TNBS (Fluka) as the reagent. Of the filtrate, 0.2 ml was added to 2.0 ml of 0.1 M sodium borate buffer (pH 9.2) and 0.8 ml of 5.0 mM TNBS solution (freshly prepared) and incubated for 30 min at 20 °C in the dark. The reaction was stopped by adding 0.8 ml of 2.0 M monobasic sodium phosphate containing 18 mM sodium sulfite (freshly prepared). The absorbance at 420 nm was measured against a blank, consisting of a not incubated reaction mixture with sodium caseinate. The absorbance was recalculated to μmol of TCA-soluble free amino groups using

standard solutions of glycine in TCA. One TNBS unit of enzyme activity was defined as the amount of enzyme required to release 1.0 µmol of TCA-soluble free amino groups per minute under standard assay conditions.

Azocasein method

The proteinase activity measurements with sulfanilamide-azocasein (Sigma) as a substrate was performed according to El-Sissi et al. (1982), with some modifications. The reaction mixture, containing 1.0 ml of 1.0 % azocasein in water, 1.0 ml of 0.2 M TrisHCl containing 2 mM CaCl$_2$ (pH 7.0) and 0.5 ml of enzyme solution, was incubated for 90 min at 37 °C. The reaction was stopped by adding 4.0 ml of 0.72 N TCA, and the precipitated azocasein was filtered using Schleicher & Schüll 589[5] red ribbon filter paper. The absorbance of the filtrate, containing the low molecular weight, red coloured azopeptides, was measured at 400 nm against a blank that had not been incubated.

4.3   Results and discussion

Supernatant containing the enzyme was diluted in 0.02 M TrisHCl buffer and assayed with the TNBS and azocasein method according to the standard procedures. The results are shown in figure 4.1. The activity measured with the TNBS method was linearly correlated with the enzyme concentration up to 2 µg / ml. At higher enzyme concentrations the absorbance was smaller than expected, possibly caused by depletion of substrate or limitations of equipment.

In another experiment the activity was determined as function of incubation time. Using the TNBS method the formation of TNBS-detectable free amino groups was measured, using the azocasein method the formation of azopeptides was followed measuring the absorbance at 400 nm (figure 4.2). The production of free amino groups per unit time was virtually constant during the experiment, meaning that the activity measured gives a fair indication of the initial velocity (Tipton, 1992). The absorbance of the blank was proportional to the dilution of the supernatant (figure 4.2), indicating that the supernatant contained substances, e.g. peptides and amino acids, causing absorbance. Also ammonium sulfate increased the absorbance in the TNBS method.

For the measurements with the azocasein method we observed a nonlinear relation between absorbance and concentration of proteinase, even at fairly low enzyme concentrations (figure 4.1). This was also found by Iversen and Jørgensen (1995). Kroll and Klostermeyer, however, did find a linear relation with the proteinase from *Pseudomonas fluorescens* 112 over a wide range of concentrations. The main difference between the method used here and that described by Kroll and Klostermeyer (1984) was the wavelength used. Kroll and Klostermeyer suggested a wavelength of 330 nm for clear solutions and 360 nm for solutions in which Maillard
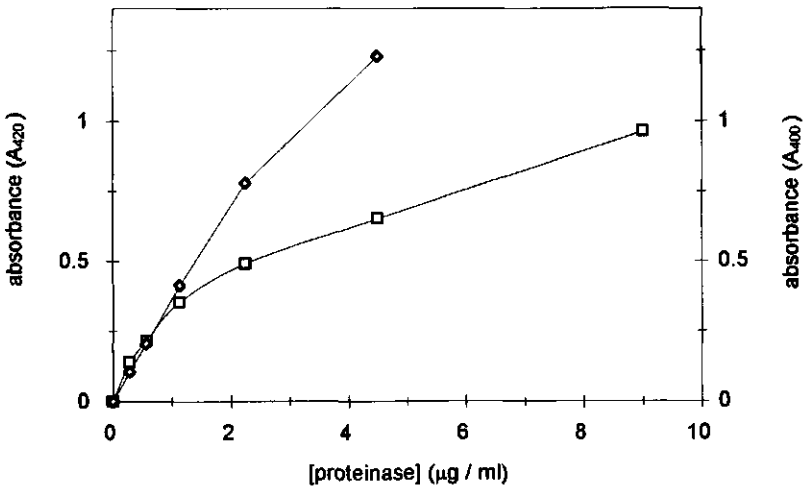
Figure 4.1:   Absorbance measured in the Azocasein and TNBS methods as a function of proteinase concentration. Azocasein: absorbance at 400 nm (□), TNBS: absorbance at 420 nm (◊).
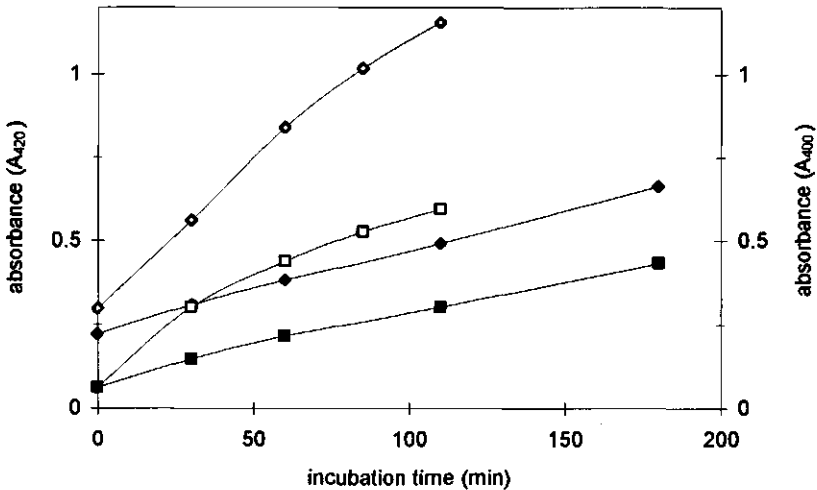
Figure 4.2:   Production of free amino groups detected by the TNBS method as function of incubation time, using 2.3 µg / ml (◊) and 0.6 µg / ml (♦) proteinase under standard conditions. Also shown is the production of azopeptides as measured at 400 nm as function of incubation time, using 2.3 µg / ml (□) and 0.6 µg / ml (■) proteinase under standard conditions .
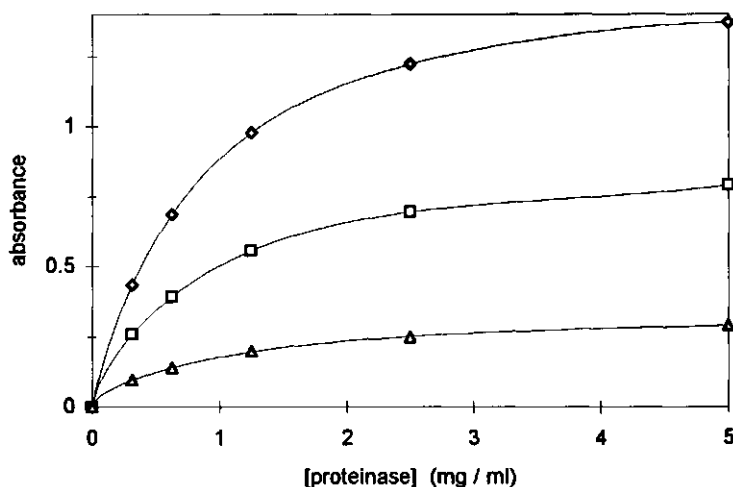
Figure 4.3:   Absorbance increase, measured with the azocasein method as a function of proteinase concentration at 360 nm (◊), 400 nm (□) and 440 nm (▲).

reactions have occurred. The maximal absorbance of digested and precipitated azocasein in TCA against 7 % TCA was at 330 nm (Kroll and Klostermeyer, 1984). Sarath et al. (1989) suggested a wavelength of 440 nm. We did experiments with the other suggested wavelengths (figure 4.3), but no linear relation was found. The absence of linearity makes it difficult to compare initial activity with residual activities after heating. Neither the dilution of the supernatant (figure 4.2), nor the presence of ammonium sulfate affected the blank value.

To determine the precision of the assays the activity of a purified enzyme solution was examined 10 times with the TNBS and the azocasein methods. The results are shown in table 4.1. The differences in absorbance (± standard deviation) between sample and blank for the TNBS and azocasein methods were 0.618 ± 0.009 (Coefficient of variation = 1.4 %) and 0.492 ± 0.010 (CV = 1.6 %), respectively.

The TNBS method appeared to be suitable for studying the heat inactivation of the proteinase from *Pseudomonas fluorescens* 22F. The response of the assay was proportional to the actual enzyme concentration, making calculation of the inactivation easy. Also the precision was acceptable. A disadvantage of the method is that it is time consuming and elaborate. Another disadvantage is the interference by substances with an amino group (e.g. amino acids and peptides present in the growth medium of *Pseudomonas fluorescens* 22F) and by ammonium sulfate (used in the purification of the proteinase), necessitating dialysis of some samples before activity can be measured.

Table 4.1:   Precision of TNBS and Azocasein method. Absorbances at 420 nm and 400 nm for TNBS method and azocasein method, respectively.

TNBS method		Azocasein method	
sample	blank	sample	blank
0.811	0.170	0.494	0.007
0.799	0.179	0.511	0.006
0.783	0.170	0.495	0.006
0.787	0.169	0.488	0.007
0.791	0.171	0.502	0.006
0.785	0.171	0.514	0.006
0.787	0.171	0.486	0.007
0.780	0.172	0.496	0.006
0.785	0.172	0.488	0.006
0.789	0.175	0.508	0.006

The azocasein method appeared not to be suitable for quantitative study, such as heat inactivation measurements of proteinases. Because the assay is less elaborate and time consuming, and not susceptible to interference of amino acids, peptides and ammonium sulfate, it can be used for qualitative work, such as checking the presence of proteolytic activity, for instance in the fractions of column chromatography.

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Chapter 5

Kinetics of heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F at 35 - 70 °C

Abstract

The extracellular proteinase from *Pseudomonas fluorescens* 22F was studied for its inactivation at 35 - 70 °C. The inactivation was modelled with computer simulation. From these simulations, it was concluded that the mechanism of inactivation could be explained by intermolecular autoproteolysis. The kinetic and thermodynamic parameters were also derived from computer simulations. The activation enthalpy $(\Delta H^{\ddagger})$ and entropy $(\Delta S^{\ddagger})$ of the unfolding reaction were 504 kJ mol^{-1} and 1252 J mol K^{-1}, respectively. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the refolding reaction were strongly temperature dependent. Sodium caseinate could stabilize against autoproteolysis. Small amounts of caseinate stabilized against unfolding of the proteinase, resulting in an increased denaturation temperature (T_d). Larger amounts of sodium caseinate, to a maximum of 1.5 %, prevented autoproteolysis by acting as a competitive inhibitor. Calcium stabilized the conformation of the enzyme molecule. Addition of 10 mM CaCl$_2$ to the enzyme solution increased T_d by approx. 4 K. EDTA did not influence T_d, but increased the rate of inactivation. o-Phenanthroline notably destabilized the proteinase, resulting in a decrease of T_d. The inactivation was dependent on the pH of the enzyme solution. The proteinase was more stable at pH 7.5, than at pH values 5.8, 7.0, and 9.6.

5.1   Introduction

Pseudomonas fluorescens, as well as several other psychrotrophic bacteria, are notorious for their secretion of thermostable proteinases (Cousin, 1982; Fairbairn and Law, 1986; Champagne et al., 1994; Shah, 1994). Even after ultra-high temperature heat treatment (e.g. 1 s at 145 °C) part of the proteolytic activity may be retained (Driessen, 1983; Kroll, 1989), so that defects in milk and milk products may occur.

Besides the thermostability of the proteinases from *Pseudomonas fluorescens* at high temperatures, a relatively high susceptibility towards thermoinactivation between 40 and 70 °C has been reported (Barach et al., 1976, 1978; Griffiths et al., 1981; Stepaniak and Fox, 1983, 1985; Kroll and Klostermeyer, 1984; Christen and Marshall, 1984, 1985; Mitchell et al., 1986; Diermayr et al., 1987; Stepaniak et al., 1987, 1991; Patel and Bartlett, 1988; Fortina et al., 1989; Kumura et al., 1993; Owusu and Doble, 1994). This inactivation is generally referred to as low temperature inactivation (LTI), because the mechanism of inactivation appears to be different from inactivation at temperatures higher than 80 °C (chapter 6 of this thesis). It should be noted that low temperature inactivation has nothing to do with cold denaturation of proteins (Privalov, 1990). One of the possible explanations for this increased inactivation is the occurrence of intermolecular autoproteolysis, which is the hydrolysis of partially unfolded proteinase molecules by its own type, as reported for many other proteolytic enzymes (Kunitz and Northrop, 1934; Kawamura et al., 1981; Adler-Nissen, 1986; Fontana, 1988; Owusu and Doble, 1994). Other possible inactivation mechanisms include intramolecular autoproteolysis, where one partly unfolded molecule would inactivate itself (Stepaniak and Fox, 1983), and aggregation with milk protein (Barach et al., 1978; Stepaniak et al., 1991). In this chapter the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* strain 22F at low temperature will be described.

5.2   Theory

Intermolecular autoproteolysis model

Autoproteolytic inactivation has been reported for various proteinases. Kunitz and Northrop (1934) already proposed a model describing the inactivation behaviour of trypsin, based on intermolecular autoproteolysis. This model has also been applied to other proteinases, sometimes in a slightly different form (Kumar and Hein, 1970; Kawamura et al., 1981; Adler-Nissen, 1986; van Boekel, 1993; Owusu and Doble, 1994). Kunitz and Northrop (1934) assumed a two-state nature

of the unfolding/refolding transition. In general, native globular protein molecules can not be digested by proteinases. In order to be susceptible to proteolytic digestion the protein molecule must unfold. Under certain conditions, for instance at certain pH values or near the denaturation temperature T_d, both native and unfolded enzyme molecules will be present in substantial amounts in the solution. If the protein considered is a proteinase, the unfolded proteinase molecules will be digested by the native ones under these conditions. The inactivation mechanism can be described by the following two reactions:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \overset{k_i}{\rightarrow} I \tag{5.1}$$

$$N + U \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} NU \overset{k_d}{\rightarrow} N + I_1 + I_2 \tag{5.2}$$
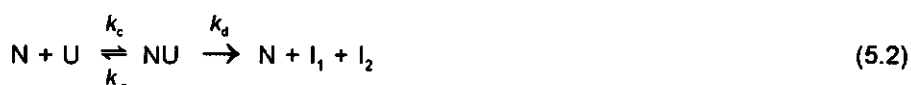
In the first reaction sequence, the unfolding/refolding transition and the irreversible transition to an inactivated state, are described, where N, U and I represent the native, unfolded and inactivated enzyme molecule, respectively, and k_u, k_f, and k_i the rate constants for the unfolding, refolding and thermal inactivation reactions, respectively. In this reaction scheme the reaction causing thermal inactivation, described in further detail in chapters 1 and 6 of this thesis, is included, but in the case of the extracellular proteinase from *Pseudomonas fluorescens* 22F the rate of this reaction is negligibly small in the temperature range studied here (chapter 6 of this thesis). In the second reaction sequence, which is the actual autoproteolysis reaction, the digestion of the unfolded molecule by the native proteinase takes place. Here, k_c, k_{-c}, and k_d are the rate constants of the formation of the complex NU, its reverse reaction, and the digestion, respectively, and I_1 and I_2 the products of autoproteolysis, being the fragments of the enzyme molecule. The autoproteolysis reaction may continue, resulting in even more fragments of the enzyme molecule. The fragments of the proteinase produced by autoproteolysis are catalytically inactive.

The autoproteolytic breakdown of proteinases (eq. 5.2) has been described with Michaelis-Menten kinetics (Adler-Nissen, 1986; Owusu and Doble, 1994). Adler-Nissen (1986) assumed one of the hydrolysis steps to be rate-limiting in the proteolytic inactivation of proteinases (k_d), in conformity with the degradation of protein substrate. Then, the overall reaction rate of autoproteolysis would be:

$$v = k_d [NU] \tag{5.3}$$

Assuming steady-state for the concentration of the complex of native and unfolded species, and assuming that [U] is so small that it may be neglected in comparison with the Michaelis-Menten constant K_m ($K_m = (k_c + k_d) / k_c$), the reaction rate would become:

$$v \ = \ \frac{k_d}{K_m} \ [N] \, [U] \tag{5.4}$$

It is questionable whether the assumption of a rate-limiting hydrolysis step holds in the case of autoproteolysis of proteinases, as this was found in systems where the concentration of substrate was very much higher than the enzyme concentration. In dilute systems it seems more likely that the formation of the complex native-unfolded proteinase is rate limiting. Also in more concentrated solutions of proteinases this would be true, if the unfolding/refolding transition is far from equilibrium. The rate of autoproteolysis may then be written as:

$$v \ = \ k_c \ [N] \, [U] \tag{5.5}$$

In both equations 5.4 and 5.5 the rate of the autoproteolysis reaction is determined by a bimolecular reaction, either with reaction rate constant k_d / K_m or k_c. Therefore equation 5.2 may be simplified to:

$$N + U \ \xrightarrow{k_a} \ N + I_1 + I_2 \tag{5.6}$$

$$v = k_a \, [N] \, [U] \tag{5.7}$$

where k_a is the apparent rate constant for autoproteolysis.

Because intermolecular autoproteolysis (eq. 5.7) is a bimolecular reaction between species at low concentration, second-order kinetics may seem obvious, as indicated, amongst others, by Diermayr et al. (1987). However, it should be noted that the overall reaction rate is determined by three simultaneously proceeding reactions, viz. unfolding, refolding and autoproteolysis. Two situations can be distinguished. In the first situation, the autoproteolytic reaction is much faster than the unfolding reaction ($k_a \gg k_u$). This may occur when the enzyme concentration is relatively high. U is digested as soon as it is formed, and its concentration is very low in steady-state. The reaction rate of autoproteolysis will then be given by:

$$v \ = \ k_u \, [N] \tag{5.8}$$

meaning that the overall inactivation is first-order with respect to N. In the second situation the unfolding reaction is much faster than the autoproteolysis ($k_u \gg k_a$), and the inactivation rate is given by eq. 5.7.

In general, a two-state denaturation model may be sufficient to describe the phenomena taking place with protein unfolding (Tanford, 1968, 1970; Lapanje, 1978). However, it has been shown that intermediates may be formed before the cooperative transition leads to a complete unfolded protein molecule. These intermediates, which may be catalytically active, can be susceptible to proteolysis (Mihalyi, 1972; Tsou, 1995). Consequently, the mechanism of autoproteolytic inactivation may be more complex:

$$N \underset{k_{f,1}}{\overset{k_{u,1}}{\rightleftharpoons}} N^+ \underset{k_{f,2}}{\overset{k_{u,2}}{\rightleftharpoons}} U \overset{k_i}{\rightarrow} I_1 + I_2 \tag{5.9}$$

$$N + N^+ \overset{k_{a,1}}{\rightarrow} N + I_1 + I_2 \tag{5.10a}$$

$$N + U \overset{k_{a,2}}{\rightarrow} N + I_1 + I_2 \tag{5.10b}$$

$$N^+ + N^+ \overset{k_{a,3}}{\rightarrow} N^+ + I_1 + I_2 \tag{5.10c}$$

$$N^+ + U \overset{k_{a,4}}{\rightarrow} N^+ + I_1 + I_2 \tag{5.10d}$$
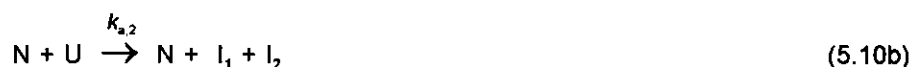
$$v = k_{a,1}[N][N^+] + k_{a,2}[N][U] + k_{a,3}[N^+]^2 + k_{a,4}[N^+][U] \tag{5.11}$$

where N^+ is a partly unfolded, but still catalytically active proteinase molecule, and $k_{u,x}$, $k_{f,x}$, and $k_{a,x}$ the rate constants for the unfolding, refolding, and autoproteolysis reactions, respectively. Inactivation can occur via digestion of both N^+ and U by N and N^+.

Intramolecular autoproteolysis model

Stepaniak and Fox (1983) proposed a model in which inactivation is caused by intramolecular autoproteolysis. In this model the enzyme is assumed to unfold in more than one step, as in eq. 5.9. A first unfolding step would result in a proteinase molecule that is partly unfolded but still catalytically active (N^+). A second unfolding step would make the proteinase inactive (U). The partially unfolded proteinase

molecule is supposed to inactivate itself. A flexible loop, which is unfolded, is attacked by the catalytic site of the same molecule, causing inactivation of the proteinase. The model is shown schematically as:

$$N \;\; \underset{k_{f,1}}{\overset{k_{u,1}}{\rightleftharpoons}} \;\; N^+ \;\; \underset{k_{f,2}}{\overset{k_{u,2}}{\rightleftharpoons}} \;\; U \;\; \overset{k_i}{\rightarrow} \;\; I \qquad\qquad (5.9)$$

$$N^+ \;\; \overset{k_a}{\rightarrow} \;\; I_1 + I_2 \qquad\qquad (5.12)$$
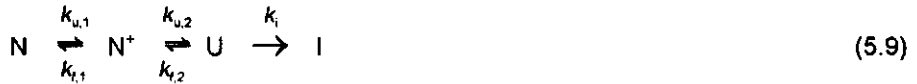
Here, k_a represents the rate constant for intramolecular autoproteolysis.

The intramolecular autoproteolytic inactivation is a monomolecular process, and because the inactivation is expected to be independent of the enzyme concentration, it may be described with first-order kinetics. When examining the inactivation at low temperature with SDS-PAGE or gel filtration, one would expect to find a fragment of the proteinase of considerable molar mass (Diermayr et al., 1987).

Aggregation model

A third mechanism of low temperature inactivation of pseudomonal proteinases in milk, based on aggregation of the proteinase to casein, was proposed by Barach et al. (1978). When C^{14}-labelled proteinase from *Pseudomonas* MC60 was heated for 10 min at 55 °C in milk, it was found that the amount of radio-active label associated with the casein increased from 12 % in the unheated sample, to 51 % in the heated sample. Upon heating the enzyme molecule unfolds, exposing more of its hydrophobic side groups to the solvent. The unfolded molecule is assumed to form an inactive enzyme-casein complex as a result of hydrophobic interactions:

$$N \;\; \underset{k_f}{\overset{k_u}{\rightleftharpoons}} \;\; U \;\; \overset{k_i}{\rightarrow} \;\; I \qquad\qquad (5.1)$$

$$U + \text{casein} \;\; \overset{k_{aggr.}}{\rightarrow} \;\; \text{proteinase-casein complex} \qquad\qquad (5.13)$$

The enzyme-casein complex could be deaggregated by treatment with 6 M urea, causing 20 to 35 % reactivation of proteolytic activity if high enzyme concentrations were used, and almost complete reactivation if low enzyme concentrations were used (Barach et al., 1978). Stepaniak et al. (1991), using ELISA techniques,

confirmed this mechanism of inactivation for the proteinase from *Pseudomonas fluorescens* P1. When this proteinase was heated in milk at 55 °C, intact molecules of proteinase were associated to the casein, forming an inactive complex. Higher concentrations of casein resulted in higher fractions of enzyme associated with the casein. On the other hand, it was also shown that the presence of increasing concentrations of casein could increasingly reduce the inactivation at 55 °C (Stepaniak and Fox, 1983; Stepaniak et al., 1991).

In this section, three possible mechanisms of low temperature inactivation were described. The mechanisms are different with respect to their kinetics. With kinetic modelling it may be possible to distinguish between these mechanisms of inactivation (section 5.4.1). Furthermore, the dependence of the inactivation on protein content and purification (section 5.4.2), metal ions (section 5.4.3), and pH (section 5.4.4) is described.

5.3   Materials and methods

Enzyme production and purification

For the experiments, the extracellular proteinase from *Pseudomonas fluorescens* 22F was used. Production and purification of the enzymes were performed as described in chapter 3 of this thesis. *Pseudomonas fluorescens* 22F was grown in skimmed milk, or tryptone-lactose medium (see section 3.2). After incubation of 8 days at 20°C, the cells were removed by centrifugation. Purification was done by ammonium sulfate precipitation, hydrophobic interaction chromatography, ultrafiltration and gel filtration. Purity was checked with SDS-PAGE.

Proteinase assay

For the determination of the proteolytic activity the TNBS method, as described in chapter 4 of this thesis, was used. The residual activity was defined as the fraction of the initial activity left after heat treatment.

Calcium ion activity

Calcium ion activity was measured using a calcium ion selective electrode (Orion, model 93-20), calibrated with 10^{-2} - 10^{-5} M $CaCl_2$, according to Geerts et al. (1983).

HPLC

HPLC measurements were performed using a Kontron 414 pump (fixed loop, 20 µl), a Marathon autosampler, a Kratos Spectroflow 757 UV-visible variable wavelength detector, and TSP PC1000 computer software. A 220 x 13 mm

Superdex 75 HR 10/30 size exclusion chromatography column (Pharmacia) was used. The eluent was 10 mM phosphate buffer pH 7.0, at a flow rate of 1.2 ml/min. Detection was performed at 214 nm.

DSC

Differential scanning calorimetry was performed using a Setaram Micro DSC. Samples of 0.9 ml 1.07 mg/ml purified proteinase in 0.02 M TrisHCl in 10 mM o-phenanthroline (oPA), pH 7.0, were heated from 20 to 110 °C at a scanning rate of 0.5 K min^{-1}. 0.02 M TrisHCl in 10 mM oPA was used as a reference. The enthalpy change of unfolding ΔH° and denaturation temperature T_d were determined according to Privalov (1979).

Fitting of models to the results

Programs written in TurboPascal 4.0, run on a IBM-compatible 80486 computer, were used to fit the autoproteolysis models to the experimental data. Ordinary differential equations (ODE's) were derived for the intermolecular and intramolecular autoproteolysis models, (eqs. 5.14 and 5.15, respectively), and were integrated numerically using Gear's algorithm for stiff ODE's (Stabler and Chesick, 1978; Chesick, 1988). Rate constants were found using Powell's direct search for nonlinear weighted least squares (Lobo and Lobo, 1991). The objective function to be minimized was the χ^2 stochastic.

Calculations for fitting the model for stabilization by sodium caseinate to the results were performed in Microsoft Excel 5.0. ODE's (eqs. 5.23 and 5.24) were integrated numerically, using a first-order Euler algorithm. The model was analyzed 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.

5.4   Results and discussion

5.4.1 Mechanism and kinetics of inactivation

The first target of the work was to establish whether the extracellular proteinase from *Pseudomonas fluorescens* 22F was inactivated in the temperature range 35 to 70 °C. For that purpose supernatant containing the proteinase, from a culture of *Pseudomonas fluorescens* 22F grown in skimmed milk, was diluted in 0.2 M TrisHCl pH 7.0, and heated for 30 min at various temperatures. In figure 5.1 the residual activity after heat treatment is given. Above 70 °C the rate of inactivation increased with temperature as expected according to Eyring's theory.
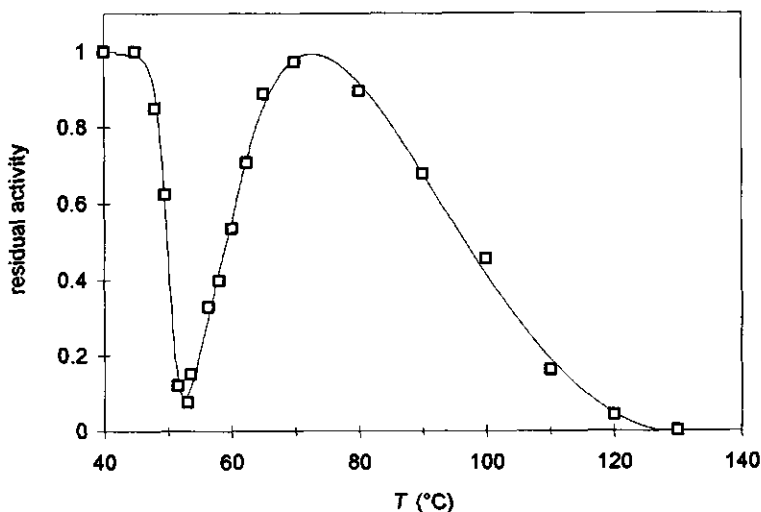
Figure 5.1:   The residual proteolytic activity of the enzyme preparation after 30 minutes of heating at the indicated temperatures.

Inactivation at temperatures above 70 °C will be discussed in chapter 6. Between 40 and 70 °C the extent of inactivation was relatively high, obviously because the mechanism of inactivation was different from the inactivation at high temperature. When the enzyme solution was heated at 70 °C, almost no inactivation occurred. Because at 70 °C no proteolytic activity was observed (chapter 3), it could be concluded that virtually all non-degraded enzyme molecules refolded to the native conformation following cooling after heat treatment.

