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Reduced β-lactoglobulin IgE binding upon *in vitro* digestion as a result of the interaction of the protein with casein glycomacropeptide

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ABSTRACT

The aim of this work was to evaluate the effect of the presence of casein glycomacropeptide (CMP) on the *in vitro* digestibility and potential allergenicity of β-lactoglobulin (β-lg)–CMP mixtures. The digestion products were analyzed by RP-HPLC and RP-HPLC–ESI-MS/MS. The potential allergenicity of the digestion products was studied by human IgE binding by inhibition ELISA with serum samples from children with clinical allergic symptoms to β-lg. No differences were observed by HPLC in the mixtures hydrolysates due to CMP–β-lg interactions. RP-HPLC–ESI-MS/MS results showed different peptides occurring in the mixtures hydrolysates. Additionally, it was observed a significant reduction of β-lg IgE binding in the presence of CMP. The disappearance of epitopes in the digested mixtures could explain the lower IgE binding observed in these systems compared to β-lg. It can be concluded that the presence of CMP in products containing β-lg may modify the digestion products that may reduce the potential allergenicity of β-lg.

1. Introduction

Casein glycomacropeptide (CMP) and β-lactoglobulin (β-lg) are present in cheese-whey at concentrations about 20% and 50% respectively. In the last years CMP has received increasing attention because of its unique composition and biological activities (Brody, 2000; El-Salam, El-Shibiny, & Buchheim, 1996; Thomä-Worringer, Sørensen, & López Fandiño, 2006) so it is of importance to incorporate this peptide in food formulations. On the other hand, whey proteins are frequently used as ingredients due to their outstanding functional properties (Foegeding, Davis, Doucet, & McGuffey, 2002) which are mainly attributed to β-lg that makes up about 50% of proteins in whey. However, it is well known the high allergenicity of cow's milk proteins (among them, β-lg is one of the most allergenic milk protein) that affects 2–3% in children and more than 1% in adults (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001; Molina, Chicón, Belloque, & López-Fandiño, 2009; Sélo et al., 1999). β-lg presents two disulfide bonds per monomer and one free cysteine group. These intramolecular bonds are responsible of maintaining the structural integrity of β-lg upon hydrolysis and heat treatment (Hambling, Mc Alpine, & Sawyer, 1992), thus contributing to its high allergenicity.

In a previous study, the effect of *in vitro* digestion in the allergenicity of egg proteins, assessing the effect of factors such as pH, pepsin-substrate ratio, presence of surfactants or bile salts has been reported (Jiménez-Saiz, Martos, Carrillo, López-Fandiño, & Molina, 2011; Martos, Contreras, Molina, & López-Fandiño, 2010). Moreover, the effects of processing (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011) and of the food matrix (Jiménez-Saiz, López-Expósito, Molina, & López-Fandiño, 2013; Jiménez-Saiz, Pizones Ruiz-Henestrosa, López-Fandiño, & Molina, 2012; Martos, López-Fandiño, & Molina, 2013) have been studied. The obtained results underlined the importance of the digestion model and the food matrix in the digestibility of the allergens and their capacity to trigger the immune response. For example, interactions between proteins may contribute to the ability of a protein or package of proteins to reach the sites of active immune sampling in the gastrointestinal mucosa, promoting or protecting their digestion, and therefore influencing the potential allergenicity of a protein (Teuber, 2002).

Regarding β-lg, its high resistance to proteolysis provides greater potential for maintaining its allergenicity, even after

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in vitro gastrointestinal digestion (Bossios et al., 2011). However, a reduction of the immunoreactivity of the digestion products of β-lg by the interaction with polysaccharides has been reported by different authors (Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010; Kobayashi et al., 2001; Mouécoucou, Frémont, Villaume, Sanchez, & Méjean, 2007) or by polymerization with transglutaminase in the presence of cysteine (Villas-Boas, Vieira, Trevizan, de Lima Zollner, & Netto, 2010), showing that although the immunogenicity of β-lg is not fully eliminated, the interaction with food components could be promising to reduce its allergenicity. Recently, it was demonstrated that β-lg and CMP strongly interact, at pH 7.0 and 3.5, in the aqueous phase and at the air–water interface forming assembled structures driven by hydrophobic and electrostatic interactions thus improving interfacial, foaming and gelling properties mainly at pH 3.5 (Martinez, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2009, 2012; Martinez, Farías, & Pilosof, 2010).

The objective of the present work was to assess if the interaction between β-lg and CMP affects the digestion products and hence the potential allergenicity of β-lg, as β-lg and CMP are present together in dairy products containing cheese whey.

2. Materials and methods

2.1. Sample preparation

BioPURE-GMP® casein glycomacropeptide (CMP) and BioPURE® β-lactoglobulin (β-lg) was provided by DAVISCO Foods International, Inc. (Le Sueur, MN, USA). Composition of CMP was: protein (dry basis) 83.0% (w/w) (N × 6.47) being CMP 90.0% (w/w) (N × 7.07) of total proteins, 0.6% (w/w) fat, 6.3% (w/w) ash and 6.0% (w/w) moisture. Composition of β-lg was: protein (dry basis) 97.8% (w/w) (N × 6.38) being β-lactoglobulin 93.6% (w/w) of total proteins, 0.3% (w/w) fat, 1.8% (w/w) ash and 5.0% (w/w) moisture.

