

Raising the pH of the pepsin-catalysed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates

D. G. SCHMIDT, R. J. G. M. MEIJER*, C. J. SLANGEN and E. C. H. VAN BERESTEIJN*

Department of Biophysical Chemistry and *Department of Nutrition, Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands

Summary

Background Hypersensitivity to cow milk protein is frequently observed in infancy. Since the pH in the infant's stomach is relatively high (pH 3–4) compared with adults (pH 2), an incomplete digestion of the milk proteins is expected to occur.

Objective The determination of the degree of hydrolysis by pepsin of the four main proteins of bovine whey, i.e. α -lactalbumin (α La), β -lactoglobulin (β Lg), bovine serum albumin (BSA) and bovine immunoglobulin G (B-IgG), in the pH range 2.0–4.0 and of the antigenic properties of the resulting hydrolysates.

Methods Whey proteins were successively hydrolysed with pepsin at pH values ranging from 2.0 to 4.0 and with pancreatic enzymes at pH 7.5 using a pH-stat. The resulting hydrolysates were characterized by their degree of hydrolysis, and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, gel permeation chromatography and immunologically by competitive enzyme-linked immunosorbent assay.

Results In general, the degree of hydrolysis, the gel electrophoretic patterns, the contents of peptides of molecular mass >5 kDa and the residual human-immunoglobulin E and G antigenicities of the hydrolysates did not differ much whether the pepsin incubation was done at pH 2.0 or 3.0. Pepsin incubation at pH 4.0, however, resulted in a decreased hydrolysis and enhanced residual antigenicity of α La, BSA and B-IgG, but not of β Lg.

Conclusion The poor and slow degradation of the antigenic epitopes of whey proteins when pepsin digestion occurs under conditions that prevail in the stomach of infants could be of much importance for the development of cow milk hypersensitivity. The immature gastrointestinal mucosal barrier of infants allows large antigenic fragments of these proteins to pass into the systemic circulation.

Keywords: α -lactalbumin, β -lactoglobulin, bovine serum albumin, bovine immunoglobulin G, pepsin, pancreatic enzymes, antigenicity

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Introduction

Most whey proteins from bovine milk are readily degraded to peptides under the influence of pepsin at

pH 2. Such a pH prevails in the fasted stage in the stomach of healthy adult humans, but it increases to values around 3 after food intake [1]. Beta-lactoglobulin (β Lg), however, largely resists proteolysis by pepsin under such conditions [2–5]. This may lead to the appearance of intact β Lg in the duodenum, where its degradation by the pancreatic enzymes may be too slow

Correspondence: Dr E. C. H. van Beresteijn, Netherlands Institute for Dairy Research (NIZO), PO Box 20, 6710 BA Ede, the Netherlands.

to prevent the absorption of large peptides, and even of the intact protein, through the intestinal epithelium. This is particularly true for infants shortly after birth, in whom the immature gastrointestinal tract is more permeable to large molecules than in adults [6–8].

When foreign large molecules, particularly large peptides and intact proteins, enter the circulation they will give rise to immunological responses which, in a number of individuals, may lead to allergic reactions. Cow milk allergy is indeed a well-known disease, occurring in about 2–7% of the newborns [9,10]. This allergy was initially ascribed to the presence in bovine milk of β Lg, a protein which is absent in human milk [11]. Gjesing *et al.* [12], however, showed that antibodies against all major milk proteins are circulating in the serum of patients suffering cow milk allergy, an observation confirmed by Otani *et al.* [13,14].

The conditions in the stomach of infants are not favourable for optimal activity of pepsin. In general the pH in the stomach of infants up to 2 months old is 3–4, a value which increases after food intake; even pH values above 6 have been reported [15,16]. This is remote from the pH optimum of pepsin, which is about 2 for most proteins, although denaturation of the substrate protein would shift this optimum to higher pH values [17]. It might therefore be expected that proteolysis by pepsin in the stomach of infants is less efficient than in that of adults, which enhances the risk of transferring intact proteins and large peptides to the circulation and consequently increases the risk of developing immunological and allergic responses.

In this paper we describe the proteolysis of the major bovine whey proteins, α -lactalbumin (α La), β Lg, bovine serum albumin (BSA) and bovine immunoglobulin G (B-IgG) under simulated physiological conditions. The proteins were successively hydrolysed with pepsin at pH 2.0, 3.0 or 4.0 and with Corolase PP[®], a mixture of pancreatic enzymes, at pH 7.5. The hydrolysates were characterized by physico-chemical and immunological techniques. The results of these analyses are discussed with respect to a possible relationship with the development of cow milk allergy.

Materials and methods

Patient serum samples

We obtained sera from 215 children (2 weeks–3.5 years) and five adults (20–35 years) of either sex, suspected of cow milk allergy, by kind donations from the Departments of Paediatrics of the Academic Hospital of the Free University, Amsterdam, and of the Wilhelmina Children's Hospital, Utrecht, the Netherlands. For this

study we selected serum of patients with a history of immediate reactions to cow milk (skin and/or gastrointestinal symptoms, urticaria and elevated milk-specific immunoglobulin E (IgE) antibody concentrations (IgE-Fast class ≥ 3 ; cow milk fluorescence allergosorbent test, IgE Fast-Plus[®] test, 3M Diagnostic Systems Inc., Santa Clara, CA, USA). In addition a positive elimination/provocation test for the children's sera, for the adults a positive skin-prick test (weal ≥ 3 mm) and an incidental positive provocation. These sera were tested for specific IgE and IgG binding to individual milk proteins by indirect enzyme-linked immunosorbent assay (ELISA). In this experiment we used the following patient sera (AS) for the immunological characterization of the whey protein hydrolysates: AS I, an adult serum with high IgE antibody titre to α La, β LgA and B, AS II, an adult serum with high IgG antibody titre to these proteins, and AS III, consisting of pooled sera from five children with high IgE and immunoglobulin G (IgG) antibody titres to BSA and B-IgG. Sera collected from eight individuals not allergic to milk proteins and with negative milk-specific IgE-FAST were pooled and served as a normal control serum (NCS). The serum samples were stored at -20°C until further analysis.

