JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Modification of Milk and Whey Surface Properties by Enzymatic Hydrolysis of Milk Phospholipids

HANNA M. LILBÆK,*,†,‡ TINE M. FATUM,‡ RICHARD IPSEN,† AND NIELS KRISTIAN SØRENSEN§

Institute of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK- Frederiksberg, Novozymes A/S, Laurentsvej 51-53, DK-2880 Bagsværd, and Chr Hansen A/S, Bøge Allé 10-12, DK-Hørsholm, Denmark

Phospholipase A1 were shown to improve foaming properties of skim milk and whey, implying that phospholipases can be useful tools for modifying the functionality of dairy products and ingredients. The ability of three fungal phospholipases and porcine pancreatic phospholipase A2 to hydrolyze milk phospholipids was investigated by using sodium deoxycholate-solubilized milk phospholipid and whole milk. The enzyme with the highest activity in milk was *Fusarium venenatum* phospholipase A1. Milk and whey were subsequently characterized using tensiometry and interfacial shear rheology. The lysophospholipids released from the fat globule membrane decreased the surface tension of skim milk and whey. A dramatic decrease in the surface shear viscous and elastic moduli indicated a shift from a protein-dominated to a surfactant-dominated interface. The surface shear moduli did not correlate with the foam stability, which was improved by phospholipase A1.

KEYWORDS: Phospholipase; milk; whey; surface tension; surface shear rheology; foaming; air−water interface

INTRODUCTION

Phospholipids are natural emulsifiers, found within the biological membranes of all living organisms and contributing to the properties of many food materials due to their surface-active character. Commercially used phospholipid emulsifiers are mainly obtained as a byproduct from vegetable oil refining and are widely used as an additive in various processed foods to stabilize emulsions alone or together with proteins (*1*). Phospholipids can be modified enzymatically using phospholipases to yield lysophospholipids, which are more water-soluble and provide improved functionality in some applications, e.g., in margarines and heat-treated emulsions (*2*, *3*). Phospholipases can also be employed for modification of phospholipids directly in different food materials during processing, to improve the stability of emulsions (*4*−*6*) or to reduce or replace emulsifiers and chemical stabilizers (*7*) due to enzymatic lysophospholipid formation in situ. The industrially relevant enzymes mostly belong to the phospholipase A1 (PLA1) [E.C.3.1.1.32] or phospholipase A2 (PLA2) [E.C.3.1.1.4] classes, according to the specific cleavage of the ester bond at the sn-1 or sn-2 position of phospholipids, respectively. Both PLA1 and PLA2 catalyze the hydrolysis of a diacyl glycerophospholipid to a lysophospholipid and a free fatty acid (FFA). Porcine pancreatic PLA2 (PpPLA2) has been employed in industrial applications for decades (*7*, *8*) and has been characterized in most detail (*8*−*11*). Recently, fungal enzymes with PLA1 and PLA2 activity have been cloned and are now commercially available (*12*−*16*), but the fungal PLA2s and in particular PLA1s have not been extensively characterized (*8*).

Milk contains approximately 0.01−0.03% (w/w) phospholipids, of which phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SPH) together make up 80−90% (*17*−*19*). The rest consists of phosphatidylserine (PS) and phosphatidylinositol (PI). Despite the modest concentration, the phospholipids have a critical role in stabilizing milk fat globules against coalescence. They are major constituents of the complex milk fat globule membrane (MFGM) but are also found in milk serum, in particular in processed milks due to release of MFGM from the fat globules by mechanical treatments such as pumping and homogenization (*20*). The serum phospholipids form vesicular structures, which have been isolated from the serum phase of heat-treated cream (*21*).

Recently, PLA1 has been introduced as a potential processing aid in cheesemaking (*22*), and a significant increase of cheese yield on a pilot as well as on an industrial scale was reported, when milk was hydrolyzed by *Fusarium venenatum* PLA1 (FvPLA1) prior to rennet coagulation (*23*, *24*). The yield increase was due to improved fat and moisture retention and was related to increased lysophospholipid retention in cheese curds, suggesting interactions of lysophospholipids with the protein matrix. Havn and co-workers (*25*) observed increased

* To whom correspondence should be addressed. Tel: (+45)2674 1198. Fax: (+45)4498 0610. E-mail: hnmt@novozymes.com.
† University of Copenhagen.
‡ Novozymes A/S.
§ Chr Hansen A/S.

10.1021/jf062705b CCC: $37.00
Published on Web 03/21/2007

Table 1. Enzyme Preparations Used in the Study

origin	specificity	abbreviation	activity (U/mL)	protein content (mg/mL)
A. oryzae	A2	AoPLA2	995	3.3
F. venenatum	A1	FvPLA1	6040	12.3
porcine pancreas	A2	PpPLA2	10030	27.4
T. borchii	A2	TbPLA2	5810	4.7

thermal stability and improved foaming of a phospholipid-containing whey protein concentrate solution after treatment with various phospholipases, suggesting a broader potential for phospholipases in dairy applications. However, at present, few published data are available on the ability of different phospholipases to hydrolyze milk phospholipids. The literature has mostly been concerned with phospholipase C (PLC) from psychrotropic spoilage bacteria (*26*−*28*), which catalyzes a different reaction from PLA1 and PLA2, as the cleavage occurs at the phosphate ester bond at the sn-3 position.

We have investigated the ability of four phospholipases (FvPLA1, two fungal PLA2s, and PpPLA2) to hydrolyze milk phospholipids and evaluated the usefulness of a titration assay to predict activity in situ in milk. To gain understanding on how phospholipases act to alter the functionality of milk-based ingredients, we further investigated the effect of a selected phospholipase on the physical characteristics of milk. In particular, we have focused on the air−water interfacial properties, which are closely related to the foam formation and stability. The ability of solutions to form foams has been related to the rate of lowering of surface tension, while the stabilization of foams against coalescence depends on the properties of the absorbed layer at the air−water interface (*29*). We compared the surface tensions of phospholipase-treated milk and whey and used surface shear rheological measurements to characterize the mechanical properties of the air−skim milk and air−whey interfaces. The results are discussed in relation to the foaming properties of the samples.

MATERIALS AND METHODS

Materials. Purified PpPLA2 (Lecitase 10L), FvPLA1 (YieldMAX PL), and the experimental enzymes *Aspergillus oryzae* PLA2 (AoPLA2) and *Tuber borchii* PLA2 (TbPLA2) expressed in *A. oryzae* (*16*) were from Novozymes A/S (Bagsværd, Denmark). Activities and protein concentrations of the enzyme preparations are presented in **Table 1**. One unit of phospholipase activity was defined as 1 μmol/min fatty acids released at 40 °C, pH 8.0, using soybean phospholipid substrate (72.8% w/w phospholipids, Sigma, St. Louis, MO). The activities were determined using a PpPLA2 standard with a defined activity. Unhomogenized whole milk was purchased from the local supermarket. Raw milk was from Arla Foods Brabrand Dairy (Brabrand, Denmark). Sodium azide was purchased from Sigma. Milk phospholipid concentrate (71.6% w/w phospholipids) was kindly donated by Arla Foods Ingredients (Nr. Vium, Denmark). Fatty acid free milk phospholipid hydrolysate was prepared by hydrolyzing milk phospholipid concentrate with PpPLA2 and separating the phospholipids by preparative chromatography using unmodified silica column. The hydrolysate was applied onto the column dissolved in hexane. After elution of the column with hexane, ethylene chloride, and acetone:hexane:acetic acid: triethylamine (897:70:18:15) to remove FFA and other minor lipid components, the lysophospholipids were eluted with methanol.

