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Original article

The effect of added proteolytic enzymes on meltability of Mozzarella cheese manufactured by ultrafiltration

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Abstract — Four proteolytic enzymes (Neutrase®, *Bacillus licheniformis* proteinase (BLP), porcine trypsin (PT), and *Fusarium oxysporum* proteinase (FOT)) were evaluated in Mozzarella cheese manufactured by ultrafiltration (UF). The UF Mozzarella cheese had impaired melting behaviour due to the incorporation of whey protein into the cheese curd. PT and FOT had no activity during incubation and storage. The melting (< 10 % diameter expansion (DE)) and sensoric behaviour of these cheeses were similar to the control UF Mozzarella without enzyme addition. Cheeses with Neutrase® showed very strong proteolytic activity and increased non-coagulable nitrogen (NCN) to > 50 % after 5 weeks. DE was ~ 45 %, but a very bitter off taste was found. BLP increased proteolysis NCN ~ 30 %), and the DE was ~ 25 % without any off flavours. Acceleration of proteolysis in UF Mozzarella by enzyme addition generally improved the meltability due to increased casein degradation, while whey protein was resistant to proteolysis. © Inra/Elsevier, Paris.

ultrafiltration / Mozzarella cheese / proteinase / proteolysis / meltability

Résumé — Effet des enzymes protéolytiques sur la capacité de fonte du fromage mozzarella produit par ultrafiltration. Quatre enzymes protéolytiques (Neutrase®, protéase de *Bacillus licheniformis* (BLP), trypsine porcine (PT) et protéase de *Fusarium oxysporum* (FOT) ont été evaluées dans un fromage mozzarella produit par ultrafiltration (UF). La mozzarella obtenue par UF avait une aptitude réduite à la fonte, du fait de l'incorporation des protéines sériques dans le caillé. La PT et la FOT n'ont montré aucune activité durant l'acidification et l'affinage. L'aptitude à l'étalement (< 10 % d'élargissement du diamètre, DE) et les propriétés sensorielles de ces fromages étaient identiques à celles de l'échantillon témoin (mozzarella UF sans ajout d'enzyme). Les fromages additionnés de Neutrase® présentaient une protéolyse accrue et une augmentation de l'azote non coagulable (NCN), de plus de 50 % après 5 semaines d'affinage. Le DE était d'envi-

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ron 45 %, mais un goût très amer s'est developpé parallèlement. La BLP a montré une augmentation de la protéolyse (NCN ~ 30 %), et le DE était d'environ 25 %, sans qu'aucun mauvais goût ne soit détecté. L'accélération de la protéolyse dans l'UF mozzarella par l'addition des enzymes a amélioré la capacité de fonte, due à une dégradation accélérée de la caséine, tandis que les protéines sériques étaient résistantes à la protéolyse. © Inra/Elsevier, Paris.

ultrafiltration / fromage mozzarella / protéase / protéolyse / aptitude à la fonte

1. Introduction

Ultrafiltration (UF) of milk for cheese production was patented in 1969 [31]. In the following years this pioneering work initiated research to adapt the new technique for production of a wide range of cheese types [9, 21, 33]. The driving force for this research was the expected yield increase, reduction in rennet consumption, the possibility of standardising the composition of the retentate with respect to both fat and protein, and also better possibilities of obtaining a continuous and automated cheese production [7, 33].

Commercially the UF technique has been successful in Denmark, where the production of Feta cheese increased from a few tons in 1975 to > 112 000 tons in 1993, mainly exported to Iran, and approaching one-third of the total Danish cheese production [1]. The fast development of this production has demanded large investments in UF production equipment, so in order to become less dependent on a single market, UF based technologies for the production of several other Danish cheese types Havarti [37], Danablu [38], and Danbo [40], respectively, were worked out. At an early stage unripened Mozzarella type cheese was expected to be a successful candidate for production by UF, and Maubois and Kosikowski [34] and later Friis [11] outlined processes for production of high moisture UF Mozzarella from full concentrated retentate. According to Fernandez and Kosikowski [10] Mozzarella with satisfactory melting and stretching properties can be manufactured from low concentrated (2:1) retentate. UF Mozzarella made from freeze-dried retentate showed poor meltability compared to conventionally produced cheese but diafiltration and storage improved the melting property [4]. The reason for the poor melting quality of UF Mozzarella has not been definitely established, but improper calcium-to-protein ratio and calcium binding to denatured β-lactoglobulin (β-Lg) has been suggested [26]. Furthermore, the presence of whey proteins has been related to impaired melting properties in the manufacture of process cheese [42]. Lawrence [26] pointed out that heating would induce complex formation between whey proteins and para κ-casein and α_{s2}-casein, thus reducing the 'flow' of the caseins. Another reason could be the less intensive proteolysis in UF cheese compared to conventional cheese [8, 39], as melting has generally been shown to be improved by proteolysis [24, 44]. Since whey protein solutions at high ionic strength readily form gels on heating [22] one could hypothesise that the whey proteins contained in the cheese would actually be able to form a gel during the oven heating.

