Factors affecting proteolytic action of *Lactococcus lactis* in cheese.

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Factors affecting proteolytic action of *Lactococcus lactis* in cheese

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THEOREMS

1. The stretching process of acid curd in the manufacture of "pasta filata" types of cheese, such as Mozzarella, is likely to cause less rennet to become inactivated than is generally assumed.

This thesis

2. The results given by Monib on the dissolution of inorganic phosphorus in the sera of reconstituted soft and hard cheeses under various physico-chemical conditions, are mutually conflicting.

A. M. M. F. Monib. The calcium-paracaseinate-phosphate complex under conditions similar to those in cheese. Thesis, Wageningen Agricultural University, 1962.

3. It is generally assumed that cheeses of the same variety but of a higher water content mature faster. It is, however, unlikely that such an effect is due to differences in the composition of the moisture in the cheese or in the attackability of the protein.

This thesis

4. The stability of proteolytic enzymes of starter bacteria that can be active in cheese is largely unknown and may well be an important variable in relation to cheese maturation.

This thesis

5. The experimental procedure used by Driessen to study the heat inactivation kinetics of alkaline milk proteinase in milk, does not allow to obtain a decisive answer on the completeness of the enzyme inactivation.

F.M. Driessen. Lipases and proteinases in milk. Thesis, Wageningen Agricultural University, 1983.

This thesis

6. It is well possible that the role of intracellular peptidases from starter bacteria in the maturation of cheese, greatly depends on the extent of lysis of these bacteria during an early stage of maturation.

This thesis

7. To prevent the loss of valuable nutrients and diminish environmental pollution with the salted whey, the traditional method of manufacture of white pickled cheese in Egyptian factories should be replaced by modern cheesemaking technologies, preferably based on ultrafiltration of the milk.

8. A cheese produced by applying methods for accelerated ripening, generally should not, how ever good it may be, be designated as a cheese of the variety from which it was derived.

9. The publications by Gouda et al. on aspects of cheese making and cheese properties should not lead to the conclusion that Gouda cheese was developed in Egypt.

A. Gouda, S. A. El-Shabrawy, A. El-Zayat & E. El-Bagoury. Egyptian J. Dairy Sci. 13 (1985) 115-119.

A. Gouda & S. El-Shibiny. Egyptian J. Dairy Sci. 15 (1987) 255-262.

A. Gouda. Egyptian J. Dairy Sci. 15 (1987) 15-23.

Theorems to thesis "Factors affecting proteolytic action of Lactococcus lactis in cheese" by Youssef Bahr Youssef, Wageningen, September 4th 1992.

PREFACE

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ABSTRACT

Bahr Youssef, Y., (1992). Factors affecting proteolytic action of *Lactococcus lactis* in cheese. Ph.D. thesis, Agricultural University, Wageningen. (pp 99, English and Dutch summaries).

Keywords:
cheese ripening, model cheese, *Lactococcus lactis ssp. cremoris HP*, protein breakdown, amino acids, cheese composition, ripening temperature, autolysis

Abstract:

Model cheeses were developed to study the behaviour of proteolytic agents involved in cheese maturation under conditions that closely resemble those in normal cheese. The models were applied to study protein breakdown by *Lactococcus lactis ssp. cremoris HP*, as a function of various ripening variables. Proteolysis was monitored by several methods; amino acid-N determinations and amino acid analyses were best suited for quantitatively following protein breakdown. Amino acid production was enhanced at a higher pH, at a lower NaCl content and at a higher ripening temperature of cheese; much larger quantities of amino acids were produced from paracasein being predigested by rennet than from untreated paracasein. Ratios of paracasein to water of 1:2.5 or 1:3.5 did not significantly affect amino acid formation by the starter strain. Compared to intact cells, lysed cells drastically enhanced amino acid production; the results pointed to lysis of a (minor) part of the starter population. It is argued that the use of these model systems can produce unequivocal results.

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CHAPTER 1

General introduction

Degradation of protein is a major biochemical process during cheese ripening, crucially determining taste and flavour formation in cheese. The importance of the extent and of the character of proteolytic processes to the development of desired characteristics of cheese have been reviewed by several authors (e.g. Castberg & Morris, 1976; Desmazeaud & Gripon, 1977; Grappin *et al.*, 1985; Rank *et al.*, 1985; Law, 1987; Fox, 1988-1989, 1989; Kamaly & Marth, 1989). Proteolysis in cheese was also intensively studied at the Dairying Laboratory of the Agricultural University at Wageningen, the Netherlands (e.g. Mulder, 1952; Ali, 1960; Kleter, 1977; Visser, 1977*c*; Jong, 1978; Noomen, 1978 *a*,*b*, 1983).

The main proteolytic sources for ripening of cheeses without a specific microbial surface flora are: rennet enzymes, being strongly active in most varieties of cheese (except those made from sweet curd submitted to a high scalding temperature, like Emmental cheese, and cheese with acid curd stretched at high temperature, like Mozzarella cheese); enzymes from the cultures of lactic acid bacteria (starter bacteria) used in cheese making and, of much less importance, indigenous milk proteinases being strongly associated with the casein in milk and thus passing almost quantitatively into cheese. Particular bacteria of the milk flora and organisms which contaminate the milk during processing, may grow out in cheese made from raw milk and contribute to proteolysis. In cheeses made from HTST-pasteurized milk (e.g. 15 s at 72°C), growth of bacteria from the raw milk which survive pasteurization or their heat-resistant enzymes, in particular enzymes from psychrotrophic bacteria, and growth of organisms which recontaminate the milk after pasteurization, may do so. Growth of micro-organisms like bacteria, yeasts and moulds on the cheese surface also affects proteolysis. This especially holds true for cheeses ripening under the influence of a surface flora specific for the variety of cheese, e.g. growth of *Penicillium camemberti* on Camembert cheese and of *Brevibacterium linens* on Limburger cheese. Development of an internal flora of moulds is desired in blue-veined cheeses, e.g. growth of *Penicillium roqueforti* in Roquefort cheese.

In each variety of cheese many enzymes of various origin, and their interactions, will determine the progress and the character of protein breakdown. This causes the study of mechanisms of proteolysis in cheese to be far from easy.

1.1 Role of starter bacteria in proteolysis of cheese

Aseptically made, chemically acidified types of Gouda cheese which were ripened solely by the action of calf rennet and of milk proteinases, completely failed to develop cheese taste and flavour. Only bitterness became apparent, its intensity being dependent on the quantity of rennet enclosed in the cheese and the time of cheese ripening (Visser, 1977*d*). The experiments clearly established the crucial role of the proteolytic system of starter bacteria in the formation of flavour compounds in cheese. This results from the degradation of casein, and in most varieties of cheese in particular from the further conversion of predominantly larger peptides formed by rennet action to small peptides and especially to amino acids. These may contribute directly to flavour and they may serve as precursors for the production of more specific aroma compounds.

The proteolytic system of starter bacteria consists of proteinases and peptidases; cell wall-associated, cell membrane-associated and intra-cellular enzymes may be distinguished. Bacterial proteolytic activity of these organisms was, however, reported to be weak compared with other groups of bacteria (Law & Kolstad, 1983). To gain knowledge on the mechanisms of protein breakdown by the bacterial cell, the nature and location of enzymes and their interactions were studied intensively (e.g. Law, 1980; Exterkate, 1984, 1990; Baankreis, 1992).

The activity of any enzyme primarily depends on its quantity in cheese. Quantities of proteolytic enzymes during cheese ripening thus are determined by the number and the proteolytic system of the starter bacteria, for instance whether or not the cell is provided with particular cell wall proteinases permitting rapid growth of the bacterium in milk (Prt^+ and Prt^- variants, respectively). Among factors affecting growth, the final number of a starter bacterium in cheese may depend on simultaneous growth of other bacteria involved in lactic acid fermentation, e.g. on commensalism between Prt^+ and Prt^- variants of strains. In addition to its quantity, the contribution of an enzyme to protein breakdown is governed by several conditions. Proteolysis is even more intricate because conditions

change during cheese maturation, some of them quite significantly. Important variables are: temperature, pH, sodium chloride content in the moisture and water content or, preferably, protein to water ratio. In varieties of cheese made traditionally through the expulsion of by far the greater part of the whey containing the serum proteins, this ratio is almost identical to the ratio of paracasein to water. Together with other factors, especially pH, this ratio largely determines the chemical composition of the cheese moisture, for instance its calcium content, and the conformation of the protein; the ratio thus may affect enzyme activity and attackability of the substrate. The water content of cheese may also directly influence the velocity of enzymic reactions through its effect on the diffusion coefficients of various compounds. Modern varieties of ripened cheese made from ultrafiltered milk contain the serum proteins in addition to paracasein, and this probably affects proteolysis. Compared with the traditional types of cheese they develop flavour less rapidly and insufficiently; the reasons are not well understood.

A significant contribution to proteolysis requires an enzyme to be quite stable in cheese; it must not or only very slowly be inactivated. Remarkably, very little work has been done on factors determining the stability of proteolytic enzymes of starter bacteria under conditions as in a ripening cheese, and it is uncertain whether they all keep their activity. Evidently, at least some enzymes must remain active, seeing of the continuous increase of e.g. amino acids during ripening.

After growth to its maximum number during cheese making a starter bacterium loses its viability, at a rate dependent on the particular strain. It is frequently assumed that the non-viable organisms also start to lyse. Autolysins, involved in the synthesis of the cell wall during growth, are supposed to degrade the protecting, rigid wall after growth. Differences in osmotic pressure inside and outside the cell then may initiate a more or less severe damage of the cell membrane. This will cause either the release of intracellular enzymes - which should favour contact of the enzymes with their potential substrates - or enhanced accessibility of the substrates to cytoplasmatic enzymes still inside lysed cells, e.g. inside leaking cells. In this way, lysis of starter bacteria may affect proteolysis in cheese.

1.2 Aim of the study

Numerous articles have been published on proteolysis in cheese, dealing with almost all aspects of interest, including the crucial importance of starter bacteria to protein breakdown and to flavour formation. In spite of this, relatively little quantitative information exists about the effects of the temperature and the composition of cheese on the action of the proteolytic system of starter bacteria during cheese maturation. The aim of this study has been to gain more knowledge on those aspects. Such research requires the use of cheese models of well defined physico-chemical, enzymic and bacterial composition. These models were based on those developed earlier at the Dairying Laboratory of this University (Noomen, 1978*b*), but adapted to the purpose of the study. Cheeses were provided with a standardized mass of cells of the mesophilic strain *Lactococcus lactis ssp. cremoris HP* (Prt^+ variant; further referred to as *L. cremoris HP*), and protein breakdown was studied as a function of the keeping time of cheeses. Various methods were applied to characterize proteolysis. The following aspects were studied: effect of temperature, pH, NaCl content, paracasein to water ratio, and lysis of cells. In most cases this concerned the proteolytic action of the starter strain towards casein that had been predegraded by rennet enzymes, corresponding to the situation in most varieties of cheese.

CHAPTER 2

Materials and methods

2.1 Materials for model cheeses

2.1.1 Cheeses without rennet and starter bacteria; control cheeses

2.1.1.1 Calcium paracaseinate - calcium phosphate complex

Wet paracaseinate particles were prepared as described by Noomen (1978*b*), from freshly drawn, bulk cow's milk obtained from the University herd. The particles were submitted to a heat-treatment aimed to destroy indigenous milk proteinase, and subsequently freeze-dried, milled, tinned and stored at room temperature. Henceforth, the powdered "milk proteinase inactivated" paracaseinate complex will be referred to as PIC.

Two batches of PIC were used in the study:

1. a batch obtained from a suspension of particles in water (ratio of sedimented wet paracaseinate to added water: 3:1), heated at 95°C for 30 min. The PIC had a water content of 4.3%, a nitrogen content of 13.09% corresponding to a protein content of 83.5%, and a calcium content of 2.66%.
2. a batch obtained from a suspension of particles in water (ratio of sedimented wet paracaseinate to added water: 1:1), autoclaved at 121°C for 15 min. Composition of this PIC was: water 1.95%; nitrogen 13.9%, corresponding to a protein content of 88.68%; calcium 2.64%.

2.1.1.2 Simulated cheese moisture at pH ~ 6.6 without lactose and non-casein N substances

The buffer of Jenness & Koops (1962) was prepared and supplied with 1% (v/v) of the trace-metal solution described by Exterkate (1979); magnesium salt, being sufficiently present in the buffer, was omitted from this solution. The mixture, in this report further defined as JK-solution, was made just before use and sterilized through Seitz-filtration

(filter EKS). For cheeses with starter bacteria, JK-solution was supplemented with vitamins, see Section 2.1.3.2.

2.1.1.3 Other materials

Lactic acid (90% w/v) and dry NaCl were used, both of analytical grade (Merck, Darmstadt, FRG). In the preparation of cheeses with autoclaved PIC the lactic acid was separately heated for 15 min at 121°C; NaCl was sterilized at 180°C for 2 h, as were all other dry materials required in the manufacture of cheeses. Cheeses were made in glass bottles with screw caps.

2.1.2 Cheeses with rennet

2.1.2.1 Preparation: see Section 2.1.1.

2.1.2.2 Rennet

Dutch commercial calf rennet (strength 1:10.000; CSK, Leeuwarden, the Netherlands) was used. The rennet was sterilized through Seitz-filtration (filter EKS).

2.1.3 Cheeses with untreated or predigested paracasein and starter bacteria

2.1.3.1 Preparation: see Sections 2.1.1 and 2.1.2 for untreated and predigested paracasein, respectively.

2.1.3.2 Bacterial cell mass

Starter strain

The mesophilic strain *L. cremoris HP* was used, obtained from the Netherlands Institute for Dairy Research (NIZO, Ede, the Netherlands). Stock cultures were kept in litmus milk at -40°C.

Casein medium

The medium was composed with UF permeate from reconstituted skim milk (Christopherson & Zottola, 1989). Reconstituted skim milk, made by dissolving 10% low heat skim milk powder in demineralized water, was ultrafiltered (DDS, NAKSKOV, Denmark) at 5°C. Sodium caseinate powder (milk protein-spray-88%, DMV Veghel, the Netherlands) was dissolved in the permeate (1% w/v), the pH adjusted to 7.0 with NH_4OH (25%), and the medium autoclaved at 121°C for 15 min. pH after sterilization was 6.6.

Cultivation of cells

An inoculum of stock culture (0.5%) was subcultured in casein medium at 20°C for 20 h. The grown culture was inoculated (0.5%) in 2 l of casein medium in a 3 l laboratory fermentor (Applikon bv, Schiedam, the Netherlands), and incubated at 20-22°C for 36-40 h. For reasons not yet understood, initial growth of the organism was strongly inhibited by stirring of the medium. Therefore, the culture was allowed to grow without stirring until the pH had decreased to about 5.5 (12-16 h). Incubation was continued for 24 h under stirring at a constant pH of 6.0, using NH_4OH (25%) as a neutralizing agent. Incubation was stopped at the late exponential/early stationary growth phase, indicated by the cease of ammonia addition (Servograph REA 160, Radiometer, Copenhagen, Denmark), and also checked by measurements of the optical density at 660 nm (spectrophotometer PMQII, Carl Zeiss, Oberkochen, FRG).

Preparation of a concentrated suspension of cells

The pH of the grown culture was adjusted to pH 7.0 with NH_4OH (25%). One percent trisodium citrate.$2H_2O$ (w/v) was added to dissolve any undissolved casein (Stadhouders *et al.*, 1969; Exterkate, 1984), and the culture was cooled to 5°C. Cells were harvested from 1200 ml of the culture by centrifugation at 13300 x g for 15 min at 5°C (Sorvall RC-5B, GSA-rotor), and washed by suspending the cell pellet in cold JK-solution followed by centrifugation at 3000 x g for 15 min at 5°C (Bench Centrifuge Mistral 400, Beun-de-Ronde B.V., Abcoude, the Netherlands, MS 420/3-rotor; enabling sterile handling of small quantities of suspension). Washing was repeated 3 times. The final pellet was resuspended in 80 ml JK-solution (concentration factor of cells 15). In order to

standardize the number of cells per ml of suspension, its optical density at 660 nm was adjusted to 50 (0.5 for a 1:100 dilution of suspension in JK-solution).

Just before its addition to cheese, the suspension was supplied with a solution of various vitamins (1% v/v) and of the vitamin ascorbic acid (0.5% v/v), as used by Exterkate (1979) in the composition of a synthetic growth medium for lactic acid bacteria. The concentrations of the respective vitamins in the moisture of a finished cheese were adjusted to those in that medium; conveniently, their concentrations in the solutions added to the suspension of cells were calculated in proportion to their dilution in the manufacture of cheeses with a different paracasein to water ratio (P/W ratio). For example, the concentration of ascorbic acid in the moisture of a finished cheese always amounted to 0.05%.

A stock solution of vitamins was pasteurized at 75°C for 10 min, and kept at 4°C; the ascorbic acid solution was freshly prepared when needed, and sterilized through Seitz-filtration (filter EKS).

Preparation of a homogenate of cells

Bacteria were lysed by treating a concentrated suspension of cells with egg-white lysozyme (10 mg per ml of suspension) at 37°C for 1 h, followed by osmotically shocking the cells with sterile K_2SO_4 (0.15 M) and additional incubation at 37°C for 1 h (Otto *et al.*, 1982). Vitamins were added at the end.

Lysozyme (Boehringer Mannheim, FRG) was dissolved in JK-solution (100 mg per ml), and sterilized by filtration before use (S & S, filter FP 030/2).

2.2 Preparation of cheese; methods to prevent growth of contaminating micro-organisms

A detailed description of the manufacture of model cheese and of general considerations involved in its preparation is given in Chapter 3.

Cheeses required the absence of growth of contaminating organisms. For control cheeses (Section 3.2.1) this condition was realized in two ways, depending on the type of PIC used in their preparation:

1. Cheeses made with PIC obtained from heated wet paracaseinate (Section 2.1.1.1 - batch 1) were γ-irradiated at Proefbedrijf Voedselbestraling (Wageningen, the Netherlands), according to the principles described by Kampelmacher (1984). The treatment was performed at -40°C under anaerobic conditions, created by flushing the contents of bottles with nitrogen gas before irradiation. Although the number of anaerobic sporeforming bacteria of PIC was less than 50 per gram, of which some spores of *Clostridium tyrobutyricum*, the intensity of irradiation had to be increased to 10-12 kGy to destroy them all; they are known to be more resistant to irradiation than sporeforming aerobic and non-sporeforming aerobic and anaerobic bacteria.
2. PIC prepared from autoclaved wet paracaseinate (Section 2.1.1.1 - batch 2) was free of anaerobic bacteria. The number of aerobic bacteria amounted to about 30 per gram, which had to be attributed to a slight contamination of the paracaseinate during further treatments, in particular during the milling of the freeze-dried material. Further contamination of cheeses made with this PIC was prevented by maintaining strictly aseptic conditions at all stages of their preparation, e.g. by use of a clean-air cabinet.

Sterile conditions were also applied in the manufacture of cheeses with rennet and/or starter bacteria (Sections 3.2.2 and 3.2.3). Additionally, the preservation of all cheese was enhanced by its composition, in particular the absence of sugar, and by the storage condition (Section 2.4). Numbers of undesired bacteria in ripening cheeses were always found zero with the methods applied (Section 2.5).

2.3 Inactivation of rennet in cheese with pH 5.2

In experiments aimed to follow the breakdown of peptides by the starter bacterium, a fixed 'pool of peptides' was obtained by preliminary action of rennet enzymes on paracasein in cheese at pH 5.2. Subsequently, rennet was inactivated by increasing the pH to 6.5 with 2.5 M NaOH, followed by heating the cheese at 70°C for 1 h.

2.4 Storage of cheese

Cheeses were stored in jars under anaerobic conditions at a particular ripening temperature. Anaerobiosis was created with the BBL Gas Pak System. Evaporation of water from cheeses was prevented by placing a beaker with JK-solution in the jar; cheeses showed no loss of weight at the ripening temperatures.

2.5 Bacteriological analysis

Numbers of aerobic and anaerobic bacteria in PIC and cheeses without starter bacteria were estimated on Plate Count Agar (Difco, Michigan, USA) enriched with 0.1% skim milk powder. PIC was analysed as described by Noomen (1978*a*). Five grams of PIC were blended with 100 ml of a 2% trisodium citrate solution at 45°C (Visser, 1977*a*). Ten ml were taken as a sample. Petri dishes were incubated aerobically and anaerobically for 3 days at 30°C. Cheeses without starter bacteria were analysed correspondingly, taking the total cheese, containing 5 g of dry PIC (Section 3.2), as a sample.

The absence of the anaerobic sporeforming *Clostridium tyrobutyricum* in cheeses was checked with RCM-lactate enrichment medium and LATA confirmatory medium, as described by Fryer & Halligan (1976). Blended samples were heated at 80°C for 10 min, and 5 ml were taken as a sample.

Numbers of starter bacteria in suspensions of cells and in cheeses were estimated with enriched Plate Count Agar (see before). Decimal dilutions of samples were made in peptone-NaCl solution, containing 1 g Bacto-peptone (Difco) and 8.5 g NaCl per litre of demineralized water. Nutrient Agar (Difco) was used to analyse suspensions and cheeses for undesirable growth of non-sugar requiring bacteria.

The absence of growth of contaminating organisms in cheeses was also checked frequently by microscopic examination and, indirectly, by measurements of pH.

2.6 N-determinations

Nitrogen was determined by the macro-Kjeldahl method according to the Netherlands standard NEN 3198 (1984).

2.6.1 Total nitrogen

Total nitrogen content of PIC was estimated with 0.5 g of paracaseinate. The PIC was dissolved in 10 ml HCl (25%) under gently heating. After cooling, the solution was made up to 100 ml with demineralized water. Nitrogen was determined in 25 ml of liquid. The casein content of PIC was calculated with the conversion factor 6.38. Quantities of total nitrogen and protein in cheeses, which always contained 5 g of dry paracaseinate, were simply calculated from the composition of PIC (Section 2.1.1.1).

2.6.2 Soluble N and amino acid-N

Determinations were performed by the methods according to Noomen (1978*a*). The extract of cheese was made by blending the entire cheese with such a quantity of 0.037 M $CaCl_2$ that the total volume of liquid, including that of cheese moisture, amounted to 150 ml. Quantities of soluble N (SN) and of amino acid-N (AN) were calculated as a percentage of the total N of cheese: % SN/TN and % AN/TN, respectively.

2.7 Analysis with HPLC

Extracts of cheese (Section 2.6.2) were fractionated with HPLC equipment (Spectra-physics, San Jose, California, USA) as described by Boekel & Walstra (1989), using range 0.02. Settings were identical for all chromatograms. Differences between fractions of peptides and of amino acids were deduced from heights and surface areas of peaks at their particular retention times (RT). Fractions were classified in high molecular weight peptides (MW > 14000), and in medium to low molecular weight peptides (MW < 14000) according to the scheme of Visser (1977*c*), and in amino acids (MW < 255). Use was made of a standard curve (see Chapter 4 - Fig. 4.1).

In comparative studies on particular series of cheese, all extracts were deep-frozen (-40°C) until the end of the experiment, and analysed in one run. Extracts showing a slight precipitate after defrosting were filtered through paper (S & S, filter 595½) before use.