Degree of Hydrolysis (DH). Milk was preheated to 35 °C for 15 min and was incuated with varying enzyme dosages for 30 min. Each enzyme concentration, including control without enzyme, was prepared in duplicate. The reaction was stopped by addition of the extraction solvent chloroform:methanol (2:1). The samples were extracted twice, and the combined extracts were evaporated to dryness in a rotary vacuum evaporator, dissolved in chloroform, and applied on Bond-Elut NH2 solid-phase extraction columns (Varian, Lake Forest, CA). The columns were washed with hexane, chloroform:2-propanol (2:1 v/v), and 2% acetic acid in ether, to remove neutral fat and FFA, respectively. Phospholipids were eluted with methanol, dried under a stream of nitrogen, and dissolved in 2:1 chloroform:methanol. Subsequently, the sample was analyzed by high-performance liquid chromatography according to the method described by Sas et al. (*30*) with minor modifications. Quantification was performed using standard curves, which were prepared using pure egg L-α-PC, 1-palmitoyl-2-oleoyl-PE, 1-palmitoyl-2-hydroxy-PC (lysophosphatidylcholine, LPC), 1-palmitoyl-2-hydroxy-PE (lysophosphatidyl ethanolamine, LPE), and milk SPH from Avanti Polar Lipids, Inc. (Alabaster, AL). The DH of vesicular milk phospholipids was determined according to the same procedure. Milk phospholipids (200 μg/g) were suspended in 20 mM sodium phosphate, pH 6.8, and sonicated until suspension was clear, before addition of 6.3 mM $CaCl_2$.

Hydrolytic Rates. The rate of milk and soy phospholipid hydrolysis was determined by titration of FFA at 40 °C, pH 8.0, using a VIT90 Video Titrator (Radiometer, Copenhagen, Denmark). Enzyme samples were diluted in demineralized water. Twenty-five milliliters of the prewarmed substrate solution, containing 15 g/L phospholipid concentrate, 3.1 mM sodium deoxycholate, and 6.3 mM $CaCl_2$, was adjusted to pH 8.0 by addition of 0.1 M NaOH. The reaction was started by adding 1 mL of enzyme solution into the substrate solution, and the release of FFA was followed during 2 min by the addition of 0.1 M NaOH. Each sample was measured in duplicate. The amount of FFA released per minute was calculated based on the NaOH consumption, using the linear part of the titration curve.

Particle Size Distribution. The particle size distribution in milk samples was determined by Malvern Mastersizer Microplus (Malvern Instruments Ltd., Malvern, United Kingdom). Particle refractive index 1.4700 (real part), 0.0100 (imaginary part), and dispersant refractive index 1.33 were used. The data were fitted to a polydisperse model, and the residual was under 2%. Each sample was measured in four replicates.

Isolation of MFGM. MFGM was isolated from freshly collected unpasteurized bovine milk according to the procedure described by Hvarregaard et al. (*31*). The MFGM isolate was suspended in 50 mM NH_4HCO_3, pH 7.2, and the protein composition was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with periodic acid−Schiff reagent (PAS) and Coomassie Blue according to standard procedures.

Preparation of Skim Milk Samples. Unhomogenized, pasteurized (74 °C, 15 s) whole milk was prewarmed to 35 °C in a water bath for 15 min. Sodium azide (0.1%) was added to prevent microbial growth. FvPLA1 (50 U/g milkfat) was added, and the milk was incubated with gentle stirring at 35 °C for 30 min, corresponding to conditions allowing maximum DH, and subsequently cooled to 4 °C. Fat was separated by centrifugation (3000*g*, 20 min, 4 °C), and the skim milk was filtered through glass wool and a paper filter (Whatman, Brentford, United Kingdom). The samples were tempered to 22 °C, and the surface tension, surface shear rheological parameters, and foaming were analyzed within 24 h after preparation.

Preparation of Whey Samples. Whey was obtained from pilot scale manufacture of low moisture part skim Mozzarella cheese (Chr. Hansen A/S, Hørsholm, Denmark). The manufacturing process is described in detail elsewhere (*23*). Four 150 L cheese vats were prepared from semiskim milk: two control vats and two vats with FvPLA1, using the dosage 5 U/g milkfat. The whey was passed through a fine sieve and frozen immediately after collection. Upon defrosting, sodium azide (0.1%) was added to prevent microbial activity. The samples were centrifuged to remove fine precipitate (3000*g*, 20 min, 4 °C), and the supernatant was filtered through glass wool and a paper filter (Whatman). The samples were tempered to 22 °C, and the surface tension, surface shear rheological parameters, and foaming were analyzed within 24 h after preparation.

Compositional Analysis. The protein content of skim milk was calculated based on the total nitrogen content, determined by LECO FP-528 nitrogen analyzer (Leco Corp., St. Joseph, MI), using factor $6.38 \times N$. Each sample was analyzed in eight replicates. The fat content

of skim milk and whey and the protein content of whey were determined by MilkoScanTM FT-120 (Foss Electric A/S, Hillerød, Denmark), calibrated using relevant International Dairy Federation reference methods (IDF 20 B,1D and 21B).

Phospholipid Analysis. The phospholipid content of milk and soy phospholipids and whey was analyzed by 31P-nuclear magnetic resonance (NMR) after extraction of the freeze-dried samples with 1:1 chloroform:methanol. The dried extracts were dispersed in 2 mL of 1:2 methanol:$CDCl_3$ and were shaken shortly with Cs-EDTA (0.2 M, pH 7.5). The separated organic phase was analyzed as described by Meneses and Glonek (*32*). Triphenylphosphate was used as an internal standard. The analysis was performed by Spectral Service GmbH (Cologne, Germany).

Surface Tension. The surface tension of skim milk and whey was measured using a Sigma 703 tensiometer (KSV Instruments Ltd., Helsinki, Finland) equipped with a Wilhelmy plate, which was cleaned by heating it in a flame until glowing prior to each measurement. Changes in surface tension were measured in a thermostated room at 22 °C for 120 min. Milk hydrolysis was performed on five independent days, and each sample was measured in duplicate. Whey from each cheese vat was measured in duplicate.

Surface Shear Rheology. A Camtel CIR-100 interfacial rheometer (Camtel Ltd., Royston, United Kingdom) was used for measuring surface shear viscous modulus (η) and surface shear elastic modulus (E) of the skim milk−air and whey−air interfaces. A 15 mL aliquot of skim milk or whey was dispensed in a 43 mm sample dish, and a 13 mm platinum De Noüy ring, cleaned by heating it in a flame until glowing prior to each measurement, was placed at the air−water surface. Prior to each measurement, a reference curve with demineralized water was recorded for 20 min, employing an oscillation frequency of 5 Hz and a strain amplitude of 2000 μrad. E and η were monitored for 12 h at 22 °C employing the same frequency and strain amplitude. Each measurement was performed in duplicate.

Foaming. Foaming was investigated using the conductometric method described by Clark et al. (*33*). In brief, 2 mL of sample solution was transferred into a jacketed glass vessel with a diameter of 0.85 mm, and foam was produced by sparging prehumidified nitrogen through the sample via a microscopic orifice in the bottom of the vessel. Conductivity was measured across two platinum electrodes situated in the inner walls of the vessel, 3.5 cm above the orifice, using a conductivity meter. Once the foam had reached the predetermined height of 5 cm, the nitrogen flow was turned off and the conductivity was measured for 10 min. The measurements were made at 22 °C and performed in triplicate. The initial conductivity was used as a measure of foamability. The foam stability was determined as the ratio of conductivity after 10 min relative to initial conductivity.

