RESEARCH ARTICLE

Comparative resistance of food proteins to adult and infant *in vitro* digestion models

Didier Dupont[1], Giuseppina Mandalari[2,3], Daniel Molle[1], Julien Jardin[1], Joëlle Léonil[1], Richard M. Faulks[2], Martin S. J. Wickham[2], E. N. Clare Mills[2] and Alan R. Mackie[2]

[1] INRA, AGROCAMPUS OUEST, UMR 1253, Science et Technologie du Lait et de l'Œuf Rennes, France
[2] Institute of Food Research, Norwich Research Park, Colney, Norwich, UK
[3] Pharmaco-Biological Department, University of Messina, Messina, Italy

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IgE-mediated allergy to milk and egg is widespread in industrialised countries and mainly affects infants and young children. It may be connected to an incomplete digestion of dietary proteins causing an inappropriate immune response in the gut. In order to study this, a biochemical model of infant gastroduodenal digestion has been developed, which has reduced levels of protease (eightfold for pepsin and tenfold for trypsin and chymotrypsin), phosphatidylcholine and bile salts, compared with the adult model. This model has been used to study the behaviour of three characterised food-relevant proteins (bovine β-lactoglobulin (β-Lg), β-casein (β-CN) and hen's egg ovalbumin), all of which are relevant cows' milk and hens' egg allergens. Digestion products were characterised using electrophoresis, immunochemical techniques and MS. These showed that ovalbumin and β-CN were digested more slowly using the infant model compared with the adult conditions. Resistant fragments of β-CN were found in the infant model, which correspond to previously identified IgE epitopes. Surprisingly, β-Lg was more extensively degraded in the infant model compared with the adult one. This difference was attributed to the tenfold reduction in phosphatidylcholine concentration in the infant model limiting the protective effect of this phospholipid on β-Lg digestion.

Keywords:
Egg / Food allergy / *In vitro* digestion / Milk / Phosphatidylcholine

1 Introduction

Understanding the fate of dietary proteins during gastrointestinal digestion has become of particular interest because of the potential role digestion may play in determining the allergenic potential of foods [1]. One important allergenic food is cows' milk, which in industrialised nations is estimated to affect approximately 2% of infants under 2 years of age [2, 3]. The major allergenic fraction of cow's milk is the casein fraction, comprising a mixture of αs_1-, αs_2-, κ- and β-casein (Bos d8). Despite their excellent digestibility, caseins are generally considered to be potent allergens [4]. The globular protein β-lactoglobulin (β-Lg, Bos d5), present in the whey fraction of the milk of most mammals, is also considered to be a major cause of allergic response to cows' milk in humans [5]. A second important allergenic food infancy and early childhood is hens' egg [6]. Almost all the allergens of egg are derived from the white and include the proteins ovomucoid, ovalbumin (OVA) and lysozyme [7]. Among these, such as OVA (Gal d2), are characteristically more resistant to gastrointestinal digestion as a consequence of the glycosylation and globular structure of this protein.

It may be that, while the bulk of food proteins are broken down into immunologically inactive fragments, very small proportions of immunologically active material may escape digestion. The extent of this digestion may also be affected by factors such as the maturity of the gut and its healthy functioning [8] and raised intragastric pH caused by anti-

Correspondence: Dr. Didier Dupont, INRA–AGROCAMPUS OUEST, UMR 1253 Science et Technologie du Lait et de l'œuf, 65, rue de St. Brieuc, 35000 Rennes, France
E-mail: didier.dupont@rennes.inra.fr
Fax: +33-2-23-48-53-50

Abbreviations: β-Lg, β-lactoglobulin; **β-CN,** β-casein; **OVA,** ovalbumin; **PC,** phosphatidylcholine

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ulcer medication, as has been shown for studies of fish allergens [9]. Indeed, poor digestion of codfish represents a risk factor for anaphylaxis in patients with allergy [10].

Furthermore, it has been shown that cows' milk whey peptides need to have a molecular weight greater than 3000 Da (around 25 residues) in order to stimulate an immune response [11], and an allergen must contain at least two IgE epitopes, each of which with a minimum of 15 amino acid residues long, in order to make the antibody binding possible [12]. Fragments combining multiple epitopes at least 1 nm in size are required to elicit histamine release [13, 14]. Thus, large stable fragments, as well as intact proteins, have the potential to trigger both initial sensitisation and phases of allergic responses.

Since cows' milk and hens' egg allergies are diseases of infancy, *in vitro* studies of digestion, even using systems, which seek to be physiologically relevant, have not, in the past, taken into account the conditions found in the immature gastrointestinal tract of infants. Although the gold standard for investigating the human digestive process is *in vivo* approaches, these are constrained for ethical and practical reasons, especially when considering infants. There is, therefore, a need to develop effective *in vitro* models of digestion, which mirror the conditions and processes that actually occur *in vivo*. Several more or less refined models of the biochemical conditions found in adults have been used to study food protein digestion [15–18]. Based on the published data, a prototype biochemical model of infant gastrointestinal digestion was developed and used to assess the digestion of model cows' milk allergens β-Lg, β-casein (β-CN) and the archetypal model allergenic protein from hens' egg, OVA. One of these, β-CN, is extensively degraded during digestion [19], whereas the others are both more resistant [19, 20]. In addition to using a biochemical assessment of digestibility, including immunoreactivity of residual proteins and peptides resulting from digestion by a panel of relevant monoclonal and polyclonal antibodies [21], we have undertaken a comprehensive mapping of proteolysis products resulting from digestion of β-CN using MS.

2 Materials and methods

2.1 Chemicals

Unless otherwise stated, chemicals were from commercial origin (Sigma, St. Louis, MO, USA). Purity of β-Lg, β-CN and OVA was $\geq$90%.

2.2 *In vitro* adult digestion model

Phospholipid vesicles were prepared using a modification of a procedure described previously [22]. The solvent was removed from a 0.94 mL aliquot of L-α-phosphatidylcholine 63.5 mM in chloroform stock solution (phosphatidylcholine (PC), egg lecithin grade 1, 99% purity, purchased from Lipid Products, South Nutfield, Surrey, UK) and dried under rotary evaporation at 5°C in order to make a thin film of phospholipids. The film of phospholipids was then suspended in 12.2 mL of warmed simulated gastric fluid (0.15 M NaCl, pH 2.5) and the suspension sonicated for 7 min using a sonication probe (Status US 200, Avestin, Canada) at 5°C in a coolant-jacketed vessel. The liposomes were then covered with a cushion of argon before equilibrating at 37°C for 20 min in a shaking incubator. Proteolysis was performed essentially as described previously [22] using triplicate incubations at 37°C. Briefly, β-Lg, β-CN or OVA was dissolved in simulated gastric fluid (1 mg/mL), mixed with PC vesicles and the pH was adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (EC 3.4.23.1, Sigma, activity: 3300 U/mg of protein calculated using haemoglobin as a substrate) was added to give 182 U of pepsin/mg of β-Lg, β-CN or OVA (0.05 mM, final concentration). Aliquots (100 μL) were removed over the 60 min digestion time course. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M ammonium bicarbonate (BDH, Pole, Dorset, UK). Before duodenal proteolysis, the pH of samples was adjusted to 6.5 by addition of 0.1 M NaOH and duodenal digestion components added to give final concentrations as follows: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM *bis*-Tris buffer pH 6.5, 0.4 U/mg of β-Lg, β-CN or OVA bovine α-chymotrypsin (activity 40 U/mg of protein using benzoyltyrosine ethyl ester as substrate), 34.5 U/mg of β-Lg, β-CN or OVA porcine trypsin (activity 13 800 U/mg of protein using benzoylarginine ethyl ester as substrate). Aliquots (100 μL) were removed over the 30-min digestion time course, and proteolysis stopped by addition of a twofold excess of soybean Bowmann–Birk trypsin–chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

2.3 *In vitro* infant digestion model

The infant digestion model was applied to the same batches of β-Lg, β-CN and OVA using the same protocol as described above but with the following modifications:

(i) pH of the gastric digestion mix was adjusted at 3.0 instead of 2.5;
(ii) the pepsin concentration in the gastric digestion mix was decreased by a factor of 8;
(iii) the duodenal digestion mix was altered by reducing the bile salt concentration by a factor of 4, whereas the PC, trypsin and chymotrypsin concentrations were reduced by a factor of 10.