2.8 Amino acid analysis

Samples to be analysed were prepared according to the principles given by Reiter *et al.* (1969) and Mondino *et al.* (1972). Equal volumes of cheese extract (Section 2.6.2) and of a 6% (w/v) solution of 5-sulphosalicylic acid in water were mixed in a 10 ml centrifugation tube. The mixture was kept for 30 min in ice to precipitate the peptides, and subsequently centrifuged below 5°C for 15 min at 8880 x g (Sorvall, SM 24 rotor). The supernatant was filtered through paper (S & S, filter 595½). The amino acid profile of the filtrate was determined with an automatic analyser (Biotrini LC 6000 E with durrum DC 6A resin, Maintal, FRG), with norleucine as an internal standard for each sample.

Samples were prepared from cheese extracts kept at -40°C until the end of a particular experiment, and analysed in one run.

2.9 Polyacrylamide gel electrophoresis (PAGE)

In some experiments the electrophoretic patterns of cheeses were determined by PAGE according to the method of Jong (1975). A 2% (w/v) solution of protein in urea buffer (pH 8.5) was made by adding a calculated amount of cheese (~ 4 g) to 40 ml urea buffer with 3 ml 50% (w/v) EDTA solution. The mixture was heated at 45°C for 30 min under agitation, cooled to room temperature and made up to 50 ml with urea buffer. A 0.5% solution was made by dilution with urea buffer.

2.10 Estimation of calcium and inorganic phosphorus

For determinations in the dry paracaseinate, 10 ml 5% ammonia was added to 0.3 g of powder. The mixture was continually stirred on a hot plate until the preparation was completely dispersed. It was then cooled and transferred to a 50 ml volumetric flask. The protein was precipitated with 15% trichloroacetic acid solution (final concentration); calcium and inorganic phosphorus were estimated on the filtrate.

For determinations in the cheese serum, the cheese was centrifuged at 10000 x g for 10 min. An aliquot of the supernatant was weighed in a 25 ml volumetric flask and treated as described before.

Calcium was determined by the method described by Have (1954), and the inorganic phosphorus according to Netherlands Standard NEN 6663 (1983).

CHAPTER 3

Manufacture of model cheeses

3.1 Introduction

Studies on the effects of ripening conditions of cheese on the proteolytic action of lactic acid bacteria require the use of suitable models of cheese. Normal cheese cannot be used as a substrate because it is not possible to standardize its composition independently as to, for instance, pH, NaCl-content or its ratio of protein to water, nor to control growth of undesired organisms at any composition of cheese, for instance at high pH and low salt content; consequently, it is not possible to vary a single ripening factor. In principle, these drawbacks also hold true for aseptic model curds as applied by Kleter (1975), Lebars *et al.* (1975), Desmazeaud *et al.* (1976), and Visser (1977*a*). Moreover, such curds do not exclude milk proteinase action from the ripening process, and consequently do not permit studies on the action of particular proteolytic enzymes in cheese without possible interaction with milk proteinase. Protein breakdown in simple model substrates, like in solutions of casein, paracasein or synthetic peptides, and also in milk, does not reflect the actual progress of proteolytic processes in cheese (e.g.: Rapp, 1969; Ohmiya & Sato, 1972; Kikuchi *et al.*, 1974; Castberg & Morris, 1976; Jong & de Groot-Mostert, 1977).

To overcome the main disadvantages of the substrates mentioned before, Noomen (1978 *a,b*) developed "cheese-like" model systems which reliably reflected the action of milk proteinase and calf rennet under various conditions in cheese. The study of the proteolytic behaviour of starter bacteria was therefore based on their action in such model cheeses. Their manufacture was, however, adapted to the aim of the particular investigation.

3.2 Manufacture of cheeses

The general outline for the manufacture and storage of cheeses used in our studies is given in Fig. 3.1. All cheeses were prepared with 5 g of PIC (dry matter) in 30 ml bottles with screw cap. In most cases, the final P/W ratio amounted to 1 : 2.5. At lower ratios (e.g. 1 : 2) it became difficult to homogeneously mix the ingredients of a cheese, whereas at high ratios (e.g. 1 : 3.5) cheeses showed separation of some moisture.

3.2.1 Control cheeses

Basic cheeses were prepared with both types of PIC (Section 2.1.1.1; batches 1 and 2), according to the scheme of Fig. 3.1 - steps 1 to 3. If using PIC-batch 1, obtained from wet paracaseinate heated at 95°C for 30 min, their manufacture was followed by step 4, γ-irradiation with 10-12 kGy, to destroy all contaminating organisms, in particular clostridia (Section 2.2).

The following ingredients were successively mixed (see also Section 2.1.1):

- lactic acid. Quantities required to arrive at a particular pH of a finished cheese were established in preliminary experiments.
- a calculated quantity of JK-solution, to adjust the P/W ratio of a finished cheese to the desired value.
- a weighed amount of NaCl, its quantity being dependent on the desired NaCl concentration in the water of a finished cheese.
- paracaseinate corresponding to 5 g of dry PIC. The paracaseinate was very well mixed with the solution and compressed. For example: with PIC-batch 1 (water content 4.3%, protein content 83.5%), 5.22 g of paracaseinate containing 4.35 g of protein were mixed with 10.7 ml of JK-solution to adjust the P/W ratio of cheese to 1 : 2.5.

3.2.2 Cheeses with untreated paracasein and starter bacteria

Basic cheeses were manufactured as described above, and γ-irradiated if PIC-batch 1 was used. The quantity of JK-solution, however, was reduced by 2 ml, and replaced by 2 ml of the concentrated suspension of cells in JK-solution with vitamins added (Section 2.1.3), to arrive at the desired P/W ratio of the finished cheese.

3.2.3 Cheeses with predigested paracasein and starter bacteria

3.2.3.1 Predigestion of paracasein by rennet

Basic cheeses were prepared with pH 5.2, without NaCl. They were provided with 1 ml of a 3% (v/v) solution of calf rennet in JK-solution (Fig. 3.1 - step 5); see also Section 2.1.2. Cheeses, at this stage with a P/W ratio of 1 : 2, were stored at 13°C for some time, under anaerobic conditions (Section 2.4). This aimed the formation by rennet of a 'pool of peptides' as a substrate for the starter bacteria (Fig. 3.1 - step 6).

3.2.3.2 Inactivation of rennet

Any further contribution of rennet to the formation and degradation of peptides after the addition of starter bacteria to cheese would make the study of net proteolytic effects of the organism impossible. The 'pool of peptides' formed at pH 5.2 was therefore fixed by inactivation of rennet by heating the cheese at 70°C for 1 h at pH 6.5 (Fig 3.1 - step 7). To this end, the pH of cheese had been adjusted with NaOH-solution (Section 2.3). Thereafter, the cheese was cooled to room temperature.

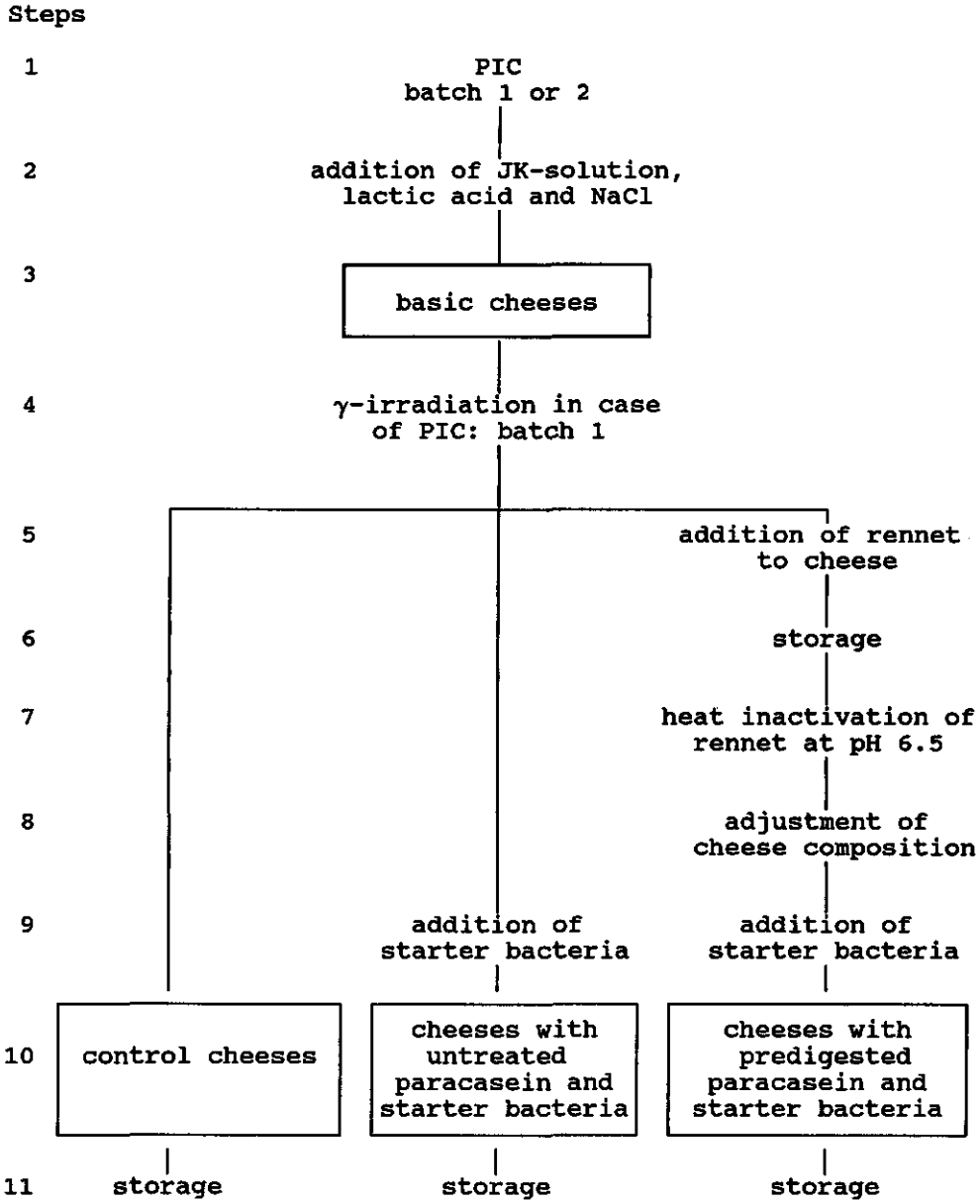


Fig. 3.1 General outline for the manufacture and storage of cheeses

3.2.3.3 Adjustment of cheese composition; addition of starter bacteria

The pH of cheese was subsequently adjusted with lactic acid (90% w/v) to a preset value, the lowest pH tested being about 4.6. The small quantities of concentrated NaOH-solution (Section 3.2.3.2) and of lactic acid used had a negligible effect on the P/W ratio in the finished cheese. Of course, the use of lactic acid to readjust the pH of cheese caused the lactate concentration in the finished cheese to vary with pH.

The composition of cheese was further adjusted to its desired P/W ratio and NaCl content (Fig. 3.1 - step 8). In the calculation of the quantity of JK-solution required, the later addition of 2 ml of a concentrated suspension of cells (Section 2.1.3, Fig. 3.1 - step 9) was taken into account. During the manufacture of series of cheese, requiring considerable time, the suspension was kept in ice. NaCl was calculated from sodium content, taking into account the sodium in the NaOH-solution used in the method to inactivate rennet.

3.2.4 Cheeses with predigested paracasein and lysed starter bacteria

Cheeses were prepared as described in Section 3.2.3, but the 2 ml of cell suspension were replaced by 2 ml of a suspension of lysed cells (Section 2.1.3.2). In parallel experiments on cheeses with intact and lysed cells, respectively, the concentration of K_2SO_4 in the suspension of intact cells was correspondingly adjusted to 0.15 M.

3.2.5 Storage of cheeses

Cheeses were stored at different temperatures and for varying periods of time according to the aim of a particular investigation, under the conditions mentioned in Section 2.4.

3.3 General considerations on cheese manufacture

Starter strain

The Prt^+ variant of *Lactococcus lactis spp. cremoris HP* was selected for our studies. The strain is very well-known and the nature of its proteolytic enzyme system has been subject of extensive study (e.g. Exterkate, 1990; Baankreis, 1992).

Paracaseinate complex as a source of protein

The chemical composition of the calcium paracaseinate - calcium phosphate complex derived from milk approximates the composition of casein after renneting of fresh milk in the manufacture of many varieties of cheese, e.g. Gouda cheese. The incorporation in cheese of paracaseinate as the source of protein thus was the obvious choice. At the various conditions in model cheeses the properties of the paracaseinate complex, in particular the dissociation of calcium and phosphate at lower pH, may be assumed to greatly resemble those in normal cheese; see below.

Simulation of cheese moisture

The investigations were aimed at studying the proteolytic action of a standardized mass of starter bacteria against untreated or predigested paracasein in cheese. Consequently, conditions in cheese should not permit growth of those bacteria, requiring the absence of any fermentable sugar. The simulation of cheese moisture at pH ~ 6.6 was therefore based on approximate contents of mineral salts, trace-metals, and of most of the vitamins in the serum of milk, without lactose and non-casein N compounds (Walstra & Jenness, 1984). JK-solution and vitamins, the latter being added to cheese together with the starter bacteria, served that purpose. As an exception, the concentration of ascorbic acid in the moisture of cheese (0.05%) did far exceed the average vitamin C concentration in milk, being about 0.003%, to favour a rapid decrease of E_h.

The pH of the cheese, its ratio of paracasein to water, and its NaCl content relative to water, strongly affect the equilibrium between undissolved colloidal calcium phosphate of paracaseinate complex and the quantities of calcium and phosphate dissolved in the cheese (Monib, 1962). Particular concentrations of calcium and phosphate in the moisture

of cheeses at different conditions thus will automatically result from the equilibrium existing at the preset composition of a particular cheese.

Inactivation of milk proteinase

Because indigenous milk proteinases are strongly associated with casein at the pH of normal milk, the enzymes almost quantitatively pass into the wet paracaseinate complex described in Section 2.1.1.1. The paracaseinate had to be freed from these enzymes to exclude their possible interactions with the proteolytic action of starter bacteria. Noomen (1978*b*) prepared cheeses from paracaseinate submitted to a heat treatment at 80°C for 30 min, which satisfactory reduced milk proteinase action in those cheeses kept at 13°C for relatively short periods of time (tested up to 1 month). According to Driessen (1983), the pasteurization of milk at 85°C for 30 min inactivates alkaline milk proteinase for about 99.99% (D-value for inactivation at 85°C: 384 s).

To establish the absence of milk proteinase activity in the paracaseinate, control cheeses (Section 3.2.1) were prepared with powders derived from wet complex heated for 30 min at 80, 85 or 95°C, and γ-irradiated. Cheeses, made with pH 6, without NaCl and with a P/W ratio 1 : 2.5, were stored at 37°C. These conditions favour the action of the enzymes (Noomen, 1978*a*). Their residual action in cheese was estimated by the increase of % SN/TN (Section 2.6.2) and with PAGE (Section 2.9). As shown in Fig. 3.2, heating of the complex at 80 and 85°C was quite insufficient to inactivate milk proteinases.

Electrophoretic patterns of cheeses stored for 1 month (slots 1 and 2) revealed a very distinct formation of 'minor caseins' below the ß-casein band. Also a slight formation of bands in front of the α_{s1}-casein band was observed, which particularly at a low pH of cheese originates from the action of acid milk proteinase (Kaminogawa & Yamauchi, 1972; Noomen, 1978*a*).

Patterns of cheeses made with paracaseinate heated at 95°C (slot 3) did not show any increase of particular bands in comparison with blank cheeses (slot 4), which suggested the complete inactivation of milk proteinases at this temperature. Electropherograms of cheese protein were not affected by γ-irradiation (see slots 4 and 5). Results with PAGE were reflected in values of % SN/TN; after 1 month of storage at 37°C the SN/TN percentage of cheeses prepared with paracaseinate heated at 80, 85 and 95°C had increased by about 19, 17 and 0.4%, respectively. These results did us believe that

paracaseinate heated at 95°C could be used in the manufacture of cheese. However, cheeses made from that preparation still showed some milk proteinase activity when kept for long time (see Section 4.3.1.3).

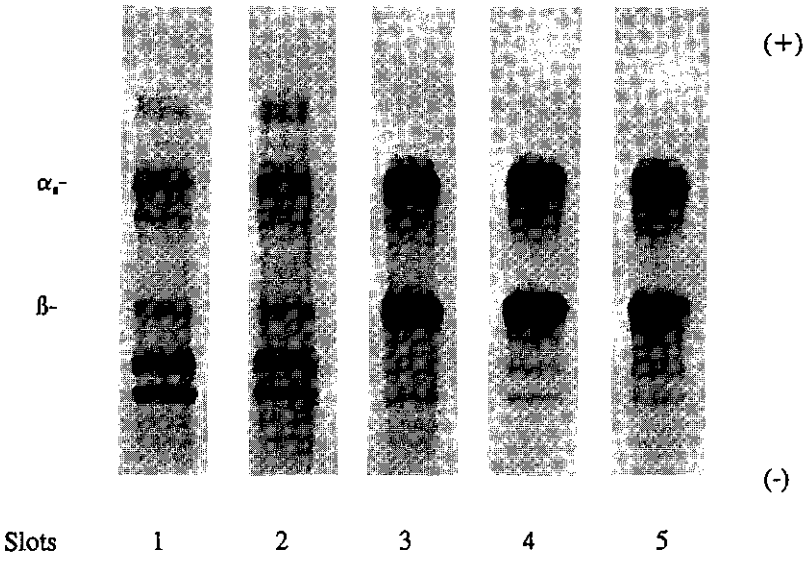


Fig. 3.2 Electrophoretic patterns of protein degradation by milk proteinases in control cheeses prepared with paracaseinate submitted to various heat treatments. Composition of cheese: P/W ratio 1:2.5, pH 6, NaCl 0%. Slots 1 to 3 inclusive: cheeses prepared with paracaseinate heated for 30 min at 80, 85 and 95°C, respectively, after storage for 1 month at 37°C. Slots 4 and 5: blank cheeses made with paracaseinate heated for 30 min at 95°C, before and after γ-irradiation, respectively.

In later experiments, the absence of active milk proteinases was achieved by heating the paracaseinate for 15 min at 121°C. During autoclavation, the ratio (by weight) of sedimented wet particles of paracaseinate to added water is rather critical (Section 2.1.1.1). Too little water causes the colour to turn brown and the consistency sticky and rubber-like, which makes the preparation of appropriate PIC impossible. The use of autoclaved paracaseinate served the simplicity of cheese manufacture, as γ-irradiation could be omitted. Moreover, cheeses were lacking the particular cooked flavour present

in cheeses submitted to irradiation. However, organoleptic examinations did not form part of our research.

At very low levels of soluble N, values of % SN/TN varied considerably. In the control cheeses with different composition and storage conditions used in the investigations, this value mostly varied between 0.4 and 1.2%.

Predigestion of paracasein by rennet

During the ripening of most varieties of cheese, the paracasein is digested by rennet to a significant degree before the proteolytic action of starter bacteria becomes apparent; relevant literature has been reviewed by Fox (1989). To simulate this condition, rennet was allowed to act in cheeses at 13°C (a normal ripening temperature for e.g. Gouda cheese) to create a 'pool of peptides' as a substrate for the starter bacteria. pH and NaCl content of the cheeses (5.2 and 0%, respectively) and the quantity of rennet used were selected to obtain a 'pool' of sufficient magnitude, arbitrarily chosen as amounting to at least 6% in terms of SN/TN, within a fairly brief storage of a few weeks. Literature reveals that calf rennet shows maximum action in cheese at pH ~ 5 at low NaCl concentration (Noomen, 1978*b*). The quantity of rennet required was established in preliminary experiments. Seitz-filtration of rennet (Section 2.1.2.2) caused its clotting power in milk to become reduced by about 20%. Taking this into account, the 0.03 ml of filtered rennet added to cheese (Section 3.2.3.1) represented about 5.75 ml of commercial rennet per kg of casein, being approximately 5 times its concentration in Gouda cheese (e.g. Visser, 1977*a*; Stadhouders *et al.*, 1977).

Inactivation of rennet in cheeses with low pH

The study of the action of the starter bacteria towards peptides produced by rennet required the inactivation of rennet before their addition to cheese. It was tried to realize that by heating of cheese. Freshly prepared cheeses with rennet and with pH 5.2 (Section 3.2.3.1) were centrifuged and their supernatants submitted to heating at 70°C for various times, ranging between 0.5 and 3.5 h. A particular quantity of a supernatant was added to pasteurized skimmed milk (15 s at 72°C) containing 200 ppm thiomersal as a preservative, and the mixture (at pH 6.5) incubated at 30°C for 2 weeks. Residual activity of rennet in supernatants was demonstrated qualitatively by clotting of milk.

Results indicated that even a heating for 3.5 h did not completely destroy rennet activity. The action of residual rennet was also reflected in the formation of soluble N in heat-treated cheeses kept at 13°C. For instance, % SN/TN in cheeses heated for 1 h at 70°C increased by about 3 and 5% during storage for 7 and 13 weeks, respectively. (Note: % SN/TN of cheese before heating amounted to ~9% after storage for 2 weeks). These findings established significant heat resistance of rennet at the pH of cheese.

Because rennet is very sensitive to heating at the pH of fresh milk (pH ~ 6.6), to inactivate rennet after its predigestion of paracasein in cheese of pH 5.2, the pH of the cheese was raised to 6.5 and the cheese heated for 1 h at 70°C (Section 3.2.3.2). Heated cheeses with a pH readjusted again to 5.2 (Section 3.2.3.3) did not show any further accumulation of soluble N during storage at 13°C for periods up to 3 months, demonstrating the effectiveness of the rennet inactivation.

Control of bacterial growth

Growth of undesired organisms in cheeses was prevented by the conditions described in Section 2.2. The use of preservatives in the manufacture of cheese was considered unacceptable, because they might affect the proteolytic system of the starter bacteria added to cheese.

Incorporation of the starter bacteria in cheese

Quantities of free amino acids and of small peptides in milk allow lactic acid bacteria to multiply a few times, but the continuation of growth depends on the production of growth factors by cell wall proteinases of Prt^+ strains (Thomas & Mills, 1981), like the strain used in our study. To force the bacterium to produce those particular enzymes it was cultivated in a casein medium (Schmidt *et al.*, 1976, 1977; Hugenholtz *et al.*, 1984). Conditions allowing its preliminary growth as in milk were realized with milk permeate as the moisture phase of the medium (Section 2.1.3.2). This also had the advantage that the substrate was freed of serum proteins, which otherwise would precipitate during sterilization of the medium, and finally would pass into cheese together with the starter bacteria added because of the serum proteins would sediment during the centrifugation applied for the preparation of a concentrated suspension of cells (Section 2.1.3.2).

Growth of the starter bacterium was stopped at the late exponential/early stationary growth phase, presumably the physiological condition of lactic acid bacteria after their growth in normal cheese. It may be that the physiological state of those bacteria affects their production of proteolytic enzymes. For *Pseudomonas fluorescens 22F*, Driessen (1983) observed significant formation of extracellular proteinase only towards the end of the exponential growth phase.

