



Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins

Gianluca Picariello^{a,b,*}, Giuseppe Iacomino^a, Gianfranco Mamone^a, Pasquale Ferranti^{a,b}, Olga Fierro^a, Carmen Gianfrani^{a,c}, Aldo Di Luccia^{a,d}, Francesco Addeo^{a,b}

^a Istituto di Scienze dell'Alimentazione (ISA), CNR, Via Roma 64, 83100 Avellino, Italy

^b Dipartimento di Scienza degli Alimenti, Università di Napoli "Federico II", Parco Gussone, Portici (NA) 80055, Italy

^c European Laboratory for the Investigation of Food Induced Diseases (ELFID), Università di Napoli "Federico II", Napoli, Italy

^d Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, Via Napoli 25, 71100 Foggia, Italy

ARTICLE INFO

Article history:

Received 30 October 2012

Received in revised form 5 January 2013

Accepted 8 January 2013

Available online 4 February 2013

Keywords:

Milk proteins
Gastrointestinal digestion
Caco-2 cell monolayers
Bioactive peptides
Cow's milk allergy
Peptide uptake

ABSTRACT

The entire panel of peptides produced from caseins (CN) and whey proteins (WP) that survive *in vitro* sequential gastro-pancreatic digestion and translocate across monolayers of Caco-2 cells, used as a model of the intestinal epithelium, has been characterised by HPLC and mass spectrometry. Among the milk-derived bioactive peptides, only minor amounts of mono-phosphorylated peptides arising from α_s1 - and β -CN were detected. The absorption behaviour of two resistant β -lactoglobulin (β -Lg) domains, β -Lg 125–135 and β -Lg 40–60, was studied in detail using synthetic peptides. The IgE-binding properties of the digests recovered from the apical and basolateral monolayer compartments were evaluated by dot-blot, using the sera of milk allergic children ($N = 5$). Outcomes indicated β -Lg 127–135 as a possible "immune sensitising factor" *in vivo*. The almost complete loss of the IgE-affinity of CN and WP after digestion points out the need to design *in vivo* experiments to track the metabolic fate of dietary proteins.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Many studies have demonstrated that peptide sequences encrypted in food proteins can significantly influence a range of physiological functions (Clare & Swaisgood, 2000; Korhonen & Philanto, 2006; Meisel, 2005; Silva & Xavier Malcata, 2005). Food-derived bioactive peptides are effective as long as they are released by proteolytic events or during gastrointestinal (GI) digestion *in vivo*. The ability of bioactive peptides to reach their target site(s) unhydrolysed is a prerequisite for exerting any physiological effects. Therefore, it is clear that the proteolytic pathways of GI digestion have a decisive impact on the formation and/or degradation of specific bioactive sequences. The actual physiological involvement of food-derived peptides is uncertain in most cases since almost all biological tests have assayed sequences released *in vitro* under conditions that are very different from those occurring *in vivo*.

Milk proteins are by far the most extensively investigated source of food-derived bioactive peptides, although active sequences have been described from several food matrices. Several experimental approaches have attempted to reproduce more or

less closely the conditions of the GI digestion of milk proteins to trace the pathways of their breakdown. However, none of these methods has yet been generally accepted and standardised (Hur, Lim, Decker, & McClements, 2011). The pattern of resistant peptides is expectedly affected by a certain degree of variability, depending on the conditions of the GI digestion model (Defernez, Mandalari, & Mills, 2010; Mandalari et al., 2009). Nevertheless, an overview of the recent literature confirms that some milk protein domains exhibit an intrinsic relative stability to hydrolysis. The domains of caseins (CN) and whey proteins (WP) that survived an *in vitro*, three-phase GI digestion, including simulated sequential gastric, duodenal and intestinal hydrolysis, have been investigated in a previous work (Picariello et al., 2010). The early extensive hydrolysis of CN, the partial resistance of β -lactoglobulin (β -Lg) to pepsin, and the resistance of a few specific protein regions were consistent with recent results regarding the degradation of milk proteins in piglets (Bouzerzour et al., 2012).

Although selected peptides may, in principle, exert their actions locally at the level of the GI tract, intestinal absorption prior to distribution to peripheral organs is generally an additional key factor that affects the bioavailability, kinetics and systemic actions of peptides *in vivo*. Peptides that are resistant to proteolytic degradation and are taken up to a significant extent are good candidates either to exert beneficial effects *in vivo* or, conversely, to act as

* Corresponding author at: Istituto di Scienze dell'Alimentazione (ISA), Consiglio Nazionale delle Ricerche (CNR), Via Roma 64, 83100 Avellino, Italy. Tel.: +39 0825 299216; fax: +39 0825 781585.

E-mail address: picariello@isa.cnr.it (G. Picariello).

“immune sensitising factors”, which are antigenic determinants that can induce sensitisation and elicit allergic reactions in predisposed individuals (Jensen-Jarolim & Untersmayr, 2006). Because the *in vivo* determination of intestinal uptake of nutrients or drugs in the intestine is technically difficult, transport studies across human colon adenocarcinoma (Caco-2) cell monolayers have been routinely utilised as an *in vitro* model to mimic absorption by the human intestinal epithelium. Despite their colorectal origin, differentiated Caco-2 cells maintain many morphological and functional traits that characterise the mature enterocytes of the small intestine, such as cell polarisation, microvillous structure, carrier-mediated transport systems, functional tight junctions between adjacent cells and expression of brush-border membrane (BBM)-associated proteases and peptidases. The intestinal absorption predicted by using Caco-2 cell monolayers is well correlated with intestinal uptake *in vivo*, at least from a qualitative standpoint (Artursson, Palm, & Luthman, 2012).

The transport of milk-derived peptides across Caco-2 cell monolayers has already been investigated using primarily single-peptide systems or simple mixtures of synthetic peptides (Cakir-Kiefer et al., 2011; Iwan et al., 2008; Regazzo et al., 2010; Sienkiewicz-Szłapka et al., 2009).

In the present work, the uptake by Caco-2 monolayers of the entire panel of peptides produced from simulated GI digestion of bovine CN and WP fractions has been evaluated. The peptides that translocated across the model epithelia were characterised by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) and reversed phase-high performance liquid chromatography (RP-HPLC) coupled to either UV or electrospray (ESI) MS/MS detection. The peptides recovered from both the apical (UP) and basolateral (DOWN) insert media, along with the HPLC-isolated parent proteins and two proteolytically stable, synthetic peptides of β -Lg were assayed for their binding affinity to IgEs derived from the sera of $N = 5$ children diagnosed with cow's milk allergy (CMA).

2. Materials and methods

An individual bovine milk sample, which was obtained from a local farm, was preliminarily assessed by HPLC and MS as being heterozygous for both the β -CN A1 and A2 and β -Lg A and B and used for this study. After manual expression, the milk was immediately frozen and stored at -20°C until use. Pepsin, trypsin, chymotrypsin, carboxypeptidase A, elastase, neutral red powder, dithiothreitol (DTT), iodoacetamide and α -cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Ammonium bicarbonate, trifluoroacetic acid (TFA) and HPLC-grade solvents were purchased from Carlo Erba (Milan, Italy) and were used without any further purification.

2.1. Fractionation of milk proteins

CN and WP were separated from skim milk (20 mL) diluted two-fold with distilled water according to the Aschaffenburg method (Aschaffenburg & Drewry, 1959). The milk was acidified to pH 4.6 with 10% (v/v) acetic acid/1 M sodium acetate buffer, incubated for 30 min at 37°C and centrifuged at $5000g$ at 4°C for 10 min. The soluble WP were isolated by loading the supernatant onto Econo-pac[®] 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and the proteins were eluted with 50 mM ammonium bicarbonate. The column effluents were monitored by UV detection at $\lambda = 280\text{ nm}$ (Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden). The WP was then quantitated by Bradford assay and freeze-dried. The CN precipitate was washed

twice with sodium acetate/acetic acid buffer and then freeze-dried.

2.2. Gastro-pancreatic digestion

In vitro sequential, gastro-pancreatic digestion of milk proteins was performed as previously described (Picariello et al., 2010). Briefly, CN and WP were dissolved in 5% formic acid at a concentration of 1 mg/mL and incubated at 37°C with pepsin (1:100 enzyme/protein ratio, w/w) for 60 min. Prior to pancreatic digestion, the samples were lyophilized and reconstituted twice with deionised water. The peptic digests were redissolved in 0.1 M sodium phosphate buffer (pH 7.0) at a concentration of approximately 1 mg/mL and further hydrolysed for 1 h at 37°C with a pool of pancreatic enzymes containing trypsin (1:100, w/w), chymotrypsin (1:100, w/w), carboxypeptidase (1:100, w/w) and elastase (1:500, w/w). The simulated pancreatic digestion was stopped by heating the tubes in a boiling water bath for 5 min. The hydrolysis of milk proteins was monitored by RP-HPLC. The HPLC profiles of the digests, analyzed immediately before and after heating, did not appreciably differ, so excluding major heat-induced modifications of peptides.

Prior to application onto the Caco-2 monolayers, the gastro-pancreatic digests were desalted on Sep-Pak C₁₈ cartridges (Waters, Milford, MA, USA) and then lyophilized after reconstitution with MilliQ water.

2.3. Synthetic peptides

β -Lg 125–135 and β -Lg 40–60, which were used as the GI-resistant model peptides, were synthesised by a solid-phase methodology using the Fmoc (9-fluorenylmethoxycarbonyl) strategy on a Pioneer peptide synthesizer (Synthesis System 9050 instrument, PE-Biosystems, Framingham, MA, USA). The purity (>95%) was assayed by both RP-HPLC and MALDI-TOF MS.

2.4. Cell culture

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Hank's Buffered Salt Solution (HBSS) were purchased from Lonza-BioWhittaker (Verviers, Belgium). The Caco-2 cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum (Gibco Life Technology), 1% nonessential amino acids (Euroclone), 2 mM L-glutamine and penicillin/streptomycin (Lonza-BioWhittaker). The Caco-2 cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The cells used in the experiments were between passages 20 and 30. Cells in log-phase growth were subcultured weekly by trypsinisation and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. The cell viability was tested using the vital dye neutral red, according to the manufacturer's instructions.

2.5. Transepithelial transport studies

For the transport experiments, the cells were seeded at a density of 4.5×10^5 cells/cm² in 6-well filter support inserts with polyethylene terephthalate membranes (0.4 μm pore size, 23.1 mm diameter, 4.2 cm² growth surface area; purchased from BD Falcon, Italy). Cell attachment was improved by first covering the transwell membrane with type I bovine collagen (Gibco, Invitrogen, Italy) according to the manufacturer's instructions. The monolayers reached confluence 4 days after plating, and the cells differentiated for at least an additional 14 days prior to the transepithelial transport experiments. The integrity of the cell layers was evaluated by transepithelial electrical resistance (TEER) measurements

using an epithelial Volt-Ohm Meter (Millicell ERS-2, Millipore, Italy). Only the Caco-2 monolayers with TEER values higher than $700 \Omega/\text{cm}^2$ were selected for the subsequent experiments. Following the TEER value measurement, the Caco-2 cell monolayers were gently rinsed twice with PBS, and transport medium (HBSS supplemented with 25 mM glucose and 10 mM HEPES, TM) was added to the apical (2 mL) and basolateral (3 mL) compartments. The inserts were incubated at 37 °C for 330 min with the peptide digests (1.5 mg). Following this incubation, the solutions from the apical and basolateral compartments were collected for RP-HPLC and RP-HPLC–ESI/MS analysis. The TEER value was measured before and after the transport experiments to ensure the integrity of the monolayers. In the same set of cells, the integrity of the layers was also evaluated by a lucifer yellow (LY) permeability assay (Mineo, Hara, Shigematsu, Okuhara, & Tomita, 2002). The fluorescence leakage was determined for LY by excitation at $\lambda = 485 \text{ nm}$ and emission at $\lambda = 530 \text{ nm}$ using a fluorescence plate reader (Bio-Rad). Transport buffer (HBSS containing 10 mM HEPES, pH 7.4) was added to the DOWN compartment. The LY was diluted in transport buffer and applied to the apical compartment at a final concentration of 100 μM . The apical to basal permeability coefficients (P_c) were calculated according to the following equation: $P_c = (V/(A \times C_i)) \times (C_f/T)$, where V is the volume of the basal chamber (mL), A is the area of the membrane insert (cm^2), C_i and C_f are the initial and final concentrations of the fluorochrome, respectively, and T is the assay time (s). The control inserts received only growth medium. In the control monolayers, no significant changes in the TEER values and LY absorption rates were observed during the entire incubation period. Moreover, cell differentiation was evaluated by alkaline phosphatase activity assayed according to the method described by Garen and Levinthal (1960) using *p*-nitrophenol phosphate as the substrate. To check for repeatability, all transport experiments were performed at least three times in duplicate assays.

