

Detection of Potentially Allergenic Material in 12 Hydrolyzed Milk Formulas

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ABSTRACT

Hypoallergenic milk formulas are used as an alternative diet for infants who have allergies to cow's milk when breast-feeding is not possible. These products are based on proteins, which have been heat-treated and hydrolyzed to a different degree in order to cleave antibody-binding structures. Even extensively hydrolyzed products have occasionally been observed to elicit allergic reactions in sensitized infants, however. Therefore, the parameters of relevance to allergenic potential require more investigation. The objective of the present study was to investigate 12 different hydrolyzed milk formulas for their contents of potentially allergenic protein material, i.e. material that may induce allergenicity or elicit allergic responses in already sensitized individuals. Analytical methods applied were gel filtration, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, immunoblotting, dot-immunobinding, and ELISA. Care was taken to assure that all protein fractions were investigated, including supernatants and precipitates following centrifugation of the milk formulas. By gel filtration, protein material with apparent molecular masses of 7 to >30 kDa was detected. Analysis by SDS-PAGE of formula precipitates showed that proteins with a molecular mass above 20 kDa were present even in some of the extensively hydrolyzed formulas. Residual antigenic β -lactoglobulin was found by ELISA in all products. By immunoblotting and dot-immunobinding with antibodies against total whey, caseins, or Kunitz soybean trypsin inhibitor, we observed antigenic material mainly in partially hydrolyzed products. We concluded that SDS-PAGE of formula supernatants and precipitates gave the most differentiated profile of hydrolyzed formulas and that this method is well suited for screening potential allergenicity.

(Key words: hydrolyzed milk formulas, SDS-PAGE, gel filtration, ELISA)

Abbreviation key: CAPS = 3-cyclohexylamino-1-propanesulfonic acid, DTT = dithiothreitol, eHF = extensively hydrolyzed formula, KSTI = Kunitz soy trypsin inhibitor, pHF = partially hydrolyzed formula.

INTRODUCTION

Cow's milk-based formulas are used when a substitute for breast-feeding is required. Such formulas may cause problems for the 2 to 3% of infants who develop an allergy to cow's milk proteins during the first year of life (15). To aid these infants, hypoallergenic formulas have been developed, the first commercial preparations of which emerged on the market in the 1940s. These products were based on extensively hydrolyzed bovine casein and tolerated by the vast majority of infants allergic to cow's milk. However, during the years, case stories of infants reacting to these extensively hydrolyzed formulas (eHF) have been reported (5, 21, 31). The products based on hydrolyzed casein have a further major drawback: acceptance is difficult because of the unpleasant taste. To address this problem, new generations of hypoallergenic formulas based on either extensively or partially hydrolyzed whey proteins have been developed. The partially hydrolyzed formulas (pHF) have a less bitter taste than eHF because of the lower concentration of very short and hydrophobic peptides, but it is now evident that a substantial number of infants with cow's milk allergy react to pHF (11, 26, 29, 30). Moreover, it has been reported that pHF may induce allergic reactions even in infants not previously sensitized to cow's milk (8). Extensively hydrolyzed products based on whey proteins have been marketed because they have a less bitter taste than extensively hydrolyzed caseins, but these products have also been reported to cause allergic reactions in infants with established cow's milk allergy (6, 26). Thus, it appears that both eHF and pHF contain potentially allergenic material. Minute amounts of protein material with at least two epitopes (including insufficiently hydrolyzed peptides), residual intact proteins, and polymers or aggregates formed during the production or reconstitution of the formula could account for allergic reactions observed in individuals sensitized to cow's milk. The detection of potentially allergenic components

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in hydrolyzed formulas is therefore important, even if they are present at only very low concentrations.

In the present study, 12 commercially available hydrolyzed milk formulas were analyzed by physicochemical and immunochemical methods to: 1) detect potentially allergenic material and 2) test whether the products could be differentiated from each other for parameters relevant for allergenicity. The term allergenicity is used here as the ability of protein components to elicit an allergic reaction and implies immunogenicity (the ability of protein components to elicit an immune response), which in turn implies antigenicity (the ability of protein components to bind preexisting antibodies). Accordingly, potentially allergenic material signifies components with the ability to give rise to production of antibodies and components with at least two antibody binding sites because this is a prerequisite for inducing an allergic response. The lower size limit will depend on the component, but the probability that a component meets these criteria increases above 3000 Da. The present study involved characterization of six eHF and six pHF, all of which are listed in the European Society of Pediatric Allergy and Clinical Immunology (ESPACI) position paper from 1993 (7), except one pHF, Aptamil Hypoantigen. All milk formulas were characterized by SDS-PAGE, native PAGE, gel filtration run under non-dissociating conditions, immunoblotting, dot-immunobinding, and ELISA. Because the aim of this study was to detect small amounts of nondegraded protein or aggregated protein fragments, some of the analytical methods were stretched by the application of a high load of protein material. Furthermore, the examination of all protein-containing fractions of the formulas was emphasized, including investigations of precipitates following centrifugation of the formulas.

MATERIALS AND METHODS

Chemicals

Proteins were from Sigma (St. Louis, MO): aprotinin (A-1153), BSA (A-7906), α -CN (C-6780), β -CN (C-6905), cytochrome *c* (C-2506), Kunitz soybean trypsin inhibitor (**KSTI**) (T-9003), α -LA (L-6010), β -LG (L-0130), lysozyme (L-6876), ovalbumin (A-7641), and ovomucoid (T-9253). Blue dextran was from Pharmacia (Uppsala, Sweden). Tricine (T-0377) and Tris-base (T-1503) from Sigma and Coomassie brilliant blue G-250 (no. 35050) from Serva (Heidelberg, Germany) were used in SDS-PAGE. For immunoblotting, 3-cyclohexylamino-1-propanesulfonic acid (**CAPS**) (C-2632), nitrobluetetrazolium (N-6876) and 5-bromo-4-chloro-3-indolyl phosphate (B-8503) from Sigma were used. Triton X-100 used in ELISA was from Serva (no. 37240) and 3,3'-5,5'-tetra-

methybenzidine (no. 8622) from Merck (Darmstadt, Germany).