Powder samples of β-lg and CMP were dissolved separately in Milli-Q ultrapure water at room temperature under agitation. The CMP:β-lg mixed systems at ratios 0:100 (pure β-lg), 25:75, 50:50, 75:25 and 100:0 (pure CMP) were prepared by mixing the appropriate volume of each protein solution up to achieve a total concentration of 4% (w/w). The pH of solutions was adjusted to 7.0 or 3.5 by using 1 N HCl or NaOH. Then, the solutions were freeze dried. Such conditions of pH were selected from the previous studied (Martinez et al., 2009, 2010, 2012) in which was verified the interactions between β-lg and CMP. Moreover, the pH 3.5 is especially interesting since is just between the pI of the glyco and aglyco fractions of CMP (gCMP and aCMP, respectively) (Kreuβ, Strixner, & Kulozik, 2009).

2.2. In vitro gastro-duodenal digestions

In vitro gastric and duodenal digestions were performed according to the procedure of Martos et al. (2010).

2.2.1. In vitro gastric digestion

CMP:β-lg solutions were dissolved in simulated gastric fluid (SGF, 35 mM NaCl pH 2.0) and preheated for 15 min at 37 °C. The *in vitro* gastric digestion started with the addition of porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma–Aldrich, MO, USA) at an enzyme/substrate ratio of 1:20 w/w (concentration: 172 units/mg of protein). Samples were incubated at 37 °C under constant agitation and pepsin reaction was stopped by adding 1 M $NaHCO_3$ up to pH 7.0. Aliquots were taken at 0, 10 and 60 min (G0, G10 and G60, respectively) at a final protein concentration of 4.78 mg/mL for G0 and 4.45 mg/mL for G10 and G60.

Gastric digestions were performed in duplicate. Aliquots G0 were diluted with Milli-Q ultrapure water to a protein concentration of 4.45 mg/mL and stored with aliquots G10 and G60 at −20 °C whereas the remaining 60 min gastric material was subjected to the duodenal digestion phase.

2.2.2. In vitro duodenal digestion

Duodenal digestions were performed on the 60 min gastric aliquots adjusted to pH 7.0. Then it was mixed with 1 M $CaCl_2$, 0.25 M bis-Tris pH 6.5, and a 0.25 M bile salts mixture containing equimolar quantities of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma). After preheating for 15 min at 37 °C, porcine pancreatic lipase (EC 232-619-9, 111,000 units/mg protein, Sigma), porcine pancreatic colipase (EC 259-490-1, Sigma), pancreatic bovine trypsin (EC 232-650-8, 10,100 BAEE units/mg protein, Sigma), and pancreatic bovine α-chymotrypsin (EC 232-671-2; 58 units/mg protein, Sigma), were added to the duodenal mix and incubated at 37 °C under agitation. Aliquots were taken at 15 and 30 min (D15 and D30, respectively) and the reaction was stopped by adding a large excess of the Bowman–Birk trypsin and α-chymotrypsin inhibitor from soybean (Sigma–Aldrich). The final composition of the mixture was: 4.76 mg/mL of protein, 12.3 mM of bile salts, 20.3 mM of bis-Tris, 7.6 mM of $CaCl_2$; the enzymes referred to the quantity of protein were: trypsin: 34.5 units/mg, α-chymotrypsin: 0.44 units/mg, lipase: 28.4 units/mg and the colipase/protein ratio was 1:895 w/w.

The duodenal digestion was performed in duplicate. Aliquots were diluted to a protein concentration of 4.45 mg/mL and stored at −20 °C before analysis.

2.3. Particle size determination

Particle size of the solutions before and after freeze drying and dissolving in SGF pH 2, was determined by dynamic light scattering (DLS) in a Zetasizer Nano-Zs (Malvern Instruments, Malvern, Worcestershire, United Kingdom), provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Measurements were carried out at a fixed scattering angle of 173° and contained in a disposable polystyrene cell. Contin's algorithm was used to obtain the particle size distributions as described elsewhere (Martinez et al., 2009).

The samples for DLS were filtered through a 0.45, 0.22 and 0.02 μm microfilter (Whatman International Ltd., Maidstone, England) before use. The assay was performed in duplicates.

2.4. RP-HPLC

The analysis of gastric and duodenal digested aliquots at a protein concentration of 2.4 mg/mL (filtered through 0.2 μm microfilter) were performed in a C_{18} Hi-Pore RP-318 (250 mm × 4.6 mm internal diameter) column (Bio-Rad, Richmond, CA, USA), in a Waters 600 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and UV detector. The digests were eluted by using double-distilled water as solvent A and HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland) as solvent B, containing trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) at 0.37% (v/v) and 0.27% (v/v) for phase A and B, respectively.

The elution was at 1 mL/min with a linear gradient of solvent B in A, from 0% to 65% in 50 min, and from 65% to 100% of solvent B in 5 min, then it was kept 100% solvent B for 4 min and finally it was returned to 100% of solvent A in 1 min. Detection was at 220 nm and data were processed by using Empower 2 Software (Waters Corporation).

