Proteomic Characterization of Specific Minor Proteins in the Human Milk Casein Fraction

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ABSTRACT: Human milk contains many bioactive proteins that are likely to support the early development of the newborn. The aim of this study was to identify whether there are specific minor proteins associated with the human milk casein micelle prepared by the acid precipitation method. Protein identification was performed by liquid chromatography tandem mass spectrometry analysis. Eighty-two proteins were identified in the casein micelle, 18 of which are not present in their whey compartment. Thirty-two of these proteins specifically associated with the casein micelle have not previously been identified in human milk or colostrum. Proteins involved in immune function comprised the major part (28%) of total proteins, and another significant part is involved in metabolism/energy production (22%). Most of the proteins were of extracellular or cytoplasmic origin (accounting for 50 and 29%, respectively). This study indicates that various soluble proteins should be considered as part of the casein compartment, prepared by the acid precipitation method. The data provide new insight not only into the proteomic profile of the human milk casein micelle and its physiological significance, but also into the proper proportion of casein and casein-associated proteins to use in infant formula.

KEYWORDS: human milk, casein, protein, proteomics, LC–MS/MS

INTRODUCTION

Human milk contains proteins and peptides that provide valuable biological functions in the newborn. These proteins are present in three compartments: the aqueous whey fraction containing the majority of soluble milk proteins, the casein micelle consisting predominantly of casein proteins, and only 4–5% in the milk fat globule membrane (MFGM) fraction.^{1–3} Most bioactive human milk proteins described to date have been found in the whey fraction; aside from the β -casein and κ -casein subunits, the human casein micelle has received little attention with regard to bioactivities.

Casein micelles are supramolecules consisting of multiple molecular entities organized by noncovalent intermolecular bonds.⁴ Casein constitutes approximately 20% of the total protein content of human milk during early lactation and ~45% during late lactation,⁵ whereas an average of 80% of total protein in bovine milk⁶ and ~70% of total protein in sheep milk⁷ is casein. The human casein fraction is a major source of calcium and phosphate for the newborn infants. Several biological activities have also been demonstrated for the major casein subunits in human milk, i.e., β - and κ -casein. β -Casein is a highly phosphorylated protein, and the closely spaced phosphoserine and phosphothreonine residues have been shown to chelate calcium and facilitate its absorption.^{8,9} Various proteolytic fragments of β -casein have been shown to have opioid activities,

which may have physiological effects on behavior, such as sleep/wake cycles.¹⁰ Human κ -casein is a heavily glycosylated protein and has been shown to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa.¹¹ Proteolytic digestion of κ -casein results in formation of a large carbohydrate-containing moiety, glycomacropeptide, which has been shown to have immune-enhancing effects.¹² There is further evidence for beneficial effects of the milk casein fraction in children. In a randomized 7 day supplementation study in prepubertal Caucasian boys, milk whey and casein fractions exhibited distinctly different growth-promoting effects by increasing fasting insulin or IGF-1 levels, respectively.¹³ Proline-rich peptides^{14,15} and c12 peptides¹⁶ with angiotensin-converting enzyme inhibitory and antihypertensive activities have been isolated from casein fractions. In addition, commercial casein hydrolysates have been found to activate the innate immune system.¹⁷

It is generally accepted that the milk casein fraction contains predominantly casein proteins and calcium phosphate.⁴ However, interactions between whey and casein proteins occur readily. In a study using diffusing wave spectroscopy (DWS), Alexander and Dalgleish found the presence of whey protein/ κ -casein aggregates, indicating a direct interaction between casein micelles and soluble whey protein complexes. They

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further confirmed that the effect was specific to the presence of casein micelles and could not arise simply from the aggregation of the whey proteins themselves.¹⁸

The objective of this study was to identify minor proteins in the human milk casein fraction prepared by the acid precipitation method.

MATERIALS AND METHODS

Milk Sample Collection

The study procedure was approved by the Institutional Review Board (IRB) at University of California Davis. Colostrum and milk samples from 1, 2, 3, 6, and 12 months of lactation were collected from 30 healthy donors. Milk was collected from one breast (at least 2–4 h after prior nursing) by manual expression (colostrum) or manual breast pump into 50 mL polypropylene containers. The complete breast was emptied and an aliquot was frozen. Mothers who delivered singleton term infants (gestational age 38–42 wks) were recruited, and mothers with maternal illnesses, such as cold, mastitis, and flu, were excluded. Samples from five women were collected at each time point. Colostrum was collected within 48 h of initiation of lactation.¹⁹ All women were exclusively breast-feeding up to 6 months, and partially up to 12 months of lactation. All samples were immediately stored at –20 °C until further analyses.