Rabbit antibodies against whey proteins (no. 45253), caseins (no. 45252), and α -LA (no. 45257) were from Riedel-de-Häen (Seelze, Germany). Rabbit antibodies against KSTI and ovalbumin were a kind gift from Hanne Frøkiær, Department of Biochemistry and Nutrition, Technical University of Denmark. Secondary antibodies were from Dako (Glostrup, Denmark): alkaline phosphatase-conjugated swine anti-rabbit antibodies (D306) were used in immunoblotting and dot-immunobinding, and peroxidase conjugated streptavidin (P397) was used in ELISA.

Milk Formulas

Milk formulas analyzed in this study are listed in Table 1. They were purchased from pharmacies or retailers in Denmark, Germany, or Austria [Nan1 (N1), Nan HA (NHA), Beba HA (BHA), Alfaré (ALF), Profylac (PRO), Nutramigen (NUT), Pregestimil (PRL)] or were kind gifts from the local representatives [Nutrilon Pepti (NP), Nutrilon Pepti Plus (NPP), Pepti Junior (PEJ), Aptamil HA (AHA), Aptamil Hypoantigen (AHY), Pregomin (PRN)]. For all products, at least two batches were analyzed unless otherwise stated. Samples of breast milk from 11 mothers were kindly provided by Arne Høst, Odense University Hospital, Denmark.

In the present study, the term "whole formulas" is used for milk formulas, which were freshly prepared in distilled water, as indicated on the package and used as such. Formulas subjected to centrifugation and filtration are specified as "precipitates" or "supernatants."

Amino Acid Analysis

The protein contents of whole formulas, fractions from gel filtration, precipitates, and supernatants obtained after centrifugation were determined by amino acid analysis by the method of Barkholt and Jensen (1). Water was drawn from a Milli-Q System equipped with an Organex cartridge from Millipore (Bedford, MA).

Gel Filtration

Gel filtration was performed on a 0.9- × 60-cm column of Sephadex G-75 (Pharmacia), which has a fractionation range of 3 to 80 kDa. All formulas were prepared as indicated on the package and centrifuged at 8000 × *g* for 15 min at room temperature. The top lipid layer was discarded, and the supernatant was filtered through a 0.45- μ m syringe filter from Sartorius (Göttingen, Germany) or through a 0.45- μ m filter followed by a 0.22- μ m filter. Each sample of 0.5 ml, containing 7.5 to 10.6 mg

Table 1. Milk formulas included in this study.

Product	Abbreviation	Manufacturer ¹	Protein basis	Degree of hydrolysis ²
Nan1	N1	A	Bovine milk proteins	Nonhydrolyzed
Nan HA	NHA	A	Whey proteins	pHF
Beba HA	BHA	A	Whey proteins	pHF
Alfaré	ALF	A	Whey proteins	eHF
Nutrilon Pepti	NP	B	Whey proteins	pHF
Nutrilon Pepti Plus	NPP	B	Whey proteins	pHF
Pepti Junior	PEJ	B	Whey proteins	eHF
Aptamil HA	AHA	C	Bovine collagen and soy proteins	pHF
Aptamil Hypoantigen	AHY	C	Whey proteins and casein	pHF
Pregomin	PRN	C	Bovine collagen and soy proteins	eHF
Nutramigen	NUT	D	Caseins	eHF
Pregestimil	PRL	D	Caseins	eHF
Profylac	PRO	E	Whey proteins	eHF

¹A: Nestlé, Vevey, Switzerland; B: Nutricia, Zoetermeer, The Netherlands; C: Milupa, Friedrichsdorf, Germany; D: Mead Johnson, Nijmegen, The Netherlands; E: ALK, Hørsholm, Denmark.

²Degree of hydrolysis according to the European Society of Pediatric Allergy and Clinical Immunology (ESPACI) position paper, 1993 (7).

of protein, was injected on the column and eluted with 100 mM NH_4HCO_3 (4 ml/h). The eluate was monitored at 280 nm, and fractions were collected. Molecular weights were calculated from a standard curve obtained by the application of blue dextran (>80,000), ovalbumin (43,000), cytochrome *c* (12,400), and aprotinin (6500).

SDS-PAGE

An Xcell II Mini-Cell system (Novex, San Diego, CA) was used with Novex precast gradient Tricine gels, 10 to 20% acrylamide, 2.6% bis-acrylamide (EC-6625). All samples were mixed 1:1 in reducing sample buffer (8% SDS, 24% glycerol, 0.1 M Tris-base, 0.02% Coomassie brilliant blue G-250, 40 mM DTT, pH 6.8) and boiled for 5 min. The cathode buffer was 0.1 M Tris-base, 0.1 M Tricine, 0.1% SDS, pH 8.25, the anode buffer was 0.2 M Tris-base, pH 8.9. The power supply (Power PAC 3000, Bio-Rad) used for electrophoresis was programmed at 35 mA constant current for 185 voltage hours (approximately 140 min).

Regardless of the staining method applied, 20 μl containing 200 μg of hydrolyzed protein was subjected to SDS-PAGE. Whole formulas and formula supernatants and precipitates, obtained after centrifugation at $8000 \times g$ for 15 min at room temperature, were analyzed. Molecular weight markers were LMW and PMW from Pharmacia, and STD, a mixture of equal amounts of α -CN, β -CN, α -LA, and β -LG.

Native electrophoresis was performed as described above, with the following modifications: SDS and DTT were omitted from all buffers, and the sample buffer was

24% glycerol, 0.2 M Tris-base, and 0.02% Coomassie brilliant blue G-250 (pH 8.8). The samples were not boiled.

Gels were Coomassie stained according to Schagger and von Jagow (32) or by silver staining according to Blum et al. (4). Silver-stained gels were developed for 3 min. For both staining techniques methanol was replaced by ethanol, and gels were immersed in 25% ethanol and 3% glycerol for 20 min before drying.