2.5. Human IgE binding by inhibition ELISA

All protocols involving human sera were approved by the Bioethical Committee of the CSIC and followed the current EU legislation (Directive 2010/63/EU). The study of human IgE binding by inhibition ELISA was made using two pool samples of 3 different sera from cow's milk allergic children with clinical allergic symptoms presenting high levels of IgE specific to milk and β-lg.

Single wells of polystyrene microtiter plates (Corning, Cambridge, MA, USA) were coated with 10 μg/mL of native β-lg solution in 0.01 M phosphate buffer (PBS) pH 7.4, and incubated overnight under refrigeration. Plates were washed with PBS containing 0.05% Tween 20 (PBST 0.05%) using the EXL50 plate washer (Biotek). This washing system was used after each incubation step. Then, nine serial dilutions (from 2 mg/mL) of each sample (D30 of different CMP:β-lg ratios previously filtered through 0.2 μm microfilter, but maintaining the same β-lg concentration) were incubated during 120 min at room temperature with patient's sera previously diluted in PBST 0.05% (1:1 v/v). At the same time, PBS containing 2.5% Tween 20 (PBST 2.5%) was used as saturating agent to avoid nonspecific binding and after 240 min of blocking, 50 μL of the mixture of sample and serum was added to each well. After 120 min of incubation, 50 μL of HRP-conjugated rabbit antihuman IgE, diluted 1:1000 in PBST 0.05%, was added per well and incubated for 60 min. Then, 50 μL of anti-rabbit IgG/HRP, diluted 1:2000 in PBST 0.05%, was added per well and incubated for 60 min. The tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Walthman, MA, USA).

Finally, 3,3′,5,5′-tetramethyl-benzidine (TMB) (Sigma) was used as substrate, and after 15–30 min of incubation, the reaction was stopped by the addition of 0.5 M sulfuric acid. Absorbance was measured at 450 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Two negative controls without serum (native β-lg at pH 7.0 and 3.5 in PBST 0.05%) and two positive controls (sera pool diluted in PBS) were included in each plate.

A non-linear adjustment of the data obtained for each dilution was applied for each sera pool and sample. The adjustment model was a sigmoid curve of inhibition dose–response with variable slope, from which the IC_{50} (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism 5 for Windows (GraphPad software, San Diego, CA). The IgE binding capacity was expressed as the percentage of the IC_{50} of the intact protein. The IC_{50} of each sample were expressed as a mean ± SD for two replicates. Significant differences ($P < 0.05$) were evaluated by analysis of variance (ANOVA) using statistical program Statgraphics Centurion XV.

2.6. Mass spectrometry analyses (HPLC–ESI-MS/MS)

Hydrolysates were analyzed by RP-HPLC MS/MS in a C_{18} Hi-Pore RP318 column (Bio-Rad) on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltoniks GmbH, Bremen, Germany) equipped with an electrospray ionization source. MS/MS spectra were acquired over the range 100–3000 m/z (depending on the m/z and the charge of the precursor ion). Spectral data of eluting peaks were interpreted using Biotools software (Bruker Daltonics). Data were analyzed against bovine β-lactoglobulin sequence (UniProtKB/SwissProt database), setting pepsin, trypsin and chymotrypsin as digestion enzymes and allowing unspecific cleavages. MS and MS/MS tolerance was set at 0.5 kDa. Precursor charge state was set at +1. Matching series of y and b ions in the obtained MS/MS spectra allowed for unambiguous identification of the parent ions detected across the chromatographic run.

3. Results and discussion

3.1. Size distributions of particle at gastric conditions

As previously mentioned, CMP and β-lg interact in the aqueous phase forming CMP:β-lg complexes (at ratio 25:75, 50:50 and 75:25) at pH 7.0 and 3.5 with diameter between 6 and 7 nm (Martinez et al., 2010). These interactions were observed by dynamic light scattering and differential scanning calorimetry and the influence of these interactions on the rheological, interfacial and foaming properties of the mixed systems were also observed in previous works (Martinez et al., 2009, 2010, 2012). In the present work, these mixed systems are studied with regard to their *in vitro* digestibility. For this reason, in order to analyze the effect of the gastric conditions on the complex formation, the mixed systems at pH 3.5 and 7.0 were freeze dried and subsequently dissolved in SGF pH 2.0 to determine the particle size (according to the procedure of Martinez et al. (2010)) and to compare with the systems before freeze dried, as in the previous works. No changes in solubility were observed as a result of freeze drying.

