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Differential influence of the degree of processing on immunogenicity following proteolysis of casein and β -lactoglobulin

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Simulated gastric fluid digestion was used to investigate how heat processing and fat content influence effects of proteolytic digestion on milk protein immunogenicity in milk with varying fat content and degree of processing. Changes in antigenicity and allergenicity of casein and β -lactoglobulin (β -LG) were demonstrated, using polyclonal antibodies and milk-allergic patient sera in immunoblot and enzyme-linked immunosorbent assay (ELISA) inhibition. Heat-induced changes in ultra-high temperature processed (UHT) milk increased stability of IgE and IgG casein epitopes to digestive proteolysis, but had little effect on specific IgE or IgG binding. Native κ -casein may be more resistant to digestion, but this was abrogated by heat treatment. Although β -LG's digestive stability decreased in UHT milk, immunogenicity was retained after hydrolysis in both processed (UHT) and non-processed (semi-skimmed) milks. Differences in fat levels in three milk matrices which had been processed similarly did not appear to influence digestibility. The possible clinical significance of these findings remains is discussed.

Keywords: simulated digestion; food processing; cow's milk; caseins; β -lactoglobulin

Introduction

Cow's milk allergy (CMA) is one of the most frequent forms of food allergy in children, with a prevalence of 2–3% (Host, 2002), and as milk and milk products form an essential part of the diet in the young child, there is a need for greater understanding of the influence of the food matrix on the digestibility of milk proteins, and how it may be affected by food processing. Cow's milk contains several allergenic proteins, characterised by their ability to elicit immunoglobulin (Ig)E-mediated allergic responses or bind to IgE from sensitised individuals. β -lactoglobulin (β -LG), a dimeric globular protein, was previously considered to be the dominant milk allergen, but it is now agreed that several milk allergens are involved in the majority of CMA cases (Wal, 2004). Casein has more recently been described as the main antigenic and allergenic cow's milk component (Bernard, Creminon, Yvon, & Wal, 1998; Docena, Fernandez, Chirido, & Fossati, 1996). The casein fraction consists of four proteins: α_{s1} -, α_{s2} -, β - and κ -casein, differing considerably in their primary structure and functional properties. They are, however, all phosphorylated and have a flexible non-compact tertiary structure without disulphide bonds (Wal, 2004). The individual caseins cross-link non-covalently to form aggregates, or micelles, which consist of a hydrophobic core and a hydrophilic surface layer composed mainly of κ -casein. Most CMA patients are

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co-sensitised to all the casein components, although cross-sensitisation may also occur (Bernard et al., 1998).

Processes in industrial food preparation and production may influence food protein allergenicity. Epitopes may be inactivated or destroyed, neoepitopes may be formed or hidden epitopes may be unmasked (Besler, Steinhut, & Paschke, 2001). Thermal processing (such as pasteurisation, ultra heat treating, sterilisation and evaporation) of milk products induces conformational changes in proteins that may affect their allergenicity by enhancing or decreasing their digestibility. Aggregates formed on heating may further increase allergenicity (Hartmann, Wal, Bernard, & Pentzien, 2007). β -LG is relatively resistant to low pH and enzymatic degradation, which may facilitate mucosal absorption of intact portions of the protein after digestion (Wal, 2004). Heat treatment, however, denatures the native structure of β -LG, which may impact upon its allergenicity. While higher temperatures ($>90^{\circ}\text{C}$) cause stable structural changes, more or less reversible denaturation occurs already at the temperature of conventional pasteurisation (72°C) (Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004). These changes may also affect β -LG's immunogenicity. In contrast, the micellar structure of casein is presumed to be modified on ingestion/digestion, as casein is susceptible to enzymatic digestion by proteases and exopeptidases but is highly heat-stable (Wal, 2004).

Stability to proteolytic digestion was previously assumed to be a basic characteristic of allergenic food proteins, allowing allergens to retain their IgE-binding ability (Taylor & Lehrer, 1996). Allergenic food proteins were found to be generally more resistant to *in vitro* pepsin digestion than other proteins using a protocol in accordance with the US Pharmacopeia (Astwood, Leach, & Fuchs, 1996). This type of study has, however, been criticised for insufficiently simulating physiological conditions and for not having used a standardised protocol (Bannon, Fu, Kimber, & Hinton, 2003). The Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology therefore recommended the use of a more detailed test protocol (FAO-WHO, 2001) and recently a more standardised protocol was established by Thomas (Thomas et al., 2004).

The food matrix and the degree of processing may also affect stability to digestion (Wal, 2004). Furthermore, there are indications that protein allergens may interact with lipid or phospholipid components (Burnett et al., 2002; Moreno, Mackie, & Mills, 2005). The impact of proteolysis on the antigenicity and allergenicity of purified cow's milk proteins (CMP) has been well documented (Astwood et al., 1996; Bannon et al., 2003; Fu, Abbott, & Hatzos, 2002; Thomas et al., 2004), but few studies have addressed the influence of food processing and complex milk matrices on its outcome. Isolated milk proteins may be hydrolysed more rapidly than proteins in native milk and stability to digestion may thus be influenced by the matrix or the degree of processing (Wal, 2004).

The aim of the present study was therefore to examine the extent to which heat processing and fat content of the matrix might influence the effects of proteolytic digestion on the antigenicity and allergenicity of the major cow's milk allergens, casein and β -LG. IgE-binding patterns were studied using immunoblot with sera from milk-allergic subjects and a polyclonal anti- β -LG antibody. Changes in immunogenicity induced by milk processing and digestive hydrolysis were further studied using ELISA inhibition, with patient serum and polyclonal antisera to β -LG, total casein and α -, β - and κ -casein.

Materials and methods

Patient sera

A serum pool, adjusted for variations in individual milk-specific serum IgE levels (ImmunoCAP IgE FEIA system, Phadia AB, Uppsala, Sweden), was prepared from the sera of 21 CMA patients who were referred to the specialist clinic at Voksentoppen, Department of Pediatrics, Rikshospitalet University Hospital, Oslo, Norway. Individual sera from eight of these patients, all of whom had previously demonstrated IgE-binding to α -, β - and κ -caseins and β -LG in an indirect screening ELISA, were also obtained. Briefly, the inclusion criteria were: (a) a confirmed clinical history of IgE-mediated CMA; (b) elevated milk-specific IgE serum level (range 10–>100 KU/L); and (c) positive responses to skin prick tests using cow's milk as antigen. Negative control sera (milk-specific IgE < 0.35 KU/L) were obtained from four healthy milk-consuming adult volunteers. Blood samples were donated after informed consent. The study was approved by the Regional Committee for Medical Research Ethics, southern Norway.