In order to find out whether this high loss of activity was caused by autoproteolysis or by another mechanism, the inactivation was followed by HPLC. Purified enzyme in 0.02 *M* TrisHCl, pH 7.0, was heated at 55.2 °C, and after 0, 20, 40, 60, and 80 s cooled in ice water. Samples were run on a gel filtration column. The proteinase eluted at 8.3 min, corresponding to an apparent molar mass of approximately 45 kDa. During the heat treatment the amount of enzyme was rapidly reduced. This reduction was proportional to the loss in proteolytic activity. At the same time new peaks eluted at 13.2 and 16.0 min, roughly corresponding with molar masses of 2 and 1 kDa, respectively, indicating that the proteinase molecules were digested into small fragments. From these results it can be concluded that autoproteolysis caused at least part of the inactivation in the temperature range 40 - 70 °C. The formation of small fragments suggests that inactivation is caused by intermolecular autoproteolysis, rather than by intramolecular autoproteolysis.
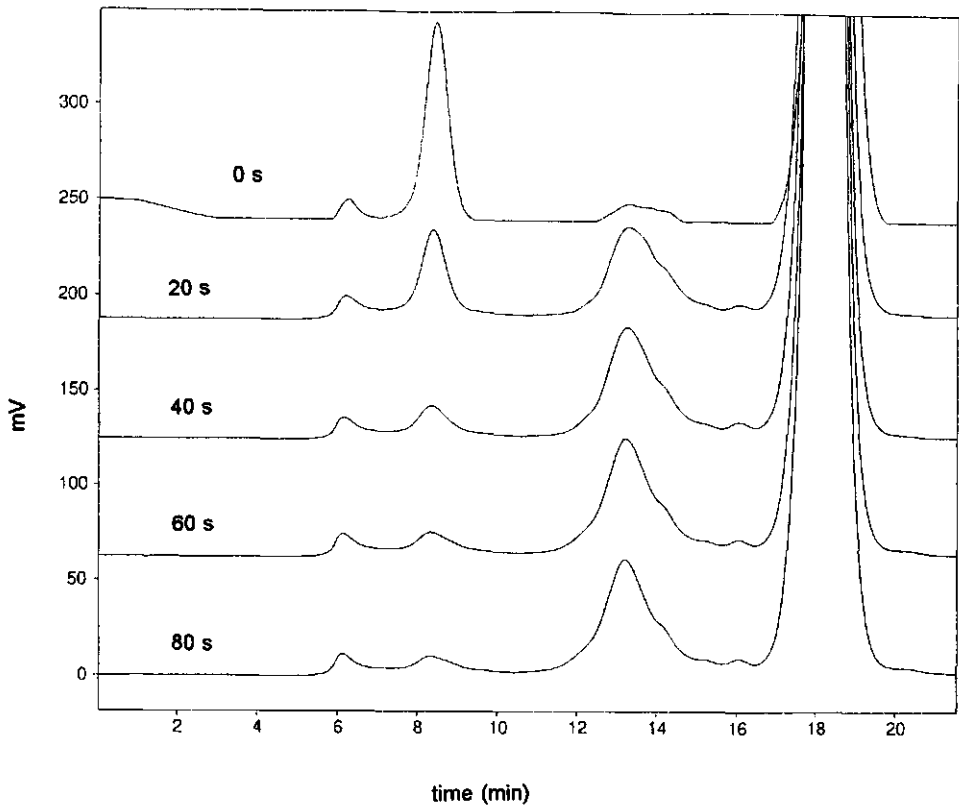
Figure 5.2:   HPLC chromatograms of purified enzyme solution heated at 55.2 °C. The proteinase
eluted at 8.3 min, the autoproteolysis products at 13.2 and 16.0 min. The large peak
at 18 min is caused by elution of Tris. Detection at 214 nm; column: Superdex 75
HR 10/30 (Pharmacia); eluent: 10 mM phosphate buffer; flow rate: 1.2 ml/min.

We tried to discriminate between intra- and intermolecular autoproteolysis by
kinetic modelling. Two types of experiments were planned. In the first experiment,
crude supernatant from a culture of *Pseudomonas fluorescens* 22F in skimmed
milk was diluted 10 times in demineralized water, and heated in a thermostatted
water bath. The pH of these enzyme preparations was 7.0. The enzyme
concentration was approximately $1 \cdot 10^{-7} M$ ($\cong$ 5 µg/ml). To overcome the problem of
heating-up periods, the supernatant was diluted with water of a temperature just
above the desired temperature. The resulting temperature was recorded and kept
constant for the period of the experiment. After the desired heating time, samples

were drawn from the enzyme solution, cooled rapidly in ice water, and the residual activity was measured. In the second experiment the same enzyme preparation was preheated for 10 s at 100 °C, and subsequently held at the desired temperatures. After the desired heating time, samples were drawn from the enzyme solution, cooled rapidly in ice water, and the residual activity was measured.

In the case of intermolecular autoproteolysis, it was assumed that in the beginning of the first experiment all enzyme molecules were in their native form (N). After bringing the enzyme solution to the desired temperature, the native enzyme N starts to unfold to form U at an initial reaction rate $v = k_u$ [N]. Then, U can be digested by the native N (if still present), to form the inactive fragments I_1 and I_2 at a reaction rate $v = k_a$ [N][U] (eq. 5.7). In the second experiment a complete transformation of the proteinase to its unfolded form U is induced by a heat treatment of 10 s at 100 °C. When the enzyme solution is brought to the desired temperature, U will refold to N at an initial reaction rate of $v = k_f$ [U], and subsequently will be inactivated with a reaction rate $v = k_a$ [N][U]. The intermolecular autoproteolysis model was simulated and fitted to the experimental data, using the following system of nonlinear differential equations:

$$\frac{d[N]}{dt} = -k_u[N] + k_f[U] \tag{5.14a}$$

$$\frac{d[U]}{dt} = k_u[N] - k_f[U] - k_a[N][U] \tag{5.14b}$$

$$\frac{d[I_1 + I_2]}{dt} = -\frac{d(\text{residual activity})}{dt} = k_a[N][U] \tag{5.14c}$$

with residual activity (t) = [N] (t) + [U] (t), as U will refold upon cooling. Initial conditions were assumed to be [N] (0) = 1 and [U] (0) = 0 for the first experiment, and [N] (0) = 0 and [U] (0) = 1 for the second.

In the case of intramolecular autoproteolysis, it was also assumed that in the beginning of the first experiment all enzyme molecules were in their native form (N). After bringing the enzyme solution to the desired temperature, the native enzyme N starts to unfold to the partially unfolded, but still active form N⁺, at an initial reaction rate $v = k_u$ [N]. Subsequently, N⁺ could inactivate itself, at a reaction rate $v = k_a$ [N⁺]. In the beginning of the second experiment all enzyme is assumed to be in unfolded form U. After cooling the enzyme solution to the desired temperature, U will refold to the partly folded, and active form N⁺, after which

inactivation can follow. The intramolecular autoproteolysis model was simulated and fitted to the experimental data, using the following system of nonlinear differential equations:

$$\frac{d\,[N]}{d\,t} = -k_{u,1}\,[N] + k_{f,1}\,[N^+] \tag{5.15a}$$

$$\frac{d\,[N^+]}{d\,t} = k_{u,1}\,[N] - (k_{f,1} + k_{u,2} + k_a)\,[\,N^+] + k_{f,2}\,[U] \tag{5.15b}$$

$$\frac{d\,[U]}{d\,t} = k_{u,2}\,[N^+] - k_{f,2}\,[U] \tag{5.15c}$$

$$\frac{d\,[I_1 + I_2]}{d\,t} = -\frac{d\,(\text{residual activity})}{d\,t} = k_a\,[N^+] \tag{5.15d}$$

with residual activity = $[N]\,(t) + [N^+]\,(t) + [U]\,(t)$, as N^+ and U will refold upon cooling. Initial conditions were assumed to be $[N]\,(0) = 1$ and $[N^+]\,(0) = [U]\,(0) = 0$ for the first experiment, and $[N]\,(0) = [N^+]\,(0) = 0$ and $[U]\,(0) = 1$ for the second.

A first impression of the results for this particular enzyme preparation is given in figure 5.3, in which the residual activity after 30 min of heating is given in an autoproteolysis curve. The extent of inactivation of the proteinase is determined by the reaction rates of the folding, refolding, and autoproteolysis reaction. According to the intermolecular autoproteolysis model, the temperature at which inactivation is maximal, roughly corresponds with the denaturation temperature T_d, as at this temperature the relative fractions of native and unfolded enzyme molecules are equal, and the product of the fractions maximal. Generally, this T_d will coincide with the temperature of the midpoint of a cooperative transition leading to a completely unfolded and inactive enzyme molecule. However, it may also mark a local transition, leading to disruption of the catalytic site of the enzyme. According to the intramolecular autoproteolysis model, inactivation is maximal when the relative fraction of N^+ is largest. Consequently, the temperature at which the inactivation is maximal, now determined by two unfolding equilibria, will be in between the two transition temperatures $T_{d,1}$ and $T_{d,2}$. The temperatures of maximal inactivation were 52 °C and 50 °C for the not-preheated and preheated enzyme, respectively. These temperatures are similar to denaturation temperatures found for other Pseudomonal proteinases (Barach et al., 1976; Leinmüller and Christophersen, 1982; Stepaniak et al., 1982; Stepaniak and Fox, 1983, 1985; Kroll and Klostermeyer, 1984; Diermayr et al., 1987; Patel and Bartlett, 1988; Owusu et al., 1991; Kumura et al., 1993; Owusu and Doble, 1994).
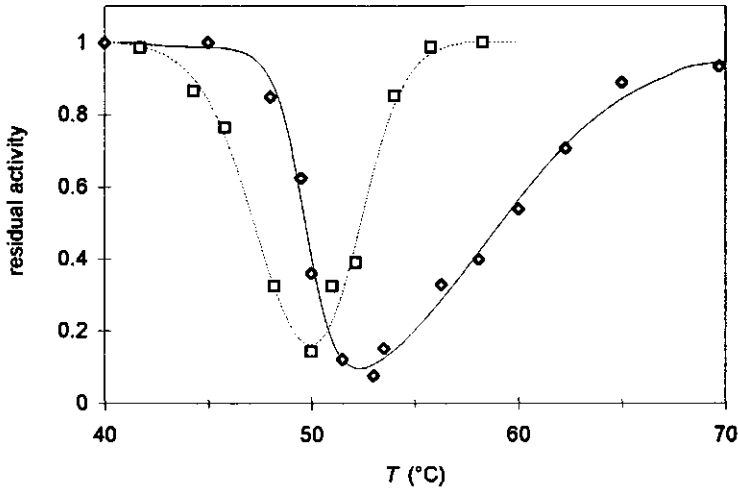
Figure 5.3:   Residual activity after holding the proteinase solution for 30 min at indicated temperature (pH 7.0), - - □ - - = preheated 10 s / 100 °C, —◇— = not preheated.

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

The effect of heating-up could also partly explain the asymmetrical curve in figure 5.3. This asymmetry is even more pronounced if no precautions are taken to shorten the heating-up period (e.g. experiments in sections 5.4.2 and 5.4.3). Other reasons for asymmetry of the autoproteolysis curve are the increase of the rate of

autoproteolysis with increasing temperature, and the finite values of the reaction rates for unfolding and refolding, as will be shown by computer simulation at the end of this section. Similar asymmetrical curves have been found by others (Barach et al., 1976; Leinmüller and Christophersen, 1982; Stepaniak et al., 1982; Kroll and Klostermeyer, 1984; Christen and Marshall, 1985; Patel and Bartlett, 1988).

At several temperatures the inactivation of the proteinase was followed in time, and both autoproteolysis models were tried to fit to the experimental results. At temperatures up to 50 °C both models could adequately describe the results. The unfolding reaction appeared to be rate limiting at these temperatures, resulting in a pseudo first-order inactivation for both models. Unfolded enzyme molecules are digested as soon as they are formed. In figure 5.4A, the results and fits are given for the experiment at 50 °C as an example. At higher temperatures the intermolecular autoproteolysis model could fit the results, while the intramolecular autoproteolysis model could not (see figure 5.4B for an example at 52 °C). Here, the autoproteolysis reaction would be more important. Obviously, an autoproteolysis model including a second-order reaction was needed to fit the results, indicating that the proteinase from *Pseudomonas fluorescens* 22F most likely was inactivated by intermolecular autoproteolysis, confirming the results of the HPLC experiments.

We determined the orders with respect to concentration and time for the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F. The order of a reaction indicates the empirical dependence of the reaction rate on concentration, and may also tell something about the mechanism of that reaction. The rate at which a concentration c of a single reactant changes with time can be written as a differential equation:

$$v = - \frac{dc}{dt} = k c^n \tag{5.16}$$

in which n is the order with respect to c. In our case, the change in residual activity with time is used for the determination of the reaction order. The orders of inactivation were determined using the differential method, as described in chapter 2 of this thesis. Supernatant from a culture in skimmed milk was diluted 10, 20, 50 and 100 times in 0.2 M TrisHCl containing 2 mM CaCl$_2$, pH 7.4. Samples of 1.25 ml were heated at 50 or 52 °C, cooled in ice water, and examined for the residual proteolytic activity with the TNBS method. The incubation time with sodium caseinate in the proteolytic assay was increased for the more diluted samples in order to obtain a large enough increase in absorbance at 420 nm. To determine

the order with respect to concentration $\tilde{n}_c$, the initial rates of inactivation were measured at the various concentrations, and log v was plotted versus log c (figure 5.5A). In the case of 52 °C, this resulted in a straight line. The slope (± 95 % confidence interval), being $\tilde{n}_c$, was estimated at 1.23 (± 0.33). A reaction order higher than 1 may suggest that inactivation by intermolecular autoproteolysis is more likely than intramolecular autoproteolysis at this temperature. At 50 °C, the relation was not linear. The inactivation appeared to be little dependent on the enzyme concentration at relatively high concentrations. This, again, may indicate that the unfolding reaction is rate limiting at this temperature. The inactivation of the 100 times diluted supernatant clearly deviated, but as stated in section 5.2, the rate of autodigestion may become increasingly important when lowering the enzyme concentration.

The order with respect to time $\tilde{n}_t$ was derived from the experiment with 10 times diluted supernatant. The rates of inactivation were measured at various times, corresponding to different concentrations of proteinase, and log v was plotted versus log c (figure 5.5B). At 50 and 52 °C, $\tilde{n}_t$ (± 95 % confidence interval) was 1.01 (± 0.12) and 1.23 (± 0.09), respectively. Again, this suggests inactivation by intermolecular autoproteolysis, with a rate limiting unfolding reaction at 50 °C, and a rate of inactivation determined by a combination of the unfolding and autoproteolysis reaction at 52 °C.

Although it was concluded that intermolecular autoproteolysis is most likely the cause of inactivation, it may be possible that also some intramolecular autoproteolysis is taking place. At sufficiently low enzyme concentrations it may be possible that intramolecular autoproteolysis is the sole cause of inactivation. Because it would be sterically difficult for a flexible loop of the molecule to be reached by the catalytic site, it seems reasonable to assume that at higher concentrations the flexible loops of other proteinase molecules will be attacked rather than its own. All in all, we did not found an indication for intramolecular autoproteolysis from our results. HPLC results, the reaction order, and computer simulations, all suggest that inactivation in the temperature range 40 - 70 °C is caused by intermolecular autodigestion.

From the inactivation experiments at several temperatures we then tried to derive the three reaction rate constants of the intermolecular autoproteolysis model, namely k_u, k_f, and k_a. It may be expected that several combinations of these reaction rate constants can fit the model to the results, since we were only able to determine the residual activity with the present method. However, preliminary calculations showed that the parameter k_u was quite critical for experiments with not preheated enzyme, while k_f was critical for experiments with preheated enzyme. Comparison of preliminary results indicated that parameter k_a usually showed about
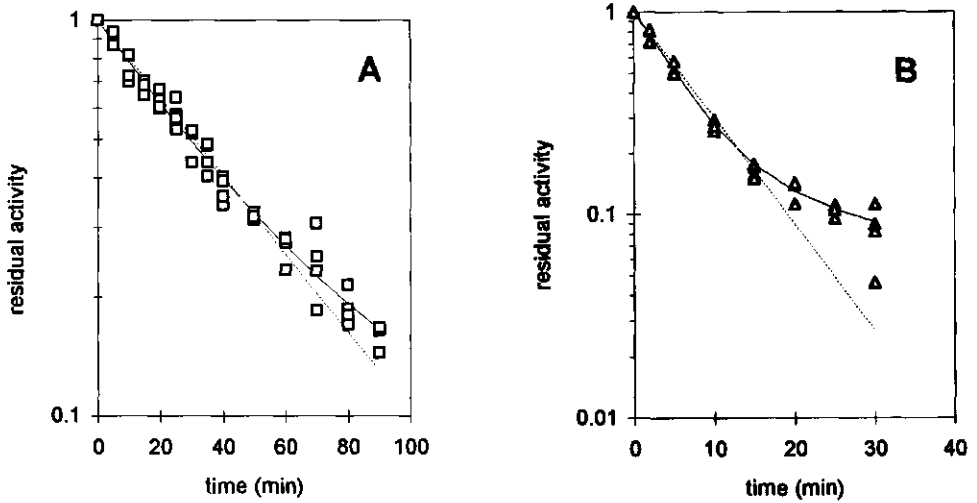
Figure 5.4:   Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F at
50 °C (□) and 52 °C (▲). Fits of intermolecular (———) and intramolecular
autoproteolysis model (- - - -) are included.
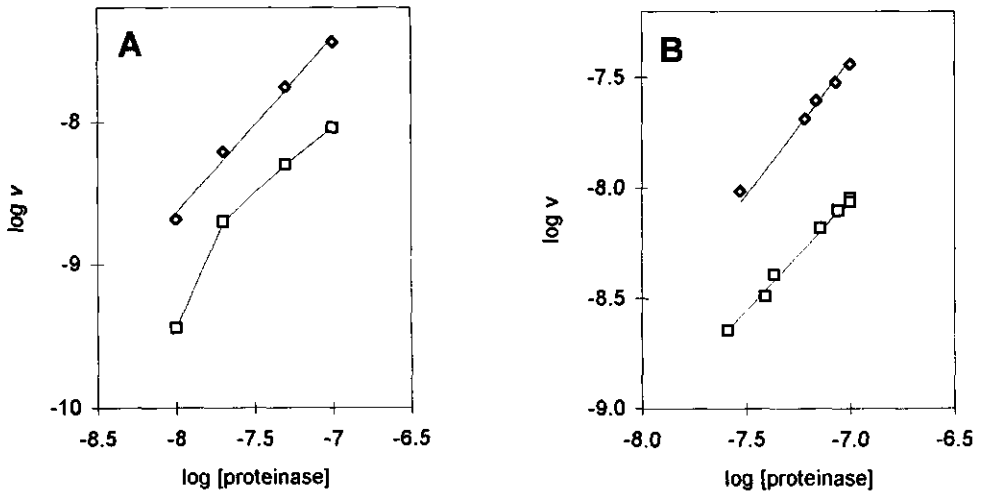
Figure 5.5:   Plot of log v versus log [proteinase], for various initial proteinase concentrations (A),
and for various proteinase concentrations measured in one run (B). □ = 50 °C;
◇ = 52 °C.

the same value for all the experiments, without much effect of the temperature. This is to be expected, as k_a reflects the proteolytic activity. Therefore the value of k_a was allowed to vary only within the range found in preliminary experiments, while forcing a Q_{10} of 2, as this was found for the digestion of sodium caseinate (chapter 3 of this thesis). In doing so, the parameter k_a was actually more or less fixed, so that the possibility for finding unique values for k_u and k_f was increased.

Table 5.1: Values of the reaction rate constants (± standard deviation) as derived from computer simulations (k_a was varied with Q_{10} = 2).

T (°C)	not preheated			preheated		
	$10^5\ k_u$ (s^{-1})	$10^5\ k_f$ (s^{-1})	$10^{-4}\ k_a$ ($M^{-1}\ s^{-1}$)	$10^5\ k_u$ (s^{-1})	$10^5\ k_f$ (s^{-1})	$10^{-4}\ k_a$ ($M^{-1}\ s^{-1}$)
48.0	12 ± 57	1833 ± 50	25	8 ± 2	233 ± 18	25
49.5	37 ± 2	233 ± 266	28			
50.0	52 ± 8	433 ± 1100	29	17 ± 17	38 ± 5	28
50.1				17 ± 7	25 ± 2	29
51.5	162 ± 6	233 ± 76	32			
52.0	283 ± 41	32 ± 90	33	200 ± 208	6 ± 2	33
52.9	200 ± 16	17 ± 73	35			
53.0	308 ± 100	33 ± 366	35			
54.0				500 ± 217	1 ± 15	38
56.3	2300 ± 683	38 ± 13	44	?	< 2	43
58.0	10500 ± 1480	142 ± 2	50			
60.0	14000 ± 2333	102 ± 17	57			
62.0	28100 ± 833	58 ± 5	66			

The results of the fitting procedure for the experiments of not-preheated and preheated enzyme are given in table 5.1. The standard deviations were taken from the covariance matrix, assuming that the errors were normally distributed (Press et al., 1990). The fits were acceptable from a statistical point of view: the reduced χ^2 ($\chi^2\ /\ \nu$) was in most cases about 1. In figure 5.6 some results of the experiments and simulations are presented.
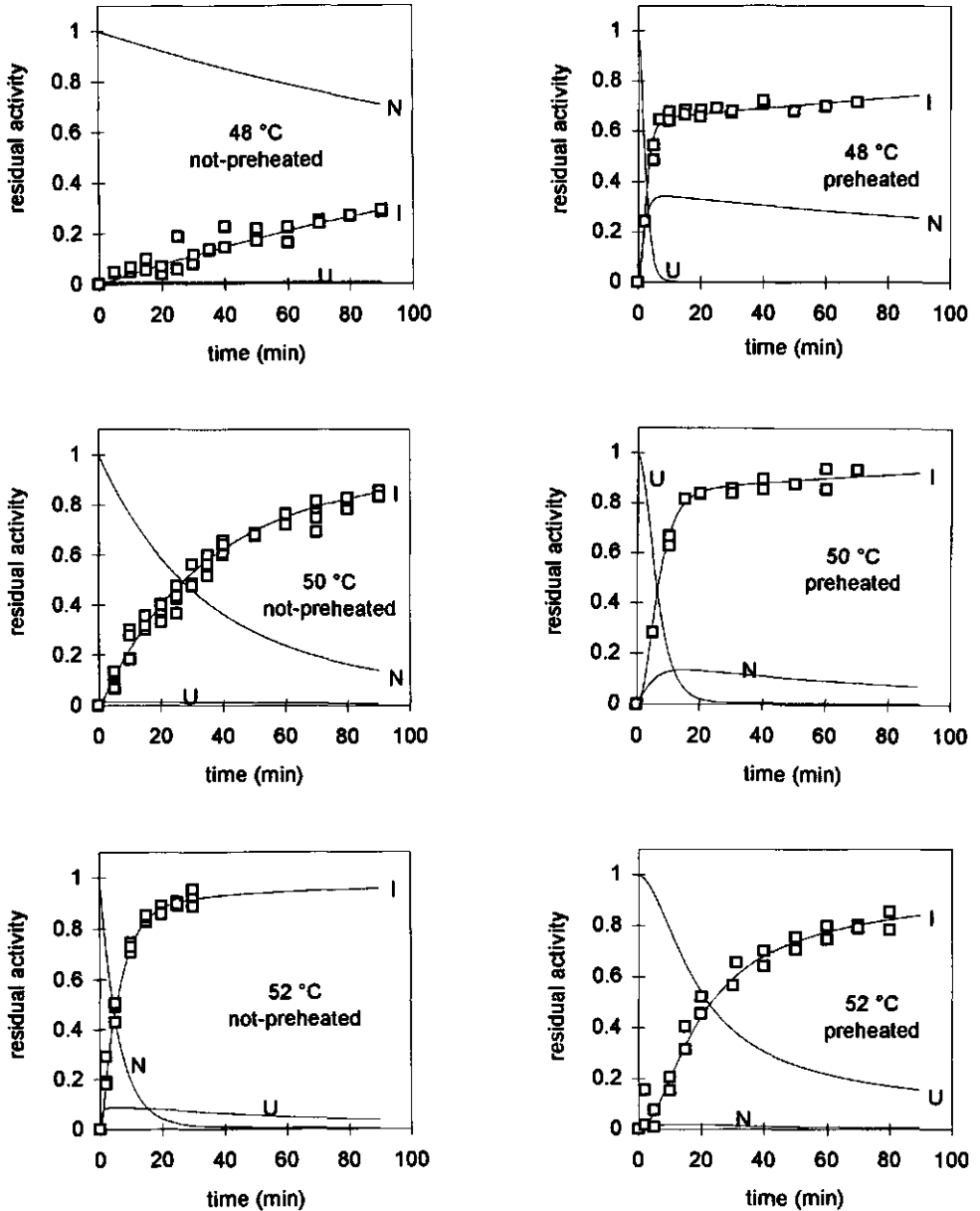
Figure 5.6:   Results of experiments (□) and simulations (——), using the intermolecular autoproteolysis model for the inactivation of the proteinase from *Pseudomonas fluorescens* 22F at 48.0, 50.0, and 52.0 °C.

The values for the rate of the unfolding reaction were found to be of the same order of magnitude for the preheated and not preheated enzyme. The rate constant sharply increased with the temperature, as is to be expected for an unfolding reaction. The temperature dependence of rate constants k_u and k_f was analyzed using the Eyring equation:

$$ k = \frac{k_b T}{h} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \tag{5.17} $$

where k_b is Boltzmann's constant $(1.38 \cdot 10^{-23}$ J K$^{-1})$, h is Planck's constant $(6.62 \cdot 10^{-34}$ J s$^{-1})$, and R is the gas constant $(8.31$ J mol^{-1} K$^{-1})$, $\Delta S^{\ddagger}$ the activation entropy, and $\Delta H^{\ddagger}$ the activation enthalpy. The Eyring plots for the unfolding reaction rate constants were linear (figure 5.7), as expected (Creighton, 1990). $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the unfolding reaction, estimated from the slope and intercept of the regression lines in the Eyring plot, are given in table 5.2.
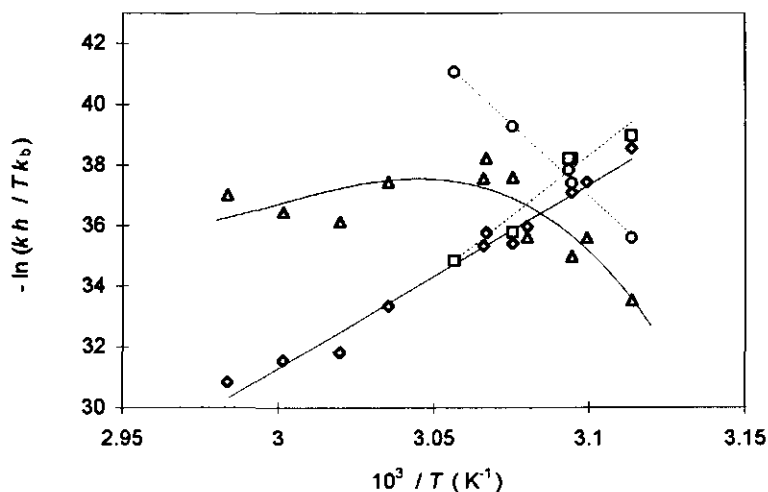
Figure 5.7:   Regression lines for the Eyring plot for reaction rate constants k_u and k_f from table 5.1: $\lozenge = k_u$, not preheated enzyme; $\triangle = k_f$, not preheated enzyme; $\square = k_u$, preheated enzyme; $\bigcirc = k_f$, preheated enzyme. h = Planck's constant; k_b = Boltzmann's constant.

Table 5.2:   Activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for the unfolding reaction ($\pm$ 95 % confidence interval)

	without preheating	with preheating
$\Delta H^{\ddagger}$ (kJ mol^{-1})	504 ($\pm$ 51)	665 ($\pm$ 291)
$\Delta S^{\ddagger}$ (J mol K^{-1})	1252 ($\pm$ 165)	1743 ($\pm$ 900)

The calculated activation enthalpy and entropy are quite normal for unfolding of proteins. The difference between the values for not preheated and preheated enzyme was not statistically significant.

Large differences were found for the values of the rate constants of the refolding reaction k_f between not-preheated and preheated enzyme. This implies that the temperature shift in the autoproteolysis curves of not preheated and preheated enzyme, as shown in figure 5.3, should be ascribed to this difference in k_f . In the case of not-preheated enzyme, k_f was of not much significance, as indicated by its very large standard deviations. The rate constant k_f decreased with temperature, suggesting that the activation enthalpy has a negative value. In the case of not-preheated enzyme, the Eyring plot of k_f was not linear: at temperature higher than 55 °C the rate of refolding increased again. It is not uncommon that non-linear Eyring plots are observed for the refolding reaction, however, in most cases the curvature is opposite. In those cases, the rate of refolding increases with temperature at low temperatures, as do most chemical reactions. The reaction rate of refolding reaches a maximum and than decreases again at higher temperatures. Two explanations have been proposed for this temperature dependence. The first explanation is that the difference in heat capacity between the folded and unfolded states, ΔC_p , due to a difference in hydration between the native and unfolded conformation, must be reflected in non-linear Eyring plots of the rates of unfolding or refolding (Creighton, 1990; Oliveberg et al., 1995). The second explanation is that refolding of protein molecules depend on the presence of meta-stable, partially folded intermediates, which would be increasingly destabilized at increasing temperature, thereby decreasing the rate of folding (Creighton, 1990). However, as said, the temperature dependence of the refolding reaction rate of the extracellular proteinase from *Pseudomonas fluorescens* 22F is different. It is not unlikely that the observed temperature dependence of refolding reaction is the result of inaccuracy of determination of k_f , due to the indirect method of determination.

From the values of the reaction rate constants for unfolding and refolding from table 5.1, a rough estimation may be made of the thermodynamic equilibrium

constant $K_d = k_f / k_u$ at various temperatures. Furthermore, the denaturation temperature T_d (T where $K_d = 1$) can be estimated. From the temperature dependence of K_d the enthalpy and entropy difference ($\Delta H°$ and $\Delta S°$) between the folded and unfolded state can be estimated using van 't Hoff's equation:

$$\ln K_d = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} \tag{5.18}$$

The calculated denaturation temperatures were 50.8 °C and 50.2 °C for not-preheated and preheated enzyme, respectively. These values correspond rather well with the estimates of T_d from figure 5.3. In figure 5.8 ln K_d is plotted versus the inverse temperature. As in the Eyring plot (figure 5.7), a non-linear temperature dependence is found for the not-preheated enzyme. In general, the enthalpy of unfolding increases with temperature due to the greater apparent heat capacity of U than of N. Again, the curvature we found was opposite. From the van 't Hoff plot $\Delta H°$, $\Delta S°$, and $\Delta G°$ may be estimated, but it must be considered that the estimated values of thermodynamic parameters will not be accurate, due to large uncertaincies in k_u and k_f. $\Delta H°$ and $\Delta S°$ were estimated from the slope of the van 't Hoff plot at temperature T_d, where ln $K_d = 0$, $\Delta G° = 0$, and $\Delta S° = \Delta H° / T_d$. Then, $\Delta G°$ was computed at 298 K. In table 5.3 the thermodynamic parameters for the unfolding reaction for the not-preheated and preheated enzyme are given. The values for the thermodynamic parameters were very large compared with those of other pseudomonal proteinases or enzymes in general (Owusu et al., 1991; Privalov, 1979). Presumably, the present indirect method of determining the thermodynamic parameters by using the reaction rates of unfolding and refolding is not very useful, due to inaccuracy in the determination of the kinetic parameters.

Table 5.3:   Thermodynamic parameters for the unfolding reaction of the extracellular proteinase from *Pseudomonas fluorescens* 22F.

