Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications

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A B S T R A C T

Resistance to proteases throughout the gastrointestinal (GI) tract is a prerequisite for milk-derived peptides to exert biological activities. In this work an *in vitro* multi-step static model to simulate complete digestion of the bovine milk proteins has been developed. The experimental set-up involved the sequential use of: (i) pepsin, (ii) pancreatic proteases, and (iii) extracts of human intestinal brush border membranes, in simulated gastric, duodenal and jejuneal environments, respectively. Enzymatic concentrations and reaction times were selected in order to closely reproduce the *in vivo* conditions. The aim was to identify the peptide candidates able to exhibit significant bioactive effects. Casein and whey protein peptides which survived the *in vitro* GI digestion have been identified by the combined application of HPLC and mass spectrometry techniques. While the permanence of the main potentially bioactive peptides from both casein and whey proteins was found of limited physiological relevance, the high resistance to proteolysis of specific regions of β-lactoglobulin (β-Lg), and especially that of the peptide β-Lg f125–135, could have implications for the immunogenic action of β-Lg in the insurgence of cow's milk allergy.

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1. Introduction

Food proteins are extensively hydrolyzed at all stages of the GI digestion before being transferred as di- or tri-peptides to the basolateral side of the intestinal enterocytes. They are then released into the interstitial space and finally absorbed. Nevertheless, it has been observed that large polypeptides or proteolysis-resistant entire proteins can enter, albeit in small amounts, the blood circulation [1]. A large amount of studies have demonstrated that milk proteins are a source of biologically active peptides (for reviews see [2–8]). Some peptide sequences, inactive within the parent protein, may act, once released during food processing or GI digestion, as regulatory compounds with hormone-like activity. The biologically active peptides have been found to exhibit a wide range of biological functions including antimicrobial, anti-hypertensive, antithrombotic, cytomodulant and opioid activity [2–8]. Most of the bioactive peptides are potentially health enhancers for nutraceu-

tical and pharmaceutical applications [5,7–9]. However, in spite of the extensive studies, data about the resistance of milk protein domains to enzymatic attack along the digestive tract are still scarce and conflicting. Most of the evaluations of peptide bioactivity, in fact, have been obtained after proteolytic release in conditions which were remote from those of the human GI tract, for instance by action of pure proteases [9,10], or by bacterial [8,11–14] and yeast [15,16] fermentation. The effective *in vivo* production of the biologically active peptides, which is an obvious requirement to perform their specific functions, remains to be established. In other terms, it has not been ascertained whether the "functional" domains of milk proteins survive the digestive events and reach concentrations of physiological significance (β-casomorphin, for example, is 250 times less potent than normorphin [17]). Therefore, whether peptides released from milk proteins could have a beneficial effect on health or, conversely, a toxicological involvement still remains an open question. Several authors have tried to address this issue developing more or less complex *in vitro* static or dynamic, mono- or multi-phase models that reproduce the GI digestion [18]. For instance, a pilot plant, named "model gut" or "dynamic gastric model", which continuously removed the digestion products from the reaction mixture, has been developed to follow the fate of the dietary proteins

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and to evaluate the release of bioactive peptides [18]. Numerous other previous studies have shown that *in vitro* digestion of milk proteins with pepsin and pancreatic enzymes well describes the suckling gastric and duodenal digestion. Usually in the experimental designs the action of the intestinal brush border membrane (BBM) enzymes has been neglected [19,20]. Activity of BBM endo- and exo-peptidases has drastic effects in gradually shortening polypeptides to oligopeptides and free amino acids [21]. On the other hand, the action of digestive proteases in the immature newborn's GI tract reaches its completeness progressively [22]. For this reason, milk protein degradation in newborns is rather variable and products of digestion are somewhat complex to be rationalized. Petrilli et al. [23] using an *in vitro* digestion model, which included a final hydrolysis step by using BBM, demonstrated that β-casomorphins did not survive the digestive degradation of buffalo β-casein (β-CN). Similarly, pig pancreatic juice did not release β-casomorphins from a pro-β-casomorphin tryptic peptide [24]. No significant amounts of β-casomorphins or other known bioactive peptides were formed during a peptic digestion of bovine β-CN under simulated gastric conditions [25]. On the contrary, Oshawa et al. [26] have recently assessed the very low susceptibility of the anti-hypertensive β-casein VPP and IPP tri-peptides, to digestive proteases and BBM enzymes expressed by Caco-2 cell cultures. Therefore, these two tri-peptides can be considered good candidates to efficiently influence the *in vivo* regulation of arterial blood pressure. Generally, the stability of specific domains of proteins to digestion is also an indicative criterion for the identification of potential epitopes involved in triggering the immunological response in food allergies [27,28]. However, the resistance of protein domains to digestive enzymes is not a sufficient condition for exerting an adverse response. Tight junctions sealing neighbouring epithelial cells together and mucus produced by goblet cells cooperate to prevent food antigens from breaching into gut barrier [29]. The complex levels of interaction of the epitopes with the food matrix also prevent predicting the harmful effect *in vivo*. Determination of milk-derived peptides directly in blood plasma would be the most appropriate strategy to individuate valuable candidates for explicating biological or toxicological actions. Chabance et al. [30], for example, targeting specific peptides, identified k-casein derived macropeptide and some N-terminal fragments of α_{s1}-casein in the bloodstream after consumption of yogurt. However, to explore the entire panel of peptides generated by milk proteins is a very challenging task because of the myriad of possibly formed peptides which, in addition, vanish within the intrinsically complex polypeptide components of blood. Furthermore, it is current opinion that the majority of the known bioactive peptides do not pass into the bloodstream. Therefore, the possible biological effects might be mediated directly in the gut lumen or through receptors on the intestinal cell wall [8]. With the aim of individuating peptide candidates to exert biological functions or toxicological effects, a static, multi-step *in vitro* model of the complete GI digestive process was developed. In this work, casein (CN) and whey proteins (WP) from bovine milk, or their purified fractions, have been sequentially hydrolyzed in simulated human physiological conditions, in terms of pH and protease(s)-to-protein ratio, using pepsin (gastric digestion), pancreatic enzymes (duodenal digestion) and BBM peptidases (small intestine digestion). The CN and WP peptides surviving the *in vitro* GI digestion were identified by mass spectrometry (MS) techniques, also combined with chromatographic separation, including matrix assisted laser desorption ionization-time of flight (MALDI-TOF), off-line HPLC coupled with *nano*-electrospray ionization (*nano*ESI), tandem MS (MSMS) and on-line *micro*HPLC/ESI-MSMS [31]. The structural features as well as the possible functional or toxicological implications of the digestion-resistant peptides are also discussed.

2. Materials and methods

Individual samples of bovine milk, obtained from a local dairy farm, were used in this study. Immediately after milking, to prevent undesired proteolysis, milk samples were frozen at −20 °C until used. The samples were screened by HPLC and mass spectrometry to individuate casein and whey protein variants. An individual milk sample heterozygous for both the β-CN A1 and A2 and β-Lg A and B was finally selected for the study. The UHT bovine milk sample was obtained from the commerce. Pepsin, trypsin, chymotrypsin and elastase, carboxypeptidase A, elestase, dithiothreitol (DTT), iodoacetamide, α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were purchased from Sigma (St. Louis, MO, USA). Ammonium bicarbonate, reagents and HPLC-grade solvents from Carlo Erba (Milan, Italy) were used without any further purification. Sequence-grade bovine alkaline phosphatase from calf intestine was supplied by Roche (Mannheim, Germany). Porcine pepsin, chymotrypsin elastase and trypsin were used assuming that no significant differences in their activity exist with respect to the human counterparts [31,32].

2.1. Fractionation of milk proteins

Milk was skimmed by centrifugation at 4500 rpm for 20 min at 2 °C (Labofuge 400R, Heraeus Instruments, USA). After fat removal, isoelectric casein was prepared by precipitation according to the Aschaffenburg's method [33]; briefly, 20 mL of skimmed milk were diluted by distilled water (20 mL) and acidified to pH 4.6 with acetic acid/sodium acetate buffer; sample was kept 30 min at 37 °C and finally centrifuged (5000 × g, 4 °C, 10 min). The casein precipitate was washed twice with a 1 M sodium acetate/10% (v/v) acetic acid buffer, pH 4.6, and than freeze-dried. Hammerstein pure isoelectric casein was prepared by three-fold precipitation at pH 4.6 from a casein solution adjusted at pH 7.0 with 0.5 N NaOH, followed each time by three-fold washing with distilled water. For purification of whey proteins, aliquots of whey (3 mL) were loaded onto C_{18} Sep-Pak cartridges (Waters, Milford, MA, USA) previously equilibrated in 0.1% trifluoroacetic acid (TFA, v/v). Proteins were washed with 10 mL 0.1% TFA (v/v) and finally eluted with 3 mL of 70% acetonitrile (v/v) containing 0.1% TFA (v/v). Solution was then evaporated in speed-vac and lyophilised.

2.2. Dephosphorylation of caseins and caseinphosphopeptides (CPP)

An aliquot of dry casein (5 mg) was dissolved in 1 mL 0.4% ammonium bicarbonate, pH 8.5 and dephosphorylation carried out overnight at 37 °C, with calf intestine alkaline phosphatase using 5 mU enzyme/mg casein. Reaction was stopped by freeze-drying. CPP dephosphorylation was carried overnight at 37 °C in 50 mM ammonium bicarbonate (20 mL) by addition of 0.05 mU alkaline phosphatase.

2.3. Preparation of human BBM proteases

BBM extracts were a kind gift of Dr. Mauro Rossi, CNR, Avellino, Italy. Extraction of BBM proteases was carried out according to the method by Shirazi-Beechey et al. [34] with little modifications [35]. Briefly, frozen surgical specimens of intestinal jejunum (1 cm) were thawed in ice-cold 50 mM mannitol, 2 mM Tris–HCl pH 7.1. Cells were removed from the connective tissue by using a Vibromixer (model E-1, Alpha Laval, UK) at max speed 2 × 1 min. The cell suspension was homogenised with an Ultraturrax T 25 (IKA, Works, Inc., USA) and diluted with $MgCl_2$ to a final concentration of 10 mM. The suspension was stirred 20 min at 0 °C and then centrifuged at

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$3000 \times g$ 15 min at 4 °C to eliminate cell debris, basolateral, membranes, nuclei, and mitochondria. The supernatant was centrifuged at $30,000 \times g$ 30 min at 4 °C. The pellet was resuspended in 300 mM mannitol, 0.1 mM $MgSO_4$, and 2 mM Tris pH 7.4 to obtain a clear protein solution that was passed several times through a 27-gauge needle; the brush-border vesicles were stored in aliquots at −80 °C. Shortly before use, the vesicles were thawed on ice and washed with the sodium phosphate buffer.

