



In vitro digestibility of bovine β -casein with simulated and human oral and gastrointestinal fluids. Identification and IgE-reactivity of the resultant peptides



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ABSTRACT

Stability during digestion is considered an important feature in determining the allergenicity of food proteins. This study aimed to provide an immunological characterisation of the digestion products of the major cow's milk allergen β -casein (β -CN) produced by *in vitro* orogastric hydrolysis with simulated and human digestive fluids. β -CN was unaffected by oral digestion, but quickly broke down during the early stages of gastric digestion. The degradation with human fluids was faster than that with commercial enzymes. There were similarities in the peptide patterns of the hydrolysates produced in both models, showing 20 peptides in common after gastric digestion. After gastroduodenal digestion, the human fluids gave less numerous and shorter peptides. The IgE binding of most of the individual sera used to the hydrolysates produced with simulated and human fluids increased at the end of the gastric phase and decreased when the duodenal digestion was completed. Two IgE-binding synthetic peptides: β -CN (57–68) and β -CN (82–93), which matched fragments released by β -CN following *in vitro* digestion with simulated and human fluids, consisted of the most immunoreactive areas of the protein. The similarities found between the *in vitro* simulated digestion system and that using human digestive fluids suggest that the former would provide a reasonably good estimation of the potential allergenicity of protein digests.

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1. Introduction

Cow's milk allergy is defined as an immunologically mediated adverse reaction to cow's milk protein and it is usually the first food allergy identified in childhood. With an incidence that ranges from 2% to 7.5% in population-based studies in different countries, allergy to cow's milk often precedes the development of other IgE-mediated allergies (Fiocchi et al., 2010). Although most children outgrow cow's milk allergy by the age of 4 years, a significant proportion of the population retains the allergy throughout life.

Milk contains approximately 20 different proteins that can be involved in allergic sensitisation, but only a few are recognised as being major allergens. Caseins (up to 80% of the total protein content) are one of the most critical milk allergens accounting for the highest circulating IgE levels (Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2009). Amongst them, β -casein (β -CN) (approximately

27% of the total milk proteins) represents a serious health risk to patients with cow's milk allergy, since 75% of the sera from patients with IgE-mediated allergy to cow's milk have IgE directed against β -CN (Shek, Bardina, Castro, Sampson, & Beyer, 2005).

One of the main aspects to consider when evaluating the allergenic potential of food allergens is the effect of gastrointestinal digestion. It is known that allergens are usually able to survive the harsh acidic environment of the stomach, tolerate the presence of surfactants and resist digestion by gastrointestinal enzymes, in order to reach the gut-associated lymphoid tissue (Mackie & Macierzanka, 2010). They might also be digested into high molecular weight peptide fragments, which retain the same, or sometimes increased, IgE binding and T-cell stimulating capacities (Lehmann et al., 2006).

Because of the difficulties involved in conducting *in vivo* experiments to investigate the gastrointestinal stability of the different food allergens, *in vitro* digestion models provide an alternative tool for this purpose (Moreno, Mellón, Wickham, Bottrill, & Mills, 2005). Regarding β -CN, there are few published studies describing β -CN digestion with physiologically relevant models using commercial enzymes (Dupont et al., 2010a,b). Alternatively, other authors have

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used human gastric and duodenal juices in order to evaluate the degradation of milk proteins during digestion (Almaas et al., 2011; Furlund et al., 2013). Although those studies have investigated different aspects of β -CN digestion, the presence of potential IgE epitopes in the digestion products has not been addressed. In this regard, peptide microarray-based immunoassays provide a promising technology because they make it possible to assay several target peptides in parallel, using small volumes of diluted sera and thus greatly reduce the biological sample cost of individual assays and allow for robust replication and statistical approaches for epitope determination (Lin et al., 2009).

The aim of this work was to provide an immunological characterisation of the digestion products of β -CN. For this purpose, *in vitro* hydrolysis of β -CN was compared using simulated and human digestive fluids, mimicking three areas of the gastrointestinal tract: the mouth, stomach and small intestine. The reactivity of IgE antibodies towards the digests was assessed. The peptide products obtained were identified by HPLC–MS/MS and the IgE-binding properties of the most relevant peptides resulting in potential epitopes were evaluated by a microarray-based immunoassay.

2. Material and methods

2.1. Reagents

Phosphatidylcholine (PC) was purchased from Larodan (Malmö, Sweden). Bovine β -CN, α -amylase (EC 3.2.1.1, 210 U/mg solid), porcine pepsin (EC 3.4.23.1, 3640 U/mg protein), pancreatic porcine lipase (EC 232-619-9; type VI-S, 47900 U/mg protein), pancreatic porcine colipase (EC 259-490-1), pancreatic bovine trypsin (EC 232-650-8, type I 10100 BAEE U/mg protein), pancreatic bovine α -chymotrypsin (EC 232-671-2; type I-S; 55 U/mg protein), Bowman–Birk trypsin–chymotrypsin inhibitor from soybean, and all the other reagents used, unless otherwise indicated, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Human digestive fluids

Saliva was collected from 3 healthy donors after 2 h fasting. Human gastric and duodenal fluids were collected by gastroscopy from 4 different patients without any gastrointestinal pathology at the Hospital Universitario La Paz (Madrid, Spain) after 8 h of fasting. Aspirates were collected on ice, and gastric and duodenal fluids were separately pooled and frozen at -20°C to stop any enzymatic reaction. Pepsin activity was assayed with haemoglobin as a substrate, and the activities of trypsin and chymotrypsin were measured using *N*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrates, respectively, yielding a pepsin activity of 728.9 U/mL in the pooled gastric fluid (pH 2.13), and trypsin and chymotrypsin activities of 167.2 and 2.8 U/mL, respectively, in the duodenal fluid (pH 7.8). The bile salts concentration in the duodenal fluid was 0.7 mM, as estimated by a commercial bile acids determination kit from Trinity Biotech (Wicklow, Ireland).

