

Increased positivity of skin test and allergenic stability of glycerinated soybean hull extracts

F. MORELL, R. CODINA* and M.-J. RODRIGO*

*Servei de Pneumologia and *Servei de Bioquímica (Unit d'Immunologia), Hospital General Vall d'Hebron, Barcelona, Spain*

Summary

Background Results obtained from previous experiences with soybean hull antigens suggest that the addition of glycerine to the extract increases positivity of the skin test.

Objective The purpose of this study was to investigate the effect of this glycerine addition and the influence of different storage temperatures on the potency of soybean hull extracts.

Methods Twenty-two asthmatic patients admitted to emergency rooms during one of the soybean-related Barcelona asthma epidemics were evaluated 2 years after the last epidemic for: (1) sensitivity to prick test with glycerinated and non-glycerinated extracts, both fresh and stored at 4 °C, – 20 °C and – 70 °C for 30 days, and (2) specific IgE to a fresh hull extract. All extracts were also studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results No differences in positivities were detected for prick tests performed the same day of preparation between non-glycerinated (5/22; 22.7%) and glycerinated (4/22; 18.2%) extracts. After 30 days storage, positivities of prick test with non-glycerinated extracts stored at 4 °C did not significantly differ from the results obtained on the first day. However, prick tests performed with glycerinated extracts stored for 30 days showed increased positivity for the extracts stored at 4 °C (7/22; 31%) ($P < 0.05$) as well as for those stored at – 20 °C and – 70 °C (9/22; 40.9%) ($P < 0.001$). This latter percentage is similar to that of specific immunoglobulin E detected by radioimmunoassay (10/22; 45.4%). Also, for the glycerinated extracts only, the quantitative response showed an increase in mean diameter of the induration at 30 days at the three temperatures, even though owing to the great standard deviation and limited number of patients, it was only significant for the extract stored at – 20 °C ($P < 0.01$). SDS-PAGE gel densitometry at 30 days demonstrated a loss of protein in the bands with a molecular weight higher than 66.2 kDa for non-glycerinated extracts stored at 4 °C.

Conclusions The results of this study confirm previous observations with soybean hull allergen extracts and indicate that (1) storage of soybean hull extracts with glycerine addition for 30 days at the concentration studied (1:100 w/v) determines a significant increase in the positivity of skin tests, and (2) glycerine addition stabilizes soybean hull extracts at any of the temperatures studied.

Keywords: soybean asthma, prick test, glycerine

Clinical and Experimental Allergy, Vol. 29, pp. 388–393. Submitted 18 March 1998; revised 29 June 1998; accepted 29 September 1998.

Introduction

Since severe asthma epidemics produced by the unloading

of soybeans in the harbour of Barcelona, Spain, were first identified [1,2], other epidemics attributed to the same aetiology have been described [3]. Soybeans are harvested in large amounts and transported and processed in many countries; thus, soybean asthma is expected to be more frequently diagnosed, thereby posing a new diagnostic

Correspondence: R. Codina, Division of Allergy and Immunology, University of South Florida, Tampa FL, USA.

challenge. The diagnosis of this condition is based on clinical features and confirmed immunologically by radio-allergosorbent tests, enzyme-linked immunosorbent assay (ELISA) and skin tests, among others [4]. Hence, reliable soybean extracts are required.

A loss of activity of allergen extracts is often initiated during the process of extract preparation, even when performed at low temperatures [5]. Two approaches have been used to study the stability of allergen extracts: (1) the evaluation of thermostability at different temperatures and periods [6], and (2) the verification of stability after the addition of different preservatives [7–9]. Glycerine is commonly added to extracts to stabilize the antigenic solution used in skin tests [10]. Although satisfactory results have been reported for the addition of glycerine to some specific antigens including some cereal allergens [11], for most of the allergens the duration of potency has not been rigorously studied [12].

In previous experiences using soybean hull extracts, an increase in positivity of the skin test performed with glycerinated extracts was observed [4,13,14], a fact that, to our knowledge, has not been reported in the literature. The present paper was designed to (1) study the effect of glycerine addition in soybean hull extracts when they are used for skin testing, and (2) assess breakdown and loss of antigenicity of soybean hull extracts with or without glycerine addition and stored at different temperatures 1 month after preparation.