Whey proteins and enzymes

Preparations of α La and of the genetic variants A and B of β Lg were purified from cheese whey by chromatography as described before [5]. We only used the A-variant of β Lg because preliminary experiments had shown that the antigenic behaviour of the two genetic variants did not differ. BSA and B-IgG were purchased from Sigma Chemicals (St Louis, MO, USA), A-4378 and I-5506 respectively. Pepsin was also from Sigma (P-7000); its activity was 550 proteinase units/mg (substrate haemoglobin). Corolase PP[®], a mixture of enzymes from porcine pancreas, was purchased from Röhm (Darmstadt, Germany); its activity was 350 proteinase units/mg (substrate casein). For a definition of the proteinase units and the determination of enzyme activities see the product information sheets from Röhm [18,19].

Enzymatic hydrolysis of whey proteins

The proteins were dissolved in distilled water and adjusted with 0.5 N HCl to pH 2.0, 3.0 or 4.0 for the incubation with pepsin. The volume was 20 mL, the protein concentration 7 g/L and the temperature 37°C . At zero time pepsin was added at a concentration of 20 mg/g protein. The pH was kept constant by the addition of 0.1 N HCl via a pH-stat. Aliquots of 1.5 mL were taken out of the reaction mixture at zero time and 30 and 90 min after the

addition of the pepsin. Enzyme inactivation in the samples was done by raising the pH to 7–8 with 1 N NaOH. After 90 min the remainder of the hydrolysis mixture was brought to pH 7.5 with 4 N NaOH and Corolase PP was added at a concentration of 40 mg/g protein. During the following hydrolysis the pH was kept constant at 7.5 by the addition of 0.1 N NaOH via the pH-stat. Aliquots of 1.5 mL were taken out after 10, 30, 60 and 150 min. The enzyme in these samples was inactivated by heating for 5 min at 95°C in a waterbath, after which the samples were cooled to room temperature with running tap water and sodium azide (0.02%) was added to all samples as a preservative.

After the last aliquots had been taken (total time of hydrolysis 240 min), the remaining reaction mixtures, about 10–11 mL, were likewise heated for 5 min at 95°C and, after cooling, subjected to ultrafiltration through an Omega 3000 Da filter (FILTRON, Northborough, MA, USA). The permeates were used without further treatment. The retentates (about 0.5 mL) were diluted threefold with distilled water. A portion of each sample was stored at –20°C until further analysis. For the *in vitro* determination of the antigenic properties the samples were used immediately without freezing, to avoid possible formation of protein aggregates.

When the hydrolysis of B-IgG with pepsin was done at pH 3.0 or 4.0 a precipitate was formed during subsequent heating at 95°C. In these samples the hydrolysis apparently has not proceeded far enough or, in contrast to the hydrolysis at pH 2.0, heat-insoluble peptides have been formed. Therefore these samples were either cooled to 4°C for direct determination of the antigenic properties or immediately frozen at –20°C.

In order to determine whether the heat treatment alone influenced the antigenicity of the proteins, solutions of the individual whey proteins were heated for 5 min at 95°C without enzymes.

Physicochemical characterization of the hydrolysates

The average degree of hydrolysis of the whey proteins in each hydrolysate was calculated from the increase of the number of primary aminogroups compared with that of unhydrolysed protein. We determined this number by the *O*-phthaldialdehyde (OPA) method [20], using *N,N*-dimethyl-2-mercaptoethyl-ammonium chloride instead of 2-mercaptoethanol [21].

The hydrolysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Phast system and high density gels (Pharmacia, Uppsala, Sweden). The total protein concentration in the samples applied to the gels was approximately 2 g/L. Two standard protein mixtures, Bio-Rad LMW standard

(Bio-Rad, Richmond, CA, USA) and BDH-44247 (BDH Laboratory Supplies, Poole, Dorset, UK) were used as molecular mass markers. After electrophoresis the proteins and peptides were stained with silver as described in the Phast GelTM Silver Kit Instruction Manual. This method is 100 times more sensitive than Coomassie blue staining.

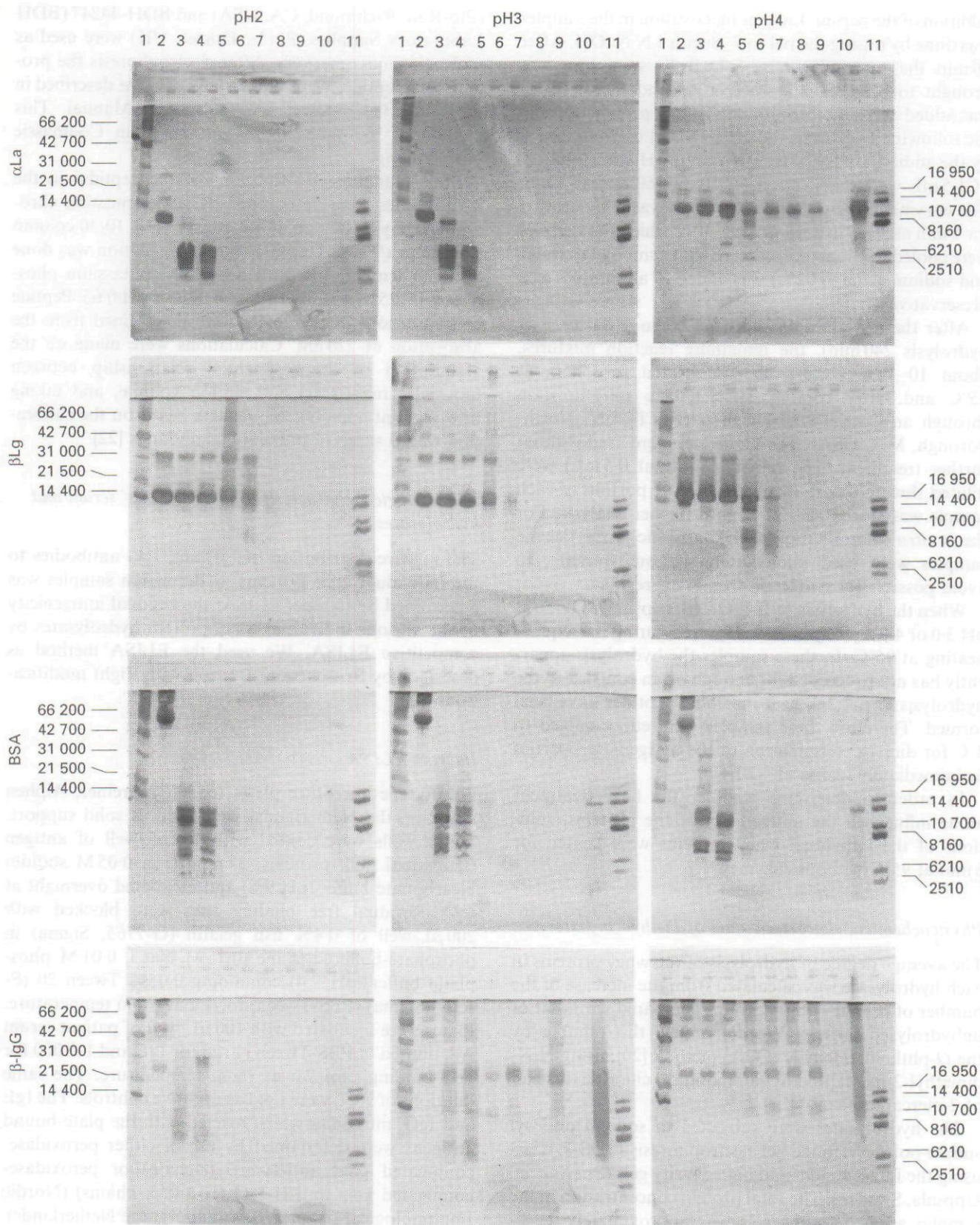
Molecular mass distributions of the peptides in the hydrolysates were determined by gel permeation chromatography (GPC) on a Superdex-75 HR 10/30 column (Pharmacia/LKB, Uppsala, Sweden). Elution was done at room temperature with a 0.125 M potassium phosphate–0.125 M sodium sulfate buffer of pH 6.65. Peptide concentrations in the eluate were determined from the absorption at 220 nm. Calculations were made on the assumption of an exponential relationship between molecular mass (M_r) and elution volume, and taking into account apparent M_r -regions based on the calibration with a series of reference compounds [22].