RESULTS AND DISCUSSION

Hydrolysis of Milk Phospholipids. When added to unhomogenized whole milk, each of the tested phospholipases transformed PE and PC to LPE and LPC, respectively, while none exhibited any activity toward SPH. Thus, the enzymes appeared to be specific to glycerophospholipids and the unability to hydrolyze SPH can be attributed to its sphingosine backbone. The glycerophospholipids PI and PS were hydrolyzed to some degree, but because of their low amounts in milk, the extent of hydrolysis could not be quantified. FvPLA1 hydrolyzed PE and PC to higher degree than the other phospholipases: 69 ± 0.5 and 67 ± 1% of PE and PC were hydrolyzed, respectively (**Figure 1**). In contrast, AoPLA2 only hydrolyzed approximately 10 and 20% of milk PE and PC, respectively, independent of the enzyme dosage in the interval of 4−100 U/g milkfat. TbPLA2 and PpPLA2 showed intermediate DH in the tested concentration range: At the highest used enzyme concentrations, TbPLA2 hydrolyzed 51 ± 3% of PE and 59 ± 3% of PC, while PpPLA2 hydrolyzed 62 ± 5% of PE and 42 ± 1% of PC. The pancreatic enzyme PpPLA2 showed a clear polar headgroup preference to ethanolamine, while the fungal PLAs hydrolyzed

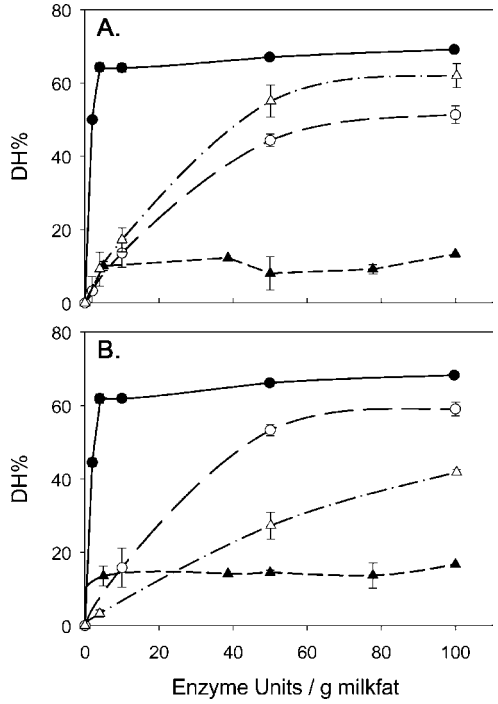

Figure 1. DH of phospholipids in whole milk. (**A**) PE and (**B**) PC. The error bars represent the standard error of the mean of two replicates. Closed spheres, FvPLA1; open spheres, TbPLA2; closed triangles, AoPLA2; and open triangles, PpPLA2.

milk PE and PC to similar extents. Mammalian secreted PLA2s have been found to preferentially hydrolyze PE in vesicles or mammalian membranes (*34*−*36*), while homologous secreted PLA2s from marine invertebrates (*34*) or snake venoms (*37*) preferably hydrolyzed PC. Hydrophilic group specificity of fungal phospholipases has not to our knowledge been reported in literature.

It is remarkable that none of the tested enzymes hydrolyzed 100% of the PE or PC present in milk. Several alternative explanations could be possible. It could be hypothesized that acyl chain selectivity of the enzymes plays a role, and PC and PE with specific fatty acids are left unhydrolyzed; however, in general, the secreted PLA2, including PpPLA2, shows little fatty acid discrimination (*9*). Alternatively, the phospholipids might be partially protected by the complex multilayer structure of the native MFGM. The MFGM contains a number of proteins that are attached to the lipid membrane in different ways, many of them heavily glycosylated (*38*). The proteins, glycosyl moieties, or other unhydrolyzable membrane components may pose a steric hindrance that leaves a part of the phospholipid inaccessible to the enzymes, even in case the lysophospholipids detach from the fat globule surface. Such a shielding effect of proteins has been illustrated by prevention of PLC-mediated hydrolysis of a dipalmitoyl PC monolayer by adsorbed α-lactalbumin (*39*). O'Mahony and Shipe (*27*) reported that PLC hydrolyzed approximately 60% of phospholipids in milk but 90% when phospholipids were suspended in buffer. We also investigated the DH of milk phospholipids in a sonicated suspension, at the phospholipid concentration and pH corresponding to whole milk. At enzyme dosages 0.35−17.6 U/mg phospholipid (corresponding approximately to 2−100 U/g milkfat), the DHs of PE and PC were in general higher as compared to the values reached in whole milk at corresponding enzyme dose (results not shown). These results may indicate that unhydrolyzable MFGM components protect phospholipids

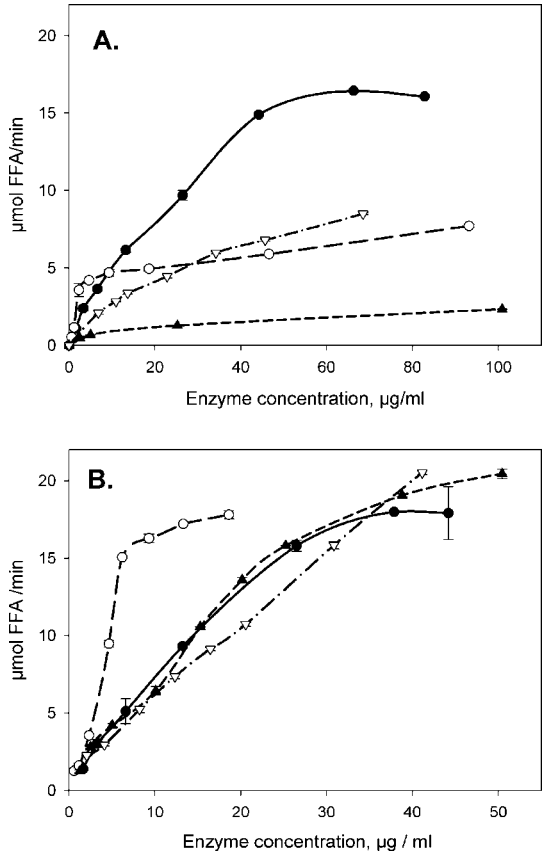

Figure 2. Hydrolytic rates of phospholipases measured by titration of FFA at pH 8.0. (**A**) Milk phospholipid substrate and (**B**) soy phospholipid substrate. The error bars represent the standard error of the mean of two replicates. Closed spheres, FvPLA1; open spheres, TbPLA2; closed triangles, AoPLA2; and open triangles, PpPLA2.

from hydrolysis by steric hindrance. However, it should be noted that the aggregation form of the substrate per se and the efficient surface concentration of hydrolyzable phospholipids also strongly influence interfacial kinetics of phospholipase reaction, and these factors are different in the native MFGM and in the suspended phospholipid vesicles (*40*).