	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})	$\Delta G°_{298 K}$ (kJ mol^{-1})	T_d (K)
not-preheated	1128	3481	91	324.0
preheated	1453	4493	114	323.3
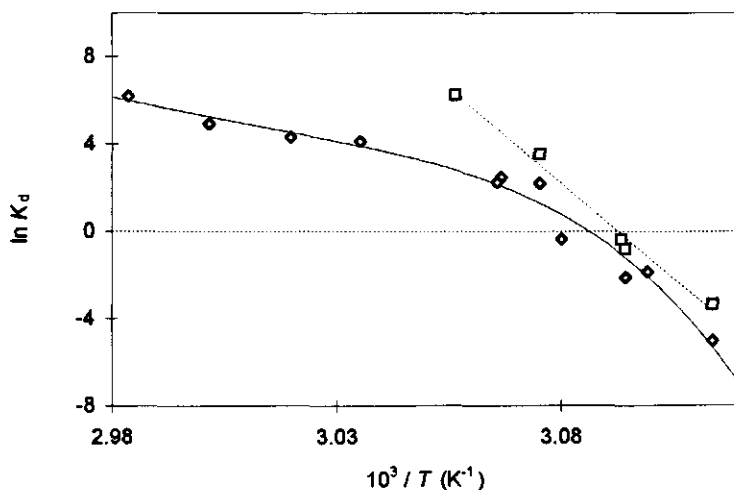
Figure 5.8:   Van 't Hoff plot of ln K_d versus inverse temperature for not-preheated (◊) and
preheated proteinase from *Pseudomonas fluorescens* 22F (☐).

We also tried to determine the enthalpy change of unfolding by differential
scanning calorimetry. In order to prevent autoproteolysis during the run, 10 mM o-
phenanthroline (oPA), an inhibitor of the proteinase (chapter 3 of this thesis), was
added to the enzyme preparation. An enthalpy change of 435 kJ mol^{-1} was found,
which is a more reasonable value than the estimates from the indirect method
described above. However, due to the addition of oPA the denaturation
temperature shifted from 51 °C to approximately 44 °C, indicating a decreased
conformational stability of the proteinase by removal of Zn^{2+}. This destabilization by
oPA was also observed in the autoproteolysis curve (figure 5.13, section 5.4.3).
Measurement of the stability of proteinases, especially metalloproteinases where
the metal ion is involved in both catalysis and conformational stability, with
differential scanning calorimetry seems to be impossible. Optical methods, like
circular dichroism or fluorescence spectrometry, will face the same problem.
Therefore, determination of thermostability of proteinases under physiological
conditions by autoproteolysis may be useful to circumvent this problem.

Having established the mechanism of inactivation and estimated the rate
constants of the unfolding, refolding, and autoproteolyis reactions, the position and
shape of the autoproteolysis curves can be explained. A symmetrical
autoproteolysis curve with a minimum at T_d will be expected, if it is assumed that
the enzyme solution is instantaneously brought to the desired temperature, that the
equilibrium between N and U is immediately established, and that the reaction rate
constant of the autoproteolytic reaction is independent of temperature (figure 5.9).

However, these assumptions are not realistic, and therefore, we performed computer simulations in order to evaluate the effects of these complicating factors. For the calculation we used the estimates of the reaction rate constants given in table 5.1.

Firstly, we evaluted the influence of the temperature dependence of k_a on the position and shape of the autoproteolysis curve. In this simulation, we assumed infinitely fast heating-up and equilibrium between N and U. Then, we calculated the residual activity after heating $1 \cdot 10^{-7}$ *M* and $1 \cdot 10^{-8}$ *M* proteinase solutions for 30 min at various temperatures. In one simulation, k_a was set at $3 \cdot 10^5$ *M*$^{-1}$ s^{-1}, in a second simulation k_a was varied with temperature with a Q_{10} of 2. The results of the simulations are shown in figure 5.9. The autoproteolysis curves calculated for a fixed k_a were symmetrical, as expected, whereas the autoproteolysis curves calculated for a temperature dependent k_a were slightly asymmetrical, because the inactivation rate increased with increasing temperatures.

It is not realistic to assume that the equilibrium between N and U is immediately established, as the reaction rate constants for unfolding and refolding have finite values. In a second simulation, estimates for the rate constants of the unfolding and refolding reactions from table 5.1 were used, and calculations were made to estimate the extent of inactivation induced by heating $1 \cdot 10^{-7}$ *M* and $1 \cdot 10^{-8}$ *M* proteinase solutions for 5, 15 and 30 min at various temperatures. The results of the simulations are shown in figure 5.10. The minimum of the autoproteolysis curves shifted towards higher temperatures, and the shape became even more asymmetrical. Obviously, this effect is most distinct if the heating time is relatively short, and the enzyme concentration is high. Consequently, the minima of autoproteolysis curves are only a rough indications of the T_d, and may only be compared if experimental conditions are equal. If the simulation was performed while assuming that in the beginning of the experiment all enzyme molecules were unfolded, and the minimum of the autoproteolysis curve was shifted towards lower temperatures. This would explain the difference between the autoproteolysis curves in figure 5.3.

Finally, the effect of heating-up on the autoproteolysis curve was simulated. Now, it was assumed that the enzyme solution was not brought to the desired temperature instantaneously, but that the temperature had to pass through a heating-up profile (Hiddink, 1975; chapter 6 of this thesis). The extent of inactivation induced by heating $5 \cdot 10^{-7}$ *M*, $1 \cdot 10^{-7}$ *M*, and $1 \cdot 10^{-8}$ *M* proteinase solutions for 30 min at various temperatures, was calculated. The results are shown in figure 5.11. The extent of inactivation at temperatures above T_d was relatively high, leading to strongly asymmetrical autoproteolysis curves. The effect was increasingly distinct with increasing enzyme concentration. It may be concluded that the shapes and positions of the autoproteolysis curves in figure 5.3 are in accordance with the intermolecular autoproteolysis model.
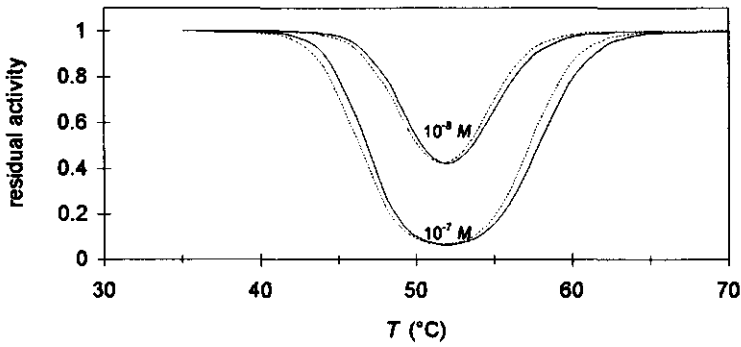
Figure 5.9:   Simulated autoproteolysis curves: - - - - - $k_a = 3 \cdot 10^{-5}\ M^{-1}\ s^{-1}$; ———— k_a as in table 5.1; further assumptions: see text.
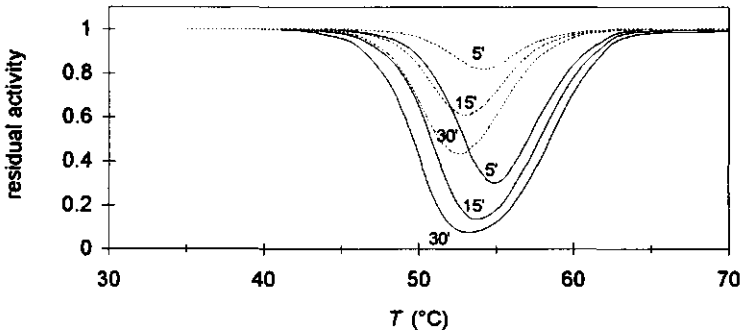
Figure 5.10:   Simulated curves for residual activity after heating the proteinase for 5, 15, and 30 min. - - - - - - : [proteinase] = $10^{-8}\ M$; ———— : [proteinase] = $10^{-7}\ M$; further assumptions: see text.
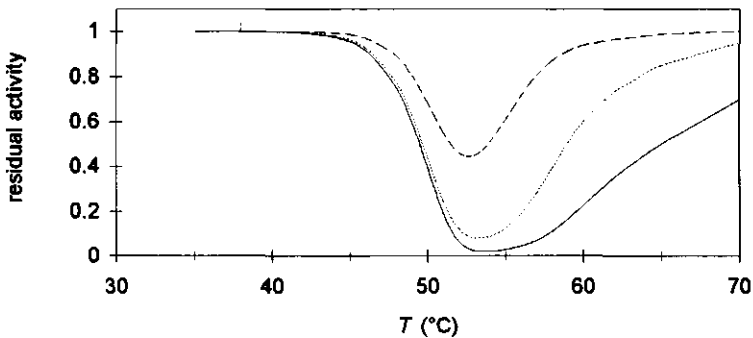
Figure 5.11:   Simulated autoproteolysis curves. — — — : [proteinase] = $1 \cdot 10^{-8}\ M$; - - - - : [proteinase] = $1 \cdot 10^{-7}\ M$; ———— : [proteinase] = $5 \cdot 10^{-7}\ M$; further assumptions: see text.

5.4.2 Influence of protein content and purification on low temperature inactivation

So far we have examined the inactivation of unpurified extracellular proteinase from *Pseudomonas fluorescens* 22F diluted in demineralized water. It has been reported that purification of proteinases from *Pseudomonas* species enhances the susceptibility of the proteinase to autoproteolysis (Barach et al., 1976; Griffiths et al., 1981; Leinmüller and Christopherson, 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; Stepaniak and Fox, 1983; Kroll and Klostermeyer, 1984; Stepaniak et al., 1991). Apparently, other proteins present stabilize the proteinase against inactivation by autoproteolysis.

For many enzymes it is found that substrate or other ligands stabilize the molecule 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, 1972). Binding of substrate or ligand molecules may also cause a conformational change of the enzyme molecule, such that susceptible peptide bonds can not be attacked or that the conformation is stabilized against unfolding (Mihalyi, 1972). In the latter case a raise in T_d is 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, also 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 acts as a competitive inhibitor against autoproteolysis.

In order to investigate the effect of proteins on the heat stability of the extracellular proteinase from *Pseudomonas fluorescens* 22F, autoproteolysis curves were made for solutions with various amounts of sodium caseinate. Purified proteinase in 0.2 *M* TrisHCl, containing 0, 0.1, 0.6, and 1.5 % sodium caseinate, was heated for 10 min at various temperatures, cooled in ice water, and, subsequently, the activity was measured. The residual activity was defined as the fraction of the activity of the unheated enzyme solution. The results are given in figure 5.12.

Purified proteinase was inactivated much faster than unpurified proteinase, as was observed for other pseudomonal proteinases (Barach et al., 1976; Griffiths et al., 1981; Leinmüller and Christopherson, 1982; Kroll, 1989; Kumura et al., 1991). Clearly, substances in the supernatant had a protective function against
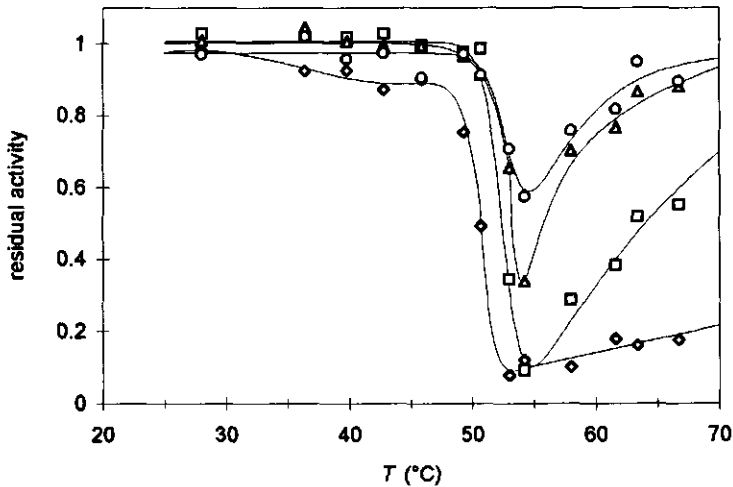
Figure 5.12:   Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F
after 10 minutes of heating at indicated temperatures. ◊ = 0 % sodium caseinate;
□ = 0.1 %; ▵ = 0.6 %; ○ = 1.5 %.

autoproteolysis. Probably, these substances are concomitant or associated proteins
or peptides, but also other substances are possible. At temperatures above T_d a
relatively large fraction of the proteinase was inactivated. Computer simulations
showed that this was mainly due to inactivation during heating-up.

The temperature of maximal inactivation shifted a little towards higher
temperature when 0.1 % sodium caseinate was added to the purified enzyme.
Addition of more sodium caseinate did not shift the autoproteolysis optimum any
further. Possibly, small amounts of sodium caseinate can stabilize the native
proteinase molecule against unfolding, resulting in an increased T_d. Furthermore, a
distinct reduction in the extent of inactivation with increasing caseinate content was
observed. This reduction must be induced by association of proteins, making it
sterically impossible to break susceptible peptide bonds, or by competitive inhibition
of the autoproteolysis reaction by substrate molecules, as explained above.

In order to check the possibility of reduced inactivation of the proteinase by
competitive inhibition of autoproteolysis by the substrate, an experiment was
performed in which enzyme inactivation and product formation were followed in
time. Sodium caseinate was added to a solution of purified proteinase in 0.1 *M*
TrisHCl in 2 m*M* CaCl$_2$, to final concentrations of 0.1, 0.3, 0.6, 1.5, and 2.5 %.
Samples of 0.4 ml of the enzyme preparations were heated at 55.3 °C, cooled in

ice water, and the residual activity was measured with the TNBS method. The blank values of the TNBS method were taken as a measure of product formation. The results are shown in figure 5.13.

Figure 5.13A clearly shows the protective function of the sodium caseinate. Addition of more than 1.5 % sodium caseinate did not give further protection. Figure 5.13B shows that at 55.3 °C the proteinase was still active, but that the activity, approximately proportional to d[P]/dt, decreased in time. Product formation in absence of sodium caseinate was negligibly small, so that the increase in absorbance at 420 nm must completely be ascribed to proteolysis of the substrate, and not to autoproteolysis. The rate of product formation did not increase further when the substrate concentration was increased higher than 1.5 %, indicating that the proteolytic reaction was inhibited by substrate. Inhibition of enzymic activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F by sodium caseinate at concentrations higher than 1 % was also found at 37 °C (chapter 3 of this thesis).

Then we tried to model the protective function of substrate by implementing substrate breakdown according to Michaelis-Menten kinetics in the autoproteolysis model. Let us first consider the proteolytic breakdown of substrate at temperatures where the enzyme is not unfolded, and no autoproteolysis occurs. The proteolytic reaction is assumed to follow unireactant Michaelis-Menten kinetics (Segel, 1975; Whitaker, 1994):

$$N + S \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} NS \overset{k_p}{\rightarrow} N + P \tag{5.19}$$

where N represents the enzyme in solution, S 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. After a period in which the complex NS is formed (the pre-steady-state, usually within seconds), the concentration [NS] is assumed to be more or less constant. In this steady-state situation:

$$\frac{d\,[NS]}{d\,t} = k_s\,[N][S] - (k_{-s} + k_p)\,[NS] \cong 0 \tag{5.20}$$
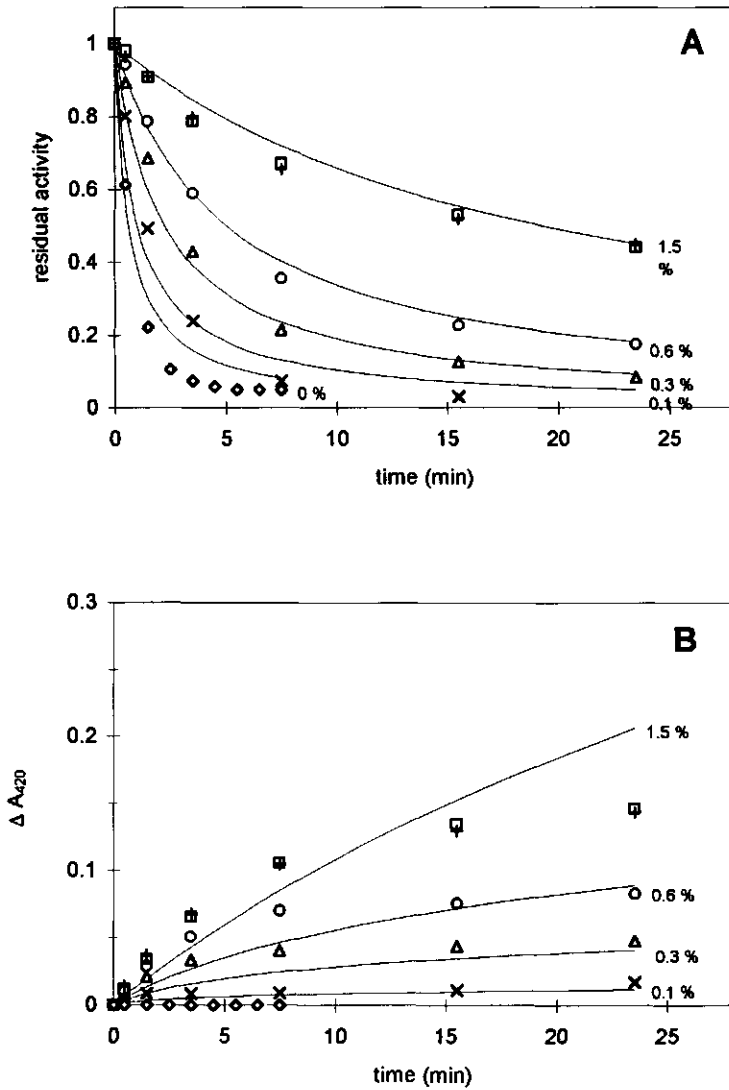
Figure 5.13: Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F
(A), and the product formation by the proteinase (B) as function of heating time at
55.3 °C.

◊ = 0 % sodium caseinate; x = 0.1 %; △ = 0.3 %; ○ = 0.6 %; + = 1.5 %; □ = 2.5 %;
———— = fit lines for model describing inhibition of autoproteolysis by substrate;
K_d = 1.13; K_m = 6.7•10^{-5} M ; k_a = 1.9•10^5 M^{-1} s^{-1} ; k_p = 2.6•10^3 s^{-1} .

Because the total amount of enzyme $[N]_{tot} = [N] + [NS]$, the fraction of enzyme molecules in the enzyme-substrate complex is:

$$[NS] = \frac{[N]_{tot}}{(1 + \{(k_{-s} + k_p)/k_s\}/[S])} = \frac{[N]_{tot}}{(1 + K_m/[S])} \tag{5.21}$$

where the Michaelis-Menten constant K_m is:

$$K_m = \frac{(k_{-s} + k_p)}{k_s} = \frac{[N][S]}{[NS]} \tag{5.22}$$

In the temperature range where autoproteolysis occurs, the situation is somewhat more complex. Now, also unfolding of native enzyme molecules, and digestion of unfolded molecules is taking place, so that an increasingly reduced amount of enzyme is available for digestion of substrate:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \tag{5.1}$$

$$N + U \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} NU \overset{k_d}{\rightarrow} N + I_1 + I_2 \tag{5.2}$$

On the other hand, the amount of enzyme available for autoproteolysis is also reduced, because it is complexed in the enzyme-substrate complex. This amount is increased as the substrate concentration increases.

For the calculations we assumed the amount of enzyme molecules in the complex NU to be negligible. Furthermore, we assumed an equilibrium between N, U and NS, so that:

$$[N](t) = \frac{[N]_{tot}(t)}{1 + K_d + ([S]/K_m)} \tag{5.23a}$$

$$[U](t) = \frac{[N]_{tot}(t) \; K_d}{1 + K_d + ([S]/K_m)} \tag{5.23b}$$

$$[NS](t) = \frac{[N]_{tot}(t) \; ([S]/K_m)}{1 + K_d + ([S]/K_m)} \tag{5.23c}$$

Ordinary differential equations for the enzyme inactivation and product formation were derived from eqs. 5.6 and 5.18, respectively:

$$- \frac{d \text{ (residual activity)}}{d\,t} = \frac{d\,[I_1 + I_2]}{d\,t} = k_a\,[\,N]\,[U] \qquad (5.24)$$

$$\frac{d\,[P]}{d\,t} = k_p\,[NS] \qquad (5.24)$$

These ODE's were integrated numerically using a first-order Euler forward algorithm. The model was analyzed 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. The results of the experiment with 2.5 % sodium caseinate were excluded from the calculation, because of the earlier mentioned inhibition of proteolytic activity by high concentrations of sodium caseinate.

The model described above was fitted to the results. The values for K_d, K_m, k_a, and k_p were estimated at 1.13, $6.7 \cdot 10^{-5}$ M, $1.9 \cdot 10^5$ M^{-1} s^{-1}, and $2.6 \cdot 10^3$ s^{-1}, respectively. The fitted lines are included in figure 5.13. In figure 5.13A, the fit appears reasonable for the inactivation of the proteinase. The calculated inactivation of the proteinase in absence of sodium caseinate deviated from the measured results, but it should be noted that the mechanism of stabilization of small amounts of substrate would be different, as indicated above. The values of the estimated parameters agree reasonably well with those found in other experiments. K_m was found $7 \cdot 10^{-5}$ M at 37 °C (chapter 3 of this thesis), k_a was estimated at $4 \cdot 10^5$ M^{-1} s^{-1} (section 5.4.1), and according to figure 5.12, K_d should be slightly larger than 1 at 55 °C.

The value of k_p is more difficult to evaluate, because this rate constant indicates the proportionality between the amount of enzyme-substrate complex and change in absorbance at 420 nm in the TNBS method. The calculated product formation, expressed in change in absorbance at 420 nm in the TNBS method, did not agree well with the experimental results. From the tangents of the product formation the activity can be estimated. The activities at 55 °C deviated significantly from the modelled activities. This may indicate that the assumption of [NU] being negligibly small is not correct. It may also indicate that competitive inhibition of autoproteolysis by substrate is not the only mechanism of protection, but that formation of a reversible complex between enzyme molecules and caseinate also helps preventing autoproteolysis.

It is interesting to compare our results with those of Stepaniak et al. (1983), who heated the proteinase from *Pseudomonas fluorescens* AFT36 at 55 °C in the presence of 0 to 10 % N, N-dimethylated casein. N, N-dimethylated casein was used as substrate, because sodium caseinate inhibited proteolytic activity above a concentration of 5 % (Stepaniak et al., 1982). At 55 °C, proteolysis of the substrate was reported to be negligible, indicating that also inactivation by autoproteolysis was not likely to have occurred. Inactivation of the proteinase by formation of an irreversible enzyme-substrate complex was proposed as a mechanism. However, the proteinase was increasingly stabilized against inactivation by increasing concentrations of substrate, suggesting the opposite. In our view, this must indicate that the proteinase also forms a reversible complex with the substrate, thereby preventing formation of the irreversible complex. The proteinase from *Pseudomonas fluorescens* P1 could digest casein in the temperature range in which autoproteolysis was taking place. Also here the formation of inactive irreversible enzyme-substrate was concluded on the basis of immunological assays (Stepaniak et al., 1991). In our case, inactivation by the formation of an irreversible enzyme-substrate complex may partly contribute to the asymmetry of the autoproteolysis curves shown in figure 5.1 and 5.3, although computer simulations showed that this asymmetry could solely be explained by intermolecular autoproteolysis.

5.4.3 Influence of metal ions on low temperature inactivation

The extracellular proteinase from *Pseudomonas fluorescens* 22F, like most of the other pseudomonal extracellular proteinases, is a metalloproteinase, with most likely a Zn^{2+} ion in its catalytic centre (chapter 3 of this thesis). Besides Zn^{2+}, also Ca^{2+} is found in most pseudomonal proteinases (Fairbairn and Law, 1986; Fox et al., 1989). Calcium is considered to play a role in the conformational stability of the proteinases (Stepaniak et al., 1983, 1985; Kroll and Klostermeyer, 1984; Patel and Bartlett, 1988; Kroll, 1989). This stabilizing effect is also found for many other proteolytic enzymes, such as thermolysin (Voordouw and Roche, 1975; Fontana, 1988), and subtilisin (Voordouw et al., 1976; Genov et al., 1995).

A supposed mechanism of stabilization by calcium against denaturation and autoproteolysis is via intramolecular calcium bridges, thereby reducing the flexibility of the protein molecule (Fontana, 1988; McPhalen et al., 1991). Stabilization by calcium can be local, preventing loops from becoming exposed and flexible, and thereby becoming susceptible to autoproteolysis (Dahlquist et al., 1976; Fontana, 1988). Calcium may also stabilize the overall structure: many Ca^{2+} binding sites include ligands from distant segments of the polypeptide chain, supplying structural

cross-linking and stability similar to that by disulfide bridges (Fontana, 1988; McPhalen et al., 1991). Obviously, which of the peptide bonds is attacked, is important for the extent of inactivation and the extent of digestion into small peptides. Enzymes subjected to limited proteolysis or autoproteolysis, where cleavage of peptide bonds in flexible loops is involved, may have molecular characteristics similar to the native molecule, but have quite different relative activities (Mihalyi, 1972; Vita et al., 1985), while in the case that cleavage of peptide bonds occurs if the enzyme molecule unfolds cooperatively, digestion to small peptides may occur.

In order to investigate whether metal ions are also important in the stabilization of the extracellular proteinase from *Pseudomonas fluorescens* 22F, autoproteolysis curves were made for supernatant from a skimmed milk culture, diluted 10 times in 0.2 *M* Tris Maleate, pH 7.4, containing various amounts of $CaCl_2$ or chelating agents. These dilutions were freshly prepared and kept at room temperature for 30 min before heat treatment. The calcium ion activity was estimated with a calcium ion selective electrode. The enzyme preparations were heated for 15 min at various temperatures, cooled in ice water, and examined for residual proteolytic activity. The residual activity was defined as the fraction of the activity of the unheated enzyme solution.

Firstly, the influence of addition of calcium was examined. Therefore, 2 m*M* or 10 m*M* $CaCl_2$ was added to the enzyme preparation. The calcium ion activities of these solutions were $1.4 \cdot 10^{-4}$ *M*, and $5.0 \cdot 10^{-4}$ *M*, respectively. The calcium ion activity of the enzyme solution containing no $CaCl_2$ was $4.7 \cdot 10^{-5}$ *M*. The calcium ion activity of this enzyme preparation was low due to the calcium binding capacity of maleate. The results are given in figure 5.14. At temperatures above T_d a relatively large fraction of the proteinase was inactivated. Computer simulations showed that this was mainly due to inactivation during heating-up. Addition of 10 m*M* $CaCl_2$ to the enzyme solution raised the denaturation temperature T_d by approximately 4 K, while addition of 2 m*M* $CaCl_2$ only had a slight effect. Clearly, calcium could stabilize the proteinase. The fact that addition of calcium raised the denaturation temperature may indicate that calcium is involved in a stabilization of a cooperative unfolding, leading to the extensively unfolded and inactive enzyme molecule U.

Moreover, experiments were performed in which the calcium ion activity of the enzyme solution was lowered by the chelating agents ethylenediamine tetraacetic acid (EDTA), and ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA). EDTA is an aspecific chelating agent for divalent metal ions, while EGTA specifically chelates Ca^{2+}. Enzyme preparations were made with 0.1 m*M* or 0.5 m*M* EDTA, or 0.5 m*M* EGTA. The calcium ion activities of these

Figure 5.14:   Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F after holding the enzyme solution for 15 min at indicated temperature. ◊ = in 0.2 *M* Tris Maleate; ∆ = in 0.2 *M* Tris Maleate containing 2 m*M* CaCl$_2$; □ = in 0.2 *M* Tris Maleate containing 10 m*M* CaCl$_2$.

solutions were $3.4 \cdot 10^{-5}$ *M*, $2.5 \cdot 10^{-5}$ *M*, and $2.4 \cdot 10^{-5}$ *M*, respectively. The autoproteolysis curves are shown in figure 5.15. Addition of neither EDTA nor EGTA could notably change the position of the minimum in the autoproteolysis curve of the extracellular proteinase from *Pseudomonas fluorescens* 22F. It appeared that somewhat more inactivation occurred at temperatures lower than T_d, if EDTA had been added to the enzyme solution. This effect seemed to be more pronounced when pre-incubation with EDTA was longer, but those results were poorly reproducible (results not shown). This may indicate that EDTA removed calcium from the enzyme molecule that stabilizes a flexible loop, making the molecule susceptible to autoproteolysis. This removed calcium would be involved in the stabilization of a local transition, not leading to an inactive enzyme molecule, rather than in a cooperative transition of the enzyme molecule. Therefore, it may indicate that a two-state denaturation model is not sufficient to describe the unfolding of the extracellular proteinase from *Pseudomonas fluorescens* 22F, but that unfolding intermediates are formed, as suggested in section 5.2.

Finally, autoproteolysis curves were made with ortho-phenanthroline (*o*PA), a specific zinc chelator and strong inhibitor of the proteinase. Supernatant was diluted 10 times in 0.2 *M* Tris Maleate, pH 7.4, containing 0.1 m*M* or 0.5 m*M* *o*PA. Calcium ion activities of these solutions were $5.3 \cdot 10^{-5}$ *M* and $5.5 \cdot 10^{-5}$ *M*, respectively. The results are given in figure 5.16. Addition of 0.1 m*M* and 0.5 m*M*

Figure 5.15:   Residual activity of the extracellular proteinase from Pseudomonas fluorescens 22F after holding the enzyme solution for 15 min at indicated temperature. ◊ = in 0.2 *M* Tris Maleate;□ = in 0.2 *M* Tris Maleate containing 0.2 m*M* EDTA; △ = in 0.2 *M* Tris Maleate containing 0.5 m*M* EDTA; ○ = in 0.2 *M* Tris Maleate containing 0.5 m*M* EGTA.
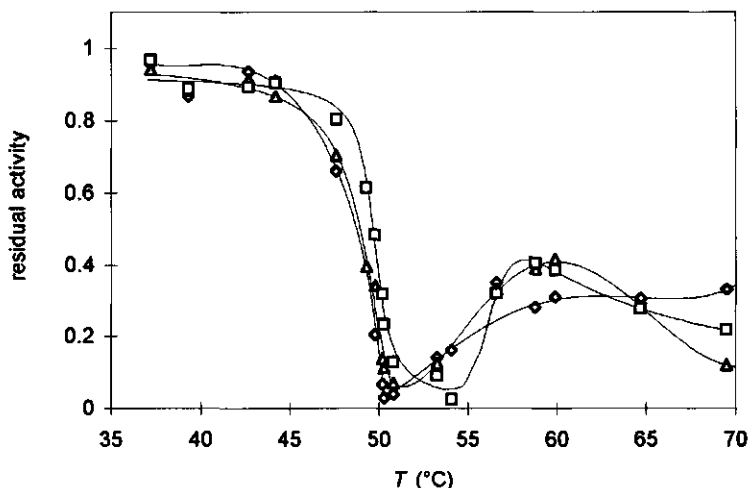

Figure 5.16:   Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F after holding the enzyme solution for 15 min at indicated temperature. ◊ = in 0.2 *M* Tris Maleate; □ = in 0.2 *M* Tris Maleate containing 0.1 m*M* oPA; △ = in 0.2 *M* Tris Maleate containing 0.5 m*M* oPA.

oPA to the enzyme solution decreased T_d by approximately 3 and 8 K, respectively. Zinc ions are considered to play role in the integrity of the catalytic site of the proteinase. The fact that T_d is lowered by addition of oPA may suggest that Zn^{2+} is involved in stabilizing a cooperative transition. A lowered T_d was also found by DSC measurements, when 10 mM oPA was added to the enzyme solution (section 5.4.1). On the other hand, it may also concern a local destabilization of the structure in the vicinity of the catalytic centre, caused by the removal of zinc, and resulting in an increased susceptibility to autoproteolysis, followed by further unfolding of the molecule. Digestion of peptide bonds in the vicinity of the catalytic site presumably leads to immediate inactivation.