Immunological characterization of patients' serum and hydrolysates

The relative distribution of IgE and IgG antibodies to the individual milk proteins in the serum samples was determined by indirect ELISA; the residual antigenicity of the various individual whey protein hydrolysates by competitive ELISA. We used the ELISA method as described by Fritsché and Bonzon with slight modifications [23].

Indirect ELISA

Polystyrene microtitre plates (655061, Greiner, Alphen a/d Rijn, the Netherlands) were used as solid support. Single wells were coated with 135 µL/well of antigen (individual milk proteins, 37 µg/mL in 0.05 M sodium bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Residual free binding sites were blocked with 200 µL/well of 0.4% fish gelatin (G-7765, Sigma) in phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4) containing 0.05% Tween 20 (P-1379, Sigma) (PBS-Tween) for 1 h at room temperature. Plates were incubated with 100 µL/well of patient serum AS, diluted in PBS-Tween (1 : 25 for IgE and 1 : 1000 for IgG binding), for 2 h at room temperature. The same dilutions of NCS were used as negative controls. The IgE and IgG antibodies which reacted with the plate-bound antigens were determined by use of either peroxidase-conjugated goat anti-H-IgE (ϵ -chain) or peroxidase-conjugated goat anti-H-IgG (H and L chains) (Nordic Immunological Laboratories, Tilburg, the Netherlands). The anti-H-IgE and anti-H-IgG antibodies were diluted



5000-fold in PBS-Tween containing 0.4% fish gelatin and were incubated for 1 h at room temperature. As enzyme substrate a solution of freshly prepared *O*-phenylene-diamine dihydrochloride (P-1526, Sigma) 1 mg/mL in 0.05 M citrate, 0.1 M potassium phosphate buffer, pH 5.0, containing 0.012% hydrogen peroxide, was used. After incubating the plates for 30 min at room temperature, 100 μ L of 2 N sulfuric acid was added. Optical densities were read at 490 nm on an automated ELISA plate reader (Dynatech MR 7000, Trade Tek, Rotterdam, the Netherlands). ELISA determinations were carried out in duplicate and measurements averaged.

Competitive ELISA

Single wells of ELISA plates were coated with 135 μ L of α La, β Lg, BSA or B-IgG (15 μ g/mL in 0.05 M sodium bicarbonate buffer, pH 9.6). In 1.5 mL glass vials, serial ninefold dilutions of the hydrolysates in PBS-Tween were incubated overnight at 4°C with a constant amount of AS (1.5% for IgE or 0.05% for IgG binding). Residual unbound IgE and IgG antibodies were determined by the ELISA method described above by applying 100 μ L of preincubated AS to each well of the coated ELISA plates. The percentage of inhibition was calculated in relation to the maximum inhibition (vials containing NCS instead of AS) and the minimum inhibition (vials without hydrolysate). For this purpose an inhibitor concentration of 1 g/L was chosen, because that was the highest concentration at which differences in inhibition of the various hydrolysates still could be detected and also the lowest concentration at which an almost 100% inhibition for all individual whey proteins could be attained.

Reliability of the experiments

Since most patients suffering cow milk allergy are infants it is difficult to get sera with high concentrations of antibodies against the different individual milk proteins. The amount of serum made available to us was not sufficient to run duplicate experiments. Therefore we determined the precision of our analytical methods, as expressed by the repeatability standard deviation (S_r)

[24] from separate experiments. For our determination of the average degree of hydrolysis S_r was 0.99% (degrees of freedom [d.f.] = 48), for the molecular mass distribution S_r was 0.29% (d.f. = 40) and for the ELISA, including between-well and between-plate variation S_r was 1.77% (d.f. = 19).

Results

Pepsin incubation at pH 2.0 and 3.0; Corolase PP incubation at pH 7.5

The electrophoretic patterns of the α La, β Lg and BSA hydrolysates (Fig. 1) were roughly the same whether the pepsin hydrolysis was done at pH 2.0 or 3.0. It is clear that, in contrast to the other proteins, β Lg was barely hydrolysed by pepsin, as is confirmed by Fig. 2, showing the increase of the average degree of hydrolysis with time of hydrolysis. During the subsequent incubation with Corolase PP, the proteins that had remained intact, and large peptides, were broken down to small fragments that could no longer be detected in gel electrophoresis. This paralleled the increase of the average degree of hydrolysis during hydrolysis (Fig. 2) and the decrease of the amount of peptides of molecular mass >5 kDa (Fig. 3). The human (H)-IgE and H-IgG binding inhibitions of the hydrolysates did not differ much whether the pepsin incubation was done at pH 2.0 or 3.0 (Figs 4 and 5). The antigenicities of the protein samples which were heated in the absence of enzymes did not differ significantly from the zero time values, which indicates that the heat treatment applied had no effect on the antigenicity of the individual whey proteins (data not shown). Ultrafiltration of the final hydrolysates virtually eliminated all residual antigenicity of α La, β Lg and BSA (Table 1).

