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Improving the Yield of Mozzarella Cheese by Phospholipase Treatment of Milk

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ABSTRACT

Part-skim Mozzarella cheese was manufactured from milk hydrolyzed with fungal phospholipase A_1 prior to renneting. The phospholipase treatment reduced fat losses in whey and cooking water and increased cheese yield as a result of improved fat and moisture retention in the cheese curd. The amount of phospholipids in the whey was reduced because of improved retention of lysophospholipids in the cheese curd. Water binding in the fresh curds and young cheeses up to 3 wk of storage was investigated by a ^{1}H nuclear magnetic resonance spin–spin relaxation technique. In the fresh curds, 2 dominant water fractions were present, characterized by average spin–spin relaxation times (T_2) of 14 and 86 to 89 ms, respectively. These 2 fractions of low- and high-molecular-mobility water were similar in all cheeses and presumed to represent water associated with the casein matrix and water present in the pores. A few hours after manufacture, cheeses made with phospholipase showed decreased T_2 of the high-mobility fraction, indicating improved water-holding capacity. It is suggested that lysophospholipids released from the fat globule membranes act as surface-active agents in the cheese curd, helping emulsification of water and fat during processing and reducing syneresis. During 3 wk of storage after manufacture, the mobility of both water fractions increased in all cheeses, but was highest in the cheeses made with phospholipase. The increase in mobility during the first weeks of storage has earlier been ascribed to structural changes in the protein matrix, which in principle could be accelerated because of the higher moisture content. However, the microstructure of phospholipase-treated cheese was investigated by confocal laser scanning microscopy and found to be very similar to the control cheese during processing and up to 28 d of storage. In addition, flowability, stretchability, and browning were acceptable and similar in all the manufactured cheeses. Thus, phospholipase hydrolysis of cheese milk improved the cheese yield without changing the cheese microstructure, and resulted in cheese with functional properties that were identical to traditional Mozzarella cheese.

Key words: Mozzarella cheese, phospholipase, low-resolution nuclear magnetic resonance, microstructure

INTRODUCTION

Maximizing cheese yield without compromising product quality is a key concern for industrial cheese manufacturers. Depending on the milk composition, cheese recipe, and manufacturing technology, 75 to 78% of milk protein and 85 to 95% of milk fat are entrapped in the cheese curd, leading to a typical total cheese yield of 9 to 15% on a weight basis (Farkye, 2004; Nielsen, 2004). The remaining protein and fat are lost in the whey and, to a lesser extent, in the brine. In the production of pasta filata Mozzarella cheese, the fat retention rarely exceeds 90% because of additional losses encountered in the hot stretching step, where the fresh curd is molded and stretched in hot water. A number of approaches have been developed to increase the cheese yield, ranging from optimization of production equipment designs such as cutting devices and of the traditional cheese-making process, to alternative approaches involving concentration of the cheese milk by microfiltration or ultrafiltration and inclusion of whey protein (Banks et al., 1987; Lelievre, 1988; Brandsma and Rizvi, 2001). Standardization of cheese milk with microfiltration or ultrafiltration retentate is being successfully applied in the industrial production of Mozzarella cheese; however, problems related to impaired functional properties and textural defects of the products are encountered if the whey protein content of the cheese is too high.

Recently, a new enzymatic method for increasing cheese yield through treating milk with phospholipase prior to cheese making was suggested (Nielsen, 2002). Phospholipase A_1 (EC 3.1.1.32) hydrolyzes the *sn*-1 ester bond of phospholipids, resulting in formation of lysophospholipids and fatty acids. Lipases that are used in the manufacture of certain cheese types often hydrolyze a broad range of triglycerides. In contrast, phos-

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pholipases suggested for use in dairy processing are more specific and have little or no activity toward di- or triglycerides. Because of the narrow specificity, flavor defects caused by the release of short-chain fatty acids are avoided, because phospholipids mainly contain nonvolatile palmitic, oleic, and stearic acids. Whereas phospholipases are new in dairy applications, several different enzymes belonging to this category are already used in other industrial-scale food processes, such as degumming of vegetable oils, modification of egg yolks, and dough improvement (Kirk et al., 2002). In milk, phospholipids are mainly present on the surface of milk fat globules, stabilizing the milk fat against coalescence. Some phospholipids are also found in milk serum in amounts depending on the mechanical processing and storage of milk (Walstra et al., 1999). The average total content of phospholipids in cow's milk is approximately 0.01 to 0.03% (wt/wt), varying in concentration and composition with the stage of lactation (Bitman and Wood, 1990). The major phospholipid components of cow's milk are phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), and sphingomyelin (**SM**), which together make up 80 to 90% of the total milk phospholipids. The remaining 10 to 20% are mainly the negatively charged phospholipids phosphatidylserine (**PS**) and phosphatidylinositol (**PI**; Bitman and Wood, 1990; Rombaut et al., 2005).

Hydrolysis leads to the release of less hydrophobic and thus more water-soluble lysophospholipids, which have a higher dynamic surface activity because of the higher unimer concentration in the aqueous phase. Lysophospholipids are excellent emulsifiers, and the oil-in-water emulsions stabilized by hydrolyzed phospholipids show improved heat stability and are less sensitive to flocculation by Ca^{2+} and Mg^{2+} (Van Nieuwenhuizen, 1981). In emulsions containing hydrolyzed egg lecithin, the increased stability against heat-induced coalescence has been attributed to lysophospholipid–protein complexes (Mine, 1997). Also α_{s1}-CN and β-LG have been shown to interact with (lyso)phospholipids and form surface-active lipoprotein complexes (Barratt et al., 1974; Wiechen et al., 1985; Sarker, 1995).

Enrichment of milk with phospholipids from buttermilk or from soy has been shown to increase the cheese yield, as well as to improve the texture of low-fat cheese (Mistry et al., 1996; Drake et al., 1998; Govindasamy-Lucey et al., 2006). The yield improvement has, in most cases, been ascribed to increased moisture retention, especially in low-fat cheeses. Enhanced fat retention has been reported only in full-fat Colby cheese manufactured with added soy lecithin (Hicks et al., 1985). To our knowledge, cheese manufacture with added hydrolyzed phospholipids has not been previously reported in the literature.

The objective of the present work was to evaluate the effect of a novel phospholipase A_1 from the fungus *Fusarium venenatum* on the yield and functional properties of semi-skim Mozzarella, and to investigate the changes in cheese microstructure and water binding caused by modification of the phospholipids. Our goal was to gain insight into the mechanism of phospholipase-mediated yield improvement in the complex cheese system.