The presence of Ca^{2+} may greatly influence the level of cell wall-associated proteolytic activity of lactococci, as it may possibly affect their attachment to the cell wall and their stability; the literature has been reviewed recently by Kamaly & Marth (1989). JK-solution (Ca^{2+} concentration 2.2 mM) used in the washings of cells (Section 2.1.3) was considered to prevent loss of bacterial proteolytic activity.

To study the proteolytic action of a standardized mass of the starter bacteria (fixed 'pool of enzymes') in cheese, the optical density at 660 nm of concentrated suspensions of cells prepared on different days were adjusted to 50 (Section 2.1.3.2). In spite of that, numbers of bacterial colony forming units (cfu) in the suspensions varied considerably. With the 2 ml of suspension used, their numbers in cheese varied between 0.4 and 14.7 x 10^9 cfu per g of casein. Corresponding numbers of starter bacteria are found in one day-old Gouda cheese (Visser, 1977*a*). The variation in cfu added to cheese did not affect results obtained in parallel experiments.

Storage conditions of cheese

Lactic acid fermentation causes the redox potential of cheese to decrease to about minus 150 mV (Galesloot, 1960). In our studies, a low E_h of cheese was achieved by storing the cheese anaerobically (Section 2.4). The rate of decrease of E_h will have been enhanced by the high concentration of ascorbic acid in the moisture of cheese (0.05%; see Section 2.1.3.2), as compared to about 0.003% in milk (Walstra & Jenness, 1984). Advantageously, anaerobic storage of cheese prevented growth of contaminating aerobic organisms, especially that of moulds. The BBL Gas Pak System applied to create anaerobiosis generates hydrogen gas and carbon dioxide. Especially CO_2 will have

diffused into the cheese. A considerable production of CO_2 is a characteristic for many varieties of cheese, e.g. for Dutch-type cheeses with a desired formation of 'eyes'. Therefore, the presence of CO_2 was considered to be a normal ripening condition for our cheeses.

CHAPTER 4

Proteolytic action of *Lactococcus cremoris HP* in model cheeses. 1. Effects of pH, NaCl concentration and ripening temperature

4.1 Introduction

It is generally agreed that the composition of the cheese and its ripening temperature considerably affect proteolysis. Surprisingly, little quantitative knowledge exists regarding the effects of those factors on the proteolytic action of starter bacteria at actual conditions of ripening cheese. Such studies require the use of models of cheese which: 1) allow their composition to be standardized; 2) reflect the conditions in normal cheese as closely as possible; and 3) enable to establish the action of the various proteolytic sources in cheeses separately, as well as combined.

Chapter 3 dealt with the preparation of models of cheese which, in our opinion, meet those requirements. They were used to study proteolysis by the lactococcus strain at various conditions as in a ripening cheese. This chapter deals with the effects of pH, NaCl concentration and ripening temperature on the degradation of untreated paracasein or of predigested paracasein in cheeses with a P/W ratio of 1:2.5. Paracasein was predigested by rennet, since this is involved in the ripening of most varieties of cheese.

4.2 Materials and methods

4.2.1 Preparation and storage of cheeses

Cheeses with a P/W ratio of 1:2.5 were prepared from paracaseinate heated at 95°C for 30 min (Section 2.1.1.1 - batch 1); basic cheeses were sterilized by γ-irradiation (Section 2.2-1). Cheeses with untreated paracasein and bacteria were made as described in Section 3.2.2 and those with predigested paracasein and bacteria according to Section 3.2.3. Their composition was adjusted to various pH and NaCl contents. Cheeses were stored (see Section 2.4) at various temperatures for various periods of time.

4.2.2 Estimation of protein breakdown

Proteolysis was estimated by: soluble N and amino acid-N, HPLC-chromatograms of extracts of cheese, amino acid analysis, and sometimes by PAGE; see the Sections 2.6 to 2.9.

HPLC-chromatograms of duplicate cheeses at the same condition showed a similar pattern. Amino acid analysis was performed on series of single cheeses.

By HPLC, fractions of peptides and amino acids were classified according to molecular weight by means of the standard curve shown in Fig. 4.1.

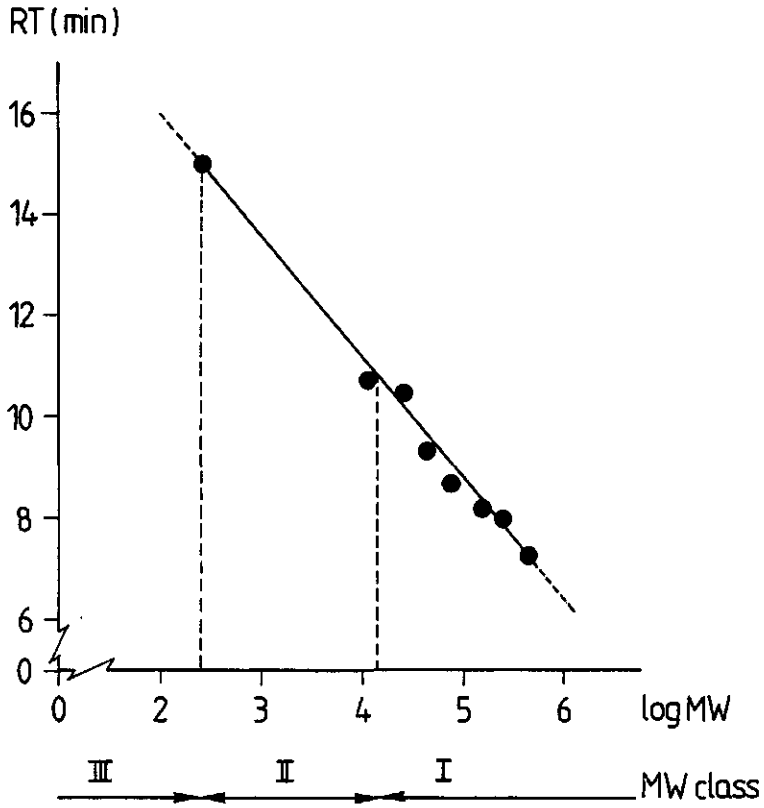


Fig. 4.1 Standard curve for the classification according to MW of N-compounds in cheese extracts. I: high-MW peptides (MW > 14000; RT < 10.9 min), II: medium to low-MW peptides (MW < 14000; RT 10.9-15.0 min), III: amino acids (MW < 255; RT > 15 min).

The curve was made with Protein Molecular Weight Standards, Kit MS II, Serva Feinbiochemica, New York, U.S.A., containing ferritin, catalase, aldolase, bovine serum albumin, chymotrypsinogen, myoglobin, cytochrome C and DNP-L-alanine, with molecular weights ranging from 450 000 to 255.

4.3 Results

4.3.1 Cheeses with untreated paracasein

The experiments were aimed at studying the breakdown of paracasein (not predigested by rennet) by a fixed mass of bacteria, as a function of pH, NaCl concentration relative to water, ripening temperature and ripening time of cheese. These effects were studied with three series of cheese, each of them made in duplicate. Series consisted of:

1. cheeses with a different pH at a fixed NaCl content and ripening temperature,
2. cheeses with a different NaCl content at a fixed pH and ripening temperature,
3. cheeses at a fixed pH and NaCl content, ripened at a different temperature.

Duplicate series were made on a single day, using the same batch of bacterial mass. Because of the considerable labour involved, the particular series 1 to 3 were made on different days. Consequently, the cells used in their preparation were from different batches, causing the number of viable bacteria (cfu) added to those series to vary (Section 2.1.3.2); see Table 4.1.

As shown in Table 4.1, the set pH of most cheese changed only slightly during keeping for 3 months. The pH of some cheeses had decreased by about 0.1 unit, which may have been caused by some hydrolysis of bimolecular and higher esters of lactic acid present in the concentrated acid used in the preparation of cheese, and which are known to hydrolyse in dilute solutions, particularly at higher temperature and higher pH (Holten *et al.*, 1971). pH values of duplicate cheeses on a particular sampling day varied within 0.04 unit, supporting the reproducibility of pH-adjustment of cheese.

Table 4.1 Production of soluble N and of amino acid-N from untreated paracasein by *L. cremoris HP* in cheeses with a P/W ratio 1:2.5, as a function of pH, % NaCl relative to water, ripening temperature and keeping time of cheese. Averaged results from duplicate series of cheese, corrected for values of blank cheeses kept without bacteria. Freshly made series of cheese contained: 1) 1.7×10^9; 2) 2.5×10^9, and 3) 14.7×10^9 cfu of bacteria per g of protein, respectively.

Composition of fresh cheese; ripening temperature			Ripening time (days)							
			32				91			
pH	% NaCl	°C	pH	SN[a]	AN[b]	AN[c]	pH	SN[a]	AN[b]	AN[c]
1) 4.74	4	13	4.72	0.42	0.24	57.14	4.64	0.61	0.37	60.66
5.24	4	13	5.22	1.05	0.53	50.48	5.22	1.68	0.72	42.86
6.02	4	13	6.02	1.43	0.91	63.64	5.91	1.89	1.47	77.78
2) 5.20	0	13	5.20	1.92	0.41	21.35	5.21	3.36	1.10	32.74
	4	13	5.23	1.36	0.43	31.62	5.23	1.95	0.83	42.56
	8	13	5.20	0.89	0.36	40.45	5.22	1.24	0.66	53.23
3) 5.20	4	13	5.23	2.22	0.73	32.88	5.23	2.90	1.08	37.24
	4	20	5.19	2.74	0.97	35.40	5.16	3.98	1.57	39.45
	4	30	5.10	3.33	1.39	41.74	5.12	5.32	2.42	45.49

a: in % TN b: in % TN c: in % SN

Cheeses were analysed after 32 and 91 days of storage. HPLC-chromatograms and patterns of amino acids after 32 days showed a tendency similar to those after 91 days, be it at a distinctly lower level of protein breakdown. Therefore, results of the 32-days period of ripening are not shown.

4.3.1.1 Effect of pH

The effect of pH on the degradation of untreated paracasein by the lactococcus strain was studied in cheeses at a pH near to 4.7, 5.2 or 6.0, containing 4% NaCl relative to water, and ripened at 13°C.

Quantities of soluble N and of amino acid-N produced at the various ripening conditions are presented in Table 4.1 - series 1. Both the values of % SN/TN and of % AN/TN were higher at a higher pH and a longer storage time of cheese. Obviously, the pH had strongly affected proteolysis. Compared with cheese ripened at pH ~ 4.7 quantities of SN and of AN at pH ~ 5.2 had increased more than twofold and at pH ~ 6.0 more than threefold. However, values of % SN/TN and of % AN/TN were rather low, amounting only to about 1.9% and 1.5%, respectively, even after 3 months of ripening.

Blank cheeses did not reveal noticeable proteolysis. During their entire period of keeping, values of % SN/TN and of % AN/TN remained at the low level of 0.6% and 0.3%, respectively, being indicative for the absence of proteolytic activity.

Results from N-determinations were reflected in HPLC-chromatograms of cheese; see Fig. 4.2. The pattern of blank cheeses (A) did not change during their keeping and corresponded to that of freshly made cheese (not shown). In cheeses with bacteria (B), medium and low-MW peptides and amino acids (RT > 15 min) had accumulated significantly, especially at high pH of cheese.

Amino acid analysis of cheeses with bacteria, ripened for 91 days, revealed the presence of numerous amino acids, be it for many of them in a quite small quantity (< 0.05 mg per g of cheese protein) particularly at a lower pH of cheese; see Fig. 4.3-A. At pH ~ 4.7, hardly any amino acids had been formed. At pH ~ 5.2, several amino acids had accumulated somewhat, up to a concentration of about 0.1 mg per g of cheese protein, whereas at pH ~ 6.0 the production of almost all amino acids had been favoured

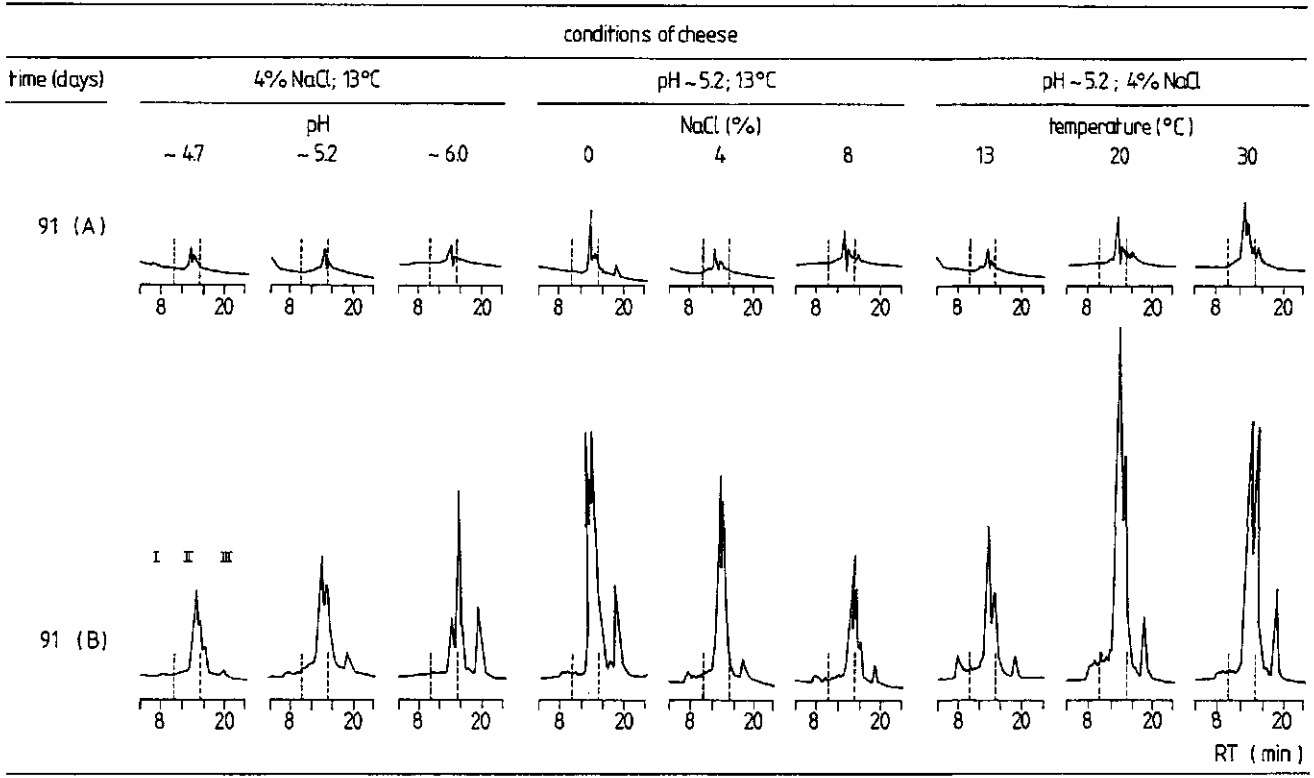


Fig. 4.2 HPLC-chromatograms of cheeses from Table 4.1, after 91 days of storage. (A): cheeses without bacteria (blanks). (B): cheeses with bacteria. Fractions were classified according to the standard curve of Fig. 4.1.

distinctly; especially leucine, glutamine, lysine, phenylalanine, tyrosine, valine and glutamic acid had accumulated, but also the quantities of alanine, arginine, histidine, isoleucine, methionine and serine had increased significantly. For the total quantities of amino acids produced at various pH; see Fig. 4.7 - B1.

Amino acids did not accumulate in blank cheeses kept for 91 days; concentrations of some of them being already detected in freshly made cheese remained far below the level of 0.05 mg per g of cheese protein.

4.3.1.2 Effect of NaCl

The effect of NaCl on the degradation of paracasein by the lactococcus strain was studied with cheeses at pH ~ 5.2, made without or with 4 or 8% NaCl relative to water. Cheeses were ripened at 13°C.

Quantities of soluble N and of amino acid-N produced in the various cheeses are presented in Table 4.1 - series 2. Both N-fractions had increased with longer storage time. Their production had been favoured in the absence of NaCl, and had decreased at an increasing salt concentration. However, the values of % SN/TN and of % AN/TN remained rather low at all conditions of ripening; see also Fig. 4.4 - effect of NaCl %. A concentration of 4% NaCl already decreased the production of SN by about 40%, and that of AN by about 25%. At 8% NaCl, those percentages amounted to about 60% and 40%, respectively, at the end of ripening.

HPLC-chromatograms of cheese reflected the results from N-determinations; see Fig. 4.2 for patterns after 91 days of ripening. Cheeses with bacteria (B) revealed the presence of medium and low-MW peptides and of amino acids (RT > 15 min), especially in cheese without salt and less so at increasing levels of NaCl.

Chromatograms of blank cheeses were not as uniform as those observed in Section 4.3.1.1. Although the quantities of SN and of AN did not increase significantly at a longer keeping time of cheese, particularly the chromatogram of cheese without NaCl did suggest that some breakdown of paracasein had taken place; see further Section 4.3.1.3.

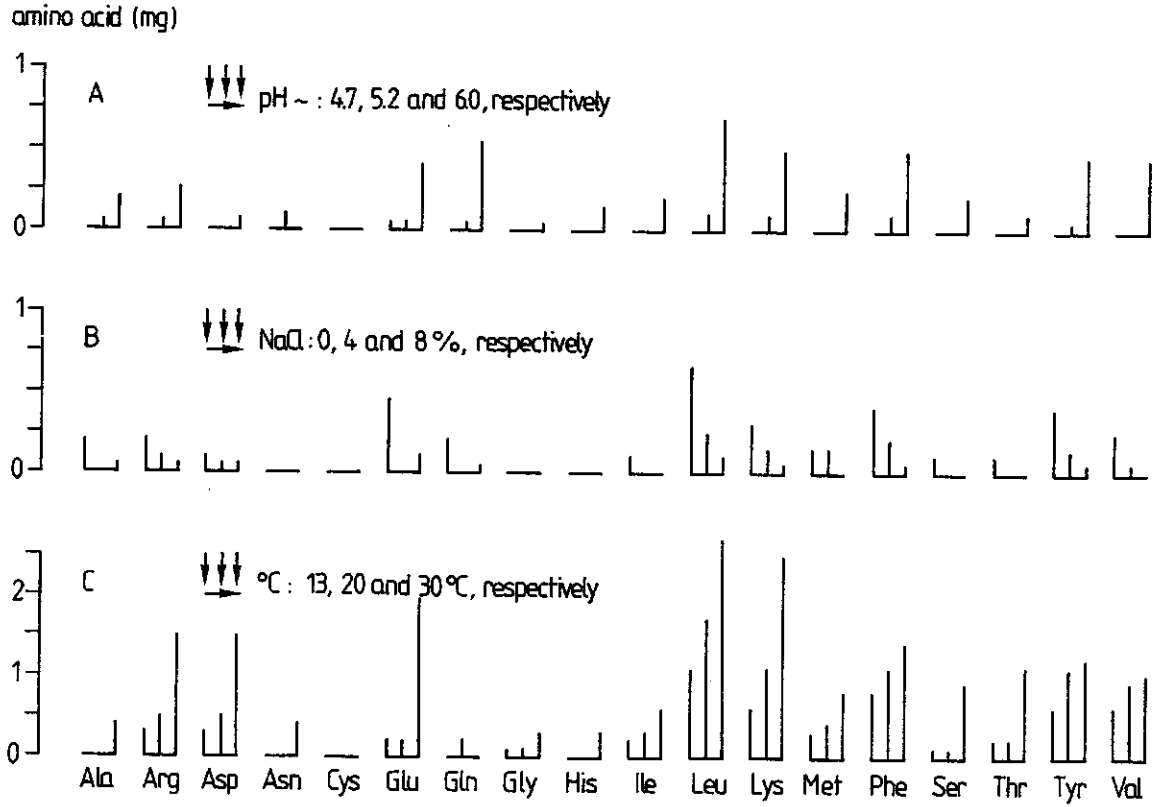


Fig. 4.3 Amino acids, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses from Table 4.1, as a function of pH (A), % NaCl relative to water (B) and temperature (C), after 91 days of ripening. At 30°C, NH_3 had accumulated to a quantity of about 0.6 mg per g of cheese protein, being about 3 times higher than the quantity at all the other ripening conditions tested. Only quantities of ≥ 0.05 mg amino acid are indicated.

Results of amino analysis are shown in Fig. 4.3 - B. Obviously, the production of most amino acids was greater in cheese without NaCl, and was already strongly reduced at 4% NaCl. For the total quantities of amino acids produced at the various NaCl contents, see Fig. 4.7 - B2. Blank cheeses behaved like those described in Section 4.3.1.1.

4.3.1.3 Effect of temperature

The effect of the ripening temperature was studied with cheeses at pH ~ 5.2, containing 4% NaCl relative to water, and ripened at 13, 20 or 30°C.

Quantities of soluble N and of amino acid-N having been produced in cheese at the various conditions are given in Table 4.1 - series 3. The concentrations of both N-fractions had increased with a longer ripening time, and their production had been distinctly stimulated at an increasing ripening temperature. For example, compared with the cheese ripened at 13°C for 91 days, those ripened at 20°C contained about 1.4 times as much SN and about 1.5 times as much AN. For cheeses ripened at 30°C, those increases amounted to about 1.8 and more than 2, respectively. The production of SN and of AN was, however, limited as compared to cheeses made with predigested paracasein (see Section 4.3.2.3).

HPLC-chromatograms of cheese supported the results of N-determinations; see Fig. 4.2 for patterns after 91 days of ripening. Cheeses with bacteria (B) showed the increased formation of medium and low-MW peptides and of amino acids (RT > 15 min) at a higher ripening temperature.

Blank cheeses, particularly those kept at 30°C, developed soluble N upon storage; after 91 days the value of % SN/TN had increased by about 1%. Accordingly, the chromatogram of the cheese indicated some proteolysis. The same was suggested before for cheese without NaCl at pH ~ 5.2, ripened at 13°C (Section 4.3.1.2). These observations point to a very slight action of milk proteinases which apparently had not been inactivated completely at heating the paracaseinate for 30 min at 95°C. A low concentration of NaCl, a higher pH and a higher temperature (up to 37°C) have been reported to stimulate those enzymes (e.g. Noomen, 1978*a*). For some ripening conditions of cheese, even a very small residual activity may become perceptible in cheese ripened

for a long time. Consequently, in later experiments the absence of active milk proteinases was achieved by autoclavation of the paracaseinate (Section 3.3).

Amino acids present in cheeses after 91 days of ripening are shown in Fig. 4.3-C. Results show that the vast majority of the individual amino acids had accumulated more at a higher ripening temperature. For results on the total quantities of amino acids produced at the various temperatures, see Fig. 4.7-B3.

4.3.1.4 Other observations

Cheeses developed high values of % AN/SN (see Table 4.1), which indicates that the bacteria predominantly produce small peptides and amino acids.

It may be asked to what extent results from AN determinations and from amino acid analysis would correspond quantitatively. Milligrams of 'amino acids + NH_3' nitrogen per g of cheese protein produced at a particular ripening condition of cheese were calculated from the values of % AN/TN in Table 4.1, and from the quantity of 'amino acids + NH_3' nitrogen derived from amino acid analysis, respectively, taking the nitrogen content of each amino acid into account; see Table 4.2. Large differences were found between the results of both calculations, although they showed the same trends. The much higher values derived from % AN/TN most probably are to be attributed to the presence of very small peptides in the AN-extracts of cheese (Farkye & Fox, 1990), which particularly at a low level of amino acid production cause the real AN-content in those extracts to become overestimated. As a consequence, at such conditions values of % AN/TN are less suitable to establish the effects of ripening variables.