Normally, proteolysis is limited in Mozzarella cheese [26]. The high stretching temperature used in the manufacturing of Mozzarella partly inactivates the rennet [5], and furthermore whey proteins are able to inhibit rennet [27] and plasmin [2, 41]. Whey proteins in cheese are not degraded by either rennet, plasmin or the enzymes from starter bacteria [16].

In this study we have investigated the relation between proteolysis and melting of UF Mozzarella cheese made with addition of proteinases with different specificities: two trypsins with specificity at the basic amino acids similar to plasmin, Neutrase®, with a broad specificity and often used to accelerate maturation of cheese [13, 25], and a serine proteinase from *Bacillus licheniformis,* specific for acidic amino acids, and claimed to improve the melting of UF cheese [6].

2. MATERIALS AND METHODS

2.1. Enzymes

The enzymes used were porcine trypsin (PT) (EC 3.4.21.4.; 6.0 Anson units (AU) g^{-1}), *Fusarium oxysporum* trypsin (FOT) (EC 3.4.21.4.; 160 casein protease units L^{-1}), *Bacillus licheniformis* proteinase (BLP, EC 3.4.21.19.; 8.25 AU g^{-1}), *Bacillus subtilis* proteinase (Neutrase®, EC 3.4.24.28; 0.5 AU g^{-1}) all obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). All indicated activities are according to the declaration by Novo Nordisk A/S.

2.2. Ultrafiltration

The manufacturing of UF Mozzarella was performed on two occasions at APV Research and Development Centre Videbæk, Denmark. Bulk milk standardised to a fat content of 2.8 % was pasteurised at 72 °C for 15 s, cooled to 5 °C and preacidified with acetic acid to pH 6.0. After holding for 2 h the milk was heated to 50 °C and concentrated using one APV spiral-wound module with Dow Chemicals GR 62 P membranes and three APV plate and frame UF 37 modules equipped with Dow Chemicals GR 60 PP membranes. In the first UF 37 module the retentate was diafiltered until the Brix value in the permeate was 2.5. In the last UF 37 module the temperature of the retentate was lowered to 45 °C, and portions of 5 L were removed continuously.

2.3. Manufacture of UF Mozzarella

To each portion of retentate (5 L) 3 g of a thermophilic DVS® starter culture (CH 1, yoghurt type, Chr. Hansen, Hørsholm, Denmark), 2.5 g of standard rennet (70% chymosin and 30% bovine pepsin, Chr. Hansen, Hørsholm, Denmark) and a solution (standardised to 0.035 AU kg^{-1} retentate) of the proteolytic enzymes was added. A control experiment was made without addition of enzyme solution. The retentate was held at 42–44 °C until pH had reached 5.4 to 5.6, then the curd was cut (2 × 2 × 5 cm) and immersed in hot water (65 °C). After 1 min 500 to 600 *g* of the curd was kneaded and stretched manually for 2 to 3 min, maintaining the temperature by occasionally dipping the curd in the hot water. The UF Mozzarella was placed with the fibres orientated longitudinally in a cheese mould (15 × 7 × 8 cm) and cooled overnight at 5 °C in a cold storage room. Each UF Mozzarella cheese was brine salted in 20% NaCl at 15 °C for 90 min, air dried and cut into four quarters (approximately 125 g) which were separately vacuum packed and placed in a cold storage at 5 °C. After 1, 2 and 5 weeks of storage, respectively, one quarter of each cheese was analysed. For comparison commercial conventionally produced Mozzarella (MD Foods, Farsø, Denmark) was analysed at the same intervals.

2.4. Composition of retentate and cheese

The retentate and cheeses were analysed for fat [18], total solids [17], and protein as Kjeldahl nitrogen × 6.38 [20]; the pH was measured in a grated cheese sample using a PHM 62 standard pH meter (Radiometer, Copenhagen, Denmark). The fat and salt content [19] in the cheese was determined only after storage for 1 week.

2.5. Proteolysis

2.5.1. Total nitrogen (TN)

10 g of grated cheese was made up to 200 mL with 0.1 mol L^{-1} trisodium citrate (pH 8.5) and 40 °C. Fat was remove by filtration through glass wool and N was determined in duplicate in 2 mL of the supernatant [20].

2.5.2. pH 4.6 soluble nitrogen (pH4.6-SN)

To 100 mL of the cheese-citrate solution from the TN determination 11 mL of water and 14 mL of 1 mol L^{-1} HCl was added while vigorously stirring, causing the pH to drop to 4.5–4.6. After 30 min rest the sample was filtered through a Whatman® n° 42 filter, and N was determined in duplicate in 10 mL of the supernatant [20]. The precipitate was used for capillary electrophoresis analysis according to Otte et al. [36].

2.5.3. Non coagulable nitrogen (NCN)

50 mL of the pH4.6-SN filtrate was heated in an Erlenmeyer flask covered with aluminium foil ~ 95 °C for 30 min, cooled to 20 °C and filtered through a Whatmann® n° 42 filter. N was determined in duplicate in 10 mL of the supernatant [20].