2.3. Human sera

For inhibition ELISA, individual serum samples from six children with proven allergy to bovine milk proteins were collected from the Hospital Gregorio Marañón (Madrid, Spain). For the peptide microarray immunoassay, twelve patients with milk allergy were recruited from the Mount Sinai Medical Center (New York, USA). The diagnosis of IgE-mediated milk allergy was made by an allergist on the basis of objective symptoms of an acute reaction after milk ingestion, together with evidence of specific IgE antibodies

Table 1

Specific IgE levels (kU/L) towards milk and casein of the sera used in the inhibition ELISA (patients 1–6), Western blotting (patients 1–4) and microarray assays (patients 7–18). ND = not determined.

Patient	Age	IgE levels (kU/L)	
		Milk	β -CN
1	2 Years	95.3	91.7
2	6 Years	98.8	90.9
3	7 Years	99.7	94.9
4	5 Years	85.6	62.4
5	1 Year	54.5	48.1
6	13 Years	24.2	32.1
7	1 Year	>100.0	ND
8	8 Months	>100.0	ND
9	4 Months	>100.0	ND
10	7 Years	>100.0	ND
11	7 Years	91.3	ND
12	11 Months	30.4	ND
13	2 Years	30.2	ND
14	9 Months	26.4	ND
15	3 Years	19.2	ND
16	4 Years	17.8	ND
17	2 Years	14.2	ND
18	10 Months	5.1	ND

against milk. The specific IgE levels of the sera used are shown in Table 1.

2.4. *In vitro* orogastroduodenal digestions with commercial enzymes

For *in-vitro* oral digestion, β -CN was dissolved in simulated oral fluid (0.005 M potassium phosphate, 0.004 M CaCl_2 , 0.04% NaCl, pH 6.5) at a concentration of 46 mg/mL. After incubation at 37°C for 15 min, α -amylase was added at the physiological ratio of 150 U/mL of simulated fluid (Bernard, 1953). Oral digestions were performed at 37°C over 2 min and stopped by decreasing the pH to 3.5 with 1 N HCl.

In-vitro gastric and duodenal digestions were performed according to Moreno et al. (2005) with some modifications (Jiménez-Saiz, López-Exposito, Molina, & López-Fandiño, 2013). For gastric digestions, simulated gastric fluid (35 mM NaCl, pH 2.0) containing phospholipid vesicles was prepared by dissolving PC at a concentration of 9.58 mg/mL, followed by sonication in ice (Martos, Contreras, Molina, & López-Fandiño, 2010). Gastric digestions were performed at pH 2.0 for 60 min at 37°C , with 182 U/mg β -CN of porcine pepsin, using the 2-min oral digests as the starting material. Aliquots were withdrawn at 15, 30 and 60 min. Duodenal digestions were performed on the 60-min gastric digests re-adjusted to pH 6.5, with the addition of: 0.25 M Bis-Tris, pH 6.5, 1 M CaCl_2 and a 0.25 M bile salt mixture containing equimolar quantities of sodium glycodeoxycholate and sodium taurocholate. After preheating at 37°C for 15 min, pancreatic porcine lipase, pancreatic porcine colipase, pancreatic bovine trypsin, and pancreatic bovine α -chymotrypsin were added to the duodenal mix at enzyme to substrate ratios of 24.7 U/mg, 1:895 mg/mg, 34.5 U/mg and 0.44 U/mg, respectively (Moreno et al., 2005). The reactions were stopped after 30 min at 37°C by adding a solution of Bowman–Birk trypsin–chymotrypsin inhibitor, at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. The final composition of the mixture was 3.3 mg/mL PC, 3.9 mg/mL β -CN, 7.4 mM bile salts, 7.6 mM CaCl_2 and 20.3 mM Bis-Tris.

2.5. *In-vitro* digestions with oral, gastric and duodenal human fluids

In-vitro protein digestion with human fluids was performed in 3 steps, simulating the oral, gastric and duodenal phases. First,

protein was dissolved in saliva (20 mg/mL, pH 6.5) and incubated at 37 °C for 2 min, followed by a pH decrease to 3.5 with 1 N HCl.

For gastric digestion, β -CN (20 mg/mL) was mixed with the pool of human gastric fluids (112 μ L and 560 μ L, respectively, pH 2.1), so that the pepsin to β -CN ratio was the same as that employed in the digestion with commercial pepsin (182 U/mg). After 1 hour of gently shaking at 37 °C, the pH was adjusted to pH 6.5 with 1 M NaHCO₃ and the gastric digest was mixed with the pool of human duodenal fluids (300 μ L and 198 μ L, respectively, pH 6.7), so that the enzyme to substrate ratio was 34.5 U/mg of trypsin and 0.49 U/mg of chymotrypsin. Aliquots were taken at different time-points during the incubation. The reaction was stopped with a solution of Bowman–Birk trypsin–chymotrypsin inhibitor from soybean, as described above.

2.6. SDS-PAGE Analyses

β -CN and its hydrolysates were diluted to 1 mg/mL in sample buffer containing 2% (w/v) SDS and 5% (v/v) β -mercaptoethanol and heated at 95 °C for 4 min. Samples were analysed on Precast Criterion XT 12% Bis-Tris or 16.5% Tris-Tricine gels and electrophoretic separations were carried out at 100 or 150 V, using XT-MES or Tris-Tricine as running buffers (Bio-Rad, Richmond, CA), respectively, in the Criterion cell (Bio-Rad). Gels were stained with Bio-Safe Coomassie G-250 (Bio-Rad).