The reaction rates obtained by titration of FFA using milk phospholipid substrate are presented in **Figure 2A**, as a function of the used enzyme concentration. By comparing the dose−response curves of the four tested enzymes, it was seen that at low enzyme concentrations (<9 μg/mL) TbPLA2 hydrolyzed milk phospholipids at highest rate. However, at higher enzyme concentrations, FvPLA1 showed significantly higher hydrolysis rates as compared to the other three enzymes, reaching a maximum rate of 16 μmol FFA/ min at 66 μg/mL. AoPLA2 hydrolyzed milk phospholipids at the lowest rate (3 μmol FFA/ min at 500 μg/mL), while TbPLA2 and PpPLA2 showed intermediate reaction rates in the tested concentration interval. The differences in hydrolytic rates were unlikely to be due to selectivity of the enzymes, as it was seen that all enzymes hydrolyzed PC and PE but not SM in milk and in milk phospholipid suspension. For comparison, hydrolytic rates were also determined using soybean phospholipids as substrate (**Figure 2B**). Interestingly, in this case, the rate vs enzyme concentration curves of AoPLA2, FvPLA1, and PpPLA2 reminded each other in the tested concentration interval. FvPLA1 showed similar rates in both substrates, while the other enzymes hydrolyzed soy phospholipids at higher rates as compared to milk phospholipids. Both soy and milk substrates were presented

Table 2. Composition of Milk Phospholipid and Soybean Phospholipid Used as Substrate in the Titration Assay

	content (mol %)			content (mol %)	
	soy	MFGM		soy	MFGM
PC	31.7	22.9	PI	18.7	7.9
LPC	1.0	0	PS	1.2	5.5
PE	28.6	28.0	PA	10.6	0
LPE	0.8	0	LPA	0.6	0
APE	2.8	0	PG	1.8	0
SPH	0	35.8	DPG	1.3	0

as mixed sodium deoxycholate bicelles (*40*), and thus, the difference in hydrolytic rates can be attributed to the phospholipid composition rather than physical aggregation form of the substrate. Soy phospholipids consisted mainly of PC and PE but also contained a considerable amount of phosphatidic acid (PA) and a higher concentration of PI as compared to milk phospholipids (**Table 2**). It is possible that the affinity of TbPLA2, AoPLA1, and PpPLA2 to the substrate was increased by the negatively charged PA and PI, while the affinity of FvPLA1 was independent of the presence of these phospholipids. It should also be noted that soybean phospholipids generally contain more unsaturated fatty acids, especially 18:2, as compared to milk phospholipids, so the possibility of fatty acid selectivity cannot be excluded. Further study of the interfacial kinetics of phospholipase hydrolysis of milk is necessary to explain the different hydrolytic rates of the enzymes in milk and soy phospholipid substrates.

When comparing the DH in milk with the hydrolytic rates determined by the titration assay (**Figures 1** and **2A**), it was apparent that the hydrolytic rates using milk phospholipid substrate could predict the ability of the phospholipases to hydrolyze phospholipids in situ in milk. The titration assay is much faster and less laborious as compared to the determination of DH in milk, and as such, it is more suited as a screening test for selection of enzymes suitable for dairy applications. The enzyme with the highest activity in milk, FvPLA1, was chosen for further experiments where the effect of hydrolysis on a number of milk properties was explored.

Stability of Fat Globules in Unhomogenized Whole Milk. Phospholipase activity affects the constituents of the MFGM, and this could have consequences for emulsion stability in milk. Microbial PLC has been shown to induce coalescence of fat globules in washed cream (*28*) but not in milk (*27*). However, enzymatic hydrolysis by any of the phospholipases used in the current study did not affect the physical appearance of whole milk as determined by the eye; that is, no aggregation, precipitation, or oil floatation ("oiling off") was observed. The particle size distribution in the FvPLA1-treated whole milk was identical to untreated whole milk. This was the case even after milk was acidified from 6.6 to 6.2 with lactic acid and stirred vigorously at 35 °C for 60 min to simulate cheese manufacturing processing conditions (results not shown). Clearly, the applied hydrolysis of MFGM phospholipids did not induce coalescence of fat globules in whole milk. This result was in accordance with our previously reported observations made using confocal laser scanning microscopy, showing unchanged fat globule size distribution in Mozzarella cheese curd manufactured from FvPLA1-treated milk (*23*).

It has been suggested that surface-active compounds formed as a result of the action of lipolytic enzymes on MFGM lipids can lead to replacement of the original MFGM from the fat globule surface (*20*). To investigate this, we isolated MFGM from untreated and hydrolyzed raw milks and studied the protein

Table 3. Phospholipid Content of Milk and Whey Samples[a]

	whole milk		skim milk		whey	
treatments	control	FvPLA1	control	FvPLA1	control	FvPLA1
n	2	2	2	2	6	6
total fat (g/L)	39.7 ± 0.3 a	40.1 ± 0.1 a	1.04 ± 0.10 b	1.30 ± 0.20 b	1.35 ± 0.04 b	1.25 ± 0.04 b
PC (μg/g)	61.6 ± 1.5 a	9.9 ± 1.1 b	35.3 ± 9.4 c	4.9 ± 0.5 d	39.2 ± 3.6 c	6.9 ± 0.1 b
LPC (μg/g)	ND	25.6 ± 3.4 a	ND	22.7 ± 2.3 a	ND	6.8 ± 0.1 b
PE (μg/g)	59.2 ± 0.5 a	14.6 ± 2.5 b	29.4 ± 5.3 c	8.21 ± 1.4 b	35.7 ± 2.9 c	ND
LPE (μg/g)	ND	20.7 ± 3.3 a	ND	23.6 ± 2.9 a	ND	ND

[a] Skim milk was separated after incubation of whole milk with 50 units FvPLA1/g milkfat. Whey originated from Mozzarella cheese manufacture. Semiskimmed cheese milk was hydrolyzed with 5 units FvPLA1/g milkfat. The values represent means of *n* replicates ± standard errors of the mean (values of *n* given in the table). Means in a row without common letters differ ($P < 0.05$). ND, none detected.

composition by native and reduced SDS-PAGE gels stained with Coomassie blue or PAS reagent (not shown). Incubation of raw milk at 35 °C for 45 min increased the casein content of the MFGM isolate, but the increase was similar in untreated and hydrolyzed milks. The protein compositions were also identical otherwise, indicating that hydrolysis of milk phospholipids did not lead to increased loss of MFGM proteins to serum (aqueous phase) or significant attachment of other milk proteins on the fat globules in raw milk. To summarize, despite the high extent to which the MFGM phospholipid constituents were hydrolyzed, the fat globules in hydrolyzed milk appeared to retain their integrity and the original membrane protein composition.

Distribution of Phospholipids and Lysophospholipids between Fat Globules and Skim Milk. In fresh raw milk, approximately 80% of the lipid phosphorus is in the fat fraction, and the rest is found in the aqueous serum (*20*). During storage and processing, increasing amounts are found in the skim milk, as MFGM becomes detached from the fat globules. In our experiment, the PE and PC content of skim milk separated from commercial unhomogenized whole milk with no enzyme treatment was approximately 50−60% of the content in the whole milk (**Table 3**). The higher amount of phospholipids in the aqueous phase as compared to raw milk is likely to be the consequence of milk cooling and processing at the dairy and the applied sample preparation procedure. The control milk samples were gently stirred at 35 °C for 45 min, similar to the enzyme-treated samples, and this procedure may have released some of the MFGM phospholipids. Upon enzymatic hydrolysis, a change in the partitioning of phospholipids between fat globule membrane and skim milk could be expected, due to the increased hydrophilicity of lysophospholipids as compared to diacylglycerolipids. The more conical shape of the lysophospholipid molecules as compared to the cylinder form of diacylglycerolipids makes them more likely to form nonlamellar structures and hence cause destabilization of membranes (*41*). In skim milk separated from FvPLA1-treated whole milk (50 U/g milkfat, 30 min of incubation at 35 °C), both LPC and LPE were present in amounts comparable with the levels detected in hydrolyzed whole milk, indicating that lysophospholipids to a very large extent were released from the fat globules.

Surface Properties. Foaming properties are closely related to the mixture of surface-active molecules that are present in the solution. In untreated skim milk and whey, casein and whey proteins are the dominating surface-active components, respectively. However, the hydrolysis of milk phospholipids by PLA1 or PLA2 results in the formation of small surface-active molecules, i.e., lysophospholipids and fatty acids. These can affect the foaming properties via changing the dynamic surface tension and altering the composition and mechanical properties of the adsorbed film at the air−water interface.