As a consequence of the reduced levels of trypsin and chymotrypsin a tenfold lower concentration of Bowmann–-Birk inhibitor was added to terminate simulated duodenal proteolysis.

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2.4 Antibodies

Eight mouse monoclonal antibodies specific for β-CN were taken from INRA's collection [21] and were directed against the following fragment of this protein: β-CN (f1–25), (f42–56) for two antibodies, (f76–93), (f133–150) for two antibodies, (f167–178) and (f184–202).

Rabbit polyclonal antibodies specific for β-Lg and β-CN were raised following the protocol previously described by Senocq *et al.* [23]. OVA-specific rabbit polyclonal antibodies were kindly provided by Dr. Didier Levieux (INRA, Theix, France).

2.5 SDS-PAGE

SDS-PAGE was performed using 10% polyacrylamide NuPAGE Novex *bis*-Tris precast gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All samples were reduced using 0.5 M DL-dithiothreitol before analysis. A measure of 10 μg of each allergen was loaded onto each lane. Gels were fixed in 50% v/v methanol, 10% v/v acetic acid and after 30 min were rinsed in deionised water before staining with Colloidal Blue Staining Kit (Invitrogen). Molecular weight markers comprised the following mix of proteins (Invitrogen): insulin α-chain (M_r = 2500 Da), insulin β-chain (M_r = 3500 Da), aprotinin (M_r = 6000 Da), α-lactalbumin (M_r = 14 200 Da), lysozyme (M_r = 14 400 Da), trypsin inhibitor (M_r = 20 000 Da), carbonic anhydrase (M_r = 31 000 Da), lactate dehydrogenase (M_r = 36 500 Da), OVA (M_r = 45 000 Da), glutamic dehydrogenase (M_r = 55 400 Da), bovine serum albumin (M_r = 66 000 Da), bovine serum albumin (M_r = 66 300 Da), phosphorylase (M_r = 97 400 Da), β-galactosidase (M_r = 116 300 Da) and myosin (M_r = 200 000 Da).

Image analysis of SDS-PAGE gels was carried out using Totallab 120 (Nonlinear Dynamics, Newcastle, UK) using an automated process supplemented with occasional manual adjustments. Each lane was first defined, and the average intensity across the width of these lanes depicted as a function of the distance in pixel from the top of the image. The background was subtracted from each profile (parameters adjusted on a case-by-case basis), after which the bands were identified. Gaussian curves were fitted to the bands, which allowed overlapping bands to be deconvoluted. The lanes of molecular weight standards were then used to reference the position of the bands. For this, lanes corresponding to a given Rf or molecular weight were drawn across the width of the gel, allowing the position of each band to then be estimated. The output of this process was a list of bands defined either by their Rf or by their molecular weight, together with an estimate of the volume under the curve corresponding to each band (volume of fitted Gaussian curve).

2.6 RP-HPLC

Samples (25 μL) were loaded onto a Phenomenex (Torrance, CA, USA) Jupiter Proteo 90 Å pore size, 4 μm particle size (250 × 4.6 mm id) column attached to a Dionex HPLC system (Dionex, Amsterdam, The Netherlands) with a diode array detector. Samples were eluted using 0.1% w/v trifluoroacetic acid in ultra-pure water as solvent A and 0.085% w/v trifluoroacetic acid in ultra-pure water/ACN (10:90, v/v) as solvent B following the method described by Moreno *et al.* [24].

2.7 Inhibition ELISA

Inhibition ELISA using β-Lg, β-CN and OVA-specific polyclonal antibodies was applied to the samples collected throughout digestion of these three proteins in order to determine the residual immunoreactivity of each protein during the digestive process. ELISA plates (NUNC, Maxisorp, Roskilde, Denmark) were coated with 0.5 μg/mL β-Lg, β-CN or OVA in 0.1 M bicarbonate buffer, pH 9.6 (100 μL *per* well) and incubated for 1 h at 37°C. Wells were rinsed between incubation steps for 15 s with four changes of 250 μL PBS, 0.05% Tween 20 (PBS-T, Sigma) using a Model 1575 Immunowash microplate washer (Bio-Rad, Hercules, CA, USA). Blocking of the remaining binding sites was performed with 250 μL fish gelatin (Sigma) at 10 g/L in PBS-T for 1 h at 37°C. Serial dilutions of β-Lg, β-CN or OVA in PBS-T were used as standards (concentrations ranging from 0 to 100 μg/mL). Samples diluted in PBS-T (four dilutions from 1:1000 to 1:5000, 75 μL) were incubated in test tubes with 75 μL of rabbit polyclonal antibodies specific for β-Lg, β-CN or OVA diluted at 1:32 000, 1:3500 and 1:5000 v:v, respectively, and incubated for 1 h at 37°C. A volume of 100 μL of the mixture was then added to each ELISA plate well and further incubated for 1 h at 37°C. The reaction was revealed by incubating 100 μL of goat anti-rabbit Ig alkaline phosphatase conjugate (Sigma) diluted 1:3000 v:v in PBS-T for 1 h at 37°C. Finally, 100 μL *p*-nitrophenyl phosphate (Sigma) at 1 g/L 1 M diethanolamine-HCl, 1 mM $MgCl_2$, 0.1 mM zinc acetate was incubated in the wells. After 30 min at 37°C, the absorbance at 405 nm was read against a blank (substrate only) using a Benchmark Plus microplate spectrophotometer (Bio-Rad).

2.8 Indirect ELISA

This method was used to detect the β-CN regions resistant to digestion using a panel of eight monoclonal antibodies (Mabs) specific for fragments as follows: β-CN (f1–25) for 1 Mab, (f42–56) for 2 Mabs, (f76–93), (f133–150) for 2 Mabs, (f167–178) for 1 Mab and (f184–202) for 1 Mab. Briefly, 100 μL of adult or infant β-CN digest diluted 1:1000 v:v in 0.1 M bicarbonate buffer, pH 9.6, was coated onto a microtitre plate (NUNC) and incubated for 1 h at 37°C. Wells were rinsed between incubation steps for 15 s with four changes of 250 μL PBS-T using a Model 1575 Immunowash microplate washer (Bio-Rad). The remaining binding sites were

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blocked by incubating 250 μL fish gelatin (Sigma) at 10 g/L PBS-T for 1 h at 37°C. Hybridoma culture supernatants were diluted 1:2 v:v in PBS-T and incubated for 1 h at 37°C. Bound mouse Ig was detected by incubating 100 μL of goat anti-mouse Ig alkaline phosphatase conjugate (Sigma) diluted 1:3000 in PBS-T for 1 h at 37°C. Following the last rinsing, 100 μL *p*-nitrophenyl phosphate (Sigma) at 1 g/L in 1 M diethanolamine-HCl, 1 mM $MgCl_2$, 0.1 mM zinc acetate was added to each well. After 30 min at 37°C, the absorbance at 405 nm was read against a blank (substrate only) and corrected according to the background signal in the absence of antigen using a Benchmark Plus microplate spectrophotometer (Bio-Rad).