Materials and methods

Subjects

Twenty-two asthmatic patients (age range: 27–64 years; mean: 51 years) admitted to the emergency room of five hospitals in Barcelona during an outbreak of severe acute soybean asthma were studied [13,14]. Patients were selected because they had all had a positive cutaneous prick test to soybean hull allergen extract at 1:10 (100 µg/mL) dilution in previous studies.

A group of 22 patients (21–63 years; mean 54 years) who had been treated at the emergency-room on a non-epidemic day, served as a control group for the prick test performed with glycerine alone at a dilution of 50%.

A blood sample was taken from each patient for specific immunoglobulin (IgE) evaluation to fresh non-glycerinated extract, which was considered as a reference extract.

Antigen extract preparation

The extract used throughout the study was prepared from soybean hulls, previously shown to be the most allergenic

part of the seed [15,16], from Toledo (OH, USA), and unloaded into the silos of the Barcelona harbour.

One hundred grams of soybean hulls were grounded with a mortar. Extraction was performed by gentle shaking overnight (1:20 w/v) with 0.2 M ammonium bicarbonate, pH 7.9. The supernatant was isolated, centrifuged at 600 × g for 15 min and dialysed exhaustively for 2 days at 4 °C with a 3500 MWCO membrane (Spectra/Por MWCO 3500, Spectrum, Houston, TX, USA) against deionized water. The extract was centrifuged at 600 × g for 20 min and the total protein content determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, USA).

The extract, part of which was 50% glycerinated (G) as well as non-glycerinated (NG), was fractionated into aliquots and stored (St) at 4 °C, –20 °C and –70 °C for 30 days.

On the day of the tests, a new fresh extract was prepared from the same hull sample with the same methodology. To avoid adsorption of the proteins on the walls of the vials, the prick test solutions were prepared in siliconated tubes.

Prick test

The skin test was performed by the same person with the usual prick technique [17] throughout the study. The results of the test were read at 15 min and considered positive when the largest and smallest diameter of the wheal divided by two was equal to or greater than 3 mm and greater than that obtained with the negative control (saline solution). The histamine positive control was greater than 3 mm in all patients.

In the present study, a 1:100 dilution (protein content of 10 µg/mL) was used instead of a 1:10 dilution (100 µg/mL) to which all patients were positive in a preliminary pilot study. The aim was to confirm quantitatively and qualitatively whether the addition of glycerine and also the storage for 30 days at different temperatures determine any modification in positivity of skin test. Therefore, each individual was simultaneously tested for the following extracts: non-glycerinated fresh (NGF); non-glycerinated stored for 30 days at 4 °C (NG-St 4), –20 °C (NG-St 20) and –70 °C (NG-St 70); the same was done for glycerinated extracts: GF: G-St 4; G-St 20 and G-St 40.

A prick test with glycerine alone at a dilution of 50%, precisely the same as that used in the preparation of glycerinated extracts, was also performed in the 22 patients and 22 asthmatic controls.

Identification of specific IgE to soybean hull allergens

Identification of specific IgE to fresh soybean hull allergens was performed by a solid phase radioimmunoassay (RIA)

assay as follows: fresh hull extract was adsorbed to high-binding Removawell strips (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA, USA). Each well was coated with 1 µg of protein in 100 µL of 0.1 M of carbonate-bicarbonate buffer, pH 9.6. The plates were incubated in a humid box overnight at room temperature and washed four times with 0.1 M of PBS, pH 7.5, containing 0.01% Tween 20 (Sigma Chemical Co., St Louis, MO, USA). One hundred µL of serum of the 22 patients diluted at 1:2 in washing buffer containing 1% BSA (Sigma Chemical Co) were added to each well, incubated overnight and washed. After the addition of 100 µL of ¹²⁵I-labelled rabbit anti-human IgE (Pharmacia Diagnostics AB, Uppsala, Sweden) diluted 1:2, the plates were incubated overnight, washed again and the radioactivity counted in a gamma counter. Binding that was 200% above the mean of four normal control sera was interpreted as positive but borderline, and binding above 400% was considered clearly positive.

SDS-PAGE

SDS-PAGE of extracts preserved under different conditions for 1 month and with freshly prepared extract was carried out in 13 cm long, 5% to 17% acrylamide linear-gradient gels according to the discontinuous system of Laemmli [18].

