

Heat Treatment of Milk During Powder Manufacture Increases Casein Resistance to Simulated Infant Digestion

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Abstract Infant formulas (IFs) are major sources of proteins for the neonate when breast-feeding is not possible. Heat treatments used during IF manufacture affect the structure of milk proteins but the impact of these structural modifications on the digestion of milk proteins and the identity of the bioactive peptides released in the gut have never been studied so far. The objective of this work was to determine the impact of two technological parameters (percentage of dry matter of the milk concentrate prior to heat treatment and intensity of the heat treatment applied to it) considered as essential in IF manufacture on the resistance of milk caseins (CNs) to digestion. Six skim milk powders were manufactured by spray-drying after having submitted 25% or 35% dry matter milk concentrates to three types of heat-treatment traditionally used in industry (80°C/20 s, 85°C/180 s, 105°C/60 s). Those six samples and a reference unheated one were characterised (particle size, composition...) and submitted to an in vitro digestion model mimicking the gastrointestinal digestion of an infant. Digested samples were characterised by SDS-

PAGE and western-blotting and by ELISA using a collection of 23 α s₁, α s₂, β and κ -CN-specific monoclonal antibodies. Kinetics of β -CN hydrolysis was studied by antibody arrays. Peptides resisting to digestion were identified using mass spectrometry. Heat-treatment of milk prior to spray-drying was shown to significantly increase the residual CNs immunoreactivity monitored after digestion. Areas showing either post-translational modifications or high hydrophobicities were identified as being the most resistant to digestion whatever the process studied was.

Keywords Milk powder · Simulated digestion · Heat treatment · Process · Infant

Introduction

Heat treatments are widely used to ensure the safety of dairy products. In the case of infant formula (IF) manufacture, they are known for generating interactions between the different constituents leading to the formation of neo-formed components such as Maillard reaction products that are suspected to have a negative impact on infant health [1]. It has also been shown that, when heated, whey proteins alone or caseins (CNs) and whey proteins can form thermally induced aggregates [2–4]. Increasing evidence shows that aggregation could lead to a modification of the protein resistance towards digestion [5]. Consequently, whey protein aggregates have been recently shown to play a key role in the sensitization of patients suffering from allergy to cow's milk [6]. Aggregation of whey proteins (β -lactoglobulin and α -lactalbumin) resulted in an inhibition of their uptake by intestinal epithelial cells in vitro and in vivo and a redirected uptake to Peyer's patches, which promoted

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significantly higher Th2-associated antibody and cytokine production in mice than their native counterparts.

IF are mainly made by dissolving dried whey proteins and caseins (CNs) in water at different percentages of dry matter (DM) and submitting the resulting milk concentrate to several heat treatments before being finally spray-dried to give powders. During IF manufacture, up to 60 different ingredients (vitamins, minerals...) can be added to the formula [7, 8].

We have recently proposed a new infant in vitro digestion model dedicated to study the resistance of milk and egg allergens to digestion [9]. This model includes two phases, a gastric phase at pH 3.0 consisting of the action of pepsin in the presence of phosphatidylcholine followed by a duodenal phase at pH 6.5 with addition of trypsin, chymotrypsin and bile salts. All the compounds are added at physiologically relevant concentrations. This static in vitro model constitutes a good screening technique allowing the comparison of different samples in the same conditions [5].

The objective of the present study was to determine how the modifications of milk protein structure induced by heat treatment during milk powder processing affect CN digestion in vitro. Two technological parameters, i.e. the percentage of DM of the milk concentrates and the intensity of the heat treatment, were considered because they are essential in IF manufacture. To reach this goal, six skim milk powders were manufactured by spray-drying after having submitted 25% or 35% DM milk concentrates to three types of heat-treatment traditionally used in industry (80°C/20 s, 85°C/180 s, 105°C/60 s). Those six samples and a reference unheated one were characterised (particle size, composition...) and submitted to in vitro digestion. Digested samples were characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA)

and western-blotting and peptides resisting to digestion identified using mass spectrometry. Heat-treatment of milk concentrates prior to spray-drying was shown to significantly increase the residual immunoreactivity of CNs monitored after digestion.

Materials and Methods

Chemicals

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

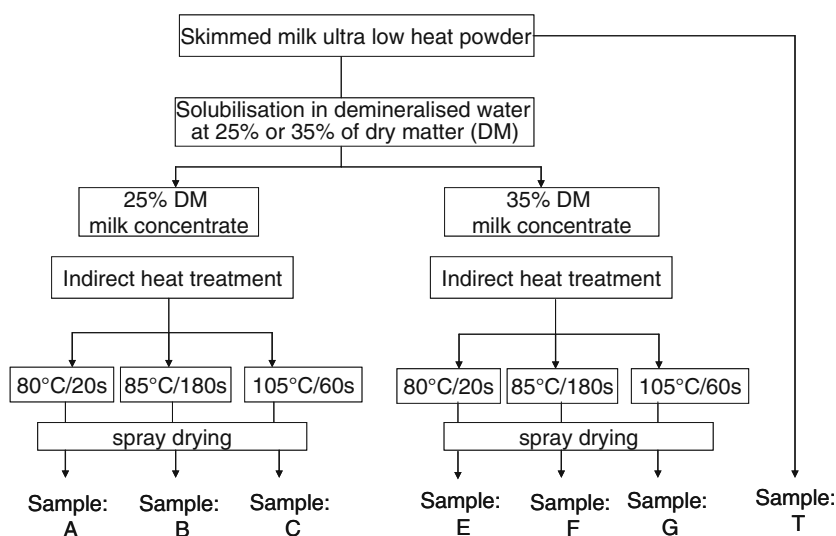
Milk Powder Manufacture

Milk powders were manufactured according to the diagram in Fig. 1. Ultra-low-heat milk powder (INRA, home-made) was used as starting material for milk powder manufacture. It was solubilised in demineralised water at 25 or 35% DM and submitted to a heat-treatment using an indirect heat treatment processing system (Microthermics UHT/HTST Lab 25 EDH, Microthermics Inc., Raleigh, NC, USA) following three industrially relevant time×temperature conditions: 80°C×20 s, 85°C×180 s and 105°C×60 s. Finally, the six manufactured powders were spray-dried in a pilot plant (GEA-PE, St Quentin-en-Yvelines, France) which can remove 5 kg of water per hour by using a two-fluid nozzle or rotary atomizer. For subsequent analyses, the seven milk powders were rehydrated at 13% (w/v) in distilled water.