2.4. Gastric-pancreatic and BBM digestion

Caseins (both native and dephosphorylated) and whey proteins were dissolved in 5% formic acid at the concentration of 1 mg/mL and incubated at 37 °C with pepsin (1:100 enzyme/protein ratio, w/w) for 60 min. Before pancreatic digestion, the samples were evaporated and washed twice with deionised water. Trypsin (1:100, w/w), chymotrypsin (1:100, w/w), elastase (1:500, w/w) and carboxypeptidase (1:100, w/w) were added in 0.1 M sodium phosphate buffer (pH 7.0) and the mixtures incubated 1 h at 37 °C. The reaction was stopped by heating 5 min in a boiling water bath. The samples from gastric-pancreatic digestion were two-fold diluted with 0.1 M sodium phosphate buffer, pH adjusted at 7.2 and incubated after BBM supplementation (650 mU/mg) up to 4 h; aliquots were picked up also after 1 and 2 h-incubation. To evaluate repeatability, each incubation was carried out at least in triplicate. A parallel incubation of the proteolytic enzymes in the opportune buffers was carried out and used as the control. The isolated β-Lg f125–135 was subjected to simulate digestion either in the same described conditions or by doubling the incubation time and the proteases-to-protein ratio.

2.5. HPLC analysis of peptide digests

To fractionate CN and WP digests and isolate the β-Lg f125–135 from the simulated GI digest, RP-HPLC was performed using a 2.0 mm i.d. × 250 mm, C_{18}, 5 μm reverse-phase column (Phenomenex, Torrance, CA, USA) with a flow rate of 0.2 mL/min on a HP1100 modular system (Agilent, Palo Alto, CA, USA). Solvent spazio A was 0.1% TFA (v/v) in water; solvent B was 0.1% TFA (v/v) in acetonitrile. The column was equilibrated at 5% solvent B. Peptide separation (25 μg for each analysis) was carried out with a gradient of 5–60% solvent B over 60 min. The column effluent was monitored by UV detection (220 and 280 nm) and each peak was manually collected.

2.6. Isolation of caseins and whey proteins

Single casein and whey protein fractions were isolated by RP-HPLC using the same chromatograph as above. In this case column was an i.d. 4.6 mm × 25 cm, C_4 reverse-phase Vydac (Hesperia, Palo Alto, CA, USA). HPLC runs (0.4 mg for each injection) were carried out with a gradient of 30–50% solvent B in the case of caseins and 35–55% solvent B in the case of whey proteins, both over 60 min at a flow rate 1 mL/min. Caseins were singly collected while the two β-Lg A and B variants were collected together. All protein fractions were finally freeze-dried.

2.7. Isolation of β-Lg f125–135

To confirm stability toward proteolytic action, β-Lg f125–135 was purified from a tryptic digest of β-Lg A and B in mixture by HPLC. Before tryptic digestion, carried out 6 h at 37 °C; enzyme to protein ratio 1:100 (w/w), β-Lg (1 mg) was reduced 1 h at 56 °C in 6 M guanidine, 0.5 M Tris–HCl, 1 mM EDTA, 10 mM DTT and alkylated by iodoacetamide for 40 min at room temperature in the dark. The β-Lg was finally desalted on Sephadex PD-10 desalting

columns (Amersham Pharmacia, Uppsala, Sweden) under gravity flow eluting with ammonium bicarbonate 50 mM pH 8.0 and protein concentration was determined with the Bradford assay. The β-Lg f125–135 was isolated by RP-HPLC using a C_{18} column (Vydac), i.d. 4.6 mm × 25 cm, applying a gradient of 5–60% solvent B over 60 min at a flow rate 1 mL/min, and finally lyophilised. A quantitative estimation of the purified peptide was obtained with the bicinchoninic acid assay (micro-BCA kit, Pierce, Rockford, IL, USA) using an absorbance microplate reader Bio-Rad Model 550 (Bio-Rad, Hercules, CA, USA) operating at λ = 560 nm. A standard solution of angiotensin I (Sigma) was used to build the calibration curve.

2.8. Enrichment of caseinphosphopeptides

CPP were enriched from an aliquot of the peptide mixture (25 μg) with manually packed TiO_2 micro-columns as described by Larsen and coworker [36]. CPP were eluted in 10 μL 50% acetonitrile (v/v) containing NH_4OH pH 10.5 and acidified with 5 μL of 5% (v/v) formic acid before mass spectrometric analysis.

2.9. MALDI-TOF MS analysis

MALDI-TOF MS experiments were carried out on a PerSeptive Biosystems (Foster City, CA, USA) Voyager DE-PRO instrument equipped with an N_2 laser (337 nm 3 s pulse width). Before MS analysis, 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). HPLC peaks (1 μL) were directly loaded on a stainless steel plate together with 1 mL of α-cyano-4-hydroxycynnamic acid matrix (10 mg/mL aqueous 50% acetonitrile/TFA 0.1%) and air-dried. For the CPP analysis, 2,5-dihydroxybenzoic acid (10 mg/mL aqueous 50% acetonitrile/phosphoric acid 1%) was used as matrix. Mass spectra were acquired in the positive ion reflectron mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with mass peptide standards (Sigma).

2.10. nanoESI-MSMS and HPLC/ESI-MSMS

ESI-MS and tandem MS data were obtained by using a Q-Star Pulsar (Applied Biosystems, Foster City, CA, USA) equipped with a nanoESI source (Proxeon, Odense, Denmark). HPLC fractions (2 μL) were diluted with 30% acetonitrile (v/v) containing 1% formic acid (v/v) and sprayed from gold-coated "medium-length" borosilicate capillaries (Proxeon). The capillary voltage used was 800 V. For fragmentation, parent ions were selected by using the quadrupole mass filter and the collision energy was 20–40 eV, depending on the size of the peptide. Mass spectra were processed by using the Analyst 1.1 software (Applied Biosystem) or manually assigned. Peptides deriving from simulate digestion of both caseins and whey proteins were also analyzed by microHPLC/ESI-MSMS by using an Integral HPLC system (PerSeptive Biosystems). In this case, peptide solutions (5 μL) were loaded with a high flow rate of 5 μL/min onto a custom made 2 cm pre-column [packed with ODS-A C_{18} (YMC, Kyoto, Japan) 5/15 μm particles]. Micro-HPLC separation was then performed at a constant flow of 5 μL/min, obtained by the pre-column split of a 0.2 mL/min flow, through a 300 μm i.d. × 15 cm analytical column, C_{18} PepMap300, 5 μm, 300 Å particles (LC Packings, Abberdaan, Netherlands) applying a linear gradient from 0 to 40% B over 60 min. Mobile phase A consisted of 0.08% TFA, 0.02% formic acid (v/v) in Milli-Q water and mobile phase B consisted of 0.08% TFA, 0.02% formic acid in acetonitrile. Eluate was directly injected into the ESI source of the Q-star mass spectrometer using automated data-dependent acquisition. Mass- and charge-dependent

collision energies were used for peptide fragmentation. A MS-TOF survey spectrum was recorded for 1 s. The two most abundant ions present in the survey spectrum were automatically mass selected and fragmented by CID (4 s/MSMS spectrum). The tandem mass spectra acquired from the HPLC/ESI-MSMS analysis were smoothed, centroided and converted to peak-list text files (.mgf file format) using the Analyst 1.1 software. Attempts to automatically identify peptide by searching the .mgf data files in the Swiss-Prot database through the Mascot search engine (version 2.1.04, Matrix Science Ltd., London, UK) were only partially successful due to the non-tryptic nature of the peptides that complicated assignment of MSMS spectra. HPLC/ESI-MSMS analysis was also scarcely informative by a quantitative point of view, since intensity of each peak in the Total Ion Current (TIC) chromatogram was strongly dependent on the nature of the peptides and their ionization efficiency. In any case, identification made with HPLC/ESI-MSMS analysis was validated by combination with the off-line *nano*ESI-MSMS spectra. Sequence analysis and peptide assignment were accomplished using the GPMAW 5.1 software (Lighthouse data, Odense, Denmark).

3. Results

The schematic of the analytical strategy used for the identification of the milk protein digests is outlined on Fig. 1. The MALDI-TOF peptide pattern generated from the digestion of the whole milk protein fraction closely reproduced those of the CN and WP individually digested (data not shown). Therefore, in order to simplify the identification of the survived peptides, digests of CN and WP were examined separately. As indicated in Fig. 1, proteins were sequentially incubated with pepsin at pH ∼ 2 to mimic the gastric environment; then peptic digests were further hydrolyzed at pH 7.2 by a pool of pancreatic enzymes, i.e. carboxypeptidase A, chymotrypsin, elastase and trypsin, to simulate the *in vivo* duodenal digestion. The series of proteolytic enzymes was chosen on according to the major roles played during the *in vivo* digestion process, while incubation time ranges according to the rough physiological permanence in the single GI sections, and to indications derived

from the digestion of vegetable proteins [35]. Then, to simulate the final degradation occurring on the polarized apical surface of the small jejuneal enterocytes, the polypeptides were further incubated with BBM enzymes which usually reduces proteins to di-, tri-, oligopeptides and free amino acids.

