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Detailed proteomic analysis on DM: insight into its hypoallergenicity

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1. ABSTRACT

Successful therapy in cow milk (CM) protein allergy rests upon completely eliminating CM proteins from the child's diet: it is thus necessary to provide a replacement food. Donkey milk (DM) has recently aroused scientific and clinical interest, above all among paediatric allergologists. A deeper knowledge of proteins in DM is necessary to evaluate the immunological and physiological properties of this natural substitute for cow's milk. The paper offers a detailed comparative analysis among the protein fractions of DM, CM and human milk, following an extensive proteomic study of the casein and whey proteins of DM performed by narrow pH range 2-DE. The detailed protein composition and structural features reported in this study provide insight into the molecular reasons for the hypoallergenicity of DM. Whole DM might constitute a valid substitute of CM in feeding children with CM protein allergy and it might also constitute the basis for formulas suitable for allergic subjects in the first year of life.

2. INTRODUCTION

Cow's milk protein allergy (CMPA) chiefly occurs in childhood, involving approximately 3% of children below the age of three (1) Successful therapy depends upon completely eliminating cow milk (CM) proteins (CMP) from the child's diet. Ideally, the replacement food should be hypo- or anallergenic, non-cross-reactive with CM, nutritionally adequate and palatable – the latter being fundamental in view of the young age of these patients (2) At present the ideal formula, meeting all these requirements at once, does not exist, and nor is there an international consensus on which formula should be considered first choice in treating CMPA when the mother's milk is not available (3).

The possibility of using milk from other mammalian species for infants and young children with CMPA has already been examined, and it has been shown that goat's and sheep's milks are contraindicated since their

proteins cross-react extensively with CMP both *in vitro* and *in vivo (*4 - 6) Mare's milk appears to be more promising, since its composition is much closer to human milk (HM) than to CM (7, 8): mare's milk has been found to be tolerated by some children with severe IgE-mediated CMPA (9); however, its availability is limited and collection is difficult. Donkeys, as well as horses, belong to the *Equus* family and this phylogenetic relationship emerges in the similarity of their milk composition (10) Donkey milk (DM) has recently aroused scientific interest above all among paediatric allergologists. DM hypoallergenic features have recently been demonstrated in 38 of 46 highly problematic and pluriallergic children (83% tolerability) (2) A similar figure (88% tolerability) was reported by Vita *et al* (11) in a study assessing the tolerability and clinical effect of DM compared with goat milk in a single-blind, controlled, randomised study on twenty-eight CMP allergic children with atopic dermatitis.

The use of natural milk rather than a formula, during the CM-free diet period, should be encouraged because of its content of functional proteins and peptides that have immunological-like properties and are able to stimulate the functional recovery and development of the neonatal intestine (12, 13)

Although several studies have been published on the composition, the physico-chemical and nutritional properties of DM protein components (14 – 16) and a few on the characterisation of genetic variants of whey proteins (17), also using proteomics techniques (18), a deeper knowledge of proteins in DM is necessary to better understand the immunological and physiological properties of this natural substitute for cow milk. The paper offers a detailed comparative analysis among the protein fractions of HM, CM and DM following an extensive proteomic study of the casein and whey proteins of DM, in order to give insight at molecular level of the clinical tolerability of DM among CM allergic children.

3. MATERIALS AND METHODS

3.1. Sample preparation

Holder-pasteurized CM (commercial), HM (milk bank of OIRM - S. Anna Hospital. Turin - Italy) and pooled DM (Montebaducco farm, Reggio Emilia - Italy) were skimmed by centrifugation at 2000g for 30' at 4°C. Fat fraction and skimmed milk were stored at -20°C until use.

3.2. Protein separation - 2DE

Total protein concentration was determined using the BCA-Kit (Pierce, Rockford, IL, USA).

Skimmed milks were diluted with rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM Tris, either 0.2% BioLyte Ampholyte (Bio-Rad, Hercules, CA) or 0.5% ampholytes (GE Healthcare Life Sciences, Uppsala, SE), 1 % DTT (and a trace of bromophenol blue) and applied to either 11 cm ReadyIPG strips (Bio-Rad, Hercules, CA) with linear pH gradient 3-6, or 13 cm strips with linear pH gradient 3-10 (GE Healthcare Life Sciences) at a uniform total protein load of 160 µg per strip.

The 3-6 pH gradient strips were focused on a Protean IEF Cell (Bio-Rad, Hercules, CA) with a rapid linear voltage slope until 30000 Vhrs were reached. The 3-10 pH gradient strips were focused on an IPGphor unit (GE Healthcare Life Sciences, Uppsala, SE) at 20°C constant temperature. During IEF the voltage was kept at 30 V and at 60 V for 6h, at 1000 V, 3000V, and 5000V for 30 minutes, at 5000-8000V for 90 minutes and at 8000 V, until 48000 Vhrs were reached.

After IEF the IPG gel strips were incubated at room temperature for 15 min in 6 M urea, 30% w/v glycerol, 2% w/v SDS, 2% DTT, 50 mM Tris HCl, pH 8.6. A second equilibration step was carried out for 15 min in the same solution, with the exception that DTT was replaced by 4.5% iodoacetamide plus a trace of bromophenol blue as tracking dye.

SDS-PAGE (Hoefer SE 600, GE Healthcare Life Sciences) was carried out on homogeneous running gels 12% T, 2.7% C (Duracryl, Genomic Solution Ltd.) The running buffer was 25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3 and running conditions were 11°C, 400 V constant voltage, 50 mA/gel until the bromophenol blue reached the bottom of the gels.

