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The antigenicity and allergenicity of microparticulated proteins: Simplesse®

H. A. SAMPSON and S. COOKE

Department of Pediatrics, Division of Allergy/Immunology, Johns Hopkins University, Baltimore, Maryland, U.S.A.

Summary

New technologies are allowing the food industry to develop products from standard foods which may not be recognized in its modified form by food allergic patients. One such product, Simplesse®, has been formulated by microparticulation of egg white and/or cows' milk proteins and is used as a fat substitute in many fat-laden foods. The purpose of this study was to determine whether the process of microparticulation altered the allergenicity/antigenicity of egg white and cows' milk proteins compared to the starting materials.

Soluble protein fractions of Simplesse® and its respective starting materials were compared to egg white, cows' milk protein, an ultra-filtered egg white/condensed milk mixture, and/or a whey concentrate by SDS-polyacrylamide gel electrophoresis. In addition, sera from 16 patients with documented egg and/or cows' milk hypersensitivity and two controls who were not allergic to egg or milk were used to assess potential allergenicity/antigenicity of these products by immunoblot (Western blot) analysis. There were heterogeneous IgE and IgG binding patterns to the food fractions among these food allergic patients suggesting differing sensitivity patterns among the individuals tested. However, utilizing both SDS-PAGE and immunoblot analyses, the major allergens in the microparticulated products were the same as those found in the starting materials, egg and cows' milk. In addition, there was no evidence of 'novel' protein fractions in the Simplesse® test materials compared to the starting materials.

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Introduction

A variety of processing techniques have long been utilized to alter the taste and texture of common foods. The physical structure of many foods may be altered simply by heating, mixing, and/or whipping. One type of structural change that may occur from such processing is microparticulation of protein. Microparticulated proteins may develop naturally in some foods or arise secondary to normal processing techniques (blending, mixing, pasteurization, pH alternation, baking) used in the food industry. Recently a technique utilizing heat and a high shear processor has been developed to coagulate food proteins into microparticles that are uniform in particle distribution, size, and quality. These microparticles are 0·1–3 μm in diameter and when placed in the mouth are perceived as fat. The resulting material, Simplesse®, may be used to replace the fat in various fat-laden foods, greatly reducing the fat, cholesterol and caloric content of these foods. Simplesse® is available in a large variety of low-fat food products in the United States and recently became available in the United Kingdom. It is being used primarily in cream-based foods, such as frozen desserts, cream cheeses, soft cheeses, sour cream, etc., and oil-based products such as mayonnaise and salad dressings, where it comprises 7% to 40% of total protein. Simplesse® has been approved for use in most European Economic Community countries, Japan, and Australia. In addition, other protein sources (e.g. soy) could be utilized in this process.

The processing of food proteins may lead to some alteration in the antigenic/allergenic composition of the natural food [1]. The specific allergenic fractions of egg and cows' milk have been isolated and well characterized [2]. The major allergenic proteins in egg are ovalbumin, ovomucoid, and ovotransferrin [3–6] and the major

Correspondence: Professor H. A. Sampson, Johns Hopkins Hospital, CMSC-1103, 600 N. Wolfe Street, Baltimore, Maryland 21205, U.S.A.

allergenic proteins in cows' milk are β-lactoglobulin, α-lactalbumin, and caseins [7–9]. These allergenic components are glycoproteins with molecular weights in the range of 12 000 to 80 000 Daltons and are largely resistant to heating and to breakdown by proteases. Simplesse®, which is made from ultra-filtered egg white and skim milk proteins or whey concentrate, is subjected to heat and high shear forces to form microparticles. The purpose of this study was to determine whether the process of microparticulation alters the antigenicity/allergenicity of the starting ingredients. Immunoblot analyses were performed with sera from individuals with documented allergic reactions to whole cows' milk and/or eggs. Additional analyses included comparisons with test articles subjected to conventional heating to determine any effects of heating on the antigenicity/allergenicity of the starting ingredients. Although the process of microparticulation represents a true technological breakthrough for diet conscious individuals, it could present a danger for egg or milk allergic patients if not appropriately labelled.