MATERIALS AND METHODS

Cheese Manufacture

Part-skim low-moisture pasta filata Mozzarella cheese was manufactured in the pilot facility of Chr. Hansen A/S in Hørsholm, Denmark, on 4 separate days. On each day, 4 vats of cheese were prepared: 2 with phospholipase and 2 control vats, with a random assignment of the vats on each day using a balanced Latin square design. Each vat contained 150 kg of milk. Pasteurized part-skim milk (74°C, 15 s) from Borup Dairy (Gørløse, Denmark) was standardized to 2.6 to 2.7% fat content by addition of pasteurized cream. Frozen Direct Vat Set starter culture STM5 containing *Streptococcus thermophilus* (Chr. Hansen A/S, Hørsholm, Denmark) was added to preheated (34.5°C) milk at the rate of 10 g/100 kg. YieldMAX PL phospholipase (Chr Hansen A/S) was added in the 2 experimental vats 20 min prior to renneting, in a dose corresponding to 5 lecitase units/g of milk fat. One hour after the addition of starter culture, 33.75 g of rennet was added to each vat (CHY-MAX Plus, strength 200 international milk-clotting units/mL; Chr. Hansen A/S). After 25 min, the curd was cut by 10-mm knives and stirred for 15 min. The temperature of the curd and whey was then increased to 41°C within 30 min while stirring, and the whey was drained off 20 min after scalding was ended. The vats were weighed before and after whey drainage. The cheese curd was cut into blocks, which were then turned and stacked every 10 min. When the pH had reached 5.1 to 5.2, the curd was milled and dry salted by adding 2% NaCl based on wet weight. Samples for microscopy and nuclear magnetic resonance (**NMR**) were collected from the milled curd before salting. The salted curd was stretched for 6 min in 70 L of 74°C cooking water by using a stretcher–extruder (Almac s.r.l., Modena, Italy). Fresh cooking water was used for each vat. The stretched curd was extruded into 6 loaves of approximately 2.5 kg, which were cooled in 5 to 7°C water for 20 min, and then immersed in saturated NaCl brine with 34% $CaCl_2$, pH 5.1, for 1.5 h. After air-drying for 30 min, the loaves were weighed and packed in vacuum-sealed bags. Two loaves from each vat were cut into 4 equal parts, which

were vacuum-packaged separately for the NMR analysis. All cheeses were stored at 2 to 4°C until analysis.

Compositional Analysis of Milk, Whey, and Cooking Water

The chemical composition of milk, whey, and cooking water (fat, protein, lactose, and total solids) was determined by a MilkoScan FT-120 (Foss Electric A/S, Hillerød, Denmark), calibrated using relevant International Dairy Federation reference methods. The phospholipid content of milk and whey was analyzed by ^{31}P NMR after extraction of the freeze-dried samples with 1:1 chloroform:methanol. The dried extracts were dispersed in a (1:2:2) mixture of methanol:$CDCl_3$:Cs-EDTA (0.2 *M*, pH 7.5). Triphenylphosphate was used as the internal standard. The analysis was performed by SpectralService GmbH (Cologne, Germany), using the reference method of the International Phospholipid and Lecithin Society. Cheese composition (fat, protein, salt, and moisture contents) and pH were analyzed 1 wk after manufacture. The composition was determined by using FoodScan Lab near-infrared spectroscopy equipment (Foss Electric A/S), calibrated using relevant International Dairy Federation reference methods. Two cheeses from each vat were shredded and each was analyzed in 3 replications. Milk, whey, cooking water, and cheese attributes were analyzed by single-factor ANOVA at $P = 0.05$.

Protein and Peptide Composition of Cheese

Cheese samples were frozen 2 d after manufacture until analysis of protein and pH 4.4-soluble peptide composition by capillary electrophoresis (**CE**) and reversed-phase HPLC (**RP-HPLC**), respectively. Samples were thawed in a refrigerator overnight and tempered to room temperature before grating. A 20% (wt/vol) cheese slurry was prepared from grated cheese in 0.5 *M* trisodium citrate buffer preheated to 45°C, and stirred on a magnetic stirrer in a 45°C water bath for 1 h. The cheese slurry was diluted to 5% (wt/vol) with demineralized water, and fat was removed by centrifugation. The supernatant was diluted 1:1 with sample buffer (2.56 g/L of DL-dithiothreitol in 10 *M* urea) and incubated at room temperature for 1 h before analysis by CE. Each sample was prepared in 3 replications. The analysis was performed at 45°C with an HP 3-D CE system (Hewlett-Packard A/S, Birkerød, Denmark) in a coated capillary column (PVA Agilent G1600-61219; Agilent Technologies, Inc., Palo Alto, CA). Separation was performed by applying a linear voltage gradient from 0 to 25 kV for 3 min, followed by 25 kV for 40 min. The running buffer consisted of 20 m*M* trisodium citrate, 190 m*M* citric acid, and 6 *M* urea, pH 3.0, with methyl hydroxypropyl cellulose (0.5 mg/mL).

For the analysis of peptide composition, the diluted cheese slurry described above was cooled to 12°C and the pH adjusted to 4.4 by adding 1 *M* HCl under vigorous shaking. A small volume of each sample was centrifuged (1,400 × *g*, 30 min, 4°C), and the clear middle phase was taken out and filtered (0.45 μm) before analysis by RP-HPLC. The analysis was performed at 30°C with a Waters 2690 separation module (Waters, Milford, MA) in a Nucleosil 300-5 C18 silica column (Macherey-Nagel, Düren, Germany). A 2-μL volume of each sample was injected in duplicate.

Degree of Phospholipid Hydrolysis in Milk

Unhomogenized organic milk with 3.5% fat, purchased from the local supermarket, was incubated with 5 lecitase units of phospholipase/g of milk fat at 35°C for 0, 0.5, 1.5, 3, and 20 h. Each incubation time was analyzed in duplicate. The reaction was stopped by addition of extraction solvent 2:1 (vol/vol) of chloroform:methanol. Samples were extracted twice with this mixture and the extracts containing phospholipids were combined, evaporated to dryness, dissolved in chloroform, and applied on Bond-Elut NH_2 solid-phase extraction columns (Varian, Harbor City, CA). The columns were rinsed with 2:1 chloroform:isopropanol (vol/vol) and 2% acetic acid in ether to remove neutral lipids and free fatty acids, respectively. Phospholipids were eluted by methanol, evaporated to dryness under N_2 flush, and quantified by the HPLC method described by Sas et al. (1999) with minor modifications.

Low-Resolution ^1H NMR

Moisture and fat contents in different types of foods can be analyzed by low-resolution ^1H NMR. The spin–spin relaxation time (T_2) is a measure of the interactions of protons with their surroundings, and has been applied to characterize the nature of water binding in cheese and other complex matrices. Protons that are chemically bound to, for example, protein structures have decreased mobility and thus shorter T_2 compared with protons in bulk water or in "structured" water in the hydration sheath surrounding proteins. A frequency distribution of the ^1H NMR signal can be interpreted as populations of protons with different degrees of mobility existing in the sample.