2.6. RP-HPLC analysis

The RP-HPLC analyses of the protein digests and peptide mixtures recovered from both the UP and DOWN compartments of the Caco-2 monolayer inserts were performed on an HP 1100 modular system (Agilent, Palo Alto, CA, USA) equipped with a 2.0 mm i.d. $\times$ 250 mm, C_{18} , 5 μm reversed-phase column (Phenomenex, Torrance, CA, USA). Solvent A was 0.1% TFA (v/v) in water, and solvent B was 0.1% TFA (v/v) in acetonitrile. Separations were performed with a 5–65% gradient of solvent B over 60 min, after a 10 min isocratic elution at 5% B at a constant flow rate of 0.2 mL/min. The column effluent was monitored by UV detection ($\lambda = 220$ and 280 nm) and the peaks were manually collected. To monitor the *in vitro* digestion, $\sim 45 \mu\text{g}$ of peptide digests was injected for each run. For analyzing the peptide mixtures recovered from the UP and DOWN compartments of the Caco-2 monolayers, 60 and 90 μL of solution were injected, respectively. Peak integration was performed using HPLC offline software (Agilent).

2.7. MALDI-TOF MS mass spectrometry

The MALDI-TOF MS experiments were performed on a PerSeptive Biosystems Voyager DE-PRO instrument (Foster City, CA, USA) equipped with an N_2 laser (337 nm, 3 ns pulse width). Prior to the MS analysis, the peptide mixtures were desalted using C_{18} Zip-Tip pre-packed micro-columns (Millipore, Bedford, MA, USA) previously equilibrated with aqueous 0.1% TFA (v/v) and eluted with 70% acetonitrile (v/v) containing 0.1% TFA (v/v). The HPLC peaks (1 μL) were directly loaded onto a stainless steel plate together with 1 mL of α -cyano-4-hydroxycinnamic acid matrix (10 mg/mL aqueous 50% acetonitrile/0.1% TFA) and air-dried. The

mass spectra were acquired in the positive ion reflector mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. The external mass calibration was performed with mass peptide standards (Sigma). The spectra were analysed with the Data Explorer software (version 4.0) that was provided with the instrument.

2.8. μ -HPLC ESI MS/MS

The peptide digests of CN and WP that were recovered from both compartments of the Caco-2 monolayers were separated by microflow (μ -)HPLC using an Integral 100Q HPLC system (PerSeptive Biosystems, Framingham, MA, USA). For each injection, 20 and 30 μL of solution recovered from the apical and basolateral sides, respectively, were analysed. The flow rate was split from 200 to 5 $\mu\text{L}/\text{min}$ using a flow splitter. The eluents were as follows: (A) 5% ACN in 0.08% FA and 0.01% TFA and (B) 95% ACN in 0.08% FA and 0.01% TFA. The peptides were separated on a capillary column, C_{18} PepMap, 300 Å, 300 mm i.d. $\times$ 150 mm, (LC Packings), using a linear gradient of 5–40% B over 60 min. For MS/MS analysis, a Q-Star Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an ESI ion source was used. The experiments were performed in IDA (intelligent data acquisition) mode. The precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: ions greater than m/z 400.0, charge state 1–3, intensity exceeding 15 counts, former target ions excluded for 60 s and ion tolerance of 50.0 milli-mass unit (mmu). The ions were fragmented by collision-induced decay (CID) using nitrogen as the collision gas. The raw μ -HPLC–MS/MS files were used to generate the Mascot generic file format (.mgf) text files with the software Analyst (ver. 1.1, Applied Biosystems). The peptides were identified with the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>), operating with the following criteria: databases: Uniprot ver. 11.01.2011; taxonomy: *Bos taurus*; type of search: MS/MS ion search; enzyme: none; variable modifications: methionine oxidation and pyro-Glu formation at the N-terminal Gln; mass values: monoisotopic; parent tolerance: 0.08 Da; MS/MS fragment tolerance: 0.2 Da; and number of maximum missed cleavages: 1. The peptide identifications were manually validated. The selected mass ions were manually assigned based on the μ -HPLC MS/MS spectra. The peptide entries were considered identified when the measured MH^+ of the parent corresponded to the expected monoisotopic value and a sequence of at least three consecutive b- or y-ions occurred in the MS/MS spectrum.

2.9. Dot-blot assays

For the dot-blot assay, the sera from five children (34–76 months of age, all male), four of whom had persistent CMA and one who outgrew the allergy, were used as a source of the specific IgE antibodies. The diagnosis of CMA was confirmed based on positive allergen-specific IgE titers, skin prick tests and controlled oral challenges. Serum specific IgE levels for common milk allergens were measured using ImmunoCAP (GEhealthcare, Uppsala, Sweden). The symptoms ranged from cutaneous to GI and respiratory manifestations. An aliquot of the peptide fractions ($\sim 0.1 \mu\text{g}$) was spotted onto a 0.45 μm Transblot nitrocellulose membrane (Bio-Rad) and air-dried. The membranes were blocked with 5% chicken ovalbumin (Sigma) in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 1 h at room temperature (RT). The membranes were then incubated overnight at 4 °C with the sera from either allergic children or healthy individuals (negative controls, $N = 2$) diluted 1/20 in TBS-T. After washing with TBS-T, a monoclonal peroxidase conjugated anti-human IgE antibody (Sigma) in blocking solution (1/10,000) was incubated with the membrane

for 1 h at RT. The membrane was extensively rinsed with TBS-T (3×10 min) and finally with TBS (1×10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare, Sweden) and X-ray films (Kodak, Chalon/Saône, France) were used for visualising the immunoreactive peptide spots at various exposure times.

3. Results

To identify the milk-derived peptides that are resistant to proteolysis, an *in vitro* sequential, multi-phase model of GI digestion that included the BBM hydrolases from human gut for simulating intestinal degradation was developed in a previous investigation (Picariello et al., 2010). Similarly, in this study, to mimic gastro-duodenal digestion, CN and WP were sequentially incubated, first with pepsin at pH ~ 2 and second with a pool of pancreatic enzymes, including carboxypeptidase A, chymotrypsin, elastase and trypsin at pH 7.2. For the transepithelial transport studies, the gastro-duodenal digests were applied onto Caco-2 monolayers that were differentiated on porous inserts. Because Caco-2 cells maintain the hydrolytic functions of mature enterocytes, the step involving the intestinal BBM endo-/exopeptidases was omitted in this study. The operating proteolytic activity of the Caco-2 cells was confirmed by applying an aliquot of purified β -casein to a control monolayer. The RP-HPLC analysis of the apical solution revealed the almost complete disappearance of the intact protein

after 180–240 min and the production of small- and medium-sized peptides (data not shown).

3.1. Transepithelial transport of milk-derived peptides: RP-HPLC analysis

The CN- and WP-derived peptides recovered in the UP and DOWN compartments of the Caco-2 monolayers after a 330 min incubation were analyzed by RP-HPLC (Figs. 1 and 2). The integrity of the monolayers was assessed by both TEER and LY measurements. In the experimental conditions, the outflow rates of LY were typically lower than 0.5%. Both the CN- and WP-derived mixtures showed a series of minor chromatographic fractions accompanying a relatively high-abundance peptide eluted at retention time (t_r) ~ 35.5 min. This peptide was collected manually and analyzed by MALDI-TOF MS and direct injection ESI-Q-TOF-MS/MS and was identified as β -Lg 127–135 ($MH^+ = 1047.5$). Consistent with our previous results, a relatively small number of milk protein regions survived the *in vitro* GI digestion. In particular, we found that the proteolytic events operate a “process of enrichment” for the β -Lg 125–135 fragment that, owing to its intrinsic resistance, was dominant in both the CN- and WP-derived peptide fractions (Picariello et al., 2010). The β -Lg-derived sequences in the CN fraction arose from contaminating WP (5–8%) that co-precipitated with the casein fraction. The β -Lg 125–135 (sequence TPEVDDEALEK), which occurred in the gastro-duodenal digests of both CN and WP

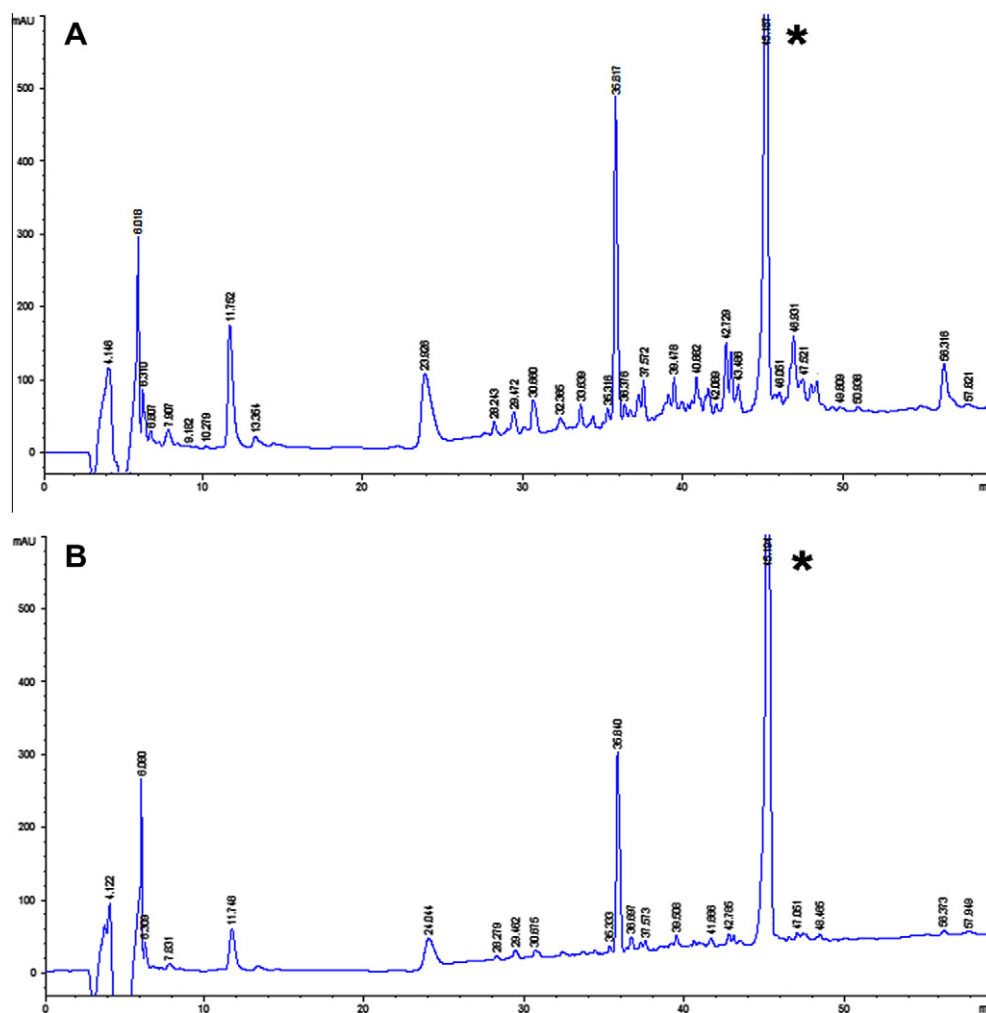


Fig. 1. RP-HPLC chromatograms of CN-derived peptides recovered from the apical (A) and basolateral (B) compartments of the Caco-2 monolayers. (*) Phenol red.