Antigen extracts containing 50 µg of protein were treated with SDS and 2-mercaptoethanol (5%), boiled for 10 min, allowed to cool and then applied to the stacking gel. A set of molecular weight standards was also run in each gel (Pre-stained SDS-PAGE Standards, Low Range, BIO-RAD Laboratories, Richmond, CA, USA).

Electrophoresis was carried out at 24 mA/gel until the tracking dye reached the bottom of the gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (BIO RAD Laboratories) and stained bands of the different hull extract were scanned for absorbance by laser densitometry (UltraScan XL laser densitometer, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analyses

The mean of prick test results was compared using the Student's *t*-test and variance analysis by Fisher-Snedecor *F*-test. *P* < 0.05 was considered significant.

Results

Prick tests

Qualitative results (Table 1) Pricks with NG-F, prepared and performed the same day, were positive in 5/22 (22.7%) patients, while those with G-F solution were positive in 4/22 (18.2%) (pNS).

For the NG extracts, no significant differences in positivity were observed between NG-F extract: 5/22 (22.7%) and NG extract after storage for 30 days, either at 4 °C: 5/22 (22.7%), -20 °C: 4/22 (18.2%) or -70 °C: 4/22 (18.2%) (pNS).

For the G extracts stored for 30 days, there was an increase in positivity compared with G-F extract: 4/22 (18.2%), when stored at 4 °C: 7/22 (31.0%) (*P* = 0.05); 9/22 (40.9%) (*P* < 0.001) at -20 °C and -70 °C.

When positivities obtained with both NG and G extracts were compared after 30 days' storage at 4 °C, differences were observed (22.7% vs. 31.0%) (*P* = 0.05). Moreover, at -20 °C and -70 °C differences were also significant (18.2% vs. 40.9%) (*P* < 0.001), showing a clear increase in positivity with the G extract.

Quantitative results (Table 2) After 30 days storage, all G extracts increased the mean diameter of the pricks, but only the differences between non-glycerinated and glycerinated extracts stored at -20 °C are statistically significant (*P* < 0.01).

Prick tests with glycerine alone were positive in 3/22 (13.6%) patients and in 2/22 (9.1%) asthmatic controls (pNS).

Evaluation of specific IgE

Solid-phase RIA assay demonstrated IgE antibody positivity in 10/22 (45.4%). IgE values for eight of these subjects reached levels, between 2000 and 32 000%. To correctly evaluate that positivity was found in only 45.4% of the asthmatic epidemic patients, it must be remembered that this study was conducted 2 years after the local authorities intervened in the unloading of soybean into the silos to prevent the release of dust into the atmosphere. Positivity of specific IgE to soybean during the epidemic outbreaks was 84.9% [2].

SDS-PAGE

Bands obtained by SDS-PAGE with fresh soybean hull extract and extracts stored at 4 °C, -20 °C and -70 °C for 30 days, with and without the addition of glycerine, are shown in Fig. 1.

For non-glycerinated extracts, kept at 4 °C for 30 days (Fig. 1, lane 4), bands with a molecular weight ranging from 66.2–97.4 kDa disappeared. These results are confirmed by gel densitometry (Fig. 2, No. 4) which also illustrates a pattern of bands similar to that of fresh extract (Fig. 2, No. 2) in extracts kept at -20 °C and -70 °C (No. 6 and 8).

For glycerinated extracts, densitometry of the gel confirms preservation of the protein bands compared with fresh extract, even for the extract stored at 4 °C (Fig. 2, No. 3, 5, 7

Table 1. Prick test results obtained with soybean hull extract: qualitative evaluation.

Extract (1:100)	Day 1	Temperature in which stored for 30 days	Skin test positivity, n (%)
Non-glycerinated	Fresh		5 (22.7)
		4 °C	5 (22.7)
		– 20 °C	4 (18.2)
		– 70 °C	4 (18.2)
Glycerinated	Fresh		4 (18.2)
		4 °C	7 (31.0)*†
		– 20 °C	9 (40.9)**††
		– 70 °C	9 (40.9)**††

* $P = 0.05$ compared with fresh non-glycerinated. ** $P < 0.001$ compared with fresh non-glycerinated. † $P < 0.05$ compared with stored non-glycerinated kept at the same temperatures. †† $P < 0.001$ compared with stored non-glycerinated kept at the same temperatures.

and 9). This can also be clearly observed, despite the distortion produced by glycerine, in the separation of extracts by SDS-PAGE (Fig. 1, lanes 3, 5, 7 and 9).