Preparation of milk proteins and milk hydrolysates

In a previous evaluation of the protocol (Thomas et al., 2004), assay pH was not found to influence digestion time. A preliminary study using the USP protocol (Board of Trustees, 1995) confirmed this observation and therefore hydrolysis was carried out at pH 2.0, only. The aim of this study was not to assess digestive stability *per se*, but to examine the effects of food matrix and processing on residual antigenicity and allergenicity of CMP. Consequently, the enzyme:protein ratio chosen reflected a desire to use the most stringent conditions.

Whole, semi-skimmed, skimmed and UHT (ultra-pasteurised and additionally lactose reduced) milks were purchased from local stores in Norway (Table 1). Total protein concentrations were determined using the DC Protein assay (Bio-Rad, Hercules, CA, USA), according to the method of Lowry. All milk samples, and stock solutions of casein and β -LG (Sigma-Aldrich, Steinheim, Germany) were diluted to 5 mg/ml with deionised water. Simulated gastric fluid (SGF) was prepared in accordance with the following protocol (Thomas et al., 2004): for each sample of milk or milk protein, freshly prepared pepsin reaction solution containing 0.95 mg (4000 U) pepsin with a specified activity of 4230 units/mg protein (Sigma-Aldrich), in 1520 μ l 0.084 N HCl/35 mM NaCl, pH 2.0 were pre-heated to 37°C in 15 ml plastic test tubes. 80 μ l of the respective test protein solution (5 mg/ml) were added to each reaction tube, giving an enzyme: protein ratio 2.4:1. The tubes were vortexed gently and incubated in a 37°C waterbath. At intervals of 1, 5, 10, 15, 30 and 60 min, 200 μ l aliquots of the individual hydrolysates were transferred into

Table 1. Nutritional content per 100 ml of milk products. Skimmed, whole and semi-skimmed milk is homogenised and pasteurised (heated to 72–80°C for at least 15 seconds). UHT (ultra-high temperature processed) is homogenised and partially sterilised (heated to a temperature in excess of 140°C for 2–4 seconds).

Milk type	Skimmed	Whole ^a	Semi-skimmed	UHT ^b
Protein	3.3 g	3.2 g	3.3 g	3.3 g
Carbohydrate	4.8 g	4.6 g	4.7 g	4.8 g
Fat	0.1 g	3.9 g	1.5g	1.5 g

^aFull cream (UK). ^bUHT also lactose-reduced.

Eppendorf tubes, the reaction was stopped by the addition of 70 µl of 200 mM NaHCO₃ and the tubes were placed on ice. Zero time point samples were prepared by adding the stop solution immediately prior to adding protein. All samples were stored at -20°C until analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of hydrolysates

Milk hydrolysates were electrophoretically separated under reducing conditions on the NuPAGE Gel system (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. The hydrolysates and controls (4.5–6.0 µg/lane) were prepared with lithium dodecyl sulphate (LDS) sample buffer and dithiothreitol (DTT) reducing agent. Separation was performed for 40 min at 200 V in 2-(N-morpholino) ethane sulphonic acid (MES) SDS running buffer, on 4–12% Bis-Tris pre-cast gels and with SeeBlue® Plus2 prestained reference standard (all from Invitrogen). Proteins were visualised using Simply Blue Coomassie brilliant blue R-250 staining (Invitrogen), and the blots scanned.

Immunoblotting

Hydrolysates of semi-skimmed and UHT milks for time points 0, 1, 5, 10 and 15 min (4.5–6.0 µg/lane), together with casein, β-LG and equine myoglobin as negative controls (all 5 µg/lane; Sigma-Aldrich) were electrophoretically separated as described above. The proteins were then electrophoretically transferred (30 V, 1h, RT) using a XCell II Blot Module (Invitrogen) onto Protran BS83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Tris-buffered saline/0.05% Triton X-100 (Roth, Karlsruhe, Germany) was used as wash buffer (3 × 15 min), between all incubation steps. The same buffer with the addition of 5% horse serum (Gibco, Paisley, Scotland, UK) was used as blocking and assay buffer. All incubations were at RT, with gentle rocking, with the exception of incubations with patient sera or chicken polyclonal anti-β-LG antibody (a kind gift from AgResearch, Ruakura, NZ) which were at 4°C. After blocking for 1 h, membrane strips were incubated overnight with patient sera (1:20) or anti-β-LG antibody (1:2000). A serum with no detectable CMP-specific IgE (CAP <0.35 KU/L) served as negative control and 250 ng/ml human IgE myeloma protein (Calbiochem, La Jolla, CA, USA) was added to this serum to provide a positive IgE-control. Blots were then incubated, respectively, with rabbit anti-human IgE (1.5 h, 1:7500; DakoCytomation, Glostrup, Denmark), followed by goat anti-rabbit IgG-horse-radish peroxidase (HRP) conjugate (45 min, 1:10,000; Zymed, San Francisco, CA, USA) or with rabbit anti-chicken-HRP (45 min, 1:5000; Sigma-Aldrich). The enzyme reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB; Zymed), the blots were scanned and band intensities were measured using the Gel-Pro® Analyzer-program (MediaCybernetics, Silver Spring, MD, USA). Bands corresponding to the three casein fractions and β-LG had been confirmed using chicken polyclonal anti-α-, β- and κ-casein and anti-β-LG antisera (AgResearch), as described previously (Sletten, Halvorsen, Egaas, & Halstensen, 2007).