Antibodies

Antibodies against native and denatured β -LG were raised in 3-mo-old male rabbits (Black Russian). β -Lactoglobulin was denatured by heating samples of 1 mg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 , and 8.1 mM Na_2HPO_4 , pH 7.4). To obtain antibodies against different forms of denatured β -LG, two samples were heated, one to 80°C and one to 95°C for 30 min. The rabbits were immunized with a mixture of these samples. Samples of native or denatured protein were diluted in 150 mM NaCl to 400 $\mu\text{g}/\text{ml}$ and mixed 1:1 in Freund's incomplete adjuvant before subcutaneous injection of 4×0.25 ml at different sites on the back of each rabbit, corresponding to 200 μg of protein. Immunizations were performed at monthly intervals, and ear-bleeds were collected 7 d after each immunization. Sera were purified by affinity chromatography on a 1.0×4.0 -cm β -LG-coupled Mini-Leak Medium column (Kem-En-Tec, Copenhagen, Denmark). Coupling of β -LG to the matrix was performed according to the instructions of the manufacturer, using 24 mg of β -LG/g dry matrix.

Affinity chromatography was performed according to the following procedure: The column was equilibrated with 10 ml of binding buffer (3.3 M NaCl, 1.65 M glycine adjusted to pH 8.8 with NaOH). Serum was diluted 1:1 in binding buffer, and 4 ml was injected on the column. The column was then washed with 10 ml of binding buffer followed by 40 ml of PBS. Antibodies were eluted with 0.1 M citric acid, pH 3, and fractions of 1 ml were collected. A flow of 0.7 ml/min was used in all steps, and the eluate monitored by UV-detection (280 nm). Fractions containing antibodies were pooled. The solution was concentrated to 3 mg of protein/ml, and the buffer was exchanged to PBS with an ultrafiltration cell (Amicon/Millipore Corp., Bedford, MA), equipped with a PM 10 filter.

Immunoblotting

SDS-PAGE gels subjected to immunoblotting were run as described above with a prestained standard (Seebue no. SP-LC5625 from Novex). Protein material was transferred onto two layers of nitrocellulose (0.1- μ m pore size, from Schleicher and Schuell, Dassel, Germany) by semi-dry electroblotting (20). Low current transfer (0.4 mA/cm² for 1.5 h) was chosen to reduce the migration of small protein fragments through the membrane. Electrobuffer was 10 mM CAPS in 10% methanol, pH 11. After blotting, the membranes were immersed in blocking buffer (50 mM Tris-base, 150 mM NaCl, 0.2% Tween 20, pH 10.3) for 10 min. Rabbit antibodies against α -LA, β -LG, whey proteins, and KSTI were diluted 1:500, and antibodies to caseins 1:2000 in incubation buffer (50 mM Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 10.3). Membranes were incubated overnight at room temperature on a rocking table. Binding of rabbit antibodies was visualized through alkaline phosphatase-conjugated swine anti-rabbit antibodies with 5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium as substrate (3).

Dot-Immunobinding

Hydrolyzed formulas were suspended in distilled water (20 mg of protein/ml), and 1 μ l of each was applied to a sheet of nitrocellulose. The N1, 0.1 mg/ml, was used as a positive control, and ovalbumin, 1 mg/ml, as a negative control. All samples were allowed to air-dry, and the membrane was blocked and treated as described above for immunoblotting. Rabbit antibodies against whey proteins, caseins, and KSTI were applied to test the products for antigenicity. The specificity of binding was evaluated by the use of rabbit antibodies against ovalbumin as a negative control, diluted 1:500.

Stained membranes were imaged by a digital CCD video camera, and the density was evaluated by image

processing using commercially available software (CREAM, Kem-En-Tec Software Systems, Denmark) as described by Jensen et al. (17). The density of dots was expressed as "units of area," following background subtraction. Staining was considered significant when the density was three times that observed for ovalbumin.

ELISA

The content of β -LG in ready-to-use formulas was determined by sandwich-ELISA. Microtiter plates (96-well Nunc MaxiSorp F96, Roskilde, Denmark) were coated with 100 μ l of affinity-purified rabbit anti- β -LG antibodies (a 1:1 mixture of antibodies to native and denatured β -LG) at a concentration of 5 μ g/ml in 50 mM carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Coatings were done overnight at 4°C, and all subsequent incubations were carried out for 1 h at room temperature on a rocking table. Hydrolyzed formulas were suspended in washing buffer (1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 0.5 M NaCl, 2.6 mM KCl, 0.3% BSA, 0.1% Triton X-100, pH 7.4) at a concentration of 140 mg/ml, and N1 at a concentration of 0.2 mg/ml. Samples were added in triplicate on plates. β -Lactoglobulin, 1 μ g/ml, was added in duplicate and used as an internal standard. Washing buffer served as a blank. Fourfold dilutions of all samples were performed, starting by transferring 33 μ l from a well with 133 μ l to each subsequent lane containing 100 μ l of washing buffer. Rabbit anti- β -LG antibodies (6 μ g/ml), biotinylated according to Harlow and Lane (13), were then added, followed by peroxidase-conjugated streptavidin, diluted 1:5000 in washing buffer. Plates were washed four times between each step in an automatic washer (WellWash 5, Denley Instruments, UK). After the last step, plates were rinsed four times with distilled water. Enzyme-catalyzed color development was accomplished by the addition of a solution of 3,3',5,5'-tetramethylbenzidine [5 mg/ml in methanol and acetone (9:1)] diluted 36-fold in substrate solution (40 mM CH₃COOH/CH₃COONa, 3.2 mM NaBO₂·H₂O₂, pH 5.0). The enzyme reaction was stopped after 10 min by the addition of 2 M H₃PO₄ and color development measured at 450 nm by an EL340 automatic plate reader (Bio-Tek Instruments, Winooski, VT).

Nonspecific binding to plates was tested by replacing antibodies in the coating step with the egg-white protein ovomucoid (5 μ g/ml in carbonate buffer, pH 9.6). The specificity of antibody binding was evaluated by the application of 1 mg/ml of BSA, ovomucoid, ovalbumin, or lysozyme instead of β -LG.

β -Lactoglobulin in the milk formulas was quantified by a comparison of titer values for the standard (0.004 to 1000 ng/ml) and samples at 0.2 and 1.2 (A_{450}) above the blank. Titer values were found by the use of a com-

mercially available software package, KinetiCalc (1991 version, Bio-Tek Instruments). Sample and standard curves were inspected for parallelism. All curves were accepted, although there seemed to be a small matrix effect for NHA and BHA. The detection limit of the assay was 0.3 ng/ml. The internal standard gave a within-run CV of 3.6% and a between-days CV of 13.3% ($N = 26$).