5.4.4 Influence of pH on low temperature inactivation

At ambient temperatures most proteins are the most stable near the isoelectric pH. At extreme pH values the proteins can be inactivated irreversibly, presumably caused by electrostatic repulsion of charged groups and the impossibility to form internal salt bridges (Tanford, 1968; Darby and Creighton, 1990). The proteinase from *Pseudomonas fluorescens* 22F was observed to be stable between pH 5 and 10 at 4 and 25 °C (chapter 3 of this thesis). In combination with high temperatures, denaturation will already occur at less extreme pH values. Besides the direct influence of pH on the stability of the proteinase, pH may also affect the rate of the autoproteolysis reaction.

We examined the influence of pH on the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F at 50 °C. Supernatant from a culture of *Pseudomonas fluorescens* 22F in skimmed milk, containing the proteinase, was diluted in demineralized water and set at various pH values with HCl and NaOH. The enzyme solutions were heated for 30 min at 50 °C, subsequently cooled in ice water, and examined for the residual activity at pH 7.4. The results are shown in figure 5.17. For comparison, pH stability curves for 1 h/25 °C and 24 h/4 °C are included.

Figure 5.17 shows a reduced stability of the proteinase between pH 6 and 10, obviously because autoproteolysis is an additional cause of inactivation. The pH stability of proteinase from *Pseudomonas fluorescens* 22F resembled that of trypsin, as observed by Kunitz and Northrop (1934). The pH stability curve could be explained as follows: at pH values below 6 and above 10, the proteinase is inactivated by the same processes that occur at the lower temperatures at extreme pH. Due to the combination of high temperature and extreme pH, this inactivation is shifted to less extreme pH values. Between pH 6 and 10 the proteinase is inactivated by autoproteolysis. As stated above, inactivation by autoproteolysis is
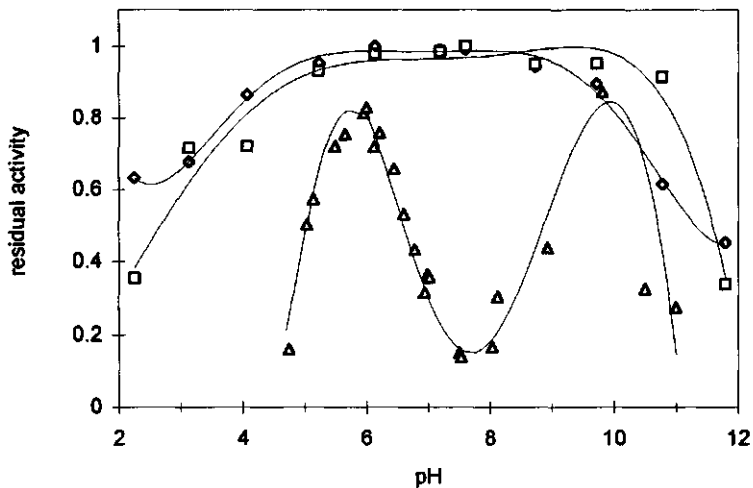
Figure 5.17:   Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F
                    at various pH. △ = 30 min at 50 °C, ◊ = 1 h at 25 °C, ☐ = 24 h at 0 °C.
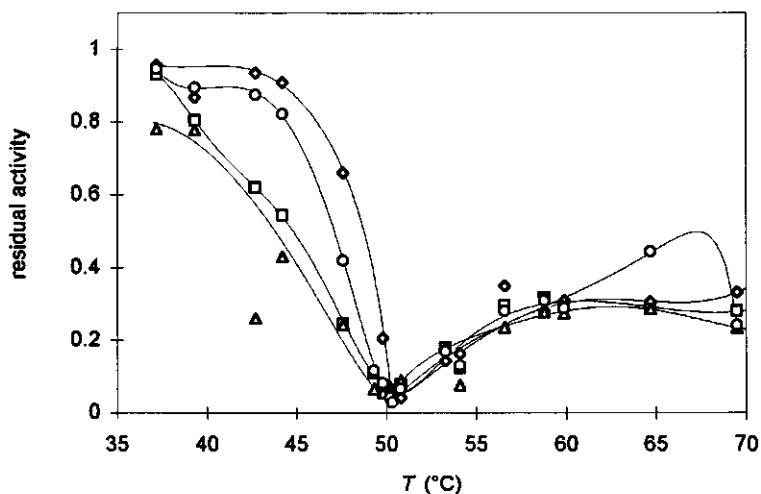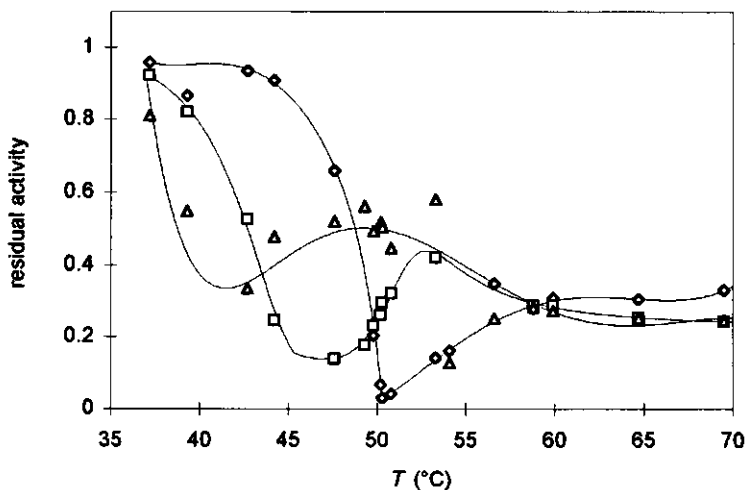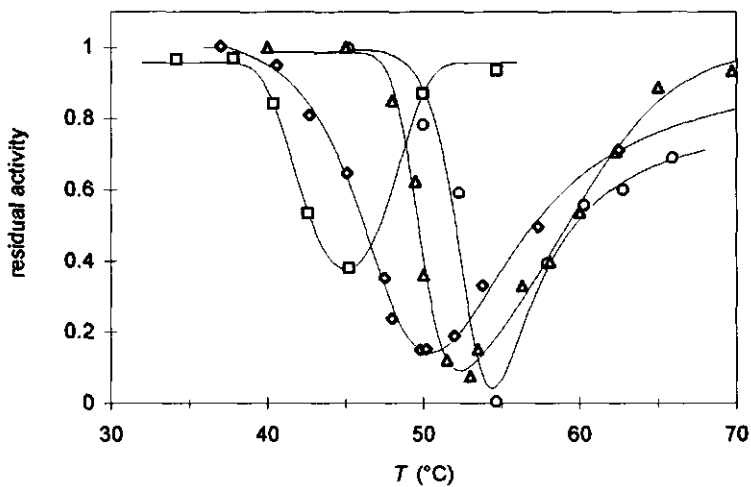

Figure 5.18:   Residual activity of the extracellular proteinase from *Pseudomonas fluorescens* 22F
                    after holding the enzyme solution for 30 min at indicated temperature. ☐ = pH 9.6, ◊
                    = pH 7.5, △ = pH 7.0, ○ = pH 5.8.

maximal when the fractions of native and unfolded enzyme molecules are equal
($T = T_d$; $K_d = 1$). At 50 °C this is approximately at pH 7.5. Between pH 6 and 7.5
and between 7.5 and 10 there is an excess of either native or unfolded enzyme
molecules, depending on the unfolding equilibrium at 50 °C and that specific pH.

This was confirmed in the following experiment: supernatant from a culture
of *Pseudomonas fluorescens* 22F in skimmed milk was diluted in demineralized
water and brought to pH 5.6, 7.0, 7.5 and 9.6 with HCl and NaOH. The enzyme
solutions were heated for 30 min at various temperatures, subsequently cooled in
ice water, and examined for the residual activity. The results are shown in figure
5.18. The temperatures of maximum inactivation were 45, 50, 52 and 54 °C for pH
values of 9.6, 7.5, 7.0, and 5.8, respectively. This pH-induced shift of denaturation
temperature is often observed (Tanford, 1968; Privalov, 1979; Eijsink, 1991). It can
be concluded that 50 °C is below the denaturation temperature T_d at pH values
between 6 and 7.5. Here, inactivation by autoproteolysis is not maximal because
there is an excess of native enzyme molecules. At pH values between 7.5 and 10,
however, 50 °C is above the denaturation temperature T_d . Here, most of the
enzyme molecules are in the reversibly unfolded form, and consequently, there is
only a relatively small amount of enzyme molecules to perform autoproteolysis.

The denaturation temperature T_d is a measure of the conformational stability
of the proteinase. The conformational stability of proteins is dependent on, among
other factors, the pH. Of the 4 pH values examined in our experiment, pH 5.8
resulted in the highest conformational stability, pH 9.6 in the lowest. However, it is
difficult to translate these results to molecular characteristics of the proteinase
molecule. Generally, a protein molecule is maximally stable at its isoelectric pH,
because the net charge of the molecule is zero, so that repulsive electrostatic
forces are minimal. However in some proteins, other stabilizing forces, like
hydrophobic interactions, may prevail over the electrostatic repulsion to varying
degrees (Tanford, 1968, 1970; Mihalyi, 1978). The isoelectric pH of the
extracellular proteinase from *Pseudomonas fluorescens* 22F was found to be 7.4
(chapter 3 of this thesis), and from figure 5.18 the optimum pH for stability would
be below about 6. The pH of the enzyme solution may also influence the binding of
calcium to the proteinase (Dahlquist et al., 1976), or availability of substrates for
competitive inhibition of autoproteolysis.

5.5   Conclusions

It can be concluded that kinetic modelling can be used for elucidation of the
mechanism of inactivation of the extracellular proteinase from *Pseudomonas
fluorescens* 22F. Inactivation by intermolecular autoproteolysis appeared to be

much more likely than intramolecular autoproteolysis. Kinetic modelling can be used for the estimation of kinetic parameters of unfolding and refolding (Mihalyi, 1972; Imoto, 1986). Because this method determines the rate constants of unfolding and refolding only indirectly, large standard deviations may be expected. However, in our case we could estimate the kinetic parameters reasonably accurately. These parameters were found to be in the expected order of magnitude. From the kinetic parameters, thermodynamic parameters of the unfolding reaction may be estimated. These estimates were subject to large deviations, because the errors in the estimation of the kinetic parameters are accumulated. In our case the values of the thermodynamic parameters were found outside the expected range. More accurate was the estimation of T_d . Because traditional techniques for the determination of the thermodynamics of unfolding, like calorimetry or optical methods, appear to be impractical for proteinases prone to autoproteolysis, indirect estimation of T_d by autoproteolysis as measure of thermostability may be useful.

With kinetic modelling, we also found a conclusive explanation for the protective role of sodium caseinate against autoproteolytic inactivation. Small amounts of caseinate appeared to stabilize the conformation of the proteinase, while larger amounts could act as an competitive inhibitor against autoproteolysis.

Calcium could stabilize the proteinase, presumably by stabilizing the native conformation against unfolding. Removal of calcium resulted in an increased rate of inactivation at temperature below T_d , suggesting a stabilizing role of calcium against unfolding of a flexible loop. Removal of zinc resulted in a lowered T_d . Possibly, zinc is involved in the stabilization of a cooperative transition. The conformational stability of the proteinase was dependent on the pH of the enzyme solution.

From the results presented in this chapter, only speculations could be made about molecular characteristics of the proteinase. Elucidation of the three-dimensional structure of the molecule may give a better understanding of the mode of action of metal ions and pH in the stabilization against inactivation of this proteinase.

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Chapter 6

Kinetics of heat inactivation of extracellular proteinase from *Pseudomonas fluorescens* 22F at 80 - 120 °C

Abstract

In this chapter the heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F at 80 - 120 °C was studied. The activation enthalpy $\Delta H^{\ddagger}$ and activation entropy $\Delta S^{\ddagger}$ of the inactivation reaction, when analyzed with a first-order 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 models were proposed and modelled to fit the data. The model with the fewest parameters being statistically acceptable, consisted of two sequential first-order irreversible reactions, and could be used for predictive modelling of the inactivation of the proteinase. A 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 statistically better and also appeared to be more in accordance with the mechanism of inactivation. The inactivation behaviour was dependent on the medium in which the bacteria were grown. Addition of sodium caseinate to the enzyme solution decreased the heat stability of the proteinase. The inactivation was dependent on pH. Calcium ion activity did not have a significant influence on the heat stability of the proteinase. Fast inactivation during heating-up caused a biphasic inactivation behaviour. Addition of sodium caseinate to the enzyme solution diminished this initial inactivation, partly by decreasing the rate of autoproteolysis. Addition of EDTA increased the extent of inactivation during heating-up, possibly because of complexation of calcium that stabilizes part of the native conformation or facilitates renaturation.

6.1   Introduction

Extracellular proteinases of psychrotrophic bacteria can be extremely stable to high temperatures; resisting ultra-high temperature sterilization, they can reduce the shelf-life of food products. Also the extracellular proteinase from *Pseudomonas fluorescens* 22F is reported to be very heat stable (Driessen, 1983; Schokker and van Boekel, 1993).

Heat inactivation of enzymes is generally shown schematically as:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \overset{k_i}{\rightarrow} I \qquad\qquad (6.1)$$

In the first 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).

Driessen (1983, 1989) found a typical heat inactivation behaviour of proteinases from *Pseudomonas 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 behaviour can also be seen in his Arrhenius plot. A relatively slow denaturation reaction has been suggested to explain this behaviour (van Boekel and Walstra, 1989), but this appeared not to be the case for this enzyme, as the unfolding of enzyme molecules from *Pseudomonas fluorescens* 22F takes place between 40 - 60 °C (van Boekel, 1993; chapter 5 of this thesis). In the present chapter an attempt is made to find an alternative model that can describe the peculiar inactivation behaviour. Also, some factors that may influence the inactivation at high temperature are evaluated.

6.2   Theory

Description of heat inactivation of the enzymes.

Enzyme inactivation is generally described by first-order kinetics; an inactivation model as given by equation 6.1 is used. In general, the rate of inactivation is determined by the unfolding and thermal inactivation reaction:

$$k_{obs} = \frac{k_i\ K_d}{1 + K_d} \qquad\qquad (6.2)$$

where k_{obs} is the apparent reaction rate constant for inactivation, and K_d the equilibrium constant. However, when the inactivation is examined at temperatures reasonably far above the denaturation temperature, the influence of the folding/refolding equilibrium is negligible, and the inactivation is only determined by the secondary reaction, leading to irreversible inactivation (Zale and Klibanov, 1983; chapter 2 of this thesis). 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, 1985). The following equations are used:

$$N \overset{k_i}{\rightarrow} I \tag{6.3}$$

$$\frac{d\,[N]}{d\,t} = -k_i\,[N] \tag{6.4}$$

$$a\,(t) = [N]\,(t)\,/\,[N]\,(t = 0) = a_0\,\exp\,(-k_i\,t) \tag{6.5}$$

with $a\,(t)$ being the residual activity, and a_0 the initial activity. In many cases such a model adequately describes the inactivation.

Alternative inactivation models

Since the inactivation of the proteinase from *Pseudomonas fluorescens* 22F was found to show a deviating inactivation behaviour (Driessen, 1983; Schokker and van Boekel, 1993), we propose alternative models to fit the experimental data better. These models, and the equations for residual activity as function of time, are given in table 6.1. 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**). Furthermore, it is assumed that the unfolding reaction has proceeded completely after heating the enzyme solution to the desired temperature (chapter 5 of this thesis). These assumptions rule out any influence of unfolding and refolding reactions on the actual inactivation. When modelling the experimental data, the inactivation during the heating-up time is neglected. The residual activity after two minutes of heating-up time ($t = 0$) is 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. By doing so, changes in concentration during the heating-up time are omitted, giving a distorted representation.

The alternative models for the inactivation of the proteinase from *Pseudomonas fluorescens* 22F can, in principle, account for an initial lag in the inactivation. More

Table 6.1:   Models of inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F. Explanation in the text.

Model	Equation
1 $N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \xrightarrow{k_1} I$	$a(t) = a_0 \exp(-k_1 t)$ (6.1)

Model 2:

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

$$a(t) = \frac{a_0}{(R_2 - R_1)} \left\{ R_2 \exp(-R_1 t) - R_1 \exp(-R_2 t) \right\} \quad (6.6)$$

$$R_1 = 0.5(p - q) \qquad R_2 = 0.5(p + q)$$

$$p = k_1 + k_{-1} + k_2 \qquad q = (p^2 - 4 k_1 k_2)^{0.5}$$

Model 3:

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

$$a(t) = a_0 \left\{ \frac{k_2}{k_2 - k_1} \exp(-k_1 t) - \frac{k_1}{k_2 - k_1} \exp(-k_2 t) \right\} \quad (6.7)$$

Model 4:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U_1$$
$$\downarrow k_1$$
$$N^* \underset{k_f^*}{\overset{k_u^*}{\rightleftharpoons}} U_2 \xrightarrow{k_2} I$$

$$a(t) = a_0 \left\{ \left(1 + \frac{\beta k_1}{k_2 - k_1}\right) \exp(-k_1 t) - \frac{\beta k_1}{k_2 - k_1} \exp(-k_2 t) \right\} \quad (6.8)$$

Model 5:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_2 \xrightarrow{k_2} I$$
$$ \searrow_{k_3} I$$

$$a(t) = a_0 \left\{ \left(\frac{k_2 - k_3 - k_1 - k_{-1}}{2q} + 0.5 \right) \exp(-R_1 t) + \right.$$

$$\left. \left(\frac{k_1 - k_2 + k_3 + k_{-1}}{2q} + 0.5 \right) \exp(-R_2 t) \right\} \quad (6.9)$$

$$R_1 = (k_2 + k_3 + k_1 + k_{-1} + q) / 2$$

$$R_2 = (k_2 + k_3 + k_1 + k_{-1} - q) / 2$$

$$q = \sqrt{(k_2 - k_3 + k_1 - k_{-1})^2 + 4 k_1 k_{-1}}$$

Table 6.1:   continued

Model	Equation
6 $N \overset{k_u}{\underset{k_f}{\rightleftharpoons}} U_1 \overset{k_1}{\rightarrow} U_2 \overset{k_2}{\rightarrow} I$ $\overset{k_3}{\longrightarrow} I$	$a(t) = a_0 \left\{ \dfrac{(k_3 - k_1)\exp(-(k_2 + k_1)t) - k_1 \exp(-k_2 t)}{(k_3 - k_2 - k_1)} \right\}$ (6.10)
7 $N \overset{k_u}{\underset{k_f}{\rightleftharpoons}} U_1$ $\quad \downarrow k_1$ $N^* \overset{k_u}{\underset{k_f}{\rightleftharpoons}} U_2$ $\quad \downarrow k_2$ $N^{**} \overset{k_u}{\underset{k_f}{\rightleftharpoons}} U_3 \overset{k_3}{\rightarrow} I$	$a(t) = a_0 \left\{ \left[1 + \dfrac{\beta_1 k_1}{k_2 - k_1} + \dfrac{\beta_2 k_1 k_2}{(k_2 - k_1)(k_3 - k_2)} \right] \exp(-k_1 t) \right.$ $+ \left[\dfrac{\beta_1 k_1}{k_1 - k_2} + \dfrac{\beta_2 k_1 k_2}{(k_1 - k_2)(k_3 - k_2)} \right] \exp(-k_2 t) +$ $\left. \dfrac{\beta_2 k_1 k_2}{(k_1 - k_2)(k_3 - k_2)} \exp(-k_3 t) \right\}$ (6.11)

complex models than described in table 6.1 could be used, but these are considered impractical because of the high number of parameters to be estimated.

Model 1 is the first order inactivation model as described above, in which the inactivation is caused by a single first-order reaction, and is included in table 6.1 for comparison. When comparing various models, 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 (eq. 6.6), the unfolded enzyme is subjected to an additional reversible reaction before it can be inactivated. This reaction could either be an additional unfolding or a chemical reaction. This model has been used before to describe the lagged inactivation (Schokker and van Boekel, 1993). Model 3 (eq. 6.7) is similar to model 2, but here k_{-1} is negligibly small. It is assumed that the rate of the reaction from U_2 to U_1 is negligible 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 non-inactivated enzyme molecules can return to the native form.

In model 4 (eq. 6.8), the secondary reaction is irreversible, even at low temperature. After cooling the enzyme solution 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 smaller than 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 is equal to that of model 3. This deterministic unidentifiability between models arises often when only one variable is to be determined, in our case residual activity (Godfrey, 1983).

In models 5 (eq. 6.9) and 6 (eq. 6.10), the proteinase can be inactivated from U_1 and U_2. In model 5 the secondary reaction is reversible, in model 6 the reaction rate from U_2 to U_1 is negligible. Then, in model 7 (eq. 6.11) 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.

Finally, there is also a general model, that uses the mean values of the measured data. For every time/temperature combination, this model gives the mean value of the results at that combination. Therefore, this model is not useful for interpolation and explanation.

Temperature dependence

This study is concerned with the description of thermal inactivation of the proteinase from *Pseudomonas fluorescens* 22F. Clearly, the rate of inactivation is influenced by temperature. In order 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:

$$k = \frac{k_b T}{h} \exp\left(\frac{\Delta S^\ddagger}{R}\right) \exp\left(\frac{-\Delta H^\ddagger}{RT}\right) \tag{6.12}$$

where k_b is Boltzmann's constant ($1.38 \cdot 10^{-23}$ J K^{-1}), h is Planck's constant ($6.62 \cdot 10^{-34}$ J s^{-1}), and 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. $\Delta H^\ddagger$ and $\Delta S^\ddagger$ of the inactivation reactions can be estimated directly, in case of model 1 by the following equation:

$$a(t) = a_0 \exp\left(-\frac{k_b T t}{h} \exp\left(\frac{-\Delta H^\ddagger}{RT}\right) \exp\left(\frac{\Delta S^\ddagger}{R}\right)\right) \tag{6.13}$$

Direct estimation of the kinetic parameters from this equation is preferable to a stepwise estimation, where reaction rate constants at constant temperature are determined, and subsequently $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are estimated by regression of ln ($k_i h / k_b T$) on reciprocal temperature, because in the stepwise method unnecessary parameters, namely the reaction rate constants, are estimated. The stepwise procedure generally 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).

Generally, when estimating activation enthalpies and entropies of the inactivation reactions 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 is reparameterized:

$$a\,(t) \;=\; a_0 \; \exp\,(-T\,X\,\exp\,(-Y\,\Delta H^{\ddagger})\,t\,) \tag{6.14}$$

$$X \;=\; \frac{k_b}{h} \; \exp\,(\,\frac{\Delta S^{\ddagger}}{R}\,)\,\exp\,(\,\frac{-\Delta H^{\ddagger}}{RT_{av}}\,) \tag{6.15}$$

$$T_{av} \;=\; \frac{\Sigma\,T}{n} \tag{6.16}$$

$$Y \;=\; \frac{1}{R}\;(\,\frac{1}{T}\;-\;\frac{1}{T_{av}}\,) \tag{6.17}$$

Fitting the results

The models were analyzed with unweighted nonlinear regression, using Marquardt's algorithm (Marquardt, 1963) or the derivative-free algorithm DUD (Ralston and Jennrich, 1978). These methods minimize the sum of squares of the difference between the predicted and measured residual activity (*SSE*):

$$SSE \;=\; \sum\,(a_{measured}\;-\;a_{predicted})^{2} \tag{6.18}$$

The algorithms calculate the set of parameters with the lowest *SSE* and their 95 % confidence intervals.

Model comparison

The strategy to discriminate among models is twofold. In the first place, the fits obtained in the various models are 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.

Besides assessment of goodness of fit, the models are compared statistically. Comparison of nonlinear models is less straightforward than of linear ones. 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, which is the sum of squares of the general model ($SSE_{general\ model}$). Therefore, the difference between the SSE of models 1 to 7 and the general model (the measuring error) is calculated as an estimate of the lack of fit of the models. If the lack of fit ($SSE_{model} - SSE_{general\ model}$) is much smaller than the measuring error ($SSE_{general\ model}$), 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 following equation:

$$f = \frac{(SSE_1 - SSE_2) / (\nu_1 - \nu_2)}{SSE_2 / \nu_2} \tag{6.19}$$

and is tested against $F_{(\nu1-\nu2),\ \nu2,\ 0.95}$. Here, SSE_1 refers to the residual sum of squares of the fit of the model with fewer parameters, one of the proposed models, SSE_2 to the residual sum of squares of the fit of the general model, and ν_1 and ν_2 to the number of degrees of freedom (number of data points minus number of parameters) of the proposed model and the general extended model, respectively (Godfrey, 1983; Motulsky and Ransnas, 1987; Bates and Watts, 1988; Mead et al., 1993). It should be noted that the F-ratio test formally may be applied only for models which are linear in their parameters, because only then the f value would be F-distributed. However, when the sample size is large, the variance ratio is also approximately F-distributed for nonlinear models (Godfrey, 1983; Bates and Watts, 1988).

For comparison of fits obtained with nonlinear regression also other optimization criteria than the F-ratio can be used, such as the residual variance s^2, Akaike's optimization criterion AIC (Hurvich and Tsai, 1989), and Schwarz' optimization criterion SC (Schwarz, 1978). These criteria compare models by their SSE, corrected for the number of parameters. The residual variance is defined as:

$$s^2 = \frac{SSE}{n - p} \tag{6.20}$$

Akaike's criterion is defined as:

$$AIC = n\ \ln\left(\frac{SSE}{n}\right) + \frac{n\ (n + p)}{(n - p - 2)} \tag{6.21}$$

Schwarz' criterion is:

$$SC = n \ln \left(\frac{SSE}{n} \right) + p \ln (n) \tag{6.22}$$

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' criteria, respectively, is the best choice from a statistical point of view. The residual variance is independent of scale, whereas Akaike's and Schwarz' criteria are scale dependent.

Data set comparison

Also for comparison of data sets fitted to the same model an *F*-ratio test is used (Motulsky and Ransnas, 1987). The sum of residual sums of squares of fits with the separate data sets ($SSE_{sep} = \Sigma SSE$) 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 analysing the sets separately is calculated as:

$$f = \frac{(SSE_{pool} - SSE_{sep}) / (v_{pool} - v_{sep})}{SSE_{sep} / v_{sep}} \tag{6.23}$$

and tested against $F_{v\,sep,\,v\,pool,\,0.95}$. Here, v_{pool} and v_{sep} are the number of the degrees of freedom of the pooled data set and the separate data sets, respectively.

6.3   Materials and methods

Production of enzymes

Pseudomonas fluorescens 22F was inoculated with different types of sterilized (15 min at 121 °C) growth media, and after incubation for 8 days at 20 °C the cells were removed by centrifugation (27,000 g, 30 min at 4 °C). Growth media used were fresh skimmed milk; 2.5 % sodium caseinate in milk ultrafiltrate; and tryptone-lactose medium (see chapter 3 of this thesis). The enzyme solution obtained from skimmed milk was stored until use at - 20 °C, the other enzyme solutions were stored at 4 °C, after addition of 0.025 % NaN_3. In some experiments (partially) purified enzyme solutions were used. The procedure for purification has been described in chapter 3 of this thesis.

Proteinase assay

The TNBS method for the determination of proteolytic activity was used, as described in chapter 4 of this thesis. The residual activity was defined as the fraction of the initial activity left after heat treatment.

Heating experiments

Enzyme solutions (2.1 ml) were heated in stainless steel tubes (7 x 120 mm), which were rotated in a thermostatted glycerol bath. After heating, the tubes were cooled immediately in ice water. Assuming that the temperature is uniform throughout the tubes the following equations can be used to describe the profile of temperature increase and decrease (Hiddink, 1975):

$$V \rho c_p \frac{dT}{dt} = \Phi A (T_w - T) \tag{6.24}$$

where

$$\frac{T_w - T}{T_w - T_0} = \exp\left(\frac{-\Phi A t}{V \rho c_p}\right) \tag{6.25a}$$

$$\frac{1}{\Phi} = \frac{1}{\alpha_{glycerol}} + \frac{d_w}{\lambda_w} + \frac{1}{\alpha_{enzyme\ solution}} \tag{6.25b}$$

where V is the volume of the liquid to be heated ($2.1 \cdot 10^{-6}$ m^3), ρ the density of the liquid (1025 kg m^{-3}), c_p the heat capacity of the liquid (3950 J kg^{-1} K^{-1}), Φ the overall heat transfer coefficient (W m^{-2} K^{-1}), A the heating surface area ($2.6 \cdot 10^{-3}$ m^2), T_w the temperature of the tube wall (K), T_0 the initial temperature (K), α the local heat transfer coefficient (W m^{-2} K^{-1}), d_w the thickness of the tube wall ($1.0 \cdot 10^{-3}$ m), and λ_w the heat conductivity of the tube wall (50 W m^{-1} K^{-1}). Because $\alpha_{glycerol}$ and $\alpha_{enzyme\ solution}$ were unknown, Φ had to be estimated, such that the calculated temperatures fitted the measured temperature profiles. In the case the tubes were not rotating, Φ was estimated at 120 W m^{-2} K^{-1}. In the case the tubes are rotated, Φ will be larger and temperature changes faster. The activity after two minutes of heating-up time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating-up.

Calcium ion activity

Calcium ion activity was determined using a calcium ion selective electrode (Orion, model 93-20) (Geerts et al., 1983). Calibration was done with 10^{-2} - 10^{-5} M CaCl$_2$.

Statistical analysis

Models were analyzed with unweighted nonlinear regression, using procedure NLIN of the package SAS® version 6.09, run on a VMS DEC 3000. For estimation of the starting values of the parameters a preliminary grid search was executed. The objective function to be minimized was the residual sum of squares *SSE* (eq. 6.18) using Marquardt's or DUD algorithm (SAS, 1991).