IgE ELISA inhibition

Phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK) containing 0.05% Tween-20 (Sigma-Aldrich) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) was used as assay/blocking buffer. The high CMP-specific IgE titre serum pool

(50 μ l) diluted in assay buffer (1:40 or 1:20 for binding inhibition to total casein or β -LG, respectively) was preincubated overnight at 4°C with 50 μ l of five- or 10-fold serially diluted milk hydrolysate (time points 0, 1, 5, 10 and 15 min) in assay buffer. High-binding ELISA plates (Corning, NY, USA) were coated overnight at 4°C, with 100 μ l of 0.05 M carbonate/bicarbonate buffer, pH 9.6 (Sigma–Aldrich) containing 2 μ g/ml of the respective caseins or 20 μ g/ml β -LG, washed three times in 200 μ l PBS–Tween 0.05% and then blocked for 1 h at RT with 150 μ l assay/blocking buffer. After washing, 100 μ l triplicates of the inhibited sera (containing 100–0.032 μ g protein/ml for whole and skimmed milks or 100–0.001 μ g/ml for semi-skimmed and UHT milks) were added to the coated wells and incubated for 1 h at RT under gentle shaking. The wash step was repeated and 100 μ l of suitably diluted rabbit anti-human IgE (DakoCytomation) were added and incubated as above. After further washing, 100 μ l of goat anti-rabbit-HRP conjugate diluted 1:5000–10 000 (Zymed) were added and incubated as previously. After a final wash, wells were developed with 75 μ l K-Blue TMB (Neogen, Lexington, KY) as substrate. The reaction was stopped by the addition of 50 μ l/well of 2 M H_2SO_4 and absorbance read at 450 nm on a 1420 VICTOR₂ multilabel plate counter (Wallac, Turku, Finland). Percentage inhibition was calculated using the following formula: % inhibition = $100 - (OD_{450} \text{ with inhibitor} \times 100 / OD_{450} \text{ without inhibitor})$.

IgG ELISA inhibition

An affinity-purified polyclonal rabbit IgG anti-total casein antibody (Sletten, Lovberg, Moen, Skarpeid, & Egaas, 2005) and polyclonal chicken IgG(Y) antisera to α -, β -, and κ -caseins and β -LG (AgResearch) were used in an IgG inhibition ELISA. PBS/Tween + 1% BSA was used as assay/blocking buffer. Rabbit anti-casein (1:100 000) and chicken anti- β -LG (1:8000), both 50 μ l in assay buffer, were preincubated overnight at 4°C with 50 μ l of 10-fold serially diluted milk hydrolysates (time points 0, 1, 5, 10 and 15 min) in assay buffer, giving final protein concentrations of 100, 10, 1, 0.1 & 0.01 μ g/ml. Chicken anti- α -casein (1:5000), anti- β -casein (1:8000) and anti- κ -casein (1:4000), were similarly preincubated with 50 μ l of 10-fold serially diluted milk hydrolysates in assay buffer, giving final protein concentrations of 50, 5, 0.5, 0.05, 0.005 and 0.0005 μ g/ml. ELISA plates were coated with 100 μ l of 0.05M carbonate/bicarbonate buffer, containing 2 μ g/ml total casein, β -LG, α -, β - or κ -casein and incubated overnight at 4°C. PBS, pH 7.6, containing 0.05% Tween-20, was used as wash buffer. The plates were washed, then blocked for 1 h at RT with 150 μ l assay/blocking buffer. The wash step was repeated and 100 μ l of inhibited antiserum were added to the wells and incubated for 1 h at RT under gentle shaking. After further washing, 100 μ l of goat anti-rabbit-HRP conjugate (Zymed) or rabbit anti-chicken-HRP conjugate (Sigma-Aldrich), appropriately diluted, were added and incubated as above. The final wash, colour development with TMB substrate and calculation of percentage inhibition and IC_{50} were performed as previously described. The concentration required for 50% inhibition of IgG binding (IC_{50}) was also calculated from the graphs.

Results

Protein composition of milk hydrolysates

The protein bands visualised by SDS-PAGE analysis of the four milk hydrolysates (from skimmed, semi-skimmed, whole and UHT milk) are shown in Figure 1. The 0 min milk samples showed bands corresponding in size to α_{s2} -casein (faintly at 25.2 kDa), the most

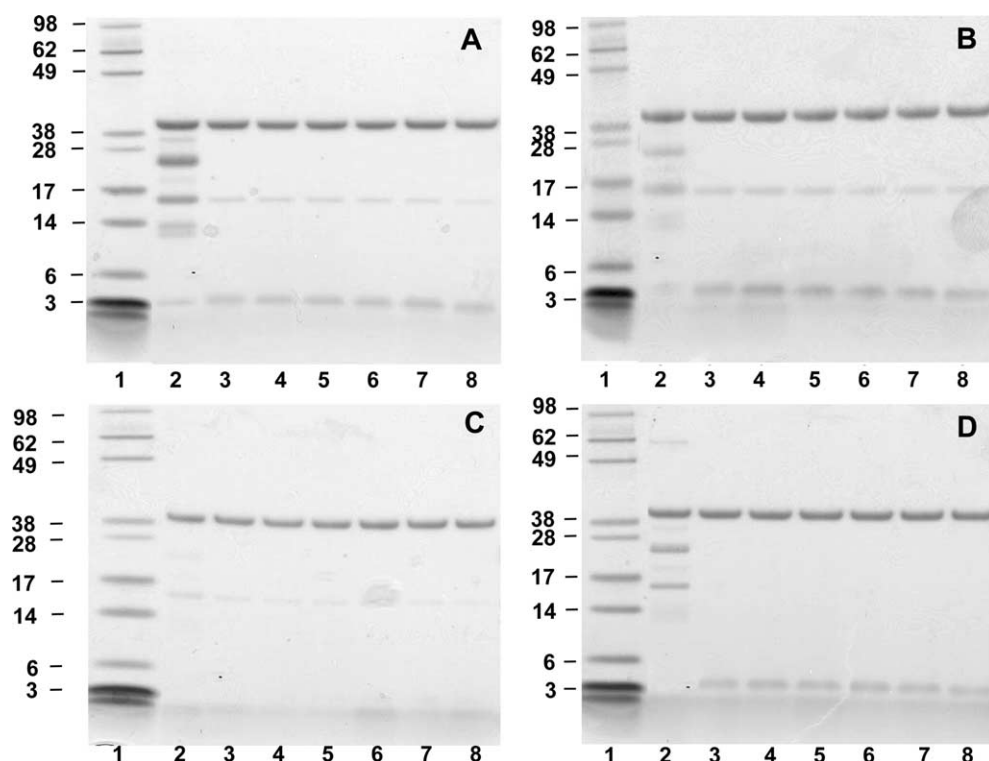


Figure 1. SDS-PAGE of hydrolysed milks run under reducing conditions: (A) skimmed milk; (B) semi-skimmed milk; (C) whole milk; (D) UHT milk. The samples were digested using SGF for 0, 1, 5, 10, 15, 30 or 60 min. Lane 1: mol. weight standard; lane 2: 0 min; lane 3: 1 min; lane 4: 5 min; lane 5: 10 min; lane 6: 15 min; lane 7: 30 min; lane 8: 60 min. Pepsin can be seen as a strongly-stained band at approx. 40 kDa.