The analysis was carried out in a 23.4-MHz Maran Ultra low-resolution ^1H NMR spectrometer (Resonance Instruments Ltd., Witney, UK), equipped with a thermostat arrangement for maintaining temperature. The spin–spin relaxation was measured using a Carr–Pur-

cell–Meiboom–Gill (**CPMG**) sequence with a dwell time of 1 μs, and the radiofrequency of 90 to 180° pulse gap (τ) of the CPMG sequence was 75.0 μs. The number of echoes was set to 2,048 and 4 scans were acquired for each measurement. WinDXP software (Resonance Instruments Ltd.) was used to fit the relaxation data into an exponential function, producing a continuous spin–spin relaxation time (T_2) distribution:

$$A(t) = a + \sum_i A_i \exp\left(-\frac{t}{T_{2,i}}\right)$$

where a is the zero drift (offset), A_i the fraction of spins in a given state and $T_{2,i}$ the spin–spin relaxation time of protons in the given state i. The T_2 values and corresponding peak heights were obtained by Gaussian fitting of the frequency distribution curve, using GraFit software v. 4.0.16 (Erithacus Software Ltd., Horley, UK); A_i values were normalized to sample weight. Samples were analyzed on the day of manufacture (unsalted curd before molding and fresh cheese after molding and brining), and at 2, 5, 7, 10, 14, and 21 d after manufacture. Cylindrical cheese samples, approximately 15 mm high, were pressed out from the center of the cheeses with 14-mm i.d. NMR tubes that were sealed with Teflon plugs to avoid evaporation. The samples were tempered at 20°C for 30 min before analysis. Two vats of each type of cheese were analyzed on each day in 3 to 5 replications, and the statistical significance of the results was determined by single-factor ANOVA at $P = 0.05$.

Confocal Laser Scanning Microscopy

Microscopy experiments were carried out using a Leica DM IRE2 inverted microscope with a TCS SP2 MP scanning unit (Leica Microsystems, Heidelberg, Germany), fitted with a Kr/Ar laser giving excitation wavelengths of 488 and 568 nm. The emission wavelengths at 500 to 535 nm and 600 to 635 nm were collected, corresponding to fluorescein isothiocyanate (**FITC**; green) and Nile Red (red) emission, respectively. A Peltier scanning stage connected with a heating unit (Linkam Scientific Instruments Ltd., Tadworth, Surrey, UK) was used to control the temperature, which was set to 20°C. Images of 512 × 512 pixels in size were recorded using a 63× water correction objective with a 1.2 numerical aperture, approximately 20 μm below the sample surface. Samples were stained by Nile Red and FITC (Molecular Probes, Eugene, OR) to visualize fat and protein, respectively. Slices approximately 2 mm thick and 10 × 10 mm were cut from the center of each cheese perpendicular to the protein fiber direction. A drop each of 0.02% Nile Red in acetone and 0.02% FITC in acetone was spread in a glass-bottomed sample dish (WillCo Wells BV, Amsterdam, The Netherlands) and allowed to dry before placing the cheese sample on top. The dish was covered with a lid to prevent drying of the sample. The staining was complete after 5 min at room temperature. Two vats of each type of cheese were studied by confocal laser scanning microscopy (**CLSM**).

Image Analysis

Twenty random images of the fresh, unsalted curd from 2 experimental and 2 control cheese vats were included in the analysis (i.e., a total of 80 images). The images collected at emission wavelengths of 500 to 535 nm (green channel) showing the protein network and at 600 to 635 nm (red channel) showing the fat fraction were studied separately. The red-channel images were corrected for unspecific staining of protein by subtracting the green-channel signal after Gaussian blurring of both channels. Noise was reduced by arithmetical averaging of the images.

The average, minimum, and maximum areas of the different fat globule units were calculated, without discriminating between clusters and fat pools. The coarseness of the images was determined to evaluate the relative amounts of fat globule clusters, which appeared rough, and coalesced fat pools, which appeared smooth. This was done by calculating the absolute value of the grayscale intensity gradient as a function of the zoom-out factor s, normalized to the total area of fat globules on each image:

$$G(s) = \frac{1}{A_{\text{total}}} \Sigma_{i,j} |\nabla I(n,m,s)| \cdot * [(|110 > \nabla I|) \cdot * (I > 20)],$$

where A_{total} is the total area of fat globule clusters and pools, s is the zoom-out factor, N and M are the dimensions of the image, and $I(n,m,s) = [I(1{:}s{:}N,\ 1{:}s{:}M,\ 0)]$. The value of $G(s)$ indicates the amount of particles of a given size s, and thus the larger the $G(s)$, the coarser the images and the higher the relative amount of clusters compared with coalesced fat. The signifier "*"represents pairwise element-by-element matrix multiplication. The relative area covered by the protein network and the P/L parameter were calculated after Gaussian blurring and background intensity correction of the green-channel images by using Adobe Photoshop v.6 and Image Processing Toolkit v.5.0 (Reindeer Graphics, Inc., Asheville, NC). The P/L parameter is related to the mesh size of the network, with finer strands yielding a higher number of crossovers from high- to low-intensity pixels. The statistical analyses were done by single-factor ANOVA with $P = 0.05$.

Functionality Tests

Cheeses manufactured on a single day (2 vats of each type) were subjected to functionality tests 1 wk after manufacture. The cheeses were grated with a coarse grater for the analyses and mixed well.

Browning. Grated cheese (16 g) was placed on top of toast bread and exposed to infrared heat (160°C for 8 min) in a balance with a drying facility (Sartorius GmbH, Goettingen, Germany). The samples were ranked by 3 assessors by visual inspection according to the degree of browning. Each vat was tested in 4 replications. Cheeses were compared using a one-tailed Wilcoxon ranked-sums test with $P = 0.05$.

Flowability. Grated cheese (30 g) was transferred into one end of a Kjeldahl tube, which was placed horizontally in a top- and bottom-heated oven and baked for 1 h at 100°C. The flowability of the samples was determined as the length of the melted mass in the tubes after cooling. Each vat was tested in 2 replications. Cheeses were compared by single-factor ANOVA with $P = 0.05$.

Stretchability. Grated cheese (30 g) was placed on slices of toast that had been cut in halves. The samples were heated at 200°C for 10 to 12 min until the cheese started to bubble. The samples were rotated in the oven every 2 min to ensure even heat distribution. After melting, the samples were stretched by fixing one-half of the toast and lifting the second half by using forceps. If the cheese could be stretched to a length of 30 cm for 6 s, the stretchability was deemed acceptable. Each vat was tested in 2 replications.