2.7. Western blotting

After SDS-PAGE separation on Precast Criterion 16.5% Tris-Tricine gels, the gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min and subjected to semidry transfer in a Trans-Blot SD (Bio-Rad, Richmond, CA) for 30 min at 18 V. Then, the nitrocellulose membranes were blocked with Tris Buffer Saline (50 mM Tris, 150 mM NaCl, pH 7.6) with Tween 20 (0.05% v/v) (TBST) containing, BSA 1% w/v, for 60 min. The membranes were washed with TBST and dipped into human serum (patients 1, 2, 3 and 4, Table 1), and diluted in TBST–BSA (0.1%), overnight at 4 °C. After a new wash, they were incubated overnight at room temperature with biotin conjugated antihuman IgE antibody (clone HP6029, Southern Biotech, Birmingham, AL; 1:500 in TBST–BSA 0.1%). After a further wash, they were incubated with HRP conjugated streptavidin for 1 h at room temperature. Finally, the membranes were rinsed and detection with chemiluminescence was developed with Amersham TM ECL TM Prime (GE Healthcare, Chalfont St Giles, UK). Image acquisition (exposure time 4 min) was performed using the VersaDoc Imaging System (Bio-Rad).

2.8. Human IgE binding by inhibition ELISA

Human IgE binding to β -CN and its digests was assessed by inhibition ELISA as previously reported (Jiménez-Saiz et al., 2013), using sera from patients 1 to 6 (Table 1).

2.9. RP-HPLC

β -CN hydrolysates were analysed in a Waters 600 HPLC system using a RP318 column (250 $\times$ 4.6 mm, Bio-Rad). Operating conditions were as follows: column at room temperature; flow rate, 1 mL/min; injection volume, 50 μ L; solvent A, 0.37 mL/L TFA in Milli-Q water and solvent B, 0.27 mL/L TFA in HPLC-grade acetonitrile. A linear gradient of solvent B in A, from 0% to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 I dual detector. The software Empower 2000 system data (Waters) was used.

2.10. Peptide sequencing by RP-HPLC–MS/MS

RP-HPLC–MS/MS analyses of the digested samples were performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbronn, Germany) with a reverse-phase RP318 HiPore column (250 $\times$ 4.6 mm i.d., Bio-Rad). The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionisation source. Solvents and gradient conditions were as explained above. The injection volume was 50 μ L, and the flow rate 0.8 mL/min. Mass spectra were recorded over the range m/z 100–3000. Using Data Analysis™ (version 4.0, Bruker Daltonik), the m/z spectral data were processed and transformed to spectra representing mass values. BioTools (Version 3.1, Bruker Daltonik) was used to process the MS(n) spectra and perform peptide sequencing.

2.11. MALDI-TOF MS

The range of peptide masses in β -CN gastric digests was analysed by MALDI-TOF MS on a Bruker Autoflex Speed spectrometer (Bruker Daltonik). The sample (0.5 μ L) was loaded on a dry matrix spot (0.5 μ L of 20 mg/mL 2,5-dihydroxybenzoic acid in acetonitrile/H₂O/trifluoroacetic acid (66/33/0.1) (v/v/v) onto a Bruker Anchorchip target and allowed to dry. All mass spectra were initially calibrated with Peptide Calibration Standard and Protein Calibration Standard I (Bruker Daltonik).

2.12. Microarray

The peptide microarray immunoassay was performed as previously described (Wang et al., 2010) with minor modifications. A library of 58 peptides consisting of 20 amino acids each overlapping by 17 (3–offset), corresponding to the primary sequence of β -CN, as well as peptides selected from *in-vitro* digestions were commercially synthesised (JPT peptide Technologies, Berlin, Germany). Amongst the peptides identified from the gastroduodenal phase with simulated and human fluids, the peptides shorter than 8 amino acids, those which were part of longer sequences and/or the peptides that overlapped with well-established epitopes of β -CN by microarray and SPOT membrane in this and in previous studies, were discarded. Peptides with 8 and 9 amino acids were extended to 10 amino acids with the preceding and following amino acids of the protein chain and the peptides over 20 amino acids were replaced by 2 shorter overlapping peptides. Immunolabelling with the patient sera described in Table 1 (patients 7–18) and data analysis were performed as described by Vickery et al. (2013). An index z -value of each peptide element was generated from the median of z -scores of the 6 replicate spots. A peptide was considered positive if its z -score exceeded 3, meaning that the signal was significantly above the background ($p < 0.003$). According to the z -score, the intensity of IgE binding was classified as low (3–5), medium (5–10) or high (>10).

3. Results

3.1. Digestibility and IgE binding of β -CN

A 2-min *in vitro* oral digestion period of β -CN, either with simulated oral fluid or human saliva, did not show any effect on the β -CN pattern as estimated by SDS-PAGE (Fig. 1a and b, lane 3). Regarding the *in-vitro* gastric digestion, both systems also showed similar trends. β -CN was quickly degraded in the early stages of digestion and no residual β -CN was observed by the end of the gastric phase (Fig. 1a and b). With commercial porcine pepsin, β -CN was broken down into three fragments with molecular masses of,

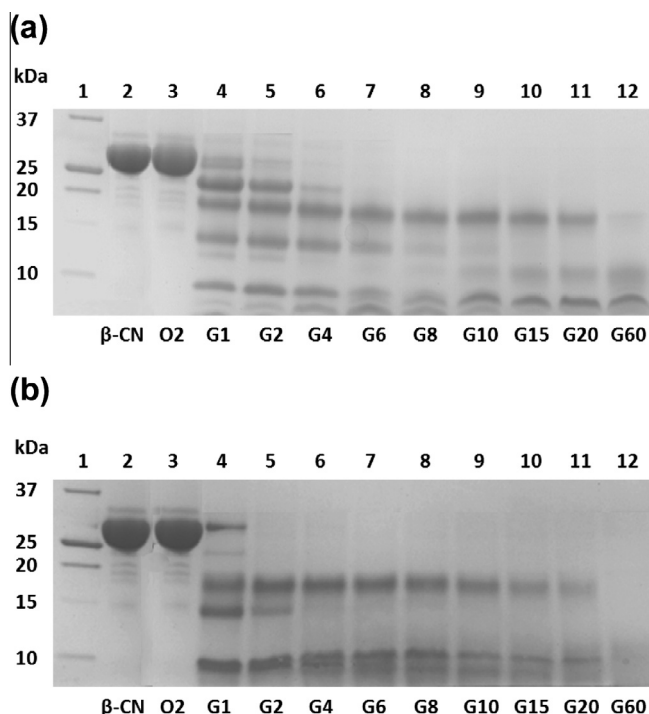


Fig. 1. SDS-PAGE patterns of β -CN after *in vitro* oral and gastric digestions with simulated (a) and human (b) gastric fluids. Electrophoresis was performed using bis-tris 12% acrylamide gels with reducing agents. Lane 1: molecular mass marker (Precision Plus Protein standards (Bio Rad), ranging in molecular mass from 10 to 250 kDa; lane 2: β -CN, lane 3: 2-min oral digests (O2); lanes 4–12: 1 (G1), 2 (G2), 4 (G4), 6 (G6), 8 (G8), 10 (G10), 15 (G15), 20 (G20) and 60-min (G60) gastric digests.

