Effect of Minor Milk Proteins in Chymosin Separated Whey and Casein Fractions on Cheese Yield as Determined by Proteomics and Multivariate Data Analysis

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ABSTRACT

The objective of this work was to find regressions between minor milk proteins or protein fragments in the casein or sweet whey fraction and cheese yield because the effect of major milk proteins was evaluated in a previous study. Proteomic methods involving 2-dimensional gel electrophoresis and mass spectrometry in combination with multivariate data analysis were used to study the effect of variations in milk protein composition in chymosin separated whey and casein fractions on cheese yield. By mass spectrometry, a range of proteins significant for the cheese yield was identified. Among others, a C-terminal fragment of β -casein had a positive effect on the cheese yield expressed as grams of cheese per 100 g of milk, whereas several other minor fragments of β -, α_{s1} -, and α_{s2} -casein had positive effects on the transfer of protein from milk to cheese. However, the individual effect of each identified protein was relatively low. Therefore, further studies of the relations between different proteins/peptides in the rennet casein or sweet whey fractions and cheese yield are needed for advanced understanding and prediction of cheese yield.

Key words: rennet casein, sweet whey protein, proteomics, protein transition number

INTRODUCTION

Within the dairy industry it is desirable to control the technological properties of milk to achieve optimal cheese yield and quality. To improve cheese yield, it is crucial to increase the amount of protein that is transferred from cheese milk into the cheese curd. Further-

more, the milk proteins, especially the caseins, contribute to the cheese structure and texture. Accordingly, in many countries, milk farmers are compensated for high amounts of milk protein to obtain higher cheese yield and quality. However, poor cheese-making properties in spite of high milk protein concentrations have been reported (Ikonen et al., 1999), and other significant variables have been suggested. Among them are concentrations (Marziali and Ng-Kwai-Hang, 1986; Wedholm et al., 2006) and genetic variants (Ng-Kwai-Hang, 1998) of individual caseins. Significant variables for the technological quality of milk have also been found within the whey protein fraction. It is documented that the B allele of β -LG is associated with greater cheese yield than the A allele (Marziali and Ng-Kwai-Hang, 1986; Schaar et al., 1985; Wedholm et al., 2006), which is due to the positive correlation between casein number and β -LG B (Schaar et al., 1985; van den Berg et al., 1992; Lundén et al., 1997).

In addition to genetic variation among milk proteins there are several posttranslational modifications such as phosphorylation, glycosylation, disulphide-bond formation, and proteolysis that occur among the milk proteins. The use of 2-dimensional gel electrophoresis (2-DGE) facilitates the separation of modification products with similar molecular masses because the proteins are not just separated according to molecular weight, but also in a second dimension according to their isoelectric points. Earlier studies by Lindmark-Månsson et al. (2005) showed that 2-DGE is a useful tool to detect variations in milk protein composition, and it is a widely used method within milk proteomics (O'Donnell et al., 2004). Using 2-DGE and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), Galvani et al. (2000, 2001) identified the major caseins and whey proteins, including their isoforms, in commercial bovine milk and in a milk powder. Holland et al. (2004) were able to detect 10

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isoforms of κ -CN from a cow heterozygous for the A and B variants of κ -CN, by 2-DGE and mass spectrometry detection. Several low-abundance bovine milk proteins have also been identified in whey and skim milk fractions by proteomic methods (Yamada et al., 2002; Smolenski et al., 2002; Fong et al., 2008). In a previous study the 2-DGE technique in combination with MS detection and multivariate data analysis was used to study the relation between milk protein composition and milk yield (Larsen et al., 2007). The same method has also been used to link proteomic profiles and quality parameters in meat (Jessen et al., 2002) and in plant science (Gottlieb et al., 2004).

During renneting, most of the whey proteins end up in the sweet whey fraction and the caseins in the rennet casein fraction. However, the separation is never complete which is believed to have consequences for the cheese yield. Earlier, when Wedholm et al. (2006) analyzed the effect of the major milk protein composition on the cheese-making properties, it was confirmed that the casein number (casein in relation to total protein) in milk had a significantly positive effect on the transfer of proteins from milk to cheese (i.e., the protein transition number). In the same study, the significant effects of the individual major caseins (α -, β -, and κ -casein) on cheese yield were demonstrated. The subject of this study was to evaluate these findings further by analyzing minor milk proteins or protein fragments in the whey and casein fractions of chymosin-treated milk and relate it to cheese yield. By use of proteomics in combination with multivariate regression, this work aimed to study the effect of variations in whey and casein composition, as separated by chymosin, between individual cows on the yield of low-fat model cheeses.

