

ORIGINAL ARTICLE

Detection by Competitive Enzyme-Linked Immunosorbent Assay of a Bovine Serum Albumin Peptide (ABBOS) in Infant Formulas Based on Hydrolyzed Cow's Milk Protein

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OBJECTIVE — Our aim was to determine whether currently available products based on hydrolyzed milk protein and a small peptide (ABBOS) in bovine serum albumin (BSA) (positions 126–144) share common antigenic sites. The commercial products are primarily developed to reduce cow's milk protein-associated allergenicity, but whether they also could be used in intervention trials to elucidate the role of BSA in the etiology of IDDM is not known.

RESEARCH DESIGN AND METHODS — A sensitive competitive enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal antibody raised against the ABBOS peptide in BSA was developed. With this method, we determined cross-reactivity between the ABBOS peptide and commercially available products: four milk protein hydrolysates and eight infant formulas based on hydrolyzed milk protein. The products were further characterized by physicochemical techniques.

RESULTS — Hydrolyzed milk protein products are found to inhibit competitively the binding of ABBOS peptide to antibody. The number of residual reactive sites varied considerably among products and was not strongly related to the degree of hydrolysis (DH) or the molecular mass distribution of the hydrolysates.

CONCLUSIONS — The presence of possible immunoreactive peptides in infant formulas based on hydrolyzed cow's milk protein cannot be adequately predicted by the DH or molecular mass distribution of the hydrolysates. Its specific determination is needed to ensure infant formulas free of cross-reactive ABBOS antibody binding sites for use in ongoing and forthcoming intervention trials to elucidate the role of BSA as a possible environmental trigger agent of IDDM.

The role of cow's milk proteins and, in particular, of bovine serum albumin (BSA) in the etiology of IDDM is controversial. It has been hypothesized that a 19-amino acid peptide (ABBOS) in BSA (positions 126–144 [1]) might serve as a trigger for the autoimmune sequence that culminates in IDDM (2–4). Antibodies to this peptide may induce a cross-reactive

pathogenic response to p69, a β -cell surface protein, culminating in islet cell destruction. Higher levels of serum IgG antibodies to BSA were detected more in newly diagnosed children with IDDM than in healthy control subjects (4–7); the bulk of the antibodies were found to be ABBOS specific (4). Others have failed to confirm these findings (8,9). Furthermore,

results on T-cell responses to BSA are not unequivocal (8,10), and tolerization with BSA reduced the prevalence of BSA antibodies but did not affect the occurrence of diabetes in the nonobese diabetic (NOD) mouse (11). The mechanistic hypothesis of Karjalainen et al. (4) extends observations on an increased immune response to a variety of cow's milk proteins (12,13) and may provide an immunological basis for the increased diabetes risk in genetically susceptible subjects (14).

The convincing indirect evidence from animal experiments and epidemiological observations in man (15) was considered sufficient to justify an international double-blind controlled study to determine whether early avoidance of cow's milk prevents IDDM in genetically susceptible newborn infants (15–18). The participating infants will be fed either a cow's milk/ABBOS-free infant formula or a cow's milk-based infant formula when supplementary feeding (i.e., in addition to breast-feeding) is required or at the time of weaning from breast-feeding. For the cow's milk/ABBOS-free formula, a casein hydrolysate was chosen mainly on the basis of peptide size in the hydrolysate and because the hydrolysate did not cause IDDM in the NOD mouse (16,17).

Enzymatic cleavage of milk proteins may eliminate the specific ABBOS epitopes of BSA, but destruction is likely to depend on enzyme specificity. Human proteinases involved in digestion as well as other food-grade enzyme preparations of plant, bacterial, or fungal proteinases are currently used to manufacture hydrolysates, and their cleavage specificity has mostly been studied in relation to the reduction of cow's milk allergenicity (19,20). To our knowledge, information on the effect of these enzyme systems on the specific ABBOS sequence in BSA and, in particular, its antigenic site(s) is not available. We therefore developed a sensitive competitive enzyme-linked immunosorbent assay (ELISA) using