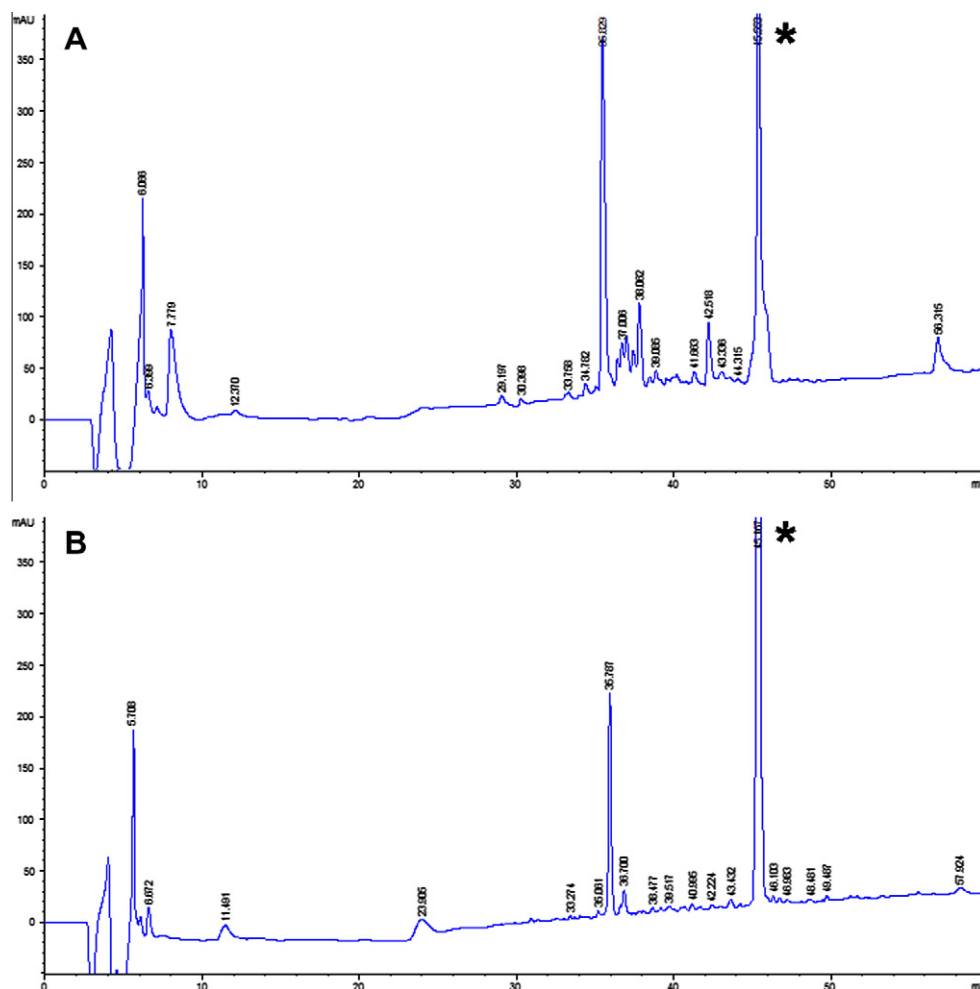


Fig. 2. RP-HPLC chromatogram of WP-derived peptides recovered from the apical (A) and basolateral (B) compartments of the Caco-2 monolayers. (*) Phenol red.

(Supplementary Data, Fig. S1) was detected only in trace amounts by HPLC-ESI MS after incubation with the Caco-2 cells in either the UP or the DOWN digests. The hydrolysis of the N-terminal TP dipeptide suggests that a prolyl dipeptidase, such as dipeptidyl peptidase IV, should be active within the Caco-2 proteolytic machinery, which is different from the BBM extracts. In this regard, it has already been established that the sequential action of the gastro-duodenal and BBM enzymes is not effective in hydrolysing dietary epitopes at the level of proline residues (Hausch, Shan, Santiago, Gray, & Khosla, 2002), differently from the Caco-2 processing. In any case, the corresponding region of β -Lg was confirmed to be by far the most resistant to digestion among the milk protein domains. Compared to the other HPLC peaks, the peptide β -Lg 127–135 exhibited an average transport rate of approximately 38% and was further enriched in the DOWN peptide fraction. Except for a few components eluted at low t_r , which were di-/tri-peptides, no additional peptides permeated the Caco-2 monolayers in a significant amount, providing evidence of a certain level of epithelial selectivity. No significant variability was observed when replicate HPLC analyses were performed to check for repeatability. To confirm the above results, β -Lg 125–135 and β -Lg 40–60, this latter encrypting a relatively resistant domain as well (Picariello et al., 2010), were chemically synthesised and utilised in the epithelial transport experiments. The synthetic peptides were applied to Caco-2 monolayers either individually (Supplementary Data, Figs. S2 and S3) or simultaneously at a 1:1 ratio (Fig. 3). These experiments confirmed that β -Lg 125–135 ($t_r \sim 37.8$ min) is almost completely converted by the Caco-2 cells to β -Lg 127–135

($t_r \sim 35.5$ min), which is in turn $\sim 38\%$ adsorbed (mean of three replicate analysis $\pm 8\%$). The β -Lg 40–60 ($MH^+ = 2469.3$) was not further processed by the Caco-2 cells; however, it was adsorbed at a very low rate (not higher than 4%).

3.2. Transepithelial transport of milk-derived peptides: mass spectrometry-based analysis

The evolution of the CN and WP fractions upon *in vitro* GI digestion and their transport across the monolayers was monitored by MS-based techniques. The MALDI-TOF MS comparisons (apical vs. basolateral Caco-2 compartments) for the CN- and WP-derived peptides are shown in the Supplementary Data (Figs. S4 and S5, respectively). The peptide mixtures that permeated across the cell epithelium were also characterised by μ -HPLC-ESI MS/MS. The comprehensive identification of the components is not a trivial issue, considering the heterogeneity of the mixtures, the relatively low-abundance of the greater part of the peptides and the almost complete lack of cleavage specificity. Therefore, MALDI-TOF MS and μ -HPLC-ESI MS/MS-based strategies were used complementarily to profile the peptide components. The peptides produced from CN and WP recovered at the DOWN side of the Caco-2 monolayers are presented in Tables 1 and 2, respectively.

Despite the somewhat expected selectivity that promoted a linearly enhanced translocation of the small-sized protein moieties (Föger, Kopf, Loretz, Albrecht, & Bernkop-Schnürch, 2008) the MALDI MS profiles for either the CN or WP peptides did not differ significantly between the two monolayer compartments. Never-

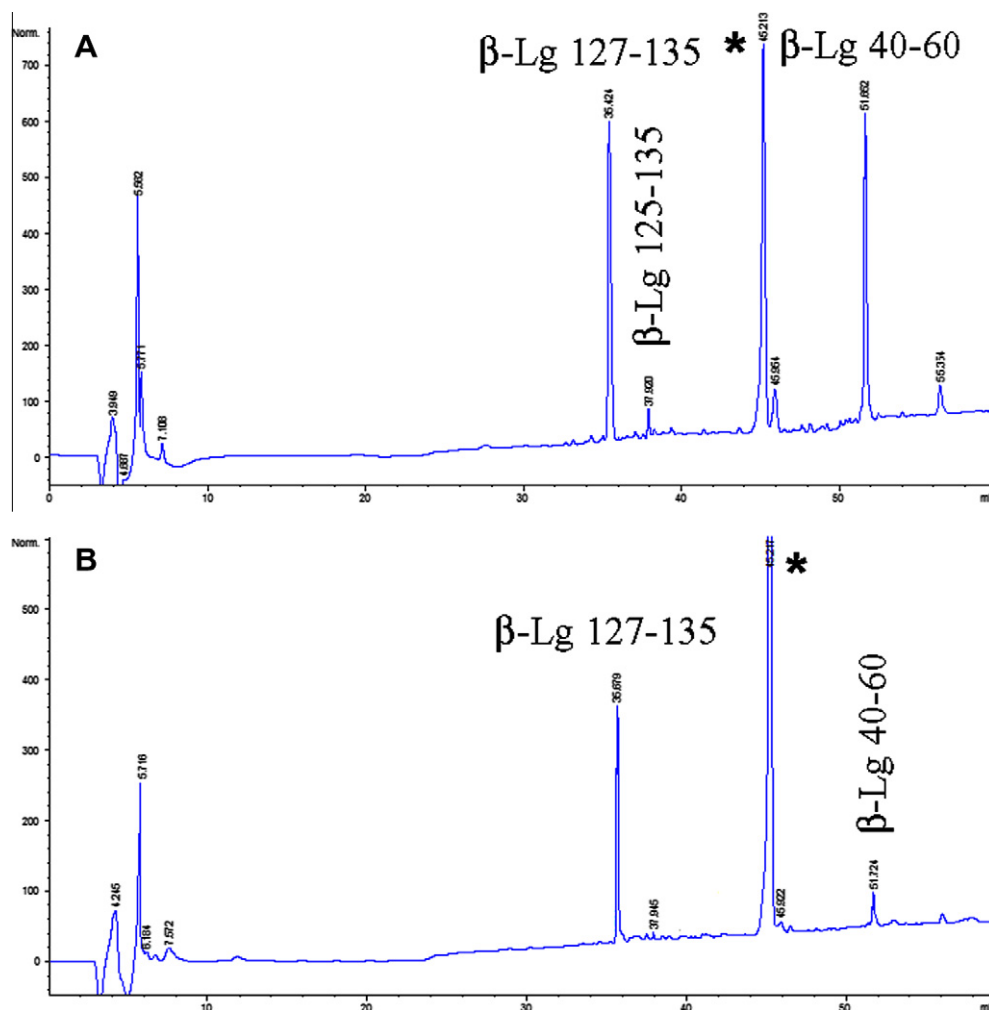


Fig. 3. RP-HPLC chromatograms of an equimolar mixture of β -Lg 125–135 and β -Lg 40–60 recovered from the apical (A) and basolateral (B) compartments of the Caco-2 monolayers. (*) Phenol red.

theless, because of the effects of ion suppression, the MS analysis did not allow us to infer quantitative information regarding the prevalence of specific peptides. Therefore, it is worth noting that the predominant peptides after *in vitro* digestion, such as β -Lg 125–135 or its shortened form, β -Lg 127–135, were not detected by MALDI-TOF MS, most likely because of the high density of acidic amino acid residues that prevent effective ionisation. The μ -HPLC-ESI MS/MS analysis confirmed that the related domain of β -Lg was clearly among the milk protein regions that were most resistant to the digestive enzymes. Because of its high sensitivity and dynamic range, MS also enabled the detection of quantitatively minor peptides. Example outputs of the software-assisted μ -HPLC-ESI MS/MS peptide identifications are displayed in the [Supplementary Data \(Figs. S6–S10\)](#). The assignment process, which was complemented and validated by the manual inspection of MS spectra, enabled the identification of peptides that arose from all the CN families except α_{s2} -CN and from either β -Lg or α -La. Both of the MS techniques indicated that the milk proteins are massively degraded by the digestive enzymes, whereas relatively few CN or WP regions partially endure digestion and cross the Caco-2 epithelium. The proline-rich central region of β -CN (residues 88–107) and the central region of α_{s1} -CN (residues 104–119) exhibited a certain relative stability. The intense signals of the fragments arising from the C-terminal region of β -CN and from the N-terminal domain of α_{s1} -CN, observed particularly in the MALDI-TOF MS spectra, should

be ascribed to a high ionisation efficiency rather than to their abundance (Bouzerzour et al., 2012; Picariello et al., 2010). Some signals, for example m/z 994.5 (β -CN 194–202), appeared only in the DOWN compartment, most likely reflecting their secondary production caused by the Caco-2 processing of longer precursors. Aside from some large-size α -La-derived fragments that were most likely resistant to hydrolysis due to their disulphide cross-linkages, no peptide at a molecular mass greater than 3 kDa was detected by MALDI MS. However, disulphides *per se* do not seem to confer resistance because we did not detect S–S cross-linked peptides of β -Lg. Notably, some mineral-binding α_{s1} - and β -CN phosphopeptides survived digestion, although to a minor extent, and partly translocated across the Caco-2 monolayers. By contrast, as also previously demonstrated, the phosphate-rich casein clusters were almost completely hydrolysed.

3.3. IgE-binding properties of CN- and WP-derived peptides

The IgE-binding properties of the CN- and WP-peptides sampled from the UP and DOWN sides of the Caco-2 monolayers were assayed by dot-blot analysis using the sera from $N = 5$ children with a documented history of CMA as the source of the specific IgEs. Among these children, one had outgrown the allergy and had acquired tolerance to milk proteins. The dot-blot analysis was performed to determine the reactivity against the RP-HPLC-

Table 1

MS-based identification of CN-derived peptides translocated across Caco-2 monolayers.

