Lait (1993) 73, 281-292

Original article

Proteolysis in cheese made with liposome-entrapped proteolytic enzymes

G Scolari, M Vescovo, PG Sarra, V Bottazzi

Istituto di Microbiologia, Università Cattolica, via Emilia Parmense 84, 29100, Piacenza, Italy

(Received 11 August 1992; accepted 24 March 1993)

Summary — The evolution of proteolysis was compared in cheeses made either with free or liposome encapsulated commercial protease and in cheese supplemented with encapsulated enzymatic extract from *Lactobacillus helveticus*. In cheese with added free neutrase, a very rapid proteolysis occurred combined with a strong bitterness defect, which was correlated with high and medium molecular weight peptide accumulation measured by gel permeation chromatography. In cheese with added *L helveticus* enzymatic extracts a lower primary proteolysis resulted except for control cheese, but the finished flavour appeared earlier than in the other enzyme supplemented cheeses.

liposome / cheese / accelerated ripening / proteinase / peptidase

Résumé — Protéolyse de fromages préparés avec des enzymes protéolytiques encapsulées dans des liposomes. *On a comparé l'évolution de la protéolyse de fromages préparés avec ajout d'une protéase commerciale, soit comme enzyme libre soit contenue dans des liposomes, ou ajout d'un extrait enzymatique de* L helveticus *renfermé dans des vésicules. Dans le fromage contenant la Neutrase libre, la protéolyse a été très rapide, mais il présentait un grave défaut d'amertume qui a été associé à l'accumulation de peptides de poids moléculaire moyen et haut, mis en évidence par chromatographie sur gel de Sephadex. Dans le fromage préparé avec l'extrait enzymatique de* L helveticus, *la protéolyse primaire était inférieure à celle des autres fromages, exclusion faite du fromage témoin, mais l'arôme final apparaissait beaucoup plus rapidement que dans les autres fromages.*

liposome / fromage / accélération de l'affinage / protéase / peptidase

INTRODUCTION

Texture and flavour are the 2 main components of cheese quality produced during the prolonged ripening process. The progressive curd transformation involves complex biochemical, physical and microbiological modifications, mainly effected by milk-clotting enzymes, indigenous milk proteinases and the enzymatic activity of starter and non-starter lactic acid bacteria (O'Keeffe *et al,* 1978; Puchades *et al,* 1989).

In order to reduce the cost of cheese storage, numerous attempts have been made to halve the maturation time of several cheese varieties by promoting their proteolytic and peptidolytic activities; this has been accomplished by the use of exogenous enzymes, increased population of starters (heat or freeze-shocked) and microbial proteinases, as reviewed by Bottazzi and Scolari (1983) and more recently by El Soda and Pandian (1991) and Kirby (1991).

However, the success of the enzyme treatment of cheese milk has been greatly impaired either by enzyme loss in the whey (rendering this by-product useless for processing in other foods) or by unwanted yield-reducing proteolysis and premature flavour changes. Liposome technology has been proposed as a tool to overcome these difficulties.

Different liposome preparations have been employed: multilamellar vesicles (MLV) (Law and King, 1985), small unilamellar vesicles (SUV) (Piard *et al,* 1986), reversed phase vesicles (REV) (Alkalaf *et al,* 1988) and recently multilamellar-microfluidized liposome (MLV-MF) (Lariviere *et al,* 1991). Kirby *et al* (1987) improved the efficiency of enzyme encapsulation using liposome prepared according to the dehydration–rehydration procedure developed by Kirby and Gregoriadis (1984) also adopted by ourselves in this study. This technique achieves the most efficient level of encapsulation without using the denaturing conditions characteristic of other entrapment methods.

In this paper the evolution of proteolysis in cheese containing added or encapsulated proteolytic enzymes either of commercial origin or extracted from *Lactobacillus helveticus* is compared.

MATERIAL AND METHODS

Strain and culture conditions

L helveticus IMPC HS1 was used in this study as source of protease and peptidase cell-free enzyme extract. *Streptococcus thermophilus* IMPC1 was utilised for cheese-making, and came from the stock collection of our institute, maintained by weekly transfers in skim milk at 42°C and grown in MRS broth (Difco) when required.

Cell free crude extract (CFCE) preparation

A 3-l culture of *L helveticus* IMPC HS1 was grown in MRS broth supplemented with 20 mmol/l $CaCl_2$ up to mid-log phase of growth. Cells were collected by centrifugation and washed with 50 mmol/l β-glycerophosphate buffer pH 7.0 in 20 mmol/l $CaCl_2$. The pellet was resuspended in 300 ml of the same buffer and cells mechanically disintegrated in a Braun disintegrator using 0.11-μm diameter Ballottini beads; unbroken cells were selectively removed by centrifugation at 1 500 *g* for 15 min at 42 °C.

Commercial enzyme preparation

Neutrase 0.5 l (0.5 AU/ml) (Novo Industri, Copenhagen, Denmark) was employed diluted to 2.5% (v/v) in order to give a comparable proteolytic activity with the CFCE.