Discussion

One of the problems when working with antigen extracts is the loss of antigenicity. This loss of potency may be due either to the action of proteolytic enzymes contained in the extract or to denaturation of the antigenic proteins, resulting in alteration of their conformational epitopes [19,20]. Only by studying each antigen individually is it possible to predict how a specific antigenic extract will be affected by these factors and establish optimal storage conditions.

In the present study on soybean hull extracts, laser densitometry of SDS-PAGE gel confirms breakdown for the NG extract stored at 4 °C for 30 days only. This fact indicates that storage at – 20 °C and – 70 °C preserves protein degradation and confirms the preserving capacity of glycerine for proteins contained in soybean hull extract.

Whereas other extract stability studies with *Alternaria*

tenuis found no variation in potency of extracts stored with or without glycerine [7,8], the results of the present study concur with those reported by Nelson *et al* [10] for *Salsola pestifer*, in which 50% of glycerinated extracts, at a protein concentration of 0.1 µg/mL, maintained their potency at 4 °C and 17 °C for 12 months. Hiatt *et al* [6] also described similar results in ragweed at even higher temperatures. Varjonen *et al* [11] reported a good stability of extracts of wheat, rye and barley flours when stored for 21 months at 4 °C in an aqueous solution containing 50% glycerol. Thus, these findings suggest that addition of glycerine to the extracts studied determines a good stability at low temperatures and even at 4 °C. This latter temperature is the one at which clinicians maintain the extracts to later perform skin and challenge tests.

The previously mentioned protein diminution detected by SDS-PAGE in NG extracts kept at 4 °C for 30 days did not affect positivity of skin test. A possible explanation could be found in the densitometric analysis of the SDS-PAGE which demonstrated degradation in high molecular weight proteins, while those of low molecular weight are conserved.

Table 2. Prick test results obtained with soybean hull extract: quantitative evaluation

Extract (1:100)	Day 1	Temperature in which stored for 30 days	Skin test positivity* (mm)
Non-glycerinated	Fresh		1.40
		4 °C	1.55
		– 20 °C	0.90
		– 70 °C	1.04
Glycerinated	Fresh		1.04
		4 °C	2.09
		– 20 °C	2.72**
		– 70 °C	1.90

* Mean diameter for the wheal (mm). ** $P < 0.01$ compared with fresh glycerinated.

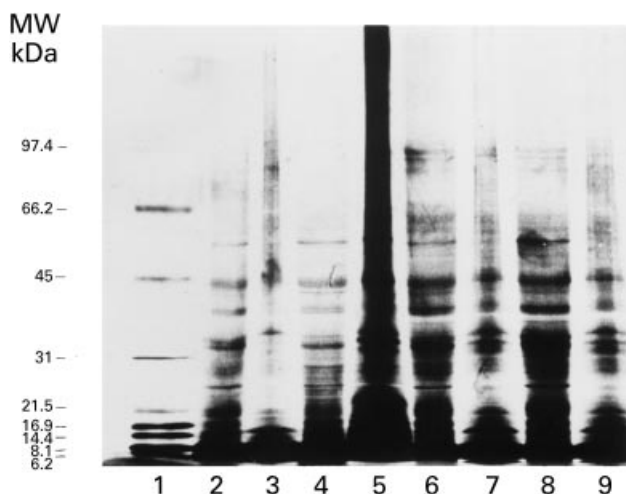


Fig. 1. SDS-PAGE of soybean hull extracts (50 µg/lane) stained with Coomassie Blue and silver nitrate. Lane 1, molecular weight markers; lane 2, fresh non-glycerinated extract; lane 3, fresh glycerinated extract; lane 4, non-glycerinated extract, 1 month at 4°C; lane 5, glycerinated extract, 1 month at 4°C; lane 6, non-glycerinated extract, 1 month at -20°C; lane 7, glycerinated extract, 1 month at -20°C; lane 8, non-glycerinated extract, 1 month at -70°C; and lane 9, glycerinated extract, 1 month at -70°C. No degradation of protein bands is observed, except for NG extract kept at 4°C for 30 days (lane 4) where a degradation in high molecular weight proteins is observed while those of low molecular weight are conserved.