Mass (M+H) ⁺	Identification	Technique of identification
491.3	β-CN 116–119	ESI-MS/MS
576.4	β-CN 134–138	ESI-MS/MS
665.4	α _{s1} -CN β-CN 156–161	ESI-MS/MS
689.5	β-CN 134–139	ESI-MS/MS
690.4	β-CN 184–189 Met-OX	ESI-MS/MS
722.5	α _{s1} -CN 17–22	ESI-MS/MS
758.4	α _{s1} -CN 25–31	ESI-MS/MS
776.4	α _{s1} -CN 155–161 pyroGlu	ESI-MS/MS
780.6	β-CN 170–176	ESI-MS/MS
791.4	β-CN 126–132	ESI-MS/MS
793.4	α _{s1} -CN 156–163	ESI-MS/MS
830.5	β-CN 177–186	MALDI and ESI-MS/MS
831.4	α _{s1} -CN 84–90	MALDI and ESI-MS/MS
871.5	α _{s1} -CN 16–22	MALDI and ESI-MS/MS
886.4	κ-CN 154–161	ESI-MS/MS
907.4	α _{s1} -CN 8–15	MALDI and ESI-MS/MS
910.5	α _{s1} -CN 125–132	ESI-MS/MS
926.4	β-CN 85–92	MALDI and ESI-MS/MS
936.4	β-CN 116–123	ESI-MS/MS
946.5	α _{s1} -CN 35–42	ESI-MS/MS
965.6	β-CN 74–82	ESI-MS/MS
977.5	β-CN 194–202 pyroGlu	MALDI and ESI-MS/MS
994.5	β-CN 194–202	MALDI and ESI-MS/MS
1023.5	β-CN 116–124	ESI-MS/MS
1025.6	β-CN 84–92	ESI-MS/MS
1041.4	β-CN 126–134	MALDI and ESI-MS/MS
1051.5	β-CN 194–203	MALDI and ESI-MS/MS
1079.6	β-CN 73–82	ESI-MS/MS
1099.6	α _{s1} -CN 14–22	ESI-MS/MS
1112.5	α _{s1} -CN 10–19	ESI-MS/MS
1124.6	β-CN 83–92	ESI-MS/MS
1140.6	α _{s1} -CN 1–9	MALDI and ESI-MS/MS
1151.6	β-CN 145–154	MALDI and ESI-MS/MS
1157.6	β-CN 193–202	MALDI and ESI-MS/MS
1177.6	α _{s1} -CN 27–36	MALDI and ESI-MS/MS
1227.7	α _{s1} -CN 13–22	ESI-MS/MS
1252.6	κ-CN 1–10 pyroGlu	ESI-MS/MS
1267.6	α _{s1} -CN 125–135	MALDI and ESI-MS/MS
1270.7	β-CN 192–202	MALDI and ESI-MS/MS
1272.6	κ-CN 113–123	ESI-MS/MS
1337.7	α _{s1} -CN 80–90	MALDI and ESI-MS/MS
1363.8	β-CN 197–209	MALDI and ESI-MS/MS
1373.6	α _{s1} -CN 174–186	ESI-MS/MS
1373.6	κ-CN 113–124	ESI-MS/MS
1377.6	β-CN 195–207	MALDI and ESI-MS/MS
1494.8	α _{s1} -CN 10–22	MALDI and ESI-MS/MS
1605.8	α _{s1} -CN 9–22 pyroGlu	MALDI and ESI-MS/MS
1622.9	α _{s1} -CN 9–22	MALDI MS
1697.2	α _{s1} -CN 105–119 1P ^a	MALDI MS
1708.9	α _{s1} -CN 105–119	MALDI and ESI-MS/MS
1759.9	α _{s1} -CN 8–22	MALDI and ESI-MS/MS
1788.9	α _{s1} -CN 105–119 1P	MALDI and ESI-MS/MS
1846.0	β-CN 153–169	ESI-MS/MS
1859.9	α _{s1} -CN 104–119 1P ^a	MALDI MS
1871.9	α _{s1} -CN 104–119	MALDI MS
1938.0	β-CN 145–161	MALDI MS
1951.9	α _{s1} -CN 104–119 1P	ESI-MS/MS
2037.0	β-CN 145–162	MALDI MS
2068.9	β-CN 144–161	MALDI and ESI-MS/MS
2084.9	β-CN 144–161 Met-OX	ESI-MS/MS
2092.0	α _{s1} -CN 125–143	ESI-MS/MS
2104.9	α _{s1} -CN 174–193	MALDI and ESI-MS/MS
2168.1	β-CN 144–162	MALDI and ESI-MS/MS
2206.1	β-CN 179–196	MALDI MS
2316.9	β-CN 33–51 1P ^a	MALDI MS
2409.0	β-CN 33–51 1P	MALDI and ESI-MS/MS
2463.9	β-CN 33–52 1P ^a	MALDI MS
2556.0	β-CN 33–52 1P	MALDI and ESI-MS/MS
2632.4	β-CN 174–195	MALDI MS

^a Metastable ion.

isolated CN and WP. Table 3 summarises the main clinical characteristics of the children enrolled in the study and the immunoreactivity patterns of the sera to the milk proteins, peptide

Table 2

MS-based identification of WP-derived peptides translocated across Caco-2 monolayers.

Mass (M+H) ⁺	Identification	Technique of identification
673.4	β-Lg 9–14	ESI-MS/MS
803.4	β-Lg 85–91	ESI-MS/MS
871.5	α-La 95–102	ESI-MS/MS
853.4	β-Lg 94–100	ESI-MS/MS
916.4	β-Lg 84–91	ESI-MS/MS
926.4	β-CN 85–92 – imp.	MALDI MS and ESI-MS/MS
1015.6	β-Lg 84–92	ESI-MS/MS
1044.6	β-Lg 83–91	MALDI MS
1047.5	β-Lg 127–135	ESI-MS/MS
1065.6	β-Lg 92–100	ESI-MS/MS
1141.5	β-Lg 16–26	MALDI MS
1245.6	β-Lg 125–135	ESI-MS/MS
1320.6	β-Lg 60–70 Var. B	MALDI MS
1378.6	β-Lg 60–70 Var. A	MALDI MS
1432.6	β-Lg 150–161	MALDI MS and ESI-MS/MS
1434.6	α-La 37–49	MALDI MS and ESI-MS/MS
1437.7	β-Lg 127–138	MALDI MS and ESI-MS/MS
1455.7	β-Lg 43–55	MALDI MS and ESI-MS/MS
1500.8	β-Lg 123–135	MALDI MS and ESI-MS/MS
1546.6	β-Lg 150–162	MALDI MS and ESI-MS/MS
1568.8	β-Lg 43–56	MALDI MS and ESI-MS/MS
1597.7	α-La 37–50	ESI-MS/MS
1618.8	β-Lg 42–55	ESI-MS/MS
1635.8	β-Lg 125–138	ESI-MS/MS
1663.8	β-Lg 124–137	MALDI MS and ESI-MS/MS
1681.9	β-Lg 43–57	MALDI MS and ESI-MS/MS
1717.9	β-Lg 41–55	MALDI MS and ESI-MS/MS
1830.9	β-Lg 41–56	MALDI MS and ESI-MS/MS
1837.9	β-Lg 10–26	MALDI MS and ESI-MS/MS
1891.0	β-Lg 123–138	MALDI MS and ESI-MS/MS
1923.0	β-Lg 43–59	MALDI MS and ESI-MS/MS
1944.0	β-Lg 41–57	MALDI MS
2100.1	β-Lg 40–57	MALDI MS and ESI-MS/MS
2252.1	β-Lg 45–63	MALDI MS and ESI-MS/MS
2313.2	β-Lg 41–60	MALDI MS and ESI-MS/MS
2316.9	β-CN 33–51 1P ^a – imp.	MALDI MS
2409.0	β-CN 33–51 1P – imp.	MALDI MS and ESI-MS/MS
2463.9	β-CN 33–52 1P ^a – imp.	MALDI MS
2556.0	β-CN 33–52 1P imp.	MALDI MS and ESI-MS/MS
2609.2	β-Lg 116–138	MALDI MS
2623.2	β-Lg 114–136	MALDI MS
2679.4	β-Lg 14–39	MALDI MS
2737.3	β-Lg 115–138	MALDI MS
2752.4	β-Lg 123–146	MALDI MS
2793.8	β-Lg 116–140	MALDI MS
2808.3	β-Lg 115–139	MALDI MS
2866.3	β-Lg 114–138	MALDI MS
2921.5	β-Lg 116–141	MALDI MS
<i>Tentative assignment of disulphide cross-linked peptides of α-La</i>		
3515.4	α-La 1–24-S-S-117–122	MALDI MS
3742.5	α-La 1–24-S-S-115–122	MALDI MS

^a Metastable ions. Imp.: casein-derived impurity.

digests and synthetic peptides. The reconstructed patterns of reactivity are shown in the [Supplementary data \(Fig. S11\)](#). Although the limited number of sera assayed impedes the inferring of statistically significant figures, all the allergic children exhibited a strong response against α_{s2}-CN. This finding is consistent with previous results indicating that α_{s2}-CN, which is lacking in human breast milk, is the most prevalent antigen of individuals with IgE-mediated CMA (Natale et al., 2004). Two sera, AS3 and AS4, clearly contained specific IgEs against α_{s1}-CN, whereas AS1 and AS3 were reactive against κ-CN and β-CN, respectively. Only one of the sera, AS3, exhibited significant sensitisation against β-Lg. AS3 displayed IgEs against α_{s1}-CN, β-CN and α-La and, remarkably, significant reactivity against *in vitro* GI digests of WP and the synthetic peptide, β-Lg 125–135. The response against β-Lg 127–135, which was collected after Caco-2 processing, appeared more intense compared to its β-Lg 125–135 precursor. The serum of the tolerant

Table 3
Clinical characteristics of allergic children diagnosed with cow's milk allergy (CMA) who were enrolled in the study and IgE-binding reactivity to milk proteins, *in vitro* GI digests and synthetic peptides assayed by dot-blot.

Serum		AS1	AS2	AS3	AS4	TS	CS
Age (months)		76	72	39	34	49	38
Symptoms ^a		U, AE	GI	U, GI	U, AE	GI	–
Total IgE (kU/L)		234	111	463	279	69	<1
Specific IgE (kU/L)		6.4	11.9	69.1	21.4	2.4	n.d.
<i>IgE-binding immunoreactivity</i>							
Spot No.	Tested sample						
1	κ-CN	+++	–	–	±	–	–
2	α _{s2} -CN	+++	++	+++	+++	±	–
3	α _{s1} -CN	±	+	+++	++	–	–
4	β-CN	±	+	+++	–	–	–
5	Ribonuclease A (negative control)	–	–	–	–	–	–
6	α-La	–	±	++	+++	++	–
7	β-Lg	–	±	++	±	–	–
8	Total CN	+++	+++	+++	+++	++	+
9	Total WP	+++	+++	+++	+++	++	+
10	CN digests – UP side	++	–	±	–	–	–
11	WP digests – UP side	++	+	+	–	–	–
12	CN digests – DOWN side	±	±	±	–	–	–
13	WP digests – DOWN side	+	–	+	–	–	–
14	Synthetic β-Lg 125–135	–	–	+	–	–	–
15	β-Lg 127–135 from Caco-2 processing (DOWN side)	–	–	++	–	–	–
16	Synthetic β-Lg 40–60	–	–	–	–	–	–

Legend: AS = sera of children with CMA; TS = serum of child that outgrew allergy (tolerant); CS = control serum of an healthy child.

^a Symptoms are referred to the time of CMA diagnosis: U = urticaria; AE = angioedema; GI = gastrointestinal discomfort.

child showed a certain degree of sensitisation against only α-La. It would be interesting to evaluate on a larger scale whether the sensitisation against the α-La epitopes may be associated with the acquisition of oral tolerance to milk proteins (Järvinen et al., 2002).

In all the sera, no discrepancy in the IgE-affinity was observed between the HPLC-isolated *N*-glycosylated α-La and non-glycosylated α-La (data not shown), thereby suggesting that glycans do not play a significant immunogenic role. Only AS1 exhibited strong reactivity against κ-CN. All the sera, including the control, were reactive against the total CN and WP fractions. This unexpected reactivity was attributed to a certain degree of aspecific cross-reactivity of the secondary anti-IgE against the bovine milk immunoglobulins and to mutual contamination of the CN and WP fractions. After simulated GI digestion, the IgE-binding capability of CN and WP was drastically reduced (AS1 and AS3) or completely lost.