2.9 Immunoblotting

The samples collected at the end of the digestion process were electrophoresed as described above. Immediately after separation, proteins and peptides were transferred onto a 0.2-μm pore size nitrocellulose membrane (Bio-Rad) at 15 V and 400 mA during 20 min using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad) and a 39 mM glycine, 48 mM Tris, 0.0375% w:v SDS and 20% v:v methanol transfer buffer. Immunodetection was conducted according to the following procedure. The membrane was incubated at room temperature for 1 h periods in PBS-T, with successively, 1% w:v gelatine, β-Lg-, β-CN- or OVA-specific rabbit polyclonal antibodies at 1:2000, 1:500 and 1:1000 v:v, respectively, and goat anti-rabbit immunoglobulin alkaline phosphatase conjugate at 1:500 v:v. The membrane was washed between each step by soaking for 5 min in four changes of the same buffer. After the last washing, the membrane was stained with Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma) at one tablet in 10 mL water.

2.10 Nano-LC-MS-MS

Samples collected at the end of duodenal digestion in both adult and infant models were analysed by LC-MS-MS in order to identify the peptides remaining after digestion. Digests were subjected to nanoscale RP-LC on a modular LC Packings Ultimate HPLC system equipped with a Famos autosampler and a Switchos microcolumn switching device (LC Packings, Dionex). Samples were previously concentrated and desalted on a 5 mm × 300 μm PepMap C18 precolumn (100 Å, 5 μm, LC Packings). The nanoscale LC eluent from the analytical column was sent to the nanoelectrospray ionisation source of a QSTARXL global hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystem, Framingham, CA, USA) operated in positive ion mode. Raw data provided in Supporting Information were automatically analysed on a local server harbouring MASCOT (Matrix Science, London, UK) and updated Swiss-Prot and NCBI-NR library databanks. Results were corroborated with ProID software (Applied Biosystem). Peptides were considered unambiguously identified when their MASCOT score indicated significant identity or extensive homology ($p < 0.05$).

2.11 Hydrophobicity profile

β-CN hydrophobicity profile at pH 3.0 was established as previously described by Sweet and Eisenberg [25].

3 Results

3.1 Development of a prototype biochemical model of infant gastrointestinal digestion

An adult *in vitro* model of gastroduodenal digestion has been described previously, which was based on *in vivo* data obtained by gastric and duodenal aspiration and from collection of effluent from ileostomy volunteers at the Institute of Food Research [17, 22]. This was modified according to the published data [26–28] to provide conditions that mimicked more closely those found in the infant gut. More precisely, gastric-phase digestion was undertaken at pH 3.0 instead of 2.5 in the adult model because it corresponds to the gastric fasting pH as it was recorded in mature [29] and preterm infants [30–32]. Pepsin, trypsin and chymotrypsin concentrations were decreased by a factor of 8, 10 and 10, respectively, compared with the adult model [33–34]. The concentration of the gastric surfactant, PC, was lowered by a factor of 10 and bile salts, consisting of a mixture of equimolar quantities (0.0125 M) of sodium taurocholate and glyco-deoxycholic acid, was reduced four-fold compared with the adult model [26].

3.2 Comparison of infant and adult models on kinetics of digestion

In general, the proteins were digested in a similar manner using both the adult and the infant model, but as might be anticipated given the lower protein:protease ratios employed the kinetics of digestion were slower for the infant model.

Thus, β-Lg, a protein known to be highly resistant to pepsinolysis [35] was not affected during gastric digestion, being virtually unaltered after 60 min under both the adult and the infant gastro-duodenal models (results not shown). The duodenal phase showed differences between the two gut models, β-Lg being more extensively degraded in the infant model with only ~56% of the intact protein remaining after 30 min duodenal digestion compared with ~72% remaining at the same time point in the adult model (Fig. 1A and B; Fig. 2A). This was surprising since the concentration of trypsin and chymotrypsin in the infant model was reduced by a factor of 10 compared with the adult

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model. This observation can be explained by the fact that the gastric PC concentration was also lowered in the infant model, reducing the protective effect of PC normally observed in the adult model [36]. The kinetics of appearance of digestion products was also different between the adult and the infant models. As previously reported [36], the M_r~12 900 and ~11 400 Da polypeptides were observed early during digestion in the presence of physiological concentrations of PC, and persisted until the end of duodenal digestion. However, M_r~12 900 Da polypeptide was not present in the infant gut model, indicating a different pathway of degradation. The appearance of an M_r~7000 Da polypeptide, along with the poorly resolved M_r~3700 Da polypeptide, was only observed in the adult model, both fragments being evident after 30 min digestion.

In contrast, β-CN was hydrolysed rapidly in both infant and adult models, the M_r 24 000 Da polypeptide corresponding to the intact protein not being detected after