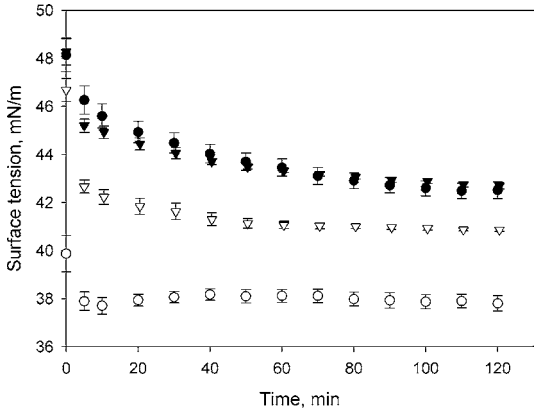

Figure 3. Surface tension of skim milk and whey measured by Wilhelmy plate method at 22 °C. The error bars show the standard error of the mean of 7–9 replicates. Closed spheres, untreated skim milk; open spheres, skim milk separated after FvPLA1 treatment; closed triangles, untreated whey from Mozzarella production; and open triangles, whey from Mozzarella production with FvPLA1.

The surface tension of untreated skim milk decreased from 48 to 43 mN/m during the 2 h measurement, along with adsorption of milk protein to the air−water interface (**Figure 3**). Whey from untreated vats showed a very similar behavior. Sodium azide, which was added to prevent microbial growth, did not affect the surface tension or other surface measurements. Hydrolysis of phospholipids by FvPLA1 caused the skim milk system to reach a stable equilibrium surface tension much more rapidly, and the value of the equilibrium surface tension was significantly lower as compared to the untreated skim milk (**Table 4**). Whey from cheese vats made using hydrolyzed milk decreased from 47 to 41 mN/m during the same period of time and reached equilibrium slightly slower as compared to hydrolyzed milk but more rapidly as compared to untreated whey. Thus, both in the skim milk and in the whey, phospholipase treatment resulted in a more rapid decrease of surface tension. The decrease of surface tension can be explained by the presence of lysophospholipids, which are able to decrease surface tension more rapidly and to lower values as compared to milk proteins. The slightly higher equilibrium surface tension of FvPLA1 whey as compared to the enzyme-treated skim milk and the slower equilibration suggest that these variables were dependent on the lysophospholipid concentration (**Table 3**). This was supported by the fact that addition of FFA-free milk phospholipid hydrolysate into the skim milk progressively decreased the surface tension in the concentration interval 26−200 μg lysophospholipids/g milk.

The mechanical properties of the air−water interfacial films were studied using interfacial shear rheology. In untreated skim

Table 4. Surface and Foaming Properties of Skim Milk and Whey[a]

	skim milk		whey	
treatments	control	FvPLA1	control	FvPLA1
equilibrium surface tension (mN/m)	42.5 ± 0.3 a	37.8 ± 0.3 b	42.7 ± 0.1 a	40.9 ± 0.1 c
E after 12 h (mN/m)	23.67 ± 0.46 a	0.02 ± 0.10 b	6.12 ± 0.02 c	–0.12 ± 0.14 b
η after 12 h (μN s/m)	117.6 ± 1.9 a	2.3 ± 0.4 b	76.0 ± 3.8 c	12.4 ± 0.6 d
initial conductivity (μS/cm)	450 ± 5 a	460 ± 6 a	no measurable foam	300 ± 14 b
foam stability after 10 min (%)	24.1 ± 1.9 a	48.6 ± 1.7 b	NA	4.0 ± 0.5 c

[a] Skim milk was separated after incubation with FvPLA1. Whey originated from Mozzarella cheese made from semiskimmed milk hydrolyzed with FvPLA1. The values after the sign ± represent standard errors of the mean. Means in a row without common letters differ ($P < 0.05$). NA, not applicable.

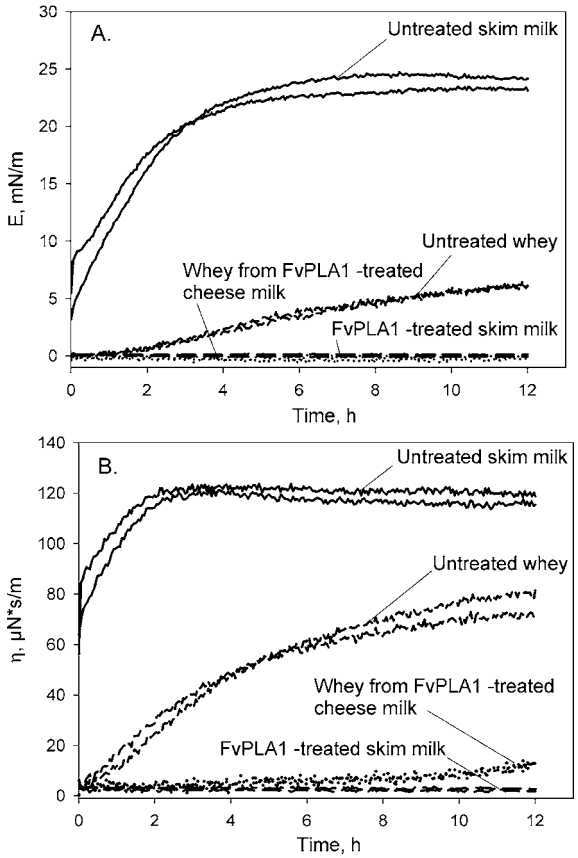

Figure 4. Surface shear rheology of skim milk and whey. Two curves for each sample are shown to illustrate repeatability. (**A**) Surface shear elastic modulus *E* vs time and (**B**) surface shear viscous modulus η vs time.

milk and whey, a surface film with measurable mechanical resistance to shear deformation developed during the 12 h measurement (**Figure 4**), concomitant with the adsorption of milk proteins from the bulk to the air−water interface. In skim milk, equilibrium values of shear elastic modulus *E* (24 mN/m) and viscous modulus η (120 μNs/m) were established much faster as compared to whey, probably due to higher bulk protein content (32.8 ± 1.0 g/L in skim milk and 8.6 ± 0.3 g/L in whey, respectively). Although the fat content of the samples was low (0.10 ± 0.02 and 0.13 ± 0.01% in skim milk and whey, respectively), the higher fat/protein ratio of whey may explain why the *E* and η remained lower than in skim milk during the entire measurement period. The effect of FvPLA1 treatment on the mechanical properties of the surface film was dramatic. In phospholipase-treated skim milk, the *E* and η remained virtually at zero during the 12 h measurement. In whey, the effect of phospholipase was very similar; however, η started to increase very slightly after 10 h of measurement.

These data clearly indicate that hydrolysis of milk by FvPLA1 brought about a shift in the characteristics of the air−water interface. The native skim milk and whey show characteristics of a typical protein-dominated system, developing an interfacial film with considerable mechanical strength to resist shear deformation. The mechanical strength of the air−water interfacial film depends on the inter-molecular cross-linking of the protein molecules at the interface. However, the presence of even small amounts of surfactants in a protein film can prevent protein−protein interactions and thus drastically weaken the strength of protein films (*42*, *43*). The lack of viscoelasticity in the air−water interfacial films of the hydrolyzed skim milk and whey samples strongly suggested that lysophospholipids and maybe also FFA (or their salts) were present in the film.