In contrast to the results with α La, β Lg and BSA, the electrophoretic pattern of the hydrolysates of B-IgG with pepsin incubation at pH 3.0 showed that total proteolysis was less than that with pepsin incubation at pH 2.0 (Fig. 1). The average degree of hydrolysis, however, was only slightly reduced (Fig. 2) and also the amount of peptides of molecular mass >5 kDa was hardly influenced whether the pepsin incubation was done at pH 2.0 or 3.0 (Fig. 3). In the latter case, however, the H-IgG binding inhibition of the hydrolysates was somewhat

Fig. 1. SDS-PAGE patterns of the hydrolysates of α La, β Lg, BSA and B-IgG obtained by incubation with pepsin at pH 2.0, 3.0 or 4.0, followed by incubation with Corolase PP at pH 7.5. In all gels, lanes 1 and 11 contain reference samples (Bio-Rad LMW standard and BDH 44247 respectively). Molecular masses in the reference samples are indicated along the gels. Samples were analysed after 0, 30 and 90 min incubation with pepsin (lanes 2–4) and after 90 min incubation with pepsin, followed by 10, 30, 60 and 150 min incubation with Corolase PP (lanes 5–8), lanes 9 and 10 contain the permeate and retentate respectively of the ultrafiltration of the final hydrolysate through a 3000 Da filter.

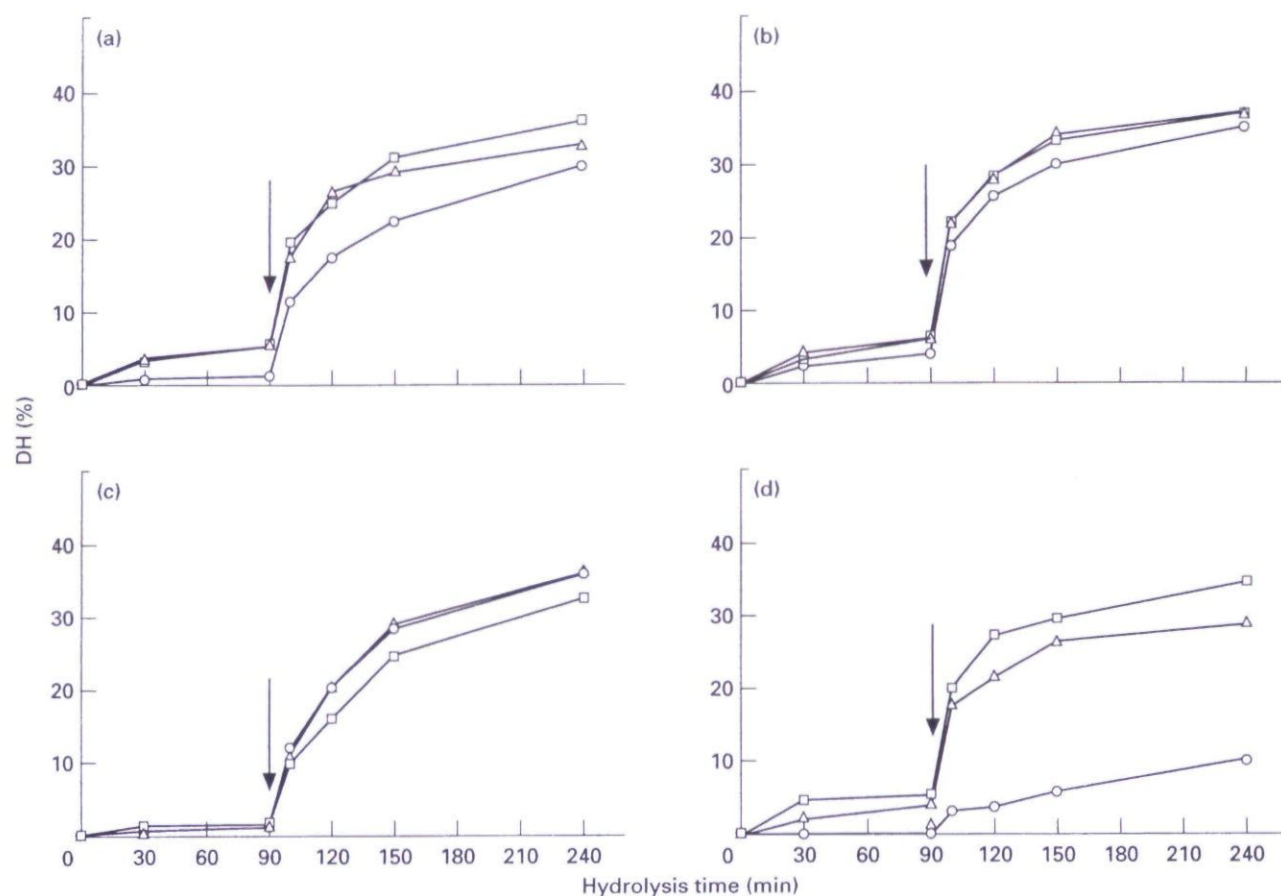


Fig. 2. Average degree of hydrolysis of hydrolysates of (a) α La, (b) BSA, (c) β Lg and (d) B-IgG during incubation with pepsin at pH 2.0 ($\square$), 3.0 (Δ) or 4.0 ($\circ$), followed by incubation with Corolase PP at pH 7.5. Arrows indicate the time of Corolase PP addition.

Table 1. Residual H-IgE and H-IgG antigenicity^a of hydrolysates of individual whey proteins, obtained by 90 min incubation with pepsin at pH 2.0, 3.0 or 4.0, followed by incubation with Corolase PP at pH 7.5 for 150 min, before and after ultrafiltration through a 3000 Da filter

Protein	Antibody	Before ultrafiltration			After ultrafiltration		
		pH 2.0	pH 3.0	pH 4.0	pH 2.0	pH 3.0	pH 4.0
α La	H-IgE	4.8	14.0	47.8	0	4.2	4.7
	H-IgG	0	0	4.2	0	0	0
β Lg	H-IgE	8.3	0	0	7.1	0	0
	H-IgG	9.0	7.4	6.4	0	1.8	0
BSA	H-IgE	0	0	57.6	0	0	0
	H-IgG	0	0	17.9	0.8	0	0
B-IgG	H-IgE	2.5	54.4	91.2	0	5.9	5.5
	H-IgG	0	0	100	0	0	0

^a Percentage inhibition of H-IgE and H-IgG binding to the individual milk proteins. The concentration of the inhibiting proteins was 1 g/L.