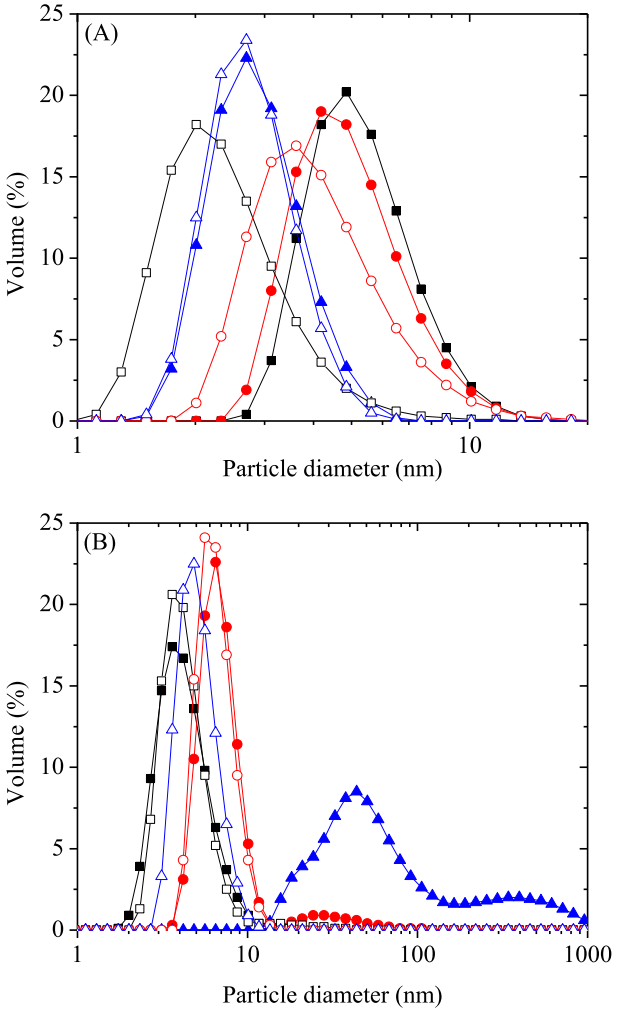

Fig. 1. Volume particle size distribution of (■,□) β-lg, (▲,△) CMP, and (●,○) CMP:β-lg mixtures at ratio 50:50 prepared at pH (A) 7.0 and (B) 3.5. Close symbols mean the solutions freeze dried and dissolved in SGF pH 2 and open symbols mean the solution before freeze drying.

Fig. 1 shows the particle size volume distributions of β-lg, CMP and one of the mixed systems at the ratio 50:50 as an example. The interactions between CMP and β-lg were demonstrated since the population corresponding to CMP almost disappeared in the distributions corresponding to the mixed system 50:50 dissolved in SGF at pH 2, either originally prepared at pH 7.0 (Fig. 1A) or at pH 3.5 (Fig. 1B). Even, no differences were observed in the volume size distributions before and after freeze drying and dissolving in SGF for β-lg and the mixed system 50:50 in Fig. 1B (solutions originally prepared at pH 3.5); however, CMP size distribution showed populations at higher size when the solution was freeze dried and dissolved in SGF at pH 2. When the solutions were originally prepared at pH 7.0, CMP was not affected by freeze drying and dissolving in SGF, but β-lg and the mixed system 50:50 showed higher size after freeze drying and dissolving in SGF.

3.2. In vitro digestibility of CMP:β-lg systems

Fig. 2A and B shows the RP-HPLC profiles of single β-lg and CMP at pH 3.5 during the *in vitro* gastric and duodenal digestion.

Native β-lg solution (Fig. 2A, G0) showed two main peaks corresponding to the genetic variants A and B (Thomä, Krause, & Kulozik, 2006) which elute with a retention time about 48–50 min (indicated as β-lgA and β-lgB in Fig. 2A, G0) and another smaller peak corresponding to α-lactalbumin (α-la) (at a retention time of 46 min). In agreement with previous reports (Mandalari et al., 2009; Mouécoucou, Villaume, Sanchez, & Méjean, 2004), β-lg was hardly hydrolyzed by pepsin during the gastric digestion (Fig. 2A, G60) and then it was gradually degraded during the duodenal digestion (DD), remaining no intact β-lg after 30 min (Fig. 2A, D30). On the other hand, CMP at t = 0 eluted at a retention time between 28 and 41 min (Fig. 2B G0) and presented two main peaks corresponding to aglyco-CMP_A (indicated as $aCMP_A$) and aglyco-CMP_B (indicated as $aCMP_B$) and several minor peaks corresponding to glycosylated forms of CMP_A and CMP_B (indicated as $gCMP_A$ and $gCMP_B$). As it was previously reported by several authors (Minkiewicz et al., 1996; Mollé & Leonil, 2005; Thomä et al., 2006) CMP is very heterogeneous arising from the existence of four genetic variants (A, B, C, E) and several phosphorylation and glycosylation sites. CMP was rapidly hydrolyzed by pepsin during the gastric digestion (Fig. 2B, G60) as shown by the disappearance of peaks at retention time 28–41 min and the appearance of peaks at retention time between 20 and 32 min. Chatterton, Rasmussen, Heegaard, Sørensen, and Petersen (2004) by studying digestion of CMP by porcine pepsine also reported that it was rapidly cleaved by gastric proteases leading to several smaller fractions. During the duodenal digestion (Fig. 2B, D30) the peak observed at 28.5 min (indicated by solid arrow) seems to be highly resistant to duodenal environment while the peak at 27.5 min (indicated by dotted arrow) in G60 considerably decreased indicating the proteolytic effect of the duodenal enzymes on this fragment.

The digestion chromatographic profiles of CMP:β-lg mixed systems prepared at pH 3.5 at ratio 25:75, 50:50 and 75:25 are shown in Fig. 2. However, the comparison of those profiles with the one of single β-lg or CMP did not allow concluding about significant peak differences as in the region below retention time 32 min many small peaks superimposed. Similar results were observed at pH 7.0 (data not shown).