At each ripening condition tested (Sections 4.3.1.1 to 4.3.1.3), the peak area of the chromatogram representing amino acids (RT > 15 min) was not well related to the total quantity of amino acids derived from amino acid analysis. Both methods showed, however, similar trends.

Analysis by PAGE did not reveal any clear change of the caseins at all ripening conditions studied. Results are therefore not shown.

Table 4.2 Milligrams of 'amino acids + NH_3' nitrogen per g of protein in cheeses from Table 4.1 after 91 days of ripening, calculated from the values of % AN/TN (A) and from the quantities of 'amino acids + NH_3' nitrogen derived from amino acid analysis (B).

Conditions of cheese											
4% NaCl, 13°C				pH ~ 5.2, 13°C				4% NaCl, pH ~ 5.2			
pH ~	4.7	5.2	6.0	NaCl (%)	0	4	8	°C	13	20	30
A	0.58	1.13	2.30		1.72	1.30	1.03		1.69	2.46	3.79
B	0.12	0.19	0.80		0.64	0.29	0.20		0.60	0.86	1.87

4.3.2 Cheeses with predigested paracasein

The experiments were aimed at studying the decomposition of predigested paracasein by a fixed mass of bacteria, as a function of pH, NaCl concentration relative to water, ripening temperature and ripening time of cheese. Cheeses were made as described in Section 3.2.3. Predigestion of paracasein was acquired by the action of rennet for 12 days at 13°C. After rennet inactivation, cheeses of various pH and NaCl content were prepared, provided with bacteria, and stored at various temperatures; see Table 4.3 for details. Bacteria numbered about 3.2×10^9 cfu per g of cheese protein, and were derived from one batch of cells. The study was performed with series of single cheeses because of the labour involved in the preparation of more than thirty cheeses on a single day.

Cheeses were analysed after 6, 32 and 91 days. HPLC-chromatograms of cheese and results of amino acid analysis revealed that proteolysis increased with storage time, but at each ripening condition the nature of the protein breakdown did not change with time. Therefore, not all the results will be reported.

The slight changes of the pH during ripening corresponded to those observed for cheese with untreated paracasein; see the Tables 4.3 and 4.1.

4.3.2.1 Effect of pH

The effect of pH on the decomposition of predigested paracasein by the lactococcus strain was studied with series of cheese at a pH near to 4.7, 5.2 or 6.2, containing 4% NaCl relative to water, and ripened at 13°C.

Values of % SN/TN and of % AN/TN estimated at the different stages of ripening are given in Table 4.3; see also Fig. 4.4 for results after 91 days. It follows that the production of SN and of AN was considerably enhanced at a higher pH and a longer keeping time of cheese. For example, after 91 days cheese at pH ~ 6.2 contained more than twice the SN and about four times the AN than did the cheese ripened at pH ~ 4.7. Qualitatively, the pH of cheese thus affected the decomposition of predigested paracasein like that of untreated paracasein (see Section 4.3.1.1). Quantitatively, however, much larger quantities of SN and of AN were produced from the predigested paracasein;

Table 4.3 Production of soluble N and of amino acid-N from predigested paracasein by *L. cremoris HP* in cheeses with a P/W ratio 1:2.5, as a function of pH, % NaCl relative to water, ripening temperature and keeping time. Values of % SN/TN and of % AN/TN have been corrected for those of blanks, amounting to 6% and 0.3%, respectively, after the inactivation of rennet. Freshly prepared cheeses contained $\sim 3.2 \times 10^9$ cfu per g of cheese protein.

Composition of fresh cheese; ripening temperature		Ripening time (days)*											
		6				32				91			
NaCl %	°C	pH	SN[a]	AN[b]	AN[c]	pH	SN[a]	AN[b]	AN[c]	pH	SN[a]	AN[b]	AN[c]
4	13	4.75	0.72	0.36	50.00	4.77	1.13	0.67	59.29	4.75	2.60	1.12	43.08
4	13 →	5.20	1.10	0.84	76.36	5.25	2.00	1.33	66.50	5.24	3.98	1.69	42.46
4	13	6.21	1.84	1.57	85.32	6.20	4.16	2.01	48.31	6.24	6.63	4.51	68.02
0	13	5.25	1.46	1.21	82.88	5.22	3.13	1.45	46.32	5.22	6.59	2.60	39.45
4	13 →	5.20	1.10	0.84	76.36	5.25	2.00	1.33	66.50	5.24	3.98	1.69	42.46
8	13	5.27	0.74	0.43	58.11	5.24	1.84	0.89	48.37	5.21	3.00	1.51	50.33
4	13 →	5.20	1.10	0.84	76.36	5.25	2.00	1.33	66.50	5.24	3.98	1.69	42.46
4	20	5.26	0.95	0.62	65.26	5.18	2.62	1.36	51.90	5.17	4.53	2.00	44.15
4	30	5.25	1.28	0.67	52.34	5.16	4.34	1.50	34.56	5.12	7.09	3.22	45.42

*: after the inactivation of rennet a: in % TN b: in % TN c: in % SN →: same series of cheese

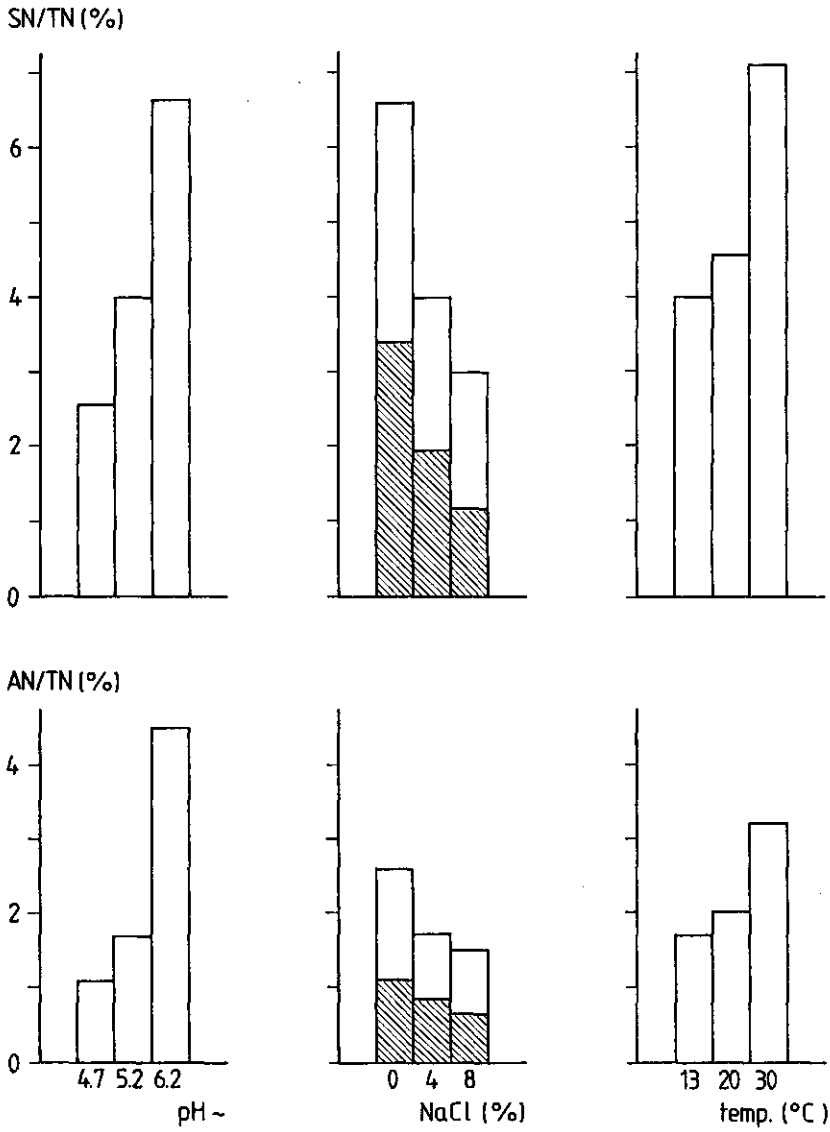


Fig. 4.4 Soluble N and amino acid-N produced by *L. cremoris HP* in cheeses from Table 4.3, as a function of pH (at 4% NaCl, 13°C), % NaCl relative to water (at pH ~ 5.2, 13°C) and ripening temperature (at 4% NaCl, pH ~ 5.2), after 91 days of ripening. Hatched areas represent the results of experiments in which paracasein had not been predigested by rennet whereas the other conditions were almost similar, see Table 4.1 - series 2. Both types of cheeses had been made with roughly $3x10^9$ bacteria (cfu) per g of cheese protein.

compare Table 4.3 and Table 4.1 - series 1. For example, after 91 days of ripening the values of % SN/TN and of % AN/TN of cheese with predigested paracasein at pH ~ 6.2 amounted to about 6.6% and 4.5%, respectively, whereas those values of cheese with untreated paracasein at almost similar conditions were only about 1.9% and 1.5%, respectively. An objection may be that these particular series of cheese were not prepared with equal numbers of viable bacteria. Cheeses with the predigested paracasein had been made with 3.2×10^9 cfu and those with untreated paracasein with 1.7×10^9 cfu per g of protein, respectively, which could have caused increased proteolysis in cheese with predigested paracasein. This difference in viable numbers, however, cannot have been the cause for the large differences in SN and AN between the two types of cheese; see the Sections 4.3.2.2 and 4.3.2.3.

HPLC-chromatograms of cheeses ripened for 6 and 91 days are shown in Fig. 4.5. They generally reflected the results from N-determinations. Far more medium and low-MW peptides and amino acids (RT > 15 min) were produced at a higher pH, especially after 91 days. The effect of ripening time on the increase of both fractions was small, being only perceptible at the higher pH values.

Chromatograms of cheese after the inactivation of rennet revealed the presence of predominantly medium and low-MW peptides. Upon keeping, blank cheeses (cheeses after the inactivation of rennet) showed no marked change of their chromatograms; examples are given in Fig. 4.5. Conveniently, their values of % SN/TN and of % AN/TN did not change significantly. These observations corroborate the effectiveness of the method to inactivate rennet.

Amino acids present after 91 days of cheese ripening are presented in Fig. 4.6-A. The formation of nearly all amino acids had been enhanced considerably at a higher pH, thus supporting the results from the AN-determinations and the HPLC-analyses. Evidently, the bacteria had produced the same amino acids from predigested paracasein as from untreated paracasein, but generally a much larger quantity of each amino acid had accumulated from the conversion of the former substrate: compare Fig. 4.6-A with Fig. 4.3-A (note the difference in scale). At pH ~ 6.2, the amino acids lysine, phenylalanine, tyrosine, valine, glutamic acid, glutamine, arginine and especially leucine had been produced abundantly. With the exception of cystine, also the others had

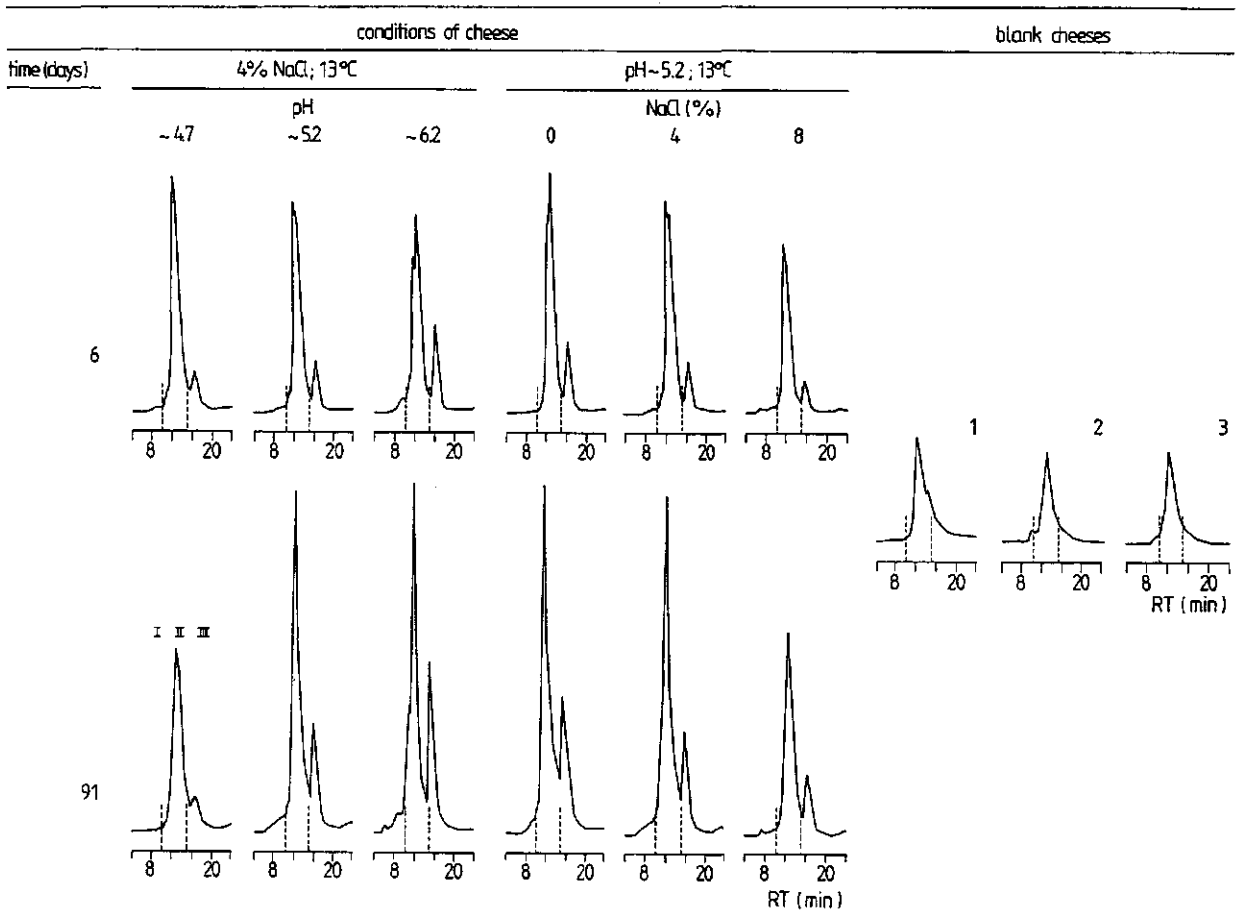


Fig. 4.5 HPLC-chromatograms of cheeses from Table 4.3, ripened for 6 and 91 days. Blank cheeses: 1. after the inactivation of rennet (zero time); 2. cheese at pH ~ 6.2 with 4% NaCl relative to water, kept at 13°C for 91 days; 3. cheese at pH ~ 5.2 without NaCl, kept at 13°C for 91 days.

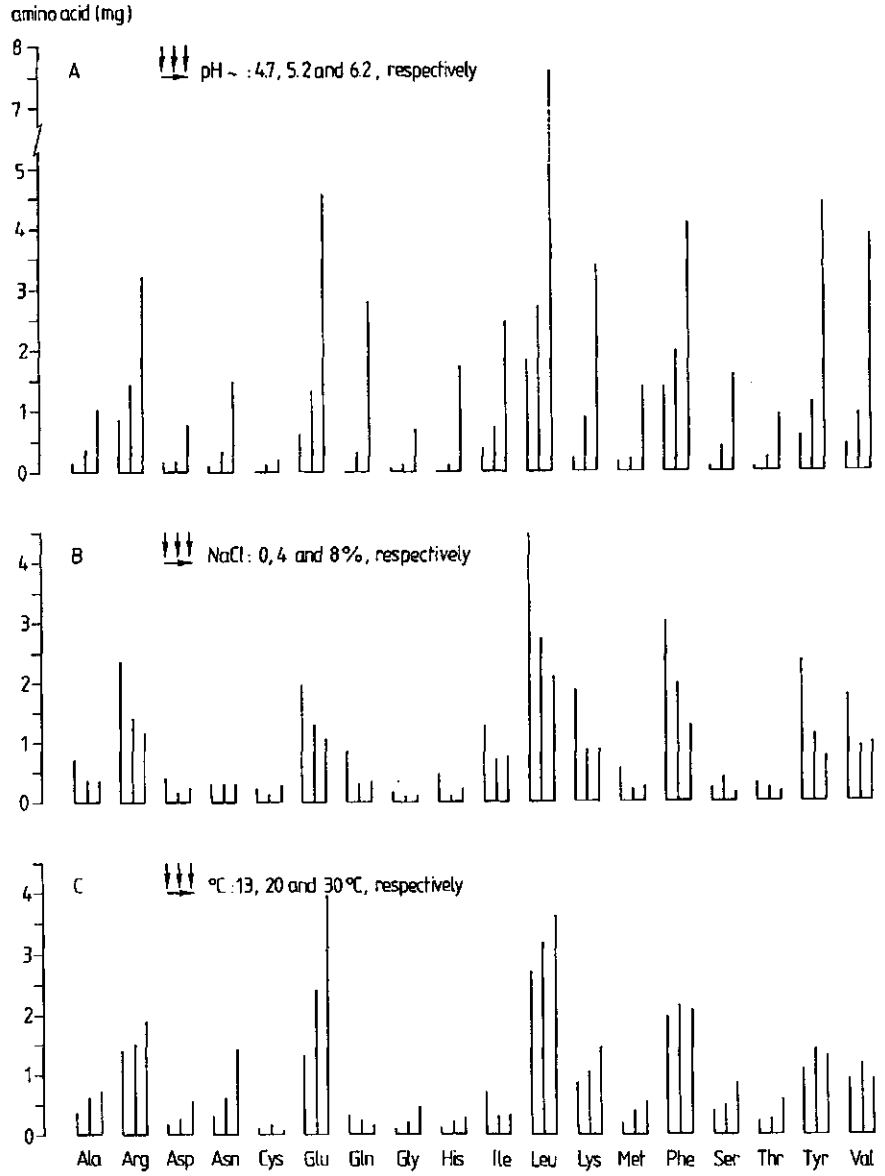


Fig. 4.6 Amino acids, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses from Table 4.3, as a function of pH (A), % NaCl relative to water (B) and temperature (C), after 91 days of ripening. At 30°C, NH_3 had accumulated to a quantity of about 2 mg per g of cheese protein, being about 5 times the quantity at all the other ripening conditions tested. Only quantities of ≥ 0.05 mg amino acid are indicated.

accumulated to appreciable quantities. At pH ~ 5.2, the production of all amino acids was reduced markedly. At pH ~ 4.7 most of the amino acids were hardly formed: leucine, phenylalanine, tyrosine, valine, isoleucine, glutamic acid and arginine having been produced most. For results on the total quantities of amino acids at various pH, see Fig. 4.7-A1.

4.3.2.2 Effect of NaCl

The effect of NaCl on the breakdown of predigested paracasein by the lactococcus strain was studied with cheese of a pH ~ 5.2, containing 0, 4 or 8% NaCl relative to water, and ripened at 13°C.

Values of % SN/TN and of % AN/TN of cheeses at their various stages of ripening are presented in Table 4.3; see also Fig. 4.4 for results after 91 days. Proteolysis had been increasingly reduced with an increasing concentration of NaCl. For example, compared with cheese ripened without NaCl for 91 days, the cheese with 4% NaCl contained about 40% less soluble N and about 35% less amino acid-N, whereas those percentages of cheese with 8% NaCl amounted to about 55% and 40%, respectively. Quantitatively, NaCl thus affected protein breakdown in cheese with predigested paracasein as in cheese with untreated paracasein (see Section 4.3.1.2). However, as already observed in the preceding Section, much more soluble N and amino acid-N were produced from the predigested substrate, as illustrated in Fig. 4.4 (effect of NaCl %). Cheeses with the different types of substrate (but otherwise with almost similar ripening conditions including a comparable number of bacteria added to cheese) appeared to accumulate much more soluble N and amino acid-N from the predigested paracasein.

HPLC-chromatograms of cheese supported the results from N-determinations, showing the formation of medium and low-MW peptides and of amino acids (RT > 15 min) to be less at a higher NaCl content, and to increase with keeping time; see Fig. 4.5.

Quantities of amino acids in cheese after 91 days of ripening are presented in Fig. 4.6-B. Results confirmed that the formation of most amino acids became increasingly reduced at a higher concentration of NaCl. For results on the total quantities of amino acids produced at various concentrations of NaCl, see Fig. 4.7-A2.

4.3.2.3 Effect of temperature

The effect of ripening temperature on the decomposition of predigested paracasein by the lactococcus strain was studied with series of cheese of pH ~ 5.2, with 4% NaCl relative to water, and ripened at 13, 20 or 30°C.

Quantities of soluble N and of amino acid-N are presented in Table 4.3; see also Fig. 4.4 for results after 91 days. Evidently, proteolysis increased with temperature and keeping time. For example, if compared with the cheese ripened at 13°C for 91 days, the cheese ripened at 30°C developed about 80% more soluble N and about 90% more amino acid-N. Again, much larger quantities of both N-fractions were produced from predigested paracasein than from untreated paracasein: compare the data in Table 4.1-series 3. These results can not be attributed to a higher number of bacteria in the cheese with predigested substrate, which might have enhanced proteolysis. Contrariwise, those particular series of cheese were made with less bacteria (in terms of cfu) than the cheeses with untreated paracasein, their numbers amounting to roughly $3x10^9$ and $15x10^9$ cfu per g of cheese protein, respectively.

HPLC-chromatograms of cheeses are not shown. By accident, the extracts were kept at a too high temperature which caused considerable precipitation of N-compounds. As a consequence, samples were not representative anymore.

Quantities of amino acids present in cheeses after 91 days of ripening are shown in Fig. 4.6-C. Results indicate that most amino acids had increasingly accumulated at a higher temperature. However, if compared with their quantity in the cheese ripened at 13°C, the accumulation of some amino acids, e.g. of phenylalanine, tyrosine and valine, seemed only slightly greater at 20 and 30°C, whereas the formation of isoleucine even seemed to have been less at these elevated temperatures. It may therefore be questioned as to what extent the concentrations of the individual amino acids in cheese, especially in cheese with predigested paracasein ripened at 30°C, represent their real production. A part of the amino acids will have been further decomposed by e.g. deamination, liberating NH_3; cheese ripened at 30°C had accumulated about 2 mg of NH_3 per g of cheese protein, being about 5 times the quantity in cheeses ripened at the lower temperatures, and indicating that about 35% of the total amino acid-N produced had been converted into ammonium-N.

