

Application of an electrophoretic methodology for the identification of low molecular weight proteins in foods

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Identification and characterisation of food proteins are core features of food allergy research. Current methods used to identify allergenic proteins in food have insufficient resolution and are unable to detect low molecular weight proteins. In this study we report the use of a simple SDS-PAGE method which allows resolution of small proteins. We have subsequently applied this method and reported presence of low molecular weight proteins in a range of hydrolysed milk formulae (Nutramigen, Pregestimil, Alfare, Pepti-Junior and Pregomin), and crude peanut protein extract. The molecular weight distribution for the peanut extract and the hydrolysates ranged between 5–200kDa and 2–17kDa respectively.

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Physical and chemical characterisation of such inhaled allergens as pollens, dust mites and animal danders are well documented (1). In comparison, the amount of work which has been carried out on identification and purification of food allergen is relatively small.

Food allergy is more common in infants and children, affecting up to 8% of preschool children (2). Three foods (cow's milk, hens' egg and peanuts) account for approximately 80% of positive food challenges in such allergic children (3–4). Food allergens leading to specific sensitisation have been demonstrated to be proteins or glycoproteins, (5) but, so far, no features have been identified that distinguish allergens as unique antigens (6). Characterisation of food allergens is germane to structural and immunological studies aimed at understanding how they stimulate IgE production.

To date most identified major food allergenic proteins have been of high molecular weight. However, it is likely that immediate Type-I reactions to foods is initiated by low molecular weight proteins. It is extremely difficult to identify these small proteins using conventional biochemical techniques such as SDS-PAGE system. There are no published data regarding specific identification of low molecular weight food proteins. Most existing methods of electrophoresis all have an insufficient resolving power for proteins below 10kDa and either not all subunits can be detected or the

reproducibility is inadequate. We have employed a simple SDS Page method originally developed by Schagger & von Jagow (7) which will allow resolution of small proteins in hydrolysed milk formulae and peanut. Although the procedure described in this paper is documented previously (7), applying it to identify low-molecular weight food-proteins is an original idea.

Material and method

Chemicals – SDS, Tricine, glycerol, acrylamide, bisacrylamide, Serva blue G, TEMED, Tris, ammonium persulphate and bromophenol blue were purchased from Sigma (Poole, UK). Molecular weight standards were purchased in the PMW kit from Pharmacia-UK (2.55–17.2 kDa) and Novex-USA (4–250kDa).

Protein extraction

Hydrolysates: Five different hydrolysates were investigated:

- 1) Casein hydrolysate I (Nutramigen, Mead-Johnston)
- 2) Casein hydrolysate II (Pregestimil, Mead-Johnston)
- 3) Whey hydrolysate I (Alfare, Nestle)
- 4) Whey hydrolysate II (Pepti-Junior, Nutricia)
- 5) Partial soy-bovine collagen hydrolysate (Milupa).