approximately, 23, 18 and 14 kDa, together with fragments of 10 kDa and less. The intact protein and the fragment of 23 kDa disappeared after about 2 and 4 min of digestion, respectively, whilst the band of 14 kDa resisted for 10 min (Fig. 1a, lanes 5, 6 and 9). The concomitant appearance of an additional band of around 12 kDa, together with lower molecular mass peptides was also observed (Fig. 1a, lanes 9–12). Human fluids yielded basically the same profile, but digestion progressed more rapidly: β -CN was degraded in only 2 min and the band of 18 kDa, the product most resistant to hydrolysis, was no longer present after 60 min of digestion (Fig. 1b, lanes 5 and 12). No bands were detected by SDS-PAGE in the gastroduodenal digests but, as estimated by the number and intensity of the small or hydrophilic peptides with low retention time in the RP-HPLC analyses, hydrolysis with pancreatic enzymes was also more rapid when human fluids were used (not shown).

Reactivity against IgE was evaluated by inhibition ELISA using the sera from 6 milk allergic patients (patients 1–6, Table 1). Fig. 2 shows that the immunoreactivity of β -CN at the end of gastric digestion increased, as detected by 4 out of the 6 sera, to values reaching 200% of the initial IgE binding of the undigested protein. Despite individual variations, and in agreement with the lowest protein degradation, the highest IgE binding corresponded to the digests produced with commercial porcine pepsin. IgE binding fell drastically at the end of the duodenal phase of the *in-vitro* digestion either with commercial or human enzymes (Fig. 2).

Western blotting was used to assess the immunoreactivity against IgE of the degradation products (Fig. 3). The different individual sera showed different specificities, but in general terms, the IgE binding of the proteolysis fragments released by pepsin during the first stages of *in-vitro* gastric hydrolyses was progressively reduced as the digestions proceeded. However, in the gastric digests with both simulated and human fluids, a band of around 6.5 kDa was recognised, even after 60 min of hydrolysis, by sera from

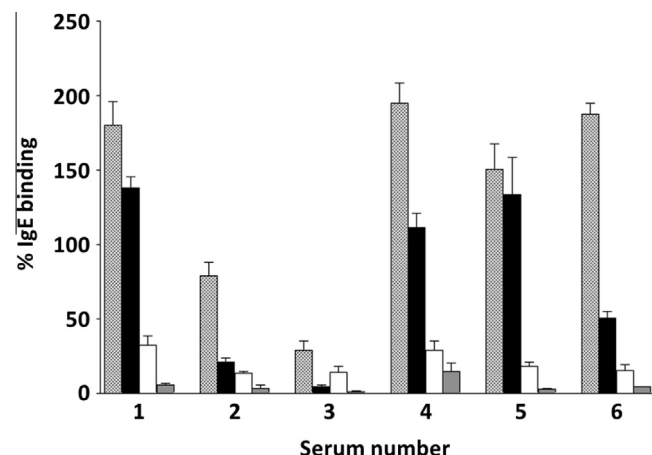


Fig. 2. IgE binding of β -CN, expressed as percentage of the EC_{50} of the intact protein as estimated by inhibition ELISA with sera from six allergic patients (patients 1–6, Table 1), after *in vitro* gastric digestion with simulated (hatched) and human (black) fluids and gastroduodenal digestion with simulated (white) and human (grey) fluids.

patients 1 and 4, which gave a high response by inhibition ELISA (Fig. 2 and Fig. 3b and c), but not by serum from patients 2 (Fig. 2 and Fig. 3d) and 3 (not shown) which gave a low response. It is, therefore, likely that this degradation product contributed to the residual IgE binding of the *in-vitro* gastric digests, although its actual relevance is probably hampered by an inefficient transfer yield in the electroblotting, as revealed by Ponceau staining of the nitrocellulose membrane (results not shown).

3.2. Peptide identification after *in-vitro* digestions and epitope recognition

RP-HPLC/MS–MS was used for the analysis of the gastric (60 min) and gastroduodenal (with a further 30 min of duodenal hydrolysis) digests of β -CN. Fig. 4 (see also Supplementary data 1 in the on line repository) shows the sequences of the 152 peptides identified, which ranged between 3 and 21 amino acids. The sequence coverage was high: 67.0% and 80.4% after gastric digestion and 82.8% and 70.3% after subsequent duodenal digestion with simulated and human fluids, respectively. The peptide patterns of the hydrolysates produced in both models were fairly similar following gastric digestion, showing 20 peptides in common, although the human fluids gave rise to a higher proportion of peptides of less than 1500 Da. Because the range of m/z scanned in RP-HPLC/MS–MS was between m/z 100–3000, the peptide mass distribution of the gastric digests was analysed by MALDI-TOF MS, that showed that 17% of the peptides produced after 60 min of gastric digestion were between 3000 to 10,000 Da. Gastroduodenal digestion with human fluids gave less numerous peptides, all shorter than 1500 Da (Fig. 4, Supplementary data 1).