RESULTS AND DISCUSSION

Hydrolysis of Milk Phospholipids

The amount of phospholipids in whole milk was determined after incubation with phospholipase to determine the degree of hydrolysis (**DH%**) caused by the enzyme as a function of incubation time (Figure 1). After 30 min of incubation at 35°C, corresponding roughly to the beginning of rennet coagulation in the Mozzarella process used in the current study, 62.9 ± 7.7% and 60.3 ± 4.1% of the major milk phospholipid species PE and PC were hydrolyzed, respectively. The amount of SM was unchanged regardless of incubation time or enzyme dose (results not shown). The inability of the used phospholipase to hydrolyze SM is probably due to the different backbone structure: PE and PC are both glycerophospholipids, whereas SM has a sphingosine backbone. The maximum DH% of PE and PC were 74.3 ± 0.8% and 71.8 ± 0.4%, respectively, and were reached after 3 h at 35°C. By increasing the enzyme dose 10-fold, the maximum DH% was unchanged, but was achieved after just 30 min (results not shown). The fact that full hydrolysis was not achieved may be due to the phospholipids being unavailable to the enzyme, possibly because of steric hindrance by the fat globule membrane proteins. On the other hand, the form in which the substrate is presented to the enzyme often has a remarkable effect on enzyme activity (Deems, 2000). At 110 min after phospholipase addition, corresponding to the time of whey drainage, the reaction had reached >93% of the maximum DH%. In the Mozzarella-making process, the temperature is increased to 41°C after cutting the curd, which speeds up the enzymatic reaction by approximately 10% without affecting the enzyme stability (T. M. Fatum, unpublished results). Thus, it is reasonable to assume that the phospholipase reaction proceeded close to the maximum obtainable DH% before whey drainage.

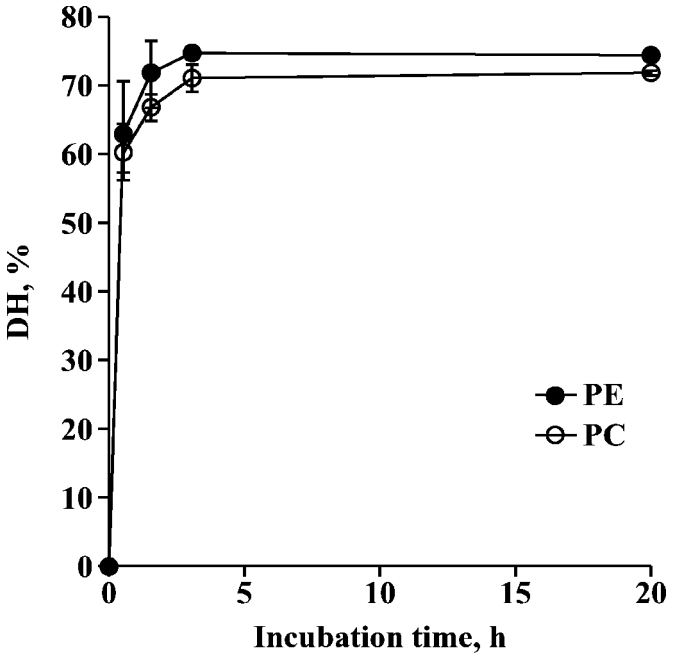

Figure 1. Hydrolysis of milk by *Fusarium venenatum* phospholipase A_1. Degree of hydrolysis (DH) of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as a function of incubation time. The error bars represent standard errors of the mean for 2 replicates.

Milk Composition

The composition of standardized cheese milk did not vary significantly between the cheese manufacturing days or between the experimental and control vats. The total protein, fat, lactose, and DM contents are shown in Table 1. The cheese milk contained, on the average, 0.023 ± 0.004% (wt/wt) of phospholipids, composed mainly of PC (28.4 mol%), PE (29.3 mol%), and SM (23.3 mol%). The figures represent the total phospholipids in

Table 1. Composition of milk, whey, stretch water, and cheese, and data on cheese yield[1]

Component	Treatment		SEM	Difference (%)	*P*-value
	Control	PL			
Standardized cheese milk					
Total protein, %	3.525	3.522	0.015	–0.09	NS
Fat, %	2.638	2.637	0.014	–0.02	NS
Lactose, %	4.690	4.696	0.013	0.12	NS
TS, %	11.565	11.544	0.038	–0.18	NS
Whey					
Total protein, %	0.848	0.843	0.010	–0.59	NS
Fat, %	0.132	0.126	0.004	–4.27	NS
Lactose, %	4.641	4.645	0.010	0.08	NS
TS, %	6.276	6.274	0.009	–0.03	NS
Total phospholipids, μM	183.0	68.4	5.0	–63.10	<0.001
Cooking water					
Total protein, %	0.238	0.241	0.002	1.32	NS
Fat, %	0.300	0.294	0.005	–2.08	NS
Cheese composition					
Total protein, %	26.09	25.36	0.15	–2.78	<0.005
Fat, %	22.19	21.87	0.06	–1.46	<0.005
NaCl, %	1.07	1.15	0.03	7.42	NS
Moisture, %	47.12	48.02	0.12	1.92	<0.001
MNFS, %	60.56	61.46	0.17	1.49	<0.005
FDM, %	41.96	42.07	0.18	0.26	NS
S/M, %	2.27	2.40	0.05	5.40	NS
pH at whey drain	6.19	6.19	0.03	0.08	NS
pH at milling	5.16	5.14	0.01	–0.33	NS
pH, 1 wk	5.33	5.34	0.02	0.21	NS
Cheese yield information					
Curd yield, %	10.80	10.92	0.09	1.07	NS
Actual yield, %	10.35	10.69	0.05	3.24	<0.001
Moisture-adjusted yield, %	10.53	10.68	0.05	1.47	NS
Fat yield, %	87.20	88.72	0.24	1.74	<0.001
Protein yield, %	76.69	77.03	0.24	0.44	NS
DM yield, %	46.43	47.10	0.11	1.45	<0.001
Phospholipid retention, %	45.4	79.3	3.2	74.7	<0.001

[1]PL = Phospholipase; SEM = standard error of the mean; NS = not significant (*t*-test, $P = 0.05$); MNFS = moisture in the nonfat substance of the cheese; FDM = fat content on a DM weight basis; S/M = salt in the moisture phase of the cheese; curd yield = yield of fresh unsalted curd before stretching; moisture-adjusted yield = yield adjusted to the target moisture content 48% (wt/wt); DM yield = recovery of total milk solids.

milk (i.e., both the membrane-bound phospholipids and those found in the milk serum). The contents and distribution are similar to previously published results on raw milk phospholipids (Bitman and Wood, 1990; Rombaut et al., 2005).

Cheese and Whey Composition

The cheese and whey compositions are shown in Table 1. Phospholipase treatment increased the overall moisture content of the cheese ($P < 0.05$), from 47.12 ± 0.10% to 48.02 ± 0.13%, as well as the moisture in nonfat solids (from 60.56 ± 0.16% to 61.46 ± 0.18%; Table 1). The composition of the DM (fat and protein content relative to the DM) was not significantly different between the control and phospholipase cheeses. The protein composition of the cheeses was determined by capillary electrophoresis 2 d after manufacture and was found to be identical in all cheeses, indicating that retention of any particular CN component was not specifically affected by the phospholipase (data not shown). Also, the pH 4.4-soluble peptide profiles obtained by RP-HPLC were essentially similar in the phospholipase and control cheeses. The detected amounts of β-LG and α-LA were low and similar in all samples.