The content of KSTI in pHF and eHF was determined by competitive ELISA. Microtiter plates were coated with 5 $\mu\text{g/ml}$ of rabbit antibodies against KSTI in carbonate buffer, pH 9.6. Plates were then washed with washing buffer four times. Milk formulas were suspended in washing buffer as described above and added in triplicate on plates, as was KSTI (25 ng/ml), which served as an internal standard. Samples were diluted twofold on plates. Plates were preincubated for 1 h, followed by the addition of biotinylated KSTI in washing buffer (30 ng/ml, 100 $\mu\text{l/well}$). Plates were washed four times, and peroxidase conjugated streptavidin was added (diluted 1:5000 in washing buffer). Color was developed as described above. All incubations, including the coating step, were performed for 1 h at room temperature on a rocking table. Maximal response was found as A_{450} in wells with washing buffer instead of sample. Wells without biotinylated KSTI served as a blank. The detection limit of the assay was 3 ng/ml.

RESULTS AND DISCUSSION

Physicochemical Methods

A range of physicochemical methods were used to determine potentially antigenic protein material in the 12 hydrolyzed formulas.

Gel filtration. The molecular weight profiles of the formula proteins were evaluated by gel filtration run under nondenaturing conditions to detect possible aggregates and molecules cross-linked by disulfide bridges. Sample preparation was required to precipitate insoluble material before gel filtration. The 12 milk formulas were subjected only to centrifugation, but not filtration before the first series of gel filtrations. After this procedure, all products gave very large front peaks: for NHA, PRO, and NUT the protein material eluting in the void volume constituted only 3.7, 0.18, and 0.07%, respectively, of total protein injected on the column. The large front peaks were therefore assumed to consist mainly of dispersed lipids and starch and, accordingly, filtration through a 0.45- μm filter reduced the peak considerably, and a 0.22- μm filter did so to an even greater extent. Gel filtration following filtration through both filters still gave front peaks with molecular masses of 30 to >80 kDa for all products, although with different peak areas

(shown for three products in Figure 1). The highest molecular mass determinable in the range 3 to 30 kDa was found by comparison with a standard run. As shown in Table 2, pHF as well as eHF contained protein material with apparent molecular masses in the range of 7 to >30 kDa. The material eluting at volumes larger than the total column volume (V_t , Figure 1) is tryptophan and tyrosine.

The possibility of detecting protein material of a molecular weight similar to that of intact proteins by gel filtration has been somewhat overlooked. Usually, only the percentage of protein material with a molecular mass >3 to 5 kDa is reported without any assignment of the highest molecular mass observed (2, 33), or the molecular weight profile is evaluated on hydrolysates rather than on complete products (19, 22, 34). Here we have found the highest molecular weight of protein material that could be determined by gel filtration of hydrolyzed products. Moreover, the presence of protein material with an apparent molecular weight >30 kDa was demonstrated even in eHF by amino acid analysis of the front peaks. Whether this material consists of intact proteins or aggregates remains to be established.

Centrifugation. Because gel filtration of hydrolyzed milk formulas requires centrifugation as part of the sample preparation, and precipitates may contain important fractions of potential allergenicity, we wanted to establish how protein material from each product was distributed between precipitate and supernatant. The effect of centrifugation on the products varied. The products N1, NHA, BHA, NP, PEJ, AHY, NUT, and PRL gave only small precipitates (in the range of 10 μl of precipitate/ml of supernatant), ALF and NP intermediate precipitates (100 $\mu\text{l/ml}$ range), whereas large precipitates (400 $\mu\text{l/ml}$ range) were seen for AHA, PRN, and PRO. Only for N1 was the concentration of protein in the precipitate considerably higher than that in the supernatant. The hydrolyzed milk formulas had similar concentrations of protein material in the supernatant and precipitate, but small differences in the amino acid profiles of precipitates and supernatants were found in NHA, BHA, AHY, NUT, and PRL (results not shown). Hence, precipitates from centrifuged products were further investigated by SDS-PAGE and immunoblotting.

SDS-PAGE. Gel electrophoresis was stretched beyond normal operating limits with respect to protein capacity. Regardless of the staining method applied, gels were loaded with the maximum amount of hydrolyzed protein (200 μg per lane) compatible with suspension in water and electrophoretic performance. The detection limits for β -LG by Coomassie and silver staining were 100 and 10 ng, respectively. Electrophoretic profiles of whole formulas and standards run under reducing conditions are shown in Figure 2a and b. The pHF are shown in lanes