3.1. Kinetics of the hydrolysis

The digestion kinetics of the CN and WP from raw milk was followed by RP-HPLC. Degradation of CN started very early and was rapidly completed (Fig. 2A–C). After 1 h-incubation with pepsin, no measurable amounts of CN remained intact. In contrast, the dominant whey proteins α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), in the peaks at retention time (t_r) ∼ 48.47, 51.00 and 51.56 min, significantly resisted to peptic attack (Fig. 2D–E). This relative resistance is probably conferred by the highly compact conformation assumed by α-La and β-Lg for the presence of 4 and 2 intramolecular disulphide bridges, respectively. Consistently with the previous data [37,38], bovine β-Lg resulted more resistant to peptic hydrolysis than α-La, which is known to assume, at acidic pH, the lesser compact "molten globule" conformation. Conversely, α-La is much less affected by pancreatic enzymes than β-Lg [39]. Pepsin-mediated peptides of CN and WP gave rise to a series of HPLC peaks corresponding to medium- and large-sized polypeptides detected by MALDI-TOF (data not shown). As expected, peptides eluted earlier than the parent protein (Fig. 2B and E) and those produced by the pancreatic enzyme pool were for the major part of short size (Fig. 2C and F). Fig. 3 shows the MALDI-TOF spectrum of the CN digests containing peptides survived the digestive simulated process, included the final step of 4 h-incubation with BBM. However intestinal digestion could be considered already completed within 2 h, since peptide pattern did not appreciably change after longer incubation times. To further restrict the consistency of the ion signal identification, each casein family was individually digested and the MALDI-TOF MS spectra of the κ-, $α_{s2}$-, $α_{s1}$- and β-CN-derived peptides were compared with the spectrum of whole CN digests. The C-terminal region of β-CN gave rise to very intense peptide signals. By contrast, that central of $α_{s1}$-CN generated few peptides
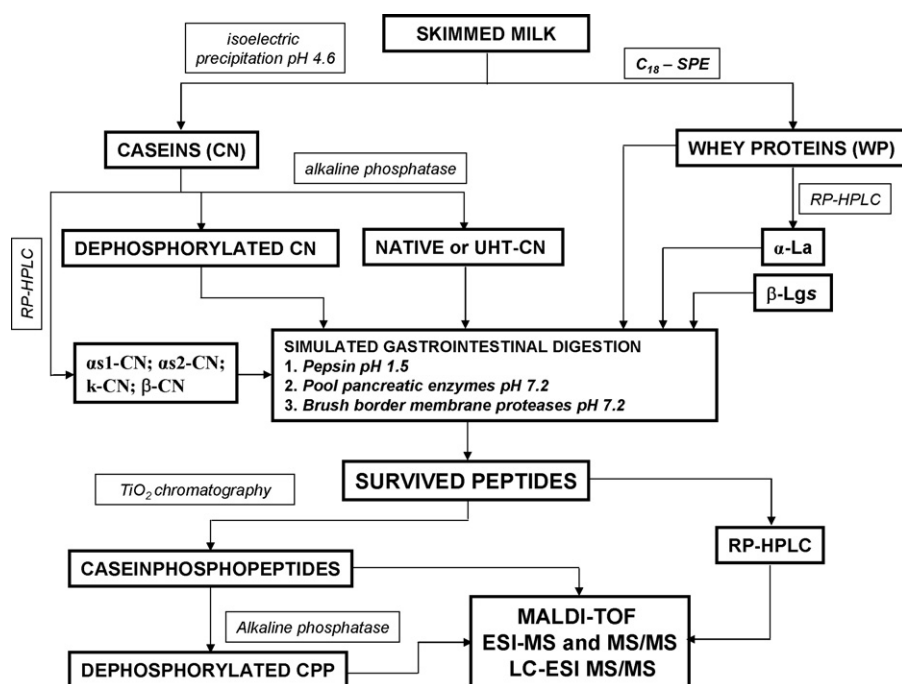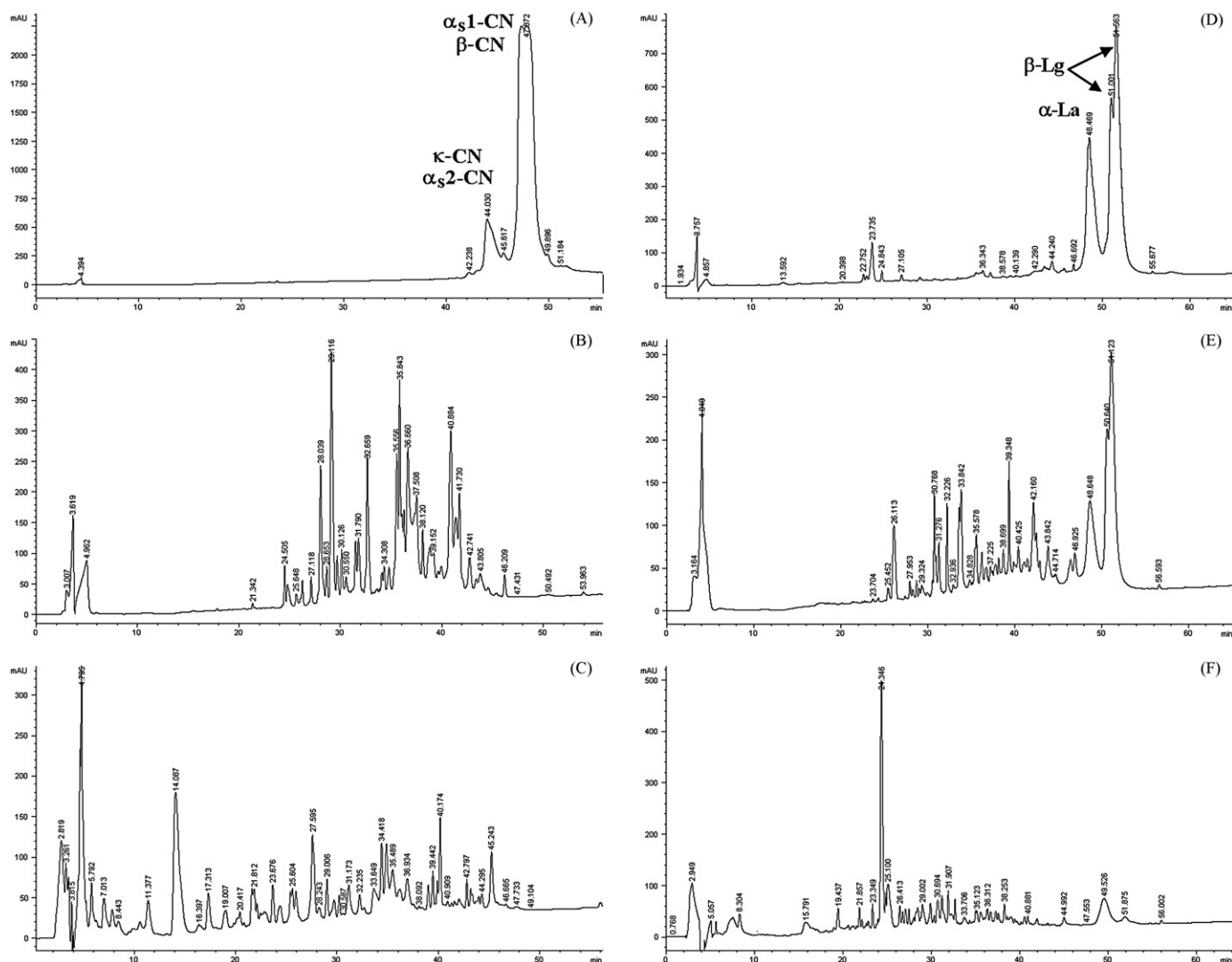

Fig. 1. Schematic overview of the *in vitro* gastrointestinal digestion process and of the analytical strategy applied to identify resistant milk-derived peptides.

Fig. 2. Kinetics of sequential release of peptide fragments from CN and WP by pepsin and pool of pancreatic proteases, followed by RP-HPLC: not hydrolyzed CN (A) and WP (D); peptic digests of CN (B) and WP (E); gastro-pancreatic digests of CN (C) and WP (F).

(Table 1). From the analysis of whole casein digests, we deduced that MALDI-TOF MS was not adequate to exhaustively profile the complex peptide mixture because of the well-known suppression effects. Fractionation of the whole casein digests by RP-HPLC allowed to detect few dominating peaks (Fig. 4) containing the peptides reported in Table 2. The most intense HPLC peak at t_r 27.95 min

was constituted by β-CN f26–41 1P (P indicates phosphorylation), which yielded a signal of low intensity, and β-Lg f125–135 as prevailing component. Further confirmation of the sequence was obtained by ESI-MSMS (Fig. 5). Although the isoelectric CN was contaminated by an amount of soluble β-Lg lower than 5%, as estimated by RP-HPLC, the β-Lg f125–135 fragment resulted one of the most
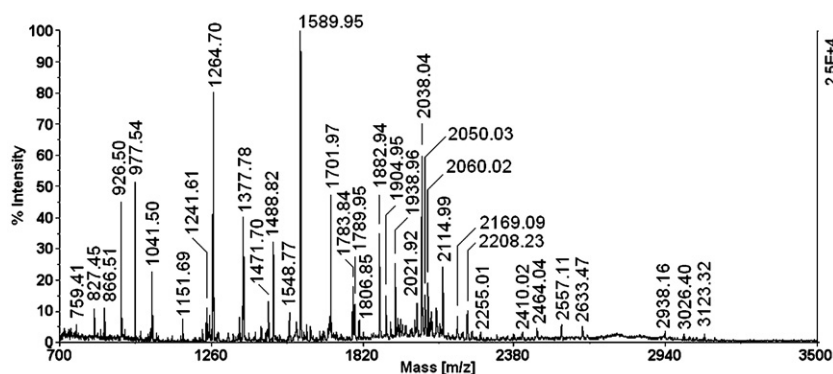
Fig. 3. MALDI-TOF MS spectrum of the CN-derived peptides survived the complete *in vitro* digestion, including the step with BBM enzymes. The main peptide signals are assigned in Table 1.

Table 1
MALDI-TOF MS analysis of the peptide mixture survived the *in vitro* gastrointestinal digestion of CN.