MATERIALS AND METHODS

Milk Sampling

Evening whole milk (10 L) from 43 Danish Holstein Friesian (SDM) cows were sampled at the experimental herd at the Faculty of Agricultural Sciences, University of Aarhus (former Danish Institute of Agricultural Sciences) as described earlier (Wedholm et al., 2006). The cows were housed in tie stalls and fed a total mixed ratio of normal energy density. All cows included in the study were healthy and milked twice a day. The cows were selected to represent a balanced material of varying levels of total milk protein concentration (from low to high). Total protein and casein concentrations of the samples were determined by a Milkoscan FT 6000 (Foss Electric, Hillerød, Denmark) and were in the range from 2.69 to 4.21 g/L and 2.05 to 3.30 g/L, respectively.

Table 1. Means and SD of cheese yield (expressed in 2 ways)

Item	g of cheese per 100 g of milk	g of dry cheese solids per 100 g of milk protein
Mean	7.90	91.57
SD	1.23	10.45
df	42.00	35.00 ¹
Minimum	5.70	67.90
Maximum	10.20	129.68

¹Number of observations reduced because of missing data.

Cheese Making and Determination of Cheese Yield

Low-fat model cheeses were produced from skimmed milk to reduce the number of variables influencing cheese yield. After 2 d of cold storage (4°C), individual milk samples were preheated to 40°C, defatted, and then heated in a pilot plate heating apparatus (72°C for 15 s) as described by Allmere et al. (1998). Four liters of skimmed milk was inoculated with a commercial starter culture (0.1 g/L of *Lactobacillus helveticus* 174 and 0.1 g/L of Probat 404, Danisco, Sweden) and incubated at 30°C for 30 min. This was followed by addition of chymosin (1.25 mL/L, Chy-max Plus with 190 International Milk Clotting Units/mL, Chr Hansen A/S, Copenhagen, Denmark) and gentle stirring. After 30 min at 30°C, the gel formed was cut into 2-cm cubes. To allow syneresis, the curd was incubated at 50°C for another 30 min during gentle stirring. The whey was removed and the curd was pressed (0.04 kg/cm²) during 20 h at room temperature. After 2 wk of storage at 10°C, the cheeses were weighed to obtain the yield. To obtain dry weight from individual cheeses, 2 to 3 g was taken from the interior of the cheeses, grated, and mixed with a fixed amount of sand. These cheese samples were incubated at 105°C overnight and then placed in a dessicator for 1 h before weighing. The cheese yield was expressed in 2 ways: in relation to amount of cheese milk used (as g of cheese per 100 g of milk) or as a sort of protein transition number (as g of dry cheese solids per 100 g of milk protein), as recommended by Emmons (1993). Means and standard deviations of cheese yield obtained from the individual milk samples are presented in Table 1.

Fractionation of Samples for 2-DGE

Milk samples for 2-DGE analysis were fractionated separately from the cheese making trial to avoid the influence of the starter culture growth. An aliquot of each individual milk sample was skimmed twice at 3,000 × g for 10 min. The skimmed milk was preheated at 30°C for 30 min and fractionated into casein and whey by addition of chymosin (2 mL/L). A higher concentration

was needed for this separation because of the higher pH in absence of starter culture. The samples were incubated at 30°C for 30 min and then centrifuged at $1,000 \times g$ for 10 min at 5°C to separate sweet whey and rennet casein, followed by recovery of the sweet whey fraction. This fraction is subsequently denoted as proteins in the whey fraction (**PWF**). The rennet casein was washed twice in cold MP-water (USF Elga Maxima system, Bucks, UK) and centrifuged at $1,000 \times g$ for 5 min at 5°C. The casein fraction was dissolved in 0.1 M tri-sodium citrate buffer, pH 8.9, to the original milk volume and stored at -20°C until further use. This casein fraction was subsequently denoted as proteins in the casein fraction (**PCF**).

