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Proteomic evaluation of milk from different mammalian species as a substitute for breast milk

ENZA D'AURIA¹, CARLO AGOSTONI¹, MARCELLO GIOVANNINI¹, ENRICA RIVA¹,
ROLF ZETTERSTRÖM², RICCARDO FORTIN³, GIAN FRANCO GREPPI³, LUIGI BONIZZZI⁵
& PAOLA RONCADA^{3,4}

¹Department of Paediatrics, San Paolo Hospital, University of Milan, Milan, Italy, ²Karolinska Hospital, Stockholm, Sweden, ³Department of Veterinary Clinical Sciences, University of Milan, Milan, Italy, ⁴Italian Experimental Institute "L Spallanzani", Milan, Italy, ⁵Department of Animal Pathology, Hygiene, and Veterinary Public Health, University of Milan, Milan, Italy

Abstract

As milk represents the main source of nutrition for infants, the question of an effective human milk substitute becomes mandatory when a formula-fed baby is allergic to cows' milk proteins. In this case, formulas containing extensively hydrolysed milk proteins should be preferred, but even such a formula may cause allergic reactions in highly sensitive patients. If there is evidence of cows' milk allergy with IgE-associated symptoms, after 6 mo of age, a soy bean formula may be recommended only when tolerance to soy protein has been established by clinical challenge. In infants with allergic reactions to cows' milk proteins, even after extensive hydrolysis, proteomic techniques coupled to immunological methods may make it possible to select other milk products that do not contain the same allergens as ordinary cow's milk. In this paper, evidence will be presented that proteomic evaluation of proteins from different mammalian species may be a suitable method of testing whether proteins from the milk of different mammalian species may be used as a substitute for untreated bovine milk.

Conclusion: Proteomic evaluation of milk from different mammalian species may not only be of help when recommending suitable feeding in cases of cows' milk allergy but also gives new insight into the background to allergic reactions caused by milk proteins.

Key Words: Cows' milk allergy, infant feeding, proteomic technique

Introduction

Cows' milk allergy is the most common food allergy in childhood, with a reported prevalence of 2–7.5% [1]. In such instances, and when breast milk is not available or may not be advisable, such as when the mother is HIV positive, the only efficient treatment is to avoid the allergen. As milk represents the main source of nutrition for infants, research to find the most valid alternative to human milk has a high priority. To be nutritionally adequate, any substitute should have the same nutritional characteristics as breast milk. In addition, it should be hypoallergenic and palatable [2].

Therapeutic formulas based on extensively hydrolysed proteins should represent the preferred choice in the treatment of cows' milk protein allergy. Such formulas, however, have several drawbacks. In addition to being expensive, they have a bitter taste, at the same time as there is a risk of cross-reactions in highly sensitive infants. These types of formula may also contain residual allergenic epitopes [3,4]. As an alternative to hydrolysed and hypoallergenic cow's milk formulas, soy bean-derived formulas and milk from mammalian species other than cows have been used. Although several extensive clinical studies have documented that formulas based on soy bean are adequate from a

nutritional point of view [5,6], they are not always safe from an allergological standpoint [7,8]. For this reason, some other mammalian milks have been proposed as alternatives to hydrolysates of cow's milk proteins and soy bean formulas.

Milk from different species varies most notably regarding protein quality and concentration but also in the content of electrolytes, various carbohydrates and lipids. A proteomic approach seems to be of great value due to its unique capacity to obtain simultaneous high-resolution analyses of the complex mixture of proteins in milk [9]. A few proteomic analyses of milk proteins have been reported for humans [10,11] and some mammalian species such as cow [12,13], goat [14] and mare [15]. However, inter-species proteomic evaluation of milk proteins has not yet been reported.

In this paper we report the results of milk proteomes from various animal species analysed by two-dimensional gel electrophoresis (2-DE) after they have been coupled to mass spectrometry (MS).

Theoretical background to proteomic analysis of milk proteins

In response to the availability of complete genome sequences for numerous organisms, the field of proteomics has been applied with the aim of developing methods which will permit functional analysis of various proteins [16,17]. Generally, proteomics means protein expression and function; two characteristics which complement each other. Protein expression relies on combined 2-DE, protein staining and MS for the separation, detection and identification of proteins [18]. In modern proteomics, 2-DE coupled with MS is the cornerstone. Such a technique permits the simultaneous evaluation of the relative abundance and modifications of numerous endogenous proteins. New proteins which are formed during discrete physiological and/or pathological states can thus be identified in this way [19].