A tendency toward lower overall fat contents in the whey from phospholipase-treated vats was seen consistently on each experimental day. Results obtained in industrial-scale trials showed similar trends toward lower fat contents in both whey and in cooking water in vats treated with phospholipase, suggesting that phospholipase may improve fat retention in the cheese curd (Høier and Lilbæk, 2006). The hydrolysis of cheese milk by phospholipase decreased the total phospholipid concentration in the whey from 183.0 ± 5.4 μM in control vats to 68.4 ± 4.5 μM in experimental vats ($P < 0.05$). The phospholipid content of control whey corresponded to 54.6 ± 4.2 mol% recovery of milk phospholipids in the whey, and thus approximately 45% retention in the cheese curd. Similar ratios have been reported by Govin-

Table 2. Phospholipid composition of cheese milk and whey mole percent of total phospholipids

Phospholipid[1]	Cheese milk[2]	SEM[3]	Whey,[4] control	Whey,[4] phospholipase	SEM[5]
PC	28.4	0.8	28.0	9.2	0.5
2-LPC	ND[6]	—	ND	12.5	1.1
PE	29.3	1.4	26.09	1.6	0.8
LPE	ND	—	ND	1.75	0.8
PS	11.6	0.4	10.0	3.6	0.5
PI	7.4	0.3	7.2	1.58	0.5
SM	23.2	1.4	28.6	69.65	2.5
Other	ND	—	ND	2.45	0.8

[1]PC = Phosphatidylcholine; LPC = lyso-PC; PE = phosphatidylethylamine; LPE = lyso-PE; PS = phosphatidylserine; PI = phosphatidylinositol; SM = sphingomyelin.

[2]Means of 2 batches.

[3]Standard error of the mean for the milk samples.

[4]Means of 6 batches.

[5]Pooled standard error of the mean for the whey samples.

[6]ND = None detected.

dasamy-Lucey et al. (2006), who found 39.4% phospholipid retention in semi-skim pizza cheese, and Turcot et al. (2001), who reported 44% average retention in low-fat Cheddar prepared from buttermilk-enriched milk. In the phospholipase vats, only 20.7 ± 2.6% of total milk phospholipids were recovered in the whey, implying increased retention of phospholipids in the curd. In particular, the lysophospholipids were retained to a higher degree compared with the native milk phospholipids, whereas the amount of SM in the experimental whey was not significantly lower than in the control, consistent with the inability of the used phospholipase to hydrolyze this phospholipid species. The compositions of the milk and whey phospholipids are shown in Table 2. The composition of phospholipids in the control whey was similar to the composition in cheese milk. On the other hand, phospholipase activity was evident from the composition of the whey from experimental vats, which contained only small percentages of PE and PC, as well as some lyso-PE and lyso-PC. The amounts of PS and PI were lower than in the control; however, lyso-PS and lyso-PI were not present in detectable amounts. The high retention of lysophospholipids in the cheese may be due to an interaction with milk protein and the incorporation of lysophospholipid–protein complexes into the cheese matrix. Wiechen et al. (1985) reported that the retention of added soy phospholipids in Camembert curd could be increased from approximately 30% to more than 80% by inducing the formation of CN–phosholipid complexes by heat. Lysophospholipids may interact with milk proteins even more readily, forming complexes at ambient temperature. However, not much attention has been directed to the topic, and the mechanisms involved are still not understood in detail. Egg lysolecithin and the synthetic lyso-PC C_{14}, C_{16}, and C_{18} were reported to form stable complexes with α_{S1}-CN at ambient temperatures, whereas unhydrolyzed egg lecithin did not interact (Barratt et al., 1974). Also, β-LG has been observed in complexes with lyso-PC in simple model systems, in which the complexes showed an increased ability to stabilize foams (Sarker, 1995). Lysophospholipids formed in hydrolysis may interact with CN or β-LG or both of these in a similar manner in the cheese curd, which could explain their increased retention.

Cheese Yield

The total cheese yield was increased by 3.2% in the phospholipase vats compared with the control vats (Table 1). A tendency toward a slightly higher yield was already seen in the fresh curd after whey drainage, but first became significant ($P < 0.05$) in the finished cheeses. The increase was partly a consequence of the higher moisture retention in the cheese curd; however, total DM yield was also improved by 1.45%. The higher DM yield was primarily due to higher fat retention, consistent with the trends that were seen toward reduced fat contents in the whey and cooking water. Although a small increase in protein retention in curds was also observed, this was not statistically significant. Similar yield improvements of 0.7 to 3.2% have been obtained in industrial-scale trials, depending on the recipe and technology used. The large-scale trials also showed that the moisture-adjusted yield was significantly increased (Høier and Lilbæk, 2006).

Several authors have reported increased cheese yields on enrichment of cheese milk with phospholipids from buttermilk, soy, or oats (Mistry et al., 1996; Drake et al., 1998; Govindasamy-Lucey et al., 2006). In most cases, the yield increase was ascribed to higher moisture retention, whereas the DM yield was less affected. In the case of added buttermilk solids, incorporation of denatured whey protein into the buttermilk concentrate was found to improve the protein yield slightly, whereas fat retention decreased because of poor retention of the added buttermilk phospholipids in the cheese curd (Govindasamy-Lucey et al., 2006). Only in the case of soy lecithin addition to full-fat Cheddar was a slight improvement observed in fat retention (Hicks et al., 1985). Irrespective of the origin of added phospholipids, the moisture content of the cheese seems to correlate with the phospholipid concentration in the curds. In our study, we also found a similar relationship between phospholipid concentration and moisture retention. The increased moisture content of the cheese may be linked to the ability of lysophospholipids to reduce syneresis, either via their intrinsic ability to bind water, or indirectly by influencing the water-holding capacity of the

protein network. Jendrasiak and Hasty (1974) have reported that the intrinsic water-binding capacity of phospholipids is increased through enzymatic hydrolysis: In particular, egg lyso-PC and lyso-PS have been reported to bind more water than their diacyl counterparts at full hydration. In aqueous environments, unmodified milk phospholipids spontaneously form lamellar phases such as vesicles and bilayers found on the fat globule membrane (Waninge et al., 2003). However, lysophospholipids have a more conical molecule shape and preferably form micellar structures rather than bilayers, and they are also found to a greater extent as unimers in solution because of their higher critical aggregation concentration. Thus, it is expected that, at least to certain extent, lysophospholipids are released from the fat globule membranes on hydrolysis and may act as emulsifiers in the cheese curd. The unimeric or micellar lysophospholipids have a higher dynamic surface activity compared with unmodified phospholipids and may more rapidly interact with hydrophobic surfaces, such as hydrophobic patches on proteins or triglycerides. The release of lysophospholipids from the fat globule surface on hydrolysis may thus also be an important factor in the yield increase mechanism, because surface-active material present in the serum phase can help to emulsify water and fat during heating of the curd before whey drainage and during stretching in hot water.