Protein Extraction

Milk samples (5 mL) were thawed at 4 °C overnight. CaCl₂ was added to a final calcium concentration of 60 mM. The pH was adjusted to 4.6, and samples were incubated at room temperature for 1 h.²⁰ The samples were then subjected to centrifugation at 13000g for 30 min twice. The top cream layer and the infranatant were removed, and the precipitates were collected as the casein fraction.

The casein pellet was washed with pH 4.6 PBS three times, then dissolved using minimal amounts of 1 N NaOH with protease inhibitors (Complete mini EDTA-free, Roche, Basel, Switzerland) added. The solution was centrifuged at 8500g for 30 min, dried, and stored at –20 °C until being reconstituted with pH 7.0 PBS buffer at 37 °C. The final concentration of casein protein was measured by the Microplate BCA protein assay kit (Pierce, Rockford, IL).

In-Solution Digestion

Proteins were digested in-solution using a standard Trypsin digestion protocol. Briefly, the proteins were dried in a vacuum centrifuge and then resuspended in 50 mM ammonium bicarbonate. The proteins were then reduced with tris(2-carboxyethyl)-phosphine (TCEP) (Pierce, Rockford, IL), alkylated by iodoacetamide, and treated with trypsin (modified trypsin, sequencing grade: Promega, Madison, WI) at a 1:50 enzyme to substrate ratio (w/w) overnight at 37 °C. The trypsin digested samples were dried and redissolved in 2% ACN/0.1% TFA for MS analysis.

LC–MS/MS

A Paradigm MG4 HPLC system (Michrom Bio Resources, Inc., Auburn, CA) coupled with a Thermo Finnigan LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA) through a Michrom Advance Captivespray source was used for protein separation and analysis. Fifteen micrograms of each digested sample were loaded onto a trap column (Zorbax 300SB-C18, 5 µm, 0.3 mm × 5 mm: Agilent Technologies

Inc., Folsom, CA) and desalted online. Peptides were then eluted from the trap and separated with a reverse-phase Michrom Magic C18AQ (200 µm × 150 mm) capillary column (Michrom Bioresources, Inc., Auburn, CA) at a flow rate of 2 µL/min. Peptides were eluted using a 90 min gradient of 2% B to 35% B over 60 min, 35% B to 80% B for 15 min, held at 80% B for 1 min, 80% B to 5% B in 1 min, and re-equilibrated for 13 min at 5% B (A = 0.1% formic acid, B = 100% acetonitrile) and directly sprayed into the mass spectrometer. The mass spectrometer was operated using a standard top 10 method, where one survey scan was followed by 10 MS/MS scans of the most intense ions eluting from the column. Dynamic exclusion was enabled.

Data Analysis

Database Searching. Tandem mass spectra were extracted by BioWorks version 3.3. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using X! Tandem (www.thegpm.org; version TORNADO (2010.01.01.4)). X! Tandem was set up to search the Uniprot human complete proteome set database (2010_07, 21 525 entries) and 110 nonhuman common laboratory contaminants from the common repository of adventitious proteins database (www.thegpm.org), plus an equal number of reverse sequences assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulphone of methionine, tryptophan oxidation to formylkynurenin of tryptophan, and acetylation of the N-terminus were specified in X! Tandem as variable modifications.

Criteria for Protein Identification. Scaffold (version Scaffold_3_00_03, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm.²¹ Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.²² Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Using these Scaffold criteria, a false discovery rate was calculated as 0.4% on the peptide level and 4.5% on the protein level using decoy/target as discussed in ref 23.

Assignment of Protein Function and Subcellular Localization. All the identified proteins were analyzed in the Human Protein Reference Database (<http://www.hprd.org/>) and UniProt Database release 15.14 (<http://www.uniprot.org/>), to obtain functional assignment and subcellular localization according to previously published evidence.