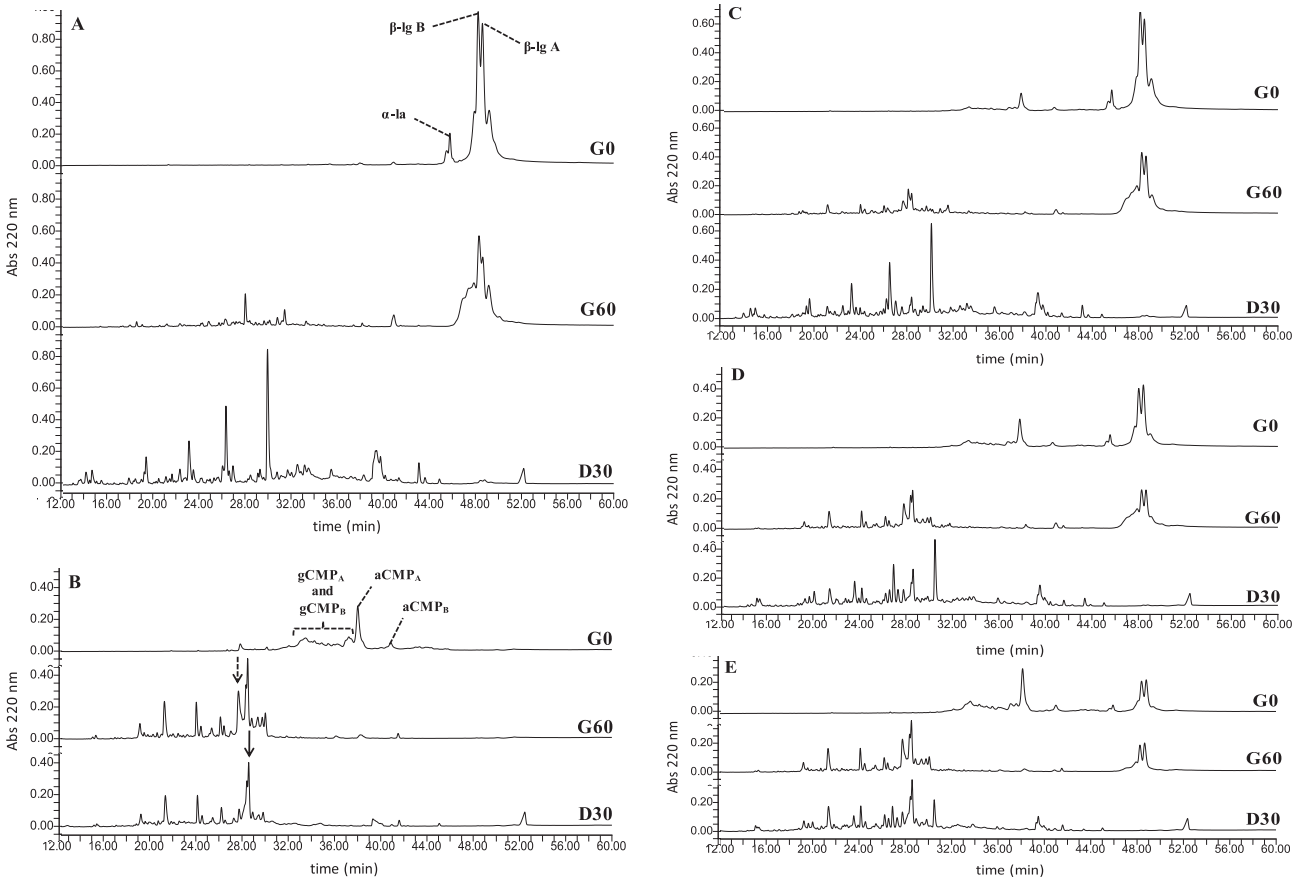

Fig. 2. RP-HPLC analysis of solutions of (A) β-lg, (B) CMP, and CMP:β-lg mixtures at ratio (C) 25:75, (D) 50:50 and (E) 75:25 subjected to gastric digestion for 0 min (G0), 60 min (G60) and followed by duodenal digestion for 30 min (D30) at pH 3.5.

Therefore, due to the low resolution of this technique to screen a possible effect of CMP:β-lg interaction on the digestion products, IgE binding of digests was further analyzed.

3.3. Human IgE binding by inhibition ELISA

Human IgE binding of the gastric and duodenal digests (D30) of single β-lg (CMP:β-lg 0:100) and CMP:β-lg 25:75 and 50:50 complexes formed at pH 3.5 and 7.0, were evaluated by inhibition ELISA (with human sera of patients allergic to β-lg) at the same β-lg concentration (Fig. 3).

