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Antibody-independent identification of bovine milk-derived peptides in breast-milk

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Exclusively breast-fed infants can exhibit clear signs of IgE or non IgE-mediated cow's milk allergy. However, the definite characterization of dietary cow's milk proteins (CMP) that survive the maternal digestive tract to be absorbed into the bloodstream and secreted into breast milk remains missing. Herein, we aimed at assessing possible CMP-derived peptides in breast milk. Using high performance liquid chromatography (HPLC)-high resolution mass spectrometry (MS), we compared the peptide fraction of breast milk from 12 donors, among which 6 drank a cup of milk daily and 6 were on a strict dairy-free diet. We identified two bovine β -lactoglobulin (β -Lg, 2 out of 6 samples) and one α_{s1} -casein (1 out of 6 samples) fragments in breast milk from mothers receiving a cup of bovine milk daily. These CMP-derived fragments, namely β -Lg (f42–54), (f42–57) and α_{s1} -casein (f180–197), were absent in milk from mothers on dairy-free diet. In contrast, neither intact nor hydrolyzed β -Lg was detected by western blot and competitive ELISA in any breast milk sample. Eight additional bovine milk-derived peptides identified by software-assisted MS were most likely false positive. The results of this study demonstrate that CMP-derived peptides rather than intact CMP may sensitize or elicit allergic responses in the neonate through mother's milk. Immunologically active peptides from the maternal diet could be involved in priming the newborn's immune system, driving a tolerogenic response.

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Introduction

Cow's milk proteins (CMP) derived from maternal diet can elicit IgE- or non IgE-mediated allergic response in exclusively breastfed infants.^{1,2} In spite of clinical evidence, definite proof on a molecular basis that CMP flow into breast milk remains missing.

In addition to the potential primary (direct) and secondary (indirect) involvement in inducing adverse reactions in a selected cohort of allergic newborns, the exposure to allergens through breast milk has relevant physiological implications, as these allergens might be involved in priming the newborn's immune system to tolerate harmless food and foreign proteins.³

The passage of low amounts of food-derived allergens into breast milk as a possible route of sensitization has been suggested.^{4–7} Using immunochemical assays, Vadas *et al.* detected Ara h 1 and Ara h 2 allergens in breast milk of lactating women who had consumed roasted peanuts.⁸ More recently, other authors identified a different peanut allergen in breast milk, *i.e.* Ara h 6, less than ten minutes after peanut ingestion.⁹ A direct correlation has been described between the dose of egg intake and the amount of intact ovalbumin in breast milk.¹⁰ On the contrary, wheat intake and levels of non-degraded gliadins in blood serum or breast milk are not correlated.¹¹ Breast milk has been described to contain a basal level of immunoreactive gliadins even before intake of wheat, suggesting a certain degree of cross-reactivity between anti-gliadin antibodies and endogenous human milk proteins, provided that the mothers strictly complied with the avoidance diet during trial.¹² Thus, in general the definition of the immunological determinants at this stage appears quite controversial, mainly due to the lack of validation of the immunochemical methods of detection, which can be biased by cross-reactivity or nonspecific antibody recognition.^{13,14}

Whether β -lactoglobulin (β -Lg) occurs in human milk has been debated for many years. The scientific community now commonly accepts that humans do not express β -Lg. However,

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variable amounts of β -Lg have been detected in human milk by immunochemical techniques, supposedly being bovine β -Lg of dietary origin.¹⁵ β -Lg is highly resistant to pepsin, the main gastric protease, while it is extensively degraded by pancreatic proteases, before possible absorption in the small intestine.¹⁶ In contrast, caseins are quickly proteolyzed under both gastric and intestinal conditions.¹⁷ Interestingly, authors who had previously attributed the detection of β -Lg to antibody nonspecific recognition^{18,19} have later described intact bovine α_{s1} -casein in human milk²⁰ and colostrum.²¹ Although the identification of α_{s1} -casein was based on mass spectrometry (MS)-based proteomics, these findings seem to conflict with the prompt digestion of caseins in the gastro-intestinal tract of the lactating mother. Indeed, the likelihood that CMP-derived peptides are transferred into milk through the mammary epithelium is much higher than for intact parent proteins. Nevertheless, no published research has attempted to detect immunologically active fragments of food allergens in human breast milk.