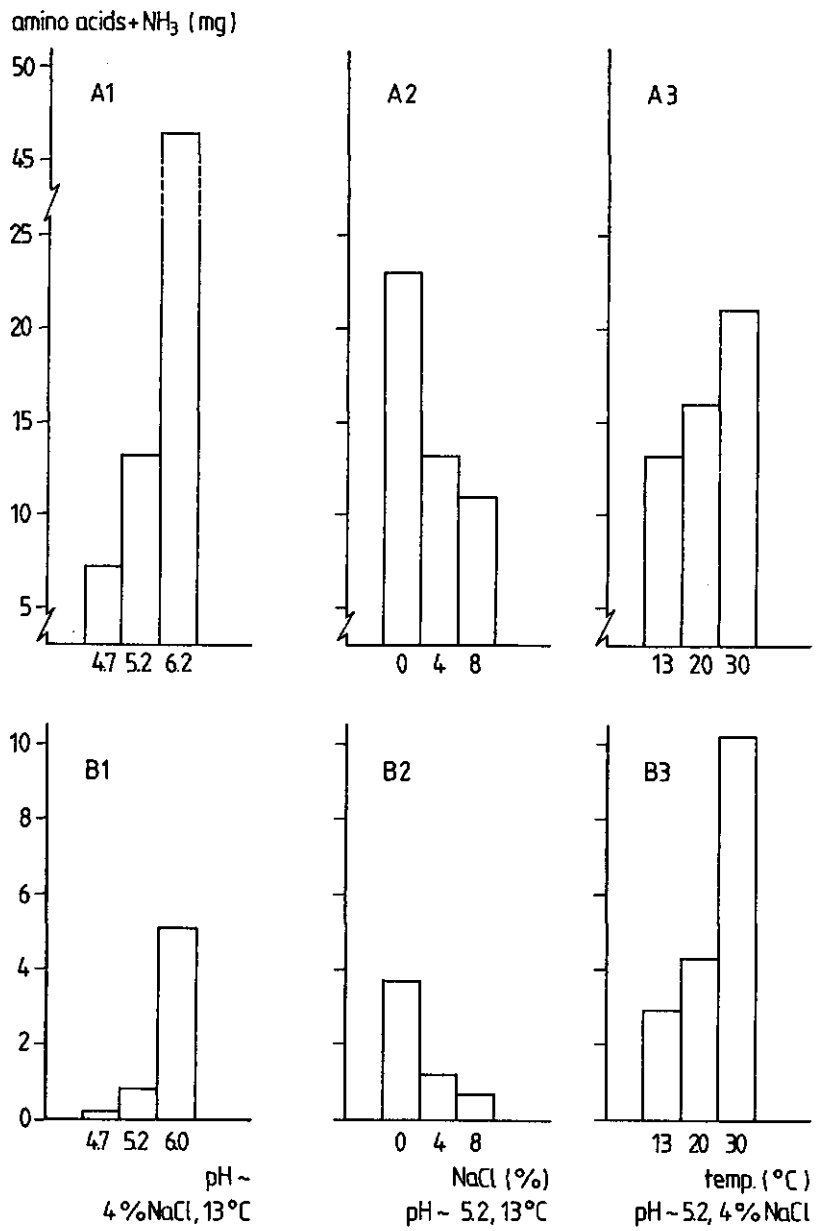


Fig. 4.7 Total amino acids + NH_3, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses with a P/W ratio 1:2.5, as a function of pH, % NaCl relative to water and temperature, after 91 days of ripening. A: cheeses with predigested paracasein from Table 4.3, and B: cheeses with untreated paracasein from Table 4.1. Bacteria (cfu) added per g of cheese protein: A_1 to A_3, 3.2×10^9; B_1, B_2, B_3: 1.7, 2.5 and 14.7×10^9, respectively.

The results indicate that the proportions of the individual amino acids in AN-extracts of cheese changed significantly at a high ripening temperature, illustrating the known fact that determinations of amino acid-N cannot be used to characterize the nature of amino acid production.

For results on the total quantities of amino acids, see Fig. 4.7-A3.

4.3.2.4 Other observations

Cheeses developed high values of % AN/SN (see Table 4.3). The proteolytic action of the bacteria thus predominantly liberated small peptides and amino acids.

At the various ripening conditions of cheeses tested, the quantities of 'amino acids + NH_3' nitrogen per g of cheese protein were calculated from the values of % AN/TN and from the quantities of 'amino acids + NH_3' nitrogen derived from amino acid analyses, respectively. Examples are given in Table 4.4. The results differ far less than those from cheeses with untreated paracasein (compare with Table 4.2), which most probably may be explained by the relatively higher contribution of amino acids to the total quantity of N-compounds contained in the AN-extracts of cheese with predigested paracasein.

Table 4.4 Milligrams of 'amino acids + NH_3' nitrogen per g of protein in cheeses from Table 4.3 (series with different NaCl %), calculated from the values of % AN/TN (A) and from the quantities of 'amino acids + NH_3' nitrogen derived from amino acid analysis (B).

Ripening time (days)									
	6			32			91		
NaCl %	0	4	8	0	4	8	0	4	8
A	1.90	1.31	0.68	2.27	2.08	1.39	4.07	2.65	2.36
B	2.22	1.62	0.89	2.47	2.17	1.41	4.10	2.22	1.97

Electropherograms of cheese (PAGE) being submitted to the action of rennet for 12 days at 13°C showed the usual patterns of α_s- and ß-casein degradation. After the inactivation of rennet, proteolysis by the bacteria of the predigested paracasein did not reveal any clear change of those patterns at all ripening conditions tested (results not shown).

As was also observed with cheeses made from untreated paracasein (Section 4.3.1.4), for cheese with predigested paracasein the peak areas of the chromatograms representing amino acids (RT > 15 min) did not correlate well with the total quantities of amino acids derived from amino acid analyses. However, either method showed the same trend.

4.4 Conclusions

Protein breakdown by *L. cremoris HP* in cheeses made from untreated paracasein or from predigested paracasein was affected considerably by pH, % NaCl and temperature. The decomposition of protein was greatest in cheese without NaCl (at pH ~ 5.2, 13°C), at a high pH (at 4% NaCl, 13°C) and at a high ripening temperature (at pH ~ 5.2, 4% NaCl), and was increasingly reduced for a higher NaCl %, a lower pH and a lower temperature. Proteolysis was much more extensive in cheeses made from predigested paracasein than in those made from untreated paracasein.

Gel electrophoresis (PAGE) proved to be unsuitable to detect any proteolytic activity of the bacterium. The effect of a particular ripening parameter on protein breakdown was reflected by the other methods used to follow proteolysis: they all showed a similar trend.

Results are discussed in Chapter 7.

CHAPTER 5

Proteolytic action of *Lactococcus cremoris HP* in model cheeses. 2. Effect of the ratio of paracasein to water

5.1 Introduction

Especially the pH of cheese, its ratio of paracasein to water, and to a lesser extent its ionic strength (mainly dependent on the NaCl content relative to water), strongly affect the equilibrium between the quantity of undissolved colloidal calcium phosphate associated with paracaseinate and the quantities of calcium and phosphate dissolved. The dissolution of calcium phosphate is higher for a lower pH. At a particular pH and NaCl content of cheese, a lower P/W ratio increases the proportions of calcium and phosphate dissolved, but simultaneously decreases the final concentrations of these constituents in the cheese moisture. Compared with a suspension of paracaseinate with a P/W ratio of 1:2, those effects were increasingly distinct for more dilute suspensions with a P/W ratio of 1:10 or 1:50 (Monib, 1962); see Table 5.3 for his results on Ca. Along with other physico-chemical factors, the P/W ratio largely determines the chemical composition of the cheese moisture and the conformation of the protein; consequently, the ratio may affect enzyme activity and attackability of the substrate.

Broadly speaking, in industrial cheesemaking the production of hard cheese (P/W ratio 1: < 1.5), or of semi-hard cheese (P/W ratio 1:1.5 to 2) or of soft cheese (P/W ratio 1:2 to 3.5) is distinguished. These ratios differ far less than those studied by Monib (1962), and therefore will affect the quantities of calcium and phosphate dissolved and their concentrations in the cheese serum less significantly. A few data in the literature, in particular studies by Raadsveld (1952) on Edam cheese, suggest that proteolysis is enhanced at a higher water content of the cheese, which may indicate an effect of a relatively small difference in the P/W ratio of cheese on the protein breakdown. Experimental evidence for this is however lacking. Virtually, no studies have been done with cheeses differing in their P/W ratio but otherwise of a similar and standardized composition, ripened under entirely controlled bacteriological conditions.

This brought us to study the effect of the P/W ratio on the protein breakdown by *L. cremoris HP* with the models of cheese described in Chapter 3. The experiments were done with cheeses with a ratio of 1:2.5 (which are far easier to prepare than those with a ratio of e.g. 1:2; see Section 3.2), and with cheeses with a ratio of 1:3.5. These various ratios prevail in varieties of cheese like Camembert and Noordhollandse Meshanger cheese, respectively. Cheeses were made from paracasein predigested by rennet, which is the usual substrate for starter bacteria in most varieties of ripened cheese.

5.2 Materials and methods

5.2.1 Preparation and storage of cheeses

The cheeses were prepared as described in Section 3.2.3 from autoclaved paracaseinate; see Section 2.1.1.1-batch 2. The paracasein was predigested by rennet for 16 days at 13°C. After rennet inactivation, the composition of cheeses was adjusted following the procedures described in Section 3.2.3.3. Cheeses were made with a pH~5.2, 4% NaCl relative to water, and with a p/w ratio of 1:2.5 or of 1:3.5. Bacterial cells were derived from one batch; their number added to each cheese amounted to 5.2×10^9 cfu per g of cheese protein. The cheeses were made in duplicate and ripened up to 80 days at 13°C under anaerobic conditions; see Section 2.4.

Control cheeses (blanks) made from untreated paracasein (pH~5.2, 0% NaCl, P/W ratio of 1:2 according to Section 3.2.3.1) or made after the inactivation of rennet (pH~5.2, 4% NaCl, P/W ratio of 1:2.5 or of 1:3.5) were stored and analysed to establish the absence of milk proteinase activity and the effectiveness of rennet inactivation, respectively.

Blank cheeses with a P/W ratio ranging between 1:2 and 1:3.5 were analysed for the quantities of calcium and inorganic phosphorus becoming dissolved and their concentrations in the cheese serum. Before analysis, those cheeses were kept overnight at 13°C to allow their composition to equilibrate. In this particular experiment, use was made of a newly prepared batch of autoclaved paracaseinate; its composition, especially its mineral content, therefore differed from the batch of Section 2.1.1.1; see also Table 5.3.

The cheeses with a P/W ratio of 1:3.5 showed some separation of moisture.

5.2.2 Estimation of protein breakdown

Proteolysis was followed by determinations of soluble N and of amino acid-N, by amino acid analysis and HPLC analysis of cheese extracts, and occasionally by PAGE; see Sections 2.6 to 2.9.

5.2.3 Calcium and inorganic phosphorus

Calcium and inorganic phosphorus in the dry paracaseinate and in the serum of cheese were determined according to the methods described in Section 2.10.

5.3 Results

The cheeses were analysed at 'zero time' (i.e. 4 h after the addition of bacteria) and after 7, 31 and 80 days of ripening. The slight changes of the pH of cheese agreed with earlier observations; see Chapter 4.

5.3.1 N-determinations

Results on the formation of soluble N and of amino acid-N are presented in Table 5.1. The results have been corrected for the values of % SN/TN and of % AN/TN of cheeses after the inactivation of rennet, on average amounting to 11.3% and 0.3%, respectively. Obviously, proteolysis was more extensive after a longer keeping time. However, in either type of cheese the increase of the values of % SN/TN or of % AN/TN were about the same, implying that the proteolytic action of the bacteria was not markedly affected by the difference in the P/W ratio studied. As established by the high values of % AN/SN, proteolysis predominantly liberated amino acids from soluble N.

Control cheeses did not accumulate soluble N and amino acid-N.

Table 5.1 Production of soluble N and of amino acid-N from predigested paracasein by *L. cremoris HP* in cheeses at pH ~ 5.2 and containing 4% NaCl relative to water, as a function of the P/W ratio and the ripening time at 13°C. Averaged results from duplicate series of cheese, corrected for values of blank cheeses after the inactivation of rennet. Freshly made cheeses contained 5.2×10^9 cfu per g of cheese protein.

P/W ratio	Ripening time in days[a]															
	0[b]				7				31				80			
	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]
1:2.5	5.22	0.00	0.15	100	5.20	1.22	0.73	60	5.26	3.68	2.48	67	5.18	6.40	3.23	50
1:3.5	5.21	0.00	0.18	100	5.25	1.17	0.81	69	5.28	3.91	2.29	59	5.19	6.11	2.95	48

a: after the inactivation of rennet b: 4 h after the addition of bacteria c: in % of TN d: in % of SN

Table 5.2 Milligrams of 'amino acids + NH_3' nitrogen per g of protein in cheeses from Table 5.1, calculated from the values of % AN/TN (A) and from the quantities of 'amino acids + NH_3' nitrogen derived from amino acid analyses (B).

Ripening time	0		7		31		80	
P/W ratio	1:2.5	1:3.5	1:2.5	1:3.5	1:2.5	1:3.5	1:2.5	1:3.5
A	0.23	0.28	1.14	1.27	3.89	3.59	5.06	4.62
B	0.83	1.02	1.43	1.52	5.76	4.83	6.48	6.06

5.3.2 Analysis by HPLC

Chromatograms of cheeses are presented in Fig. 5.1. They generally reflected the results of N-determinations.

Upon keeping, control cheeses made from untreated paracasein did not show any change on the chromatogram. This corroborated the absence of residual milk proteinase, which activity still was observed in blank cheeses made from paracaseinate heated for 30 min at 95°C; see Section 4.3.1.2). The chromatograms of control cheeses made from predigested paracasein were neither affected by the keeping time of cheese, which established that rennet had been inactivated completely.

Chromatograms of cheeses with bacteria showed the quantities of medium and low-MW peptides and of amino acids (RT > 15 min) to increase with ripening time. On any sampling day, patterns of the two types of cheese nicely corresponded, indicating that proteolysis was not affected significantly by the P/W ratio in the range studied.

5.3.3 Amino acid analysis

Results of the determinations of amino acids are presented in Fig. 5.2. They show that the quantities of the amino acids increased with the ripening time of cheese. Already 4 h after the addition of bacteria the amino acids arginine, isoleucine, leucine and phenylalanine had accumulated significantly. At the end of storage arginine, glutamic acid, lysine, phenylalanine, tyrosine, valine, and especially leucine had accumulated most, which largely reflected earlier results; see Fig. 4.6-A. Contrary to the results of all the other experiments during our studies, proline did accumulate in the cheese ripened for 80 days. During the entire ripening period, cystine was only produced at a very low level and has been omitted from the figure.

Obviously, the production of each amino acid was not affected substantially by the difference in the P/W ratio. Several of the amino acids accumulated at an almost equal rate in both types of cheese. The formation of others tended to be somewhat less at the ratio of 1:3.5, which might have some significance because of the values of % AN/TN of cheese with that ratio were also found to be somewhat lower after 31 and 80 days of ripening; see Table 5.1.

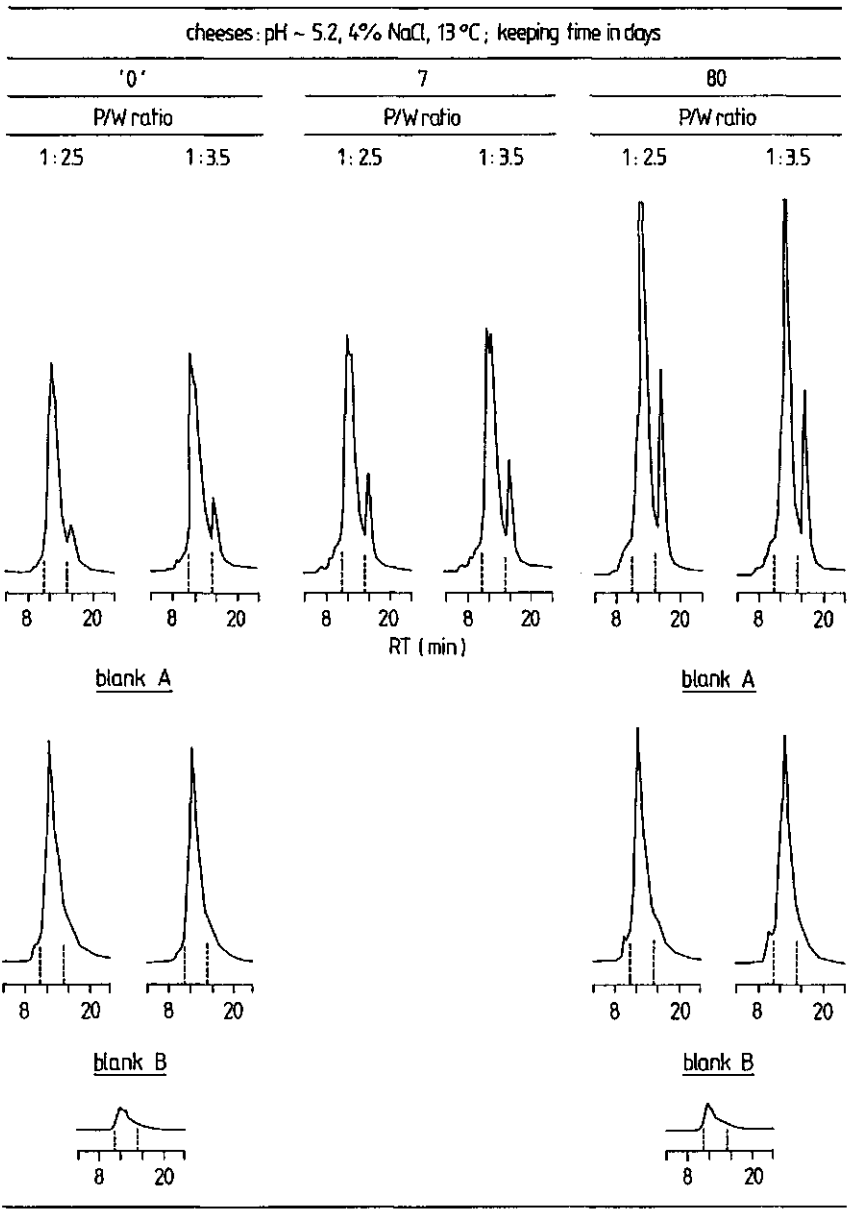


Fig. 5.1 HPLC-chromatograms of cheeses from Table 5.1; results after 31 days have been omitted. '0' means 4 h after the addition of bacteria to cheese. Blank cheeses: A. made from predigested paracasein; B. made from untreated paracasein (note: P/W ratio of 1:2, 0% NaCl). Fractions were classified according to the standard curve of Fig. 4.1.

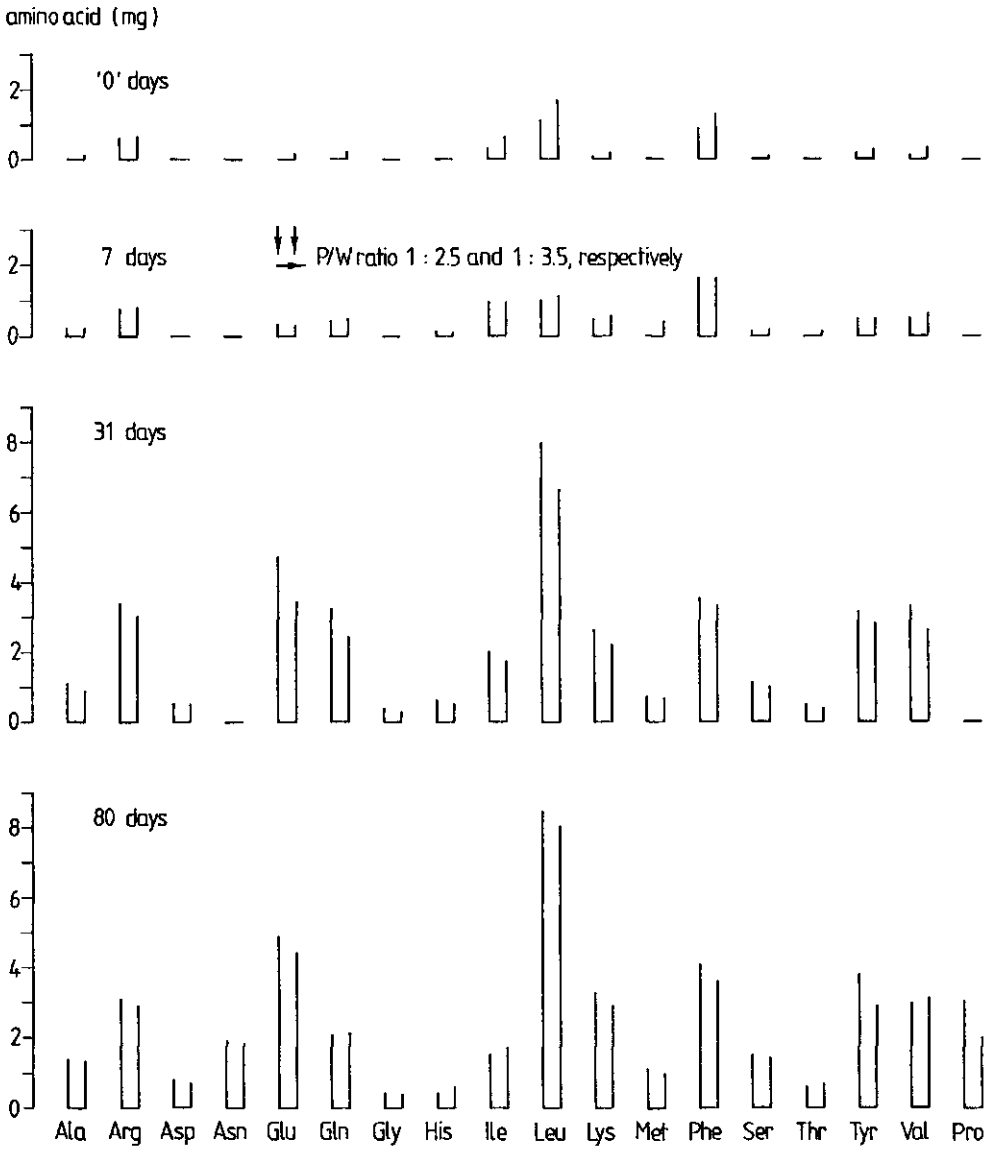


Fig. 5.2 Amino acids, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses from Table 5.1, as a function of the P/W ratio and ripening time of cheese. NH_3 accumulated from ~0.3 mg to ~0.7 mg per g of cheese protein at the end of ripening. Only quantities of ≥0.05 mg amino acid are indicated.

Exceptionally, the values of 'amino acids + NH_3' nitrogen per g of cheese protein calculated from the results of amino acid analyses considerably exceeded those calculated from the values of % AN/TN, but they showed the same trend; see Table 5.2.

5.3.4 Gel electrophoresis

Electropherograms of cheese are shown in Fig. 5.3. The pattern of control cheeses made from untreated paracasein did not change with keeping time (slots 1 and 2). Cheeses with paracasein submitted to the action of rennet showed the usual patterns of casein degradation; in particular α_{s1}-casein and, to a lesser extent, ß-casein becoming more decomposed with time (slots 3 to 5). Electropherograms of control cheeses with inactivated rennet were similar at zero time and after 80 days of keeping (slots 6 and 7). Like the other methods to follow proteolysis, the results of PAGE established the absence of proteolytic activity in blank cheeses.

The proteolytic action of the bacteria on predigested paracasein was not reflected in the electropherograms of cheese; patterns were not affected by the P/W ratio and the ripening time of cheese (slots 8 and 9).