Measured MW[a] (MH⁺)	Theoretical MW[a] (MH⁺)	Identification	Sequence
827.45	827.42	β-CN 85–91	PPFLQPE
926.50	926.50	β-CN 85–92	PPFLQPEV
977.54	977.49	β-CN 183–190	RDMPIQAF
1041.50	1041.52	β-CN 126–134	TLTDVENLH
1151.69	1151.69	β-CN 197–207	VLGPVRGPFPI
1241.61	1241.62	β-CN 43–52	DELQDKIHPF
1264.69	1264.78	β-CN 197–208	VLGPVRGPFPII
1377.78	1377.79	β-CN 195–207	EPVLGPVRGPFPI
1488.82	1488.79	β-CN 194–207 pyro-Glu	pyroEEPVLGPVRGPFPI
1491.77	1490.87	β-CN 195–208	EPVLGPVRGPFPII
1589.95	1589.95	β-CN 195–209	EPVLGPVRGPFPIIV
1701.97	1701.99	β-CN 194–209 pyro-Glu	pyroEEPVLGPVRGPFPIIV
1782.84	1782.99	β-CN 193–208	YQEPVLGPVRGPFPII
1789.95	1789.92	α$_{s1}$-CN 105–119 1P	KVPQLEIVPNSpAEER[b]
1882.94	1882.07	β-CN 193–209	YQEPVLGPVRGPFPIIV
1938.96	1938.98	β-CN 145–161	HQPHQPLPPTVMFPPQS
2021.92	2022.01	α$_{s1}$-CN 125–142	EGIHAQQKEPMIGVNQEL
2038.04	2038.04	β-CN 145–162	HQPHQPLPPTVMFPPQSV
2050.04	2050.01	β-CN 149–167 pyro-Glu	pyroQPLPPTVMFPPQSVLSLSQ
2060.02	2060.08	β-CN 134–150	HLPLPLLQSWMHQPHQP
2114.99	2114.19	β-CN 189–207	AFLLYQEPVLGPVRGPFPI
2169.09	2169.09	β-CN 144–162	MHQPHQPLPPTVMFPPQSV
2208.23	2207.11	β-CN 179–196	PYPQRDMPIQAFLLYQEP
2255.01	2255.31	β-CN 190–209	FLLYQEPVLGPVRGPFPIIV
2410.02	2410.03	β-CN f33–51 1P	FQSpEEQQQTEDELQDKIH[b]
2464.04	2464.10[c]	β-CN f33–52[c]	FQSpEEQQQTEDELQDKIHPF[b]
2557.11	2557.10	β-CN f33–52 1P	FQSpEEQQQTEDELQDKIHPF[b]
2633.47	2633.37	β-CN 174–195	PQKAVPYPQRDMPIQAFLLYQE
2938.16	2937.27[c]	β-CN 1–25 2P[c]	RELEELNVPGEIVESpLSpSpSpEESITR[b]
3026.40	3030.27[c]	β-CN 1–25 3P[c]	RELEELNVPGEIVESpLSpSpSpEESITR[b]
3123.23	3123.27	β-CN 1–25 4P	RELEELNVPGEIVESpLSpSpSpEESITR[b]

[a] MW are those of the most abundant isotopic ions.
[b] Sp indicates a phosphorylated Ser.
[c] Metastable ion.

abundant components in the CN digests. This peptide escaped to direct detection by MALDI-TOF probably because the presence in its sequence of five acidic out 11 amino acid residues prevented an efficient ionization. Nevertheless, β-Lg f125–135 gave an intense ESI-MS signal, also accompanied by the confirmative Na⁺ and K⁺ adduct ions. Although β-Lg f125–135 lacks aromatic amino acids the HPLC peak exhibited a significant absorption at λ = 280 nm. This could indicate the presence of a minor contaminating unassigned component(s). To confirm the predominant contribute of β-Lg f125–135 to the peak no. 6, Hammerstein grade pure isoelectric CN isolated from raw skimmed milk was also submitted to digestion. In this case, the HPLC at t_r 27.95 min did not longer include β-Lg f125–135 and the peak area, measured by integration of the λ = 220 nm chromatogram, decreased by ∼30% (Fig. 6).

In addition to this the β-Lg f94–99 in low-abundance was identified in the fraction no. 7. On the basis of chromatographic peak area, no other peaks overcame 5% parent protein intensity, except for early eluting short oligopeptides. This demonstrates that caseins were extensively hydrolyzed during the *in vitro* simulated GI digestion. From both whole CN and β-CN the most intense MALDI-TOF
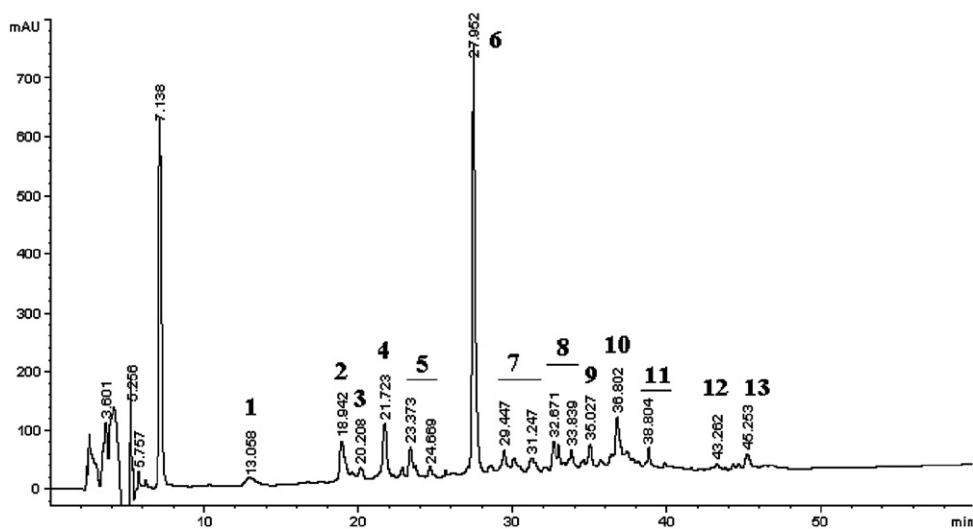
Fig. 4. RP-HPLC separation of the CN-derived peptides survived the *in vitro* gastrointestinal digestion. The fractions were manually collected and analyzed by *nano*ESI-MSMS. The identified peptides are reported in Table 2.

Table 2
Assignment of the CN sequences survived the *in vitro* gastrointestinal digestion by off-line *nano*ESI-MSMS analysis of the RP-HPLC fractions (Fig. 4).

HPLC fraction (t_r, min)	Ion detected m/z (charge)	Calculated MW (MH$^+$)[a]	Theoretical MW (MH$^+$)[a]	Identification	Sequence
Fractions t_r = 5.25, 5.76, 7.14	MW < 400				
1 (13.06)	618.35 (3)	1853.05	1852.97	α_{s1}-CN 4–19	HPIKHQGLPQEVLNEN
	580.32 (3)	1739.98	1738.92	α_{s1}-CN 4–18	HPIKHQGLPQEVLNE
2 (18.94)	416.17 (2)	831.33	831.38	α_{s1}-CN 84–90	EDVPSER
	589.28 (1)	589.28	589.28	β-CN 129–133	DVENL
	612.25 (2)	1229.49	1223.53	α_{s1}-CN 110–119 P	EIVPNSpAEER[b]
3 (20.21)	626.79 (2)	1252.57	1252.64	β-CN 137–146	LPLLQSWMHQ
	669.30 (2)	1337.60	1337.57	α_{s1}-CN 51–61	DQAMEDIKQME
4 (21.72)	680.30 (1)	680.3	680.28	α_{s1}-CN 157–163	DAYPSGA
	545.71 (2)	1090.41	1090.39	α_{s2}-CN 138–146 P	TVDMESpTEV[b]
	764.32 (1)	764.32	764.30	α_{s1}-CN 172–178 pyro-Glu	pyroEYTDAPS
	698.05 (3)	2092.15	2092.05	α_{s1}-CN 125–143	EGIHAQQKEPMIGVNQELA
	674.37 (3)	2021.09	2021.01	α_{s1}-CN 125–142	EGIHAQQKEPMIGVNQEL
	894.98 (2)	1788.97	1788.89	α_{s1}-CN 105–119 P	KVPQLEIVPNSpAEER[b]
5 (23.37–24.67)	989.82 (2)	1978.64	1978.93	α_{s2}-CN 142–157 P	ESpTEVFTKKTKLTEEE[b]
	496.27 (1)	496.27	496.28	β-CN 132–135	NLHL
	915.07 (3)	2743.21	2743.33	κ-CN 79–103	VPAKSCQAQPTTMARHPHPHLSFMA
	948.78 (3)	2844.34	2844.38	κ-CN 78–103	TVPAKSCQAQPTTMARHPHPHLSFMA
6 (27.95)	499.25 (2)	997.50	997.52	κ-CN 148–156	VIESPPEIN
	563.76 (2)	1126.51	1126.56	κ-CN 147–156	EVIESPPEIN
	623.28 (2)	1245.55	1245.58	β-Lg 125–135	TPEVDDEALEK
	686.62 (3)	2057.94	2057.99	β-CN 26–41 P	INKKIEKFQSpEEQQQT[b]
7 (29.44–31.25)	700.34 (2)	1399.67	1399.66	α_{s1}-CN 181–193	DIPNPIGSENSEK
	743.87 (2)	1486.69	1486.69	α_{s1}-CN 180–193	SDIPNPIGSENSEK
	674.34 (1)	674.34	674.31	β-CN 184–189	DMPIQA
	725.36 (1)	725.36	725.33	β-Lg 94–99	VLDTDY
8 (32.67–33.84)	489.28 (2)	977.55	977.53	β-CN 194–202 pyro-Glu	pyroEEPVLGPVR
	668.38 (1)	668.39	668.35	α_{s1}-CN 181–186	DIPNPI
	642.30 (2)	1283.60	1283.57	β-CN 114–124	YPVEPFTESQS
	679.71 (3)	2037.13	2037.03	β-CN 145–162	HQPHQPLPPTVMFPPQSV
	723.39 (3)	2168.18	2168.08	β-CN 144–162	MHQPHQPLPPTVMFPPQSV
9 (35.03)	491.23 (3)	1471.66	1471.70	β-CN 41–52	TEDELQDKIHPF
	621.30 (2)	1241.59	1241.61	β-CN 43–52	DELQDKIHPF
	705.04 (3)	2113.11	2113.18	β-CN 189–207	AFLLYQEPVLGPVRGPFPI
	803.69 (3)	2409.05	2409.02	β-CN 33–51 P	FQSpEEQQQTEDELQDKIH[b]
	981.28 (3)	2941.84	2941.56	β-CN 174–198	PQKAVPYPQRDMPIQAFLLYQEPVL
10 (36.80)	719.43 (2)	1437.85	1437.81	α_{s1}-CN 11–22	LPQEVLNENLLR
	893.81 (2)	1786.62	1786.69	β-CN 35–48 P	SpEEQQQTEDELQDK[b]
	852.73 (3)	2556.19	2556.09	β-CN 33–52 P	FQSpEEQQQTEDELQDKIHP[b]
11 (38.80)	487.25 (3)	1459.74	1459.89	β-CN 80–92 K$^+$ adduct	TPVVVPPFLQPEV
	711.40 (2)	1421.80	1421.80	β-CN 80–92	TPVVVPPFLQPEV
	1053.03 (2)	2105.06	2104.97	α_{s1}-CN 174–193	TDAPSFSDIPNPIGSENSEK
	744.91 (2)	1488.81	1488.82	β-CN 194–207 pyro-Glu	pyroEEPVLGPVRGPFPI
12 (43.26)	926.44 (1)[c]	926.44	926.49	β-CN 85–92	PPFLQPEV
	1093.11 (2)	2185.23	2185.22	β-CN 73–92	NIPPLTQTPVVVPPFLQPEV
	1957.89 (1)[c]	1957.89	1958.09	β-CN 75–92	PPLTQTPVVVPPFLQPEV
	776.96 (2)	1552.92	1552.84	β-CN 80–93	TPVVVPPFLQPEVM
	870.86 (3)	2610.58	2610.45	β-CN 69–92	SLPQNIPPLTQTPVVVPPFLQPEV
	914.54 (3)	2741.62	2741.49	β-CN 69–93	SLPQNIPPLTQTPVVVPPFLQPEVM
13 (45.25)	1781.8 (1)[c]	1781.78	1781.99	β-CN 193–208	YQEPVLGPVRGPFPII
	1880.9 (1)[c]	1880.88	1881.06	β-CN 193–209	YQEPVLGPVRGPFPIIV
	689.37 (2)	1377.74	1377.79	β-CN 195–207	EPVLGPVRGPFPI
	1993.9 (1)[c]	1993.96	1994.14	β-CN 192–209	LYQEPVLGPVRGPFPIIV
	2106.97 (1)[c]	2106.97	2107.22	β-CN 191–209	LLYQEPVLGPVRGPFPIIV
	1363.70 (1)[c]	1363.70	1363.84	β-CN 197–209	VLGPVRGPFPIIV
	1490.77 (1)[c]	1490.77	1490.87	β-CN 195–208	EPVLGPVRGPFPII
	1717.97 (1)[c]	1717.97	1717.99	β-CN 194–209	QEPVLGPVRGPFPIIV
	795.50 (2)	1590.00	1589.95	β-CN 195–209	EPVLGPVRGPFPIIV

