Proteolysis in cheese made with liposome-entrapped proteolytic enzymes

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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.

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) (Lavriere 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 CaCl₂ 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 CaCl₂. 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% (w/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 α-
phthalaldehyde and \( \beta \)-mercaptoethanol with \( \alpha \)

- 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 \( 10^{-3} \) \( \mu \)mol/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 \( \text{Na}_2\text{PO}_4 \), 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 evaluated 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 \( x \) 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 1. 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 acid method.
Table I. Amount of encapsulated and free enzyme added to cheese milk.
Quantité d'enzyme ajoutée au lait, soit libre soit dans les liposomes.

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Enzyme preparation</th>
<th>Amount (ml/l cheese milk)</th>
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<tbody>
<tr>
<td>A</td>
<td>Without addition</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Free 2.5% Neutrase</td>
<td>5.00</td>
</tr>
<tr>
<td>C</td>
<td>2.5% Neutrase-entrapped</td>
<td>5.00</td>
</tr>
<tr>
<td>D</td>
<td><em>L. helveticus</em> HS1 CFCE-entrapped</td>
<td>5.00</td>
</tr>
<tr>
<td>T</td>
<td>CF-entrapped</td>
<td>2.00</td>
</tr>
</tbody>
</table>

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

酸 (TNBS) 方法 (Polychroniadou, 1988)，并且表示为420 nm的吸光度。奶酪样品（5 g）每周去除一次，共35天，并在42 °C下使用0.5 mol/l sodium citrate buffer, pH 7.0, 使用一个Osterizer搅拌机以最大速度搅拌1 min

可溶性氮在pH 4.4

在pH 4.4的可溶性氮是通过75 ml的柠檬酸悬浮液提取并根据Gripon et al (1975)进行确定的；不溶性部分通过电泳法进行检测。

70%乙醇可溶性氮

4.4 ml的前一分段被稀释到11 ml的冷却绝对乙醇以提供70%的最终乙醇浓度，并在1 x 10⁴ g for 20 min at 0 °C离心。

磷酸钨酸 (PTA) 可溶性氮

磷酸钨酸性可溶性氮部分是根据Gripon et al (1975)获得的。

pH 4.4可溶性氮的凝胶过滤

pH 4.4可溶性氮部分被冷冻干燥，然后将与原始提取物相等的25 ml的馏分在水、缓冲液中溶解，加载到Sephadex G50 (Pharmacia) 柱 (1.6 x 100 cm)，用83 mmol/l acetic acid以流速为17 ml/h透过柱，并在280 nm处监测。收集的（5 ml）50 ml被分池用于单个峰的进一步分析。

聚丙烯酰胺凝胶电泳 (PAGE)

尿素-PAGE的pH 4.4不溶性部分在0.34 mmol/l β- mercaptoethanol存在下根据Erhardt (1989)进行的。样品在8 mol/l urea, 0.78 mol/l β-mercaptopethanol, 0.061 mol/l Tris-HCl pH 6.8中溶解。

在Sephadex排除的各部分的聚丙烯酰胺电泳使用Laemmli (1970)的20% acrylamide浓度作为运行胶。样品缓冲液是用8 mol/l urea, 6% SDS和2% β-mercaptopethanol在填充凝胶缓冲液中准备的。

RESULTS AND DISCUSSION

细胞外原粗提液（CFCE）中的酶活性

*pL. helveticus* IMPC HS1 CFCE的蛋白酶和肽酶活性分析表明121 U/ml的活性。
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>
<thead>
<tr>
<th>Ripening time (d)</th>
<th>% CF released</th>
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<tr>
<td>Liposomes with CF + Neutrase</td>
<td>Liposomes with CF + CFCE</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
</tr>
<tr>
<td>21</td>
<td>56</td>
</tr>
<tr>
<td>35</td>
<td>67</td>
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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
Accelerated cheese ripening by liposomes

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 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.
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.
Accelerated cheese ripening by liposomes

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-
selective 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.

REFERENCES