Taking together the 20-amino acid synthetic peptides, the IgE binding areas identified with the peptide microarray covered almost the whole β -CN sequence, although the area of the protein that was recognised by most sera corresponded to that between residues 31–101 and 124–209 (Fig. 4). The IgE-binding patterns of the 12 printed peptides selected from the gastroduodenal digests with simulated and human fluids are also shown in Fig. 4, and Table 2 compares their binding frequency to the individual sera with that of matching sequences from the 20-amino acid peptide library. The highest IgE binding (58% of patients) corresponded to the β -CN positions 57–93 [β -CN (57–68), β -CN (69–80), β -CN (73–84) and β -CN (82–93)]. Peptides belonging to the region β -CN (144–163) and to the C-terminus [β -CN

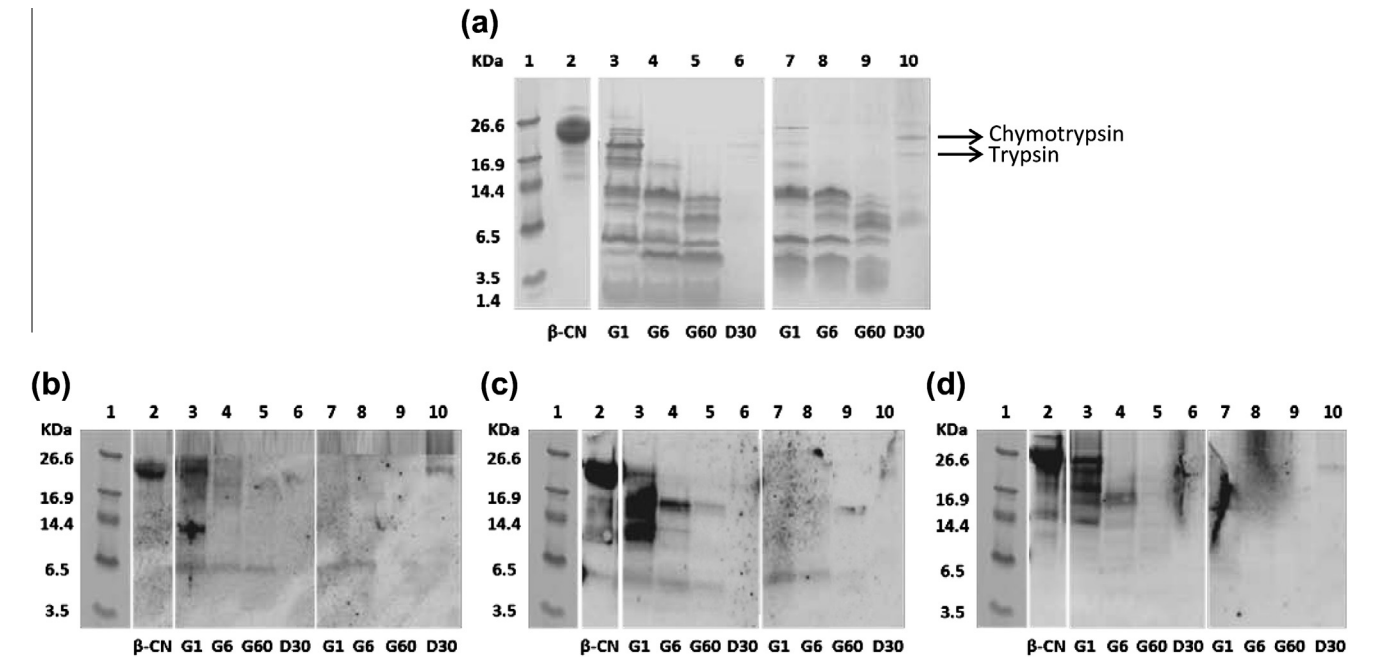


Fig. 3. SDS-PAGE patterns (a) and Western blotting (b–d) of β -CN after *in vitro* gastric and gastroduodenal digestions with simulated (lanes 3–6) and human (lanes 7–10) gastric fluids. Electrophoresis was performed using tris-tricine 12% acrylamide gels with reducing agents. Lane 1: molecular mass marker containing triosephosphate isomerase (26.6 kDa), myoglobin (16.9 kDa), α -lactalbumin (14.4 kDa), aprotinin (6.5), insulin b chain (3.4 kDa) and bacitracin (1.4 kDa); lane 2: β -CN; lanes 3–5 and 7–9: 1 (G1), 6 (G6), and 60-min (G60) gastric digests; lane 6 and 10: gastroduodenal digests (60 min of gastric followed by 30 min of duodenal digestion, D30). Sera from patients 1 (b), 4 (c) and 2 (d), described in Table 1, were used.