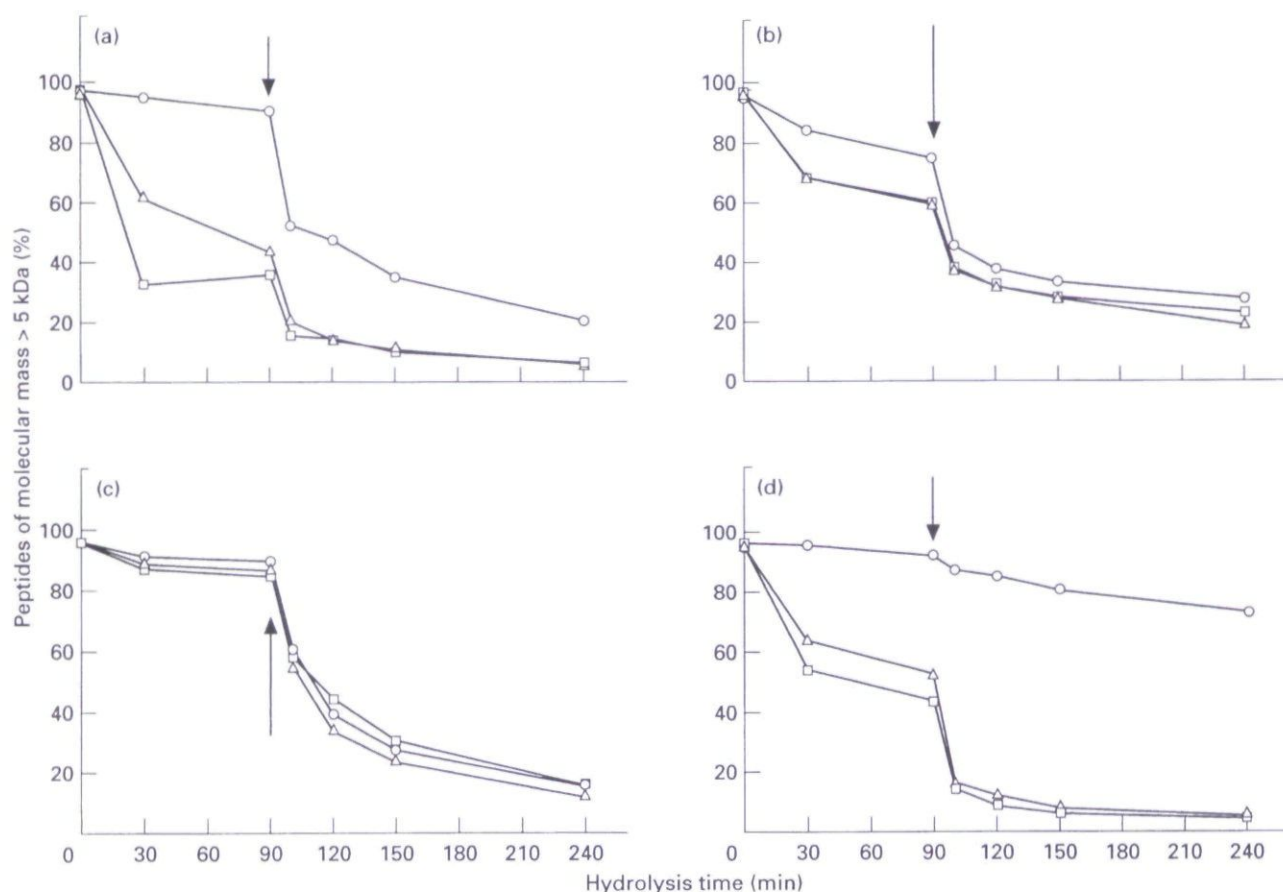


Fig. 3. Amount of peptides of molecular mass >5 kDa as a function of the total time of hydrolysis during incubation with pepsin at pH 2.0 (□), 3.0 (Δ) or 4.0 (○), followed by incubation with Corolase PP at pH 7.5. Arrows indicate the time of Corolase PP addition. (a) α La. (b) BSA. (c) β Lg. (d) B-IgG.

greater (Fig. 5) and the H-IgE binding inhibition was considerably greater (Fig. 4 and Table 1).

Pepsin incubation at pH 4.0; Corolase PP incubation at pH 7.5

When the pepsin incubation was carried out at pH 4.0 most of the electrophoretic patterns differed considerably from those obtained after hydrolysis at pH 2.0 or 3.0 (Fig. 1). Not only was α La hardly hydrolysed by pepsin, but the following hydrolysis with Corolase PP was also less efficient, which is also clear from the lower values of the degree of hydrolysis (Fig. 2), the increased amounts of peptides of molecular mass >5 kDa (Fig. 3) and the enhanced binding inhibition of H-IgE antibodies (Fig. 4). Figs 1–5 show that, in contrast, the overall hydrolysis of β Lg was only slightly influenced by the pH of the pepsin incubation.

BSA seemed to be hydrolysed well at pH 4.0, as is demonstrated by Figs 1 and 2, but overall H-IgE and

H-IgG binding inhibition of the hydrolysates was considerably increased (Figs 4 and 5 and Table 1), which agrees with the presence of some peptide material of molecular mass around 14 kDa in the electrophoretic pattern of the ultrafiltration retentate (Fig. 1). It is worth noting that at all pH values the amount of peptide material of molecular mass >5 kDa was larger in the final hydrolysate of BSA than in that of α La and β Lg (Fig. 3).

Figures 1–5 and also Table 1 most convincingly demonstrate that the hydrolysis of B-IgG at pH 4.0 was very small. Neither the pepsin incubation at pH 4.0, nor the subsequent incubation with Corolase PP at pH 7.5 resulted in an appreciable hydrolysis or change in antigenic properties of B-IgG.