Lysophospholipids are most likely the primary surface-active component causing the change of surface characteristics of skim milk and whey, rather than the FFA. This is because of the higher aqueous solubility and thus higher bulk concentration of lysophospholipids as compared to the long-chained fatty acids. The latter are very sparingly soluble, even when they exist predominantly as salts in the pH of milk and whey (*20*). However, fatty acids may be solubilized to some extent in the aqueous medium in the presence of lysophospholipids and may have a modulating effect on the surface tension, decreasing it to a larger extent than lysophospholipids alone (*44*). In addition to "free" lysophospholipids and FFA, both of these may bind to milk proteins and form surface-active complexes, which also may compete for adsorption at the air−water interface. β-Lactoglobulin (β-lg) and bovine serum albumin are known to bind fatty acids in the hydrophophobic binding pocket (*45*, *46*), and binding of LPC with milk proteins has also been reported (*47*−*49*).

Foaming. Foaming of skim milk and whey was investigated by using microconductivity. This technique measures foam density, which depends on the quantity of liquid entrained in the foam lamellae and plateau borders (*50*). The drainage rate from the lamellae determines the foam stability. The decrease of foam conductivity with draining time is illustrated in **Figure 5**. The initial foam conductivity in skim milk was similar in the untreated and hydrolyzed samples; however, the foam formed in the hydrolyzed milk was significantly more stable as compared to the control (**Table 4**). The untreated whey sample did not form measurable foam even when nitrogen was passed through the liquid for extended periods of time. In contrast, the whey originating from FvPLA1-treated cheese milk could be foamed up and formed a transient type of foam that rapidly disappeared after gas flow was stopped. The poorer foaming properties of whey as compared to skim milk are likely to be related to the lower total protein content. Also, the higher fat/protein ratio may play a role, as fat content of whey protein concentrates has been found to negatively correlate with the foaming properties (*51*).

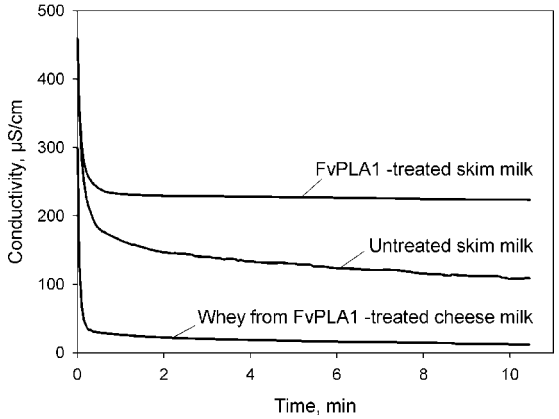

Figure 5. Foam stability of skim milk and whey, determined by conductivity of the foam during 10 min of drainage. The lines represent averages of 4–7 measurements. The coefficient of variation was <10% for the FvPLA1-treated skim milk sample and <30% for the whey and control skim milk samples. The control whey did not foam and is not shown.

Initial foam formation has been related to the dynamic surface tension, i.e., the rate of surface tension decrease (*29*). Although the surface tension decrease was faster after FvPLA1 treatment both in milk and in whey, only whey showed significantly increased initial foamability. This may be explained by the difference in time scales: The surface tensions were measured at time scales of minutes while the adsorption of surface active compounds onto nascent air bubbles occurs at time scales of milliseconds. Rouimi et al. (*52*) have reported that at very short time scales, milk proteins, especially caseins, adsorbed to air−water surface faster as compared to sucrose ester (a low molecular weight. This could possibly explain why additional surfactant (lysophospholipid) does not significantly affect the initial foam formation in skim milk. The protein composition, not merely the total content, may also be important. In skim milk, caseins contribute strongly to the initial foam formation, while whey proteins, especially β-lg, dominate in whey. Because of their more disordered and flexible structure as compared to the globular whey proteins, caseins adsorb faster to the surface of nascent air bubbles. This was illustrated by Zhang et al. (*53*), who showed that the protein adsorbed on air−water interfaces of foam made of mixed milk proteins was primarily casein and to lesser amount whey proteins. The adsorption of globular proteins requires partial unfolding of the tertiary structure (*53*). While the protein content and composition are most likely to explain the differences in the foaming behavior of whey and skim milk, the fat content, although this was low, could have some importance. It could be suggested that the lysophospholipids solubilize some of the lipids in micelles and in this way remove the lipid from the surface. This could possibly improve foaming in whey, which had a higher fat/protein ratio as compared to skim milk.

The foam stability strongly depends on the characteristics of the adsorbed surface layer. The coalescence stability of protein foams has been correlated to the mechanical strength of the adsorbed protein layer at the air−water interface (*54*). In contrast, small molecular weight surfactants stabilize foams by their high lateral mobility in the adsorbed film, which allows rapid equalization of surfactant concentration gradients in the film. These two mechanisms of foam stabilization are mutually incompatible, and consequently, mixed surfactant−protein systems often show reduced foam stability (*29*). However, charged surfactants, in particular lysophospholipids, have also been found to stabilize protein foams and emulsions. Sarker et al. (*47*) showed that egg lyso-PC could stabilize a β-lg foam when the molar ratio of LPC to β-lg was in the interval of 0.5−7. Larger relative amounts of LPC caused foam destabilization. The estimated R in whey from FvPLA1-treated milk was 0.06, when 50% of the whey protein is assumed to be β-lg (*55*). This was 1−2 orders of magnitude lower than the concentration ratios needed for stabilization in the reported model system, and thus, the low relative amount of lysophospholipids could explain the small observed effect on foam stability in whey. In hydrolyzed skim milk, the estimated ratio of LPC to β-lg was 0.2 (based on 12% β-lg in milk; *55*), but taking LPE into account, the total lysophospholipid:β-lg ratio was approximately 0.4. Interaction of LPE with proteins may contribute to stabilization in a similar way to LPC; however, to our knowledge, this has not yet been investigated. Sarker et al. showed binding of egg-LPC to β-lg in bulk solution and suggested that foam stabilization was related to complexation. Similarly, Wilde et al. (*56*) reported enhanced foaming properties of the wheat protein puroindoline in the presence of LPC and showed formation of a LPC−protein complex. Increased thermal stability of emulsions made with PLA2-hydrolyzed egg yolk has also been attributed to formation of surface-active lysophospholipid−protein complexes at the emulsion droplet interface (*57*, *58*). Also, not only β-lg but also caseins may interact with lysophospholipids, yielding surface-active complexes, which could play a role in stabilization of bubbles against coalescence (*48*).

The observed increased foam coalescence stability of the hydrolyzed skim milk, where a mixture of milk proteins, lysophospholipids, and fatty acids and probably also lipid−protein complexes were present, did not correlate with the reduced shear viscoelasticity of the mixed interfacial films of these samples. The foam stabilization thus seems to occur via a different mechanism than in pure protein films. Sarker et al. (*47*) reported that addition of egg-LPC ($R < 1$) decreased surface dilatational elasticity but increased the dilatational viscosity. Also, Fruhner et al. (*59*) found by using oscillating bubble technique at high frequencies that dilatational viscosity was necessary to form foams formed from surfactant solutions. However, these dilatational techniques are different from the shear rheology, which was used in the current study, as they measure a response to surface area change, while shear rheology measures directly the strain resulting from applied mechanical stress. Wustneck et al. (*60*) reported that the surface shear viscosity progressively decreased upon addition of ionic surfactants in β-lg and β-casein solutions, also in the region where adsorption of surface-active surfactant−protein complexes occurred. It could be hypothesized that in skim milk, casein initially adsorbs at the air bubble surface, but lysophospholipids and surface-active lysophospholipid−protein complexes become dominating rapidly after the foam is formed. Protein−protein interactions are prevented by the lysophospholipids and lipid−protein complexes, and foam stabilization thus occurs essentially via similar mechanism as for small molecular weight surfactants. The complex formation between milk proteins, lysophospholipids, and FFA and the mechanisms leading to foam stabilization need further investigation in a system where the concentrations of the different compounds can be controlled.