6.4   Results and discussion

6.4.1   Modelling experimental results with first-order inactivation kinetics

For the investigation of the kinetics of heat inactivation, supernatant obtained from a culture grown in skimmed milk medium, containing the enzyme, was used. For the heating experiments the supernatant was diluted 10 times in water to a final enzyme concentration of approximately $1.0 \cdot 10^{-7}$ *M*. The enzyme solution was heated as described above. Results are shown in figure 6.1A. The inactivation data were analyzed with the first-order inactivation model (model 1), as described above. Firstly, 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 6.2.

From these results the activation enthalpy $\Delta H^\ddagger$ and entropy $\Delta S^\ddagger$ were calculated by linear regression of ln $(k_i h / k_b T)$ against the reciprocal temperature. The estimates of the kinetic parameters (± 95 % confidence interval) were $\Delta H^\ddagger = 85.0 \pm 80.2$ kJ mol^{-1} and $\Delta S^\ddagger = -81.6 \pm 215.3$ J mol^{-1} K^{-1}, respectively. The stepwise determination of the kinetic parameters indeed led to large confidence intervals, as predicted. Figure 6.2 shows the Eyring-plot for the rate constant of the inactivation reaction.

The kinetic parameters were also estimated directly by unweighted nonlinear regression (eqs. 6.14 - 6.17). 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) were found to be 84.5 ± 5.0 kJ mol^{-1} and - 83.2 ± 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 *Pseudomonas 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 6.1A the calculated inactivation curves are included. In figure 6.1B the studentized residuals of the fit are shown.
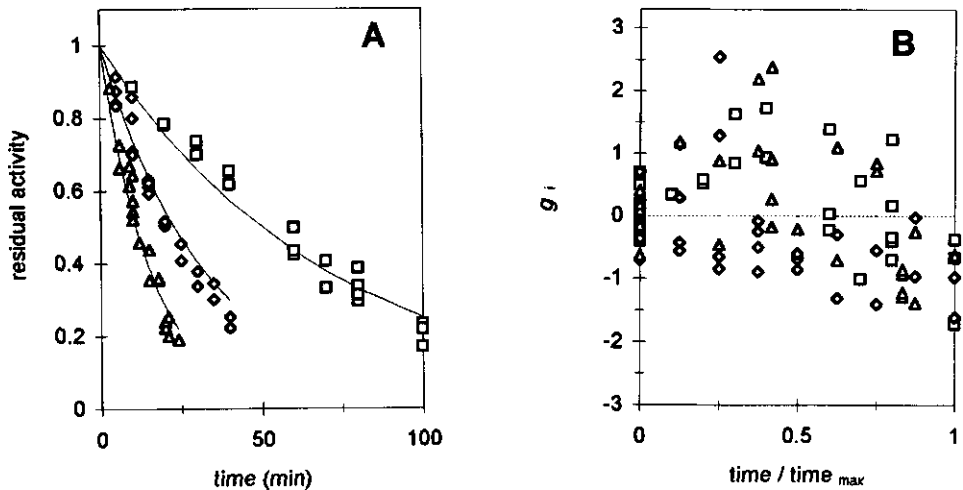
Figure 6.1: Influence of temperature on inactivation of proteinase from *Pseudomonas fluorescens* 22F, grown in skimmed milk, lines calculated for model 1, eq. 6.12 (A) and studentized residuals ($g_i = e_i / s_y$, where $e_i = y_i - \hat{y}$ and $s_y^2 = \Sigma(y_i - \hat{y})^2 / (n - p)$) (B). $\Diamond$ = 90 °C; $\square$ = 100 °C; $\triangle$ = 110 °C.
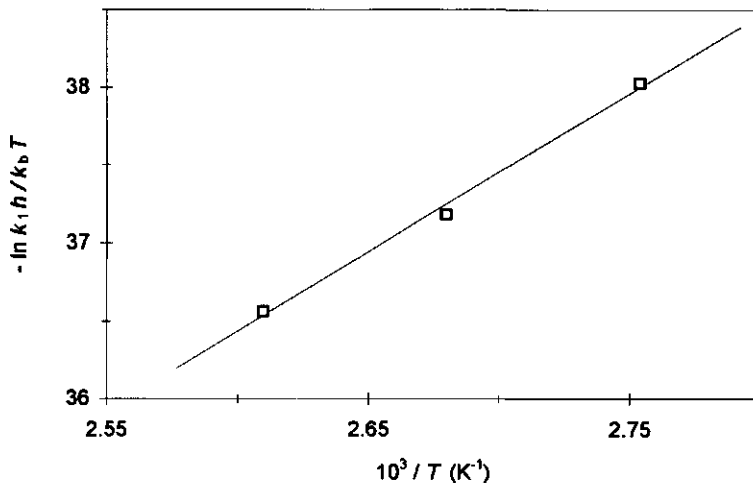
Figure 6.2: Eyring-plot for the rate constants of thermal inactivation of the proteinase from *Pseudomonas fluorescens* 22F, grown in skimmed milk. k_i = reaction constant calculated for first order inactivation, h = Planck's constant ($6.62 \cdot 10^{-34}$ J s), k_b = Boltzmann's constant ($1.38 \cdot 10^{-23}$ J K^{-1}), and T = temperature (K). Line was calculated with eq. 6.12.

Table 6.2:   Inactivation rate constants and initial activities (± 95 % confidence intervals) as estimated with inactivation model consisting of a single first-order reaction (model 1). Enzyme solution used was supernatant from a culture of *Pseudomonas fluorescens* 22F, grown in skimmed milk, diluted in demineralized water, pH 7.0. n is the number of observations, *SSE* the residual sum of squares.

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

It can be seen from figure 6.1B that the distribution of the residuals of the fit with model 1 seems reasonable, but 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-order reaction. This was confirmed by the order of the reaction. The reaction orders with respect to concentration and time were determined as described in section 5.4.1. For the estimation of the reaction order with respect to concentration enzyme preparations were heated at 100 °C. The enzyme preparations used were undiluted supernatant from a culture in skimmed milk, and supernatant diluted 10 and 100 times in 0.2 *M* TrisHCl containing 2 m*M* CaCl$_2$. For the estimation of the reaction order with respect to time, results from the experiment with 10 times diluted supernatant were used. The reaction orders with respect to concentration and time (± 95 % confidence interval) were found 0.78 (± 0.68) and 0.75 (± 0.06), respectively. The confidence interval of the reaction order with respect to concentration was quite large, due to the small number of observations. However, these reaction orders indicate that the reaction can not be described with a single first-order reaction, but that intermediates must be present in the reaction sequence (Hill, 1977). Driessen (1983), investigating the inactivation of the same proteinase, when heated as a complete culture in skimmed milk medium, also found such inactivation behaviour. In section 6.4.2 other inactivation models will be discussed. For comparison of our data with those of others, we will assume that the first-order inactivation model is correct, because other authors also assumed this.

Driessen (1983) performed similar experiments with proteinases from *Pseudomonas fluorescens* 22F. Instead of diluted supernatant, Driessen heated complete cultures in skim milk in which the bacteria had grown. Although non first-order inactivation was found by Driessen, the kinetic parameters were nevertheless

Table 6.3: Kinetic data of heat inactivation of proteinases from various *Pseudomonas fluorescens* strains

Strain	MC60	AR11	B52	AFT36	112	P38	MFP1	22F	22F
Ref.	Barach & Adams (1977)	Alichanidis & Andrews (1977)	Richardson (1981)	Stepaniak & Fox (1983)	Kroll & Klostermeyer (1984)	Owusu & Doble (1994)	Fortina et al. (1989)	Driessen (1983)	This work
Purification[a]	p	p	p	p	np	np	np	np	np
Heating menstrua	buffer[b]	buffer[c]	buffer[d]	SMUF[e]	skimmed milk peptone		milk	culture in skim milk[f]	supernatant in water
Temp. range	110-150	120-160	110-150	70-150	130-150	100-145	90-135	70-130	90-110
$\Delta H^{\ddagger}$ (kJ mol⁻¹)	77.0	90.7	100.5	84.5	115.1	32.8	72.4	97.8	83.6
$\Delta S^{\ddagger}$ (J mol⁻¹ K⁻¹)	- 89.5	- 52.8	- 33.7	- 66.5	- 2.5	- 201	- 110	- 48.8	- 85.4
$\Delta G^{\ddagger}$ (kJ mol⁻¹)[g]	112.1	111.5	113.7	110.7	116.0	111.8	114.9	116.0	117.4

a: np = not purified; p = purified
b: 0.1 M TrisHCl in 10 mM CaCl$_2$, pH 7.0
c: 0.05 M KH$_2$PO$_4$, pH 6.5
d: 0.02 M TrisHCl in 10 mM CaCl$_2$, pH 7.5
e: Simulated Milk Ultrafiltrate
f: Complete skim milk culture (5d, 20 °C)
g: at 120 °C

calculated using first-order kinetics. Our results are more or less comparable to those from 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 6.3). 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 inactivation data of proteinases of other *Pseudomonas fluorescens* strains, some of which have been partly recalculated using original data from the publications (table 6.3). It must be noted that comparison of the inactivation data is difficult. Firstly, because the experimental conditions were different, secondly, 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 modelling of the heat inactivation of proteinases from *Pseudomonas 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. In order to deal with this problem, it would be recommendable to study the inactivation behaviour of proteinases from many different *Pseudomonas fluorescens* strains under standard conditions, using the direct method to estimate kinetic parameters, as described above.

6.4.2 Modelling experimental results with alternative inactivation models

From the residual plot of the first-order inactivation model it was concluded that the fit was not adequate. Alternative inactivation models (table 6.1) were tried to fit the data, using unweighted nonlinear regression. The results of the simulations are given in table 6.4 and shown in figures 6.3 to 6.8. The fits obtained in the various models were examined for the distribution of the residuals. Obviously, the general model had the most random distribution of residuals. The measurement errors were homoscedastic (figure 6.3), so there was no necessity to perform transformation or weighting of the errors.

Problems occurred when performing the statistical analysis of the larger models. When analyzing the inactivation data with model 5 no convergence was obtained, because of the high correlation between the parameters. For model 7 a solution was found, but obviously 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 6.4). Most estimates of the parameters in these models were not significant, as zero was in the confidence interval. As was stated in section 6.2, 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 reparameterization. However, also high correlation was found between the kinetic parameters determining different reactions rates, especially when estimating models consisting of many parameters. A high $|\rho|$ value 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 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 behaviour with respect to heating time and temperature. In models 2 and 3 this nonrandomness was still observed, although to a lesser extent. Models 4 and 6 showed random behaviour. Finally, the residuals in model 7 showed that the residuals for the 90 °C experiment were not randomly distributed, obviously because the calculated residual activity dropped rapidly to 0.88 as result of a fast first reaction.

Besides examination of the distribution of the residuals, the models were compared statistically. The different models were tested against the general model using the lack of fit test. In addition, the residual variance, and Akaike's and Schwarz' criteria were calculated for the different models (table 6.5). 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 this 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' criteria it was concluded that models 4 and 6 were the best models to describe the inactivation.

Model selection

The selection of a model depends on the purpose of the study. As stated in chapter 2, mathematical modelling 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 modelling it is recommendable to choose the model in which the fewest parameters are estimated (Ockham's razor), because it is the easiest model to use. Moreover, this model would be the most stable, because the parameters are less correlated. Furthermore, the
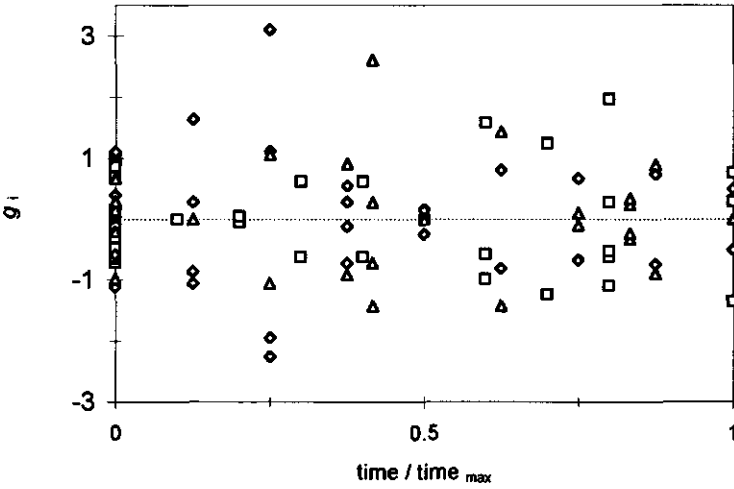
Figure 6.3: Studentized residuals for inactivation data analyzed with general model. ◊ = 90 °C; □ = 100 °C; △ = 110 °C.
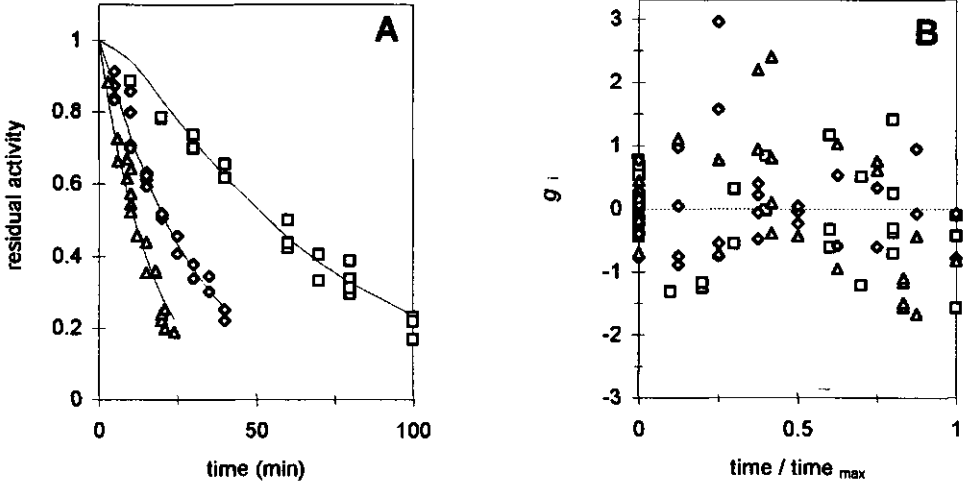
Figure 6.4: Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, analyzed with model 2, eq. 6.6 (A), and the studentized residuals (B). ◊ = 90 °C; □ = 100 °C; △ = 110 °C.
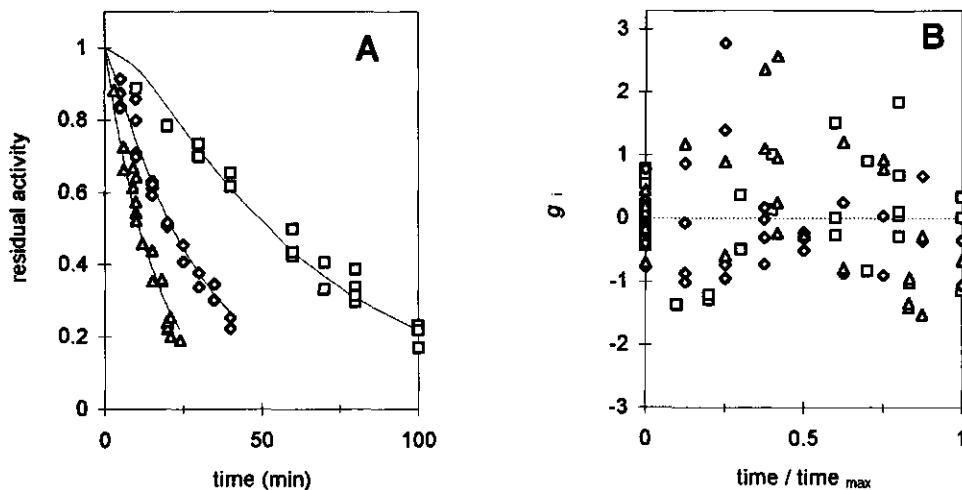
Figure 6.5:   Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, analyzed with model 3, eq. 6.7 (A), and the studentized residuals (B). $\Diamond$ = 90 °C; $\square$ = 100 °C; $\triangle$ = 110 °C.
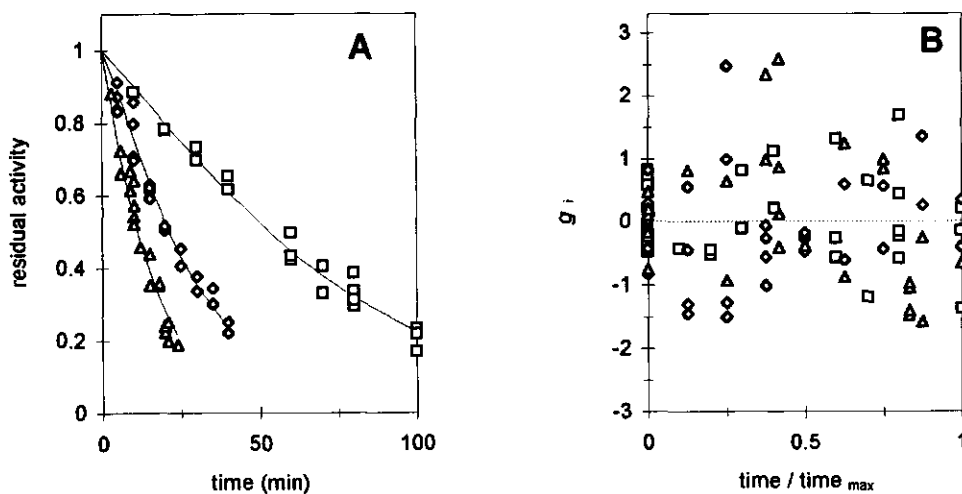
Figure 6.6:   Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, analyzed with model 4, eq. 6.8 (A), and the studentized residuals (B). $\Diamond$ = 90 °C; $\square$ = 100 °C; $\triangle$ = 110 °C.
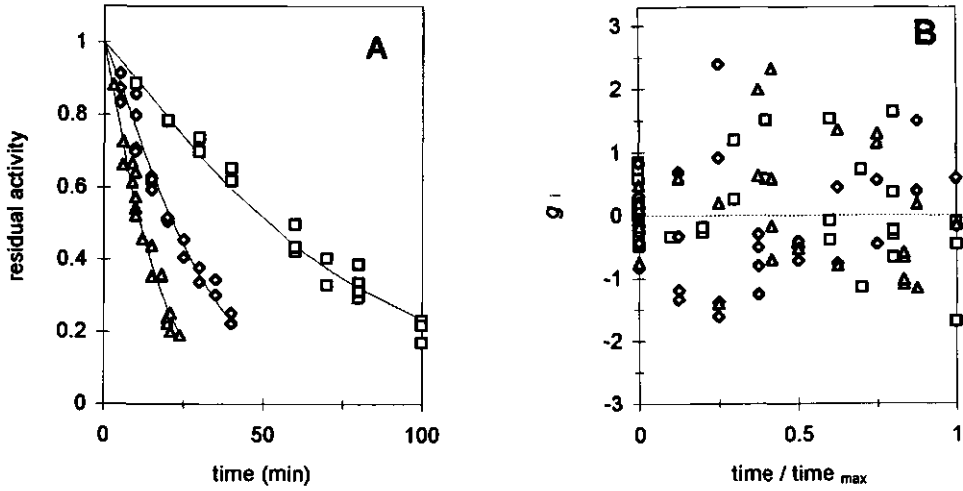
Figure 6.7:   Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, analyzed with model 6, eq. 6.10 (A), and the studentized residuals (B). $\Diamond$ = 90 °C; $\square$ = 100 °C; $\triangle$ = 110 °C.
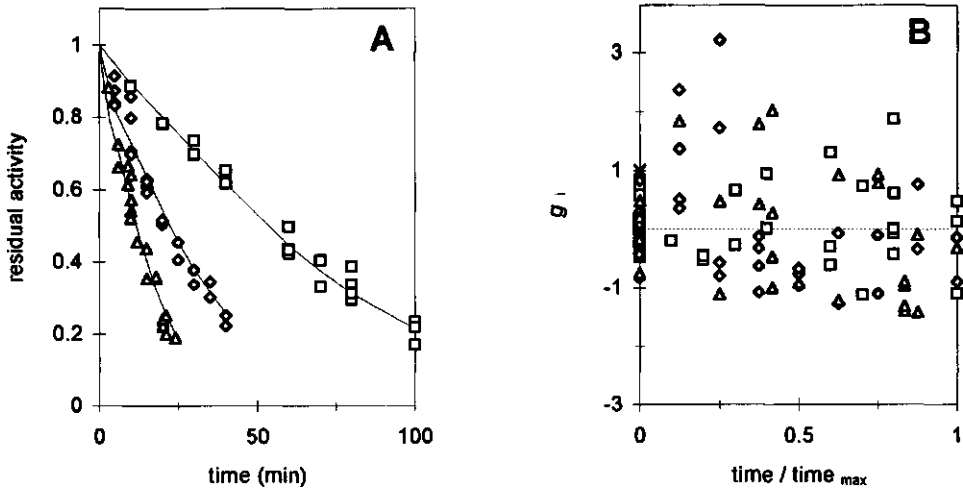
Figure 6.8:   Inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, analyzed with model 7, eq. 6.11 (A), and the studentized residuals (B). $\Diamond$ = 90 °C; $\square$ = 100 °C; $\triangle$ = 110 °C.

Table 6.4: Kinetic parameters (± 95% confidence interval) for the alternative models describing the inactivation of the proteinase from *Pseudomonas fluorescens* 22F. Included are the correlation matrices and the calculated sum of squares of the errors (SSE).

Model	Estimate (± 95% confidence interval)	Correlation matrix						SSE
1	$\Delta H_1^{\ddagger}$ = 84.5 (± 5.0) kJ mol⁻¹	1						0.143
	$\Delta S_1^{\ddagger}$ = -83.1 (± 13.6) J mol⁻¹ K⁻¹	-0.01	1					
2	$\Delta H_1^{\ddagger}$ = 267.9 (± 829.2) kJ mol⁻¹	1						0.112
	$\Delta S_1^{\ddagger}$ = 436.8 (± 511.2) J mol⁻¹ K⁻¹	-0.19	1					
	$\Delta H_{-1}^{\ddagger}$ = 55.4 (± 873.2) kJ mol⁻¹	0.82	-0.70	1				
	$\Delta S_{-1}^{\ddagger}$ = -161.8 (± 1425.3) J mol⁻¹ K⁻¹	0.48	-0.95	0.88	1			
	$\Delta H_2^{\ddagger}$ = 62.5 (± 724.1) kJ mol⁻¹	-0.99	0.18	-0.80	-0.46	1		
	$\Delta S_2^{\ddagger}$ = -140.8 (± 1951.1) J mol⁻¹ K⁻¹	0.06	-0.99	0.61	0.91	-0.04	1	
3	$\Delta H_1^{\ddagger}$ = 258.5 (± 101.7) kJ mol⁻¹	1						0.115
	$\Delta S_1^{\ddagger}$ = 411.6 (± 278.8) J mol⁻¹ K⁻¹	0.87	1					
	$\Delta H_2^{\ddagger}$ = 71.8 (± 7.2) kJ mol⁻¹	-0.14	0.31	1				
	$\Delta S_2^{\ddagger}$ = -116.3 (± 20.0) J mol⁻¹ K⁻¹	-0.43	-0.67	-0.62	1			
4	$\Delta H_1^{\ddagger}$ = 55.8 (± 17.4) kJ mol⁻¹	1						0.099
	$\Delta S_1^{\ddagger}$ = -157.9 (± 48.1) J mol⁻¹ K⁻¹	-0.91	1					
	$\Delta H_2^{\ddagger}$ = 221.2 (± 44.4) kJ mol⁻¹	0.19	-0.48	1				
	$\Delta S_2^{\ddagger}$ = 298.1 (± 129.0) J mol⁻¹ K⁻¹	0.68	-0.84	0.69	1			
	β = 0.61 (± 0.17)	-0.53	0.52	-0.23	-0.10	1		

Table 6.4: continued:

Model	Estimate (± 95% confidence interval)	Correlation matrix								SSE
5	No convergence									-
6	$\Delta H_1^\ddagger$ = 173.1 (± 278.8) kJ mol⁻¹	1	0.91	0.94	-0.96	-0.98	0.61			0.096
	$\Delta S_1^\ddagger$ = 154.9 (± 755.3) J mol⁻¹ K⁻¹		1	0.95	-0.96	-0.86	-0.37			
	$\Delta H_2^\ddagger$ = 5.4 (± 114.0) kJ mol⁻¹			1	-0.99	-0.91	0.57			
	$\Delta S_2^\ddagger$ = -288.0 (± 313.5) J mol⁻¹ K⁻¹				1	0.91	-0.60			
	$\Delta H_3^\ddagger$ = 89.5 (± 103.8) kJ mol⁻¹					1	-0.57			
	$\Delta S_3^\ddagger$ = -73.1 (± 281.2) J mol⁻¹ K⁻¹						1			
7	$\Delta H_1^\ddagger$ = 453.7 (± 151.3) kJ mol⁻¹	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) J mol⁻¹ K⁻¹		1	-0.48	-0.67	0.90	0.84	0.48	-0.61	
	$\Delta H_2^\ddagger$ = 56.4 (± 125.0) kJ mol⁻¹			1	0.17	-0.77	-0.48	0.19	0.02	
	$\Delta S_2^\ddagger$ = -152.8 (± 349.5) J mol⁻¹ K⁻¹				1	-0.62	-0.92	-0.77	0.97	
	$\Delta H_3^\ddagger$ = 94.7 (± 138.3) kJ mol⁻¹					1	0.87	0.23	-0.49	
	$\Delta S_3^\ddagger$ = -51.1 (± 380.4) J mol⁻¹ K⁻¹						1	0.56	-0.83	
	β_1 = 0.88 (± 0.15) kJ mol⁻¹							1	-0.89	
	β_2 = 0.72 (± 0.96) J mol⁻¹ K⁻¹								1	

Table 6.5:   Evaluation of models describing the inactivation of proteinase from *Pseudomonas fluorescens* 22F with *F*-ratio test, residual variance (s^2), Akaike's criterion (*AIC*) and Schwarz' criterion (*SC*).

model	n	p	SSE	f	F_{tab}	$10^3 \, s^2$	AIC	SC
general	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 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 acceptable model with the fewest parameters is model 3.

When the purpose of modelling 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 in order to be able to distinguish also between very complex models. From the models studied in this section, three were acceptable. Models 4 and 6 were found to be the best models according to the residual variance, and Akaike's and Schwarz' criteria. Both models described the data adequately, as the distribution of the residuals showed random behaviour. 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, as given in this chapter, is carried out with a number of data sets in order to find out 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 *Pseudomonas fluorescens* 22F in the temperature range 90 - 110 °C.

In order 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 results of Driessen (1983). In the first place, the kinetic parameters found in our experiments (table 6.4) were used for modelling the inactivation data from Driessen (figure 6.9). There is some discrepancy between the modelled 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}. The results of the modelling are given in figure 6.10. The results of this fit were reasonable. At 80 °C and 90 °C, and to a lesser extent at 70 °C and 100 °C, the logarithmic curves are not linear, indicating that non first-order inactivation occurs. 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.

Although Driessen also perceived the lagged inactivation behaviour at 90 and 100 °C, he determined the reaction rates of the inactivation at all temperatures as if they were obeying first-order kinetics. Subsequently, Driessen estimated the kinetic parameters from the Arrhenius equation (eq. 2.10). It is interesting to note that in Driessen's Arrhenius plot, in which the relationship between the reaction rate constant for inactivation and the temperature is given, there appears to be a break in the curve at 85 °C, whereas a linear relation would be expected if only one reaction is rate limiting (figure 6.11). We also determined reaction rates from the calculated curves of figure 6.10, neglecting the lagged inactivation and assuming first order inactivation kinetics, and compared these with Driessen's results. We also found the deviation from linearity, obviously because the different reactions are rate limiting in different temperature regimes. Also Barach and Adams (1977) have reported a nonlinear slope in the Arrhenius plot at 139 °C for the inactivation of proteinase from *Pseudomonas* sp. MC60, indicating an accelerated inactivation above this temperature or an inactivation mechanism consisting of more than one reaction.

From the kinetic data presented above it is not yet possible to speculate about the mechanism, except that the inactivation is not caused by a single reaction, but by a sequence of at least two reactions. Additional information would have to be collected from heating experiments under various conditions. In the following sections, the influence of growth medium, pH, calcium ion activity, and protein concentration on thermal inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F is described.
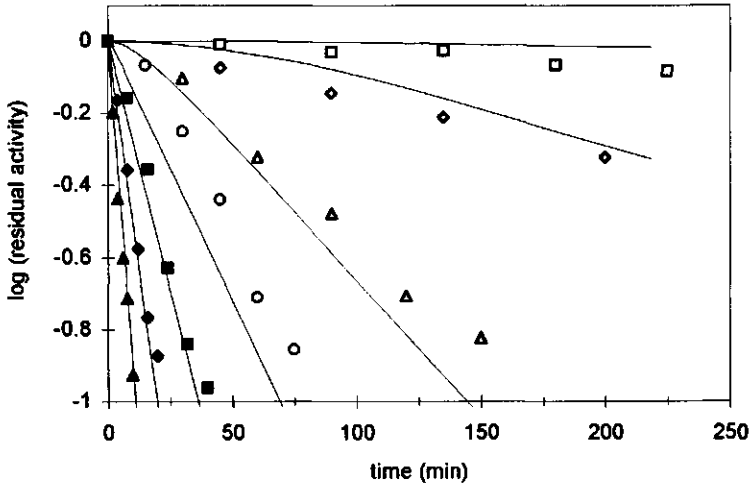
Figure 6.9: Calculated inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, using model 3 with $\Delta H_1^{\ddagger} = 258.5$ kJ mol^{-1}, $\Delta S_1^{\ddagger} = 411.6$ J mol^{-1} K^{-1}, $\Delta H_2^{\ddagger} = 71.8$ kJ mol^{-1}, and $\Delta S_2^{\ddagger} = -116.3$ J mol^{-1} K^{-1}. Inactivation data from Driessen (1983):□ = 70 °C; ◊ = 80 °C; Δ = 90 °C; O = 100 °C; ■ 110 °C; ◆ = 120 °C;▲ = 130 °C.
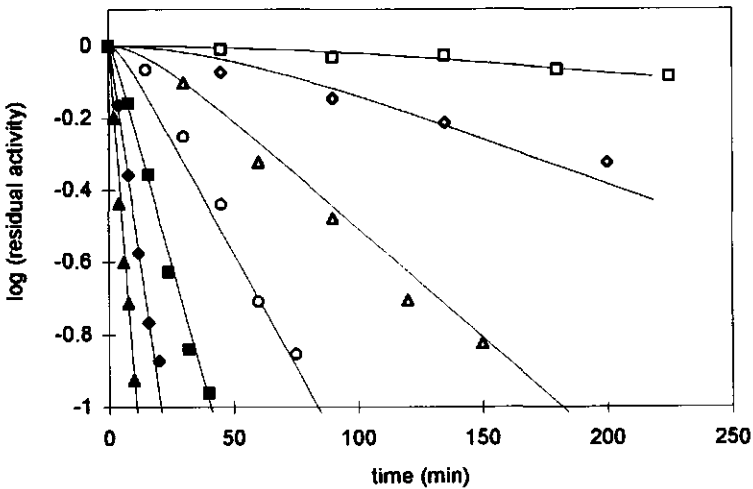
Figure 6.10: Calculated inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F, using model 3 with $\Delta H_1^{\ddagger} = 139.3$ kJ mol^{-1}, $\Delta S_1^{\ddagger} = 81.2$ J mol^{-1} K^{-1}, $\Delta H_2^{\ddagger} = 79.8$ kJ mol^{-1}, and $\Delta S_2^{\ddagger} = -96.2$ J mol^{-1} K^{-1}. Inactivation data from Driessen (1983): □ = 70 °C; ◊ = 80 °C; Δ = 90 °C; O = 100 °C; ■ 110 °C; ◆ = 120 °C;▲ = 130 °C.
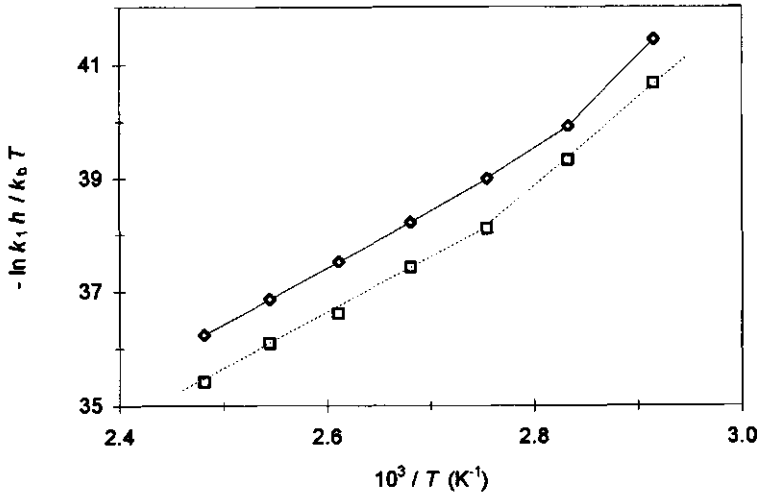
Figure 6.11: Eyring-plot for the rate constants of the calculated thermal inactivation of the proteinase from *Pseudomonas fluorescens* 22F (□). Included are rate constants from Driessen (1983) (◊). k_i = reaction constant calculated for first-order inactivation, h = Planck's constant ($6.62 \cdot 10^{-34}$ J s), k_b = Boltzmann's constant ($1.38 \cdot 10^{-23}$ J K^{-1}), and T = temperature (K).