2.5.4. Whey protein nitrogen (WPN)

WPN was calculated as the difference between pH4.6-SN and NCN.

2.5.5. 12% TCA soluble nitrogen (TCA-SN)

To 25 mL of the cheese-citrate solution from TN determination 25 mL of a 24% trichloracetic acid (TCA) was added under vigorous stirring. After 30 min of rest the sample was filtered through a Whatman® n° 42 filter, and N was determined in duplicate in 10 mL of the supernatant [20].

2.6. Melting test

From the Mozzarella cheese samples slices (5 mm thick) were cut longitudinally to the fibres. From each of four slices from the inner part of the cheese one cylindrical sample (diameter = 30 mm) was cut. All four samples were placed on a glass plate covered with aluminium foil and equilibrated at room temperature for 5 min. The foil was removed and the glass plate was placed in a prewarmed oven at 160 °C for 150 s. After cooling to ambient room temperature, the diameter was measured by a pair of callipers as the average of four measurements at predetermined places on each cylinder. The diameter expansion (DE) in percent was then calculated relative to the diameter of the unmelted cheese.

2.7. Sensory evaluation

The unmelted cheeses were evaluated for off-flavours by three members of the scientific staff at the Institute, all experienced in sensory evaluation of cheese. The intensity of the off-flavour was graded on a 9-point scale (0 = no off-flavour to 8 = extreme off-flavour). When possible the type of off-flavour was stated.

2.8. HPLC

The HPLC equipment consisted of a Waters 600 E Multisolvent Delivery System, a Waters 700 Satellite Wisp Injector and a Waters H90 Programmable Multiwavelength Detector (Waters Division of Millipore Milford, MA, US). Data were processed with Millenium™ 2010 version 2.10 software (Millipore Corporations, Waters Chromatography Division, Milford, MA, USA).

Size exclusion HPLC was performed on a TSK Gel G 2000 SWXL column (inner diameter 7.8 mm, length 300 mm) preceded by a guard column TSK Gel SWXL® (inner diameter 6 mm, length 40 mm) both from Tosohaas Gmbh, Stuttgart, Germany. The following conditions were used: injection volume, 20 µL; flow rate, 0.8 mL min^{-1}; solvent 0.01 mol L^{-1} K_2HPO_4, 0.09 mol L^{-1} KH_2PO_4 and 0.15 mol L^{-1} Na_2SO_4, pH 5.65. Total elution time was 23 min and the absorbance was recorded at 220 nm. Creatinin from Merck (Darmstadt, Germany) and bovine serum albumin A-4378, β-lactoglobulin (β-Lg) L-0130, α-lactoglobulin (α-La) L-6010, insulin B chain I-6383, and angiotensin II A-0289 from Sigma Chemicals (St Louis, USA) were used as standards for the MW determination.

2.9. Capillary electrophoresis

CE was conducted on a Waters Quanta™ 4000 Capillary Electrophoresis System (Waters, Division of Millipore, Milford, MA, USA) with an untreated fused-silica capillary

(J & W Scientific, Falcom, CA 95630-4714, USA, No 190-4714; inner diameter 50 μm, total length 60 cm, effective length 52.5 cm). The precipitate after lowering pH to 4.6 were analysed and assignment of peaks to casein components was done according to Otte et al. [36]. The supernatant (pH4.6-SN) was analysed and the assignment of α-La and β-Lg was done according to Otte et al. [35].

3. RESULTS AND DISCUSSION

The average gross composition of the UF Mozzarella cheeses from the two experiments is listed together with the composition of the retentates (*table I*). With 48.6% and 47.1% moisture, respectively, and fat in dry matter of 46.7% the cheeses can be classified as low moisture Mozzarella according to US standards [23]. During incubation of the retentate until the pH had dropped to 5.5–5.4, syneresis occurred, thus increasing the dry matter content. Syneresis was most pronounced in the second experiment where the dry matter increased from 47.6% in the retentate to 52.9% in the cheeses. The reason for the larger increase in the second experiment was probably that the acidification was slower, and therefore the incubation time, until the desired pH was reached, was almost doubled compared to the first experiment. With the more extensive syneresis in the second experiment more of the native and soluble whey protein was lost, causing a lower whey protein content in the cheese (*table I*).

In both experiments the 90 min of salting time was too short to reach 1.5% salt, which has been recommended to obtain the best melt property [12]. Conventionally produced Mozzarella cheese had DE of 25–40% at the experimental conditions, while DE for UF Mozzarella was much less, regardless of the diafiltration step, which was recommended by Covacevich and Kosikowski [4]. The diameter expansion of UF cheese without proteinase added (control) and cheeses with FOT added were below 10% throughout the storage time (*figure 1*). However, the BLP treated cheese melted significantly better with ~ 20% DE after 1 week in experiment 2 (with ~ 5.4% whey protein), and increasing to 37% after 5 weeks of storage (*figure 1*). In experiment 1, with ~7.1% whey protein in the cheese, DE in BLP treated cheese was less, approaching only 25% after 5 weeks. By comparing results from the two experiments in *figure 1* it is seen that DE was systematically

Table I. The average composition of traditional Mozzarella, the retentate, and the UF Mozzarella cheeses after 1-week storage.