The aim of the current study was to assess the occurrence of CMP-derived peptides in breast milk using an antibody-independent method, such as high performance liquid chromatography (HPLC) coupled with high-resolution MS. *Via* HPLC-MS, the peptide fractions of mature (after colostrum-to-milk transition) breast milk samples from mothers regularly consuming bovine milk and other dairy products were compared to those from mothers on a strict dairy-free diet.

Materials and methods

Samples of mature breast milk (40 mL) were obtained through a breast pump equipped with plastic disposable pump sets from 12 non-atopic healthy donors (median age: 32 years old) who delivered at term, 2–4 weeks after parturition. All subjects were from across the Campania region in Italy. A written informed consent was provided by the mothers before the milk collection.

Six of the samples were collected from women who drank one cup of pasteurized bovine milk daily and regularly consumed other dairy products. Six control samples were collected from women on a strict milk- and dairy product-free diet since at least one week, opportunely instructed to avoid any possible sources of CMP. Breast milk of milk-drinking mothers was collected 2 hours after a regular milk load (200 mL). A skilled operator at the Department of Translational Medicine of the University of Naples “Federico II” (Italy) supervised the administration of bovine milk, sample collection and code labelling. Within less than 3 min after milk expression, the operator added a serine-protease inhibitor (Pefabloc®, Sigma, St Louis, MI, USA) to each sample (1 mM final concentration) and immediately froze the samples of milk at -20 °C. High purity grade solvents and chemicals were from Sigma.

Peptide extraction

Aliquots of each milk sample were skimmed by centrifugation (3000g for 15 min at 4 °C) and the upper fat layer was removed

with a spatula (repeated twice). Proteins of skimmed milk were precipitated (at 4 °C) with 12% (w/v) trichloroacetic acid (TCA) final concentration and soluble peptides were solid-phase extracted using Sep-Pak C18 pre-packed columns (Waters, Milford, USA). After loading, peptides were extensively washed with 0.1% trifluoroacetic acid (TFA) and eluted with 70% acetonitrile containing 0.1% TFA. The organic solvent was evaporated in a vacuum centrifuge (SpeedVac, Savant) and peptides were finally lyophilized.

Competitive ELISA for β -Lg fragments

The presence of β -Lg-derived peptides in the breast milk samples was assayed using the RIDASCREEN® competitive ELISA kit, specifically developed for the detection of hydrolyzed fragments of β -Lg (R4901, R-Biopharm AG, Darmstadt, Germany). The assay was carried out according to the manufacturer's instruction, using intact bovine β -Lg as a standard. The samples were assayed in triplicate. Standard points fit a cubic spline curve that was built using the Ridasoft Win software vers. 1.91 (R-Biopharm AG). Breast milk was assayed at several dilutions, including 1/500 (recommended by the manufacturer), 1/200, 1/100, and 1/50 (v/v). To exclude matrix effects, control breast milk samples spiked with variable amounts of β -Lg were used as the positive control. The manufacturer's declared limit of detection and limit of quantification were 0.115 ppm and 5 ppm, respectively, with <1% cross-reactivity with caseins or other whey proteins.

Western blotting detection of intact β -Lg

The 12% TCA insoluble breast milk proteins were three-fold washed with diethyl ether and assayed for intact β -Lg by western blot analysis. The proteins were dissolved in Laemmli sample buffer at 1 mg mL⁻¹ and 10 μ g were separated by SDS-PAGE using 12% acrylamide precast gels (Bio-Rad Laboratories, Hercules, CA, USA). Gels were run in duplicate; after separation, one gel was stained using G-250 Coomassie Blue Silver staining while the other was electroblotted onto nitrocellulose membranes using a Trans-Blot Cell (BioRad) at 400 mA at 4 °C for 1 h. The membranes were blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin (Sigma) in a Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C, with immunoaffinity purified anti- β -Lg IgG polyclonal antibody developed in rabbit (Abcam Ltd, Cambridge, UK) previously diluted 1/10 000 in TBS-T. Monoclonal peroxidase-conjugated mouse anti-rabbit IgG antibody (Sigma) diluted in TBS-T (1/10 000) was applied to the membrane for 1 h at room temperature. The membrane was extensively rinsed with TBS-T (3 \times 10 min) and finally with TBS (1 \times 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times ranging from 0.5 to 5 min. A sample of bovine whey was used as the positive control. The positive control contained 2 μ g or 1–100 pg of β -Lg for the SDS-PAGE and western blot analyses, respectively.