Molecular weight markers were the Low Molecular Weight Electrophoresis Calibration Kit (GE Healthcare Life Sciences, Uppsala, SE), CandyCane (Invitrogen, Carlsbad, CA, USA) molecular weight markers (a mixture of glycosylated and non-glycosylated protein standards) and PeppermintStick Phosphoprotein Molecular Weight Standards (Invitrogen, Carlsbad, CA, USA)

3.3. Staining and imaging

A series of stains was applied to the gel, as follow: first, the gels were always stained with Pro-Q Emerald 488 Glycoprotein Gel Stain (Invitrogen, Carlsbad, CA, USA) or Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instruction; the gels were then stained with both SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) stain and Blue G250 colloidal (GE Healthcare Life Science, Uppsala, SE) for total protein profile.

Gels were imaged using a ProXPRESS™ 2D Proteomic Imaging System (PerkinElmer, Shelton, CT, USA), a multi-wavelength imaging platform equipped with a 16-bit slow-scan monochrome CCD camera cooled to -35°C and the ProSCAN 4.0 image acquisition software.

All gel images were acquired with 16-bit gray scale depth. Fluorescent stains were imaged using a Xenon arc and the top illumination mode of the instrument. Pro-Q Diamond gel stain was imaged using 540/25 nm excitation and 590/35 nm emission filters; Pro-Q-Emerald was imaged using 480/30 nm excitation and 530/30 nm emission filters; SYPRO Ruby protein gel stain was imaged using 460/80 nm excitation and 650/150 nm emission filters. A UV-to-visible light converter was employed to image the Brilliant Blue G colloidal stain, using the

installed neutral density emission filter. Images were typically acquired at a resolution of 100 µm.

Each 16-bit TIFF image was subsequently analyzed using Progenesis 220 Software (Non linear Dynamics, Newcastle, UK) Gel images, pseudocolored blue and yellow, respectively for Brilliant Blue G colloidal and Pro-Q Emerald or Pro-Q Diamond stains, were aligned and automatically overlaid using the warping algorithm to determine total protein expression as well as post-translational modification levels on the same gel.

3.4. Protein identification

DM proteins separated by 2DE were identified either by N-terminal sequencing, after passive adsorption on PVDF membrane, or by MALDI TOF, MALDI TOF-TOF, LC-nano-ESI-ion trap, or by a combination of two of them.

For passive adsorption, spots of interest were excised and dried in Speed Vac. The gel pieces were reswollen in 200mM Tris/HCl, 2% SDS, pH 8.5. After swelling, distilled H_2O and a small piece of prewetted (in methanol) PVDV membrane were added. After 24h, methanol was added to a final concentration of 10%. Adsorption was allowed for about 4-5 days at RT. The membrane was washed 5 times with 10% methanol vortexing. The membranes were air dried and analyzed on an Applied Biosystems 492 Procise sequencer in pulse-liquid mode (Applied Biosystems, Foster City, CA, U.S.A.) All chemicals were from Applied Biosystems.

For MALDI TOF and LC-nano-ESI-ion trap analysis, "in gel" digestion was performed: portions of the gel containing protein spots of interest were cut and destained overnight in a solution of 25 mM ammonium bicarbonate and 50% ethanol, after which the gel was washed three times with 25 mM ammonium bicarbonate for 15 minutes and three times with acetonitrile under stirring and, finally, dried in a Speedvac. Dried gels were rehydrated with modified trypsin (Promega, Madison, WI, USA), in 25 mM ammonium bicarbonate overnight at 37°C under shaking.

For MALDI-TOF mass spectrometry, 0.5 µl of each peptide mixture obtained from "in gel" digestion were applied to a target disk and allowed to dry; 0.5 µl of matrix solution (alpha-cyano-4-hydroxycinnamic acid in 30% acetonitrile, 0.1% TFA) were then applied to the dried sample and allowed to dry under vacuum. Spectra of protein digests were obtained using an Ultraflex II MALDI-TOF-TOF (Bruker Daltonik, Bremen, Germany) MS-Fit (http://prospector.ucsf.edu/) software was used to interpret the MS spectra by the peptide mass fingerprinting (PMF) method and Mascot Search software (http://www.matrixscience.com/) to interpret MS/MS spectra.

For LC-nano-ESI-ion trap analysis (LC MS/MS) (LC/MCD-Trap-XCT-Ultra, Agilent-Technologies), the peptide mixtures were separated on a Reverse Phase C18 column (Zorbax 300SB-C_{18}; 150×0.075 3.5µm), using a 5-70% acetonitrile gradient in 0.1 % formic acid over 55 minutes, with a flow rate of 0.3 µl/min. The spectra were acquired in *Data dependent scan* modality and analyzed using Mascot Search (http://www.matrixscience.com/) and Spectrum Mill (Agilent Technologies, Palo Alto, CA) software.

4. RESULTS AND DISCUSSION

Detailed analytical data on DM protein composition, such as to enable a comparative study at the molecular level with HM and CM, are still to a great extent lacking. In this study, classical proteomic techniques were applied to characterize the main DM proteins and their isoforms, with particular focus on the proteins with pH between 3.5 and 6, given that all the major CM allergens (caseins and the main whey proteins, alpha-lactalbumin and beta-lactoglobulin) are focalized in this pH range.

The results obtained for protein composition of DM are presented and discussed in comparison with HM and CM, with special regard to the immuno-allergological implications.