Enzymatic activity estimation

The proteolytic activity of CFCE, either free or encapsulated, and of commercial enzyme was assessed by the spectrophotometric method of Church *et al* (1983), reading the absorbance at 340 nm of the reaction products of *o*-

phthaldialdehyde and β-mercaptoethanol with α-amino groups released by hydrolysis. Peptidolytic activity was evaluated according to El Soda *et al* (1978) using leucine *p*-nitroanilide (LNA) (Sigma Chemical Co, St Louis, MO, USA) as substrate. Enzymatic activities were expressed as amount of enzyme providing an increase of 10^{-3} AU at 340 nm per h under the experimental conditions for proteolysis, and hydrolyzing 10^{-3} μmol/min LNA for peptidolysis.

Liposome preparation

Vesicles were prepared with porcine cholesterol and egg phosphatidylcholine (Sigma Chemical Co) in a 1:1 molar ratio according to Gregoriadis (1976).

Neutrase solution or *L helveticus* HS1 CFCE was encapsulated in liposome vesicles by the dehydration–rehydration method (DRV) (Kirby and Gregoriadis, 1984), as indicated by Kirby *et al* (1987). Liposomes were separated from non-encapsulated enzyme by washing in saline phosphate buffer (PBS) (0.14 mol/l NaCl, 3 mmol/l KCl, 10 mmol/l Na_3PO_4, pH 7.4) and centrifuging (12 000 *g*, 40 min).

In order to evaluate the encapsulation efficiency, the washed liposomes were destroyed with 1% Triton X-100 and the released enzymatic activity compared with that of the original preparation in presence of the same percentage of Triton X-100.

Estimation of liposome retention and rupture in cheese

A solution of 150 mmol/l carboxyfluorescein (CF, Fluka Chemicka, Buchs, Switzerland) purified according to Weinstein *et al* (1984), was encapsulated in liposomes under the same conditions as those for enzymes. In order to determine the influence of entrapped material on liposome stability, 2 separate liposome preparations were made including the same quantity of CF in both the enzymatic extracts.

Curd liposome retention was evaluted preparing cheese containing encapsulated CF as indicated below.

Curd (10 g) was homogenized in 20 ml deionized water at 50°C for 10 min by Lab-Sonic 1510 Sonicator (Brown, Melsugen, Germany). Liposomes in equal volumes of serum curd suspension were dissolved by treatment with 1% Triton X-100, and the resulting solutions clarified by centrifugation (1.4×10^4 *g* for 15 min). The fluorescence of the supernatants was measured by a Jasco FP55 spectrofluorimeter (492 nm and 530 nm excitation and emission wavelength respectively). The extent of liposome rupture in cheese was evaluated weekly in 2 cheese samples homogenized as previously described: one in 20 ml PBS and the other in 20 ml 1% Triton X-100, in order to detect the released and total CF content respectively. The suspensions were then clarified and the fluorescence measured as stated previously.

Cheese-making

Five experimental types of cheese were manufactured in 3 different trials with the same lot of pasteurized milk according to the Taleggio cheese procedure. Cheese milk heated to 41°C was inoculated (2%) with a *Streptococcus thermophilus* culture; after 45 min (milk pH 6.3) the appropriate amount of encapsulated and free enzyme preparations for each experiment were added, as shown in table I. Milk was then coagulated by adding 0.2% of a commercial rennet and cut curd was pressed at 1.2, 2.4, 3.6 g/cm^2 in the 1st, 2nd and 3rd h respectively. After brine salting for 15 min, cheese curd was immersed in a solution (20%) of potassium sorbate in order to prevent mould growth. Cheeses were stored at 8°C and 85% relative humidity and turned over daily. Two additional cheeses were manufactured in the same manner for the experiments with liposomes containing the mixture of CF and enzymatic extracts.

Monitoring of proteolysis during cheese ripening

Soluble nitrogen estimation

Nitrogen content of soluble nitrogen fractions was determined by the trinitrobenzenesulphonic

Table I. Amount of encapsulated and free enzyme added to cheese milk.
Quantité d'enzyme ajoutée au lait, soit libre soit dans les liposomes.

Cheese	*Enzyme preparation*	*Amount (ml/l cheese milk)*
A	Without addition	–
B	Free 2.5% Neutrase	5.00
C	2.5% Neutrase-entrapped	5.00
D	*L helveticus* HS1 CFCE-entrapped	5.00
T	CF-entrapped	2.00

CFCE : cell-free crude extract; CF: carboxyfluorescein.

acid (TNBS) method (Polychroniadou, 1988), and expressed as absorbance at 420 nm. Cheese samples (5 g) were removed weekly up to 35 d and homogenized at 42 °C in 25 ml of 0.5 mol/l sodium citrate buffer, pH 7.0, with an Osterizer blender at maximum speed for 1 min.

Soluble nitrogen at pH 4.4

Soluble nitrogen at pH 4.4 was extracted from 75 ml of the citrate suspension and determined according to Gripon *et al* (1975); the insoluble fraction was examined electrophoretically.

70% Ethanol soluble nitrogen

4.4 ml of the previous fraction was diluted with 11 ml chilled absolute ethanol to give a 70% final ethanol concentration, and centrifuged at 1 x 10^4 *g* for 20 min at 0 °C.

Phosphotungstic acid (PTA) soluble nitrogen

The phosphotungstic acid fraction was obtained according to Gripon *et al* (1975).

