DETECTION OF BOVINE ALPHA-S1-CASEIN IN TERM AND PRETERM HUMAN COLOSTRUM WITH PROTEOMIC TECHNIQUES

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Due to increased social awareness of allergens and population hyper-sensitization, the reported incidence of allergic reactions to food allergens has increased over the past two decades. Cow's milk proteins (CMPs) are among the most common food allergens. The aim of this study was to use proteomics techniques to investigate cow's milk allergens in both full-term human colostrum and in preterm newborns' mothers – where both groups showed no prior allergen detection -- in order to understand whether cow's milk allergens could be a cause of sensitization established through lactation. The most relevant finding was the detection of the intact bovine alpha-S1-casein in both term and preterm colostrum. Using techniques detailed in this paper and which allowed for direct protein identification, β-lactoglobulin was not detected in any of the colostrum samples. According to our results, bovine alpha 1 casein is considered a major cow's milk allergen, is readily secreted in human milk, and so could be considered a possible cause of sensitization in exclusively breastfed infants.

The incidence of allergic reactions to and the number of allergens found in food has increased during the last two decades (1). Cow's milk proteins (CMPs) are among the most common food allergens. Cow's milk allergy (CMA) is often encountered during the first year of life. However, it is also observed in about 0.5% of exclusively breastfed infants. This may be connected to the fact that small amounts of CMPs can be secreted in human milk (2-6).

Cow's milk contains more than twenty-five different proteins, but only whey proteins α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), and lactoferrin, as well as four caseins, have been identified as allergens (7). The casein fraction is composed of αS1-, αS2-, β-, and κ-casein, of which αS1-casein appears to be the major allergen, according to IgE and T-cell recognition data (8-11).

It has been demonstrated that αS1-casein as well as αS1-casein-derived peptides exhibit IgE reactivity, whereas the intact αS1-casein induce strong basophilic degranulation (12). These results suggest that primarily intact αS1-casein or larger IgE-reactive portions may be responsible for IgE-mediated symptoms of food allergy.

Until now the detection of the above-mentioned food allergens in human milk has been achieved by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoblotting techniques (13). However, both these methods potentially interact multiple proteins present in the sample, and do not account for possible chemical modifications or the proteolytic digestion of the proteins (13).

Key words: Alpha-1 casein, proteomics techniques, human milk, cow's milk allergens

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

The aim of our study is to use direct proteomic techniques to investigate cow's milk allergens in human colostrum, and, after a pre-treatment of the samples in order to equalize the concentration of the different proteins, to understand which allergens may be the primary cause of sensitization during lactation.

MATERIALS AND METHODS

Sample collections and preparation

Term colostrum samples were collected from 62 healthy mothers delivering at term (36 completed weeks of gestation). Preterm colostrum samples were collected from eleven healthy mothers who delivered prematurely (between 25 and 30 weeks of gestational age). Samples were collected using an electric breast pump from mothers following a non-restricted diet that included cow's milk and derivatives, just after breastfeeding their own babies.

Samples were gathered through the fourth day after delivery and frozen and stored at -80°C immediately after collection. Before analysis, samples were defrosted at room temperature. Two different pools of 245 mL were created, one of Colostrum of mothers delivering Term infants (CT) and another one of Colostrum of mothers delivering Preterm infants (CP). Then in each pool (CT and CP) five complete protease inhibitor cocktail tablets were added.

The pool was mixed for 30 min at 4°C, then both CT or CP samples were divided into aliquots and centrifuged 1000 g at 4°C for 30 min to remove the lipid phase and cell fraction. The casein phase was removed by ultracentrifugation at 176,000 g at 4°C for 70 min, and the whey phase obtained by filtering the remaining sample through a 0.22 m Stercup (Millipore) filter in order to remove the last contaminants.

The CT and CP total protein concentrations of the pooled samples were determined by bicinchoninic acid (14) assay, where bovine serum albumin was used as a standard. A total volume remaining was 204 mL of which the whey fraction named Start CT/Start CP sample was separated and divided into aliquots to be frozen at -40°C.