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BLg, β -lactoglobulin; BSA, bovine serum albumin; DH, degree of hydrolysis; DMA, N,N-dimethyl-2-mercaptoethyl-ammonium chloride; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; NIZO, Netherlands Institute for Dairy Research; NOD, nonobese diabetic; NRS, normal control serum; OPA, o-phthalaldehyde; PBS, phosphate-buffered saline; S_r, repeatability standard deviation.

rabbit antiserum against the synthesized ABBOS peptide of BSA to study whether various commercially available products based on hydrolyzed whey protein or casein competitively inhibit the binding of ABBOS peptide to antibody. The molecular characteristics of the hydrolysates were determined by physicochemical methods.

RESEARCH DESIGN AND METHODS

Materials

The peptide FKADEKKFWGKLYEIARR (ABBOS) corresponding to residues 126–144 in BSA (1) was synthesized with an additional COOH-terminal cysteine residue not present in the natural sequence, allowing conjugation to a carrier protein, keyhole limpet hemocyanin (KLH), using maleimido benzoic acid (Zeneca, Cambridge Research Biochemicals, Cheshire, U.K.). The amino acid positions (126–144) for the ABBOS peptide in BSA used in this study differ from those (152–168) used by others (2–5). These authors used preproalbumin (GenBank, accession number: A03232), which is 24 amino acids longer than albumin, as the starting point for numbering.

BSA (A-4378) was purchased from Sigma (St. Louis, MO). To investigate the possibility that the ABBOS peptide is hidden within the tertiary structure of BSA and therefore inaccessible for antibody binding, BSA was pretreated by heating for 10 min at 95°C. β -lactoglobulin (β Lg) was purified from cheese whey by chromatography, as described before (21). Soy isolate (Purina protein) was purchased from Hofhuis Pentacon BV (Bunschoten, The Netherlands) and Nutrilon soya, an infant formula based on soy protein, from Nutricia (Zoetermeer, The Netherlands).

Four commercially available milk protein hydrolysates and eight infant formulas based on milk protein hydrolysates were investigated. Because The Netherlands Institute for Dairy Research (NIZO) is a research institute privately owned by the Dutch dairy industry, the products were coded for reasons of confidentiality. Hydrolysates 1 to 4 and infant formulas 5 to 11 were based on hydrolyzed whey protein; formula 12 was based on hydrolyzed casein. The study was carried out because of interest in the subject from the Dutch dairy companies.

Rabbit antisera

For the production of IgG antibodies

directed to the specific ABBOS peptide in BSA, two rabbits (one male R487, one female R482) were immunized with 100 μ g of the conjugated ABBOS peptide (ABBOS-KLH) in 1 ml phosphate-buffered saline solution (PBS) (0.15 mol/l NaCl in 0.01 mol/l phosphate buffer, pH 7.4) mixed with 1 ml complete Freund's adjuvant (638607, Difco, Detroit, MI). On days 28, 56, and 84, the rabbits were boosted with 100 μ g ABBOS-KLH in 1 ml PBS mixed with 1 ml incomplete Freund's adjuvant (638606, Difco). The antigen was injected at two different sites. After three boosters, the IgG antibody titer against the ABBOS peptide showed no further increase. The rabbit serum taken before the immunization served as the normal control serum (NRS). The serum samples were stored at -20°C .

Chemical and immunological characterization of the hydrolysates

The average degree of hydrolysis (DH) of the whey proteins in each hydrolysate was calculated from the increase of the number of primary amino groups compared with that of unhydrolyzed protein. We determined this number by the *o*-phthaldialdehyde (OPA) method (22), using *N,N*-dimethyl-2-mercaptoethyl-ammonium chloride (DMA) instead of 2-mercaptoethanol (23). For sample preparation, the hydrolysates and infant formulas were dissolved in distilled water to a protein concentration of ~ 5 g/l. After 2 h at room temperature, the solutions were centrifuged for 7 min at 10,000g to separate fat and other undissolved material. The intermediate layer was used to determine the DH of the samples. In spite of the centrifugation step, most of the infant formulas still showed some turbidity, which could interfere with the determination of the DH. Therefore, we measured the absorbance at 340 nm, not against water, but against a blank containing the sample in the assay buffer without the OPA and DMA. Because the number of primary amino groups of the protein preparations used for the manufacture of the hydrolysates was unknown, we determined the average number of primary amino groups per molecule protein in a large variety of whey protein concentrates and in casein. We found 17.6 (repeatability standard deviation [S_e] = 1.4 and degrees of freedom [df] = 14) and 16.3 primary amino groups per mole of protein, respectively.