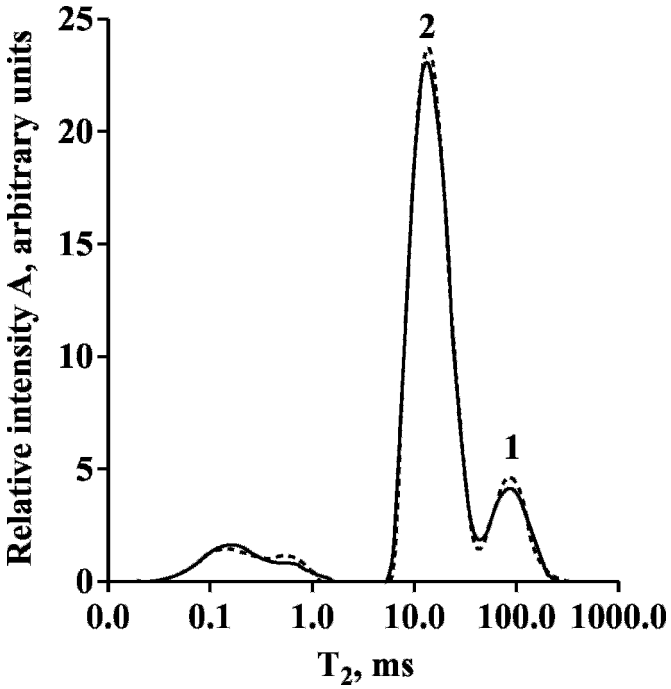

Figure 2. A continuous spin–spin relaxation time (T_2) distribution of fresh Mozzarella cheese curd, measured by low-resolution ^1H nuclear magnetic resonance. Solid line = control cheese; dotted line = cheese made from phospholipase-treated milk.

Water Binding

The water present in complex biological matrices such as foods may exist in an infinite number of states, with T_2 relaxation times ranging from tens of microseconds to hundreds of milliseconds (Ruan and Chen, 1998). The less mobile the water is, the shorter is the relaxation time, because the protons have less freedom of movement and the signal decay after a magnetic pulse is faster. The relative amounts of different states of water in a given matrix can be presented as a continuous T_2 distribution, where peaks represent fractions with different degrees of mobility. Frequency distributions of T_2 in fresh, unsalted Mozzarella curds were dominated by 2 peaks with average T_2 of 86 to 89 ms ($T_{2,1}$) and 14 ms ($T_{2,2}$) (Figure 2). Additionally, 1 to 2 low-intensity peaks were observed at shorter relaxation times in the range of 0.01 to 0.1 ms. It should be noted that each of the above relaxation times represents an average value for a group of water molecules with slightly different mobilities. The T_2 frequency distributions of phospholipase and control curds were similar before salting and stretching (Table 3). However, the stretching step affected water binding in the phospholipase and control cheeses to different extents, and consequently, the young cheeses analyzed a few hours after manufacture showed significantly different T_2 distributions. In all cheeses, stretching and salting caused a reduction of the mobilities of both dominating water fractions. On the other hand, a change occurred in the relative amounts of different water states, and a larger fraction of water was found in the high-mobility state immediately after manufacture. At this stage, phospholipase cheese showed significantly lower mobility of the $T_{2,1}$ fraction compared with the control, suggesting that the water-holding capacity was increased by phospholipase. The result is supported by the moisture yield data and underlines the role of the stretching step in the yield increase caused by phospholipase.

During 3 wk of storage after manufacture, the frequency distributions showed a development toward higher amounts of less mobile water: The relative intensity of the less mobile peak 2 increased, whereas the more mobile peak 1 decreased to a constant level after 10 d (Figure 3). Despite the higher water content in experimental cheeses, the intensities of the peaks were not significantly different from those of the control cheese. This was not surprising, considering the small difference in moisture contents. In all cheeses, both $T_{2,1}$ and $T_{2,2}$ relaxation times increased between 5 and 21 d of storage (Figure 4).

After 5 d, phospholipase cheeses showed significantly increased mobility of both dominating fractions com-

Table 3. Spin–spin relaxation times (T_2) and the corresponding relative peak intensities (A) of the 2 dominating states in fresh, unsalted Mozzarella curd and in the young cheese a few hours after manufacture

	Before stretch		After stretch		
Item	Control	PL[1]	Control	PL	SEM[2]
Relaxation time $T_{2,1}$, ms	86.18[a]	88.89[a]	56.33[b]	47.89[c]	2.66
Relaxation time $T_{2,2}$, ms	13.98[a]	14.45[a]	12.55[b]	13.60[b]	0.36
Peak intensity A_1	4.50[a]	4.44[a]	6.25[b]	7.10[c]	0.34
Peak intensity A_2	23.70[a]	24.85[a]	12.37[b]	9.55[c]	0.55

[a–c]Means in row with different superscripts differ ($P < 0.05$).

[1]PL = Phospholipase.

[2]Pooled standard error of the mean.

pared with the control. This could be interpreted as an accelerated reorganization of the cheese structure, possibly as an indirect effect of the higher moisture content.

Our results support the findings of Kuo et al. (2001), who reported that water in young *pasta filata* Mozzarella cheese existed in 2 dominating states. A decrease in the amount of higher mobility water and a simultaneous increase in lower mobility water during the first 10 d after manufacture were observed, similar to our results, and were interpreted as an increase in hydration of the CN network and a decrease in the amount of pore water. Thus, we assigned the $T_{2,1}$ peak to the high-mobility water present in whey pockets of the cheese and the $T_{2,2}$ peak to the moisture associated with the CN matrix. We also expected to detect a signal from liquid fat, because our measurements were performed at 20°C, where a considerable fraction of the milk triglycerides are in liquid state in the cheese. Liquid fat has a T_2 on the order of 100 ms, detectable by the CPMG sequence applied in the current study. We suggest that the signal overlaps with the signal of mobile water in the whey pores, and consequently, the $T_{2,1}$ peak is composed of these 2 contributions. The temperature dependence of the $T_{2,1}$ peak