The following chemicals were used. Sodium dodecyl sulfate (SDS), glycine, agarose, mineral oil, dithiothreitol reducing agent (DTT), tris, urea, IPG Strips pH 3-10NL 17cm, Bio-Lyte 3–10 ampholytes and Precast Tris-HCl 8-16% 17 cm gel (Bio-Rad Laboratories). NuPAGE® MES SDS Running Buffer (20X), NuPAGE® LDS sample buffer (20X), Mark 12 unstained standard, NuPAGE® Novex 4-12% Bis-Tris gel X 10 wells, NuPAGE® Novex 4-12% Bis-Tris ZOOM® gel X IPG well, ZOOM® Strips pH 3-10NL 7cm, Sypro Ruby protein stain and ZOOM® Anpholytes pH 3-10 (Invitrogen). Thiourea, (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS), ethanol, methanol and iodoacetamide (Sigma-Aldrich). Glycerol, DeStreak reagent and low molecular weight calibration kits for electrophoresis (GE Healthcare). Porcine Trypsin (Promega). BCA protein assay kit and comassie plus protein assay kit (Pierce). Complete protease inhibitor cocktail tablets (Roche Diagnostics). Comassie brillant blue G-250 (USB). Acetonitrile and phosphoric acid (Riedel de Haen). Amonium bicarbonate, acetonitrile with 0.1% formic acid and water with 0.1% formic acid (Fluka). Acetic acid and Bromophenol blue (Merk).

Proteominer sample treatment

The enhancement of low abundant proteins within the Start CT or start CP sample was achieved by using ProteoMiner techniques (15-19). Two different hexapeptide ligand libraries, which each had a different terminating primary amine (E-library) or carboxyl group (S-library) (17) were used. Separate 200 mL of Start CT or Start CP samples were thawed, the pH of each sample was measured, and salt was added to each sample to reach the ionic strength of PBS which was used for the equilibrations of hexapeptides ligand library beads.

Two mL of hexapeptides ligand E-library beads was added to 200 mL Start CT and to 200 mL Start CP sample previously introduced in two separate Nalgene bottles. Each mixture was shaken under vertical rotation for 2 h. The remaining Start CT (or Start CP) sample was separated using centrifugation from the hexapeptide ligand E-library beads bound to the proteins, and was transferred into a clean Nalgene bottle where 2 mL of ligand S-library was added (previously equilibrated in PBS). After 2 h of gentle vertical rotation the hexapeptides ligand S-library beads bound to the proteins were collected by centrifugation. The two libraries' (E,S) beads bound to the proteins were loaded into two different spin columns (Supelco, 10 mL) and extensively washed with PBS (6*10 beads volumes) before proceeding to the final elution steps.

Bound proteins were eluted from both library beads, in four steps, using four different eluents characterized by an increasing hydrophobicity degree. The eluents used were: 1M NaCl solution, TUC solution (2 M thiourea, 7.7 M urea, 4% Chaps), UCA solution (8 M urea, 2% Chaps, 0.1 M citric acid) and HOM solution (6.6% IPA, 3.3% ACN, 3% Ammonia). All eluates were collected, dialyzed against 0.1 M ammonium carbonate and lyophilised. The amount of protein in each eluate was determined by coomassie plus protein assay kit.

2D-page analysis

The lyophilized eluates of each sample (Start CT and

Start CP) was solubilized in 2D sample buffer containing: 2 M thiourea, 7 M urea, 4% Chaps, 10 mM Tris, 1% DTT 4l/mL Destreak solution, 0.2% v/v carrier ampholytes pH 3–10 (20-22). The two end concentration samples of Start CT and Start CP were 2mg/mL and 10mg/mL, respectively.

Mini gels: 60 g of total protein from the original Start CT sample, 60 g of total protein of each eluate sample obtained from the Start CT proteominer treatment and 60 g of the in-silica sample obtained by mixing 15 g of TUC E, UCA E, TUC S, UCA S eluates were loaded into 7 cm IPG Strip 3-10NL.