Milk Powder Characterisation

Total solids content was calculated by weight loss after drying a 1.5-g sample of the powder mixed with sand in a

Fig. 1 Diagrammatic representation of the milk powders manufacture



forced air oven at 105 °C for 5 h. CN and whey protein contents were determined according to [10]. Whey protein heat-denaturation was calculated as the percentage of soluble whey proteins in the heated powders compared to the one in the unheated sample. Colour measurement indicating the development of browning reactions was estimated on powders rehydrated at 13% (w/v) in comparison to the unheated sample as previously described by [11].

Particle Size

The particle size distributions in the milk powders rehydrated at 13% (w/v) were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) with two laser sources. The refractive indexes used for casein micelles were 1.439 and 1.439 at 633 and 466 nm, respectively, and 1.333 for water [12].

In Vitro Infant Digestion Model

Prior to digestion, phospholipid vesicles were prepared using a modification of a procedure previously described [13]. Indeed it has been previously reported that phosphatidylcholine is produced in significant amounts by the gastric mucosa [14, 15] and increases the hydrophobicity and consequently the protection of the gastric mucosa. Solvent was removed from a 0.94 mL aliquot of L- α -phosphatidylcholine 63.5 mM in chloroform stock solution (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 Inc., Canada) at 5°C in a coolant-jacketed vessel. The liposomes were then covered with a cushion of nitrogen before being equilibrated at 37°C for 20 min in a shaking incubator. Proteolysis was performed essentially as previously described [9] using triplicate incubations at 37°C. The concentrations of digestive enzymes, bile salts, surfactants, etc. were chosen according to the data available in the literature on newborns consuming real foods (mainly infant formula) [9]. Prior to digestion, the six manufactured milk powders and the reference unheated powder were rehydrated at 13% (w/v) in 0.15 M NaCl, pH 6.5. Then, 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 protein (0.05 mM, final concentration). Aliquots (100 μ L) were

removed after 0, 1, 2, 5, 10, 20, 40 and 60 min gastric digestion. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M ammonium bicarbonate (BDH, Poole, 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 protein bovine α -chymotrypsin (activity 40 U/mg of protein using benzoyltyrosine ethyl ester, BTEE, as substrate), 3.45 U/mg of total protein porcine trypsin (activity 13,800 U/mg of protein using benzoylarginine ethyl ester, BAEE, as substrate). Aliquots (100 μ L) were removed after 0, 1, 2, 5, 10, 15 and 30 min duodenal digestion and proteolysis stopped by addition of a twofold excess of soybean Bowman-Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

Antibodies

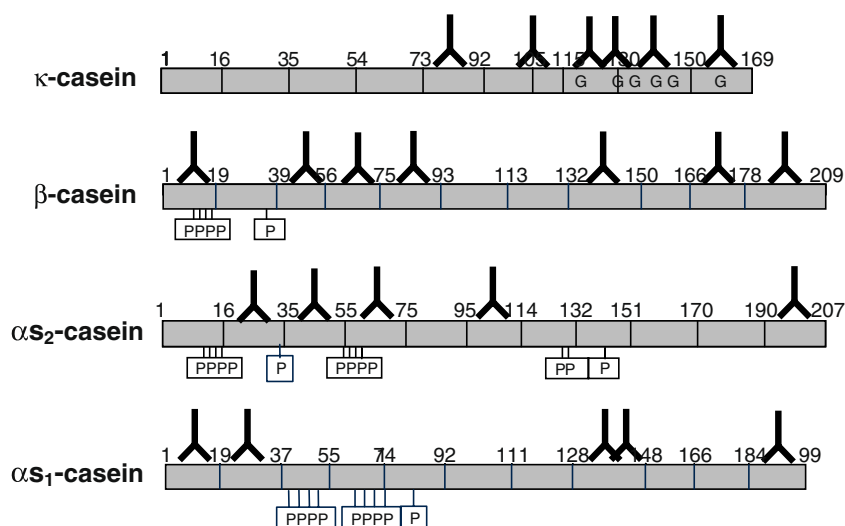
Twenty-three mouse monoclonal antibodies specific for α ₁-, α ₂-, β - and κ -CN were taken from INRA's collection [16] in order to cover as much of the CNs sequences as possible. The specificity of these antibodies is represented in Fig. 2.

A total CN-specific rabbit polyclonal antibody with broad specificity used as a capturing reagent for the antibody microarray experiments (see below) was obtained from GeneTex Inc. (Irvine, CA, USA).

SDS-PAGE

Samples taken at different stages of the digestion were analysed in duplicate by SDS-PAGE. Digested samples (10 μ L) were added to 290 μ L 62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol and bromophenol blue and heated at 70°C for 10 min. Samples (15 μ L) were loaded onto a 4–12% polyacrylamide NuPAGE Bis-Tris Novex precast gel (Invitrogen, Carlsbad, CA, USA). A continuous buffer system was used consisting of 50 ml of 20 $\times$ NuPAGE MES SDS running buffer with 950 ml of ultra-pure water (Invitrogen). NuPAGE antioxidant (0.5 ml) was added to 200 ml buffer and used to fill the inner tank, the other 800 ml being used to fill the outer tank. Gels were run for 35 min at 120 mA per gel and 200 V and were stained with Colloidal Blue Staining Kit (Invitrogen) during 3 h at room temperature, according to the manufacturer's recommendations. Gels were destained in deionized water during 7 h at room temperature. The Mark 12 unstained standard (Invitrogen) was used as protein standard.

Fig. 2 Specificity of the monoclonal antibodies recognising α_{S1} -, α_{S2} -, β - and κ -casein used in the present study



Indirect ELISA

This method was used to detect the α_{S1} -, α_{S2} -, β - and κ -CN area resistant to digestion using 23 monoclonal antibodies specific for these 4 CNs. Briefly, 100 μ L of digested milk powders were diluted 1:2,000 in 0.1 M bicarbonate buffer, pH 9.6 and coated onto a micro-titre plate (NUNC) and incubated 1 h at 37°C. Wells were rinsed between incubation steps for 15 s with four changes of 250 μ L phosphate-buffered saline, 0.5% Tween 20 (PBS-T) using a Model 1575 Immunowash microplate washer (Bio-Rad). The remaining binding sites were blocked by incubating 250 μ L fish gelatin (Sigma) at 10 g/L 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 μ L of goat anti-mouse Ig alkaline phosphatase conjugate (Sigma) diluted 1:3,000 in PBS-T for 1 h at 37°C. Following the last rinsing, 100 μ L *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. After 30 min at 37°C, the absorbance at 405 nm was read against a blank and corrected according to the background signal in the absence of antigen using a Benchmark Plus microplate spectrophotometer (Bio-Rad). Analyses were performed in duplicate.

