LIPASES AND PROTEINASES IN MILK

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LIPASES AND PROTEINASES IN MILK

OCCURRENCE, HEAT INACTIVATION, AND THEIR IMPORTANCE FOR THE KEEPING QUALITY OF MILK PRODUCTS

Proefschrift

ter verkrijging van de graad van

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Stellingen

1. Gebreken in melkprodukten door activiteit van melklipoproteïnelipase en/of melkproteïnase kunnen worden voorkomen door de melk een adequate hittebehandeling te geven.
   Dit proefschrift.

2. Activiteit van bacteriële lipasen en proteïnasen in melkprodukten kan alleen worden voorkomen door maatregelen te nemen die leiden tot het verhinderen van groei van gramnegatieve bacteriën in de melk waaruit deze produkten worden gemaakt.
   Dit proefschrift.

3. De aanbeveling „Eet gevarieerd", die voortkomt uit vrees voor voedingsdeficiënties, lijkt in strijd met de uitkomsten van proeven met ratten door Ross & Bras (1974). Deze dieren bleken betere levensverwachtingen te hebben wanneer ze steeds hetzelfde voeder kregen dan wanneer ze vrije keuze hadden uit verschillende soorten voeder.
   Ross, M. H. & Bras, G. (1974), Dietary preference and diseases of age. Nature, 250: 263-265.

4. De stabiliteit van een continue cultuur van yoghurtbacteriën in melk in het pH-gebied van 6,3 tot 5,5 is te verklaren doordat *Streptococcus thermophilus* voor een snelle groei in melk afhankelijk is van de door de langzamer groeiende *Lactobacillus bulgaricus* gevormde kleine peptiden en aminozuren.
   Driessen, F. M. (1981), Protocooperation of yogurt bacteria in continuous cultures. In: Mixed culture fermentations. Eds. M. E. Bushell & J. H. Slater. Acad. Press, London — New York — Toronto — Sydney — San Francisco, p. 99-120.

5. Tijdens de bereiding van yoghurt wordt de groei van *Lactobacillus bulgaricus* in melk gestimuleerd door koolzuur dat door *Streptococcus thermophilus* wordt geproduceerd.
   Driessen, F. M., Kingma, F. & Stadhouders, J. (1982), Evidence that *Lactobacillus bulgaricus* in yogurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. Neth. Milk Dairy J., 36: 135-144.

6. Om bij het toepassen van een gesloten systeem voor het verhitten van melk voor de yoghurtbereiding een goede procesbeheersing mogelijk te maken, is het noodzakelijk de melk te ontluchten.

7. Het is niet waarschijnlijk dat de significante toeneming van de proteolytische activiteit die Noomen (1975) vaststelde in melk die 15 s op 72°C was gepasteuriseerd, kan worden verklaard door een gedeeltelijke inactivering van een natuurlijke proteïnaseremmer.
   Noomen, A. (1975), Proteolytic activity of milk protease in raw and pasteurized cow's milk. Neth. Milk Dairy J., 29: 153-161.

8. Monsters rauwe melk van dezelfde bacteriesamenstelling en met een zelfde kiemgetal kunnen desondanks verschillen in bacteriële kwaliteit.

9. Karnemelk heeft na bewaren in een gasdoorlatende verpakking een betere smaak dan na bewaren in een gasdichte verpakking, doordat in het eerste geval de aromacomponent diacetyl beter behouden blijft.

10. Het afbreken van caseïne door middel van proteolytische enzymen met het doel colloïdaal calciumfosfaat in caseïnemicellen te bestuderen kan aanleiding geven tot foutieve conclusies.
    Holt, C., Hasnain, S. S. & Hukins, D. W. L. (1982), Structure of bovine milk calcium phosphate determined by X-ray absorption spectroscopy. Biochim. Biophys. Acta, 719: 299-303.

11. Bij manipulatie van het genetisch materiaal van melkzuurbacteriën teneinde betere zuursels voor de zuivelbereiding te verkrijgen, dient rekening te worden gehouden met het feit dat deze bacteriën in zuursels elkaar beïnvloeden.
    Wouters, J. T. M. & Stadhouders, J. (1982), Genetica van melkzuurbacteriën; een weg tot nieuwe toepassingen? Zuivelzicht, 74: 1071-1073 en 1172-1174; Voedingsmiddelentechnologie, 15: (24) 19-21 en (26) 26-28.

12. Het verdient overweging de hoogte van de hondenbelasting — naar analogie van de wegenbelasting — te bepalen naar het gewicht van de hond.

Proefschrift F. M. Driessen
Lipases and proteinases in milk. Occurrence, heat inactivation, and their importance for the keeping quality of milk products.
Wageningen, 2 september 1983

Voor Floortje en Krijn

CONTENTS

ABSTRACT

Driessen, F.M. (1983). Lipases and proteinases in milk. Occurrence, heat inactivation and their importance for the keeping quality of milk products. Doctoral thesis, Agricultural University Wageningen (157 pp., English and Dutch summaries).

The occurrence and heat inactivation of native and bacterial lipases and proteinases in milk were studied.

Production of these enzymes by Gram-negative psychrotrophic bacteria in milk was found to take place towards the end of exponential growth and in the stationary growth phase.

Kinetics of heat inactivation in milk of milk lipoprotein lipase, alkaline milk proteinase and lipases and proteinases of some Gram-negative bacteria are given.

The effects of residual lipolytic and proteolytic activity on the keeping quality of milk products were studied. In order to prevent activity of native lipases and proteinases, the milk should be heated sufficiently. The only way of preventing activity of bacterial lipases and proteinases in milk is to take measures against the growth of Gram-negative bacteria in the milk to be processed.

VOORWOORD

Bij het verschijnen van dit proefschrift wil ik al degenen bedanken die hebben bijgedragen aan de totstandkoming ervan.

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Het bestuur en de directie van het NIZO ben ik erkentelijk voor het feit dat zij mij in de gelegenheid hebben gesteld deze promotie te realiseren.

Grote waardering heb ik voor wat mijn collega's van het NIZO hebben bijgedragen. In dit verband wil ik met name noemen: Fedde Kingma, Bart Nannings en Cees van der Waals wegens de onmisbare hulp bij het uitvoeren van de beschreven experimenten; Henk van Brakel en Joop Mondria die de figuren en afbeeldingen hebben vervaardigd; Jacques Maessen en Gosse Stel voor de hulp mij geboden bij het persklaar maken van het manuscript en Gera van Cleef-Bos en Martine van Mazijk voor het verzorgen van het typewerk.

I want to express my gratitude to Dr. Malcolm Anderson, National Institute for Research in Dairying, Reading U.K., for revising the manuscript.

Ten slotte dank ik Wilma die met de haar kenmerkende directe, kritische en relativerende opmerkingen mij steunde en waakte over het juiste evenwicht tussen inspanning en ontspanning.

Chapter 1

INTRODUCTION

1.1 Scope

The keeping quality of raw milk is comparatively poor. For the preservation of milk and milk products a heat treatment in the dairy is often applied. However, liquid milk and milk products may deteriorate after heat treatment. Sometimes rapid deterioration occurs through microbial growth as a consequence of recontamination or insufficient heat treatment. The composition of the milk also changes as a result of chemical reactions. In addition milk may slowly deteriorate from the effect of the residual activity of enzymes, such as those which are the subject of this study: lipases and proteinases.

Lipases act upon emulsified fat (triacylglycerides). During this hydrolysis, di- and monoglycerides and finally glycerol are formed together with free fatty acids (FFA). The build up of FFA may produce a rancid or soapy flavour. In some products (e.g. cheese) a moderate lipolysis may be desirable for flavour.

Proteinases act upon milk proteins, to form smaller peptides and ultimately amino acids. Certain small peptides may induce a bitter or astringent taste in milk. The action of proteinases may also change rheological properties and defects such as gelation can occur.

Lipases and proteinases in milk may originate from two sources, namely from:
- the milk itself (indigenous);
- micro-organisms growing in the milk (exogenous).

Some of these enzymes have a considerable thermoresistance, and therefore they may be important to the keeping quality of milk products, even when these products are sterilized. Deterioration depends on residual enzyme activity and the conditions of storage. This study deals with the following topics:
- the presence of indigenous lipase and proteinase in milk;
- the circumstances favourable for production of exogenous lipases and proteinases in milk;
- the thermoresistance of these enzymes in milk;
- consequences of (residual) activity of these enzymes for the keeping quality of milk and milk products;
- recommendations concerning the prevention of lipolysis and proteolysis in milk and milk products.

1.2 Milk enzymes

Raw milk contains many enzymes. It is not yet clear whether enzymes in milk

serve some purpose or whether they should be considered as extraneous material introduced into milk during the secretory process (Johnson, 1974). However, the enzymes produced by bacteria can not be considered obligate components of milk (see Section 1.3). Comprehensive reviews have been published on the role and significance of about forty five enzymes which have been reported to be found in normal milk (Jenness & Patton, 1959; Shahani, 1966; Groves, 1971; Shahani et al., 1973; Johnson, 1974; Fox & Morrissey, 1981).

Some milk enzymes may be of importance in certain aspects of milk technology. They are responsible for the development of specific flavour compounds or defects in milk and milk products. On the other hand, the heat lability of certain enzymes furnishes the basis for important tests to assess the extent of heat treatment to which a sample of milk may have been subjected. Thus, the destruction of alkaline phosphatase is widely used as a test for the adequacy of pasteurization (IDF/FIL-Standards 63:1971). It is remarkable that milk does not deteriorate more rapidly in view of the wide spectrum of enzymes present in milk and the natural substrates that milk contains for several of these enzymes. It has to be considered, however, that:

- most enzymes in milk occur in very low concentrations;
- many equilibria in milk do not favour enzymatic activity;
- some changes in milk are not or hardly noticed organoleptically or physically.

In addition some of these enzymes do not work in the form in which they exist in milk. This is particular true for the milk enzymes in this study, lipoprotein lipase and alkaline milk proteinase. The substrate for milk lipoprotein lipase, milk fat, is protected by a membrane and proteinase in milk exists for a greater part in the zymogen form.

All enzymes can be inactivated by heat treatments. The heat inactivation of these enzymes is described more fully in this study.

1.3 Occurrence of lipolytic and proteolytic bacteria in milk and milk products

Milk and milk products may contain a variety of micro-organisms capable of secreting lipases and proteinases which subsequently may alter these products. The introduction of refrigerated storage of bulk raw milk at the farm for two or three days and the subsequent storage of bulk raw milk at the dairy factory for an other day at low temperature favours the development of a psychrotrophic bacterial flora in milk. Spoilage of the milk by mainly Gram-negative bacteria rather than by lactic acid bacteria has become possible (Stadhouders, 1973; Thomas, 1974a, b). The Gram-negative bacteria in particular produce extracellular lipases and proteinases that may be remain (partly) active after the usual heat treatments applied in the manufacture of dairy products. Investigations of the psychrotrophic flora in bulk tank milk have shown that as

far as Gram-negative rod-shaped bacteria are concerned the dominant genus is usually *Pseudomonas* with lower proportion of *Acinetobacter (Achromobacter/Alcaligenes)*, *Flavobacterium* and coliform organisms (Thomas, 1974b). Also psychrotrophic thermoduric spore formers have been isolated from pasteurized milk, all representatives of the genus *Bacillus* (Langeveld et al., 1973; Priest, 1977). The optimum growth temperature of psychrotrophic Gram-negative bacteria may lie between 20 and 30 °C (Harder & Veldkamp, 1971), and according to Eddy (1960) they are able to grow below 5 °C and according to Kandler (1966) below 10 °C. The genera referred to above originate mainly from water and soil and get into the milk via improperly cleaned equipment (Witter, 1961).

The thermoresistance of the Gram-negative psychrotrophic microflora of raw milk is generally low. Assuming that a satisfactory reduction of the microflora is equivalent to a decrease to 10^{-10} it can be calculated that a conventional high-temperature short-time pasteurization (HTST) treatment will kill all the organisms whose thermoresistance is known (Cogan, 1979). For practical application a thermization treatment of 10 s at 65 °C is sufficient to eliminate these psychrotrophic bacteria (Gilmour et al., 1981; Stadhouders, 1982).

In milk products two main groups of bacterial lipolytic enzymes can be defined, namely lipases from micro-organisms added during a production process, and lipases from contaminating micro-organisms. Examples of the first group are found in numerous cheese varieties, and also in some fermented milks which are inoculated with micro-organisms that cause development of a specific flavour (Kosikowski, 1977). In addition lactic acid bacteria may also be responsible for limited proteolysis in cheese (Stadhouders & Veringa, 1973). However, lipases from the first group of microbial lipases will not be considered further.

This study deals with the lipases originating from Gram-negative psychrotrophic bacteria, especially lipases having a high heat stability. These enzymes can remain active after the thermal death of the bacteria and as a consequence flavour defects may develop in products. These defects are described as soapy or rancid. It has been known for a long time that Gram-negative bacteria can produce thermoresistant lipases (Söhngen, 1912; Stadhouders & Mulder, 1960), and that during cold storage of raw milk the lipolytic flora will increase (Witter, 1961; Stewart et al., 1975; Chapman et al., 1976; Muir et al., 1978).

In general bacterial proteinases are mainly produced by the same Gram-negative psychrotrophic bacteria that produce lipases. Comprehensive reviews on these proteinase producing bacteria have been written by Law (1979), Suhren (1981) and Fox (1981). In addition to the thermolabile bacteria some thermoduric bacteria can produce extracellular proteinases (Washam et al.,

1977). Psychrotrophic spore formers are also well known for their ability to produce extracellular proteinases (Priest, 1977), but their total numbers and rates of growth are usually too low to be of importance in milk. Bacterial proteinase activity can cause flavour defects in milk and milk products. These defects can be described as astringent and bitterness. Textural failures can also be a consequence of proteolysis, namely coagulation or gelation of milk or milk products.

1.4 Kinetics of heat inactivation of enzymes

Enzymes are globular proteins and possess a specific secondary and tertiary structure. The native conformation of the enzyme, maintained mainly by hydrophobic interactions, hydrogen bonding and often by some disulfide linkages and internal salt bridges, can be destroyed by heat treatments. The peptide chain will unfold (partially). The unfolding as such is a reversible process, but heat denaturation is often found to be irreversible. This can probably be attributed to the release of reactive side groups, that now may react at high temperatures. Reorganisation of S-S bridges may also occur. These changes in the protein prevent refolding to the native state. Arguments for this assumption are supported by accompagnying changes in physico-chemical characteristics of the peptide chain such as intrinsic viscosity, sedimentation and diffusion rate, circular dichroism and optical rotation dispersion (Tanford, 1968). Since enzym activation is dependent on the native globular structure of the enzyme, enzymes may lose their activity. Despite the reactions of side groups being responsible for the irreversibility, it is often the unfolding of the peptide chain that is the rate determining step. Both inactivation of enzymes and denaturation of globular proteins follow similar kinetic parameters. Enthalpy and entropy are usually large, while the constant of inactivation depends on external conditions such as pH, ionic strength and in some cases the presence of substrate for the enzyme. The kinetics of heat inactivation of enzymes, as described in this section, hold for one kind of molecule, but not, for instance for isozymes. Sometimes denaturation of globular proteins may occur in two stages.

The denaturation generally proceeds according to a first order reaction and the denaturation rate is given by:

$$-dc/dt = k'c \qquad (1.1)$$

or, after integration:

$$\ln c_0/c = k't \qquad (1.2)$$

in which k' represents the reaction constant (s^{-1}), t the reaction time (s), c the concentration of the native protein at time t , and c_0 its original concentration. It is seen in Eq. (1.2) that there is a linear relation between

log c and t :

$$t = \frac{2.303}{k'} \log \frac{c_0}{c} \qquad (1.3)$$

The time required to destroy 90 % of the activity, where $c = 0.1\ c_0$ is defined as D (decimal reduction time). Thus Eq. (1.3) gives:

$$D = \frac{2.303}{k'} \qquad (1.4)$$

k' depends on many parameters, among which temperature is an important factor. According to Eyring's theory of the activated complex:

$$k' = kT/h \exp -\Delta G^*/RT \qquad (1.5)$$

where k is Boltzmann's constant ($1.38\ 10^{-23}$ J.K^{-1}) and h is Planck's constant ($6.62\ 10^{-34}$ Js), T is absolute temperature (K), R is the gas constant (8.314 J.K^{-1}.mol^{-1}) and ΔG^* is the Gibbs free energy of activation for the reaction. According to the second law of thermodynamics:

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad (1.6)$$

where H is enthalpy and S is entropy.

Substitution of Eq. (1.6) in (1.5) gives:

$$k' = kT/h \exp(-\Delta H^*/RT) \exp(\Delta S^*/R) \qquad (1.7)$$

It is generally assumed that ΔH^* and ΔS^* are independent of T and this may be more or less true for a limited range of T . Consequently, for this assumption the plot of log (k'/T) versus $1/T$ should give a straight line, the slope of which is $\Delta H^*/R$. Hence, we know ΔH^*. With the experimental value of k' the free energy of activation (ΔG^*) follows from Eq. (1.5):

$$\Delta G^* = RT[\ln (k/h) + \ln T - \ln k'] = 8.314\ T\ (23.76 + \ln T - \ln k') \qquad (1.8)$$

ΔS^* can be calculated from Eq. (1.6)

For the denaturation of an enzyme the activation entropy ΔS^* is large and positive. The unfolding of the peptide chain is a consequence of the release of numerous bonds per molecule. The release of these bonds is accompanied by considerable increase in the conformational entropy of the unfolding peptide chain. This is also true for the activation enthalpy ΔH^*, that is largely caused by the disruption of numerous weak bonds, such as hydrophobic interactions. *Table* 1.1 contains examples of protein denaturation and enzyme inactivation. It is seen that despite the large and varying values of ΔH^* and ΔS^* (increasing with molecular weight), ΔG^* is fairly constant. Using measurements of the absolute rate of inactivation at various temperatures information can be obtained about the contribution of ΔH^* and ΔS^* to the reaction rate. In the case of inactivation as a consequence of denaturation of the enzyme, the mentioned values had to be more or less in accordance with those of the denaturation of proteins with comparable molecular weights.

In practice one often does not determine k' , but rather the time needed to

Table 1.1 Activation enthalpy (≈energy) (ΔH^*), entropy (ΔS^*) and free energy (ΔG^*) in the heat denaturation of some proteins (Walstra & Jenness, 1983).

Protein	Molecular weight	ΔH^* (kJ.mol^{-1})	ΔS^* (J.mol^{-1}.K^{-1})	ΔG^* (kJ.mol^{-1})
Insulin	5 800	150	100	113
Hemoglobin	17 000	316	640	103
Chymosin	31 000	375	870	94
Alkaline phosphatase	40 000	450	1 000	108
Lactoperoxidase	82 000	775	1 950	106

inactivate an enzyme [see Eq. (1.4)]. It is usual to plot log D against the temperature T (in °C), and often a straight line is obtained if the temperature range is not too wide. This relation is derived from the Arrhenius theory, according to which the reaction constant k' can be written as:

$$k' = C \exp(-E_a^*/RT) \qquad (1.9)$$

where C is designated as the 'frequency factor'. According to Eq. (1.9) there is also a linear relation between ln k' and the reciprocal temperature T (K). As shown previously this relation is, however, not precise [see Eq. (1.5)], but because of several small uncertainties the curve gives a good fit for practical purposes. The deviation is often less than 1 % of the theoretically better approximation according to Eyring, because in the relation:

$$\Delta H^* = E_a^* - RT \approx E_a^* \qquad (1.10)$$

the factor RT (≈ 2.7 kJ.mol^{-1}) is neglected (see also *Table 1.1*). For reasons of general acceptance the plot log k' versus $1/T$ has been used in this study.

The parameters Q_{10} and Z, which are often used to characterise the effect of a heat treatment, are explained briefly as follows.

The temperature dependence of a reaction can be given as Q_{10}, defined as the factor by which dc/dt is increased if the temperature is raised by 10 °C. By comparing k'_T and k'_{T+10}, Eq. (1.9) gives:

$$\ln \frac{k'_{T+10}}{k'_T} = \ln Q_{10} = E_a^*/RT.(T + 10) \approx 10\, E_a^*/RT^2 \qquad (1.11)$$

For the denaturation of a protein a Q_{10} of approximately 100 is quite normal, while for chemical reactions Q_{10} is usually 2 to 3 (Booy, 1964). However, from

Eq. (1.11) it is seen that Q_{10} depends on the temperature. For reactions with Q_{10} = 2 and 100 at 320 K this gives E_a^* of about 60 kJ.mol^{-1} and 390 kJ.mol^{-1}, respectively.

The Z value is defined as the temperature rise (K) needed to increase the reaction rate by a factor 10. Consequently, from Eq. (1.11) follows:

$$Z = 10/\ln Q_{10} \approx RT^2/E_a^* \qquad (1.12)$$

The Z value depends also on the temperature.

1.5 Description of the heat treatments used in this study

1.5.1 Heat treatments with longer holding times

1.5.1.1 Heat treatments below 100 °C in glass tubes

At temperatures below 100 °C samples of skim milk or culture fluid were heated in glass tubes with an inner bore of 10 mm and a wall thickness of 1.0 mm. Samples were preheated in a water or oil bath maintained at 15 °C above the desired temperature. The temperature was controlled with a thermocouple fitted in the centre of the tubes. During the preheating period, which lasted about 40 s, the tubes were shaken. At the desired temperature the tubes were quickly transferred to a thermostatically controlled water bath (± 0.1 °C) and held at this temperature for various periods of time. The samples for 'zero time' were cooled immediately after preheating.

1.5.1.2 Heat treatments below 100 °C in capillary tubes

The rather long preheating time of the heat treatment described in the previous section can be shortened by applying the capillary technique, in which capillary tubes with an inner bore of 2.4 to 2.9 mm, filled with 0.1 ml of sample were heated in a water bath set at the desired temperature (± 0.1 °C). The preheating time was assumed to be 7 s (Stern et al., 1952; Stern & Proctor, 1954; Franklin et al., 1958).

1.5.1.3 Heat treatments above 100 °C

At temperatures above 100 °C samples of culture fluid were heated in a mini-sterilizer, consisting of a stainless steel vessel with an inner diameter of 50 mm and a height of 300 mm. This vessel was supplied with an airtight screw cap through which a stirrer shaft with an air lock was mounted. The stirrer shaft with 6 propellor blades was rotated at 1 300 rpm. The samples were preheated in an oil bath maintained at 15 °C above the desired temperature. The temperature was controlled with a thermocouple, fitted 10 mm from the wall and 30 mm above the bottom of the tube. At the desired temperature the equipment was

quickly transferred to a thermostatically controlled oil bath (± 0.1 °C) and held at this temperature for various periods of time. Than the sample was cooled in a water bath. Samples for 'zero time' were cooled immediately after preheating. For heating at 110, 120 and 130 °C the time needed for preheating was 110, 132 and 157 s, respectively.

1.5.2 Heat treatments with a flow-through mini-pasteurizer

Pasteurization treatments of samples of skim milk or culture fluid were carried out with a flow-through mini-pasteurizer, developed at the Netherlands Institute for Dairy Research. This equipment was built up with several stainless steel spirals, submerged in thermostatically controlled water baths. The residence times of the samples in the preheater and heater were 17 and 3 s, respectively. The length of the holding section was varied by the use of different spirals. The cooling spiral was submerged in cold water.

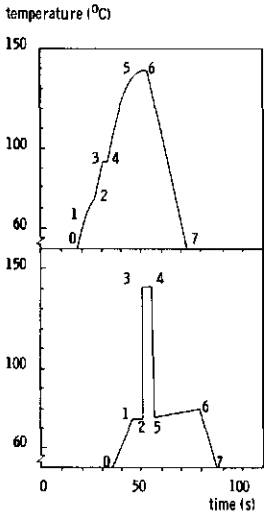

Figure 1.1 (Top) time-temperature profile of an indirect UHT-sterilizer. 0-1: preheater; 1-2:homogenizer 71-74 °C; 2-3:heating in counter current, 74-95 °C; 3-4:holding section, 2 s at 95 °C; 4-5:steam heater, 95-139 °C, 17.5 s; 5-6:holding section, 2.4 s at 139 °C; 6-7:cooler in counter current, 139-50 °C, 21.6 s.

Figure 1.2 (Bottom) time-temperature profile of a direct UHT-sterilizer. 0-1:preheater; 1-2:holding section, 5.1 s at 75 °C; 2-3:steam injection, 75-140 °C, ~ 0 s; 3-4:holding section, 5.1 s at 140 °C; 4-5:cooling by evaporation, 140-70 °C, ~0 s; 5-6:homogenizer, 76-79 °C, 2.5 s; 6-7:cooler, 79-20 °C, 15 s.

1.5.3 Description of the ultra-high-temperature (UHT)-sterilizers

The UHT-sterilization procedure is carried out commercially either by a direct or an indirect heating system. Direct heating is based upon steam injection into the product, followed by an evacuation to remove the injected water vapour. During indirect heating the product is separated from the steam by a wall, usually by the use of concentric tubes. In this latter procedure heating and cooling of the product proceed more slowly. The course of temperature with time in both heat treatments is illustrated in *Figures* 1.1 and 1.2 (Hallström & Dejmek, 1977). The way in which keeping quality is affected by the sterilization procedure was one of the objectives of this study. In the following sections descriptions are provided of the equipment used in this study for the UHT-sterilization treatment. Because the heat inactivation of the alkaline milk proteinase was studied in detail with an Alfa Laval VTIS sterilization plant, this plant is described more extensively.

1.5.3.1 The Alfa Laval VTIS sterilization plant and its operation

Layout of the plant . A diagram of the Alfa Laval VTIS sterilization plant used for the experiments is shown in *Figure* 1.3. Milk was pumped through the preheater with a centrifugal pump. In the preheater the milk was heated to about 80 °C. A high pressure pump then pumped the preheated milk into a steam

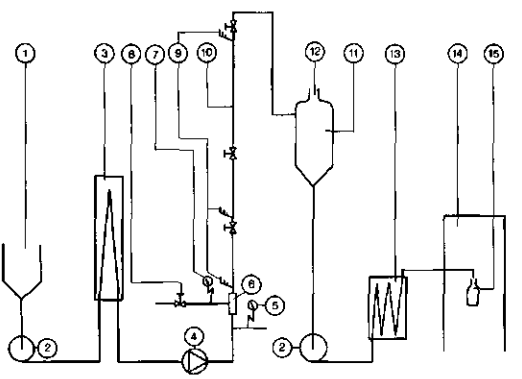

Figure 1.3 General lay out of the Alfa Laval VTIS sterilization plant used in this study. 1. Milk inlet; 2. centrifugal pump; 3. preheater; 4. high pressure pump; 5. holding pressure gauge; 6. direct steam injector; 7. steam pressure gauge; 8. steam control valve; 9. thermocouple; 10. holding tube with three back pressure valves; 11. flash chamber; 12. outlet vapour to condensor and vacuum pump; 13. cooler; 14. laminar flow cabinet; 15. sampling point.

injector, where it was heated instantaneously to the temperature of sterilization. Then the milk flowed through a holding tube with a total length of 1.8 m and an inner diameter of 10 mm. During the operation one of three back pressure valves (type Saunders) was adjusted to maintain the desired pressure during the sterilization treatment. The other two valves were then fully opened to minimize the pressure drop across the first valve. The milk flowed through the vacuum chamber, in which low pressure was maintained by a vacuum pump. At this low pressure milk evaporates thus cooling it until the temperature approximates that of the preheated milk. This treatment restores the original dry matter content of the milk. The milk was then removed from the bottom of the vacuum chamber by a centrifugal pump to the cooler and on to a sampling point in the laminar flow cabinet.

At three places along the holding tube provision was made to measure the milk temperature with copper-constantan thermocouples.

From other investigations it is known that for direct steam injection heating several operation conditions must be fulfilled (Burton, 1958; Stroup et al., 1972; Edgerton et al., 1970). An important condition is the back pressure in the holding tube. If the pressure is too low there will not be complete condensation of the injected steam into the milk, and this will cause a variation of the temperature in the holding tube. Furthermore a certain excess back pressure is necessary to prevent separation of dissolved gas as a consequence of the rise in temperature. Experimentally, it emerged that the back pressure in the holding tube should be at least 0.2 bar above the product saturation vapour pressure. Although constant temperatures were attained with such a pressure excess, the holding pressure was mostly maintained at 0.5 bar above the saturation pressure.

Plant operation. The plant was sterilized by operating it with water at a temperature above 130 °C for at least 30 min. To switch from sterilization of the plant to normal operation, back pressure control was taken over by one of the back pressure valves. The required temperature and pressure conditions were adjusted. The steam supply was controlled by the steam control valve to give the operating temperature. The vacuum pump was started and the flow adjusted with a positive displacement pump to give a flow of liquid at the sampling point of 60 litres per h. When the plant conditions were stable at the required pressure and temperature the circulating water was replaced by milk. If necessary slight corrections of the pressure and temperature were made. The samples were taken after ample time for the milk to pass through the plant.

When more than one temperature treatment was used the highest temperature was applied first followed in succession by the lower ones to avoid contamination of the highly sterilized product. Before setting a new holding time the temperature

was increased by opening the steam pressure control valve. The new holding time was adjusted by using another expansion valve to maintain the back pressure, keeping the product pressure and temperature constant. At this new holding time the desired plant operating conditions were adjusted and when the conditions were stable again a second experiment was carried out as described above.

1.5.3.2 Reliability of the Alfa Laval VTIS sterilization plant for studying the heat inactivation of enzymes in milk

The true residual enzymatic activity after a certain heat treatment will be less than the measured one, as a consequence of the spread in the residence time in the holding section, and also of the profile of heating and cooling. All milk particles cannot flow at the same rate. In general, the spread of the velocities about the mean will depend on the degree of turbulence in the liquid flow and the geometry of the equipment. It was calculated that the spread in residence time was negligible. However, this calculation was checked, in which use was made of a bacteriological method, based upon the determination of the thermal death rates (TDR) of bacterial spores at various heating temperatures. At the same temperatures and holding times the thermal inactivation of spores determined with the capillary technique according to Stern & Proctor (1954) was compared with the thermal inactivation of spores by direct heating with the sterilizer.

Bacterial spores. A suspension of *Bacillus stearothermophilus var. calidolactis* strain 6241 from the collection of the Netherlands Institute of Dairy Research was streaked on the surface of an Oxoid nutrient agar (ONA) plate containing 10 ng Mn^{2+}/kg. The agar plates were incubated at 63 °C for 3 days in plastic bags, to prevent drying out. Spores and vegetative cells were harvested by rinsing the plates with a quarter strength Ringer solution. Spore suspensions were prepared by heating the solution at 85 °C for 15 min and further dilution with skim milk.

Thermal death rates with the capillary technique . The capillary tubes (see Section 1.5.1.2) containing skim milk with approximately 10^5 spores/ml were heated in a glycerol bath and further cooled in water. The capillary tubes were disinfected in a solution of chlorine (0.2 ml/l; v/v) and placed in a 10 ml sterile flask with 4 ml quarter strength Ringer solution. The tubes were crushed with a flamed glass bar. The residual number of spores was determined with DTS-agar after incubation (in plastic bag) for 3 days at 63 °C (Busta, 1967). When a spore count less than 10 per ml was expected, the spore suspension in milk was centrifuged for 15 min at 12 000 *g* after addition of 1 % sodium-citrate

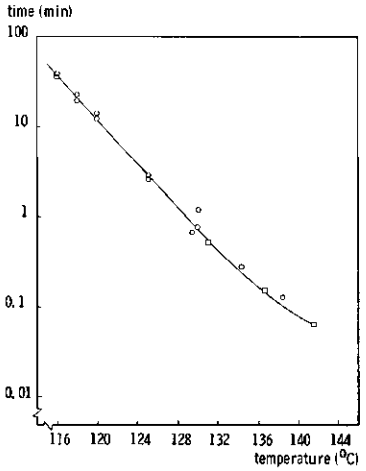

Figure 1.4 Decimal reduction time (DRT) curve for spores of *Bacillus stearothermophilus var. calidolactis* strain 6241.
O—O Determined with a capillary technique.
□—□ Determined with direct heating with the Alfa-Laval VTIS sterilizer.

(w/w) to concentrate the spores. The sediment was washed with quarter strength Ringer solution, and the spores were counted. In this way a reduction of spores up to 10^{-6} could be estimated.

At various temperatures holding times were chosen in such a way that TDR could be determined. The decimal reduction time (DRT) curve, given in *Figure* 1.4, was logarithmic, although a 'tail' was observed at high temperatures.

Thermal death rates with direct heating. Skim milk with approximately 10^5 spores/ml was heated at various temperatures. The holding times were chosen in such a way that TDR could be determined. The DRT values are shown also in *Figure* 1.4.

The results of these experiments prove that the Alfa Laval VTIS sterilizer is suitable for studying the thermal inactivation of enzymes.

1.5.3.3 The Stork Sterideal VTS sterilization plant and its operation

Layout of the plant. A diagram of the Stork Sterideal VTS sterilization plant used for the experiments, is given in *Figure* 1.5. Milk was pumped through a preheater with a pressure pump and there the milk was heated with steam to about

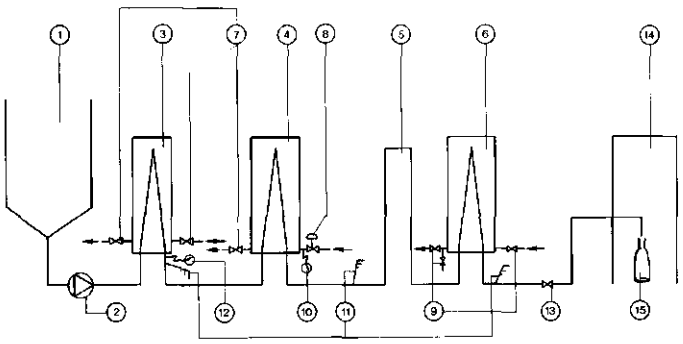

Figure 1.5 General lay out of the Stork Sterideal VTS sterilization plant used in this study. 1. Milk inlet; 2. high pressure pump; 3. preheater; 4. sterilizer; 5. holding section; 6. cooler; 7. steam valve; 8. steam control valve; 9. water valve; 10. steam pressure gauge; 11. thermocouple; 12. product pressure gauge; 13. back pressure valve; 14. laminar flow cabinet; 15. sampling point.

80 °C. Then the milk flowed into the sterilizer, in which the milk was gradually heated to the sterilization temperature. There was no holding tube between the preheater and the sterilizer. The sterilizer was connected with exchangable holding tubes of about 5, 15, 30 or 45 s. After the sterilization treatment milk was cooled with tap water. At the end of the plant milk was collected at a sampling point in a laminar flow cabinet. During operation the back pressure valve (type Saunders) was adjusted to maintain the desired pressure during the sterilization procedure.

The amount of steam for preheating and the amount of tap water for cooling were controlled by hand. The amount of steam for the sterilizer was controlled automatically. Milk temperature at the end of the preheater, sterilizer and cooler was measured with copper-constantan thermocouples. Plant pressure during the sterilization treatment was measured with a pressure gauge.

Plant operation . The plant was sterilized with water by operating at a temperature above 130 °C for at least 30 min. The required temperature and pressure were adjusted. The steam supply was controlled by a steam control valve to give the operating temperature. The capacity was about 100 l per h. When the plant conditions were stable at the required pressure and temperature the circulating water was replaced by milk. If necessary, slight corrections of the temperature and pressure were made.

When more than one sterilization temperature was used, the highest temperature was applied first followed in succession by the lower ones, to avoid contamination.

When the holding section was changed, the operating procedure for the plant was re-started.

Acknowledgement . S. Bouman, G. Hup and J. Stadhouders are acknowledged for permission of publishing their results concerning the characterization of the profile of heating of the Alva Laval VTIS sterilization plant.

Chapter 2

MILK LIPOPROTEIN LIPASE

2.1 Introduction

Raw milk contains endogenous lipolytic activity. Since the discovery of both the enzyme and a means of determining its activity by Rice & Markley (1922) this lipase has been the subject of many studies. Bovine milk has considerable potential lipolytic activity but very little lipolysis occurs in most samples of raw milk. In milk the fat globules - droplets of 0.2 - 10 μm in diameter - are surrounded by a membrane of amphipolar molecules, proteins and phospholipids, which protects the triglycerides against enzyme attack. The enzyme probably originates from capillary endothelial cells in the mammary gland and is thought to be secreted into the alveolar lumen together with milk proteins and lipids.

For a long period of time it was thought that milk contained several lipolytic enzymes (glycerol-ester hydrolases: EC 3.1.1.3). This conclusion was based upon variation in the relative lipolytic activity towards different substrates (Frankel & Tarassuk, 1956 a, b). According to the location of activity in milk, Tarassuk & Frankel (1957) distinguished between a naturally active or "membrane lipase" and a "plasma lipase". Cows' milk was also found to contain a lipoprotein lipase (EC 3.1.1.34), most of which was observed in skim milk. However, the triglycerides of cream were not hydrolysed by this lipoprotein lipase (Korn, 1962).

In the seventies new evidence about the nature of the milk lipase system became available. Several groups of research workers discovered that milk lipase was in fact the same enzyme as the lipoprotein lipase described by Korn (1962), and that this enzyme was the principal endogenous lipase of normal bovine milk.

Lipolysis in raw milk is promoted by the addition of blood serum (Jellema & Schipper, 1975). The defect known as spontaneous rancidity may be connected with the presence of an activator in the milk (Driessen & Stadhouders, 1974 and 1975). This activator is probably part of the high density lipoprotein fraction of skim milk, which appears to have immunological determinants in common with high density lipoproteins from cows' blood (Castberg & Solberg, 1974). These observations are in agreement with the conclusion that the major lipolytic enzyme in bovine milk is a lipoprotein lipase (Olivecrona et al., 1975). It was shown with immunological techniques that milk lipoprotein lipase is structurally related to the lipoprotein lipase present in post-heparin blood plasma (Hernell et al., 1975). However, bovine colostrum has a low lipolytic activity with characteristics other than those of a lipoprotein lipase (Driessen, 1976a).

2.2 Biochemistry of milk lipoprotein lipase

Milk lipoprotein lipase exists as a dimer with a molecular weight of about 100 000 daltons (Iverius & Östlund-Lindqvist, 1976). Chemical analysis shows that the enzyme is a glycoprotein containing 8.3 % carbohydrate (Iverius & Östlund-Lindqvist, 1976). Bovine milk contains 1 - 2 mg enzyme per litre, that is 10 - 20 nM (Olivecrona, 1977). Milk lipoprotein lipase can have a very high turn-over rate. With 1,3-diolein as a substrate, at pH 8.5 and 25 °C, a turn-over rate of more than 3 000 s^{-1} was measured (Olivecrona, 1977). With this turn-over rate, milk would be rancid in 10 - 20 s. In practice this does not occur.

The enzyme is highly surface-active. Most of it is located in the milk plasma (Castberg et al., 1975). The largest proportion being associated with the casein micelles. Lipase-casein binding is mainly through ionic forces, but hydrophobic interactions may also contribute (Downey & Murphy, 1975). Bovine milk lipoprotein lipase binds with high affinity to heparin, and this property can be used for the purification of the enzyme by affinity chromatography, on a column of heparin-substituted agarose (Olivecrona et al., 1971; Egelrud, 1973).

Heparine-bound lipoprotein lipase is catalytically active and can be stimulated by the cofactor apolipoprotein C II, henceforth called cofactor. Therefore, it can be postulated that milk lipoprotein lipase, in addition to its active site, has at least three other functional regions:

- an interface recognition site;
- a region for interaction with the cofactor;
- a region for interaction with heparin and other polyanions (Bengtsson & Olivecrona, 1981).