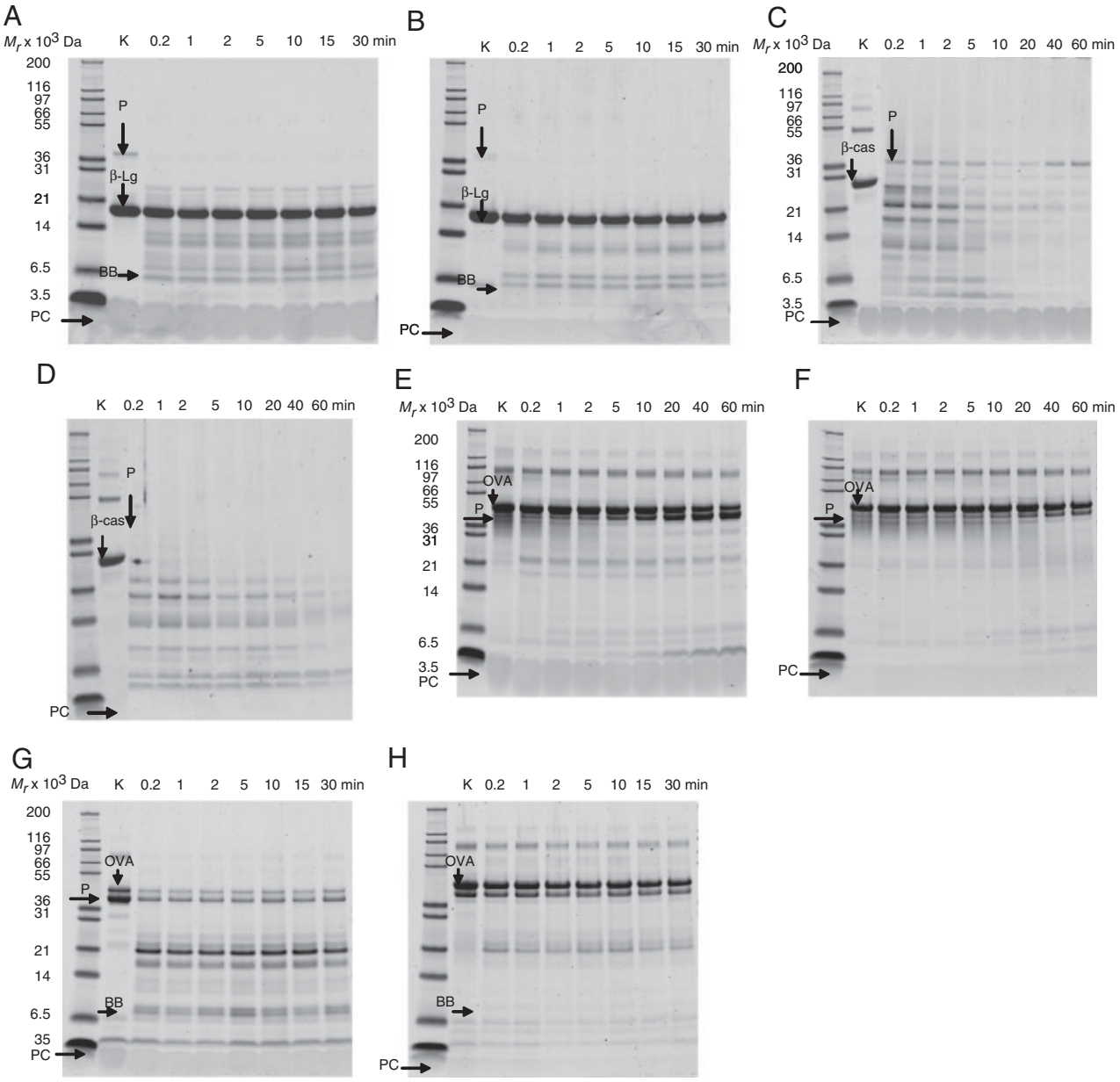

Figure 1. SDS-PAGE analysis of β-Lg subjected to duodenal proteolysis using the adult (A) or the infant (B) *in vitro* gastro-duodenal digestion model, β-CN subjected to gastric proteolysis using the adult (C) or the infant (D) *in vitro* gastro-duodenal digestion model and OVA subjected to gastric proteolysis using the adult (E) or the infant (F) *in vitro* gastro-duodenal digestion model, OVA subjected to duodenal proteolysis using the adult (G) or the infant (H) *in vitro* gastro-duodenal digestion model. SDS-PAGE was performed under reducing conditions. P, pepsin; soybean Bowmann–Birk inhibitor and K, no enzyme added.

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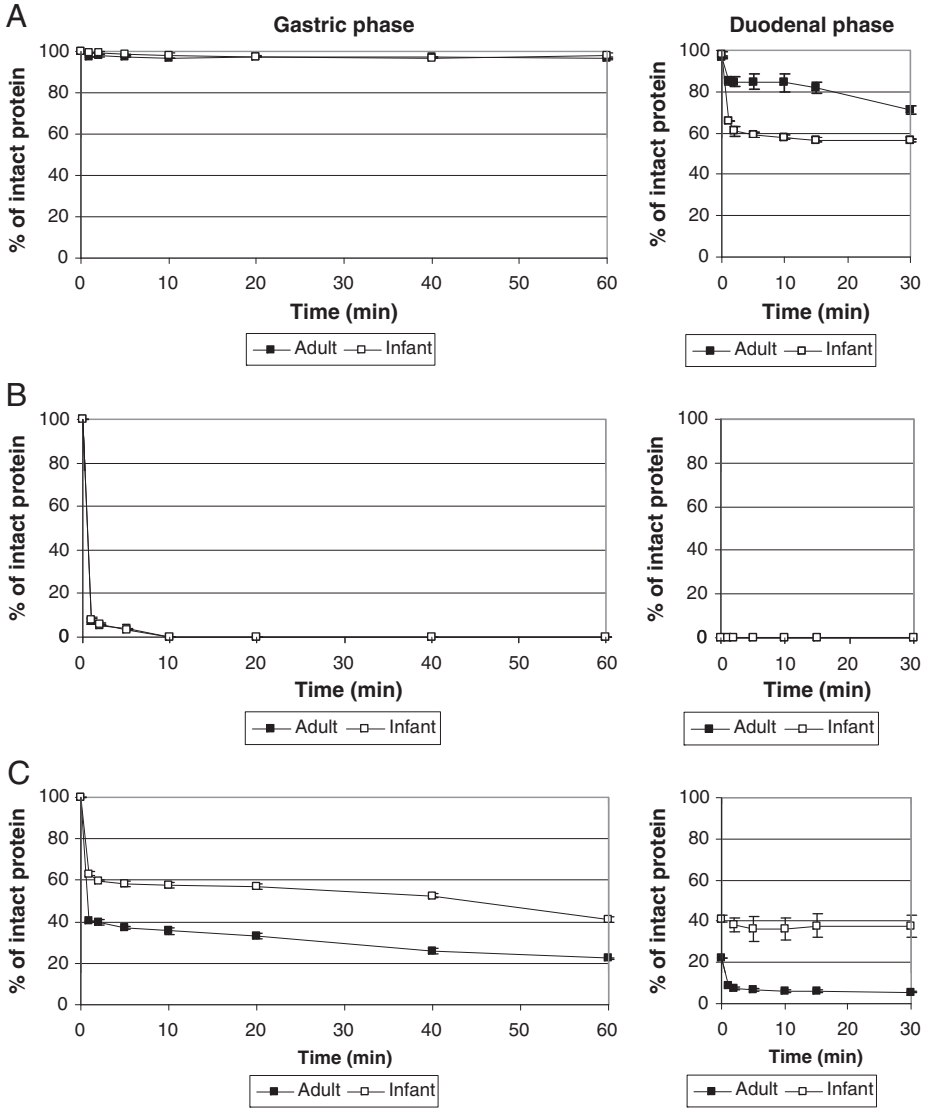

Figure 2. Digestion of β-Lg (A), β-CN (B) and OVA (C) using an infant (□) or an adult (■) *in vitro* gastro-duodenal model. Digestion was determined by densitometric analysis of the intact parent protein band on two replicate SDS-PAGE gels. Residual proteins were calculated as a percentage remaining after 60 min gastric or 30 min duodenal digestion. Data point represents means ($n = 2$) ± standard error.

10 min gastric digestion (Fig. 1C and D; Fig. 2B). Digestion products of M_r∼22 500, 19 000 and 12 800 Da, appearing after 1 min digestion, showed more resistance, disappearing after 40 min gastric digestion and remaining detectable throughout the gastric stage of digestion in the infant model, indicating that β-CN was more rapidly cleaved by the adult model. Polypeptides of M_r∼11 200, 5700 and 4400 Da were observed after 1–2 min digestion in the adult model, all being degraded by 20 min. In contrast, intermediate M_r∼7400 Da and ∼4400 Da polypeptides were present in the infant model of digestion, persisting for up to 60 min.

OVA showed an intermediate behaviour, being resistant to pepsinolysis in both models (Fig. 1E and F) but was more rapidly hydrolysed in the adult than in the infant one, with percentages of intact OVA remaining after the gastric stage of 22.3 and 41.1%, respectively (Fig. 2C). As for β-Lg, the kinetics of appearance of digestion products was different between the two models: while the M_r∼21 700 and 17 800 Da polypeptides were present in the adult model, the latter disappearing after 60 min digestion, an intermediate M_r∼20 600 Da polypeptide was observed in the infant model, increasing in intensity toward the end of digestion. Lower M_r∼5400 and 4600 Da polypeptides were present both in the adult and in the infant model, persisting until the end of digestion with the exception of the M_r∼4600 Da polypeptide, which disappeared after 40 min in the adult model.

During the duodenal phase, the percentage of intact OVA decreased further with the adult model whereas it remained constant with the infant one (Fig. 1G and H). Prominent intermediate digestion products of M_r∼20 700, 17 000, 12 500, 10 400 and 6500 Da were observed during the early stages of adult digestion (1–2 min) and persisted after 30 min. In the infant model, only the intermediate digestion products of M_r∼20 700, 5700 and 5000 Da were present, all of which remained until the end of digestion.