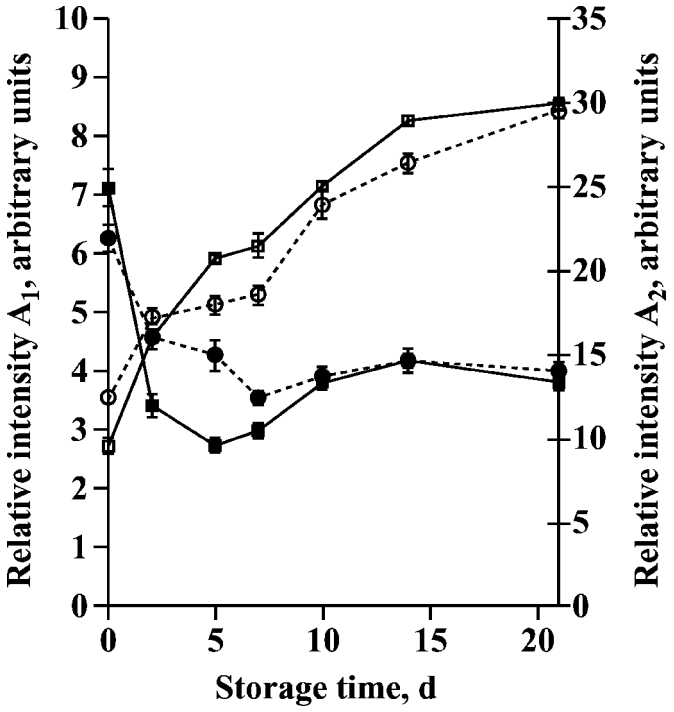

Figure 3. Development of the peak intensity (A) of the 2 dominating peaks in semi-skim Mozzarella cheese at 3 wk after manufacture. ● = A_1 of control cheese; ■ = A_1 of phospholipase (PL) cheese; ○ = A_2 of control cheese; □ = A_2 of PL cheese. The error bars represent standard errors of the mean for 3 to 5 replicates.

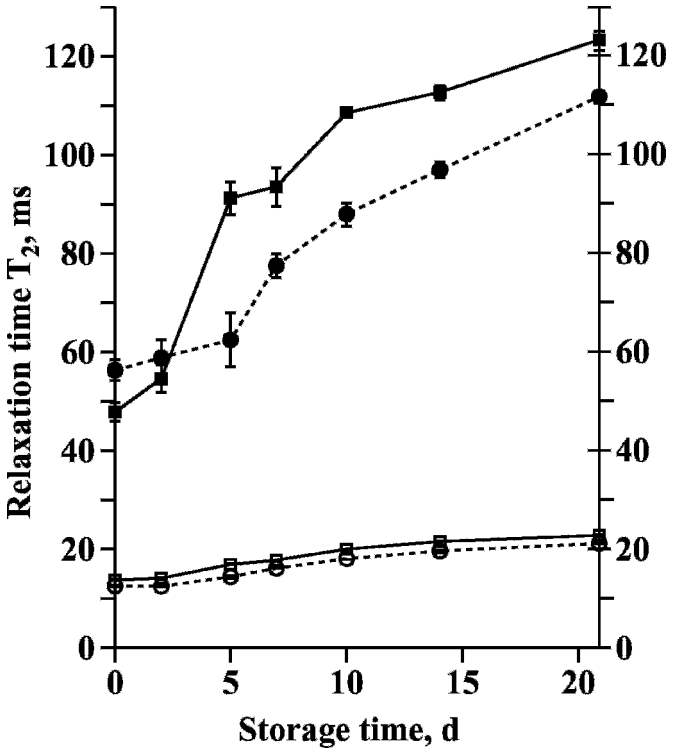

Figure 4. Development of T_2 relaxation times of the 2 dominating peaks in semi-skim Mozzarella cheese at 3 wk after manufacture. ● = $T_{2,1}$ of control cheese; ■ = $T_{2,1}$ of phospholipase (PL) cheese; ○ = $T_{2,2}$ of control cheese; □ = $T_{2,2}$ of PL cheese. The error bars represent standard errors of the mean for 3 to 5 replicates.

intensity supports the suggested double assignment, because the peak was absent from frozen samples (–23°C) but increased in intensity and relaxation time when the temperature was raised from +2 to +40°C, an interval at which milk fat melting takes place. Hubbard et al. (2005) reported a fat-related signal at T_2 relaxation times similar to the high-mobility water peak in Mozzarella cheese. The signals could be separated based on their diffusion coefficients by using a 2-dimensional Laplace inversion NMR technique.

Cheese Microstructure

Fresh Cheese Curd Before Salting and Stretching. The fresh cheese curd was characterized by an open, spongy protein network structure, as seen in Figure 5A and 5E. The curd granule junctions were visible as smooth sections of the otherwise porous matrix. Fat globules were incorporated into the protein matrix in different ways: as single globules, as clusters of globules, and as coalesced pools of fat, which were also observed to line some of the larger pores in the protein network. The distribution of fat varied considerably within a single vat, complicating the comparison of experimental and control cheeses. Image analysis of the fat globule distribution did not show significant differences between the experimental and control cheeses with respect to the apparent size of the fat globule clusters and coalesced pools of fat, or with respect to their relative amounts (data not shown). Both of these measurements exhibited a large standard deviation, expressing the natural inhomogeneity of cheese microstructure. The results showing that fat globule aggregation or coalescence is unaffected by phospholipase seem surprising, considering the extent of modification of the membrane stabilizing the fat globules. However, supportive data were obtained by static light-scattering measurement of unhomogenized whole milk, showing that the particle size distribution and the apparent average diameter $D_{[4,3]}$ of the fat globules (3.54 ± 0.05 μm) were not affected by incubation with phospholipase. It thus seems that modification of the fat globule surface phospholipids by phospholipase A_1 does not induce destabilization of the fat globules. This probably depends on enzyme specificity: Shimizu et al. (1980) previously reported coalescence of milk fat globules by microbial phospholipase C. Image analysis of the protein network of the fresh curd did not show significant differences in the relative area covered by protein or the coarseness of the network structure. In summary, the fresh phospholipase cheese curds could not be distinguished from the controls, with respect to either the fat or the protein network microstructure, by using CLSM.