The pH optimum of bovine milk lipoprotein lipase against triacylglyceride emulsions is between 8.0 and 9.0, and the optimum temperature ranges from 35 to 40 °C (Brockerhoff & Jensen, 1974).

A number of different proteins can inhibit lipase activity. This inhibition is probably caused by competition of these proteins with the cofactor (Bengtsson & Olivecrona, 1977). Activity is also inhibited by the reaction products, long chain fatty acids. Therefore, for the determination of lipase activity a fatty acid acceptor, such as Ca^{2+} or serum albumin, should be included in the assay mixture (Scow & Olivecrona, 1977).

Milk lipoprotein lipase acts predominantly on the fatty acids in the α-position of triacylglycerides (Brockerhoff & Jensen, 1974). Experiments with equimolar quantities of triacylglycerides show that there is a preferential hydrolysis of the triacylglycerides with lower carbon numbers (Jensen et al., 1964). One reason for this apparent specificity may be that short chain fatty acids tend to be more frequently situated in the α-position. Alternatively,

since the diffusion rate of low molecular weight triacylglycerides in fat is higher than those of larger triacylglyceride molecules, so the low molecular weight triacylglycerides preferentially move close to the water-fat interface where lipolysis takes place.

2.3 Thermoresistance of milk lipoprotein lipase

Trout (1950) found it necessary to pasteurize the milk, either before or immediately after homogenization, in order to avoid lipolysis. Lipolytic activity seems to be destroyed by a conventional high-temperature short-time (HTST) pasteurization. The conditions required to inactivate the enzyme have been investigated by various research workers. A survey of these results, shown in *Table* 2.1, does not indicate a clear relationship between heat treatment and residual activity, because individual results are concerned with only one time-temperature combination and are obtained from different experimental conditions. Therefore the heat inactivation of milk lipoprotein lipase was investigated in more detail.

Table 2.1 Survey of the thermoresistance of the milk lipolytic enzyme, measured by various investigators.

Authors	Inactivation of the milk lipase after heating for:	Calculated $\log \frac{\text{initial activity}}{\text{residual activity}}$
Hetrick & Tracy (1948)	5 s at 85 °C	12.10
Harper & Gould (1959)	17.6 s at 82 °C	19.66
Sjöström (1959)	25 s at 80 °C	16.67
Nilsson & Willart (1961)	20 s at 80 °C	13.34
Andersen & Kjaergaard (1962)	16 s at 76 °C*	3.80
Shipe & Senyk (1981)	16.7 s at 76 °C*	3.97

*Sufficient for pasteurized homogenized milk.

2.3.1 Materials and methods

Milk. Various samples of milk were drawn aseptically from a limited number of cows. The bacterial counts of these samples of milk were in all cases below 400 per ml. The cream was separated by centrifugation in sterilized tubes at 3 300 *g*

for 15 min at 4 °C. Approximately 50 % of the membrane material originally associated with the fat globules was found in the resultant skim milk (Koops & Tarassuk, 1959).

Heat treatments. At temperatures below 65 °C aliquots of skim milk were heated in glass tubes according to the method described in Section 1.5.1.1.

At temperatures above 65 °C pasteurization treatments were carried out in a flow-through mini-pasteurizer according to the method described in Section 1.5.2.

Assay of lipase activity by the pH stat method. Activity measurements were made using a substrate of butter oil, emulsified with gum arabic as recommended by Parry et al. (1966). The composition of the substrate was as follows: 10 g gum arabic; 10 g butter oil; 0.67 g NaCl; 4.4 g bovine serum albumin, and 400 IE heparin in 100 g distilled water. Emulsions were prepared with a Branson sonicator, Model S 125, operated at full power for 3 min while keeping the sample container in ice. This treatment produced fat globules with a size ranging from 0.5 to 2.3 µm.

During the experiments the pH stat was connected to a Metrohm precision potentiometer, type E 353 B. The pH was kept constant at 8.75 by the automatic addition of 0.02 N NaOH. The temperature was regulated by a thermostatically controlled water bath at 37.0 $\pm$ 0.1 °C. One unit of lipolytic actvity is defined as the number of micro-equivalents of alkali required per minute to titrate the FFA released per ml of sample while maintaining a given pH. The number of units was calculated from the initial slope of the recorded pH stat curve.

Pasteurized milk. Aseptically drawn milk was pasteurized at various temperatures and subsequently homogenized at 20 MPa and 50 °C. Sterilized bottles were filled aseptically with this milk and stored at 7 °C. During storage the milk fat acidity was measured by the modified BDI-method (Driessen et al., 1977). For the duration of the experiment the number of viable micro-organisms was estimated on Plate Count agar to which 1 % skim milk had been added (PCM-agar). The poured plates were incubated for three days at 30 °C. Only the results from milk with counts below 100 per ml were taken into account.

2.3.2 Results

2.3.2.1 Heat inactivation of milk lipoprotein lipase

The thermoresistance of the lipase system in skim milk was investigated over a temperature range of 50 to 70 °C. For each temperature the logarithm of the

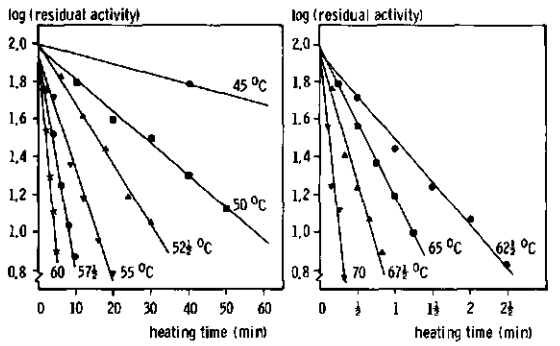

Figure 2.1 Inactivation of the milk lipoprotein lipase by various heat treatments.

residual enzyme activity was plotted against the heating time. The results are given in *Figure* 2.1. From these data linear regression lines were calculated, together with D (decimal reduction time) and k' (constant of inactivation). These values were expressed as (reciprocal) seconds. The kinetic and thermodynamic parameters of the inactivation of the enzyme are given in *Table* 2.2. The Q_{10} at 70 °C was calculated to be 16.7. The reaction kinetics are discussed in Section 1.4.

Table 2.2 Kinetic and thermodynamic parameters of the heat inactivation of lipoprotein in skim milk.

Temperature (°C)	$10^3/T$ (K^{-1})	D (s)	Reaction rate constant, k' (10^{-3} s^{-1})	Activation: Gibbs free energy, ΔG^* ($kJ.mol^{-1}$)	Activation: enthalpy, ΔH^* ($kJ.mol^{-1}$)	Activation: entropy, ΔS^* ($J.mol^{-1}.K^{-1}$)
45	3.145	11 150	0.2	100.6	231.5	411.6
50	3.096	3 470	0.7	99.0	231.5	410.2
52.5	3.072	1 880	1.2	98.1	231.4	409.5
55	3.049	940	2.4	97.0	231.4	408.8
57.5	3.026	510	4.5	96.1	231.4	409.4
60	3.003	270	8.5	95.1	231.4	409.3
62.5	2.981	126	18.3	93.7	231.4	410.4
65	2.959	74	31.0	92.9	231.3	409.5
67.5	2.937	43	53.3	92.1	231.3	408.8
70	2.915	16	142.2	90.0	231.3	412.0

Inactivation of milk lipoprotein lipase by various heat treatments appeared to follow first order reaction kinetics (see *Figure* 2.1). There is also a linear relationship between k' and T. The results are given in *Table* 2.2, and presented as an Arrhenius plot in *Figure* 2.2. This plot is characterized by the equation:

$$\log k' = -12.220\ (10^3/T) + 34.667 \qquad (r^2 = 0.996) \qquad (2.1)$$

For the milk lipoprotein lipase the relationship between D and temperature T' (°C) is given in *Figure* 2.3. The plot is characterized by the equation:

$$\log D = -0.112\ T' + 9.136 \qquad (r^2 = 0.998) \qquad (2.2)$$

Eqs. (2.1) and (2.2) can be used to predict the relative lipolytic activity of heat-treated milk.

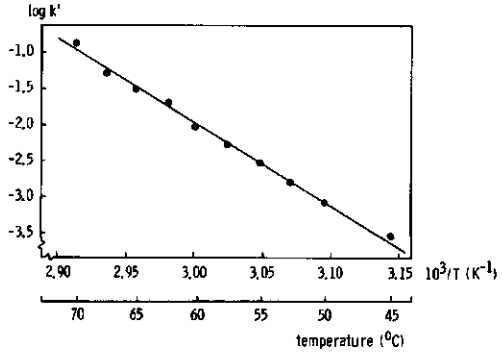

Figure 2.2 Arrhenius plot of the inactivation of the milk lipoprotein lipase.

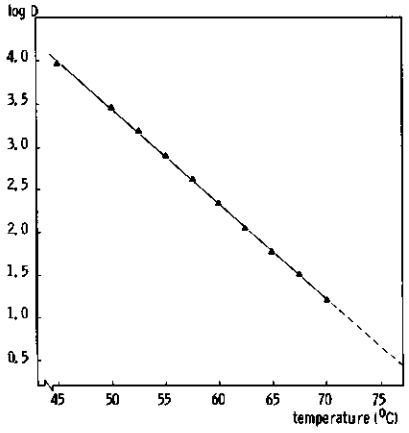

Figure 2.3 Thermal destruction curve for the milk lipoprotein lipase.

2.3.2.2 Lipolysis in pasteurized homogenized milk

In view of the thermoresistance of milk lipoprotein lipase it can be expected that some lipolysis will occur in pasteurized homogenized milk. From Eq. (2.2) it can be calculated that at a typical temperature for HTST pasteurization, for instance 72 °C, the *D* for inactivation is 11.8 s. Therefore, residual lipolytic activity in pasteurized homogenized milk heated at various temperatures for 10 s, was investigated. The results of a series of experiments are plotted in *Figure* 2.4.

It is obvious from these experiments that lipolysis can occur in homogenized milk, pasteurized at 72 °C. Within 3 or 4 days this milk is likely to have a rancid taste and therefore the keeping quality will be reduced. To ensure good keeping quality milk should be pasteurized at a higher temperature, for example at 78 °C for 10 s. No milk lipoprotein lipase activity is measured after a heat treatment of 10 s at 85 °C.

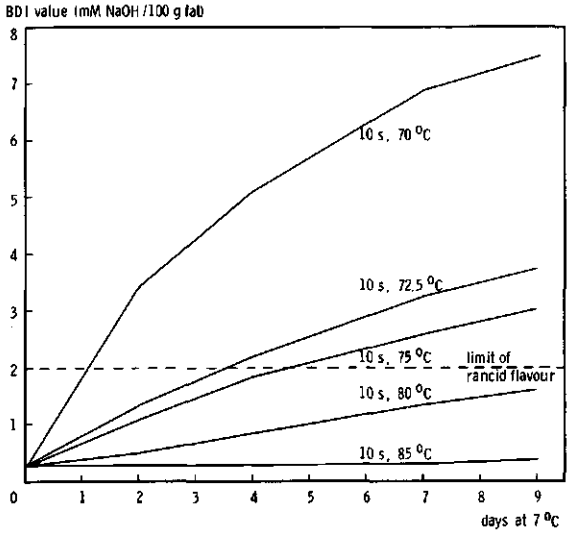

Figure 2.4 Course of the acidity of the fat during storage at 7 °C of homogenized whole milk, pasteurized at various temperatures.

2.3.3 Discussion

In milk the fat is protected against lipolysis by the intact fat globule membrane. When this membrane is somehow damaged lipolysis can occur. The intensity of the fat splitting activity in milk will depend on the residual activity of the enzyme after the heat treatment and on several other factors.

Inactivation of milk lipoprotein lipase follows the kinetics of a first order reaction. The Gibbs free energie during the heat treatment was approximately 93 $kJ.mol^{-1}$ at 65 °C.

As previously stated, milk has a high potential lipolytic activity. It can be calculated that under optimum conditions for lipolysis, milk would be rancid in 10 to 20 s. From the kinetics of inactivation, as measured in this study, the number of *D* ($1/10^n$, in wich n = number of *D*) necessary for inactivation according to various investigators was calculated. These values are given in *Table* 2.1 column 3. For complete inactivation milk must be heated until approximately n = 15 had been reached. Although the amount of lipoprotein lipase in milk is high, the number of *D* for total inactivation is such that it may be assumed that Eqs.(2.1) and (2.2) should not be extrapolated too far beyond the range of experimental conditions. In this study a heat treatment of 10 s at 85 °C, appeared to be sufficient for complete elimination of lipolysis in pasteurized homogenized milk. However, in this milk the conditions were not optimal for lipolysis. The milk is stored cold and has a suboptimal pH. In these conditions lipolysis appears to be inhibited sufficiently when the milk has been heated to an equivalent of 3.9 *D* (Andersen & Kjaergaard, 1962; Shipe & Senyk, 1981). This observation was confirmed in this study, a heat treatment for 10 s at 78 °C corresponds to 4.0 *D*.

Homogenization of raw milk causes very rapid lipolysis and hence a soapy, rancid flavour detectable within 5 to 10 min of the treatment (Mulder & Walstra, 1974).

The activation energy for the denaturation of globular proteins depends on the molecular weight of the protein, assuming that, per unit weight approximately the same number of amino acid residues is available for bonds responsible for globular conformation of the protein. For the denaturation of a globular protein with a molecular weight of 50 000 the ΔH^* and ΔS^* are at least 500 $kJ.mol^{-1}$ and 1 000 $J.mol^{-1}.K^{-1}$, respectively (Walstra & Van der Haven, 1979). Since the results given in *Table* 2.2 are less than half these values, it is unlikely that the inactivation of lipoprotein lipase in milk can be fully explained by destruction of the globular structure of the protein.

2.4 Milk lipoprotein lipase activity in milk and milk products

2.4.1 Raw milk

When the membrane is damaged, lipolysis will occur in raw milk without addition of a cofactor (Deeth & Fitz-Gerald, 1976). Therefore, lipolysis can be expected in milk products where the milk fat substrate is present in an attackable form. This substrate activation can be done in three ways: homogenization, foaming and

temperature manipulation. Homogenization and foaming will cause a mechanical damage of the membrane by which the fat loses its protection against lipolysis more or less. By temperature manipulation the membrane may be damaged physically, which results in an increased content of free fatty acids during further cold storage of the milk (Claypool, 1965).

The defect in raw milk known as spontaneous rancidity may be connected with the presence of an activator in the milk. This activator may arise in milk by leakage from the blood (Castberg & Solberg, 1974).

Lipolysis may occur predominantly in raw milk of cows in an advanced stage of lactation. Feeding may also influence the lipolysis in milk. A low energy ration is mainly related to an abnormal decrease in milk production and an increase in susceptibility to rancidity (Jellema, 1975).

Physiological processes may evoke lipolysis in raw milk. Especially hormone involvement in milk lipolysis has been proposed because of its possible influence upon secretion of activator substances and lipase into the milk (Bachman, 1982).

2.4.2 Butter

The flavour of butter is readily impaired when lipolysis occurs. Since butter is usually made from cream pasteurized at a high temperature, it is generally considered that milk lipoprotein lipase does not play a role in increased acidity of the fat during storage. Lipolysis occurring in butter is therefore mainly attributed to lipolytic micro-organisms (Sjöström, 1959; Deeth & Fitz-Gerald, 1976).

2.4.3 Cheese

The hydrolysis of fat is a normal part of cheese ripening and some of the fatty acids liberated during the ripening are essential flavour constituents of cheese. Enzymes that may hydrolyse fat during the cheesemaking process can originate from rennet, milk, starter organisms, adventitious bacteria or from added lipases.

The fact that lipolysis occurs in cheese made from aseptically drawn raw milk demonstrates that milk lipoprotein lipase contributes to the hydrolysis of fat in cheese made from raw ~~milk~~ (Stadhouders & Mulder, 1957). In contrast, when milk is heated at the time-temperature combination favoured by cheese factories in the Netherlands, it has been observed that the residual activity is of little importance in the hydrolysis of the cheese fat (Stadhouders & Mulder, 1960; Reiter et al., 1969; Kleter, 1976 and 1977). However, measurements of the thermoresistance of milk lipoprotein lipase in this study show that about 17 % of the activity could survive a normal cheese milk pasteurization treatment,

i.e. 10 s at 72.°C. The apparent conflict between this observation and that from factory results, indicates that the influence of milk lipoprotein lipase on lipolysis in cheese is not fully understood. It is possible that the low pH of cheese (approximately 5.2) is of significance in this phenomenon, since milk lipoprotein lipase is labile at low pH (Frankel & Tarassuk, 1956b; Driessen, 1976). Therefore a number of important questions remain unanswered including: why lipolysis occurs in cheese made from raw milk, and why during cheese ripening lipolysis continues at low pH.

2.4.4 Other dairy products

Lipolysis can also be expected in cream; ice cream (mix); milk powder and evaporated milk. During the last two decades few papers on lipolysis in these products have been published and the review by Sjöström (1959) still appears to be up to date. He reported that for ice cream mix milk should be heated for more than 30 min at 68 °C or 25 s at 80 °C. These heat treatments are in agreement with those necessary for pasteurized homogenized milk and for cream. For milk powder similar heat treatments are needed to prevent lipolysis by milk lipoprotein lipase. The heat treatments applied during the manufacture of sterilized products like evaporated milk are such that milk lipoprotein lipase is completely inactivated.

2.5 Concluding remarks

Milk contains high concentrations of lipoprotein lipase. However, little milk fat is hydrolysed in raw milk because it is protected by the fat globule membrane. Mechanical damage of this membrane or the presence of a cofactor of the enzyme in the milk or both, initiates lipolysis in raw milk.

To prevent defects caused by milk lipoprotein lipase it is recommended that milk is heated to at least 78 °C with a holding time of 10 s. It is of practical importance to respect this fact, and also to prevent that homogenized milk (returns) is mixed either with raw or with low pasteurized milk or reconstituted low heat milk.

Chapter 3

LIPASES OF GRAM-NEGATIVE BACTERIA IN MILK AND MILK PRODUCTS

3.1 Introduction

In this study the lipases originating from Gram-negative bacteria are henceforth called lipases. If lipases from other micro-organisms are involved, reference is made in the text.

During the last two decades cold storage of milk in bulk tanks on the farm has increased considerably, especially in the Netherlands. Consequently, the bacterial flora present in raw milk has changed in favour of organisms that can grow at low temperature. These organisms are the so called psychrotrophic bacteria. A survey of the occurrence of lipolytic bacteria in milk and milk products is given in Section 1.3.

There are relatively few reports on what proportion the milk flora produces lipase, but results suggest that approximately one third of the isolates from cold stored milk is lipolytic (Stadhouders & Mulder, 1958; Kishonti & Sjöström, 1970; Muir et al., 1979). The lipolysis which occurs on storage of milk is according to Muir et al. (1978) correlated with the total count of psychrotrophic bacteria before storage. However, milk with relatively high bacterial counts ($> 1.7 \cdot 10^7$ ml^{-1}) were used in this study.

Lipase is secreted by bacteria during their growth in milk. It is generally assumed that lipases produced by psychrotrophic bacteria are exclusively extracellular, and it is certainly true that they can be almost completely recovered from culture supernatants (Law, 1979). Rancidity can be produced by adding the culture supernatant of a pre-grown lipolytic strain, but not by washed cells, to milk and pasteurizing it immediately before cheese-making (Law et al., 1976). The amount of lipase produced per cell by *Pseudomonas fluorescens* varies with the temperature and reaches a maximum at 8 °C. Little lipase activity was observed in cultures incubated at 30 °C, although the bacterial counts were high (Andersson, 1980a).

This chapter deals with the practical significance of these lipases, especially the conditions for enzyme production, the heat resistance of the enzymes and lipolysis in milk products.

3.2 Biochemistry of bacterial lipases

Specificity. The lipases secreted by Gram-negative rod-shaped bacteria are glyceryl ester hydrolases (EC 3.1.1.3). Natural fats are insoluble in water and

lipases act upon emulsified fat at the oil/water interface. The enzymatic reaction rate is therefore not related to the substrate concentration, but to the interfacial area of the emulsion (Desnuelle, 1961). The lipase produced by *Pseudomonas fragi* attacks primarily the α-position of the triacylglyceride, but has very slight, if any, action on the β-position (Alford et al., 1964). However, the lipase from *P. fluorescens* is reported to hydrolyse blood serum triglycerides completely to glycerol and free fatty acids (FFA) (Sugiura & Oikawa, 1977), and it is assumed that the fatty acids change their position from β- to α- by isomerization.

Characterization. There are relatively few reports on the characterization of these enzymes. The lipase of *P. fluorescens* appears to be a single chain protein containing neither sugar nor lipid residues. Its molecular weight is estimated to be approximately 33 000 daltons and its isoelectric pH is 4.46 (Sugiura & Oikawa, 1977). The lipase is inhibited by heavy metals (Co, Ni, Zn, Fe, Cu, Hg, Ag and Cd) (Lawrence, 1967; Landaas & Solberg, 1978). Activity is completely inhibited by EDTA, but partly restored by the addition of Ca or Mg (Landaas & Solberg, 1978). The mechanism of action of the Ca^{2+}-ion is not clear. It is possible that calcium may act as an acceptor of released fatty acids (Shah & Wilson, 1965) since long chain fatty acids inhibit lipase activity (Scow & Olivecrona, 1977). However, according to Ota & Yamada (1967) the lipase required one Ca^{2+}-ion for one active site of the enzyme.

Specific -SH reagents such as p-chlormercuribenzoic acid, iodoacetic acid and potassium ferricyanide, have no effect, indicating that -SH groups are not part of the active centre of microbial lipases (Patel et al., 1964; Landaas & Solberg, 1978). Fungal lipases may contain methionine and histidine in their active sites (Motai et al., 1966). Lawrence and coworkers (1967) observed that micrococcal lipase was inhibited by organophosphorus compounds and by 2,4-dinitrofluorobenzene in alkaline solution and found that metal ion inhibition of the enzyme was overcome by histidine which suggests that the active centre may contain both serine and imidazole groups.

Temperature. Bacterial lipases are most active within the temperature range of 30 to 40 °C (Nashif & Nelson, 1953a; Lawrence, 1967; Landaas & Solberg, 1978), but there is still substantial activity at low temperatures (Alford & Pierce, 1961; Andersson, 1980a). These lipases are very stable at temperatures below 8 °C, and at 20 °C still 50 % of the original activity can remain after a storage of about 3 months (Andersson, 1980a).

Effect of the pH. A rough distinction can be made between microbial lipases originating from yeasts or fungi and from bacteria. The optimum pH of most fungal or yeast lipases appears to be at acid pH values, while for most bacterial lipases it is at neutral or alkaline pH values. Crude lipases of *P.fluorescens* and *P. fragi* are most active at pH 6.5 and 7.6, respectively (Law et al., 1976). Other investigators report an optimum pH of 8.0 for the lipase activity of a fluorescent pseudomonad (Landaas & Solberg, 1978). The extracellular lipase of *Achromobacter lipolyticum* has an optimum pH of 7.0 (Khan et al., 1964).

The variation in optimum pH for lipolytic activity, as reported by various investigators, may be due to differences between species or strains of micro-organisms. In addition pH may influence not only the activity of the enzyme but also the stability of the emulsion, since the rate of lipolysis depends on the interfacial area. It has been suggested that the optimum pH depends upon the nature of the substrate, the buffer solution and other external conditions (Nashif & Nelson, 1953a; Rottem & Razin, 1964).

Water activity (a_w). According to Andersson (1980a) the rate of lipolysis is fairly constant until a water activity of 0.54 is reached. Above this a_w lipolysis increases until a three-fold activity is reached at an a_w of 0.85. Furthermore, lipolysis can occur in milk powder ($a_w < 0.6$) stored at 30 °C for 18 months, when it is manufactured from milk which has been heat-treated at low temperature or was of low bacteriological quality (Kjaergaard Jensen & Sloth Hansen, 1972).

3.3 Production of lipases by Gram-negative bacteria in batch cultures

3.3.1 Introduction

The lipolytic bacterial count increases in raw milk during cold storage. It is of importance, however, to realize that bacteria have a long lag phase (about 3 days) in cold milk, otherwise it would not be possible to store milk at the farm (Stadhouders, 1982). Bacteria produce lipases during their growth (Muir et al., 1979) and the amount of lipase present in the medium depends on several factors which regulate the production of lipase. For pseudomonads the amount of enzyme produced per cell at 5 °C equals that produced at 20 °C, and a maximum is found at 8 °C. Increasing the temperature above 8 °C has commonly a depressing effect on lipase production (Nashif & Nelson, 1953b; Alford & Elliott, 1960; Andersson, 1980a). *Achromobacter lipolyticum* produces the maximum amount of lipase at 21 °C (Kahn et al., 1964). Aeration promotes lipase production per cell as in a shallow layer culture and the maximum yield per cell occurs at

neutral pH (Nashif & Nelson, 1953b; Alford & Elliott, 1960). However, according to Rowe & Gilmour (1982) the production of lipase by *Pseudomonas spec.* is induced by a low oxygen content in a simulated milk medium.

The addition of refined oils to nutrient broth delays the growth of *P. fluorescens* and its lipase production, but the final cell density and amount of lipase are approximately the same as in unsupplemented nutrient broth (Andersson, 1980b). Pseudomonads appear to require a source of organic N for lipase production, though not for growth. *P. fluorescens* and *P. fragi* produce lipase in peptone media or synthetic media containing free amino acids, although the amino acids are a less effective N source (Law, 1979). Despite the abundance of information on the production of lipases by Gram-negative bacteria in synthetic media, there remains a need for investigations in milk to establish the effects of specific measurable parameters such as physiological stage and temperature on relative lipase activity. These considerations are studied and the results discussed in the following sections.

3.3.2 *Materials and methods*

Lipolytic bacteria. All the organisms used in the experiments came from the Netherlands Institute for Dairy Research and originate from raw milk. The strains involved in this investigation were: *Pseudomonas fluorescens* 22F, *P. fluorescens* R11, *P. putrefaciens* R48, *P. fragi* R67, *Alcaligenes viscolactis* 23a1, *A. viscolactis* 23a2, *Achromobacter spec.* 1-10 and *Serratia marcescens* D2.

Cultivation. Cultures, freshly grown at 20 °C, were inoculated in 150 ml portions of UHT-sterilized homogenized whole milk in 500 ml Erlenmeyer flasks, and incubated at 7 °C. The initial bacterial count was approximately 10^3 ml^{-1}. One strain, *P. fluorescens* 22F, was inoculated in heated skim milk (5 min at 105 °C), and during incubation the culture was monitored for growth and lipase production. Growth was measured by a plating technique with Plate Count agar containing 1 % skim milk (PCM-agar). The plates were incubated for three days at 30 °C.

Activities of the lipases. The lipase activity of *P. fluorescens* 22F was determined by the pH stat method of Parry et al. (1966). For the estimation a Metrohm precision potentiometer E353B was used. The reaction vessel contained 10 ml substrate to which 1 ml of culture was added. The culture was centrifuged at 49 500 *g* for one hour, and lipase present in the supernatant was estimated. The pH was maintained at the optimum value 8.75 (see Section 3.3) by automatic

delivery of 0.02 N NaOH, and the temperature maintained at 37.0 ± 0.1 °C with a thermostatically controlled water bath. The substrate for the lipase assay consisted of 15 % butter oil emulsified in a 10 % gum arabic solution. Emulsions were prepared with a Branson sonic emulsifier, Model S125, using it at full power for 3 min on ice. Fat globule size in the substrate ranged from 0.5 to 2.3 µm as determined by microscopic measurements. The initial slope of the recorded curve was taken as a measure of lipolytic activity, and an unit of activity defined as the number of micromoles of NaOH required per minute to maintain the given pH.

Lipase activities during the growth of the other cultures were estimated as follows. Growth of the cultures in commercially UHT-sterilized whole milk was determined after a fixed incubation time at 7 or 10 °C, depending on the growth rate at low temperature of the particular strain. The culture was pasteurized for 30 min at 63 °C and subsequently incubated for 5 days at 37 °C. Cultures were then checked for sterility and the increase in acidity of the fat during the 5 days incubation period was taken as a measure of lipase activity. The acidity of the fat was determined according to the modified BDI method (Driessen et al., 1977).

3.3.3 Results

The relationship between growth and lipolytic activity of Pseudomonas fluorescens 22F. The relationship between growth of *P. fluorescens* 22F in milk and the lipolytic activity measured with the pH stat is given in *Figure* 3.1. Bacterial lipase could only be measured in the late exponential growth phase and

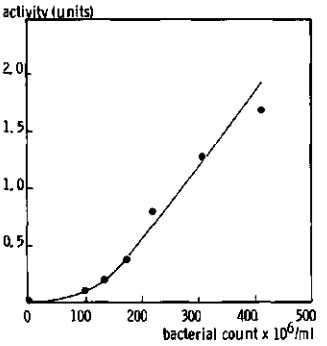

Figure 3.1 Relation between lipase activity and bacterial count of *Pseudomonas fluorescens* 22F at 7 °C. The activity was measured with the pH stat method.

in the stationary phase of growth, which means at rather high bacterial numbers. When the activity per cell was calculated, however, it appeared that the production of lipase was fairly constant over the range in which activity could be measured. As the lipase activity was measured for very short incubation times, approximately 5 min, it can be expected that these bacteria also produce lipase at an earlier stage of growth. Therefore other experiments were done in milk with a longer period of incubation.

Production of lipase by some Gram-negative bacteria in milk. Various Gram-negative bacteria were cultivated in commercially UHT-sterilized whole milk. During their growth at 7 or 10 °C the production of lipase was measured by determining the change of the acidity of the fat over a period of 5 days at 37 °C.

The different *Pseudomonas* species grew well at 7 °C, and their growth curves were very similar to each other. Lipase production could only be measured

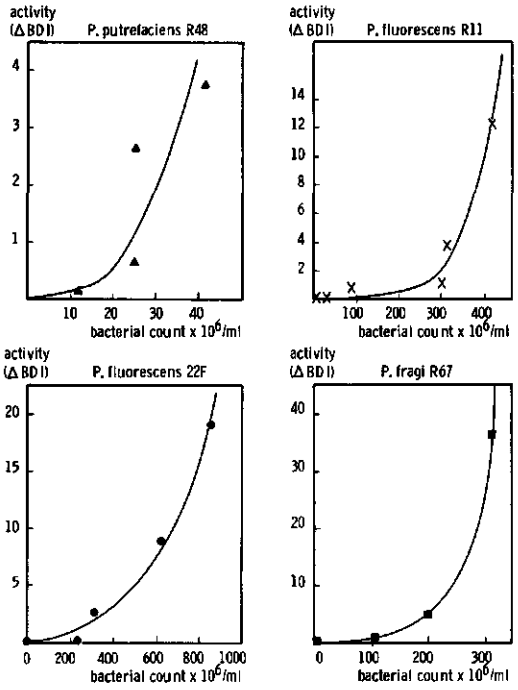

Figure 3.2 Relation between lipase activity and bacterial count of some pseudomonads cultivated in UHT-sterilized whole milk at 7 °C. The acidity of the milk fat was determined after 5 days at 37 °C.

towards the end of the exponential phase of bacterial growth. Results are given in *Figure* 3.2. It clearly appeared that the measured lipolytic activity (ΔBDI) increased far more than the corresponding bacterial count. The most lipolytic strain in this investigation was *P. fragi* R67. This strain showed no measurable lipolytic activity when the bacterial count was $8.0 \cdot 10^6$ ml^{-1}. Detectable activity was measured at a bacterial count of $1.2 \cdot 10^8$ ml^{-1} (96 h). Between 96 and 120 h of incubation the bacterial count increased two fold, while the lipolytic activity increased seven times. This indicates that lipase accumulated in the stationary phase of bacterial growth. Production of lipase at 7 °C by other bacteria showed the same trends, although the activities were less pronounced.

Bacteria growing at 10 °C showed results comparable with those growing at 7 °C. These results are given in *Figure* 3.3. The most lipolytic strain of this group was *Alcaligenes viscolactis* 23a2. This strain showed lipolytic activity towards the end of the exponential growth phase, when the bacterial number exceeded $8.0 \cdot 10^6$ ml^{-1}. Considerable activity was measured at a bacterial count

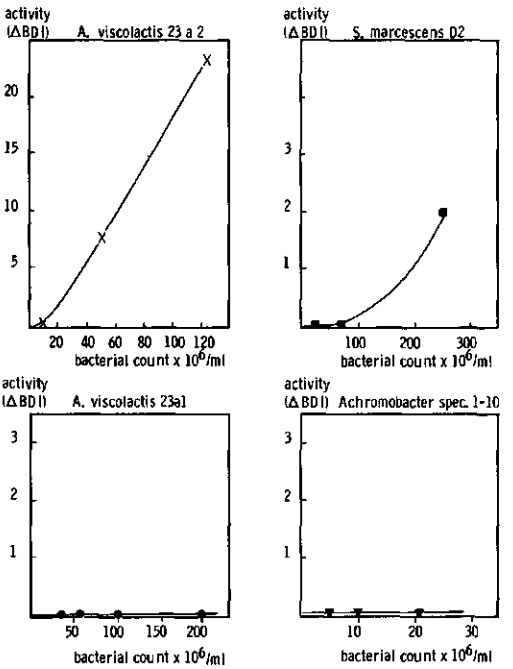

Figure 3.3 Relation between lipase activity and bacterial count of some Gram-negative bacteria cultivated in UHT-sterilized whole milk at 10 °C. The acidity of the milk fat was determined after 5 days at 37 °C.

of $1.5 \cdot 10^7$ ml^{-1}. *Achromobacter spec.* 1-10 and *A. viscolactis* 23a1 did not produce lipase in milk at 10 °C, although at 20 °C enzyme was produced by *A. viscolactis* 23a1 (see Section 3.5). The lipolytic activity of *Serratia marcescens* D2 could not be measured during the stationary phase of growth. The milk became transparent as a result of accompanying proteolytic activity and the fat could not be liberated.

3.3.4 Discussion

The production of lipase of the Gram-negative bacteria involved in this investigation took place only towards the end of exponential growth or at stationary growth phase when the bacterial count exceeded $8.0 \cdot 10^6$ ml^{-1}. This count is much higher than the value usually found in the Netherlands. Applying the pH stat method for the measurements of the lipase activities, the results suggested that there was some accumulation of lipolytic activity from the end of the exponential phase to the stationary phase of growth. Confirmation of such an accumulation was shown by the more sensitive measurement of lipase activity.

This observation is of practical interest. The physiological state of the bacteria appears to be of importance for the production of extracellular lipase. In a fully-grown culture the lipase is accumulated, while during growth in milk there is little or no measurable activity. From this point of view it is of importance to avoid dead spaces in processing lines very carefully. From the previous discussion it also appears that milk or parts of milk should not be stored for too long a period. In both cases the bacteria may have the opportunity to grow out unlimited and consequently lipase can be produced. From the bacterial number in the raw milk alone, it is not possible to predict the lipolytic activity. This is only possible if is known how this number is achieved, and which bacteria are present in the milk.

3.4 Production of lipase by Pseudomonas fluorescens 22F in continuous cultures

3.4.1 Introduction

From the experiments described in Section 3.3 it appeared that in Gram-negative bacteria used, extracellular lipase was produced towards the end of the exponential growth phase. As already stated this fact is of great practical interest. Therefore the relationship between the growth of *P. fluorescens* 22F and lipase production was investigated in a different way, namely with a continuous culture system. By maintaining the bacterial number at a constant level the particular phenomena characteristic for that growth phase can be shown more clearly than in batch cultures, were every growth phase is only a transition stage.

3.4.2 Materials and methods

Culture of P. fluorescens 22F. *P. fluorescens* 22F was inoculated in peptone-meat extract broth and incubated for 24 h at 20 °C. This culture was then stored in ice and the bacterial number estimated by direct microscopic count.

Continuous cultures. A Biolafitte 2 l fermenter (Gourdon, Maison-Lafitte, France) was used. The vessel was filled with 1 litre of skim milk, heated for 5 min at 105 °C, and kept at 20.0 $\pm$ 0.1 °C in a water bath. The milk was inoculated with approximately 10^4 *P. fluorescens* 22F per ml. After a 16 h incubation period the bacterial number was estimated by direct microscopic count and when the desired number was reached, the inlet pump was started. The dilution rate was chosen in a way that the culture remained in steady state. The culture was kept in steady state for at least 9 h. The bacterial numbers during steady state were estimated on Plate Count agar containing 1 % skim milk (PCM-agar).

Lipase activity. A 5 ml portion of milk from the fermentation vessel was added to 125 ml commercially UHT-sterilized homogenized whole milk. To this mixture 1 ml of a thimerosal solution (Fluka AG, Buchs, Switzerland) containing 13 mg/ml was added. The mixture was incubated for 24 h at 37 °C, and the increase of the free fatty acids was estimated according to the modified BDI-method (Driessen et al., 1977). For reference 5 ml water or raw milk were added to the UHT-sterilized milk.

Table 3.1 Relation between bacterial count of *Pseudomonas fluorescens* 22F in continuous culture at 20 °C and the production of extracellular lipase.

sample	dilution rate (h^{-1})	bacterial count (ml^{-1})	increase of free fatty acids during storage for 24 h at 37 °C (meq/100 g fat)
water	-	-	0.00
raw milk	-	-	5.40
1	0.34	$1.2\ 10^7$	0.00
2	0.32	$4.3\ 10^7$	0.00
3	0.29	$2.0\ 10^8$	0.04
4	0.28	$5.0\ 10^8$	0.64

3.4.3 Results and discussion

The results of the experiments with the continuous cultures are given in *Table* 3.1. These experiments clearly show that the extracellular lipase of *P. fluorescens* 22F is only produced towards the end of the exponential growth phase. These results are in agreement with those obtained from the batch process and are shown in *Figure* 3.2. There are no reasons to believe that the other strains involved in this investigation will show characteristics differing from those of the tested strain.

3.5 Thermal activation and inactivation of extracellular lipase of Gram-negative bacteria

3.5.1 Introduction

The influence of temperature on lipolysis by Gram-negative bacteria has been studied by numerous workers (Witter, 1961; Lawrence, 1967; Cogan, 1977; Law, 1979), but in many cases the results have been poorly quantified. The inactivation rate of the extracellular lipases have not been characterized over a wide range of temperature, but only for some heat treatments of practical interest. The lipases of *Pseudomonas fluorescens* and *P. fragi* are reported to remain wholly or partly active after heat treatment at 63 °C for 30 min, which is a low-temperature long-time pasteurization treatment (Law et al., 1976). At high-temperature short-time pasteurization treatments, the lipases of a number of different species of psychrotrophic bacteria isolated from dairy products are thermoresistant (Stadhouders & Mulder, 1960; Griffiths et al., 1981). Some of these enzymes are so thermoresistant that they are not (fully) inactivated by UHT-sterilization treatments (Björklund; 1970).

Since the lipases of these organisms are of importance in connection with fat hydrolysis in many dairy products more exact data concerning the effect of the temperature on the activity and the inactivation of the lipases of Gram-negative bacteria are needed. These data are presented in the following sections.

3.5.2 Materials and methods

Lipolytic bacteria. All the organisms used in the experiments came from the pure-culture collection of the Netherlands Institute for Dairy Research and originate from raw milk. They were screened for their lipolytic capacity as described later. The strains involved in this investigation were: *Pseudomonas fluorescens* 22F and 31H, *P. putrefaciens* R48, *P. fragi* 14-2, *Alcaligenes viscolactis* 23a1 and 23a2, *Escherichia coli* ATTC 11246, *Flavobacterium spec.* 28-2, *Achromobacter spec.* 23 D and *Serratia marcescens* D2.