After 4 h of passive rehydration, the IEF was run with a starting low Voltage (200V) and received a gradually increased Voltage, up to 2000V, with currents limited to 50uA/strip. The IEF was stopped when the value of 6000 V for each strip was reached, and the temperature was set at 20°C. The IPG strips were equilibrated under gentle shaking for 2x15 min, first in 10 mL of a reducing solution containing 6 M Urea, 2% SDS, 30% glycerol, 50 mM tris-HCl (pH 8.8), 2% DTT w/v, 4µg/mL Destreak (modified 23-24), and then in 10 mL of the reducing solution where the reducing agents were replaced by 260 mM Iodoacetamide alkylation solution (29-30). Once the reduction and akylation of the strips had been carried out, the IPG strips were loaded into NuPAGE® 4-12% Bis-Tris polyacrylamide gel. A run was performed using the NuPAGE® MES SDS Running Buffer under constant voltage (200V) using a maximum of 120 mA per gel. The gels were stained by Coomassie colloidal blue overnight and the image was acquired using a GS 800 densitometer.

17x20 Gels

30 g of TUC E, UCA E, TUC S, UCA S eluates were mixed in equal proportions in order to obtain the in-silica sample named Treated CT. 40 g of TUC E, UCA E, TUC S eluates were mixed in order to obtain the in-silica sample named Treated CP. 120 ug of total protein from the Start CT, Start CP, Treated CT and Treated CP samples, respectively, was loaded into 17 cm IPG strips 3-10NL and actively rehydrated for 12 h. The IEF was run with a starting voltage of 250 V, which increased gradually in 5 steps, up to 10.000 V. The IEF was stopped when the last step reached 60.000 V. The IPG strips were equilibrated as mentioned in Mini Gels (methods above) and then loaded onto precast polyacrilamide 8-16% gels. A run was then conducted under constant voltage (200V).

The gels first were stained by Sypro Ruby and then stained with Coomassie colloidal blue. The image was acquired by ProXPRESS 2D proteomic imaging system (Perkin Elmer) and the images were analyzed by Progenesis same spot software (Nonlinear dynamics). Two replicates for each term colostrum sample (Start CT and Treated CT) and preterm colostrum sample (Start CP and Treated CP) were generated.

In-gel tryptic digestion of proteins

All 2D-Page spots under the 45KDa from Start CT, Start CP, Treated CT and Treated CP samples were manually excised and transferred to Eppendorf tubes. The gel pieces were distained overnight in a solution of 40% v/v ethanol in 50 mM ammonium bicarbonate and then washed three times with 25 mM ammonium bicarbonate solution at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature for 15 min three times. The solvent mixture was removed and gel pieces dried.

The gel pieces were rehydrated with 20 g/µl modified trypsin in 25 mM ammonium bicarbonate solution for 1 h at 37°C. An additional minimal volume of 25 mM ammonium bicarbonate solution and 10% v/v acetonitrile in 25 mM ammonium bicarbonate solution was added to the samples and then incubated overnight at 37°C in a shaking incubator. After digestion the supernatants were collected and analyzed by mass spectrometry.

Protein identification by tandem mass spectrometry

Peptides eluted from the in-gel digests were analyzed by HPLC MS/MS using an integrated Agilent 1100-Nanoflow LC system connected to an MS detector Ion-Trap XCT-Ultra (Agilent Technologies, Palo Alto,CA) with an on-line orthogonal nanoelectrospray source. For LC separation, samples (1 µl) were injected and separated on a nanocapillary C18 column (Zorbax 300SB-C18, 150 mm*75 µm, 3.5 µm; Agilent) with a flow rate of 300 l/min and a 5% to 55% linear gradient of acetonitrile in 5% water with 0.1% formic acid over 50 min. In all cases, peptide samples were analyzed in the positive ion mode under the following hardware and software condition: drying gas flow: 5 L/min; drying gas temperature: 300°C; capillary Voltage: 1400-1700 V; skim 1: 40 V; capillary exit: 181 V; trap drive: 97; ICC: on; maximum accumulation time: 300 ms; smart target: 125,000; MS scan range: 80-220s; full scan mode: AutoMs(n); number of parents: 4; averages: 2; fragmentation amplitude: 1.3 V; smart Frag: on, 30-200%; active exclusion : on, 2 spectra, 1min; prefer 2+: on; MS/MS scan range : 100-2200 m/z; ultra scan on; ICC target : 125000; threshold : 74000.