Functional proteomics for the identification and characterization of proteins by a proteomic approach, e.g. by the analysis of target proteins instead of entire proteomes, is used for many directed proteomic analyses.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [20], in which the proteins are separated by isoelectric focusing (IEF) in the first dimension according to their charge (pI), and by SDS-PAGE in the second dimension according to their size (Mr), gives a unique capacity to resolve complex protein mixtures such as those in milk and can simultaneously analyse up to 1000 gene products. Once separated and stained, a special protein can be identified after digestion with trypsin or another protease, the process of which is followed by estimation of the

molecular masses of the different peptides by the use of mass spectroscopy (matrix-assisted laser desorption ionization/time of flight, MALDI-TOF) and peptide mass fingerprint (PMF) [21]. The unique PMF for each protein makes it possible to identify them and also to compare the theoretical mass fingerprints with the primary sequence of every known protein.

Material and methods

Fresh mature milk of the studied species was collected at an appropriate time from healthy individuals. The milk was immediately frozen at -20°C until analysed. When analysed, the milk samples were centrifuged at 10 000 *g* for 5 min at 4°C . The fat layer was then carefully removed [22]. After protein quantification with 2D-Quant Kit (Amersham Biosciences), 1000 μg of protein was subjected to micropreparative gels and 100 μg to analytical gels after dilution to 300 μl with a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 1.6% Ampholine of pH 4–6.5. After a short vortex, the samples were subjected to ingel-rehydration loading overnight for 20 h at 20°C using pH 4–7L 11-cm-long homemade IPG strips (4% T, 3% C). For analytical gels, pH 3–10NL 18-cm-long IPG strips (Amersham Biosciences) were used. After full incorporation of the samples, IPG strips were transferred to a MANIFOLD-equipped IPGphor II (Amersham Biosciences). IEF was then performed at 20°C by a series of increasing voltage "steps" as follows: 50 V for 2 h, 100 V for 2 h, 500 V for 2 h, 1000 V for 3 h, 3000 V for 3 h, 4000 V for 3 h, 5000 V for 3 h, 6000 V for 2 h, and 8000 V which was held constant until a total of 65 000 V/h.

After the first dimension, IPG strips were equilibrated twice for 15 min under gentle stirring with a solution containing 6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8 and 30% glycerol. To the first equilibrium, 1% DTT was added and to the second 2.5% iodoacetamide and a trace of bromophenol blue. The second dimension was performed by the use of homemade 10–15% acrylamide gradient vertical SDS-PAGE slab gels of dimension $170 \times 160 \times 1.5$ mm on a Ruby SE-600 electrophoresis system (Amersham Biosciences) for micropreparative gels. For analytical gels, a second dimension separation was carried out on homemade 14% acrylamide vertical SDS-PAGE slab gels of dimension $200 \times 200 \times 1$ mm. The samples were run overnight (Ettan Dalt six, Amersham Biosciences) and limited to 1.5 W/gel [23].

IPG strips were put on top of the SDS gels which were poured up to 1 cm from the top of the plates and then sealed with 1.5 ml of a solution containing 0.5% low-melting-point agarose diluted in hot SDS running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS). Markers from Invitrogen (50 μl