4.1. Protein fraction

Although the total protein content of DM is reported to be quite similar to that of HM (14), hence lower than in CM, differences in protein distribution are already clear at low definition, as shown in Figure 1, where the milk proteins were separated in the first dimension over a broad pH range (3 to 10) DM proteins, numbered in Figure 1, were identified by MALDI-TOF peptide mass fingerprint analysis and are listed in Table 1. Due to the scanty information available on the donkey genome and proteome, identifications reported in Table 1 were achieved thanks to the high degree of identity between horse and donkey proteins: 27 out of 33 identifications were by identity with peptides from horse proteins and only six by matching with already-known donkey proteins.

At this level of resolution, only alphas1- and beta-casein molecules could be identified in the casein family, while the two beta-lactoglobulin variants, beta-lactoglobulins I and II, already described in DM, could be separated and identified, in spots 6 and 10 of Figure 1 respectively (Table 1)

Both to achieve a higher resolution among the acidic proteins and to load a higher protein amount so as to visualize minor components, 2D electrophoretic maps were drawn using a narrower pH gradient (3 to 6) in the first dimension, as shown in Figure 2. Proteins contained in numbered spots in Figure 2 are listed in Table 2.

Proteins were identified by identity or homology search after N-terminal sequencing (16 spots), by MALDI-TOF peptide mass fingerprint analysis (9 spots) or by LC-ESI MS/MS analyses (11 spots), as shown in Table 2.

The peptide mass fingerprint analysis of the (two) proteins, contained in spot 36, clearly indicated the

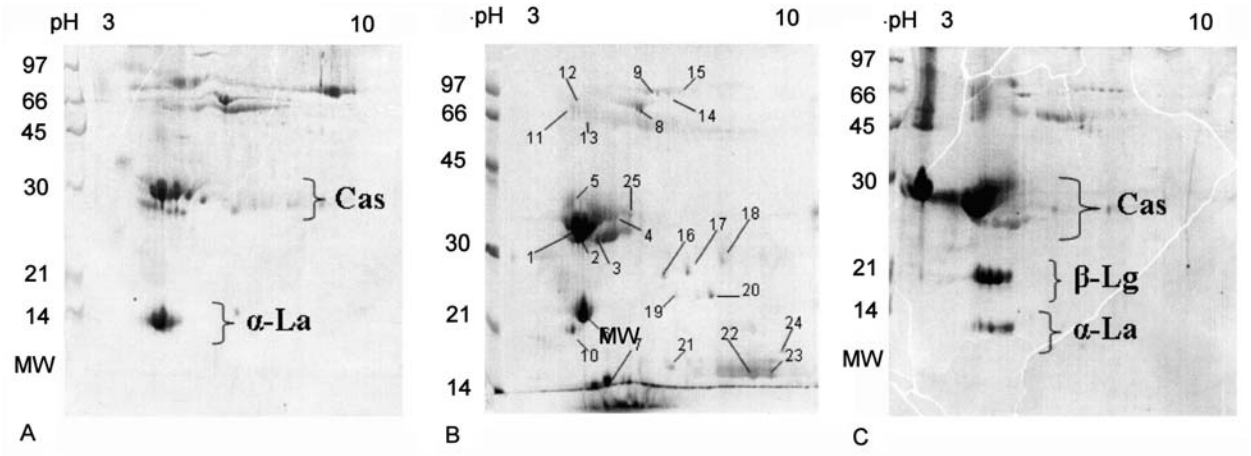

Figure 1. Human, Donkey and Cow's milk proteins separated by 2D electrophoresis over a broad pH range (3 to 10) Cas, Caseins; alpha-La, alpha-Lactalbumin ; beta-Lg, beta-Lactoglobulin (A, Human milk; B, Donkey's milk; C, Cow's milk).

presence of donkey beta-casein, by matching of 6 peptides covering 23% of the molecule, while to confirm the presence and to identify the second protein component in the spot as a homologue of horse kappa-casein, fragmentation by MALDI TOF/TOF of the peptide at 1584.85 m/z was necessary (Table 2)

From the total of 39 spots analyzed, six different protein species were identified: alpha-s1-casein, alpha-s2-casein, beta-casein, kappa-casein, alpha-lactalbumin and beta-lactoglobulin, expressed as multiple isoforms arising from both post translational modifications (PTMs) and the presence of genetic variants, and only 4 spots out of 39 showed the co-presence of two or more proteins while 35 contained only one component.

The diverse arrays of PTMs that occur on proteins play an important part in regulating protein structure and function. Among the several biological functions reported for each milk protein, increased calcium absorption has for example been associated with the phosphorylated forms of the casein fraction (19), while oligosaccharides bearing sialylated lacNAc or lacdiNAc antennae at their terminal ends - responsible for the pH shift of the glycosylated isoforms - have been reported to manifest immunosuppressive effects by specifically blocking adhesive and activation-related events mediated by CD22, the human B cell receptor (20)

In the case of CM allergy, structural post-translational modifications, such as phosphorylation of alpha-s2- and beta-casein (21) and glycosylation of kappa-casein (22), have been shown to influence the IgE binding of these proteins.