HPLC-Orbitrap MS/MS

HPLC separation was performed on a Waters NanoAcquity UHPLC (Waters Corporation) with a Proxeon nanospray source. The digested peptides were reconstituted in 3% acetonitrile/0.1% formic acid and roughly 3 μg of each sample was loaded onto a 100 μm \times 25 mm Magic C18 100 \AA 5U reverse phase trap, where they were desalted online before being separated on a 75 micron \times 150 mm Magic C18 200 \AA 3U reverse phase column. Peptides were eluted using a gradient of 0.1% formic acid (A) and 100% acetonitrile (B) with a flow rate of 300 nL min^{-1} . A 60 min gradient was run with 5% to 35% B over 50 min, 35% to 80% B over 3 min, 80% B for 1 min, 80% to 5% B over 1 min, and finally held at 5% B for 5 minutes. Each sample run was followed by a 60 min column wash. Mass spectra were collected on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) in a data-dependent mode with one MS precursor scan followed by 15 MS/MS scans. A dynamic exclusion of 20 s was used. MS spectra were acquired with a resolution of 70 000 and a target of 1×10^6 ions or a maximum injection time of 30 ms. MS/MS spectra were acquired with a resolution of 17 500 and a target of 5×10^4 ions or a maximum injection time of 50 ms. Peptide fragmentation was performed using high-energy collision dissociation with a normalized collision energy value of 27. Unassigned charge states as well as ions $>+6$ were excluded from MS/MS fragmentation.

Database search and peptide identification

LC-MS/MS raw data was converted to .mgf format and analyzed by the offline search engine X!Tandem²² as described previously,²³ with some modifications. Briefly, mass spectral data were searched against the bovine proteome (downloaded from uniprot.org) to identify naturally-occurring peptides which could have originated from a bovine protein. An error tolerance of 10 and 20 ppm was specified for precursor and fragment m/z values, respectively. A minimum e value of 0.01 was required of all peptide matches, corresponding to a 99% confidence score. Oxidation of methionine was allowed as a potential modification, and phosphorylations of serine and threonine were allowed as potential modifications in a single refinement step. The output of the software was refined to exclude peptides which could have originated from a human protein. Since there is a significant overlap between human and bovine milk protein sequences, it was necessary to refine the data further by identifying peptides that matched both proteomes. The output of the software was entered into the Uniprot BLAST search engine (uniprot.org/blast) and searched against mammalian proteomes, with a maximum allowable e value of 10. Peptides that were an exact match to a human sequence under these conditions were removed from the final results list. Next, a list of bovine milk proteins was assembled from prior proteomic studies.^{24–26} Peptides not originating from one of these proteins were also excluded from the final results.

To further characterize the samples and assess the performance of the peptide extraction, mass spectral data were also searched for naturally-occurring human milk peptides. The search employed the same parameters used to identify bovine milk-derived peptides and referenced a library of proteins previously identified in proteomic studies of human milk.^{27–30}

3D molecular modelling

The molecular structure prediction of β -Lg was carried out using the on-line resource I-TASSER (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>), provided by the Department of Computational Medicine and Bioinformatics, University of Michigan, USA.

Results and discussion

LC-high resolution tandem mass spectrometry

Human milk is intrinsically rich in oligopeptides³¹ which are released from milk proteins by endogenous proteases.^{32,33} These peptides are present at an even higher amount in pre-term than in term breast milk.³⁴ The tandem MS-based identification of foreign peptides (if any) occurring at trace levels in breast milk challenges the dynamic range of MS alone. Therefore, prior to MS analysis, peptides must be separated with high resolution chromatography. Although the identification of possible bovine milk-derived peptides is restricted to a relatively low number of abundant proteins (casein and major whey proteins), it requires an untargeted approach, due to the lack of any predictable specificity of protein cleavage. The complexity of such a combination of “deep” and “untargeted” peptidomics is probably the reason why food allergen-derived peptides have not been detected in human milk so far.