Two-Dimensional Gel Electrophoresis

The rennet sweet whey and rennet casein fractions were analyzed on separate 2-DGE gel sets consisting each of 43 gels. The first dimension of protein separation was carried out in immobilized 11-cm IPG strips (pH 4 to 7, BioRad, Hercules, CA), whereas 8 to 16% gradient Criterion gels (BioRad) were used for the second dimension. For analytical gels subjected to image analysis a volume of the sweet whey protein fraction corresponding to 50 µg was applied, whereas for analysis of rennet caseins 40 µg was analyzed. For preparative gels used for MS analysis a volume corresponding to 370 µg of sweet whey protein sample or 200 µg of rennet caseins was applied. The total protein and whey protein contents were based on the Milkoscan determinations. The desired amount of sample was dissolved at 1:10 vol/vol in denaturing buffer consisting of 6 M urea, 2 M thiourea, 1.5% (wt/vol) pharmalyte (GE Healthcare, Uppsala, Sweden), 0.8% (wt/vol) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, CHAPS (Applichem, Darmstadt, Germany) 1% (wt/vol) dithioerythritol in water and incubated for 2 h at room temperature. The reduced proteins were further diluted in a rehydration buffer to a final volume of 185 µL. The rehydration buffer consisted of the same substrates, in same concentrations as the reducing buffer, but with pharmalyte (5 µL/mL) instead of 1% dithioerythritol. Running conditions for the 2-DGE gels were essentially as described by Lametsch and Bendixen (2002). Analytical gels were silver-stained according to Lametsch and Bendixen (2002), whereas preparative gels for MS were stained according to Shevchenko et al. (1996).

Image Analysis

The 2-DGE gels were photographed by a Vilber Lourmat digital camera (ImageHouse, Copenhagen, Denmark) equipped with Gel Pro analyzer software.

The gel spots were detected and quantified with ImageMaster 2D platinum software (Amersham Pharmacia Biotech, Uppsala, Sweden). After initial analysis using automated spot detection and segmentation, all images were manually checked and the spots were matched by comparing the relative positions of the individual spots on each gel. The spots were quantified by adding the pixel intensities within the spot boundary, and the spot volumes were calculated. To overcome gel-to-gel variations in spot intensities due to technical variations related, for example, to the staining procedure, the relative spot volumes were calculated for each separate spot on each gel, and these values were used in further data analysis.

In Gel Digestion, Desalting, and Concentration of Protein Spots

Protein spots of significance were subjected to in-gel digestion by addition of trypsin essentially as described by Jensen et al. (1998). Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture from each spot before MS analysis as described by Lametsch et al. (2002). For MALDI-TOF analysis the peptides were eluted in 0.5 µL of matrix solution (15 to 20 g/L of α -cyano-4-hydroxycinnamic acid; Sigma Aldrich, St. Louis, MO, in 70% acetonitrile) directly onto the MALDI target plate (Bruker Daltonics GmbH, Bremen, Germany). For liquid chromatography quadrupole time-of-flight (**Q-TOF**) MS analysis the peptides were eluted in 0.5 µL 70% acetonitrile.

Identification of Milk Proteins by MALDI-TOF Mass Spectrometry

Mass spectra were obtained using a Bruker Ultraflex MALDI-TOF tandem mass spectrometer in reflection mode. A peptide calibration standard (0.2 µL, Bruker Daltonics GmbH) containing 7 standard peptides ranging in molecular masses from 1,046.54 to 3,147.47 Da was spotted separately onto the MALDI target plate. The ion accelerating voltage was 25 kV with a delay time of 40 ns. The laser frequency was 50 Hz and 200 laser shots were accumulated for each spectrum. For tandem mass spectrometry (**MS/MS**) lift mode the ion accelerating voltage was 19 kV. Proteins were identified by peptide mass fingerprinting by mass searches in the database Swiss Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland), or by fractionation of their parent ions in MS/MS mode followed by mass searches in the database. For both applications the MS/MS ion search program Mascot (Matrix Science, Boston, MA) was used. In this program the experimental mass value, obtained from MS or MS/MS, is compared with

calculated peptide masses from a database. A scoring algorithm is used to identify the closest match. Significant protein identifications (protein scores above 62, $P < 0.05$) were reported and manually verified.

