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ABSTRACT

The objective of this work was to determine if processing could modify the resistance of casein to digestion in infant. A range of different dairy matrices were manufactured from raw milk in a pilot plant and submitted to *in vitro* digestion using an infant gut model. Digestion products were identified using mass spectrometry and immunochemical techniques. Results obtained showed that caseins were able to resist digestion, particularly κ- and αs_2-casein. Resistant areas were identified and corresponded to fragments hydrophobic at pH 3.0 (gastric conditions) and/or carrying post-translational modifications (phosphorylation, glycosylation). Milk processing led to differences in peptide patterns and heat treatment of milk tended to increase the number of peptides found in digested samples. This highlights the likely impact of milk processing on the allergenic potential of caseins.

Introduction

Milk allergy mainly affects children through their first contacts with non-human milk products. Fortunately, up to 85% of them outgrow their allergy in the first 5-10 years of life [1]. Most if not all the milk proteins are potential allergens. Whey proteins like β-lactoglobulin (β-lg), α-lactalbumin, bovine serum albumin and lactoferrin that account for 20% of the total milk proteins are mostly globular proteins and several IgE-binding epitopes have been identified on these proteins in the past [2]. However caseins (CNs) which represent c.a. 80% of the milk proteins have also been shown to be major allergens [3]. This is quite surprising if we consider that for eliciting an allergic response, they must be partly resistant to the enzymatic degradation that occurs during digestion. CNs have a very flexible structure and are therefore extremely sensitive to proteolysis. Indeed, purified bovine CNs were shown to be rapidly cleaved by digestive proteases when submitted to various in vitro digestion models [4-7].

Several hypotheses have been raised to explain the resistance of CNs to digestion. The presence of phosphorylated sequences that could also explain the cross-sensitization found in several patients [8], the protection of CNs by fat, heat-denatured whey proteins or by the dairy matrix and the resistance of CNs to digestion due to the immaturity of the both infant immune and digestive systems can all potentially contribute to CNs allergenicity.

Recently, Roth-Walter et al. [9] showed that triggering of an anaphylactic response towards milk proteins requires two phases (1) sensitisation by thermally-induced milk protein aggregates through Peyer's patches and (2) efficient transfer of milk protein across the epithelial barrier. Although, this was only demonstrated for whey protein aggregates, we need to keep in mind that heat-treatment of milk will also result in the

formation of aggregates between casein micelles and whey proteins via the formation of disulfide bonds between κ- and/or αs_2-casein and whey proteins [10, 11].
Heat treatment of milk could therefore be partly responsible for sensitization via the formation of milk protein aggregates. Hence understanding how milk processing, and particularly heat treatment, affects the digestion of milk proteins is of great importance. Most of the studies conducted so far on CN *in vitro* digestions were made using adult gut models which is almost irrelevant since the pathology mainly affects children. Therefore, we recently proposed a new infant *in vitro* digestion model dedicated to study the resistance of milk and egg allergen to digestion [7].
The objective of the present work was to determine if processing could modify the resistance of CNs to digestion and to identify resistant regions capable of eliciting an allergic response in infant. To reach this goal, a set of dairy samples was manufactured from a raw whole milk used as a reference sample and submitted to simulated digestion using the infant gut model. Milk protein hydrolysis was investigated using SDS-PAGE, immunoassays and mass spectrometry. Results obtained showed that milk processing increased CNs resistance to digestion. Some resistant area were identified and compared to known IgE-epitopes.

Materials and methods

Chemicals

Unless otherwise stated, chemicals were from commercial origin (Sigma, St-Louis, MO, USA).