In the current case, the identification of bovine milk-derived peptides in breast milk could be further complicated by the homology among the corresponding milk specific proteins. However, the most abundant protein components of human milk, β -casein, α_{s1} -casein and α -lactalbumin share 53, 31 and 74% homology, respectively, with their bovine counterparts, thereby offering a fair margin to discriminate peptides according to their origin. As the intent of the research is to identify peptides unique to bovine milk occurring in human milk (that may induce sensitization or allergic reaction to bovine milk), peptides completely homologous between the two species were irrelevant to our search.

On average, each sample of milk contained more than 1100 endogenous human milk protein-origin peptides ($e \leq 0.01$). These human milk peptides were derived from mostly β -casein, osteopontin, and toll-like receptor 9. Overall, 11 peptides were identified as matching bovine milk protein-derived peptides, while they did not match protein sequences of human milk (Table 1). All raw data and X!Tandem peptide results are available in the ProteomeXchange database with identifier PXD003657.

Table 1 Bovine milk-derived peptides identified in breast milk samples. M* = oxidized methionine

Peptide sequence	Protein of origin (position)	Uniprot protein ID	Number of times identified	Mothers on milk-free diet (N = 6)	Milk-drinking mothers (N = 6)
GLKRSRKKF	Osteopontin (159–167)	P31096	5	1	4
YGLKRSRKKF	Osteopontin (158–167)	P31096	1	—	1
VAPGALLGLGNLTHLSLKYNLLEVPRLPPLSLDTL	Toll-like receptor 9 (189–224)	Q512M5	3	1	2
VAPGALLGLGNLTHLSLKYNLLEVPRLPPLSLDT	Toll-like receptor 9 (189–223)	Q512M5	1	—	1
YVEELKPTPEGD	β -Lactoglobulin (42–54)	P02754	2	—	2
YVEELKPTPEGDLEIL	β -Lactoglobulin (42–57)	P02754	1	—	1
RCLQATLTQDSTYGNEDCLYLNIVWPQGR	Bile salt-activated lipase (81–109)	P30122	1	1	—
SDIPNPIGSENSEKTTM*PLW	α_{s1} -Casein (180–197)	P02662	1	—	1
ASVAVPEQLSVVTRVANLPLVS	Perilipin-2 (2–23)	Q9TUM6	1	1	—
AKDVTVCVGHIIGA	Xanthine dehydrogenase/oxidase (656–669)	P80457	1	—	1
YPNLCQLCKGEGENQ	Lactotransferrin (185–199)	P24627	1	—	1

The most significant finding was the identification of a α_{s1} -casein fragment and two peptides from β -Lg in the milk of lactating mothers who drank bovine milk. These peptides were not detected in any control samples. In relation to the β -Lg-derived peptides, one breast milk sample contained β -Lg (f42–54) and β -Lg (f42–57), while an additional sample contained only β -Lg (f42–54). The α_{s1} -casein (f180–197) was found in an independent sample and it probably survived digestion, because it belongs to a relatively proteolysis-resistant region of the protein.³⁵ No additional casein peptides were detected, most likely due to their prompt and extensive degradation by the gastro-intestinal system of the mother. The MS/MS spectrum of β -Lg (f42–57) is shown in Fig. 1 with the assignment of the most intense fragments, also manually validated. Notably, this spectrum did not match any protein sequence in the human database. After identification, the CMP-derived peptides were also searched in the remaining samples using a targeted approach (peptide ion extraction) unsuccessfully.

The most frequently identified bovine milk protein peptides were those from osteopontin, a well-described milk protein,