abundant α_{s1} - and β -caseins (close together at 24 and 23.6 kDa), κ -casein (faintly at 19 kDa), β -LG (18 kDa) and α -lactalbumin (14 kDa). The SeeBlue Plus2 molecular standard gave only an approximation of the actual molecular size of the proteins. In general, the casein bands were not visible following onset of digestion in all the milk types. β -LG bands at all six digestion time points, as well as the 0 min control, were visible in skimmed, semi-skimmed and whole milks but not in UHT milk (Figure 1) but a slight reduction in band intensity may suggest modest β -LG degradation in the first 5 minutes in all four milk types. Subsequently, the band intensity for β -LG appeared to remain stable, with the exception of in UHT milk, where degradation apparently continued. The decrease in the casein and β -LG bands was accompanied by the appearance of diffuse protein bands at approximately 3 kDa, which may reflect the presence of degradation products (Figure 1).

IgE-binding patterns to hydrolysates of semi-skimmed and UHT milks with immunoblotting

Hydrolysates of semi-skimmed and UHT milks were immunoblotted and developed using the high CMP-specific IgE titre serum pool. Protein bands corresponding in size to the caseins (19–25 kDa), β -LG (18 kDa) and α -lactalbumin (14 kDa) were observed, as well as

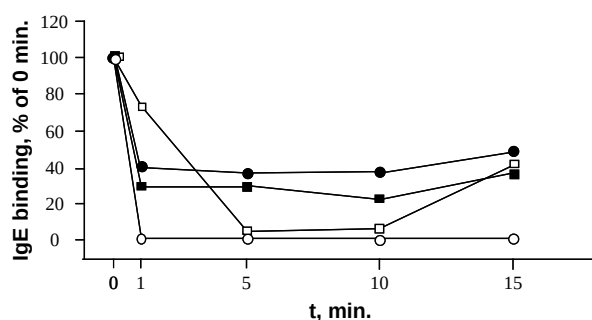


Figure 2. Immunoblot of hydrolysed milks, using a milk-allergic patient serum pool ($n=21$). Band intensities for casein and β -LG in the immunoblot were measured using Gel-Pro software and are expressed as % of intensity at 0 min for β -LG or total casein. Semi-skimmed milk: ●: β -LG; ○: casein; UHT milk: ■: β -LG; □: casein.

bands at 67 kDa, corresponding in size to BSA, and which had not been previously observed in SDS-PAGE (data not shown).

The serum pool IgE-binding intensities for the β -LG bands (Figure 2), measured using Gel-Pro, showed a sharp decrease at 1 min in both semi-skimmed and UHT milks, and then remained relatively stable thereafter, ranging from 37–48% of the 0 min level in semi-skimmed milk, and from 22–36% of the 0 min level in UHT milk. The IgE-binding intensities for total casein (the sum of α -, β - and κ -caseins) behaved similarly, decreasing sharply at 1 min and remaining stable, at between 0.6 and 1.2% of the 0 min level. However, the pattern for UHT milk differed somewhat; a relatively strong binding intensity of 74% of the 0 min level was measured at 1 minute, thereafter declining to 5–6% of the 0 min level at 5 and 10 minutes, so apparently increasing to 40% of the 0 min level at 15 minutes.

IgE-binding to the hydrolysates varied considerably in immunoblots of the individual sera of 8 allergic patients (data not shown). The Gel-Pro analysis of the IgE-

Table 2. Inhibitor concentration in $\mu\text{g/ml}$ required for 50% inhibition (IC_{50}) of IgE-binding: (a) to β -lactoglobulin.

Digestion (min)	Skimmed milk	Whole milk	Semi-skimmed milk	UHT milk
0	6.3	3.0	1.6	5.0
1	1.1	1.2	0.9	4.1
5	n.d.	n.d.	1.4	6.9
10	1.6	2.3	0.6	2.2
15	n.d.	n.d.	1.0	5.4

(b) to casein.

Digestion (min)	Skimmed milk	Whole milk	Semi-skimmed milk	UHT milk
0	0.03	0.02	0.04	0.09
1	31	7.7	50	0.22
5	n.d.	n.d.	67.	48
10	71	34	65	47
15	n.d.	n.d.	62	76

binding intensities from the patient sera showed a similar trend to that observed in the serum pool, i.e. low levels of residual casein-binding activity after 1 minute of digestion in semi-skimmed milk (1–13% of 0 min level, in 5 out of 8 sera), and in three sera after 15 min. In contrast, levels of residual casein-binding at 1 minute were considerably higher for UHT milk compared to semi-skimmed milk in all eight sera (four $\geq 100\%$, four between 15 and 90%) and in seven of the sera, binding activity was seen at 15 min. None of the patient sera had IgE-binding to the negative control myoglobin and none of the control sera or the myeloma IgE-spiked control serum exhibited any IgE-binding.

IgE ELISA inhibition of binding to β -LG and casein

The IC_{50} of the milk hydrolysates for inhibition of IgE-binding to β -LG was remarkably stable in all four milk types, even after 15 minutes (Table 2(a)). IgE-antibody reactivity to β -LG was demonstrated at a similar level in all milk types and all time points. In contrast, the IC_{50} for IgE-binding to total casein is far less stable in all four milk types (Table 2(b)). At one minute of digestion, the IC_{50} for skimmed and semi-skimmed milk had increased over 1000-fold, and by nearly 400-fold in whole milk, indicating a large loss in allergenic activity. This increase in required inhibitor was maintained throughout the other time points. In UHT milk, however, IC_{50} had only increased by 2.5 times at 1 minute. This was no longer the case at 5, 10 or 15 minutes minutes, when the IC_{50} had increased at least 500-fold (Table 2(b) and Figure 3).