The IgE binding significantly decreased (the IC_{50} increased) with increasing the CMP content at pH 3.5, from 7 μg/mL at CMP:β-lg 0:100 (pure β-lg) to 54 μg/mL at CMP:β-lg 50:50 for the serum pool 1, and from 12 μg/mL CMP:β-lg 0:100 to 120 μg/mL at CMP:β-lg 50:50 for serum pool 2. At pH 7.0, the reduction of IgE binding was only significant for the 50:50 ratio. Nevertheless, for both mixed systems studied (25:75 and 50:50) IgE binding was much lower at pH 3.5 than at pH 7.0. Since the IgE binding was evaluated at the same β-lg concentration, the differences found would correspond to the effect of the presence of CMP. In a previous work (Martinez et al., 2010) we reported that at pH 7.0, both CMP and β-lg present a ζ-potential around -20 mV, therefore, besides the hydrophobic interactions, only weak electrostatic interactions can take place between them. Contrarily, at pH 3.5 the aCMP is below its pI (4.15) and ζ-potential is about + 5 mV, while the gCMP is above its pI (3.15) and ζ-potential is about -5 mV, because the negative charge imparted by the sialic acid residue (Kreuß et al., 2009), and β-lg is far below its pI and ζ-potential is around + 20 mV (Harnsilawat, Pongsawatmanit, & McClements, 2006). Therefore at pH 3.5, in addition to the hydrophobic interactions, β-lg strongly interact with gCMP reinforcing the complexes which would provoke different CMP:β-lg digests with a lower immunoreactivity than at pH 7.0.

Thus, *in vitro* D30 digests of CMP:β-lg 50:50 resulted in the lowest reactivity against IgE from sera of allergic patients, although the detectable IgE binding response suggested the presence of peptides containing intact binding sites recognizable by serum IgE. Hence, we decided to evaluate the peptides sequence of the digests by mass spectrometry to test whether the released peptides contained allergenic epitopes.

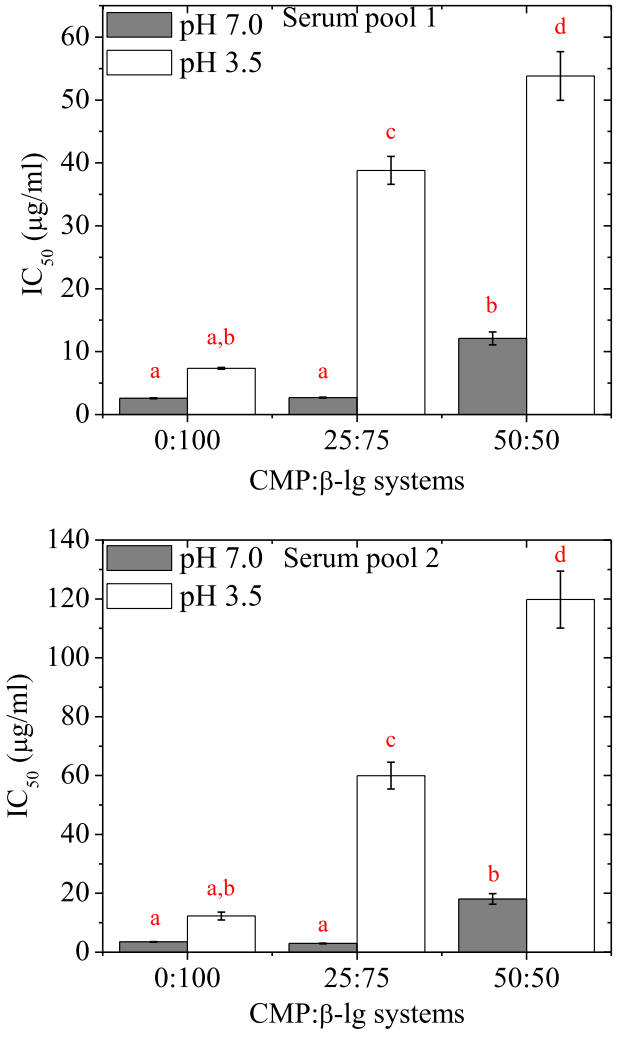

Fig. 3. Human IgE-binding by inhibition ELISA, expressed as IC_{50}, of β-lg and the mixed CMP:β-lg solutions at ratio 25:75 and 50:50, at pH 7.0 and 3.5, after the complete gastric and duodenal digestion (D30) tested with 2 different pools of sera of milk-allergic patients with specific IgE to β-lg. Error bars are standard deviations of mean values of triplicates. Mean value with different letters were significantly different ($P < 0.05$).

Table 1
Peptide sequence of the D30 digests of (A) single β-lg pH 7.0 and (B) CMP:β-lg 50:50 pH 7.0, as determined by LC–ESI-IT-MS/MS.