4. Discussion

Tracking the metabolic fate of dietary proteins is one of the key aspects to understanding the effects of food on human health. Therefore, it is not surprising that there has recently been a considerable increase in the number of studies attempting to define the degradation routes of proteins during digestion. In this regard, milk has been used as both a case study and a model source of dietary proteins and is by far the most extensively investigated food. Despite the large number of *in vitro* and *in vivo* models that have been developed to mimic the phases of physiological digestion, the variability of factors that affect the metabolic processes provides an incomplete picture with regard to the protein domains that reach the jejunum and ileum, where they can be absorbed. Regardless of the specific model of GI digestion, some selected milk-derived peptides have emerged as intrinsically resistant to proteolysis.

In general, both CN and WP are extensively hydrolysed at the end of gastric and duodenal sequential digestion. Only β-Lg is known to exhibit a certain resistance to peptic hydrolysis. Our current results confirm that only a few selected regions of CN and WP survive *in vitro* proteolysis, although to a minor extent. If low num-

bers of α_{s1}-CN and β-CN mono-phosphorylated peptides are the exception, the presence of the most commonly claimed bioactive peptides of milk on the basolateral side of the Caco-2 monolayers was negligible. For instance, the opioid-like β-casomorphins of the β-CN 60–68 strategic region were not detected in either the UP or DOWN compartments. Although synthetic human β-casomorphins can translocate across Caco-2 monolayers (Iwan et al., 2008), their resistance to GI digestion is doubtful, considering that their degradation by either gastro-pancreatic and BBM enzymes (Petrilli et al., 1984) or dipeptidyl peptidase IV from Caco-2 cells was complete in less than 60 min (Iwan et al., 2008). By contrast, the ability of caseinphosphopeptides to translocate across the intestinal epithelium could have important implications because of their role as mineral carriers.

β-Lg 127–135 was a unique peptide that was able to endure proteolysis and be translocated across the Caco-2 cells in considerable amounts. This peptide is encrypted in an acidic protein domain that is hypothesised to represent one of the antigenic determinants of the “major milk allergen”, β-Lg (Bos d 5). Notably, the affinity of the β-Lg 127–135 peptide for the specific IgEs of a child with serum highly immune-reactive to β-Lg (AS3) was higher than the affinity of the β-Lg 125–135 precursor that was unprocessed by the Caco-2 cells. These findings suggest the involvement of the relevant β-Lg region in eliciting immunoreactions in CMA individuals and highlight the possible role of intestinal processing in unmasking or delivering the epitopes to mucosal immunocompetent cells.

In spite of the limited number of sera from CMA children that were screened, a high variability was observed in the dot-blot immune-reactivity against the milk proteins and derived peptides. Whereas intact casein sub-types were immune-reactive for four of the five serum samples, one serum, i.e. AS3, exclusively showed a significant response against β-Lg. Notably, the serum of the child who outgrew the milk allergy exhibited reactivity only against α-La. We have examined only one CMA tolerant child and the immunoreactivity against α-La-derived epitopes requires further investigation to establish if it may be a possible prognostic factor associated with the acquisition of milk tolerance. Only AS1 displayed a residual IgE reactivity to CN and WP digests, whereas

the remaining sera almost completely lost their IgE-binding affinity, with the exception of a faint response of AS3. It is important to note that the IgE-reactivity of AS1 to the Caco2-processed CN and WP was markedly reduced if compared to that of the parent proteins. These observations confirm that the intestinal permeability of large-sized peptides, which is in part attributable to an immature development of the digestive system and to a slack bowel barrier in infants, could be a primary aspect of the mechanism of immuno-sensitisation. The well-established sentry-like role of dendritic cells (DCs) also suggests that sensitisation could arise at the level of the intestinal lumen, where immunogenic dietary peptides can be sampled by dendrites that protrude across the mucosa out of the epithelium (Rescigno et al., 2001). In any case, considering that DCs express tight junctions, the mechanism by which allergens make contact with circulating IgEs when they elicit systemic immune reactions remains to be elucidated. Therefore, in addition to the possible role of migrating DCs (Smit et al., 2011), transport of large peptides across the epithelium is certainly occurring. These hypotheses are consistent with Roth-Walter et al. (2008), who demonstrated that an allergic reaction to milk proteins requires sensitisation through Peyer's patches and an efficient transfer of soluble proteins across the gut epithelial barrier.

Indeed, the divergence between *in vitro* models and GI physiology suggests that the production of larger polypeptides and a higher degree of peptide uptake should most likely occur *in vivo* because of a series of concomitant factors: (1) food matrices have a strong impact on both protein degradation and uptake of food allergens, with potential consequences for the sensitising capacity and clinical symptoms of allergic individuals (Schulten, Lauer, Scheurer, Thalhammer, & Bohle, 2011). For instance, using the *in vivo* animal model it has been demonstrated the persistence of large fragments of WP in the jejunum at an advanced stage of the digestion of whole milk (Bouzerzour et al., 2012); (2) due to the colonic origin of cells (Matsson et al., 2005), the monolayers of Caco-2 have TEER values that are much higher compared to those for the human bowel epithelium (Legen, Salobir, & Kerč, 2005) and, consequently, peptides and drugs are generally absorbed by the model monolayers at a much slower rate than by the human jejunum (Artursson et al., 2012; Föger et al., 2008); (3) the gut-specific absorptive surfaces, which are difficult to define because of epithelial crypts and villi, are thought to be much wider than the cell monolayers; (4) a supplementary passive paracellular transport of specific peptides *in vitro* may be obstructed by saturation events; (5) additional mechanisms of active transcytosis can operate *in vivo*. Furthermore, mechanisms of translocation can differ as a function of the peptide structural properties, including charge, hydrophobicity, size and hydrogen-bonding potential (Regazzo et al., 2010). An overlooked contribution of conformational epitopes to IgE binding that has been demonstrated for other food allergens (Albrecht et al., 2009) appears unlikely for milk proteins, particularly for caseins that are rheomorphic proteins and lack a defined conformation (Dupont et al., 2010). Other factors, such as the production of mucus *in vivo*, which can be partially simulated by the co-culture of Caco-2 and goblet-like HT29 cells, the regulation of both electrolyte and water flux, the time of intestinal transition and peptide-peptide interactions could impact the discrepancy in the absorption rates of predictive cell models with respect to the human gut.

Conclusion

A model has been developed to follow the degradation and adsorption of dietary proteins after ingestion. This model can be used to verify the survival and delivery of bioactive peptides and to test the possible role of peptides in the onset of allergic reac-

tions. The results of this study indicate that selected milk-derived peptides are capable of crossing the gut epithelium and triggering an allergic response. By contrast, the reactions of CMA children are not consistent with the loss of IgE reactivity to milk proteins after digestion and epithelial transport. The probable enhanced degree of adsorption in the human jejunum supports the concept that large-size polypeptides could breach the gut epithelium *in vivo*, thereby reaching the *lamina propria* and, most likely, the blood circulation. These outcomes point out the need to design appropriate *in vivo* bioavailability assays to track the metabolic fate of dietary proteins and to validate the actual involvement of milk derived-peptides in physiopathological events.

Acknowledgements

This work was in part supported by a dedicated grant from the Italian Ministry of Economy and Finance to CNR and ENEA for the project “Innovazione e sviluppo del Mezzogiorno – Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare” – Legge n.191/2009. Mass spectrometry experiments were carried out at the CeSma-Pro Bio facility of the Institute of Food Science – CNR, Avellino (Italy). The Authors thank the American Journal Experts (www.journalexperts.com) for text revision.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.01.063>.

References

- Albrecht, M., Kühne, Y., Ballmer-Weber, B. K., Becker, W. M., Holzhauser, T., Lauer, I., et al. (2009). Relevance of IgE binding to short peptides for the allergenic activity of food allergens. *J. Allergy Clin. Immunol.*, 124, 328–336.
- Artursson, P., Palm, K., & Luthman, K. (2012). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery Reviews*. <http://dx.doi.org/10.1016/j.addr.2012.09.005>.
- Aschaffenburg, R., & Drewry, J. (1959). *Proceedings of 15th International Dairy Congress* (Vol. 3, p. 1631), London.
- Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huërou-Luron, I., et al. (2012). *In vivo* digestion of infant formula in piglets: Protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition*, 1, 1–10.
- Cakir-Kiefer, C., Miclo, L., Balandras, F., Dary, A., Siligout, C., & Le Roix, Y. (2011). Transport across Caco-2 cell monolayer and sensitivity to hydrolysis of two anxiolytic peptides from α s1-casein, α caseozepine, and α s1-casein-f91–97: Effect of bile salts. *Journal of Agricultural and Food Chemistry*, 59, 11956–11965.
- Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus. *Journal of Dairy Science*, 83, 1187–1195.
- Defernez, M., Mandalari, G., & Mills, E. N. (2010). Quantitative assessment of multi-laboratory reproducibility of SDS–PAGE assays: Digestion pattern of beta-casein and beta-lactoglobulin under simulated conditions. *Electrophoresis*, 31, 2838–2848.
- Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., et al. (2010). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition & Food Research*, 54, 1677–1689.
- Föger, F., Kopf, A., Loretz, B., Albrecht, K., & Bernkop-Schnürch, A. (2008). Correlation of *in vitro* and *in vivo* models for the oral absorption of peptide drugs. *Amino Acids*, 35, 233–241.
- Garen, A., & Levinthal, C. (1960). A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochimica Biophysica Acta*, 38, 470–483.
- Hausch, F., Shan, L., Santiago, N. A., Gray, G. M., & Khosla, C. (2002). Intestinal digestive resistance of immunodominant gliadin peptides. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 283, G996–1003.
- Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry*, 125, 1–12.
- Iwan, M., Jarmolowska, B., Bielkiewicz, K., Kostyra, E., Kostyra, H., & Kaczmarek, M. (2008). Transport of micro-opioid receptor agonists and antagonist peptides across Caco-2 monolayer. *Peptides*, 29, 1042–1047.
- Järvinen, K. M., Beyer, K., Vila, L., Chatchatee, P., Busse, P. J., & Sampson, H. A. (2002). B-cell epitopes as a screening instrument for persistent cow's milk allergy. *The Journal of Allergy and Clinical Immunology*, 110, 293–297.
- Jensen-Jarolim, E., & Untersmayr, E. (2006). Food safety: *In vitro* digestion tests are non-predictive for allergenic potential of food in stomach insufficiency. *Immunology Letters*, 102, 118–119.

- Korhonen, H., & Philanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16, 945–960.
- Legen, I., Salobir, M., & Kerč, J. (2005). Comparison of different intestinal epithelia as models for absorption enhancement studies. *International Journal of Pharmaceutics*, 291, 183–188.
- Mandalari, G., Adel-Patient, K., Barkholt, V., Baro, C., Bennet, L., Bublin, M., et al. (2009). In vitro digestibility of beta-casein and beta-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. *Regulatory Toxicology and Pharmacology*, 55, 372–381.
- Matsson, P., Bergström, C. A., Nagahara, N., Tavelin, S., Norinder, U., & Artursson, P. (2005). Exploring the role of different drug transport routes in permeability screening. *Journal of Medicinal Chemistry*, 48, 604–613.
- Meisel, H. (2005). Biochemical properties of peptides encrypted in bovine milk proteins. *Current Medicinal Chemistry*, 12, 1905–1919.
- Mineo, H., Hara, H., Shigematsu, N., Okuhara, Y., & Tomita, F. (2002). Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions in vitro. *Journal of Nutrition*, 132, 3394–3399.
- Natale, M., Bisson, C., Monti, G., Peltran, A., Garoffo, L. P., Valentini, S., et al. (2004). Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Molecular Nutrition & Food Research*, 48, 363–369.
- Petrilli, P., Picone, D., Caporale, C., Addeo, F., Auricchio, S., & Marino, G. (1984). Does casomorphin have a functional role? *FEBS Letters*, 169, 53–56.
- Picariello, G., Ferranti, P., Fierro, O., Mamone, F., Caira, S., Di Luccia, A., et al. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Science*, 878, 295–308.
- Regazzo, D., Mollé, D., Gabai, G., Tomé, D., Dupont, D., Leonil, J., et al. (2010). The (193–209) 17-residues peptide of bovine β -casein is transported through Caco-2 monolayer. *Molecular Nutrition & Food Research*, 54, 1428–1435.
- Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., et al. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology*, 2, 361–367.
- Roth-Walter, F., Berin, M. C., Arnaboldi, P., Escalante, C. R., Dahan, S., Rauch, J., et al. (2008). Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy*, 63, 882–890.
- Schulten, V., Lauer, I., Scheurer, S., Thalhammer, T., & Bohle, B. (2011). A food matrix reduces digestion and absorption of food allergens in vivo. *Molecular Nutrition & Food Research*, 55, 1484–1491.
- Sienkiewicz-Szłapka, E., Jarmołowska, B., Krawczuk, S., Kostyra, E., Kostyra, H., & Bielikowicz, K. (2009). Transport of bovine milk-derived opioid peptides across a Caco-2 monolayer. *International Dairy Journal*, 19, 252–257.
- Silva, S. V., & Xavier Malcata, F. (2005). Caseins as source of bioactive peptides. *International Dairy Journal*, 15, 1–15.
- Smit, J. J., Bol-Schoenmakers, M., Hassing, I., Fiechter, D., Boon, L., Bleumink, R., et al. (2011). The role of intestinal dendritic cells subsets in the establishment of food allergy. *Clinical and Experimental Allergy*, 41, 890–898.

Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins

Gianluca Picariello^{a,b,*}, Giuseppe Iacomino^a, Gianfranco Mamone^a, Pasquale Ferranti^{a,b}, Olga Fierro^a, Carmen Gianfrani^{a,c}, Aldo Di Luccia^{a,d}, Francesco Addeo^{a,b}

^a *Istituto di Scienze dell'Alimentazione (ISA) - CNR, Via Roma 64, 83100 Avellino, Italy*

^b *Dipartimento di Scienza degli Alimenti, University of Naples "Federico II," Parco Gussone, Portici (NA) 80055, Italy*

^c *European Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples "Federico II," Naples, Italy*

^d *Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, Via Napoli 25, 71100 Foggia, Italy*

***Corresponding author:**

Dr. Gianluca Picariello, Istituto di Scienze dell'Alimentazione (ISA) – Consiglio Nazionale delle Ricerche (CNR), Via Roma 64, 83100 Avellino, Italy.

Email: picariello@isa.cnr.it

Tel.: +39 0825 299216; **Fax:** +39 0825 781585

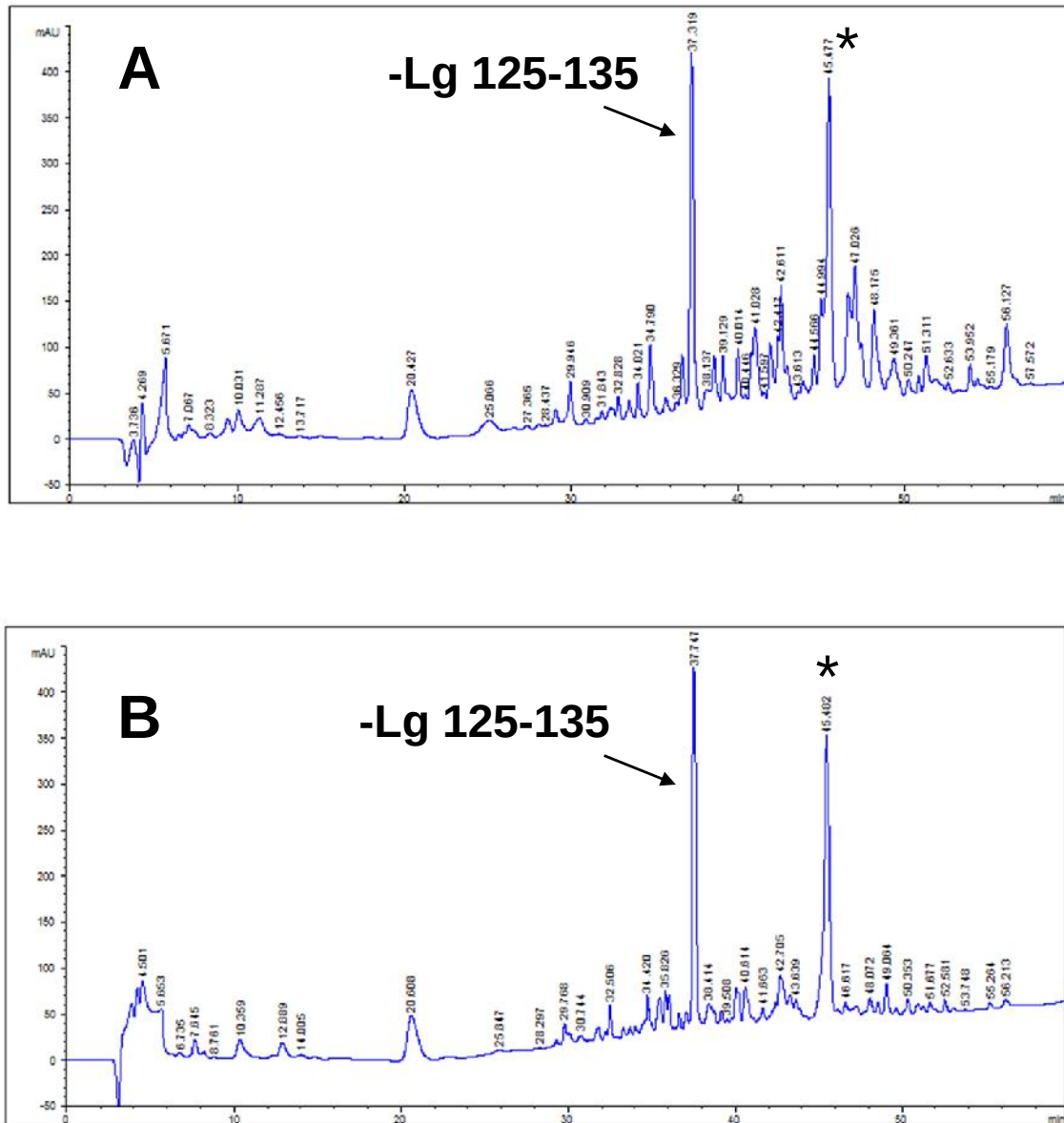


Figure S1. RP-HPLC analysis of the gastro-pancreatic digests of CN (A) and WP (B) before application to Caco-2 monolayers (time=0 peptide digests). *phenol red.

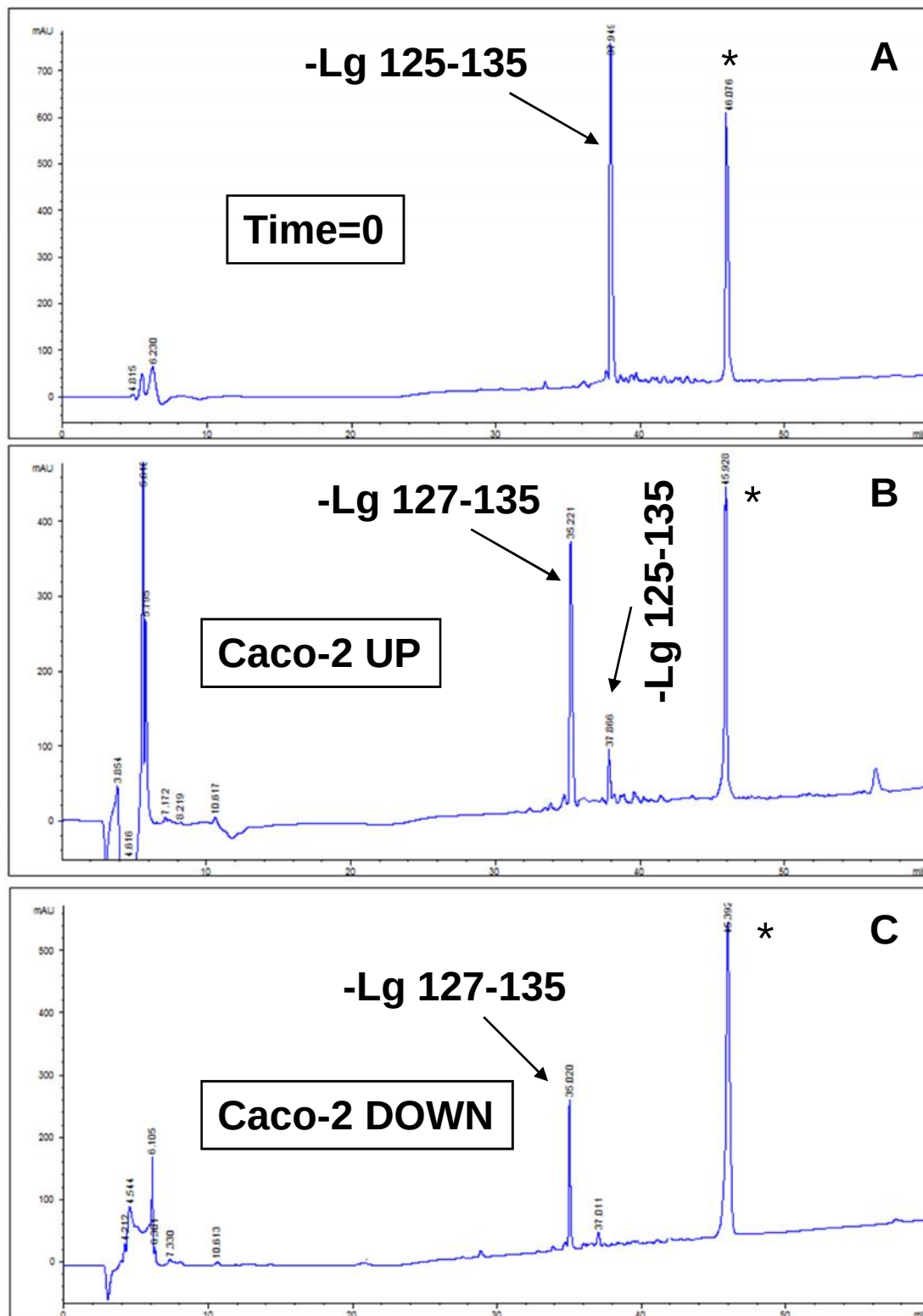


Figure S2. RP-HPLC analysis of synthetic β -Lg 125-135 applied to the Caco-2 monolayers (A) time=0; (B) recovered from the apical (B) and basolateral (C) compartment after 330 min. Caco-2 enterocytes process the peptide and remove the TP N-terminal dipeptide. The β -Lg 127-135 permeates monolayers at a rate of ~38%. *phenol red.