Antibody Microarray

This technique was applied for characterising the residual immunoreactivity of CN epitopes throughout the digestion process of the unheated milk powder sample. An affinity-purified rabbit polyclonal anti-CN antibody at 1 mg/ml was dialysed against distilled water overnight at room temperature. Its Ig concentration was determined by measuring the absorbance at 280 nm. This antibody was then spotted on a microscope glass slide covered with nitrocellulose (PATH

TM plus Protein Microarray Slide, Gentelbio, USA) using a piezo non-contact printer (Piezotarray, Perkin-Elmer, Shelton, CT, USA) according to the protocol already described by [17]. Arrays of nine spots were spotted on three different glass slides. They were separated from each other by depositing a frame with a wax machine (SlideImprinter, The Gel Company, San Francisco, CA, USA) at the surface of the slide. Spotted glass slides were then stored under vacuum at 4°C until further use. Before use, spotted glass slides were gently warmed during 30 min at room temperature and rinsed in PBS-T under agitation at room temperature. They were dried by centrifugation (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) at 900 rpm during 1 min. All the steps were then performed at room temperature.

Slides were saturated in PBS-T, 1% BSA (Sigma) during 1 h, rinsed 3 times in PBS-T during 3 min and dried by centrifugation as described above. Unheated milk powder samples after 0, 1, 2, 5, 10, 20, 40 and 60 min gastric digestion and 0, 1, 2, 5, 10, 15 and 30 min duodenal digestion were diluted at 1 μ g/ml protein in 100 μ L sample buffer (PBS, 1% Brij 35, 1% Tween 20, 1% protease inhibitors from Roche Diagnostics, Mannheim, Germany), 250 μ L of IgG cocktail (home-made) and 650 μ L water.

One microlitre of each diluted sample was added on each nine-spot arrays using a pipette (P10, Gilson Inc., Middleton, WI, USA) without any contact between the tip and the slide. Glass slides were incubated in a humidified chamber under gentle stirring during 1 h at room temperature. They were rinsed three times for 3 min with PBS-T and dried by centrifugation at 900 rpm (Eppendorf) for 2 min.

Then, 1 μ L of culture supernatant containing anti- β -CN monoclonal antibodies specific for β -CN (f1–25), (f42–56), (f76–93), (f133–150), (f167–178) and (f179–209) fragments, diluted one-half in PBS-T, 0.1% BSA was added onto each array without touching the slide with the pipette

tip and incubated 1 h at room temperature in a humidified chamber under gentle agitation.

Slides were rinsed three times during 3 min in PBS-T and dried by centrifugation at 900 rpm (Eppendorf) during 2 min and 1 μ l of a goat anti-mouse Ig, Alexa 546 conjugate (Molecular Probes, Invitrogen) diluted 1/400 in PBS, 1% Tween 20, 1% Brij-35, 0.1% BSA was added on each array without touching the nitrocellulose with the pipette tip. Slides were incubated 1 h at room temperature in a humidified chamber under gentle agitation, dried by centrifugation at 900 rpm during 3 min and scanned at 575 nm using a microarray scanner (LS-Reloaded, TECAN, Durham, NC, USA).

Immunoblotting

Further analysis of digested samples by immunoblotting and LC-MS-MS was performed on digested milk powders T (unheated), E (35% DM, 80°C×20 s) and G (35% DM, 105°C×60 s) collected at the end of the gastro-duodenal digestion process. After being electrophoresed as described above, proteins and peptides from digested samples were transferred onto a 0.2 μ m 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% SDS and 20% 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% gelatine, α s₁-, β - and κ -CN-specific polyclonal antibodies at 1:2,000, 1:500 and 1:1,000, respectively, or a mixture of α s₂-CN monoclonal antibodies specific from the area 35–75 (diluted 1:2). Reaction was revealed using either goat anti-rabbit (for polyclonal antibodies) or goat anti-mouse (for monoclonal antibodies) immunoglobulin alkaline phosphatase conjugate at 1:500 and Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) as substrate.

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

Digested samples T, E and G 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 [9]. 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 home-made 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 asparagine 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 percentage of DM and the intensity of the heat-treatment on the residual immunoreactivity remaining after gastro-duodenal digestion of rehydrated milk powders was tested by variance analysis using the R software package [18] running on the UNIX® system.

Results

Milk Powder Characterisation

Table 1 shows the protein composition and some characteristics of the manufactured heat powders. Heat treatment resulted in a decrease of the soluble whey proteins and an increase in the CN content corresponding to the aggregation of heat-denatured whey proteins on the CN micelle. Heat treatment also resulted in the development of browning

Table 1 Protein composition and characteristics of the milk powders

	T	A	B	C	E	F	G
Caseins (g/100 g powder)	27.67	28.70	30.45	32.78	28.68	30.05	32.93
Whey Proteins (g/100 g powder)	6.38	5.90	4.37	2.08	6.15	5.07	2.12
Heat-denatured WP (in%)	0	7.3	31.5	67.4	3.6	20.5	67
Colour		+0.37	+1.55	+4.17	+0.66	+2.97	+4.92
Total Solid (in%)	95.4	96.0	94.9	96.8	97.1	97.9	97.2

reactions modifying the colour of the rehydrated heat-treated powders.

Characterisation of Particle Size in Rehydrated Milk Powders

After manufacture, milk powders were rehydrated at 13% (w/v) in distilled water. Mean diameter of the particles found in the unheated sample was c.a. 160 nm corresponding to the mean diameter of the CN micelle (Fig. 3). As the intensity of the heat treatment increased, mean diameter of the particles increased to reach values of c.a. 224 nm for 35% DM due to aggregation of the heat-denatured whey proteins onto the CN micelle. Particles of mean diameters around 5 μm appeared in the 35% DM sample heated 60 s at 105°C.