Cultivation. The organisms were inoculated in 150 ml portions of heat-treated skim milk (5 min at 105 °C) in 500 ml Erlenmeyer flasks and incubated for 5 days at 20 °C. The cultures were then checked by plating techniques for contamination. More intensely heated milk cannot be adjusted with sufficient accuracy to a defined pH, which interferes with pH stat estimations of lipase activity. The cultures were centrifuged at 49 000 *g* for one hour. Lipase activity in the supernatant was estimated in each culture to check for the extracellular nature of the enzyme. No activity was found with washed or disrupted washed cells.

Activity of the lipases. The assay was as described in Section 3.3.2. The reaction vessel contained 10 ml substrate to which 1 ml of culture supernatant was added.

Heat treatments. Complete cultures were heated at different temperatures for different periods of time. Pasteurization treatments with short holding times were carried out in a flow-through mini-pasteurizer according to the method described in Section 1.5.2.

Pasteurization treatments with longer holding times were carried out by the capillary technique according to the method described in Section 1.5.1.2.

Heating above 100 °C was done with a mini-UHT-sterilizer according to the method described in Section 1.5.1.3.

The pH of the various cultures at the time of heating is given in Section 3.5.3 since the heat stability of lipases depends on the pH of the culture (Björklund, 1970).

3.5.3 Results

3.5.3.1 The optimum pH of the lipase of Pseudomonas fluorescens 22F

In connection with the results reported in the next Section, the effect of the hydrogen ion activity on the activity of the lipase was measured both at 37 and 50 °C. These relationships are given in *Figure* 3.4. For both temperatures optimum activity of the enzyme was found at approximately pH 8.75. All subsequent activity measurements with the lipase of *P. fluorescens* 22F were carried out at this pH.

3.5.3.2 Effect of the temperature on the lipase activity of Pseudomonas fluorescens 22F

The effect of temperature on lipase activity was expressed in an Arrhenius plot. This plot, shown in *Figure* 3.5, indicates a remarkable relationship. At

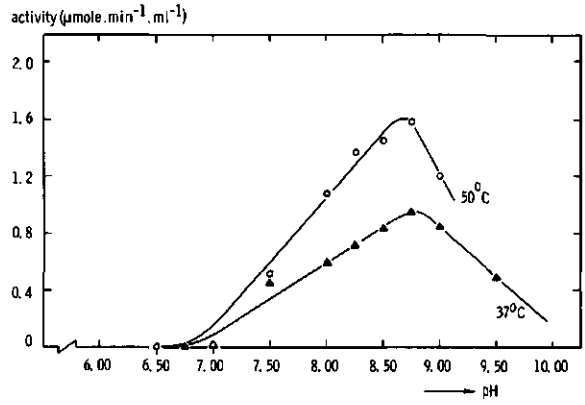

Figure 3.4 Effect of pH on the activity of the extracellular lipase of *Pseudomonas fluorescens* 22F.

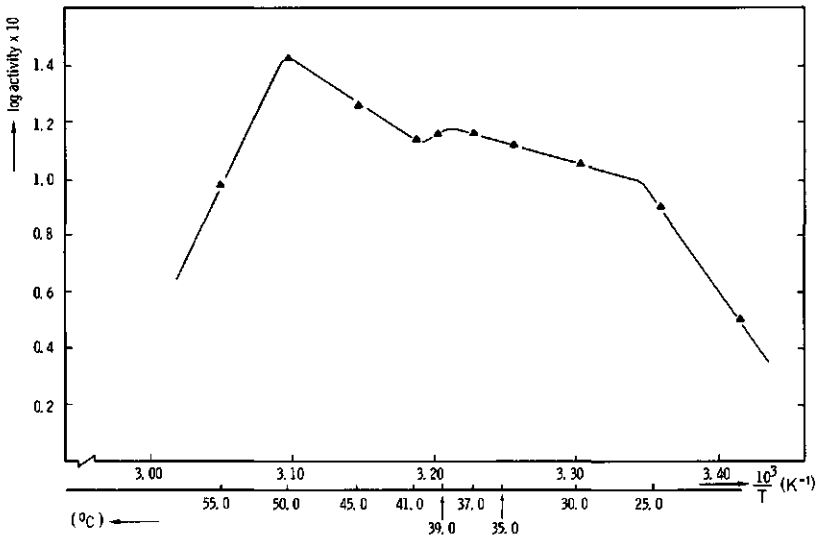

Figure 3.5 Effect of temperature on the activity (v) of the extracellular lipase of *Pseudomonas fluorescens* 22F at pH 8.75.

low temperatures an apparent activation energy (E_a^*) was found of 150.4 kJ.mol^{-1}. Between 25 and 37 °C the E_a^* was 21.8 kJ.mol^{-1}. After a small decrease in activity a new linearity was observed corresponding to an E_a^* of 56.6 kJ.mol^{-1}. The curve in *Figure* 3.5 shows the probability of two optima,one at about 50 °C and the other, a relative one, at about 37 °C. The same relationship between activity and temperature for the extracellular lipase of pseudomonads has reported by other workers (Björklund, 1970; Petricca & Harper, 1970).

3.5.3.3 Heat stability of the lipase of Pseudomonas fluorescens 22F

Several cultures were heated by the capillary technique for different periods of time at 50.0, 52.5, 55.0, 57.5 and 60.0 °C. Exposure of the culture to temperatures above 50 °C resulted in a two-stage loss of lipolytic activity. Inactivation of the lipase took place until a certain limit was reached. Heating for a longer period showed only a small decrease in lipolytic activity. The regression lines are shown in *Figure* 3.6. Obviously two different processes take place, the first stage of inactivation is relatively fast, the second stage relatively slow.

In order to determine the relatively slow rate of inactivation high temperatures were needed for characterization of the regression lines. A culture was heated for different periods of time at 85, 90 and 95 °C, and in another experiment for different periods of time at 110, 120 and 130 °C. These results are shown in *Figure* 3.7. Both stages of inactivation are according to a first-order reaction.

Table 3.2 Kinetic and thermodynamic parameters of the first stage of inactivation of the extracellular lipase of *Pseudomonas fluorescens* 22F at relatively low temperatures.

tempera-ture of heating (°C)	$10^3/T$ (K^{-1})	D (s)	reaction constant, k' (s^{-1})	activation: free energy, ΔG^* (kJ.mol^{-1})	activation: enthalpy, ΔH^* (kJ.mol^{-1})	activation: entropy, ΔS^* (J.mol^{-1}K^{-1})
50.0	3.096	30 000	$7.7\ 10^{-5}$	104.8	424.4	989
52.5	3.072	12 700	$1.8\ 10^{-4}$	103.3	424.4	986
55.0	3.049	3 060	$7.5\ 10^{-4}$	100.2	424.4	988
57.5	3.025	870	$2.7\ 10^{-3}$	97.5	424.4	989
60.0	3.003	288	$8.0\ 10^{-3}$	95.2	424.3	989

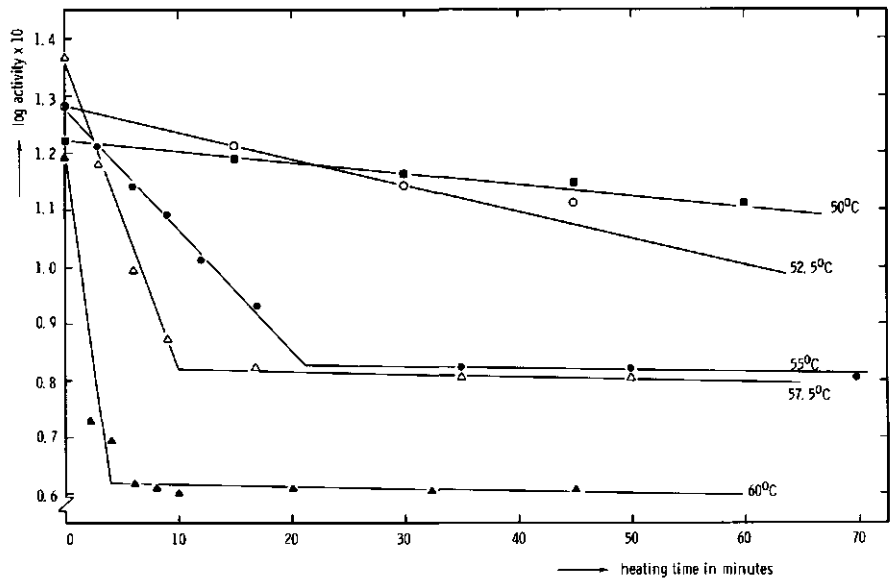

Figure 3.6 Regression lines of the loss of activity (v) of the extracellular lipase of *Pseudomonas fluorescens* 22F at relatively low temperatures.

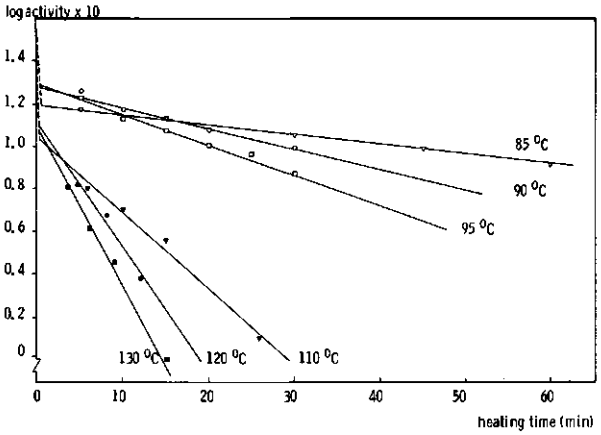

Figure 3.7 Regression lines of the loss of activity (v) of the extracellular lipase of *Pseudomonas fluorescens* 22F at relatively high temperatures.

From these regression lines values for D and k' were calculated and these are quoted in *Tables* 3.2 and 3.3. The Q_{10} values for the first and the second stages of inactivation are 103.9 and 2.86 measured at 60 and 95 °C, respectively. From the data in these tables Arrhenius plots were made for the first and second stages of inactivation (*Figures* 3.8 and 3.9, respectively). The equations of these plots, calculated by the method of the least squares, are:

for the first stage of inactivation:

$$\log k_1' = -22.306\ (10^3/T) + 64.880 \qquad (r^2 = 0.995) \qquad (3.1)$$

for the second stage of inactivation:

$$\log k_2' = -3.502\ (10^3/T) + 6.187 \qquad (r^2 = 0.969^*) \qquad (3.2)$$

For the lipase of *P. fluorescens* 22F the relationship between D value and temperature is given in *Figure* 3.10. The plots are characterized by the equations:

for the first stage of inactivation:

$$\log D_1 = -0.208\ T' + 14.933 \qquad (r^2 = 0.995) \qquad (3.3)$$

for the second stage of inactivation:

$$\log D_2 = -0.024\ T' + 6.000 \qquad (r^2 = 0.960^*) \qquad (3.4)$$

in which T' is the temperature in °C.

Table 3.3 Kinetic and thermodynamic parameters of the second stage of inactivation of the extracellular lipase of *Pseudomonas fluorescens* 22F at relatively high temperatures.

temperature of heating (°C)	$10^3/T$ (K^{-1})	D (s)	reaction constant, k' (s^{-1})	activation: free energy ΔG^* ($kJ.mol^{-1}$)	activation: enthalpy ΔH^* ($kJ.mol^{-1}$)	activation: entropy ΔS^* ($J.mol^{-1}K^{-1}$)
85.0	2.793	12 000	$1.9\ 10^{-4}$	113.7	64.0	-139
90.0	2.755	5 880	$3.9\ 10^{-4}$	113.2	64.0	-136
95.0	2.717	4 200	$5.5\ 10^{-4}$	113.7	63.9	-135
110.0	2.611	1 800	$1.3\ 10^{-3}$	115.8	63.8	-136
120.0	2.545	1 200	$1.9\ 10^{-3}$	117.6	63.7	-137
130.0	2.481	840	$2.7\ 10^{-3}$	119.5	63.6	-139

* The deviation which is not explained by the regression ($\sqrt{1-r^2}$) is rather high for the second stage of inactivation (18 to 20 %) and suggests that values should not be extrapolated beyond the range of the experimental conditions.

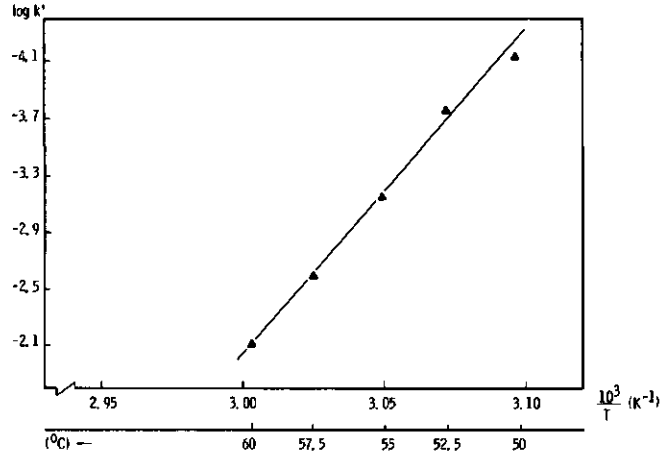

Figure 3.8 Arrhenius plot of the first stage of inactivation of the extracellular lipase of *Pseudomonas fluorescens* 22F.

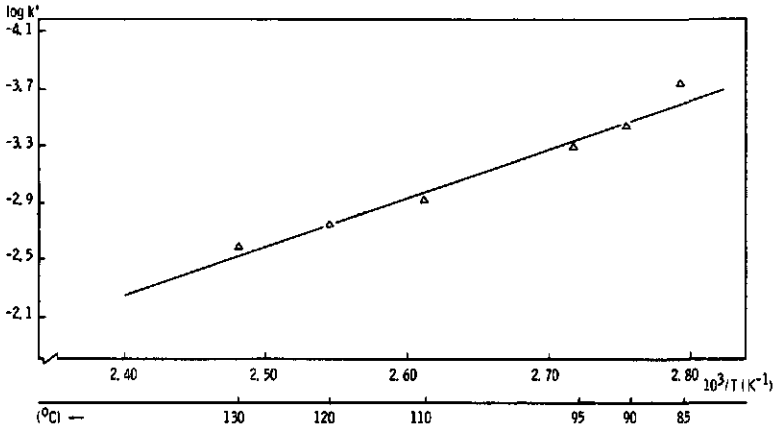

Figure 3.9 Arrhenius plot of the second stage of inactivation of the extracellular lipase of *Pseudomonas fluorescens* 22F.

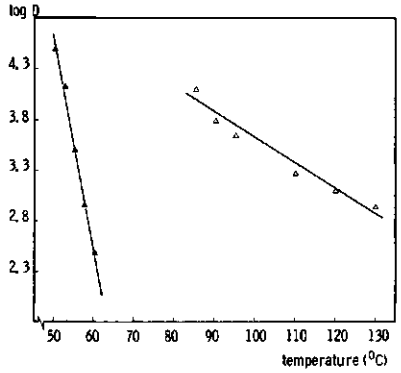

Figure 3.10 Thermal destruction curve for the extracellular lipase of *Pseudomonas fluorescens* 22F.

▲——▲ First stage of inactivation or low temperature inactivation.

△——△ Second stage of inactivation or high temperature inactivation.

The Z values for the first and the second stages of inactivation of the lipase of *P. fluorescens* 22F are 4.8 and 41.7 °C, respectively.

Eqs. (3.1) to (3.4) can be used to predict the effect of specific heat treatments on the residual activity of the lipase of *P. fluorescens* 22F.

3.5.3.4 Effect of a mild preheating on the lipase activity of Pseudomonas fluorescens 22F

From the Arrhenius plots given in *Figures* 3.8 and 3.9, it appears that preheating the lipase at a relatively low temperature destroys either a heat-sensitive enzyme, or a heat-sensitive conformation of the enzyme during the first stage of inactivation. In one experiment the lipases were heated for 10, 20 or 30 min at 54.4 °C and the activity was estimated over a temperature range of 20 to 55 °C. The D for the first stage of inactivation, calculated from Eq. (3.3) is 4 150 s (69 min), and that for the second stage of inactivation, calculated from Eq. (3.4) is 49 500 s (825 min). The effect of these mild heat treatments, as far as 30 min at 54.5 °C, on the second stage of inactivation is therefore negligible.

After these preheating treatments the relative temperature optimum at 37 °C almost disappeared and only one optimum remained at 50 °C. In addition, low temperature activation was deminished. These results are plotted in *Figure* 3.11. The calculated E_a^* values are given in *Table* 3.4. It is clear from these results that the enzyme activity with the most rapid inactivation corresponds with the relative optimum at 37 °C. It is debatable whether *P.*

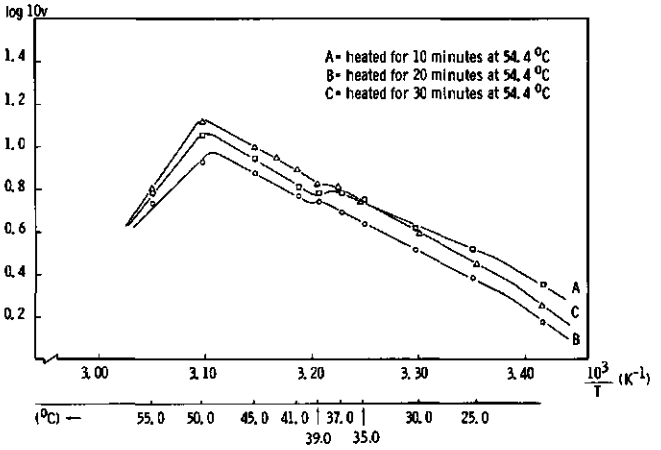

Figure 3.11 Effect of temperature on the activity (v) of the extracellular lipase of *Pseudomonas fluorescens* 22F after various heat treatments.

Table 3.4 Apparent average energy of activation for the lipolytic activity after preheating the culture of *Pseudomonas fluorescens* 22F at 54.5 °C for different periods of time.

heating time at 54.5 °C (min)	E_a^* 37 (kJ.mol^{-1})	E_a^* 50 (kJ.mol^{-1})
0	23.9	56.5
10	44.0	58.4
20	49.1	52.7
30	50.4	50.4

fluorescens 22F produces one enzyme consisting of a thermolabile and a thermostabile conformation or a thermolabile and a thermostabile enzyme. Both enzymes or conformations of one enzyme have the same pH optimum.

3.5.3.5 Effect of temperature on the activitity and inactivation of lipases of other Gram-negative bacteria

No extracellular lipase was found in the culture fluids of *Escherichia coli* ATTC 11246 and *Flavobacterium spec.* 28-2. All the other cultures involved in this study showed lipolytic activity.

Table 3.5 Effect of temperature on the lipolytic activity of some Gram-negative bacteria isolated from raw milk. E_a^* = apparent average activation energy.

organism	temperature range					
	low temperature		medium temperature		high temperature	
	T' (°C)	E_a^* (kJ.mol^{-1})	T' (°C)	E_a^* (kJ.mol^{-1})	T' (°C)	E_a^* (kJ.mol^{-1})
P.fluorescens 22F	<25	150.4	25-37	21.8	41-50	56.6
P.fluorescens 31H	<24	96.8	24-37	28.1	41-52	100.6
P.fragi 14-2	<15	n.d.1	15-37	45.3	41-50	84.6
P.putrefaciens R48	<15	n.d.1	15-37	34.4	absent	absent
A.viscolactis 23a2	<27	82.5	27-37	33.5	41-52	43.6
A.viscolactis 23a1	<22	112.3	22-37	49.4	absent	absent
S.marcescens D2	<20	110.6	20-37	43.6	41-50	- 2)
Achromobacter spec 23 O	<23	96.0	23-58	53.6	absent	absent

1) n.d.= not determined

2) no E_a^* was found, a wide temperature optimum.

pH optima. In connection with the results reported in this section, the effect of the hydrogen ion activity on the lipase activity was measured either at 37 or 50 °C or at both temperatures, according to the species investigated. For all the species the pH was found to be optimal at approximately 8.75. Subsequent experiments were therefore carried out at this pH.

Effect of temperature on the enzyme activity. In order to express the effect of the temperature on the activities of the lipase, Arrhenius plots were made. From a summary of the results given in *Table* 3.5, it appears that *P. fluorescens* 31H, *P. fragi* 14-2 and *A. viscolactis* 23a2 show a relationship between the activity of their lipases and temperature, which is similar to that for *P. fluorescens* 22F. The other strains, *P. putrefaciens* R48, *A. viscolactis* 23a1, *Achromobacter spec.* 23 O and *Serratia marcencens* D2, possess a single linear relationship with temperature.

Heat stability of the lipases. All the cultures were exposed to a temperature of 74 °C, a normal temperature for milk pasteurization. The relationship between lipase activity and time of exposure at 74 °C was examined by regression analysis. From the regression lines D values were calculated and are given

Table 3.6 *D* for the inactivation at 74 °C of the extracellular lipases of some Gram-negative bacteria isolated from raw milk.

organism	pH of the culture	*D* value of the first inactivation (s)	*D* value of the second inactivation (s)	residual activity at knee in curve (%)
P.fluorescens 31H	7.50	100	4 000	55
P.fragi 14-2	6.52	16	1 050	14
P.putrefaciens R48	6.80	44	absent	-
A.viscolactis 23 a2	6.90	96	985	31
A.viscolactis 23 a1	7.21	35	absent	-
Achromobacter spec 23 0	7.00	1 980	absent	-
S.marcescens D2	6.90	23	absent	-

in *Table* 3.6. Those strains whose lipases show both a relative and an absolute optimum in relation to the temperature have a two-stage heat inactivation. Both stages of inactivation appear to follow a first-order reaction. The strains whose lipases show a simple relation with temperature, also have a single-stage heat inactivation, according to first-order kinetics.

3.5.4 Discussion

The lipase(s) of *P. fluorescens* 22F appear(s) to have a relative temperature optimum at 37 °C and an absolute optimum at about 50 °C. A similar observation was made by Björklund (1970) for *P. fluorescens* and by Petricca & Harper (1970) for *Achromobacter lipolyticum* . The Arrhenius plot (see *Figure* 3.5) shows a large deviation from linearity at low temperatures, where the apparent energy of activation is rather high (150.4 $kJ.mol^{-1}$), a value equivalent to the low-temperature activation of an enzyme (Brandts, 1967). By increasing the temperature, two other E_a^* values were found. This observation may be consistent with the following two possibilities: there are two enzymes, or there is one enzyme with two active conformations. Both enzymes or conformations show optimum activity at pH 8.75.

By heating the enzymes at 54.4 °C for 10, 20 or 30 min the apparent $E_a^*{}_{37}$ increased, while the $E_a^*{}_{50}$ did not change very much. The transition point at about 37 °C becomes less distinct when increasing the pre-heat treatment, because the relative contribution of E_a^* 37 at lower temperatures is decreased.

Exposure of the culture to temperatures above 50 °C resulted in a two-stage loss of activity. The initial decrease in activity was very rapid, Q_{10} for inactivation being 103.9. This high value is consistent with the denaturation of a protein (Booy, 1964). The decrease in the second stage was rather slow, Q_{10} being 2.86 at 95 °C. This value indicates a chemical reaction (Booy, 1964). The second stage of inactivation is therefore not likely to be caused by the denaturation of a native protein. There is a large difference in the entropy of inactivation for the two stages of lipolytic activity. The ΔS^* of the second stage may even be negative. Clearly, the mechanism of inactivation of these two enzymes or conformations of one enzyme are different.

It is possible that by heating the lipase of *P. fluorescens* 22F to a sufficient high temperature, the native form changes into a temperature-stable configuration, that is still enzymatically active. However, the change in free energy during the heat treatment was approximately 100 $kJ.mol^{-1}$, a value which is often found for protein denaturation (Payens, 1962). For a complete understanding of the heat inactivation of the lipolytic activity, isolation and purification of the enzyme would be necessary. The phenomena are also observed with acetoacetate decarboxylase obtained from *Clostridium acetobutylicum*, which also undergoes a two-stage thermal inactivation. According to Autor & Fridovich (1970), the native decarboxylase may be modified in a still active conformation. This conformation showed kinetics of denaturation different from those of the native enzyme. Cogan (1979) suggested that the first stage of inactivation concerns an extracellular lipase, and the second slow stage a more heat resistant intracellular enzyme. This suggestion is not acceptable because the experiments were done with the supernatant of the culture. Furthermore it has been shown by isoelectric focusing of the lipase of *P. fluorescens* that isoenzymes are absent (Andersson et al., 1979). According to Andersson et al. (1979) the two stages of inactivation in skim milk are probably the result of the formation during heating of a complex of the lipase with components in the skim milk.

The extracellular lipases of Gram-negative bacteria may be very thermoresistant. As appears from *Tables* 3.5 and 3.6, the thermal inactivation as described for *P. fluorescens* 22F is quite common for extracellular lipases of other Gram-negative bacteria. A biphasic temperature-activity curve is coupled with two stages of thermal inactivation. The second stage is always slow compared to the first stage. However, if Gram-negative bacteria produce an extracellular lipase not showing inactivation in two stages, the heat stability is always high compared with the heat stability of the native milk lipase (see Chapter 2). A normal pasteurization treatment is not sufficient for inactivation of the lipolytic activity originating from Gram-negative bacteria. These lipases

may therefore be active not only in pasteurized food products, but even in more intensely heated ones. The effect of these lipases in cheese and sterilized milk was studied and the results are reported in the next Sections.

This work clearly shows that bacterial lipases as crude enzymes may be highly thermoresistant. However, in some experiments it appeared that the lipase of *P. fluorescens* 22F was also stable when it was in the ammonium sulphate precipitated form, but stability decreased with further purification. It is possible that in the crude preparation the enzyme may associate with some substance which in turn may have a protective action on the enzyme structure. The purification process may eliminate this factor (Lu & Liska, 1969). Exposure of the purified enzyme to 40 °C for 10 min caused a complete loss of activity (Mencher & Alford, 1967).

3.6 Lipolysis caused by Gram-negative bacteria in cheese

3.6.1 Introduction

The widespread practice of bulk cooling and storage of milk on the farm for two or three days, followed by storage of about one day at the dairy plant has heightened the significance of psychrotrophic bacteria (Stadhouders, 1969). It follows that such organisms may contribute to the lipolytic off-flavour of milk products by the production of thermoresistant extracellular lipases. These lipases are resistant to pasteurization treatments, which kill the parent organisms (Cogan, 1979). Several workers have shown that psychrotrophic bacteria can be responsible for hydrolysis of the milk fat during cheese ripening (Stadhouders, 1956; Pinheiro et al., 1965; Csiszar & Romlehner-Bakos, 1956; Lawrence, 1967; Kishonti & Sjöström, 1970).

In Section 3.3 it was shown that psychrotrophic bacteria produce lipase in milk. It may be expected that during the cheesemaking process most of the bacterial lipase is lost, because from 100 parts of milk only 10 parts of cheese are made. Furthermore, the heat stability of the bacterial lipases have been characterized in laboratory experiments. It is not certain whether such experiments on a limited scale can predict the residual activity of the lipases after cheesemaking. These aspects were therefore investigated.

3.6.2 Materials and methods

Lipolytic bacteria. The organisms used in the experiments came from the Netherlands Institute for Dairy Research and originate from raw milk. The strains involved in this investigation were: *Pseudomonas fluorescens* 22F and *Alcaligenes viscolactis* 23a1.

Cultivation. The organisms were cultivated as described in Sections 3.3.2 and 3.4.2.

Activity of the lipases. The activities of the lipases were determined as described in Sections 3.3.2 and 3.4.2

Measurements of lipase heat stability in culture fluid. Heat stability of the lipases in culture fluids was determined as described in Section 3.4.2.

Cheese manufacture. Cheese was made from seven portions of milk as follows. Each of 5 portions of 100 l raw milk was inoculated with 150 ml of a culture containing *Alcaligenes* or *Pseudomonas* bacteria and subsequently pasteurized at various temperatures. As a control, 80 l of pasteurized milk was inoculated with 120 ml of the culture, and another quantity of 80 l pasteurized milk was not inoculated.

The acidity of the cheese fat was followed during ripening according to the method of Sandelin (1939).

The rate of fat hydrolysis in cheese is directly proportional to the ripening time (Raadsveld & Mulder, 1949) and to the amount of enzyme present (Stadhouders, 1956). It may be assumed that the rate of fat hydrolysis in cheese will be related to the amount of lipase left after heating.

Table 3.7 Thermo-resistance of the lipase of *Alcaligenes viscolactis* strain 23a1. pH stat technique.

pasteurization		activity of the supernatant	retention of activity (%)
time (s)	temperature (°C)	(μmole.min^{-1}.ml^{-1})	(%)
control (not pasteurized)		0.89	100
10	50.0	0.82	92
10	58.5	0.74	83
10	66.0	0.56	63
10	72.0	0.37	42
10	78.0	0.15	17
10	84.0	0.06	7
10	90.0	0.00	0

3.6.3 Results

3.6.3.1 Heat stability of the lipase of Alcaligenes viscolactis 23a1

Supernatant of culture fluid was pasteurized at various temperatures for 10 s, and activity was measured immediately after pasteurization. The results are given in *Table* 3.7.

The thermoresistance of the lipase produced by this *Alcaligenes* strain was also studied in a cheesemaking experiment. The results are given in *Table* 3.8.

From the *Tables* 3.7 and 3.8 it is clear that the lipase is indeed partly inactivated during heating for 10 s at 74 °C. When heated for 10 s at 90 to 92 °C, the lipase is completely inactivated. The activity of *A. viscolactis* 23a1 lipase which remained after the heat treatment both in the laboratory test and the cheesemaking experiment are shown in *Figure* 3.12. There is good agreement between the results of both experiments. It again appears that the lipolytic Gram-negative bacteria present in raw milk are of importance in the hydrolysis of the fat in cheese made from this milk after pasteurization.

3.6.3.2 Lipase of Pseudomonas fluorescens 22F in cheese

The distribution of *P. fluorescens* 22F lipase between curd and whey after cheesemaking was investigated. 0.15 % culture supernatant was added to cheese milk. After each stage of the cheesemaking process the activity of the lipase was determined. From the results of these experiments, given in *Table* 3.9, it appears that only a small part of the lipase activity is retained in the whey.

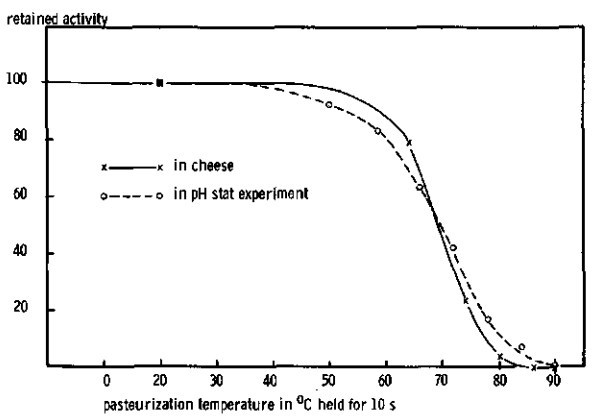

Figure 3.12 Relative retention of the activity (%) of the extracellular lipase of *Alcaligenes viscolactis* 23a1 after various heat treatments.

Table 3.8 Thermo-resistance of the lipase of *Alcaligenes viscolactis*, strain 23a1. Cheese experiment.

treatment of the milk	bacterial count* in raw cheese milk after inoculation (ml^{-1})	pasteurization treatment time (s)	pasteurization treatment temp. (^{o}C)	bacterial count after pasteurization (ml^{-1})	acidity of the cheese fat after 6 weeks of ripening (meq/100 g/fat)	retention of the activity (%)
not inoculated	-	10	74	$3.0\ 10^{2}$	0.87	-
inoculated after pasteurization	-	10	74	$2.8\ 10^{6}$	8.27	100
inoculated before pasteurization	$3.8\ 10^{6}$	10	64	$3.6\ 10^{4}$	6.73	79
inoculated before pasteurization	$3.8\ 10^{6}$	10	74	$7.8\ 10^{2}$	2.44	21
inoculated before pasteurization	$3.6\ 10^{6}$	10	80	$1.8\ 10^{2}$	1.13	3.5
inoculated before pasteurization	$3.5\ 10^{6}$	10	86	$1.0\ 10^{2}$	0.78	-1.2
inoculated before pasteurization	$3.9\ 10^{6}$	10	92	$4.0\ 10^{1}$	0.80	-0.9

* Initial count of raw milk: $8.9 \cdot 10^{4}$ per ml.

Table 3.9 Distribution of the extracellular lipase of *Pseudomonas fluorescens* 22F during the cheesemaking process.

experiment	weight of milk + culture (g)	activity of the lipase ($\mu eq.ml^{-1}.min^{-1}$)	weight after curdling whey (g)	weight after curdling curd (g)	activity after curdling whey ($\mu eq.ml^{-1}.min^{-1}$)	activity after curdling curd ($\mu eq.ml^{-1}.min^{-1}$)
1	303	0.59	265	38	0.21	5.20
2	200	0.55	173	27	0.17	4.15
3	200	0.41	176	24	0.17	5.16

Table 3.10 Cheese made from milk, to which *Pseudomonas fluorescens* 22F has been added.

inoculated before or after pasteurization	number of bacteria in the milk before pasteurization (ml^{-1})	after pasteurization (ml^{-1})	heated for 10 s at (°C)	increase of the acidity of the cheese fat/week (meq/100 g fat)
-	$5.5\ 10^4$	$2.7\ 10^3$	72	0.02
after	$5.5\ 10^4$	$1.0\ 10^7$	72	1.67
before	$8.6\ 10^6$	$1.1\ 10^1$	92	1.72
before	$8.6\ 10^6$	$1.9\ 10^3$	82	1.51
before	$8.6\ 10^6$	$4.0\ 10^3$	75	1.51
before	$8.6\ 10^6$	$1.0\ 10^4$	68	1.59
before	$8.6\ 10^6$	$1.1\ 10^4$	62	1.52

Apparently the bacterial lipase is attached to the casein micelles. The lipolytic activity of the curd and the whey together was higher than that of the original cheese milk.

Inactivation of *P. fluorescens* 22F lipase by pasteurization of the cheese milk was investigated. 0.15 % culture supernatant was added to the milk. Cheese was made from this milk and fat hydrolysis during ripening was followed. The results of this experiment, given in *Table* 3.10, are to some extent unexpected, because they do not show any differences in cheese fat acidity between heat treatments. This seems to indicate that it is of no consequence whether the lipase is pasteurized with the milk or not. However, measurements with a pH stat at pH 8.75 showed that about 10 % of the activity of the thermolabile lipase was destroyed by a mild heat treatment of 10 s at 62 °C and about 99 % at a heat treatment of 10 s at 72 °C. These facts were not seen in cheese lipolysis during this experiment.

3.6.4 Discussion

There was good agreement between pH stat measurements and the cheesemaking experiments with the lipase of *A. viscolactis* 23a1. However, pH stat experiments did not predict the observed lipolysis in cheese when the lipase of *P. fluorescens* 22F is involved. This lipase possesses two stages of inactivation, indicating the existence of a thermolabile and a thermostable enzyme or enzyme form (see Section 3.5). It can be calculated from Eqs. (3.2) and (3.4) that the

residual activity of the thermolabile part of the lipase is about 90 and 1 % after heating for 10 s at 62 and 72 °C, respectively, while the residual activity of the thermostable part of the lipase is for both heat treatments about 100 %. However, the actual increase of the acidity of the cheese fat during ripening was the same for all cheeses. Apparently the conditions in cheese do not favour the thermolabile part of the enzyme.

According to Law et al. (1976) cheese made from milk in which one or more strains of lipolytic Gram-negative bacteria had been allowed to mutiply to > 10^7 bacteria per ml, became rancid during its ripening. These results suggest that lipase is produced in the late exponential growth phase, which agrees with the results given in Sections 3.3 and 3.4. Therefore growth of bacteria in milk does not necessary mean that sufficient lipase is produced to cause defects. Growth at a storage temperature of 4 °C is limited (Langeveld & Cuperus, 1980). If the number of lipase producing bacteria has greatly increased during storage of raw milk, there is a risk of cheese becoming rancid after a time (Stadhouders, 1969). The lipolysis is enhanced by accumulation of the extracellular lipase in curd during cheesemaking. In addition when, in exceptional cases, milk is contaminated with a fully grown culture, for example when small amounts of milk are left behind in tanks and spoil during further storage, it is possible that cheese made from such contaminated milk may become rancid.

3.7 Activity of Pseudomonas fluorescens 22F lipase in UHT-sterilized milk

3.7.1 Introduction

Since the extracellular lipase of *Pseudomonas fluorescens* 22F appears to partly resist heat treatments above 100 °C, it may be expected that lipolysis caused by these enzymes can also occur in UHT-sterilized whole milk. This aspect was investigated and the results are reported in this Section.

3.7.2 Materials and methods

Aseptically drawn raw milk was inoculated with 0.15 % culture supernatant of *P. fluorescens* 22F. This culture supernatant was prepared as described in Section 3.5.2. The milk was homogenized at 20 MPa at 70 °C and then sterilized with indirect heating (Stork, Sterideal) for 45 s at 143 °C. The milk was packed in sterilized bottles and stored at various temperatures. During storage the acidity of the fat was determined according to the modified BDI method (Driessen et al., 1977).

3.7.3 Results and discussion

Results of the experiments are shown in *Figure* 3.13. As expected, strong

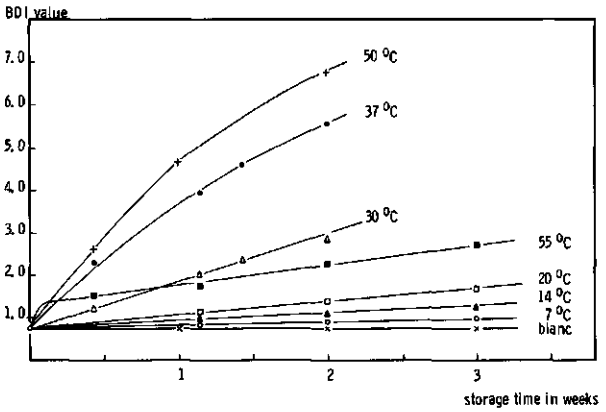

Figure 3.13 Acidity of the fat of homogenized UHT-sterilized (45 s at 143 °C) whole milk during storage at various temperatures. A fully-grown culture of *Pseudomonas fluorescens* 22F (0.15 %) was added to the milk.

hydrolysis of milk fat can occur, especially at higher storage temperatures. When the BDI value exceeds 1.5, milk becomes rancid (Kuzdzal-Savoie, 1979). The rate of rancidity development is related to the effect of temperature (Q_{10}) on the activity of the lipase. The Q_{10} is approximately 3.0.

The measurements of the lipolytic activity with short incubation times showed a very low activity at the pH of milk (see *Figure* 3.4). Nevertheless this experiment shows that lipolysis can take place in UHT-sterilized whole milk during longer storage. This is also shown by Adams & Brawley (1981).

The inoculation of the milk with culture fluid is equivalent to a heavy bacterial contamination. With a lower initial inoculation the lipolytic activity will be proportionally lower.

Even if the milk is sterilized in two stages, namely a UHT-sterilization procedure followed by a heat treatment of the filled bottles at about 105 °C, some bacterial lipases, if present, are likely to remain partly active; hence the development of rancidity is still possible. Even in evaporated milk, which has been heated far more intensively, lipolysis can occur (Radaeva et al.,1966).