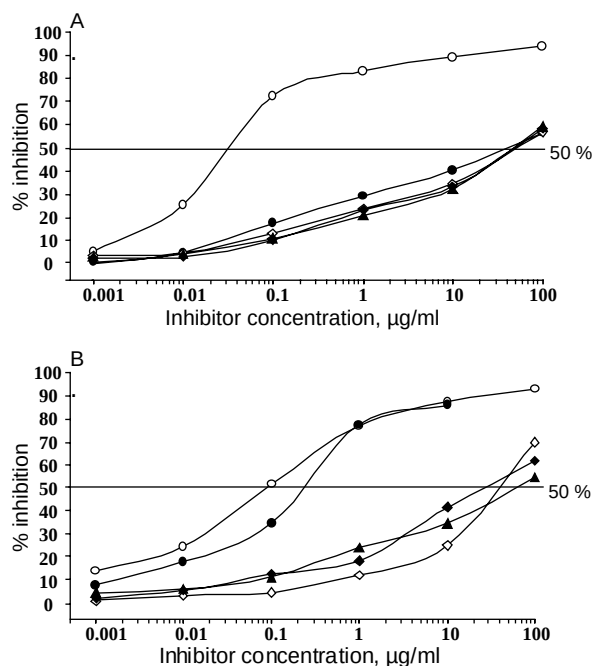


Figure 3. Inhibition of IgE-binding to casein by: (A) semi-skimmed milk and (B) UHT milk hydrolysates. The milk samples were digested in simulated gastric fluid (SGF) for 0 min: $\circ$; 1 min: $\bullet$; 5 min: $\diamond$; 10 min: $\blacklozenge$; 15 min: $\blacktriangle$. Range of SEM for triplicate values for semi-skimmed milk at 0 min: 0.0–3.0; 1 min: 0.2–0.8; 5 min: 0.1–1.1; 10 min: 0.2–1.6; 15 min: 0.3–1.6. SEM for triplicate values for UHT milk at 0 min: 0.0–1.0; 1 min: 0.1–1.4; 5 min: 0.0–1.1; 10 min: 0.2–1.0; 15 min: 0.2–1.2.

Table 3. Inhibitor concentration in $\mu\text{g/ml}$ required for 50% inhibition (IC_{50}) of: (a) anti- β -LG IgG(Y) binding to β -LG in inhibition ELISA.

Digestion (min)	Skimmed milk	Whole milk	Semi-skimmed milk	UHT milk
0	1.5	1.9	0.3	0.5
1	2.1	2.2	0.7	1.0
5	1.4	3.0	0.9	1.8
10	1.6	2.8	0.8	1.3
15	2.1	2.2	0.9	2.7

(b) Anti-total casein IgG binding to casein^a.

Digestion (min)	Skimmed milk	Whole milk	Semi-skimmed milk	UHT milk
0	3.6	1.9	3.5	3.0
1	158	148	80	11
5	204	212	135	170
10	333	311	162	219
15	458	304	219	363

^aWhere $\text{IC}_{50} >$ the maximum inhibitor concentration of 100 $\mu\text{g/mL}$, the values were estimated from the curves.***IgG(Y)-binding patterns to hydrolysates in immunoblots***

Consistent staining for anti- β -LG antibody binding to β -LG was seen in all four milk types, irrespective of time point and changes in total protein band intensity (data not shown). In addition, diffuse staining approximately between 18 kDa and 28 kDa was observed in semi-skimmed milk at all time points, in particular at 0 min. UHT milk also showed the same diffuse staining from 18–28 kDa, at 0 min and weakly at 1 min. Binding intensities measured using Gel-Pro confirmed the relative stability of β -LG in hydrolysed skimmed, semi-skimmed and whole milks, whereas a decrease of around 40% was seen in UHT milk (data not shown).

IgG ELISA inhibition of binding to immobilised β -LG and casein

The hydrolysate concentrations required to inhibit polyclonal anti-casein and β -LG antibody binding to immobilised β -LG and casein, by 50% (IC_{50}) were calculated for each series of hydrolysates at 0, 1, 5, 10 and 15 minutes (Tables 3(a) and (b)). The data illustrate the relatively similar IC_{50} for binding to β -LG for all digestion time points in skimmed and whole milks (Table 3(a)). The IC_{50} increased only slightly from 0–1 min in semi-skimmed milk and remained stable thereafter (Figure 4A), whereas the IC_{50} for UHT milk, although relatively stable for the first few minutes, continued to increase to 5 times at 15 minutes, as seen in Figure 4B. The IC_{50} for inhibition of binding to casein increased dramatically from 0 to 1 minute, thereafter to increase steadily after 5, 10 and 15 minutes in skimmed, whole and semi-skimmed milks (Table 3(b)). However, in UHT milk, the IC_{50} increased only slightly from 0–1 min, but then increased more rapidly from five minutes onwards. Curves showing the inhibition of binding to casein by semi-skimmed and UHT milks suggested that the casein in UHT milk appeared to be less rapidly hydrolysed than in semi-skimmed milk (Figures 5A and B).