In Fig. 1 the electrophoretic patterns of the unhydrolysed whey proteins (the lanes 2 in the gels) suggest that the proteins contain some impurities. The α La sample may be slightly contaminated by β Lg and B-IgG and some effect of cross-contamination may have occurred in

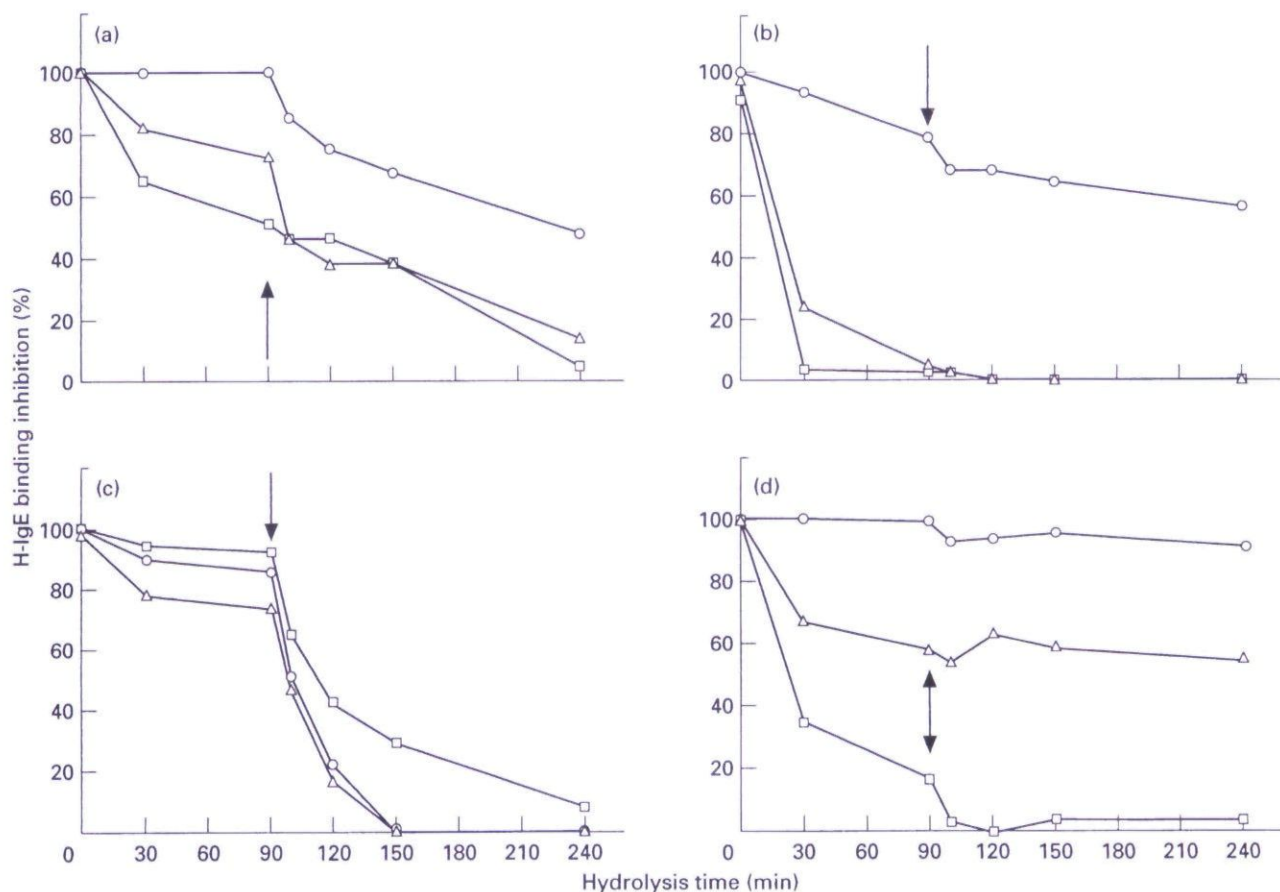


Fig. 4. Residual H-IgE antigenicity (percentage inhibition of H-IgE binding at a protein inhibitor concentration of 1 g/L) as a function of the total time of hydrolysis during incubation with pepsin at pH 2.0 (□), 3.0 (Δ) or 4.0 (○), followed by incubation with Corolase PP at pH 7.5. Arrows indicate the time of Corolase PP addition. (a) α La. (b) BSA. (c) β Lg. (d) B-IgG.

the immunological analyses. In the electrophoretic pattern of β Lg the band corresponding to a molecular mass somewhat higher than 31000 represents the dimer of β Lg. Similarly, the different bands in the electrophoretic patterns of BSA and B-IgG also represent various states of aggregation of these proteins.

Discussion

Our results clearly show that incubation of α La, BSA and B-IgG with pepsin at pH 4.0 not only leads to a reduced hydrolysis compared with incubation at lower pH values, but also to a decreased susceptibility to subsequent hydrolysis by Corolase PP at pH 7.5. In contrast, the very limited hydrolysis of β Lg is hardly influenced by the pH of the pepsin incubation. Not only is the pH in the stomach of infants higher than in that of adults but also acid production is delayed, so the pH in the stomach will remain at a high level for a considerable time after food intake; pH values well above 4 up to 3 h

after food intake have been reported [15,16]. In addition secretion of pepsin in infants is low [16]. We thus conclude that in the stomach of infants whey proteins are hardly hydrolysed and that they will pass almost unchanged into the duodenum, as suggested earlier [15]. Except for β Lg the subsequent proteolysis by the pancreatic enzymes is reduced and it is to be expected that large peptides and even intact protein molecules will survive for a sufficient time to be transferred to the circulation. The persistence in the gastrointestinal tract of high levels of epitopes of the whey proteins, which, after absorption, are capable of binding preformed H-IgE antibodies or inducing an immunological IgE response, might be of considerable importance for the development of cow's milk allergy. This is particularly true in young infants having an immature epithelial cell surface barrier, so that comparatively large amounts of various macromolecules are able to cross the intestinal epithelium [6–9, 25–27].