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 mol/l potassium phosphate 0.125 mol/l sodium sulfate buffer of pH 6.65. Peptide concentrations in the eluate were determined from the absorbance 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 (24). For sample preparation, the products were dissolved in the elution buffer to a protein concentration of ~ 2 g/l. Before application to the column, the samples were centrifuged for 7 min at 10,000g and then filtered through a Millipore filter cartridge with pore size 0.22 μ m.

The anti-ABBOS titers of the rabbit sera against the ABBOS peptide and BSA were determined by indirect ELISA. We used the ELISA method, as described by Fritsché and Bonzon (25), with modifications. Briefly, polystyrene microtiter plates (655061, Greiner, Alphen a/d Rijn, The Netherlands) were used as solid support. Wells were coated with 135 μ l of antigen (the unconjugated ABBOS peptide or non-treated BSA; 37 μ g/ml in 0.05 mol/l bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Residual free binding sites were blocked with 200 μ l/well of 0.8% (wt/vol) fish gelatin (G-7765, Sigma) in PBS containing 0.05% (vol/vol) Tween-20 (Sigma) (PBS-Tween) for 1 h at room temperature. Plates were incubated with 100 μ l/well of rabbit anti-ABBOS serum diluted in PBS-Tween-20 (1:1000) for 2 h at room temperature. The same dilution of NRS was used as a negative control. The IgG ABBOS-antibodies that reacted with the plate-bound antigens (ABBOS or non-treated BSA) were determined by use of goat anti-rabbit IgG (H and L chains) peroxidase-conjugated antibodies (Nordic Immunological, Tilburg, The Netherlands). The anti-rabbit IgG antibodies were diluted 5,000-fold in PBS-Tween-20 containing 0.8% (wt/vol) fish gelatin and were incubated for 1 h at room temperature. As peroxidase substrate a freshly prepared solution of 1 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) in 0.024 mol/l citrate and 0.05 mol/l potassium phosphate buffer, pH 5.0, containing 0.04%