3.8 Lipolysis by Gram-negative bacteria in other milk products

3.8.1 Butter

An increased level of free fatty acids in butter can be ascribed to the activity of the milk lipase or to lipolytic micro-organisms. As is shown in Chapter 2

milk lipase is inactivated by the pasteurization treatment of the cream during the manufacture of butter. According to Deeth et al. (1979), however, organoleptic grading, indicated that butter produced from lipolyzed cream was usually quite acceptable even when the free fatty acids level was abnormally high. The lipolysis occurring in butter is caused chiefly by lipolytic micro-organisms. A distinction has to be made between sour and sweet cream butter. The microbial flora of both types of butter differs markedly. Growth of the micro-organisms depends strongly on the distribution of moisture in the butter. If this distribution is good, there will be hardly any growth. With bad distribution in sour cream butter, yeasts and moulds may grow and in sweet cream butter Gram-negative bacteria will grow (Storgårds & Hietaranta, 1949; Sjöström, 1959; Deeth & Fitz-Gerald, 1976). Butter in which free fatty acids were produced by lipolysis after manufacture were severely down graded at quite low acid degree values. Bacterial lipases which release the short chain acids with or without the longer chain acids will cause the most noticeable effects on flavour (Deeth et al., 1979). Poor keeping quality of cold stored butter can be predicted after 6 to 7 days storage from the lipolytic bacterial count (Schwartz & Ciblis, 1965).

The presence of lipolytic bacteria in butter is not the only reason why butter can become rancid. If butter is made from milk with high lipolytic counts rancidity can develop. According to Kishonti & Sjöström (1970) the extracellular lipase of a *Pseudomonas* strain migrates partly into the cream during the buttermaking process. When cream with addition of a culture of *Pseudomonas* was heated to 90 °C for 2 min, the butter made subsequently became rancid in two days at 5 °C.

3.8.2 Milk powder

It is generally accepted that the growth of bacteria and most yeasts virtually stops below a water activity (a_w) of 0.9, while moulds do not grow at $a_w < 0.7$, and so a product like milk powder with $a_w < 0.6$ will not spoil by bacterial growth. Lipolysis in whole milk powder with added lipase of *Pseudomonas fluorescens* can occur, in spite of the low a_w, but the rate of lipolysis is low (Andersson, 1980a). It was found that the quality of whole milk powder was influenced by storage of the original raw milk for 3 days at 5 °C. Fat hydrolysis took place in this powder during a period of storage of 18 months. The acid degree in the fat increased from 0.77 to 1.14 meq alkali/100 g fat (Kjaergaard Jensen, 1971).

Bacterial lipases in milk powder can cause flavour problems in products made from the powder, such as chocolate, recombined evaporated milk and recombined (sweet) condensed milk (Vos & Mol, 1979).

3.8.3 Pasteurized liquid milk products

As a consequence of the usually low concentration of bacterial lipase in milk, defects caused by this type of lipolysis are expected only in products which are stored for a long period of time at ambient temperatures, and in which the fat is present in an attackable form. Pasteurized milk products are kept for a short period of time at low temperatures. Therefore rancidity is unlikely to occur in these products. There are no reports available concerning the effects of lipases from psychrotrophic bacteria in pasteurized liquid milk products.

Increased levels of free fatty acids in pasteurized liquid milk products can be dued mostly to (residual) activity of milk lipase. This particular point is described in Chapter 2.

3.9 Survey of lipolysis in milk and milk products

The lipolytic enzymes described in the Chapters 2 and 3 are able to cause rancid or soapy off-flavours in milk and milk products. For the majority of the cases it is possible to prevent this enzymatic spoilage by taking appropriate measures. A summary of the occurrence of lipases and their effects on dairy products is given in *Table* 3.11.

Table 3.11 Survey of the lipolysis in milk and milk products.

	lipolysis caused by:	
	milk lipoprotein lipase	bacterial lipase
Occurrence:	raw milk; low pasteurized milk and milk products.	cheese; evaporated milk; UHT-sterilized milk.
Defects:	rancid or soapy liquid milk; initial rancidity in cheese and other milk products.	rancidity during ripening of cheese or storage of milk and milk products.
Remedy:	sufficiently high temperature of pasteurization; prevention of damage of the fat globule; prevent mixing of milk containing lipase with milk containing "attackable" fat globules.	use milk of good bacteriological quality; milk storage at low temperature (< 4 °C) and for at most 3 days; milk had to be worked up within 24 h after delivery, if not: a thermization treatment of the milk has to be applied.

Chapter 4

MILK PROTEINASE

4.1 Introduction

Raw milk contains both native proteolytic enzymes, and under some circumstances proteinases produced by bacteria. The presence of naturally occurring proteolysis in milk was first described at the turn of the century (Babcock & Russell, 1897 and 1900). Since then numerous investigators have confirmed the presence of native proteinases in the milk from several species (Visser, 1981).

One of the enzymes, alkaline milk proteinase which shows maximum activity at slightly alkaline pH, resembles closely the bovine plasmin (Kaminogawa et al., 1971; Halpaap et al., 1977). It has been suggested that this alkaline proteinase may actually be plasmin transported from bovine plasma across mammary epithelial cells (Kaminogawa et al., 1972). The alkaline milk proteinase is heat-stable and survives low-temperature short-time pasteurization conditions (Noomen, 1975).

In addition to the alkaline proteinase an acid proteinase with maximum activity at pH 4.0 has been reported in milk (Kaminogawa et al., 1969; Kaminogawa & Yamauchi, 1972). It has been suggested that in addition to alkaline milk proteinase an acid milk proteinase, acting preferentially on α_{s1}-casein under favourable conditions, contributes to protein breakdown in certain soft cheeses (Noomen, 1978). The significance of the acid milk proteinase for milk and milk products is described by Kaminogawa et al., (1978). This acid proteinase is heat-labile (Kaminogawa & Yamauchi, 1972). This enzyme has not been considered in this investigation.

4.2 Biochemistry of the alkaline milk proteinase

Purification. During the purification steps of the milk proteinase system there is always a risk of contamination with proteinase originating from bacteria. Therefore various bacteriostatic agents have been employed as preservatives in order to study only the endogenous milk proteinases. Information on this matter is provided by the review of Humbert & Alais (1979).

The native proteinase is found to be largely associated with the casein micelles in milk and can be extracted from the micelles at low pH values (Reimerdes & Klostermeyer, 1974). It has also been shown that milk fat globule membrane preparations from fresh cows' milk contain proteinase activity comparable to that of bovine casein on an equivalent protein basis (Hofmann et al., 1979). The enzyme has been isolated at various degrees of purity. The

purification process usually starts with an acidification step to precipitate the casein from the milk at pH 4.6 or centrifugation to separate the casein micelles. This step is followed by precipitation with ammonium sulphate, generally at 40 % (w/v). The enzyme is further purified by different techniques such as dissociation with 30 % (v/v) dimethylformamide, dialysis against buffer solutions and various chromatographic treatments. These methods are comprehensively reviewed by Humbert & Alais (1979). A highly purified proteinase can be obtained by applying affinity chromatography on lysine-sepharose (Deutsch & Mertz, 1970; Reimerdes & Petersen, 1978).

Characterization. According to the general classification of proteinases by Hartley (1960) the alkaline milk proteinase is a serine proteinase, since diisopropylfluorophosphate (DFP) is a most effective inhibitor of proteolysis and phenylmethylsulfonylfluoride (PMSF) is less effective (Hofmann et al., 1979). The enzyme is inhibited by soybean trypsin inhibitor (STI) but not by a chymotrypsin inhibitor like tosylphenylalanine chloromethylketone (TPCK) (Chen & Ledford, 1971; Hofmann et al., 1979; De Koning & Kaper, 1981). There is general agreement that milk proteinase is a serine proteinase with trypsin like activity (EC 3.4.21.7).

The alkaline proteinase in bovine milk occurs mainly (greater than 80 %) as plasminogen (Rollema et al., 1981; Richardson & Pearce, 1981). Plasminogen is the zymogen of the proteolytic enzyme plasmin, the enzyme responsible for the dissolution of fibrin clots in blood. The milk proteinase zymogen is strongly activated by urokinase (Kaminogawa et al., 1972).

The plasmin of bovine origin has not been fully characterized. There are indications that the mechanism of activation of the bovine plasmin resembles that of human and rabbit plasmin. The molecular weight of human plasminogen is 81 000 daltons (Barlow et al., 1969), and the molecular weight of the rabbit plasminogen 89 000 - 94 000 daltons. The activation of single chain rabbit plasminogen to plasmin, by urokinase, has been shown to involve cleavage of two peptide bonds in the plasminogen molecule. Cleavage of the first bond by urokinase in the interior of the molecule, results in a plasmin molecule containing two chains. The heavy chain, molecular weight of 66 000 - 69 000 daltons, is disulfide linked to a light chain of molecular weight of 24 000 - 26 000 daltons. Plasmin autolytically removes a N-terminal peptide of molecular weight of 6 000 - 8 000 daltons from the heavy chain (Castellino & Sodetz, 1976). The amino acid sequences of human and rabbit plasminogen and plasmin have been partly solved (Robbins et al., 1973; Castellino & Sodetz, 1976).

Blood contains various inhibitors of plasmin and milk proteinase is strongly inhibited by blood serum (Halpaap, 1978). It is possible that one or more of

these specific inhibitors penetrate from the blood into milk since milk serum inhibits milk proteinase to a much lesser extent (Halpaap, 1978). Three inhibitory components have been found in milk. While the proteinase in milk is associated with casein micelles, the inhibitors are associated with whey proteins (Reimerdes et al., 1976). The inhibitory components in milk are specific for the proteolysis of casein (Kiermeier & Semper, 1960b). The cleavage of α_{s2}- and β-casein by plasmin can be inhibited when adding denaturated β-lactoglobulin (Snoeren et al., 1980).

Specificity. The alkaline milk proteinase is trypsin like. It cleaves peptide bonds on the C-terminal side of Arg and Lys residues. The enzyme attacks β- and α_{s2}-caseins at about the same rate (Snoeren & Van Riel, 1979). Breakdown products obtained from β-casein result from cleavage of the peptide bonds Lys(28)-Lys(29), Lys(105)-His(106) and Lys(107)-Glu(108) (Gordon et al., 1972; Groves et al., 1972 and 1973; Reimerdes, 1976). These peptides are generally called γ-caseins. The complementary fragments of the β-casein are found in milk as the so called proteose peptone fractions 5, 8F and 8S (Andrews, 1979). Proteolysis of α_{s2}-casein results in the production of positively charged peptides with higher electrophoretic mobility than α_{s2}-casein (Snoeren & Van Riel, 1979). α_{s1}-Casein is degraded two or three times more slowly than β-casein (Noomen, 1975). Plasmin degrades α_{s1}-casein B to a peptide with electrophoretic mobility slightly slower than α_{s1}-casein B and several bands with faster electrophoretic mobilities (Eigel, 1977). Whey proteins and κ-casein are resistant to cleavage by this enzyme (Eigel, 1977; Chen & Ledford, 1971; Yamauchi & Kaminogawa, 1972).

Temperature. The optimum temperature for the activity of the milk proteinase is generally found at 37 °C (Kiermeier & Semper, 1960a; Humbert & Alais, 1979). The enzyme is stable at -10 °C (Kiermeier & Semper, 1960a). After storage for 4 days at 4 °C the loss of activity is about 10 % (Halpaap, 1978).

pH. This milk proteinase has a maximum activity at slightly alkaline pH. The optimum in milk is generally found at pH 8, though some investigators have reported slightly lower optimum values (Humbert & Alais, 1979). Milk proteinase is stable over a wide range of pH. Chen & Ledford (1971) found a good stability between 1.5 and 9. Because of this feature an acid extraction at pH 2.0 may be a part of the purification procedure (Reimerdes & Klostermeyer, 1974).

4.3 Thermoresistance of the milk proteinase

The milk proteinase is considered to be fairly thermoresistant and it is not

destroyed by heating for 30 min at 60 °C (Dulley, 1972). It has been stated that milk proteinase cannot resist UHT sterilization treatments (Nakai et al., 1964; Cheng & Gelda, 1974), but conversely that according to Kiermeier & Semper(1960a) it is inactivated after 2 min at 70 °C. Ged & Alais (1976) showed proteolytic activity in milk sterilized by friction. It has been suggested that the milk proteinase may be reactivated after the heat treatment (Hostettler et al., 1957; Hostettler, 1972; Samuelsson & Holm, 1966). This study is an attempt to characterize the thermoresistance of the milk proteinase in milk by determining the proteinase activity in milk heat-treated at different temperatures for different times.

4.3.1 Materials and methods

Milk. Various milk samples were drawn aseptically from a limited number of cows. The bacterial colony counts of this milk, determined with poured plate techniques on Plate Count agar containing 1 % skim milk (PCM-agar), incubated for 3 days at 30 °C, was in all cases below 400/ml. The fat was separated by centrifugation in sterilized tubes at 3 300 *g* for 15 min at 4 °C.

Heat treatments. At temperatures below 100 °C aliquots of skim milk were heated in glass tubes according to the method described in Section 1.5.1.1. Heat treatments above 100 °C were performed with direct heating (Alfa Laval, VTIS) according to the description given in Section 1.5.3.1.

A series of treatments at a particular temperature was carried out with the same batch of milk.

Proteolytic activity measured with polyacrylamide gel electrophoresis (PAE). The heat-treated milk was preserved with 0.01 % thimerosal (Fluka AG, Buchs, Switzerland) and incubated at 37 °C for 72 h under anaerobic conditions (Noomen, 1975). After the incubation the samples were checked for bacterial spoilage. Precipitates of the caseins were prepared by adjusting milk diluted with distilled water (1:2; v/v) to pH 4.6 with 0.5 N HCl. These precipitates were dissolved at pH 7.0 and dialysed against distilled water at 4 °C for 16 h. The solutions were freeze-dried, weighed and subsequently submitted to analysis. The maximum weight loss of the precipitates during incubation was 10 %. This loss is probably caused by an increase in the non-precipitable protein fractions. Proteinase activity was quantitatively measured by analysing the freeze-dried precipitates by PAE (De Jong, 1975) using E-C vertical gel cell (E-C Apparatus Corp., Philadelphia). The gels were stained and de-stained according to Hillier (1976). Proteinase activity was quantitatively determined using densitometry (De

Jong, 1975). The optical density of the stained protein bands was determined at 620 nm with a Shimadzu CS 900 densitometer (Seisakusho Ltd., Kyoto, Japan). A sample of raw milk which was cooled immediately, was taken as a blank. The protein concentrations were calculated with a Hewlett Packard calculator (type 9830A) connected with a digitizer (type 9864A).

Proteolytic activity measured with a spectrophotometric method. A spectrophotometric method for the quantitative determination of plasmin and plasminogen in bovine milk with the chromogenic substrate D-valyl-L-leucyl-L-lysyl-4-nitroanilide (S2251) (AB Kabi Diagnostica, Stockholm, Sweden) was used (Rollema et al., 1983). The absorbance at 405 nm was measured as a function of time with a Cary 219 spectrophotometer equipped with a thermostated cell holder and a microcell adapter accessory. The spectrophotometer was interfaced with an HP 85 desk top computer (Hewlett Packard). Each absorbance measurement was the average of ten readings taken within one second. The change in absorbance per unit of time was taken as a measure of proteolytic activity.

Starch gel electrophoresis. Horizontal starch bed gel electrophoresis was carried out according to the method of Schmidt & Both (1975).

4.3.2 Results

Reliability of the determination of proteolysis with PAE. The activity of milk proteinase was determined by calculating the increase in the amount of γ-caseins related to the total caseins in the PAE gel pattern, expressed as the percentage of all coloured bands in the gel. The increase appeared to be approximately proportional to the incubation time (see *Figure* 4.1). According to De Jong (1975) only a very small proportion of the other degradation products of β- and α_s-caseins have *Rf* values comparable with these caseins. It was assumed that the proteolytic activity as reported below was proportional to the amount of proteolytic enzyme present in the sample. This holds true only for low concentrations of γ-caseins. By the further breakdown of β-casein a relatively too high percentage of γ-caseins is calculated ($\beta_t = \beta_o - \gamma_t$, in which β_o is the initial concentration of β-casein, and β_t and γ_t are the concentrations of β- and γ-caseins at time t). For this reason the curve will not be linear (see *Figure* 1.4).

Thermoresistance of milk proteinase. Proteolysis of heat-treated milk during storage can be followed by PAE. The decrease in the concentration of γ-caseins

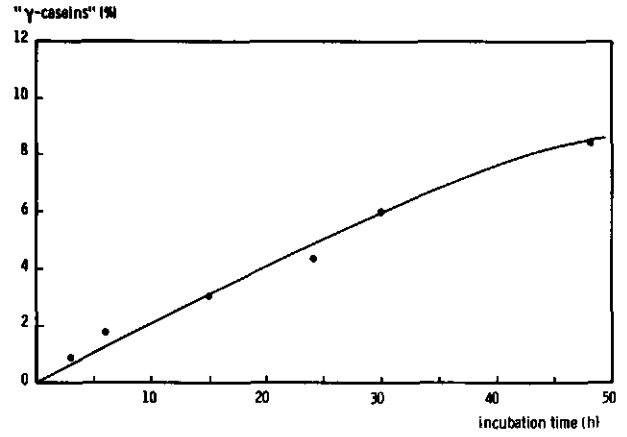

Figure 4.1 Amount of γ-caseins during the incubation time as a percentage of total caseins in polyacrylamide gel electrophorsis pattern.

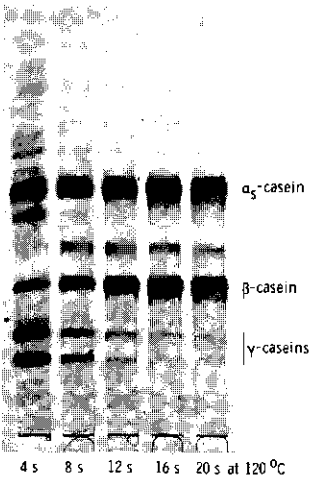

Figure 4.2 Breakdown of proteins in milk. Polyacrylamide gel electrophoresis pattern of milk, sterilized one to five times at 120 °C for 4 s with direct heating (Alfa Laval, VTIS) and incubated at 37 °C for 72 h.

as a function of the heating time can be seen very clearly. An example is given in *Figure* 4.2, in which the γ-caseins were used as indicator of proteolytic activity in milk.

Heat inactivation of milk proteinase appeared to follow a first order reaction. The results are plotted in *Figures* 4.3A and 4.3B. For ease of

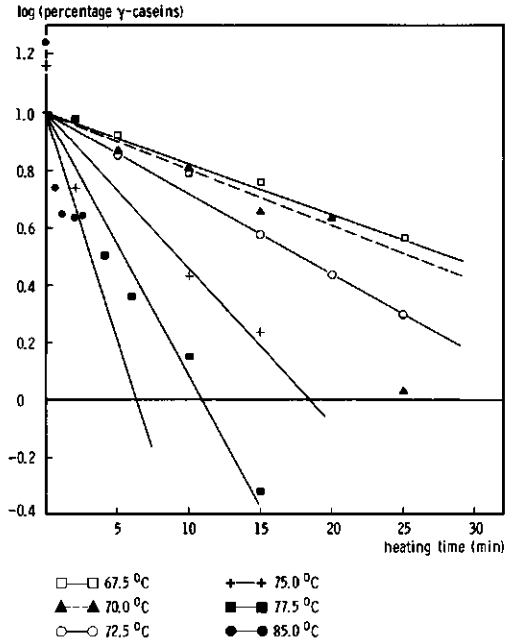

Figure 4.3A Inactivation of milk proteinase. Regression lines giving the formation of γ-caseins in aseptically drawn milk after heating at lower temperatures for longer times.

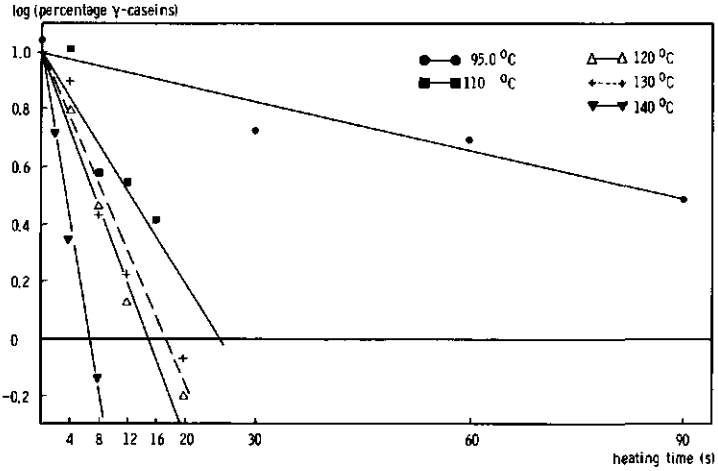

Figure 4.3B Inactivation of milk proteinase. Regression lines giving the formation of γ-caseins in aseptically drawn milk after heating at higher temperatures for shorter times.

assessment the amounts of γ-caseins at 'zero time' for each heating temperature were taken to be 10 %. From these data the regression lines were calculated, together with D (decimal reduction time) and k' (rate constant of inactivation). The values are given in *Table* 4.1. It appears from these data that below 95 °C the residual activity decreased with increasing temperature. In the measurements above 110 °C residual activity was less dependent of temperature. Because of these unexpected results, residual plasmin and plasminogen activities were determined in at various temperatures and various times directly heated UHT-milk using a spectrophotometric method (Rollema et al., to be published). These results, given in *Table* 4.2, show that the residual activities of both plasmin and plasminogen did hardly depend on the temperature (compare results for the same heating time), but that they were dependent on holding time (see also Section 4.4).

An Arrhenius plot has been calculated for the inactivation of the milk proteinase activity for the temperature range between 67.5 and 95 °C (see *Figure* 4.4):

$$\log k' = -6.126\ (10^3/T) + 14.864 \quad (r^2 = 0.923) \qquad (4.1)$$

The Q_{10} of the denaturation of the proteinase at pasteurization temperatures is approximately 3.0, while this value at sterilization temperatures is approximately 1.4. The relationship between D and temperature is given in *Figure* 4.5. This plot is characterized for the temperature range between 67.5 and 95 °C by the equation:

$$\log D = -0.049\ T' + 6.761 \quad (r^2 = 0.919) \qquad (4.2)$$

in which T' is the temperature in °C.

From Eq (4.1) or (4.2) the proteolytic activity of milk heated to temperatures below 95 °C can be predicted.

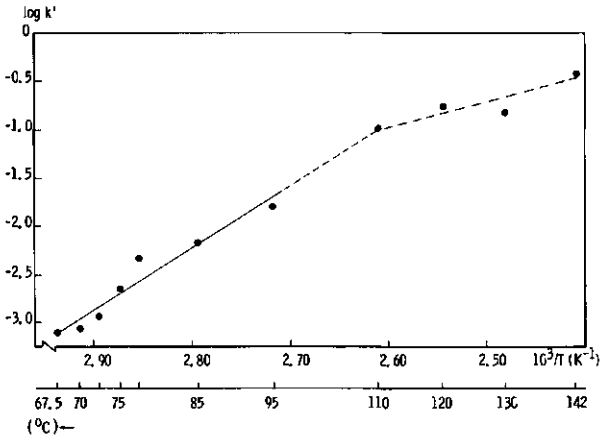

Figure 4.4 Arrhenius plot of the inactivation of milk proteinase.

Table 4.1 Kinetics and thermodynamics of heat inactivation of alkaline proteinase in milk.

temperature (°C)	$10^3/T$ (K^{-1})	D (s)	reaction rate constant, k' (s^{-1})	activation: free energy ΔG^* ($kJ.mol^{-1}$)	activation: enthalpy ΔH^* ($kJ.mol^{-1}$)	activation: entropy ΔS^* ($J.mol^{-1}.K^{-1}$)
67.5	2.937	3 336	$6.9\ 10^{-4}$	104.4	114.5	29.7
70.0	2.915	2 856	$8.1\ 10^{-4}$	104.7	114.4	28.3
72.5	2.894	2 142	$1.1\ 10^{-3}$	104.7	114.4	28.1
75.0	2.874	1 092	$2.1\ 10^{-3}$	103.5	114.4	31.3
77.5	2.853	516	$4.5\ 10^{-3}$	102.1	114.4	35.1
85.0	2.793	384	$6.0\ 10^{-3}$	103.4	114.3	30.4
95.0	2.717	174	$1.3\ 10^{-2}$	104.0	114.2	27.7
110	2.611	25.2	$9.1\ 10^{-2}$			
120	2.545	15.0	$1.5\ 10^{-1}$			
130	2.481	17.4	$1.3\ 10^{-1}$			
142	2.410	7.2	$3.2\ 10^{-1}$			

Table 4.2 Residual units plasmin/plasminogen in direct at various temperatures during various times heated UHT-sterilized milk, determined with a spectrophotometric method. The activity the raw milk was 13/75.

time (s)	units plasmin/plasminogen at a temperature of ... °C					
	110	117	125	132	140	147
4.6	8/32	8/32	8/23	9/36	7/31	7/21
8	5/28	5/33	6/22	8/29	6/21	5/20
12	4/16	3/13	4/16	5/19	6/18	5/16
16	4/15	5/16	5/19	6/20	5/16	4/19
20	4/16	5/20	5/20	5/15	3/11	-/-
24	~1/~2	~1/~1	~1/~1	-/-	1/3	1/3

note:-/- no detectable activity.

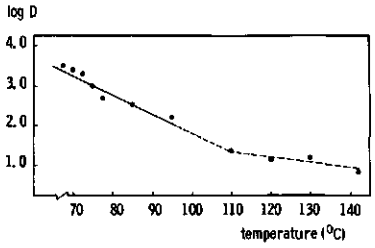

Figure 4.5 Thermal destruction curve of milk proteinase.

4.3.3 Discussion

The methods used in this study allow only a rough approximation of the residual activity of milk proteinase and the results have been affected by variable conditions. However, it is clear that milk proteinase is extremely stable to heat. From the experimental results it is not clear whether the heat treatment modifies the substrate, thus reducing hydrolysis, or whether milk proteinase is reactivated after the heat treatment (Samuelsson & Holm, 1966; Hostettler et al., 1957; Hostettler, 1972). The Q_{10} values are very low for the inactivation of an enzyme by heat denaturation.

Inactivation of the milk proteinase at temperatures above 110 °C could not be understood. It is striking that in the UHT-milk produced by direct heat treatment measurements of the proteolytic activity using PAE and spectrophotometric techniques showed in some cases a higher D or residual activity when heated at higher temperatures. Possible explanations for this fact could not be given. Hence, this phenomenon was studied in more detail (see Section 4.4). In earlier reports is has been stated that milk proteinase cannot withstand UHT sterilization treatments (Nakai et al., 1964; Cheng & Gelda, 1974). This is not in accordance with the data from the present investigation. An explanation of these different results could be provided by the fact that milk proteinase acts very specifically (Reimerdes, 1976). It may be possible that haemoglobin should not be used as a substrate for the enzyme (Nakai et al., 1964). Furthermore the proteinase activity cannot be measured accurately from an increase of the non-protein nitrogen (NPN) in milk during storage as done by Cheng and Gelda (1974), because the γ-caseins are precipitated with 12 % TCA and milk proteinase can only cause a small increase in NPN.

According to the results of this study, milk proteinase will be completely inactivated in milk products sterilized in bottles for 15 min at 120 °C. Defects caused by milk proteinase are usually not noticed in pasteurized milk products. It is assumed that commonly used pasteurization temperatures and length of storage are such that proteolysis is limited. Milk proteinase is of importance

for UHT-sterilized products intended to have a long shelf-life at ambient temperature.

At present there are two regular procedures for UHT sterilization, namely the direct and the indirect method. The cumulative heat treatments given may differ markedly for these two processes, using the same holding time and the same temperature (Burton & Perkin, 1970; Hallström & Dejmek, 1977). Until now, the essential purpose of UHT sterilization of milk has been the complete destruction of bacterial spores, together with minimum occurrence of browning and cooked flavour. Little or no attention has been paid to the inactivation of enzymes. In this respect the characteristics of the thermal inactivation of milk proteinase are of practical interest, since this enzyme may cause instability of UHT-sterilized products during storage. This particular aspect was studied in more detail. The results of this investigation are reported in the next section.

4.4 The activity of milk proteinase in UHT-sterilized milk

From the previous section it is clear that the milk proteinase may partly resist certain UHT sterilization treatments. The activity remaining after the UHT sterilization treatment could be of importance for the keeping quality of this milk. This section deals with the influence of the residual activity of milk proteinase on keeping quality. All experiments were performed in such a way that enzymes originating from bacteria could not interfere with the final results.

4.4.1 Materials and methods

Milk. Where necessary, milk was drawn aseptically from a limited number of cows. This milk, handled as described in Section 4.3.1, had a bacterial colony count below 400 ml^{-1}, estimated on Plate Count agar containing 1 % skim milk (PCM-agar) after 3 days at 30 °C. Only skim milk was used.

UHT sterilization. The milk was UHT sterilized either by a direct heating process (Alfa Laval, VTIS) or an indirect heating process (Stork, Sterideal VTS). The milk was filled aseptically into sterilized bottles in a laminar flow cabinet (Bassaire, J. Bass, Crawley, Sussex, UK) and was stored at 20 or 30 °C for periods up to 12 weeks.

Bacterial recontamination. After the various periods of storage the milk was checked for bacterial recontamination. Streaks of milk were made on PCM-agar plates which were incubated for 3 days at 30 °C. Any contaminated milk was discarded in this study.

Proteolysis. Proteolysis in UHT-sterilized milk during storage was followed using horizontal bed starch gel electrophoresis (Schmidt & Both, 1975). The activity of the proteinase was measured quantitatively by densitometry as described in Section 4.3.1. The non-casein nitrogen (NCN) and non-protein nitrogen (NPN) fractions were prepared according to the method of Harwalkar & Vreeman (1978). The amounts of NCN and NPN were determined with the accelerated Kjeldahl method (Koops et al., 1975).

Viscosity. The viscosity of the milk and the apparent viscosity of the gelled milk were measured at 20 °C with a Brookfield Synchro Lectric viscosity meter, type LVT, connected with a Brookfield Helipath Stand (Brookfield Eng., Lab., Massachusetts, USA). The viscosity meter was calibrated with Brookfield calibration liquids. Spindel C was used at 60 rpm.

Proteinase inhibitors. In some reference experiments proteolysis in UHT-sterilized milk was inhibited by the addition of 0.17 trypsin inhibitor units (TIU) aprotinin.ml^{-1} (Sigma, St. Louis, USA) and 5 mM diisopropylfluorophosphate (DFP) (Fluka AG, Buch, Switzerland) (De Koning et al., to be published).

Turbidity test. The turbidity test according to Asschaffenburg was used to control the presence of native whey proteins after the UHT sterilization (Schulz, 1965). The result was considered to be positive when the filtrate in a reagens tube was turbid.

Organoleptic characteristics. The milk was judged during storage by a pannel of experts.

4.4.2 Results

4.4.2.1 Influence of sterilization temperature

The influence of sterilization temperature on the keeping quality of milk was studied, using a constant holding time. UHT sterilization treatments were applied at 134, 137, 140, 143 and 146 °C with holding times of 4.8 and 4.7 s for the direct (Alfa Laval, VTIS) and the indirect (Stork, Sterideal VTS) heating systems respectively. Fresh skim milk was used with a total count of $3.8 \cdot 10^4$ ml^{-1}. The sterilized milk was stored in half-litre bottles for periods up to 12 weeks at 20 or 30 °C. The turbidity tests of all the sterilized samples were positive.

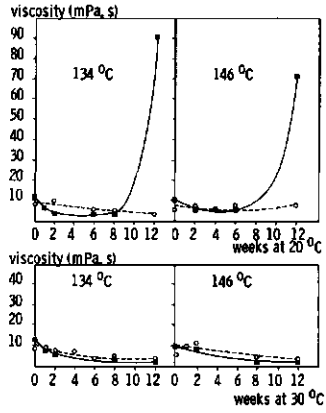

Figure 4.6 Apparent viscosity of at 134 or 146 °C UHT-sterilized milk during storage at 20 and 30 °C.

■—■ sterilized with direct heating (Alfa Laval, VTIS) holding time 4.8 s;

O--O sterilized with indirect heating (Stork, Sterideal VTS), holding time 4.7 s.

Viscosity of the milk. The appearance of the skim milk changed during the period of storage. The milk became transparent and gelled in some cases. Samples of milk, sterilized by direct heating gelled after storage at 20 °C, but not when stored at 30 °C. Samples sterilized by indirect heating did not gel during the 12 week storage period. The viscosity during storage is given in *Figure* 4.6. In this figure only the lowest and the highest sterilization temperatures are given; the temperature in between showed a similar viscosity change. The higher the temperature, the smaller was the increase of the viscosity. Viscosity decreased at first and this decrease was more pronounced in the samples sterilized at the lower temperatures. Viscosity of milk sterilized at 146 °C with the indirect system remained constant during the period of storage.

The viscosity of milk stored at 30 °C decreased during the 12 week storage period and this decrease was more pronounced than in the milk stored at 20 °C.

Milk flavour during storage. During the storage period milk flavour deteriorated. The milk became bitter, an indication that proteolysis had taken place. The occurrence of the bitter flavour was related to sterilization temperature, type of heating and storage temperature. The bitter flavour was first noticed in the milk sterilized by direct heating and later in that sterilized by indirect heating. Development of the bitter flavour in the

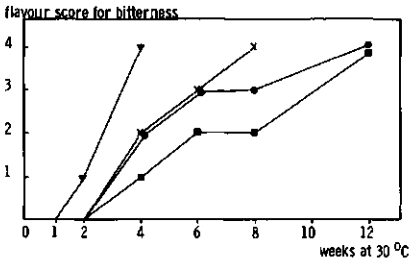

Figure 4.7 Development of bitterness at for 4.8 s directly heated UHT-sterilized milk (Alfa Laval, VTIS). Scale for bitterness: 0-4, 0 = no off-flavour and 4 = strong off-flavour.
▼—▼ sterilized at 134 and 137 °C;
X—X sterilized at 140 °C;
●—● sterilized at 143 °C;
■—■ sterilized at 146 °C.

directly heated milk during storage at 30 °C is given in *Figure* 4.7.Occurrence and intensity of the off-flavour depended on sterilization temperature.

It was striking that a sterilized milk flavour arose after an 8 week storage period at 30 °C, in milk sterilized by indirect heating at 143 and 146 °C. This off-flavour indicates to an increase in the Maillard reaction, which would be expected from the intensity of the sterilization treatment combined with the high temperature of storage. This milk was not bitter.

At the moment when the milk became bitter, there was a change in appearance. The milk became more or less transparent.

Proteolysis in milk during storage

In all the UHT-sterilized milk specific protein fractions were degraded during storage. The most rapid degradation was noticed for α_{s2}-casein and β-casein, while α_{s1}-casein was degraded more slowly. Proteolysis of κ-casein and of the whey proteins α-lactalbumin, β-lactoglobulin and serumalbumin was not observed.

On starch gel electrophoretograms the *Rf* values of α-lactalbumin and α_{s1}-casein are very close to each other, and therefore total proteolysis of α_{s1}-casein could not be measured. Proteolysis caused the appearance of some new fractions of smaller proteins on the gels, especially γ-caseins ('minor caseins') and proteose peptone '5' and '8'. On the negatively charged side of the gel, close to the origin, residues of α_{s2}-casein were found in bands. Para-κ -casein-like compounds were never found, indicating that the κ-casein had not been broken down.

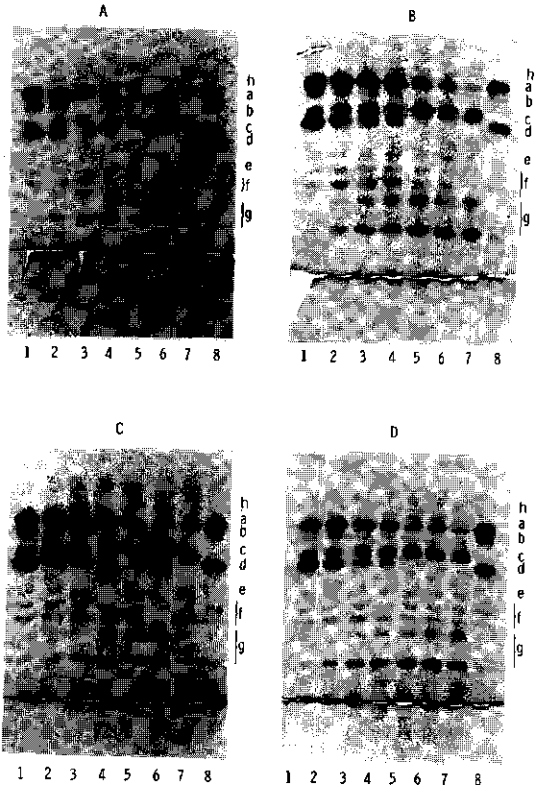

Figure 4.8 Starch gel electrophoresis patterns of fractions of proteins of UHT-sterilized milk during storage at 20 °C.

A. Directly heated for 4.8 s at 134 °C (Alfa Laval, VTIS);

B. Directly heated for 4.8 s at 146 °C (Alfa Laval, VTIS);

C. Indirectly heated for 4.7 s at 134 °C (Stork, Sterideal VTS);

D. Indirectly heated for 4.7 s at 146 °C (Stork, Sterideal VTS).

Codes:

a. α_{s1}-casein and α-lactoglobulin;
b. α_{s2}-casein;
c. β-lactoglobulin;
d. β-casein;
e. serum albumin;
f. κ-casein;
g. γ-caseins;
h. proteose peptone '5';
i. proteose peptone '8'.

1. fresh;
2. stored for one week;
3. stored for two weeks;
4. stored for four weeks;
5. stored for six weeks;
6. stored for eight weeks;
7. stored for twelve weeks;
8. reference: total caseins.

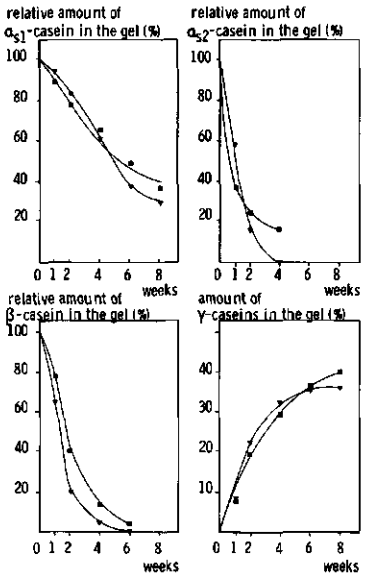

Figure 4.9 Proteolysis in directly heated UHT-sterilized milk (Alfa Laval, VTIS) during storage at 20 °C.
▼—▼ 4.8 s at 134 °C;
■—■ 4.8 s at 146 °C.

It is striking that proteolysis in the UHT-sterilized milk appeared to be almost independent of sterilization temperature, but on the other hand depended greatly on the system of heating. Milk heated by the direct process was broken down more rapidly than that heated by the indirect method. An example is given in *Figure* 4.8, in which for each process results the lowest and highest temperatures are given. The temperatures in between showed a similar degree of proteolysis, and this can also be seen from the densitometric analyses of the starch electrophoresis gels. These results are given in the *Figures* 4.9 and 4.10, for direct and indirect heating systems, respectively. The results are for milk stored at 20 °C. After storage at 30 °C proteolysis was more rapid.

There was no direct relationship between proteolysis as measured by starch gel electrophoresis and the occurrence of gelation or a bitter taste. Gelation was noticed after a 12 week storage period at 20 °C and the bitter taste after 6 to 12 weeks.

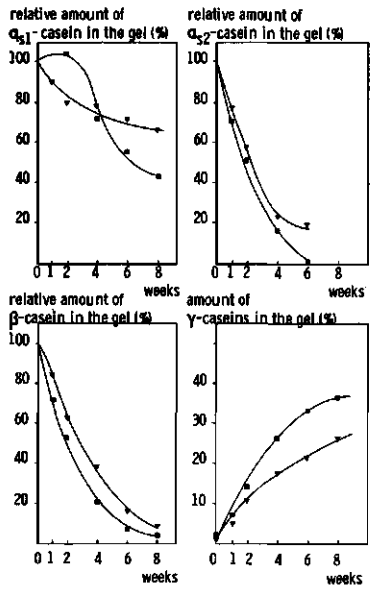

Figure 4.10 Proteolysis in indirectly heated UHT-sterilized milk (Stork, Sterideal VTS) during storage at 20 °C.
▼—▼ 4.7 s at 134 °C;
■—■ 4.7 s at 146 °C.