Gel filtration of pH 4.4 soluble nitrogen

The pH 4.4 soluble nitrogen fraction was freeze-dried and an amount corresponding to 25 ml of the original extract was redissolved in distilled water, loaded onto a Sephadex G50 (Pharmacia) column (1.6 x 100 cm), eluted with 83 mmol/l acetic acid at a flow rate of 17 ml/h and monitored at 280 nm. Fractions collected (5 ml) were pooled for individual peaks and freeze-dried for further analysis.

Polyacrylamide gel electrophoresis (PAGE)

Urea–PAGE of pH 4.4 insoluble fraction was performed in the presence of 0.34 mmol/l β-mercaptoethanol according to Erhardt (1989). Samples were dissolved in 8 mol/l urea, 0.78 mol/l β-mercaptoethanol, 0.061 mol/l Tris–HCl pH 6.8.

Discontinuous sodium dodecylsulphate (SDS) electrophoresis of the Sephadex-eluted fractions was carried out according to Laemmli (1970), using a 20% acrylamide concentration for the running gel. The sample buffer was prepared dissolving 8 mol/l urea, 6% SDS and 2% β-mercaptoethanol in the stacking gel buffer.

RESULTS AND DISCUSSION

Enzymatic activity in cell-free crude extract (CFCE)

Analysis of proteolytic and peptidolytic activity of *L helveticus* IMPC HS1 CFCE showed 121 U/ml for proteolytic activity.

Very similar results were obtained by Hickey *et al* (1983) in cell extracts of some *L helveticus* strains for L-leucyl aminopeptidase activity.

While the dehydration–rehydration process did not affect *L helveticus* HS1 CFCE enzymatic activity, Triton X-100 liposome treatment resulted in a 80–90% loss of peptidolytic activity.

Efficiency of enzyme encapsulation, liposome retention and rupture in cheese

Encapsulation rate as evaluated by proteolytic activity release in 3 separate trials was 22% (mean value); CF experiments showed that 42% (mean value) of the liposomes was retained in curd.

Although the percentage of liposome retention in curd is comparable with the 17% obtained by Law and King (1985) with reverse-phase evaporation vesicles, and higher than the 60.3% reported by Alkalaf *et al* (1988) using multilamellar vesicles, it was far from the value found by Kirby *et al* (1987) in experiments (83–92%) with dehydrated–rehydrated vesicles as used by ourselves.

However, it is well know that liposome retention in curd is affected by the composition of the vesicles and cheese technology (Piard *et al,* 1986), vesicle dimension (Kirby *et al,* 1987), and vesicle surface charge (Alkalaf *et al,* 1989). Thus our low values could probably be attributed to the high coagulation temperature.

Results concerning liposome rupture in cheese (table II) showed a different behaviour between the vesicles containing CFCE and those prepared with commercial enzyme. After 7 d, CF released by CFCE containing liposomes was 51%, while 19% was released by liposomes with neutrase.

As the pH and salt content in both cheeses were the same, it could be assumed that the CFCE by *L helveticus* altered the surface tension in the liposomes and adversely affected vesicle stability.

Evolution of soluble nitrogen (SN) during cheese ripening

The release of SN at pH 4.4 (fig 1) was linear with ripening time in B cheese, while in C and D type cheeses it increased significantly only after the 1st and 2nd week respectively. No further differences were found in primary proteolysis of any cheese with added enzyme after 20 d; SN content in control cheese was the lowest throughout the ripening period.

Soluble nitrogen in 70% ethanol is correlated with medium and low molecular weight peptide content (Kuchroo and Fox, 1982). A very similar increase was shown in Neutrase-supplemented cheeses, either

Table II. Carboxyfluorescein (CF) release from liposomes prepared with CF and Neutrase or CF and cell free crude extract mixtures during cheese ripening.
Libération de carboxyfluorescéine (CF) par les liposomes contenant les mélanges CF et Neutrase ou CF et extrait enzymatique de L helveticus *au cours de l'affinage des fromages.*

Ripening time (d)	*% CF released*	
	Liposomes with CF + Neutrase	*Liposomes with CF + CFCE*
7	19	51
14	45	63
21	56	65
35	67	78