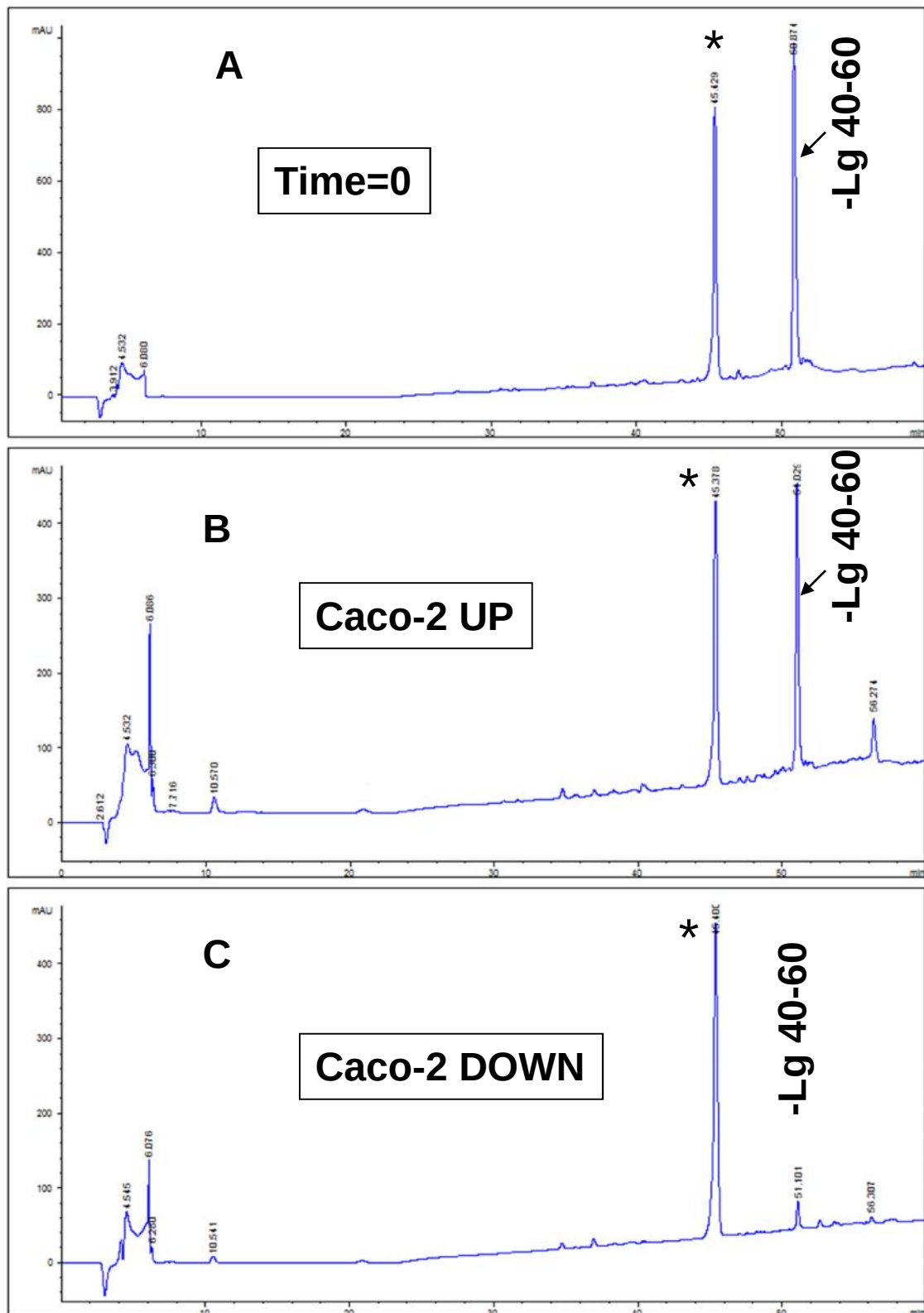


Figure S3. Synthetic β -Lg 40-60 applied to the Caco-2 monolayers (A) time zero; (B) recovered from the apical (B) and basolateral (C) compartment after 330 min. The peptide β -Lg 40-60 is not processed by Caco-2 and permeates monolayers at a rate not higher than 4%. The peak at $t_r \sim 56.3$ min is a cell-released blank substance. *phenol red.

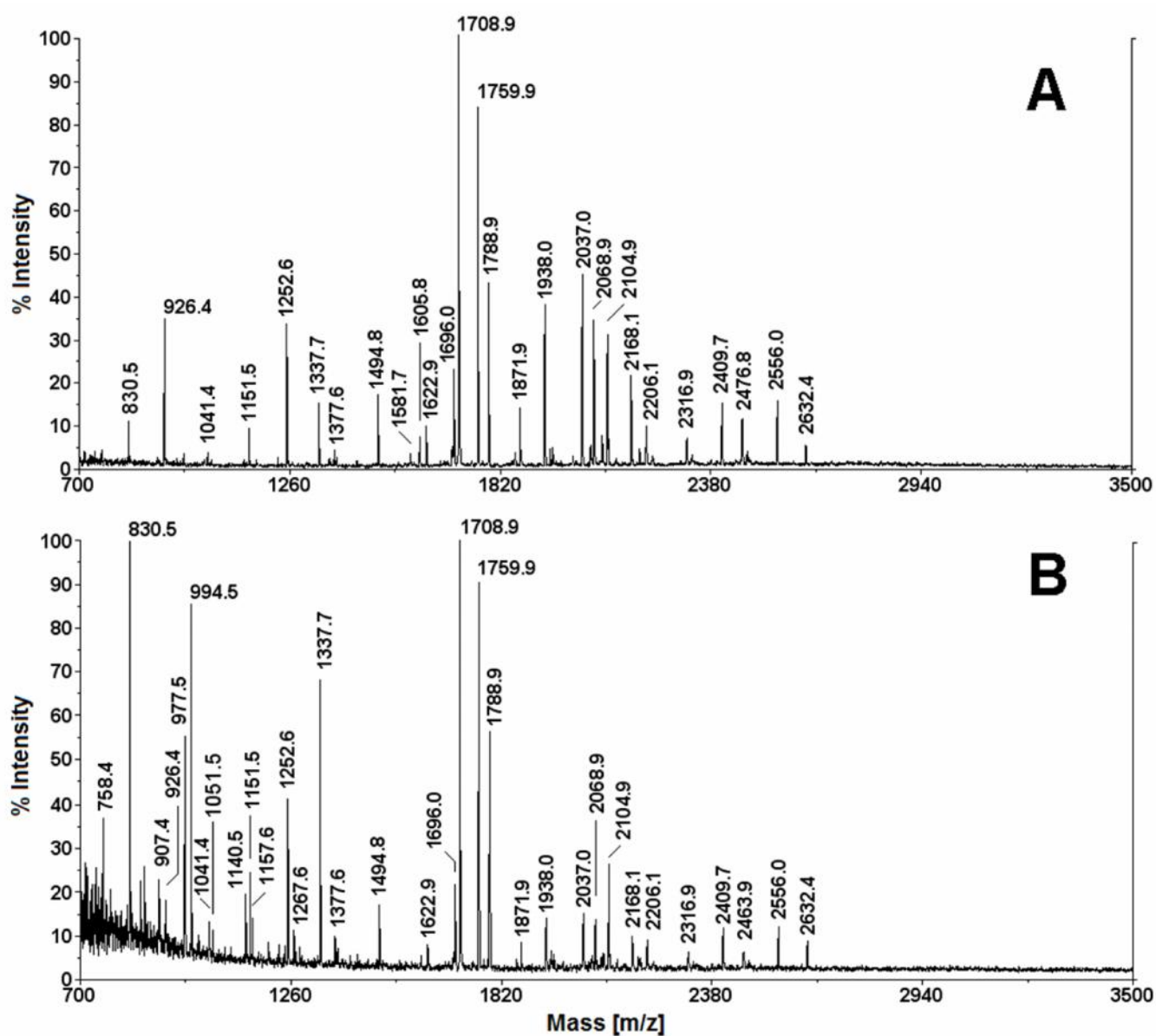


Figure S4. MALDI-TOF mass spectra of CN-derived peptides recovered from the apical (A) and basolateral (B) compartments of the Caco-2 monolayers.

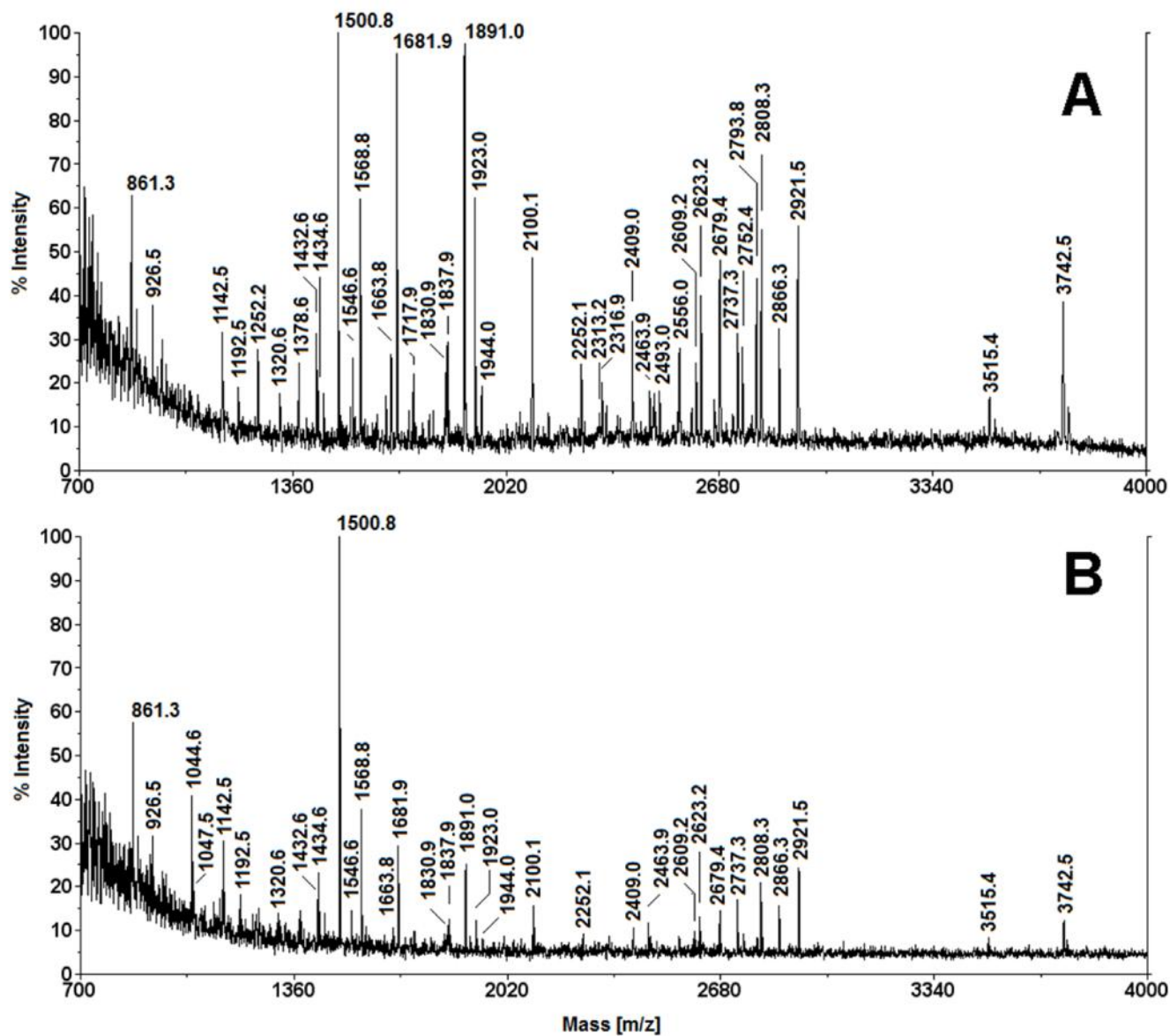


Figure S5. MALDI-TOF mass spectra of WP-derived peptides recovered from the apical (A) and basolateral (B) compartments of the Caco-2 monolayers.

1 Acc. #: [P02662](#) Uniprot ID: [CASA1](#) BOVIN Species: BOVIN Name: Alpha-S1-casein
Organism: Bos taurus Gene: CSN1S1 Existence: Evidence at protein level Version: 2
Protein MW: 24529.1 Protein pI: 5.0 Protein Length: 214

1 MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV APFPEVFGKE KVNELSKDIG SESTEDQAME DIKQMEAESI
81 SSSEEIVPNS VEQKHIQKED VPSEYLGYL EQLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM IGVNQELAYF
161 YPELFRQFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP IGSENSEKTT MPLW

Num Unique	% Cov	Best Disc Score	Best Expect Val
19	40.7	3.55	3.5e-7

M+H	m/z	z	Da	DB Peptide	Variable Mods	RT	Score	Expect	# in DB
1708.8926	570.3024	3	-0.010	KVPQLEIVPNSAEER		23.14	30.2	3.5e-7	1
1494.8055	747.9064	2	-0.011	GLPQEVLNENLLR		30.94	30.4	1.0e-6	1
1337.6729	669.3401	2	-0.0040	HIQKEDVPSEY		13.21	28.6	1.3e-6	1
1227.6505	614.3289	2	-0.0094	QEVLNENLLR		25.91	33.5	6.2e-6	1
1871.9567	624.6571	3	-0.0098	YKVPQLEIVPNSAEER		26.74	19.9	7.5e-6	1
907.4477	454.2275	2	-0.0077	HQGLPQEV		18.32	21.0	2.0e-5	1
1759.9220	587.3122	3	-0.0077	HQGLPQEVLNENLLR		27.92	31.6	3.2e-5	1
1272.6023	636.8048	2	-0.0041	DAPSFSDIPNPI		33.61	22.6	7.7e-5	1
910.4721	455.7397	2	-0.0010	EGIHAQQK		11.27	21.9	1.3e-4	1
1112.5477	556.7775	2	-0.0053	GLPQEVLNEN		26.39	20.9	1.7e-4	1
1373.6489	687.3281	2	-0.0047	TDAPSFSDIPNPI		32.66	20.3	4.5e-4	1
1099.6139	550.3106	2	0.0016	EVLNENLLR		25.55	19.9	5.3e-4	1
946.5117	473.7595	2	-0.0044	EKNELSK		12.89	17.2	6.9e-4	1
722.5000	722.5000	1	0.13	LGYLEQ		23.01	20.9	0.0015	1
1283.5861	642.2967	2	-0.0094	EGIHAQQKEPM	Oxidation@11	14.47	15.6	0.0016	1
1384.6849	692.8461	2	-0.011	QLEIVPNSAEER		22.06	17.4	0.0019	1
871.5019	436.2546	2	0.0011	LNENLLR		20.86	15.8	0.0070	1
758.4125	379.7099	2	-0.0015	NENLLR		16.91	18.1	0.0094	1
2104.9199	1052.9636	2	-0.024	TDAPSFSDIPNPIGSENSEK		28.28	15.3	0.0094	1

Figure S6. μ -HPLC ESI-Q-TOF MS/MS-based identification of α_s1 -casein-derived peptides that are transported across the Caco-2 monolayers.