The resulting MS/MS spectra were exported from the DataAnalysis for LC/MSD Trap version 3.3 software package (BrukerDaltonik GmbH) using default parameters for AutoMS(n) and compound "export". The resulting mgf files were then submitted to MASCOT searching against the publicly available NCBI protein database for "All" and "homo sapiens", Swiss-Prot protein database for "All" and "homo sapiens". Database searches were conducted allowing for up to one missed trypsin cleavage and using

the assumption that the peptides were monoisotopic, carbamidomethylated at the cysteine residues as fixed modification and oxidized at methionine residues as variable modification. Peptide tolerances of ± 1.2 Da for MS and fragmentation tolerances of ± 0.6 Da for MS/MS was the window of error allowed for matching the peptide mass values. MASCOT uses a probability based "MOWSE (molecular weight search) Score" to evaluate data obtained from tandem mass spectra. Protein matches were only claimed if at least two distinct peptides were detected per protein, with MOWSE scores being higher than 59 ($P<0.05$).

RESULTS

The sensitive detection of relatively rare and new types of proteins contained in the whey fraction of colostrum (Start samples) was made through the introduction of an additional pre-fractionation step. This step theoretically increases the concentration of the most diluted proteins and simultaneously reduces the concentration of the proteins present at high concentration (Proteominer Treatment) (15-19).

Evaluation of the ProteoMiner treatment performance was achieved through a qualitative comparison between the two dimensional gel electrophoresis of the Start CT sample versus the six different eluates obtained from the treatment of the whey fraction of human colostrum with the Proteominer technology.

Fig. 1A shows the two dimensional electrophoresis maps of the Start Term Colostrum sample (Start CT on the left upper image) and its E-eluates as NaCl E (on the upper right image), TUC E (on the left lower image) and UCA E (on the right lower image).

Fig. 1B shows the two dimensional electrophoresis maps of the Start CT sample (on the right upper image) and its S-eluates as NaCl S (on the upper left image), TUC S (on the right lower image) and UCA S (on the right lower image). Fig. 1 A and B immediately show that the two types of libraries (E and S) demonstrate complementary behaviour, indeed different classes of proteins were enriched from the two distinct type of libraries. Four of the most abundant proteins, serum albumin, Ig alpha-1 chain C region, lactoferrin and secretory component, seem to be strongly reduced in concentration in all E-eluates apart from NaCl E eluate (Fig. 1A), whereas, -lactalbumin is absent in each of E-eluates (Fig. 1A) and S-eluates (Fig. 1B). A close analysis of Fig. 1 A and B highlight interesting areas of enrichment. An increased number of spots can easily be visualized in 4 of the 6 eluates coming from E and S hexapeptide libraries. The greatest number of new proteins show in E-eluates, especially the TUC E and UCA E (Fig. 1A), whereas, most of the proteins visualized in each of S-eluates (Fig. 1B) seem to be redundant and the number of new species of proteins is less than the number found in E-eluates.

Careful observation of the 2D maps across all six eluates obtained from the treatment of the term colostrum sample, has established that the most significant eluates in terms of number of non-redundant new proteins were found in the eluates TUC E, UCA E, TUC S and UCA S.

The in-silica sample, called Treated CT, was made by merging the TUC E, UCA E, TUC S and UCA S eluates together. Fig. 2 shows this in the two-dimensional electrophoresis maps of the Start CT and the Treated CT sample, respectively.

The two maps in Fig. 2 show different protein patterns: the 2D-Page map of the treated sample exhibits a larger number of spots compared to the map of the Start CT sample, especially in the area under 50 KDa.

The fraction of non-alkaline proteins is predominant (Fig. 2). Furthermore, two areas of interest, where a larger number of spots are displayed, are highlighted. The first is found under 14.4 kDa and the second is localized between 14.4 KDa and 31 kDa.