Materials and methods

Patient population

Since the initial studies by May [10], the double-blind placebo-controlled oral food challenge (DBPCFC) has become accepted as the 'gold standard' for diagnosing food allergy. Utilizing this method [11,12], 124 patients have been identified with IgE-mediated hypersensitivity to egg and 54 patients with IgE-mediated hypersensitivity to cows' milk. Sera from 13 representative patients (from whom ample quantities of sera were available) with documented symptomatic food allergy to egg and/or milk were used for immunoblot analyses of the Simplesse® formulated from milk and egg (Simplesse-1), egg, milk, and egg white/cows' milk concentrate. Seven patients were clinically symptomatic to both egg and cows' milk, three were symptomatic to egg, and three to milk. Sera from six milk allergic patients were used for evaluation of Simplesse derived from whey concentrate (Simplesse-2), whey and milk. Specific IgE antibodies to egg and/or cows' milk were demonstrated by positive prick skin tests in all cases. Sera from two normal controls with no evidence of symptomatic allergy to egg or cow's milk also were examined. Both normal controls were previously shown to possess very small amounts of IgG specific antibodies to milk by a sensitive enzyme-linked immunoassay.

Microparticulated protein test materials

Five Simplesse-1 test materials (Simplesse-1 premix, Simplesse-1 premix gently heated [163°C.; custard-like material], Simplesse-1 treated with extreme heat, Simplesse-1, and Simplesse-1 with extra sugar), ultra-filtered egg white, condensed skim milk, Simplesse-2 test materials (Simplesse-2 premix, Simplesse-2 premix + cooking, and Simplesse-2), and whey concentrate were received frozen (−12°C) from The NutraSweet Company. Fresh eggs and whole milk were purchased locally; egg white was carefully separated from the yolk and cows' milk was defatted by centrifugation.

Soluble protein extracts were prepared in a standard fashion by adding 3 g of each sample to 30 ml (1:10 weight:volume) of PBS (phosphate-buffered saline), pH 7·4, and rotated at 4°C for 14 hr. Sample supernatants were first centrifuged at 3000 times gravity for 10 min to remove large particulate matter. Supernatants were then centrifuged a second time at 17 000 times gravity for 15 min to remove all particulate matter. Supernatants were sterile filtered (0·22 μm filter) and then analysed for protein content utilizing the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, California, U.S.A.). Following protein determination, aliquots of samples were frozen at −20°C until used for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

All protein extracts (5 Simplesse-1 test materials, a mixture of 2 parts ultra-filtered egg white and 1 part condensed milk (E+M), 3 Simplesse-2 test materials, whey concentrate, egg white and cows' milk) were adjusted to a concentration of 1 mg/ml and then mixed with equal volumes of 2Xs SDS sample buffer (0·125 M of Tris HCl (pH 6·8) buffer containing 2% SDS, 10% B-mercaptoethanol, 10% glycerol, bromophenol blue, and pyronin Y), and boiled for 10 min.

The protein extracts were resolved in a discontinuous SDS-PAGE system as previously published [13]. Briefly, the running gel was prepared from a stock of 33·5% acrylamide and 0·3% *N*, *N*′-bisacrylamide to a final concentration of 13·5% acrylamide in 1 M Tris-hydrochloride, pH 9·1. The stacking gel was prepared from a stock of 30% acrylamide and 0·44% *N*,*N*′-bisacrylamide to a final acrylamide concentration of 3% in 0·5 M Tris-hydrochloride, pH 6·8. Both gels contained 0·1% SDS and were polymerized with 3% ammonium persulphate and *N*,*N*,*N*′,*N*′-tetramethylenediamine. The electrode tank buffer was 25 mM Tris, 192 mM glycine with 0·1% SDS. Previous studies demonstrated that 25 μg of the protein extracts were the maximum amount of protein that could be added to each lane to provide maximum sensitivity without encountering protein diffusion into neighbouring lanes.