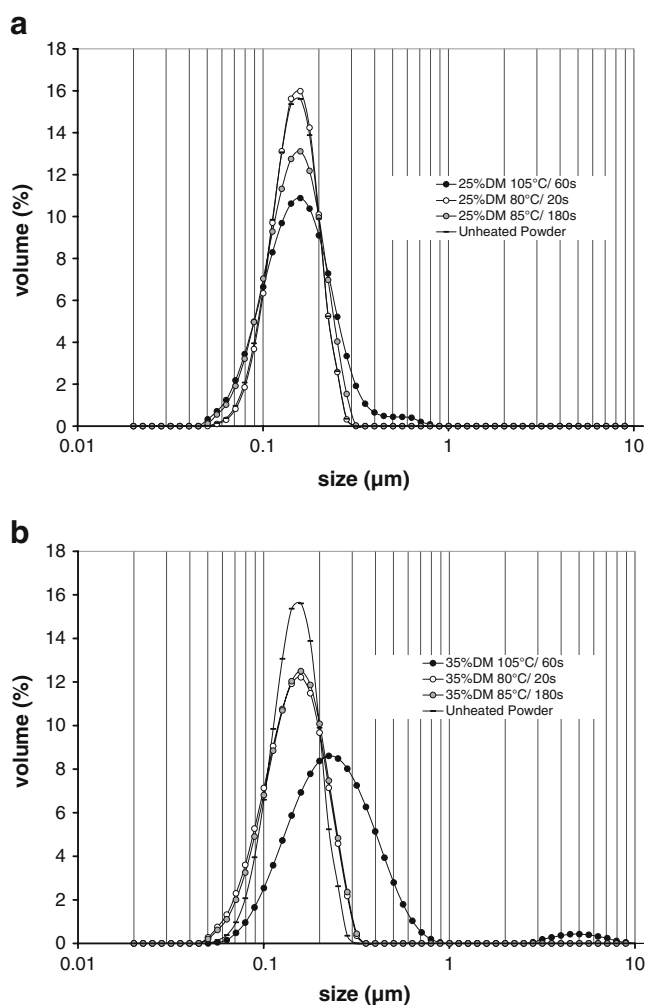


Fig. 3 Particle size (in nm) determined by light scattering in the 25% DM (a) or 35% DM (b) milk powders rehydrated at 13% (w/v). Prior to spray-drying samples had been unheated (solid line) or heated at 80°/20 s (white circle), 85°/180 s (grey circle) or 105°/60 s (black circle)

Analysis of Digested Samples by SDS-PAGE and Western-Blotting

Figure 4 shows SDS-PAGE analysis of samples A, B, C, E, F, G and T submitted to gastro-duodenal digestion. Hydrolysis of CNs was faster than that of β -lactoglobulin. Indeed, even at the end of gastro-duodenal digestion, the band corresponding to β -lactoglobulin was still visible for all the samples. Bands corresponding to intact CNs disappeared throughout digestion but the kinetics of hydrolysis appeared to be strongly influenced by the processing conditions. Sample G (35% DM, 105°C/60 s) showed the highest resistance of CNs to digestion together with sample C to a lesser extent. Bands corresponding to intact CN were even visible for sample G during the duodenal phase whereas the other samples showed total disappearance of CN bands confirming that this sample showed higher resistance to CN digestion. SDS-PAGE is a global analysis that may lack sensitivity and specificity. Therefore, samples T, E and G collected at the end of the gastro-duodenal digestion were further analysed by western-blotting with CN-specific antibodies. Figure 5 shows the western-blotting experiments performed on the samples at the end of gastro-duodenal digestion. Lanes corresponding to unheated sample (T) and 35% DM samples heated at 80°C/20 s (E) and 105°C/60 s (G) collected at the end of gastro-duodenal digestion were transferred onto nitrocellulose and specifically revealed using polyclonal antibodies directed towards αs_1 -, αs_2 -, β - and κ -CN. Because of the higher sensitivity of western-blotting compared to SDS-PAGE, intact bands and degradation products of αs_1 -, αs_2 - and β -CN were observed in all the samples analysed. Bands at higher Mw were observed only with αs_2 - and κ -CN, these bands being visibly more intense in the highly heated sample G. Since both αs_2 - and κ -CN are the only CNs able to bind whey proteins via disulfide bridges, it is probable that these high Mw bands correspond to CN–whey protein aggregates as it has previously been demonstrated [4, 19, 20].

Identification of the Casein Area Resistant to Digestion Using a Collection of Specific Monoclonal Antibodies

Residual immunoreactivity of the seven milk powders submitted to simulated gastro-duodenal digestion was estimated by indirect ELISA using 23 CN-specific monoclonal antibodies. In order to improve clarity, only results obtained with samples T, E and G are shown in Fig. 6a,b,c,d.

For αs -CN, resistant areas were revealed using monoclonal antibodies specific for the αs_1 -CN (f129–151; Fig. 6a) and αs_2 -CN (f36–55) and αs_2 -CN (f56–75; Fig. 6b). For β -CN, the three areas β -CN (f1–25), β -CN