To depict the PTM (glycosylation and phosphorylation) pattern of the proteins from the three milks, a multiplexed proteomic approach was used. Figure 3 shows the visualization of phosphorylated proteins after specific staining with Pro-Q Diamond Phosphoprotein Gel Stain. Figure 4 shows the visualization of glycosylated proteins after specific staining with Pro-Q Emerald 488 Glycoprotein Gel Stain. Identification of the phosphorylated and glycosylated proteins in DM (Figure 3 - Donkey and Figure 4 - Donkey, respectively), shown in Table 3, was performed by spot matching, as described in Material and Methods - Staining and imaging.

4.2. DM protein features

The overall casein content of DM is much lower than that of CM (see Figure 1), being less than 50% of the total protein content (16) and thus approaching the casein/whey protein ratio of HM.

4.2.1. alpha-s1-casein

Donkey alpha-s1-casein was identified as the sole protein in 9 spots out of 12, while in the remaining three spots it was identified together with beta-casein (spot 11), beta-casein plus kappa-casein (spot 12) and with kappa-casein (spot 37) All but two donkey alpha-s1-casein isoforms appear as phospho- or glyco-proteins, or as a combination of the two (Table 3) In the two major spots containing alpha-s1-casein, namely spots 9 and 10 in Fig.2, the protein appears as both phosphorylated and glycosylated forms, in spite of the fact that neither human nor bovine alpha-s1-caseins have been reported to be glycosylated. The presence of alpha-s1-casein in DM was already reported by Vincenzetti *et al.* (23) after N-terminal sequencing identification, without reporting of any PTMs. In CM, alpha-s1-casein has been reported to be one of the major casein components and also one of the major allergens, with some linear epitopes being related to the persistence of CM allergy in adulthood (24) Cocco *et al.*, (25) found that even single amino acid substitutions, in specific linear epitopes of alpha-s1-casein, could drastically reduce the binding capacity of IgE from the sera of CM allergic patients.

The complete amino acid sequence of donkey alpha-s1-casein is not yet known, but horse alpha-s1-casein shows a higher identity to human alpha-s1-casein (42.9%) than the bovine protein (32.4%) This higher similarity with the human counterpart could contribute to explaining the already-cited hypoallergenicity of equine milks (9, 2)

Table 1. Identification of DM proteins numbered in Figure 1

Spot	T. pI	T. MW kDa	Protein (Accession number)	Mass Spectrometry MALDI TOF. N. matching peptides (coverage%)
1	6.02	25305	alpha-s1casein (Q8SPR1 Horse)	5 (21)
	5.78	25511	beta-casein (Q9GKK3 Horse)	5 (24)
2	6.02	25305	alpha-s1casein (Q8SPR1 Horse)	7 (27)
	5.78	25511	beta-casein (Q9GKK3 Horse)	5 (21)
3	6.02	25305	alpha-s1casein (Q8SPR1 Horse)	10 (32)
	5.78	25511	beta-casein (Q9GKK3 Horse)	4 (21)
4	6.02	25305	alpha-s1casein (Q8SPR1 Horse)	9 (30)
	5.78	25511	beta-casein (Q9GKK3 Horse)	5 (21)
5	5.78	25511	beta-casein (Q9GKK3 Horse)	6 (21)
6	5.0	20345	beta-lactoglobulin I precursor (variant B) (Donkey) (17)	12 (66)
7	5.11	14669	alpha-lactalbumin (P28546 Donkey)	10 (84)
8	5.89	70490	serum albumin precursor (Q5XLE4 Donkey)	37 (64)
9	8.3	75922	lactoferrin (O77811 Horse)	9 (15)
10	4.7	18263	beta-lactoglobulin-2 (BETA-LG-2) (beta -lactoglobulin II, minor monomeric) (P19647 Donkey)	9 (74)
11	5.78	25511	beta-casein (Q9GKK3 Horse)	10 (22)
	8.3	75922	lactoferrin (O77811 Horse)	14 (22)
12	5.78	25511	beta-casein (Q9GKK3 Horse)	8 (17)
	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	8 (12)
13	5.78	25511	beta-casein (Q9GKK3 Horse)	6 (17)
14	-	-	Not identified	-
15	6.8	78095	serotransferrin precursor (P27425 Horse)	5 (8)
16	5.78	25511	beta-casein (Q9GKK3 Horse)	6 (19)
			lactotransferrin precursor (O77811 Horse)	6 (11)
17	5.78	25511	beta-casein (Q9GKK3 Horse)	8 (26)
18	5.78	25511	beta-casein (Q9GKK3 Horse)	3 (14)
19	5.78	25511	beta-casein (Q9GKK3 Horse)	4 (18)
20	5.78	25511	beta-casein (Q9GKK3 Horse)	4 (18)
21	5.78	25511	beta-casein (Q9GKK3 Horse)	3 (14)
22	5.78	25511	beta-casein (Q9GKK3 Horse)	3 (14)
	8.41	14688	lysozyme C (P11375 Donkey)	3 (36)
23	8.41	14688	lysozyme C (P11375 Donkey)	8 (71)
24	5.78	25511	beta-casein (Q9GKK3 Horse)	3 (14)
25	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	5 (18)

T. pI and T. MW = Theoretical pI and Theoretical molecular mass of the reference proteins

4.2.2. alpha-s2-casein

Donkey alpha-s2-casein was only identified in two very weak spots (spots 8 and 35 in Figure 2) by N-terminal sequence homology with pig alpha-s2-casein (Table 2), as the mare's milk alpha-s2-casein sequence is not yet known. Both donkey alpha-s2-casein isoforms seem to be phosphorylated (Table 3) It could be speculated that the unidentified spots 6 and 7 in Figure 2 might also contain donkey alpha-s2-casein, not identified because of a lack of homology with known alpha-s2-caseins; be that as it may, it is evident that alpha-s2-casein is a very minor component of the casein family in DM (Figure 2 and Table 2)