Identification of Milk Proteins by Q-TOF Mass Spectrometry

Automated liquid chromatography electrospray ionization MS/MS was performed using a hybrid Q-TOF mass spectrometer (Bruker Daltonics) and an Ultimate nano-HPLC system (LC Packings, Amsterdam, the Netherlands) mounted with a vented-column setup. Reversed phase columns (pre-column 2 cm, 75 μm id; separation column 12 cm, 50 μm internal diameter) were packed in-house with ReproSil-Pur C18-AQ 3- μm resin (Dr. Maisch GmbH, Ammer-Buch-Entringen, Germany) using a high-pressure vessel. Aliquots of the tryptic peptides corresponding to 25% of each gel spot were injected onto the pre-column with a flow rate of 3 $\mu\text{L}/\text{min}$ and subsequently eluted at 175 nL/min using a 35 min gradient of 5 to 40% acetonitrile in 0.6% acetic acid and 0.005% heptafluorobutyric acid. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition selecting the 3 most abundant precursor ions. The tandem MS data was deconvoluted and deisotoped and exported in a mascot generic format before database searching. Protein homologs were identified by use of an in-house Mascot database search engine using the SwissProt database or the NCBI nr database (May 2007). Tandem MS search parameters had a mass accuracy of ± 0.025 Da; methionine oxidation, serine and threonine phosphorylation, and carbamidomethyl allowed as variable modifications. Cleavage specificity was specified as semitryptic to validate any sequence processing. Results from the automatic database search were evaluated in accordance with the Paris Publication Guidelines (2007). Significant protein identifications (protein scores above 75, minimum 2 unique peptides identified, $P < 0.05$) were reported and manually verified.

Partial Least Squares Regression Analysis

Partial least squares regression 1 (PLS-1) analysis was carried out using the software The Unscrambler ver. 9.0 (CAMO ASA, Oslo, Norway) to study the effect of 2-DGE spot variations on the response Y-variables, cheese yield expressed as grams of cheese per 100 g of milk or as grams of dry cheese solids per 100 g of milk protein. The rennet casein and sweet whey protein fractions on the 2-DGE gels were analyzed separately and composed the regressors (X-variables) in the PLS-

1 model. When analyzing the effect of the sweet whey protein composition on the 2 types of cheese yield the relative volume of each spot, as determined by image analysis, was used as continuous X-parameters, whereas discriminant regressors were used in the model for analysis of the effect of rennet casein composition on the same traits. In the discriminant model, the protein spot in the sample was denoted by a binary variable: 1 if present or 0 if absent, as indicated by image analysis (Radzikowski et al., 2002). The background for application of a discriminant model for the analysis of the spots in the rennet casein fraction is further discussed in the Results and Discussion section. Standardized (centered: $\mu = 0$, and normalized: $1/\text{SD}$) variables and full cross validation were used. Spots contributing little to the model were removed by jack-knifing (Martens and Martens, 2000) until an optimally calibrated and validated model was achieved (i.e., when the calibration and validation curves became parallel). The optimal number of principal components and significant ($P < 0.05$) regression coefficients were identified using the uncertainty test.

RESULTS AND DISCUSSION

2-DGE Separation and MS-Identification of Milk Proteins

To be able to relate the distribution of casein and whey proteins between the 2 fractions to the cheese yield, the skimmed milk samples were fractionated by addition of chymosin. The rennet casein and sweet whey fractions were subsequently separately analyzed. Examples of 2-DGE separations of proteins in the casein fraction (PCF) and in the sweet whey fraction (PWF) are shown in Figures 1 and 2, respectively. It is evident, that due to the similarities in molecular masses and pI values of the caseins, these were more poorly separated than the whey proteins. The positions of major individual milk proteins in the 2-DGE gels are indicated. The identifications of major caseins and whey proteins were carried out by MALDI-TOF MS by peptide mass fingerprinting, by MS/MS analysis of peptides generated from major proteins excised from the gels (results not shown) or by comparison with earlier published 2-DGE pictures of the caseins (Holland et al., 2004) and whey proteins (Larsen et al., 2007; Fong et al., 2008), and are indicated in Figures 1 and 2. The major proteins identified in the rennet casein fraction included β -CN, α_{s1} -CN, and α_{s2} -CN monomers. The α_{s2} -CN dimer was not seen due to the inclusion of reducing agent in the sample buffer. Due to the addition of chymosin, most of the κ -CN was cleaved into para- κ -CN and caseinomacropепptide (CMP). Para- κ -

Figure 1. Two-dimensional gel electrophoresis of proteins in the rennet casein fraction (PCF) isolated from 1 individual cow. Proteins with a significant influence on cheese yield (expressed as g of cheese per 100 g of milk or as g of dry cheese solids per 100 g of milk protein) are marked with white circles, and indicated by their ID numbers from the image analysis. Protein spots identified by mass spectrometry are further indicated by names. The positions of other major proteins are indicated by protein names given in bold face.