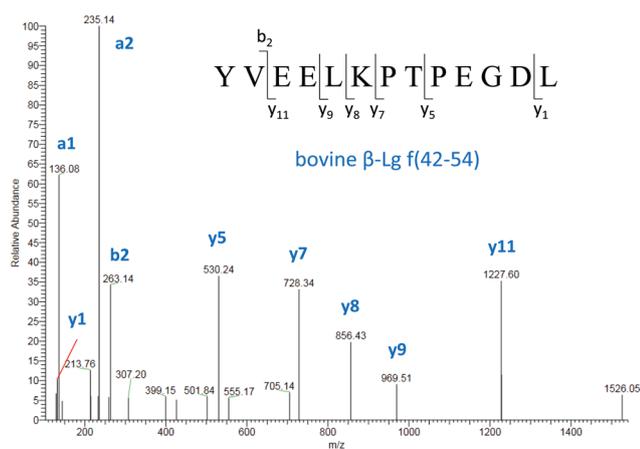


Fig. 1 Identification of β -Lg (f42–54) through the MS/MS spectrum of the peptide precursor $[M + 2H]^{2+} = 745.37$. The most intense y- and b-/a-fragments are assigned.

which also occurs in human milk.^{29,36} The peptide derived from bovine osteopontin has a closely related sequence in human osteopontin (GLKRSRKKF in bovine *versus* GLRSKSKKF in human), exhibiting the same molecular weight and differing only because of a positional K/R inversion. Hydrolytic events also release a significantly high number of osteopontin peptides in human milk.³⁴ A manual inspection of the spectra confirmed that the lack of the fragmentation ions at the level of the K or R residues resulted in a software mismatch, thereby justifying the identification of this peptide as of bovine origin rather than human and its detection even in the control samples (milk from mothers on milk-free diet). Toll-like receptor 9 is a very minor protein of milk and it seems quite improbable that some specific domains could survive maternal digestion and flow into breast milk at a relatively high amount. Indeed, a peptide from Toll-like receptor 9 was also detected in a control sample. The peptides from xanthine dehydrogenase and lactoferrin (1 peptide each) identified in samples from milk-drinking mothers are probable candidate fragments of bovine origin. However, the confidence of their identification was low because they both contain free C residues, which under not-reducing conditions are expected to be engaged in disulfide bonds. A similar observation might hold for a peptide from bile salt-activated lipase, which contains two C residues and was identified in one control sample. The bovine and human sequences of bile salt-activated lipase in that region only differ by an N(bovine) \rightarrow D(human) substitution inducing a shift of 1 mass unit besides an isobaric L \rightarrow I. For the reasons above, the peptides from minor milk proteins, differently from those from β -Lg and α_{s1} -casein, are most likely false positive identifications, thereby emphasizing that software-assisted characterization should be carefully and critically evaluated. In the current case, the presence or absence of these peptides has only technical relevance, because they should not be involved in immunological mechanisms.

Competitive ELISA for β -Lg fragments

A competitive ELISA was applied to detect β -Lg peptides in breast milk samples. The ELISA kit is specifically designed to detect peptides arising from processed β -Lg in food matrices.

Standard β -Lg solutions fit a cubic spline curve. Only one sample gave a positive response, with an apparent β -Lg concentration of 2.1 ppm; however, this concentration was below the limit of quantification of the assay (5 ppm). The ELISA β -Lg-positive sample was a control and therefore the presence of β -Lg peptides should be ruled out. In all the remaining samples, assayed at varying sample dilutions, β -Lg peptides were undetectable, at a 0.115 ppm limit of detection. The inconsistency of the ELISA results demonstrates that immunological methods suffer from bias, most likely due to the specificity of the antibody reactivity. The pitfalls of the ELISA-based methods in the detection and quantification of allergens in complex food matrices have been recently reviewed.¹⁴

Western blotting detection of intact β -Lg

The SDS-PAGE and parallel western blot analyses of the breast milk samples are shown in Fig. 2. Bovine whey proteins were used in both cases as a positive control for β -Lg. Estimated amounts of β -Lg were 2 μ g for the SDS-PAGE analysis and 1–100 pg for the ECL reagent based detection, respectively. The estimated limit of detection of β -Lg by the western blot was well below 0.1 ppm (1 pg with respect to 10 μ g total protein). β -Lg was clearly detected in the whey protein extract with both the analyses, while no band ascribable to it was detected in any of the breast milk samples. These results confirmed the absence of intact bovine β -Lg or derived large immunoreactive fragments at detectable amounts.