Only a small amount of new types of proteins appears in the alkaline region of the Treated CT sample. A reduction in the number of spots can be pointed out in the 2D map of the Treated CT sample, in the area above 45 KDa (Fig. 2).

The numbers in the map show the manually excised spots that were digested with trypsin and subsequently submitted to mass spectrometry analysis for their identification; spot number 39 resulted one of the major CM allergens: bovine Alpha-S1-casein (Fig. 3; Table I).

Moreover, among the proteins identified in Fig. 3, some proteins and their isoforms not previously reported in human milk, were detected: No. 16, Galectin-7; No. 17, 14-3-3 protein epsilon; No. 19, 14-3-3 protein beta/alpha, 14-3-3 protein eta and

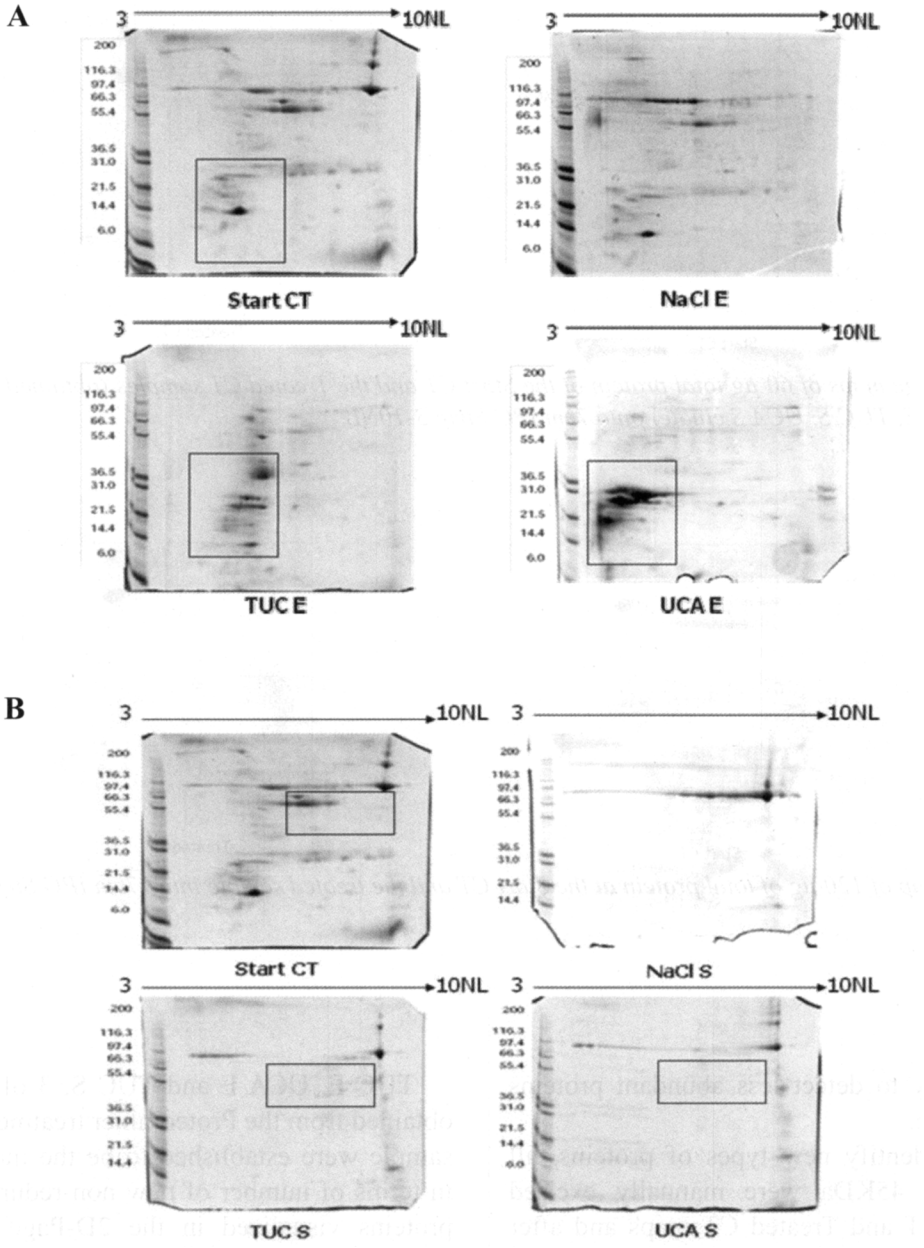

Fig. 1. A) *Mini 2D-Page maps made loading 60µg of total protein of the Start CT sample and of its E-eluates onto 7cm IPG Strip 3-10NL. The red rectangles on the TUC E and UCA E eluates maps highlight the enriched areas of these eluates compared to the corresponding red underlined area in the Start CT sample.* **B)** *Mini 2D-Page maps showing vertical loading of each 60µg of total protein of the Start CT sample and its S-eluates onto 7cm IPG Strip 3-10NL. The red rectangles on the TUC S and UCA S eluates maps highlight the enriched areas of these eluates compared to the corresponding red underlined area in the Start CT sample.*