Immunoblotting

The study protein fractions were transferred from the polyacrylamide gels on to nitrocellulose paper overnight

by electrophoretic transfer at 0·15 A in 50 mM Tris-glycine (pH 9·1) containing 20% methanol at room temperature [13]. The nitrocellulose blots were blocked in 0·5% porcine gelatin in phosphate buffered saline, pH 7·4, with 0·05% Tween 20 and 0·001% merthiolate for 5 to 6 hr at room temperature. The blots were then probed for antigen-specific IgE or IgG binding to the transferred protein bands. The nitrocellulose blots were incubated for 3 hr at room temperature on a rocking platform in patient or control sera diluted in PBS Tween with 0·5% porcine gelatin and 0·001% merthiolate (1:25 for IgE and 1:500 for IgG), and then washed for 5 min each with five exchanges of 0·05% Tween 20 in PBS. The blots were then incubated for 2 hr at room temperature with 1:2000 dilution of adsorbed, affinity purified, biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, U.S.A.) or 1:15000 goat anti-human IgG (Jackson Laboratories, West Grove, Pennsylvania, U.S.A.), in 0·5% porcine gelatin in PBS, and the wash step repeated. The blots were then reacted with enzyme solution (1:1000 dilution of Horseradish Peroxidase-Avidin D [Vector Laboratories, Inc., Burlingame, California, U.S.A.]) in 0·5% porcine gelatin in PBS for 1 hr. After repeating the wash step, the blots were developed for 2–3 minutes in 10 mM potassium phosphate, pH 6·8, with 1 mM EDTA containing 0·009% H_2O_2 and 0·3 mg/ml 3,3′-diaminobenzidine. The reaction was stopped by washing the developed blots in PBS.

Results

Food extract protein content

The protein content of all test materials is shown in Table 1. The quantities of water-extractable protein from the cooked materials (Simplesse-1 premix + gentle and extreme heat, Simplesse-1, Simplesse-2 + cooking, and Simplesse-2) are significantly less than that from the raw starting materials. However, sufficient extractable protein was available from all samples for testing.

SDS-PAGE and electrophoretic transfer to nitrocellulose

Figures 1 and 2 demonstrate the protein fractions of each sample following SDS-PAGE and electrophoretic transfer to nitrocellulose followed by staining with amido black. Comparison of the amido black stained nitrocellulose to the silver nitrate stained acrylamide gel demonstrated good transfer of all protein fractions to the nitrocellulose. Silver nitrate staining of an acrylamide gel following the electrophoretic transfer to nitrocellulose revealed no residual protein, indicating complete transfer of the protein fractions. Figure 1 demonstrates the main protein fractions identified in the Simplesse-1 test

Table 1. The protein concentration of each Simplesse-1 extract test material and the calculated percentage of protein extracted from the starting material

Sample	Protein content (mg/ml)	Yield (%)
Simplesse-1 premix	7·6	62
Simplesse-1 premix + gentle heat	1·3	10
Simplesse-1 premix + severe heat	1·3	10
Simplesse-1	2·9	24
Simplesse-1 + extra sugar	2·6	25
Egg + milk concentrate*	9·3	—
Simplesse-2 premix	6·1	36
Simplesse-2 premix + cooking	0·8	4
Simplesse-2	1·4	8
Whey concentrate	7·8	45
Egg	7·5	74
Milk	1·7	52

*Ultra-filtered egg white: condensed milk = 2:1.