4.4.2.2 Influence of sterilization holding time

The effect of the holding time during the sterilization process on the keeping quality was studied at a constant sterilization temperature. Sterilization was performed at 140 °C and the holding times were 0 (without a holding section), 4, 8 and 16 s. A holding time of "0 s" was insufficient to obtain a sterile product with the direct heating process. Therefore the results of this series of experiments were discarded.

In this experiment fresh skim milk was used. The total bacterial count of this skim milk, estimated on PCM-agar after 3 days at 30 °C was $2.4 \cdot 10^4$ ml^{-1}. After the sterilization treatment the milk was filled aseptically in 500 ml sterilized bottles and stored at 20 or 30 °C. The turbidity tests of all samples were positive.

The viscosity of the milk during storage. Holding time at temperature of sterilization affects the change in milk viscosity during storage. Short holding times favour the occurrence of gelation. In contrast with the previous experiment (cf. Section 4.4.2.1) milk sterilized by indirect heating also gelled, but only if the holding section had been removed from the equipment. Milk sterilized by the direct process gelled when the holding times were 4 and 8 s. The gel of the milk which was sterilized for 4 s was firmer than that of the milk sterilized for 8 s. In all cases viscosity showed an initial decrease. These results are given in *Figure* 4.11.

The UHT-sterilized milk stored at 30 °C showed an initial viscosity decrease, but not a clear increase of the apparent viscosity during further storage. It was noticed that in the milk sterilized for 4 s by the direct heating process a slight viscosity increase occurred, but this milk did not gel during the storage period.

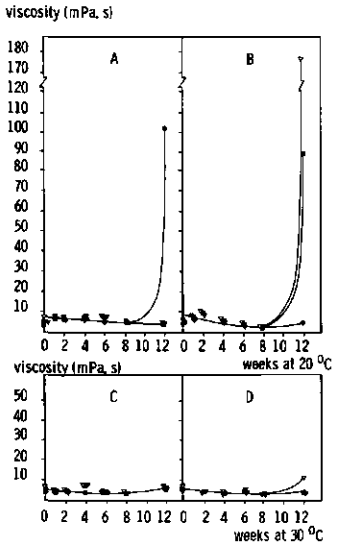

Figure 4.11 Apparent viscosity of UHT-sterilized milk during storage at 20 and 30 °C.
A and C: indirect heating (Stork, Sterideal VTS);
B and D: direct heating (Alfa Laval, VTIS);
●—● 0 s at 140 °C;
▽—▽ 4 s at 140 °C;
■—■ 8 s at 140 °C;
▼—▼ 16 s at 140 °C.

In reference experiments milk was sterilized by direct heating for 2 and 16 s at 140 °C. The proteolysis in this milk was inhibited by addition of aprotinin and DFP. This milk showed neither an initial decrease of viscosity nor an increase of apparent viscosity during a storage period of 12 weeks at 20 °C.

Milk flavour during storage. The flavour of the sterilized milk deteriorated during storage. The milk turned bitter, an indication that proteolysis had taken place. The occurrence of the bitter flavour could be delayed by increasing the holding time during sterilization. A heating time of 16 s at 140 °C was sufficient to ensure that milk retained satisfactory organoleptic characteristics during a 12 week storage period at 20 °C. In general keeping quality of UHT-sterilized milk improves when sterilization is carried out by the indirect rather than the direct heating process, if the same temperature and short holding time are used. Longer holding times during sterilization result in a better keeping quality, but also in a more intense cooked flavour. Therefore the acceptability of freshly sterilized milk, processed by the indirect process for 16 s was slightly lower. The results of these experiments are given in *Figure* 4.12.

Indirect heating for 8 and 16 s resulted in 'sterilized' flavour during storage at 30 °C. This failure probably points to an increase in Maillard reactions which would be expected from the intensity of the sterilization treatment, combined with the high temperature of storage. This milk did not become transparent during storage, while the milk held for 4 s with indirect heating did.

Milk sterilized by direct heating with a holding time of 16 s developed neither a bitter, nor a sterilized flavour, and did not become transparent during the period of storage at 20 or 30 °C. Milk sterilized by this system with shorter holding times became somewhat transparent and turned bitter.

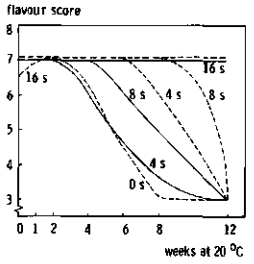

Figure 4.12 The taste of at 140 °C UHT-sterilized milk during storage.
—— direct heating (Alfa Laval, VTIS);
--- indirect heating (Stork, Sterideal VTS).
Scale 3-8: 3= very poor taste; 8= very good taste.

Proteolysis in milk during storage. Proteolysis in the milk corresponding to the description given in Section 4.4.2.1, proceeded faster in the directly heated than in the indirectly heated milk for equal holding times. Contrary to the results for constant holding time and increasing temperature - where only a slight effect of temperature was measured - proteolysis decreased with increasing holding times at 140 °C. Results are given in *Figure* 4.13, which shows starch gel electrophoresis patterns for the shortest and longest holding times. These samples were stored at 20 °C.

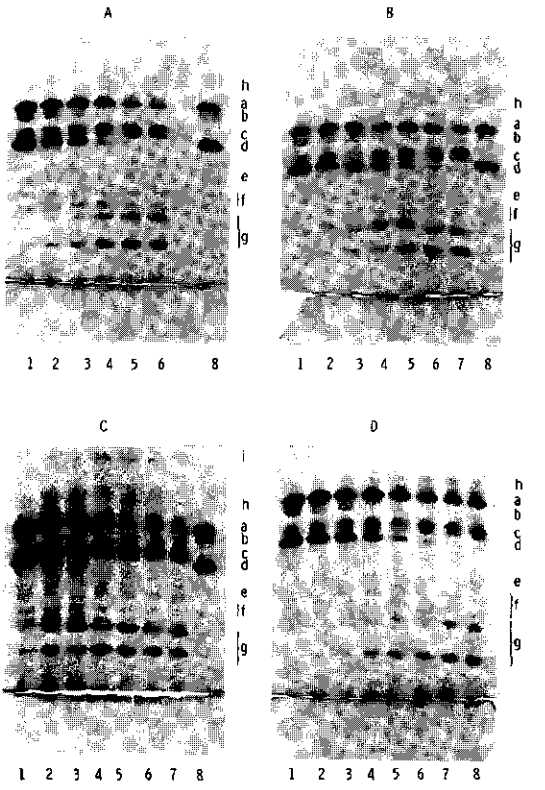

Figure 4.13 Starch gel electrophoresis patterns of the proteins of UHT-sterilized milk during storage at 20 °C.
A. direct heating for 4 s at 140 °C (Alfa Laval, VTIS);
B. direct heating for 16 s at 140 °C (Alfa Laval, VTIS);
C. indirect heating for 0 s at 140 °C (Stork, Sterideal VTS);
D. indirect heating for 16 s at 140 °C (Stork, Sterideal VTS).
Codes as in *Figure* 4.8.

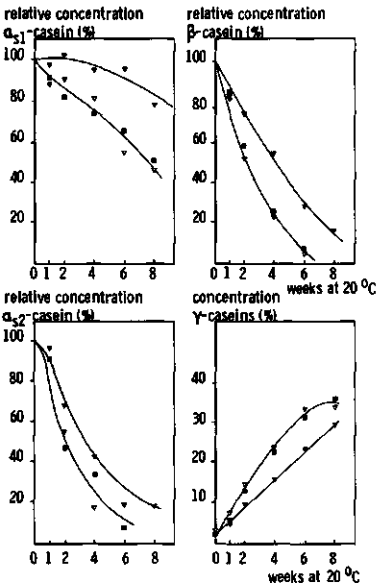

Figure 4.14 Proteolysis in directly heated (140 °C) UHT-sterilized milk (Alfa Laval, VTIS) during storage at 20 °C. Heating time: ▽, 4 s; ■, 8s; ▼, 16 s.

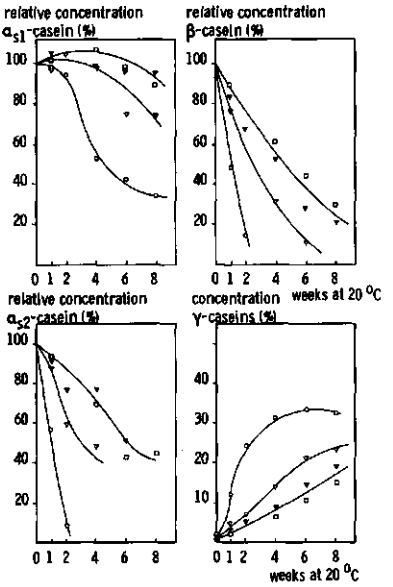

Figure 4.15 Proteolysis in indirectly heated (140 °C) UHT-sterilized milk (Stork, Sterideal VTS) during storage at 20 °C. Heating time: ○, 0 s; ▽, 4 s; □, 8 s; ▼, 16 s.

Densitometric analyses of the electrophoresis patterns are given in *Figures* 4.14 and 4.15. The results are for milk stored at 20 °C. At 30 °C proteolysis proceeded more rapidly. In general proteolysis can be reduced by increasing the holding time. However, holding times of 4 and 8 s with direct heating and also 8 and 16 s with indirect heating caused no significant differences.

In all samples proteolysis occurred. From the proteolysis seen on starch gel electrophoretograms, it was not possible to predict whether the milk would turn bitter of whether it would gel.

No proteolysis was found in UHT-sterilized milk to which proteinase inhibitors had been added.

4.4.3 Discussion

The total input of heat into milk during sterilization is determined by the equipment. The aim of milk sterilization is the destruction of bacterial spores. A major advantage of the introduction of the ultra-high-temperature short-time procedure was that additional effects, such as browning, development of a sterilization flavour, losses of vitamines, etcetera, could be considerably reduced (Hostettler et al., 1957; Schaafsma, 1979). However, the possibility that other processes such as enzymatic reactions could still occur in UHT-sterilized products was not taken into account. The storage temperature of these products is often high, and the period of storage may be considerable. Residual enzyme activities can be of importance in determining the keeping quality of the product.

4.4.3.1 Influence of sterilization temperature

It was striking that sterilization temperature had little effect on the residual activity of milk proteinase. The differences in sterilization temperature showed only minor effects on the change of viscosity and on proteolysis of the milk during storage. This was the same for both the direct and the indirect heating systems (see also Section 4.3). In contrast with these findings, the temperature of sterilization was of great importance in the development of off-flavours during storage. This observation points to a complex situation. The measured proteolysis could not be correlated directly to the flavour deterioration of the milk. It is necessary for proteins to be broken down far beyond the level which can be observed with the starch gel electrophoresis technique before any bitter flavour will manifest itself and the appearance of the milk will change (tranparency, gelation).

According to the results described in Section 4.3.2 there is no clear relationship between residual proteolysis and temperature of heating in the area

of UHT sterilization. At 120 °C D was found to be 15.0 s and at 130 °C 17.4 s! These values indicate that the temperature in this area is of minor importance for the residual activity of milk proteinase. The same conclusion can be drawn from the results reported in Section 4.4.2. It is very likely that the calculated Arrhenius plot for the inactivation can only be applied for temperatures below 100 °C. It is not advisable to extrapolate Eqs. (4.1) and (4.2) beyond a temperature range of 67.5 to 95 °C. Residual activities at temperatures above 100 °C may be read directly from *Figure* 4.5, which gives, for example, at 140 °C log D = 1.00 or D = 10 s. The course of milk proteinase inactivation at temperatures above 100 °C may be explained by a number of possibilities, such as reactivation of the milk proteinase after the heat treatment or a modification of the enzyme by the removal of one or more prostethic groups or proteolytic enzymes other than the milk proteinase being active. Special attention should be paid to the formation of active proteinase from plasminogen after the heat treatment (Rollema et al., 1981).

The period of storage of UHT-sterilized products may be long. It appeared that the spoilage of the product occurred slowly and therefore the experimental period had to be fairly long, for instance several weeks. A decrease in the length of an experiment can easily lead to wrong conclusions.

4.4.3.2 Influence of sterilization holding time

For a particular holding time the stability and flavour of the milk sterilized by indirect heating were superior to those of the milk sterilized by direct heating. The quality of the product during the period of storage depended on the sterilization treatment and on the storage temperature. It was noticed that milk sterilized by the indirect process for 8 and 16 s at 140 °C and subsequently stored at 30 °C developed a sterilized off-flavour. It was assumed that Maillard reactions had occurred during storage at 30 °C. It is conceivable that these reactions prevented gelation during storage at 30 °C.

Proteolysis in milk differed according to the sterilization procedure. With indirect heating small differences were observed in proteolysis and in the formation of γ-caseins during storage, if the holding times were 8 or 16 s at 140 °C. A similar finding was observed for the direct heating process if the holding times were 4 or 8 s at 140 °C. This agrees with the results given in Section 4.4.2.1, which show an imperfect correlation between the starch gel electrophoretograms and the temperature-time combination of the sterilization process. This relation was far less clear than is generally assumed until now.

The experiments with proteinase inhibitors added to the UHT-sterilized milk showed clearly that typical storage defects, such as transparency and gelation

of milk, did not occur when proteolysis is prevented. Therefore, it is of great practical importance to be aware of the role of the residual milk proteinase activity during the manufacture of UHT-sterilized milk and milk products.

4.5 Preheating for 60 min at 55 °C

Since some American investigations had indicated that the keeping quality of UHT-sterilized milk could be lengthened by a mild heat treatment of the raw milk, experiments were carried out to find the cause of this phenomenon (Barach et al., 1976b; Barach et al., 1978; West et al., 1978). The assumption that the proteinases originating from bacteria would be inactivated by this heat treatment is not likely because of the low bacterial numbers in the milk showing this phenomenon (Speck & Adams, 1976). It may be possible that a partial inactivation of the alkaline milk proteinase causes an improvement in keeping quality.

4.5.1 Materials and methods

Milk. In this investigation aseptically drawn milk was used. This milk,which had a bacterial count of less than 500 ml^{-1}, was handled as described in Section 4.3.1.

Heat treatment. The milk was skimmed and divided into two portions. Portion 1 was heated for 60 min at 55 °C and portion 2 was worked up without any pretreatment. The milk was sterilized with an Alfa Laval VTIS sterilizer (direct heating) for 2 s at 142 °C and aseptically filled into 500 ml sterile bottles. The milk was stored at 20 and 30 °C for periods up to 11 weeks. After the heat treatmente the samples appeared to possess undenaturated whey proteins according to the test of Asschaffenburg (Schulz, 1965).

Proteolysis. The analyses were performed as described in the Section 4.4.1.

Viscosity. The analyses were performed as described in the Section 4.4.1.

Organoleptic characteristics. During storage the milk was assessed by a pannel of experts.

4.5.2 Results

Viscosity. The viscosity of the milk samples during storage is plotted in *Figure* 4.16. Preheating prevented gelation for at least 11 weeks at 20 °C.

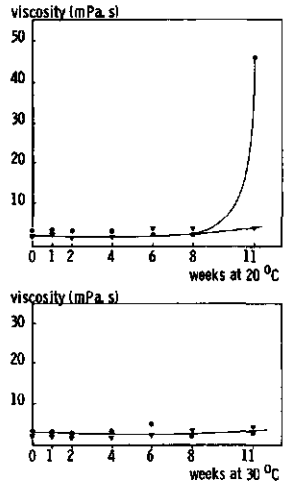

Figure 4.16 Apparent viscosity of for 2 s at 142 °C directly heated UHT-sterilized milk (Alfa Laval,VTIS) during storage at 20 and 30 °C.

●—● milk not treated;

▼—▼ milk preheated for 60 min at 55 °C.

The milk sterilized without preheating gelled by the end of the storage period. Samples stored at 30 °C did not gel, which agrees with the results reported in the Section 4.4.

Proteolysis. The heat treatment of 60 min at 55 °C considerably inhibited proteolysis in UHT-sterilized milk. This is shown in *Figure* 4.17. These samples were stored at 20 °C. At 30 °C proteolysis occurred more rapidly. A more quantified picture is given by the densitometric analyses of the different protein patterns of the starch gel electrophoresis preparations. These results are plotted in *Figure* 4.18.

Organoleptic characteristics. After the heat treatment of 60 min at 55 °C milk flavour remained good for a longer period than those of the untreated milk. Evaluation of the flavour during storage at 20 °C is given in *Figure* 4.19. Defects such as transparency and bitterness were noticed at a later time when milk was preheated. The decrease in flavour score was caused by bitterness. Keeping quality at 20 °C was better than that at 30 °C.

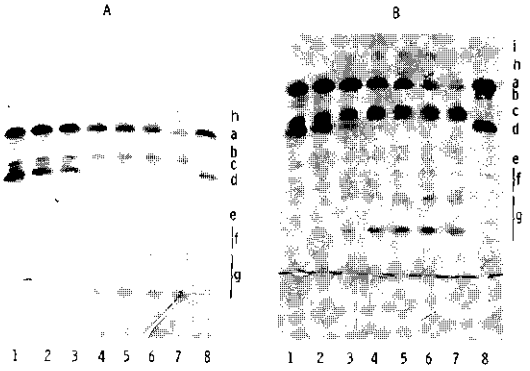

Figure 4.17 Starch gel electrophoresis patterns of for 2 s at 142 °C directly heated UHT-sterilized milk (Alfa Laval, VTIS) during storage at 20 °C.

A. Milk preheated for 60 min at 55 °C.

B. Milk not treated.

Codes as described in *Figure* 4.8.

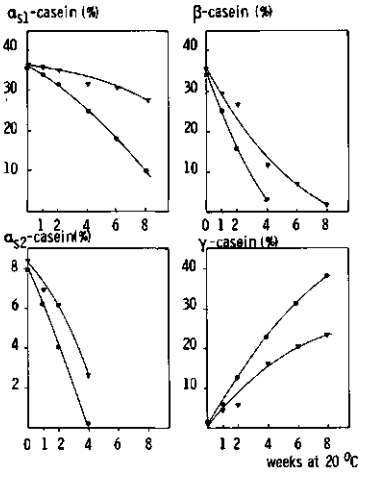

Figure 4.18 Proteolysis in for 2 s at 142 °C directly heated UHT-sterilized milk (Alfa Laval, VTIS) during storage at 20 °C.

●—● milk not treated;

▼—▼ milk preheated for 60 min at 55 °C.

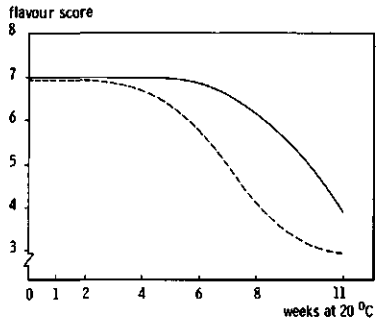

Figure 4.19 The taste of for 2 s at 142 °C directly heated UHT-sterilized milk (Alfa Laval, VTIS) during storage at 20 °C.
- - - milk not treated;
—— milk preheated for 60 min at 55 °C.
Scale: 3-8; 3= very poor taste; 8= very good taste.

4.5.3 Discussion

Preheating milk for 60 min at 55 °C improved the keeping quality of UHT-sterilized milk. Gelation did not occur within 11 weeks, proteolysis, bitterness and transparency of the milk were retarded. The assumption that proteinases of bacterial origin are partly inactivated by this preheating treatment does not hold, because the experimental material was aseptically drawn milk with very low bacterial counts (Barach et al., 1976). In this type of milk only the natural milk proteinases play a role, and it is concluded that the native proteolytic activity is (partly) inhibited. At 55 °C D of the milk proteinase was 167 min, as extrapolated from Eq. (4.2), Section 4.3.2. A heat treatment of 60 min affected the activity of this enzyme. The residual activity was calculated to be about 45 %, which agrees with the differences in breakdown of the protein fractions of the preheated and untreated milk (see *Figure* 4.18).

The heat treatment at 55 °C for 60 min is difficult to apply in dairy practice. More convenient temperature-time combinations can be calculated with Eq. (4.2). Hence the same effect can possibly be achieved by preheating milk for 1.4 min at 90 °C, which means that common plate heat exchangers can be modified for this particular purpose.

4.6 The activity of milk proteinase in UHT-sterilized custard

It has been consistently noticed in practice that the viscosity of UHT-sterilized custard, after an initial increase, gradually decreases after a storage period of about one week at ambient temperatures. This instability of

the product is undesirable. The cause of the defect is not known. It is known that modification of the starch does not solve the problem.

Since the results of Sections 4.3 and 4.4 show that a native milk proteinase may partly resist the commercial UHT-sterilization treatments of milk and milk products, it can be assumed that the viscosity of the product could decrease as a consequence of this proteolysis. This assumption was investigated and the results are given in this section.

4.6.1 Materials and methods

Milk. In order to exclude the effect of proteinase activity from bacteria in the raw milk, experiments were done with aseptically drawn milk. The colony count of this milk (estimated with Plate Count agar containing 1 % skim milk, so called PCM-agar, and counted after three days at 30 °C) was $5.1 \cdot 10^2$ ml^{-1}. The fat was separated by centrifugation in a separator which had been sterilized before use. The colony count of the skim milk was $4.0 \cdot 10^2$ ml^{-1}.

Other experiments were performed with bulk collected milk, which had a total count of $3.0 \cdot 10^4$ ml^{-1}.

Manufacture of the custards. The custards were prepared using a direct heating UHT-sterilization plant (Alfa Laval, VTIS), equipped with different temperature holders for sterilization times ranging from 2 to 36 s. The temperature of sterilization was 142 °C. The composition of the custards in the experiment with aseptically drawn milk is given in *Table* 4.3, and that of custards in other

Table 4.3 Composition of the custards in experiments with aseptically drawn milk.

component (%, w/w)	holding time at 142 °C (s)				
	0.7	2.0	4.4	8.4	18.0
skim milk	85.55	86.2	86.8	86.8	86.8
sugar	8.0	8.0	8.0	8.0	8.0
snowflake 06308[1])	5.5	4.95	4.4	4.4	4.4
globe 03401[2])	0.80	0.72	0.64	0.64	0.64
butter essence	0.075	0.068	0.060	0.060	0.060
gelloid J[3])	0.03	0.027	0.024	0.024	0.024
agar KBL 80	0.050	0.045	0.040	0.040	0.040

[1])modified maize starch. [2])native maize starch. [3])carrageenan

Table 4.4 Composition of the custards in experiments with bulk collected milk.

component (%, w/w)	experiment number		
	1-4-7	2-5-8	3-6-9
skim milk	86.3	84.95	-
sugar	8.0	8.0	8.0
snowflake 06308	4.9	6.3	5.3
globe 03401	0.7	0.7	0.7
butter essence 150373	0.05	0.05	0.05
gelloid 3	0.03	-	0.03
agar KBL 80	0.05	-	0.05
SMUF[1])	-	-	81.6
lactose	-	-	4.3

[1])SMUF: skim milk ultrafiltrate buffer (Jenness & Koops, 1962).

experiments in *Table* 4.4. Variations in the recipes were applied to adjust the viscosity to a generally acceptable level (6.0 Pa.s). The mixtures were kindly given by CPC Corn Products (Sas van Gent, The Netherlands).

The custards were packed aseptically in polyethylene beakers in a laminar flow cabinet. The beakers were closed with an aluminium lid and subsequently stored at 20 °C for periods up to 16 weeks. The packaging materials had been sterilized by γ-radiation (Gammaster, Ede, The Netherlands).

Bacterial contamination. After various periods of storage the beakers were tested for bacterial contamination. Streaks were made om PCM-agar. The plates were incubated for three days at 30 °C. Only sterile products were used for further investigations.

Proteinase activity. The degrees of proteolysis during storage were followed by gel electrophoresis in horizontal starch beds as described in Section 4.3.1. A sample of total caseins prepared from fresh raw milk was taken as a blank.

Viscosity. Two instruments were used to measure the apparent viscosity of the custards at 20 °C: a Brookfield Synchro Lectric viscometer, type LVT, (Brookfield Eng. Lab., Massachusetts, USA) and a Haake roto viscometer, type RVII (Haake Mess-Technik GmbH u. Co, Karlsruhe, Germany).

The Brookfield viscometer was equipped with a Brookfield Helipath Stand. All

measurements were performed with the bar-type spindle C at 6 or 60 rpm, depending on the viscosity. The penetration rate of the spindle in the beakers was 2.2 cm per minute.

The viscosity measurements with the Haake roto viscometer were done with the MVI concentric cylinders. The change in viscosity at a constant shear rate of 150 s^{-1} was measured as a function of time. In all cases the apparent viscosity reached an equilibrium value after 10 minutes shearing.

The difference between the values obtained by both instruments gives an indication of the contribution and formation of weak network structures in custards on storage.

Table 4.5 Concentrations of β- and γ-casein in the custards during the period of storage. The concentrations are expressed as the surface of the scanned peak in cm^2.

holding time at 142 °C (s)	period of storage (weeks)				
	0	1	4	8	12
		concentration of β-casein			
0.7	3.81	3.24	0.82	0.34	0.12
2.0	3.74	3.38	1.14	0.46	0.12
4.4	3.70	3.37	0.92	0.34	0.10
8.4	3.92	3.97	1.73	1.10	0.34
18.0	3.65	3.51	2.55	2.27	1.48
		concentration of γ-casein			
0.7	0.11	0.25	0.60	1.00	1.03
2.0	0.07	0.23	0.63	0.96	1.12
4.4	0.11	0.19	0.49	0.96	1.02
8.4	0.10	0.12	0.31	0.78	0.98
18.0	0.06	-	0.15	0.35	0.47
		ratio of γ/(residual) β-caseins			
0.7	0.02	0.08	0.73	2.92	8.67
2.0	0.03	0.07	0.55	2.10	9.42
4.4	0.03	0.06	0.54	2.81	10.30
8.4	0.02	0.03	0.18	0.71	2.91
18.0	0.02	-	0.06	0.15	0.32

4.6.2 Results

Experiments with aseptically drawn milk. In the first series of experiments, custards were made from aseptically drawn milk. This was done in order to show the behaviour without the intervention of bacterial enzymes. The recipes were as normally used in practice.

Some of the milk proteins were broken down during storage of the custards. Depending on the heat treatment, the α_S- and β-caseins were degraded to various degrees, accompanied by the appearance of γ-caseins. The κ-caseins were not broken down to a degree that was visible in the gel and no para-κ-casein-like compounds were found. The concentrations of β- and γ-casein and the ratio of γ/β (residual)-caseins are given in *Table* 4.5. It is clear from this table that the custards sterilized with holding times up to 4.4 s had undergone considerable proteolysis. Custards sterilized with holding times of 8.4 and 18.0 s showed moderate and slight proteolysis respectively.

During the period of storage at 20 °C viscosity was measured at various times with the Brookfield viscometer. The results of the measurements are given in *Figure* 4.20. The custards showed large initial differences in viscosity, which confuses the interpretation of the results. All the custards showed an initial increase in viscosity followed by a decrease. This was most apparent in the custard prepared with a holding time of 2.0 s, but occurred to a lesser extent in those prepared with holding times of 4.4 and 8.4 s. After storage for 10 to 12 weeks a slight to sharp increase in the viscosity was measured in custards prepared with a holding time of up to 8.4 s.

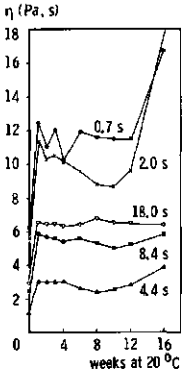

Figure 4.20 Apparent viscosity of UHT-sterilized custards, prepared with holding times of 0.7, 2.0, 4.4, 8.4 and 18.0 s at 142 °C during a period of storage of sixteen weeks at 20 °C, measured with the Brookfield viscometer at 20 °C.

The custards prepared with a holding time of 0.7 s showed some variation in viscosity during the storage period. After 12 weeks of storage this custard showed wheying off and visible gelation.

The viscosity of the custard prepared with a holding time of 18 s was stable over a storage periode of 16 weeks at 20 °C.

The custard prepared with a holding time of 0.7 s developed a very glassy appearance during the storage period. Those custards prepared with holding times of 2.0 and 4.4 s became slightly glassy. At the end of the storage period, the taste of the custards prepared with holding times up to 8.4 s became bitter. The custard prepared with a holding time of 18 s was not bitter after twelve weeks of storage.

Omission of custard ingredients. Although the experiment with the aseptically drawn milk suggested that proteolysis and viscosity were related, the results were not conclusive. The custard prepared with a holding time of 4.4 s showed considerable proteolysis without the expected drop in viscosity. In order to obtain a better understanding of the relation between the breakdown of milk proteins by milk proteinase and changes in the viscosity of the custards a

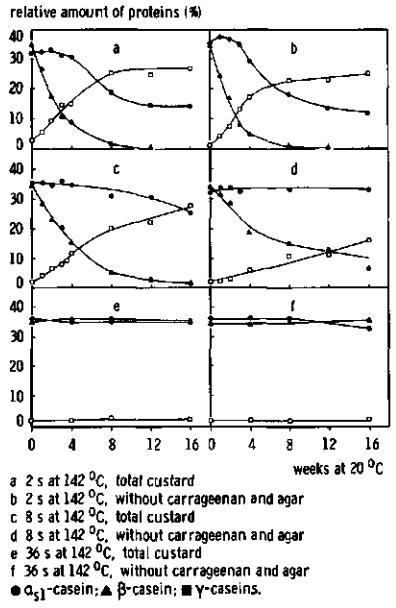

Figure 4.21 Proteolysis of the α_{s1}- and β-casein in UHT-sterilized custards during storage at 20 °C.

second set of experiments was carried out with bulk collected milk. The recipes were varied in such a way that the effect of starch, carrageenan and milk proteins could be distinguished (see *Table* 4.4).

Proteolysis of the UHT-sterilized custards corresponded to that previously reported. Considerable proteolysis was measured in the custard sterilized for 2 s at 142 °C, and moderate proteolysis was found in the custard sterilized for 8 s. When the custard was sterilized for 36 s, proteolysis was absent. The presence of carrageenan and agar was of no significance. These results are shown in *Figure* 4.21. It is strange that the β-casein was initially degraded in the custards prepared with holding times of 2 and 8 s, whilst the α_{s1}-casein in these custards was broken down only after a storage period of four weeks.

The changes in the apparent viscosity of the custards on storage at 20 °C, measured with the Brookfield and Haake viscometers, are depicted in *Figures* 4.22 and 4.23 respectively. The custards in which considerable proteolysis was found also showed a sharp decrease in viscosity after storage for one week. Compared with the first experiment, where aseptically drawn milk was used, the decrease in viscosity was more pronounced and qualitatively similar results were subsequently found for the custards prepared with a holding time of 8 s. After storage for 8 weeks a similar increase in viscosity was found when measured with the Brookfield viscometer. No increase in viscosity was measured for the custard sterilized for 2 s at 142 °C. The increase in viscosity was not found after shearing for 10 minutes in the Haake roto viscometer. This suggests that the increased viscosity in *Figure* 4.22 for custards with a holding time of 8 s is

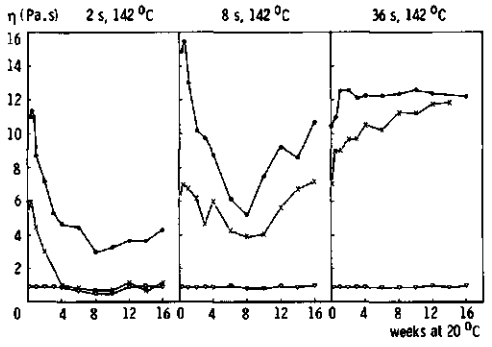

Figure 4.22 Apparent viscosity of UHT-sterilized custards during storage at 20 °C, measured with the Brookfield viscometer at 20 °C.

●—● custard prepared with the usual recipe;

X—X carrageenan omitted from the recipe;

▽—▽ the milk replaced by skim milk ultrafiltrate (SMUF) buffer and lactose.

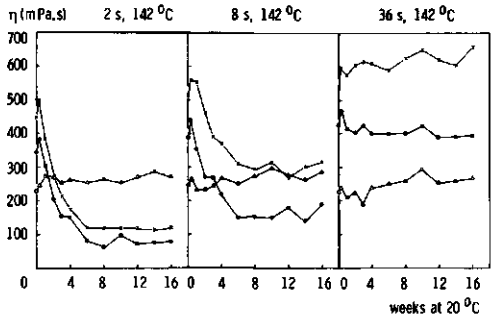

Figure 4.23 Viscosity of UHT-sterilized custards during storage at 20 °C measured with the Haake roto viscometer at 20 °C after 10 min shearing.

●—● custard prepared with the usual recipe;

×—× carrageenan omitted from the recipe;

Δ—Δ the milk replaced by skim milk ultrafiltrate (SMUF) buffer and lactose.

due to weak structures. The custard prepared with a holding time of 36 s was stable if carrageenan and agar were present. Without these stabilizers the viscosity gradually increased. The Haake results also show that the stabilizers were primarily responsible for the formation of a weak gel, which is easily disrupted by shearing forces. In contrast to the Brookfield results the viscosity of the custards without stabilizers was higher, due to the higher concentration of starch in the recipe.

When the milk proteins were omitted a much lower viscosity was observed (< 1 Pa.s). All these samples were stable on storage. The apparent viscosity after shearing of the custards in which strong proteolysis took place was lower than in the solutions without proteins.

4.6.3 Discussion

The results clearly show that proteolysis can occur in commercial UHT-sterilized custards. These custards attained a 'glassy' appearance and a bitter taste, which can probably be explained by an extensive proteolysis. These phenomena are comparable with the changes in UHT-sterilized skim milk in which some milk proteinase activity is left after the sterilization treatment.

The proteolysis in the custards must be caused by the residual activity of a milk proteinase, as indicated by the patterns from starch gel electrophoresis. This proteolysis is limited after a holding time of 8 s at 142 °C and is negligible after holding times longer than 18 s at 142 °C.

In the experiments with bulk collected milk there were slight differences in proteolysis in the custards with and without carrageenan. These differences may have been caused by differences in experimental conditions, although the UHT-sterilization apparatus was cleaned and re-assembled after each run.

The difference between the results found with the Brookfield and those found with the Haake viscometer (see *Figures* 4.22 and 4.23, respectively) indicates a contribution of weak network structures towards the consistency of the custards. The results suggest an essential structural role of the milk proteins in this product, in particular the interaction seems to be very dependent on β-casein. Furthermore it is tempting to relate the degree of α_{s1}-casein breakdown to the occurrence of a minimum in the viscosity-time curve. Gelation after storage for 8 weeks was only found for the custard sterilized for 8 s at 142 °C in which hardly any α_{s1}-casein was hydrolysed. Similar results were found after shorter holding times in the first experiment.

Viscosity measured with the Haake viscometer after shearing the custards in which proteolysis took place was lower than the viscosity of the custards without milk proteins. Possible explanations for this observation could be a drop in the swelling of the starch caused by an interaction between milk proteins and starch or a degradation of the starch by amylase activity. Work published by Guy and Jenness (1958) showed that native milk α-amylase is not heat-stable, whilst Wütrich et al. (1964) reported that milk amylases were not completely destroyed by uperization and sterilization.

The role of carrageenan in the consistency of custards was not as large as expected. Addition of the carrageenan increased the viscosity, but variation in viscosity during the storage period was not influenced by carrageenan. There is no explanation for the gradual increase of the viscosity of the custard prepared without carrageenan with a holding time of 36 s.

The possibility that the starch contained amylase activity was investigated by varying the recipes. When the custards were prepared without milk proteins, the viscosity was stable but low. There was no indication of amylase activity in these experiments. This indicates an important role of the proteins in determining the consistency of the custards.

The custards prepared with holding times of 18 s and 36 s at 142 °C remained stable. It is therefore recommended that attention is payed to the inactivation of native milk proteinase during the manufacture of UHT-sterilized custards.

Acknowledgement

A. van Hooydonk is acknowledged for his cooperation in measuring and discussing the rheological properties of the custards.

4.7 Activity of milk proteinase in UHT-sterilized evaporated milk

It is known that the heat stability of evaporated milk may be rather poor. The thermostability of the product can be improved by the addition of orthophosphate. Furthermore, polyphosphates retard gelation in concentrated milk during storage (Herreid & Wilson, 1963). In order to avoid the possible influence of phosphate on the heat stability of the milk proteinase, the use of this compound in the present work was avoided. As a consequence long holding times could not be applied because of impairing the stability of the product. Therefore the required heat treatments were carried out with normal milk.

4.7.1 Materials and methods

Milk. Milk was drawn aseptically from a limited number of cows. This milk had a bacterial count of $2.8 \cdot 10^3$ ml^{-1} and was handled as described in Section 4.3.1. After skimming and pasteurization the bacterial count was 4.0 ml^{-1}. The bacterial counts were estimated on Plate Count agar containing 1 % skim milk (PCM-agar) after a period of incubation of 3 days at 30 °C.

Manufacture of evaporated milk. The skim milk was sterilized for 0.7, 8 and 16 s at 142 °C with the Alfa Laval VTIS sterilizer (direct heating). The milk was evaporated at 45 °C until a solid-not-fat content of 22 % had been reached. The bacterial counts were $5.0 \cdot 10^3$, $1.2 \cdot 10^3$ and $9.5 \cdot 10^3$ ml^{-1} for the respective heat treatments. The evaporated milk was sterilized for 2 s at 142 °C and filled aseptically in 250 ml sterilized flasks and stored at 28 °C. After the various periods of storage the samples were checked for bacterial recontamination as described in Section 4.4.1.

Alcohol stability. Alcohol stability tests were performed with evaporated milk and evaporated milk diluted with water (1:1). One ml alcohol (96, 83, 72 and 62 %; v/v) was added to a series of test tubes with 1 ml of sample. The contents were mixed and judged immediately and again after 5 min. The highest concentration of alcohol at which the sample did not coagulate is given in the results.

Proteolysis. Protein fractions in the evaporated milk were separated according to Harwalkar & Vreeman (1978) and analyses were performed as described in Section 4.4.1.

Viscosity. The analyses were performed as described in the Section 4.4.1.

Table 4.6 Alcohol stability of UHT-sterilized evaporated skim milk during storage at 28 °C. The highest concentration of alcohol at which the sample did not coagulate is given.

period of storage	0.7 s at 142 °C		8 s at 142 °C		16 s at 142 °C	
	whole	diluted (1:1)	whole	diluted (1:1)	whole	diluted (1:1)
fresh	62	72	<62	72	<62	62
two days	62	83	<62	72	<62	72
one week	72	96	<62	62	<62	62
two weeks	<62	72	-	62	-	62
three weeks	<62	62	-	<62	-	<62
four weeks	-	<62	-	<62	-	<62

4.7.2 Results

Alcohol stability. The results are given in *Table* 4.6. The data given in this table indicate that the diluted evaporated milk is more stable than the original one. During storage there appeared to be an initial increase in stability followed by a decrease. There was no difference in alcohol stability during storage of the evaporated milk prepared from milk sterilized for 8 and for 16 s, but these samples were less stable than that sterilized for 0.7 s.

Viscosity. There was a clear difference in the change of viscosity of the evaporated milk prepared from milk sterilized for 0.7 s and that sterilized for 8 or 16 s. The evaporated milk prepared from milk sterilized for 0.7 s showed an initial sharp decrease in viscosity and gelled after only 5 weeks of storage at 28 °C. The evaporated milk prepared from milk sterilized for 8 and 16 s showed little change in viscosity and gelation did not occur after storage for 6 weeks at 28 °C. The measurements were stopped after 6 weeks because the samples were not stable. The UHT-sterilized evaporated milk showed poor stability, also caused by the omission of polyphosphate. The results are plotted in *Figure* 4.24.

pH. In all samples an initial decrease of the pH was noticed, while during further storage the pH increased gradually (see *Figure* 4.25).