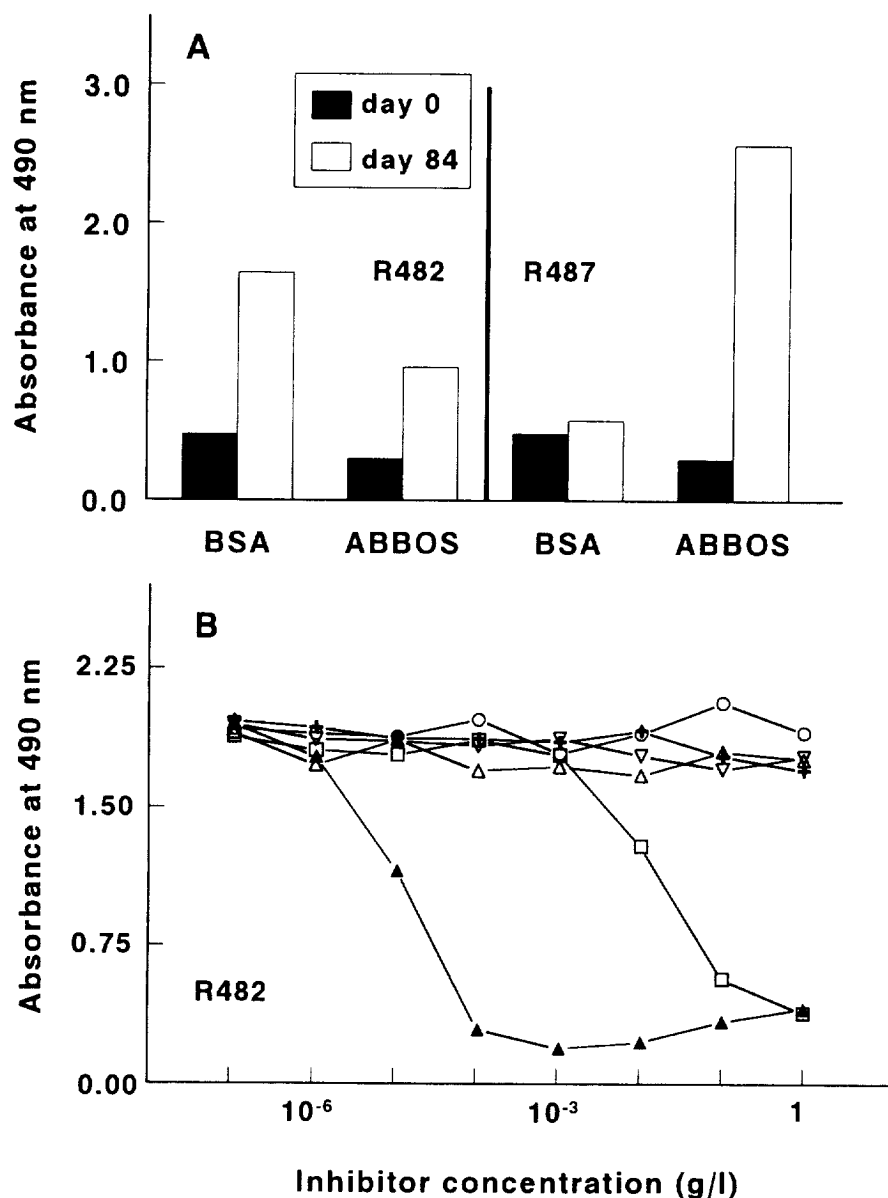


Figure 1—A: IgG binding of nontreated BSA and ABBOS-peptide to rabbit anti-ABBOS serum (R482 and R487) before (solid bars) and after (open bars) immunization. The protein concentration of the coating antigen was 37 µg/ml; sera were diluted 1,000-fold. B: ABBOS-binding inhibition assay. Inhibition of the binding to rabbit IgG anti-ABBOS antibodies (R482) of ABBOS-peptide (▲), nontreated BSA (▽), heat-treated BSA (□), heat-treated soy-isolate (△), heat-treated Nutrilon soya (+), and heat-treated βLg (○). Protein concentration of the coating antigen (ABBOS) was 15 µg/ml. Absorbance values are from duplicate measurements.

(vol/vol) hydrogen peroxide, was used. After the plates had been incubated for 30 min at room temperature, 100 µl of 2N sulfuric acid was added. Optical densities were measured 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 results were averaged.

The inhibitory binding potency of the various whey protein hydrolysates and the

milk protein hydrolysate in the infant formulas was determined by competitive ELISA. The products were dissolved in distilled water to a protein concentration of 6.7 g/l (stock solution). To expose possible hidden reactive sites in large fragments, the samples were also heated for 10 min at 95°C in a water bath, followed by cooling to room temperature with running tap water. In 1.5-ml glass vials, serial dilutions of the stock solution of the products

were incubated overnight at 4°C in PBS-Tween-20 with equal amounts (0.033%, vol/vol) of rabbit anti-ABBOS serum. Solutions without the inhibitor served as positive controls; solutions containing NRS instead of rabbit anti-ABBOS serum served as negative controls. Residual unbound IgG anti-ABBOS antibodies were determined by the indirect ELISA method described, by application of 100 µl of preincubated serum to each well of ELISA plates coated with 135 µl of unconjugated ABBOS peptide (15 µg/ml in 0.05 mol/l sodium bicarbonate buffer, pH 9.6). The percentage of inhibition was calculated in relation to the maximum inhibition (vials containing NRS instead of rabbit anti-ABBOS) and the minimum inhibition (vials without milk protein hydrolysate). For this purpose, an inhibitor concentration of 1 g/l was chosen because it was the lowest concentration at which an almost 100% inhibition for both the ABBOS peptide and intact heat-treated BSA was attained (Fig. 1B). The specificity of the anti-ABBOS antibodies was ascertained through the use of soy isolate, infant formula Nutrilon soya, and βLg as inhibitor proteins up to a maximum concentration of 10 g/l. Because of poor solubility, it was not possible to test higher concentrations.