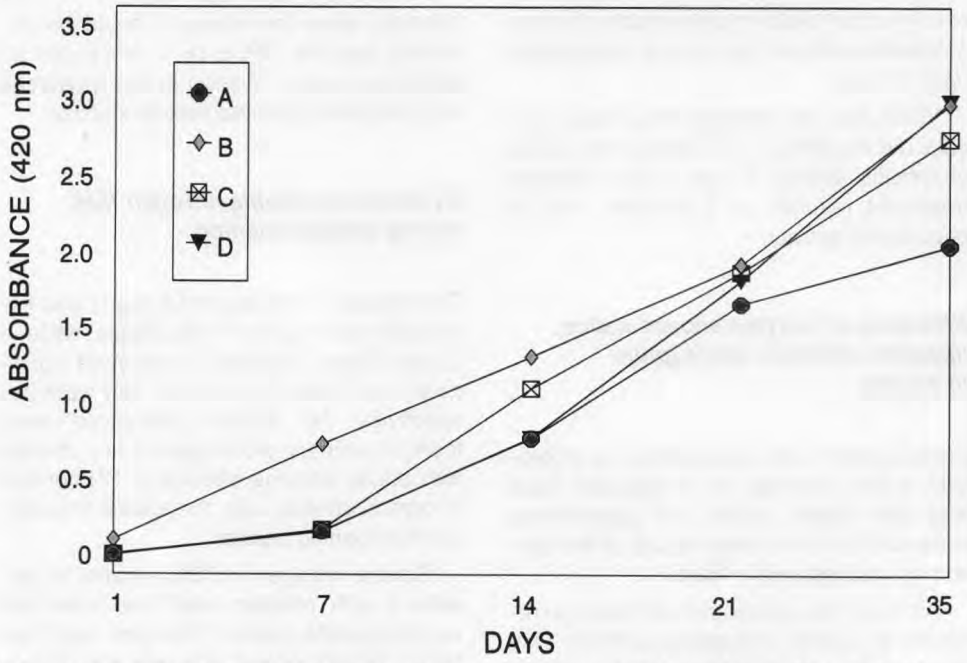

Fig 1. Content of soluble nitrogen, expressed as absorbance at 420 nm, from : A) control cheese, without enzyme addition; B) cheese with 2.5% free Neutrase; C) cheese with entrapped 2.5% Neutrase; D) cheese with entrapped *L helveticus* extract.
Quantité d'azote soluble à pH 4,4, exprimé par l'absorbance à 420 nm, de : A) fromage témoin, sans ajout d'enzymes; B) fromage avec Neutrase libre; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus *encapsulé.*

free or encapsulated (fig 2); the higher value of the former indicates the incidence of commercial proteinase at this stage of proteolysis. The entrapped CFCE had the same effect in D type cheese, but 7 d later; however, at the end of ripening, the content of soluble nitrogen in 70% ethanol exceeded that of cheese C, probably due to the bacterial proteolytic contribution. The lack of homogeneity of such data suggests the need to go beyond a threshold value in primary proteolysis, before the appearance of peptidase activity (Law and Wigmore, 1983; Stadhouders *et al,* 1988). This threshold is more rapidly reached when primary proteolysis is faster (cheeses B and C).

Five percent PTA-soluble nitrogen depends on the small peptide (MW < 600 Da) and amino acid content (Rank *et al,* 1985). While no linear relationship exists between the amount of ethanol-soluble nitrogen and small PTA-soluble proteolysis products (Lin *et al,* 1987), it is generally recognized that this second nitrogen fraction is very closely related to the peptidolytic activity originating from starter and non-starter flora (Bartels *et al,* 1987; Ardo *et al,* 1989), and as flavour precursor to cheese flavour development (Olson, 1990). As expected, in cheese containing CFCE liposomes (D), the peptidolytic activity was more evident than in the other cheeses (fig 3); higher levels of PTA-SN were detectable from the

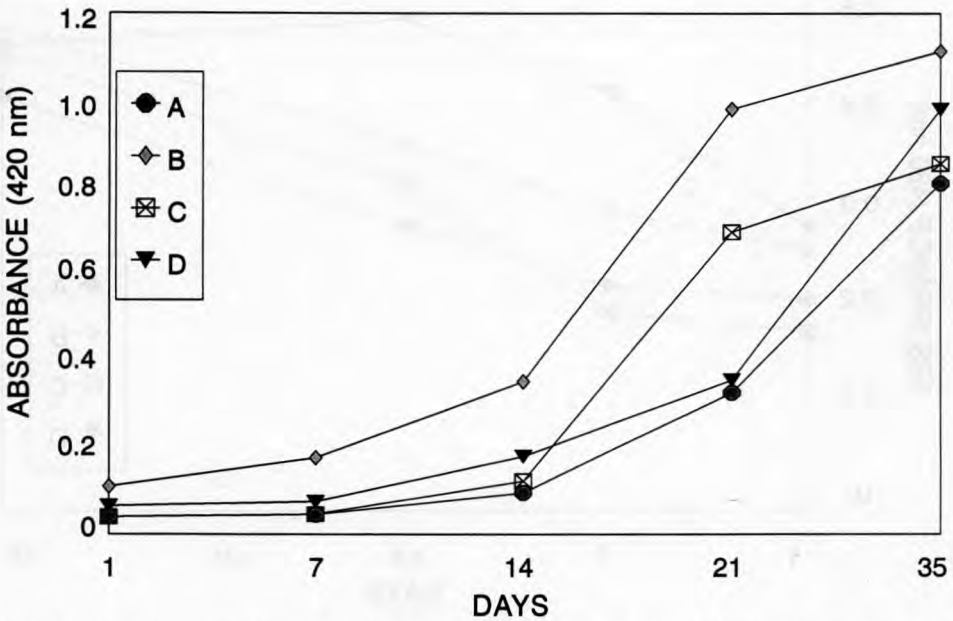

Fig 2. Content of nitrogen soluble in 70% ethanol, expressed as absorbance at 420 nm, from : A) control cheese, without enzyme addition; B) cheese with 2.5% free Neutrase; C) cheese with encapsulated 2.5% Neutrase; D) cheese with encapsulated *L helveticus* extract.
Quantité d'azote soluble dans l'éthanol 70%, exprimé par l'absorbance à 420 nm de : A) fromage témoin, sans ajout d'enzymes; B) fromage avec Neutrase; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus *encapsulé.*

1st week and the maximum value was reached after 14 d of cheese-making. In the free-Neutrase cheese (B) the small peptide and amino acid yield was proportional to the storage time; conversely, only after 14 d the C type cheese reach PTA-SN levels comparable to those of the type B cheese due to the lack of immediately available ethanol-soluble nitrogen (fig 2). This behaviour was in accordance with the liposome rupture time occurring from the 1st to the 2nd week as detected by CF release. The PTA-SN evolution in D type cheese showed the highest value just from the beginning of ripening. The dissimilar behaviour of C and D cheeses could be attributable to a different liposome time rupture, as demonstrated by data in table II.