Table I. Composition de la mozzarella, des rétentats UF et des mozzarella UF maturées 1 semaine.

	1. Experiment		2. Experiment		Conventional control
	Retentate	Mozzarella	Retentate	Mozzarella	Mozzarella
Dry matter (%)	48.1	51.4 ± 0.70	47.6	52.9 ± 1.02	53.3 ± 0.86
Total protein (%)	22.2	23.4 ± 0.82	23.1	24.5 ± 0.90	23.2 ± 2.13
Whey protein (% of total protein)		7.1 ± 1.95		5.4 ± 1.56	–
Fat (%)	19.5	24.0 ± 0.58	17.7	24.7 ± 0.62	22.6 ± 0.22
Salt (%)	–	0.8 ± 0.10		0.7 ± 0.07	1.6 ± 0.23
pH		5.46 ± 0.03		5.53 ± 0.06	5.67 ± 0.12

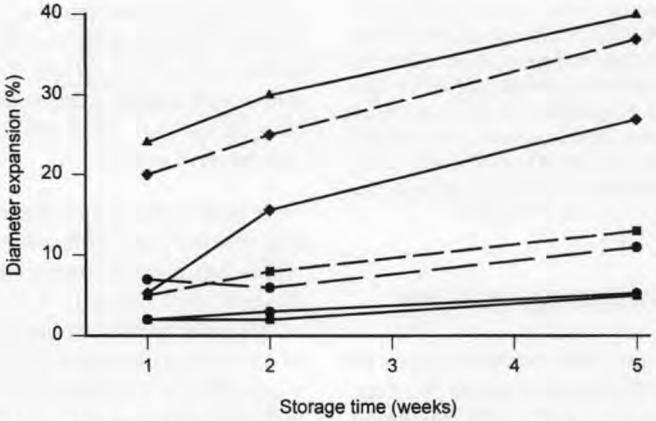

Figure 1. Melting of Mozzarella cheese (diameter expansion of a cylindrical sample after heating). (▲), commercial Mozzarella without whey protein; (●), UF Mozzarella (control); (■), UF Mozzarella treated with *Fusarium oxysporum* proteinase; (◆), UF Mozzarella treated with *Bacillus licheniformis* proteinase. Full line, UF Mozzarella with ~7.1% whey protein; dotted line, UF Mozzarella with ~ 5.4% whey protein.

Figure 1. Fonte de la mozzarella (expansion du diamètre d'un échantillon cylindrique après chauffage). (▲) Mozzarella commerciale sans protéines sériques ; (●) mozzarella UF (échantillon témoin) ; (■) mozzarella UF traitée avec protéinase de *Fusarium oxysporum ;* (◆) mozzarella UF traitée avec la protéinase de *Bacillus licheniformis*. Tracé plein : mozzarella UF avec environ 7,1 % de protéines sériques. Tracé pointillé : mozzarella UF avec environ 5,4 % de protéines sériques.

highest in cheeses with the lowest level of whey protein in accordance with the results of Savello et al. [42]. Although differences in the mineral balance also conceivably could contribute to differences in meltability, it was probably not very important here, as the two effects were counteracting each other. In experiment two the acidification time was longer and the extent of syneresis larger, which could lead to a larger loss of calcium and improved meltability. However, in this experiment the final pH was slightly higher, which would produce the opposite result, as less calcium would be solubilised.

Proteolysis in the conventional Mozzarella cheese was low and during the storage time the NCN increased to 11.3% of total N, a level quite similar to the water soluble N level reported by Creamer [5]. After 1 week of storage the NCN of the UF control was higher than NCN in traditional Mozzarella. This may be due to the content of proteose peptones and glucomacro-peptide, which do not coagulate during the heat precipitation of whey proteins by the NCN method. Another reason may be related to the hydrolysis of protein by the starter enzymes during the first week of storage as evidently these enzymes are active causing the amount of TCA-SN to increase to 7.4% after 5 weeks of storage (*table II*). In the cheeses treated with the two types of trypsin the NCN slightly increased during the storage, but the rate of increase was independent of presence of the two trypsins and similar to the UF control (*table II*). A further fivefold increase in the addition of the two trypsins still had no effect on proteolysis (results not shown). Probably, this lack of effect was due the low storage temperature and a low pH, far from the optimum conditions of these enzymes (pH 8–9 and

Table II. Formation of non-coagulable nitrogen (NCN), 12% TCA soluble nitrogen (TCA-SN) in percent of total nitrogen (TN), and TCA-SN/ NCN in UF Mozzarella cheese treated with different enzymes.