Proteomics Data Set

The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash: yuwIOhIRIjzPHypjUYyqWHk4HU1RtNAImjGYMwuYe0chMf7YMl9ZagFTkGzGZ5ShqFWkl4bZEBXeXC8liHh90WmZWMAAAAAAAAAAZPa==.

Table 1. Proteins Identified in Acid-Precipitated Human Milk Casein^a

serial number	protein name	accession number	MW (kDa)	number of unique peptides	sequence coverage (%)
1	14-3-3 protein zeta/delta	P63104	28	3	14
2	actin, cytoplasmic 1	P60709	42	9	36
3	alcohol dehydrogenase	P14550	37	3	12
4	alpha-1-antichymotrypsin	P01011	48	7	25
5	alpha-1-antitrypsin	P01009	47	4	14
6 ^b	alpha-2-macroglobulin-like protein 1	A8K2U0	161	2	4.3
7	alpha-amylase 1	P04745	58	2	7.4
8	alpha-enolase	P06733	47	4	15
9	alpha-lactalbumin	P00709	16	15	52
10	alpha-S1-casein	P47710	22	16	79
11	apolipoprotein A-I	P02647	31	4	22
12	apolipoprotein D	P05090	21	2	14
13 ^b	ATP-binding cassette subfamily G member 2	Q9UNQ0	72	3	6.4
14	beta-1,4-galactosyltransferase 1	P15291	44	5	20
15	beta-2-microglobulin	P61769	14	3	35
16	beta-actin-like protein 2	Q562R1	42	2	12
17	beta-casein	P05814	25	44	83
18	bile salt-activated lipase, carboxyl ester lipase	P19835	79	23	34
19	butyrophilin subfamily 1 member A1	Q13410	59	24	41
20	carbonic anhydrase 6	P23280	35	5	30
21	chitinase-3-like protein 1	P36222	43	2	7.3
22 ^b	chloride intracellular channel protein 1	O00299	27	2	12
23	chordin-like protein 2	Q6WN34	47	10	33
24	clusterin	P10909	52	15	31
25	complement C3	P01024	187	13	14
26	complement C4—B	P0C0L5	193	19	15
27 ^b	cytosolic nonspecific dipeptidase	Q96KP4	53	2	4
28 ^b	endoplasmic	P14625	92	2	3.1
29	fatty acid synthase	P49327	273	41	24
30	fatty acid-binding protein, heart	P05413	15	8	57
31	galectin-3-binding protein	Q08380	65	4	12
32 ^b	glutathione peroxidase 3	P22352	26	2	13
33	glyceraldehyde-3-phosphate dehydrogenase	P04406	36	3	13
34	golgi-associated plant pathogenesis-related protein 1	Q9H4G4	17	2	17
35 ^b	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	P62873	37	2	5.3
36	haptoglobin	P00738	45	2	9.1
37 ^b	heat shock protein beta-1	P04792	23	2	13
38 ^b	heat shock protein HSP 90-alpha	P07900	85	3	4.4
39	Ig alpha-1 chain C region	P01876	38	15	59
40	Ig alpha-2 chain C region	P01877	37	2	29
41	Ig gamma-1 chain C region	P01857	36	2	10
42	Ig kappa chain C region	P01834	12	8	89
43	Ig kappa chain V—III region SIE	P01620	12	2	38
44	Ig lambda-2 chain C regions	P0CG05	11	6	86
45	Ig lambda-7 chain C region	A0M8Q6	11	2	48
46	Ig mu chain C region	P01871	49	5	18
47	immunoglobulin J chain	P01591	18	4	26
48	kappa-casein	P07498	20	16	55
49	lactadherin	Q08431	43	17	57
50	lactoferrin	P02788	78	86	82
51	leucine-rich alpha-2-glycoprotein	P02750	38	2	13
52 ^b	leucine-rich repeat-containing protein 16C	Q6F5E8	155	2	2.2
53	lipoprotein lipase	P06858	53	5	20