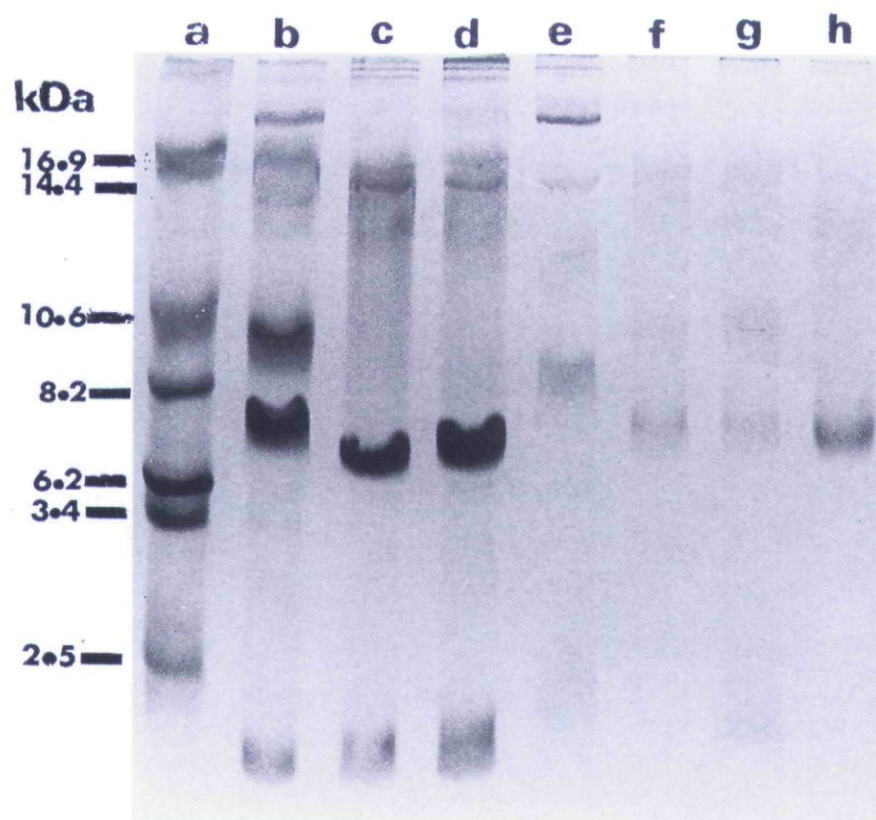


Fig. 1. Separation of hydrolysates. The low molecular weight markers are shown in lane a. Lane b is Alfare (20mg) and lanes c and d are Pepti-junior (10 and 15mg respectively). Lane e demonstrates the electrophoretic mobility of Pregomin (5mg) while lanes f-g represent Nutramigen (10 and 20 mg respectively) and lane h is Pregestimil (20mg).

Each formula was reconstituted and 500 ml of each was extracted with diethyl ether as previously reported (8). Following lipid extraction each milk was dialysed against distilled water for 24 h with three changes of dialysate. The dialysing tube used had a molecular weight cut-off of 1.3kDa (Medicell International LTD, London, UK). The resulting solution was freeze dried and protein content was measured. Each protein extraction was repeated 5 times and the reproducibility of protein extraction was above 95%. Protein extracts were stored at -20°C until used in the study.

Peanut extracts

Defatted crude peanut extract, CPE, (Sigma Chemicals, Poole, UK) was subjected to FPLC employing methods similar to those described previously (9). Briefly the CPE was dialysed against phosphate buffered saline and 100mg was loaded onto the Mono Q 10/10 column. A stepwise salt gradient of sodium chloride was applied. Seven fractions were obtained. Each fraction corresponding to a peak was pooled, dialysed and freeze-dried. Protein concentrations were determined using the general method of Bradford (10).

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

Gel preparation – Stock solutions prepared for gel electrophoresis are listed in Table 1. The composition of these acrylamide mixtures and of all gels is defined by the letters T and C according to Hjerten (11). The composition of separating 'spacer', and stacking gels is given in Table 2. The gel was composed of a small-pore gel (16.5% T) overlaid by a 10% T, 3% C spacer gel (2–3cm) that again was overlaid by the 4% T, 3% C stacking gel (1–2cm). The small pore gel and the short 10% T spacer gel (without glycerol) were polymerised together within 15 min by addition of 100 μl of 10% ammonium persulphate and 10 μl TEMED to 30 ml of each

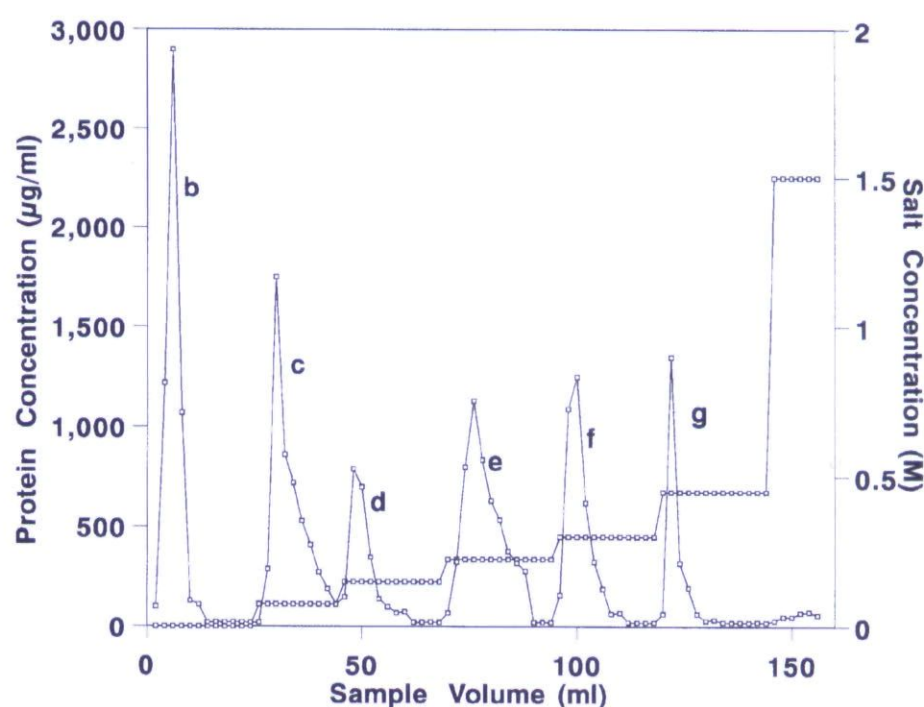
Table 1 : Stock solutions for SDS-PAGE (7). T represents the percentage concentration of acrylamide and bisacrylamide while C denotes the concentration of the crosslinker