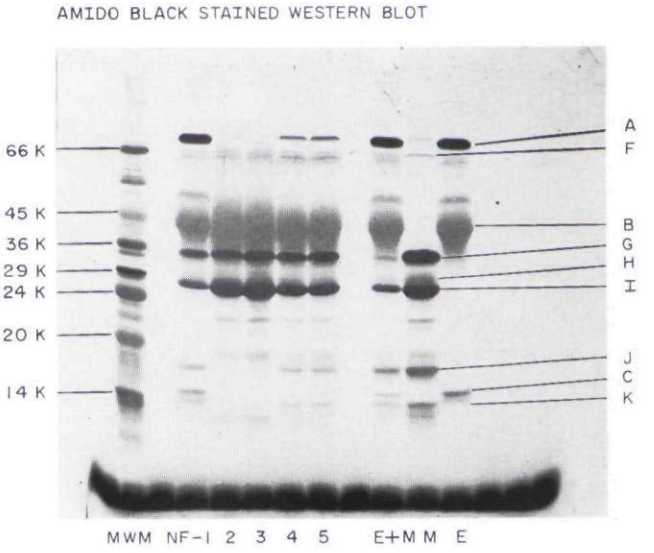


Fig. 1. Protein fractions of Simplesse-1 test materials produced by SDS-PAGE followed by electrophoretic transfer to nitrocellulose and stained with amido black. The lane orientation is the same for immunoblots in Fig. 4(a–c). MWM, molecular weight markers. NF-1, Simplesse-1 premix. NF-2, Simplesse-1 premix + gentle heat. NF-3, Simplesse-1 premix + severe heat. NF-4, Simplesse-1. NF-5, Simplesse + extra sugar. E + M, ultra-filtered egg white + condensed skim milk. M, milk. E, egg. A, ovotransferrin. B, ovalbumin. C, lysozyme. F, bovine serum albumin. G, α-casein. H, β-casein. I, κ-casein. J, β-lactoglobulin. K, α-lactalbumin.

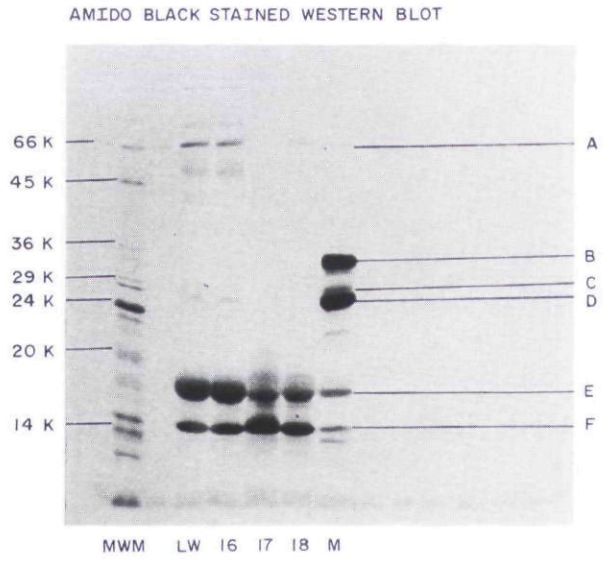


Fig. 2. Protein fractions of Simplesse-2 test materials, whey concentrate, and whole cow's milk produced by SDS-PAGE followed by electrophoretic transfer to nitrocellulose. The lane orientation is the same in Fig. 5 immunoblot. MWM, molecular weight markers. LW, liquid whey. 16, Simplesse-2 premix. 17, Simplesse-2 premix + cooking. 18, Simplesse-2. A, bovine serum albumin. B, α-casein. C, β-casein. D, κ-casein. E, β-lactoglobulin. F, α-lactalbumin.

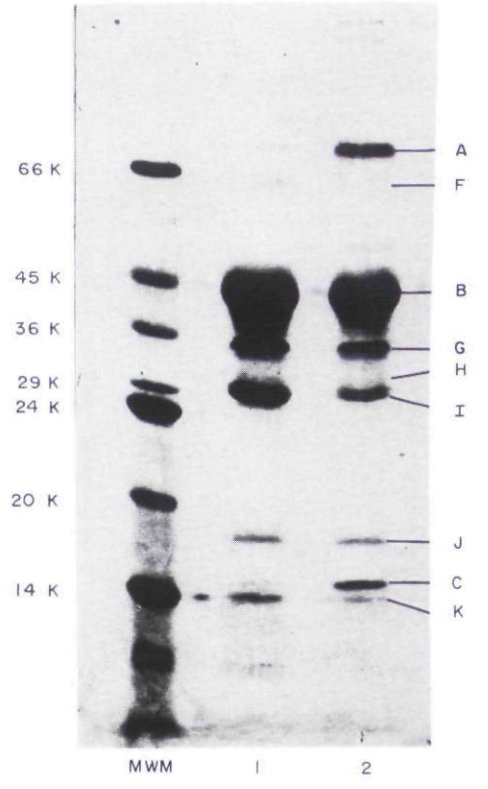


Fig. 3. SDS-PAGE comparing non-extracted Simplesse-1 dissolved directly in SDS sample buffer (1) and PBS-extracted (i.e. water-soluble) Simplesse-1 proteins (2).