6.4.3 Influence of growth medium on the kinetics of thermal inactivation

Pseudomonas fluorescens 22F was grown in different media. Because in most of our experiments we used unpurified enzyme, it is not unlikely that the inactivation behaviour was affected by components of the various growth media. In order to examine the influence of the growth media, inactivation data of enzyme solutions obtained from various growth media were compared. Growth media used were fresh skimmed milk; 2.5 % sodium caseinate in milk ultrafiltrate; and tryptone-lactose medium. The supernatant obtained from the culture in skimmed milk was diluted 10 times in demineralized water, the other supernatants were diluted in 0.2 M Tris Maleate containing 2 mM CaCl$_2$, pH 7.4. Heating experiments were performed as described above. The results are shown in figure 6.12. The inactivation was analyzed with model 4 (eq. 6.8). The results are summarized in table 6.6.

It appeared that the inactivation of the proteinase varied among experiments. The proteinase produced in skimmed milk appeared to be slightly more heat stable. Furthermore, the temperature dependence was different, as can be seen from the kinetic parameters. To test whether this difference was statistically significant, we used an F-ratio test (Motulsky and Ransnas, 1987), as described in section 6.2. The results are given in table 6.7.
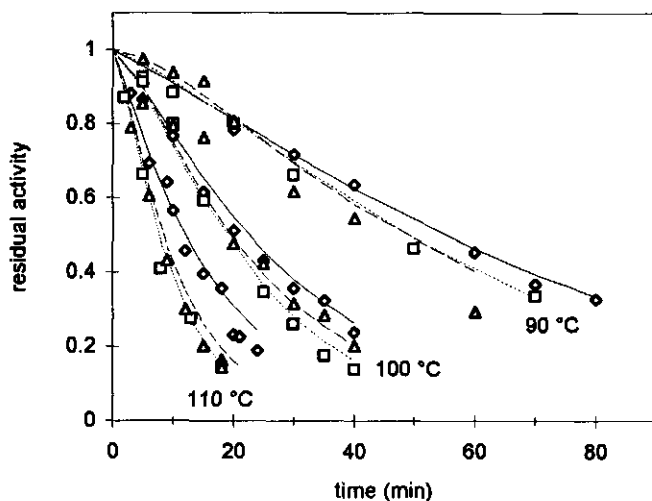
Figure 6.12: Heat inactivation of proteinase from *Pseudomonas fluorescens* 22F obtained from various growth media. - - - □ - - - = skimmed milk; ——◇—— = sodium caseinate in milk ultrafiltrate; — ——△— —— = tryptone-lactose medium. Lines calculated with model 4.

Table 6.6: Kinetic parameters of thermal inactivation of proteinase from *Pseudomonas fluorescens* 22F, grown in various media (described above), analyzed with model 4. Activation enthalpy $\Delta H^\ddagger$ (in kJ mol^{-1}) and entropy $\Delta S^\ddagger$ (in J mol^{-1} K^{-1}) ± 95 % confidence interval.

growth medium	$\Delta H_1^\ddagger$ (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
skimmed milk	56 ± 18	- 158 ± 48	221 ± 44	298 ± 129	0.61 ± 0.17
caseinate in ultrafiltrate	86 ± 11	- 74 ± 33	145 ± 22	91 ± 67	0.74 ± 0.14
tryptone-lactose medium	92 ± 18	- 60 ± 47	124 ± 63	41 ± 177	0.93 ± 0.78
pooled	99 ± 12	- 42 ± 34	108 ± 71	- 5 ± 198	0.56 ± 0.42

Table 6.7:   Results of the F-ratio test, comparing separate fits with the fit of the pooled data set.

data set	SSE	n	v	f	F
skimmed milk	0.099	67	62		
caseinate in milk ultrafiltrate	0.057	46	41		
tryptone-lactose medium	0.176	53	48		
SSE_{sep}	0.332	166	151	17.8	3.3
SSE_{pool}	0.727	166	161		

It may be concluded that the difference in thermal inactivation of the proteinase in the various enzyme solutions is statistically significant. Of course, the next question to be answered is why the proteinases, produced by the same bacteria, but in different growth media, were inactivated differently. An explanation could be that different growth media induce the bacteria to produce different proteinases, either because the proteinase has different characteristics, or because more than one or another proteinase is produced. Most studies on the expression of proteinases by *Pseudomonas fluorescens* report the production of a single proteinase, although the production of more than one proteinase is observed for some strains (Fairbairn and Law, 1986a; McKellar, 1989). We also found a single proteinase produced in detectable amounts by *Pseudomonas fluorescens* 22F, when grown in tryptone-lactose growth medium (chapter 4 of this thesis).

Another explanation of the variation in thermal stability of the proteinases produced in the various media could be the presence or absence of one or more substances during the heating experiment. One could think of the buffer in which the supernatant was diluted, of substances from the growth medium, or of substances produced by the bacteria, e.g. inhibitors or activators. A few factors that could possibly give raise to variation are investigated in the following sections. We did not investigate the effects of growth medium on enzyme production and thermal stability in more detail. Although important and interesting, we considered this beyond the scope of this thesis.

6.4.4 Influence of pH on the kinetics of thermal inactivation

To our knowledge, studies of the influence of pH on the rate of thermal inactivation of proteinases from *Pseudomonas fluorescens* or from other psychrotrophic bacteria, have not been published. For other enzymes it is known that the rate of irreversible inactivation is dependent on pH (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). As described in chapter 1, inactivation of enzymes can be
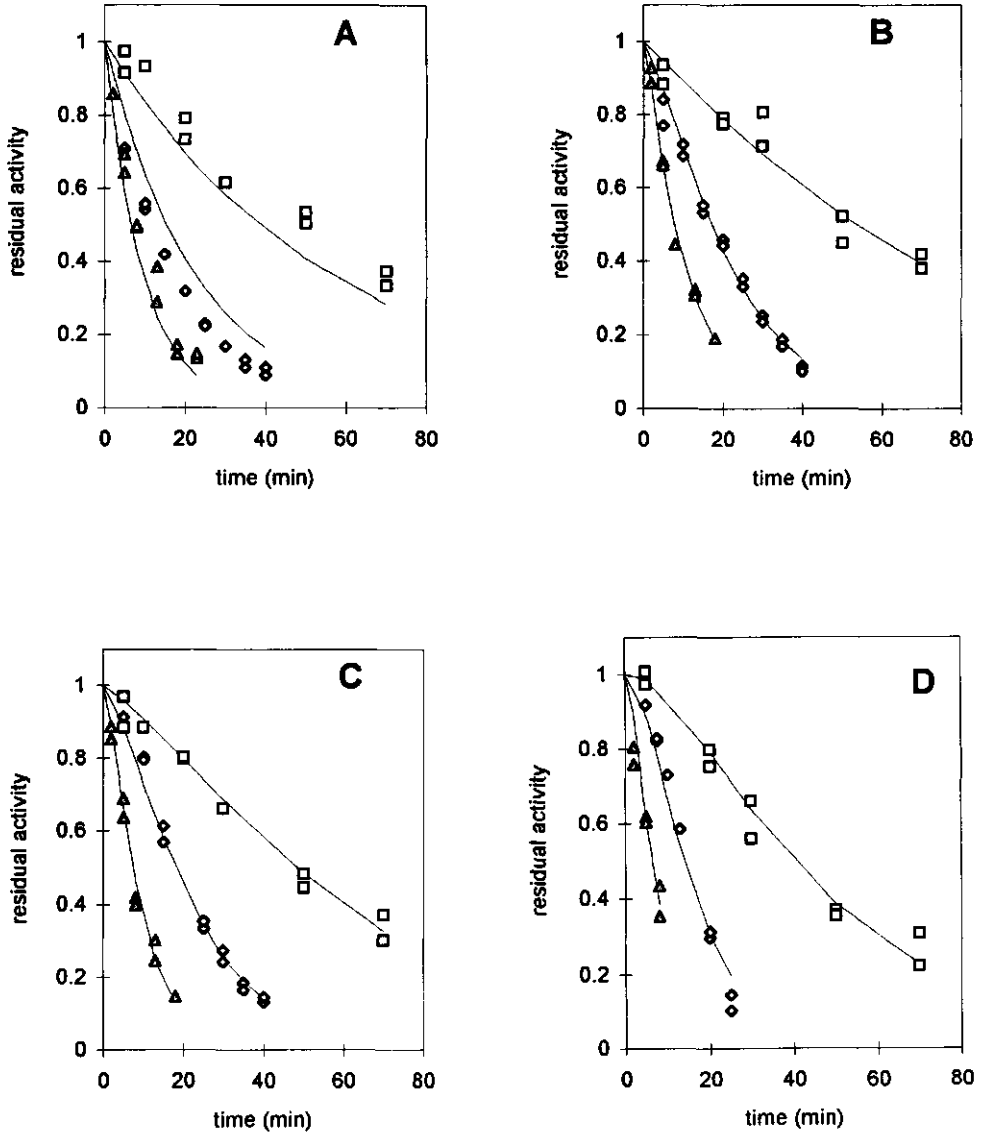
Figure 6.13: Influence of pH on the inactivation of extracellular proteinase from *Pseudomonas fluorescens* 22F. □ = 90 °C; ◊ = 100 °C; ▵ = 110 °C; A = pH 5.5; B = pH 6.5; C = pH 7.5; D = pH 8.6; Lines calculated with model 4.

caused by various reactions, each of which having its own pH and temperature dependency. Consequently, to study the influence of pH on the inactivation, experiments must be performed at several temperatures.

In order to investigate the influence of pH on inactivation kinetics, heating experiments were performed at 90, 100, and 110 °C. The enzyme solution used for these experiments was the raw supernatant from a culture of *Pseudomonas fluorescens* 22F, grown in 2.5 % sodium caseinate in milk ultrafiltrate. The supernatant was diluted 10 times with 0.2 M Tris Maleate buffer in 20 mM $CaCl_2$, pH 5.5, 6.5, 7.5, and 8.6, heated as described in section 6.3, cooled in ice water, and subsequently, the residual activity was measured. Although the pH during incubation with sodium caseinate deviated slightly from 7.0, due to the influence of the heating buffer, the activity of the proteinase was not materially changed (chapter 4 of this thesis).

The results are shown in figure 6.13. When heated at 90 °C, the inactivation of the proteinase was slightly faster at pH 8.6 than at other pH values. At 100 °C, the inactivation did not show the typical behaviour as described in section 6.3.3 in the case of pH 5.5 and 6.5, and presumably first-order inactivation kinetics would apply better. At pH 7.5 and 8.6, the lagged inactivation behaviour was found. The inactivation at pH 5.5 and 6.5 appears to be fastest, but when looking at the inactivation in the final stage of the heating experiment, inactivation was faster at pH 7.5 and 8.6. Finally, at 110 °C the inactivation was relatively slow at pH 5.5 when compared with the other pH values. Summarizing the results, there seems to be a tendency that the rate of inactivation increased with increasing pH.

We have analyzed the inactivation with the model of two sequential reactions of which the first leads to an enzyme molecule with a lower relative specific activity β (model 4). In the cases where first-order kinetics would give better results, model 4 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. Of course, this will also be reflected in the confidence intervals of the kinetic parameters. The kinetic parameters of the inactivation are given in table 6.8. The results were analyzed statistically with an F-ratio test (table 6.9).

The difference in inactivation at different pH values was statistically significant, when analyzed with model 4. However, as stated above, model 4 is not always the best description of the inactivation, and consequently the confidence intervals 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). Klibanov and coworkers analyzed the inactivation of the enzymes with first-order kinetics, and found orders of magnitude of difference in the rate constants of inactivation between pH 4 and 8. This difference was considered to

Table 6.8:   Kinetic parameters (± 95 % confidence interval) for the inactivation at various pH.

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
pooled	53 ± 17	- 162 ± 46	245 ± 40	361 ± 112	0.73 ± 0.12

Table 6.9.   Statistical analysis of inactivation data at various pH.

pH		SSE	n	ν	f	F
5.5		0.029	36	31		
6.5		0.049	37	32		
7.5		0.037	32	27		
8.6		0.072	26	21		
	SSE_{sep}	0.187	131	111	11.8	1.8
	SSE_{pool}	0.481	131	126		

be a result of the fact that at different pH values different reactions are the cause of the inactivation. The various reactions that can lead to inactivation of an enzyme and their pH dependencies are:
- Deamidation of asparagine (Asn) and glutamine (Gln), resulting in the formation of aspartic acid (Asp) and glutamic acid (Glu), respectively. This reaction is relatively fast at high temperature and pH (Zhang et al., 1993ab; Riha et al., 1996), and could considerably contribute to inactivation of enzymes (Ahern and Klibanov, 1985; Zale and Klibanov, 1986). The Arrhenius activation energy for soy protein deamidation is 111.3, 102.6 and 86.9 kJ mol^{-1} at pH 5.0, 7.0 and 9.0, respectively (Zhang et al., 1993b).
- Exchange of disulfide bonds and destruction of cystine residuals by β-elimination contributes considerably to enzyme inactivation in alkaline environment (Ahern and

Klibanov, 1988; Kosen, 1992; Volkin and Middaugh, 1992). Rate constants for β-elimination at 100 °C are 0.8 h^{-1}, 0.06 h^{-1}, and 0.005 h^{-1} at pH 8, 6 and 4, respectively (Volkin and Klibanov, 1987). Because most proteinases from *Pseudomonas fluorescens* do not contain any cysteine (Mayerhofer et al., 1973; Barach and Adams, 1977; Richardson, 1981; Diermayr and Klostermeyer, 1984; Mitchell et al., 1986), this reaction probably is not very important in our case.

- Hydrolysis of the polypeptide backbone at aspartic acid residues can contribute to thermal inactivation of enzymes under acidic conditions (pH 2 - 4), and to a much lesser extent at higher pH (Volkin and Middaugh, 1992). We did not study the inactivation of the proteinase in this low pH range.

- The 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. 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.

- Aggregation is also assumed to be pH dependent. At the isoelectric pH aggregation is presumed maximal, because electrostatic repulsion between the molecules is minimal.

The isoelectric pH of the extracellular proteinase from *Pseudomonas fluorescens* 22F was estimated to be pH 7.4 (chapter 4 of this thesis). At this pH inactivation was not faster than at other pH values, which may indicate that aggregation or incorrect refolding of the molecules play no important role in the inactivation of the proteinase. The fact that activity was largely recovered after prolonged heating of the enzyme at 70 °C, confirmed this conclusion. Considering that cysteine residues are probably not present in the enzyme molecule, it is not strange that pH has only a small influence on inactivation. Therefore, an inactivation involving deamidation of one or more asparagine or glutamine residues appears the most probable mechanism. Also for the proteinase from *Pseudomonas fluorescens* 112 deamidation was suggested to be the mechanism for thermal inactivation, because heating at 90 °C caused a lower 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 did not always lead to a completely inactivated molecule. Often a molecule is found with a lower relative specific activity (Ahern and Klibanov, 1985; Zale and Klibanov, 1986; Ahern et al., 1987; Tomizawa et al., 1994). This would be in agreement with the simulation study in section 6.3.2. Model 4, describing a partial inactivation by a first reaction followed by complete inactivation by another, was found to be one of the two best models according to the residual variance, and Akaike's and Schwarz' criteria. The relative specific activity of 0.6 of the partially inactivated molecule is in accordance with those found by Klibanov and coworkers.
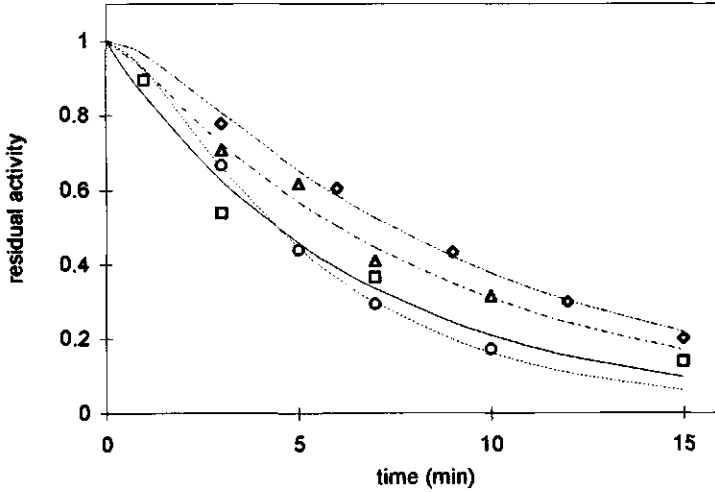
Figure 6.14: Thermal inactivation of the proteinase from *Pseudomonas fluorescens* 22F, when heated at 110 °C. ——□—— 0.15 mM CaCl$_2$; --—◊---- 20 mM CaCl$_2$; ——△——— 280 mM CaCl$_2$; ----O----- 0.5 mM EDTA; mean values of duplicates
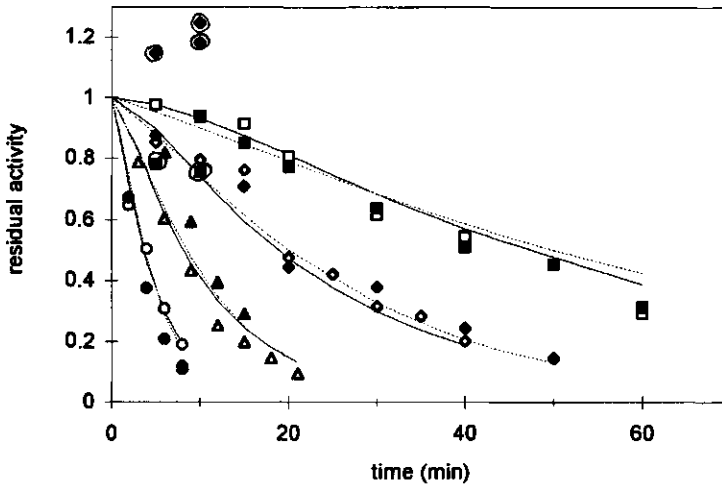
Figure 6.15: Inactivation of extracellular proteinase from *Pseudomonas fluorescens* 22F as function of calcium ion activity. □ = 90 °C; ◊ = 100 °C ; △ = 110 °C; O = 120 °C; open markers = aCa^{2+} 2.2•10^{-3} M, closed markers = aCa^{2+} 8•10^{-8} M; ——— = aCa^{2+} 2.2•10^{-3} M, - - - - - = aCa^{2+} 8•10^{-8} M; mean values of duplicates.

6.4.5 Influence of calcium ion activity on the kinetics of thermal inactivation

As mentioned before, comparison of inactivation kinetics from various literature sources is difficult, because of variation in experimental conditions. In some publications it has been reported that the calcium content of the heating menstrua can influence the rate of inactivation of extracellular proteinases from *Pseudomonas fluorescens* at high temperatures (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 a reduced heat stability in the UHT region, indicating that divalent cation bridges may be involved in the stability of the proteinases. The native calcium content of milk was found 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 the influence of calcium ion activity was investigated. Crude supernatant from a culture of *Pseudomonas fluorescens* 22F grown in tryptone-lactose medium, was diluted in 0.2 M TrisHCl buffer, pH 7.0, containing either 0.15 mM $CaCl_2$, 20 mM $CaCl_2$, 280 mM $CaCl_2$, or 0.5 mM EDTA. The calcium ion activities of these enzyme preparations, as measured with a calcium ion selective electrode, were 1.1$\bullet 10^{-4}$ M, 2.2$\bullet 10^{-3}$ M, 3.8$\bullet 10^{-2}$ M, and 9.5$\bullet 10^{-6}$ M, respectively. The enzyme solutions were heated at 110 °C, cooled in ice water, and examined for residual activity. The results are given in figure 6.14. 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 be caused by displacement of Zn^{2+} by Ca^{2+}, causing a decreased activity, as was suggested Barach et al. (1976), but it may also be caused by the increased ionic strength. Lowering the calcium ion activity caused some destabilization.

In order to study the influence of the calcium ion activity in more detail, supernatant was diluted in 0.2 M Tris Maleate buffer, pH 7.4, containing either 20 mM $CaCl_2$ or 0.5 mM EDTA. The calcium ion activities of these dilutions were 2.2$\bullet 10^{-3}$ M and 8$\bullet 10^{-8}$ M, respectively. The enzyme solution containing EDTA was kept on ice prior to the heat treatment, because it was found that addition of EDTA could cause a shift in temperature range in which inactivation of the proteinase from *Pseudomonas fluorescens* 22F by autoproteolysis takes place (chapter 5), and high concentrations of EDTA might make the proteinase susceptible to autoproteolysis even at room temperature. The enzyme solutions were heated at 90, 100, 110 and 120 °C, cooled in ice water, and subsequently examined for residual activity. The results are given in

figure 6.15. Some of the residual activities in the experiment with EDTA had unexpected values (marked with a circle). These were considered outlyers and were omitted for statistical analysis. The results were analyzed with model 4 as described in section 6.2 (table 6.10 and 6.11):

Table 6.10: Kinetic parameters (± 95 % confidence interval) for the inactivation at various calcium ion activities, according to model 4.

aCa^{2+}	$\Delta H_1^{\ddagger}$ (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
$2.2 \cdot 10^{-3}$ M	92 ± 17	- 60 ± 47	124 ± 631	41 ± 177	0.93 ± 0.78
$8 \cdot 10^{-8}$ M	89 ± 74	- 61 ± 211	108 ± 57	-17 ± 158	0.82 ± 1.04
pooled	101 ± 91	- 35 ± 254	91 ± 238	- 56 ± 648	0.64 ± 0.21

Table 6.11: Statistical analysis of inactivation data at various calcium ion activities.

aCa^{2+}	SSE	n	v	f	F
$2 \cdot 10^{-3}$ M	0.176	53	48		
$8 \cdot 10^{-8}$ M	0.206	35	30		
SSE_{sep}	0.382	88	78	0.43	2.5
SSE_{pool}	0.393	88	83		

It may be concluded that the 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, we found that the extent of inactivation during heating-up did vary a lot. This will be discussed in section 6.4.7.

6.4.6 Influence of protein content and purification on the kinetics of thermal inactivation

In chapter 5 of this thesis it was concluded that protein has a strong protective influence on the inactivation of proteinase from *Pseudomonas fluorescens* 22F in the temperature range 40 to 60 °C. The influence of accompanying proteins on inactivation at high temperatures appears to vary among extracellular proteinases from various *Pseudomonas fluorescens* strains. Some proteinases have been reported to be 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.

In order to investigate the influence of sodium caseinate and skimmed milk on the inactivation kinetics, experiments were performed at 80, 95, 105 and 120 °C, and 80 and 120 °C, respectively. For the experiments unpurified supernatant from a culture of *Pseudomonas fluorescens* 22F in tryptone-lactose medium was used. The supernatant was diluted 10 times in 0.2 M Tris Maleate in 2 mM CaCl$_2$, pH 7.4, and to the enzyme solution 1.8 % sodium caseinate or 6.7 % skim milk powder was added. The results are shown in figure 6.16. Addition of sodium caseinate to the enzyme solution resulted in a slightly increased rate of inactivation of the proteinase. This may be caused by aggregation of enzyme molecules with caseinate molecules. Addition of skimmed milk powder did not influence the heat stability of the proteinase. The results of the inactivation experiments in absence and presence of sodium caseinate were analyzed with model 4, as described in section 6.2. The effect of sodium caseinate on the thermal inactivation was statistically significant. Besides the effect of caseinate on thermal inactivation, also strong influence of proteins on the inactivation during heating-up was found (section 6.4.7).

The purity of the enzyme is also reported to have a strong effect on the inactivation kinetics (Barach et al., 1976; Richardson, 1981; Leinmüller and Christopherson, 1982; Kumura et al., 1991). According to Kroll (1989), 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, as was concluded in chapter 5 of this thesis.
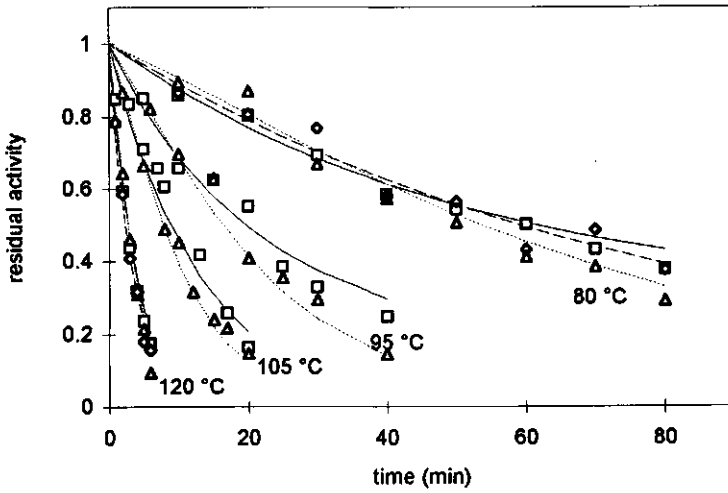
Figure 6.16: Influence of sodium caseinate and skimmed milk powder on the heat inactivation of proteinase from *Pseudomonas fluorescens* 22F. ——□—— = supernatant diluted in 0.2 M Tris Maleate in 2 mM $CaCl_2$, pH 7.4; —— ◇ —— = 6.7 % skimmed milk added to enzyme solution; - -▵- - = 1.8 % sodium caseinate added to enzyme solution. Mean values of at least duplicates.
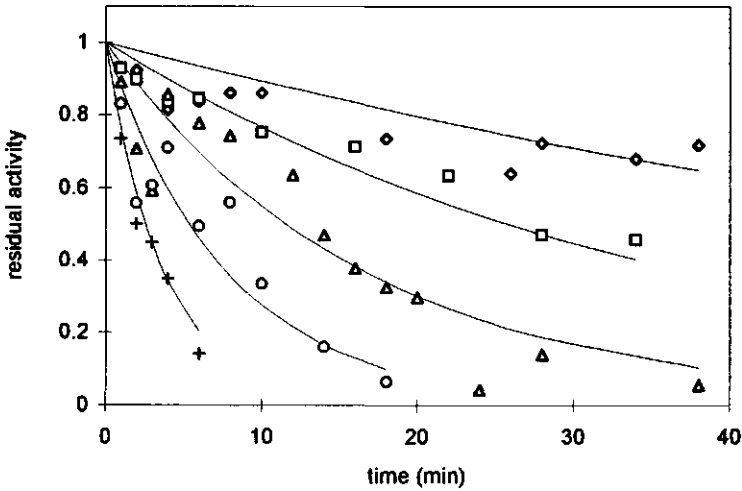
Figure 6.17: Inactivation of purified extracellular proteinase from *Pseudomonas fluorescens* 22F. ◇ = 80 °C; □ = 90 °C; ▵ = 100 °C; ○ = 110 °C; + = 120 °C; mean values of at least duplicates.

To investigate the influence of purification on the heat stability of the extracellular proteinase from *Pseudomonas fluorescens* 22F, purified proteinase was diluted in 0.2 *M* Tris Maleate, pH 7.4, to a final concentration of $1.7 \cdot 10^{-7}$ *M*, and inactivation experiments at 80 to 120 °C were performed. The results are shown in figure 6.17. The results were poorly reproducible and many unexpected results were found. The poor reproducibility may be due to variation in the extent of inactivation during heating-up. Because of the absence of accompanying protein, small differences in the temperature-time profile may cause relatively large differences in the extent of inactivation (section 6.4.7). As a consequence of the large errors it appeared impossible to analyze the results with model 4. Therefore, the results were analyzed with first-order inactivation kinetics. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (± 95 % confidence interval) of this reaction were 89.8 ± 7.4 kJ mol^{-1} and - 63.4 ± 21.6 J mol^{-1} K^{-1}, respectively. These kinetic parameters are comparable with those found for the inactivation of unpurified proteinase. The difference in kinetic parameters was not statistically significant, also because of the poor reproducibility of the inactivation of purified proteinase.