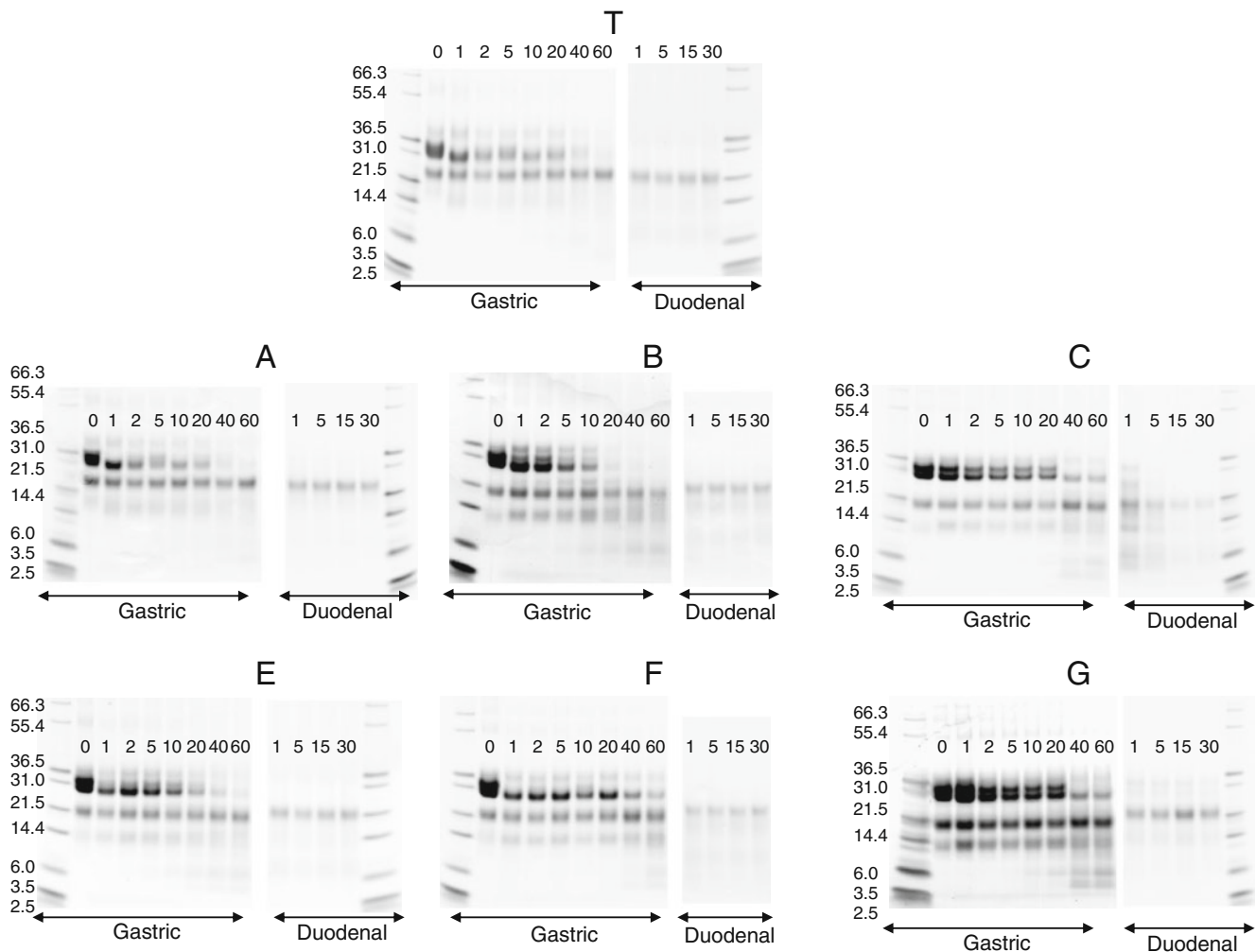


Fig. 4 SDS-PAGE analysis of samples **a**, **b**, **c**, **e**, **f**, **g** and **t** submitted to gastro-duodenal digestion

(f76–93) and β -CN (f133–150) were identified as the most resistant to digestion in all the samples studied with residual immunoreactivities reaching up to 90% at the end of gastro-duodenal digestion (Fig. 6c). Finally, for κ -CN, the highest residual immunoreactivity was found with the

monoclonal antibody specific for the κ -CN (f112–130; Fig. 6d).

It has to be pointed out that among the areas showing the highest resistance to gastro-duodenal digestion, three showed post-translational modifications. β -CN (f1–25)

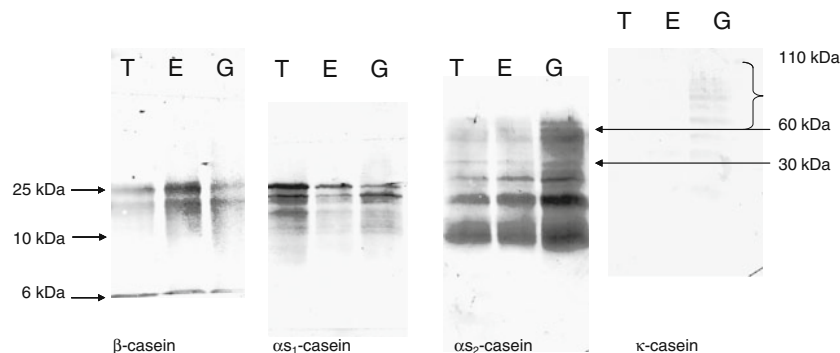
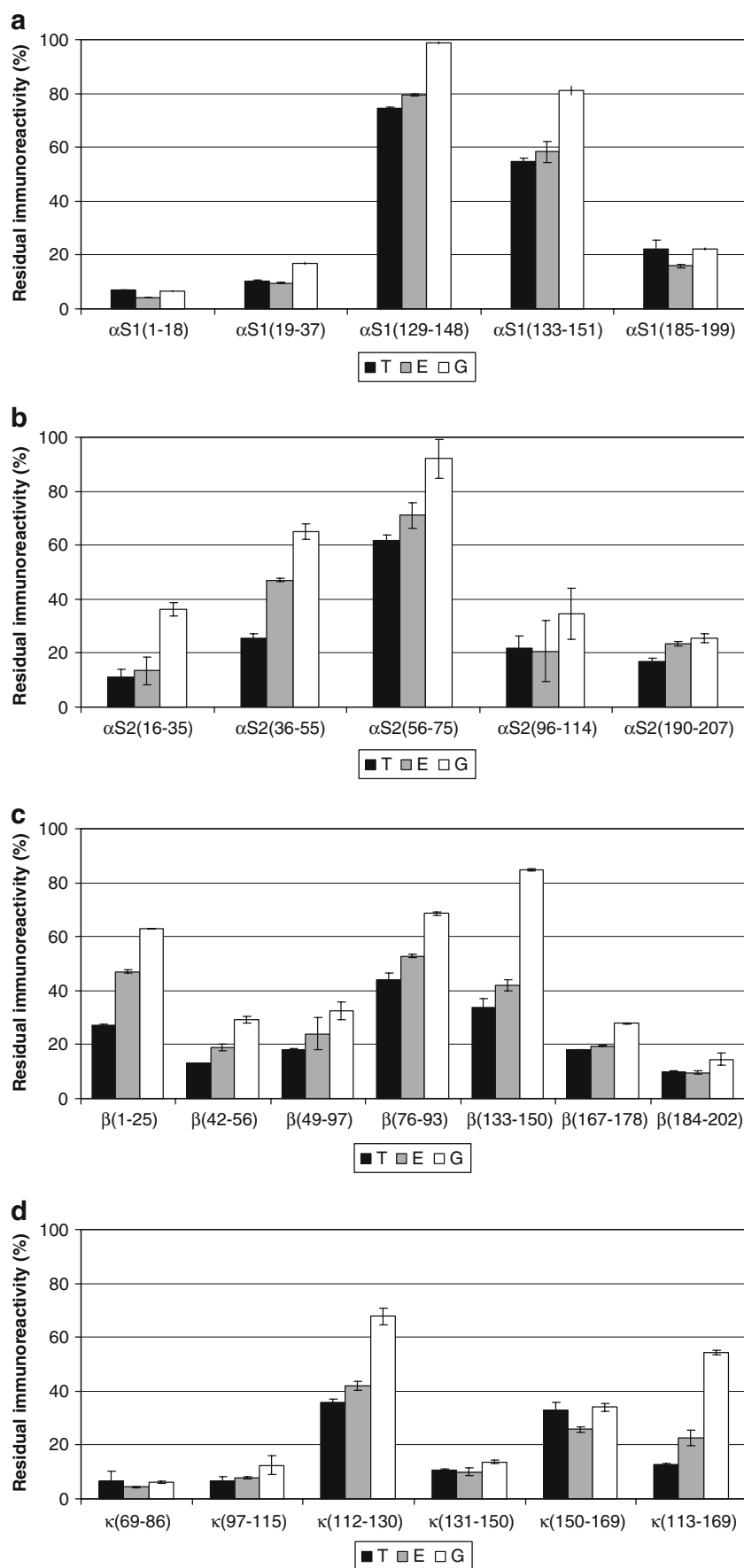