CN is expected to be associated with the rennet casein fraction, whereas the CMP ends up in the sweet whey fraction. The hydrophobic para- κ -CN (residue 1 to 105) has a molecular mass of approximately 13 kDa, whereas the polar, negatively charged CMP (residue 106 to 169) has a mass of 8 to 10 kDa, depending on the extent of glycosylation (Fox, 1988). Due to the low molecular mass of CMP, this fragment is not expected to be retained in the 2-DGE gels used. The position of para- κ -CN was not identified in this study and may be focused outside the pI-range used here for the gels. However, the importance of para- κ -CN for the transfer of protein from milk to cheese has been documented earlier and was suggested to be slightly better for prediction of cheese yield than simply the casein concentration (Emmons et al., 2003). Therefore, in the present study we focused on the significance of fragments apart from para- κ -CN that could be related to cheese yield. In spite of the addition of chymosin, some intact κ -CN spots (according to the molecular mass) were present

in the casein fraction, in addition to some remnants of BSA (Figure 1). Among PWF, the positions of the following major proteins were identified by MALDI-TOF MS: β -LG, α -LA, BSA, proteose peptone component 3, and lactoferrin (Figure 2). The protein α -LA was seen to separate into various forms with different pI values. This could be due to the presence of different glycosylated forms of the molecule (Slangen and Visser, 1999) but was not further studied. Furthermore, the position of the added chymosin was identified by MALDI-TOF MS. During renneting, most of the chymosin ends up in the whey fraction. However, the proportion that is retained in the rennet casein varies between cheese varieties and has an influence on the cheese texture and flavor (Bansal et al., 2007).

PLS-1 Analyses

The PLS is a good tool for analysis of complex data sets with a large number of x-variables that covariate

Figure 2. Two-dimensional gel electrophoresis of proteins in the sweet whey fraction (PWF) prepared from 1 individual cow. Proteins with a significant influence on cheese yield (expressed as g of cheese/100 g of milk or as g of dry cheese solids/100 g of milk protein) are marked with white circles and indicated by their ID numbers from the image analysis. Protein spots identified by mass spectrometry are further indicated by names. The positions of other major proteins are indicated by protein names given in bold face. PP3 = proteose peptone component 3; Ig LC = immunoglobulin G λ -light chain; LF = lactoferrin.

and has earlier been applied in other proteomic studies (Jessen et al., 2002; Gottlieb et al., 2004). In this study, a PLS-1 model was used to analyze the effects of variations in PCF and PWF on the cheese yield. In the case of analyzing the regressions between PCF and cheese yield, a binary annotation of the spots, instead of continuous values of the relative spot volumes, was used. By using this binary annotation for PCF instead of measured continuous values for relative spot volumes, the multivariate model was improved (data not shown). The reason for this was due to difficulties in the quantification of some of the major proteins in the rennet casein fraction, caused by some of the casein spots being very abundant. This complicated the quantification of the silver-stained caseins, a staining which has a relatively low dynamic range (Lopez et al., 2000). Therefore, the PLS-model for the PCF was selected only to indicate whether presence of a particular protein in the casein fraction had an effect on the cheese

yield or not. When analyzing the effect of PWF on the cheese yield, however, the range of protein spots was more evenly distributed within the 2-DGE gels, and the relative spot volumes could be used.

The results from the regression analyses are presented in Tables 2 and 3. Using one principal component, PCF explained 63% of the total variation in cheese yield expressed as g of cheese per 100 g of milk (GCM), whereas 58% of the total variation in cheese yield expressed as g of dry cheese solids per 100 g of milk protein (GDMP) was explained by 2 principal components (Table 2). With 2 and 1 principal components, respectively, PWF explained 92 and 52% of the total variation in the same traits, respectively (Table 3). All of the PCF (23 spots) that were significant for GDMP had positive effects on this trait (Table 2), whereas significant PWF had positive (3 spots) and negative (4 spots) effects (Table 3). This showed that a range of protein spots in the rennet casein fraction

Table 2. Significant weighted regression coefficients between proteins in the casein fraction (PCF) and the cheese yield (expressed in 2 ways)

Spot ID ¹	g of cheese per 100 g of milk [1 PC ² explains 63% of the total variation in Y (R ² = 0.63)]	g of dry cheese solids per 100 g of milk protein [2 PC explain 58% of the total variation in Y (R ² = 0.58)]
5		0.045*
7		0.040*
11		0.038**
13		0.035**
14		0.033*
26		0.037*
38		0.033***
40		0.028**
51		0.027**
54		0.025**
57		0.021*
64		0.026*
65		0.025*
76	0.060*	0.025*
77		0.027*
78		0.028**
84		0.029**
85	0.057*	0.027**
91	-0.048*	0.039***
101	0.051*	
110		
119	0.066*	0.032*
123		
132		0.028**
136	-0.077*	0.034**
201		
258		0.028**

¹Spot identification (ID) in 2-dimensional gel electrophoresis.²PC = principal component. R² is the correlation coefficient between measured and predicted y variables.**P* < 0.05, ***P* < 0.01, ****P* < 0.001.

were positively associated with the transfer of proteins from milk to cheese. Looking at cheese yield expressed as GCM, 4 PCF (Table 2) and 17 PWF (Table 3) were positively associated with this trait, whereas 2 PCF (Table 2) and 6 PWF (Table 3) were negatively associated with this type of cheese yield.