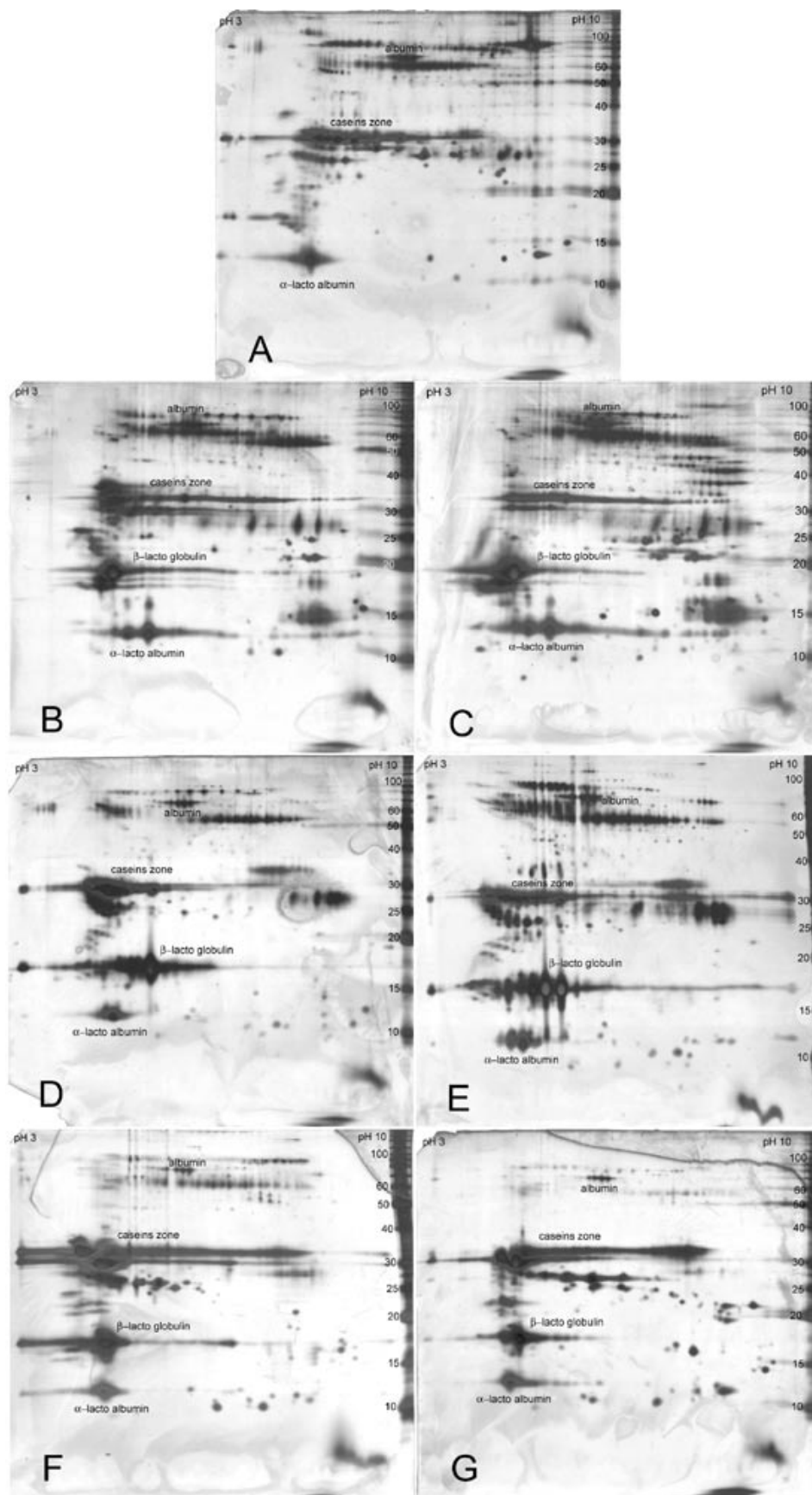


Figure 1. Two-dimensional electrophoresis (silver stained) of breast milk (A), and milk from horse (B), donkey (C), goat (D), sheep (E), cow (F) and water buffalo (G). In the figure, the major protein zones have been identified by matching and mass spectrometry, as described in the text.