14-3-3 protein gamma. These new proteins are not discussed in this paper.

The reduction of the number of the spots, in the area above 45 KDa, is not a surprising event, in fact it is related to the Proteominer treatment of the Start CT sample which greatly reduced the concentration of immunoglobulins, one of the most abundant class of proteins present in human colostrum. This process

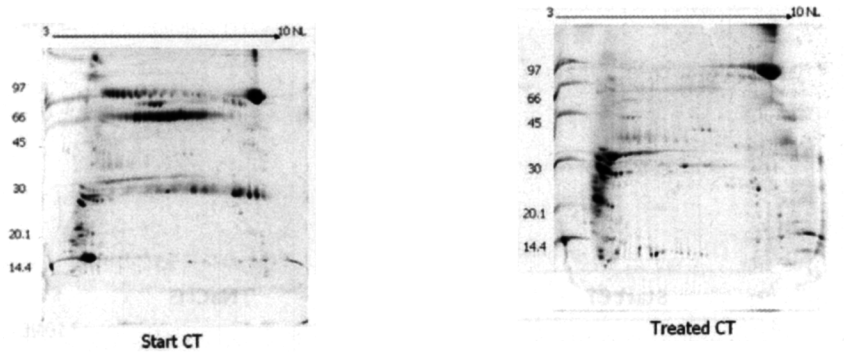

Fig. 2. *Mini 2D-Page maps of 60 μg total protein of the Start CT and the Treated CT samples (obtained mixing 15μg of each TUC E, UCA E, TUC S, UCA S eluate) onto 7cm IPG Strip 3-10NL.*

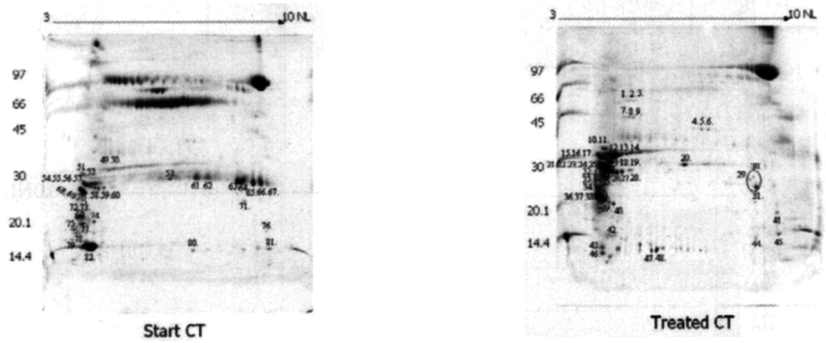

Fig. 3. *2D-Page map of 120 μg of total protein at the Start CT and the treated sample into 17cm IPG Strip 3-10NL.*

allows the ability to detect less abundant proteins within the sample.

In order to identify new types of proteins, all spots under the 45KDa were manually excised from the Start CT and Treated CT maps and after proteolytic digestion were further analyzed using mass spectrometry methods.

Most of the proteins identified were different isoforms of caseins, that have not been previously detected in colostrum except for trace amounts especially in skimmed colostrums.