Protein fragment	Related allergenic epitopes (immunoreactivity)*	
(A)		
β-lg (1–8)	β-lg (1–8) (intermediate)	
β-lg (8–19)	β-lg (9–14) (low)	
β-lg (8–20)	β-lg (9–14) (low)	
β-lg (9–14)	β-lg (9–14) (low)	
β-lg (9–20)	β-lg (9–14) (low)	
β-lg (22–34)	β-lg (25–40) (intermediate)	
β-lg (33–40)	β-lg (25–40) (intermediate)	
β-lg (38–47)	β-lg (25–40) (intermediate) and β-lg (41–60) (high)	
β-lg (41–57)	β-lg (41–60) (high)	
β-lg (42–54)	β-lg (41–60) (high)	
β-lg (43–54)	β-lg (41–60) (high)	
β-lg (43–57)	β-lg (41–60) (high)	
β-lg (43–60)	β-lg (41–60) (high)	
β-lg (43–61)	β-lg (41–60) (high)	
β-lg (71–89)	β-lg (78–83) (very low) and β-lg (84–91) (low)	
β-lg (83–91)	β-lg (84–91) (low)	
β-lg (83–93)	β-lg (84–91) (low)	
β-lg (92–102)	β-lg (92–100) (low)	
β-lg (94–100)	β-lg (92–100) (low)	
β-lg (94–101)	β-lg (92–100) (low)	
β-lg (123–130)	β-lg (125–135) (very low)	
β-lg (123–131)	β-lg (125–135) (very low)	
β-lg (123–135)	β-lg (125–135) (very low)	
β-lg (123–136)	β-lg (125–135) (very low)	
β-lg (123–138)	β-lg (125–135) (very low)	
β-lg (125–136)	β-lg (125–135) (very low)	
β-lg (125–138)	β-lg (125–135) (very low)	
β-lg (134–138)	β-lg (127–144) (high)**	
(B)		
β-lg (8–14)		β-lg (9–14) (low)
β-lg (8–19)		β-lg (9–14) (low)
-lg (22–34)		β-lg (25–40) (intermediate)
β-lg (41–57)		β-lg (41–60) (high)
β-lg (42–54)		β-lg (41–60) (high)
β-lg (43–57)		β-lg (41–60) (high)
β-lg (83–91)		β-lg (84–91) (low)
β-lg (83–93)		β-lg (84–91) (low)
β-lg (92–100)		β-lg (92–100) (low)
β-lg (123–136)		β-lg (125–135) (very low)
β-lg (123–138)		β-lg (125–135) (very low)
β-lg (125–135)		β-lg (125–135) (very low)
β-lg (125–136)		β-lg (125–135) (very low)
β-lg (125–138)		β-lg (125–135) (very low)

* From Sélo et al. (1999).
** From Järvinen et al. (2001).

3.4. Mass spectrometry analyses (HPLC–ESI-MS/MS)

To assess whether the reduction in IgE binding was related to the disappearance of β-lg reactive epitopes upon digestion in the presence of CMP, the identification by mass spectrometry of the main peptide peaks of D30 digests of single β-lg and CMP:β-lg 50:50 was performed. MS and MS/MS data of detected peptide ions were matched to the spectra of theoretically cleaved peptides in the β-lactoglobulin sequence. This comparison allows unambiguous identification of the β-lactoglobulin fragments present in the digested samples. The results are shown in Tables 1 and 2, where the peptide sequences obtained from the digestions were compared to β-lg fragments identified as epitopes by Sélo et al. (1999) with different levels of immunoreactivity. Other studies have reported β-lg epitopes (Adams et al., 1991; Heinzmann, Blattmann, Spuergin, Forster, & Deichmann, 1999; Järvinen et al., 2001); however most of them are included in the epitopes described by Seló et al. (1999). The number of identified peptides with previously described epitopes was lower for both CMP:β-lg 50:50 (14 and 16 at pH 7.0 and pH 3.5, respectively) compared to 28 and 32 in the β-lg digests.

A comparison of the results for the four systems is presented in Table 3 in which it is possible to observe the resistant and/or degraded peptides after digestion. Peptides containing whole epitopes with high immunoreactivity level were only observed for the β-lg pH 3.5 digests (41–60 and 149–162); while for the other systems, the epitopes with high immunoreactivity were interrupted or missing. β-lg digests at pH 7.0 showed also a sequence (134–138) that is not included in the epitopes described by Sélo et al. (1999), but it is related with a high immunoreactive epitope (127–144) described by Järvinen et al. (2001), therefore it was included in Table 1.

The sequence (1–8) with intermediate immunoreactivity was present in the digestion products of β-lg at both pHs but not in the digestion products in the presence of CMP. However, in the mixed systems and also in β-lg digestion products numerous epitopes with low and very low immunoreactivity were present.

Therefore, MS/MS revealed that different peptides are formed from β-lg in the presence of CMP. The disappearance of epitopes in the digested mixtures of CMP:β-lg could explain the lower IgE binding observed in these systems compared to single β-lg (Fig. 3). In fact, the only whole epitope present in mixed systems at both pH were of low or very low immunoreactivity (Table 3).

Regarding the reduction of the allergenicity because of the interactions between bioplymers it was reported that the conjugation of β-lg with polysaccharides of high molecular weight during heat-processing is very effective in reducing the immunogenicity of β-lg (Hattori, Numamoto, Kobayashi, & Takahashi, 2000; Hattori et al., 2004; Kobayashi et al., 2001). Mouécoucou et al. (2007) investigate the influence of gum arabic and low methylated pectin (charged polysaccharides) and xylan (un-charged polysaccharides) on the *in vitro* digestibility of β-lg and on the *in vitro* IgG- and IgE-binding of its digestion products and they demonstrated that, even without heating non-specific interactions between polysaccharides (mainly that low methylated pectin and xylan) and β-lg influenced protein digestibility and reduced the IgG/IgE binding after hydrolysis. They attributed the difference in response between IgG- and IgE-binding peptides to the destruction of IgE epitopes or to their inaccessibility associated with change of conformation in the presence of these polysaccharides.

Table 2
Peptide sequence of the D30 digests of (A) single β-lg pH 3.5 and (B) CMP:β-lg 50:50 pH 3.5, as determined by LC–ESI-IT-MS/MS.