Table 1. Continued

serial number	protein name	accession number	MW (kDa)	number of unique peptides	sequence coverage (%)
54	lysozyme C	P61626	17	2	27
55	macrophage mannose receptor 1	P22897	166	4	5.1
56 ^b	malate dehydrogenase, cytoplasmic	P40925	36	2	8.7
57	monocyte differentiation antigen CD14	P08571	40	5	29
58	mucin-1	P15941	122	2	2
59	mucin-4	Q99102	232	4	3.1
60	osteopontin	P10451	35	3	12
61	peptidyl-prolyl cis—trans isomerase A	P62937	18	3	27
62	perilipin-2	Q99541	48	30	76
63	perilipin-3	O60664	47	9	40
64 ^b	platelet glycoprotein 4	P16671	53	3	9.1
65	polymeric immunoglobulin receptor	P01833	83	22	39
66	prolactin-inducible protein	P12273	17	2	15
67	protein disulfide-isomerase	P07237	57	2	3.9
68 ^b	protein QN1 homologue	Q5TB80	162	2	2.8
69	rab GDP dissociation inhibitor beta	P50395	51	2	7
70 ^b	ras-related protein Rab-18	Q9NP72	23	3	18
71 ^b	rho-related GTP-binding protein RhoC	P08134	22	2	14
72	serum albumin	P02768	69	10	21
73	sulfhydryl oxidase 1	O00391	83	2	3.7
74 ^b	syndecan-4	P31431	22	2	9.1
75	tenascin	P24821	241	58	36
76	thrombospondin-1	P07996	129	3	4.5
77	triosephosphate isomerase	P60174	27	3	19
78	UTP—glucose-1-phosphate uridylyltransferase	Q16851	57	3	9.3
79	vitronectin	P04004	54	2	6.3
80	xanthine dehydrogenase/oxidase	P47989	146	39	41
81	zinc-alpha-2-glycoprotein	P25311	34	3	20
82 ^b	zymogen granule protein 16 homologue B	Q96DA0	23	2	12

^a Accession numbers were assigned according to UniProt database Release 15.14. The complete identification statistics can be found in the Scaffold located in the proteome data set uploaded to the Tranche proteome commons repository. Serial number of proteins not previously detected in human milk whey are in bold. ^b Proteins not found in the whey counterpart.

RESULTS AND DISCUSSION

In this study, data from milk casein collected at different time points of lactation were pooled and compared with a previous study, in which we analyzed the whey²⁴ and milk fat globule membrane²⁵ counterparts of the same samples. In the casein fractions of this study, 82 proteins with molecular weights ranging from 11 to 273 kDa were identified (Table 1). Eighteen of these proteins were not present in their whey counterpart and 32 of them have previously not been identified in human milk or colostrum.

This study was a first attempt to examine whether there are soluble proteins associated with the casein micelle prepared by an acid-induced casein precipitation method, which is widely used for casein isolation. The data clearly indicated that other than caseins, there are specific minor proteins trapped in the micelle, which could be due to the following reasons: (1) Casein micelles are highly hydrated spheres,²⁶ with approximately 4 g of water/g of total protein.²⁷ Water within the casein micelle not only explains the considerable conformational flexibility, but also serves as a medium for soluble proteins; however, it is unlikely that the proteins identified were only “trapped”, as they were not

found in the whey fraction, suggesting specific association to the casein micelle. (2) Upon acid treatment, casein micelles start to aggregate when the pH goes below the isoelectric point of around 4.9,²⁸ but this also introduces formation of whey protein aggregates or whey gelation with the casein micelles, resulting in “whey protein-coated casein micelles”, similar to the situation of other denaturation procedures, such as heat treatment or changing the salt content (ionic strength) of the aqueous phase.^{29–31} It is unlikely, however, that these whey aggregates would be so specific that there are no remainders in the whey fraction. Thus, it is likely that the unique whey proteins instead are specific components of the casein micelle and are associated with casein subunits during synthesis/excretion of milk by the mammary gland.

Therefore, the casein should rather be called the “casein fraction” or “casein complex”, taking into account soluble proteins associated with casein. It should be noted that structural transitions constantly occur in the milk casein fraction,⁴ and the structure of the casein micelle results directly from interactions with nonmicellized components of the milk; results from this study may possibly represent this interaction. It is difficult to determine what the true “physiological” proteome of the casein micelle is, partly because the micelles range over a fairly wide

Figure 1. Pie graph representation of functional characterization of unique proteins identified in human milk casein. Numbers indicate the percentage share of each category. The abbreviations used are C;S (cell communication/signal transduction); I (immune response); G;M (cell growth and/or maintenance); T (transport); P (protein metabolism); M;E (metabolism/energy pathways); N/A (unknown).

range of size distribution and are difficult to isolate in “native” form without changing equilibria, even with differential centrifugation techniques. Thus, we resorted to the “classical” casein micelle isolation procedure, using acid, but optimized the conditions to avoid coprecipitation of other proteins.