Table II. Formation d'azote non coagulable (NCN), d'azote soluble à 12 % dans le TCA (TCA-SN) en pourcentage de la matière azotée totale (TN), et TCA-SN/NCN dans la mozzarella UF traitée avec des enzymes différentes.

	NCN/TN (%)			*TCA-SN/TN (%)*			*TCA-SN/NCN (%)*		
	1 week	*2 weeks*	*5 weeks*	*1 week*	*2 weeks*	*5 weeks*	*1 week*	*2 weeks*	*5 weeks*
UF Control	9.6	11.8	16.0	3.5	5.5	7.4	36.5	46.6	46.3
Neutrase	25.9	35.4	51.8	7.7	11.3	15.9	29.7	31.9	30.6
Trypsin	11.0	12.0	13.8	4.3	5.4	6.7	39.1	45.0	48.6
BLP	20.3	24.2	30.3	7.7	10.2	13.9	37.9	42.1	45.9
FOT	13.5	12.5	16.3	5.3	5.9	8.4	39.2	47.2	51.5
Traditional Mozzarella	7.7	7.4	11.3	2.1	1.4	1.8	27.3	18.9	15.9

45 °C [14]), or some of the whey proteins inhibited the trypsins which belong to the same serine type of enzymes as plasmin, that has been shown to be inhibited by whey protein [2, 3].

From 1 to 5 weeks of storage NCN increased from 20% to 30% in the BLP treated UF Mozzarella (*table II*). Although the storage temperature was far from 60 °C and 70 °C, which is the optimum temperature for BLP on casein and whey protein substrates, respectively [29], the high level of proteolysis compared to trypsin probably was caused by the ability to hydrolyse in an acidic environment as demonstrated for proteinases with similar specificity [43]. Neutrase® was the most proteolytic enzyme, and increased NCN to more than 50% in 5 weeks (*table II*). However, NCN data alone do not clarify whether the stronger proteolysis was due to activity of the BLP and Neutrase alone, or partly was a result of better substrate availability for the starter enzymes. The development of small peptides and free amino acids as measured by the TCA-SN content showed that in all UF Mozzarella cheeses TCA-SN increased during storage (*table II*). Although the absolute content of TCA-SN in the Neutrase® treated Mozzarella was not different from the BLP treated cheeses, the strong proteolytic activity of Neutrase® produced excessive amount of peptide substrate (NCN), causing the proportion of TCA-SN to NCN to be constant at ~ 30% during storage. In all other UF Mozzarella cheeses the ratio TCA-SN to NCN increased from ~ 36% to ~ 50% due to degradation of some of the peptides in the NCN fraction by the peptidases of the starter bacteria.

Generally proteolysis improved melting of UF Mozzarella cheese (*figure 2*), and thus supported the findings of Kindstedt et al. [24] concerning conventionally produced Mozzarella cheeses. However, it is clear from *figure 2* that the relation between melting ability and proteolysis depends strongly on the specificity of the enzyme. NCN had to reach 45–50% of total N before DE corresponded to that of the conventional cheese (without whey protein). Neutrase® treated cheeses showed very good melting properties at 50% NCN, but the unmelted cheeses were very soft, and one cheese with extreme proteo-

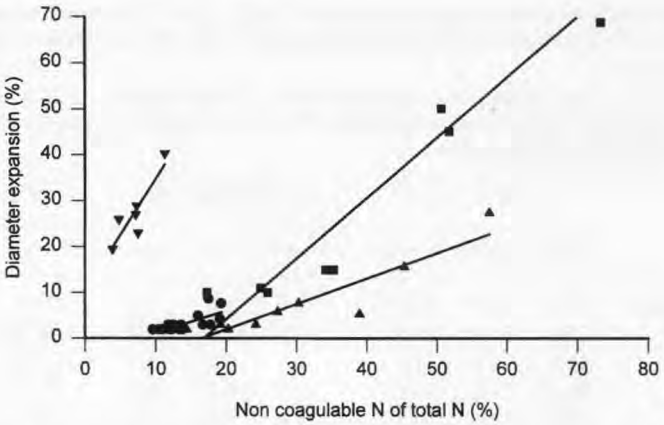

Figure 2. Melting (diameter expansion of a cylindrical sample after heating) of Mozzarella cheese as a function of proteolysis (non-coagulable nitrogen). (▼), Commercial Mozzarella without whey protein; (●), UF Mozzarella (control) and UF Mozzarella treated with trypsin); (■), UF Mozzarella treated with Neutrase®; (▲), UF Mozzarella treated with *Bacillus licheniformis* proteinase. Regression lines of each group are indicated.