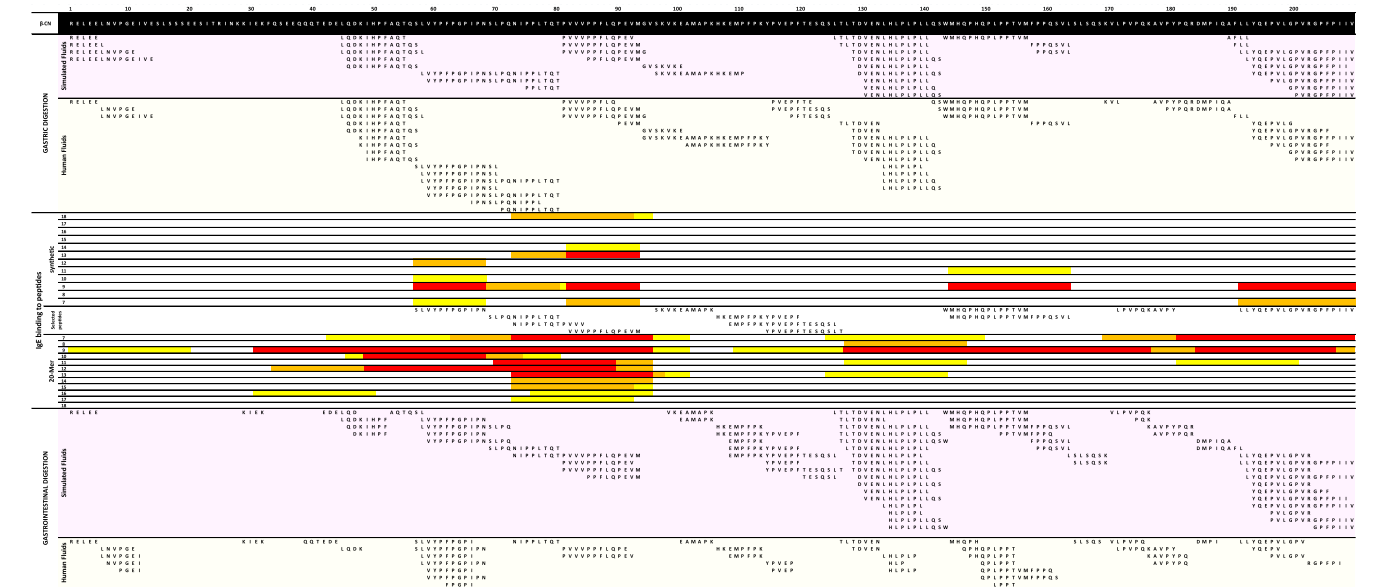


Fig. 4. Peptide sequences, identified by RP-HPLC–MS/MS, in the gastric (60 min) and gastroduodenal digests of β -CN (60 min of gastric followed by 30 min of duodenal digestion), with simulated and human fluids, and IgE binding, estimated by peptide microarray with 12 sera of milk allergic patients (patients 7–12, Table 1), of 12 peptides from simulated digestions and 58 20-mer peptides from a peptide library that spans the whole β -CN sequence. The intensity of the IgE binding, low (light gray), medium (dark gray) and high (black) is shown for each individual serum.

(191–209)] showed a moderate IgE binding, being in both cases recognised by approximately 16% of the patients included in the study, whilst peptides corresponding to the regions between positions 96–126 [β -CN (96–105), β -CN (106–119), β -CN (108–125) and β -CN(114–126)], β -CN (143–156) and β -CN (171–180) did not react with any of the patient's sera employed in the study.

4. Discussion

Given the importance of the resistance of food proteins to gastrointestinal digestion in their capacity to modulate the immune response, this work compared the digestibility of β -CN, one of the main cow's milk allergens, in two different *in vitro* systems using simulated and human fluids, and assessed the

Table 2

IgE-binding (expressed as the number of allergic patients with a positive response towards each peptide) to synthetic peptides derived from *in vitro* digestions of β -CN and related 20-mer peptides from a peptide library that spans the whole β -CN sequence. The matching β -CN fragments identified in the *in vitro* gastric (60 min, G60) and gastroduodenal digests (60 min of gastric followed by 30 min of duodenal digestion, D30) with simulated and human digestive fluids by RP-HPLC–MS/MS are also shown, highlighting the part of the sequence that coincides with the synthetic peptide.

Synthetic peptides derived from digestions	β -CN 20-mer peptides	β -CN fragments	Reactive patients	RP-HPLC-MS/MS identified peptides			
				Simulated fluids		Human fluids	
				G60	D30	G60	D30
SLVYFPFGPIPN		57-68	4/12				
	IHPFAQTQSLVYFPFGPIPN	49-68	4/12	LVYFPFGPIPNSLPQNIPLTQT	LVYFPFGPIPN	SLVYFPFGPIPNSL	SLVYFPFGPI
	FAQTQSLVYFPFGPIPNSLP	52-71	4/12	VYFPFGPIPNSLPQNIPLTQT	LVYFPFGPIPNSLPQ	LVYFPFGPIPNSL	SLVYFPFGPIPN
	TQSLVYFPFGPIPNSLPQNI	55-74	4/12		VYFPFGPIPN	VYFPFGPIPNSL	LVYFPFGPI
SLPQNIPLTQT		69-80	1/12				
	PFPGPIPNSLPQNIPLTQT	61-80	4/12	LVYFPFGPIPNSLPQNIPLTQT	SLPQNIPLTQT	LVYFPFGPIPNSLPQNIPLTQT	NIPPLTQT
	GPINSLPQNIPLTQTPVV	64-83	1/12	VYFPFGPIPNSLPQNIPLTQT		VYFPFGPIPNSLPQNIPLTQT	VYFPFGPIPNSLPQNIPLTQT
	PNSLPQNIPLTQTPVVPP	67-86	0/12	PPLTQT		IPNSLPQNIPLPQNIPLTQT	VYFPFGPIPN
NIPPLTQTPVVV		73-84	2/12				
	PNSLPQNIPLTQTPVVPP	67-86	0/12	LVYFPFGPIPNSLPQNIPLTQT	SLPQNIPLTQT	LVYFPFGPIPNSLPQNIPLTQT	NIPPLTQT
	LPQNIPLTQTPVVPPFLQ	70-89	2/12	VYFPFGPIPNSLPQNIPLTQT	NIPPLTQTPVVPPFLQPEVM	VYFPFGPIPNSLPQNIPLTQT	PVVVPPFLQPE
	NIPPLTQTPVVPPFLQPEV	73-92	10/12	PPLTQT	PVVVPPFLQPEV	IPNSLPQNIPLPQNIPLTQT	PVVVPPFLQPEV
VVVPPFLQPEVM		82-93	5/12				
	PLTQTPVVPPFLQPEVMGV	76-95	10/12	PVVVPPFLQPEV	NIPPLTQTPVVPPFLQPEVM	PVVVPPFLQ	PVVVPPFLQPE
	QTPVVPPFLQPEVMGVSKV	79-98	3/12	PVVVPPFLQPEVM	PVVVPPFLQPEV	PVVVPPFLQPEVM	PVVVPPFLQPEV
	VVVPPFLQPEVMGVSKVKEA	82-101	3/12	PVVVPPFLQPEVMG	PVVVPPFLQPEVM	PVVVPPFLQPEVMG	
SKVKEAMAPK		96-105	0/12				
	EVMGVSKVKEAMAPKHKEMP	91-110	0/12	GVSKVKE	VKEAMAPK	GVSKVKE	EAMAPK
				SKVKEAMAPKHKEMP	EAMAPK	GVSKVKEAMAPKHKEMPFPKY	
						AMAPKHKEMPFPKY	
HKEMFPKYPVEPF		106-119	0/12				
	EAMAPKHKEMPFPKYPVEPF	100-119	0/12	SKVKEAMAPKHKEMP	HKEMFPK	GVSKVKEAMAPKHKEMPFPKY	HKEMFPK
	APKHKEMPFPKYPVEPFES	103-122	0/12		HKEMFPKYPVEPF	AMAPKHKEMPFPKY	EMFPK
	HKEMFPKYPVEPFESQSL	106-125	0/12		EMFPK	YPVEPF	YPVEPF
EMPFPKYPVEPFESQSL		108-125	1/12				
	HKEMFPKYPVEPFESQSL	106-125	0/12	SKVKEAMAPKHKEMP	HKEMFPK	GVSKVKEAMAPKHKEMPFPKY	HKEMFPK
					HKEMFPKYPVEPF	AMAPKHKEMPFPKY	EMFPK
					EMFPK	PVEPFTE	YPVEPF
YPVEPFESQSLT		114-126	0/12				
	MPFPKYPVEPFESQSLTLT	109-128	1/12		HKEMFPKYPVEPF	PVEPFTE	YPVEPF
					EMFPKYPVEPFESQSL	PVEPFESQSL	PVEPF
					YPVEPF	PVEPFESQSL	
WMHQPHQLPPTVM		143-156	0/12				
	LLQSWMHQPHQLPPTVMFP	139-158	1/12	WMHQPHQLPPTVM	WMHQPHQLPPTVM	QSWMHQPHQLPPTVM	MHQPH
	SWMHQPHQLPPTVMFPQ	142-161	1/12		MHQPHQLPPTVM	SWMHQPHQLPPTVM	QPHQLPPT
					MHQPHQLPPTVMFPQSVL	WMHQPHQLPPTVM	PHQLPPT
MHQPHQLPPTVMFPQSVL		144-163	2/12				
	HQPHQLPPTVMFPQSVLS	145-164	1/12	WMHQPHQLPPTVM	WMHQPHQLPPTVM	QSWMHQPHQLPPTVM	MHQPH
				FPQSVL	MHQPHQLPPTVM	SWMHQPHQLPPTVM	QPHQLPPT
				PPQSVL	MHQPHQLPPTVMFPQSVL	WMHQPHQLPPTVM	PHQLPPT
LPVPQKAVPY		171-180	0/12				
	LSLSQSKVLPVQKAVPYQ	163-182	1/12		VLVPQK	AVPYQQRDMPQIA	VLVPQK
	SQSKVLPVQKAVPYQQRDM	166-185	1/12		PQK	PYPQQRDMPQIA	LPVPQKAVPY
	KVLPVQKAVPYQQRDMPQI	169-188	2/12		KAVPYQR		KAVPYQ
LLYQEPVLGVPVRGPFPIIV		191-209	2/12				
	FLYQEPVLGVPVRGPFPIIV	190-209	2/12	AFLL	LLYQEPVLGVPVR	FLY	LLYQEPVLGVP
				FLL	LYQEPVLGVPVRGPFPIIV	YQEPVLG	YQEPV
				LLYQEPVLGVPVRGPFPIIV	LYQEPVLGVPVR	YQEPVLGVPVRGPF	PVLGPV