[a] Monoisotopic mass.
[b] Sp indicates a phosphorylated Ser.
[c] Assigned by MALDI-TOF MS analysis of the HPLC fractions.
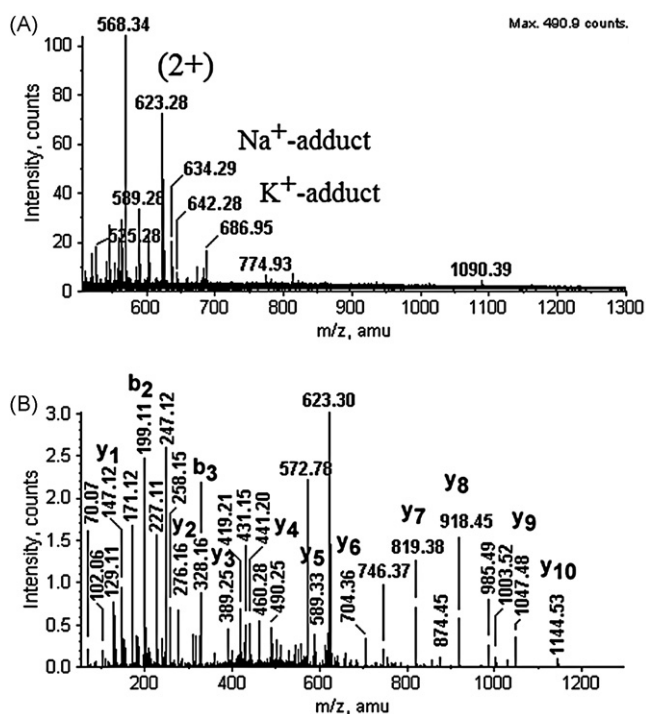
Fig. 5. (A) *nano*ESI-MS spectrum of the chromatographic fraction no. 6 at t_r 27.96 min. The double-charged peptide ions m/z 623.28 and its Na$^+$ and K$^+$ adducts are predominant components. (B) *nano*ESI-MSMS fragmentation of the selected ion m/z 623.28: identification of the β-Lg f125–135 by sequence reconstruction. The main y- and b-ions are labelled.

signal at m/z 1589.90 corresponding to β-CN f195–209 was formed (see Fig. 3). Other C-terminal fragments of β-CN, also detected in mixture by MALDI-TOF, were retrieved in fractions no. 9 and 12, as shown in Table 2. The expected free amino acids produced in the digestion were detected neither by mass spectrometry because of their low molecular mass, nor by HPLC analysis at 220 nm, because of the lack of UV-absorbing peptide bonds. Some other oligopeptides were identified by off-line ESI-MSMS. However, it is likely that the small size range peptides (MW < 400) eluting throughout the first HPLC elution window escaped to MS identification. Therefore, this approach may be adequate for the identification of medium- and high-mass peptides, while the profiling of low molecular weight ones still remains incomplete. The patterns of

the CN digests from UHT milk, although derived from a bulk milk, did not appreciably differ from that of raw milk (Fig. 6), supporting the previous observation that proteases would most likely act in a similar manner on either the native or the heated caseins [25]. In many cases, assignment of methionine-containing peptides from UHT milk was confirmed by the presence of low-intense satellite peaks at +16 Da reflecting the oxidation of the methionine residue [40]. Small amounts of intact α-La and β-Lg were detected in the UHT-CN digests, corresponding to proteases-resistant WP. Probably, the heat-denatured α-La and β-Lg, co-precipitated with CN fraction [41], refold in conformations in which sensitive bonds are less accessible to proteases [42].

### 3.2. Isolation of phosphopeptides

Several β-, α$_{s1}$-, and α$_{s2}$-CN-derived phosphorylated peptides were detected in the CN digests and identified by off-line *nano*ESI-MSMS analysis. They were contained in some of the most prominent HPLC peaks (e.g. fractions no. 2, 4 and 10 of Fig. 4). The anionic character of the phosphate may confer them a marked resistance to certain proteases. Even though data about their intestinal absorption are conflicting, CPP have been detected in the distal small ileum of humans orally administered of milk or crude CPP preparations [43]. Primary CPP were found in long-term maturing cheese in mixture with N- and C-terminally truncated counterparts [44]. The aminopeptidase activity partially shortened CPP provided that the N-terminal residue was not phosphorylated. In other words, while N-terminal SerP makes the peptides resistant to aminopeptidase, the dephosphorylation of the residue may render them readily prone to hydrolysis. Discrepancies could occur between *in vivo* and *in vitro* behaviour of CPP to enzyme activity. Compared to free CPP, iron–CPP complexes were less susceptible to alcalase and pancreatic proteases [45] and, therefore, resistant to luminal digestion. The susceptibility of CPP and iron–CPP complexes to alkaline phosphatase is one of the key points for assessing the brush border mechanisms of absorption of complexed iron [46]. In general, from the analytical point of view, the non-phosphorylated peptides tend to suppress the signal of phosphopeptides in mixtures. Indeed, few minor low-intense signals of CPP were individuated in the MALDI-TOF spectra (Fig. 3, Table 1). Therefore, in order to identify CPP the selective isolation on TiO$_2$ micro-column was carried out [36]. In this way, a number of CPP was identified by MALDI-TOF MS in the CN digests (Fig. 7) as shown in Table 3. CPP identification was confirmed by the presence of broad fragment ion signals at mass values reduced by 93 Da besides
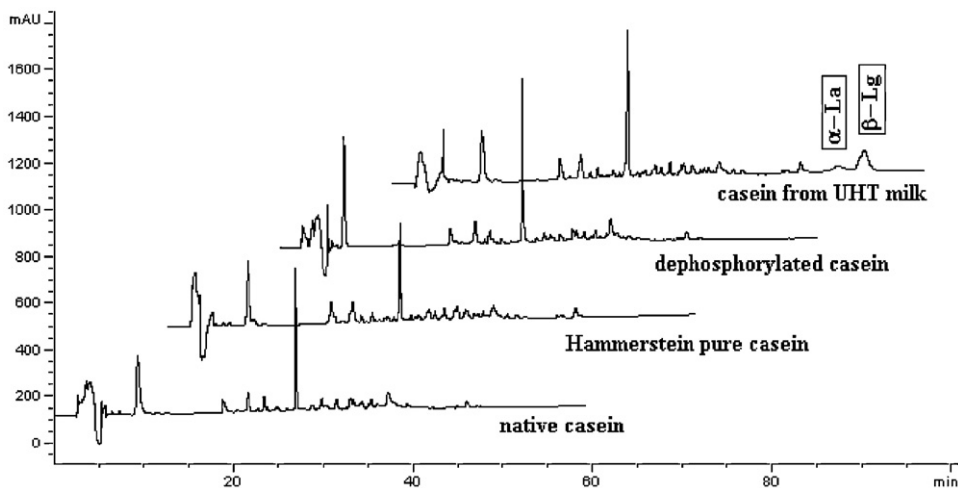
Fig. 6. Comparative RP-HPLC analysis of the peptides survived the *in vitro* digestion of isoelectric native, Hammerstein pure, dephosphorylated and UHT-derived CN samples.
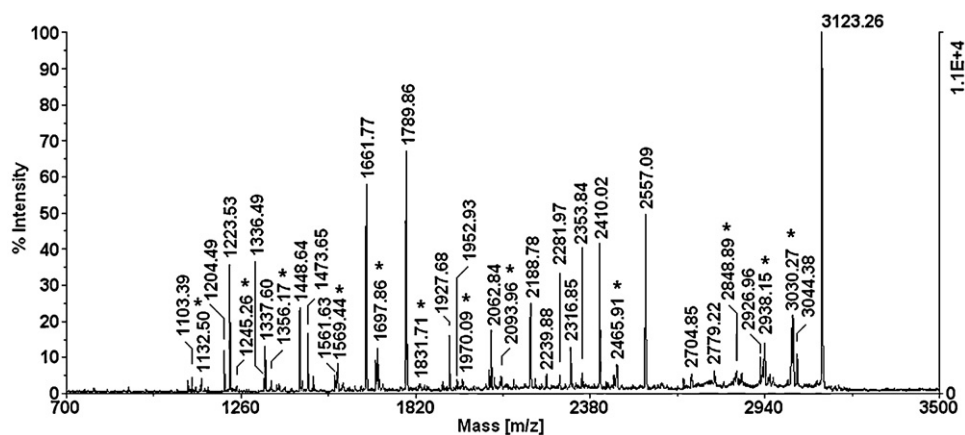
Fig. 7. MALDI-TOF MS analysis of CPP enriched by TiO$_2$ chromatography. The broad ions signals due to metastable decomposition of phosphate groups are labelled with (*). The most represented CPP components are assigned in Table 3.