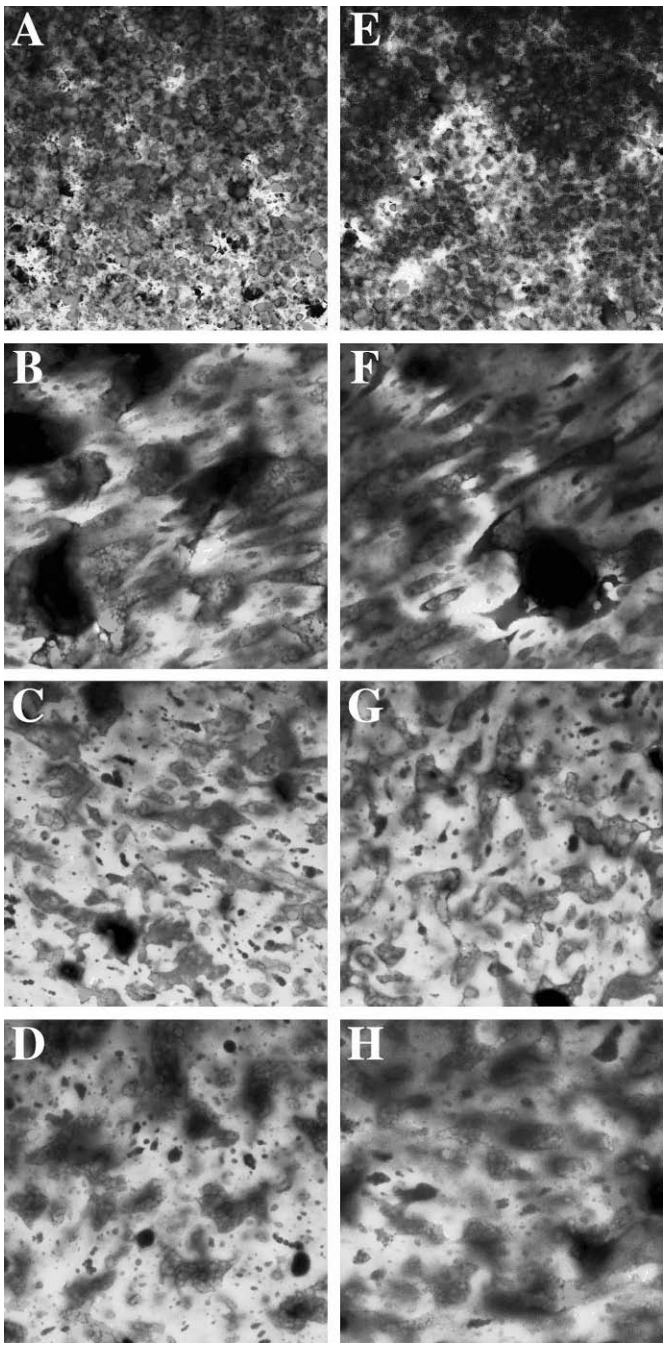

Figure 5. Development of the microstructure in semi-skim Mozzarella cheese at 4 wk after manufacture. A to D: control cheese; E to H: cheese made from phospholipase-treated milk. A, E: fresh, unsalted cheese curd prior to stretching; B, F: after 3 d; C, G: at 14 d; D, H: at 28 d. The image size is 240 × 240 μm.

Development of Microstructure During Storage. The microstructure of the cheeses was investigated by confocal microscopy on 4 occasions during storage up to 28 d. As expected, stretching of the curd profoundly changed the microstructure: The protein network was

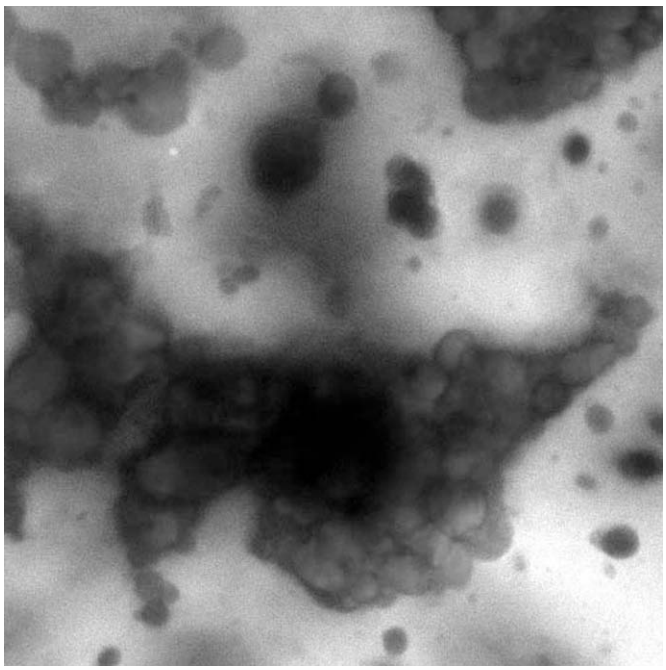

Figure 6. Fat globules in semi-skim Mozzarella cheese prepared with phospholipase at 28 d after manufacture. The image size is 60 × 60 μm.

reorganized into a filamentous structure, and whey channels and fat globule clusters were arranged along the protein fibers. Individual fat globules were also observed residing within the protein matrix (Figure 5B and 5F). The clustered fat globules showed a moderate degree of partial coalescence, and the individual fat globules were distinguishable in all samples. During the 3-wk storage time covered by the current study, the structure of the protein network grew tighter and appeared smoother as storage time increased. Whey channels diminished in size, indicating swelling of the CN and binding of the moisture to the protein structure (Figure 5C, D and 5G, H). The aforementioned changes in the microstructure of Mozzarella cheese in the first weeks after manufacture were first observed by McMahon (1995) by using scanning electron microscopy, and Auty et al. (2001) described a similar development by using CLSM. The phospholipase cheese showed characteristic development of the microstructure comparable to the control Mozzarella cheese in all aspects. The fat globules in the curd retained a similar appearance throughout the entire period of the study, and individual fat globules could still be clearly distinguished within the fat clusters in 3-wk-old cheeses (Figure 6).

Functionality

Low-moisture Mozzarella such as the one manufactured in the current study is mostly used as an ingredient in food, for instance in pizza, where its most important functionality is the melting behavior. We evaluated the flowability, stretchability, and browning of the phospholipase cheeses 1 wk after manufacture. The phospholipase and control cheeses showed a similar extent of melting (13.4 ± 0.4 cm and 12.8 ± 0.1 cm in length, respectively) and all cheeses could be stretched to over 30 cm for at least 6 s, which had been set as the criterion for acceptable stretchability. The 2 cheeses were also indistinguishable with respect to browning. In summary, despite the higher moisture content in phospholipase cheese, the functional properties were not affected. We also studied the texture of the cheeses by uniaxial compression 4 wk after manufacture, and again found no significant differences between the cheeses (results not shown).

CONCLUSIONS

We conclude that the hydrolysis of milk phospholipids with phospholipase A_1 prior to renneting significantly increased the cheese yield through better moisture and fat retention during whey drainage and stretching. The fungal phospholipase A_1 from *F. venenatum* hydrolyzed the major milk phospholipids PE and PC, but exhibited no activity toward SM. Lysophospholipids were retained in the curds in higher amounts compared to native phospholipids, possibly because of interaction with CN and subsequent incorporation into the cheese matrix. We propose that the observed yield improvement is a result of improved emulsification and water-holding capacity as a consequence of the lysophospholipids present in the curd. We found no significant differences in the cheese microstructure during processing or during the first weeks of storage, nor any differences in functionality (melting, stretching, and browning), indicating that these factors were largely unaffected by phospholipid hydrolysis. Despite the enzymatic modification of the fat globule membrane, the fat globules retained their original size and appearance. Because yield improvement is the prime issue for industrial-scale cheese production, the applied novel enzyme technology was evaluated as a valuable method for increasing cheese yield without adversely affecting the product quality. Further yield improvements might be obtained by combining the use of phospholipase with enrichment of cheese milk with buttermilk phospholipids to increase the amount of lysophospholipids in the curd. This could facilitate not only further improvement in fat retention, but also higher protein retention. The interaction of lysophospholipids with CN and the surface activity of the lysophospholipid–protein complexes should be studied in more detail to understand the mechanism leading to yield improvements.

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