Table 6.12:   Kinetic parameters (± 95 confidence interval) for the inactivation in 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
pooled	76 ± 15	- 92 ± 50	92 ± 20	- 55 ± 60	0.73 ± 0.67

Table 6.13:   Statistical analysis of inactivation data of heating experiments in presence and absence of 1.8 % sodium caseinate

[caseinate]		SSE	n	ν	f	F
-		0.130	65	60		
1.8 %		0.135	69	64		
	SSE_{sep}	0.265	134	124	10.7	2.0
	SSE_{pool}	0.389	134	129		

6.4.7 Inactivation during heating-up

Until now the inactivation occurring during the heating-up period was neglected, and residual activities were calculated as fraction of the activity after the heating-up period of 2 min. However, it is interesting to evaluate the extent of inactivation occurring during the heating-up period, because the temperature is lower than the temperature at which the experiment is performed, and the rate of inactivation may be different. Assuming that the temperature dependence of the reaction rate behaves according to Eyring's theory, inactivation would be slower than at the final heating temperature. However, during the heating of the enzyme solution, the temperature also passes the temperature regime in which autoproteolysis occurs (chapter 5 of this thesis). Prolonged residence time in the critical zone for low temperature inactivation may cause increased inactivation by autoproteolysis, as electrophoresis experiments showed for other proteinases from other *Pseudomonas fluorescens* strains (Barach and Adams, 1977; Richardson, 1981; Diermayr et al., 1987). Consequently, the inactivation in the first minutes is dependent on factors influencing both autoproteolytic and thermal inactivation. In this section the influence of protein content, purification, and calcium ion activity on the inactivation will be discussed. Inactivation data were taken from experiments described in sections 6.4.5 and 6.4.6. Of these experiments the relative activities of unheated samples, compared with samples heated for 2 min, are given in table 6.14. These values give information about the amount of proteinase that was inactivated during the heating-up period.

Table 6.14:   The relative activity (± standard deviation) after 2 min of heating-up time compared to that of the unheated enzyme solution. Results taken from experiments described in sections 6.4.5 and 6.4.6.

T (°C)	Unpurified. 0.2 M Tris Mal + 2 mM CaCl$_2$	Unpurified. 0.2 M Tris Mal + 2 mM CaCl$_2$ + 1.5 % caseinate	Unpurified. 0.2 M TrisHCl + 20 mM CaCl$_2$	Unpurified. 0.2 M TrisHCl + 0.5 mM EDTA	Purified. 0.2 M Tris Mal
80	0.62 ± 0.09	0.98 ± 0.03	0.66 ± 0.02	-	0.75 ± 0.14
90	-	-	0.80 ± 0.11	0.52 ± 0.06	0.74 ± 0.09
95	0.74 ± 0.10	0.92 ± 0.08	-	-	-
100	-	-	0.77 ± 0.09	0.36 ± 0.03	0.66 ± 0.32
105	0.73 ± 0.02	0.90 ± 0.06	-	-	-
110	-	-	0.79 ± 0.06	0.57 ± 0.01	0.63 ± 0.18
120	0.67 ± 0.03	0.72 ± 0.07	0.68 ± 0.08	0.60 ± 0.08	0.51 ± 0.09

        In our study, in most cases the inactivation during heating-up was relatively fast as compared with inactivation at the final heating temperature. This was also found for many other proteinases from *Pseudomonas fluorescens* strains (Barach et al., 1976; Barach and Adams, 1977; Richardson, 1981; Leinmüller and Christophersen, 1982; Patel et al., 1983; Stepaniak and Fox, 1983; Kroll and Klostermeyer, 1984; Yan et al., 1985; Fairbairn and Law, 1986b; Diermayr et al., 1987; Azcona et al., 1988). However, in many of these reports the inactivation during heating-up was not taken into account when analyzing the kinetics of thermal inactivation, which led to misinterpretation of the mechanism or the kinetic parameters.

        The extent of inactivation of the proteinase was strongly affected by the composition of the heating menstrua. Presence of 1.5 % sodium caseinate largely reduced the inactivation. This stabilizing effect of proteins against inactivation during heating-up was also found by Barach et al. (1976), Richardson (1981) and Yan et al. (1985). Assuming that the fast inactivation is caused by autoproteolysis during heating-up, influence of concomitant proteins is expected, as these reduce the rate of autodigestion (chapter 5 of this thesis). Other explanations for the protective action of proteins could be the stabilization of a critical conformation near the active site by an enzyme-substrate complex or facilitation of refolding.

        The calcium ion activity also appeared to have strong influence on the inactivation during heating-up. Reduction of the calcium ion activity increased the initial inactivation of the proteinase (table 6.14). This difference was not expected, because the rates of autoproteolysis and thermal inactivation are not affected by calcium ion activity, as was concluded in sections 5.4.3 and 6.4.5 of this thesis, respectively. A mechanism for the protective effect of calcium was proposed by Barach and Adams (1977). Most of the extracellular proteinases from *Pseudomonas fluorescens* strains are metalloproteinases, containing a zinc ion in the active site. Except for this catalytic metal ion, calcium ions are present as structural elements, forming salt bridges. These salt bridges are supposed to be important for regeneration of the native conformation of the proteinase, rather than maintenance of native structure during heating. Calcium, and other divalent metal ions, would stabilize part of the native structure near the active site of the proteinase, allowing rapid and accurate enzyme renaturation. Possibly, partial unfolding by heat is necessary for removal of the divalent metal ions by EDTA. Another mechanism for initial inactivation in the presence of EDTA was found for thermolysin, the extracellular metalloproteinase from *Bacillus thermoproteolyicus* (Vita et al., 1985; Fassina et al., 1986; Fontana, 1988). In presence of EDTA, susceptibility to autoproteolysis of the native thermolysin molecule is increased, and the molecule can be hydrolysed into 3 peptide chains. These parts do not fall apart, but form a complex with a relative specific activity lower than that of the native molecule. Upon heating the complex falls apart, leading to irreversible inactivation.

Assuming that the increased inactivation is caused by autoproteolysis, then the effect is expected to be more pronounced if the heating temperature is relatively low (80 - 90 °C), because the time necessary to pass the critical temperature range for autoproteolysis is relatively long (eqs. 6.24 and 6.25). This was only found for unpurified proteinase in absence of sodium caseinate. From the results it appears as if autoproteolysis is not the sole cause of the relatively fast inactivation during heating up. Therefore, we made an estimate of the loss of proteolytic activity by autoproteolysis and thermal inactivation during heating-up based on reaction rate constants found earlier. The temperature change as function of time was estimated using equations 6.24 and 6.25. For estimating the inactivation in the temperature range 40 - 70 °C, reaction rate constants for unfolding, refolding and autoproteolysis at various temperatures were estimated by interpolation of results in table 5.1. The influence of sodium caseinate on the rate of inactivation by autoproteolysis was calculated as described in section 5.4.2, with K_m is $6.7 \cdot 10^{-5}$ M. For temperatures above 70 °C, we assumed inactivation by a single first-order reaction with $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of 84.5 kJ mol^{-1} and - 83.2 J mol^{-1} K^{-1}, respectively. The inactivation as a function of time was calculated numerically, using a first-order Euler forward algorithm, with steps of 0.1 s. The results of the calculations are given in table 6.15. As an example, the calculated inactivation during heating-up and cooling to 100 °C in the presence and absence of 1.5 % sodium caseinate, is shown graphically in figure 6.18.

Table 6.15:   Calculated inactivation of proteinase from *Pseudomonas fluorescens* 22F during heating-up and cooling in the presence and absence of sodium caseinate as fraction of initial activity (see text). Between brackets the contribution of autoproteolysis and thermal inactivation, respectively.

T (°C)	1.5 % caseinate	No caseinate
80	0.013 (0.006 / 0.007)	0.054 (0.047 / 0.007)
100	0.035 (0.004 / 0.031)	0.061 (0.031 / 0.030)
120	0.121 (0.003 / 0.118)	0.139 (0.023 / 0.116)

Of course, this calculation is a rough approximation of the initial inactivation. Temperature-time profiles of the solution in the tubes were measured in rest, while in the actual experiments the tubes were rotated in the glycerol bath, so that the heat transfer would have been faster and thermal inactivation more important. Furthermore, the reaction rate constants for unfolding, refolding, and autoproteolysis from table 5.1 may be useful to describe the inactivation in the experiments of section 5.4.1, though they can not accurately describe the unfolding equilibrium, as was concluded in
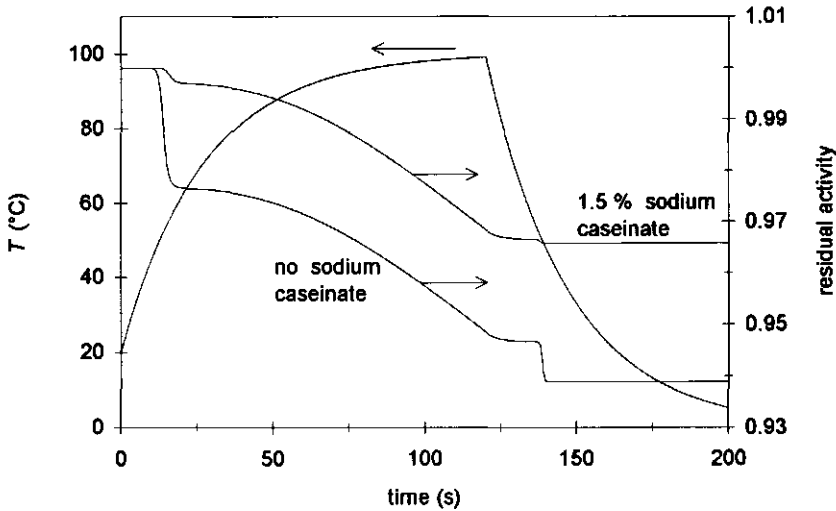
Figure 6.18:   Calculated inactivation during heating-up to 100 °C and cooling to 0 °C in the presence
and absence of 1.5 % sodium caseinate as function of time (see text).

chapter 5 of this thesis. The temperature dependence of the unfolding equilibrium is
expected to be less than calculated in section 5.4.1, so that inactivation by
autoproteolysis is underestimated in these calculations. Finally, the reaction rate
constants used were derived for the inactivation of an enzyme preparation of
supernatant from a culture skimmed milk diluted in demineralized water. Besides
having another calcium ion activity and ionic strength, the enzyme preparation used
in section 5.4.1 also had a slightly higher protein content than the enzyme
preparations used in these experiments. As concomitant proteins diminish the rate of
autoproteolysis, the extent of inactivation is underestimated in our calculations,
especially in case of purified enzyme. Still, this approximation indicates that
autoproteolysis and thermal inactivation can not completely explain the loss of
proteolytic activity during the first 2 min, especially in the case that purified proteinase
is used or EDTA is added to the enzyme solution, and that other mechanisms would
also play a role in the strong initial inactivation.

6.5   Conclusions

From the results of our experiments several conclusions can be drawn on the
kinetics of the thermal inactivation of extracellular proteinase from *Pseudomonas
fluorescens* 22F. The first conclusion is that the inactivation of this proteinase could
not be adequately described with a single first-order reaction. The simplest significant

model that could describe the inactivation, as deduced from the lack of fit test, was a model consisting of two consecutive reactions. This model also could successfully describe the results of Driessen (1983), who studied the inactivation of the same proteinase, and may be used for predictive modelling of the inactivation caused by heat treatment for this particular proteinase. Two other statistically acceptable models gave a better description of the results, according to the residual variance, and to Schwarz' and Akaike's criteria, the first model consisting of two consecutive irreversible reactions, where the first reaction leads to a partially inactivated enzyme molecule with a specific activity of approximately 0.6, the second model consisting of two consecutive reactions leading to two different unfolded proteinase molecule species, that can independently be inactivated. The first of these models appeared more consistent with literature. The model was useful to describe the results of inactivation experiments in which the conditions during heating were varied. A main disadvantage of this model is that, due to the extra parameters to be estimated and their intercorrelation, the confidence intervals of the kinetic parameters are large. This makes prediction of the inactivation with the model more difficult, especially when the influence of experimental conditions is examined. The model is not useful for describing the inactivation of other proteinases from *Pseudomonas fluorescens* strains, because the inactivation behaviour found for the proteinase from *Pseudomonas fluorescens* 22F, was not found for other proteinases. In most cases, approximate first-order inactivation kinetics was found (when inactivation during heating-up was excluded from analysis). Obviously, in such cases, the model describing the inactivation by a single first-order reaction is the best choice for prediction.

From the results of the lack of fit test alone, it was not possible to draw conclusions about the mechanism of thermal inactivation, as various models could possibly 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. Circumstantial evidence was taken from experiments in which the influence of calcium ion activity, pH, protein content, and enzyme concentration was examined.

Addition of proteins appeared to cause a slightly increased thermal inactivation. Possibly, part of the inactivation of the proteinase, when heated in milk, is caused by aggregation to casein. However, this is not the most important cause of inactivation. The inactivation of purified proteinase was poorly reproducible, possibly due to large differences in the extent of inactivation during heating-up and cooling-down, but appeared not to be very different from the inactivation of unpurified proteinase.

We did not find significant influence of calcium ion activity on the kinetics of inactivation. The pH of the enzyme solution had a slight influence on the rate of thermal inactivation. This finding rules out that the inactivation is caused by reactions

like 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 rules out inactivation caused by hydrolysis of the peptide chain. It appeared 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 lowering of the isoelectric pH after heating of proteinase at 90 °C. Assuming that deamidation of residues is the main cause of inactivation, a model consisting of two consecutive reactions, where the first reaction leads to an enzyme molecule with a lower specific activity (model 4, section 6.2) seems to be reasonable to describe the inactivation.

The inactivation of the proteinase during heating-up was relatively fast as compared with inactivation at the final heating temperature. The extent of inactivation appeared to be dependent on the composition of the heating menstrua. Inactivation was especially fast if EDTA was added to the enzyme solution or if purified enzyme was used. Kinetic modelling of the inactivation in the heating-up and cooling-down period showed that autoproteolysis and thermal inactivation could not completely explain the loss of proteolytic activity. Calcium and concomitant proteins may stabilize part of the native conformation or facilitate fast and accurate refolding.

Summarizing, kinetic modelling can be an important tool in elucidating the mechanism of enzyme inactivation. However, without complete insight in primary and higher structures of the protein 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. Furthermore, it was concluded that it is very difficult to compare inactivation results from literature with each other. In order to make predictions on the inactivation of pseudomonal proteinases, it is necessary to standardize heat inactivation experiments, both for experimental setup and statistical analysis.

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Chapter 7

Use of urea in studying the mechanism of thermal inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F

Abstract

The rate of inactivation at 100 °C of the extracellular proteinase from *Pseudomonas fluorescens* 22F was increased when 6 *M* urea was added to the enzyme solution before heat treatment. Also at lower urea concentrations the inactivation rate was increased. Addition of 6 *M* urea to the enzyme solution after heat treatment increased the rate of inactivation. This effect was not found when the urea concentration was below 1 *M*. It was concluded that cyanate, formed from urea at high temperature, was the cause of the increased inactivation, as addition of cyanate could increase the inactivation rate, while a treatment to remove the cyanate could prevent increased inactivation. The use of urea appears not to be suitable for the elucidation of the mechanism of thermal inactivation of enzymes.

7.1   Introduction

Ahern and Klibanov (1988) described a method to analyze the reaction pathways that can cause inactivation of enzymes. They distinguished between changes in the primary structure of the enzyme molecule and changes in higher orders of structure. Changes in the primary structure include hydrolysis of peptide bonds, destruction of amino acids, Maillard reactions and reshuffling of disulfide bonds. Except for the reshuffling of disulfide bonds, these changes are irreversible. Changes in higher orders of structure include aggregation and formation of incorrectly refolded structures. Ahern and Klibanov considered these changes as potentially reversible, because by using strong denaturants like urea, acetamide or guanidine hydrochloride, this type of inactivation could be prevented or the enzyme could be reactivated.

Presence of strong denaturants causes proteins to unfold (Tanford, 1968; Lapanje, 1978). This denaturation is highly reversible: when the denaturant concentration is lowered, for instance by dialysis or dilution, the protein will generally renature. Potentially reversible inactivation can be prevented by the addition of strong denaturant to the enzyme solution prior to heating. When the enzyme is cooled after heat treatment, refolding of the molecule is impossible due to the presence of the denaturant. When the denaturant is subsequently removed the enzyme molecule will refold to its native form. Because aggregation and incorrect refolding as result from the heat treatment will not occur, any observed lasting inactivation must have been caused by changes in the primary structure (Ahern and Klibanov, 1988).

Furthermore, when the enzyme has been heated in the absence of the strong denaturant, the inactivation caused by incorrect refolding or aggregation may be undone by addition of denaturant after the heat treatment. The denaturant causes the enzyme molecules to unfold. When subsequently the denaturant is removed, the molecules would renature to the native conformation (Tanford, 1968; Klibanov and Mozhaev, 1978; Ahern and Klibanov, 1988). Inactivation caused by reshuffling of disulfide bonds can be undone or prevented in a similar way by reduction followed by oxidation. The principle of reactivation of enzymes by use of denaturants, reductors and oxidators can be used to distinguish between processes involved in thermal inactivation of enzymes (Ahern and Klibanov, 1985; Zale and Klibanov, 1986; Tomazic and Klibanov, 1988ab).

In this chapter we tried to apply the methods described by Ahern and Klibanov (1988) in order to reveal the cause of heat inactivation of the proteinase from *Pseudomonas fluorescens* 22F.

7.2   Materials and methods

Production of enzymes

Pseudomonas fluorescens 22F was inoculated in sterilized growth medium consisting of 2.5 % sodium caseinate in milk ultrafiltrate. After incubation at 20 °C for 8 days the cells were removed by centrifugation (27,000 g, 30 min at 4 °C). The enzyme solution obtained was stored at 4 °C after addition of 0.025 % NaN_3.

Proteinase assay

The TNBS method for the determination of proteolytic activity was used, as described in chapter 4 of this thesis. The residual activity was defined as the fraction of the initial activity left after heat treatment.

Heating experiments

Enzyme solutions (2.0 ml) were heated in stainless steel tubes (internal diameter 7 mm), which were rotated in a thermostatted glycerol bath. After heating, the tubes were cooled immediately in ice water. The activity after two minutes of heating-up time ($t \approx 0$) was considered to be the initial activity, thereby eliminating the effects of heating-up.

7.3   Results and discussion

The objective of this study was the determination of the mechanism of the irreversible inactivation of the proteinase from *Pseudomonas fluorescens* 22F, using the method described by Ahern and Klibanov (1988). The denaturant chosen for this purpose was urea, because acetamide and guanidine hydrochloride showed strong interference with the TNBS method.

Firstly, the activity as a function of the urea concentration was determined by addition of urea to the reaction mixture (figure 7.1). At urea concentrations higher than 3 M the proteinase was almost completely inactive. The proteolytic activity was completely restored after the urea concentration was decreased by dilution. At relatively low urea concentrations the activity on sodium caseinate was increased, possibly due to a higher accessibility of the substrate for proteolytic attack.

The inactivation of the proteinase in the presence and absence of urea was determined at 100 °C. In the first experiment up to 6 M urea was added to the enzyme solution before the heat treatment. In the second experiment up to 6 M urea was added to the enzyme solution after the heat treatment and this solution was kept for 10 min at room temperature. Finally, an experiment was performed in the absence of urea. The determination of proteolytic activity involved a 5 time
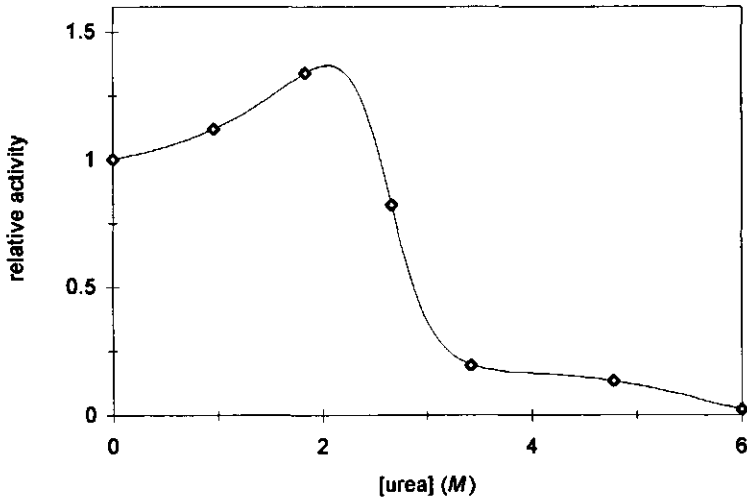
Figure 7.1:   Relative activity of proteinase from *Pseudomonas fluorescens* 22F as function of the urea concentration. Activity in absence of urea taken as relative activity = 1.

Figure 7.2:   The influence of 6 *M* urea on the inactivation of proteinase from *Pseudomonas fluorescens* 22F at 100 °C. Heated in the presence of urea (□), urea added after heat treatment (△), no urea (◊). Activity after 2 min heating-up time (t_0) taken as relative activity = 1.

dilution, so that the urea concentration during incubation with sodium caseinate was 1.2 M in the first two experiments. Residual activities were calculated using the activity after two min heating-up time as reference. The results are shown in figure 7.2.

The inactivation in the presence of urea was significantly faster when compared with the inactivation in the absence of urea, which is in contradiction with the theory of Ahern and Klibanov (1988). Also when heated at lower concentrations of urea an increased inactivation of the proteinase was observed (table 7.1).
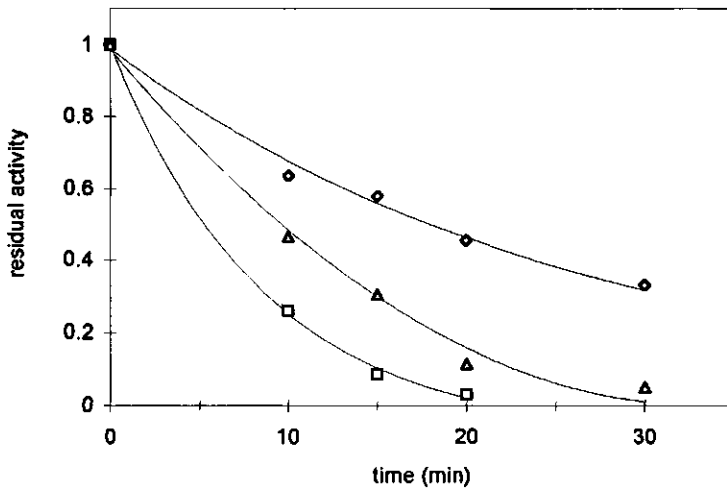
Table 7.1:   Residual activity of proteinase from *Pseudomonas fluorescens* 22F after heat treatment of 20 min at 100 °C in the presence of urea. Activity after 2 min heating-up time (t_0) taken as relative activity = 1.

[urea] (M)	residual activity
0	0.45
0.25	0.37
0.5	0.24
1.0	0.20

When urea was added to the heated enzyme solution after the heating experiment, also an increased rate of inactivation of the proteinase was observed. However, when urea was added in such a low concentration that the proteinase would not unfold completely, almost no increased inactivation was observed (table 7.2).

Table 7.2:   Residual activity of proteinase from *Pseudomonas fluorescens* 22F after heat treatment of 20 min at 100 °C. Urea added after heat treatment. Activity after 2 min heating-up time (t_0) taken as relative activity = 1.

[urea] (M)	residual activity
0	0.45
0.25	0.45
0.5	0.43
1.0	0.43

It appeared that urea is reacting with the proteinase at high temperature causing inactivation. The effect of urea on the activity of the proteinase when

added after the heat treatment was concentration dependent. The fact that urea only enhanced the rate of inactivation if its concentration was high enough to unfold the enzyme, suggests that the enzyme molecule is altered during the heat treatment in such a way that correct refolding after urea denaturation is hindered.

Stark et al. (1960) investigated the reactions of cyanate in aqueous solutions with amino acids and proteins, causing inactivation of bovine pancreatic ribonuclease. Because urea can hydrolyze to form ammonium carbonate, with cyanate as the sole intermediate (Warner, 1942), it is very well possible that the inactivation of the proteinase is due to reactions with cyanate, especially at high temperatures when the equilibrium shifts towards cyanate.

Potassium cyanate was added to the enzyme solution, and prior to the determination of the proteolytic activity the mixture was incubated for 60 min at 37 °C. The activity was compared with a sample to which no cyanate was added. The results show that cyanate indeed inactivated the proteinase (table 7.3). Probably, cyanate formed from urea at high temperatures caused an extra inactivation of the proteinase.

Table 7.3:   Relative activity of proteinase from *Pseudomonas fluorescens* 22F after incubation with potassium cyanate during 60 min at 37 °C. Activity in absence of cyanate taken as relative activity = 1.

[cyanate] (M)	relative activity
0	1
0.05	0.46
0.1	0.19

A method to decrease the concentration of cyanate in urea solutions is an acidification-neutralisation treatment. A solution of 6 M urea was heated for 20 min at 100 °C, acidified to pH 2.0 with HCl, and then neutralised to pH 7.0 with NaOH. This solution will be referred to as treated urea. An enzyme solution was incubated for 15 min at room temperature with 1.2 M urea. In the first experiment with unheated urea; in the second experiment with heated and untreated urea; and finally, in the third experiment with heated and treated urea. Results are shown in table 7.4. Treatment of the heated urea prevented the inactivation of the proteinase, suggesting that cyanate formed during heating was indeed responsible for the extra inactivation.

Table 7.4: Relative activity of proteinase from *Pseudomonas fluorescens* 22F in presence of 1.2 *M* urea. Urea added after heat treatment and incubated for 60 min at 37 °C. Activity in the presence of unheated urea taken as relative activity = 1. Means of six experiments.

	relative activity
unheated urea	1
heated and treated urea	0.99
heated urea	0.39

The use of urea to distinguish causes of heat inactivation of proteins, as proposed by Ahern and Klibanov (1988), appeared not to be suitable, at least not for our enzyme preparation. Cyanate, the product of urea, reacts with amino acids and proteins. The reactions with cyanate are carbamylation of lysine and cysteine to form homocitrulline (ε-carbamyllysine) and S-carbamylcysteine, respectively (Stark et al., 1960). We did not use other denaturants, such as acetamide and guanidine hydrochloride, to test the ideas of Ahern and Klibanov, because of the interference with the TNBS method, but they may perhaps be more useful in this respect. Use of treated urea to reactivate enzymes may be applicable in some cases, dependent on the reactions that have taken place during the heat treatment. The proteinase from *Pseudomonas fluorescens* 22F showed extra inactivation, and distinction between irreversible and potentially reversible inactivation could not be made.

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Chapter 8

General discussion

8.1 Introduction

The aim of this study was to obtain a better understanding of the kinetics of heat inactivation of the proteinases from psychrotrophic bacteria. To that end the inactivation of extracellular proteinase from *Pseudomonas fluorescens* 22F was studied. It was concluded that the proteinase can be inactivated by two different mechanisms, depending on temperature. To arrive at this conclusion, kinetic modelling was applied.

In section 8.2, a general discussion on the heat stability of the proteinases and its consequences for the industry will be given. Besides predicting residual activity, kinetic modelling can also be used as a tool for elucidation of the mechanism of enzyme inactivation. Modelling appeared to be a powerful tool in qualitative and quantitative description of the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F. In section 8.3, possibilities and limitations of kinetic modelling of enzyme inactivation will be discussed.

8.2 Inactivation of proteinases from psychrotrophic bacteria

Extracellular enzymes of psychrotrophic bacteria, such as proteinases, lipases and phospholipases, are notorious for their heat stability. Most of them resist the thermal processes applied in the food industry, such as HTST pasteurization (High Temperature Short Time) or UHT sterilization (Ultra-High Temperature). Residual activity of psychrotrophs proteinases is associated with several technological problems occurring during processing and storage of foods, such as formation of bitter off-flavours and gelation of milk products. In order to gain more insight in the heat stability of the proteinases from psychrotrophic bacteria, the kinetics of thermal inactivation of one of these proteinases was studied.

The proteinase from *Pseudomonas fluorescens* 22F was taken as an example, because *Pseudomonas fluorescens* strains are the most common psychrotrophs found in milk, and because this particular proteinase was found to

be very heat stable (Driessen, 1983). The proteinase appeared to have similar molecular characteristics and properties as other extracellular proteinases from *Pseudomonas fluorescens* strains (chapter 3 of this thesis). The heat stability of the proteinase, and its dependence on external variables was not always found to be similar for extracellular proteinases from other *Pseudomonas fluorescens* strains. In our view, these differences are often due to differences in experimental conditions or in interpretation, and we presume that our conclusions on heat stability and heat inactivation, drawn for the extracellular proteinase from *Pseudomonas fluorescens* 22F, may be extrapolated to other pseudomonal proteinases.

In the dairy industry several kinds of heat treatment are applied. These treatments include thermization (15 s / 65 °C), HTST (15-20 s / 72-75 °C), sterilization in container (20-30 min / 115-120 °C), and UHT treatment (a few seconds / 135-150 °C). In figure 8.1 typical temperature profiles for in-container sterilization and direct and indirect UHT treatment are given. Calculations were made to estimate the effect of these heat treatments on the inactivation of the extracellular proteinase from *Pseudomonas fluorescens*. Kinetic parameters were taken from tables 5.1 and 6.4, and calculations were performed as in section 6.4.7, assuming an initial enzyme concentration of $1.0 \cdot 10^{-7}$ M and an inactivation behaviour in milk similar to that in 1.5 % sodium caseinate in 0.2 M TrisHCl. The calculated extents of inactivation caused by in-container sterilization, indirect UHT, and, direct UHT treatment were estimated to be approximately 90 %, 20 %, and 10 %, respectively. Profiles of the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F are included in figure 8.1.

Clearly, the proteinase from *Pseudomonas fluorescens* 22F is not completely inactivated by either heat treatment. The extent of inactivation by in-container sterilization was highest. However, along with inactivation of enzymes, several undesirable chemical reactions, such as the Maillard reaction responsible for off-flavour and colour development, proceed to a considerable extent (Walstra and Jenness, 1984).