materials, the 2:1 egg/milk concentrate, milk, and egg, and Fig. 2 the main fractions in the Simplesse-2 test materials and milk. The major fractions in egg white identified by this technique consist of ovotransferrin, ovalbumin, and lysozyme (ovomucoid is not apparent by amido black staining as previously reported [6]); and the major fractions identified in milk are bovine serum albumin, α-, β-, κ-casein, β-lactoglobulin, and α-lactalbumin. Electrophoresis of Simplesse-1 and Simplesse-2 test materials revealed the same protein fractions seen in native egg white and cows' milk. No protein fractions were identified that were not present in cows' milk and egg. Previous studies have demonstrated that as little as 2·5 ng of an isolated protein can be detected by this method [14].

Since only a small fraction of the total protein of a sample was found in the water-soluble, extracted protein materials, whole Simplesse-1 was dissolved directly in the SDS-sample buffer and loaded onto the gel to assure that relevant less soluble proteins in Simplesse were not being lost during the extraction process. As shown in Fig. 3, all protein bands present in the non-extracted product (no. 1) also were present in the extracted material (no. 2).

Figure 4 represents immunoblot analyses with sera from three of the 13 egg and/or cows' milk allergic patients examined. The left-hand figure shows IgE-specific antibodies to the Simplesse-1 test material fractions, egg, milk and egg white/cow's milk concentrate, and the right-hand figure demonstrates IgG specific antibodies to these same fractions. Similarly, Fig. 5 depicts an immunoblot analysis of Simplesse-2 with sera from one of the six subjects with cows' milk allergy. Examination of all patients' immunoblots demonstrated some variations in the binding intensity of IgE and IgG antibodies to the major cows' milk and/or egg protein fractions. All patients and the two normal controls demonstrated some milk- and egg-specific IgG antibodies to the major egg and milk protein fractions in all test materials. However, only the cows' milk and egg allergic patients had specific IgE antibodies to the various cows' milk and egg protein fractions. A comparison of patient sera antibody binding to Simplesse-1 and Simplesse-2 test materials and to milk and egg proteins revealed no apparent differences in antibody binding to proteins in the Simplesse test materials compared to native egg and cows' milk.

Discussion

Approximately 2–3% of the population experience food allergic reactions at some time in their life. In the