Identified PCF

The positions in a 2-DGE gel of the pinpointed PCF significant for cheese yield expressed as GCM or GDMP are presented in Figure 1. Most of the identified PCF corresponded to minor proteins or protein fragments with apparent significance for the cheese yield, and not to intact, major caseins. This was expected due to the use of a discriminant PLS-model, as mentioned above (i.e., a model that does not take into account the intensity of the spot, but whether it is present or not). A further reason for this is the fact that chymosin, which was added, not only cleaves the well-known Phe₁₀₅–Met₁₀₆ bond in κ -CN, but also cleaves a range of other peptide bonds on the casein molecules, especially in α _{s1}-CN (McSweeney et al., 1993, 1995).

The range of individual rennet caseins or sweet whey proteins that were significantly associated with any of the 2 types of cheese yield was aimed for identification by MALDI-TOF or Q-TOF mass spectrometry. Of the 23 spots in PCF of significance for cheese yield expressed as GDMP, 12 individual proteins could be identified by MS, as shown in Tables 4 and 5 and indicated in Figure 1. Four of the identified PCF corresponded to different forms of α _{s2}-CN, with spot identifications (ID) 26, 38, 54, and 119, all with positive effect on this type of yield. The identification of spot ID 26, as α _{s2}-CN, is in accordance with the position of intact α _{s2}-CN reported earlier (Holland et al., 2004). In contrast to spot 26, spots 38, 54, and 119 all had lower molecular mass and a higher pI compared with intact α _{s2}-CN and thus represent proteolysis products of α _{s2}-CN, probably by chymosin or some indigenous milk enzyme (like, for example, plasmin). Chymosin has been reported to cleave α _{s2}-casein at 8 sites in the hydrophobic regions of the molecule (McSweeney et al., 1994). Regardless of the type of enzyme, these cleavage products corresponded to larger protein fragments in the PCF, which thereby could explain their positive association with the cheese

Table 3. Significant weighted regression coefficients between proteins in the whey protein fraction (PWF) and cheese yield (expressed in 2 ways)

Spot ID ¹	g of cheese per 100 g of milk [2 PC ² explain 92% of the total variation in y ($R^2 = 0.92$)]	g of dry cheese solids per 100 g of milk protein [1 PC explains 52% of the total variation in y ($R^2 = 0.52$)]
53	0.052*	
57		0.052*
116	-0.069	
128	-0.045*	
166	0.042*	
210	-0.053*	
211	0.049*	
213	0.066*	0.051*
231	0.062*	
267	0.057*	
268	0.041*	
271	0.069*	
282	0.050*	
286	-0.056*	
347	0.045*	
359	0.052*	
383	0.094**	
409		-0.046*
496	0.058**	
497	0.066*	
538		-0.069*
545	0.058	
546		-0.064
559	-0.069*	
567	0.054*	
609		-0.051*
621		0.079*
639	-0.056**	
648	0.041**	

¹Spot identification (ID) in 2-dimensional gel electrophoresis.²PC = principal component. R^2 is the correlation coefficient between measured and predicted y variables.* $P < 0.05$; ** $P < 0.01$.

yield (i.e., these fragments would not appear on the gel if they were further degraded). Further identified spots of significance for GDMP included spot number 40, 51 and 57, which were found to contain β -CN. These β -CN forms most probably corresponded to different phosphorylated forms and perhaps also genetic variants of β -CN. However, the degree of phosphorylation or the exact amino acid sequences could not be verified from the identification method used.