Buffer	Tris (M)	Tricine (M)	pH	SDS (%)
Anode buffer	0.2	—	8.9a	—
Cathode buffer	0.1	0.1	8.25b	0.1
Gel buffer	3.0	—	8.45a	0.3
Acrylamide-bisacrylamide mixture	Percentage acrylamide (w/v)		Percentage bisacrylamide (w/v)	
49.5% T, 6% C	46.5		3.0	

^a Adjusted with HCl

^b No correction of the pH, which is around 8.25

Fig. 2. FPLC peak profile of crude peanut extract. Each peak from FPLC was pooled and lyophilised for 2–3 days. Following protein concentration determination schematic peak profile was obtained.



mixture. The 10% T gel again was overlaid with water and replaced by 4% stacking gel following polymerisation the water was replaced by the 4% T stacking-gel (7).

Sample preparation – The protein samples were incubated for 30 min at 40°C in 4% SDS, 12% glycerol (w/v), 50mM Tris, 28 mercaptoethanol (v/v), 0.01% Serva blue G adjusted with HCl to pH 6.8. 5–20mg of hydrolysate proteins and 50mg of peanut extracts were applied to each well of the gel. Electrophoresis was performed at room temperature and the runs started at 30V. When the sample had completely entered the stacking gel (approximately 1 hour) the voltage was increased to 105V for 16h.

Fixing, staining, and destaining – The protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 60min, before they were stained with 0.025% Serva blue G in 10% acetic acid for 1h. Destaining of the gels was achieved using 10% acetic acid for 2h, with four changes(7).

Results

Hydrolysed formulae – The electrophoretic distribution of hydrolysed formulae is shown in Fig 1. Each formula is visible in SDS-PAGE with a characteristic pattern. The hydrolysed whey formulae, Alfare and Pepti-Junior are shown in lanes b and c-d. It can be noted that these formulae present a number of polypeptides above 6kDa and have one major band at about 2kDa. Although our data is similar to other researchers, (12) for higher molecular weight peptides, the lower molecular weight peptides are often eluted using conventional SDS-PAGE and have not been characterised. Six major bands, ranging in molecular weight between 2–17 kDa, can be observed in Alfare. Pepti-Junior presented four well defined bands concentrated in the area of 7kDa, possibly α -lactalbumin. Pregomin presented five major bands (lane e) ranging in molecular weight from 2–17.5kDa. The profile for Nutramigen and Prege-stimil is shown in lanes f–h. These clearly show a diffuse area with a residue concentrated in one or two bands. Although some of the low molecular weight proteins found in hydrolysates are larger than is claimed by the manufacturers, they are in agreement with other researchers who have reported presence of these larger proteins in hydrolysates (12).

Peanut proteins – The FPLC fractions obtained are shown in Fig 2. The electrophoretic distribution of FPLC fractions of crude peanut extracts is shown in Fig 3. Aliquots of the seven freeze-dried fractions clearly demonstrate the complexity of proteins and

Table 2 : Composition of separating, "spacer" and stacking gels (7)

Composition of separating, "spacer" and stacking gels

	Stacking gel 4% T, 3% C	"Spacer" gel 10% T, 3% C,	Separating Gel 16.5% T, 6% T
49.5% T, 6% solution	–	–10 ml	
Gel buffer	3.1 ml	10 ml	10 ml
Glycerol	–	–	4g
Add water to a final volume of	12.5 ml	30 ml	30 ml