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3.3 Evolution of the protein immunoreactivity during digestion

The residual immunoreactivity of β-Lg, β-CN and OVA was determined, throughout digestion, by inhibition ELISA using polyclonal antisera raised to the intact proteins (Fig. 3). The immunoreactivity of β-Lg was unaltered by gastric digestion in both models, more than 94% of the residual immunoreactivity being retained after 60 min gastric digestion (Fig. 3A). The duodenal phase resulted in a decrease of β-Lg immunoreactivity, which was more significant in the infant model (47.3% at the end of digestion) than in the adult one (68.3% remaining after digestion). The decrease in β-Lg immunoreactivity occurred during the first 2 min of duodenal digestion using the infant model whereas it was more gradual in the adult model.

β-CN was extremely, rapidly hydrolysed by the two models, its residual immunoreactivity being reduced to 8.1% of the starting intact protein after 2 min gastric digestion with the infant model and being completely lost after 20 min gastric digestion with the adult model (Fig. 3B). Surprisingly, the β-CN immunoreactivity was not abolished following digestion with the infant model and even increased at the end of the gastric/beginning of the duodenal phases to reach 20% of the intact protein. This was a consistent observation in replicate digestion experiments, for which we currently lack an explanation. However, the concomitant presence of M_r~7400 and ~4400 Da polypeptides as observed by SDS-PAGE persisting for up to 60 min in the infant model of digestion, tend to confirm the resistance of β-CN fragments throughout the gastric phase. Finally, the immunoreactivity decreased during the duodenal digestion in the infant model such that only 2.7% of β-CN immunoreactivity remained at the end of the gastro-duodenal process.

OVA immunoreactivity gradually decreased during both gastric and duodenal digestion with both infant and adult models but was more extensively degraded with the latter

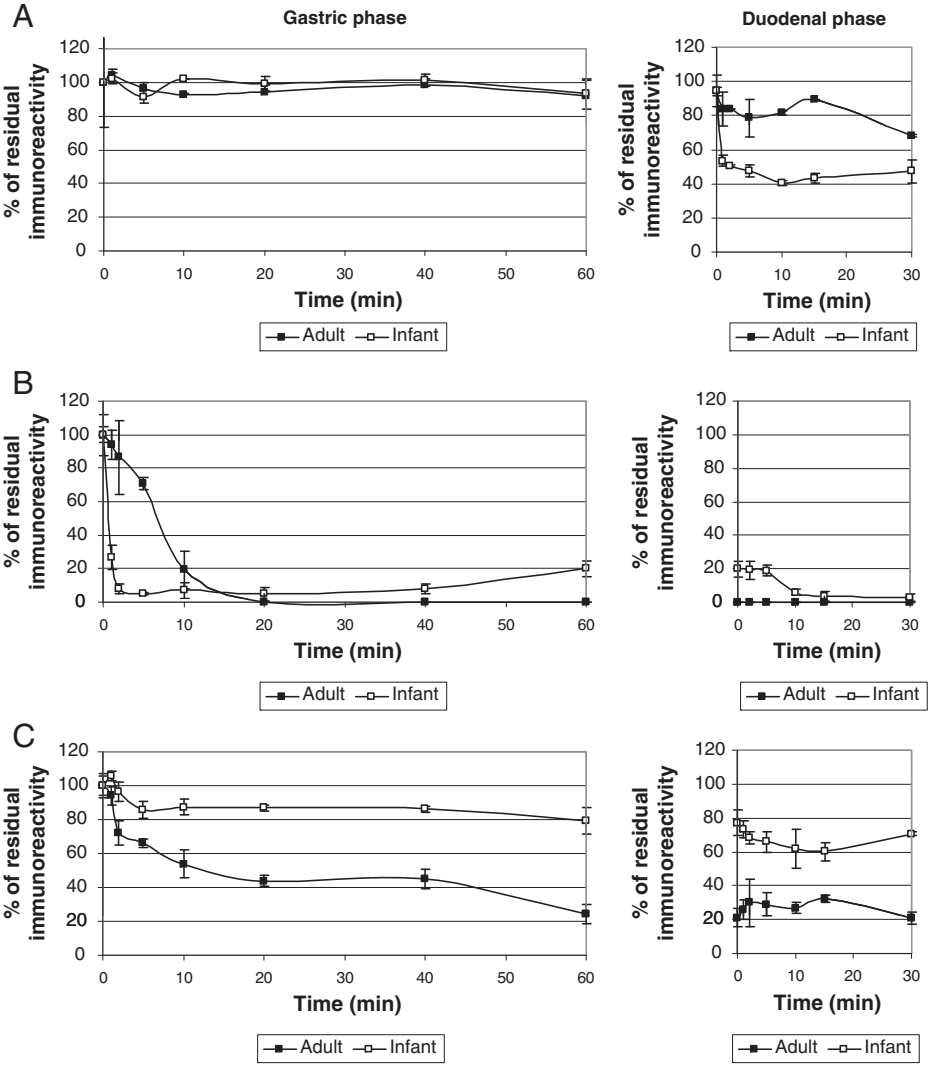

Figure 3. Evolution of β-Lg (A), β-CN (B) and OVA (C) residual immunoreactivity during *in vitro* gastric (left) and duodenal (right) digestion using an infant (□) or an adult (■) model, as determined by inhibition ELISA (data are the results of three independent determinations made in duplicates).

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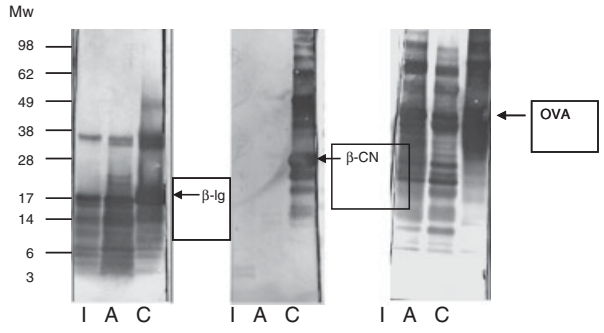

Figure 4. Western blotting of (from left to right) β-Lg, β-CN and OVA digested using the infant (lanes I) or the adult (lanes A) gut model. Lanes C correspond to the undigested samples. Bands were revealed using β-Lg-, β-CN- and OVA-specific rabbit polyclonal antibodies as described in Section 2.

(Fig. 3C). Indeed, the OVA residual immunoreactivity was 70.6 and 21.1% at the end of digestion with the infant and adult models, respectively.

Immunoblotting confirmed the differences in resistance to digestion of the three model proteins (Fig. 4) and showed that the anti-β-Lg and anti-OVA antibody preparations both recognised a spectrum of polypeptides resulting from digestion. Thus, undigested β-Lg was recognised, as indicated by the two major bands stained at M_r 18 and 36 kDa corresponding to monomeric and dimeric forms of this protein. Immunoreactive digestion products obtained with the adult model, showed several bands of different intensities running around M_r 36, 21, 18, 13 and 6 kDa. The pattern of products obtained with the infant model was slightly different, lacking the $M_r \sim 21$ kDa polypeptides with the other polypeptides being less abundant in the adult model as indicated by their weak staining. Immunoreactivity of β-CN was extensively reduced by digestion using both the adult and the infant models. No polypeptide digestion products were detected by the anti-β-CN antiserum in the adult digest, whereas two faint bands immunoreactive polypeptides were visible around M_r 3 kDa in the infant digest. Finally, intact OVA showed a strong immunoreactive band around M_r 42 kDa, which was accompanied by a band running around M_r 80 kDa, which probably corresponds to OVA dimers. Following digestion using the adult model, immunoreactive polypeptides were observed around M_r 42, 40, 24, 20, 18, 14 and 10 kDa, many of which were observed in infant model digests but were much more weakly stained.