Milk and dairy products

A 50-litres batch of raw milk collected in local farms was kindly provided by the Coopérative Laitière de Tourmont (France). Determination of fat, total protein and lactose was achieved using a Milkoscan infrared spectrophotometer. From this milk,

αs_1-, β- and κ-CN were purified as described previously [12]. A yogurt, three raw (whole, homogenised, skimmed), two pasteurised (whole, homogenised) and three sterilised (whole, homogenised, semi-skimmed) milks were manufactured at INRA's pilot plant in Poligny (France). Milk homogenisation was performed on a APV homogeniser apparatus (LAB 60 type, COMPAS sarl, Voisins le Bretonneux, France). Pasteurisation time and temperature were chosen in order to be as close as possible to the conditions used in industry. Therefore, milk was heated during 30 s at 82°C using a laboratory tubular exchanger (INRA homemade). Milk sterilisation was done on pasteurised milk (30 s/82°C) by autoclaving the flasks at 120°C during 10 min. For yogurt manufacture, 30 g of milk powder were mixed with 708 g of skimmed milk and 292 g of whole milk in order to reach objectives in term of fat (12.5 g/l), protein (44.4 g/l) and lactose (66.8 g/l). The mix was incubated at 20°C during 33 min under gentle stirring. Then, the mix was pasteurised at 92°C during 10 min in a water bath and FYS 11 starters (Danisco A/S, Copenhagen, Denmark) consisting of a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* were added at 3%. The mix was aliquoted into eight 100 ml pots and coagulation occurred after 2.5 h incubation at 45°C. After coagulation, yogurts were cooled and stored at 4°C until utilization.

<u>In vitro</u> infant digestion model

Prior to digestion, phospholipid vesicles were prepared as described previously [13]. Proteolysis was performed essentially as previously described [7] using triplicate incubations at 37°C. The concentrations of digestive enzymes, bile salts, surfactants… were chosen according to the data available in the literature on the newborn consuming real foods (mainly infant formula) [7]. Prior to digestion, samples (milks and yogurt) were diluted to 1 mg CN/ml in 0.15M NaCl, pH 6.5 in order to reduce the quantities of enzymes and surfactants necessary for conducting simulated digestions but the enzyme / substrate ratio (i.e. the digestive proteases / dietary

proteins) was set in order to remain physiologically relevant. Therefore, digestion of purified proteins and/or more complex food matrices can be studied with this model as long as the enzyme / substrate ratio remains constant. Then, diluted samples were mixed with PC vesicles and the pH was adjusted to 3.0 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (EC 3.4.23.1, Sigma, activity: 3,300 U/mg of protein calculated using haemoglobin as a substrate) was added to give 22.75 U of pepsin/mg of total CN (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). Then, pH of samples subsequently subjected to duodenal proteolysis was adjusted to 6.5 by addition of 0.1 M NaOH and components added to give final concentrations as follows: 1 mM sodium taurocholate, 1 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.04 U/mg of total CN bovine α-chymotrypsin (activity 40 U/mg of protein using benzoyltyrosine ethyl ester, BTEE, as substrate), 3.45 U/mg of total CN porcine trypsin (activity 13,800 U/mg of protein using benzoylarginine ethyl ester, BAEE, as substrate). Aliquots (100 µL) were removed over the 30 min digestion time course, and proteolysis stopped by addition of a two-fold excess of soybean Bowmann-Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

Antibodies

Twenty eight mouse monoclonal antibodies specific for αs_1-, αs_2-, β- and κ-CN were taken from INRA's collection [14] in order to cover as much of the sequence of the CNs as possible. The specificity of these antibodies is represented in Fig. 1.

Rabbit polyclonal antibodies specific for αs_1-, β- and κ-CN were raised following the protocol previously described by Senocq *et al* [15].

SDS-PAGE

Samples taken at different stages of the digestion were analysed by SDS-PAGE as described previously [7].

Inhibition ELISA

Inhibition ELISA using αs_1-, β- and κ-CN specific polyclonal antibodies was applied to the samples collected throughout digestion of these 3 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 αs_1-, β- and κ-CN 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 phosphate-buffered saline, 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 αs_1-, β- and κ-CN in PBS-T were used as standards (concentrations ranging from 0 to 100 µg/mL). Digested and undigested 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 αs_1-, β- and κ-CN diluted at 1:3000, 1:7000 and 1:67000 respectively and incubated for 1 h at 37°C. One hundred microlitres 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 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 were incubated in the wells. After 30 min at 37°C, the absorbance at 405 nm was read against a blank using a Benchmark Plus microplate spectrophotometer

(Bio-Rad). Results were expressed as percentage of residual immunoreactivity in comparison with the one of the undigested sample.