Protein fragment	Related allergenic epitopes (immunoreactivity)*
(A)	
β-lg (1–8)	β-lg (1–8) (intermediate)
β-lg (8–14)	β-lg (9–14) (low)
β-lg (8–19)	β-lg (9–14) (low)
β-lg (9–14)	β-lg (9–14) (low)
β-lg (9–20)	β-lg (9–14) (low)
β-lg (22–34)	β-lg (25–40) (intermediate)
β-lg (33–40)	β-lg (25–40) (intermediate)
β-lg (40–61)	β-lg (41–60) (high)
β-lg (41–57)	β-lg (41–60) (high)
β-lg (41–58)	β-lg (41–60) (high)
β-lg (42–54)	β-lg (41–60) (high)
β-lg (43–54)	β-lg (41–60) (high)
β-lg (43–57)	β-lg (41–60) (high)
β-lg (43–61)	β-lg (41–60) (high)
β-lg (71–82)	β-lg (78–83) (very low)
β-lg (83–89)	β-lg (84–91) (low)
β-lg (83–91)	β-lg (84–91) (low)
β-lg (83–93)	β-lg (84–91) (low)
β-lg (84–93)	β-lg (84–91) (low)
β-lg (92–102)	β-lg (92–100) (low)
β-lg (94–100)	β-lg (92–100) (low)
β-lg (94–101)	β-lg (92–100) (low)
β-lg (123–130)	β-lg (125–135) (very low)
β-lg (123–131)	β-lg (125–135) (very low)
β-lg (123–135)	β-lg (125–135) (very low)
β-lg (123–136)	β-lg (125–135) (very low)
β-lg (123–138)	β-lg (125–135) (very low)
β-lg (125–135)	β-lg (125–135) (very low)
β-lg (125–136)	β-lg (125–135) (very low)
β-lg (125–138)	β-lg (125–135) (very low)
β-lg (149–162)	β-lg (149–162) (high)
β-lg (150–162)	β-lg (149–162) (high)
(B)	
β-lg (22–34)	β-lg (25–40) (intermediate)
β-lg (38–47)	β-lg (25–40) (intermediate) and β-lg (41–60) (high)
β-lg (41–57)	β-lg (41–60) (high)
β-lg (42–54)	β-lg (41–60) (high)
β-lg (43–54)	β-lg (41–60) (high)
β-lg (43–57)	β-lg (41–60) (high)
β-lg (43–60)	β-lg (41–60) (high)
β-lg (76–91)	β-lg (78–83) (very low) and β-lg (84–91) (low)
β-lg (83–93)	β-lg (84–91) (low)
β-lg (94–100)	β-lg (92–100) (low)
β-lg (96–107)	β-lg (92–100) (low) and β-lg (102–124) (high)
β-lg (123–136)	β-lg (125–135) (very low)
β-lg (123–138)	β-lg (125–135) (very low)
β-lg (125–135)	β-lg (125–135) (very low)
β-lg (125–136)	β-lg (125–135) (very low)
β-lg (125–138)	β-lg (125–135) (very low)

* From Sélo et al. (1999).

Table 3
β-lg epitopes after digestion of single β-lg and the system CMP:β-lg 50:50 at pH 7.0 or 3.5.

Allergenic epitopes (immunoreactivity)*	pH 7.0		pH 3.5	
	Single β-lg	50:50	Single β-lg	50:50
β-lg (1–8) (intermediate)	X	–	X	–
β-lg (9–14) (low)	X	X	X	–
β-lg (25–40) (intermediate)	/	/	/	/
β-lg (41–60) (high)	/	/	X	/
β-lg (78–83) (very low)	/	–	/	X
β-lg (84–91) (low)	X	X	X	X
β-lg (92–100) (low)	X	X	X	/
β-lg (102–124) (high)	–	–	–	/
β-lg (125–135) (very low)	X	X	X	X
β-lg (127–144) (high)**	/	–	–	–
β-lg (149–162) (high)	–	–	X	–

Symbols: –: missing or not detected epitope; X: present epitope, /: interrupted epitope.

* From Sélo et al. (1999).

** From Järvinen et al. (2001).

4. Conclusions

It was observed an important reduction of the IgE binding of β-lg in the presence of CMP that could be attributed to the breakdown of allergenic determinants of β-lg in the presence of CMP, as revealed by MS/MS analysis. The effect was more significant for CMP:β-lg systems at pH 3.5 than at pH 7.0, maybe because of at pH 3.5 the electrostatic interactions between β-lg and CMP are very strong reinforcing the hydrophobic interactions of the complexes which would allow to obtain different peptides with lower immunoreactivity than a neutral pH.

Therefore, the presence of CMP in cheese whey, a widely used ingredient, may help to reduce the Ig-E binding of β-lg, as well as providing bioactive properties (Brody, 2000), by favoring the cleavage of IgE epitopes by digestive enzymes. However, it is important to underline that the interaction between CMP:β-lg and the relative influence of CMP on β-lg potential allergenicity was evaluated in standard solutions; so it would be interesting, for future studies, to investigate the effect of other proteins or fat, which are present, for example in dairy products in which CMP could be included as an ingredient of high added value.

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