These data support the observation by SDS-PAGE and ELISA that β-Lg was more extensively degraded during the duodenal stage by the infant compared with the adult model, in contrast to OVA, which showed a more extensive proteolysis with the adult than with the infant model, whereas β-CN proved to be the most rapidly degraded protein in both digestion models. The changes in immunoreactivity do not precisely mirror the loss of intact protein, suggesting that the immunoreactivity may be influenced by changes in protein structure caused by partial proteolysis, recognition of peptides and peptide aggregates, as well as intact protein.

3.4 Identification of the area of resistance in β-CN using specific monoclonal antibodies

Since β-CN was extensively degraded to low $M_r < 3$ kDa peptides, a panel of 8 Mabs of known specificity able to recognise short segments of polypeptide sequences was used to map the residual peptides in the β-CN digests (Fig. 5). With the adult model, peptides corresponding to residues 76–93 were recognised by Mab (f76–93) and survived gastric digestions with immunoreactivity of 70.8% β-CN remaining. Peptides corresponding to residues 1–25 and 167–178 returned 8.7 and 10.3% of the β-CN immunoreactivity, as detected by the relevant Mab probes. At the end of duodenal digestion peptides corresponding to residues 76–93 were further degraded. A slight residual immunoreactivity (7.9% of intact β-CN) was observed for peptides corresponding to residues 167–178, which did not seem to be affected by duodenal digestion.

These data suggest that hydrolysis of β-CN in the infant model was less extensive, with residual immunoreactivity after gastric digestion being identified for fragments corresponding to residues 76–93 (52.3%), 133–150 (44.8–47%), 42–56 (19–19.4%), 1–25 (12.7%), 167–178 (9.6%) and 184–202 (8%), respectively. After the duodenal digestion peptides corresponding to residues 76–93 showed the highest residual immunoreactivity of 10.7%.

3.5 Identification of low-molecular-weight peptides in digests by LC-MS-MS

The method of choice for mapping the evolution of complex mixtures of peptides is LC-MS-MS. Thus, digested samples of the three model proteins used in this study were subjected to such analysis to allow the identification of short peptides with molecular masses between 450 and 2500 Da. Figure 6 shows a typical example of the total ionisation current profile observed when digested β-CN was analysed (Fig. 3A), corresponding MS spectrum at retention times between 33.125 and 32.453 min (Fig. 7B), MS-MS spectrum of the ion 796.6 (Fig. 6C). Only the peptides identified unambiguously in digested β-Lg, β-CN and OVA are shown in Fig. 7A, B and C, respectively. The limitations of the technique mean that large peptides that are resistant to proteolysis during digestion will not ionise or fragment during LC-MS-MS and so could not be identified.

For β-Lg digested using the adult model, 37 peptides were identified with molecular masses ranging from 472 to 1315 Da covering most of the sequence (Fig. 7A). Among

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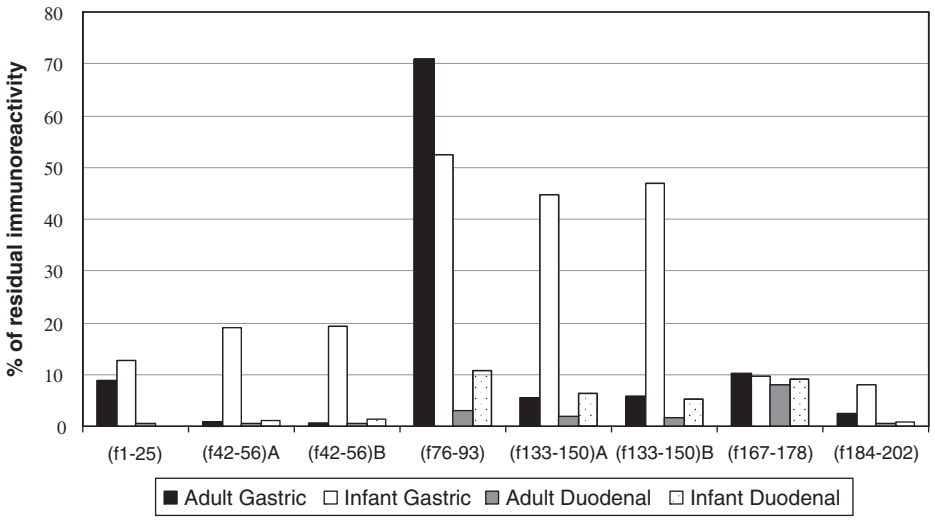

Figure 5. Residual immunoreactivity of β-CN after the gastric phase of the infant (□) and the adult (■) model, the duodenal phase of the infant (▨) and the adult (▩) model (results are expressed in % of residual immunoreactivity compares to the intact protein before digestion).

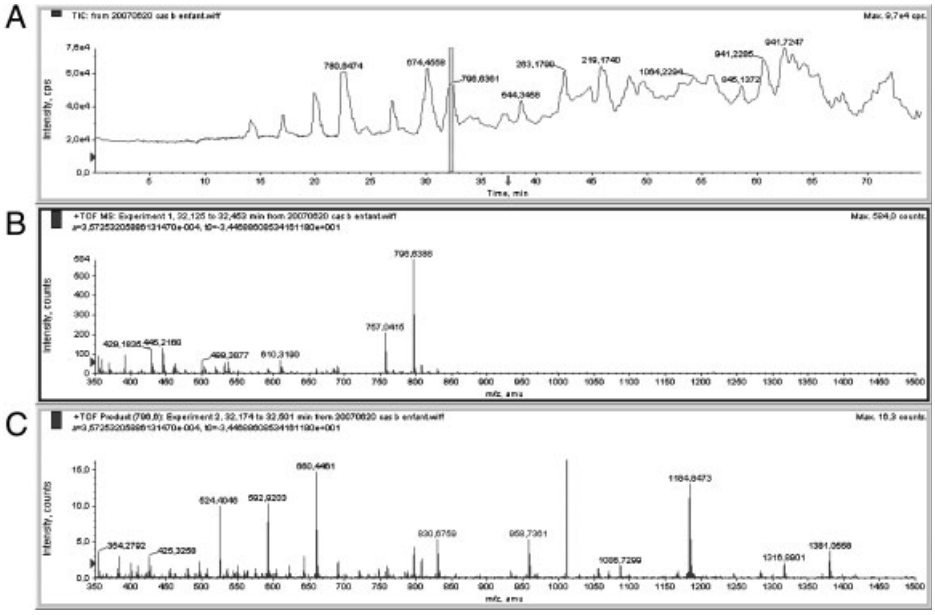

Figure 6. Peptide identification using LC-MS-MS. Typical example of the total ionisation current profile observed when β-CN submitted to gastric followed by duodenal digestion with the infant model was analysed (A), corresponding MS spectrum at a retention time of 32.125 to 32.453 min (B), MS-MS spectrum of the ion 796.6 (C).

those, 36 were found common to both variants A and B, whereas peptide corresponding to ^{110}SAEPEQSLV^{118} originated only from variant A. This suggests that the replacement of an alanine residue (variant B) by a valine (variant A) resulted in the appearance of an extra peptide in the digested samples. Among the 37 peptides corresponding to fragments of β-Lg A, 7 showed an oxidised methionine residue. Oxidation of methionine residues is observed very often in LC-MS-MS when samples are kept in the presence of oxygen before analysis (data not shown) and does not necessarily correspond to a biological oxidation of the residues during *in vitro* digestion. In β-Lg digests obtained with the infant model, 36 peptides with molecular masses ranging from 545 to 1191 Da were identified unambiguously in both variants, 4 showing an oxidised methionine residue. Of the 37 peptides identified in the adult digest, 19 were found specifically in the adult and not in the infant digest. Most of the peptides with the highest molecular masses were specific to the model used to prepare them and probably relate to the protective effect of PC [36].