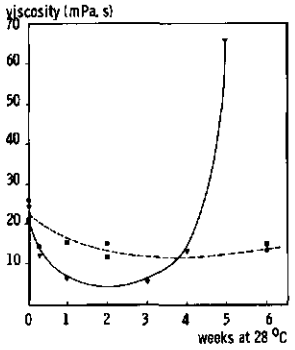

Figure 4.24 Viscosity of evaporated skim milk during storage at 28 °C. The sterilization treatments were performed at 142 °C with direct heating (Alfa Laval, VTIS).
▼—▼ milk sterilized for 0.7 s;
●--● milk sterilized for 8.0 s;
■--■ milk sterilized for 16.0 s.
The evaporated milk was sterilized again for 2 s at 142 °C before aseptic filling.

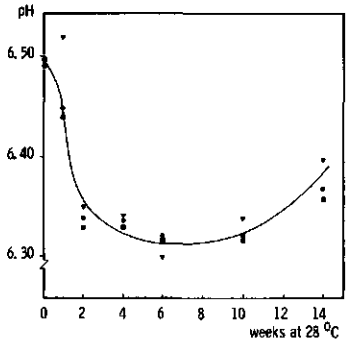

Figure 4.25 The pH of evaporated skim milk during storage at 28 °C. The sterilization treatments were performed at 142 °C with direct heating (Alfa Laval, VTIS).
▼—▼ milk sterilized for 0.7 s;
●—● milk sterilized for 8.0 s;
■—■ milk sterilized for 16.0 s.
The evaporated milk is sterilized again for 2 s at 142 °C before aseptic filling.

Proteolysis. According to the starch gel electrophoresis patterns the caseins were broken down in the evaporated milk prepared from milk sterilized for 0.7 s at 142 °C. The content of the γ-caseins increased during storage, whilst β-casein disappeared. These results are plotted in *Figure* 4.26.

The non-casein nitrogen (NCN) content increased during storage and this increase was greatest in the evaporated milk prepared from milk sterilized for 0.7 s at 142 °C. For evaporated milk prepared from milk sterilized for 8 and 16 s at 142 °C the increase was slow. The results are plotted in *Figure* 4.27.

The whey proteins were not broken down during the period of storage, but there was an increase in the nitrogen content of the two fractions containing whey proteins and proteose peptone in the evaporated milk prepared from milk sterilized for 0.7 s at 142 °C. The fractions increased from 0.030 % to 0.087 and 0.050 %, respectively, after ten weeks of storage at 28 °C. Nitrogen content remained constant (0.035 and 0.021 % respectively) in the evaporated milk sterilized for 8 and 16 s at 142 °C.

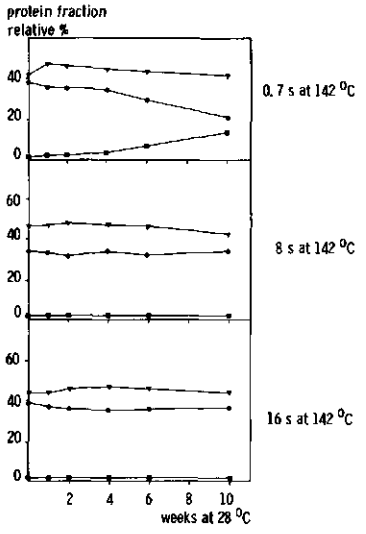

Figure 4.26 Breakdown of caseins in evaporated skim milk during storage at 28 °C. The milk was sterilized at 142 °C respectively for 0.7, 8.0 and 16.0 s with direct heating (Alfa Laval, VTIS). The evaporated milk was sterilized again for 2 s at 142 °C before aseptic filling.

▼—▼ α_{s1}-casein;

●—● β-casein;

■—■ γ-caseins.

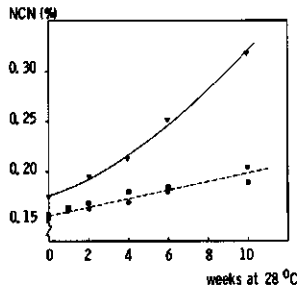

Figure 4.27 The percentage non-casein nitrogen (NCN) in evaporated skim milk during storage at 28 °C. The evaporated milk was sterilized as described in *Figure* 4.25.
▼—▼ sterilized for 0.7 s;
●--● sterilized for 8.0 s;
■--■ sterilized for 16.0 s.

The non-protein nitrogen (NPN) remained constant at 0.062 ± 0.004 % for all the samples during ten weeks of storage at 28 °C. The proteolysis of the caseins could not be detected from the NPN values.

4.7.3 Discussion

The stability of UHT-sterilized evaporated milk appeared to be rather poor. A reduction in the intensity of the sterilization treatment resulted in an accelerated rate of gelation. On the other hand, increasing the intensity of the heat treatment was followed by an accelerated decrease in the stability of the evaporated milk.

The alcohol stability decreased by increasing the holding time of the sterilization treatment from 0.7 to 8 s. During early storage the alcohol stability increased, which is at variance with the results of Harwalkar & Vreeman (1978), who reported that the alcohol stability gradually decreased with time. The reasons for this difference in behaviour are unclear. During further storage the alcohol stability decreased. The evaporated milk made from milk sterilized for 8 and 16 s showed comparable alcohol stability. The evaporated milk made from milk sterilized for 0.7 s was more stable.

Proteolysis was only detected in the evaporated milk prepared from milk sterilized for 0.7 s. This product also showed an increase in NPN content during the period of storage. It was remarkable that the evaporated milk prepared from milk sterilized for 8 and 16 s at 142 °C showed almost no proteolysis and only a slight increase of the NCN content during storage at 28 °C. This result is not

in agreement with the results reported in the Sections 4.3.2 and 4.4.2. It is possible that the way in which the evaporated milk was manufactured is the cause of this discrepancy, since the milk was sterilized for a second time after the evaporation step. The total heat treatment was thus increased considerably by this second process. Another possibility may be that a reduction in heat stability of the milk proteinase was caused by the increased dry matter content of the evaporated milk.

The percentage of the NPN did not change during the period of storage. Milk proteinase splits the casein into rather large fragments (Reimerdes, 1976). These compounds are precipitated by the treatment with 12 % TCA and do not contribute to the NPN value. The remaining smaller compounds are apparently too low in concentration to influence the total NPN value as measured with the micro Kjeldahl method.

These experiments clearly show that UHT-sterilized evaporated milk is not stable. Without the addition of, for instance, polyphosphate the keeping quality of this product is very limited (Harwalkar & Vreeman, 1978). Preheating the milk was not a final solution, but it certainly effected an improvement.

8. Concluding remarks

Ultra-high-temperature short-time sterilized milk and milk products have a limited keeping quality as a consequence of the residual activity of the alkaline milk proteinase. The keeping quality of the UHT-sterilized milk and milk products is determined mainly by the holding time at the temperature of sterilization. The temperature itself is of lesser importance, if the bacterial spores are killed. This phenomenon is a consequence of the low Q_{10} of inactivation by heating milk proteinase at temperatures above 100 °C.

The view that the UHT sterilization process must be applied at a high temperature for a very short time is based upon the occurrence of defects resulting from Maillard reaction products or sterilized flavour. However, the range of time-temperature combinations between the traditional sterilization in an autoclave and the UHT process have not yet been studied in detail. Investigations in this particular field may be of significance, particularly for the manufacture of special milk products such as custards and evaporated milk, with UHT-sterilization equipment.

Chapter 5

PROTEINASES OF GRAM-NEGATIVE BACTERIA IN MILK AND MILK PRODUCTS

5.1 Introduction

In this study the proteinases originating from Gram-negative bacteria are henceforth called proteinases. If proteinases from other micro-organisms are involved, reference is made in the text.

The composition of the bacterial flora in raw milk, stored at low temperature is reviewed in Section 1.3. In general the same bacterial species produce both extracellular lipases and extracellular proteinases (see Section 3.1).

5.2 Biochemistry of bacterial proteinases

Specificity. Among the psychrotrophic Gram-negative bacteria, representatives of the genus *Pseudomonas* appear to be the most proteolytic organisms. It has been shown that the proteolytic activity in a *P. fluorescens* culture consists of aminopeptidase enzymes too (Reimerdes et al., 1979).

A similar pathway has been observed for casein degradation by the extracellular proteinases of eight psychrotrophic bacteria from raw milk with initial specific hydrolysis of β- and κ-caseins, followed by extensive non-specific hydrolysis (Debeukelar et al., 1977; Richardson & Te Whaiti, 1978; Law et al., 1977 and 1979; Gebre-Egziabher et al., 1980). Whey proteins were reported not to be degraded to a detectable level (Debeukelar et al., 1977; Law et al. 1977 and 1979), but other investigators found a decrease of α-lactalbumin and β-lactoglobulin in milk as a consequence of growth of psychrotrophic bacteria (Adams et al., 1976; Skean & Overcast, 1960; Gebre-Egziabher et al., 1980). This discrepancy is probably a consequence of the variety of bacteria able to grow in milk.

From experiments with a series of eleven synthetic 4-nitroanalides with free amino acids, it appeared that the proteolytic enzymes from *P. fluorescens* possess a high specific activity for L-Phe-4-nitroanilide.

On the other hand, N-acetyl-L-alanin-4-nitroanilide is a good substrate for milk proteinase. It was suggested that the activity ratio for these two substrates may provide an indication of the cause of milk spoilage.

The substrate specificity of *P. fragi* ATTC 4973 was investigated by the determination of the amino acid compositions of peptides produced by hydrolysis

of insulin chains. The proteinase did not exhibit a well defined specificity except that it appeared to have a preference for bonds on the N-terminal side of hydrophilic residues such as those of aminoethylcysteine, serine, threonine, glutamine and the bonds of smaller residues, particular glycine (Noreau & Drapeau, 1979).

Characterization. There are various reports on the characterization and purification of the extracellular proteinases of pseudomonads. The initial steps for purification of the enzymes usually involve centrifugation to remove the cells, followed by a precipitation procedure with ammonium sulphate at about 50 % of saturation. The resolved precipitate is dialysed and further purified by chromatographic techniques and gel filtration. The proteinase produced by *P. fluorescens* P26 was reported to have a molecular weight of approximately 23 000 daltons (Mayerhofer et al., 1973), those produced by *P. fluorescens* AR-11 and M5 approximately 40 000 daltons (Alichanidis & Andrews, 1977; Marshall & Marstiller, 1981), that produced by *P. fluorescens* B52 46 900 daltons (Richardson, 1981) and that produced by *P. fragi* ATTC 4973 approximately 50 000 daltons (Porzio & Pearson, 1975; Noreau & Drapeau, 1979). Although the range of the reported molecular weights of the proteinases of the different pseudomonads is wide, according to Richardson (1981) it has to be assumed that these enzymes exist exclusively as a monomer. It is also suggested that these enzymes can be autodigested or generate several active forms from a common precursor (Noreau & Drapeau, 1979).

The extracellular proteinases of pseudomonads are characterized as metalloproteinases, the enzymes requires Zn for activity (Porzio & Pierson, 1975; Barach et al., 1976a; Noreau & Drapeau, 1979; Richardson, 1981). In contrast, inhibition of the extracellular proteinase of *P. fluorescens* AR-11 was reported by Alichanidis & Andrews (1977) in the presence of 10 mM Zn^{2+}-ions. This difference may be explained by the high concentration of Zn^{2+}-ions since activation was found at a concentration of 1 mM Zn^{2+}-ions (Porzio & Pearson, 1975). It must be kept in mind that milk contains only 0.05 mM Zn. The proteinase of *P. fragi* was also activated by 1 mM Ca, Co, Mn and slightly activated by Fe (Porzio & Pearson, 1975; Noreau & Drapeau, 1979). The proteinases of pseudomonads were inhibited by Ag, Al, Cd, Cu, Hg, and Pb (Malik, 1975; Alichanidis & Andrews, 1977). These enzymes were also inhibited by 1, 10-phenantheoline and ethylenediaminetetraacetic acid (EDTA) in concentrations higher than 1.0 mM and by other chelating agents (Noreau & Drapeau, 1979; Richardson, 1981). Activity was restored by Ca^{2+}-ions (Porzio & Pearson, 1975; Barach et al., 1976). This sensivity for chelating agents is typical of metalloproteinases.

Temperature. The proteinases of pseudomonads are most active within the temperature range of 37 to 45 °C (Adams et al., 1975; Alichanidis & Andrews, 1977; Richardson, 1981). Maximum enzyme production per unit cell mass occurs during the late exponential and early stationary phases of growth of *P. fluorescens* at 20 °C (McKellar, 1981). According to McKellar (1981) the proteinase production per cell of *P. fluorescens* was at 5 °C as high as that at 20 °C, while Juffs (1976) found a decline in this production when the incubation temperature was reduced from 20 °C to 5 °C. At 30 °C there was considerable growth but only slight proteinase production (Juffs, 1976).

The proteinase is very stable. After storage for three months at room temperature considerable activity of the proteinase of *P. fluorescens* MC 60 was still found (Adams et al., 1975).

The effect of pH. The proteinases of *P. fluorescens* and of *P. fragi* were most active at pH 6.5 - 8.0 and around pH 8.0, respectively (Mayerhofer et al., 1973; Adams et al., 1975; Alichanidis & Andrews, 1977; Noreau & Drapeau, 1979). These enzymes had considerable activity at the pH of normal milk.

Although the proteinase of *P. fluorescens* B52 had maximum activity at pH 7, it was classified as an alkaline proteinase since an activity of at least 70 % of the maximum was obtained between pH 6.0 - 10.5 (Richardson, 1981). The proteinase of *P. fluorescens* P26, however, had less than half the maximum activity at pH 5.5 and pH 8.5, indicating a neutral proteinase. This discrepancy reinforces the fact that these bacteria produce various extracellular proteinases.

5.3 Production of proteinases by Gram-negative bacteria

The proteolytic flora increases in raw milk during cold storage (Cogan, 1977; Law, 1979; Suhren, 1981) and the bacteria produce proteinases during their growth. Until now there have been few reports available on the relationship between growth of Gram-negative bacteria in milk and proteinase production. Law et al. (1977) found that no proteolytic activity was produced by *P. fluorescens* AR-11 when its number had increased to $8.0 \cdot 10^5$ ml^{-1}, but definite activity when the number reached $8.0 \cdot 10^6$ ml^{-1}. Adams et al. (1976) found spoilage of UHT-sterilized milk after the bacterial count of raw milk exceeded 10^8 ml^{-1}. Cousin & Marth (1977) found proteolysis in pasteurized milk made from raw milk with a total bacterial count of $7.0 \cdot 10^6$ ml^{-1} and with a total count of pseudomonads of $4.0 \cdot 10^6$ ml^{-1}. Bacterial counts below 10^6 ml^{-1} obtained by the addition of a culture of *P. fluorescens* 22F to raw milk led, however, to spoilage after UHT-sterilization (Driessen, 1976b).

The production of extracellular proteinases by bacteria may depend on the physiological state of the bacteria (Wiersma, 1978). This aspect is discussed in the following Sections.

5.3.1 Production of proteinase by Pseudomonas fluorescens 22F in batch cultures

5.3.1.1 Materials and methods

Milk and incubation of the milk. In this investigation only fresh skim milk was used with a low bacterial count. This milk was pasteurized for 5 min at 105 °C in order to avoid the activity of milk proteinase, and inoculated with 0.001 % of a peptone-meat extract culture of *P. fluorescens* 22F fully grown at 20 °C. Portions of 100 ml inoculated milk were put into 500 ml Erlenmeyer flasks and incubated at 7 °C. At various times bacterial counts were estimated on Plate Count agar containing 1 % skim milk (PCM-agar) after incubation for 3 days at 30 °C.

Estimation of proteolysis. At various times the bacterial growth was stopped by heating the milk for 5 min at 75 °C. Tubes with 10 ml of this milk were brought under anaerobic conditions by the use of 0.5 ml satured Na_2CO_3 and 0.5 ml pyrogallol (20 %; v/v) on a cotton wool plug. The tubes were closed with a rubber stopper. This milk was preserved by the addition of 1 ml 0.11 % thimerosal (w/v)(Fluka AG, Buchs, Switzerland)(Noomen, 1975) and incubated for 16 h at 37 °C.

Proteolytic activity was determined by measuring the breakdown and formation of casein compounds, using a densitometer (De Jong, 1975) after protein separation by starch gel electrophoresis (Schmidt & Both, 1975).

In another experiment sterilized milk was inoculated with various quantities of a culture of *P. fluorescens* 22F fully grown at 20 °C and the proteolytic activity was measured as mentioned above.

5.3.1.2 Results

The relationship between the growth of *P. fluorescens* 22F in milk and the proteolytic activity is given in *Figure* 5.1. In particular, β- and κ-caseins were broken down, while para-κ-caseinlike compounds were formed. The formation of the latter compounds is shown in the figure. The caseins of the α_s-group and the whey proteins were not degraded to any measurable extent. No formation of para-κ-caseinlike compounds was noticed unless the bacterial count exceeded $5.0 \cdot 10^7$ ml^{-1}.

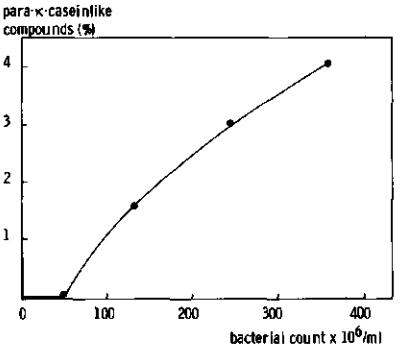

Figure 5.1 Relation between proteinase activity and bacterial count of *Pseudomonas fluorescens* 22F at 7 °C in heated milk (5 min at 105 °C). The activity is expressed as amount of para-κ-caseinlike compounds produced after 16 h at 37 °C, related to the total amount of protein.

In an experiment where milk was inoculated with varying quantities of a culture of *P. fluorescens* 22F fully grown at 20 °C, proteolysis was measured after 16 h at 37 °C. The bacterial count of the culture was $1.0 \cdot 10^9$ ml^{-1}. The results are given in *Figure* 5.2. Inoculation with 0.05 % culture, which represented approximately $5.0 \cdot 10^5$ bacteria per ml, resulted in definite proteolysis. The κ-casein disappeared and para-κ-casein was formed. β-Casein was also clearly degraded, while γ-caseins were formed. α_s-Casein was not broken

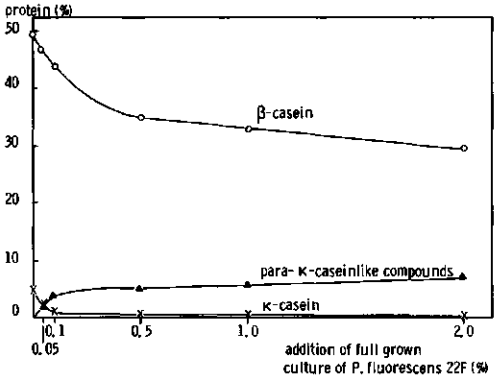

Figure 5.2 Proteolysis in milk after addition of different amounts of a culture of *Pseudomonas fluorescens* 22F fully grown at 20 °C, measured after 16 h at 37 °C.

down and was only slightly hydrolysed at greater additions of culture. Densitometric analysis of the gels revealed that the addition of 0.1 % culture fluid caused the κ-casein to almost totally disappear after the incubation period. When 2 % or more of the culture fluid was added, γ-caseins and para-κ -caseinlike compounds were degraded to such an extent that they were no longer detectable on the gel at the end of the incubation period. It is thus likely that considerable quantities of proteinase had accumulated in the culture fluid.

It is also important, however, to realize that the period of incubation for the measurement of the enzymatic activity (16 h at 37 °C) is short compared with the shelf-life of UHT-sterilized products (e.g. three months at ambient temperatures). For this reason another more sensitive experiment was carried out (see Section 5.3.3).

5.3.2 Production of proteinase by Pseudomonas fluorescens 22F in continuous cultures

5.3.2.1 Materials and methods

Culture of Pseudomonas fluorescens 22F. *Pseudomonas fluorescens* 22F was inoculated in peptone-meat extract broth and incubated for 24 h at 20 °C. Then this culture was stored in ice and the bacterial count was estimated by direct microscopic counting.

Continuous cultures. A Biolafitte 2 l fermenter (Gourdon, Maisons-Lafitte, France) was used. The vessel was filled with 1 litre of skim milk, heated for 5 min at 105 °C, and kept at 20.0 ± 0.1 °C in a water bath. The milk was inoculated with approximately 10^4 *P. fluorescens* 22F per ml. After a 16 h incubation period the bacterial count was estimated by direct microscopic counting and when the required number of bacteria had been reached, the inlet pump was started. The dilution rate was chosen in a way that the culture remained in a steady state for at least 9 h.

The bacterial numbers during steady state were estimated on Plate Count agar containing 1 % skim milk (PCM-agar).

Proteolysis. The proteolytic activity was measured as described in Section 5.3.1.1.

5.3.2.2 Results

The results of the experiments with the continuous cultures are given in *Table* 5.1. These experiments clearly show that the extracellular proteinase

Table 5.1 Relation between the bacterial count of *Pseudomonas fluorescens* 22F in continuous culture at 20 °C and the production of extracellular proteinase. The proteolytic activity is expressed as the disappearance of κ-casein and the formation of para-κ-caseinlike compounds in percentages of the total protein.

sample	dilution rate (h^{-1})	bacterial count (ml^{-1})	κ-casein (%)	para-κ-caseinlike compounds (%)
water	-	-	3.8	0
1	0.34	$1.2\ 10^7$	3.7	0
2	0.32	$4.3\ 10^7$	2.9	1.0
3	0.29	$2.0\ 10^8$	2.5	3.3
4	0.28	$5.0\ 10^8$	0	8.1

of *P. fluorescens* 22F is only produced towards the end of the exponential growth phase. Then the production per cell remained approximately constant. These results are in full agreement with those obtained from the batch cultures and shown in *Figure* 5.1.

5.3.3 Influence of the bacterial flora of raw milk on the keeping quality of UHT-sterilized milk

5.3.3.1 Materials and methods

Bacterial growth in cold stored milk. Raw skim milk was divided into three portions. One portion was sterilized immediately, one portion after a period of storage of 48 h at 6 °C, and one portion after a period of storage of 55 h at 6 °C followed by 64 h at 4 °C. The milk was sterilized for 4 s at 142 °C (Alfa Laval, VTIS). The sterilized milk was filled aseptically in bottles of half a litre and stored at 20 °C up to 12 weeks.

Proteolysis. The proteolysis was measured with starch gel electrophoresis as described in Section 5.3.1.1. Non-casein nitrogen (NCN) and non-protein nitrogen (NPN) fractions were prepared according to the method of Harwalkar & Vreeman (1978), and the nitrogen was quantified by the accelerated Kjeldahl method of Koops et al. (1975).

Bacterial flora. The composition of the bacterial flora was determined using several specific characteristics. A number of colonies was isolated from the PCM-agar plates and stored in tubes with Difco nutrient agar. The isolated strains were investigated for the following characteristics:

- colour of the colony;
- oxidase reaction according to Kovacs (1956);
- catalase reaction;
- shape and mobility of the bacteria;
- fermentation of glucose according to Hugh & Leifson (1953);
- Gram staining.

Organoleptic characteristics. The organoleptic characteristics of the milk were judged by a panel of three trained persons.

5.3.3.2 Results

Bacterial growth in cold stored milk. The bacterial flora of the raw milk during storage was determined, and the results are given in *Table* 5.2. The flora of the fresh milk (total bacterial count $2.4 \cdot 10^4$ ml^{-1}) consisted mainly of *Streptococceae*, but after two days at 6 °C pseudomonads predominated.

Proteolysis in the UHT-sterilized milk during storage. In milk sterilized in its fresh state and in that sterilized after two days of cold storage, no bacterial

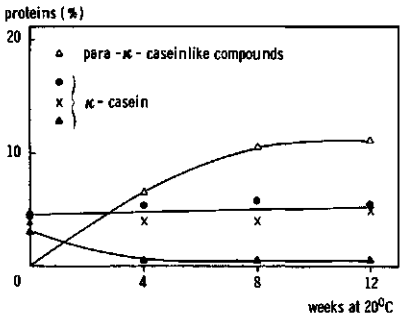

Figure 5.3 Proteolysis in UHT-sterilized milk during storage at 20 °C.
Conditions of the samples before sterilization were:
●—● fresh raw milk (viable count $2.4 \cdot 10^4$ ml^{-1});
x—x milk stored for two days at 6 °C (viable count $3.0 \cdot 10^6$ ml^{-1});
▲—▲, △—△ milk stored for two days at 6 °C and three days at 4 °C (viable count $7.3 \cdot 10^6$ ml^{-1}).

Table 5.2 Analysis of the bacterial flora of fresh and cold stored milk immediately before the UHT-sterilization. The bacteria were estimated on PCM-agar after 3 days at 30 °C (total count) and after 10 days at 7 °C (psychrotrophic count).

condition of the milk	bacterial count (ml^{-1})	number of colonies analysed	composition of the main flora	(%)
total count				
fresh	$2.4\ 10^4$	30	*Streptococceae*	(93)
			other bacteria	(7)
48 h at 6 °C	$1.0\ 10^6$	29	*Pseudomonas spec.*	(52)
			Achromobacteriaceae	(24)
			other bacteria	(23)
55 h at 6 °C				
and 64 h at 4 °C	$7.3\ 10^6$	24	*Pseudomonas spec.*	(83)
			Coliform bacteria	(17)
psychrotrophic count				
fresh	$3.2\ 10^3$	27	*Pseudomonas spec.*	(67)
			Achromobacteriaceae	(22)
			other bacteria	(11)
48 h at 6 °C	$1.4\ 10^6$	29	*Pseudomonas spec.*	(90)
			Achromobacteriaceae	(10)
55 h at 6 °C				
and 64 h at 4 °C	$1.0\ 10^7$	19	*Pseudomonas spec.*	(68)
			Achromobacteriaceae	(5)
			Coliform bacteria	(26)

proteolysis was detected and the milk remained good. The milk that was cold stored for five days already showed some proteolysis at the outset of the experiment and proteolysis markedly increased during storage after sterilization. The whey proteins were not degraded by the bacterial proteinases, while the caseins were broken down. Quantitative results are given in *Figure* 5.3. The α_{s2}- and β-caseins were degraded very rapidly, as a result of the activity of the native milk proteinase and values for these caseins are not

given in *Figure* 5.3. It is remarkable that κ-casein was only broken down in the milk cold stored for 5 days before sterilization. It is striking that in milk where the bacterial count had increased to $3.0 \cdot 10^6$ ml^{-1} no proteolysis occurred, while proteolysis was apparent when the bacterial count had increased to $1.0 \cdot 10^7$

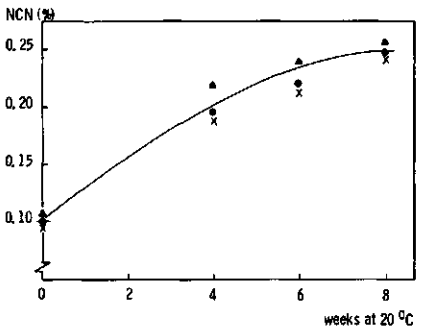

Figure 5.4 Non-casein nitrogen (NCN) in UHT-sterilized milk during storage at 20 °C. Conditions of the samples before sterilization were:
●—● fresh raw milk;
X—X milk stored for two days at 6 °C;
▲—▲ milk stored for two days at 6 °C and three days at 4 °C.
(Bacterial counts as in *Figure* 5.3)

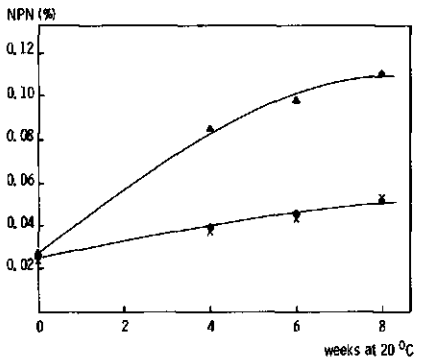

Figure 5.5 Non-protein nitrogen (NPN) in UHT-sterilized milk during storage at 20 °C. Conditions of the samples before sterilization were:
●—● fresh raw milk;
X—X milk stored for two days at 6 °C;
▲—▲ milk stored for two days at 6 °C and three days at 4 °C.
(Bacterial counts as in *Figure* 5.3)

ml^{-1}. This fact indicates the production of proteinase towards the end of the exponential growth phase.

Non-casein nitrogen (NCN) increased equally in the sterilized milk made from fresh and cold stored milk. This increase was predominantly caused by the activity of native milk proteinase, as indicated by the results plotted in *Figure* 5.4.

The non-protein nitrogen (NPN) content increased in all samples, most rapidly in the sterilized milk stored for five days. These results are given in *Figure* 5.5.

It was striking that the total bacterial count after five days ($7.3 \cdot 10^6$ ml^{-1}) was much lower than is generally found in heated milk (see e.g. *Figure* 5.1). This phenomenon may be caused by the presence of thermolabile bacteriostatic compounds in the raw milk.

Organoleptic characteristics. After a period of storage of 4 weeks at 20 °C the taste of the sterilized milk made from fresh and milk stored for two days was good. The sterilized milk made from milk stored for five days turned bitter and this milk was destabilized after 6 weeks at 20 °C.

5.3.4 Discussion

Only towards the end of the exponential growth phase of *Pseudomonas fluorescens* 22F did its proteinase appear in the milk at the time when bacterial count was above 10^7 per ml. This number is beyond the practical value as usually found in the Netherlands.

Addition of small amounts of a fully grown culture to milk can be sufficient to spoil the UHT-sterilized product. These results are in agreement with those of Gebre-Egziabher et al. (1980). Addition of 0.1 % of the fully grown culture was enough to make the milk unsuitable for use. Such an addition increased the bacterial number to 10^6 per ml, a value which is only occasionally found in dairy practice.

During storage of milk pseudomonads appeared to become the main flora. These bacteria are commonly strongly proteolytic (Law, 1979). In agreement with the preceeding results there appeared to be no difference in proteolysis between freshly sterilized milk and milk sterilized after storage for 48 h. A bacterial count of $3.0 \cdot 10^6$ per ml caused by growth in milk, is too low to result in measurable proteolysis. After storage for five days bacterial numbers had increased to $7.3 \cdot 10^6$ per ml, and in the UHT-sterilized milk made from this milk proteolysis was observed. The applied technique for the measurement of the proteinase activity is certainly sensitive enough to detect 3.0/7.3 of the observed amount of proteolysis of the latter milk. This supports the suggestion

that proteinase is only produced towards the end of the exponential growth phase.

Bacterial proteinases did not influence the NCN content of milk to a measurable extent. The residual activity of milk proteinase interfered with the results of the experiments concerning the keeping quality. However, it was striking that proteolysis of UHT-sterilized milk as a consequence of bacterial activity was accompanied by an NPN increase and the formation of para-κ-casein, while the native milk proteinase caused an NCN increase. This is a clear difference with diagnostic value for the detection of the cause of certain practical failures.

These observations are of practical interest. The physiological state of the bacteria is important for the production of extracellular proteinase. In fully grown culture the proteinase is present, while during growth there is little or no measurable activity.

The number of bacteria in raw milk can arise in two ways:

1. by growth after a small contamination;
2. by dilution with spoiled milk.

In the Netherlands, growth of bacteria in milk after a small contamination does not normally exceed the critical number. When milk, in exceptional cases, is contaminated with a fully grown culture, for example when small amounts of milk are left behind in tanks and spoil during further storage, it is possible that milk with a final, relatively low, bacterial number ($>5.0 \cdot 10^5$ ml^{-1}) has become unsuitable for the manufacture of UHT-sterilized milk. From this point of view it is important to avoid dead spaces in the processing lines and to collect all the milk very carefully. A dead space or milk residues of half a litre per 1 000 l can cause problems. From the number of bacteria in raw milk alone it is not possible to predict the keeping quality of a UHT-sterilized product. It is necessary to know how this number has been achieved, and which bacteria are present in the milk.

5.4 Thermal inactivation of the extracellular proteinases of some Gram-negative bacteria

5.4.1 Introduction

The extracellular proteinases produced by pseudomonads are known to be very thermoresistant. These enzymes can have a substantial residual activity in UHT-sterilized milk as described in the previous Sections. Until now, however, the research in this field has concentrated on *Pseudomonas fluorescens* and *P. fragi*. The reports on this subject have been reviewed several times (Cogan,

1977; Law, 1979; Suhren, 1981). The proteinase of *Aeromonas spec.* B31 also appears to be very thermoresistant (Richardson & Te Whaiti, 1978). Little is known about proteolysis by psychrotrophic bacteria, other than pseudomonads, which may grow in milk during cold storage. Therefore also the thermoresistance of extracellular proteinase of two other Gram-negative psychrotrophic bacteria was determined in more detail.

5.4.2 Materials and methods

Proteolytic bacteria. The organisms used in these experiments came from the collection of the Netherlands Institute for Dairy Research and originate from raw milk. The strains were *Pseudomonas fluorescens* 22F, *Achromobacter spec.* 1-10 and *Serratia marcescens* D2.

Cultivation. 100 ml portions of heated skim milk (5 min at 105 °C) in Erlenmeyer flasks were inoculated with a broth culture (0.1 %) of the organisms and incubated for three days at 20 °C. The bacterial count was estimated with Plate Count agar containing 1 % skim milk (PCM-agar) after three days at 30 °C.

Heat treatments. Complete cultures were heated at different temperatures for different periods of time. For temperatures below 100 °C treatments were performed in a glass tube containing 15 ml of culture according to the method described in Section 1.5.1.1. Then the tube was cooled in ice. The reference sample (holding time zero) was placed in ice immediately after preheating.

After these heat treatments the cultures were centrifuged at 27 000 *g* for 15 min at 4 °C. The clear supernatant was stored in the freezer at -35 °C until further investigation. During this frozen storage there appeared to be no decrease in enzymatic activity.

Proteolytic activity. The activity was measured according to the method described by Richardson & Te Whaiti (1978). The reaction mixture was as follows:
2 ml of a 2 % sodium caseinate solution;
1 ml 0.2 M tris-HCl in 0.02 M $CaCl_2$, pH 7.4;
0.1 ml 0.41 % thimerosal solution (Fluka AG, Buchs, Switzerland).
This mixture was incubated for 10 min in a water bath at 37 ± 0.1 °C. Then 1 ml of thawed supernatant was added and incubated for one hour at 37 °C. The reference was not incubated. The reaction was stopped by addition of 4 ml 10 % TCA, and the mixture then filtered through Whatman no. 40 filterpaper. The increase of the extinction at 280 nm was taken as a measure of the activity.

5.4.3 Results

5.4.3.1 Heat resistance of the proteinase of Pseudomonas fluorescens 22F

Exposure of the culture to temperatures above 70 °C resulted in a loss of proteolytic activity. This loss appeared to follow first order kinetics. Inactivation of the extracellular proteinase of *P. fluorescens* 22F was determined from 70 to 130 °C at intervals of 10 °C. The relationship between

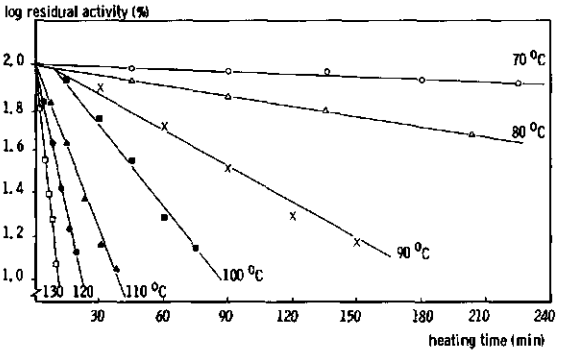

Figure 5.6 Regression lines of the loss of activity of extracellular proteinase of *Pseudomonas fluorescens* 22F during heating.

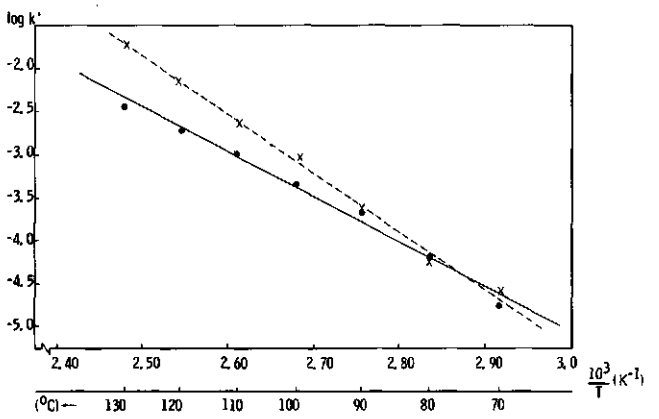

Figure 5.7 Arrhenius plots of the inactivation of the extracellular proteinases of:

●—● *Pseudomonas fluorescens* 22F;

X--X *Achromobacter spec.* 1-10.

Table 5.3 Kinetic and thermodynamic parameters of the inactivation of the extracellular proteinase of *Pseudomonas fluorescens* 22F.

temperature of heating (°C)	$10^3/T$ (K^{-1})	D (s)	reaction rate constant, k' (s^{-1})	activation: free energy ΔG^* ($kJ.mol^{-1}$)	activation: enthalpy ΔH^* ($kJ.mol^{-1}$)	activation: entropy ΔS^* ($J.mol^{-1}.K^{-1}$)
70	2.915	$1.45\ 10^5$	$1.58\ 10^{-5}$	115.9	98.0	-52.2
80	2.833	$3.73\ 10^4$	$6.17\ 10^{-5}$	115.4	98.0	-50.4
90	2.755	$1.07\ 10^4$	$2.15\ 10^{-4}$	115.0	97.9	-47.1
100	2.681	$5.28\ 10^3$	$4.37\ 10^{-4}$	116.0	97.8	-48.8
110	2.611	$2.28\ 10^3$	$1.01\ 10^{-3}$	116.6	97.7	-49.3
120	2.545	$1.32\ 10^3$	$1.75\ 10^{-3}$	117.9	97.6	-51.7
130	2.481	$6.60\ 10^2$	$3.48\ 10^{-3}$	118.7	97.5	-52.6

heating time and residual activity for each temperature was determined by regression analysis using the method of least squares. From these results shown in *Figure* 5.6, D and k' values were calculated and are quoted in *Table* 5.3. The Q_{10} of inactivation varied from 3.9 to 2.0 for the measured range of temperature.

From the data in *Table* 5.3 an Arrhenius plot was drawn for the inactivation

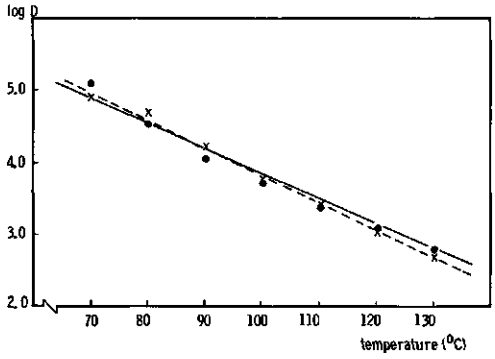

Figure 5.8 Thermal destruction curves of the extracellular proteinases of:
●——● *Pseudomonas fluorescens* 22F;
X- - -X *Achromobacter spec.* 1-10.

of this proteinase. The plot is shown in *Figure* 5.7. The linear regression equation of this plot is:

$$\log k' = -5.272\ (10^3/T) + 10.711 \quad (r^2 = 0.985) \qquad (5.1)$$

The relationship between D and the temperature is given in *Figure* 5.8. This plot is characterized by the equation:

$$\log D = -0.038\ T' + 7.612 \quad (r^2 = 0.974) \qquad (5.2)$$

in which T' is the temperature in °C.