While bovine alpha-s2-casein comprises approximately 10% of the total casein fraction of CM (26), HM does not contain a alpha-s2-casein-like protein, as the human gene encodes only a 27-aa peptide encompassing the signal peptide and the first potential phosphorylation site, due to a premature stop codon (27)

Järvinen *et al.* (28), using synthetic decapeptides synthesized on a SPOTs membrane, reported binding of IgE from sera of individuals with persistent CM allergy to residues 33-42, 87-96 and 159-168 of bovine alpha-s2-casein, while Natale *et al.* (29) found that 90% of 20 CMP allergic patients, aged 4 to 14 months, had serum IgE against bovine alpha-s2-casein, indicating bovine alpha-s2-casein to be one of the major allergens of CM. The very low abundance of alpha-s2-casein in DM could also contribute to explaining its hypoallergenicity.

4.2.3. beta-casein

Donkey beta-casein was identified in 19 of the 39 spots analyzed, by extensive peptide matching with horse beta-casein Q9GKK3, (Table 2) Some donkey beta-casein isoforms appear to be phosphorylated (spots 24, 30-32, 34, 36, Table 3), like the horse, human and bovine protein, or both phosphorylated and glycosylated, as a peculiar feature of DM (spots 13-19, Table 3)

The presence of beta-casein in DM was already reported by Vincenzetti *et al.* (23) after N-terminal sequencing identification, without reporting of any PTMs. As in HM, where beta-casein can account for up to 80% of the total caseins (30), beta-casein is also the predominant protein in DM.

Highly phosphorylated casein with a low molecular mass from Haflinger mare's milk, accounting for 4.0% of the total casein content, has been isolated and identified as a truncated form of the full-length equine beta-casein (31) The phosphorylated isoforms of donkey beta-casein, found in spots 31 and 32 (Figure 2), with an apparent MW around 17 kDa, could be the donkey homologues of this low MW beta-casein variant, as none of the matching peptides (Table 2) fall in the 50-181 residues region of the 226 amino acid full-length protein.

Table 2. DM proteins identified after 2DE separation in the pH range 3-6 (Figure 2)

Spot	T. pI	T. MW kDa	Protein (Accession number)	Identification	
				Mass Spectrometry: MALDI TOF n.matching peptides (coverage %); LC-MS/MS peptide sequence	Edman Degradation N-terminal sequence
1	5.11	14222	alpha-lactalbumin (P28546 Donkey)	-	KQFTKCELSQVL
2	5.11	14222	alpha-lactalbumin (P28546 Donkey)	-	KQFTKCELSQVL
3	4.70	18262	beta-lactoglobulin I variant B (Donkey) (17)	-	TNIPQS
4	4.79	18528	beta-lactoglobulin I (P13613 Donkey)	-	TNIPQTM
5	4.70	18262	beta-lactoglobulin II (P19647 Donkey)	-	TDIPQTM
6	-	-	Not identified	-	-
7	-	-	Not identified	-	-
8	5.78	25866	alpha-s2 casein (P39036 Pig)	-	KHEXTNIXQEKY
9	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	-	RPKL
10	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	-	RPKLPE
11	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	6 (19)	-
	5.78	25511	beta casein (Q9GKK3 Horse)	5 (22)	
12	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	11 (45)	-
	5.78	25511	beta casein (Q9GKK3 Horse)	4 (19)	
	8.03	18845	kappa casein (P82187 Horse)	3 (14)	
13	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVPSEE
14	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVSSEE
15	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVSSEP
16	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVSSE
17	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVV
18	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVV
19	5.78	25511	beta casein (Q9GKK3 Horse)	-	REKEELNVV
20	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	EDMLYQHTLEQLR	-
				LIASENSEK	
				LIASENSEKTDIIPEW	
21	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	LIASENSEK	-
				LIASENSEKTDIIPEW	
22	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	LC-MS/MS	-
				LIASENSEK	
				LIASENSEKTDIIPEW	
23	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	KFPSFALEYINELNR	-
24	5.78	25511	beta casein (Q9GKK3 Horse)	FKHEGQQQR	-
				FKHEGQQQR	
				QILNPTNGENLR	
				VAPFPQPVVPYPQR	
25	5.78	25511	beta casein (Q9GKK3 Horse)	FKHEGQQQR	-
				QILNPTNGENLR	
26	5.78	25511	beta casein (Q9GKK3 Horse)	FKHEGQQQR	-
				QILNPTNGENLR	
				VMPFLK	
27			Not identified	-	-
28	5.78	25511	beta casein (Q9GKK3 Horse)	ETILPK	-
				SPIVPFSER	
				FKHEGQQQR	
				QILNPTNGENLR	
				VAPFPQPVVPYPQR	
29	5.78	25511	beta casein (Q9GKK3 Horse)	ETILPK	-
				QDKISR	
				SPIVPFSER	
				FKHEGQQQR	
				QILNPTNGENLR	
30	5.78	25511	beta casein (Q9GKK3 Horse)	6 (21)	-
31	5.78	25511	beta casein (Q9GKK3 Horse)	6 (21)	-
32	5.78	25511	beta casein (Q9GKK3 Horse)	4 (12)	-
33	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	TDIIPEW LIASENSEKTDIIPEW	-
34	5.78	25511	beta casein (Q9GKK3 Horse)	SPIVPFSER FKHEGQQQR QILNPTNGENLR	-
35	5.78	25866	alpha-s2 casein (P39036 Pig)	-	KHEXTNIXQEKY
36	5.78	25511	beta casein (Q9GKK3 Horse)	6 (23)	-
	8.03	18845	kappa casein (P82187 Horse)	2 (11)	
				MALDI TOF-TOF of peptide at 1584.85 m/z YIPIYYVLNSSPR	
37	6.02	25305	alpha-s1 casein (Q8SPR1 Horse)	9 (33)	-
	8.03	18845	kappa casein (P82187 Horse)	3 (18)	