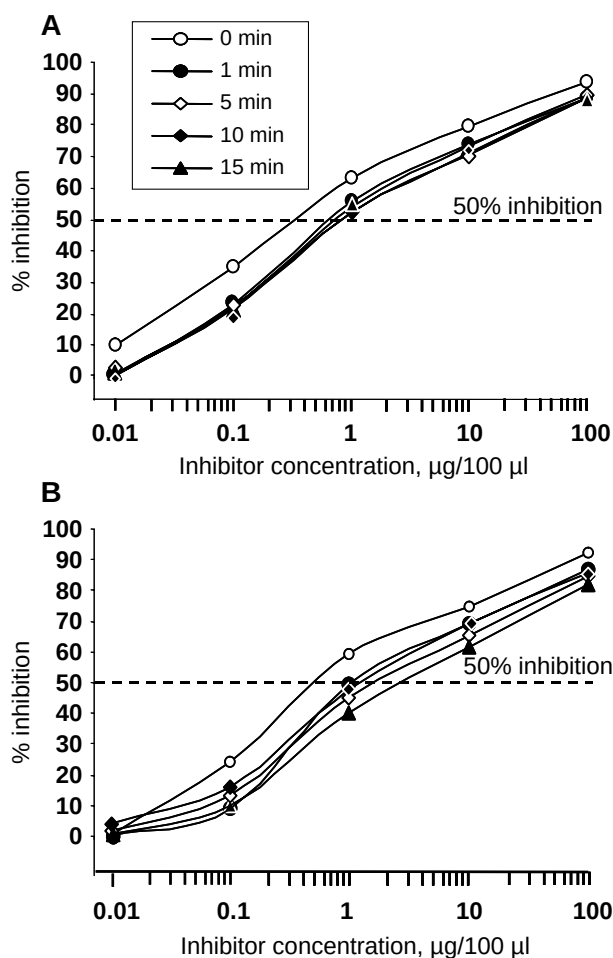


Figure 4. Inhibition of IgG binding to β -lactoglobulin by: (A) semi-skimmed milk and (B) UHT milk hydrolysates. The milk samples were digested in simulated gastric fluid (SGF) for 0 min: ○; 1 min: ●; 5 min: ◇; 10 min: ◆; 15 min: ▲. Range of SEM for triplicate values for semi-skimmed milk at 0 min: 0.2–2.4; 1 min: 0.4–1.0; 5 min: 0.2–1.4; 10 min: 0.2–1.7; 15 min: 0.7–1.5. SEM for triplicate values for UHT milk at 0 min: 0.1–2.8; 1 min: 0.3–2.9; 5 min: 0.4–4.4; 10 min: 0.3–1.9; 15 min: 0.2–4.9.

IgG ELISA inhibition of binding to α -, β and κ -caseins

The hydrolysate concentrations required to inhibit polyclonal anti- α -, β - and κ -casein antibody binding to immobilised antigen by 50% (IC_{50}) were calculated as previously described for semi-skimmed and UHT milk hydrolysates (Figure 6). The inhibitor concentrations for the three casein fractions varied, being considerably higher for κ -casein, compared with α - and β -casein. The IC_{50} for α - and β -casein in semi-skimmed milk increased, respectively, from 7 to 52 $\mu\text{g}/100\ \mu\text{l}$ (α -casein) and 11 to 48 $\mu\text{g}/100\ \mu\text{l}$ (β -casein) between 0 and 1 minute, then remained at the same level thereafter. In UHT milk, the IC_{50} for α - and β -casein at 0 minute were 6 and 13 $\mu\text{g}/100\ \mu\text{l}$, respectively, increasing only slightly at 1 minute, but thereafter increasing seven- to 10-fold between 5 and 15 minutes. The pattern differed for κ -casein: in semi-skimmed milk, at 0 minute the IC_{50} was 137 $\mu\text{g}/100\ \mu\text{l}$,

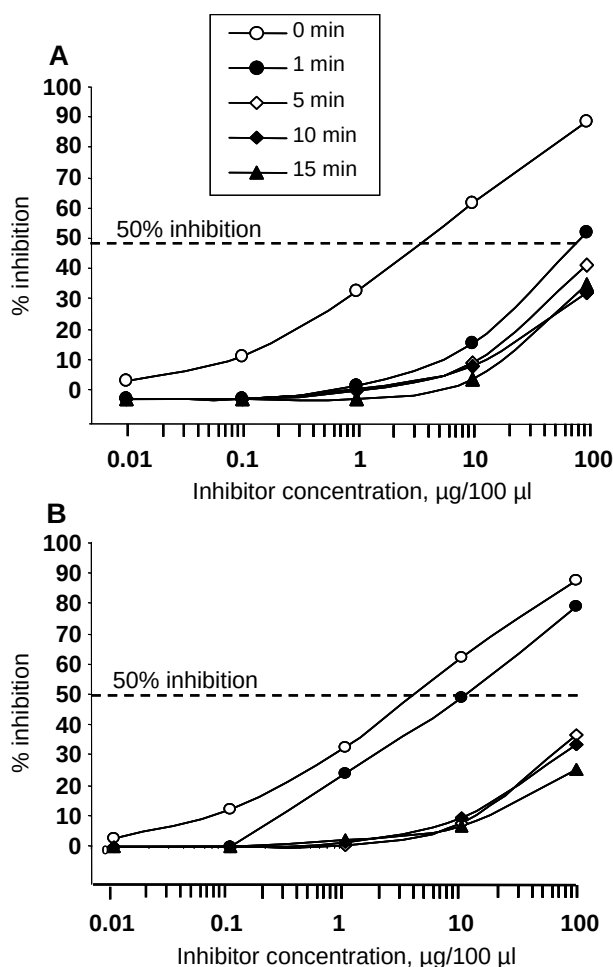


Figure 5. Inhibition of IgG binding to casein by: (A) semi-skimmed milk and (B) UHT milk hydrolysates. The milk samples were digested in simulated gastric fluid (SGF) for 0 min: ○; 1 min: ●; 5 min: ◇; 10 min: ◆; 15 min: ▲. Range of SEM for triplicate values for semi-skimmed milk at 0 min: 0.5–2.4; 1 min: 0.4–2.1; 5 min: 0.4–1.4; 10 min: 0.4–4.6; 15 min: 0.4–2.2. SEM for triplicate values for UHT milk at 0 min: 0.2–1.4; 1 min: 0.3–1.3; 5 min: 0.5–2.2; 10 min: 0.2–2.7; 15 min: 0.3–3.2.

remaining almost unchanged at 1 minute, and thereafter increasing gradually to 330 μg/100 μl at 15 minutes. The IC_{50} for UHT milk was considerably less at 0 minute (55 μg/100 μl), increasing gradually to 265 μg/100 μl at 15 minutes. The results are presented here as the relative increase in IC_{50} compared to 0 minute (Figure 6).