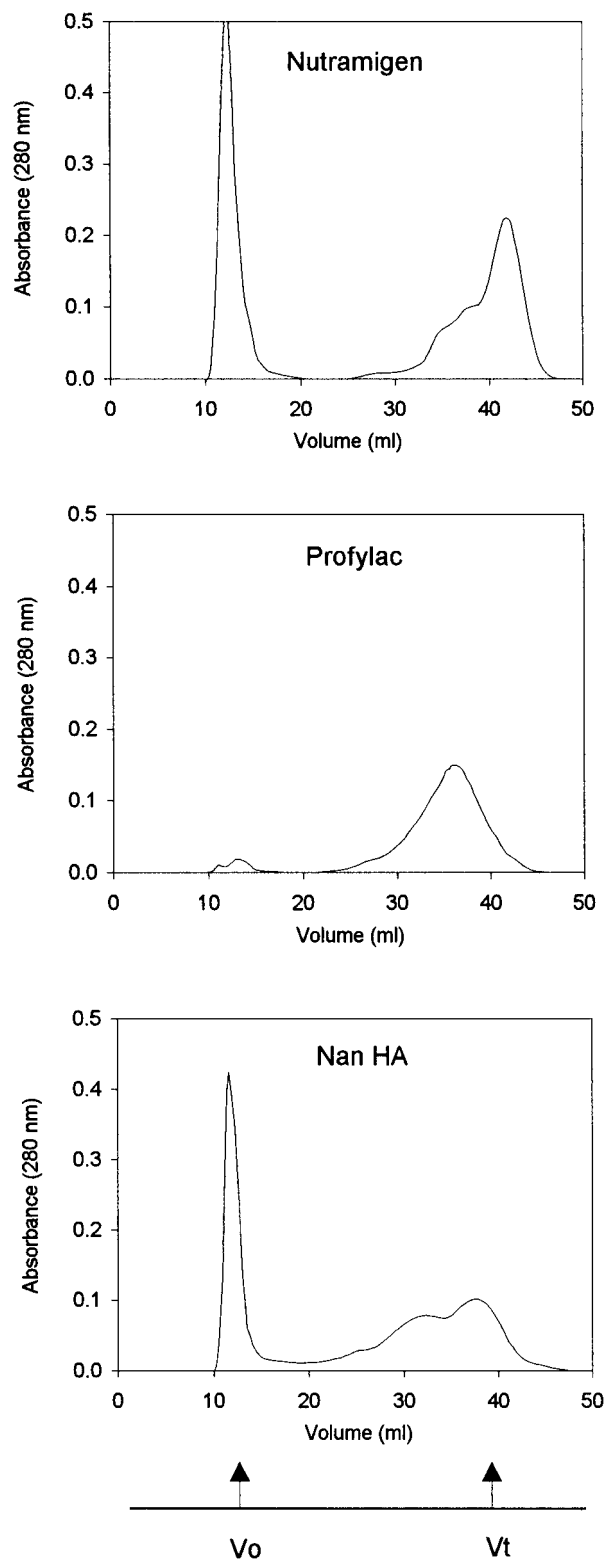


Figure 1. Chromatograms for hydrolyzed formulas, analyzed by gel filtration on a Sephadex G-75 column following filtration through a 0.22- μ m filter. Amount of protein applied: 8.3 mg (Nan HA), 7.6 mg (Profylac), and 10.6 mg (Nutramigen). V_0 is the void volume, V_t is the total column volume.

4 to 9 and the eHF in lanes 11 to 16. Intensive staining was seen for NHA, BHA, AHA, and AHY, indicating the presence of intact proteins or protein fragments. Although only partially hydrolyzed, NP and NPP did not stain appreciably by either staining method. The extensively hydrolyzed products PEJ, NUT, and PRL were also not stained. However, staining was observed for some eHF: by Coomassie staining, PRO and ALF were shown to contain protein material of an apparent molecular weight up to 12,000, and considerable staining was seen in the lane with PRN. It is remarkable that bands from PRO and ALF were barely visible following silver staining except for a 60-kDa band for ALF (Figure 2b). SDS-PAGE run in absence of reducing agent gave similar patterns.

SDS-PAGE of whole formulas, run under reducing conditions without preceding extraction or centrifugation procedures, revealed that some pHF and eHF contain protein material of apparent molecular weight well above 3000 to 5000, which is regarded as the upper limit acceptable for eHF (9, 18, 22, 34). The presence of high molecular weight material (>5000) in hydrolyzed milk formulas has also been demonstrated in other studies on single or few milk formulas, but contradictory results have been obtained. Some studies have shown no staining of eHF such as NUT (10, 24, 30), whereas protein material of molecular weight up to 70,000 has been found in other studies (28). These findings can be attributed to the application of widely different procedures, including sample preparation (extraction, centrifugation, addition of reducing agent, heat treatment), properties of gels, buffer systems, the amount of protein loaded onto the gel, and staining techniques. To be able to compare the molecular weight distribution in a range of products, the analyses should be made under identical conditions. Subjecting 12 hydrolyzed milk formulas prepared in the same way to SDS-PAGE on precast gels stained by two commonly used methods has allowed us to draw the following conclusions. Firstly, protein material in the formulas of an apparent molecular weight >5000 is visible in gels stained by silver, indicating a substantial amount of high molecular weight material in some of the products. Secondly, it appears that electrophoretic patterns obtained after SDS-PAGE are more dependent on the manufacturer (Table 1) than on the reported degree of hydrolysis (7) or the protein source. Thus, proteins in products from Milupa were intensively stained by both Coomassie and silver staining, whereas products from Nutricia and Mead Johnson contained no detectable high molecular weight material. Only the formulas from Nestlé were different in accordance with the declared degrees of hydrolysis. Thirdly, the results obtained by SDS-PAGE of pHF and eHF are highly dependent on the staining procedure. In special cases, silver

Table 2. Highest determinable molecular weight and residual antigenicity of hydrolyzed milk formulas as determined by gel filtration, ELISA, and dot-immunobinding.

Product	Gel filtration highest determinable M_r	ELISA ¹		Dot-immunobinding ²		
		μg of β -LG/L ready-to-use formula	μg of KSTI/L ready-to-use formula	Anti-whey	Anti-casein	Anti-KSTI
Nan1	>30,000	1,320,000	170	***	***	—
Nan HA	>30,000	48,180	<3	***	**	—
Beba HA	>30,000	96,250	<3	***	**	—
Alfaré	14,000	198	<3	—	—	—
Nutrilon Pepti	11,000	207	<3	—	**	—
Nutrilon Pepti Plus	12,000	18	<3	—	**	—
Pepti Junior	12,000	13	<3	—	***	—
Aptamil HA	>30,000	77	12	—	—	*
Aptamil Hypoantigen	14,000	359	<3	*	***	—
Pregomin	>30,000	4.7	<3	—	—	*
Nutramigen	7000	1.1	<3	—	—	—
Pregestimil	7000	5.0	<3	—	—	—
Profylac	12,000	23	<3	—	—	—

¹The content of β -LG was determined by sandwich-ELISA, the content of Kunitz soy trypsin inhibitor (KSTI) by competitive ELISA. The results are from one batch only. The specificity of the sandwich-assay was investigated by the application of four different proteins at a concentration of 1 g/L: Bovine serum albumin, ovalbumin, lysozyme, or ovomucoid. The first three proteins did not bind antibodies above the detection limit, whereas ovomucoid showed weak binding of antibodies corresponding to 0.37 μg of β -LG per liter.

²Asterisks (*) denote the relative density of staining as determined by densitometry, and lines (—) denote nonsignificant staining, as determined relative to a negative control (ovalbumin).

staining may be less sensitive than Coomassie staining. This was clearly demonstrated for PRO. We therefore recommend the use of Coomassie staining as well as silver staining for the evaluation of the molecular weight profile of hydrolyzed formulas.