Our study shows that PLA1 radically changes the surface properties of milk through release of surface-active lysophospholipids from the MFGM and that the altered surface properties have an impact on the functionality of dairy foods in practice. The results indicate that phospholipase technology may offer an interesting new tool for tailoring dairy products with altered functionality, such as improved foam stability, and could

partially or fully replace emulsifiers in applications where these are traditionally added. Phospholipases may be applied directly in the conventional production processes of dairy foods or may have interesting perspectives in modification of milk or whey fractions to yield dairy-based ingredients with novel functional properties.

ABBREVIATIONS USED

β-lg, β-lactoglobulin; FFA, free fatty acid; DH, degree of hydrolysis; E, surface shear elastic modulus; η, surface shear viscous modulus; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MFGM, milk fat globule membrane; NMR, nuclear magnetic resonance; PAS, periodic acid−Schiff reagent; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA1, phospholipase A1; PLA2, phospholipase A2; PLC, phospholipase C; AoPLA2, *Aspergillus oryzae* PLA2; FvPLA1, *Fusarium venenatum* PLA1; PpPLA2, porcine pancreatic PLA2; TbPLA2, *Tuber borchii* PLA2; PS, phosphatidylserine; SPH, sphingomyelin.

ACKNOWLEDGMENT

We thank Jan Trige Rasmussen and Pernille Borre Arskog for their help with the MFGM isolation and foam experiments, respectively.

LITERATURE CITED

(1) Bos, M. A.; Nylander, T.; Arnebrandt, T.; Clark, D. C. Protein/emulsifier interactions. In *Food Emulsifiers and Their Applications*; Hasenhuettl, G. L., Hartel, R. W., Eds.; Chapman & Hall: New York, 1997.

(2) van Nieuwenhuyzen, W. The industrial uses of special lecithins: A review. *J. Am. Oil Chem. Soc.* **1981**, *58*, 886−888.

(3) Van der Meeren, P.; El Bakry, M.; Neirynck, N.; Noppe, P. Influence of hydrolysed lecithin addition on protein adsorption and heat stability of a sterilised coffee cream simulant. *Int. Dairy J.* **2005**, *15*, 1235−1243.

(4) Dutilh, C. E.; Groger, W. Improvement of product attributes of mayonnaise by enzymic hydrolysis of egg yolk with phospholipase A2. *J. Sci. Food* **1981**, *32*, 451−458.

(5) Aura, A. M.; Forssell, P.; Mustranta, A.; Suortti, T.; Poutanen, K. Enzymatic hydrolysis of oat and soya lecithin: Effects on functional properties. *J. Am. Oil Chem. Soc.* **1994**, *71*, 887−891.

(6) van Nieuwenhuyzen, W.; Szuhaj, B. F. Effects of lecithins and proteins on the stability of emulsions. *Fett* **1998**, *100*, 282−291.

(7) Kirk, O.; Borchert, T. V.; Fuglsang, C. C. Industrial enzyme applications. *Curr. Opin. Biotechnol.* **2002**, *13*, 345−351.

(8) Ulbrich-Hoffmann, R. Phospholipases. In *Enzymes in Lipid Modification*; Bornscheuer, U. T., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2000.

(9) Dennis, E. A. Phospholipases. In *Lipid Enzymology*; Boyer, P. D., Ed.; Academic Press: New York, 1983.

(10) Arni, R. K.; Ward, R. J. Phospholipase A2−A structural review. *Toxicon* **1996**, *34*, 827−841.

(11) Yuan, C. H.; Tsai, M. D. Pancreatic phospholipase A(2): New views on old issues. *BBA-Mol. Cell. Biol. Lipids* **1999**, *1441*, 215−222.

(12) Soragni, E.; Bolchi, A.; Balestrini, R.; Gambaretto, C.; Percudani, R.; Bonfante, P.; Ottonello, S. A nutrient-regulated, dual localization phospholipase A(2) in the symbiotic fungus Tuber borchii. *EMBO J.* **2001**, *20*, 5079−5090.

(13) Shiba, Y.; Ono, C.; Fukui, F.; Watanabe, I.; Serizawa, N.; Gomi, K.; Yoshikawa, H. High-level secretory production of phospholipase A(1) by Saccharomyces cerevisiae and Aspergillus oryzae. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 94−101.

(14) Watanabe, I.; Koishi, R.; Yao, Y.; Tsuji, T.; Serizawa, N. Molecular cloning and expression of the gene encoding a phospholipase A(1) from Aspergillus oryzae. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 820−826.

(15) Loeffler, F.; Plainer, H.; Sproessler, B.; Frickenstein, H. O. Vegetable oil enzymatic degumming process by means of aspergillus phospholipase. U.S. Patent 6001640, 1999.

(16) Stringer, M. A.; Fatum, T. M.; Patkar, S. A. Phospholipase and method of producing it. WO Patent 2004/097012 A2, 2004.

(17) Bitman, J.; Wood, D. L. Changes in milk fat phospholipids during lactation. *J. Dairy Sci.* **1990**, *73*, 1208−1216.

(18) Morrison, W. R.; Jack, E. L.; Smith, L. M. Fatty acids of bovine milk glycolipids and phospholipids and their specific distribution in the diacylglycerophospholipids. *J. Am. Oil Chem. Soc.* **1965**, *42*, 1142−1147.

(19) Rombaut, R.; Camp, J. V.; Dewettinck, K. Analysis of phospho- and sphingolipids in dairy products by a new HPLC method. *J. Dairy Sci.* **2005**, *88*, 482−488.

(20) Walstra, P.; Jenness, R. *Dairy Chemistry and Physics*; John Wiley & Sons, Inc.: New York, 1984.

(21) Waninge, R.; Nylander, T.; Paulsson, M.; Bergenstahl, B. Milk membrane lipid vesicle structures studied with Cryo-TEM. *Colloids Surf., B* **2003**, *31*, 257−264.

(22) Nielsen, P. M. Process for producing cheese. U.S. Patent 6399121 B1, 2002.

(23) Lilbæk, H. M.; Broe, M. L.; Høier, E.; Fatum, T. M.; Ipsen, R.; Sørensen, N. K. Improving yield of Mozzarella cheese by phospholipase treatment of milk. *J. Dairy Sci.* **2006**, *89*, 4114−4125.

(24) Høier, E.; Lilbaek, H. M. *Phospholipase, A New Enzyme in Cheese Making*; Proceedings of Enzymes for Food Conference in Rennes, France, May 2006, CBB Développement; pp 31−36.

(25) Havn, S. S.; Ipsen, R.; Nielsen, P. M.; Lilbaek, H. M. Improving the foaming properties and heat stability of whey protein concentrates by phospholipase treatment. *Milchwissenschaft* **2006**, *61*, 188−191.

(26) Koka, R. A.; Weimer, B. C. Influence of growth conditions on heat-stable phospholipase activity in Pseudomonas. *J. Dairy Res.* **2001**, *68*, 109−116.

(27) O'Mahony, J. P.; Shipe, W. F. Hydrolysis of the lipoprotein fractions of milk by phospholipase C. *J. Dairy Sci.* **1972**, *55*, 408−412.

(28) Shimizu, M.; Yamauchi, K.; Kanno, C. Effect of proteolic of milk fat globule membrane proteins on stability of the globules. *Milchwissenschaft* **1980**, *35*, 9−12.

(29) Wilde, P. J. Interfaces: Their role in foam and emulsion behaviour. *Curr. Opin. Colloid Interface Sci.* **2000**, *5*, 176−181.