The most important result achieved in this study is the detection of bovine alpha-S1-casein in human colostrum (Fig. 3), one of the major allergens found in bovine milk.

As described for the term colostrum sample, an evaluation of the Proteominer treatment of the preterm colostrum sample was made.

TUC E, UCA E and TUC S, 3 of the 8 eluates obtained from the Proteominer treatment of Start CP sample were established to be the most significant in terms of number of new non-redundant types of proteins visualized in the 2D-Page (unpublished results). Therefore these eluates were merged to make the in-silica CP sample named Treated CP.

The numbers in the map show the manually excised spots that were digested with trypsin and subsequently submitted to mass spectrometry analysis. Spot number 1 and 18: bovine alpha-S1-casein; spot number 19: Serum amyloid P-component (Table I).

Fig. 4 clearly shows two protein patterns (Start CP *vs* Treated CP samples), which reflect important differences in the number of spots displayed and the displayed spots' locations. The Proteominer treated sample CP contains a great number of spots.

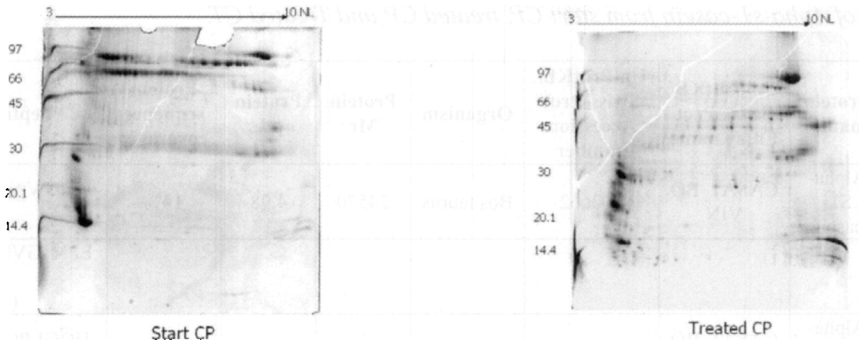

Fig. 4. *2D-Page maps of 120 µg of total protein of the Start CP sample and Treated sample into 17 cm IPG Strip 3-10NL. 40 µg of TUC E, UCA E, TUC S eluates were mixed in order to obtain the in-silica sample named treated CP.*

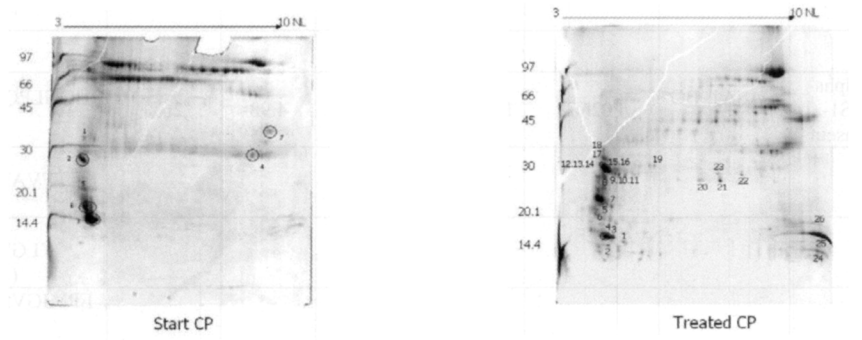

Fig. 5. *2D-Page maps of 120 µg of total protein showing the Start CP and Treated CP sample in two different 17 cm IPG Strip 3-10NL.*

According to the results obtained from the 2D-Page of the treated CT sample, a greater number of medium-sized spots in the area below 45 KDa are also evident in the treated CP sample. The majority of these spots are localized in the non-alkaline region, specifically around the 30KDa, and in the area under 14.4 kDa found in the human colostrum samples collected from mothers delivering at term. A few new spots also appeared in the alkaline region.

The Proteominer treatment was responsible of the reduction of the immunoglobulins in the area above 45 KDa (Fig. 4). Most of the proteins identified (Fig. 5) in the preterm colostrum sample were different isoforms of caseins not previously reported.