Discussion

The apparent decrease in protein band intensities following digestion, shown by electrophoretic separation of the hydrolysed proteins, suggested that casein was rapidly degraded in hydrolysates from all four milk types. Only small amounts of residual protein remained after 1 minute, which is in agreement with earlier findings on pepsin digestion of

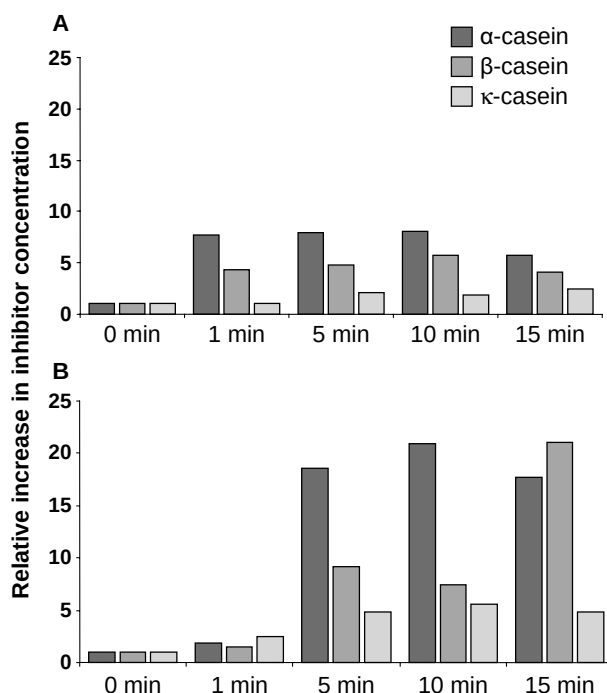


Figure 6. Relative increase in inhibitor concentration in $\mu\text{g/ml}$ required for 50% inhibition (IC_{50}) of IgG binding to α -, β - or κ -casein by: (A) semi-skimmed milk and (B) UHT milk hydrolysates. The milk samples were digested in simulated gastric fluid (SGF) for 0, 1, 5, 10 and 15 min.

casein (Astwood et al., 1996). Proteolytic degradation of casein also resulted in a decrease in specific IgE-binding, observed in both immunoblot and inhibition ELISA, using a cow's milk-allergic serum pool. This could be clearly seen in both semi-skimmed and UHT milks. However, binding activity was not totally abolished, but remained at a relatively low level thereafter. Immunoblots using individual patient sera showed considerable variation in IgE-binding patterns between subjects, presumably reflecting differences in the individuals' patterns of sensitisation to the various CMPs (Docena, Rozenfeld, Fernandez, & Fossati, 2002). However, there was a marked difference in the casein-specific IgE-binding patterns between the two products, consisting of a general trend to more persistent and somewhat higher levels of casein-specific IgE-binding in UHT milk following digestion, compared to semi-skimmed milk. The gradual reduction in casein-specific IgE-binding activity in UHT milk, shown by the more modest increase in IC_{50} , suggested that the UHT process has rendered the IgE epitopes more stable to digestion. It remains to be seen whether this apparent increased stability, shown using the peptic digestion model, is sufficient to be of clinical significance in milk-allergic subjects, and *in vitro* studies using effector cells, such as the basophil activation test, might be a logical next step in such an investigation.

The ELISA inhibition studies using a polyclonal antiserum to total casein showed that IgG epitopes in UHT milk are also less rapidly destroyed by proteolysis than those in semi-skimmed milk, further supporting a process-induced increase in digestive stability. Moreover, proteins do not need to be intact to induce allergic reactions and peptide fragments as small as 3 kDa have been shown to be sufficient to cross-link two IgE molecules on the mast cell membrane and activate IgE responses (Huby, Dearman, &

Kimber, 2000; van Beresteijn et al., 1994). Immunoblotting may be insufficiently sensitive to detect smaller immunogenic peptides, but inhibition ELISA has the advantage of detecting total antibody-binding. IC₅₀ data from the IgE inhibition ELISA, using a serum pool, suggested that even the more extensively hydrolysed fractions contained residual immunoreactive peptides, with IgE epitopes that are more resistant to pepsin digestion. This might explain reported residual IgE-binding activity and allergic reactions to hypoallergenic casein and whey hydrolysates (Docena et al., 2002; Restani et al., 1995). Sensitisation to casein, and subsequent allergic reactions in CMA patients on the ingestion of milk, clearly imply that casein is not totally degraded *in vivo* and it thus follows that milk-allergic individuals have IgE which probably recognises epitopes found in partially degraded milk. Immunoreactive IgE- and IgG-binding epitopes in the caseins have been characterised (Chatchatee et al., 2001b; Chatchatee, Jarvinen, Bardina, Beyer, & Sampson, 2001a). Interestingly, casein peptides have been found in the stomach, duodenum and blood of milk-consuming healthy subjects (Chabance et al., 1998) and a number of these peptides have sequences corresponding to IgE epitopes identified on α s1-, β - and κ -casein (Chatchatee et al., 2001a; Chatchatee et al., 2001b).

The apparent increase in casein-specific IgE-binding intensity in UHT milk at 15 minutes of digestion suggested the accumulation of larger amounts of IgE-binding protein at this time-point. This might reflect the exposure of κ -casein previously masked by denatured β -LG, which complexes to κ -casein at 70–90°C but subsequently dissociates at higher temperatures (Singh, 2004). The high molecular weight IgE-binding bands, at approximately 67 kDa, might indicate the presence of BSA-specific IgE. However, Docena et al. (2002) (Chatchatee et al., 2001a; Chatchatee et al., 2001b) reported similar bands, and concluded that BSA would be fully hydrolysed. Furthermore, they showed that these 67 kDa bands were casein aggregates, using casein-specific monoclonal antibodies, suggesting the formation of conformational IgE-binding epitopes on aggregation.