(30) Sas, B.; Peys, E.; Helsen, M. Efficient method for (lyso)-phospholipid class separation by high-performance liquid chromatography using an evaporative light-scattering detector. *J. Chromatogr.* **1999**, *864*, 179−182.

(31) Hvarregaard, J.; Andersen, M. H.; Berglund, L.; Rasmussen, J. T.; Petersen, T. E. Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. *Eur. J. Biochem.* **1996**, *240*, 628−636.

(32) Meneses, P.; Glonek, T. High resolution 31P-NMR-spectroscopy of extracted phospholipids. *J. Lipid Res.* **1988**, *29*, 679−689.

(33) Clark, D. C.; Wilde, P. J.; Wilson, D. R. Destabilization of alpha lactalbumin foams by competitive adsorption of the surfactant Tween 20. *Colloids Surf.* **1991**, *59*, 209−224.

(34) Kishimura, H.; Hayashi, K. Characterization of phospholipase A2 from the pyloric ceca of two species of starfish, *Coscinasterias acutispina* and *Plazaster borealis*. *Food Chem.* **2005**, *92*, 407−411.

(35) Diez, E. Fatty acid and phospholipid selectivity of different phospholipase A-2 enzymes studied by using a mammalian membrane as substrate. *Biochem. J.* **1994**, *301*, 721−726.

(36) Diez, E.; Louis-Flamberg, P.; Hall, R. H.; Mayer, R. J. Substrate specificities and properties of human phospholipases A2 in a mixed vesicle model. *J. Biol. Chem.* **1992**, *267*, 18342−18348.

(37) Ibrahim, S. A.; Sanders, H.; Thompson, R. H. The action of phospholipase A on purified phospholipids, plasma and tissue preparations. *Biochem. J.* **1964**, *93*, 588−594.
(38) Mather, I. H. A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *J. Dairy Sci.* **2000**, *83*, 203−247.
(39) Hanssens, I.; Van Cauwelaert, F. H. Shielding of phospholipid monolayers from phospholipase C hydrolysis by alpha-lactalbumin adsorption. *Biochem. Biophys. Res. Commun.* **1978**, *84*, 1088−1096.
(40) Deems, R. A. Interfacial enzyme kinetics at the phospholipid/water interface: Practical considerations. *Anal. Biochem.* **2000**, *287*, 1−16.
(41) Jorgensen, K.; Davidsen, J.; Mouritsen, O. G. Biophysical mechanisms of phospholipase A2 activation and their use in liposome-based drug delivery. *FEBS Lett.* **2002**, *531*, 23−27.
(42) Bos, M. A.; van Vliet, T. Interfacial rheological properties of adsorbed protein layers and surfactants: A review. *Adv. Colloid Interface Sci.* **2001**, *91*, 437−471.
(43) Clark, D. C.; Husband, F.; Wilde, P. J.; Cornec, M.; Miller, R.; Kragel, J.; Wustneck, R. Evidence of extraneous surfactant adsorption altering adsorbed layer properties of beta-lactoglobulin. *J. Chem. Soc. Faraday Trans.* **1995**, *91*, 1991−1996.
(44) Fujita, S.; Suzuki, K. Surface activity of the lipid products hydrolyzed with lipase and phospholipase A2. *J. Am. Oil Chem. Soc.* **1990**, *67*, 1008−1014.
(45) Sawyer, L.; Kontopidis, G. The core lipocalin, bovine beta-lactoglobulin. *Biochim. Biophys. Acta* **2000**, *1482*, 136−148.
(46) Brown, J. R.; Shockley, P. Serum albumin: Structure and characterization of its ligand binding sites. In *Lipid-Protein Interactions*; Jost, P. C., Griffith, O. H., Eds.; John Wiley: New York, 1982.
(47) Sarker, D. K.; Wilde, P. J.; Clark, D. C. Competitive adsorption of a-lysophosphatidylcholine/beta-lactoglobulin mixtures at the interfaces of foams and foam lamellae. *Colloids Surf., B* **1995**, *3*, 349−356.
(48) Barratt, M. D.; Austin, J. P.; Whitehurst, R. J. The influence of the alkyl chain length of lecithins and lysolecithins on their interaction with alpha s1-casein. *Biochim. Biophys. Acta* **1974**, *348*, 126−135.
(49) Brown, S. D.; Baker, B. L.; Bell, J. D. Quantification of the interaction of lysolecithin with phosphatidylcholine vesicles using bovine serum albumin: relevance to the activation of phospholipase A2. *Biochim. Biophys. Acta* **1993**, *1168*, 13−22.
(50) Wilde, P. J. Foam measurement by the microconductivity technique: An assessment of its sensitivity to interfacial and environmental factors. *J. Colloid Interface Sci.* **1996**, *178*, 733−739.
(51) Vaghela, M. N.; Kilara, A. Foaming and emulsifying properties of whey-protein concentrates as affected by lipid-composition. *J. Food Sci.* **1996**, *61*, 275−280.
(52) Rouimi, S.; Schorsch, C.; Valentini, C.; Vaslin, S. Foam stability and interfacial properties of milk protein-surfactant systems. *Food Hydrocolloids* **2005**, *19*, 467−478.
(53) Zhang, Z.; Dalgleish, D. G.; Goff, H. D. Effect of pH and ionic strength on competitive protein adsorption to air/water interfaces in aqueous foams made with mixed milk proteins. *Colloids Surf., B* **2004**, *34*, 113−121.
(54) Izmailova, V. N.; Yampolskaya, G. P.; Tulovskaya, Z. D. Development of the Rehbinder's concept on structure- mechanical barrier in stability of dispersions stabilized with proteins. *Colloids Surf., A* **1999**, *160*, 89−106.
(55) Fox, P. F.; McSweeney, P. L. H. *Dairy Chemistry and Biochemistry*, 1st ed.; Blackie Academic & Professional: London, United Kingdom, 1998.
(56) Husband, F.; Wilde, P. J.; Marion, D.; Clark, D. C. Comparison of the foaming and interfacial properties of two related lipid-binding proteins from wheat in the presence of a competing surfactant. In *Food Macromolecules and Colloids*; Dickinson, E., Lorient, D., Eds.; Royal Society of Chemistry: London, 1995.
(57) Mine, Y.; Kobayashi, H.; Chiba, K.; Tada, M. Phosphorus-31 NMR study on the interfacial adsorptivity of ovalbumin promoted by lysophosphatidylcholine and free fatty acids. *J. Agric. Food Chem.* **1992**, *40*, 1111−1115.
(58) Mine, Y.; Chiba, K.; Tada, M. Effect of phospholipids on conformational change and heat stability of ovalbumin: Circular dichroism and nuclear magnetic resonance studies. *J. Agric. Food Chem.* **1993**, *41*, 157−161.
(59) Fruhner, H.; Wantke, K. D.; Lunkenheimer, K. Relationship between surface dilational properties and foam stability. *Colloids Surf. A* **2000**, *162*, 193−202.
(60) Wustneck, R.; Kragel, J.; Miller, R.; Wilde, P. J.; Clark, D. C. The adsorption of surface-active complexes between beta-casein, beta-lactoglobulin and ionic surfactants and their shear rheological behaviour. *Colloids Surf., A* **1996**, *114*, 255−265.

Received for review September 21, 2006. Revised manuscript received December 18, 2006. Accepted January 4, 2007. Chr. Hansen A/S, Novozymes A/S, and the Danish Ministry of Technology, Development and Science are acknowledged for funding the research. We also acknowledge the Biotechnology and Biological Sciences Research Council for funding through the core strategic grant to the Institute of Food Research in Norwich, United Kingdom, where the foaming experiments were performed.

JF062705B