Indirect ELISA

This method was used to detect the αs_1-, αs_2-, β- and κ-CN area resistant to digestion using 28 monoclonal antibodies specific for these 4 CNs. Briefly, 100 µL of digested and undigested whole raw, pasteurised and sterilised milks and yogurt were diluted 1:2000 in 0.1 M bicarbonate buffer, pH 9.6 were coated onto a micro-titre plate (NUNC) and incubated 1 h at 37°C. The remaining binding sites were blocked by incubating 250 µL fish gelatin (Sigma) at 10 g/L in PBS-T for 1 h at 37°C. Hybridoma culture supernatants were diluted 1:2 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-Aldrich) at 1 g/L in 1 M diethanolamine-HCl, 1 mM $MgCl_2$, 0.1 mM zinc acetate were incubated in the wells and plates were read as described above.

Immunoblotting

The whole raw, pasteurised and sterilised milks and yogurt digested samples collected at the end of the gastro-duodenal 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) as previously described [7]. The membrane was then incubated at room temperature for 1 h periods in PBS-T, with, successively, 1% gelatine, αs_1-, β- and κ-CN specific polyclonal antibodies at 1:2000, 1:500, and 1:1000 respectively or a mixture of αs_2-CN monoclonal antibodies specific from the area 36-75 (diluted 1:2). Reaction was revealed using either goat anti-rabbit (for polyclonal antibodies) or goat anti-mouse

(for monoclonal antibodies) immunoglobulin alkaline phosphatise conjugate at 1:500 and Fast 5-bromo-4-chloro-3indolyl phosphate/nitro blue tetrazolium (Sigma) as substrate.

Nano-Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

The yogurt and raw, pasteurised and sterilised whole milks digested samples collected at the end of gastro-duodenal digestion were analysed by LC/MS/MS in order to identify the peptides remaining after digestion. Digested samples were subjected to nanoscale reverse phase liquid chromatography as previously described [7]. The online separated peptides were analysed by ESI Q-TOF–MS/MS using a QSTARXL global hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystems, Framingham, CA) operated in positive ion mode.

To identify peptides, all data (MS and MS/MS) were submitted to MASCOT (v.2.1). The search was performed against a homemade database dealing with major milk proteins which represents a portion of the Swissprot database (http://www.expasy.org). No specific enzyme cleavage was used and the peptide mass tolerance was set to 0.3 Da for MS and 0.15 Da for MS/MS. Three variable modifications (phosphorylation on serine and threonine, oxydation of methionine and deamidation of asparagines and glutamine residues) were selected. For each peptide identified, a minimum MASCOT score corresponding to a p-value below 0.05 was considered as a prerequisite for peptide validation with a high degree of confidence.

Statistical analysis

The effect of the casein type and the processing conditions on the residual immunoreactivity remaining after gastro-duodenal digestion of dairy products was tested by variance analysis using the R software package [16] running on the UNIX® system.

Hydrophobicity profile

CN hydrophobicity profiles at pH 3.0 and 6.5 were established as previously described by Sweet and Eisenberg [17].

Results

Analysis of digested samples by SDS-PAGE

After dilution of the samples in the digestion buffer, the decrease of the pH to 3.0 resulted in the flocculation of all samples. The aggregates which were visible rapidly disappeared when pepsin was added and the reaction medium was clear after 20-40 min gastric digestion.

Within each type of milk (raw, pasteurised and sterilised) all the different samples (whole, homogenised, skimmed and/or semi-skimmed) submitted to in vitro digestion showed the same pattern in SDS-PAGE (data not shown). Therefore, in order to improve the clarity only the whole milk samples are shown as representative of their respective types.

Gastric phase

Fig. 2 shows the electrophoretic patterns obtained after submitting whole raw (a), pasteurised (b), sterilised milks (c) and yogurt (d) to gastric *in vitro* digestion. In raw and pasteurised milks, the intact CNs bands disappeared after 20-40 min and bands corresponding to low Mw compounds (between 3 and 6 kDa) appeared concomitantly at the bottom of the gels in the samples. In contrast, patterns obtained with whole sterilised milks strongly differed with those obtained with both raw and pasteurised milks. Bands corresponding to intact CNs were hardly visible and smears appeared on the gels. Sterilisation may have caused extensive protein denaturation

or protein-lipid interactions altering the separation of proteins in SDS-PAGE. Finally, pattern of yogurt submitted to gastric digestion was similar to those obtained for raw and pasteurised milks, i.e. with a disappearance of intact CNs after 20 min digestion and the concomitant appearance of low Mw compounds.