For β-CN, 70 peptides with molecular masses ranging from 561 to 1730 Da were identified in the adult digest (Fig. 7B). Among these, a single oxidised methionine residue was found in 11 peptides, whereas a further 2 peptides had two oxidised methionine residues. In the infant digest, only 48 peptides with molecular masses ranging from 567 to 1451 Da were identified. Twenty-nine peptides were common to both the adult and the infant digests.

May be as a consequence of its glycosylated state only 20 peptides with molecular masses ranging from 514 to

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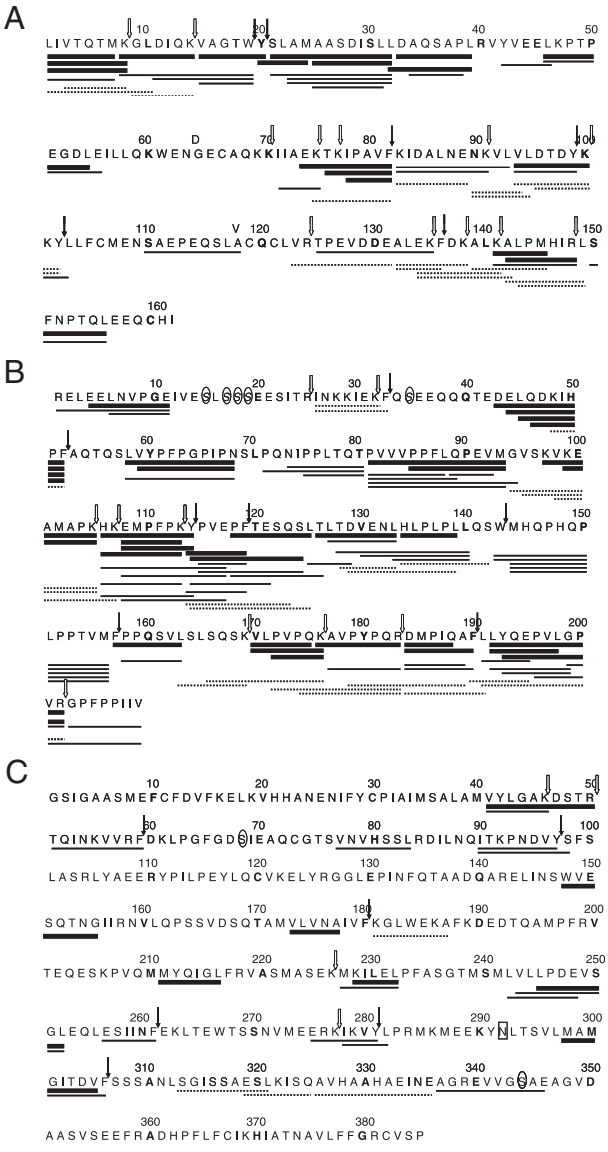

Figure 7. Peptides identified by LC-MS-MS at the end of β-Lg (A), β-CN (B) and OVA (C) gastric followed by duodenal phases digestion. Peptides found with both infant and adult models are represented as thick black horizontal bars. Peptides found only in the adult or the infant model are represented by black continuous and dashed lines, respectively. Trypsin (⇓), and chymotrypsin (⇓) cleavage sites hydrolysed during digestion are indicated within the sequence. Phosphorylated and glycosylated residues are surrounded by an oval or a rectangle, respectively.

1109 Da could be identified in the OVA adult digest (with 2 peptides and 1 peptide showing 1 and 2 oxidised methionine residues, respectively; Fig. 7C), whereas 11 peptides with molecular masses ranging from 514 to 1161 Da were found in the infant digest (with 1 peptide having 1 oxidised methionine). Seven peptides were found to be identical in both infant and adult digests.

Tables 1–3 summarise the trypsin and chymotrypsin typical cuts that generated specific identified peptides from β-Lg, β-CN and OVA, respectively. Differences in digestion products obtained between adult and infant models result from differences in both trypsin and chymotrypsin cleavages. The generation of peptide products by these proteases used in combination is complex, as in the relationship between parent–daughter peptides where two related peptides may actually originate from different peptides. In many instances, predicted cleavages could not be experimentally verified. There are several hypotheses that could explain the absence of such cleavages. It can be due to a limited access of the area for the proteases because of steric hindrance, or that some of the peptides have not been detected in digested samples by LC-MS-MS because of their low concentration. In addition, we have previously found that currently available tools for predicting pepsin cleavage are unreliable (unpublished data). Although the present results are difficult to compare with the previous studies because of differences in the digestion conditions (pH, enzyme/substrate ratio) some similarities can be emphasised.

4 Discussion

It had been anticipated that the lower concentrations of proteases used in the infant model would systematically result in less extensive degradation of the three model proteins used in this study β-Lg, β-CN and OVA. Although this was so for β-CN and OVA, in this study β-Lg was more extensively degraded by the infant than the adult digestion model. This is probably a result of the reduction in the protective effect that gastric PC has on native β-Lg retarding duodenal digestion by trypsin and chymotrypsin, which is not observed for β-CN [36]. The protective effect of PC is dose dependent and the reduction of PC in the infant model to give a 0.6:1 molar ratio of PC:protein affected the kinetics of digestion. We have recently shown that ~90% of β-Lg was degraded by trypsin and chymotrypsin at a 1:1 molar ratio of PC to protein in the adult gut model [36]. This indicates that the lower concentrations of duodenal proteases in the infant gut model together with the PC-affected β-Lg digestion. The significance of this difference regarding digestion of milk proteins will relate to the effectiveness of any thermal processing used to treat milk in denaturing β-Lg since thermal denaturation abolishes the protective effect of PC [36].

OVA was found to be more extensively hydrolysed during digestion using the adult compared with the infant model. However, after both processes some large fragments remain uncleaved. Since a relatively low number of short peptides were identified in OVA-digested samples by MS, it suggests that the large fragments generated by *in vitro* digestion retained sufficient structure to allow them to resist to further digestion by proteases and thus renders them

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Table 1. List of trypsin and chymotrypsin theoretical cleavage sites on β-Lg and the observation of their hydrolysis (↙) by the adult or infant model

Protein	Protease	Cleavage sites	Adult	Infant
β-Lg	Trypsin			
		K8-G9	↙	↙
		K14-V15	↙	↙
		R40-V41		
		K47-P48		
		K60-W61		
		K69-K70		
		K70-I71	↙	
		K75-T76	↙	↙
		K77-I78	↙	↙
		K83-I84		
		K91-V92	↙	
		K100-K101	↙	↙
		K101-Y102		
		R124-T125	↙	
		K135-F136	↙	
		K138-A139		↙
		K141-A142	↙	↙
		R148-L149	↙	↙
	Chymotrypsin			
		W19-Y20	↙	↙
		Y20-S21	↙	↙
		Y42-V43		
		W61-E62		
		F82-K83	↙	↙
		Y99-K100	↙	
		Y102-L103		↙
		F105-C106		
		F136-D137		↙
		F151-N152		