PTA-SN accumulation in control cheese showed a similar behaviour to that of the Neutrase-supplemented cheeses, but at lower levels.

In accordance with the high peptidolytic activity (fig 3), informal flavour assessment confirmed that D cheese had already attained the finished flavour at the 1st stage of ripening; B and C type cheeses were strongly affected by a bitter defect, as also observed by Aston *et al* (1983) in Cheddar cheese and Alkalaf *et al* (1988) in Saint-Paulin cheese at the first month of ripening.

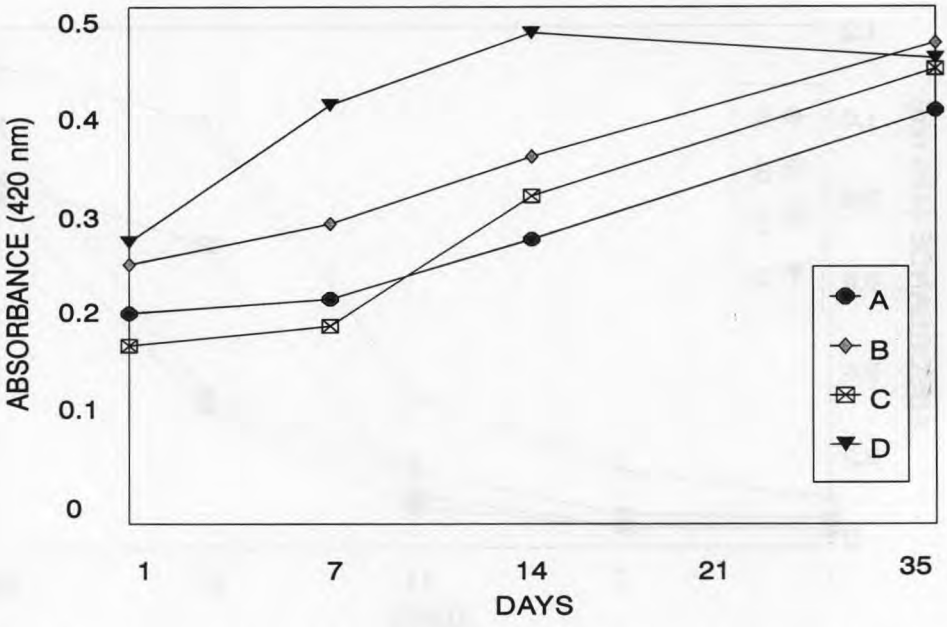

Fig 3. Content of nitrogen soluble in phosphotungstic acid, expressed as absorbance at 420 nm, from: A) control cheese, without enzyme addition; B) cheese with 2.5% free Neutrase; C) cheese with entrapped 2.5% Neutrase; D) cheese with entrapped *L helveticus* extract.
Quantité d'azote soluble dans l'acide phosphotungstique, exprimé par l'absorbance à 420 nm de : A) fromage témoin, sans ajout d'enzymes; B) fromage avec Neutrase libre; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus *encapsulé.*

Gel permeation of pH 4.4 soluble nitrogen

Sephadex G50 gel filtration of various pH 4.4 soluble extracts showed a similar distribution of 280 nm absorbing substances after the first week.

The proteolytic activity in the B type cheese was associated with the increase in the medium-high molecular weight products (peaks in zone II; fig 4a). After 35 d, elution patterns became more complex (fig 4b); type A and D cheeses respectively showed the lowest and the highest amounts of small hydrolysis products (peaks in zone V), confirmed by the absorbance level of the PTA-SN fraction. As the medium and high molecular weight peptides has been considered a prerequisite for the bitter taste (Otagiri *et al,* 1985), in B type cheese bitterness development was correlated with the intensity of peaks in zone II. Similar observations have been made by Bartels *et al* (1987) for Gouda cheese and Ardo *et al* (1989) for Swedish semi-hard cheese.