The detection of bovine alpha-s1-casein found in the colostrum collected from mothers delivering prematurely is a significant finding, especially as it was found in the treated sample and also in the sample that was not treated with the Proteominer technology.

DISCUSSION

The most relevant finding in this study was the detection of bovine alpha-S1-casein in human colostrum. It is well known that bovine alpha-S1-casein is one of the major allergens in bovine milk. According to our results, this protein may be able to cross the gastrointestinal barrier, to reach the mammary gland and to be secreted into human milk. For this reason this protein could contribute to the CMPs' sensitization observed in breastfed infants.

It is generally accepted that foreign proteins with allergenic activity are secreted in human milk (6, 25, 26). In previous studies, -lactoglobulin, (6, 27) one of the major cow's milk allergen, was detected intact in

Table I. *Identification of Apha-s1-casein from start CP, treated CP and Treated CT.*

Spot no	Sample Name	Protein name	UniProtKB/ Swiss-Prot Entry name	UniProtKB/ Swiss-Prot Accession number	Organism	Protein Mr	Protein p.I.	Protein sequence coverage:	Peptide sequence
39 (Fig.3)	Treated CT	Alpha-S1-casein	CASA1_BOVIN	P02662	Bos taurus	24570	4.98	14%	FFVAPFPEVFGK.E (38-49)
									EPMIGVNQELAYFYPELFR.Q (148-166)
I (Fig.5)	Start CP	Alpha-S1-casein	CASA1_BOVIN	P02662	Bos taurus	24570	4.98	17%	HQGLPQEVLNENLLR.F (23-37)
									FFVAPFPEVFGK.E (38-49)
									YLGYLEQLLR.L (106-115)
18 (Fig.5)	Treated CP	Alpha-S1-casein	CASA1_BOVIN	P02662	Bos taurus	24570	4.98	26%	HQGLPQEVLNENLLR.F (23-37)
									FFVAPFPEVFGK.E (38-49)
									YLGYLEQLLR.L (106-115)
									EPMIGVNQELAYFYPELFR.Q (148-166)

human milk (6, 28, 29) in estimated concentrations of nanograms per litre during their peak concentration by immunoassay-based techniques (6, 29-31). Such techniques are very sensitive methods for detecting food allergens, yet they have the disadvantage of facilitating cross-reactions between other proteins that may present in the sample and which share the same epitopes (13). Moreover, these methods focus on the detection of the intact protein and do not take into account the possible chemical modification or proteolytic digestion of proteins (13).

In our study, using Proteomic techniques, β-lactoglobulin was not detected in preterm or in term human colostrum, despite the fact that mass spectrometry methods have high sensitivity, specificity and reproducibility, mainly using a pre-fractionation step to increase the concentration of the most diluted proteins and simultaneously reduce the concentration of proteins present at high concentration (ProteoMiner). For this reason this method can be considered more reliable than immunoassay methods in the detection of hidden allergens at very low concentration.

According to our results, bovine alpha-1-casein could be considered the only cow's milk allergen that is readily secreted in human milk. As a consequence this protein could be the cause of sensitization to cow's milk in exclusively breastfed predisposed infants-

It is interesting to note differences observed in preterm colostrum compared to term colostrum samples. Firstly, in preterm colostrum the detection of bovine alpha-1-casein was found both in the Start and treated CP samples, whereas in term colostrum, it was only found in the treated sample. This finding indicates a higher concentration of bovine alpha-1-casein in preterm colostrum. A clear explanation of this observation is lacking. One possibility could

be the different membrane permeability observed in mothers who deliver prematurely compared to that in mothers who deliver at term.

It is well known that preterm birth is often associated with an inflammatory condition (e.g. maternal infections or foetal growth restriction) (32-34). Such inflammatory conditions could create different permeability of the gastrointestinal tract as well as the mammary gland that allow intact proteins such as bovine alpha-s1-casein to be secreted in human milk.

Further investigations are needed in order to clarify the clinical meaning of our findings, and in particular, whether specific IgE against bovine alpha-1-casein actually preside in the sera of exclusively breastfed symptomatic infants.

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