SDS-PAGE of centrifuged formulas. The electrophoretic profiles of precipitates and supernatants are shown in Figure 3a and b. High molecular weight material was found in all precipitates, and a marked difference between the electrophoretic pattern for supernatants and precipitates was observed. Contrary to the results from SDS-PAGE of whole formulas (Figure 2b), protein fragments were detectable in the precipitates from NP and NPP. For the extensively hydrolyzed products, NUT, PRL, and PRN distinct bands appeared corresponding to molecular weights 20,000 and 23,000 (Figure 3b), illustrating a high degree of protein-enrichment by simple centrifugation. We have recently identified these two protein-bands in NUT as zeins, water-insoluble corn proteins (12). When NUT was subjected to precipitation with trichloroacetic acid before SDS-PAGE, as described by Restani et al. (28) we observed the same two bands (results not shown). These bands seem to be identical to the bands described by Restani et al. (28), and probably also to the bands detected by Hoffman and Sampson through binding of patients' IgE (14).

The results of SDS-PAGE of precipitates suggest that all 12 milk formulas are potentially allergenic, as even minute amounts of high molecular weight material are sufficient to elicit allergic responses and they all seem to

contain material of molecular weight above 5000. These findings may explain the rare occasions of allergic infants reacting to eHF. Identification of insoluble protein material present in the precipitates from hydrolyzed milk formulas may be helpful in the elucidation of the allergic responses to these products.

Native PAGE. Native PAGE of the hydrolyzed milk formulas was performed to detect possible aggregates and high molecular weight material cross-linked by disulfide bonds. In keeping with the results from SDS-PAGE, native PAGE showed that products from the same manufacturer gave similar electrophoretic patterns, and there was no indication of aggregated material (results not shown).

Immunochemical Methods

Immunoblotting. Products were investigated for residual antigenicity of whey proteins, caseins, and the soybean protein KSTI. Prevention of migration of small peptides through the membrane was attempted by the use of low current during electroblotting. Nevertheless, only pHF showed distinct binding of antibodies. For the two products NHA and BHA a sharp band corresponding to a molecular weight of 36,000 was seen. This band may represent the dimer of undegraded β -LG. The application of rabbit anti-casein, anti- β -LG, and anti- α -LA antisera gave essentially the same patterns as anti-whey antiserum. Incubation with anti-KSTI antibodies showed staining only in the PRN lane and only to a low degree (results not shown). Electroblotting of formula

precipitates and incubation with anti-whey antibodies did not reveal additional bands.

Considering the relatively large amount of protein material visualized by Coomassie staining of gels run by SDS-PAGE, immunoblotting was expected to give more intense staining than observed, especially for the precipitates. Immunoblotting depends, however, not only on the amount of protein in the gel and the binding of antibodies but also on electrotransfer and binding capacity of protein of the membrane. Furthermore, the immunoblot pattern observed will depend on whether polyclonal or monoclonal antibodies are used. We have utilized polyclonal antibodies with a broad specificity to detect as

many epitopes as possible. When monoclonal antibodies are employed, the epitopes present may be underestimated. Yet, Restani et al. (27) succeeded in demonstrating the presence of antigenic protein material in protein-enriched pHF and eHF by the use of monoclonal anti-casein antibodies. The use of patients' sera in immunoblotting has also allowed the detection of allergenic material in hydrolyzed formulas through IgE-binding to epitopes (14, 28).

Dot-immunobinding. Dot-immunobinding was applied as an alternative to immunoblotting. Formulas were applied directly onto nitrocellulose-membranes. The relative staining with rabbit anti-whey, anti-casein,