Fig. 5 Western-blotting of (from the left to the right) unheated (**T**), heated at 80°C/20 s (**E**) and 105°C/60 s (**G**) milk powders submitted to gastro-duodenal digestion. Bands were revealed using rabbit polyclon-

al α S₁-, β - and κ -casein specific antibodies whereas α S₂-casein antibodies correspond to a mixture of mouse monoclonal antibodies recognising the fragments f(36–55) and f(56–75)

Fig. 6 Residual immunoreactivity of α S₁- (a), α S₂- (b), β - (c) and κ -CN (d) fragments determined in unheated (*T*), heated at 80°C/20 s (*E*) and 105°C/60 s (*G*) milk powders after gastro-duodenal digestion (results are expressed in percent of residual immunoreactivity compared to the undigested sample)



and α s₂-CN (f36–75) both contain four phosphoserine residues whereas κ -CN (f112–130) has one glycosylation site.

Finally, statistical analysis of these data showed a highly significant effect of the intensity of the heat-treatment on CN residual immunoreactivity ($p=0.00001$) with values higher for a treatment of 60 s at 105°C. Percentage of DM also had a significant effect ($p=0.00556$) with higher values for 35% DM than 25% DM and there was also a significant interaction between the two parameters ($p=0.03262$). In summary, the highest residual immunoreactivities, i.e. the highest resistance to in vitro digestion, were observed for samples at 35% DM heated for 60 s at 105°C.

Kinetics of β -Casein Digestion Monitored by Antibody Microarrays

Antibody microarrays were developed in order to follow the kinetics of hydrolysis of different areas in the β -CN sequence during digestion of the unheated milk powder. Figure 7 shows the evolution of the residual immunoreactivity of the seven areas of β -CN throughout digestion. Three distinct behaviours were observed depending on the area monitored on β -CN. Areas (f167–178) and (f179–209) were rapidly and extensively hydrolyzed during the first minutes of gastric digestion leading to a total disappearance of the signal at the end of the gastric phase. Fragment (f42–56) was partially hydrolysed during gastric phase and then fully digested during the duodenal phase. In contrast, areas (f1–25), (f76–93) and (f133–150) partially resisted both gastric and duodenal phases leading to a significant residual immunoreactivity after the whole digestion process.

Identification of Peptides in Digested Samples Using LC-MS-MS

LC-MS-MS was applied to samples E, G and T in order to identify the peptides remaining after gastro-duodenal digestion. Figure 8 shows the peptides unambiguously

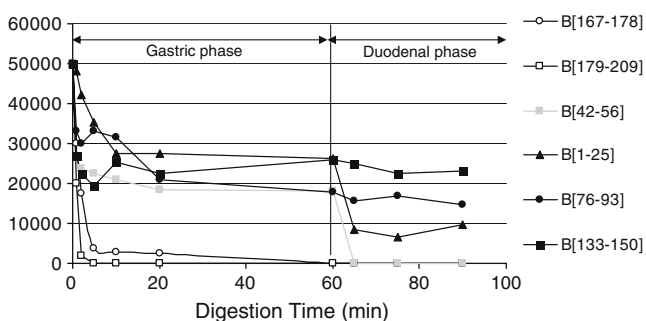


Fig. 7 Residual immunoreactivity after gastro-duodenal digestion of unheated milk powder (T) of different fragments of β -casein as determined by antibody microarrays (results are expressed in arbitrary units)

identified and generated by α s₁- (a), α s₂- (b), β - (c) and κ -CN (d) hydrolysis.