5.3.5 Calcium and inorganic phosphorus

Results of the estimations of calcium and inorganic phosphorus are presented in Table 5.3; they established that the lower the P/W ratio of cheese, the more calcium and inorganic P dissolved, accompanied by a decrease of their concentrations in the cheese serum. The results thus showed a same trend as those from Monib (1962), but obviously differences in the P/W ratios of cheese as applied in practical cheesemaking much less affect the dissolution of calcium phosphate and the composition of the cheese moisture than do more dilute suspensions of paracaseinate.

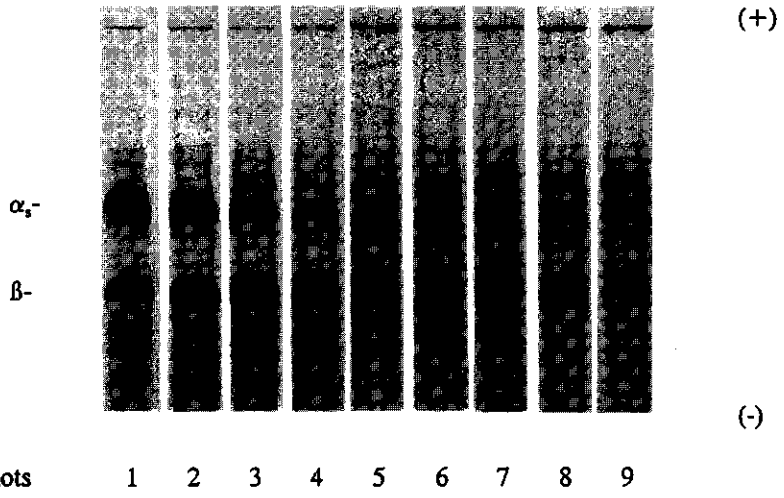


Fig. 5.3 Electrophoretic patterns of model cheeses. Slots 1 and 2: control cheeses with untreated paracasein, at zero time and after 80 days at 13°C, respectively. Slots 3 to 5: cheeses with paracasein submitted to rennet action for 8 h, 32 h or 16 days at 13°C. Slots 6 and 7: control cheeses with predigested paracasein after the inactivation of rennet, kept for 14 and 80 days at 13°C.
Slots 8 and 9: cheeses made with rennet - inactivated, predigested paracasein plus starter bacteria, ripened for 80 days at 13°C at P/W ratios of cheese adjusted to 1:2.5 and 1:3.5, respectively.

Table 5.3 Quantities of calcium and inorganic phosphorus dissolved from paracaseinate, expressed in percentages of total Ca and inorganic P (A), and their concentrations (% w/w) in the serum of cheese (B), as a function of the P/W ratio of cheese at pH ~ 5.2 with 4% NaCl relative to water. The dry paracaseinate contained 3.50% Ca and 1.12% inorganic P. For comparison, results by Monib (1962) on determinations of calcium in suspensions of paracaseinate at almost corresponding pH and NaCl content are included.

P/W ratio	1 : 2		1 : 2.5		1 : 3.5	
	A	B	A	B	A	B
calcium	31.90	0.67	32.22	0.54	34.23	0.42
inorganic P	20.83	0.14	24.22	0.13	28.01	0.11
Monib (1962)	1 : 2		1 : 10		1 : 50	
	A	B	A	B	A	B
calcium	30.60	0.54	69	0.18	96	0.05

5.4 Conclusions

In model cheeses with a different P/W ratio (1:2.5 or 1:3.5) but at otherwise similar conditions, the breakdown of predigested paracasein by *L. cremoris HP* proceeded correspondingly. Obviously, the proteolytic system of the bacterial strain at these ratios was not affected significantly by the water content, or by the quantity of calcium-phosphate dissolved or by the chemical composition of the cheese moisture.

Results are further discussed in Chapter 7.

CHAPTER 6

Proteolytic action of *Lactococcus cremoris HP* in model cheeses. 3. Effect of lysis of cells

6.1 Introduction

It is generally agreed that proteinases and peptidases of starter bacteria are essential for cheese ripening. Their concerted action produces a wide variety of substances that are crucial for cheese flavour, like peptides, amino acids, keto acids, alkanones, alkanals, alkanols, sulfhydryls, amines, NH_3. A proportion of the proteinase activity and most of the peptidase activity is located in the cytoplasm of the cells.

After their growth during cheese making, starter bacteria more or less quickly lose their viability. During cheese ripening, they are frequently said to exhibit autolysis. Put simple, this process may be described as the degradation of the peptidoglycan layer of the cell wall by autolysins, which causes in particular the membrane of cells with an osmotic pressure higher than that of their environment to burst or to attain increased permeability. As a consequence, intracellular (proteolytic) enzymes are released into the cheese matrix or, otherwise, the uptake of particular extracellular compounds by the damaged cell may be facilitated. In either case, those enzymes will act more directly on their potential substrates, which in turn may affect the nature and the extent of protein breakdown in cheese.

The literature reports that cell-free starter enzymes function under the conditions prevailing in cheese. This was particularly established in trials with Cheddar cheese, which were aimed at studying the mechanism of aroma formation (e.g. Law *et al.*, 1976). Considerable quantities of free amino acids were produced in δ - gluconic acid lactone - acidified (GAL) cheese containing lysozyme-sensitized cells (LSC) of a starter culture only, and their production was much higher in starter acidified cheese made with the same, but live culture plus LSC than in cheese made with the live culture alone, at a same number of viable cells in the freshly made cheese. LSC were added to the milk and their lysis in cheese was assured by the combined effect of lysozyme and salt added at milling the curd. Such studies greatly contribute to the understanding of particular aspects

of cheese maturation. They are, however, not adequate to study differences in the proteolytic behaviour of (at least initially) intact starter cells or of their lysed counterparts under conditions as in a ripening cheese. For instance, as already mentioned by the authors (Law *et al.*, 1976) the lysozyme treatment of starter cells, combined with centrifugation to concentrate the LSC before their addition to the milk, caused the partial loss of proteolytic activity, being attributable to the loss of cell wall-bound proteinase. Furthermore, the physical and chemical properties of chemically acidified cheese differ from those of starter acidified cheese. Initial proteolysis by rennet proceeds more quickly in GAL cheese (O'Keeffe *et al.*, 1975) and the redox potential of GAL cheese is much higher than that of starter acidified cheese, amounting to ~ +300 mV (Law *et al.*, 1976) and ~ -150 mV (Galesloot, 1960), respectively. Moreover, the two types of cheese inevitably differ somewhat as to pH, NaCl % relative to water, number of cells incorporated in the curd, etc.. All of these factors may affect the rates and equilibria of enzymic reactions in cheese. It must therefore be concluded that the experiments performed on 'real' cheese were not suitable for quantitatively studying the effect of lysis of starter bacteria on proteolysis.

Such a quantitative approach ought to be possible with the models of cheese as described in Chapter 3, by studying the proteolytic action of a fixed mass of initially intact cells of *L. cremoris HP* and that of a same mass of lysed cells containing all the proteolytic activity of the non-lysed cells, respectively, in cheeses of an otherwise similar composition. The cheeses were made from paracasein predigested by rennet, being the usual substrate for starter bacteria in most varieties of ripened cheese.

6.2 Materials and methods

6.2.1 Preparation and storage of cheeses

The cheeses were prepared as described in Section 3.2.3, from autoclaved paracaseinate; see Section 2.1.1.1-batch 2. The paracasein was predigested by rennet for 20 days at 13°C. After the inactivation of rennet, the composition of cheeses was adjusted following the procedures described in Section 3.2.3.3 and Section 3.2.4. The concentrate of bacterial cells was treated with lysozyme and the cells were lysed (as observed by

microscopic examination) by an osmotic shock with 0.15 M K_2SO_4 (Section 2.3.1.2); the concentration of K_2SO_4 in the suspension of intact cells was adjusted correspondingly. Bacterial cells were derived from one batch. The mass of live or lysed cells added to each cheese corresponded to $0.4x10^9$ cfu per g of cheese protein. The cheeses were made with a pH~5.3, 4% NaCl relative to water, and a P/W ratio of 1:2.5. Control cheeses with predigested paracasein (after rennet inactivation) plus lysozyme and K_2SO_4 in particular served to verify the absence of proteolytic activity of the lysozyme preparation. The cheeses were made in duplicate and were ripened up to 70 days at 13°C under anaerobic conditions; see Section 2.4.

Preliminary experiments on the breakdown of protein by cells of the lactococcal strain learned that proteolysis was not affected by the presence of ~0.03 M K_2SO_4 (final concentration) in the serum of cheese, as a result of the procedure applied to lyse the cells.

6.2.2 Estimation of protein breakdown

Proteolysis was followed by determinations of soluble N and of amino acid-N, and by amino acid analysis; see Sections 2.6.2 and 2.8. Gel electrophoresis (PAGE) and HPLC-analysis of cheese extracts were not applied because of their limited value in quantitatively following protein breakdown; see e.g. Sections 5.3.4 and 2.3.2.4.

6.3 Results

The cheeses were analysed at 'zero time' (i.e. 2 h after the addition of intact or lysed cells), and after 7, 28 and 70 days of ripening. Cheeses showed only minor changes in pH during keeping; see Table 6.1.

Table 6.1 Production of soluble N and of amino acid-N from predigested paracasein by initially intact (A) or lysed cells (B) of *L. cremoris HP* in cheeses at a pH~5.3, with 4% NaCl relative to water and a P/W ratio of 1:2.5, ripened for various periods at 13°C. Averaged values of duplicate cheeses, corrected for values of blank cheeses after the inactivation of rennet. In every cheese, the mass of intact or lysed cells added corresponded to 0.4×10^9 cfu per g of cheese protein.

	Ripening time in days[a]															
	0[b]				7				28				70			
	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]	pH	SN[c]	AN[c]	AN[d]
A	5.34	0.21	0.10	48	5.33	1.43	0.52	37	5.32	2.43	0.75	31	5.33	3.15	2.12	67
B	5.35	0.25	0.08	32	5.31	2.46	1.11	45	5.32	3.73	2.31	62	5.34	5.37	4.30	80

a: after the inactivation of rennet b: 2 h after the addition of intact or lysed cells c: in % of TN d: in % of SN

6.3.1 N-determinations

Results on the formation of soluble N and of amino acid-N are given in Table 6.1. The results have been corrected for the values of % SN/TN and of % AN/TN of cheese after the inactivation of rennet, which on average amounted to 17.5% and 0.3%, respectively. The results established that in both types of cheese proteolysis was increased with ripening time, but that the production of soluble N and of amino acid-N in cheese with the homogenate of bacterial cells considerably exceeded their formation in cheese to which intact cells had been added; e.g. after 70 days of ripening, the cheese with lysed cells contained about 1.7 as much soluble N and about 2.0 as much amino acid-N. This is to be attributed to the effect of lysis because the control cheeses with lysozyme and K_2SO_4 showed no increase of the values of % SN/TN and of % AN/TN during keeping, which established that the lysozyme preparation did not contribute to protein breakdown.

6.3.2 Amino acid analysis

Results of the determinations of free amino acids are presented in Fig. 6.1; they reflect those of amino acid-N determinations. With either type of cheese, the individual amino acids accumulated with ripening time, be it for each amino acid much more rapidly in the cheese containing the lysed cells. The patterns of amino acids produced in the cheese made with intact bacteria corresponded well with our earlier results (compare Fig. 6.1 with Figs. 4.6-A and 5.2); differences as to their overall quantities produced in the various experiments are to be attributed primarily to a different number of bacteria added to cheese, and to varying periods applied to predigest the paracasein by rennet, causing the composition of the 'pool of peptides' to vary. Cystine was produced at a very low level and has been omitted from Fig. 6.1; proline was not detected at all.

The total quantities of amino acids produced during the ripening of cheeses are illustrated in Fig. 6.2. They show a drastic effect of a homogenate of cells on protein breakdown, the production of amino acids being increased by a factor of about 3, compared with the cheese made with intact cells. In terms of amino acid formation, lysis of cells did not seem significantly affect the proteolytic pattern. Results of rough

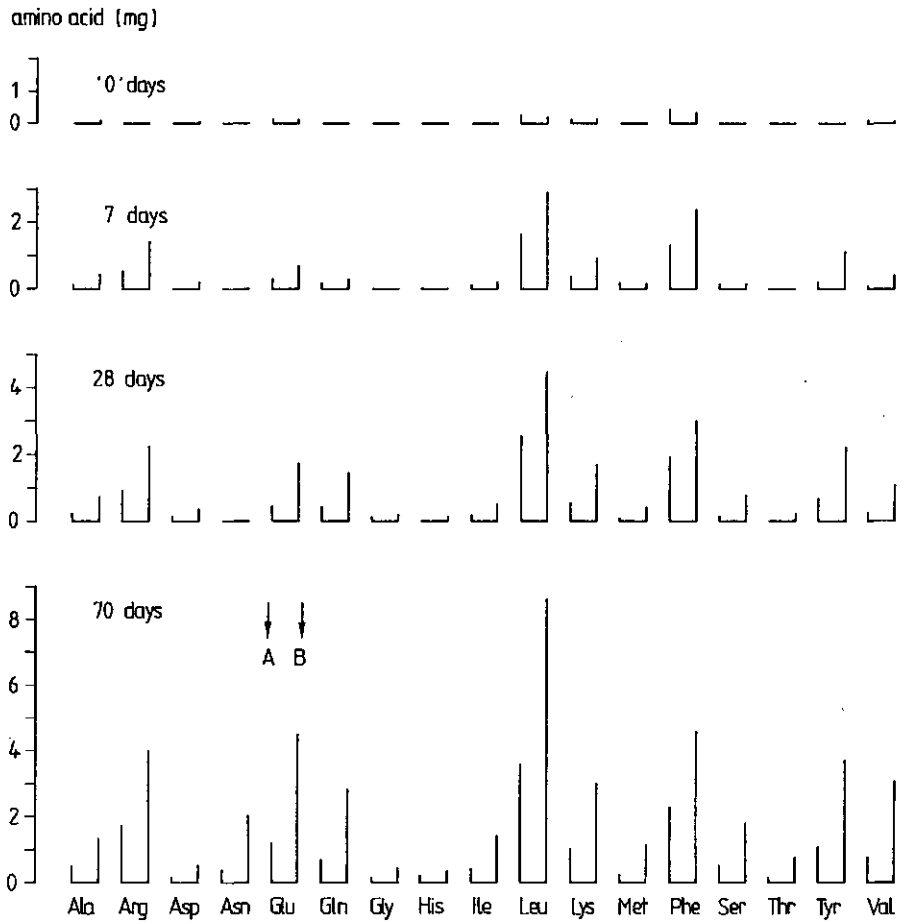


Fig. 6.1 Amino acids, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses from Table 6.1. A: cheese made with intact cells; B: cheese made with lysed cells. In either type of cheese, NH_3 accumulated from ~0.3 mg to ~0.5 mg of cheese protein at the end of ripening. Only quantities of ≥0.05 mg amino acid are indicated.

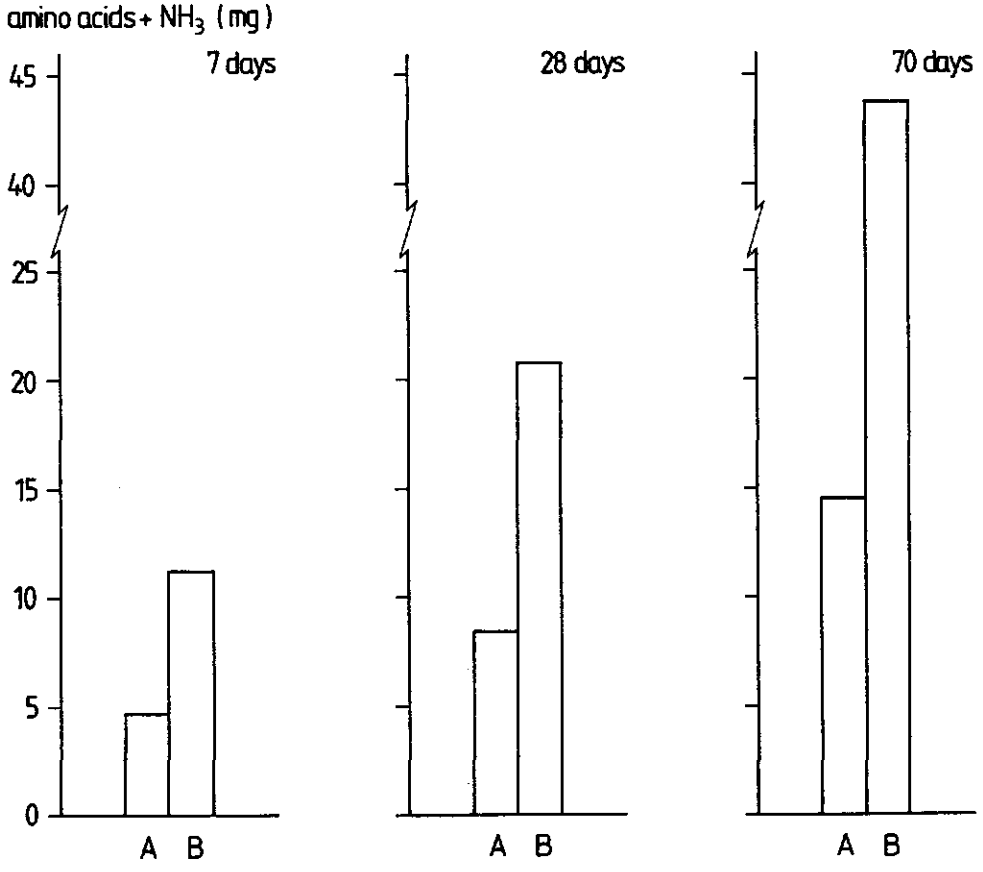


Fig. 6.2 Total amino acids + NH_3, in mg per g of cheese protein, produced by *L. cremoris HP* in cheeses from Table 6.1. A: cheese made with intact cells; B: cheese made with lysed cells. Results at 'zero time' have been omitted. The data were derived from Fig. 6.1.

Table 6.2 Milligrams of individual amino acids, expressed as a percentage of the total milligrams of amino acids produced, in cheeses from Table 6.1, at various periods of ripening. A: cheese made with intact cells; B: cheese made with lysed cells. Calculations were made from the data of the Figs. 6.1 and 6.2.

amino acid	Ripening time in days					
	7		28		70	
	A	B	A	B	A	B
Ala	2	3	3	3	3	3
Arg	10	12	11	10	12	10
Asp	-	1	1	2	1	1
Asn	-	-	-	-	2	5
Glu	5	6	5	8	8	10
Gln	3	3	5	6	5	6
Gly	-	-	1	1	1	1
His	-	-	-	1	1	1
Ile	2	1	2	3	2	3
Leu	35	26	30	21	24	20
Lys	5	8	6	8	7	7
Met	-	1	1	2	1	3
Phe	28	23	22	14	15	10
Ser	2	2	2	4	3	4
Thr	-	-	-	1	1	1
Tyr	5	10	7	11	8	8
Val	3	4	4	5	6	7

- : concentration of amino acid <0.05 mg per g of cheese protein

calculations suggest that the amino acids were produced proportionally in each type of cheese; see Table 6.2; more research would be needed to establish this more accurately.

In this study, the values of 'amino acids + NH_3' nitrogen per g of cheese protein calculated from the results of amino acid analyses agreed reasonably well with those calculated from the values of % AN/TN; see Table 6.3.

Table 6.3 Milligrams of 'amino acids + NH_3' nitrogen per g of protein in cheeses from Table 6.1, calculated from the values of % AN/TN (C) and from the quantities of 'amino acids + NH_3' nitrogen derived from amino acid analyses (D). A: cheese made with intact cells; B: cheese made with lysed cells.

	Ripening time in days							
	0[a]		7		28		70	
	A	B	A	B	A	B	A	B
C	0.16	0.13	0.82	1.74	1.18	3.62	3.32	6.74
D	0.33	0.37	0.83	1.83	1.38	3.18	2.36	6.41

a: 2 h after the addition of intact or lysed cells.

6.4 Conclusions

In terms of the production of soluble N and particularly that of amino acids, a lysed mass of *L. cremoris HP* drastically enhanced proteolysis in cheese made from predigested paracasein, as compared to the action of a same mass of intact cells added to cheese of otherwise similar composition.

Results are further discussed in Chapter 7.

CHAPTER 7

Proteolytic action of *Lactococcus cremoris HP* in model cheeses. 4. General discussion

Over the years, much efforts has been spent in describing proteolysis in cheese, as is evident from the numerous publications. Because of the complexity of both the substrate and the enzyme systems involved in cheese maturation, many investigations are performed with simple substrates like milk, solutions of casein or synthetic peptides to elucidate the actions of the various proteolytic agents. Literature on the action of starter bacteria, extracts of cells or purified proteinases and peptidases has been reviewed by Law & Kolstad (1983), Kamaly & Marth (1989), Fox (1988-1989, 1989) and Laan *et al.* (1989). Although overwhelming fundamental knowledge has been gained this way, results of studies with substrates showing conditions far removed from those in cheese need not reflect the activity of enzyme systems in cheese itself. In fact, reliable conclusions from such studies can only be drawn if the model used is representative for cheese in the essential aspects. Aseptic model curds, based on the conversion of milk into chemically or starter acidified 'real' cheese, meet those conditions to a considerable extent (Mabbitt *et al.*, 1955; Lebars *et al.*, 1975; Kleter, 1975; Visser, 1976, 1977*a*). Their manufacture is, however, far from simple, requiring aseptic milking of cows or the use of intensively heated milk provided with preservatives to prevent the growth of undesired micro-organisms in the made cheese, aseptic cheesemaking techniques requiring special equipment, etc. Heating intensities sufficient to inactivate alkaline milk proteinase (e.g. 16 s at 140°C; Driessen, 1983) preclude rennet coagulation of the milk. As a consequence, such models do never exclude the activity of milk proteinase from the ripening process, and particularly at conditions that favour its action in cheese (a fairly high pH, or a low NaCl concentration or a fairly high temperature) interaction with other enzyme systems cannot be excluded. Moreover, those models make it almost impossible to independently vary a particular ripening factor, since the change in one factor inevitably affects the composition in other respects, e.g. by differences in pH, % NaCl relative to water, redox potential, etc. However, the use of such curds is the obvious

choice for studying aspects of ripening that require sensoric or rheological assessment of cheese.

For studies not focused on cheese flavour and consistency, simpler substrates have been used by Noomen (1978*a,b*). That author developed models of cheese, composed of paracaseinate, water, lactic acid and NaCl, which as closely as possible simulate the physico-chemical and other conditions for the paracasein as in cheese; the models were preserved by merthiolate and anaerobic storage. At that time, they were used for a quantitative study of the proteolytic action of rennet and of milk proteinase, as a function of parameters like pH, % NaCl relative to water, temperature and keeping time of cheese. At the present, these models were adapted for studying the proteolytic behaviour of starter bacteria in cheese, as a function of some important ripening variables. The results of the investigations are discussed below.

7.1 Model cheeses

The application of the models as described in the Chapters 2 and 3 to study protein breakdown under conditions as in cheese proved to be very satisfactory. They combine a relatively simple preparation with a precisely adjustable cheese composition, can be kept for a very long time without any preservative, and enable establishment of the effect of variation in one ripening variable on the action of a particular proteolytic agent in cheese, in the presence of *L. cremoris HP*. The main problems encountered in their manufacture concerned the elimination of the activity of milk proteinase associated with the paracaseinate, and the inactivation of rennet enzymes after they had produced a 'pool of peptides' as a substrate for the starter bacteria.