Figure 2. Fonte de la mozzarella (expansion du diamètre d'un échantillon cylindrique après chauffage) en fonction de la protéolyse (azote non coagulable). (▼) Mozzarella commerciale sans protéines sériques ; (●) mozzarella UF (échantillon témoin) et mozzarella UF avec trypsine ; (■) mozzarella UF traitée avec la Neutrase® ; (▲) mozzarella UF traitée avec protéinase de *Bacillus licheniformis*. Les droites de régression de chaque groupe sont indiquées.

lysis (> 70% NCN) was almost liquid and difficult to handle in the cutting devices before the melting test. Trypsin treated UF Mozzarella cheese in our study was mild without any off-flavour similar to the UF control throughout the storage period, probably due to a low level of proteolysis by the trypsin at pH 6.0. High proteolysis and bitterness was found in UF cheese when trypsin, chymotrypsin and papain were added to retentate with a pH similar to milk and thus much closer to the optima of the enzymes [32]. Neutrase® treated cheeses were bitter, right from week one. The bitterness continued to develop, resulting in extreme bitterness in all 5-week-old Neutrase® treated cheeses. This findings was in line with the low TCA-SN to NCN level (*table II*) found in these cheeses indicating that starter peptidases were unable to degrade the bitter peptides into small non-bitter peptides and free amino acids. In few BLP-treated cheeses acidic and broth off-flavours were detected after 2 weeks of storage, but these flavours disappeared during the following 3 weeks, and bitterness was not detected at any of the sampling times. The difference between the BLP and Neutrase® treated cheese in relation to development of bitter peptides was not governed by degree of hydrolysis, but was caused by enzyme specificity. Neutrase® treatment will result in hydrophobic and basic amino acids located at the terminal position, which has been shown to convey much more bitterness to peptides than the acidic amino acids in the terminal position [28], that will result from BLP treatment.

Evaluation of the degradation of the individual caseins was done by CE. The electropherogram of the conventionally produced Mozzarella (results not shown)

and control UF Mozzarella (5-week-old, *figure 3A*) showed peaks for the original caseins α_{s1}-, α_{s0}-, βA1- and β A2-casein, for α_{s1}-I peptide, the first degradation product from α_{s1}-casein caused by rennet [15]. According to Otte et al. [36] γ-caseins, resulting from plasmin activity on β-caseins [15], migrate in front of the β-caseins, but only a small amount of these degradation products was seen, indicating that plasmin was not active in the UF control cheese. The UF Mozzarella cheese treated with the two trypsins (results not shown) and the UF control had similar pro-

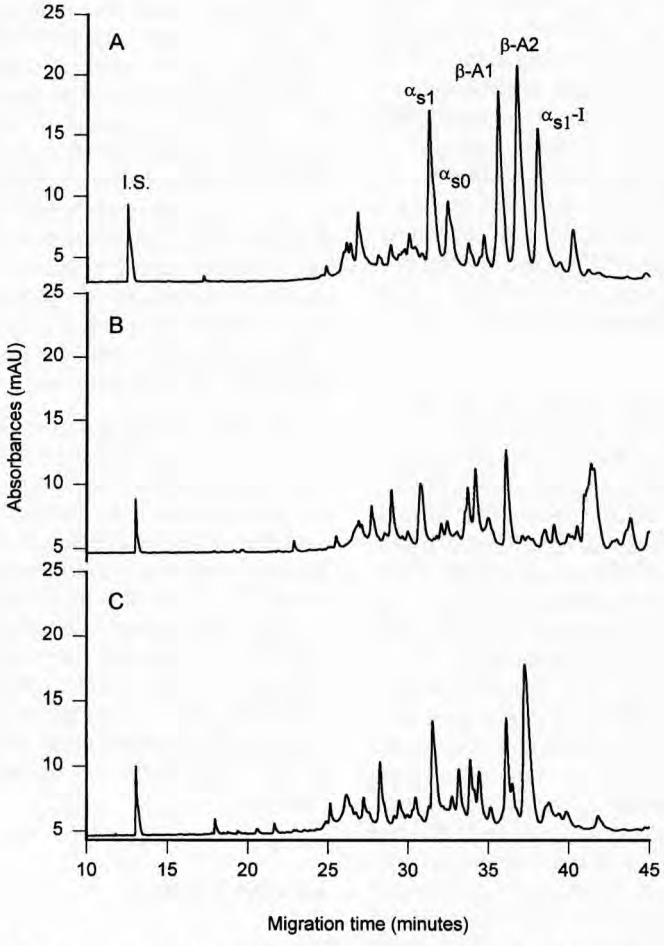

Figure 3. Capillary electrophoresis of the pH 4.6 insoluble fraction of UF Mozzarella cheese. **A.** 5-week-old UF Mozzarella without enzyme (control). **B.** 5-week-old UF Mozzarella treated with Neutrase®. **C.** 5-week-old UF Mozzarella treated with *Bacillus licheniformis* proteinase. I.S., internal standard. Absorbance at 220 nm.