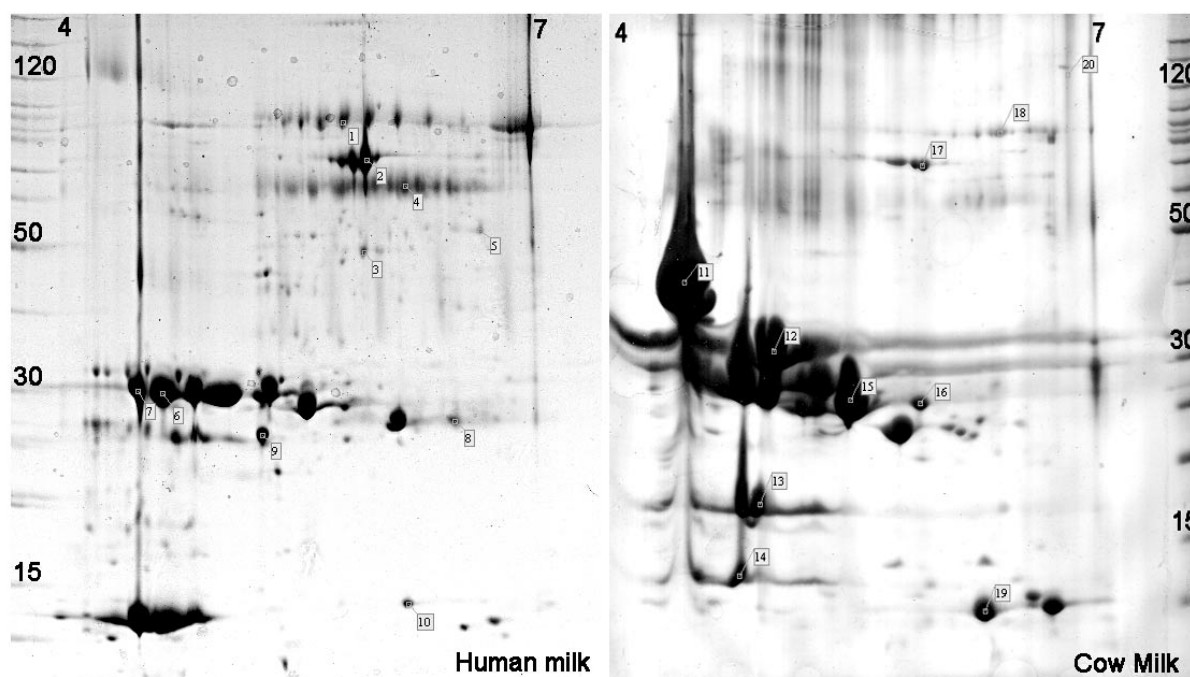


Figure 2. Two-dimensional electrophoresis (colloidal Coomassie G-250 stained) of human (A) and bovine (B) milk. The numbers correspond to proteins which have been identified by MALDI-TOF MS, as shown in Table I.

on paper pads) of 10–220 kDa molecular weight were applied on one end of the IPG strips. In the second dimension protocol, the 15 mA/gel was run for 20 min at 100 mA until the bromophenol blue front-line came out of the gels. Analytical gels had been stained with silver nitrate as shown in Figure 1, while micro-preparative gels were stained with colloidal Coomassie Brilliant Blue G-250, as shown in Figure 2.

Coomassie blue-stained spots were excised from 2-DE gels followed by digestion of the proteins by trypsin gel. Excised gel pieces were de-stained by several washings with 50%/50% 5 mM NH_4HCO_3 /acetonitrile (ACN). The gel slices were then rehydrated for 45 min at 4°C in 20 μl of a digestion buffer containing 10 ng/l trypsin and 5 mM NH_4HCO_3 [24]. After the protease solution had been removed, the volume was adjusted with 5 mM NH_4HCO_3 until the gels were covered. Digestion was then performed overnight at 37°C. For the mass spectrometer, a Tofspec SE (Micromass, Manchester, UK) equipped with a delayed extraction unit was used. The laser wavelength was 337 nm and the accelerating voltage 20 kV [25].

Peptide spectra were obtained in a reflectron mode in the range of 800–4000 Da. The peptide solution was loaded on a MALDI target plate after mixing 1.5 μl of each solution with the same volume of a matrix solution which was prepared by dissolving 10 mg/ml α -cyano-4-hydroxycinnamic acid solution in 40% acetonitrile-0.1% trifluoroacetic acid (vol/vol) and then allowed to dry. External calibration was performed by using the fragment ions from the standard

peptides (adrenocorticotrophic hormone 18–39 and angiotensin I). Each mass spectrum was then generated by the accumulation of data from 100–120 laser shots [26]. Database searches were performed with the peptide masses against the SWISSPROT or the non-redundant NCBI database using the search programs Peptident [27] and ProFound [28], respectively. In the searches, the following parameters were used: taxa Homo sapiens, Bos Taurus or other mammalian, protein molecular mass ranging from 10 to 100 kDa, trypsin digest, monoisotopic peptide masses, out of which one or two had not been cleaved by trypsin and mass deviation of 500 ppm. Chemical modifications such as carbamidomethylation of cysteine and oxidation of methionine were taken into account. To improve the quality of the identification of bovine proteins, Peptident was found to be more suitable for the identification of secreted mature proteins, as previously described [12].