In our experiments β Lg was barely hydrolysed by

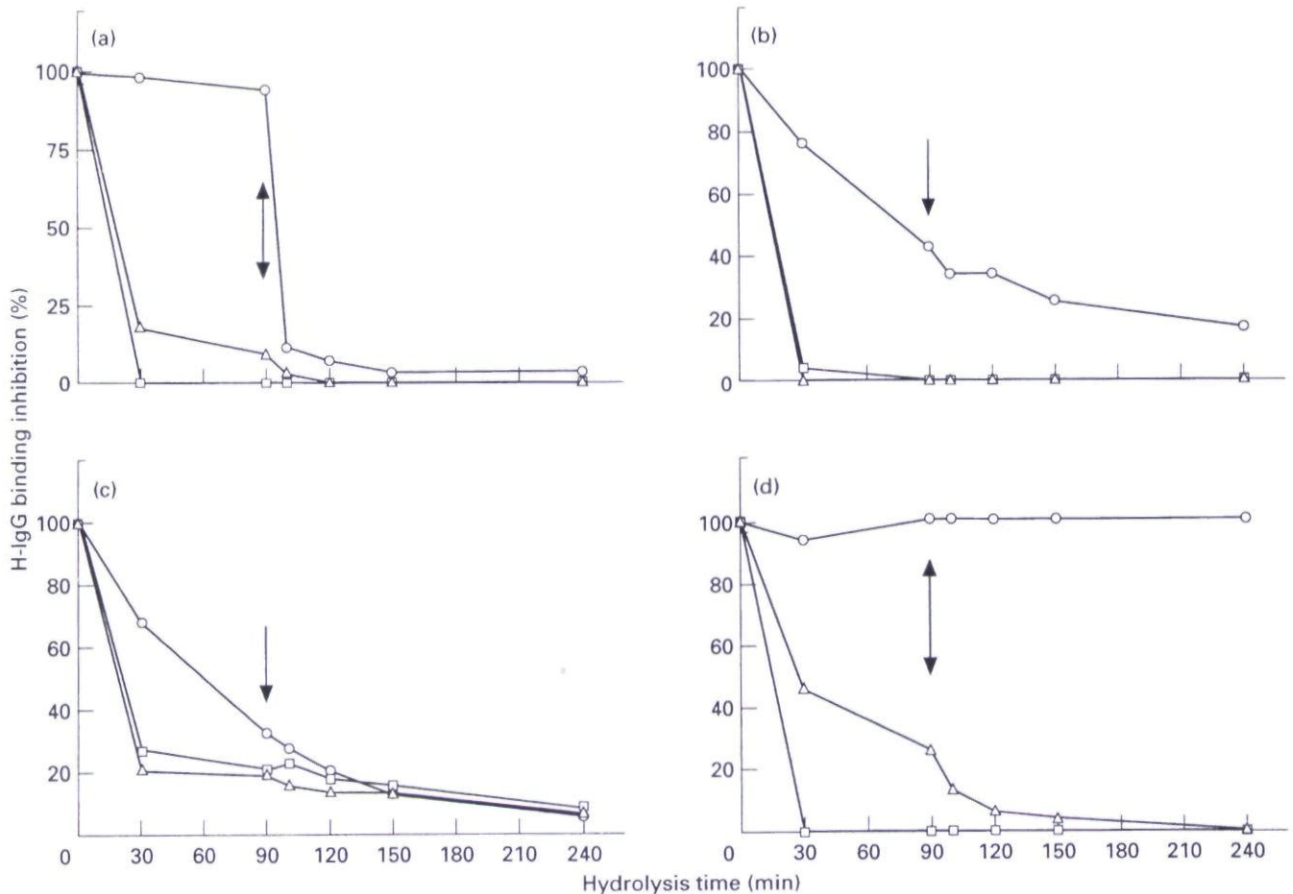


Fig. 5. Residual H-IgG antigenicity (percentage inhibition of H-IgG binding at a protein concentration of 1 g/L) as a function of the total time of hydrolysis during incubation with pepsin at pH 2.0 ($\square$), 3.0 (Δ) or 4.0 ($\circ$), followed by incubation with Corolase PP at pH 7.5. Arrows indicate the time of Corolase PP addition. (a) α La. (b) BSA. (c) β Lg. (d) B-IgG.

pepsin, independent of the pH of the pepsin incubation, a result which agrees with other observations [2–5]. The subsequent hydrolysis by pancreatic enzymes, however, was very efficient and in general the residual H-IgE and H-IgG antigenicities of the final hydrolysates were less than those of the other whey proteins (Figs 4 and 5 and Table 1). The physiological digestion process is apparently well able to destroy potentially antigenic epitopes of this protein, although under our experimental conditions the pancreatic enzymes need an hour to accomplish their complete destruction. Therefore it is desirable to eliminate antigenic epitopes completely from whey protein hydrolysates which are to be used in infant formulas for children with an increased risk of allergy. Table 1 shows that ultrafiltration through a 3000 Da filter is an efficient means of achieving this goal, which agrees with our earlier results [28].

As mentioned above the residual H-IgE and H-IgG antigenicity of B-IgG is hardly influenced by the successive hydrolyses by pepsin at pH 4.0 and pancreatic

enzymes at pH 7.5, conditions which prevail in the gastrointestinal tract of the young. In calves the surviving epitopes contribute to the passive immunization of the newborn by entering into the gut lumen or, after absorption, into the circulation. In this respect it is worthwhile noting that the level of immunoglobulins in colostrum is much enhanced compared to that later in the lactation [29]. It must be recognized, however, that this immunization process, which occurs in ruminants, does not take place in human infants, who receive their immunity from the intrauterine transport of maternal antibodies [6].

Figures 4 and 5 also show that after the pepsin incubation at pH 4.0 the H-IgE and H-IgG antigenicity of the BSA hydrolysates remains high. Apparently some antigenic epitopes survive the enzymatic treatment, which agrees well with the electrophoretic pattern of the ultrafiltration retentate (Fig. 1) and the amount of peptides of molecular mass >5 kDa (Fig. 3). This reduced proteolysis might be due to the incompleteness

of the helical-disordered transition in BSA, which starts around pH 5 [30], leaving the peptide bonds in BSA less accessible to pepsin.

The surviving H-IgG epitopes of BSA might be of importance for the triggering of the auto-immune process leading to insulin-dependent diabetes mellitus. In the mechanism which, according to Karjalainen *et al.* [31], underlies this process the so-called ABBOS(126–144) peptide, which comprises the amino acid residues 126–144 in BSA, plays a leading part. This hypothesis, however, has been challenged by Atkinson *et al.* [32]. Definite conclusions can only be made when the results of a large intervention study, undertaken by Åkerblom *et al.* [33] become available. In the future we will analyse for the presence of the ABBOS(126–144) peptide in our hydrolysates.