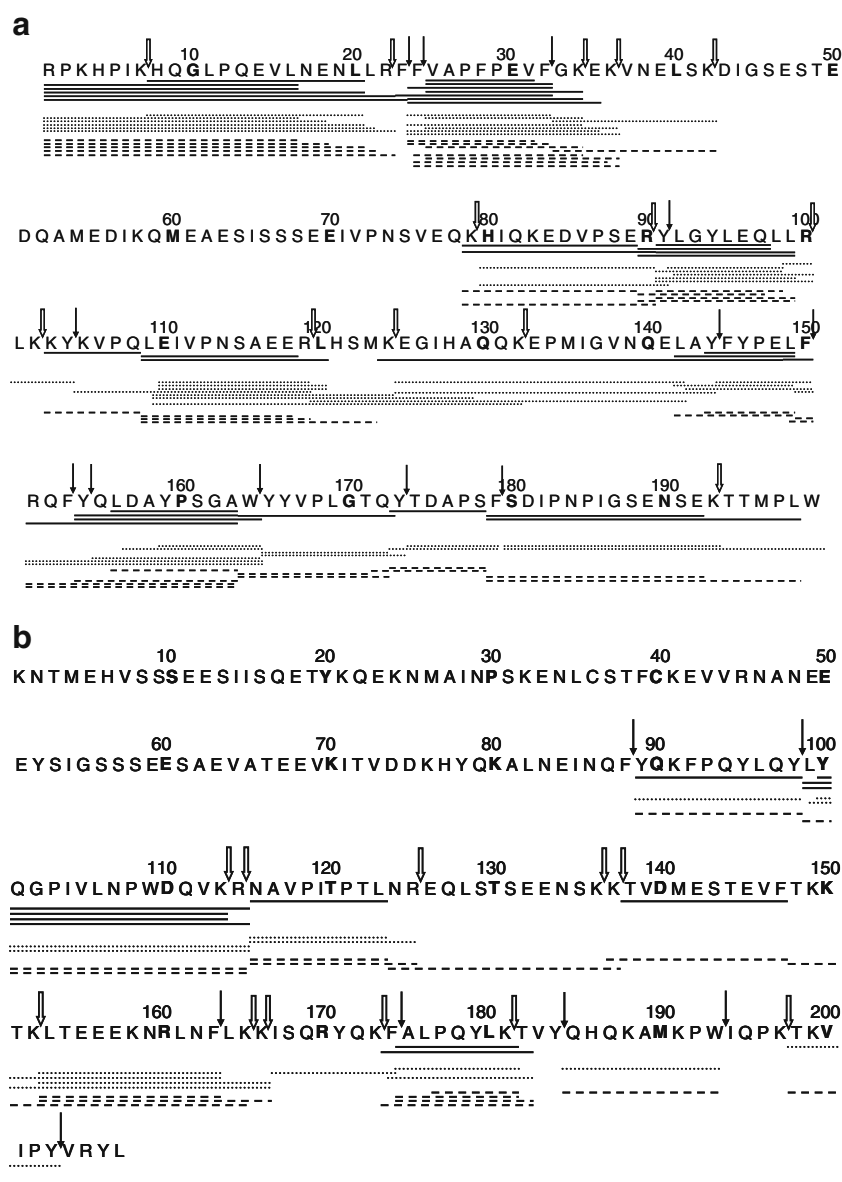
Peptides generated by α s₁-CN hydrolysis covered most of the sequence with the exception of area 43–78 that did not give birth to any peptides. α s₂- and κ -CN hydrolysis led to the appearance of a low number of identifiable peptides. This is probably because these two proteins are about three times less abundant than the two major α s₁- and β -CNs. No peptides were identified within area κ -CN (f1–16), κ -CN (f80–95), κ -CN (f106–124), κ -CN (f138–161) and α s₂-CN (f1–89), the two latter showing post-translational modifications

Finally, for β -CN, 58, 66 and 69 peptides were unambiguously identified in digested samples T, G and E, respectively. Identified peptides showed Mw ranging from 645 to 3,408 Da and covered 74.6% to 80.5% of the sequence. No peptides were identified in the area β -CN (f53–80) and only two in the β -CN (f1–25).

Discussion

The results presented in this paper clearly show that processing parameters used during milk powder manufacture can modify the resistance of CNs to simulated infant digestion. Among the parameters tested, the intensity of the heat treatment applied to the milk concentrate showed the most significant impact. The hypothesis that heat treatments applied to milk increase the resistance of CN to digestion has already been raised by [21] and was recently confirmed by [22] who observed that raw milk was digested significantly faster with human proteolytic enzymes than pasteurised and high-heated milk. In a previous study, we already showed on a set of dairy samples (milks and yoghurt) that heat treatment was increasing the resistance of CNs to in vitro digestion [5]. α s₂- and κ -CN were shown to be the most modified by the heat treatment and aggregation of denatured whey protein onto the CN micelle via the formation of disulfide bounds was the hypothesis we raised to explain these observations. In the present case, a high heat treatment applied to the sample before spray-drying resulted in the appearance of high molecular weight aggregates that could be observed by light scattering techniques (Fig. 3). Since no lipids were present in the manufactured milk powders, these aggregates were probably protein aggregates. This idea was also reinforced by the western-blotting experiments we performed on the digested samples. Indeed, revelation of the bands with α s₂- and κ -CN-specific antibodies showed high molecular weight bands appearing with a higher intensity in digested highly heated samples (Fig. 5). Besides heat treatment, the percentage of DM of the milk concentrate prior to heat treatment also influenced CNs resistance to digestion. Among the conditions tested, the highest resistances were

Fig. 8 Identification of peptides derived from α s₁- (a), α s₂- (b), β - (c) and κ -casein (d) by LC-MS-MS after gastro-duodenal digestion of unheated (*solid line*), heated at 80°C/20 s (*dotted line*) and 105°C/60 s (*dashed line*). Trypsin (down double arrow), and chymotrypsin (down arrow) cleavage sites hydrolysed during digestion are indicated within the sequence