Z for the inactivation of the extracellular proteinase of *P. fluorescens* 22F is 26.4 °C.

Equations (5.1) and (5.2) can be used to predict the effect of certain heat treatments on the relative activity of the extracellular proteinase of *P. fluorescens* 22F.

5.4.3.2 Heat resistance of the proteinase of Achromobacter spec. 1-10

Exposure of the culture to temperatures above 70 °C resulted in a loss of activity. This loss appeared to follow first order kinetics. The inactivation of the extracellular proteinase of *Achromobacter spec.* 1-10 was determined from 70 to 130 °C at intervals of 10 °C. The relationship between heating time and residual activity for each temperature was examined by regression analysis using the method of least squares. From these results shown in *Figure* 5.9. D and k' values were calculated and are quoted in *Table* 5.4. The Q_{10} of inactivation varied from 3.2 to 2.3 in the measured range of temperatures.

Table 5.4 Kinetic and thermodynamic parameters of the inactivation of the extracellular proteinase of *Achromobacter spec.* 1-10.

temperature of heating (°C)	$10^3/T$ (K^{-1})	D (s)	reaction rate constant, k' (s^{-1})	activation: free energy ΔG^* ($kJ.mol^{-1}$)	activation: enthalpy ΔH^* ($kJ.mol^{-1}$)	activation: entropy ΔS^* ($J.mol^{-1}.K^{-1}$)
70	2.915	$7.39\ 10^4$	$3.12\ 10^{-5}$	114.0	98.1	-46.4
80	2.833	$5.19\ 10^4$	$4.43\ 10^{-5}$	116.4	98.1	-51.8
90	2.755	$1.64\ 10^4$	$1.40\ 10^{-4}$	116.3	98.0	-50.4
100	2.681	$5.28\ 10^3$	$4.37\ 10^{-4}$	116.0	97.9	-48.5
110	2.611	$2.82\ 10^3$	$8.17\ 10^{-4}$	117.2	97.8	-50.7
120	2.545	$1.08\ 10^3$	$2.13\ 10^{-3}$	117.3	97.7	-49.9
130	2.481	$4.80\ 10^2$	$4.80\ 10^{-3}$	117.6	97.6	-49.6

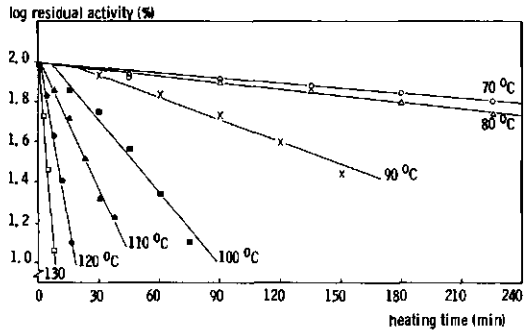

Figure 5.9 Regression lines of the loss of activity of extracellular proteinase of *Achromobacter spec.* 1-10 during heating.

From the data in *Table* 5.4 an Arrhenius plot was drawn for the inactivation of this proteinase. This plot is also shown in *Figure* 5.7. The linear regression equation of this plot is:

$$\log k' = -5.275\ (10^3/T) + 10.732 \quad (r^2 = 0.988) \qquad (5.3)$$

The relation between D and the temperature is given in *Figure* 5.8. This plot is characterized by the equation:

$$\log D = -0.038\ T' + 7.630 \quad (r^2 = 0.991) \qquad (5.4)$$

Z for inactivation of the extracellular proteinase of *Achromobacter spec.* 1-10 is 26.2 °C.

5.4.3.3 Heat resistance of the proteinase of Serratia marcescens D2

Exposure of the culture to temperatures above 50 °C resulted in loss of activity, but not always following first order kinetics. Inactivation of the proteinase took place until a certain limit was reached. This limit was not constant. The relationship between heating time and residual activity for each temperature was examined by regression analysis using the method of least squares. The regression lines are shown in *Figure* 5.10.

Obviously three different reactions or 'stages' resulted from the heat treatments. The first stage shows a relatively fast, the second stage a fluctuating and the third stage a slow rate of inactivation. From the regression lines D and k' were calculated and are quoted in *Table* 5.5. Q_{10} for the fast and slow stages of inactivation was 5.9 and 2.4, calculated for 60 and 90 °C respectively. Q_{10} for the fluctuating inactivation was not calculated, since the measured D was too variable. It was assumed that fast and slow inactivations of the proteolytic activity were interfering in the intermediate area, causing these rather inconsistent results. This stage was not studied in more detail.

Table 5.5 Kinetic and thermodynamic parameters of the inactivation of the extracellular proteinase(s) of *Serratia marcescens* D2.

temperature of heating (°C)	$10^3/T$ (K^{-1})	D (s)	reaction rate constant, k' (s^{-1})	activation: free energy ΔG^* ($kJ.mol^{-1}$)	activation: enthalpy ΔH^* ($kJ.mol^{-1}$)	activation: entropy ΔS^* ($J.mol^{-1}.K^{-1}$)
fast inactivation (I)						
50	3.096	$4.20\ 10^4$	$5.48\ 10^{-5}$	105.7	154.5	151.1
55	3.049	$1.80\ 10^4$	$1.28\ 10^{-4}$	105.0	154.5	150.9
60	3.003	$4.50\ 10^3$	$5.12\ 10^{-4}$	102.8	154.4	155.0
65	2.959	$3.30\ 10^3$	$6.98\ 10^{-4}$	103.5	154.4	155.2
70	2.915	$1.38\ 10^3$	$1.67\ 10^{-3}$	102.6	154.3	150.7
fluctuating inactivation (II)						
65	2.959	$1.47\ 10^4$	$1.57\ 10^{-4}$	-	-	-
70	2.915	$6.06\ 10^3$	$3.80\ 10^{-4}$	-	-	-
80	2.833	$1.19\ 10^4$	$2.04\ 10^{-4}$	-	-	-
85	2.793	$9.12\ 10^3$	$2.52\ 10^{-4}$	-	-	-
90	2.755	$6.00\ 10^3$	$3.84\ 10^{-4}$	-	-	-
slow inactivation (III)						
80	2.833	$5.70\ 10^4$	$4.04\ 10^{-5}$	113.3	104.3	-25.5
85	2.793	$4.80\ 10^4$	$4.80\ 10^{-5}$	117.8	104.4	-37.4
90	2.755	$1.50\ 10^4$	$1.54\ 10^{-4}$	116.0	104.2	-32.5
95	2.717	$1.16\ 10^4$	$1.99\ 10^{-4}$	116.8	104.1	-34.5
100	2.681	$1.01\ 10^4$	$2.29\ 10^{-4}$	118.0	104.1	-37.3

The transition points were varying with the temperature of heat inactivation. The residual activity at the first transition point was about 30 % at 70 °C, but more than 40 % at temperatures above 80 °C. The residual activity at the second transition point was varying from 22 to 30 % with increasing temperatures (see *Figure* 5.10).

From the data in *Table* 5.5 Arrhenius plots were drawn of the fast and the slow stages of inactivation. These plots are shown in *Figure* 5.11. The linear regression equations of these plots are:

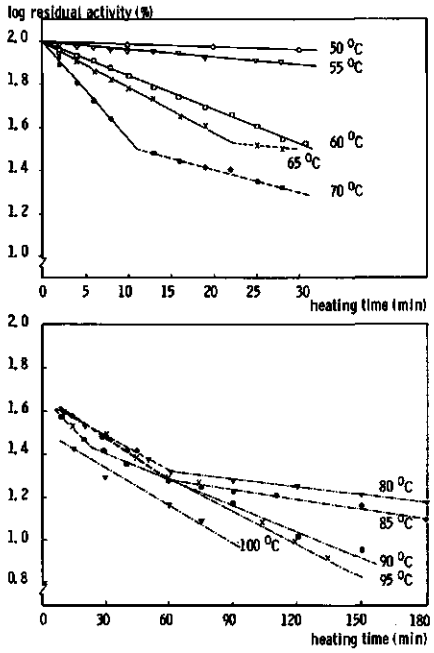

Figure 5.10 Regression lines of the loss of activity of extracellular proteinase of *Serratia marcescens* D2 during heating.

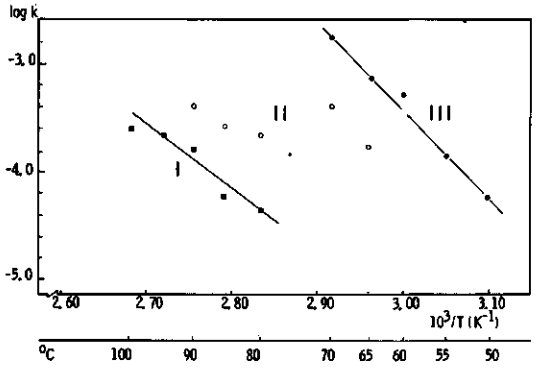

Figure 5.11 Arrhenius plots of the inactivation of the extracellular proteolytic activity of *Serratia marcescens* D2.

I : fast inactivation;

II : fluctuating inactivation;

III: slow inactivation.

for the fast stage of inactivation:

$\log k_1' = -8.209\ (10^3/T) + 21.189 \quad (r^2 = 0.973)$ (5.5)

for the slow stage of inactivation:

$\log k_2' = -5.601\ (10^3/T) + 11.462 \quad (r^2 = 0.894*)$ (5.6)

The relationship between D and temperature is given in *Figure* 5.12. These plots are characterized by the equations:

for the fast stage of inactivation:

$\log D_1 = -0.074\ T' + 8.282 \quad (r^2 = 0.970)$ (5.7)

for the slow stage of inactivation:

$\log D_2 = -0.042\ T' + 8.152 \quad (r^2 = 0.891*)$ (5.8)

Z for the inactivation of the extracellular proteolytic activity of *Serratia marcescens* D2 was 13.5 and 27.0 °C for the fast and the slow inactivation respectively.

To determine whether the specificity for breakdown of caseins by the extracellular proteinase(s) of *S. marcescens* D2 was dependent on a heat treatment, the culture fluid was heated for 30 min at 55, 70 or 95 °C, respectively. The reference sample was not heated. After these heat treatments the activities with both the fast and the fluctuating inactivation rates were

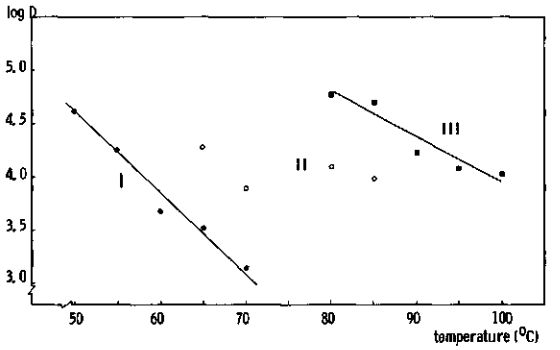

Figure 5.12 Thermal destruction curves of the extracellular proteolytic activity of *Serratia marcescens* D2.

I : fast inactivation;

II : fluctuating inactivation;

III: slow inactivation.

*The deviation which is not explained by the regression ($\sqrt{1-r^2}$) is rather high for the second stage of inactivation (about 33 %) and suggests that values should not be extrapolated beyond the range of the experimental conditions.

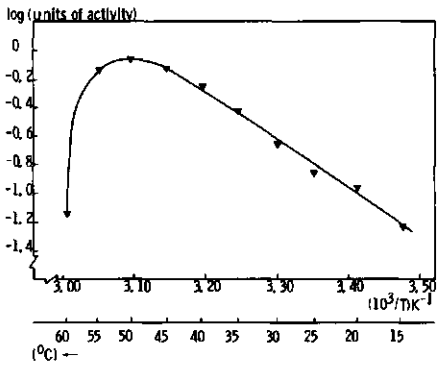

Figure 5.13 Effect of temperature on the activity of the extracellular proteinase of *Serratia marcescens* D2.

eliminated. Proteolysis was measured as described in Section 5.3.1.1. The gel electrophoretograms of all samples appeared to be the same. It was concluded that the specificity for breakdown of caseins was not changed by these particular heat treatments.

The effect of increasing temperature on the activity of the proteinase of *S. marcescens* D2 was expressed in an Arrhenius plot. This plot, shown in *Figure* 5.13, indicates a simplé relationship between activity and temperature between 15 and 50 °C. The activation energy (E_a^*) was 61.4 kJ.mol^{-1}.

5.4.4 Discussion

The bacterial proteinases involved in this investigation appeared to be very stable to heat. The kinetic parameters of the inactivation of the proteinases of *Pseudomonas fluorescens* 22F and *Achromobacter spec.* 1-10 were similar. The change of free enthalpy during the heat treatment was approximately 100 kJ.mol^{-1}, a value which is characteristic for protein denaturation (Payens, 1962). However, Q_{10} for inactivation was approximately 3. This value points to a 'chemical' reaction (Booy, 1964). Heat denaturation of proteins is characterized by a high ΔH^*, compensated by a high ΔS^*. In the measurements ΔS^* was even negative. This fact suggests that heat inactivation of these bacterial proteinases is not caused by a destruction of the secondary structure of the enzyme molecules. According to the data, in which a large similarity is measured for the extracellular proteinases of two different species, it is very likely that these proteinases are the same. This hypothesis is strongly supported by the results of several investigators, who reported comparable data for the extracellular proteinases of different *Pseudomonas* species and

an *Aeromonas* species (Mayerhofer et al., 1973; Adams et al., 1975; Kishonti, 1975; Malik, 1975; Richardson & Te Whaiti, 1978; Richardson, 1981). For *Pseudomonas* 21B, MC6D and B52 Z values were calculated to be 44.5, 32.5 and 29.9 °C, respectively (Adams et al., 1975; Kishonti, 1975; Richardson, 1981).

According to the results of this study, residual activity of bacterial proteinases can be expected even in milk autoclaved for 15 min at 120 °C. It is of practical importance to prevent production of these enzymes, especially in milk used for the manufacture of products with a potentially long storage life at ambient temperature.

The extracellular proteolytic activity of *S. marcescens* D2 is rather complicated. There is a clear inactivation of activity at relative low temperatures which levels out at about 30 % of the original proteolytic activity. The second stage of inactivation is seen, where the relationship with temperature is not clear. It may be possible that the first and third stages of inactivation interfered with this stage. The third stage of inactivation showed similarity with the previous discussed proteinases of *P. fluorescens* 22F and *Achromobacter spec.* 1-10.

Both specificity for the breakdown of casein and temperature activation of the extracellular proteolytic activity of *S. marcescens* D2 (results not shown) suggest a simple proteinase. However, the heat inactivation suggests a far more complicated proteolytic system. There is a similarity with the heat inactivation of the extracellular lipase of *P. fluorescens* 22F (see Section 3.5). But for the extracellular proteinase of *S. marcescens* D2 the most likely explanation of the phenomena, in view of the previous results, is that by heating the proteinase to sufficient high temperature, the native form changes into a temperature-stable conformation or forms a complex with components in the skim milk, that is still enzymatically active (see also Section 3.5.4), or a transition of preenzyme to active enzyme during heating at moderate temperatures. For a complete understanding of the heat inactivation of this proteinase, isolation and purification of the enzyme would be necessary.

5.5 Proteolysis by Gram-negative bacteria in other milk products

5.5.1 Pasteurized milk

Although bacterial proteinases can have substantial activity at low temperatures, e.g. at 4 °C 25 % of that at the optimum temperature (Driessen, 1976), and the pH of milk is close to the optimum pH for proteinase activity, defects in pasteurized milk caused by these enzymes have not previously been described.

It is likely that the period of storage is too short to produce noticeable off-flavours. As small peptides are responsible for bitterness, more advanced proteolysis is necessary for an off-flavour to occur (Visser et al., 1975).

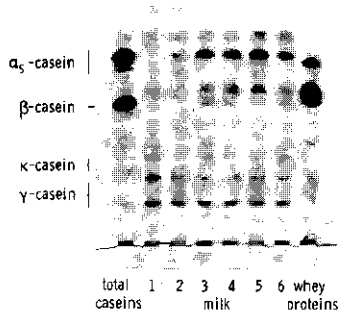

Figure 5.14 Starch gel electrophoretograms of six samples of milk. The milk was sterilized by direct heating for 3 s at 142 °C and stored for 6 weeks at 20 °C.

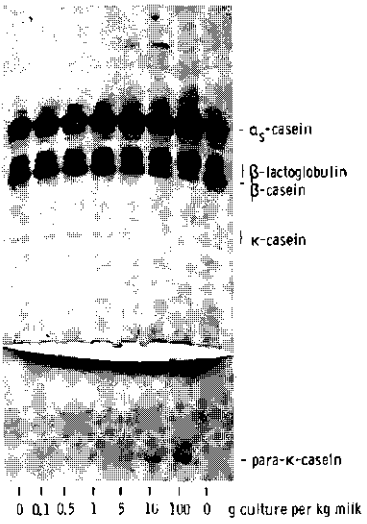

Figure 5.15 Starch gel electrophoretograms of milk to which a culture of *Pseudomonas fluorescens* 22F was added. The milk was sterilized by indirect heating for 45 s at 142 °C and stored for 3 weeks at 20 °C.

5.5.2 Sterilized milk

The effect of bacterial proteinases on the keeping quality of UHT-sterilized milk was discussed in Section 5.3. Proteolysis of this product as a consequence of bacterial growth is accompanied by an NPN increase, the disappearence of κ-casein and the formation of para-κ-casein. By these characteristics proteinases of Gram-negative bacteria differ from the native milk proteinase, which causes the disappearence of α_{s2}- and β-caseins, the formation of γ-caseins and an NCN increase (see Chapter 4). Typical starch gel electrophoretograms of both types of proteolysis are shown in *Figure* 5.14 and 5.15. The clear differences between both proteolyses have diagnostic value for the detection of the cause of certain practical failures.

The production of bacterial proteinase is often accompanied by the production of bacterial lipase. In practice lipolysis in milk is detected in an earlier stage than proteolysis. Therefore, initial deterioration of milk is caused by the development of a rancid off-flavour. Defects such as bitterness and gelation will only be noticed afterwards.

5.5.3 Cheese

In cheesemaking experiments, in which milk was contaminated with a fully grown culture of some Gram-negative bacteria (see Section 3.6) no proteolytic off-flavour could be detected in the cheese during ripening and storage. This is in accordance with results reported for Cheddar cheese. The Kjeldahl figures of the nitrogen content of maturing cheeses were unaffected by psychrotrophic counts in raw milk, while only small differences were found in the patterns of proteolysis as determined by electrophoresis. None of the cheeses developed off-flavour related to excessive protein breakdown and there were no significant changes in the yields or quality of these cheeses through the proteolytic activity of these bacteria (Law et al., 1979).

5.5.4 Other dairy products

Until now there have been no reports available dealing with the effect of bacterial proteinases of psychrotrophic bacteria on the keeping quality of other dairy products.

5.6 Survey of proteolysis in milk and milk products

The proteolytic enzymes described in Chapter 4 and 5 are able to cause a bitter off-flavour, gelation and destabilization in milk and milk products. In the majority of cases it is possible to take the measures necessary to prevent enzymatic spoilage. A survey of this matter is given in *Table* 5.6.

Table 5.6 Survey of proteolysis in milk and milk products.

Proteolysis		
caused by:	milk proteinase	bacterial proteinase
occurrence in:	UHT-sterilized milk products	UHT-sterilized milk products
defects:	- bitter off-flavour - transparency of skim milk - glassy appearance of skimmed custard - destabilisation	- bitter off-flavour - gelation - separation of whey
prevention:	sterilization treatment sufficiently high, e.g. 16 s at 142 °C.	- manufacture of milk of good bacteriological quality - cold storage (<4 °C) of the milk for maximal 3 days at the farm and 1 day at the factory - prevention of contamination of the thermizized milk - effective cleaning of processing lines.

SUMMARY AND CONCLUSIONS

The occurrence and heat inactivation of native and bacterial lipolytic and proteolytic enzymes present in milk were investigated. The consequences of different heat treatments as applied in the dairy industry to improve the keeping quality of milk and milk products are discussed.

Milk lipoprotein lipase

The thermoresistance of milk lipoprotein lipase was characterized in the temperature range of 45 to 70 °C. The inactivation of this lipase follows first order kinetic parameters.

In milk the fat is protected against lipolysis by an intact fat globule membrane. When this membrane is somehow damaged, lipolysis may occur. The intensity of lipolysis depends on residual enzyme activity after the heat treatment and on several other factors. It was found that lipolysis in pasteurized homogenized milk could be reduced sufficiently by heat treatments corresponding to $\geq 3.9\ D$, for instance 16 s at 76 °C. No milk lipoprotein lipase activity was measured after heating for 10 s at 85 °C.

Lipolysis by milk lipase can be expected if there is residual activity left in the dairy product after heat treatment and the fat is in an attackable form. However, the role of milk lipoprotein lipase in fat hydrolysis during cheese ripening is not fully understood. After a cheese milk pasteurization treatment of 10 s at 72 °C the considerable residual lipolytic activity (about 17 %) was of little importance in determining the fat acidity of the cheese.

Lipases of Gram-negative bacteria in milk and milk products

Appreciable production of lipases by Gram-negative bacteria in batch and continuous cultures only took place towards the end of exponential growth and during the stationary growth phase, when bacterial count exceeded $8.0 \cdot 10^6$ ml^{-1}. In a fully grown culture lipase accumulated, while during growth in milk there was no or little measurable activity. It is therefore important to avoid the presence of long stored milk residues in the equipment both at the farm and the dairy factory in order to prevent bacterial growth to such an extent that lipases may accumulate.

Exposure of some cultures to temperatures above 50 °C resulted in a two stage loss of lipolytic activity. This phenomenon has been elucidated for the extracellular lipase of *Pseudomonas fluorescens* 22F. The presence of two stages

in the heat inactivation of the enzyme was connected with a change in apparent activation energy of the enzyme action near 37 °C. These observations are consistent with two explanations: either that there are two enzymes or that there is one enzyme with two different active conformations. Heat inactivation of both stages followed first order reaction kinetics.

These complicated temperature relations were quite common for extracellular lipases of Gram-negative bacteria. If a second stage of inactivation was found, thermoresistance was always very high. For the extracellular lipase of *P. fluorescens* 22F, *D* was calculated to be 4.2 min at 150 °C. Enzymes showing a simple heat inactivation curve exhibit less thermoresistance, although it was always such that considerable residual activity was left after a normal pasteurization treatment of milk.

Lipase of *P. fluorescens* 22F accumulated 10-fold in the curd during cheese manufacture. Therefore cheese may be rather sensitive to rancidity caused by bacterial growth in raw milk.

There was good agreement between lipolysis in cheese and residual activity of the extracellular lipase from *Alcaligenes viscolactis* 23a1 in heated cheese milk. Such a relationship was not observed for the residual activity of the extracellular lipase from *P. fluorescens* 22F. Lipolysis in cheese was only related to the thermal stable part of the lipolytic activity.

In UHT-sterilized milk lipolysis caused by extracellular lipases of Gram-negative bacteria can occur, especially at higher storage temperatures.

If lipolysis occurs in butter, it can almost certainly be ascribed to growth of lipolytic micro-organisms in the product.

Rancidity in pasteurized milk products is unlikely to be caused by bacterial lipases. These products are kept for too short a period of time to permit the development of an off-flavour, particularly at the low storage temperatures commonly applied.

Milk proteinase

Thermoresistance of alkaline milk proteinase in aseptically drawn milk was studied. At temperatures below 100 °C thermal inactivation followed first order reaction kinetics. The interpretation of results is, however, complicated by a particular transition of pre-enzyme to active enzyme during heating at moderate temperatures.

In the temperature range from 100 to 142 °C heating temperature was of minor importance. Milk proteinase partly resisted certain UHT-sterilization treatments. The keeping quality of UHT-sterilized milk thus may be reduced by residual activity of native milk proteinase.

The relation between sterilization temperature and proteolysis in milk during storage was studied by starch gel electrophoresis. It was found that for a holding time of 4.8 s sterilization temperature had little effect on residual activity of milk proteinase.

Organoleptic characteristics of UHT-sterilized milk during storage depended much closer on heating time than on sterilization temperature. A heating time of 16 s at 140 °C with direct or indirect heating was sufficient to ensure that milk retained satisfactory organoleptic characteristics during 12 weeks storage at 20 °C. However, under these heating conditions milk proteinase was not completely inactivated.

Longer holding times during sterilization improved keeping quality, but also resulted in a more frequent occurrence of cooked flavour defect. Shorter holding times during sterilization resulted in more proteolysis during storage and in bitterness and transparency of the milk. Sometimes gelation occurred during storage at 20 °C, but not at 30 °C. When proteolysis was inhibited by aprotinin and diisopropylfluorophosphate (DFP) transparency and gelation did not occur during storage.

The effective heat-treatment during UHT-sterilization depends markedly on the type of sterilizer, since the course of temperature with time differs between direct and indirect heating. In this study comparable keeping quality of directly heated milk and indirectly heated milk could be obtained by varying the holding time of the sterilization treatment. It is therefore desirable that the manufacturer of UHT-sterilized milk should adapt the conditions of the sterilization process to the required keeping quality and flavour.

Viscosity of UHT-sterilized custard may not remain constant. During storage at 20 °C custard may become gradually thinner. This phenomenon appeared to coincide with proteolysis by residual activity of milk proteinase. In addition to the decrease in viscosity, the custard became bitter and acquired a glassy appearance. After 16 weeks storage at 20 °C gelation and instability of the product was noticed on some occasions. Custards prepared with holding times of 18 and 36 s at 142 °C remained stable. It is recommended that during the manufacture of UHT-sterilized custard attention is paid to residual milk proteinase activity.

Residual milk proteinase activity may contribute to gelation of UHT-sterilized evaporated milk.

The keeping quality of UHT-sterilized milk was improved considerably after preheating the milk before sterilization for 60 min at 55 °C. During 11 weeks storage at 20 °C gelation did not occur, bitterness and transparency of the milk were retarded and proteolysis was decreased. Milk proteinase may be inhibited or partly inactivated by this heat treatment.

Proteinases of Gram-negative bacteria in milk and milk products

Proteinase produced by *Pseudomonas fluorescens* 22F in milk could only be observed towards the end of exponential growth, when the bacterial count exceeded 10^7 ml^{-1}. During stationary growth the production of proteinase per cell remained approximately constant. These results were confirmed in batch and continuous cultures with other Gram-negative bacteria. It is therefore important to avoid the presence of long stored milk residues in the equipment both at the farm and the dairy factory to prevent bacterial growth to such an extent that proteinases may appear in milk.

Exposure of cultures of *P. fluorescens* 22F and *Achromobacter spec.* 1-10 to temperatures above 70 °C resulted in simple inactivation of proteolytic activity according to first order reactions kinetics. These proteinases were very heat stable. However, exposure of a culture of *Serratia marcescens* D2 to temperatures above 50 °C resulted in a loss of proteolytic activity not always following first order kinetics. A three stage loss of activity was observed. The first stage showed a relatively fast and the third stage a relatively slow rate of inactivation. The intermediate stage was assumed to be due to a interaction between the slow and fast rates of inactivation. Judged by the breakdown patterns of caseins and temperature-activity profile it was suggested that it concerns one enzyme, although with a complex heat inactivation and there is a possibility that a transition of pre-enzyme to active enzyme takes place during heating at moderate temperatures, or the native form changes into a temperature-stable conformation of the enzyme.

Bacterial proteinases may be of importance for (UHT)-sterilized milk quality. During storage of this milk, gelation and increasing bitterness may occur as a consequence of growth of proteolytic bacteria in raw milk.

Proteolysis caused by bacterial enzymes was accompanied by a non-protein nitrogen (NPN) increase and the formation of para-κ-casein, while alkaline milk proteinase caused a non-casein nitrogen (NCN) increase and the formation of γ-caseins. This is a clear difference with diagnostic value for the detection of the cause of certain defects of practical significance.

SAMENVATTING EN CONCLUSIES

In het onderzoek zijn het voorkomen en de hitte-inactivering bestudeerd van zowel de lipasen en proteïnasen die van nature in de melk aanwezig zijn als van die welke door bacteriën in de melk zijn geproduceerd. Ook wordt besproken welke gevolgen de verschillende hittebehandelingen die in de zuivelindustrie worden toegepast, hebben voor de houdbaarheid van melk en melkprodukten.

Melklipoproteïnelipase

De thermoresistentie van het melklipoproteïnelipase werd gekarakteriseerd voor het temperatuurgebied van 45 tot 70 °C. De inactivering van dit lipase verliep volgens een reactie van de eerste orde.

In melk wordt het vet tegen lipolyse beschermd door het intacte vetbolletjesmembraan. Wanneer dit membraan op een of andere manier wordt beschadigd, kan vetsplitsing optreden. De intensiteit van de vetsplitsing hangt voornamelijk af van de na de hittebehandeling overgebleven activiteit van het enzym en verder van verscheidene andere factoren. De vetsplitsing in gepasteuriseerde gehomogeniseerde melk bleek voldoende te kunnen worden gereduceerd door een hittebehandeling overeenkomend met $\geq 3{,}9\ D$ (D = de decimale reductietijd: tijd nodig om 90 % van het enzym te inactiveren), bij voorbeeld 16 s bij 76 °C. Restactiviteit van het melklipoproteïnelipase kon niet meer worden vastgesteld na verhitting gedurende 10 s bij 85 °C.

Lipolyse door dit enzym kan worden verwacht indien na de hittebehandeling in het melkprodukt restactiviteit is overgebleven en het vet in aantastbare vorm aanwezig is. Over de rol van het melklipoproteïnelipase in de hydrolyse van het vet tijdens de kaasrijping is echter niet voldoende bekend. Na een kaasmelkpasteurisatie van 10 s bij 72 °C bleef nog een aanzienlijke restactiviteit over (ongeveer 17 %), maar die is in de praktijk van weinig betekenis voor de zuurtegraad van het vet van de kaas.

Lipase van gramnegatieve bacteriën in melk en melkprodukten

Gramnegatieve bacteriën bleken zowel in ladingsgewijze als in continue culturen in melk pas aan het einde van de exponentiële en tijdens de stationaire groeifase meetbare hoeveelheden lipasen te produceren. Het aantal bacteriën bedroeg op dat moment meer dan $8{,}0 \cdot 10^6$ ml^{-1}. In een volgroeide cultuur hoopte

het lipase zich op, terwijl tijdens de groei geen of weinig activiteit kon worden vastgesteld. Het is daarom van belang te vermijden dat resten melk of van een melkprodukt in de apparatuur op de boerderij of in de fabriek achterblijven om zo groei van bacteriën tot het niveau waarop lipasen worden geproduceerd en zich ophopen, te voorkomen.

Sommige culturen die werden blootgesteld aan temperaturen boven 50 °C vertoonden een verlies aan lipolytische activiteit in twee stappen. Dit verschijnsel is uitgewerkt voor het extracellulaire lipase van *Pseudomonas fluorescens* 22F. De aanwezigheid van twee stappen in de hitte-inactivering van het enzym was gekoppeld aan een verandering in de schijnbare energie van activering van enzymwerking bij ongeveer 37 °C. Voor deze waarnemingen kunnen twee verklaringen worden gegeven: er zijn twee enzymen, of een enzym bezit verschillende actieve conformaties. De hitte-inactivering van beide stappen verliep volgens reacties van de eerste orde.

Deze gecompliceerde verbanden met de temperatuur, zoals beschreven voor *P. fluorescens* 22F, kwamen vrij algemeen voor bij lipasen die afkomstig zijn van andere gramnegatieve bacteriën. Lipasen waarvan de inactivering in twee stappen verliep, waren altijd zeer thermoresistent. Voor het extracellulair lipase van *P. fluorescens* 22F werd bij 150 °C een *D* waarde van 4,2 min berekend. Indien de hitte-inactivering in een enkele stap verliep, was de thermoresistentie van het lipase nog zo hoog dat na een gebruikelijke pasteurisatiebehandeling van de melk nog een aanzienlijke restactiviteit overbleef.

Het lipase van *P. fluorescens* 22F hoopte zich 10-voudig op in de wrongel tijdens de kaasbereiding. Daardoor kan de kaas tamelijk gevoelig zijn voor het gebrek rans ten gevolge van groei van de bacteriën in de rauwe melk. Er bestond een goede overeenkomst tussen de mate van lipolyse in kaas en de restactiviteit van het extracellulaire lipase van *Alcaligenes viscolactis* 23a1 in verhitte kaasmelk. De mate van lipolyse vertoonde geen verband met de restactiviteit van het lipase van *P. fluorescens* 22F; in dit geval kon de vetsplitsing in kaas alleen gerelateerd worden met het thermostabiele deel van de lipolytische activiteit.

In UHT-gesteriliseerde melk kon lipolyse door extracellulaire lipasen van gramnegatieve bacteriën optreden, vooral wanneer de melk bij hogere temperaturen werd bewaard.

Wanneer in boter vetsplitsing optreedt, kan deze vrijwel met zekerheid worden toegeschreven aan de groei van lipolytische microörganismen.

Het is onwaarschijnlijk dat ransheid in gepasteuriseerde melkprodukten wordt veroorzaakt door bacteriële lipasen. De periode dat deze produkten - bovendien bij lage temperaturen - worden bewaard is te kort om dit gebrek tot uiting te laten komen.

Melkproteïnase

De thermoresistentie van de natuurlijke melkproteïnase in aseptisch gewonnen melk werd bestudeerd. Bij temperaturen onder 100 °C verliep de inactivering van dit proteïnase volgens een reactie van de eerste orde. Het interpreteren van de resultaten wordt echter bemoeilijkt door een bijzondere transitie van pre-enzym tot actief enzym tijdens het verhitten bij gematigde temperaturen. In het temperatuurgebied van 100 tot 142 °C was de temperatuur van verhitten een minder belangrijke factor. Van de melkproteïnase bleek na bepaalde UHT-sterilisatiebehandelingen nog een zekere restactiviteit aanwezig te zijn. Hierdoor kan in UHT-gesteriliseerde melkprodukten eiwitafbraak ontstaan waardoor de houdbaarheid wordt beperkt.

De werking van de natuurlijke melkproteïnase in relatie tot de hittebehandeling die de melk had ondergaan werd bestudeerd met zetmeelgel-elektroforese. Bij een heethoudtijd van 4,8 s bleek de hoogte van de sterilisatietemperatuur van weinig invloed te zijn op de restactiviteit van de melkproteïnase. De organoleptische eigenschappen van de UHT-gesteriliseerde melk tijdens bewaren bleken sterk afhankelijk te zijn van de toegepaste heethoudtijd en weinig afhankelijk van de toegepaste sterilisatietemperatuur. Toepassing van 16 s heethoudtijd bij 140 °C was voor zowel verhitting met een indirekt als met een direkt systeem voldoende om goede organoleptische eigenschappen te behouden tijdens een bewaarperiode van 12 weken bij 20 °C. Bij deze condities van steriliseren werd de melkproteïnase niet geheel geïnactiveerd. Het toepassen van langere heethoudtijden tijdens het steriliseren resulteerde in een betere houdbaarheid, maar ook in een frequenter voorkomen van het gebrek kooksmaak. Het toepassen van kortere heethoudtijden tijdens het steriliseren gaf aanleiding tot voortschrijdende eiwitafbraak tijdens het bewaren, het toenemen van een bittere smaak en het ophelderen van de melk. Tijdens bewaren bij 20 °C geleerde de melk soms, bij 30 °C echter niet.

Wanneer proteolyse werd geremd door aprotinine en diisopropylfluorfosfaat (DFP) helderde de melk niet op en geleerde de melk niet tijdens de bewaarperiode.

De totale verhittingstijd tijdens het steriliseren hangt sterk af van de gebruikte apparatuur. De temperatuur-tijdcombinatie bij verhitting met de direkte methode en die bij verhitting met de indirekte methode vertonen opvallende verschillen. In deze studie kon een vergelijkbare houdbaarheid van direkt en indirekt gesteriliseerde melk worden verwezenlijkt door de heethoudtijd tijdens het steriliseren aan te passen. De producent van UHT-gesteriliseerde melk zou de condities van het steriliseren kunnen aanpassen aan de gewenste houdbaarheid van het produkt.

De viscositeit van UHT-gesteriliseerde vla is niet constant. De vla wordt

dunner tijdens bewaren bij 20 °C. Dit verschijnsel bleek samen te gaan met eiwitafbraak ten gevolge van restactiviteit van de natuurlijke melkproteïnase. Naast een daling van de viscositeit veroorzaakte deze eiwitafbraak een bittere smaak en een glazige consistentie. Aan het einde van de bewaarperiode van 16 weken trad zelfs destabilisering en gelering van het produkt op. Indien de vla bereid werd met heethoudtijden van 18 en 36 s bij 142 °C traden de gebreken niet op. Daarom is het aan te bevelen bij de bereiding van UHT-gesteriliseerde vla te letten op de inactivering van de melkproteïnase.

Restactiviteit van de melkproteïnase kon een versnelde gelering van UHT-gesteriliseerde geëvaporeerde melk te weeg brengen.

Door melk gedurende 60 min bij 55 °C voor te verhitten kon de houdbaarheid van de daarvan bereide UHT-gesteriliseerde melk aanzienlijk worden verbeterd. Tijdens een bewaarperiode van 11 weken bij 20 °C trad geen gelering op, het ophelderen van de melk en het bitter worden verliepen trager en de proteolyse schreed minder ver voort. Waarschijnlijk werd de melkproteïnase geremd of gedeeltelijk geïnactiveerd door de voorafgaande hittebehandeling.

Proteïnasen van gramnegatieve bacteriën in melk en melkprodukten

Pseudomonas fluorescens 22F produceerde in ladingsgewijze en continue culturen in melk pas proteïnase aan het einde van de exponentiële en tijdens de stationaire groeifase. Het aantal bacteriën bedroeg op dat moment meer dan 10^7 ml^{-1} . Het is daarom van belang te vermijden dat resten melk of van een melkprodukt in de apparatuur op de boerderij of in de fabriek achterblijven om te voorkomen dat bacteriën uitgroeien tot het niveau waarop proteïnasen worden geproduceerd.

Culturen van *P. fluorescens* 22F en *Achromobacter spec.* 1-10 die werden blootgesteld aan temperaturen boven 70 °C vertoonden een verlies aan proteolytische activiteit in een stap. Van al deze culturen verliep de hitte-inactivering volgens een reactie van de eerste orde. Hun proteïnasen waren zeer thermoresistent.

Het blootstellen van een cultuur van *Serratia marcescens* D2 aan temperaturen boven 50 °C resulteerde in een niet altijd volgens een reactie van de eerste orde verlopend verlies aan proteolytische activiteit. De inactivering bleek zich in drie stappen te voltrekken. De eerste stap verliep relatief snel en de derde stap relatief langzaam. Van de tussenliggende stap werd aangenomen dat deze een overgangssituatie vormde van de snelle naar de langzame inactivering. Het afbraakpatroon van caseïne en het temperatuur-activiteitprofiel wijzen erop dat het gaat om een enzym, weliswaar met een complexe hitte-inactivering en een transitie van pre-enzym tot actief enzym tijdens verhitten bij gematigde temperaturen.

De bacteriële proteïnasen kunnen van belang zijn voor (UHT-)gesteriliseerde

melk. Tijdens het bewaren kan deze melk in toenemende mate bitter worden en geleren als gevolg van groei van proteolytische bacteriën in rauwe melk.

Proteolyse ten gevolge van bacteriële enzymen ging gepaard met het toenemen van het gehalte aan niet-eiwitstikstof (NPN) en de vorming van para-κ-caseïne, terwijl de natuurlijke melkproteïnaseactiviteit werd vergezeld van het toenemen van het gehalte aan niet-caseïnestikstof (NCN) en de vorming van γ-caseïnen. Dit karakteristieke verschil doet een criterium aan de hand bij het diagnosticeren van de oorzaak van bepaalde gebreken die in de praktijk kunnen voorkomen.