T. pI and T. MW = Theoretical pI and Theoretical molecular mass of the reference proteins.

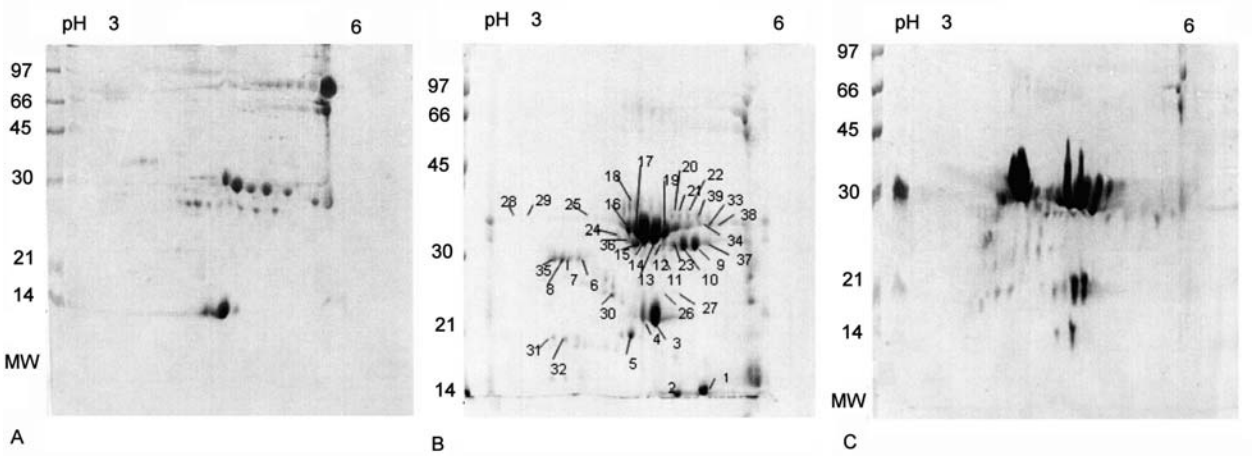

Figure 2. 2DE maps of defatted human, donkey's and cow's milk. First dimension on strips with narrow pH gradient (3 to 6) (A, Human milk; B, Donkey's milk; C, Cow's milk).

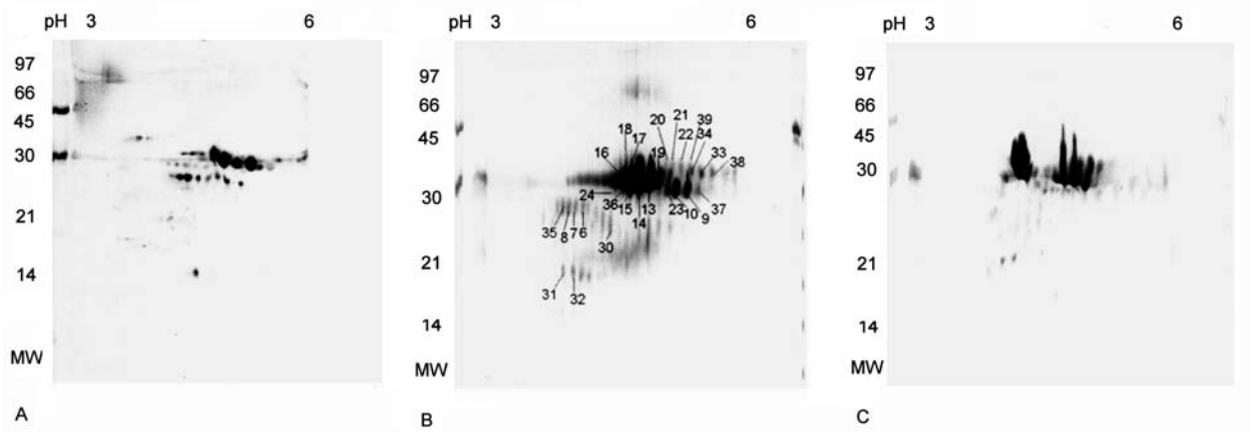

Figure 3. Pro-Q Diamond Phosphoprotein Gel Stain after 2DE separation of Human, Donkey and Cow's milk proteins (A, Human milk; B, Donkey's milk; C, Cow's milk).

The sensitizing capacity (specific IgE binding) of bovine beta-casein has been demonstrated to be rather low (15%) in a population of 20 CMP allergic subjects in a study by Natale *et al.* (29) In an another study, Bernard *et al.* (21) reported that bovine beta-casein shares with the other bovine casein fractions a common epitope in the highly conserved major site of phosphorylation, corresponding to amino acid residues 17-21 (SerP-SerP-SerP-Glu-Glu) of the bovine beta-casein and that, to some extent, IgE binding is affected by the degree of phosphorylation. The same peptide in mare's milk beta-casein appears to be significantly different (SerP-SerP-Asn-Glu-Pro), and this feature could also contribute to explaining the hypoallergenicity of mare's milk.