Table 2. List of trypsin and chymotrypsin theoretical cleavage sites on β-CN and the observation of their hydrolysis (↙) by the adult or infant model

Protein	Protease	Cleavage sites	Adult	Infant
β-CN	Trypsin			
		R1-E2		
		R25-I26		↙
		K28-K29		
		K29-I30		
		K32-F33		↙
		K48-I49		
		K97-V98		
		K99-E100		
		K105-H106	↙	↙
		K107-E108	↙	↙
		K113-Y114	↙	↙
		K169-V170	↙	↙
		K176-A177	↙	↙
		R183-D184	↙	↙
		R202-G203	↙	↙
	Chymotrypsin			
		F33-Q34		↙
		F52-A53	↙	↙
		Y60-P61		
		F62-P63		
		F87-L88		
		F111-P112		
		Y114-P115	↙	↙
		F119-T120	↙	↙
		W143-M144	↙	
		F157-P158	↙	
		Y180-P181		
		F190-L191	↙	↙
		Y193-Q194		
		F205-P206		

inaccessible to identification using this MS technique. This may be in part due to the presence of *N*-glycans linked at Asn-292, which represent up to 3% of the protein by weight. These glycans, coupled with the presence of an intramolecular disulphide bond and two phosphorylation sites at residues 68 and 344 may both reduce protease accessibility to potential cleavage sites and will affect the ability of any peptides to ionise and fragment during LC-MS-MS.

β-CN was extensively degraded during the gastric phase in both models of digestion, an observation consistent with the fact that pepsin has a preference for mobile poorly structured polypeptides, such as casein [19]. However, only the adult model resulted in a total disappearance of the protein whereas presence of a low level of resistant peptides, which persisted throughout digestion with the infant model. The region corresponding to residues 76–93 was shown to be particularly persistent. A hydrophobicity profile of β-CN shows that this area is very hydrophobic at pH 3.0 (Fig. 8), a factor which may contribute to its resistance to pepsinolysis either through changes in conformation or aggregation. At the beginning of the duodenal phase, when the pH is raised to 6.5, this area will become more hydrophilic and more accessible for proteases. It has three potential cleavage sites for chymotrypsin residues (77, 87 and 88) and our data confirmed it was cleaved by this protease. The region corresponding to residues 133–150 is also very hydrophobic at pH 3.0 and it may be protected from pepsinolysis for similar reasons to residues 76–93. Both these regions are contained within a 6 kDa peptide corresponding to residues 94–149, which has been found to resist pepsinolysis when presented in an emulsified form further supporting the role for hydrophobic interactions in protecting regions from pepsinolysis [37].

MS analysis also showed that fragments of β-CN with molecular masses up to 1451 Da (β-CN (f81–93)) could be found after simulated gastroduodenal digestion. It is interesting to note that peptide β-CN (f83–92) that is contained within this sequence has been reported as one of the major serum IgE-binding epitopes of β-CN in a panel of

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Table 3. List of trypsin and chymotrypsin theoretical cleavage sites on OVA and the observation of their hydrolysis (✓) by the adult or infant model

Protein	Protease	Cleavage sites	Adult	Infant
OVA	Trypsin			
		K16-E17		
		K19-V20		
		K46-D47	✓	
		R50-T51	✓	✓
		K55-V56		
		R58-F59		
		K61-L62		
		R84-D85		
		K92-P93		
		R104-L105		
		R110-Y111		
		K122-E123		
		R126-G127		
		R142-E143		
		R158-N159		
		K181-G182		
		K186-A187		
		K189-D190		
		R199-V200		
		K206-P207		
		R218-V219		
		K226-M227	✓	
		K228-I229		
		K263-L264		
		R276-K277		
		K277-I278	✓	
		K279-V280		
		R284-M285		
		K286-M287		
		K290-Y291		
		K322-I323		
		R339-E340		
		R359-A360		
		K369-H370		
		R386-C387		
		F10-C11		
		F12-D13		
		F15-K16		
		F28-Y29		
		Y29-C30		
		Y42-L43		
		F59-D60	✓	
		F65-G66		
		Y97-S98	✓	
		F99-S100		
		Y106-A107		
		Y111-P112		
		Y117-L118		
		Y125-R126		
		F134-Q135		
		W148-V149		
		F180-K181		✓
		W184-E185		
		F188-K189		
		F198-R199		
		Y212-Q213		
		F217-R218		
		F234-A235		
		F261-E262	✓	
		W267-T268		
		Y281-L282	✓	
		Y291-N292		
		F306-S307		✓
		F358-R359		
		F364-L365		
		F366-C367		
		F378-F379		
		F379-G380		

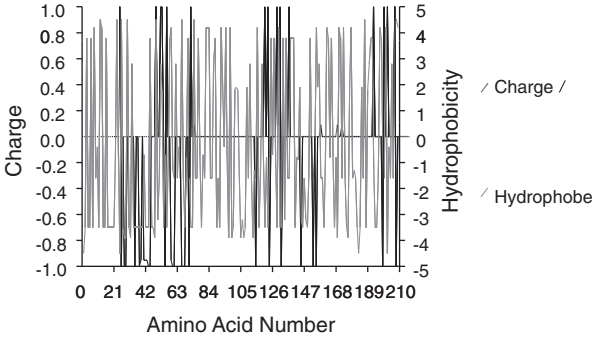

Figure 8. β-CN hydrophobicity profile as calculated at pH 3.0 according to Sweet and Eisenberg [25].

milk-allergic children [38]. Studies of allergenicity of the readily digestible allergen of peanut, Ara h 1, have indicated that aggregation of peptides may play a role in retaining allergenic activity [39]. It is possible that such phenomena would

also apply to casein peptides and could explain the apparent increase in immunoreactivity of casein digests observed with a polyclonal anti-β-CN antiserum at the end of the gastric and the beginning of the duodenal phases in the infant model.

For β-lg, the major IgE epitopes reported in the literature correspond to areas where peptides resistant to digestion were identified by LC-MS-MS in the present study, *i.e.* 47–60, 67–78, 75–86, 127–140, 141–152 [40] and 75–84, 141–150 [41]. Finally, some of the peptides identified in the digested OVA sample such as 53–60, 77–84, 178–187, 242–252, 251–259, 275–280, 322–343 have been reported as carrying IgE epitopes [42–43].

In conclusion, we report here the development of an infant gut model for studying the *in vitro* digestion of purified food proteins. This model is static and pH is kept

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constant during the whole digestion process, which might not perfectly reflect what happens during the digestion of complex foods. Indeed, it has been shown that the buffering capacity of food, particularly milk, causes a significant increase in pH during the gastric phase that will probably lower the enzyme activities [29–34]. Therefore, the present model must be considered as a screening technique allowing the comparison of different samples digested under the same conditions and that has been applied to purified proteins with limited buffering capacities. Dynamic models integrating pH variations in the stomach and peristalsis would give a clearer idea of what really happens *in vivo* and will be developed in the future. Furthermore, this study has been conducted on native proteins and it is clear that in ''real food'', proteins will be submitted to processing conditions (heat treatment, acidification, hydrolysis, *etc.*) that will significantly affect their structure and consequently their resistance to digestion. All these considerations have been taken into account in a joint project between INRA and IFR that is currently under publication.

The next step will then consist of validating this model against data collected *in vivo*. For ethical reasons, validation toward human would not be possible to carry out. Therefore, use of animal models such as piglets or young rats will be the only alternative. Such work is currently in progress.

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The authors have declared no conflict of interest.

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