Reliability of the results

All analytical determinations were done in duplicate and results averaged. The precision of the analytical methods, expressed as the S_r , was determined from separate experiments (26). For the ELISA, including between-well and between-plate variation, S_r was 1.77% (df = 19); for the DH determination, S_r was 0.99% (df = 48), and for the molecular mass distribution, S_r was 0.29% (df = 40).

RESULTS

Characterization and selection of rabbit antiserum

The two antisera (R482 and R487) were both reactive with the synthetic 20-amino acid ABBOS peptide. However, R482 but not R487 reacted with plate-bound nontreated BSA (Fig. 1A). It appeared that some kind of denaturation of BSA was needed for the antibody-antigen binding. This was clearly demonstrated in an inhibition ELISA with nontreated BSA and heat-treated BSA for antiserum R482 (Fig. 1B). By comparison, the avidity of the specific ABBOS antibodies of R487 for the

Table 1—Inhibitory binding potency, DH, and percentage of peptides with $M_r > 5$ kDa of four commercially available whey protein hydrolysates and of milk protein hydrolysate present in eight infant formulas

Sample	Inhibition of IgG binding to ABBOS-peptide	DH	$M_r > 5$ kDa
ABBOS-peptide	92		
Nontreated BSA	0		
Heat-treated BSA	91		
Hydrolysate			
1	19	13	24
2	1	30	6
3	50	30	7
4	12	30	7
Formula			
5	24	33	14
6	46	16	27
7	32	12	26
8	45	6	44
9	16	24	15
10	40	14	23
11	62	1	56
12	26	52	5

Data are %. Hydrolysates 1 to 4 are whey protein hydrolysates; formulas 5 to 11 are infant formulas based on hydrolyzed whey protein; formula 12 is based on hydrolyzed casein. The inhibition of IgG binding was determined with rabbit IgG anti-ABBOS antibodies. Concentration of the protein inhibitors was 1 g/l.

ABBOS region in heat-treated BSA was considerably lower (data not shown) and antiserum R482 was selected for the ABBOS immunoassay. The antiserum did not react with highly purified β Lg, soy isolate, or the infant formula Nutrilon soya (Fig. 1B).

Chemical and immunological characterization of the hydrolysates

The DH of the protein fraction varied from 1 to 52% in the various milk protein hydrolysates. In parallel, the presence of peptides with $M_r > 5$ kDa varied from 56 to 5%. As could be expected, these two chemical characteristics were highly correlated ($r = 0.87$, $P < 0.001$). The values of the DH and the molecular mass distributions of the protein fraction in the samples of formulas 7 and 10 agreed with those of hydrolysate 1, from which these formulas were manufactured, demonstrating the reliability of their determination (Table 1).

Figure 2 shows typical examples of the inhibition curves for the ABBOS peptide, for heat-treated intact BSA and for a number of the hydrolyzed milk protein products. The binding inhibition of IgG-ABBOS antibodies to BSA or its peptides present in the hydrolyzed milk protein

products is presented in Table 1. The results showed a considerable variation in binding inhibition and thus in residual reactive sites among the products. Hydrolysate 2 could be considered free of peptides with cross-reactive antibody binding sites but the number of residual reactive sites could be relatively high in other hydrolysates or infant formulas. Size and structure of these reactive peptides are unknown. Milk protein hydrolysates displaying a similar extent of hydrolysis or comparable amounts of peptides greater than 5 kDa could differ considerably in residual antibody binding sites. It is obvious that higher protein concentrations of BSA or protein in formulas (10^3 and 10^5 , respectively) were needed to obtain the same inhibition as caused by the ABBOS peptide as a consequence of a lower ABBOS peptide concentration per gram protein (Fig. 2).