Alternative heat treatments for more effective inactivation of psychrotrophic enzymes have been proposed, based on the relatively fast inactivation in the temperature range 40 to 70 °C (Barach et al., 1976; Bucky et al., 1988). Before or after UHT treatment the milk is heated, for example, for 60 min at 55 °C. Indeed, such treatment may lead to an inactivation of proteinases and lipases by 50 to 90 % (West et al., 1978; Kroll and Klostermeyer, 1984; Bucky et al., 1988). The alternative heat treatment has some disadvantages: along with inactivation of the enzymes, milk proteins are digested (chapter 5 of this thesis). It was found that dual heat treatment was not effective in preventing age gelation (Kocak and
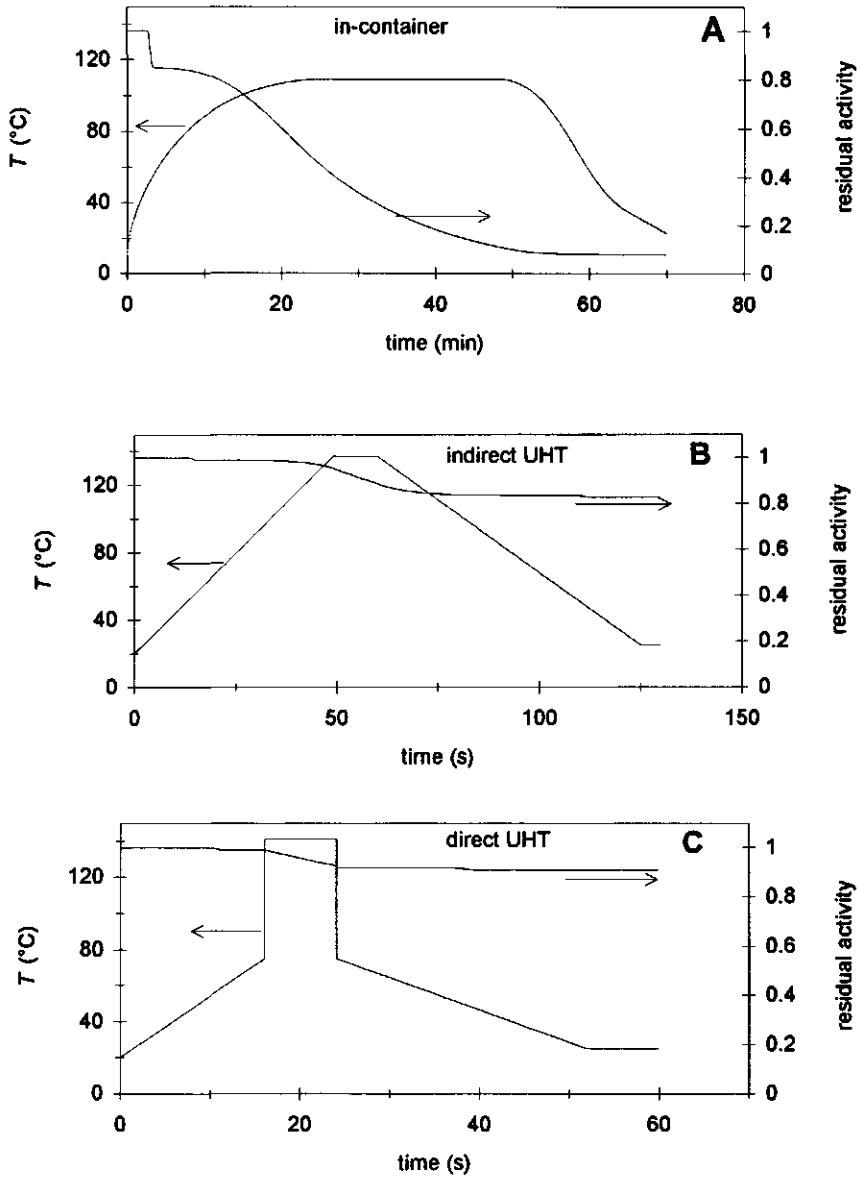
Figure 8.1: Profiles of temperature and heat inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F by in-container sterilization (A), indirect UHT (B), and direct UHT treatment (C).

Zadow, 1985). Proteinases and lipases of different species from psychrotrophic bacteria have different temperatures of maximum inactivation (Kroll and Klostermeyer, 1984), and some proteinases from *Pseudomonas fluorescens* strains have been reported to be less sensitive or even insensitive to autoproteolysis following a treatment at UHT temperatures (Griffiths et al., 1981; Stepaniak and Fox, 1983; Diermayr et al., 1987), thereby reducing the effectiveness of the treatment.

It can be concluded that no heat treatment can adequately inactivate proteinases from psychrotrophic bacteria without undesirable side effects. Therefore, it is necessary to prevent psychrotrophs to produce their extracellular proteinases and lipases. This can be done by keeping the number of psychrotrophic bacteria low, amongst others, by a hygienic milk production, low storage temperatures and thermization (recently reviewed by Champagne et al., 1994; Shah, 1994).

8.3   Kinetic modelling of enzyme inactivation

Kinetic modelling is used for many purposes in food science and technology, both for predicting changes during processing and storage, and for elucidation of complex reactions. Predictive modelling is widely used as a tool to optimize the quality of foods, such as organoleptic properties, nutritional value, shelf-life and safety conditions. It has been applied to predict microbial growth as function of time, temperature, and other parameters, such as pH and water activity (e.g. Zwietering, 1993; Rowe, 1993; Willocx et al., 1993; Griffiths, 1994; Ross, 1994), or to predict the extent of chemical changes or enzyme inactivation by heat treatment (e.g. Kessler and Fink, 1986; Luna et al., 1986; Rustom et al., 1996; de Jong, 1996). In food science, to a lesser extent kinetic modeling is used for the elucidation of complex reactions (e.g. Berg, 1993). Kinetic modelling has proven to be a powerful tool for both goals, but it also has its difficulties and limitations.

In this study, it was established, by making use of kinetic modelling, that inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F in the temperature range 40 - 70 °C could be explained better by intermolecular autoproteolysis than by intramolecular autoproteolysis or by the formation of an irreversible complex with casein (section 5.4.1). It was also established, again by making use of kinetic modelling, that sodium caseinate could stabilize against autoproteolysis by acting as a competitive substrate (section 5.4.2). We also found indications for the dependence of the conformational stability of the proteinase on calcium ion activity and chelating agents (section 5.4.3), and on pH (section 5.4.4). However, the very fact that the inactivation was caused by autoproteolysis could

only be proved with HPLC experiments, and elucidation of the primary and higher structures of the proteinase would be needed to confirm the exact role of metal ions and pH on its conformational stability. It would be helpful to use spectroscopic or calorimetric methods to follow conformational changes in the proteinase molecules, but these methods are not feasible for proteinases, because autoproteolysis would occur during measurement, or, if inhibitors are added to the enzyme preparation to prevent this, the conformational stability is affected. For the inactivation of the proteinase in the high temperature range it appeared even more that kinetic modelling has its limitations. We were able to find mathematical models that could describe the thermal inactivation in a statistically acceptable way, but the mechanism of inactivation could not be elucidated by making use of kinetic modelling. Therefore, as with low temperature inactivation, other techniques will be needed to precisely elucidate the mechanism of inactivation.

A difficulty in our study was that only one property could be measured, namely the residual activity, which, in turn, is the sum of native and reversibly unfolded enzyme molecules, as a function of time. If more variables can be determined simultaneously (i.e. multiresponse modelling), kinetic modelling can be a much more powerful tool for the elucidation of reaction mechanisms (e.g. Berg, 1993). Furthermore, when performing computer simulations, the initial conditions were assumed such that the fraction of one molecular species was 1, the fractions of all the other species 0. However, because heating-up times were relatively long, reactions may already have proceeded, and this assumption may not be correct. In the case of inactivation by a single first-order reaction this would be no problem, but if the inactivation is caused by a series of reactions, this may lead to wrong estimation of the kinetic parameters and misinterpretation of the mechanism. To a certain extent the proceeding of reactions can be accounted for by integrating over time and temperature (section 6.4.8), but this method is subject to errors. It would be better to shorten the heating-up time, for example by heating the enzyme solutions in capillaries.

When analyzing our results with the proposed inactivation models, in many cases large confidence intervals were found. The first condition for an accurate estimation of the kinetic parameters is that the method to determine activities or concentrations is precise and reproducible: the conditions of the experiments must be properly specified, the analytical method must properly represent the extent of the reaction, and the effects to be measured must be much greater than the experimental error. In our study, the experimental conditions were not always the same for all experiments. *Pseudomonas fluorescens* 22F was grown in various media to produce the proteinase, and in many cases unpurified enzyme was used in the experiments. It appeared that these results could not be compared (e.g.

section 6.4.3), so that the influence of pH, calcium ion activity, and protein content on the kinetics of heat inactivation could only be evaluated per factor, but their interaction could not. It would have been better to have used purified enzyme in the same buffer for all experiments, in order to circumvent this problem. However, complete purification of proteinases is complicated, as one enzyme molecule acts as substrate for another, and the solution will deteriorate in time. The reproducibility of experiments with purified enzyme appeared to be poor, presumably because of it great sensitivity for differences in heating-up profiles and impurities (e.g. section 6.4.6). The variation in experimental conditions among studies described in the literature was even larger, and therefore, comparison of kinetic parameters of inactivation of proteinases from various *Pseudomonas fluorescens* strains is even more difficult.

In chapter 4, two proteolytic activity assays were evaluated: the azocasein and TNBS method. With these substrates it is impossible to measure the absolute activity, because the proteins have various peptide bonds that can be attacked, with different susceptibilities to hydrolysis. Using low molecular weight substrates would hamper translation to practical situations. To be useful for kinetic modelling, it is practical that the response measured in the assay is proportional to the enzyme activity, and that the response is proportional to the assay time. Within certain limits, the TNBS method fulfilled these conditions and it could be used for estimation of the heat inactivation. The azocasein method did not satisfy the conditions, and was therefore only used for checking the presence of proteolytic activity. The coefficient of variation of the TNBS method was approximately 1.4 %, which is reasonably accurate. However, even relatively small errors in this determination may hinder accurate estimation of kinetic parameters. Moreover, samples were subjected to heat treatment, which is another source of errors that results in variation in kinetic parameters. Presumably, this variation is mainly caused during heating and cooling the sample, because this was the least reproducible part of the experiment. The variation appeared largest for purified enzyme preparations and enzyme preparations to which EDTA had been added.

Another cause of the large confidence intervals of the kinetic parameters is the intercorrelation of the kinetic parameters. Reparameterization could reduce the strong correlation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ that determine a single reaction rate, but still high correlation was found between the kinetic parameters determining different reactions rates, especially when estimating models consisting of many parameters (section 6.4.2). As a consequence of the large confidence intervals of the kinetic parameters, it was difficult to find conclusive answers concerning the mechanism of thermal inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F.

Three main conclusions can be drawn from this work. Firstly, kinetic modelling of inactivation of proteinases is difficult, because it is impossible to use completely pure enzyme preparations due to autoproteolysis, furthermore, it is impossible to measure the absolute activity because various peptide bonds with different susceptibility to hydrolysis can be attacked, and finally because only one variable, namely the residual activity, can be measured. Secondly, kinetic modelling can be a powerful method to predict enzyme inactivation as function of temperature and time. And thirdly, kinetic modelling as such can not elucidate the exact mechanism of enzyme inactivation if only one variable can be determined, but it does allow rigorous checking of a proposed reaction scheme.

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List of symbols, constants, and abbreviations

Included in the following list are symbols, constants, and abbreviations used throughout this thesis. Other, less frequently used symbols are defined as used.

Symbols

a	enzyme activity	$M^{1-\tilde{n}}\ s^{-1}$
a_0	enzyme activity at $t = 0$	$M^{1-\tilde{n}}\ s^{-1}$
AIC	Akaike's optimization criterion	-
c	concentration	M
c_p	Heat capacity	$J\ kg^{-1}\ K^{-1}$
D	decimal reduction time	s^{-1}
E_a	activation energy	$J\ mol^{-1}$
f	Fisher's criterion used in variance test	-
F	Fisher's criterion used in variance test	-
g_i	studentized residual	-
ΔG°	change in Gibbs energy	$J\ mol^{-1}$
$\Delta G^\ddagger$	change in Gibbs energy of activation	$J\ mol^{-1}$
ΔH°	change in enthalpy	$J\ mol^{-1}$
$\Delta H^\ddagger$	change in activation enthalpy	$J\ mol^{-1}$
I	irreversibly inactivated enzyme molecule	-
k	reaction rate	$M^{1-\tilde{n}}\ s^{-1}$
k_0	pre-exponential factor in Arrhenius equation	-
k_a	reaction rate constant of autoproteolysis	$M^{-1}\ s^{-1}$
k_i	reaction rate constant of irreversible thermoinactivation	s^{-1}
k_u	reaction rate constant of unfolding	s^{-1}
k_f	reaction rate constant of refolding	s^{-1}
K_d	equilibrium constant native and unfolded enzyme	-
K_m	Michaelis-Menten constant	M
n	number of observations	-
$\tilde{n}$	reaction order	-
$\tilde{n}_c$	reaction order with respect to concentration	-
$\tilde{n}_t$	reaction order with respect to time	-

N	enzyme molecule in native state	-
N^*, N^{**}	partially inactivated enzyme molecule	-
N^+	partially unfolded, but still catalytically active enzyme molecule	-
Q_{10}	factor for temperature dependence of reaction rates	-
p	number of parameters estimated in model	-
s^2	residual variance	-
ΔS°	change in entropy	J mol^{-1} K^{-1}
$\Delta S^\ddagger$	change in activation entropy	J mol^{-1} K^{-1}
SC	Schwarz' optimization criterion	-
SSE	residual sum of squares	-
t	time	s
T	temperature	K
T_d	denaturation temperature	K
U	enzyme molecule in unfolded state	-
v	reaction rate	mol s^{-1}
V	volume	m^3
V_{max}	maximum velocity of enzyme reaction	mol s^{-1}
β	relative specific activity factor	-
ν	number of degrees of freedom	-

Constants

h	Planck's constant	$6.62 \cdot 10^{-34}$ J s
k_b	Boltzmann's constant	$1.38 \cdot 10^{-23}$ J K^{-1}
R	universal gas constant	8.31 J mol^{-1} K^{-1}

Abbreviations

EDTA	ethylenediaminetetraacetic acid
TNBS	trinitrobenzenesulfonic acid
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane

Summary

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*. Psychrotrophic bacteria as such do not pose a very serious problem for 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. Residual activity of the enzymes is associated with several technological problems during processing and storage of dairy products, such as loss of yield, formation of bitter or soapy off-flavours, and gelation of milk.

The aim of this thesis was to obtain more fundamental knowledge about the kinetics of inactivation of the extracellular enzymes of psychrotrophs, in order to gain insight in the mechanism of inactivation and to predict the residual activity after heat treatment. Generally, enzyme inactivation is described by a reversible unfolding reaction leading to an inactive enzyme species, followed by an irreversible reaction leading to inactivation. However, in many cases the inactivation is much more complex. Kinetic modelling can be a powerful tool in qualitative and quantitative description of the inactivation.

In this work the inactivation of the extracellular proteinase from *Pseudomonas fluorescens* 22F was studied. This proteinase was taken as an example, because *Pseudomonas fluorescens* is the most common psychrotroph found in milk, and because this particular proteinase has been found to be very heat stable. The proteinase was purified to electrophoretic homogeneity by ammonium sulfate precipitation, hydrophobic interaction chromatography, ultrafiltration and gel filtration. The molecular characteristics and properties of the proteinase appeared to be similar at those of other extracellular proteinases from *Pseudomonas fluorescens* strains. The proteinase was characterized as a 50 kDa alkaline metalloproteinase, with an optimum pH for activity on sodium caseinate of 9.5-10. The optimum temperature of the enzyme was approximately 50 °C. V_{max} and K_m were estimated at 1560 TNBS units/ml and 0.24 % (0.11 mM) sodium caseinate, respectively (chapter 3).

For kinetic modelling of enzyme inactivation, it is essential that the method for determining the activity is accurate and reproducible. In chapter 4, two

proteolytic activity tests were evaluated: the azocasein and TNBS method. Within certain limits, the TNBS method fulfilled the conditions of accuracy and reproducibility, and could be used for estimation of the heat inactivation. The azocasein method did not satisfy the conditions, and was therefore only used for checking the presence of proteolytic activity.

The mechanism of inactivation of the proteinase was dependent on temperature. In the temperature range 35 - 70 °C, a relatively fast inactivation was found (chapter 5). It was concluded, by making use of kinetic modelling, that the inactivation was most likely caused by intermolecular autoproteolysis. Sodium caseinate could stabilize against inactivation by autoproteolysis. Small amounts of caseinate stabilized against unfolding of the proteinase, resulting in an increased denaturation temperature (T_d). Larger amounts of sodium caseinate, to a maximum of 1.5 %, diminished autoproteolysis by acting as a competitive inhibitor. Calcium stabilized the conformation of the enzyme molecule. Addition of 10 mM CaCl$_2$ to the enzyme solution increased T_d by approximately 4 K. EDTA did not influence T_d, but appeared to increase the rate of inactivation. o-Phenanthroline markedly destabilized the proteinase, resulting in a decrease of T_d. The unfolding of the proteinase was dependent on the pH of the enzyme solution. The proteinase was more stable at pH 7.5 than at pH values 5.8, 7.0, or 9.6.

In the temperature range 80 - 120 °C the heat inactivation of the proteinase was caused by another mechanism (chapter 6). Generally, in this temperature regime heat inactivation is caused by formation of incorrect conformations, due to processes such as hydrolysis of the peptide chain, destruction of amino acids, and aggregation. The inactivation of the proteinase could not be described by a single reaction; therefore, alternative models were proposed and modelled to fit the data. The model with the fewest parameters being statistically acceptable, consisted of two sequential first-order irreversible reactions, and could be used for predictive modelling the inactivation. A model consisting of two consecutive irreversible reactions, where the first reaction leads to a partially inactivated enzyme molecule with a specific activity of approximately 0.6, was statistically better and also appeared to be more in accordance with the assumed mechanism of inactivation. The inactivation behaviour was dependent on the medium in which the bacteria had been grown. Addition of sodium caseinate to the enzyme solution decreased the heat stability of the proteinase. The inactivation was dependent on pH. Calcium ion activity did not have a significant influence on the heat stability of the proteinase. Fast inactivation during heating-up caused a biphasic inactivation behaviour. Addition of sodium caseinate to the enzyme solution diminished this initial inactivation, partly by decreasing the rate of autoproteolysis. Addition of EDTA increased the extent of inactivation during heating-up, possibly because of

complexation of calcium that stabilizes part of the native conformation or facilitates renaturation.

To elucidate the mechanism of inactivation of the proteinase at high temperature in more detail, urea was added to the enzyme solution, in order to prevent aggregation and formation of incorrect conformations (chapter 7). However, the rate of inactivation at 100 °C was increased when 6 M urea was added to the enzyme solution before heat treatment. Also at lower urea concentrations the inactivation rate was increased. Addition of 6 M urea to the enzyme solution after heat treatment enhanced inactivation. This effect was not found if the urea concentration was below 1 M. It was concluded that cyanate, formed from urea at high temperature, was the cause of the increased inactivation, as addition of cyanate could increase the inactivation rate, while a treatment to remove the cyanate could prevent increased inactivation. Therefore, the use of urea appears to be unsuitable for elucidation of the mechanism of thermal inactivation of enzymes.

Having established the kinetics of heat inactivation of the proteinase, an estimate was made of the impact of various heat treatments on the inactivation of this proteinase (chapter 8). In-container sterilization, indirect UHT treatment, and direct UHT treatment resulted in approximately 90 %, 20 %, and 10 % inactivation of the proteinase, respectively. The extent of inactivation of proteinases from other *Pseudomonas fluorescens* strains is expected to be approximately similar. Alternative heat treatments, based on the relatively fast inactivation at low temperatures, also appeared not to be effective. Therefore, it is necessary to prevent psychrotrophs from producing their extracellular proteinases by keeping the number of psychrotrophic bacteria low, amongst others, by hygienic milk production, low storage temperatures and thermization.

Kinetic modelling is used for many purposes in food science and technology, both for predicting changes during processing and storage, and for elucidation of complex reactions. Also in this study kinetic modelling has proven to be a powerful tool for both goals, but it also has its difficulties and limitations, because it is impossible to use completely pure enzyme preparations due to autoproteolysis, furthermore, it is impossible to measure the absolute activity because various peptide bonds with different susceptibility to hydrolysis can be attacked, and finally because only one variable, namely the residual activity, can be measured. Kinetic modelling should be used in combination with analytical techniques, so that the nature of products can be determined. Kinetic modelling is increasingly powerful if more variables can be measured and the assays for the determination of activities are more accurate.

Samenvatting

Het bij lage temperaturen bewaren van melk op de boerderij of in de zuivelfabriek, heeft een bacteriële flora van voornamelijk psychrotrofen tot gevolg. De meest voorkomende psychrotrofe bacteriën zijn *Pseudomonas*-soorten, met name *Pseudomonas fluorescens*-stammen. De psychrotrofe bacteriën zelf zijn geen belangrijk probleem voor de zuivelindustrie, omdat ze door de gebruikelijke hittebehandelingen, zoals pasteurisatie, vrijwel allemaal gedood worden. Proteinasen, lipasen en fosfolipasen, die door de bacteriën in de melk worden uitgescheiden, veroorzaken echter wel problemen. Deze extracellulaire enzymen kunnen namelijk zeer hittestabiel zijn, zodanig zelfs dat ze bestand zijn tegen sterilisatie. Restactiviteit van de enzymen kan technologische problemen veroorzaken tijdens de verwerking en opslag van zuivelproducten, zoals opbrengstverlies, smaakafwijkingen en gelering van melk.

Het doel van dit onderzoek was om meer fundamentele kennis te verwerven over de kinetiek van de hitteïnactivering van extracellulaire enzymen van psychrotrofen, en zodoende inzicht te krijgen in het mechanisme van de inactivering, en om voorspellingen te doen over inactiverende werking van verschillende hittebehandelingen. In het algemeen kan enzyminactivering beschreven worden als een reversibele ontvouwingsreactie die resulteert in een niet actief enzymmolecuul, gevolgd door een irreversibele reactie die resulteert in een voorgoed geïnactiveerd molecuul. In veel gevallen is de inactivering echter ingewikkelder. Kinetisch modelleren kan een belangrijk hulpmiddel zijn om de inactivering kwalitatief en kwantitatief te beschrijven.

In dit onderzoek is de inactivering van de extracellulaire proteinase van *Pseudomonas fluorescens* 22F bestudeerd. Dit enzym is gekozen als voorbeeld omdat *Pseudomonas fluorescens* de meest voorkomende psychrotrofe bacterie in melk is, en omdat in eerder onderzoek is aangetoond dat deze specifieke proteinase zeer hittestabiel is. De proteinase werd gezuiverd met behulp van ammoniumsulfaatprecipitatie, hydrofobe interactiechromatografie, ultrafiltratie en gelfiltratie. De proteinase had vergelijkbare moleculaire karakteristieken als extracellulaire proteinasen van andere *Pseudomonas fluorescens*-stammen. Het bleek een metalloproteinase met een molecuulgewicht van 50 kDa te zijn, met een pH-optimum voor de afbraak van natriumcaseïnaat rond 9.5 - 10. De optimum-temperatuur was circa 50 °C, en V_{max} en K_m werden geschat op respectievelijk 1560 TNBS-eenheden / ml en 0.24 % natriumcaseïnaat (hoofdstuk 3).

Voor kinetisch modelleren is het belangrijk dat de bepalingsmethode voor de activiteit nauwkeurig en reproduceerbaar is. In hoofdstuk 4 worden twee methoden

voor bepaling van de proteolytische activiteit, namelijk de azocaseïne-methode en de TNBS-methode, vergeleken en getoetst op geschiktheid voor het onderzoek. De TNBS-methode voldeed aan de eisen van nauwkeurigheid en reproduceerbaarheid en kon dus gebruikt worden voor de bepaling van de hitteïnactivering. De azocaseïne methode voldeed niet aan de eisen en werd daarom alleen gebruikt om aanwezigheid van proteolytische activiteit aan te tonen.

Het mechanisme van hitte-inactivering van de proteinase bleek afhankelijk van de verhittingstemperatuur. In het temperatuurgebied 35 - 70 °C werd een relatief snelle inactivering gevonden (hoofdstuk 5). Met behulp van kinetisch modelleren kon worden aangetoond dat intermoleculaire autoproteolyse het meest waarschijnlijke mechanisme van inactivering is. Natriumcaseïnaat kon de inactivering beïnvloeden; lage concentraties stabiliseerden tegen ontvouwing van het proteinasemolecuul, resulterend in een hogere denaturatietemperatuur (T_d), terwijl hogere concentraties, tot een maximum van 1.5 %, de autoproteolyse-reactie vertraagden door middel van competitieve remming. Toevoeging van 10 mM CaCl$_2$ aan de enzymoplossing verhoogde T_d met circa 4 °C. EDTA had geen invloed op T_d, maar versnelde wel de inactivering. o-Phenanthroline destabiliseerde de conformatie van de proteinase aanzienlijk. Het ontvouwingsevenwicht van de proteinase was pH afhankelijk. Bij pH 7.5 was de proteinase stabieler dan bij pH 5.8, 7.0 en 9.6.

In het temperatuurgebied 80 - 120 °C wordt de inactivering van de proteinase veroorzaakt door een ander mechanisme (hoofdstuk 6). In dit temperatuurgebied wordt inactivering vaak veroorzaakt door processen als hydrolyse van peptidebindingen, afbraak van aminozuur-residuen, aggregatie en de vorming van foutieve conformaties. De inactivering van de proteinase kon niet worden verklaard met een enkele reactie. Daarom werden andere, complexere mechanismen voor de inactivering voorgesteld en gemodelleerd zodat ze resultaten konden beschrijven. Het model met de minste parameters dat nog statistisch significant was, bestond uit twee opeenvolgende, irreversibele eerste-ordereacties. Dit model kon gebruikt worden om de hitteïnactivering te voorspellen. Een model, bestaande uit twee opeenvolgende, irreversibele eerste-ordereacties, waarvan de eerste reactie resulteert in een gedeeltelijk geïnactiveerde proteinase met een relatieve specifieke activiteit van ongeveer 0.6, was vanuit statistisch oogpunt beter, en leek ook meer in overeenkomst met het mechanisme van inactivering. De inactivering was afhankelijk van het medium waarin de bacteriën gegroeid hadden. Toevoegen van natriumcaseïnaat aan de enzymoplossing versnelde de inactivering enigszins. De inactivering was afhankelijk van de pH van de enzymoplossing. De calciumion-activiteit van de enzymoplossing had geen invloed op de hittestabiliteit van de proteinase. De inactivering was relatief snel in de opwarmperiode, wat resulteerde in een schijnbaar twee-fasige inactivering. Toevoegen van natriumcaseïnaat aan de enzymoplossing verminderde de inactivering tijdens

opwarmen, onder andere door de snelheid van autoproteolyse te verlagen. Toevoegen van EDTA aan de enzymoplossing vergrootte de hoeveelheid tijdens het opwarmen geïnactiveerde proteinase, mogelijk door complexering van calciumionen, die wellicht een gedeelte van de natieve conformatie stabiliseren of terugvouwing vergemakkelijken.

Om de inactivering van de proteinase bij hoge temperaturen verder uit te zoeken, werden experimenten uitgevoerd waarin ureum aan de enzymoplossing werd toegevoegd, omdat aanwezigheid van sterke denaturanten aggregatie en vorming van foutieve conformaties zou voorkomen (hoofdstuk 7). Wanneer de proteinase werd verhit bij 100 °C, in aanwezigheid van 6 M ureum, werd een relatief snelle inactivering gevonden. Toevoegen van 6 M ureum na de hittebehandeling resulteerde ook in verhoogde inactivering. Dit effect werd niet gevonden wanneer de concentratie ureum lager dan 1 M was. Er werd geconcludeerd dat cyanaat, gevormd uit ureum bij hoge temperaturen, de oorzaak van de verhoogde inactivering was, omdat toevoegen van cyanaat aan de enzymoplossing resulteerde in versnelde inactivering, terwijl de verwijdering van cyanaat uit de oplossing resulteerde in minder inactivering. Het gebruik van ureum blijkt dus niet geschikt voor het ophelderen van het mechanisme van hitteïnactivering van enzymen.

Nadat de kinetiek van de hitteïnactivering van de extracellulaire proteinase van *Pseudomonas fluorescens* 22F was vastgesteld, kon er een schatting gemaakt worden van het effect van verschillende hittebehandelingen op de inactivering van de proteinase (hoofdstuk 8). Stand-sterilisatie, indirecte UHT-verhitting en directe UHT-verhitting resulteerde in respectievelijk 90 %, 20 %, en 10 % inactivering van de extracellulaire proteinase van *Pseudomonas fluorescens* 22F. Voor proteinasen van andere *Pseudomonas fluorescens*-stammen zal dit vergelijkbaar zijn. Alternatieve hittebehandelingen, gebaseerd op de relatief snelle inactivering bij lage temperaturen, lijken ook niet geheel effectief, zodat het belangrijk is om te voorkomen dat psychrotrofe bacteriën de extracellulaire enzymen uitscheiden, en wel door hygiënische melkproductie, lage opslagtemperaturen en thermisatie.

In de levensmiddelentechnologie wordt kinetisch modelleren gebruikt, zowel voor het voorspellen van veranderingen die optreden tijdens verwerking en opslag, als voor opheldering van complexe reactiemechanismen. Ook in dit onderzoek bleek kinetisch modelleren een belangrijk en bruikbaar hulpmiddel, ook al heeft het zijn moeilijkheden en beperkingen. Kinetisch modelleren moet in combinatie gebruikt worden met analytische methoden, zodat de aard van de producten bepaald kan worden. Het meten van meerdere variabelen en het gebruik van nauwkeurige methoden om activiteiten te bepalen, maken kinetisch modelleren een krachtig hulpmiddel.

Curriculum vitae

Erik Peter Schokker werd op 15 juni 1966 te Ede geboren. In 1984 behaalde hij het diploma voorbereidend wetenschappelijk onderwijs aan het Christelijk Streek Lyceum te Ede. In hetzelfde jaar begon hij aan zijn studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. De afstudeerrichting was zuivelkunde. De stageperiode werd uitgevoerd bij zuivelcoöperatie Noord-Nederland te Warga, Universitas Brawijaja te Malang, Indonesië, en Université Nationale du Bénin, te Cotonou, Bénin. Het doktoraaldiploma werd hem uitgereikt in augustus 1991.

Van 1 mei 1992 tot 30 april 1996 was hij aangesteld als assistent in opleiding bij de sektie Zuivel en Levensmiddelennatuurkunde van de Landbouwuniversiteit, nu Geïntegreerde Levensmiddelentechnologie en Fysica geheten. In deze periode werd het in dit proefschrift beschreven onderzoek uitgevoerd.

Vanaf 10 februari 1997 zal hij als post-doc gaan werken op de University of Guleph, te Guelph, Canada.

Nawoord

Dit proefschrift is het resultaat van vier jaar onderzoek bij de sectie Zuivel en levensmiddelennatuurkunde. Het is tot stand gekomen met medewerking en hulp van anderen. Bij het gereedkomen van het proefschrift wil ik iedereen daarom bedanken die heeft bijgedragen aan de totstandkoming ervan.

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