Inactivation of milk proteinase

Alkaline milk proteinase is very stable to heat. A rather severe heating of cheese milk at 110°C for 10s (UHT-milk) as applied by Lebars *et al.* (1975) and Desmazeaud *et al.* (1976) in the manufacture of aseptic models curds, inactivates the enzyme insufficiently, whereas more intense heating inhibits the formation of an appropriate rennet coagulate of the milk. Driessen (1983) studied the heat-inactivation of alkaline milk proteinase in milk. Although the methods used allowed only a rough approximation of the residual activity of

the enzyme, his results suggested that the enzyme would be inactivated completely by heating the paracaseinate at 95°C for 30 min, which theoretically would cause even a 10^{10}-fold reduction of enzyme-activity (D-value for inactivation at 95°C: 174 s). However, cheeses made from this paracaseinate still showed some milk proteinase activity after a long keeping time (Section 4.3.1.3). Driessen (1983) studied the residual activity of alkaline milk proteinase by incubating heat-treated milk at 37°C for 72 h, and determined that activity by calculating the increase in the amount of γ-caseins related to the total caseins in the PAGE gel pattern (as measured by densitometry) of all coloured bands in the gel. Although being valuable for studying the kinetics of enzyme-inactivation, these experimental conditions obviously do not permit a decisive conclusion on the completeness of milk proteinase inactivation. This may be attributed to the relatively short period of incubating the milk, to the limited accuracy of PAGE to distinguish enzyme-activities at a very low level, and, possibly, to a further decomposition of γ-caseins by milk proteinase to compounds not appearing on the gel. Autoclaving the paracaseinate inactivated milk proteinase completely: see Section 5.3.

Inactivation of rennet

Our results indicated that some rennet activity even may survive the heating of cheese at pH 5.2 for some hours at 70°C (Section 3.3), which implies a significant heat resistance of rennet at a low pH. This corresponds to the literature. Several authors reported the heat resistance in cheese whey to be higher at a lower pH (Struble & Sharp, 1939; Hyslop *et al.*, 1979; Thunell *et al.*, 1979). Results of Creamer (1976) and of Di Matteo *et al.* (1982) indicate that some rennet activity survives the kneading and stretching process of acid curd (pH 5.2-5.4) as applied in the manufacture of Mozzarella cheese; the temperature of the curd during stretching may reach 58°C (Fox & Guinee, 1987). Our results obtained when heating the cheese at 70°C therefore suggest that at the milder stretching conditions of Mozzarella curd, more rennet activity may survive than is frequently assumed. The extent of rennet inactivation in such 'pasta filata' types of cheese will depend on the pH of the curd at stretching, the temperature and duration of the process, and the rate at which the temperature of cheese is allowed to decrease thereafter.

At the pH of milk rennet is very sensitive to heating. Pasteurization of milk for 20 s at 72°C already completely inactivates the enzymes (Visser, 1977*b*). Garnot & Molle

(1987) studied the inactivation of chymosin in milk of pH 6.5 - 6.75, submitted to heating conditions as normally applied during the scalding of curd in the manufacture of Swiss cheese. They deduced that the enzyme will be completely inactivated during scalding for 1 h at 53 ± 2°C, if the pH of the curd is higher than 6.5, and that the scalding of curd at pH 6.5 for 1 h at 53°C will already reduce the activity of chymosin to about 1%. These results agree with those of Matheson (1981), who could not detect any residual activity of rennet in Swiss cheese. Accordingly, to inactivate rennet after its predigestion of paracasein in cheese of pH 5.2, the pH of the cheese was raised to 6.5 and the cheese heated for 1 h at 70°C (Section 3.3).

Absence of microbial growth

Microbial growth in the model cheeses was entirely prevented by the way of manufacture, the composition and the storage conditions of cheese (Sections 2.2 and 2.4). Numbers of undesired bacteria in ripening cheeses were always found to be zero by the methods applied (Section 2.5).

7.2 Methods used to monitor protein breakdown

Of the methods used, HPLC and PAGE techniques applied in our investigations turned out to be unsuitable to quantitatively follow proteolysis.

At the various ripening conditions of cheese, the areas of peaks on HPLC-chromatograms of cheese extracts representing amino acids did not correlate well with results of amino acid analysis, but they always showed the same trends (Sections 4.3.1.4 and 4.3.2.4).

Electropherograms of ripened cheese (PAGE), made from untreated or predigested paracasein, did not clearly reveal proteolytic activity of the starter bacterium (Section 5.3.4). Although proteolysis was manifest from the production of amino acids, the electrophoretic patterns did not change during the ripening of cheese for 3 months (gels were, however, not evaluated densitometrically). Visser & de Groot-Mostert (1977) used the same PAGE technique in their studies on the ripening of Gouda cheese, and did not detect any substantial variation among the patterns of aseptic rennet-free (ARF) cheeses, made with various starter cultures and ripened for 6 months. Patterns of α_{s1}-casein

degradation in particular showed the accumulation of a breakdown product in front of the α_{s1}-zone with the same mobility as α_{s1}-I-casein. The decomposition of ß-casein became apparent by the formation of γ-caseins with a lower mobility than ß-casein, being characteristic for milk proteinase activity; however, breakdown products with a higher mobility than ß-casein did not appear. Aseptic rennet- and starter-free (ARSF) cheeses, containing milk proteinase as the only proteolytic source, developed the same patterns. Both α_{s1}-casein and ß-casein were, however, markedly less degraded in the latter cheeses than in ARF cheeses, as measured by densitometer readings of the gels. At the pH of e.g. Gouda cheese (pH ≈5.2), an acid milk proteinase liberates a product from α_{s1}-casein with the same electrophoretic mobility as α_{s1}-I-casein (Kaminogawa & Yamauchi, 1972; Noomen, 1978*a*). We therefore believe that the formation of an α_{s1}-I-casein-like band in ARF cheeses was entirely due to the action of milk proteinase, which may explain the inability of the PAGE technique used in our studies to deduce proteolytic activity of the starter bacterium from the formation of particular bands.

Quantitatively, the proteolytic behaviour of the lactococcus strain was characterized best by the formation of amino acid-N and by amino acid analysis. The standard deviation of AN/TN (and of SN/TN) between replicates nearly always were below 0.1 percentage units. The deviations for the individual amino acid concentrations were much higher, but the relations between various amino acids within one sample were quite reproducible. In most of the experiments, the quantities of 'amino acids + NH_3' nitrogen per g of cheese protein, calculated from the values of % AN/TN and from the results of amino acid analyses, respectively, corresponded reasonably to very well; see e.g. Table 6.3. It is generally agreed that amino acid formation crucially affects flavour development in ripening cheese; for instance, for Cheddar cheese, Aston *et al.* (1983*a*) found the accumulation of free amino acids (phosphotungstic acid-soluble nitrogen) and that of free amino acids and trichloro acetic acid-soluble peptides (TCA-soluble tyrosine), to correlate reasonably well with flavour intensity, and considered these fractions useful as indicators for maturation.

7.3 Effect of pH, NaCl and temperature

Generally, in model cheeses made from untreated or predigested paracasein the lactococcus strain produced the individual amino acids in larger quantities at a higher pH (tested within the range pH ~ 4.7 to ~ 6.2, at 13°C and 4% NaCl), or at a lower % NaCl relative to water (tested from 0% to 8% NaCl, at pH ~ 5.2 and 13°C), or at a higher temperature (tested from 13 to 30°C, at pH ~ 5.2 and 4% NaCl); see Figs. 4.3 and 4.6 (note the difference in scale). These results are consistent with the literature dealing with the effects of those respective parameters on the proteolytic activity of whole cells or cell-free extracts of lactococci, or of their purified proteinases and peptidases, in substrates like solutions of casein or of isolated α_{s1}-, ß- or κ-casein, and in buffer solutions with synthetic peptides. The proteolytic system of *Lactococcus lactis* is very complex (Thomas & Pritchard, 1987), and is known to comprise proteinases, endopeptidases, amino peptidases and di/tripeptidases. All the enzymes of *lactococci* characterized to date, including those of *L. cremoris HP*, show optimum proteolytic activity at pH ≈ 6 or higher (several of them even at pH ≈ 8.5), at 0% NaCl or at a very low salt concentration, and at ~ 30°C or higher, up to ~ 50°C (e.g. Sullivan *et al.*, 1973; Law *et al.*, 1974; Exterkate, 1975; Pahkala *et al.*, 1984; Baankreis, 1992). Increased proteolysis by *lactococci* at a higher temperature is also evident in normal cheese. Elevated storage temperatures enhance the production of amino acid-N, as, for instance, being reported for Manchego cheese (Nuñez *et al.*, 1986; Gaya *et al.*, 1990) and in particular for Cheddar cheese (e.g. Aston *et al.*, 1983*a,b*, 1985; Fedrick *et al.*, 1983, 1986). No studies seem to have been published on the rates of amino acid production in cheeses made with various NaCl to water ratio but of otherwise similar composition, or in cheeses which differed in pH only; indirectly, the studies of Raadsveld (1952) with Edam cheese established a retarding effect of NaCl, and a stimulating effect of a higher pH, on the formation of amino acids. Such studies are very difficult to perform with real cheese, because it is hardly possible to vary one ripening variable without changing others (see in 7.4). On the other hand, results obtained from in vitro experiments on simple substrates do not need to reflect the action of a particular proteolytic agent under conditions as in a ripening cheese, and extreme caution should be exerted when trying to extrapolate such results to the situation in cheese (e.g. Jong & de Groot-Mostert, 1977; Noomen, 1978*b*; Baankreis,

1992). Model cheeses as used in this thesis may be considered to fill the gap between real cheese and simple substrates, enabling to study particular aspects of proteolysis in cheese in a rather simple way that nevertheless allows interpretation of results under conditions as in cheese.

The observed effects of pH, NaCl and temperature on amino acid formation (see e.g. Fig. 4.7) are to be attributed to their specific influence on the activity of the enzymes involved in the proteolytic system of the bacterium. It cannot be ruled out that lysis of (some) bacterial cells - presumably being favoured at a high pH, a low NaCl concentration and a high temperature (within the range of cheese conditions studied) - enhances amino acid formation; see Fig. 6.2.

Cheeses ripened with bacteria developed high values of % AN/SN; see the Tables 4.1, 4.3, 5.1 and 6.1. These results indicate that the bacteria predominantly produce amino acids and very low-MW peptides. Far larger quantities of amino acids were liberated from predigested paracasein than from untreated paracasein (see Fig. 4.7, note the difference in scale), which implies that the proteolytic system of the bacterium especially converts soluble products resulting from rennet action into low-MW peptides and amino acids. Rennet is known to hardly produce amino acids. The results establish a preponder contribution of starter bacteria to the 'depth of proteolysis' of cheese, which agrees with the literature results (e.g. Visser, 1977*c*).

At all ripening conditions of cheese tested, the same amino acids were detected, this being most evident from the patterns of cheeses made with predigested paracasein because of the larger quantities of amino acids being produced. By exception, proline was detected in only one experiment (Section 5.3.3). Calculations from the results of Fig. 4.6 suggest that variations in pH or in NaCl concentration did not change the relative proportions of the individual amino acids significantly, whereas a higher ripening temperature tended to do so, particularly at 30°C (results not shown); this is presumably due to increased decomposition of amino acids at a high temperature; see Section 4.3.2.3. Also variations in the P/W ratio of cheese did not seem to alter the mentioned proportions considerably (see Fig. 5.2) and neither did lysis of cells; see Table 6.2. These aspects need, however, to be studied in more detail.

Taking a concentration of $\geq$0.5 mg amino acid per g of cheese protein as a criterion, at the conditions of e.g. a ripening Gouda cheese (pH$\approx$5.2, 4% NaCl relative

to water, ripened at 13°C) the amino acids arginine, glutamic acid, isoleucine, Leucine, lysine, phenylalanine, tyrosine and valine may exceed that level, leucine being produced most abundantly; see Fig. 4.6. Leucine, isoleucine, tyrosine, valine, and to a lesser extent phenylalanine, are known for their bitterness. In addition to bitter peptides produced, these amino acids may contribute to the bitterness-producing behaviour of *L. cremoris HP* as observed during the ripening of traditional cheese made with this strain as a starter organism (Visser, 1977*b*).

The quantity of amino acids increased with ripening time, establishing that proteolytic enzymes of the bacterium remained active at the various conditions of model cheese. In this respect, model cheeses resemble the conditions in normal cheeses, which show a continuous increase of amino acids during ripening. In order to unravel the proteolytic system of lactic acid bacteria, that of lactococci still being most intensively studied to date, much research is spent on the detection, the characterization and the location of proteinases and peptidases (possibly) involved in cheese ripening (e.g. Baankreis, 1992). Their functioning in cheese is mostly deduced from their activities towards specific substrates in solutions, at conditions quite different from those in cheese. For a significant contribution to proteolysis, an enzyme must be quite stable in cheese; it must not be inactivated or only very slowly so. Remarkably, very little work has been done on factors determining the stability of proteolytic enzymes of starter bacteria under conditions as in a ripening cheese, and it is uncertain whether they all keep their activity or lose it faster or slower. Law *et al.* (1974) established the activity of crude dipeptidase preparations from *L. cremoris* NCDO 924 to be sufficiently stable to persist in Cheddar cheese for at least 3 months. Extracts of strains of *Lactobacillus casei*, a lactic acid bacterium which is not used in starter cultures but can frequently be isolated from ripened cheeses, exhibited amino peptidase activity in Cheddar cheese which remained constant during the first 2 months and than started to decrease (El Abboudi *et al.*, 1992). Partly purified extracellular amino peptidase from *Brevibacterium linens*, an organism involved in the maturation of surface ripened cheese like Munster, was found highly stable in Cheddar cheese, negligible amounts of the enzyme activity being lost during 3 month's maturation. The enzyme was, however, quite unstable in a buffer at pH 5.2. Possibly, the cheese environment exerts a stabilizing effect on the enzyme (Hayashi *et al.*, 1990). This

observation, again, implies the necessity for studying the stability and the activity of particular enzymes under conditions that resemble those in cheese as closely as possible.

7.4 Effect of the ratio of paracasein to water

Results of Chapter 5 established that in model cheeses with a P/W ratio of 1:2.5 or 1:3.5, *L. cremoris HP* hydrolysed predigested paracasein at the same rate. Apparently, the differences in water content of cheese, causing the quantity of dissolved calcium phosphate and the chemical composition of the cheese moisture somewhat to vary (Section 5.3.5), did not affect significantly the proteolytic system of the bacterial strain.

It is frequently postulated that higher moisture levels of cheese enhance maturation; irrefutable evidence to this is, however, lacking. With respect to the rate of protein degradation in cheese without a specific surface or internal microbial flora, like in Gouda cheese, one has principally to deal with possible effects of the water content on the activity of rennet, milk proteinase and starter bacteria, respectively, and enzymic interactions.

The proteolytic activity of rennet on casein is very much dependent on the physical state of the casein, which depends on the experimental conditions of pH, temperature and ionic strength (Ledford *et al.*, 1966; Fox, 1969, 1970; Fox & Walley, 1971). Fox & Guiney (1973) demonstrated that the component proteins of a casein system at pH 6.6 became progressively less susceptible to proteolysis at a higher degree of aggregation of the system, as studied with isolated α_{s1}- casein and ß-casein, sodium caseinate, colloidal phosphate-free milk and native skim-milk micelles. α_{s1}-casein is quite resistant to proteolysis in milk, but becomes susceptible when the micellar structure is disrupted by the removal of colloidal calcium phosphate (Fox, 1970), which occurs in cheese as a result of its decreasing pH during manufacture. According to Creamer (1976), ß-casein degradation depends on its degree of association, monomeric ß-casein being the only substrate susceptible to proteolysis by rennet; in cheese, where the casein is at a high concentration and the NaCl concentration is high, little monomeric ß-casein is assumed to be present. These factors may explain that in, for instance, Cheddar and Gouda cheese, α_{s1}-casein is much more rapidly degraded by rennet than ß-casein. Milk proteinase

activity may also contribute substantially to the degradation of ß-casein in these varieties of cheese (Creamer, 1975).

Related to the effect of the paracasein to water ratio of cheese on the action of rennet, the work of Phelan *et al.* (1973) is of interest. Water was added to fresh unsalted Cheddar curd to give increases of 0, 25, 50, 75 and 100% over the initial water content (40%). From the protein content of their experimental cheese (~25%), we calculate the P/W ratios by these additions to vary from 1:1.6 to 1:2, 1:2.4, 1:2.8 and 1:3.2, respectively. As determined by gel electrophoresis on polyacrylamide gels, ß-casein underwent progressively more proteolysis as the water content increased up to a ratio of 1:2.4; still higher water levels gave only a slight further increase in proteolysis. Their results suggest an effect of the water content of cheese on the action of rennet at P/W ratios of cheese frequently applied in industrial cheesemaking. However, the experiments were performed with unsalted curd and at 2°C. As studied with model cheeses, already low concentrations on NaCl reduce the rennet degradation of ß-casein (Noomen, 1978*b*), whereas its decomposition is favoured by low temperatures (Fox, 1969). Therefore, a significant effect of the P/W ratio on the rate of degradation of that casein under the conditions prevailing in cheese is still doubtful. Studies on the effect of a different ratio in cheese or in cheese-like systems on the rennet degradation of α_{s1}-casein seem not to have been reported.This also holds true for the breakdown of caseins by milk proteinase activity.

Virtually nothing is known about the effect of a different P/W ratio of cheese on the proteolytic activity of starter bacteria. The study of Raadsveld (1952) on Edam cheese seems to be the only one dealing with the 'depth of proteolysis' as affected by the water content of cheese. Manufacturing procedures of the experimental cheeses were adapted to equalize as much as possible the other conditions, predominantly the pH of cheese and its ratio of NaCl to water. Water contents varied between 48 and 40%; roughly calculated, these conditions caused the P/W ratio to amount to 1:2.1 at the highest moisture level, and to 1:1.2 at the lowest water content. Results clearly established that proteolysis, in terms of amino acid-N and ammonium-N formation, was favoured in the high moisture cheese. It is, however, not justified to ascribe these findings to a higher water content *per se*, enhancing protein breakdown by the starter organisms. Apart from their water

content, the variants of cheese differed, or probably did so, in several other respects, such as:

1. pH and NaCl concentration. Inevitably, already the freshly made cheeses varied more or less in pH and NaCl to water ratio. During ripening, the pH of cheese increased considerably (but not at a similar rate in the variants of cheese), and so did the NaCl concentration because of different rates of drying out of cheese. Conditions for the proteolytic activity of the starter organisms thus were far from constant.

2. rennet concentration. As normally practised, in making drier cheese a higher scalding temperature was applied, which decreases the quantity of rennet incorporated into cheese (Stadhouders & Hup, 1975). The high moisture cheese was scalded at a lower temperature, and in its manufacture water was added to the curd-whey mixture in order to prevent development of a too low pH. Not unlikely, the addition of water decreased the quantity of rennet carried into the curd. The rennet concentration in cheese is affected greatly by a lower pH, favouring the adsorption of rennet onto casein (Stadhouders & Hup, 1975). A different rate of growth, and consequently of lactic acid production by the starter bacteria in the variants of cheese, being likely because of the different temperature regimes applied during manufacture, may also have caused their rennet concentration to differ considerably. It may be assumed that a larger quantity of rennet in cheese accelerates the production of peptides which are converted into amino acids by the starter bacteria; see under 7.3. (Too much rennet induces, however, bitterness.) Remarkably however, results of Visser (1977*c*) on the proteolysis in normal aseptic Gouda cheese, made with *L. cremoris Z8* or *L. cremoris HP* as a starter, showed the accumulation of amino acid-N to be almost independent of the rennet concentration.

3. starter population. The variants of cheese may have differed in numbers of starter cells after their growth, causing their 'pool of starter enzymes' to vary.

4. lysis of starter cells. Very likely, (some) autolysis of the starter population is involved in cheese ripening, enhancing the production of amino acids; see in 7.5. The extent of lysis in cheese is presumably determined by the temperature-time regime during manufacture, the rate and extent of lactic acid formation, and the rate of NaCl diffusion into the cheese; a higher water content of cheese accelerates the uptake of NaCl during brining. Differences in these aspects may have caused the degree of lysis of cells in the variants of cheese to vary.

5. growth of non-starter organisms. The cheeses were made from raw milk. Undoubtedly, non-starter bacteria will have developed and may have contributed to amino acid-N and ammonium-N formation, their maximum numbers and proteolytic activity being markedly affected by pH and NaCl concentration, which differed significantly between the variants of cheese.

These factors clearly show several difficulties that may be encountered in studying the effect of a particular parameter on proteolysis in normal cheese.

Slurried cheese systems, as introduced by Kristoffersen *et al.* (1967) and refined by Singh & Kristoffersen (1970, 1971, 1972) quickly develop a typical cheese flavour. For instance, Cheddar cheese slurried at pH 5.3 in ~3% NaCl solution to ~40% cheese solids together with reduced glutathione and some other additives (e.g. riboflavin), develops full flavour in 4 to 5 days when incubated at 30-35°C, with daily agitation. The process causes the P/W ratio of normal Cheddar cheese, ~1:1.6, to change to ~1:3.5 in the slurry. An effect of the higher water content on enhanced flavour formation is, however, uncertain. The high incubation temperature of slurries as such considerably stimulates the formation of free amino acids (see Fig. 4.7), and their increased production does not necessarily correlate directly with flavour development (e.g. Law et al., 1976). No studies have been reported on the accumulation of free amino acids in slurries of a different P/W ratio and otherwise similar composition.

For the moment, we assume that the relatively slight variations in the chemical composition of cheese moisture and, consequently, in the attackability of the substrate at the P/W ratios of cheese as applied in industrial cheesemaking, do not significantly affect the activity of proteolytic enzymes involved in cheese maturation. This may explain that the proteolytic activity of *L. cremoris HP* was hardly affected at the different P/W ratios of cheese studied. Strain-dependency might be involved, but we consider it unlikely. A higher water content of normal cheese may, however, enhance proteolysis because of the higher diffusion coefficients of compounds, enhancing the velocity of enzymic reactions. Contrary to our model cheese, which is like a concentrated suspension, normal cheese has a semi-solid gel structure. This difference may cause the velocity of enzymic reactions to be less dependent on the P/W ratio of model cheese than in that of a normal cheese at otherwise similar conditions, but it would need further study.