Electrophoretic analysis

No detectable differences appeared in the urea–PAGE electrophoretic pattern of pH 4.4 insoluble nitrogen fractions (fig 5) from liposome-supplemented cheeses C and D

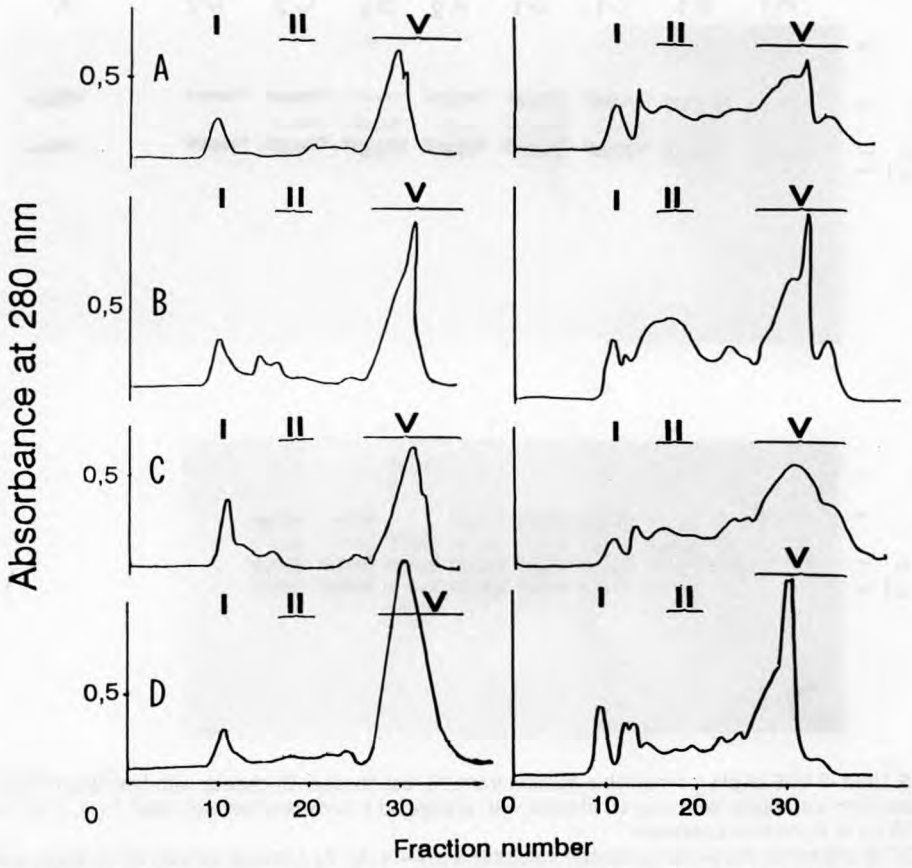

Fig 4. Sephadex G50 chromatography of pH 4.4 soluble fraction from: A) control cheese, without enzyme addition; B) cheese with 2.5% free Neutrase added; C) cheese with 2.5% entrapped Neutrase; D) cheese with entrapped *L helveticus* extract 1) after 7 d; 2) after 35 d.
Chromatographie sur gel Sephadex G50 de la fraction soluble à pH 4,4 de: A) fromage témoin, sans ajout d'enzymes; B) fromage avec Neutrase libre; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus *encapsulé. 1) après 7 jours; 2) après 35 jours.*

compared to the control. The B type cheese showed a progressive disappearance of β-casein and a simultaneously increasing intensity of a single band (X) of similar electrophoretic mobility to γ-caseins; this fragment probably corresponds to the electrophoretic band, termed 'Y' by Fernandez Garcia *et al* (1988) and 'N' by Alkalaf *et al* (1989) produced by Neutrase action on β-casein.

The first Sephadex chromatographic fraction (high MW peptides), subjected to SDS–PAGE showed an increase in the number and intensity of electrophoretic bands, particularly in the low molecular weight range, as ripening time proceeded (fig 6). The peptide patterns were rather similar except for the presence of 2 low MW peptides (G, M) in B cheese and, with a faint intensity, in C cheese; these rela-