immunochemical properties of the resulting digests. To this aim, the products of digestion of β -CN in both systems were identified by RP-HPLC–MS/MS and the IgE binding epitopes were characterised by using a peptide microarray-based immunoassay.

The results showed that β -CN was unaffected by oral digestion either with simulated oral fluid or human saliva, but quickly degraded afterwards in simulated and human stomach fluids, leaving no intact protein at the end of the gastric phase. This is in accordance with previous *in vitro* studies performed with commercial

porcine pepsin in simulated gastric fluid (Dupont et al., 2010a,b). SDS-PAGE analyses revealed a faster degradation of β -CN when digested with human fluids (Fig. 1). Even if there is not a consensus on the physiologically representative enzyme-to-protein ratios that should be used in a digestibility study, both digestion systems were standardised in terms of their proteinase activity on the basis of previously proposed physiologically relevant models (Moreno et al., 2005). Therefore, the variations encountered could be attributed to differences in the specificity and efficiency between the commercial digestive enzymes of porcine (pepsin) and bovine origin (trypsin and α -chymotrypsin) and the human digestive enzymes, as well as to the presence of peptidases, mainly carboxypeptidases (Lyons & Fricker, 2011), in the human pancreatic extracts, which could account for the most extensive *in-vitro* duodenal liberation of free amino acids from the peptides previously formed during the gastric phase (Figs. 3a and 4). On the other hand, it is known that interaction with physiological surfactants, such as phospholipids and bile salts, plays a key role in protein digestion (Mackie & Macierzanka, 2010). PC, a phospholipid secreted by the gastric mucosa, was included in our simulated gastric system, although it has been reported that it does not affect the kinetics of pepsinolysis of β -CN in solution (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). Bile acids, that solubilise dietary fats, particularly lipolysis products of triglycerides, through the formation of mixed micelles with phospholipids and fatty acids, also facilitate the proteolysis by pancreatic enzymes of several dietary proteins, such as β -lactoglobulin (Gass, Vora, Hofmann, Gray, & Khosla, 2007) and ovalbumin (Martos et al., 2010). However, in view of the low concentration of bile acids present in the human duodenal fluid, probably due to prolonged fasting (Kalantzi et al., 2006), it is not likely that it could favour the duodenal proteolysis of the bigger peptides generated from β -CN by pepsin hydrolysis.