Table 3
MALDI-TOF MS identification, after selective enrichment by TiO$_2$ micro-columns, of CPP resistant to the *in vitro* gastrointestinal digestion.

Measured mass (MH$^+$)[a]	Theoretical mass (MH$^+$)[a]	Identification
1103.39	1103.40	β-CN f33–40 1P
1204.49	1204.44	β-CN f33–41 1P
1223.53	1223.20	α$_{s1}$-CN f110–119 1P
1333.46	1333.49	β-CN f33–42 1P
1337.60	1336.61	α$_{s1}$-CN f109–119 1P
1448.64	1448.32	β-CN f33–43 1P
1473.65	1473.46	β-CN f30–40 1P
1561.63	1561.73	α$_{s1}$-CN f107–119 1P
1661.77	1660.75	α$_{s1}$-CN f106–119 1P
1789.86	1788.92	α$_{s1}$-CN f105–119 1P
1952.93	1952.09	α$_{s1}$-CN f104–119 1P
2062.84	2061.05	β-CN f30–45 1P
2188.78	2188.96	β-CN f29–45 1P
2281.97	2281.96	β-CN f35–52 1P
2316.85	2317.35	β-CN f29–46 1P
2353.84	2354.00	β-CN f35–53 1P
2410.02	2410.02	β-CN f33–51 1P
2557.09	2557.09	β-CN f33–52 1P
2779.22	2778.88	β-CN f30–51 1P
2926.96	2926.06	β-CN f30–52 1P
3044.04	3042.94	β-CN f1–25 3P
3123.01	3122.96	β-CN f1–25 4P

[a] MW are those of the most abundant isotopic ions.

the parent ions, formed because of the metastable decomposition of HPO$_3$ and H$_3$PO$_4$ in the reflector positive mode MALDI-MS (Fig. 7) [47]. The number of phosphate groups was inferred measuring the mass shift after dephosphorylation of native CPP with alkaline phosphatase. The identified CPP in the CN digests sub-

stantially arose from the N-terminal moiety of β-CN, i.e. 1–25 and 33–55, and α$_{s1}$-CN 104-119 including the SerP[115] site. β-CN f1–25 occurred either 3P or 4P, reflecting the phosphorylation of β-CN. Mass spectrometric data confirmed that multi-phosphorylated peptides containing the Ser(P)-Ser(P)-Ser(P)-Glu-Glu cluster from α$_{s1}$- and α$_{s2}$-CN did not survive the *in vitro* digestion in detectable amounts. In consideration of the above findings, the role of the BBM alkaline phosphatase would deserve a deeper investigation.

3.3. Dephosphorylated caseins

Since CPP were among the major casein peptides resistant to enzyme digestion, this could due to an intrinsic property of the peptide domains or, on the contrary, to the protective role played by the phosphate groups. In separate experiments, CN was dephosphorylated by alkaline phosphatase prior undergoing to the *in vitro* digestion. The RP-HPLC pattern of the dephosphorylated CN digests is compared with that of the native one in Fig. 6, while the MALDI-TOF spectrum of the final enzyme digest is reported in Fig. 8. RP-HPLC analyses did not indicate relevant changes in the peptide pattern. Similarly, most of the dominant signals in the MALDI spectra were the same recorded for native CN digests. Two new prominent signals were detected, i.e. β-CN f33–52 (*m/z* 2477.26) and β-CN f1–25 (*m/z* 2803.57), respectively 1P and 3-4P in native CN. In contrast, α$_{s1}$-CN f104–119 1P, significantly digestion-resistant, was missing in the digests of dephosphorylated CN. The β-CN f26–41 1P, identified in the fraction no. 6 of the native CN digests, was also missing in dephosphorylated CN. These observations suggest that the peptides rising from the phosphorylated N-terminal regions of β-CN are intrinsically stable to hydrolysis,
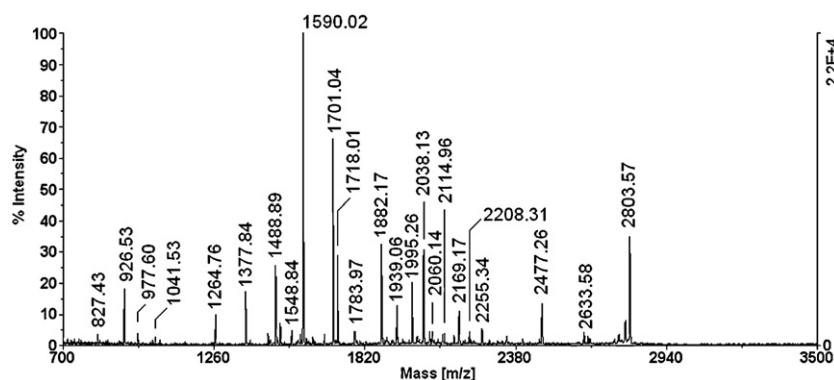
Fig. 8. MALDI-TOF MS spectrum of the *in vitro* gastrointestinal digests of dephosphorylated CN.
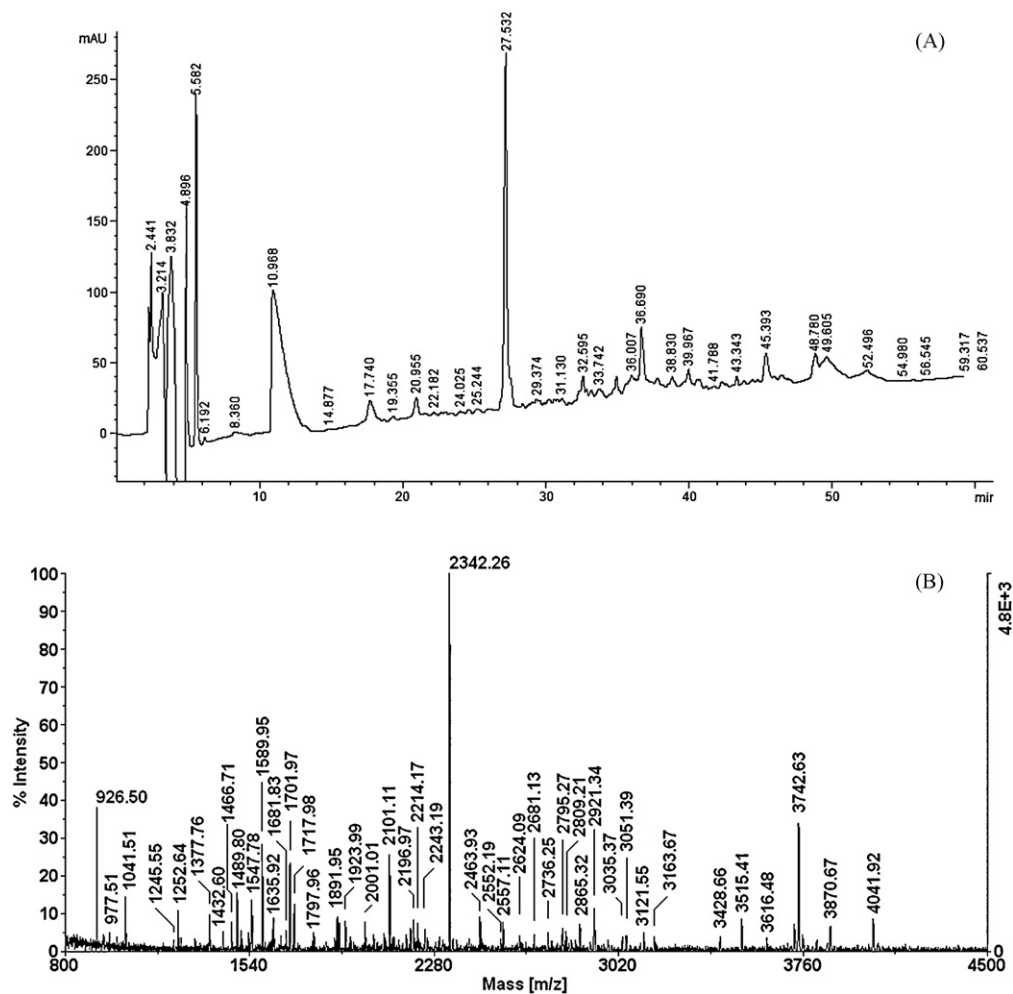
Fig. 9. RP-HPLC separation (A) and MALDI-TOF MS (B) of the WP peptides survived the *in vitro* gastrointestinal digestion. The HPLC fractions were manually collected and further analyzed by *nano*ESI-MSMS. The identified peptides are reported in Table 4.

due to their acidic character enhanced by the presence of phosphate groups. The main HPLC peak at $t_r = 27.96$ min still contained the β-Lg f125–135 peptide regardless of the casein, native or dephosphorylated.

3.4. Digestion of whey proteins

The RP-HPLC chromatogram and MALDI-TOF MS spectrum of *in vitro* WP digests are shown in Fig. 9A and B, respectively. Within the complex signal pattern of the MALDI spectrum, it was possible to assign only a limited number of peptides (Table 4). The most representative peptides isolated by HPLC were identified by off-line *nano*ESI-MSMS. Only a few low intensity peptide ions remained unassigned. The identification of many peptides was probably complicated by *S-S* cross-links of the native proteins, especially for those derived from α-La which contains 4 disulfide bridges. α-La derived species could be the result of higher order cross-linked peptides, in which up to three or four stretches might be joined together. MALDI-TOF comparison of the WP and single α-La and β-Lg digests (data not shown) allowed to restrict the identity of specific peptides to either two-parent proteins. For instance, those in the *m/z* range 3400–4050, tentatively assigned as shown in Table 4, certainly derived from α-La, whereas almost all the other were from β-Lg, as also confirmed by ESI-MSMS analysis. It is worth of note that α-La and β-Lg ($t_r = 49.60$ and 52.60 min, respectively), although

in trace amounts, can survive digestion in intact form (Fig. 9A). In the same condition, the most intense HPLC peak ($t_r = 27.53$ min) was the β-Lg f125–135. Several other peptides rising from the same protein region were among the most represented components of the digests. For instance, the β-Lg f125–138, also detected in the MALDI spectra (MH[+] = 1635.77), was identified by *nano*ESI-MS in the HPLC peak at t_r 36.69 min.