Strong differences were also observed for the band at 18kDa corresponding to β-lg. This protein was indeed shown to be highly resistant to digestion in non-heated samples. However, heat treatments applied to milk for manufacture of pasteurised, sterilised milks and yogurts resulted in an increased digestibility of β-lg.

Duodenal phase

Fig. 3 shows the pattern obtained in SDS-PAGE when whole raw (a), pasteurised (b), sterilised milk (c) and yogurt (d) were submitted to gastro-duodenal digestion. All the digested samples showed an absence of bands corresponding to intact CNs. The bands at low Mw already observed at the end of the gastric phase seemed to resist the duodenal phase of digestion. Finally, β-lg gave an intense band in the digested raw milks, whereas its intensity was less in pasteurised milks and yogurt, and the protein was not detectable in digested sterilised milks.

Determination of casein residual immunoreactivity after digestion by inhibition ELISA and western-blotting

Fig. 4 shows that some residual immunoreactivity was detectable for αs_1-, β- and κ-CN in all the samples after gastro-duodenal digestion. Indeed, even though intact CNs were hardly visible by SDS-PAGE in whole sterilised milk, ELISA shows that the proteins were detectable in this sample at similar levels than in the other samples. αs_1-CN residual immunoreactivity was significantly higher in digested yogurt than in the other dairy samples ($p<0.001$) and higher in digested sterilised milk than in raw or pasteurised milk although this was not statistically significant

(p=0.0728). β- and κ-CN residual immunoreactivities were higher in digested yogurt than in the other dairy samples (p<0.001). Therefore, it looks like that heat treatment of milk increases CN residual immunoreactivity, although the difference in microstructure between the yogurt and raw milk may also play a role. We have previously demonstrated the relationship existing between the residual immunoreactivity of a protein and the extent of its proteolysis [18]. Since residual immunoreactivity can be correlated with the resistance of CNs to digestion, our data show that milk processing into yogurt (and sterilised milks to a lesser extent) increases CN resistance to in vitro digestion. It has however to be emphasized that these residual immunoreactivities of CNs were obtained by ELISA with polyclonal antibodies, i.e. probes that are able to detect intact proteins as well as fragments of a proteins. Therefore, a 50% residual immunoreactivity does not mean that 1 molecule out of 2 present in the sample are still intact; the SDS-PAGE shows that it is much less than that.

Western-blotting of the digested raw, pasteurised and sterilised milk and yogurt samples revealed strong differences between the patterns of the digested samples that were not observable by SDS-PAGE probably because revelation of the bands is much more sensitive with specific antibodies. It confirmed an impact of the processing conditions on the composition of the digested samples (Fig. 5). It also confirmed the extensive degradation of αs_1-CN. Indeed no bands were detected with the αs_1-CN specific antibody except a faint one at 32 kDa on the digested yogurt. It is however interesting to note that smears were observable for both the digested yogurt and sterilised milk and that high-molecular weight bands were visible around 60 kDa in all samples. A mixture of monoclonal antibodies specific for fragments 16-35 and 36-55 of αs_2-CN revealed a major band at 10 kDa that was much more intense in pasteurised milk and yogurt than in raw milk. A second band around 17 kDa was only present in the digested pasteurised milk and yogurt. Intense smears were observed

when these two monoclonal antibodies were applied to the digested sterilised milk. For β-CN a band at 3 kDa was visible in all samples, whereas one at 4.3 kDa was mainly observed in the digested sterilised milk. In contrast, κ-CN showed several bands in all the samples but at different molecular weight. Digested raw and pasteurised milks showed bands at 25 and 16 kDa whereas digested sterilised milk mainly showed one intense band around 60 kDa. Digested yogurt showed bands at 60, 25, 16 and 9 kDa.