Figure 3. Électrophorèse capillaire de la fraction de l'UF mozzarella qui est non soluble à pH 4,6. **A**. Mozzarella UF maturée 5 semaines sans enzyme (échantillon témoin). **B**. Mozzarella UF maturée 5 semaines avec la Neutrase®. **C**. Mozzarella UF maturée 5 semaines avec la protéinase de *Bacillus licheniformis*. I.S. : norme interne. Absorbance à 220 nm.

tein profiles with only very little degradation of the original caseins during the storage, in accordance with the low increase of NCN in these cheeses (*table II*). In the Neutrase® treated UF Mozzarella only a small amount of α_{s1}-casein and βA1-casein of the original caseins was left after 5 weeks of storage (*figure 3B*). This corresponded well with the high level of NCN (*table II*), and the very soft texture found in that cheese. In the BLP-treated UF Mozzarella (*figure 3C*) degradation of the caseins was significantly larger than in the control cheeses, and the α_{s1}-I peptide was practically absent, while α_{s1}-casein was present in significant amounts (*figure 3C*), which indicates that the α_{s1}-I peptide was degraded very fast in this cheese, compared to the control UF cheese. Contrary to Neutrase, rate of proteolysis of βA1-casein by BLP was higher than that of βA2-casein.

The electropherogram of the pH 4.6 soluble fraction of control UF Mozzarella after 5 weeks of storage showed few peptides, thus confirming the limited proteolysis in this cheese (*figure 4A*). Similar profiles were found in UF Mozzarella treated with PT and FOT (results not shown). A large number of degradation products were found in the pH 4.6 soluble fraction of Neutrase® treated UF Mozzarella (*figure 4B*), as expected from the very intensive proteolysis. Probably all of the degradation products were derived from casein degradation as β-Lg and α-La were still present in similar quantities as the control cheeses. This indicated that Neutrase® did not hydrolyse β-Lg and α-La when incorporated in cheese, although β-Lg is hydrolysed by Neutrase® in solution at pH 7.5 [30]. In BLP-treated cheese the soluble fraction also contained a number of degradation products, probably derived from casein, as high amounts of β-Lg and α-La were present after 5 weeks of storage (*figure 4C*). This indicated that BLP did not hydrolyse β-Lg and α-La in cheese either, although whey protein concentrate at pH 7.5 to 10.0 [29] and solutions of β-Lg at pH 7.5 are hydrolysed by BLP [30].

SE HPLC confirmed the presence of whey proteins, as two peaks corresponding to β-Lg (MW: 36 000, retention time: 12 min) and α-La (MW: 14 400, retention time: 13 min), respectively were seen in the chromatogram (*figure 5B, C*). The large peak eluting at 15 min contained small peptides and free amino acids and this peak was also seen in the pH 4.6-SN supernatant of the conventionally produced Mozzarella (*figure 5A*). No difference was found in β-Lg content of the BLP treated and the control UF Mozzarella cheeses (*table III*), again indicating that α-Lg was resistant to hydrolysis by BLP in cheese. However, the accumulation of small peptides and free amino acids was higher in the BLP-treated cheeses (*figure 5C*). In view of BLP's preference for casein over whey protein [29], this was undoubtedly related to casein hydrolysis.

The peak eluting at a time characteristic of α-La was higher in BLP-treated cheese, presumably because rather large degradation products derived from the casein hydrolysis co-eluted with α-La. This assumption was confirmed by a continuous increase in this peak's area during the ripening period. The formation of small peptides and free amino acids increased (peak 3 in *figure 5C* and *table III*), indicating that starter peptidases were active, corresponding to the high amount of TCA-SN (*table II*) found in these cheeses.

4. CONCLUSION

UF Mozzarella was manufactured with the same gross composition as conventional Mozzarella cheese, but meltability of the cheese was decreased as incorporation of whey protein was increased. Generally, proteolysis improved the meltability, but proteinases with different specificities gave very different results. Two trypsin-type

enzymes were not active at the pH in the cheese, while Neutrase improved meltability considerably by degrading casein fast, but also caused the cheeses to become very bitter. BLP was less active, but nevertheless improved the meltability without accompanying bitterness. None of the enzymes were able to hydrolyse whey protein incorporated in the cheese.

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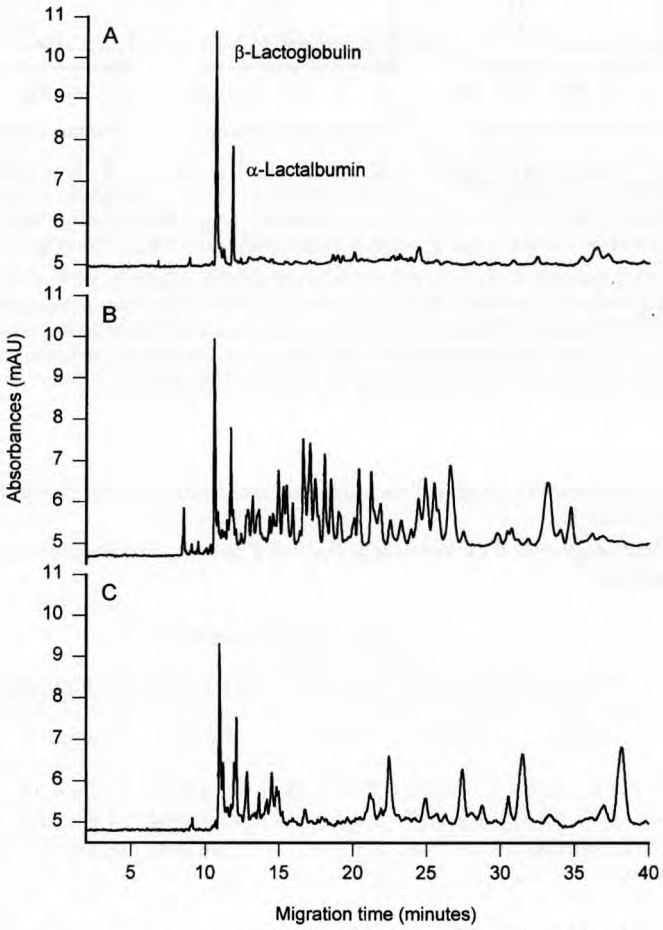