CONCLUSIONS— Current commercially available milk protein hydrolysates are generally used in hypoallergenic infant formulas for the prevention and treatment of cow's milk allergy. Because of better palatability most formulas contain hydrolyzed whey protein. The processing techniques usually include heat treatment, enzymatic cleavage, and subsequent ultrafiltration. Although the processing techniques of the various hydrolysates analyzed in this study are unknown, we presume that they will certainly differ in starting material, heat treatment, choice of proteolytic enzymes, and pore size of the ultrafiltration membrane. This resulted, as our data indicate, in considerable variation in chemical characteristics and residual ABBOS epitopes in the tested milk protein hydrolysates and infant formulas. Apparently, epitopes of the ABBOS peptide in BSA can survive the enzymatic treatment and may not be removed during the subsequent ultrafiltration step. Even extensively hydrolyzed milk protein hydrolysates (average DH $> 30\%$, corresponding to an average chain length of 3–4 amino acids) still contain fair amounts of peptide fragments with $M_r > 5$ kDa, long enough to include the ABBOS epitope. The presence of residual antibody binding sites in undefined peptide fragments of the hydrolysates could not be predicted from their chemical characteristics. DH and amount of peptides with $M_r > 5$ kDa were similar for hydrolysates 2, 3, and 4 but binding inhibition varied from 1

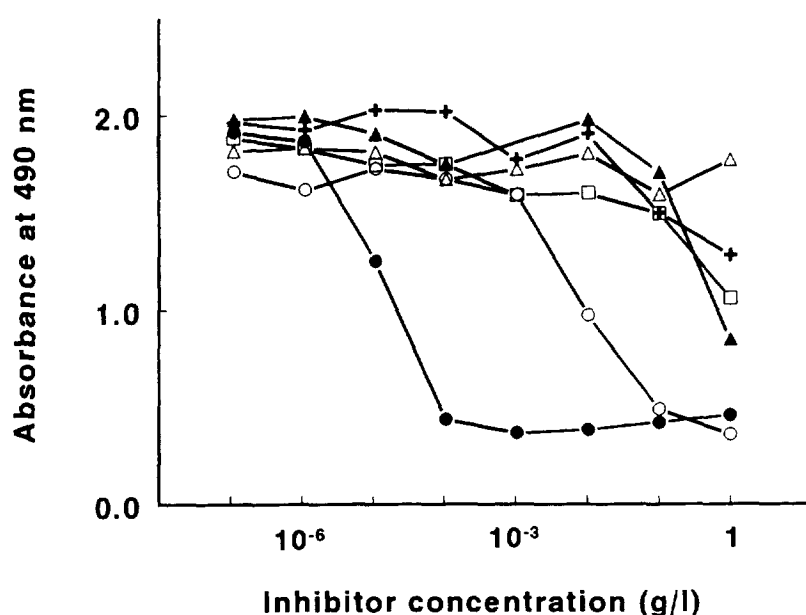


Figure 2—ABBOS-binding inhibition assay. Inhibition of the binding to rabbit IgG anti-ABBOS antibodies (R482) of ABBOS-peptide (●), heat-treated BSA (○), hydrolysate 2 (△), hydrolysate 3 (□), formula 10 (+), and formula 11 (▲). Protein concentration of the coating antigen (ABBOS) was 15 μ g/ml. Absorbance values are from duplicate measurements.

to 50%. Furthermore, although formula 11 showed the lowest DH, binding inhibition was only slightly higher than for hydrolysates of formulas with a considerably higher DH. Reasonably, it may be deduced that the specificities of the proteinases used for their manufacture have played a dominant role. Although the amino acid sequence of the ABBOS peptide is known, it would be a challenge to define its antigenic site(s) and to look for food-grade enzymes with specific cleavage sites for the ABBOS epitope sequence. Complete removal of peptide fragments with cross-reactive antigenic sites by ultrafiltration may require membranes with low molecular mass cut-off values which lowers the efficacy of the ultrafiltration process and the yield of the final product.