The antigens which triggered the soybean asthma outbreaks in Barcelona, are known to have a molecular weight below 14 kDa [15].

Concerning the skin test results, the present study confirms previous observations [4,13,14] that glycerine addition to hull extract increases positivity of NG extract. Compared with F extract, an increase in positivity of G extract stored for 30 days at 4°C was observed, being even more pronounced for the extracts stored at -20°C and -70°C. Results found with G extracts stored for 30 days at -20°C and -70°C were also significantly more positive than those found with NG extracts stored at the same temperatures, which further supports a role of glycerine in positivity of skin test. This effect is also verified in the quantitative evaluation of prick test results, but only becomes significant when mean diameters are compared after storage at -20°C, probably due to the large standard deviation and limited number of patients.

The positivity effect of glycerine may be exerted on an individual's skin because the encounter of the antigen and the individual's antibodies is facilitated. However, the positivities of fresh extract, both G and NG prepared the same day, do not significantly differ. Hence, it can be assumed that the effect of glycerine addition takes place during the storage period.

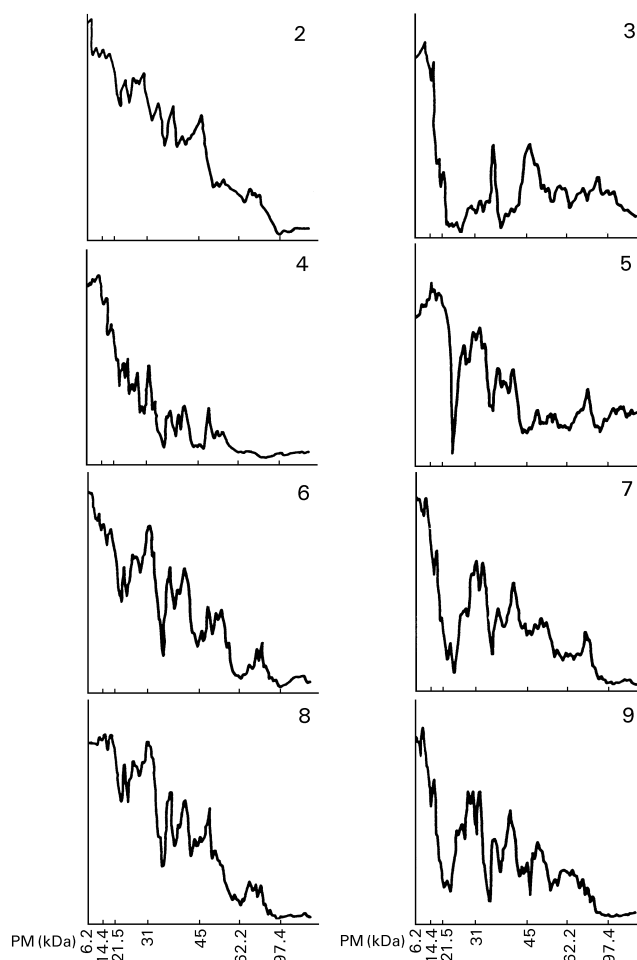


Fig. 2. Laser densitometry of the SDS-PAGE gel of Fig. 1. Numbers 2, 4, 6 and 8 correspond to the non-glycerinated fresh extract, kept for 1 month at 4°C, -20°C and -70°C, respectively; numbers 3, 5, 7 and 9 are identical extracts but are glycerinated. The pattern number 4 confirms the results obtained by SDS-PAGE for NG extract kept at 4°C for 30 days observed in Fig. 1.

Previous observations corroborate the glycerine effect found in the present study. In the pilot study, referred to in Material and Methods, an increase in mean diameter of the wheal from 1.4 mm obtained with fresh extract to 5.6 mm with G extract was verified even after only 24 h of storage. Also in a second study [4], an increase in skin test response after the addition of glycerine to the extract was detected: NG prick tests were positive in 29/90 (32.2%) subjects and for the G ones in 52/95 (57.7%), $P = 0.003$; NG extracts were used up to 10 days after preparation, whereas G extracts were used up to 1 month after preparation. This same effect of glycerine addition was also observed in other experiences [13,14].