Identification of the casein area resistant to digestion using a collection of specific monoclonal antibodies.

A collection of 28 different monoclonal antibodies specific for αs_1-, αs_2-, β- or κ-CN was used to identify by indirect ELISA the areas that were resistant to *in vitro* digestion. Fig. 6 shows the residual immunoreactivity observed with these 28 antibodies on digested raw, pasteurised and sterilised milks and yogurt.

Most of the αs_1-CN specific monoclonal antibodies gave low residual immunoreactivity in all the analysed samples confirming that this protein is extensively hydrolysed during digestion. Antibodies specific for αs_1-CN (f149-166) and αs_1-CN (f185-199) showed a slightly higher residual immunoreactivity.

Areas of resistance (f36-55 and f56-75) on αs_2-CN were also revealed by specific antibodies on all the digested samples studied and to a lesser extent αs_2-CN (f191-207) was also resistant in all samples.

For β-CN, the most resistant area in all the samples studied was the one recognized by the β-CN (f76-93) specific antibody. The other β-CN specific antibodies gave residual immunoreactivities lower or close to 10%.

For κ-CN, residual immunoreactivity was found with the 4 antibodies specific for the C-terminal half of the molecule, for all samples the highest response was obtained with the κ-CN (f131-150) specific antibody. Some differences in the pattern

of the different samples were observed. Indeed, digested yogurt reacted almost exclusively with κ-CN (f131-150) specific antibody whereas residual immunoreactivity was found with κ-CN (f98-115), κ-CN (f116-130) and κ-CN (f151-169) specific antibodies in digested raw, pasteurised and sterilised milks. It has to be emphasized however that for this CN, only one antibody is specific for the N-terminal moiety of the protein and therefore other resistant areas could be present in this half. These results confirmed that κ-CN C-terminal moiety is resistant to *in vitro* digestion.

In summary, the highest residual immunoreactivities were observed with antibodies αs_2-CN (f36-55), αs_2-CN (f56-75), β-CN (f76-93), κ-CN (f131-150) and κ-CN (f151-169), regardless of the sample. Given that the antibodies used in the study did not fully cover the whole CN sequences, other area not monitored here could also have resisted to digestion. From the percentage of residual immunoreactivity, it appears that both κ-CN and αs_2-CN are the most resistant CNs to *in vitro* digestion followed by β-CN, αs_1-CN being extensively hydrolysed.

Identification of peptides in digested samples using LC-MS-MS

LC-MS-MS was applied to digested raw, pasteurised and sterilised milks and yogurt in order to identify the remaining peptides. Only the peptides identified unambiguously are presented here.

Fig. 7 shows a diagrammatic representation of the peptides originating from αs_1-(a), αs_2-(b), β-(c) and κ-CN (d) identified by LC-MS-MS in the digested raw, pasteurised and sterilised milks and yogurt. For αs_1-CN, only 19 and 16 peptides were identified in the digested raw and pasteurised milks respectively whereas a higher number of peptides were identified in the more heavily processed dairy matrices (42 and 51 peptides identified in the digested sterilised milk and yogurt respectively). The data obtained with immunoassays (higher residual immunoreactivity found in high heated digested samples) suggest that the lower

number of peptides identified in the low heated milks could be attributed to a higher degree of proteolysis leading to shorter fragments unidentifiable by LC-MS-MS. Almost no peptides were found in the area 44-79 which is a highly phosphorylated area, with phosphoserine residues in 46, 48, 64, 66, 67, 68 and 75.

There are several reasons why part of a sequence can not be detected by LC-MS-MS:

- the area can be extensively hydrolysed leading to the formation of small peptides with Mw lower than 500 Da and therefore not validated during MASCOT database search
- the area can be resistant to digestion leading to fragments larger than 2500 Da i.e. not detectable in our conditions
- post-translational modifications can lower the abundance of peptides in the digested samples leading to concentrations too low to be detected or limit the ability of peptides to ionise or fragment.

In the present case, the absence of bands specific for large αs_1-CN fragments in western-blotting (Fig. 5) as well as the weak residual immunoreactivity detected by ELISA with αs_1-CN specific monoclonal antibodies (Fig. 6) suggested that these phosphorylated fragments were extensively hydrolysed.