It is clear that the high pH in the stomach of infants has an adverse effect on the digestion of α -La, BSA and B-IgG by pepsin. Acid infant formulas could possibly counteract a detrimental rise of the pH in the stomach after food intake. Such formulas might therefore be preferred in cases where increased risks of allergy or diabetes mellitus exist.

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References

- 1 Fordtran JS, Locklear TW. Ionic constituents and osmolality of gastric and small-intestine fluids after eating. *Am J Dig Dis* 1966; 11:503–21.
- 2 Yvon M, Van Hille I, Pélissier J-P, Guilleateau P, Toullec R. In vivo milk digestion in the calf abomasum. II. Milk and whey proteolysis. *Reprod Nutr Dev* 1984; 24:835–43.
- 3 Macron-Genty D, Tomé D, Dumontier AM, Kheroua O, Desjeux JF. Permeability of milk protein antigens across the intestinal epithelium in vitro. *Reprod Nutr Dev* 1989; 29:717–23.
- 4 Scanff P, Savalle B, Miranda G *et al.* In vivo gastric digestion of milk proteins. Effect of technological treatments. *J Agric Food Chem* 1990; 38:1623–9.
- 5 Schmidt DG, Markwijk BW van. Enzymatic hydrolysis of whey proteins. Influence of heat treatment of α -lactalbumin and β -lactoglobulin on their proteolysis by pepsin and papain. *Neth Milk Dairy J* 1993; 47:15–22.
- 6 Walker WA. Intestinal transport of macromolecules. In: Johnson LR ed. *Physiology of the gastrointestinal tract*. New York: Raven Press, 1981; 2:1271–89.
- 7 Savilahti E, Kuitunen M. Allergenicity of cow milk proteins. *J Pediatr* 1992; 121:S12–20.
- 8 Kuitunen M, Savilahti E, Sarnesto A. Human α -lactalbumin and bovine β -lactoglobulin absorption in infants. *Allergy* 1994; 49:354–60.
- 9 Jakobsson I, Lindberg TA. A prospective study of cow's milk allergy in Swedish infants. *Acta Paediatr Scand* 1979; 68:853–9.
- 10 Høst A, Husby S, Østerballe O. A prospective study of cow's milk allergy in exclusively breast-fed infants. *Acta Paediatr Scand* 1988; 77:663–70.
- 11 Bell K, McKenzie HA. β -Lactoglobulins. *Nature* 1964; 204:1275–9.
- 12 Gjesing B, Østerballe O, Schwartz B, Wahn U, Löwenstein H. Allergen specific IgE antibodies against antigenic components in cow milk substitute. *Allergy* 1986; 41:51–6.
- 13 Otani H, Dong XY, Hara T *et al.* Human serum antibodies to cow milk proteins in infants and children having clinically allergic symptoms. *Milchwissenschaft* 1989; 44: 131–4.
- 14 Otani H, Dong XY, Hara T *et al.* Specificities to cow milk proteins of human serum antibodies from clinically allergic patients. *Milchwissenschaft* 1989; 44:267–70.
- 15 Mason S. Some aspects of gastric function in the newborn. *Arch Dis Child* 1962; 37:387–91.
- 16 Koldovsky O. Development of human gastrointestinal functions: Interaction of changes in diet composition, hormonal maturation, and fetal genetic programming. *J Am Col Nutr* 1984; 3:131–8.
- 17 Schlamowitz H, Peterson LU. Studies on the optimum pH for the action of pepsin on 'native' and denatured bovine serum albumin and bovine hemoglobin. *J Biol Chem* 1959; 234:3137–45.
- 18 Röhm. Analysemethoden. Proteinase units (casein). Product information sheet EI-2-01-D, 26.01.88. Röhm AG, Darmstadt, Germany, 1988.
- 19 Röhm. Proteinase units (casein). Product information sheet EI-2-02-D, 27.01.88. Röhm AG, Darmstadt, Germany, 1988.
- 20 Church FC, Porter DH, Catignani GL, Swaisgood HE. An o-phthalaldehyde spectrophotometric assay for proteinases. *Anal Biochem* 1985; 146:343–8.
- 21 Frister H, Meisel H, Schlimme E. OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component. *Fresenius Z Anal Chem* 1988; 30:631–3.
- 22 Visser S, Slangen CJ, Robben AJPM. Determination of molecular mass distributions of whey protein hydrolysates by high-performance size exclusion chromatography. *J Chromatogr* 1992; 599:205–9.
- 23 Fritsché R, Bonzon M. Determination of cow milk formula allergenicity in the rat model by in vitro mast cell triggering and in vivo IgE induction. *Int Arch Allergy Appl Immunol* 1990; 93:289–93.
- 24 International Organisation for Standardization. Precision

- of test methods. Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests. In: ISO Standards Handbook 3. Statistical methods, 3rd edition. Switzerland: ISO, 1989: 413–61.
- 25 Robertson DM, Paganelli R, Dinwiddie R, Levinsky RJ. Milk antigen absorption in the preterm and term neonate. *Arch Dis Child* 1982; 57:369–72.
- 26 Jakobsson I, Lindberg T, Lothe L, Axelsson I, Benediktsson I. Human α -lactalbumin as a marker of macromolecular absorption. *Gut* 1986; 27:1029–34.
- 27 Axelsson I, Jakobsson I, Lindberg T et al. Macromolecular absorption in preterm and term infants. *Acta Paediatr Scand* 1989; 78:532–7.
- 28 Beresteijn ECH van, Peeters RA, Kaper J et al. Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. *J Food Protect* 1994; 57:619–25.
- 29 Guidry AJ, Butler JE, Pearson RE, Weinland BT. IgA, IgG1, IgG2, IgM, and BSA in serum and mammary secretion throughout lactation. *Vet Immunol Immunop* 1980; 1:329–41.
- 30 Lin VJC, Koenig JL. Raman studies of bovine serum albumin. *Biopolymers* 1976; 15:203–18.
- 31 Karjalainen J, Martin JM, Knip M et al. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N Engl J Med* 1992; 327:302–7.
- 32 Atkinson MA, Bowman MA, Kuo-Jang K et al. Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. *N Engl J Med* 1993; 329:1853–8.
- 33 Åkerblom HK, Dosch H-M, Robinson BH et al. Is dietary intervention for the prevention of insulin-dependent diabetes mellitus feasible? In: Laron Z, Karp M eds. *Prediabetes — Are we ready to intervene?* *Pediatr Adolesc Endocrinol*. Basel: Karger, 1993; 23: 97–104.

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