Results

Two-dimensional electrophoresis of skimmed milk from some mammalian species is shown in Figure 1. On the 2-DE gels of each milk sample, four main protein spots have been highlighted (albumin, caseins, β -lactoglobulin and α -lactalbumin). One or more minor alterations were seen due to post-translational modifications such as proteolysis, phosphorylation and glycosylation. From the protein patterns in the 2-DE gels from the species studied, it is evident that animals

Table I. Proteins from human and bovine milk which have been separated and identified by means of MALDI-TOF MS.

Protein	NCBI/Swiss prot acc. no.	pI calc.	MW (kDa) calc.	Spot number (see Fig. 2)	Matched peptides	Sequence coverage (%)
<i>Human milk</i>						
Poly-Ig receptor	gi 514366	5.38	76.44	1	24	40
Serum albumin	gi 4389275	5.69	67.99	2	27	62
	gi 4389275	5.69	67.99	3	19	52
Immunoglobulin alpha-1 in heavy chain constant region	gi 184749	6.06	38.41	4	14	51
Adipophilin fragment	gi 34577059	6.34	48.27	5	20	57
Beta-casein	gi 288098	5.52	25.29	6	5	29
	gi 288098	5.52	25.29	7	5	29
Immunoglobulin kappa light chain, VLJ region	gi 21669355	6.81	29.16	8	5	33
Alpha S1-casein	gi 1359714	5.17	20.88	9	8	34
Fatty acid-binding protein	gi 227994	7.00	14.77	10	10	68
<i>Bovine milk</i>						
Alpha-S1 casein	P02662	4.98	24.57	11	7	37
Alpha-S2 casein	P02663	8.54	26.17	12	6	41
Beta-lactoglobulin	P02754	5.02	20.27	13	6	65
Alpha-lactalbumin	P00711	4.93	16.69	14	7	42
Kappa casein	P02668	5.93	18.97	15	11	41
	P02668	5.93	18.97	16	11	41
Serum albumin	P02769	5.82	71.24	17	24	47
Lactoferrin	P24627	8.69	80.00	18	12	27
S100 calcium-binding protein	P79342	5.50	11.23	19	3	24
Xanthine dehydrogenase	gi 1620375	7.97	148.87	20	22	17

which are phylogenetically related, such as sheep/goat, cow/water buffalo, and horse/donkey, have quite similar milk protein expression. On the other hand, some differences are found which may be due to the fact that milk proteins occur in heterogeneous populations of isoforms which are subjected to post-translational modifications.

Milk from various species differs qualitatively and quantitatively. Thus, protein expression of human and cow/water buffalo milk differs remarkably, whereas human milk is more similar to that of horse/donkey and goat/sheep. Among potentially allergenic milk components, β -lactoglobulin, which is present in all studied mammalian milks, is almost absent in human milk. Furthermore, this protein is very heterogeneous in goat and sheep milk. Also, the casein population is quite heterogeneous, with rich components in milk from cow, water buffalo, sheep and horse, whereas it is relatively poor in milk from humans and donkey.

Figure 2 shows 2-DE maps of human and bovine milk after quantitative staining with Coomassie G-250. Each map has been labelled with numbers of some of the proteins which have been identified by means of MALDI-TOF MS as shown in Table I.

Discussion

Milk from various mammalian species has been proposed to be suitable as a substitute in feeding those

infants who develop severe allergic reactions when fed formulas based on soy bean or cow's milk, even if the proteins have been extensively hydrolysed. However, dietary compliance (especially in the case of hydrolysates) may also be poor because of an unpleasant taste. Although milk from mare [29] and donkey [30] has been used in a few infants with cow's milk allergy, their protein profile has not been fully examined.

Two-dimensional electrophoresis is an important means of obtaining information about the composition of milk proteins. Thus, in a 2-DE map, it is possible to separate and discriminate different individual proteins, but also to get information about their subsets of isoforms and post-translational modifications, for instance, by phosphorylation and glycosylation [12]. Moreover, 2-DE technology can easily be coupled to MS as a suitable technique for the identification of single protein spots.

By proteomic studies of the composition of proteins in milk, detailed information may be obtained not only about the content of different proteins but also about the heterogeneity and complexity of different milks. By coupling such findings to immunological techniques such as 1-D SDS-PAGE, an in-depth screening of the allergenicity of milk samples in relation to the reaction of patients can be performed. When most of the milk proteins from different species have been identified and mapped, homologous epitopic sequences may be analysed by means of immunoblotting in 2-DE.

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