ELISA inhibition studies of the UHT and semi-skimmed milk hydrolysates, using chicken polyclonal antisera specific to the casein fractions, demonstrated unexpected differences between α - and β -casein, and κ -casein. In general, the inhibitor concentration was considerably higher for κ -casein, compared with α - and β -casein, but this probably reflected varying affinities of the three antisera for their respective antigens. The IC₅₀ for α - and β -caseins increased only moderately after one minute of hydrolysis in UHT milk, whereas the increase in IC₅₀ for semi-skimmed milk was considerably greater, suggesting a similar pattern of increased digestive stability following heat processing to that seen in total casein. After 5 minutes, however, the decrease in stability for UHT milk was approximately double that seen in semi-skimmed milk, in all three casein fractions. The IC₅₀ for β -casein increased substantially at 15 minutes of digestion, suggesting that it may be particularly susceptible to heat-induced changes in stability.

Surprisingly, the stability of κ -casein showed a somewhat different pattern following UHT processing. Although the IC₅₀ for κ -casein increased on proteolysis, the decrease in IgG(Y) reactivity with proteolysis time was more gradual than in α - or β -casein. However, the trend to decreased reactivity, showed by increasing IC₅₀, was considerably less in semi-skimmed milk compared to UHT milk. These results suggested that κ -casein may be more resistant to digestion in the context of the matrix, and that heat treatment impairs this resistance. One possible explanation for this reflects the structure of the micelle, in which casein sub-micelles contain either α - and β -caseins or α - and κ -caseins; those containing κ -casein are at the surface (Walstra, 1999). Heat treatment of milk at temperatures of 70–90°C may cause denaturation of whey proteins, exposing reactive groups such as free thiols. Resulting thiol–disulphide reactions with κ -casein may lead to

proteolytically more stable β -LG complexing with κ -casein at the surface of micelle, the latter thus being partially protected from the effects of proteolysis (Vasbinder, Alting, & de Kruif, 2003).

A further explanation of why κ -casein appears to be more resistant to proteolysis than α - and β -caseins, may be due to the presence of linear epitopes on κ -casein, which increase resistance to digestion. This has been shown to be the case for κ -casein-specific IgE, where a highly significant correlation between ELISA- and immunoblot-detected κ -casein-specific IgE values in CMA patients suggested that the dominant κ -casein IgE epitopes were linearised (Sletten et al., 2007). This is in agreement with linear κ -casein epitopes recognised by IgE from patients with IgE-mediated CMA (Chatchatee et al., 2001b). However, it remains to be confirmed whether IgG epitopes are also linear and whether this contributes to increased digestive stability in κ -casein.

β -LG in skimmed, semi-skimmed and whole milks was relatively stable for up to one hour of digestion, confirming earlier studies (Astwood et al., 1996). The apparently increased tendency to UHT milk degradation from 15 to 60 minutes seen in the present study suggested that the brief duration of heat processing in UHT milk is nonetheless sufficient to reduce digestive stability. Prolonged heat denaturation (one hour at 80°C) made purified β -LG more susceptible to pepsin hydrolysis (Kitabatake & Kinekawa, 1998). However, immunoblotting performed in the present study, using both CMA patient sera and a polyclonal antibody to β -LG, demonstrated similar levels of IgE/IgG(Y) binding activity in all four milk types and at all time points, indicating that the protein has retained a high degree of its immunogenic capacity after hydrolysis, and that the degree of processing appeared to have little effect on the stability of the IgE or IgG epitopes. Inhibition ELISA studies of IgE and IgG(Y) binding to β -LG in semi-skimmed and UHT milks confirmed antibody-binding stability after hydrolysis in two milk types of similar composition but differing in subsequent processing. These observations offer further support to the presence of predominantly linear epitopes in β -LG.

UHT milk is homogenised and sterilised (at 140°C for 1–2 sec), to destroy pathogens, but this brief period of heating apparently had limited consequences for specific IgE and IgG binding in the present study. It has been shown that the degree of heat-induced impairment of IgE-binding to β -LG varies with both time and temperature, and that allergenic β -LG epitopes are stable to at least 90°C (Ehn et al., 2004), suggesting that predominantly linear IgE epitopes are involved. This has been further supported by inhibition studies using synthetic peptides (Jarvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). However, heating to temperatures as high as 140°C may destroy the structure of β -LG, causing it to dissociate from κ -casein and micelle stability to be correspondingly reduced (Singh, 2004). Prolonged heat treatment at 90°C inhibited IgE-binding to milk (Ehn et al., 2004), and boiling milk for at least 10 min gave a marked reduction (casein) or loss (β -LG) of IgE-binding activity (Besler et al., 2001).

Varying levels of fat in the matrices of three milk types, which had been processed similarly, did not appear to influence digestibility, as demonstrated using SDS-PAGE. No differences were observed in the digestion patterns for either β -LG or casein in skimmed, semi-skimmed or whole milk hydrolysates, and neither did the binding patterns obtained using an anti- β -LG polyclonal antibody in immunoblots differ. This is in agreement with a previous study, where no difference in levels of inhibition of IgE-binding to skimmed and whole milks was seen, using a CMA patient serum pool (Ehn et al., 2004). However, β -LG and β -casein have been shown to interact with lipids in model gastric emulsions, and when adsorbed to emulsion interfaces, appear to resist pepsin digestion (Burnett et al., 2002). It is therefore surprising that whole milk hydrolysates, containing nearly 4% fat, did not show

any differences compared to skimmed milk, with only 0.1% fat. Interaction with lipids has not been shown to influence casein's resistance to digestion, whereas processing had a modest effect (Dupont, Mackie, & Mills, 2007). In general, interactions between allergens and other food components, are poorly understood, in particular their effects on the allergenicity of food proteins (Breiteneder & Mills, 2005).

In conclusion, heat processing, but not differences in the matrix fat content, affected patterns of proteolytic degradation in total casein, and the casein fractions α -, β - and κ -casein. Heat-induced changes, such as those found in UHT milk, rendered both IgE and IgG epitopes more stable to digestive proteolysis. Native κ -casein in the casein micelle may be initially more resistant to digestion than α - or β -casein, but heat treatment eliminated the difference in stabilities between the fractions after five minutes. Heat treatment had little effect on the immunogenicity of β -LG; a high degree of reactivity to both IgE and IgG is retained in both processed (UHT) and non-processed (semi-skimmed) milks after hydrolysis, supporting the suggestion of presence of predominantly linear epitopes in β -LG. Heat processing alone, however, appears to have little effect on specific IgE or IgG binding. The clinical significance of these findings remains to be established.

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