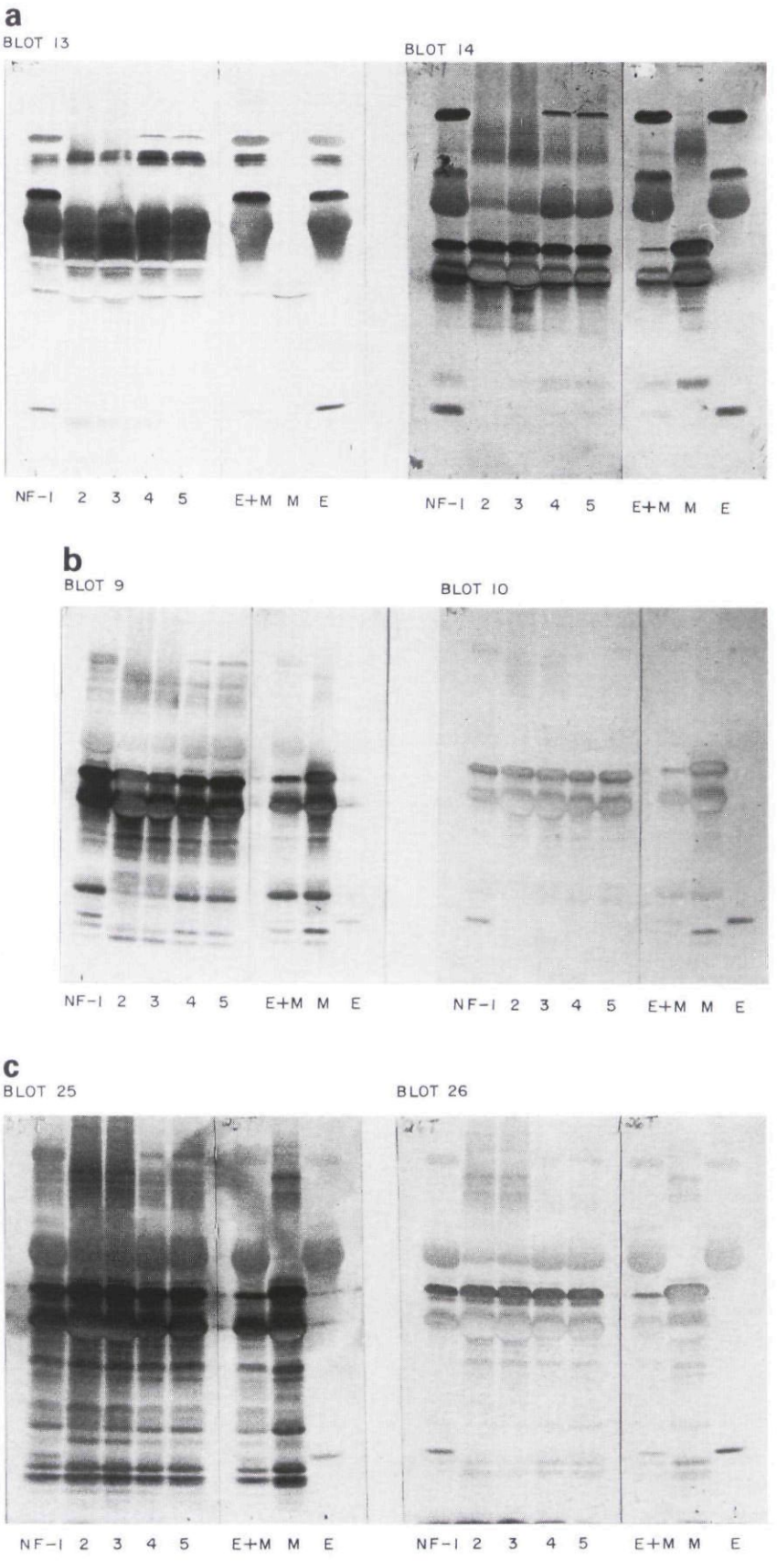


Fig. 4. Immunoblot analyses of the sera from 3/13 patients with documented allergy to egg and/or milk. The Simplesse-1 test materials are in the same orientation as shown in Fig. 1. The left-hand figure shows IgE-specific antibodies to the test materials, and the right-hand figure demonstrates IgG-specific antibodies to these same fractions. (a) Egg allergic patient. (b) Milk allergic patient. (c) Egg and milk allergic patient.

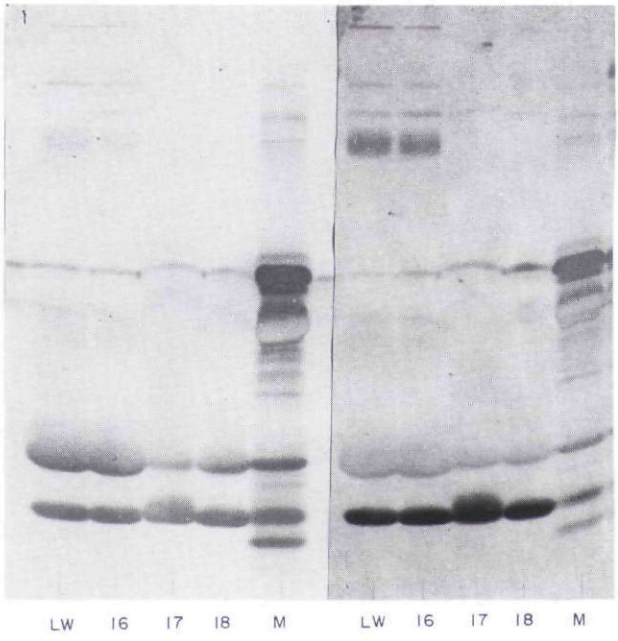


Fig. 5. Immunoblot analysis of Simplesse-2 test materials using sera from a cows' milk allergic patient. The Simplesse-2 test materials are in the same lane orientation as shown in Fig. 2. The left-hand figure shows IgE-specific antibodies and the right-hand figure demonstrates IgG-specific antibodies to the test materials.