Peptide identifications have been assisted by the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>) and manually validated.

1 Acc. #: [P02666](#) Uniprot ID: [CASB](#) BOVIN Species: BOVIN Name: Beta-casein
Organism: Bos taurus Gene: CSN2 Existence: Evidence at protein level Version: 2
Protein MW: 25107.5 Protein pI: 5.3 Protein Length: 224

1 MKVLILACLIV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS EEQQTEDEL QDKIHPPAQT QSLVYPFPGP
81 IPNSLPQ**NIP** **PLTQT**VPV**VP** **PFLQ**PEVMGV SKVKEAMAPK HKEMPFPKYP **VEPF**TESQSL TLTDVENLHL PLPLLQSW**MH**
161 **QPHQ**LPPTV **MFPP**QSVLSL SQSKVLPVPQ KAVPYQORDM PIQAFLL**YQE** **PVLGP**VRGPF PIIV

Num Unique	% Cov	Best Disc Score	Best Expect Val
13	25.4	2.04	1.8e-5

M+H	m/z	z	Da	DB Peptide	Variable Mods	RT	Score	Expect	# in DB
1270.6961	635.8517	2	-0.0096	LYQEPVLGPVR		25.65	23.1	1.8e-5	1
2068.9829	690.3325	3	-0.0089	MHQPHQPLPPTVMFPPQS		26.52	27.6	5.1e-5	1
1953.9533	651.9893	3	-0.0036	HQPHQPLPPTVMFPPQS	Oxidation@12	26.15	24.1	6.6e-5	1
994.5563	497.7818	2	-0.0058	QEPVLGPVR		19.46	17.4	7.5e-5	1
1079.5831	540.2952	2	-0.013	NIPPLTQTPV		27.56	25.2	9.3e-5	1
936.4135	468.7104	2	-0.0087	VEPFTESQ		21.71	21.8	1.1e-4	1
1157.6025	579.3049	2	-0.014	YQEPVLGPVR		22.77	19.2	1.1e-4	1
1023.4539	512.2306	2	-0.0045	VEPFTESQS		20.98	24.0	4.1e-4	1
1025.5589	513.2831	2	-0.0038	VPPFLQPEV		33.01	23.6	7.4e-4	1
1151.5821	576.2947	2	-0.0067	HQPHQPLPPT		14.59	17.1	8.3e-4	1
2084.9588	695.6578	3	-0.015	MHQPHQPLPPTVMFPPQS	Oxidation@1 13	26.62	18.8	9.4e-4	1
2100.9737	700.9961	3	-0.0086	MHQPHQPLPPTVMFPPQS	Oxidation@1;Oxidation@13	27	15.8	0.0022	1
965.5541	483.2807	2	-0.0063	IPPLTQTPV		26.98	17.1	0.015	1

Figure S7. μ -HPLC ESI-Q-TOF MS/MS-based identification of β -casein-derived peptides that are transported across the Caco-2 monolayers.

Peptide identifications have been assisted by the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>) and manually validated.

1 Acc. #: P02668 Uniprot ID: CASK BOVIN Species: BOVIN Name: Kappa-casein
Organism: Bos taurus Gene: CSN3 Existence: Evidence at protein level Version: 1
Protein MW: 21269.5 Protein pI: 6.3 Protein Length: 190
 1 MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPQ YVLSRYPSYG LNYQQKPVA LINNQFLPYP
 81 YYAKPRAVRS PAQILQWQL SNTVPAKSCQ AQPTTMRHP HPHLSFMAIP PKNQDKTEI PTINTIASGE PTSTPTTEAV
 161 ESTVATLEDS PEVIESPPEI NTVQVISTAV

Num Unique	% Cov	Best Disc Score	Best Expect Val
3	15.8	0.84	3.6e-4

M+H	m/z	z	Da	DB Peptide	Variable Mods	RT	Score	Expect	# in DB
1252.5833	626.7953	2	-0.0041	QEONQEPIR	Gln->pyro-Glu@1	16.18	21.3	3.6e-4	1
886.4029	443.7051	2	-0.0061	ESPPEINT		18.43	22.6	7.3e-4	1
1373.6735	687.3404	2	-0.0086	NQDKTEIPTINT		21.59	15.7	0.0035	1

Figure S8. μ -HPLC ESI-Q-TOF MS/MS-based identification of κ -casein-derived peptides that are transported across the Caco-2 monolayers. Peptide identifications have been assisted by the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>) and manually validated.

1 Acc. #: [P02754](#) Uniprot ID: [LACB_BOVIN](#) Species: BOVIN Name: Beta-lactoglobulin
Organism: Bos taurus Gene: LGB Existence: Evidence at protein level Version: 3
Protein MW: 19883.4 Protein pI: 4.9 Protein Length: 178

1 MKCLLLALAL TCGAQLIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE ELKPTPEGDL EILLQKWENG
81 ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL FCMENSAEPE QSLACQCLVR TPEVDDEALE KFDKALKALP
161 MHIRLSFNPT QLEEQCHI

Num Unique	% Cov
16	31.5

M+H	m/z	z	Da	DB Peptide	Variable Mods	RT	Start	End	Score
1890.9695	630.9947	3	0.0084	VRTPEVDDEALEKFDK		24.36	139	154	36.3
1717.8663	859.4368	2	0.0067	VYVEELKPTPEGDL		26.63	57	71	34.0
1437.7001	479.9049	3	0.0086	EVDDEALEKFDK		24.08	143	154	29.9
1500.7799	500.9315	3	0.0087	VRTPEVDDEALEK		20.54	139	151	36.9
1455.7375	728.3724	2	0.0081	VEELKPTPEGDL		22.44	59	71	32.6
1047.4947	524.2510	2	0.0054	EVDDEALEK		22.81	143	151	28.0
853.4393	427.2233	2	0.0046	VLDTDYK		17.44	110	116	15.8
1015.5615	508.2844	2	0.0099	IDALNENKV		20.55	100	108	25.5
1245.5899	623.2986	2	0.0028	TPEVDDEALEK		20.22	141	151	21.2
916.4807	458.7440	2	0.0037	IDALNENK		15.61	100	107	21.2
1618.8029	809.9051	2	0.0091	YVEELKPTPEGDL		24.86	58	71	19.6
1065.5851	533.2962	2	0.0012	VLVLDTDYK		25.42	108	116	15.8
673.3973	337.2023	2	0.0047	GLDIQK		16.59	25	30	15.2
1044.5803	522.7938	2	0.0059	KIDALNENK		14.85	99	107	22.0
803.3901	402.1987	2	3.4e-4	DALNENK		12.09	101	107	18.8
1568.8273	784.9173	2	0.011	VEELKPTPEGDL		27.76	59	72	16.9

Figure S9. μ -HPLC ESI-Q-TOF MS/MS-based identification of β -Lactoglobulin-derived peptides that are transported across the Caco-2 monolayers. Peptide identifications have been assisted by the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>) and manually validated.

1 Acc. #: [P00711](#) Uniprot ID: [LALBA](#) BOVIN Species: BOVIN Name: Alpha-lactalbumin
Organism: Bos taurus **Gene:** LALBA **Existence:** Evidence at protein level **Version:** 2
Protein MW: 16246.8 **Protein pI:** 4.9 **Protein Length:** 142
 1 MMSFVSLLLV GILFHATQAE QLTKEVFRE LKDLKGYGGV SLPEWVCTTF HTSGYDTQAI VQNNDSTEYG LFOINNKIWC
 81 KDDQNPHSSN ICNISCDFEL DDDLTDIMC VKKILDKVGI NYWLAHKALC SEKLDQWLCE KL

Num Unique	% Cov
3	15.5

M+H	m/z	z	Da	DB Peptide	Variable Mods	RT	Start	End	Score
1434.6631	717.8352	2	0.014	DTQAIQNNDSTE		18.41	56	68	23.7
871.5425	436.2749	2	0.0089	ILDKVGIN		21.49	114	121	15.6
1597.7233	799.3653	2	0.013	DTQAIQNNDSTEY		23.42	56	69	16.1

Figure S10. μ -HPLC ESI-Q-TOF MS/MS-based identification of α -Lactalbumin-derived peptides that are transported across the Caco-2 monolayers. Peptide identifications have been assisted by the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>) and manually validated.

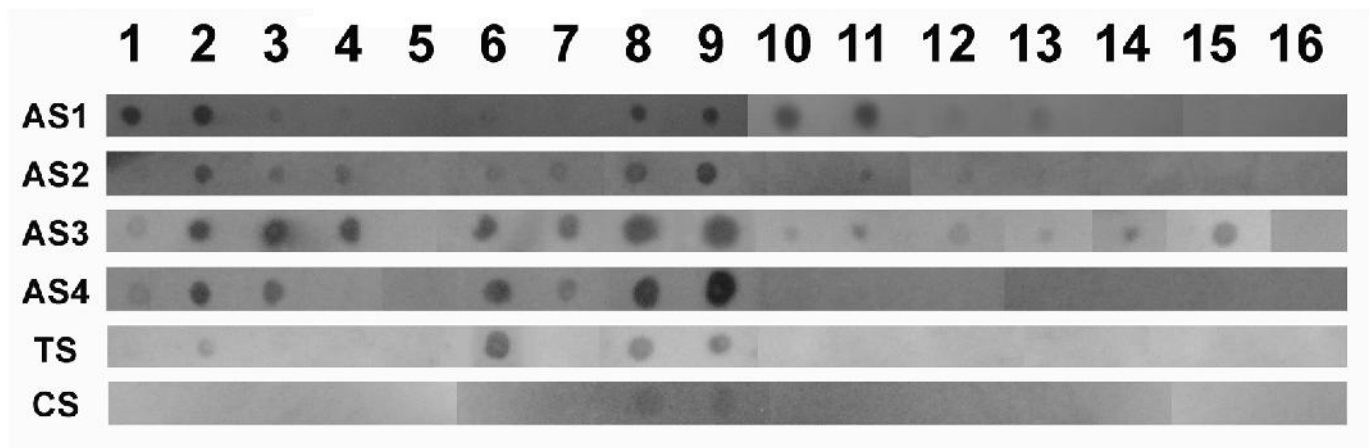


Figure S11. Dot-blot analysis for assaying the IgE-reactivity of milk proteins, CN and WP digests translocated across Caco-2 monolayers, and synthetic β -Lg peptides. Patterns were graphically reconstructed from ECL-impressed films.

Sera of children diagnosed with cow's milk allergy were used as the source of specific IgE.

Legend: AS = sera of children with cow's milk allergy; TS = serum of child that outgrew allergy (tolerant); CS = control serum of an healthy child.

Tested samples: 1 = κ -CN; 2 = α s2-CN; 3 = α s1-CN; 4 = β -CN; 5 = negative control (ribonuclease A); 6 = α -La; 7 = β -Lg; 8 = Total CN; 9 = Total WP; 10 = CN digests applied to Caco-2 – UP side; 11 = WP digests applied to Caco-2 - UP side; 12 = CN digests applied to Caco-2 - DOWN side; 13 = WP digests applied to Caco-2 - UP side; 14 = Synthetic β -Lg 125-135; 15 = β -Lg 127-135 arising from the Caco-2 processing (DOWN side of monolayers); 16 = Synthetic β -Lg 40-60.