There was a high degree of correlation between the digestion products released by simulated and human fluids, particularly after gastric digestion (Fig. 4, Supplementary data 1). The peptides identified by RP-HPLC-MS/MS after pepsin digestion covered most of the β -CN sequence, except for the area between residues 14 and 45, which comprises 5 phosphoserine groups at positions 15, 17, 18, 19 and 35. The resistance of casein phosphopeptides to gastrointestinal digestion has been demonstrated *in vitro* and *in vivo*; therefore, the absence of peptides corresponding to this part of the protein is probably due to the low ionisation efficiency of highly electronegative phosphorylated peptides during mass spectrometry analysis (García-Nebot, Alegría, Barberá, Contreras, & Recio, 2010). Following gastroduodenal digestion with simulated fluids, 68 peptides were found, many of which were either totally or partially coincident with fragments previously identified from gastroduodenal hydrolyses of β -CN using *in vitro* systems that mimic adult and an infant models of digestion (Dupont et al., 2010a,b). Gastroduodenal digestion with the human fluids gave 45 peptides, most of which corresponded to fragments shorter than the ones produced by the simulated fluids, as a result of a more extensive hydrolysis by the pancreatic extract as discussed above. Therefore, whilst other studies have shown large differences in the peptide sequences generated from hydrolysis of milk proteins when human and non-human digestive enzymes were compared (Almaas et al., 2011; Furlund et al., 2013), our results suggest that, in view of the similarities between the peptides released by both systems, the *in-vitro* digestion model utilising commercial enzymes (Moreno et al., 2005) provides a good estimation of the allergenicity of a protein following gastrointestinal digestion.

Accordingly, the IgE binding of the hydrolysates produced with both simulated and human fluids was similar, showing, in 4 out of 6 individual sera used, an increase at the end of the gastric phase, followed by an important decrease when the duodenal digestion was completed, particularly when using human enzymes (Fig. 2).

The increased IgE-reactivity of β -CN found after the gastric phase, despite the fact that the protein was rapidly degraded upon pepsin digestion, suggests that, even if β -CN has a very flexible structure with no disulphide bonds and its major IgE-binding epitopes are assumed to be mainly sequential (Cocco, Järvinen, Han, Beyer, & Sampson, 2007), some IgE-binding epitopes were unmasked following hydrolysis. In fact, caseins constitute a frequently mentioned example of digestion-sensitive proteins that are nonetheless able to sensitise and elicit allergic reactions through the gastrointestinal tract (Wal, 1998). This could be attributed to the formation of IgE-binding protein fragments, such as that with molecular mass of approximately 6.5 kDa, which bound the sera of patients most immunoreactive towards the gastric digests (Fig. 3b and c). In addition, MALDI-TOF MS revealed the presence in the gastric digests of peptides with molecular masses between 3000 and 10,000 Da that could carry IgE epitopes, but might not be detected by Western blotting following SDS-PAGE.

With the development of microarray technology and peptide synthesis techniques, peptide microarray-based immunoassays have proven very useful for epitope mapping of milk allergens (Lin et al., 2009). The use of 58 overlapping peptides showed multiple IgE-binding epitopes distributed throughout the β -CN sequence (Fig. 4), with the major immunoreactive areas, i.e. fragments 31–101 and 124–209, being related with the most hydrophobic parts of the protein: 55–92, 133–150 and 151–202 (Leaver & Dalgleish, 1990). This agrees with the results of previous studies using the peptide microarray technique (Cerecedo et al., 2008; Savilahti et al., 2010) and the SPOTs membrane technique (Chatchatee et al., 2001). Nevertheless, it should be noted that epitope diversity and binding affinity depend heavily on patient selection, especially on the severity of cows milk allergy and, in fact, provide prognostic information that may allow distinction between transient and persistent food allergy (Wang et al., 2010).

Two IgE-binding synthetic peptides: β -CN (57–68) and β -CN (82–93), related to fragments released from β -CN following *in vitro* digestion with simulated and human fluids, were coincident with the most immunoreactive areas of the protein, in terms of frequency and intensity of IgE binding (Fig. 4, Table 2), as well as with sequences previously identified as IgE-binding epitopes, particularly β -CN (57–66), β -CN (52–74) and β -CN (83–92) (Cerecedo et al., 2008; Chatchatee et al., 2001). Dupont et al. (2010a,b) also reported the appearance of β -CN (81–93) after simulated gastrointestinal digestion of β -CN.

Six synthetic peptides that resembled products of gastroduodenal digestion, β -CN (96–105), β -CN (106–119), β -CN (108–125), β -CN (114–126), β -CN (143–156) and β -CN (171–180), did not react with any of the patients' sera employed in the study. However, in some cases, IgE-binding was detected when these sequences were included in the 20 amino acid-peptides from the library (see for instance β -CN (143–156) and β -CN (171–180) in Fig. 4 and Table 2), thus highlighting the influence of peptide length and the nature of nearby amino acids, in addition to the individual patient specificity, in determining the affinity for IgE antibodies (Cocco et al., 2007; Collis, Brouwer, & Martin, 2003). This raises the question of whether the IgE-binding fragments identified after gastroduodenal digestion constitute a full epitope or only a part of it. Furthermore, their small molecular mass makes it unfeasible that they contain more than a single IgE binding epitope, suggesting marginal biological activity in terms of basophil activation properties (Albrecht et al., 2009). However, immunisation of rats with small peptides, such as those arising from peanut allergen Ara h 1 digestion, induces antibody responses, which could be attributed to their aggregation into complexes of larger sizes (Bøgh, Barkholt, Rigby, Mills, & Madsen, 2012).

Taking into account the limitations inherent in the simulation of *in-vivo* gastrointestinal conditions, which are characterised by

large inter-individual variations in gastric and duodenal secretions and intra-individual differences, due to the type and amount of food consumed or the time of the day (Hur, Lim, Decker, & McClements, 2011), and the heterogeneous recognition of critical amino acids in β -CN by individual milk allergic patients (Cocco et al., 2007), the similarities found between the *in-vitro* simulated digestion system with commercial enzymes used in this work, broadly employed in studies aimed to assess the digestibility of food allergens, and that using human digestive fluids, suggest that the former would provide a reasonably good estimation of the potential allergenicity of protein digests. In fact, by using a peptide microarray-based immunoassay, similar IgE-binding epitopes were found within the products of the digested β -CN utilising both systems, which matched the regions recognised in the peptide library of 58 fragments covering the entire β -CN sequence.

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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.2013.07.110>.

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