paediatric population, eggs and milk are the most common food allergens whereas in the adult population, peanuts, tree nuts, and seafood comprise the most frequent offenders [15]. The best understood food allergic reactions are secondary to the development of IgE antibodies to specific food antigens. Once an individual becomes sensitized (i.e. makes food-specific IgE antibodies), ingestion of the food may lead to a variety of symptoms: cutaneous—urticaria, morbilliform rash, eczema; respiratory—nasal congestion, rhinorrhoea, and sneezing, laryngeal oedema, wheezing; gastrointestinal—abdominal pain, nausea, vomiting, and diarrhoea; and anaphylactic shock and death [15–17].

SDS-PAGE provides a sensitive method for analysing individual protein fractions comprising allergens, including those found in pollens, dust mites, and foods [6,18]. Proteins are reacted with the anionic detergent, sodium dodecylsulphate (SDS), to form negatively charged complexes. Proteins are denatured and solubilized by their binding of SDS and therefore migrate according to their charge and weight. Subsequent quantitative transfer of component bands from high resolution polyacrylamide gels to nitrocellulose (immunoblotting or Western Blotting) immobilizes the protein fractions and allows access for assessment of enzymatic and binding activities. This combination of SDS-PAGE with immunoblotting techniques permits antigenic/allergenic components of various protein mixtures (e.g. foods) to be precisely defined by reacting patient sera (which contain antibodies of various immunoglobulin isotypes) with protein fractions transferred to nitrocellulose [18].

Examination of the fractionated Simplesse, cows' milk, and egg revealed protein fractions of identical molecular weight and charge. As demonstrated in a study of hydrolysed cows' milk protein infant formulas, the absence of demonstrable bands on SDS-PAGE gels and immunoblots correlates with an inability to provoke an allergic response [14]. In addition, it was demonstrated that SDS-PAGE with silver staining could detect protein fractions at a concentration of 2·5 ng, a concentration below which allergic individuals appear unlikely to experience an allergic reaction.

These studies confirmed that patients clinically allergic to egg and/or cows' milk possess IgE and IgG antibodies to multiple protein fractions in egg and cows' milk, as well as the microparticulated egg/cows' milk proteins, Simplesse-1 and Simplesse-2. The major allergenic fractions seen in Simplesse-1 and Simplesse-2 test materials appear identical to those identified in native egg and cows' milk. The intensity of staining of protein bands on the immunoblot directly correlates with the number and affinity of food fraction-specific immunoglobulin molecules present in patient sera. Therefore, there is no evidence that these microparticulated proteins have more accessible antigen epitopes leading to increased antibody binding activity, or that the major allergenic epitopes of cows' milk and egg are destroyed.

Use of microparticulated protein in place of fats in many food products provides a significant advance in preventative health management of cardiovascular disease. For example, a frozen dessert made with Simplesse would contain less than one gram of fat, be considered low in cholesterol, and have half the calories of a super premium ice cream containing 16% butterfat. However, the *in vitro* tests conducted in this study indicate that egg allergic or cows' milk allergic individuals ingesting Simplesse® are likely to experience allergic reactions similar to those provoked by the ingestion of 'native' egg or whole cows' milk. Since this process will present egg and milk proteins in an unfamiliar form, and since the greatest danger to patients with food allergy is the unknowing ingestion of a food allergen [16,17], it is paramount that all food products containing these microparticulated proteins, such as Simplesse®, be clearly labelled to indicate the presence of egg white and cows' milk, and that food allergic patients be warned about the composition of Simplesse®.

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