4.2.4. kappa-casein

kappa-casein is the only"calcium-insensitive" protein of the casein family, i.e. it is not precipitated by Ca^{2+} ions. In CM and HM, it has been demonstrated that casein micelles are formed through the stabilizing properties of kappa-casein when it interacts with the complex to form a colloidal suspension, although with important differences between the two milks due to the different molar ratios among each casein species and to the different degree of PTMs (32)

Although mare's kappa-casein were already sequenced in 2001 (33) and estimated to represent about 7% of the total casein fraction in this milk (7), the presence of a donkey kappa-casein has never yet been reported. We were only able to identify donkey kappa-casein in three very weak spots (spots 12, 36 and 37 in Figure 2) and always accompanied either by beta-casein, or by alpha-s1-casein, or both (Table 2) Spots 36 and 37 seem to contain phosphorylated and glycosylated proteins, respectively, but at present we cannot say whether these PTMs are carried by the kappa-casein or by the accompanying proteins. Interestingly, in spot 12, where all three proteins were identified, none of them seem to be phosphorylated nor glycosylated, while in mare's milk, Egito *et al.* (34) report that equine kappa-casein seems to be more glycosylated than bovine kappa-casein, and that the non-glycosylated kappa1-casein was not detected in this milk.

Table 3. Post translational modifications of DM proteins, as numbered in Figure 2

Spot (see Figure 2)	Protein	P = phosphorilation (Figure 3) G = glycosylation (Figure 4)
1	alpha-lactalbumin	G
2	alpha-lactalbumin	G
3	beta-lactoglobulin I variant B	G
4	beta-lactoglobulin I	G
5	beta-lactoglobulin II	G
6	Not identified	P
7	Not identified	P
8	alpha-s2 casein	P
9	alpha s1-casein	P - G
10	alpha s1-casein	P - G
11	alpha-s1 casein	-
	beta casein	-
12	alpha-s1 casein	-
	beta casein	-
	kappa casein	-
13	beta casein	P - G
14	beta casein	P - G
15	beta casein	P - G
16	beta casein	P - G
17	beta casein	P - G
18	beta casein	P - G
19	beta casein	P - G
20	alpha-s1 casein	P
21	alpha-s1 casein	P
22	alpha-s1 casein	P
23	alpha-s1 casein	P - G
24	beta casein	P
25	beta casein	-
26	beta casein	-
27	Not identified	-
28	beta casein	-
29	beta casein	-
30	beta casein	P
31	beta casein	P
32	beta casein	P
33	alpha-s1 casein	P
34	beta casein	P
35	alpha-s2 casein	P
36	beta-casein	P
	kappa-casein	P
37	alpha-s1 casein	G
	kappa-casein	G
38	alpha-s1 casein	P
39	alpha-s1 casein	P

Bernard *et al.* (26) report that, in a population of 58 CMP allergic children, 85% were sensitive to whole casein, and that higher amounts of specific IgE were directed against alpha-s1- casein or beta-casein than against kappa-casein, while in a study by Natale *et al.* (29), 50% of 20 CMP allergic patients had specific IgE against bovine kappa-casein.

The amount of kappa-casein in DM appears to be lower than in mare's milk and far lower than in CM or HM (7), and this might contribute to explaining the specific hypoallergenicity of DM.

4.2.5. alpha-lactalbumin

alpha-Lactalbumin plays a key role in lactose synthesis in the mammary gland and provides a major source of essential amino acids in HM, where it represents the major whey protein. The overall primary structure of human alpha-lactalbumin (LALBA_HUMA) does not differ greatly from either the bovine protein LALBA_BOVI (93/123 identical and 115/123 similar residues) or the donkey counterpart LALBA_EQUA (93/123 identical and 118/123 similar residues)

Donkey alpha-lactalbumin was identified in two spots, namely spots 1 and 2 in Figure 2, and both isoforms appear to be slightly glycosylated (spots 1 and 2, Figure 4) Donkey alpha-lactalbumin lacks the usual glycosylation consensus site for these proteins, the ^{45}Asn-Gly/Gln-Ser triplet, due to a ^{47}Ser →Lys substitution, but glycosylation might occur at the unusual triplet ^{71}Asn-Ile-Cys, as has already been reported for human alpha-lactalbumin (35)

Although alpha-lactalbumin is not always considered one of the major CM protein allergens, the presence of conformational epitopes was considered by Natale *et al.* (29), and was found to be of critical importance in 11 of 19 patients in a study by Maynard *et al.* (36), which also demonstrated the presence of sequential epitopes exposed through protein denaturation. Interestingly, Adams *et al.* (37) identified a sequential epitope in the bovine alpha-lactalbumin region ^{5}Lys – ^{18}Tyr recognized by specific IgE of CM allergic patients. The amino acid sequence of the 14 residues comprising this region shows only 85.6% similarity (57.1% identity) between human and bovine alpha-lactalbumin, while the