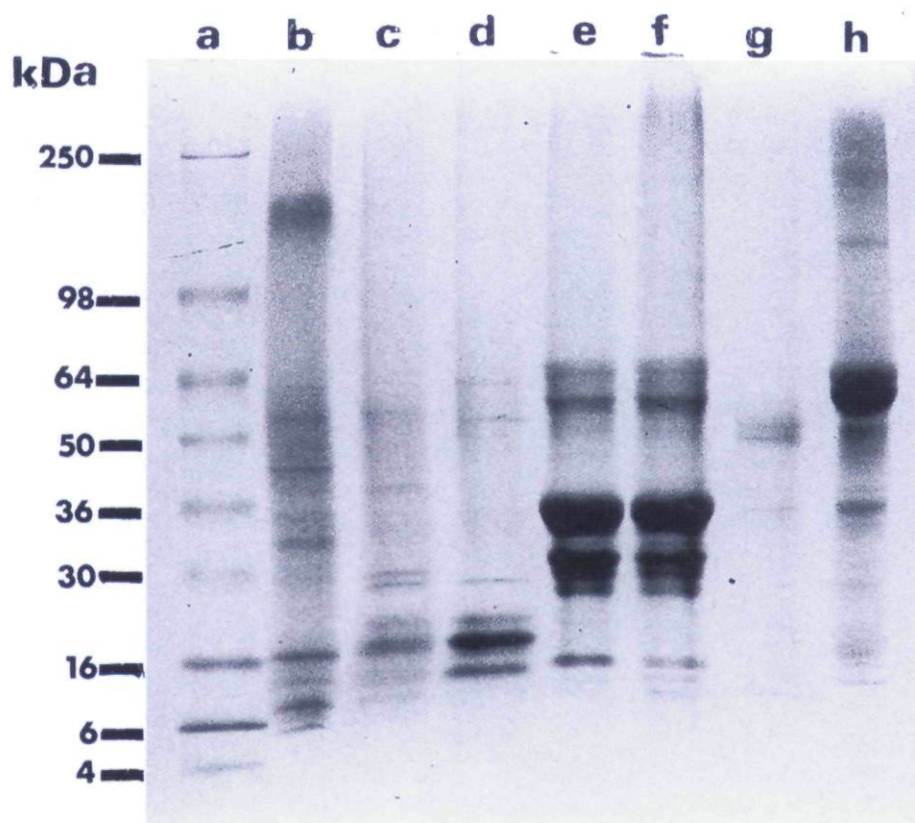


Fig. 3. SDS-PAGE of crude peanut extract fractions obtained from FPLC (lanes b-h obtained from FPLC, 50 μ each). Lane a demonstrates the molecular weights markers.

polypeptides in peanuts. The molecular weight are distributed between 5–200kDa with strong bands at 7, 10, 17, 21, 30, 35 and 65kDa. This electrophoretic pattern was highly reproducible and the same range of proteins were noted when the procedure was repeated 20 times.

Discussion

Characterisation of many allergens has been achieved by using gel electrophoresis. Unfortunately most of the systems employed so far have a poor resolution for separation of low molecular weight proteins which are usually eluted with the running buffer.

The novel application of electrophoresis system described here proved to be able to resolve all the proteins of the tested hydrolysates and crude peanut extract. The superiority of this method for resolving lower molecular weight proteins, is based mainly on the introduction of Tricine as the trailing ion and on the tailoring of the stacking limits as narrow as possible within the low-molecular-mass range. With Tricine, a stacking and destacking of small proteins is achieved at the same pH as in the separating gel and at low acrylamide concentrations.

In this study, by using a moderate concentration of acrylamide (16.5%T, 6%C), we achieved resolution of low molecular weight proteins superior to

that of other published methods of electrophoretic separation. The technique described here allows isolation of low molecular weight proteins which previously have been beyond the limits of resolution of current techniques.

The lower molecular weight peptides separated by employing this SDS-PAGE may play an important part in immunological responses of allergic individuals. These separated proteins can be used in Western blots, isolated, purified, separated and used to raise monoclonal antibodies which will facilitate further elaboration of specific T cell and IgE reactivity. Looking at the SDS-PAGE profile of the hydrolysate formulae investigated, it is interesting to note that they all share a band around the region of 7kDa which could possibly be α -lactalbumin or one of its by-products. In principle, one should not expect these moderate size proteins in extensively hydrolysed infant formulae, if the process has been carried out successfully. If future work shows that some of these lower molecular weight proteins are allergenic, recommendations for using some hydrolysates in management of cow's milk allergy has to be re-evaluated. We believe that because of their rapid solubility, low molecular weight proteins may be responsible for immediate contact symptoms which are very characteristic of peanut and cow's milk allergy.

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