Other relatively abundant surviving peptides derived from the β-Lg 42–60 region, in which five acidic amino acids and two Pro residues occur, including the trypsin non-cleavable bond Lys[47]-Pro. Peptides from this protein domain were recently characterized in significant amounts after a sequential *in vitro* digestion of β-Lg performed with pepsin and chymotrypsin/trypsin [48].

3.5. Stability of β-Lg f125–135 to digestion

The tryptic β-Lg f125–135 peptide having the (R)TPEVDDEALEK sequence was individually subjected to the simulated GI digestion and the extent of proteolysis determined by RP-HPLC. Up to two-fold incubation times and protease concentration including the sequential action of pepsin, pancreatic and BBM enzymes were used for comparison with the control. Less than 5% β-Lg f125–135 was degraded, which confirmed once again the exceptional resistance of this protein region to proteolytic attacks.

Table 4
Identification by MALDI-TOF MS in mixture and *nano*ESI-MSMS of the HPLC fractions of the WP-derived peptides surviving the *in vitro* gastrointestinal digestion (see Fig. 9A).

Measured MW (MH⁺)	Theoretical MW (MH⁺)	Identification	Sequence
MALDI-TOF MS based identification			
2552.19	2552.27	β-Lg 15–39	VAGTWYSLAMAASDISLLDAQSAPL
2681.13	2680.36	β-Lg 14–39	KVAGTWYSLAMAASDISLLDAQSAPL
2809.21	2808.43	β-Lg 13–39	QKVAGTWYSLAMAASDISLLDAQSAPL
2921.34	2921.51	β-Lg 12–39	IQKVAGTWYSLAMAASDISLLDAQSAPL
3035.37	3036.54	β-Lg 11–39	DIQKVAGTWYSLAMAASDISLLDAQSAPL
3051.39	3051.53	β-Lg 11–39 *Met-ox*	DIQKVAGTWYSLAMAASDISLLDAQSAPL
1891.95	1891.00	β-Lg 25–42	AASDISLLDAQSAPLRVY
2101.11	2101.12	β-Lg 40–57	RVYVEELKPTPEGDLEIL
2342.26	2342.27	β-Lg 40–59	RVYVEELKPTPEGDLEILLQ
1681.83	1681.89	β-Lg 43–57	VEELKPTPEGDLEIL
1794.85	1794.97	β-Lg 43–58	VEELKPTPEGDLEILL
1923.99	1924.03	β-Lg 43–59	VEELKPTPEGDLEILLQ
2001.01	2001.79	β-Lg 105–122 *intramolecular S-S*	FCMENSAEPEQSLACQCL *Var. B*
1245.55	1245.58	β-Lg 125–135	TPEVDDEALEK
1635.92	1635.77	β-Lg 125–138	TPEVDDEALEKFDK
Tentative assignment of α-La derived peptides			
3515.41	3514.81	α-La 1–24–*S-S*–117–122	EQLTKCEVFRELKDLKGYGGVSLP-*S-S*-QWLCEK
3616.48	3615.78	α-La 1–24–*S-S*–115–121	EQLTKCEVFRELKDLKGYGGVSLP-*S-S*-LDQWLCE
3742.63	3742.92	α-La 1–24–*S-S*–115–122	EQLTKCEVFRELKDLKGYGGVSLP-*S-S*-LDQWLCEK
3870.67	3871.02	α-La 1–24–*S-S*–114–122	EQLTKCEVFRELKDLKGYGGVSLP-*S-S*-KLDQWLCEK
Impurities of β-CN			
926.50	926.50	β-CN 85–92	PPFLQPEV
977.54	977.49	β-CN 183–190	RDMPIQAF
1041.51	1041.52	β-CN 126–134	TLTDVENLH
1377.76	1377.79	β-CN 195–207	EPVLGPVRGPFPI
1589.95	1589.94	β-CN 195–209	EPVLGPVRGPFPIIV
1701.97	1700.99	β-CN 194–209 *pyro-Glu*	pyroEEPVLGPVRGPFPIIV
1717.98	1717.99	β-CN 194–209	QEPVLGPVRGPFPIIV
1882.05	1882.06	β-CN 193–209	YQEPVLGPVRGPFPIIV
2463.93	2464.09	β-CN 33–52[b]	FQSpEEQQQTEDELQDKIHP
2557.11	2557.09	β-CN 33–52 1P	FQSpEEQQQTEDELQDKIHP

t_r (min)	Measured MW (MH⁺)	Theoretical MW (MH⁺)	Identification	Sequence
RP-HPLC ESI-MS based identification				
4.90				
5.58	<400 Da			
10.97				
17.74	1834.93	1834.94	β-Lg 84–99	IDALNE
20.95	2495.08	2495.38	β-Lg 83–103	KIDALNENKVLVLDTDYKKYL
	2963.34	2963.53	β-Lg 13–40	QKVAGTWYSLAMAASDISLLDAQSAPLR
25.23	1500.75	1500.75	β-Lg 123–135	VRTPEVDDEALEK
27.53	1245.59	1245.58	β-Lg 125–135	TPEVDDEALEK
29.37	1323.65	1323.70	β-Lg 48–59	PTPEGDLEILLQ
	1376.67	1376.66	β-Lg 42–53	YVEELKPTPEGD
	2021.01	2021.03	β-Lg 24–42	MAASDISLLDAQSAPLRVY
	2307.12	2307.30	β-Lg 138–157	KALKALPMHIRLSFNPTQLE
31.13	1372.70	1372.66	β-Lg 123–134	VRTPEVDDEALE
	1454.73	1455.72	β-Lg 43–55	VEELKPTPEGDLE
32.59	1326.68	1326.68	β-Lg 43–54	VEELKPTPEGDL
	1437.67	1437.67	β-Lg 127–138	EVDDEALEKFDK
35.08	1618.80	1618.78	β-Lg 42–55	YVEELKPTPEGDLE
36.00	1489.73	1489.73	β-Lg 43–54	VEELKPTPEGDL
36.69	1635.77	1635.77	β-Lg 125–138	TPEVDDEALEKFDK
38.83	1568.80	1568.80	β-Lg 43–56	VEELKPTPEGDLEI
(7)	1663.79	1663.78	β-Lg 124–137	RTPEVDDEALEKFDK
	1838.00	1837.95	β-Lg 10–26	LDIQKVAGTWYSLAMAA
39.86	1731.87	1731.87	β-Lg 42–56	YVEELKPTPEGDLEI
42.15	2100.14	2100.12	β-Lg 40–57	RVYVEELKPTPEGDLEIL
45.39	1681.88	1681.89	β-Lg 43–57	VEELKPTPEGDLEIL
	1844.94	1844.95	β-Lg 42–57	YVEELKPTPEGDLEIL
	2051.13	2051.13	β-Lg 43–60	VEELKPTPEGDLEILLQK
48.78	1944.02	1944.01	β-Lg 41–57	VYVEELKPTPEGDLEIL
	2242.20	2242.16	β-Lg 36–55	SAPLRVYVEELKPTPEGDLE
	2313.25	2313.25	β-Lg 41–60	VYVEELKPTPEGDLEILLQK
49.60	1923.02	1923.03	β-Lg 43–59	VEELKPTPEGDLEILLQ
	2341.26	2341.26	β-Lg 40–59 unhydrolyzed β-Lg[a]	RVYVEELKPTPEGDLEILLQK

Table 4 (*Continued*)

t_r (min)	Measured MW (MH$^+$)	Theoretical MW (MH$^+$)	Identification	Sequence
	1320.75	1320.75	β-CN 81–92	VFKIDALNENKV
	1421.80	1421.80	β-CN 80–92	AVFKIDALNENKV
52.50	2071.17	2071.05	β-Lg 129–146	DDEALEKFDKALKALPMH
	2184.22	2184.14	β-Lg 129–147	DDEALEKFDKALKALPMHI
	2610.44	2610.35	β-Lg 125–147 unhydrolyzed β-Lg[a]	TPEVDDEALEKFDKALKALPMHI

[a] Identified by MALDI-TOF MS.

[b] Metastable ion.

4. Discussion

4.1. In vitro digestion of caseins and resistance of bioactive peptides

Casein digestion by pepsin started with the release of β-CN f190–209, β-CN f191–209, β-CN f192–209, β-CN f193–209 peptides, all from the C-terminal region of β-CN. At the end of 1 h-incubation, the intact casein entirely shifted to medium- and large-size peptides as shown in Fig. 2A. Peptic peptides were excellent substrates for pancreatic enzymes, and digestion was achieved within 1 h. Because of the cleavage specificity of pancreatic enzymes, kinetics of medium-size oligopeptides formation has been demonstrated closely dependent on the charge. Peptides containing neutral and basic amino acids are degraded more rapidly than those containing acidic residues [19]. At the end, the simulated GI digestion of CN could be considered complete, as neither HPLC nor off-line MALDI-TOF MS was able to detect intact proteins. Indeed, only relatively few selected casein regions, in very low amounts, survived proteolysis. Although the *in vivo* digestion is a much more complex process, it is likely that most of the bioactive peptides would not survive intact, suggesting that they may need protection against gastric or intestinal degradation to exert their functional effects. In particular, the opioid agonists β-casomorphins, especially β-casomorphin-7 (β-CN f60–66), which rise from the "strategic" region of β-CN, were missing. Although contrasting with the data of other authors who identified β-casomorphins in a gastro-pancreatic digest of bovine β-CN [49], this result is consistent with previous targeted investigation which demonstrated the complete degradation of β-casomorphin-7 by digestive enzymes [23]. According to our findings, it is hard to justify the hypothesis that the common bovine β-caseins variants A1 and A2, differing for the single amino acid substitution His^{67} → Pro^{67}, may persistently exert any differential influence on the insurgence of diabetes type I, ischaemic (or coronary) heart disease, schizophrenia and autism (for recent reviews covering this issue see [50–52]). Obviously, it cannot be excluded that infants, once they have ingested milk, produce a different peptide pattern as a consequence of the lack or limited amount of specific proteases by the immature GI apparatus [22]. A precursor (fragment 114–124) of a different β-CN-derived opioid peptide, the neocasomorphin-6 (i.e. β-CN f114–119), was found in trace amount in the fraction no. 8 of the CN digest (Table 2). Phosphate groups do not confer themselves stability to a peptide region towards hydrolytic enzymes. This is also consistent with the literature results [53] showing that multi-phosphorylated α$_{s1}$- and α$_{s2}$-CN peptides were hydrolysis-labile in contrast to the significantly resistant β-CN f1–25 3/4P and f33–52 1P, even dephosphorylated. On the other hand, using a different procedure for *in vitro* digestion, the cluster regions of α$_{s1}$- and α$_{s2}$-CN were found to survive proteolysis of milk-based infant formulas [54]. It is noteworthy that peptides from the N-terminal region of β-CN accumulate during cheese ripening, resisting to the proteolytic system of acid lactic bacteria [44]. The stability of this protein region justifies its supposed involvement in some physiological processes such as negative feedback control of milk secretion [55]. β-CN 1–28 would act as a potent blocker of K$^+$ channels in the apical membrane of mammary epithelial cells. The exception is the α$_{s1}$-CN 104-119 1P peptide, missing in the digests of dephosphorylated CN.