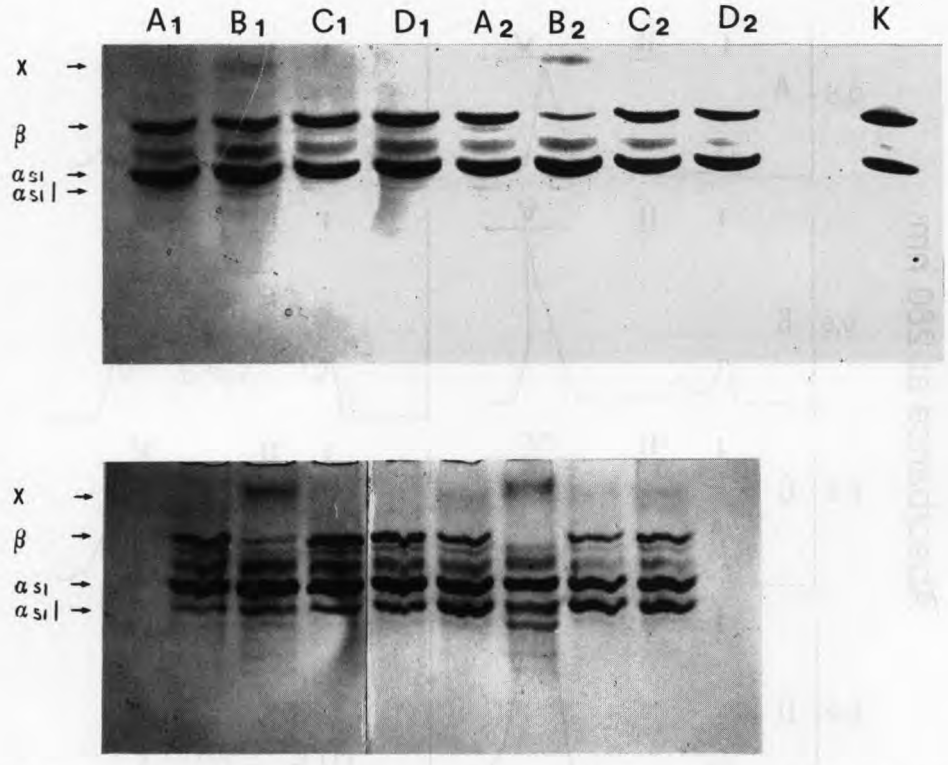

Fig 5. Urea–PAGE of pH 4.4 insoluble fraction from: A) test cheese; B) cheese with free Neutrase; C) cheese with entrapped Neutrase; D) cheese with entrapped *L helveticus* extract, after 1 (1), 7 (2), 14 (3), 35 (4) d. K: sodium caseinate.
PAGE en présence d'urée de la fraction insoluble à pH 4,4 de: A) fromage témoin; B) fromage avec Neutrase libre; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus *encapsulé, après 1 (1), 7 (2), 14 (3), 35 (4) jours. K: caséinate de sodium.*

tively small components were already detectable in the first week of ripening but later disappeared, probably degraded by the bacterial peptidases. *L helveticus* CFCE proteinases could be responsible for the reciprocal evolution of electrophoretic band intensity of S and V doublets during D type cheese maturation.

CONCLUSION

Liposome technology demonstrated its efficacy in achieving a reduction in the cheese-ripening period. In spite of this, cheese ripening by vesicle-entrapped enzymes suffers from serious problems such as large-scale liposome production and a high level of encapsulation. Furthermore, the present work emphasizes the determinant role played by the proteolytic enzyme system of *L helveticus* in the acceleration mechanism of cheese ripening.

To avoid development of bitterness, particular care should be taken in balancing proteolytic and peptidolytic activity. In order to render the liposome-ripening methods attractive it is necessary to reach a se-

lective rupture of liposomes in a manner other than that of the unsuccessful temperature-sensitive liposomes (El Soda *et al*, 1989); this could enable the subsequent release of bacterial proteinases and peptidases. To successfully reduce cheese-ripening time by liposome technology, and to circumvent unwanted hydrolytic processes, more knowledge must be gained both of casein breakdown or amino-acid catabolism by *Lactobacillus* enzymes and of their kinetics during ripening.

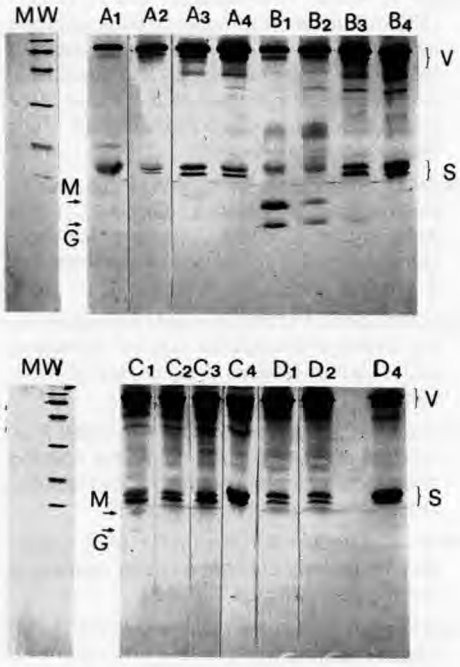

Fig 6. SDS–PAGE of fraction I from Sephadex G50 gel chromatography of: A) control cheese; B) cheese with free Neutrase; C) cheese with entrapped Neutrase; D) cheese with entrapped *L helveticus* extract; after one (1), 7 (2), 14 (3) and 35 (4) d. MW: molecular weight markers (97.4; 66.2; 45; 31; 21.5; 14.4 kDa).
SDS PAGE de la première fraction de la chromatographie sur gel de Sephadex: A) fromage témoin, sans ajout d'enzymes; B) fromage avec Neutrase libre; C) fromage avec Neutrase encapsulée; D) fromage avec extrait de L helveticus. *Après 1 (1), 7 (2), 14 (3), 35 (4) jours. MW : marqueurs de poids moléculaire (97,4; 66,2; 45; 31; 21,5; 14,4 kDa).*

REFERENCES

Alkalaf W, Piard JC, El Soda M, Gripon JC, Desmazeaud M, Vassal L (1988) Liposome as proteinase carrier for the accelerated ripening of Saint-Paulin type cheese. *J Food Sci* 53, 1674-1679

Alkalaf W, El Soda M, Gripon JC, Vassal L (1989) Accelerated cheese ripening with liposome entrapped proteinase: influence of liposome net charge. *J Dairy Sci* 72, 2233-2238

Ardo Y, Larsson PO, Mansson HL, Hedenberg A (1989) Studies of peptidolysis during early maturation and its influence on low-fat cheese quality. *Milchwissenschaft* 44, 485-490

Aston JW, Durward JG, Dulley JR (1983) Proteolysis and flavour development in Cheddar cheese. *Aust J Dairy Technol* 38, 55-59