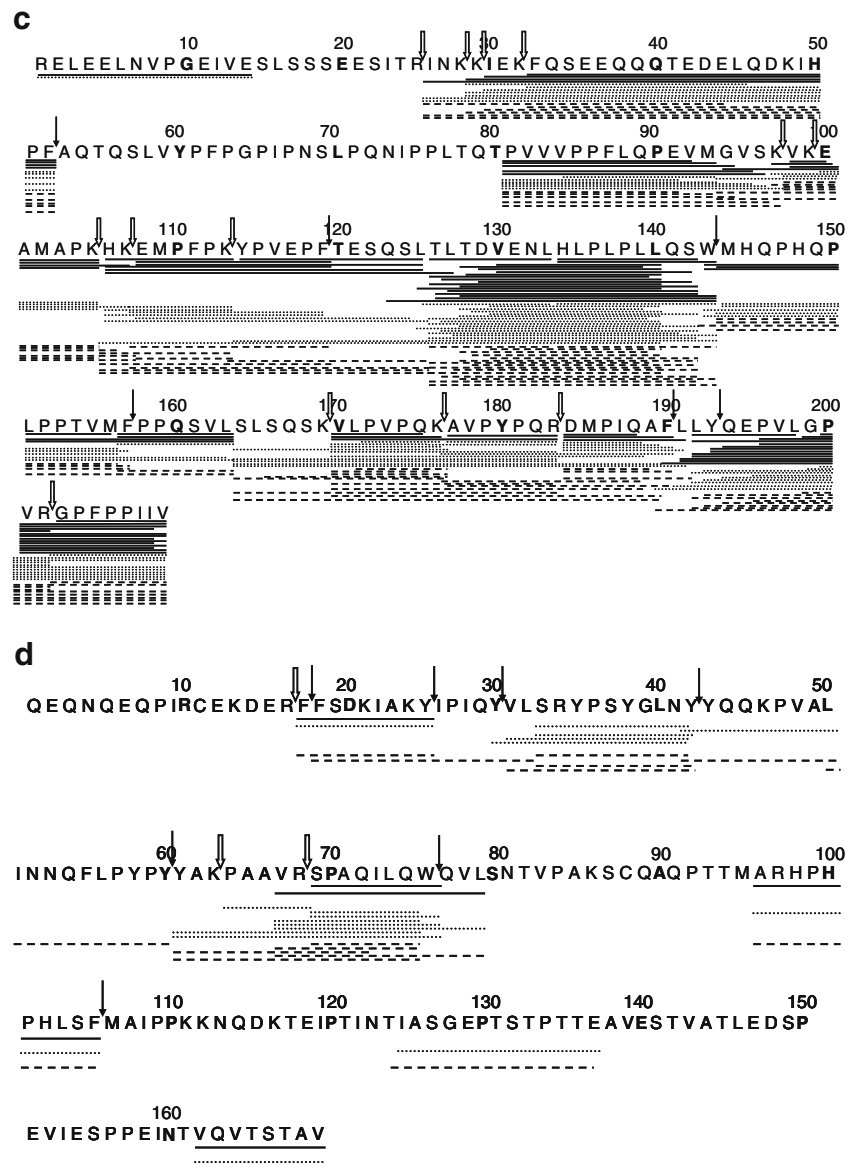
observed with milk concentrate at 35% DM heated at 105°C/60 s. This result is of great importance when we consider that in industry 45% DM milk concentrates are commonly used for IF manufacture and heated using drastic conditions (sometimes even higher than 105°C/60 s). Unfortunately, heat-treatment at 105°C/60 s of 45% DM concentrates in our pilot plant led to milk clotting in our heat exchanger rendering the manufacture of these formulas, impossible. It is therefore probable that protein aggregation will be even more important in industrial products and this raises some important questions regarding the biological consequences for a newborn consuming these products.

Indeed, CNs resistance to digestion could lead to the persistence of peptides in the gut capable of eliciting immune responses. Pathologies such as allergy to milk could be reinforced by the high resistance of milk allergens to digestion

especially for neonates who have both immature digestive and immune systems. The area showing the highest residual immunoreactivities were the fragments α s₁(f133–151), α s₁(f185–199), α s₂(f36–75), β (f1–25), β (f76–93) and κ (f112–130; Fig. 6). These areas are characterised by a high hydrophobicity at pH 3.0 (gastric conditions) and/or the presence of post-translational modifications. These two parameters have already been identified as playing a key role in the resistance of CN to in vitro digestion [5].

Kinetics of digestion of β -CN were shown to be dependent on the area of the molecule we were looking at. Indeed, use of protein array technologies revealed different behaviours: (1) total cleavage of the fragment during the gastric phase at the C-terminal end of the molecule (f167–178 and f179–209), (2) partial hydrolysis during the gastric phase followed by a total cleavage during the duodenal one (f42–56), (3) partial

Fig. 8 (continued)



hydrolysis during the gastric phase followed by an incomplete hydrolysis during the duodenal phase giving birth to resistant fragments (f1–25), (f76–93) and (f133–150; Fig. 7). It has however to be mentioned that some slight discrepancies were observed between the residual immunoreactivities determined at the end of the gastro-duodenal digestion using ELISA and antibody arrays. Indeed, area (f167–178), (f179–209) and (f42–56) appeared to be extensively hydrolysed according to the two types of analysis but residual immunoreactivities lower than 20% were found by ELISA whereas no residual immunoreactivities were observed with antibody arrays. These differences are probably due to a lower sensitivity of the antibody array technology.

Finally, a complete mapping of the peptides remaining after gastro-duodenal digestion was obtained (Fig. 8). Most of the sequences of the four caseins were covered by the peptides

obtained. However, some areas, i.e. α s₁-CN (f43–58), α s₂-CN (f1–89), β -CN (f53–80), κ -CN (f1–16), κ -CN (f80–95), κ -CN (f106–124), κ -CN (f138–161) and β -CN (f1–25) to a lesser extent did not give birth to any detectable peptides. Several reasons can explain the absence of identified peptides in a certain area; it can be due to (1) a resistance of this area to digestion leading to the persistence of a large non ionisable fragment, (2) an extensive hydrolysis of the area leading to short peptides, (3) the presence of post-translational modifications hardly detectable by LC-MS-MS, (4) a low abundance of the peptides generated by the hydrolysis of a minor protein. Area β -CN (f53–80) has been shown to be particularly hydrophobic making the accessibility to digestive proteases poor [5]. Fragment β -CN (f1–25) is characterised by the presence of post-translational modifications with phosphoserine residues in position 15, 17, 18, 19

and 35. It is therefore possible that peptides originated from this area were not detected by LC-MS-MS either because phosphorylated peptides are hardly detected with this technique or because of the protecting effect of phosphorylation towards digestion.

Some drawbacks in our study have to be mentioned. The in vitro digestion model used in the present study is static and pH is kept constant during the whole digestion process which might not perfectly reflect what happens during in vivo food digestion. 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 [23]. Nevertheless, since the objective of the study was to determine the effect of processing parameters on the resistance of CN to digestion, working at constant pH still allowed the comparison between samples digested in the same conditions. Furthermore, the pH 3 at which in vitro digestions were performed roughly corresponds to the gastric pH of fasting infants [24] and was chosen in order to increase the proteolysis events that will occur during digestion allowing the identification of areas resistant to digestion even at such drastic conditions. Also, due to the action of gastric juice, the solubilised milk powders may clot at their arrival into the stomach modifying the accessibility of certain cleavage sites to digestive proteases and slowing down the gastric emptying. Therefore, the present model must be considered as a screening technique allowing the comparison of different samples digested in the same conditions and allowing the identification of technological parameters having an impact on protein resistance to digestion. Dynamic models integrating pH variations in the stomach and peristalsis would give a sharper idea of what really happens in vivo and are currently being developed.

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