For αs_2-CN, no peptides were identified in the 1-88 (with the exception of peptide I_{71}-K_{80} that was observed in the digested sterilised milk) and 126-150 regions (Fig. 7b). Fragment 1-88 also corresponds to a phosphorylated area with phosphoserine residues in position 8, 9, 10, 16, 31, 56, 57, 58 and 61. Western-blotting showed an intense band around 10 kDa that was specifically detected by monoclonal antibodies specific from the αs_2-CN N-terminal moiety (Fig. 5), results that were confirmed by ELISA (Fig. 6). Taken together these results showed that the sequence αs_2 (f1-88) can resist *in vitro* digestion and does not generate shorter peptides in sufficient amounts to be detected by mass spectrometry.

β-CN digestion led to a very significant number of peptides in all samples covering almost the whole sequence of this protein (Fig 7c). Only digestion of area 1-42 led to a low number of peptides in sterilised milk or yogurt. β-CN shows phosphorylated serine residues in position 15, 17, 18, 19 and 35. Since ELISA with monoclonal probes indicates a low residual immunoreactivity of fragment 1-19, it is probable that the absence of peptide identified by LC-MS-MS is rather due to an extensive hydrolysis of this fragment during digestion.

Compared to the other CNs, κ-CN gave a lower number of identified peptides (Fig. 7d). Areas 1-16, 77-95, 106-137 and 147-160 gave no peptides. Part of this absence of detected peptides may be attributed to post-translational modifications. Indeed, κ-CN shows a site of phosphorylation in S_{149} and glycosylation sites in positions 131, 133, 135, 136 (variant A) and 142. κ-CN is also characterized by a pyro-Glu residue at its N-terminal extremity that, when hydrolyzed, would lead to uncharged peptides, i.e. undetectable by the LC-MS-MS technique we used. However, western-blotting analysis (Figure 5 d) showed several bands corresponding to intact quite large fragments of κ-CN that partly resisted digestion and probably gave peptides in concentrations too low to be detected by mass spectrometry and/or too short to be validated during MASCOT database search. Furthermore, ELISA with specific monoclonal antibodies on digested samples showed a high residual immunoreactivity of the C-terminal part of κ-CN confirming the partial resistance of this area to *in vitro* digestion.

Caseins hydrophobicity profiles at pH 3.0 and their area resistant to digestion

Fig. 8 shows the charge and hydrophobicity profiles of αs_1-, αs_2-, β- and κ-CN at pH 3.0 (a) and 6.5 (b), i.e. the pH used for the gastric and duodenal phases of *in*

vitro digestion respectively. It shows that for αs_1-CN the most hydrophobic part at pH 3.0 is 149-199 and it was the one that showed a slightly higher resistance to digestion for αs_1-CN. For αs_2-CN, the N-terminal moiety is more hydrophobic than the C-terminal one and, again, it was this area that showed the highest residual immunoreactivity after *in vitro* digestion. For β-CN, the main hydrophobic part is located in 55-92 area and the area showing the highest residual immunoreactivity after in vitro digestion was the 76-93. Finally, for κ-CN most of the hydrophobicity is located in the 120-169 zone which corresponds to the area that was found resistant to *in vitro* digestion. Therefore, it looks as if there may be a relationship between hydrophobicity at pH 3.0 and resistance to *in vitro* digestion. For the duodenal phase, when pH is raised to 6.5, the hydrophobic areas mentioned above become more hydrophilic and more accessible to digestive enzymes and typical trypsin and chymotrypsin cuts can be observed after peptide identification by LC-MS-MS. This is particularly the case for αs_1-CN (f149-199) for which 6 chymotrypsin cuts were observed (Fig. 7a). However, αs_2-CN (f1-88) showed only 2 trypsin cuts appearing during duodenal digestion (Fig. 7b), β-CN (76-93) only one typical chymotrypsin cut (Fig. 7c) whereas κ-CN (f106-169) showed none (Fig. 7d). It is interesting to note that the 3 latter areas were the most resistant observed by ELISA (Fig. 6).