Figure 4. Capillary electrophoresis of the pH 4.6 soluble fraction of UF Mozzarella cheese. **A**. 5-week-old UF Mozzarella without enzyme (control). **B**. 5-week-old UF Mozzarella treated with Neutrase®. **C**. 5-week-old UF Mozzarella treated with *Bacillus licheniformis* proteinase. Absorbance at 220 nm.

Figure 4. Électrophorèse capillaire de la fraction soluble à pH 4,6 de la mozzarella UF. **A**. Mozzarella UF maturée 5 semaine sans enzyme (échantillon témoin). **B**. Mozzarella UF maturée 5 semaines avec la Neutrase®. **C**. Mozzarella UF maturée 5 semaines avec la protéinase de *Bacillus licheniformis.* Absorbance 220 nm.

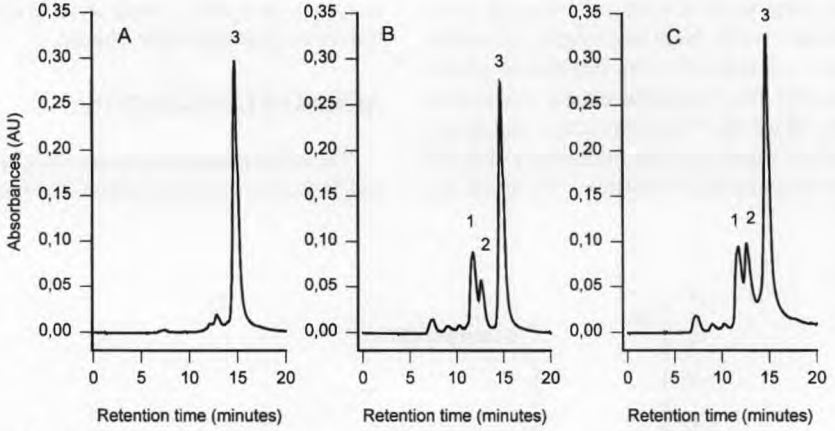

Figure 5. Size exclusion-HPLC of the pH 4.6 soluble fraction of UF Mozzarella cheese. **A.** 5 weeks old Mozzarella. **B.** UF Mozzarella without enzyme (control). **C.** 5 weeks old UF Mozzarella treated with *Bacillus licheniformis* protease. 1, β-lactoglobulin (MW ~ 36 000); 2, α-lactalbumin (MW ~14 400), and 3, small peptides (MW 5–10 000). Absorbance at 220 nm.

Figure 5. Chromatographie d'exclusion de la fraction soluble à pH 4,6 de la mozzarella UF. **A**. Mozzarella UF maturée 5 semaines. **B**. Mozzarella UF maturée 5 semaines sans enzyme (échantillon témoin). **C**. Mozzarella UF maturée 5 semaines avec une protéinase de *Bacillus licheniformis*. 1) β-Lactoglobuline (poids moléculaire ~36 000), 2) α-Lactalbumine (poids moléculaire ~14 400), 3) petits peptides (poids moléculaire 5–10 000). Absorbance 220 nm.

Table III. Size exclusion-HPLC of the pH 4.6 soluble nitrogen fraction of Mozzarella cheese (mean area ± standard deviation).

Tableau III. Chromatographie d'exclusion de la fraction d'azote soluble de la mozzarella (valeur moyenne ± écart type).

	Peak area (arbitrary units)					
	UF control Mozzarella (5 cheeses)			BLP treated UF Mozzarella (2 cheeses)		
	1 week	2 weeks	5 weeks	1 week	2 weeks	5 weeks
β-Lg	4.2 ± 0.30	4.3 ± 0.32	4.2 ± 0.32	4.2 ± 0.49	4.2 ± 0.57	3.7 ± 1.3
α-La	2.7 ± 0.19	2.8 ± 0.17	2.9 ± 0.31	4.8 ± 0.64	4.9 ± 0.64	5.6 ± 0.78
Small peptides	12.9 ± 0.87	13.0 ± 0.81	13.6 ± 1.3	16.5 ± 0.92	16.4 ± 1.3	16.7 ± 1.9

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