Among the range of minor spots found to be positively associated with GDMP (Figure 1 and Table 2) 4 spots with ID 64, 65, 78, and 85 were identified by MS to contain α_{s1} -CN. As they were located in a region below the position of the intact α_{s1} -CN cluster (Figure 1), at positions corresponding to lower molecular masses than intact α_{s1} -CN, these spots corresponded to proteolysis products of α_{s1} -CN, probably by chymosin. Chymosin is known to cleave α_{s1} -CN at several sites, initially to α_{s1} -I (f25 to 199) and later to α_{s1} -II (f25 to 169) and further products (Fox, 1988). Protein spot ID 5, in the casein fraction, corresponded to BSA (Figure 1) and was positively associated with cheese yield

expressed as GDMP (Table 2). The presence of minor whey proteins in the casein fraction most probably confirms that some whey proteins were entrapped in the precipitated caseins formed after cleavage by chymosin. The fact that BSA was significant for GDMP (i.e., the protein transition number) would imply that a higher amount of the whey proteins were entrapped in the cheese and thereby increased the transfer of total protein from milk to cheese.

Further spots in the PCF of significance for cheese yield expressed as GCM included spot ID 110, which had a positive effect. This spot contained β -CN, and due to its location far below the position of intact β -CN (Figure 2), this spot corresponded to a fragment of β -CN generated probably by chymosin, or eventually an indigenous milk protease. The peptide mass fingerprinting obtained from this spot were composed of peptides derived from positions 107 to 209 in the β -CN molecule, whereas no peptides were identified from the N-terminal part of the molecule. This indicated that this β -CN fragment was the result of a proteolytic cleavage in the N-terminal part of the β -CN molecule.

Table 4. Proteins from the rennet casein or the sweet whey fraction with significant effects on cheese yield identified by matrix-assisted laser desorption time-of-flight (MALDI-TOF) or tandem (MS/MS) mass spectrometry

Spot ID	Protein	Sequence coverage ¹	Matched peptides ²	Theo. pI ³	Theo. Mw ⁴ (kDa)	SWISS-PROT access key ⁵
Casein fraction						
5	BSA	22	11	5.8	71	P02769
26	α_{s2} -CN ⁶		1 (ALNEINQFYQK)	4.6	26	P02663
38	α_{s2} -CN	23		4.6	26	P02663
54	α_{s2} -CN	20		4.6	26	P02663
119	α_{s2} -CN	32	11	4.6	26	P02663
Whey fraction						
57	Lactoferrin	20	15	8.7	50	P24627
116	Vitamin D-binding protein	34	15	5.2	55	Q3MHN5
210	β -LG	43	7	4.8	20	P02754
211	β -LG	41	8	4.8	20	P02754
213	β -LG	54	8	4.8	20	P02754
409	α -LA	34	5	4.9	17	P00711
567	α -LA	43	8	4.9	17	P00711
609	BSA	16	8	5.8	71	P02769

¹The minimum coverage of the matched peptides in relation to the full-length sequence.

²The number of matched peptides in the database search.

³Theoretical pI of the full-length protein.

⁴Theoretical molecular mass (Mw) of the full length protein.

⁵Primary accession key in the SWISS-PROT database.

⁶The protein was identified by MS/MS of the parent α_{s2} -casein ion 1,367.696 m/z.

The positive regression between this β -CN fragment and cheese yield is interesting, but the background for this is not known and would require further studies.

Identified PWF

The locations of PWF significant for the cheese yield in a 2-DGE gel of separated whey proteins are presented in Figure 2. In addition to the major β -LG spot of native molecular mass located in the lower region of the 2-DGE gel (spot ID 359) that had a positive effect on GCM (Table 3 and Figure 2), 2 other spots containing β -LG were identified by MS (Table 4). These had significant effects on cheese yield expressed as GCM, one negatively (spot ID 210) and one positively (spot ID 211) and probably corresponded to complexes containing β -LG, due to their higher molecular masses as compared with the major β -LG spot. The β -LG variant being negatively associated with cheese yield (spot ID 210) appeared at a lower pI on the 2-DGE gel (Figure 2). It is very likely that this protein spot corresponded to the A-variant of β -LG, which due to an extra aspartic acid, has a more negative net charge compared with the B-variant. It was not possible, however, to derive this from the MS data because the relevant peptides covering these amino acids were not included in the peptide mass finger printings. In the present study, a third variant of β -LG (spot ID 213, Tables 3 and 4 and Figure 2) had a positive effect on the cheese yield expressed as GDMP. This variant most probably cor-

responded to a modification product of β -LG B, which has a more positive net charge compared with the A-variant. Most likely, also the major β -LG spot (spot ID 359) corresponded to the B-variant of β -LG, which in earlier findings was found to be positively associated with both types of cheese yield (Wedholm et al., 2006).