Discussion

This study shows that food processing, and particularly heat treatment of milk, increases CNs resistance to simulated infant digestion. Inhibition ELISA and western-blotting with polyclonal antibodies revealed a lower resistance to hydrolysis of the CNs in unheated samples than in samples submitted to high heat treatments such as sterilised milk and yogurt. Mass spectrometry analysis of the digested samples showed that a higher number of peptides were identifiable in the digested heavily processed dairy products (sterilised milks and yogurt) than in the original raw milk.

The lower number of peptides identified in the low-heated milks was therefore attributed to a higher degree of proteolysis leading to short undetectable peptides. The hypothesis that heat-treatments applied to milk increase the resistance of CN to digestion has already raised by Chatterton et al. [19] and was recently confirmed by Almaas et al. [20] who observed that raw milk was digested significantly faster with human proteolytic enzymes than pasteurised and high-heated milk.

Our results emphasize the complementarity of LC-MS-MS and immunoassays for estimating the resistance of protein area to digestion. Indeed, only few peptides were detected by LC-MS-MS in the 2 large areas αs_2-CN (f1-88) and κ-CN (f106-169) that were found to be highly immunoreactive when specific monoclonal probes were used.

Analysis of digested samples by western-blotting showed that among CNs, αs_2- and κ-CN were the ones giving most, if not all, the largest fragments at the end of digestion, which was quite surprising since they are present in milk at lower concentrations than β- and αs_1-CN (ratio 1:1:3:3 for κ, αs_2, αs_1, β). Therefore, the persistence of peptides from κ- and αs_2-CN must be a consequence of their structure rather than abundance. Both of these proteins have at least two cystein residues in their sequence compared to αs_1- and β-CN, which have none. Consequently when milk is submitted to severe heat treatments, κ- and αs_2-CN have been shown to generate heat-induced aggregates with whey proteins through the formation of disulfide bridges with the cystein residues of β-lactoglobulin and α-lactalbumin [10, 11, 21]. Although the resistance of these protein aggregates to digestion has not yet been investigated, it is probable that aggregation with whey proteins will result in an increased protection of κ- and αs_2-CN. It has recently been demonstrated that whey protein aggregation could play a key role in milk allergic patient sensitization [9]. However, the exact role of casein-whey protein aggregates on sensitization has not been investigated so far.

Use of a collection of highly specific monoclonal antibodies allowed the identification of 3 major areas of resistance, β-CN (f76-93), κ-CN (f106-169) and αs_2-CN (f36-75) that are hydrophobic at pH 3.0, i.e. in the conditions used for the gastric digestion. This high hydrophobicity is a factor which may contribute to resistance of these areas to pepsinolysis either through changes in conformation or aggregation. At the beginning of the duodenal phase, when the pH is raised to 6.5, these areas will become more hydrophilic and more accessible for proteases. Depending on the number of potential trypsin and chymotrypsin cleavage sites, they will be hydrolyzed to different extents. It is interesting to note that identification of the peptides by LC-MS-MS revealed only one typical chymotrypsin cut for β-CN (76-93) whereas none was observed for αs_2-CN (f36-75) and κ-CN (f106-169). This is probably why these 3 areas were the most resistant observed by ELISA. Another possible explanation is that the two resistant fragments κ-CN (f106-169) and αs_2-CN (f36-75) are known to carry several post-translational modifications. κ-CN (f106-169) has up to 6 glycosylation sites (in position 121, 131, 133, 136, 142 and 165) whereas αs_2-CN (f36-75) contains 4 phosphoserine residues (in position 56, 57, 58 and 61). Peptides carrying post-translational modifications have been previously shown to highly resist to in vivo gastro-duodenal digestion and their presence has been detected in intestinal fluids. Indeed, CN phosphopeptides have been identified in ileostomy fluids collected from human volunteers fed with milk [22]. Moreover, κ-CN (f106-169) has been previously shown to be particularly resistant to digestion. Ledoux et al. [23] found it in the jejunum of volunteers consuming ^{15}N labelled dairy diets. This peptide was also shown to resist digestion and be absorbed at the intestinal level and was subsequently detected in plasma [24]. All these results tend to reinforce the relevance of the in vitro digestion model we used in the present study.