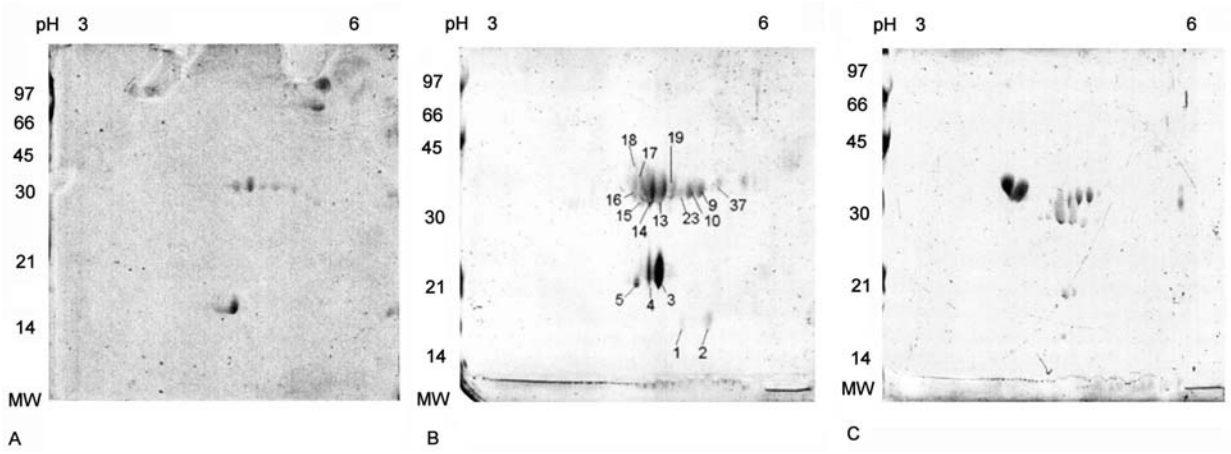

Figure 4. Pro-Q Emerald 488 Glycoprotein Gel Stain after 2DE separation of Human, Donkey and Cow's milk proteins (A, Human milk; B, Donkey's milk; C, Cow's milk).

human and the donkey proteins show 100% similarity (78.6% identity) in the same region. This may also help to explain the hypoallergenicity of DM.

4.2.6. beta-lactoglobulin

Donkey beta-lactoglobulin was found in spots 3, 4 and 5 in Figure 2 and identified as beta-lactoglobulin I - variant B (20), beta-lactoglobulin I (P13613) and beta-lactoglobulin II (P19647), respectively (Table 2) Among the three novel genetic variants recently described for DM (17), only beta-lactoglobulin I B could here be identified with certainty.

In the past, beta-lactoglobulin was thought to be present only in the milk of ruminants (cow, buffalo, goat and sheep), and hence to be the major responsible for allergic reactions to CM. Following the discovery and characterization of mare milk beta-lactoglobulin (38), this protein was found in the majority of milks, but not in HM, guinea pig, rabbit, or rodent milks. Since then, the role of CM beta-lactoglobulin in eliciting allergic reactions has been re-evaluated and it is now generally accepted that caseins are the major CM protein allergens (29, 24)

In CM, beta-lactoglobulin is the major protein component, accounting for more than 50% of the whey fraction (39) In DM, beta-lactoglobulin still represents the major whey protein component, although its amount has been reported to be less than 30% of the total whey proteins (16)

Since its first isolation, beta-lactoglobulin has been an enigma: although it is abundant in the whey fraction of all milks in which it has been found, its function is still not clear, except for the transport of small hydrophobic molecules, as is the case of other components of the lipocalin family such as retinol binding protein (RBP) and glycodelin (formerly placental protein 14)

Glycodelin has been referred to as the human "glycosylated homologue" of beta-lactoglobulin (40) It had not been described in any species other than man, but recently it was shown to occur in the baboon uterus (41) To date, beta-lactoglobulin has been reported in three primate species: cynomolgus *(Macaca fascicularis)* (42), rhesus monkey (*Macaca mulatta)* (43) and baboon (*Papio hamadryas*) (44), the latter in contrast to the theory that expression of beta-lactoglobulin and of glycodelin were mutually exclusive.

Baboon beta-lactoglobulin has been shown to be more similar to human glycodelin than to bovine beta-lactoglobulin A, but no carbohydrate was detected by the Oxford Glycosystems GlycoTrack kit on baboon beta-lactoglobulin.

The most interesting result of the Multiplexed proteomic analysis performed in this study on DM proteins is the apparent glycosylation of the three beta-lactoglobulin isoforms (Figure 4, spots 3, 4 and 5) This finding was confirmed by the PAS staining method (result not shown), following Kapitany and Zebrowsky (45) Preliminary data obtained by mass spectrometry analysis on the protein of spot 3, after treatment with PNGase F (Sigma), also appears to confirm the presence of N-glycosylation (s) borne by donkey beta-lactoglobulin.

The pairwise comparison of donkey beta-lactoglobulin I and human glycodelin shows an identity of 54.4% in a 160 residues overlap, close to the identity between donkey's and CM beta-lactoglobulin (56.9%) and higher than the identity between human glycodelin and CM beta-lg (43.9%) If the ongoing studies on DM beta-lactoglobulin I confirm a glycosylation pattern for this protein homologue to human glycodelin, this strong structural similarity might also help to explain the overall hypoallergenicity of DM.

In conclusion, the structural similarities between human and DM proteins could contribute to explain the results of clinical studies indicating DM as a valid substitute of CM for feeding allergic children. These observations might also constitute the rational for the production of starting formulas based on DM.

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Abbreviations: 2DE, two dimensional electrophoresis; CM, cow milk; CMPA, cow milk protein allergy; DM, donkey milk; HM, human milk

Key Words: Cow, Milk, Protein, Allergy, Fatty Acids, Milk Proteins, Mass Spectrometry, Nutritional Value

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