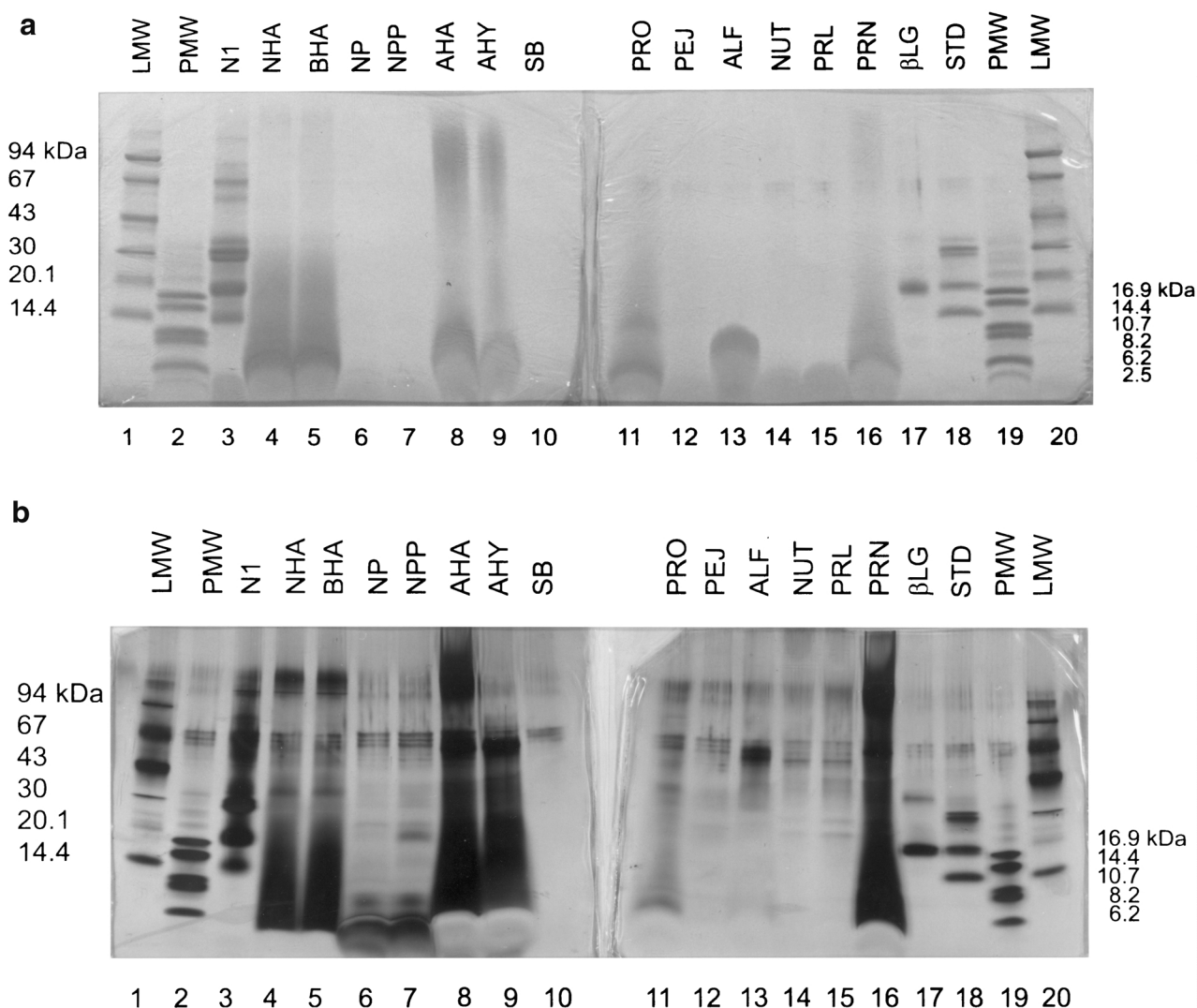


Figure 2. SDS-PAGE of six partially hydrolyzed formulas (left) and six extensively hydrolyzed formulas (right) run under reducing conditions. The material in each lane is indicated above the lane, abbreviated according to Table 1. LMW and PMW are Pharmacia standards. STD is a mixture of equal amounts of α -CN, β -CN, α -LA, and β -LG. SB is sample buffer. a) Coomassie stained gels. The protein amounts in lanes 1 and 20: 7.5 μ g; lanes 2 and 19: 10 μ g; lane 3: 20 μ g; lanes 4 to 9 and 11 to 16: 200 μ g; lane 17: 2 μ g, lane 18: 4 μ g. b) Silver-stained gels. The protein amounts in lane 1 and 20: 0.8 μ g; lane 2 and 19: 2 μ g; lane 3: 1 μ g; lanes 4 to 5: 20 μ g; lane 6 to 9 and 11 to 16: 200 μ g; lane 17: 0.2 μ g; lane 18: 0.4 μ g. SDS-PAGE run in the absence of reducing agent gave similar patterns.

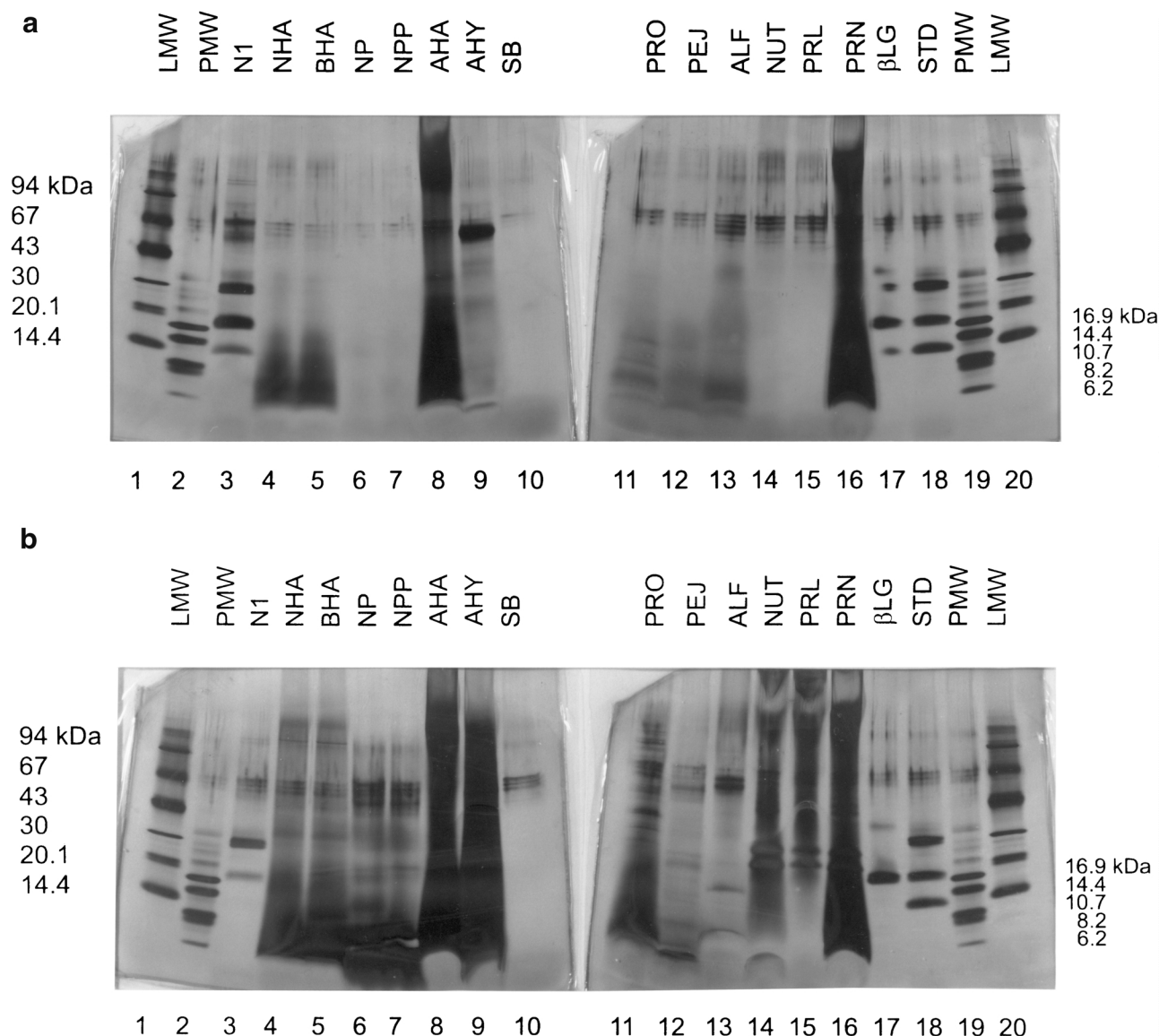


Figure 3. Silver-stained SDS-PAGE of supernatants and precipitates following centrifugation of six partially hydrolyzed formulas (left) and six extensively hydrolyzed formulas (right). The material in each lane is indicated above the lane, abbreviated according to Table 1. LMW and PMW are Pharmacia standards. STD is a mixture of equal amounts of α -CN, β -CN, α -LA, and β -LG. SB is sample buffer. a) Supernatants after centrifugation. b) Precipitates after centrifugation. The protein amounts in lane 1 and 20: 0.8 μ g; lane 2 and 19: 2 μ g; lane 3: 1 μ g; lanes 4 to 5: 20 μ g; lane 6 to 9 and 11 to 16: 200 μ g; lane 17: 0.2 μ g; lane 18: 0.4 μ g. The concentrations of protein material in precipitates and supernatants were determined by amino acid analysis.