Human milk casein mainly consists of β -casein and κ -casein, and in contrast to bovine milk, α -caseins are usually considered absent or present in very low concentrations in human milk.^{32,33} We found β -casein, κ -casein, and α_{S1} -casein, but not α_{S2} -casein. Thus, α_{S2} -casein could be considered to be absent from human milk. Biological functions have been suggested for bovine α -caseins, but it is uncertain whether the human form will have any activity at this low concentration.

The protein profile was further explored by grouping the proteins into nine categories according to their functions (Figure 1). Compared to the casein fractions' whey counterpart, no proteins that function in nucleobase, nucleoside, nucleotide, and nucleic acid regulation were detected, and percentages of proteins involved in transport, cell communication/signal transduction, protein metabolism, and growth/maintenance are similar. Proteins involved in metabolism/energy production were lower in casein by 5.5% (22.0 in whey vs 16.5% in casein), and although proteins involved in immune function are somewhat lower in casein (34.8 in whey vs 28.0% in casein), immunity regulation is still the major functional group in casein, among which Golgi-associated plant pathogenesis-related protein 1 (PR-1) has not previously been identified. Tissue screening has revealed that high expression of PR-1 is found in monocytes, leukocytes, lung, spleen, and embryonic tissue, and the protein localizes to lipid-enriched microdomains in the Golgi complex.³⁴ Interestingly, while proteins involved in cell communication/signal transduction comprise 18.3% of the total proteins, 40% of them are located at the plasma membrane, including lactadherin, macrophage mannose receptor 1, mucin 1, mucin 4, Rho-related GTP-binding protein RhoC, and syndecan-4 (Table S1, Supporting Information). The major function of caseins is general transport, and aside from caseins, four new proteins were detected with general transport functions: platelet glycoprotein 4 (CD36) is suggested to transport long-chain fatty acids across the cell membrane and regulates cellular fatty acid uptake;³⁵ chloride intracellular channel protein 1 is a chloride ion permeable protein transiently expressed in the cell membrane in

Figure 2. Pie graph representation of cellular localization distribution of unique proteins identified in human milk casein. Numbers indicate the percentage share of each category. N/A (unknown).

response to certain stimuli, e.g., oxidative stress;³⁶ ATP-binding cassette subfamily G member 2, isoform 1, functions in the efflux of xenobiotic compounds and exhibits broad substrate specificity;^{37–39} and Ras-related protein Rab-18 is strongly suggested to be involved in ER–Golgi trafficking.⁴⁰ It is possible that some of these proteins exert bioactivities in the breast-fed infant; however, it is likely that some of them reflect metabolic activities in the mammary gland occurring during milk biosynthesis.

The soluble proteins identified from milk casein were also divided into five groups based on their subcellular localization (Figure 2). Similar to what we have previously observed in the aqueous whey fraction, extracellular proteins are the predominant components of the casein fraction, in which they comprise 50.0% of total proteins. Cytoplasmic proteins (29.3%) and plasma membrane proteins (14.6%) were proportionally higher than in their whey counterpart (data not shown). It should be noted that there was one nucleus protein reported in this study, chloride intracellular channel protein 1, which is a chloride ion permeable protein transiently expressed in the cell membrane in response to certain stimuli, e.g., oxidative stress.³⁶

In summary, this study is the first to examine the composition of minor proteins in the human milk casein fraction prepared by an acid precipitation method. Results obtained showed that the functions of casein minor proteins span a broad spectrum, which may help to provide a better understanding of the physiological functions of the human milk casein fraction and may also provide suggestions for improved infant formulas by using selected fractions of bovine milk proteins. Some bovine milk proteins (e.g., lactoferrin and TGF- β) have been shown to exert bioactivities similar to those of their human milk counterparts and may therefore be considered for addition to infant formula. A better understanding of the human casein fraction and the biological activities of its constituents may help, as both specific protein constituents from bovine milk as well as different casein fractions prepared by methods such as acid precipitation or rennet treatment are commercially available.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: Names, functions, and locations of proteins identified in human milk casein fraction. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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