4.2. Exceptional stability to proteolysis of β-Lg fragments: toxicological implications

Although survived to the *in vitro* GI digestion in detectable amounts (Table 4), WP were extensively degraded by the digestive proteases. In particular, the regions of β-Lg where some potentially active peptides are located, i.e. β-Lg f9–20, β-Lg f92–105 and β-Lg f92–105 [56] were missing or in trace amounts at the end of the digestive process, in agreement with the previous observations [57]. As expected, β-Lg was highly resistant to peptic hydrolysis as it exhibited a remarkable stability at low pH [58]. The particular folding makes β-Lg resistant to pepsin, as the cleavage sites (hydrophobic or aromatic amino acid side chains) are well buried inside the β-barrel, forming a strong hydrophobic core [59]. Furthermore, contrary to common belief, GI digestibility of β-Lg has been found even to decrease with a high degree of protein denaturation [42]. This is in agreement with the findings that β-Lg co-precipitated with casein in UHT milk remains partly unhydrolyzed by digestive proteases (Fig. 6). Native α-La is more sensitive to pepsin [60] probably because of the folding in the "molten globule" conformation at acidic pH. Conversely, at pH 7.2, β-Lg was found more susceptible than α-La to the pancreatic enzymes. This was confirmed by the detection of minor amounts of intact α-La at the end of the simulated GI process including BBM enzymes, while the two β-Lg A and B variants were almost completely hydrolyzed. The exceptional resistance to the proteolysis of the β-Lg f125–135 deserves a deeper investigation on β-Lg, considered by far the most important major cow's milk allergen. It is now recognised, however, that the allergic patients are sensitised to several milk proteins at a time including α$_s$-caseins and α-La [61,62]. It must be underlined that there is no β-Lg counterpart in human milk. Therefore, it is not surprising that β-Lg may react as an allergen. Digestion in simulated gastric fluids is a commonly used indicative criterion to predict the allergenic potential of food proteins [27]. The stability to proteolysis of some specific protein domains seems to be a prerequisite for crossing the gut mucosal barrier to sensitise the immune system and/or elicit an allergic response. On the other hand, the persistence of specific proteins alone is a poor predictor, because it is not a sufficient condition to elicit an immunogenic action, intended as the ability to induce an antigen-antibody IgE class production. Furthermore, the stability to *in vitro* digestion does not take into account the effects that can be significant *in vivo* by the whole food matrix. Immunogenicity is strictly related to allergenicity and the terms could substantially be exchangeable, but the use of "allergenicity" should be restricted to cases where the immunological responses induced by the protein lead to the IgE mediated elicitation of pathological symptoms. In the case of β-Lg, denaturation *per se* does not appear to be sufficient to reduce the immunogenic properties, and pepsin or pepsin–trypsin hydrolysis could enhance the IgE affinity of allergic patients [62]. Therefore, the β-Lg immunoreactivity has been attributed to specific antigenic determinants previously identified

Cow	^{125}Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys^{135}
Buffalo	^{125}Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys^{135}
Sheep	^{125}Thr-Pro-Glu-Val-Asp-***Asn***-Glu-Ala-Leu-Glu-Lys^{135}
Goat	^{125}Thr-Pro-Glu-Val-Asp-***Lys***-Glu-Ala-Leu-Glu-Lys^{135}
Donkey β-Lg (I)	^{125}Thr-***Gln-Met***-Val-Asp-***Lys***-Glu-***Ile-Met***-Glu-Lys^{135}
Donkey β-Lg (II)	^{125}Thr-***Gln-Lys***-Val-Asp-***Lys***-Glu-***Val-Met***-Glu-Lys^{135}

Fig. 10. Sequence alignment of the β-Lg f125–135 from milk of different species.

in linear epitopes [63]. Two major β-Lg CNBr-peptides exhibited major antigenic activity, e.g. β-Lg f41–61 and β-Lg f125–145 [64], which corresponds to the regions of β-Lg found more stable to digestion. Selo et al. attributed to three tryptic peptides (β-Lg f41–60, f102–124 and f149–162) the role of major antigenic epitopes within β-Lg [65]; β-Lg f41–60 was also identified as one of the main IgE binding immunoreactive epitopes [66]. These findings are partially consistent with our results. According to a predictive model [67], the hydrophilic regions are predominantly surface orientated and therefore potentially more antigenic. The computer assisted mapping of the β-Lg antigenic profile showed as major possible epitope determinants those located within 124th and 134th residues [68]. This is also in agreement with the hydrophilic profile involving antigenic activity described by Tokita [64]. The model of the β-Lg is configurationally consistent with the spatial conformation of the sequences 124–134 and 41–60 which share a random coil structure located between β-sheets, orienting these domains on the β-Lg surface. The β-sheets conformation is in turn, generally, specific for the IgE recognition [69]. Studies in animal models have demonstrated that the tryptic peptide f125–135 and its longer form f125–138 are both responsible for inducing specific oral tolerance to native β-Lg thus suggesting that they can represent the most relevant antigenic protein determinant [70]. Using synthetic peptides as main potential epitopes and the radio-allergosorbent test (RAST) against the sera of children found hypersensitive to milk, β-Lg f124–134 was demonstrated responsible for up to 61% IgE binding of allergic patients' sera toward whole β-Lg [68], and, in addition, responsible of the cross-reactivity with α-La [68]. More recently, using two different epitope mapping techniques, e.g. PEPSCAN and "phage display" ELISA, both β-Lg f124–134 and β-Lg f41–60 were recognised as the main epitopes [70], thus confirming previous model-based predictions [71,72]. In a more comprehensive study of epitope mapping of β-Lg in which 77 overlapping decapeptides were used, it was demonstrated that one of the strongest epitope is located in the 127–144 region [73]. The immunodominant regions of β-Lg for the most part survived the *in vitro* digestion. However, epitope binding could be variable and allergenicity individually dependent [73,74]. This could also justify divergent findings about localization of the β-Lg epitopes [75]. Thus, in spite of the extensive investigation carried out, the role of conformational epitopes of β-Lg in developing milk allergy is far from being definitively understood and need to be further investigated. The highly hydrophilic peptide β-Lg f125–135 showed excellent stability towards hydrolysis also dependent on the presence of five acidic residues (in addition to the terminal carboxyl group) and one Pro over total 11 residues. Therefore, β-Lg f125–135 has the most important allergenic potential amongst the β-Lg determinants for its high resistance to proteolysis. An alignment of the 125–135 sequence of β-Lg from different species (Fig. 10) indicates that bovine and water buffalo are highly conserved. Asp^{130} is substituted by Asn^{130} in ovine and by Lys^{130} in goat β-Lg, while there are five amino acid substitutions in donkey β-Lg f125–135. The Asp^{130} → Lys^{130} substitution, besides lowering the acidic character of the peptide,

drastically changes its susceptibility to proteolysis by introducing a trypsin cleavage site for caprine β-Lg. An earlier *in vitro* digestive proteolysis of goat β-Lg with respect to its bovine counterpart has already been observed, whereas no differences in susceptibility to proteolysis between the caseins of the two species subsist [76]. This is also consistent with the observation that the disappearance of the negative charge carried by Asp^{130} (cow and buffalo) and the substitution of Asn^{130} (sheep) with Lys^{130} (goat) is directly responsible for binding the neighbouring protein domains to monoclonal antibodies [77]. Therefore, although casein is undoubtedly implicated in the induction of allergenic response, the reduced or null allergenicity of goats' or donkeys' compared to that of cows' milk might depend, at least partially, on the structural features of β-Lg.

5. Conclusion

According to the *in vitro* batch GI model, CN and WP are heavily degraded by digestive proteases. While this model is applicable for adult humans, no conclusive statement can be inferred for infants at the moment. A more articulate digestive process and the effect of the food matrix *in vivo* should also be taken into consideration. Our findings demonstrate that *in vivo* detection of protease resistant peptides is essential to validate the presumed effects of milk bioactive peptides and that most of them should be protected from the GI proteolytic enzymes when orally administered.

The unusually high resistance to proteolysis of a β-Lg region, already supposed to be responsible for antigenicity, assumes relevant importance to account for the relationship between structure and biological activity. In perspective, the actual involvement of the protease resistant peptides in antigenicity and/or allergenicity has to be confirmed through appropriate experimental protocols, for instance assaying the binding properties to the IgE from serum of allergic infants/patients. The focus is on milk allergy and, hence, on planning suitable strategies to improve milk digestibility and tolerance. In particular, making use of the proteolytic system of lactic acid bacteria [78] or of proteases from non-human source, like papain or bromelain, the antigenicity of specific peptides could be reduced. As peptide permeation can be chemically enhanced or reduced by surfactant-like agents, strategies can also be planned to modulate the delivery and absorption of selected sequences [79]. This leads to the conclusion that susceptibility of milk proteins to proteases should be more carefully evaluated if milk-based "functional foods" are to be produced. Even though the implications of the GI digestion survival of specific regions of milk proteins are speculative argumentations, the described findings provide basis for further confirmation.

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