Bartels HJ, Johnson ME, Olson NF (1987) Accelerated ripening of Gouda cheese. I. Effect of heat-shocked thermophilic lactobacilli and streptococci on proteolysis. *Milchwissenschaft* 42, 139-144

Bottazzi V, Scolari GL (1983) Maturazione accelerata e nuove prospettive per il settore caseario. Prima parte : stato attuale delle conoscenze e campi di applicazione. *Sci Tecn Latt Casearia* 31, 103-122

Church C, Swaisgood HE, Porter DH, Catignani GL (1983) Spectrophotometric assay using o-phthaldehyde for determination of proteolysis in milk and isolated milk proteins. *J Dairy Sci* 66, 1219-1227

El Soda M, Bergère JL, Desmazeaud MJ (1978) Detection and localization of peptide hydrolase in *Lactobacillus casei*. *J Dairy Res* 45, 519-524

El Soda M, Johnson M, Olson N (1989) Temperature-sensitive liposomes, a controlled release system for the acceleration of cheese ripening. *Milchwissenschaft* 44, 213-214

El Soda M, Pandian S (1991) Recent developments in accelerated cheese ripening. *J Dairy Sci* 74, 2317-2335

Erhardt G (1989) Isolierung und Charakterisiung von Casein Fraktionen Sowie dren Genetische Varianten in Schweinemilch. *Milchwissenschaft* 44, 17-20

Fernandez-Garcia E, Ramos M, Polo C, Juarez M, Olano A (1988) Enzyme accelerated ripening of Spanish hard cheese. *Food Chem* 28, 63-80

Gregoriadis G (1976) Enzyme entrapment in liposomes. *In: Methods in Enzymology*. Academic Press, New York, vol 17

Gripon JC, Desmazeaud MJ, Le Bars D, Bergère JL (1975) Étude du rôle des microorganismes et des enzymes au cours de la maturation des fromages. *Lait* 55, 502-516

Hickey MW, Hillier AJ, Jago JR (1983) Peptidase activities in lactobacilli. *Aust J Dairy Technol* 38, 118-123

Kirby CJ (1991) Microencapsulation and controlled delivery of food ingredients. *Food Sci Technol Today* 5, 74-78

Kirby CJ, Gregoriadis G (1984) A simple procedure for preparing liposomes capable of high encapsulation efficiency under milk conditions. *In: Liposome Technology* (Gregoriadis, ed) CRC Press, Boca Raton, FL, vol I

Kirby CJ, Brooker BE, Law BA (1987) Accelerated ripening of cheese using liposome-encapsulated enzyme. *Int J Food Sci Technol* 22, 355-375

Kuchroo CN, Fox PF (1982) Fractionation of the water-soluble nitrogen from Cheddar cheese: chemical methods. *Milchwissenschaft* 37, 651-653

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* 227, 680-685

Larivière B, El Soda M, Soucy Y, Trépanier G, Paquin P, Vuillemard JC (1991) Microfluidized liposome for the acceleration of cheese ripening. *Int Dairy J* 1, 111-124

Law BA, Wigmore AS (1983) Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *J Dairy Res* 50, 519-525

Lin JCC, Jeon IJ, Roberts HA, Milliken GA (1987) Effects of commercial food grade enzymes on proteolysis and textural changes in granular Cheddar cheese. *J Food Sci* 52, 620-625

O'Keeffe AM, Fox PF, Daly C (1978) Proteolysis in Cheddar cheese: role of coagulant and starter bacteria. *J Dairy Res* 45, 465-477

Olson NF (1990) The impact of lactic acid bacteria on cheese flavor. *FEMS Microbiol Rev* 87, 131-148

Otagiri K, Nosho Y, Shinoda I, Fukui H, Okai H (1985) Studies on a model of bitter peptides including arginine, proline and phenylalanine residues. I. Bitter taste of di- and tripeptides, and bitterness increase of the model peptides by extension of the peptide chain. *Agric Biol Chem* 49, 1019-1026

Piard JC, El Soda M, Alkhalaf W, Rousseau M, Desmazeaud M, Vassal L, Gripon JC (1986) Acceleration of cheese ripening with liposome-entrapped proteinase. *Biotechnol Lett* 8, 241-246

Polychroniadou A (1988) A simple procedure using trinitrobenzensulfonic acid for monitoring proteolysis in cheese. *J Dairy Res* 55, 585-596

Puchades R, Lemieux L, Simard RE (1989) Evolution of free amino-acids during the ripening of Cheddar cheese containing added lactobacilli strains. *J Food Sci* 54, 885-888

Rank TC, Grappin R, Olson NF (1985) Secondary proteolysis of cheese during ripening: a review. *J Dairy Sci* 68, 801-805

Stadhouders J, Toepoel L, Wouters JTM (1988) Cheese making with prt^- and prt^+ variants of N streptococci and their mixture. Phage sensitivity, proteolysis and flavour development during ripening. *Neth Milk Dairy J* 42, 183-193

Weinstein JN, Ralston E, Leserman LD, Klausner RD, Dragsten P, Henkart P, Blumenthal R (1984) Self quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome–cell interaction. *In: Liposome Technology* (Gregoriadis G, ed) CRC Press, Boca Raton, FL, vol III