REFERENCES

Adams, D.M., Barach, J.T. & Speck, M.L. (1975). Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci., 58: 828-834.

Adams, D.M., Barach, J.T. & Speck, M.L. (1976). Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins on ultrahigh temperature treatment. J. Dairy Sci., 59: 823-827.

Adams, D.M. & Brawley, T.G. (1981). Heat resistant bacterial lipases and ultra-high temperature sterilization of dairy products. J. Dairy Sci., 64: 1951-1957.

Alford, J.A. & Elliott, L.E. (1960). Lipolytic activity of microorganisms at low and intermediate temperatures. I. Action of *Pseudomonas fluorescens* on lard. Food Research, 25: 296-303.

Alford, J.A. & Pierce, D.A. (1961). Lipolytic activity of microorganisms at low and intermediate temperatures. II. Activity of microbial lipases at temperatures below 0 °C. J. Food Sci., 26: 518-524.

Alichanidis, E. & Andrews, A.T. (1977). Some properties of the extracellular protease produced by the psychrotrophic bacterium *Pseudomonas fluorescens* AR-11. Biochim. Biophys. Acta, 485: 424-433.

Andersen, K.P. & Kjaergaard Jensen, S.G. (1962). Lipase activity in milk and some dairy products. 136. Beretning fra Statens Forsøgsmejeri.

Andersson, R.E., Hedlund, C.B. & Jonsson, U. (1979). Thermal inactivation of heat-resistant lipase produced by the psychrotrophic bacterium *Pseudomonas fluorescens*. J. Dairy Sci., 62: 361-367.

Andersson, R.E. (1980a). Microbial lipolysis at low temperatures. Applied and Environmental Microbiol., 39: 36-40.

Andersson, R.E. (1980b). Lipase production, lipolysis, and formation of volatile compounds by *Pseudomonas fluorescens* in fat containing media. J. Food Sci., 45: 1694-1701.

Andrews, A.T. (1979). The formation and structure of some proteose peptone components. J. Dairy Res., 46: 215-218.

Autor, A.P. & Fridovich, I. (1970). The thermal inactivation of acetoacetate decarboxylase. J. biol. Chem., 245: 5223-5227.

Babcock, S.M. & Russell, H.L. (1897). Unorganized ferments of milk: a new factor in the ripening of cheese. Centralbl. f. Bakt. u. Parasitenk., 3: 615-620.

Babcock, S.M. & Russell, H.L. (1900). Galaktase, das der Milch eigentümliche proteolytische Ferment, seine Eigenschaften und seine Wirkung auf die Proteide der Milch. Centralbl. f. Bakt. u. Parasitenk., 6: 45-50; 79-88.

Bachman, K.C. (1982). Effect of exogenous estradiol and progesterone upon lipase activity and spontaneous lipolysis in bovine milk. J. Dairy Sci., 65: 907-914.

Barach, J.T., Adams, D.M. & Speck, M.L. (1976a). Stabilization of a psychrotrophic *Pseudomonas* protease by calcium against thermal inactivation in milk at ultrahigh temperature. Appl. Env. Microbiol., 31: 875-879.

Barach, J.T., Adams, D.M. & Speck, M.L. (1976b). Low temperature inactivation in milk of heat-resistant proteases from psychrotrophic bacteria. J. Dairy Sci., 59: 391-395.

Barach, J.T., Adams, D.M. & Speck, M.L. (1978). Mechanism of low temperature inactivation of a heat-resistant bacterial protease in milk. J. Dairy Sci., 61: 523-528.

Barlow, G., Summaria, L. & Robbins, K.C. (1969). Molecular weight studies on human plasminogen and plasmin at the microgram level. J. biol. Chem., 244: 1138-1141.

Bengtsson, G. & Olivecrona, T. (1977). Stimulation and inhibition of milk (lipoprotein) lipase by proteins from egg yolk lipoproteins. Eur. J. Biochem., 79: 225-231.

Bengtsson, G. & Olivecrona, T. (1981). Heparin-bound lipoprotein lipase is catalytically active and can be stimulated by apoprotein CII. FEBS Letters, 128: 9-12.

Björklund, A. (1970). Some lipolytic psychrophylic *Pseudomonas* bacteria and their hydrolysis of edible fats. Thesis, Technical University, Otaniemi, Finland.

Booy, H.L. (1964). Inleiding tot de fysische biochemie, 2nd ed., F. Bohn NV, Haarlem.

Brandts, J.F. (1967). In: A.H. Rose (Ed.), Thermobiology, Academic Press, New York, San Francisco, London, p. 25.

Brockerhoff, H. & Jensen, G.G. (1974). Lipolytic enzymes. Acad. Press, New York, San Francisco, London, p. 188-129.

Busta, F.F. (1967). Thermal inactivation characteristics of bacterial spores at ultrahigh temperatures. Appl. Microbiol., 15: 640-645.

Burton, H. (1958). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. J. Dairy Res., 25: 75-84.

Burton, H. & Perkin, A.G. (1970). Comparison of milks processed by the direct and the indirect methods of ultra-high temperature sterilization. 1. An experimental ultra-high-temperature sterilizer and its characteristics. J. Dairy Res., 37: 209-218.

Castberg, H.B. & Solberg, P. (1974). The lipoprotein lipase and the lipolysis in bovine milk. Meieriposten, 63: 961-975.

Castberg, H.B., Egelrud, T., Solberg, P. & Olivecrona, T. (1975). Lipases in bovine milk and the relationship between lipoprotein lipase and tributyrate hydrolyzing activities in cream and skim milk. J. Dairy Res., 42: 255-266.

Castellino, F.J. & Sodetz, J.M. (1976). Rabbit plasminogen and plasmin isoenzymes. Methods in Enzymology, 45: 273-286.

Chapman, H.R., Sharpe, M.E. & Law, B.A. (1976). Some effects of low-temperature storage of milk on cheese production and Cheddar cheese flavour. Dairy Ind. Int., 41: 42-45.

Chen, J.H. & Ledford, R.A. (1971). Purification and characterization of milk protease. J. Dairy Sci., 54: 763.

Cheng, W.S. & Gelda, C.S. (1974). Proteolysis in ultra-high-temperature treated and canned ten percent cream. J. Dairy Sci., 57: 1502-1504.

Claypool, L.L. (1965). Studies on milk lipase activation. Thesis, University of Minnesota.

Cogan, T.M. (1977). A review of heat resistant lipases and proteinases and the quality of dairy products. Irish J. Food Sci. Techn., 1: 95-105.

Cogan, T.M. (1979). Heat resistant lipases and proteinases and the quality of dairy products. IDF Bull. A-Doc., 43: 25-31.

Cousin, M.A. & Marth, E.H. (1977). Psychrotrophic bacteria cause changes in stability of milk to coagulation by rennet or heat. J. Dairy Sci., 60: 1042-1047.

Csiszar, J. & Romlehner-Bakos, A. (1956). Über die Hitzeresistenz der durch *Pseudomonas fluorescens* erzeugten Lipase. Proc. 14th Int. Dairy Congress, II(I): 48-56.

Debeukelar, N.J., Cousin, M.A., Bradley, R.L. & Marth, E.H. (1977). Modification of milk proteins by psychrotrophic bacteria. J. Dairy Sci., 60: 857-861.

Deeth, H.C. & Fitz-Gerald, C.H. (1976). Lipolysis in dairy products: a review. Austr. J. Dairy Technol., 31: 53-64.

Deeth, H.C., Fitz-Gerald, C.H. & Wood, A.F. (1979). Lipolysis and butter quality. Austr. J. Dairy Technol., 34: 146-149.

Desnuelle, P. (1961). Pancreatic lipase. Adv. in Enzymology, 23: 129-161.

Deutsch, D.G. & Mertz, E.T. (1970). Plasminogen: purification from human plasma by affinity chromatography. Science, 170: 1095-1096.

Downey, W.K. & Murphy, R.F. (1975). Partitioning of the lipolytic enzymes in bovine milk. Ann. Bull. IDF, 86: 19-23.

Driessen, F.M. & Stadhouders, J. (1974). A study of spontaneous rancidity. Neth. Milk Dairy J., 28: 130-145.

Driessen, F.M. & Stadhouders, J. (1975). The lipolytic enzymes & co-factors responsible for spontaneous rancidity in cow's milk. Ann. Bull. IDF, 86: 73-79.

Driessen, F.M. (1976a). A comparative study of the lipase in bovine colostrum and in bovine milk. Neth. Milk Dairy J., 30: 186-196.

Driessen, F.M. (1976b). Eiwitafbraak in stromend gesteriliseerde melk en melkprodukten. Zuivelzicht, 68: 514-515.

Driessen, F.M., Jellema, A., van Luin, P.J.F., Stadhouders, J. & Wolbers, G.J.M. (1977). The estimation of the fat acidity in raw milk. An adaption of the BDI method, suitable for routine assays. Neth. Milk Dairy J., 31: 40-55.

Dulley, J.R. (1972). Bovine milk proteinase. J. Dairy Res., 39: 1-9.

Eddy, B.P. (1960). The use and meaning of the term "psychrophilic". J. appl. Bact., 23: 189-190.

Edgerton, E.R., Jones, V.A. & Warren, J.A. (1970). Effect of process variables on the holding time in an ultrahigh-temperature steam injection system. J. Dairy Sci., 53: 1353-1357.

Egelrud, T. (1973). Studies on the enzyme lipoprotein lipase. Thesis. University of Umeå, Sweden.

Eigel, W.N. (1977). Effect of bovine plasmin on α_{s1}-B and κ-A caseins. J. Dairy Sci., 60: 1399-1403.

Fox, P.F. (1981). Proteinases in dairy technology. Neth. Milk Dairy J., 35: 233-253.

Fox, P.F. & Morrissey, P.A. (1981). Indigenous enzymes of bovine milk. In: Enzymes and food processing. Eds. G.G. Birch, N. Blakebrough & K.J. Parker. Appl. Sci. London, 213-238.

Frankel, E.N. & Tarassuk, N.P. (1956a). The specificity of milk lipase. II. Kinetics, and relative lipolytic activity in different milks. J. Dairy Sci., 39: 1517-1522.

Frankel, E.N. & Tarassuk, N.P. (1956b). The specificity of milk lipase. III. Differential inactivation. J. Dairy Sci., 39: 1523-1531.

Franklin, J.G., Williams, D.J. & Clegg, L.F.L. (1958). Methods of assessing the sporicidal efficiency of an ultra-high-temperature sterilizing plant. III. Laboratory determinations of the heat resistant spores of *Bacillus subtilis* in water and milk. J. appl. Bact., 21: 51-57.

Gebre-Egziabher, A., Humbert, E.S. & Blankenagel, G. (1980). Hydrolysis of milk proteins by microbial enzymes. J. Food Protection, 43: 709-712.

Ged, J. & Alais, C. (1976). Activé protéasique persistant dans les laits stérilisés. Le Lait, 56: 645-656.

Gilmour, A., Macelhinney, R.S., Johnston, D.E. & Murphy, R.J. (1981). Thermisation of milk. Some microbiological aspects. Milchwissenschaft, 36: 457-461.

Gordon, W.G., Groves, M.L., Greenberg, R., Jones, S.B., Kalan, E.B., Peterson, R.F. & Townend, R.E. (1972). Probable identification of γ-, TS-, R- and S-caseins as fragments of β-casein. J. Dairy Sci., 55: 261-263.

Griffiths, M.W., Phillips, J.D. & Muir, D.D. (1981). Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. J. appl. Bact., 50: 289-303.

Groves, M.L. (1971). Minor milk proteins and enzymes. In: Milk Proteins. Vol. II. Chemistry and molecular biology. Ed. H.A. McKenzie, Acad. Press, New York-London, p. 367-418.

Groves, M.L., Gordon, W.G., Kalan, E.B. & Jones, S.B. (1972). Composition of bovine γ-caseins A^1 and A^3 and further evidence for a relationship in biosynthesis of γ- and β-caseins. J. Dairy Sci., 55: 1041-1049.

Groves, M.L., Gordon, W.G., Kalan, E.B. & Jones, S.B. (1973). TS-A^2, TS-B, R- and S-caseins: their isolation, composition, and relationship to the β- and γ-casein polymorphs A^2 and B. J. Dairy Sci., 56: 558-568.

Guy, E.J. & Jenness, R. (1958). Separation, concentration and properties of alpha-amylase from cows' milk. J. Dairy Sci., 41:13-27.

Hallström, B. & Dejmek, P. (1977). Optimierung und vergleichende Beurteilung der UHT-Anlagen. Milchwissenschaft, 32: 324-327.

Halpaap, I. (1978). Vergleichende Characterizierung von Rinderplasmin und einer Proteinase aus Kuhmilch. Thesis, Technischen Hochschule Aachen.

Halpaap, I., Reimerdes, E.H. & Klostermeyer, H. (1977). Milchproteinasen. VI. Vergleichende Isolierung von Plasminogen aus Rinderblut und einer Proteinase aus Kuhmilch. Milchwissenschaft, 32: 341-346.

Harder, W. & Veldkamp, H. (1971). Competition of marine psychrophilic bacteria at low temperatures. Ant. v. Leeuwenhoek, 37: 51-63.

Harper, W.J. & Gould, I.A. (1959). Some factors affecting the heat-inactivation of the milk lipase enzyme system. XVth Int. Dairy Congress, I: 455.

Hartley, B.S. (1960). Proteolytic enzymes. Ann. Rev. Biochem., 29: 45-72.

Harwalkar, V.R. & Vreeman, H.J. (1978). Effect of added phosphates and storage on changes in ultra-high temperature short-time sterilized concentrated skim milk. 1. Viscosity, gelation, alcohol stability, chemical and electrophoretic analysis of proteins. Neth. Milk Dairy J., 32: 94-111.

Hernell, O., Egelrud, T. & Olivecrona, T. (1975). Serum-stimulated lipases (lipoprotein lipases). Immunological crossreaction between the bovine and the human enzymes. Biochim. Biophys. Acta, 381: 233-241.

Herreid, E.O. & Wilson, H.K. (1963). Sterile concentrated milk. Manuf. Milk Prod. J., 54: 14-15, 31.

Hetrick, J.H. & Tracy, P.H. (1948). Effect of high-temperature short-time heat treatments on some properties of milk. II. Inactivation of the lipase enzyme. J. Dairy Sci., 31: 881-887.

Hillier, R.M. (1976). The quantitative measurement of whey proteins using polyacrylamide-gel electrophoresis. J. Dairy Res., 43: 259-265.

Hofmann, C.J., Keenan, T.W. & Eigel, W.N. (1979). Association of plasminogen with bovine milk fat globule membrane. Int. J. Biochem., 10: 909-917.

Hostettler, H., Stein, J. & Bruderer, G. (1957). Zur Frage des Nachdickens von sterilisierter Milch. Landwirtschaftliches Jahrbuch der Schweiz, 71: 143-164.

Hostettler, H. (1972). IDF monograph on UHT milk. 2. Appearance, flavour and texture aspects. Ann. Bull. IDF, part V: 6-34.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bact., 66: 24-26.

Humbert, G. & Alais, C. (1979). Review of the progress of dairy science: the milk proteinase system. J. Dairy Res., 46: 559-571.

Iverius, P.-H. & Östlund-Lindqvist, A.-M. (1976). Lipoprotein lipase from bovine milk. Isolation procedure, chemical characterization, and molecular weight analysis. J. biol. Chem., 251: 7791-7795.

Jellema, A. (1975). Note on susceptibility of bovine milk to lipolysis. Neth. Milk Dairy J., 29: 145-152.

Jellema, A. & Schipper, C.J. (1975). Influence of physiological factors on the lipolytic susceptibility of milk. Ann. Bull. IDF, 86: 2-6.

Jenness, R. & Patton, S. (1959). Principles of dairy chemistry. J. Wiley & Sons Inc., New York, p. 182-203.

Jenness, R. & Koops, J. (1962). Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J., 16: 153-164.

Jensen, R.G., Sampugna, J., Pereira, R.L., Chandan, R.C. & Shahani, K.M. (1964). Specificity of milk lipase for a butyryl triglyceride. J. Dairy Sci., 47: 1012-1013.

Johnson, A.H. (1974). The composition of the milk. In: Fundamentals of dairy chemistry. Eds. B.H. Webb, A.H. Johnson & J.A. Alford. The Avi Publ. Comp. Inc., Westport, Connecticut. p. 31-42.

De Jong, L. (1975). A quantitative electrophoretic method of studying cheese ripening. Neth. Milk Dairy J., 29: 162-168.

Juffs, H.S. (1976). Effects of temperature and nutrients on proteinase production by *Pseudomonas fluorescens* and *Ps. aeruginosa* in broth and milk. J. appl. Bact., 40: 23-32.

Kaminogawa, S., Yamauchi, K. & Tsugo, T. (1969). Properties of milk protease concentrated from acid-precipitated casein. Jap. J. Zootech. Sci., 40: 559-565.

Kaminogawa, S., Sato, F. & Yamauchi, K. (1971). Purification and some properties of milk protease. Agric. Biol. Chem., 35: 1465-1467.

Kaminogawa, S., Mizobuchi, H. & Yamauchi, K. (1972). Comparison of bovine milk protease with plasmin. Agric. Biol. Chem., 36: 2163-2167.

Kaminogawa, S., & Yamauchi, K. (1972). Acid protease of bovine milk. Agric. Biol. Chem., 36: 2351-2356.

Kaminogawa, S. Yamauchi, K., Sasaki, S. & Koga, Y. (1978). Degradation of casein components by milk acid protease. Proc. 20th. Int. Dairy Congress, IE: 313.

Kandler, O. (1966). Zur Definition der "psychrophilen" Bakterien. Milchwissenschaft, 21: 257-261.

Khan, I.M., Chandan, R.C., Dill, C.W. & Shahani, K.M. (1964). Production and properties of the extracellular lipase of *Achromobacter lipolyticum*. J. Dairy Sci., 47: 675.

Kiermeier, F. & Semper, G. (1960a). Über das Vorkommen eines proteolytischen Enzyms und eines Trypsin-Inhibitors in Kuhmilch. I. Mitteilung. Zur proteolytische Wirkung. Ztschr. f. Lebensm. -Unters. u. Forschung, 111: 282-307.

Kiermeier, F. & Semper, G. (1960b). Über das Vorkommen eines proteolytischen Enzyms und eines Trypsin-Inhibitors in Kuhmilch. II. Mitteilung. Zur inhibitorischen Wirkung. Ztschr. f. Lebensm. -Unters. u. Forschung, 111: 373-380.

Kishonti, E. & Sjöström, G. (1970). Influence of heat resistant lipases and proteases in psychrotrophic bacteria on product quality. 18th Int. Dairy Congress, IE: 501.

Kishonti, E. (1975). Influence of heat resistant lipases and proteases in psychrotrophic bacteria on product quality. IDF Doc. 86: 121-124.

Kjaergaard Jensen, G. (1971). The influence of some processing aspects upon the properties and keeping quality of whole milk powder. 187. Beretning Statens Forsøgsmejeri-Hillerød.

Kjaergaard Jensen, G. & Slot Hansen, P. (1972). Experiments concerning the importance of pasteurizing by production of milk powder. 195. Beretning Statens Forsøgsmejeri-Hillerød.

Kleter, G. (1976). The ripening of Gouda cheese made under strict aseptic conditions. 1. Cheese with no other bacterial enzymes than those from a starter streptococcus. Neth. Milk Dairy J., 30: 254-270.

Kleter, G. (1977). The ripening of Gouda cheese made under strictly aseptic conditions. 2. The comparison of the activity of different starters and the influence of certain lactobacillus strains. Neth. Milk Dairy J., 31: 177-187.

De Koning, P.J. & Kaper, J. (1981). The effect of protease inhibitor and the Maillard reaction on the gelation of UHTST-concentrated casein micelle solution. Neth. Milk Dairy J., 35: 323-327.

Koops, J., Klomp, H. & Elgersma, R.H.C. (1975). Rapid determination of nitrogen in milk and dairy products by colorimetric estimation of ammonia following an accelerated digestion procedure. Neth. Milk Dairy J., 29: 169-180.

Koops, J. & Tarassuk, N.P. (1959). The effect of various processing treatments on the partition of phosphatides between the fat phase and the milk plasma. Neth. Milk Dairy J., 13: 180-188.

Korn, E.D. (1962). The lipoprotein lipase of cow's milk. J. Lipid Res., 3: 246-250.

Kosikowski, F. (1977). Cheese and fermented milk foods. Edwards Brothers Inc., Ann Arbor, Michigan.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature, 178: 703.

Kuzdzal-Savoie, S. (1979). Determination of free fatty acids in milk and milk products. IDF Bull. A-Doc 43: 52-65.

Landaas, A. & Solberg, P. (1978). Production and characterization of lipase from a fluorescent pseudomonad. 20th Int. Dairy Congress, E: 304-305.

Langeveld, L.P.M., Cuperus, F. & Stadhouders, J. (1973). Bacteriological aspects of the keeping quality at 5 °C of reinfected and non-reinfected pasteurized milk. Neth. Milk Dairy J., 27: 54-65.

Langeveld, L.P.M. & Cuperus, F. (1980). The relation between temperature and growth rate in pasteurized milk of different types of bacteria which are important to the deterioration of that milk. Neth. Milk Dairy J., 34: 106-125.

Law, B.A., Sharpe, M.E. & Chapman, H.R. (1976). The effect of lipolytic Gram-negative psychrotrophs in stored milk on the development of rancidity in Cheddar cheese. J. Dairy Res., 43: 459-468.

Law, B.A., Andrews, A.T. & Sharpe, M.E. (1977). Gelation of ultra high temperature sterilized milk by proteases from a strain of *Pseudomonas fluorescens* isolated from raw milk. J. Dairy Res., 44: 145-178.

Law, B.A. (1979). Reviews on the progress of dairy science: enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res., 46: 573-588.

Law, B.A., Andrews, A.T., Cliffe, A.J., Sharpe, M.E. & Chapman, H.R. (1979). Effect of proteolytic raw milk psychrotrophs on the Cheddar cheese making with stored milk. J. Dairy Res., 46: 497-509.

Lawrence, R.C. (1967). Microbial lipases and related esterases. Part II. Estimation of lipase activity, characterization of lipases. Recent work concerning their effect on dairy products. Dairy Sci. Abstr., 29: 59-70.

Lawrence, R.C., Fryer, T.F. & Reiter, B. (1967). The production and characterization of lipases from a *Micrococcus* and a pseudomonad. J. gen. Microbiol., 48: 401-418.

Lu, J.Y. & Liska, B.J. (1969). Lipase from *Pseudomonas fragi*. I. Purification of the enzyme. Appl. Microbiol., 18: 104-107.

Malik, A.C. (1975). Heat stable proteases from strains of *Pseudomonas fluorescens*. Thesis, Univ. of Wisconsin, Madison, Wisconsin, USA.

Marshall, R.T. & Marstiller, J.K. (1981). Unique response to heat of extracellular protease of *Pseudomonas fluorescens* M5. J. Dairy Sci., 64: 1545-1550.

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

McKellar, R.C. (1981). The induction by skim milk of the extracellular protease of *Pseudomonas fluorescens*. Abstr. Joint Ann. Meeting Can Soc. of Microbiology.

Mencher, J.R. & Alford, J.A. (1967). Purification and characterization of the lipase of *Pseudomonas fragi*. J. gen. Microbiol., 48: 317-328.

Motai, H., Ichishima, E. & Yoshida, F. (1966). Purification and properties of lipase from *Torulopsis*.

Muir, D.D., Kelly, M.E., Phillips, J.D. & Wilson, A.G. (1978). The quality of blended raw milk in creameries in South-West Scotland. J. Soc. Dairy Technol., 31: 137-144.

Muir, D.D., Phillips, J.D. & Dalgleish, D.G. (1979). The lipolytic and proteolytic activity of bacteria isolated from blended raw milk. J. Soc. Dairy Technol., 32: 19-23.

Mulder, H. & Walstra, P. (1974). The milk fat globule. p. 186-187. Pudoc, Wageningen, the Netherlands.

Nakai, S., Wilson, H.K. & Herreid, E.O. (1964). Assaying sterile concentrated milk for native proteolytic enzymes. J. Dairy Sci., 47: 754-757.

Nashif, S.A. & Nelson, F.E. (1953a). The lipase of *Pseudomonas fragi*. I. Characterization of the enzyme. J. Dairy Sci., 36: 459-470.

Nashif, S.A. & Nelson, F.E. (1953b). The lipase of *Pseudomonas fragi*. II. Factors affecting lipase production. J. Dairy Sci., 36: 471-480.

Nilsson, R. & Willart, S. (1961). Lipolytic activity in milk. II. The heat inactivation of the fat-splitting in milk. Report Milk and Dairy Res. No 64, Alnarp, Sweden.

Noomen, A. (1975). Proteolytic activity of milk protease in raw and pasteurized cow's milk. Neth. Milk Dairy J., 29: 153-161.

Noomen, A. (1978). Activity of proteolytic enzymes in simulated soft cheeses (Meshanger type). 1. Activity of milk proteinase. Neth. Milk Dairy J., 32: 26-48.

Noreau, J. & Drapeau, G.R. (1979). Isolation and properties of the protease from the wild-type and mutant strains of *Pseudomonas fragi*. J. Bact., 140: 911-916.

Olivecrona, T., Egelrud, T., Iverius, P.-H. & Lindahl, U. (1971). Evidence for an ionic binding of lipoprotein lipase to heparin. Biochem. Biophys. Res. Comm., 43: 524-529.

Olivecrona, T., Egelrud, T., Hernell, O., Castberg, H. & Solberg, P. (1975). Is there more than one lipase in bovine milk? Ann. Bull. IDF, 86: 61-66.

Olivecrona, T. (1977). Biochemical aspects of lipolysis in bovine milk. IDF Bull., A-Doc 43: 18-24.

Ota, Y. & Yamada, K. (1967). Lipase from *Candida paralipolytica*. Part III. Further studies on the activation of the enzyme systems with bile or calcium salts. Agr. Biol. Chem., 31: 809-816.

Parry, R.M., Chandan, R.C. & Shahani, K.M. (1966). A rapid and sensitive assay for milk lipase. J. Dairy Sci., 49: 356-360.

Patel, V., Goldberg, H.S. & Blenden, D. (1964). Characterization of leptospiral lipase. J. Bacteriol., 88: 877-884.

Payens, T.A.J. (1962). Algemene en bijzondere aspecten van de verhitting van melkeiwitten. Chem. Weekblad, 58: 219-223.

Petricca, A.V. & Harper, W.J. (1970). Some characteristics of selected microbial lipases. Centennial Res. Ser. Dep. Dairy Technol., Ohio State University, 6: 27-44.

Pinheiro, A.J.R., Liska, B.J. & Parmelee, C.E. (1965). Heat stability of lipases of selected psychrophilic bacteria in milk and Purdue Swiss-type cheese. J. Dairy Sci., 48: 983-984.

Porzio, M.A. & Pearson, A.M. (1975). Isolation of an extracellular neutral proteinase from *Pseudomonas fragi*. Biochim. Biophys. Acta, 384: 235-241.

Priest, F.G. (1977). Extracellular enzyme synthesis in the genus *Bacillus*. Bact. Reviews, 41: 711-753.

Raadsveld, C.W. & Mulder, H. (1949). Influence of the temperature on the ripening of Edam cheese. Neth. Milk Dairy J., 3: 117-141 and 222-230.

Radaeva, I.A., Bogdanov, V.M. & Shtal'berg, S.M. (1966). Rancid flavour in sweetened condensed milk. Dairy Sci. Abstr., 28: 2090.

Reimerdes, E.H. & Klostermeyer, H. (1974). Milchproteasen. I. Micellenassoziierte Protease-Anreicherung und Hydrolyse von Caseinen. Milchwissenschaft, 29: 517-523.

Reimerdes, E. (1976). Eiweissabbau in Milch. Molkerei-Ztg. Welt der Milch, 30: 785-788.

Reimerdes, E.H., Klostermeyer, H. & Sayk, E. (1976). Milch-Proteinasen. VII. Fraktionierung von Komponenten des Proteinasen-Inhibitor-Systems der Milch. Milchwissenschaft, 31: 329-334.

Reimerdes, E.H. & Petersen, F. (1978). Purification of a milk proteinase by affinity chromatography. Proc. 20th. Int. Dairy Congress, IE: 311-312.

Reimerdes, E.H., Petersen, F. & Kielwein, G. (1979). Milchproteinasen. 9. Proteinasespektren von Caseinmicellen, Milchserum, Rinderblutserum und *Pseudomonas fluorescens*. Milchwissenschaft, 34: 548-551.

Reiter, B., Sorokin, Y., Pickering, A. & Hall, A.J. (1969). Hydrolysis of fat and protein in small cheeses made under aseptic conditions, J. Dairy Res., 36: 65-76.

Rice, F.E. & Markley, A.L. (1922). Proof of the presence of lipase in milk and a new method for the detection of the enzyme. J. Dairy Sci., 5: 64-82.

Richardson, B.C. & Te Whaiti, I.E. (1978). Partial characterization of heat-stable extracellular proteases of some psychrotrophic bacteria from raw milk. N. Zld. J. Dairy Sci. Techn., 13: 172-176.

Richardson, B.C. & Pearce, K.N. (1981). The determination of plasmin in dairy products. N. Zld. J. Dairy Sci. Techn., 16: 209-220.

Richardson, B.C. (1981). The purification and characterization of a heat-stable protease from *Pseudomonas fluorescens* B52. Nw. Zld. J. Dairy Sci. Techn., 16: 195-207.

Robbins, K.C., Bernabe, P., Arzadon, L. & Summaria, L. (1973). The primary structure of human plasmin: light (B) chain active center histidine sequence. J. biol. Chem., 248: 1631-1633.

Rollema, H.S., Visser, S. & Poll, J.K. (1981). On the determination, purification and characterization of the alkaline proteinase from bovine milk. Neth. Milk Dairy J., 35: 396-399.

Rollema, H.S., Visser, S. & Poll, J.K. (1983). Spectrophotometric assay of plasmin and plasminogen in bovine milk. Milchwissenschaft, 38: 214-217.

Rottem, S. & Razin, S. (1964). Lipase acitivity of *Mycoplasma*. J. gen. Microbiol., 37: 123-134.

Rowe, M.T. & Gilmour, A. (1982). Growth, enzyme production and changes in oxygen tension occurring curing batch cultivation of psychrotrophic *Pseudomonas fluorescens* strains. Milchwissenschaft, 37: 597-600.

Samuelsson, E.G. & Holm, S. (1966). Technological principles for ultra-high-heat treatment of milk. Proc. 17th. Int. Dairy Congress, B1: 57-65.

Sandelin, A.E. (1939). Fettets sönderdelning vid de hårda ostarnas mogning. Nordisk Mejeri-Tidsskrift, 5: 102-104.

Schaafsma, G.J. (1979). De invloed van verschillende hittebehandelingen en bewaaromstandigheden op de voedingswaarde van melk. Melk in relatie tot gezondheid, 6 (3): 1-23.

Schmidt, D.G. & Both, P. (1975). Methods of gel electrophoresis of milk proteins. Ed. H. Swaisgood. North Carolina State University, Raleigh, USA, p. 7-9.

Schulz, M.E. (1965). Das grosse Molkerei-Lexikon. Volksw. Verlag Kempten p. 272.

Schwartz, G. & Ciblis, E. (1965). Beitrag zur Vorausbestimmung der haltbarkeit von im Kühlhaus gelagerter Sauerrahmbutter. Molkerei und Käserei-Ztg., 16: 605-612.

Scow, R.O. & Olivecrona, T. (1977). Effect of albumin on products formed from chylomicron triacylglycerol by lipoprotein lipase in vitro. Biochim. Biophys. Acta, 487: 472-486.

Shah, D.B. & Wilson, J.B. (1965). Egg yolk factor of *Staphylococcus aureus*. II. Characterization of the lipase activity. J. Bacteriol., 89: 949-953.

Shahani, K.M. (1966). Milk enzymes: their role and significance. J. Dairy Sci., 49: 907-920.

Shahani, K.M., Harper, W.J., Jensen, R.G., Parry, R.M. & Zittle, C.A. (1973). Enzymes in bovine milk: a review. J. Dairy Sci., 56: 531-543.

Shehata, T.E. & Collins, E.B. (1971). Isolation and identification of psychrophilic species of *Bacillus* from milk. Appl. Microbiol., 21: 466-469.

Shipe, W.F. & Senyk, G.F. (1981). Effects of processing conditions on lipolysis in milk. J. Dairy Sci., 64: 2146-2149.

Sjöström, G. (1959). Lipase problems in milk and dairy products. A review. Report No 58, Alnarp, Sweden.

Skean, J.D. & Overcast, W.W. (1960). Changes in the paper electrophoretic protein patterns of refrigerated skim milk accompanying growth of three *Pseudomonas* species. Appl. Microbiol., 8: 335-338.

Söhngen, N.L. (1912). Fat-splitting by bacteria. Zentralbl. Bakt. II Abt., 31: 292-293.

Speck, M.L. & Adams, D.M. (1976). Heat resistant proteolytic enzymes from bacterial sources. J. Dairy Sci., 59: 786-789.

Snoeren, T.H.M. & van Riel, J.A.M. (1979). Milk proteinase, its isolation and action on α_{s2}- and β-casein. Milchwissenschaft, 34: 528-531.

Snoeren, T.H.M., van Riel, J.A.M. & Both, P. (1980). Enkele eigenschappen van het uit UHT-gesteriliseerde melk geïsoleerde melkproteïnase. Zuivelzicht, 72: 42-43.

Stadhouders, J. (1956). De hydrolyse van vet bij de kaasrijping in verband met de smaak van kaas. Thesis, Wageningen.

Stadhouders, J. & Mulder, H. (1957). Fat hydrolysis and cheese flavour. I. The enzymes responsible for the hydrolysis of fat in cheese. Neth. Milk Dairy J., 11: 164-183.

Stadhouders, J. & Mulder, H. (1958). Fat hydrolysis and cheese flavour. II. Micro-organisms involved in the hydrolysis of fat in the interior of the cheese. Neth. Milk Dairy J., 12: 237-263.

Stadhouders, J. & Mulder, H. (1960). Fat hydrolysis and cheese flavour. IV. Fat hydrolysis in cheese from pasteurised milk. Neth. Milk Dairy J., 14: 141-147.

Stadhouders, J. (1969). Het gebrek "hydrolytisch rans" in kaas. NIZO-Mededelingen, 5: 1-16.

Stadhouders, J. (1973). Bacteriologische aspecten van tankmelk. NIZO-Mededelingen, 8: 24-40.

Stadhouders, J. & Veringa, H.A. (1973). Fat hydrolysis by lactic acid bacteria in cheese. Neth. Milk Dairy J., 27: 77-91.

Stadhouders, J. (1982). Cooling and thermization as a mean to extend the keeping quality of raw milk. Kieler Milchwirtschaftliche Forschungsberichte, 34: 19-28.

Stern, J.A., Herlin, M.A. & Proctor, B.E. (1952). An electronic method for continuous determination of rapid temperature changes in thermal death-time studies. Food. Res., 17: 460-465.

Stern, J.A. & Proctor, B.E. (1954). A micro-method and apparatus for the multiple determinations of rates of destruction of bacteria and bacterial spores subjected to heat. Food Techn., 8: 139-143.

Steward, D.B., Murray, J.G. & Neill, S.D. (1975). Lipolytic activity of organisms isolated from refrigerated bulk milk. Ann. Bull. IDF, 86: 38-50.

Storgårds, T. & Hietaranta, M. (1949). Microbial oxidation of butter fat. XIIth Int. Dairy Congress, II: 280-283.

Stroup, W.H., Parker, R.W., Dickerson, R.W. & Read, R.B. (1981). Temperature variations in the holding tube during ultra high-temperature pasteurization by steam injection. J. Dairy Sci., 55: 177-182.

Sugiura, M. & Oikawa, T. (1977). Physicochemical properties of a lipase from *Pseudomonas fluorescens*. Biochim. Biophys. Acta, 489: 262-268.

Suhren, G. (1981). Occurrence and levels of heat resistant proteinases and their effects on UHT-treated dairy products. IDF Bull. F-Doc., 85: 1-8.

Tanford, C. (1968). Protein denaturation. Adv. Protein Chem., 23: 121-282.

Tarassuk, N.P. & Frankel, E.N. (1957). The specificity of milk lipase. IV. Partition of the lipase system in milk. J. Dairy Sci., 40: 418-430.

Thomas, S.B. (1974a). The influence of the refrigerated farm bulk tanks on the quality of the milk at the processing dairy. J. Soc. Dairy Technol., 27: 180-189.

Thomas, S.B. (1974b). The microflora of bulk collected milk. Part 2. Dairy Industries, 39: 279-282.

Trout, G.M. (1950). Homogenized milk. Michigan State College Press, East Lansing.

Visser, S., Slangen, K.J. & Hup, G. (1975). Some bitter peptides from rennet-treated casein. A method for their purification, utilizing chromatographic separation on silica gel. Neth. Milk Dairy J., 29: 319-334.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. Neth. Milk Dairy J., 35: 65-88.

Vos, E.A. & Mol, J.J. (1979). Het verstuivingsdroogproces, in het bijzonder van melk. NIZO-Mededelingen, 12: p. 90.

Walstra, P. & van der Haven, M.C. (1979). Melkkunde. Een inleiding in samenstelling, structuur en eigenschappen van melk. Landbouwhogeschool Wageningen, p. 148.

Walstra, P. & Jenness, R. (1983). Dairy chemistry and physics. Wiley, New York.

Washam, C.J., Olson, H.C. & Vedamuthu, E.R. (1977). Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. J. Food Protection, 40: 101-108.

West, F.B., Adams, D.M. & Speck, M.L. (1978). Inactivation of heat resistant proteases in normal ultra-high temperature sterilized skim milk by a low temperature treatment. J. Dairy Sci., 61: 1078-1084.

Wiersma, M. (1978). Regulation of extracellular proteolytic enzyme synthesis in Vibrio SA 1. Thesis, RU Groningen.

Witter, L.D. (1961). Psychrophilic bacteria - a review. J. Dairy Sci., 44: 983-1015.

Wüthrich, S., Richterich, R. & Hostettler, H. (1964). Untersuchungen über Milchenzyme. II. Mitteilung. Enzyme in zentrifugierter und hitzebehandelter Kuhmilch. Ztschr. Lebensm. -Unters. u. Forschung, 124: 345-348.

Yamauchi, K. & Kaminogawa,S. (1972). Decomposition of milk proteins by milk protease. Agric. Biol. Chem., 36: 249-254.

CURRICULUM VITAE

De auteur werd geboren op 21 december 1942 te Nijmegen. Na het behalen van het diploma HBS-B aan het Canisius College te Nijmegen vervulde hij de militaire dienstplicht. Na 1 jaar in dienst te zijn geweest van de AKU te Arnhem (in de functie van materiaalanalist) maakte hij in september 1964 een aanvang met de studie biologie aan de Katholieke Universiteit te Nijmegen. In september 1969 behaalde hij het doctoraal examen met als hoofdvak zoölogie en als bijvakken botanie en cyto-histologie.

Sedert december 1969 is hij als wetenschappelijk medewerker verbonden aan de afdeling microbiologie (hoofd: Dr.Ir. J. Stadhouders) van het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede. Hier werd hij belast met het onderzoek met betrekking tot gefermenteerde consumptiemelkprodukten en microbiële enzymen in melk en melkprodukten. Aldaar werd het in dit proefschrift beschreven onderzoek verricht.