Significance of the presence of bovine milk derived peptides in breast milk

β -Lg and α_{s1} -casein are major allergens of bovine milk.³⁷ For the first time, fragments released from allergenic proteins have been identified in human milk. The detection of allergen-derived peptides in breast milk opens a new perspective to monitor potential offending components arising from the maternal diet. Milk proteins are extensively processed along the gastro-intestinal tract of the mother, before intestinal absorption and systemic distribution. Although caseins are more abundant than whey proteins in bovine milk (roughly 80:20 w/w), they undergo a very early degradation in the stomach and then further in the intestine.¹⁷

In contrast, β -Lg is highly resistant to gastric pepsin digestion and large peptides derived from it could escape duodenal degradation. The β -Lg (f42–54) and β -Lg (f42–57) peptides belong to a two β -sheet domain that is located in the lipid binding calix of the native protein (Fig. 3). In milk, β -Lg is most likely complexed with some small hydrophobic ligands, such as fatty acids or retinoids that could limit the access of proteases to the sensitive bonds. As assessed with I-TASSER simulations, β -Lg conformation should not considerably vary between free and fatty acid-complexed forms. The tertiary structure is also stable at the gastric acidic pH.³⁸ Thus, even in the non-complexed protein the calix is buried inside the hydrophobic core of the protein and should be spared by proteolysis at least in part.

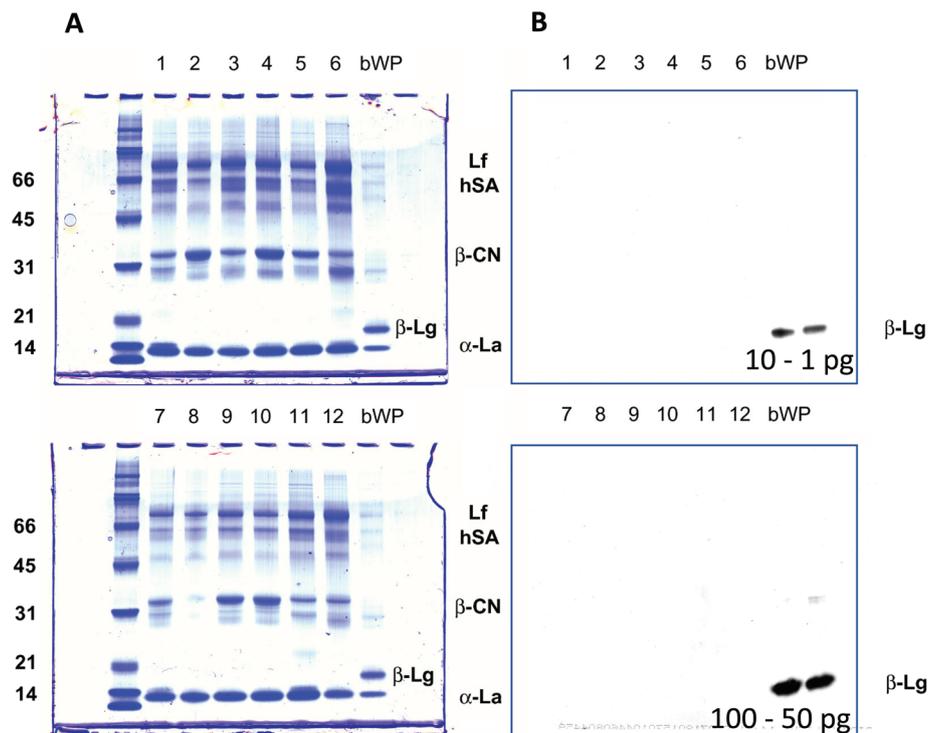


Fig. 2 SDS-PAGE electrophoresis (A) and anti- β -Lg western blotting (B) of the 12 samples of human milk analyzed. Bovine whey proteins (bWP) were loaded as the β -Lg positive control. For the western blotting analysis, the estimated amount of β -Lg in bWP control was 10 and 1 pg (upper panel) and 100 and 50 pg (lower panel).