Two different spots, identified by MS as α -LA, were significantly associated with cheese yield; one was negatively associated with GDMP (spot ID 409; Table 3 and Figure 2), whereas one was positively associated with GCM (spot ID 567). It is possible that these forms of α -LA may represent different glycosylated forms of α -LA because a minor part of the molecule is glycosylated, of which some forms contain the negatively charged sialic acid (Slangen and Visser, 1999), but this was not further studied. Of the identified minor whey proteins, lactoferrin (spot ID 57) was positively associated with GDMP, whereas BSA (spot ID 609) was negatively associated with the same trait. The latter is in agreement with the results from the casein separation (i.e., the presence of BSA in the rennet casein fraction was found to have a positive impact on GDMP). Lactoferrin identified in spot ID 57 had a lower pI compared with the theoretical pI for the full length protein (Table 4 and Figure 2). Pepsin is known to split off a fragment of 25 amino acids from lactoferrin, known as lactoferricin (Yamauchi et al., 1993), and it is possible that chymosin is responsible for this cleavage too. Furthermore, spot ID 116 (identified as vitamin-D binding protein, Table 4) and spot ID 286 (immunoglobulin G λ -light chain,

Table 5. Proteins from the rennet casein or the sweet whey fraction with significant effects on cheese yield identified by electrospray quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS)

Spot ID	Protein	Sequence coverage ¹	Unique MS/MS queries ²	Theo. pI ³	Theo. Mw ⁴ (kDa)	Access key
Casein fraction						
38	α_{s2} -CN	38	10	4.60	26	P02663 ⁵
40	β -CN	9	3	5.13	23	P02666 ⁵
51	β -CN	35	13	5.13	23	P02666 ⁵
54	α_{s2} -CN	25	5	4.60	26	P02663 ⁵
57	β -CN	55	18	5.13	23	P02666 ⁵
64	α_{s1} -CN	49	15	4.90	23	P02662 ⁵
65	α_{s1} -CN	28	6	4.90	23	P02662 ⁵
78	α_{s1} -CN	31	6	4.90	23	P02662 ⁵
85	α_{s1} -CN	35	7	4.90	23	P02662 ⁵
110	β -CN	26	19	5.13	23	P02666 ⁵
119	α_{s2} -CN	60	17	4.60	26	P02663 ⁵
Whey fraction						
282	IgG λ -light chain	37	15	7.53	24	15088675 ⁶
286	IgG λ -light chain	62	22	7.53	24	15088675 ⁶

¹The minimum coverage of the matched peptides in relation to the full-length sequence.²The number of unique matched peptides in the database search.³Theoretical pI of the full length protein.⁴Theoretical molecular mass (Mw) of the full length protein.⁵Primary accession key in the SwissProt database.⁶Primary accession key in the NCBIInr database.

Table 5) were negatively associated with cheese yield expressed as GCM. However, the explanations for the significant regressions between lactoferrin, vitamin-D binding protein, or immunoglobulin G λ -light chain and cheese yield is not known.

Each of the identified PCF or PWF had a low individual effect on the cheese yield. It is possible, though, that a combination of all significant PCF and PWF could be used to predict cheese yield from the composition of chymosin separated whey and casein. It has to be stressed that the major caseins, which were not evaluated in this study, also contribute significantly to the variation in cheese yield (Wedholm et al., 2006) and therefore should be included in a prediction model. However, further studies on a more comprehensive material combined with cheese-making trials in full scale are needed to strengthen the results found in this study. To conclude whether chymosin is responsible for the casein cleavage products identified in this study, it would be relevant in a future study to compare 2-DGE pictures of chymosin-separated caseins with those of acid precipitated.

CONCLUSIONS

Several proteins in either the rennet casein or the sweet whey fractions had positive or negative effects ($P < 0.05$) on the yield of model cheeses made from the individual milk samples. In the rennet casein fraction, several protein spots were found to have positively

significant effects on cheese yield expressed as GDMP. Some of these spots were identified, by MS, to contain different fragments of α_{s1} -, α_{s2} -, and β -CN. Among the proteins in the rennet casein fraction that had positively significant effects on cheese yield, expressed as GCM, a specific β -CN fragment was identified. In the sweet whey fraction, several whey proteins had either positive or negative effects ($P < 0.05$) on the cheese yield. Of the major whey proteins identified, a spot containing β -LG had a positive effect on GCM and was concluded to be β -LG B. Further identified significant whey proteins included α -lactalbumin, lactoferrin, vitamin D-binding protein, immunoglobulin G λ -light chain, and BSA. The explanations for associations between these proteins and the cheese yield are not yet fully understood.

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