7.5 Effect of lysis of bacterial cells

In model cheese of standard composition (P/W ratio of 1:2.5, 4% NaCl relative to water, pH ~ 5.3) kept at 13°C, the formation of amino acids by a homogenate of bacterial cells far exceeded that by the same mass of intact cells added to the cheese; see Fig. 6.2. Amino acid production by the cell homogenate was possibly even underestimated, because the lysozyme present may negatively affect peptidase activity (Noomen; unpublished results). These results point to a predominant contribution of intracellular peptidases to amino acid formation in cheese, as was also observed in other work (e.g. Law *et al.*, 1974, 1976; Baankreis, 1992). In the cheese made with intact cells, the rate of amino acid formation appeared to be almost constant during the keeping time, which points to no ongoing lysis of the bacterial population during cheese ripening. It may be argued that, like in normal cheese, released intracellular enzymes may remain localized near to lysed cells which could restrict the access of substrate, whereas in cheese containing a homogenate of cells all enzymes are homogeneously distributed. However, the diffusion coefficients of substrates and degradation products, and even of enzymes, cannot differ considerably between both types of model cheese; see in 7.4. It may be hypothesized that amino acid production in the cheese with added intact cells was largely due to the activity of peptidases of a limited number of cells that were susceptible to lysis at the moment of their addition to cheese, and that were osmotically shocked upon their exposure to the quite different conditions of cheese, notably 4% NaCl. In this line of thought, the susceptibility of cells to lyse will have been largely determined by the weakness of their cell wall, notably by the degree at which their wall was already degraded through autolysin action during strain cultivation (being affected by growth temperature and time, and NaCl concentration), and existing differences in osmotic pressure inside and outside susceptible cells. In ageing metabolically-inactive cells, these differences presumably soon become negligible. When applied to normal cheese, these considerations may imply that amino acid production by starter peptidases is primarily dependent on the extent of lysis of the starter population in the young cheese. Such lysis is probably affected most by the autolytic properties of the starter strain(s) used in cheese making, the conditions during cheese manufacture affecting autolysin action (temperature-time regime, rate of lactic acid formation and final pH of cheese), and the rate at which NaCl is taken up during salting.

Further research would be needed to establish these points. For the time being, we believe that our results may indicate lysis of only a part of the starter population in cheese, which supports the opinion of Baankreis (1992) as deduced from his studies on Gouda cheese.

7.6 Summary of results

Results of our studies are summarized in Fig. 7.1. Differences in degree of proteolysis between cheeses at similar pH, NaCl concentration and ripening temperature probably may be largely attributed to variations in bacterial mass added to cheese and in the extent of predigestion of paracasein by rennet enzymes. At almost equal ripening conditions of cheeses A1 (pH ~ 5.2) and B, amino acid production was considerably enhanced in cheeses B. Assuming the extent of lysis of bacterial cells to have been about the same in both cheeses, cheeses B may have shown enhanced proteolysis because their paracasein had been predigested more extensively by rennet (for 16 days, compared with 12 days for cheeses A1); with time, rennet decomposes paracasein progressively into smaller peptides (Visser, 1977*c*), being the preferred substrate for the starter enzymes. The same considerations may hold true when comparing proteolysis in cheeses A1 (pH ~ 5.2) and C (intact cells). A significant smaller quantity of bacterial mass (in terms of cfu) in cheeses C apparently did not affect amino acid production. However, in cheeses C the paracasein had been predigested for 20 days, which enhanced proteolysis, as argued above.

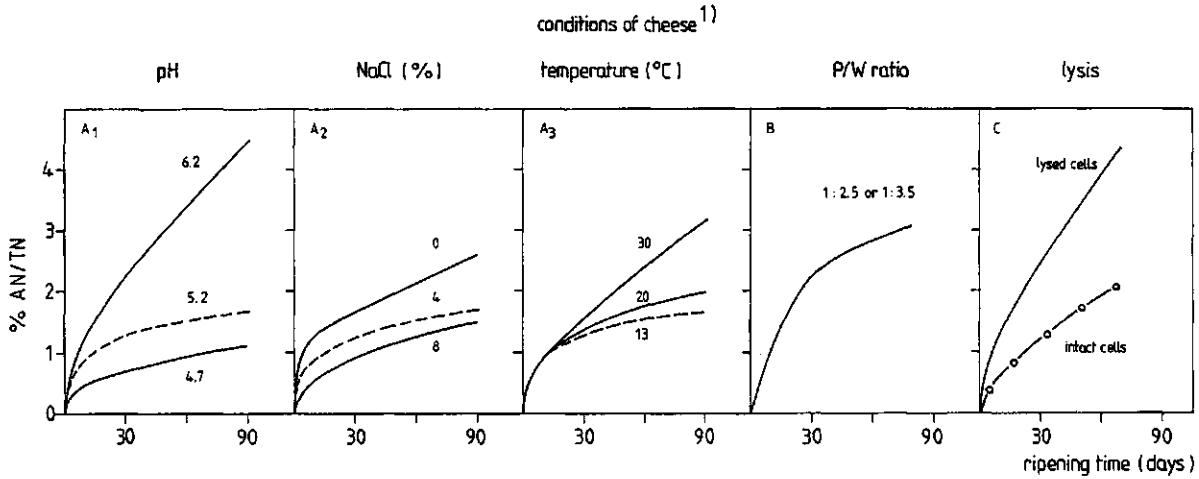
It may be objected that in our studies a fixed 'pool of peptides' from predigested paracasein served as a substrate for the starter bacteria, whereas in normal cheese rennet and those organisms are acting simultaneously. This difference, however, does not affect the overall conclusions of our work.

7.7 Possible other applications of model cheese

The model may be used to further study protein breakdown by various proteolytic agents under conditions closely resembling those in cheeses with a different physico-chemical composition and other ripening conditions: for instance, the effect of very low to very high ratios of paracasein to water on the production of amino acids by a particular starter

strain, or its cell-extract, may be investigated extensively. The models also permit to study the proteolytic behaviour of various mesophilic and thermophilic starter strains or of other organisms incorporated in cheese, the proteolytic effect of various milk coagulants and that of enzyme preparations added to cheese to enhance its maturation, etc. Furthermore, the models may be applied to study the formation of volatile and non-volatile flavour compounds, and may aid in unravelling the cause for the insufficient development of flavour in cheeses made from ultrafiltered milk, by studying proteolysis in models in which part of the paracaseinate is replaced by serum proteins to simulate the composition of UF-cheese. Model cheeses, or their separated moisture, may moreover serve to study lysis of starter cells, and the stability in cheese of enzymes (assumed to be) involved in cheese maturation.

For particular studies, the models may be provided with lactose, permitting the starter bacteria to multiply some times as they do in normally produced cheese.



P/W ratio	1 : 2.5	1 : 2.5	1 : 2.5	varied	1 : 2.5
pH	varied	~ 5.2	~ 5.2	~ 5.2	~ 5.3
2) NaCl (%)	4	varied	4	4	4
bact. mass. (10^9 cfu/g)	3.2	3.2	3.2	5.2	0.4
3) temp. (°C)	13	13	varied	13	13

1: cheeses A, B and C were made with paracacein predigested by rennet at 13 °C for 12, 16 and 20 days, respectively.
2: NaCl concentration relative to water
3: ripening temperature of cheese

Fig. 7.1 **Overview of results on the degradation of predigested paracasein by *L. cremoris HP* in model cheese expressed in percentages of AN/TN, as a function of various ripening parameters and the keeping time of cheese. Data were taken from: Table 4.3 and Fig. 4.7-A for cheeses A1 to A3; Table 5.1 and Fig. 5.2 (calculated values) for cheeses B; Table 6.1 and Fig. 6.2 for cheeses C.**

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SUMMARY

Degradation of protein is paramount for flavour formation in a ripening cheese. Proteolysis is affected by the nature and quantities of proteolytic enzymes (and their interactions), the physico-chemical composition of cheese and other ripening conditions, temperature in particular. In normal cheese, several proteolytic agents act simultaneously. Models based on real cheese made by aseptic cheesemaking techniques, may serve to unravel the contributions of separate or combined enzyme systems to protein breakdown, but are difficult to prepare and never exclude the activity of milk proteinase from the ripening process. Moreover, it is almost impossible to standardize their composition, i.e. to vary one ripening variable without changing others. On the other hand, the frequently used simple substrates, like solutions of casein or synthetic peptides, show conditions far removed to those in cheese, and results need not reflect the activity of enzyme systems in cheese itself. In fact, reliable conclusions from such studies can only be drawn if the model is representative for cheese in the essential aspects.

To meet the problems of studying proteolysis with real cheese as well as simple substrates, model cheeses were developed that enable the study of particular aspects in a rather simple way that nevertheless allows interpretation of results under conditions as in cheese. They are prepared with 'milk proteinase inactivated', calcium paracaseinate-calcium phosphate complex (freeze-dried powder), lactic acid, NaCl, simulated cheese moisture, and provided with the enzyme system(s) to be studied.

The elimination of milk proteinase activity was achieved by autoclaving the wet paracaseinate for 15 min at 121°C. The models combine a relatively simple preparation method with a precisely adjustable cheese composition, enable establishment of the effect of variation in one ripening variable on the action of a particular proteolytic agent, and can be microbiologically controlled for a very long time without any preservative added.

The models were applied to study the proteolytic action of a standardized bacterial mass of the starter strain *Lactococcus lactis ssp. cremoris HP* (Prt^+ variant), as a function of the following variables (while keeping other conditions similar): degradation of untreated or predigested paracasein, as affected by pH, or NaCl content (relative to water) or temperature; degradation of predigested paracasein, as a function of the ratio of paracasein

to water (P/W ratio); and the decomposition of predigested paracasein, as affected by the addition of lysed, rather than intact cells, to cheese. Predigestion of paracasein was achieved by rennet action, and provides the usual substrate for starter bacteria in most varieties of ripening cheese.

After rennet action onto paracasein, and before the incorporation of bacterial mass in cheese, the produced 'pool of peptides' was fixed by heating the cheese at pH 6.5 for 1 h at 70°C. At a low pH of cheese, rennet showed considerable heat resistance (suggesting that during the stretching of acid curd at 55-58°C, as applied in the manufacture of 'pasta filata' types of cheese, a higher rennet activity may remain than is frequently assumed).

Protein breakdown was monitored by the formation of soluble N and amino acid-N, by HPLC and amino acid analysis. Amino acid-N determinations and amino acid analyses were best suited for quantitatively characterizing proteolysis by the starter strain. However, all methods always showed the same trends. Polyacrylamide gel electrophoresis (PAGE) failed to indicate proteolytic activity of the bacterium, as far as the formation of particular bands of degradation products of α_s-casein and ß-casein was concerned.

In cheeses (P/W ratio 1:2.5) made from untreated or predigested paracasein, protein breakdown was enhanced at a higher pH (tested within the range pH ~4.7 to ~6.2, at 4% NaCl and 13°C), or at a lower NaCl content (tested from 0% to 8% NaCl relative to water, at pH ~5.2 and 13°C), or at a higher temperature (tested from 13 to 30°C, at pH ~5.2 and 4% NaCl). Much larger quantities of amino acids were liberated from predigested paracasein than from untreated paracasein, implying that the proteolytic system of the bacterium especially contributes to the 'depth of proteolysis' by the conversion of soluble N products produced by rennet, into low-MW peptides and amino acids. These results agree with those reported on the proteolytic behaviour of starter bacteria, or of their isolated enzymes, as deduced from studies with real cheese and simple substrates.

The effect of the water content of cheese on proteolysis was studied with cheeses at pH ~5.2, containing 4% NaCl relative to water, and with a P/W ratio of 1:2.5 and 1:3.5, respectively. These ratios caused the chemical composition of the cheese moisture somewhat to vary; at the higher water content more colloidal calcium phosphate associated with the paracaseinate became dissolved, accompanied by lower concentrations of dissolved calcium and phosphate in the moisture. These variations, which may also affect the attackability of

the substrate, did not significantly affect the proteolytic activity of the starter strain: proteolysis in both types of cheese proceeded at almost the same rate. It is assumed that relatively slight variations in the composition of cheese moisture, arising from different P/W ratios of cheese applied in industrial cheesemaking, as such do not significantly affect the activity of proteolytic enzymes of starter bacteria (and other enzymes) involved in cheese maturation. Presumably, other factors are responsible for the frequently postulated enhanced proteolysis in cheese with a higher water content. For instance, a higher moisture level in cheese may enhance the velocity of enzymic reactions because of higher diffusion coefficients of compounds, whereas in model cheeses, which are more like a concentrated suspension, significant differences in diffusion rates are unlikely. Further research, which also must include the effect of higher P/W ratios than studied in this thesis, would be needed to elucidate the effect of the water content of cheese on protein breakdown.

The effect of lysis of bacterial cells on proteolysis was studied with model cheeses at pH ~ 5.3, containing 4% NaCl relative to water and with a P/W ratio of 1:2.5. Compared to intact cells, the incorporation of a same mass, but lysed, bacterial cells drastically enhanced amino acid formation, establishing the predominant effect of the activity of intracellular peptidases. These results support the idea that only a (minor) part of the starter population in model cheese exhibit lysis. It is assumed that the formation of amino acids in normal cheese also is primarily determined by lysis of a limited number of starter bacteria in the young cheese, the extent of lysis being affected by the autolytic properties of the strain(s) used, the conditions during cheese manufacture determining the activity of autolytic enzymes that weaken the cell wall, the final NaCl concentration of cheese and, more in particular, the rate at which NaCl is taken up during salting. These aspects would warrant a separate study.

The quantity of amino acids produced increased during keeping of the cheese, establishing that enzymes of the bacterium remained active at the various ripening conditions; it is, however, uncertain whether they all kept their activity or lost part of it. Very little is known about the stability of proteolytic enzymes of starter bacteria in cheese. Because of its importance for the mechanisms of protein breakdown in real cheese, research onto this aspect would be useful. To this end, our model cheeses may be used fruitfully.

Variations in pH, or % NaCl relative to water, did not significantly affect the relative proportions of the individual amino acids produced, and neither did the variation in the P/W ratio of cheese or lysis of starter cells. A higher ripening temperature of cheese, 30°C in particular, seemed to change those proportions; obviously, a large quantity of total amino acids was decomposed, liberating NH_3.

Possible other applications of the model cheese developed are discussed.

SAMENVATTING

De afbraak van eiwit is van overheersend belang voor de aroma-vorming in rijpende kaas. De proteolyse wordt beïnvloed door de eigenschappen en hoeveelheden van proteolytische enzymen (en hun interacties), door de fysisch-chemische samenstelling van kaas en door overige rijpingsomstandigheden, in het bijzonder de rijpingstemperatuur. In normaal bereide kaas zijn verscheidene proteolytische bronnen gelijktijdig werkzaam. Modellen van kaas die zijn gebaseerd op met behulp van aseptische technieken bereide echte kaas, kunnen dienen om de bijdragen van afzonderlijke of gecombineerde enzymsystemen tot de eiwitafbraak te ontrafelen; hun bereiding is echter moeilijk en sluit nimmer de bijdrage van melkproteïnase tot het rijpingsproces uit. Bovendien is het vrijwel onmogelijk om hun samenstelling te standaardiseren, d.w.z. om één rijpingsfactor te variëren zonder andere te wijzigen. Aan de andere kant komen de omstandigheden in veelvuldig gebruikte eenvoudige substraten, zoals in oplossingen van caseïne of van synthetische peptiden, weinig overeen met die in kaas, en de met die substraten verkregen resultaten behoeven daarom niet de activiteiten van enzymsystemen in kaas weer te geven. Verantwoorde conclusies uit dergelijke onderzoekingen zijn eigenlijk alleen mogelijk wanneer het model op essentiële punten representatief is voor kaas.

Zowel het bestuderen van proteolyse met echte kaas als met eenvoudige substraten levert dus problemen op. Om deze te overkomen zijn kaasmodellen ontwikkeld die het mogelijk maken om aspecten van eiwitafbraak te onderzoeken op een vrij eenvoudige manier, welke niettemin extrapolatie van de resultaten naar omstandigheden als in kaas mogelijk maakt. Deze modellen worden gemaakt van 'van actief melkproteïnase ontdaan' calciumparacaseïnaat-fosfaat (gevriesdroogd poeder), melkzuur, NaCl en gesimuleerd kaasvocht, en voorzien van een of meer te bestuderen enzymsystemen.

Het inactiveren van melkproteïnase werd bereikt door het natte paracaseïnaat gedurende 15 minuten bij 121°C te steriliseren. De modellen combineren een betrekkelijk eenvoudige wijze van bereiding met een precies in te stellen samenstelling, maken het mogelijk het effect van veranderen van één rijpingsfactor op de werking van een bepaalde proteolytische enzymbron vast te stellen, en kunnen voor onbepaalde tijd zonder een conserveringsmiddel worden bewaard.

De modellen werden toegepast voor het bestuderen van de proteolytische werking van

een gestandaardiseerde massa cellen van de melkzuurbacterie *Lactococcus lactis ssp. cremoris HP* (Prt^+ variant), als functie van de volgende variabelen (waarbij de overige omstandigheden gelijk werden gehouden): de afbraak van paracaseïne, respectievelijk van voorverteerde paracaseïne, als functie van pH, NaCl-gehalte (berekend op water) en temperatuur; de afbraak van voorverteerde paracaseïne als functie van de paracaseïne-water verhouding (P/W), en de afbraak van voorverteerde paracaseïne bij toevoeging van gelyseerde of intacte bacteriecellen aan de kaas. Voorverteerde paracaseïne, verkregen door stremselwerking, is het gebruikelijke substraat voor melkzuurbacteriën in de meeste rijpende kaassoorten.

Na het voorverteren van paracaseïne, en vóór het doseren van bacteriemassa in de kaas, werd de gevormde 'bron van peptiden' gefixeerd door de kaas bij pH 6,5 gedurende 1 uur bij 70°C te verhitten. Bij een lage pH van de kaas bleek stremsel erg hitte-resistent te zijn (hetgeen doet vermoeden dat bij het kneden van zure wrongel bij 55-58°C, zoals toegepast tijdens de bereiding van kaassoorten van het type 'pasta filata', meer actief stremsel behouden blijft dan veelal wordt verondersteld).

De eiwitafbraak werd gevolgd door bepaling van oplosbaar N en aminozuur-N, met HPLC en met aminozuur-analyse. Bepalingen van aminozuur-N en analyses van aminozuren bleken het geschiktst om de proteolyse door de bacteriestam kwantitatief te karakteriseren. Alle methoden lieten echter dezelfde trend zien. Polyacrylamide gelelectroforese (PAGE) toonde geen proteolytische activiteit van de bacterie aan, althans niet in de zin van het zichtbaar worden van specifieke banden van afbraakprodukten van α_s- en ß-caseïne.

In kazen (met een P/W 1:2,5) gemaakt met paracaseïne of met voorverteerde paracaseïne, werd de proteolyse gestimuleerd bij een hogere pH (onderzocht bij pH ~ 4,7 tot pH ~ 6,2; bij 4% NaCl en 13°C), bij een lager NaCl gehalte (0% tot 8% NaCl berekend op water, bij pH ~ 5,2 en 13°C), en bij een hogere temperatuur (13 tot 30°C, bij pH ~ 5,2 en 4% NaCl). Uit voorverteerde paracaseïne werden veel grotere hoeveelheden aminozuren gevormd dan uit paracaseïne, hetgeen er op duidt dat het proteolytische systeem van de bacterie vooral bijdraagt tot de 'diepte van de rijping', door het omzetten van door stremsel gevormde oplosbare stikstofverbindingen tot peptiden met laag molecuulgewicht en aminozuren. Deze resultaten sluiten aan bij wat in de literatuur wordt vermeld over het proteolytische gedrag van melkzuurbacteriën of van daaruit geïsoleerde enzymen, verkregen uit onderzoek met echte kaas en eenvoudige substraten.

De invloed van het watergehalte van de kaas op de proteolyse werd bestudeerd in

kazen met een pH ~ 5,2, een NaCl gehalte van 4% berekend op water, en een paracaseïne-water verhouding van respectievelijk 1:2,5 en 1:3,5. Deze verschillende verhoudingen veranderden de chemische samenstelling van het kaasvocht enigszins; bij het hogere watergehalte loste meer van het aan paracaseïnaat gebonden kolloïdale calciumfosfaat op, en waren de concentraties van opgelost calcium en fosfaat in het kaasvocht lager. Deze verschillen, die ook de aantastbaarheid van het substraat kunnen beïnvloeden, hadden geen significant effect op de proteolytische activiteit van de bacteriestam: in beide typen kaas vond de eiwitafbraak met een nagenoeg gelijke snelheid plaats. Waarschijnlijk zullen de betrekkelijk geringe wijzigingen in de samenstelling van kaasvocht, als gevolg van de verschillende paracaseïne-water verhoudingen van kaas zoals die bij de industriële bereidingen worden toegepast, de activiteit van proteolytische enzymen van zuurselbacteriën (en die van andere enzymen) bij de kaasrijping niet wezenlijk beïnvloeden. Waarschijnlijk zijn andere factoren verantwoordelijk voor de veelal veronderstelde snellere proteolyse bij een hoger watergehalte van de kaas. Zo kan bijvoorbeeld een hoger gehalte in echte kaas de snelheid van enzymreacties doen toenemen door een snellere diffusie van verbindingen, terwijl in de kaasmodellen, die geen gelstructuur bezitten maar meer op geconcentreerde suspensies gelijken, significante verschillen in diffusiesnelheden niet waarschijnlijk zijn. Verder onderzoek, dat ook de invloed van hogere paracaseïne-water verhoudingen moet omvatten dan die welke zijn bestudeerd, zou nodig zijn om het effect van het watergehalte van de kaas op de proteolyse op te helderen.

De invloed van lysis van bacteriecellen op de eiwitafbraak werd bestudeerd in kaasmodellen met een pH ~5,3, een NaCl gehalte van 4% berekend op water en een paracaseïne-water verhouding van 1:2,5. Vergeleken met een massa intacte cellen, verhoogde eenzelfde massa gelyseerde cellen de aminozuurproduktie zeer sterk, hetgeen wijst op een overheersend effect van intracellulaire peptidasen. De resultaten ondersteunen de gedachte dat slechts een (klein) deel van de zuurselbacterie-populatie in het kaasmodel lyseerde. Wellicht dat ook de aminozuurvorming in normale kaas in hoofdzaak wordt beheerst door het lyseren van een beperkt aantal zuurselbacteriën in de nog jonge kaas, waarbij de mate van lysis wordt bepaald door de autolytische eigenschappen van de gebruikte stam(men), de omstandigheden tijdens de kaasbereiding die de activiteit van autolytische, celwand verzwakkende, enzymen beïnvloeden, het uiteindelijke NaCl gehalte van kaas en met name de snelheid waarmee NaCl tijdens het zouten wordt opgenomen. Deze aspecten zouden een

afzonderlijk onderzoek vergen.

De hoeveelheid aminozuren nam toe tijdens bewaring van de kaas, hetgeen bevestigt dat bacteriële enzymen activiteit behielden onder de verschillende rijpingscondities; het is evenwel onzeker of alle enzymen actief bleven, of dat hun activiteit in meerdere of mindere mate verloren ging. Over de stabiliteit van proteolytische enzymen van zuurselbacteriën in kaas is zeer weinig bekend. Vanwege het belang ervan voor de mechanismen van eiwitafbraak in echte kaas, zou onderzoek naar dat aspect nuttig zijn. Daartoe zouden onze kaasmodellen vruchtbaar kunnen worden gebruikt.

Verschillen in de pH van kaas of in het NaCl-gehalte hadden geen significant effect op de verhoudingen van de hoeveelheden gevormde individuele aminozuren; verschillen in de paracaseïne-water verhouding en lysis van bacteriecellen hadden dat evenmin. Bij een hogere rijpingstemperatuur, vooral bij 30°C, leken die verhoudingen te wijzigen; een groot deel van de totaal gevormde hoeveelheid aminozuren werd daarbij verder omgezet, onder vorming van NH_3.

Mogelijke andere toepassingen van de ontwikkelde kaasmodellen worden bediscussieerd.

CURRICULUM VITAE

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