Have these areas that are resistant to digestion been associated with food allergy phenomena? A closer look at the literature available on CNs IgE epitopes,

shows that it is quite difficult to give a clear answer to that question. For β-CN, Vila et al. [25] found in sera from 36 allergic children that most of the IgE epitopes were located at the C-terminal extremity of this protein and corresponded to area 151-160, 167-176, 175-184 and 193-202. In another study, Chatchatee et al. [26] identified 6 major (1-16, 45-54, 57-66, 83-92, 107-120 and 135-144) and 3 minor (149-164, 167-178, 173-184) IgE epitopes in persistant cow milk allergy patients. Among those, epitope 83-92 was the most frequently recognized (found in 13 patients out of 15) and lies within the area we found resistant to digestion in the present study.

In the same paper, 8 IgE epitopes were identified on κ-CN (9-26, 21-44, 47-68, 67-78, 95-116, 111-126, 137-148, 149-166), the later 3 corresponding to part of the caseinomacropeptide (f106-169) we identified as being resistant to digestion.

Several studies have been published on the identification of αs_1-CN IgE epitopes [25-31]. The epitopes identified were numerous covering almost the whole sequence of the protein (Fig. 9) making the designation of IgE immuno-dominant epitopes difficult which is also the case for αs_2-CN.

Finally, it also has to be mentioned that the relevance of IgE binding to short linear peptides for allergenic activity of food allergens is quite controversial. In a recent study, Albrecht et al. [32] demonstrated that peptides identified as major sequence epitopes on two food allergens (Ara h 2 from peanut and Pen a 1 from shrimp) show little contribution to the IgE binding of the allergens studied. It is probably also the case for most of the linear IgE epitopes already described. However, since CNs are rheomorphic proteins lacking a defined conformational structure, they are likely to induce antibody responses against sequential structures.

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Figure captions

Figure 1. Specificity of the monoclonal antibodies recognizing αs_1-, αs_2-, β- and κ-casein used in the present study.

Figure 2. SDS-PAGE analysis of whole raw (a), pasteurised (b), sterilised milks (c) and yogurt (d) submitted to gastric *in vitro* digestion. Lanes K correspond to the samples before gastric digestion.

Figure 3. SDS-PAGE analysis of whole raw (a), pasteurised (b), sterilised milk (c) and yogurt (d) submitted to gastro-duodenal *in vitro* digestion. Lanes K correspond to the samples before duodenal digestion.

Figure 4. Residual immunoreactivity of αs_1- (■), β- (▪)and κ-casein (□) as determined by inhibition ELISA using rabbit polyclonal antibodies in whole raw, pasteurised and sterilised milks and yogurt after gastro-duodenal digestion.

Figure 5. Western-blotting of (from the left to the right) raw whole, pasteurised, sterilised milks and yogurt submitted to gastro-duodenal digestion. Bands were revealed using polyclonal αs_1-(a), αs_2-(b), β- (c) and κ-casein (d) specific antibodies. αs_1-, β- and κ-CN specific antibodies are rabbit polyclonal antibodies whereas αs_2-casein antibodies correspond to a mixture of mouse monoclonal antibodies recognizing the fragments f(36-55) and f(56-75).

Figure 6. Residual immunoreactivity of αs_1-, αs_2-, β- and κ -CN fragments determined in digested raw, pasteurised, sterilised milks and yogurt after gastro-duodenal digestion (results are expressed in % of residual immunoreactivity compares to the undigested sample)

Figure 7. Identification of peptides derived from αs_1-(a), αs_2-(b), β-(c) and κ-casein (d) by LC-MS-MS after gastroduodenal digestion of (from bottom to top) whole raw (. . . .), pasteurised (_ _ _), sterilised milks (**. . . .**) and yogurt (_____). Trypsin (⇩), and chymotrypsin (↓) cleavage sites hydrolysed during digestion are indicated within the sequence.

Figure 8. αs_1-, αs_2-, κ- and β-casein hydrophobicity profile as calculated at pH 3.0 (a) and 6.5 (b) according to Sweet and Eisenberg [17]

Figure 9. αs_1–casein IgE epitopes as identified in different recent studies