The elimination of cow's milk immunoreactive epitopes has been studied in relation to enzyme specificity. In a previous study we determined the effect of different enzyme systems on the survival of BSA epitopes using serum of patients allergic to cow's milk containing high concentrations of IgE and IgG antibodies to BSA and found that the elimination of epitopes in BSA is mainly determined by enzyme specificity rather than by DH or M_r distribution (19). Analyzing the same commercially available products we found, in agreement with this study, a considerable variation in reduction of IgG epitopes of BSA (20).

It might be assumed that casein is a more suitable milk protein source than whey protein for the production of infant formulas free of peptides with cross-reactive ABBOS antibody binding sites. However, it should be realized that commercial casein preparations always contain trace amounts of whey proteins including BSA. Elimination of the ABBOS epitope(s) is, therefore, a prerequisite. Enzymes used for the hydrolysis of casein may not necessarily have specific cleavage sites for the ABBOS epitope sequence. This resulted, as our data demonstrated for formula 12 based on extensively hydrolyzed casein, in incomplete removal of peptide fragments containing cross-reactive antigenic sites of the ABBOS peptide (Table 1).

Hydrolysates containing reactive peptide fragments may cross the intestinal epithelium, giving rise to immunological responses. This may be particularly true for young infants, in whom the immature gastrointestinal tract is more permeable to large molecules than in adults (27–29). So

far the number of antigenic sites needed to induce an immune response is not known. It is not inconceivable that there may be a threshold dose. Above this dose it may be immunogenic and below it may be either nonsignificant or even protective by suppressing the antibody immune response (tolerization). In this study we only showed cross-reactivity between peptide fragments in the hydrolysates and the ABBOS peptide. Whether these peptides will induce B- or T-cell activation *in vivo* is as yet not certain. Further, it should be noted that after oral feeding milk protein hydrolysates undergo physiological digestion. We have previously shown that for hydrolysates produced with proteinases different from those of the intestinal tract this may lead to a further reduction or even complete elimination of residual epitopes responsible for immunological reactions in patients allergic to cow's milk (19). Therefore, the clinical significance of the residual ABBOS antibody binding sites in the tested products certainly needs further investigation by *in vitro* studies, such as lymphocyte stimulation tests and *in vivo* studies such as ABBOS immunogenicity after oral feeding of the hydrolysates to experimental animals. However, the presented *in vitro* ELISA could be used for primary screening of existing and newly developed hydrolysates.

The inhibitory binding potency was higher in formulas 7 and 10 as compared with hydrolysate 1, from which these formulas were manufactured. As the antigenic site of the ABBOS peptide is not exactly known, it is very well possible that during processing new sequential or conformational antigenic sites, resembling the amino acid sequence of the ABBOS epitope, are formed through peptide aggregation, lipophilization, or Maillard reactions (30–32). Analyses of residual cross-reactive antibody binding sites should, therefore, involve the final product and not only the protein constituent of the product.

The rabbit antiserum reactive with both the ABBOS peptide and BSA (R482) only recognized pretreated BSA. Unfolding of the molecule occurs after extensive heat treatment (33). During the absorption of BSA to the solid surface of an ELISA plate it may also undergo different levels of denaturation. The sequence of the ABBOS epitope is apparently hidden within the tertiary structure of BSA. As a consequence, unfolding is needed for antibody binding and without pretreatment,

accessibility of the epitope for enzymatic digestion is likely to be difficult.

The results of this study indicate that peptide fragments with possible immunoreactive sites can be present in commercially available infant formulas based on hydrolyzed milk proteins. The choice for use in ongoing and forthcoming intervention trials to elucidate the role of BSA as a possible environmental triggering agent of IDDM cannot be made on the basis of chemical parameters only. To ensure a product free of ABBOS antibody binding sites its specific determination is a prerequisite. If a relationship with IDDM becomes more apparent, then it will be a challenge for the dairy industry to develop new technologies to eliminate selectively the possible immunoreactive ABBOS epitope from BSA.

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