and anti-KSTI antibodies is depicted in Table 2, demonstrating staining for all pHF, although to a different extent. Incubation with anti-KSTI showed only weak binding of AHA and PRN, both based on soy protein. Nan HA and BHA were easily recognized by anti-whey antibodies, AHY bound anti-whey antibodies weakly, and AHA, NP and NPP did not bind these antibodies at all. Incubation with anti-casein antibodies, on the other hand, revealed staining not only for NHA, BHA, and AHY, but also for the three products from Nutricia, NP,

NPP and PEJ, although none of these products is based on casein. Moreover, PEJ is an eHF. It may be that the glycomacropeptide from κ -casein, which follows the whey fraction during precipitation of casein with rennet, is present in the products. The presence of epitopes recognized by anti-casein antibodies in NP and PEJ is supported by the studies of Restani and coworkers (27).

ELISA. Proteins were quantified by ELISA methods. The residual antigenicity of β -LG was determined, since β -LG is a major cow's milk allergen (35); for hydrolyzed

milk formulas based on whey proteins, the content of residual β -LG also provides information about the extent of hydrolysis.

Using a sandwich ELISA, we demonstrated the presence of β -LG in all milk formulas (Table 2) in accordance with results from other investigations (23, 24). In N1, the conventional formula, 1320 mg/L of β -LG was found, corresponding to 9% of the protein content of the formula. β -Lactoglobulin was also detected in all hydrolyzed products, regardless of the protein source. For NHA and BHA, the content of β -LG was reduced approximately 15-fold relative to N1, whereas the content was reduced more than 260,000-fold for NUT, PRL, and PRN. For comparison, we determined the content of β -LG in breast milk from 11 lactating women. Each sample was analyzed individually. The median value was 1.6 μ g/L and the range was <0.3 to 19.8 μ g/L, illustrating the variation between individuals as reported also by Høst et al. (16), who demonstrated the presence of 0.9 to 150 μ g β -LG/L in 19 out of 20 mothers, median value 4.2 μ g of β -LG/L. As can be seen from Table 2, the β -LG level is <19.8 μ g/L in five and <150 μ g/L in seven out of 12 products, indicating thorough hydrolysis. Nonetheless, the presence of β -LG in products based on proteins other than whey proteins calls for some consideration. Residual β -LG in NUT, based on caseins, can be explained by coprecipitation of whey proteins during isolation of the casein fraction. Contamination during processing may also occur. The presence of β -LG in the soy-based formulas PRN and AHA is probably a result of contamination. It should be stressed, however, that the content of residual antigenicity of proteins might be overestimated by ELISA methods involving polyclonal antibodies due to: 1) the detection of many peptides rather than one intact polypeptide chain; 2) nonparallelity between standard and sample curves as a result of differences in affinity towards native proteins and peptides, and 3) nonparallelity of curves due to matrix effects. Overestimation might account for the relatively high level of residual whey protein reported for NUT in investigations using competitive ELISA, i.e., 25.6 μ g/g of protein, corresponding to 466 μ g/L in ready-to-use formula reported in (10) and 911 μ g/L reported in (14).

Small amounts of KSTI were found in the conventional formula N1 and in AHA; the latter product was based partly on soy protein. The presence of KSTI in N1 may be attributed to soy in cow's feed.

Combined results. With the results from all methods taken together, the 12 products could be classified into four groups: *a*) NHA, BHA; *b*) AHY, AHA; *c*) ALF, NP, NPP, PEJ, PRO, PRN; and *d*) NUT, PRL, ranked from highest to lowest allergenic potential. This classification is in agreement with clinical reports accessible for some of the products. Thus, provocation tests have revealed

reactions to NHA, ALF, and NUT in decreasing order (29). Similar results have been found with skin prick testing of individuals who are allergic to cow's milk and by the application of radio-allergo-sorbent tests on patients' sera (25). In accordance with these observations and with the serious adverse reactions to NHA in some cow's milk allergic infants (8), only eHF are approved for infants with established cow's milk allergy (7). It is therefore of great commercial interest to have a hydrolyzed product classified as eHF rather than pHF. In the present study, we were able to classify milk formulas into four groups (*a* to *d*) and not into two (eHF and pHF). For instance, group *c* includes pHF as well as eHF. These findings suggest that some products may qualify as eHF, if investigated properly in clinical trials. It should be remembered, however, that a product labeled eHF is not synonymous with a nonallergenic product, since anaphylactic reactions to products in group *a* (11) as well as in group *d* (31) have been observed.

CONCLUSIONS

In this study, 12 hydrolyzed formulas were investigated by physicochemical and immunochemical methods. Analyses of formula precipitates after centrifugation and of formula supernatants by gel filtration under non-dissociating conditions showed that the 12 products did not contain substantial amounts of aggregated or polymerized material. Results from gel filtration, SDS-PAGE and ELISA suggest, however, that both eHF and pHF contain potentially allergenic protein material.

SDS-PAGE was found to be best suited for screening of potential allergenicity. Centrifuging formulas followed by SDS-PAGE of precipitates greatly improved the possibility of detecting protein material with molecular weights as for intact proteins. The use of both Coomassie and silver staining ensured detection of bands that would otherwise have been overlooked. A comparison of electrophoretic patterns revealed that products from the same company seemed to be more alike than products from different companies based on the same proteins or products with the same declared degree of hydrolysis. Dot-immunobinding and immunoblotting did not provide further information, but the application of sensitive ELISA techniques is mandatory for detecting contaminating potentially allergenic proteins in the formulas.

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