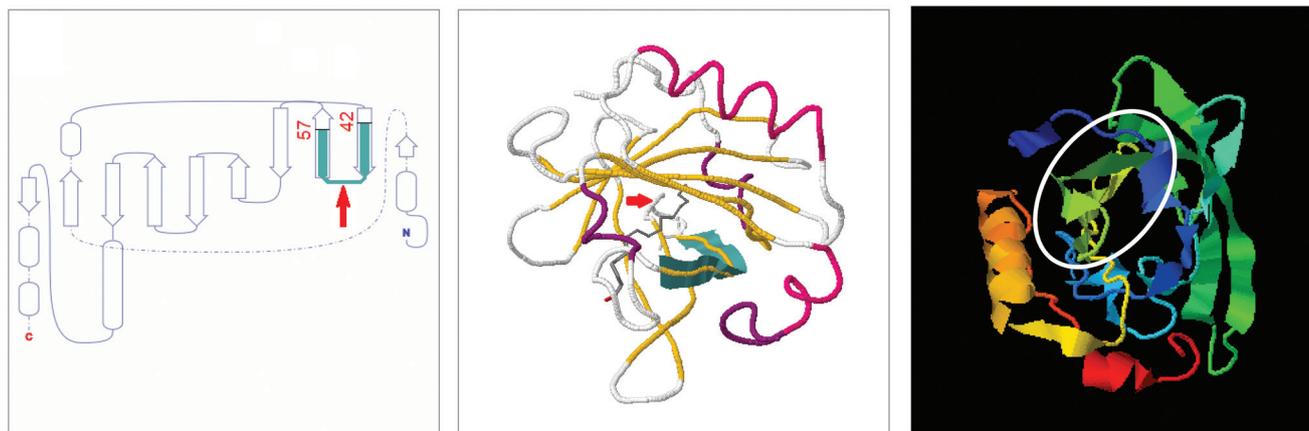


Fig. 3 3D structure of non-complexed β -Lg. The β -Lg 42–57 domain is highlighted in the unrolled model (left panel) and indicated in the X-ray crystal structure of monomer β -Lg (middle panel, PDB data bank). The molecular structure prediction (right panel) performed with I-TASSER confirms that β -Lg 42–57 belongs to the lipid binding calix of the protein.

To this purpose, the peptides of the region 40–60 of β -Lg are relatively resistant to the simulated gastric-duodenal-intestinal digestion of milk proteins, also including peptidases from Caco-2 cells³⁹ and the jejunal brush border membrane.³⁵ The high local number of acidic residues could also explain the resistance of this protein domain to digestion.¹⁷

Through breast milk, peptides from maternal diet can induce early and occult sensitization to food. Alternatively, the early exposure to low amounts of immunogenic epitopes could result in tolerogenic and protective responses.^{40,41} The immunological mechanisms that might underlie these events are still unclear, further complicated by the variability in the frequency and concentration of foreign peptides/proteins in breast milk. Indeed, in our study β -Lg peptides were detected only in two milk samples, thus confirming that the kinetics of dietary components is affected by high individual variability,¹⁵ most likely depending on varying gastro-intestinal digestion capacity or intestinal permeability as well as integrity of the mammary tissue. The secretion of β -Lg peptides into breast milk could also be related to long-term consumption of cow's milk, as higher levels of the protein have been reported in the milk of subjects who are used to consuming milk in large amounts.⁴²

Conclusions

Immunologically active peptides derived from CMP in the maternal diet, rather than intact allergens, could be responsible of adverse responses in exclusively breast-fed newborns. These findings also indirectly prove that dietary protein fragments, albeit in low amounts, can survive gastro-intestinal digestion to be absorbed into the bloodstream and distributed to peripheral organs. Data herein are in line with the very recent detection of gluten-derived immunogenic peptides in the urine of celiac subjects.⁴³

We did not accurately quantify the β -Lg peptides in breast milk samples. However, we can infer from the MS spectral

count that they occur at a very low relative abundance. Whether trace amounts of these peptides are able to sensitize and elicit allergic responses or contribute to priming the immune system, promoting the acquisition of the oral tolerance, remains unknown. From this perspective, the time course of dietary peptide appearance in breast milk and the minimum amount of a dietary protein inducing a detectable presence of peptides in breast milk are important next steps for this research. In consideration of the very low amounts (if any) of potentially immunogenic protein fragments in breast milk, our data support the current guidelines according to which maternal elimination diets during pregnancy and lactation are not recommended as a strategy to prevent childhood allergies of 'at risk' infants.^{7,44}

Conflict of interest

The authors have declared no conflict of interest.

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