In the group of non-epidemic asthmatic patients

(considered non-sensitive to soybean) used as controls in the referred study [4], prick tests with NG extracts were positive in 6/135 (4.4%) subjects and with G extracts in 7/107 (6.5%); thus, it seems that glycerine addition to the extract increases positivity only in skin tests of patients sensitive to soybean antigens.

If the results of the present study are confirmed, they may yield clinical implications in skin testing as well as in other in-vivo immunological provocation tests. In fact, the effect of glycerine on the extract permitted the sensitivity of prick test to be as great as that obtained by RIA or amplified ELISA, as shown in a previous [4] and in the present study, in which the percentage of positivity of prick test performed with stored G extract was very similar to the percentage of positivities obtained by RIA.

In conclusion, although further work should be conducted to corroborate these results, the present study suggests that addition of glycerine stabilizes soybean hull extracts even at 4 °C and increases positivity of prick test.

Acknowledgements

We thank Mercedes Catalán, technical assistant, for her invaluable technical support, Andrew Campbell and Rosa Llòria for their editorial assistance, and Miss Christine O'Hara for her help with the English version of the paper. This study was supported by the FIS (Fondo Investigaciones Sanitarias) n°89/0924–3.

References

- Antó JM^a, Sunyer J, Rodríguez Roisín R, Suarez M, Vázquez L and the Toxicologic Committee. Asthma epidemics associated with soybean dust released during harbor unloading activities. *N Engl J Med* 1989; 320:1097–120.
- Sunyer J, Antó JM^a, Rodrigo MJ, Morell F. Epidemic asthma and soybean IgE antibodies. *Lancet* 1989; 1:179–82.
- González R, Zapatero L, Caravaca F, Carreira J. Identification of soybean proteins responsible for respiratory allergies. *Int Arch Allergy Appl Immunol* 1991; 95:53–7.
- Morell F, Codina R, Rodrigo M^aJ, Sunyer J, Antó JM^a, Reed CE. Diagnosis of soybean asthma. *J Allergy Clin Immunol* 1995; 96:320–4.
- Volkin DB, Klibanov AM. Minimising protein inactivation. In: Creighton TE, ed. *Protein function, a practical approach*. IRL Press, Oxford, 1990.
- Hiatt CW, Baer H, Hooton ML. Kinetics of thermal decay of antigen E from short ragweed pollen. *J Biol Standarization* 1977; 5:39–44.
- Vijay HM, Young NM, Bernstein IL. Studies on *Alternaria* allergens. VI. Stability of the allergen components of *Alternaria tenuis* extracts under a variety of storage conditions. *Int Arch Allergy Appl Immunol* 1987; 83:325–8.
- Dockhorn R, Grater WC. The stability of diluted *Alternaria* extract. Report from Committee Am College Allergists On Standardization Allergic Extracts 1978; 40:203–5.
- Nelson HS. The effect of preservatives and dilution on the deterioration of Russian thistle (*Salsola pestifer*), a pollen extract. *J Allergy Clin Immunol* 1979; 63:417–25.
- Bernstein IL. Proceedings of the task force guidelines for standardizing old and new technologies used for the diagnosis and treatment of allergic diseases. *J Allergy Clin Immunol* 1988; 82 (Suppl.):487–26.
- Varjonen E, Björkstén and Savolainen J. Stability of cereal allergens. *Clin Exp Allergy* 1996; 26:436–43.
- Van Metre TE, Franklin Adkinson N, Kagey-Sobotka A, Marsh DG, Norman PS, Rosenberg GL. How should we use skin testing to quantify IgE sensitivity? *J Allergy Clin Immunol* 1990; 86:583–6.
- Antó JM^a, Sunyer J, Reed CE et al. Allergen avoidance in preventing soybean epidemics asthma: an intervention study. *N Engl J Med* 1993; 329:1760–3.
- Sabrià J, Antó JM^a, Sunyer J et al. Clinical and functional characteristics of patients two years after being affected by the soybean asthma epidemic in Barcelona. *Thorax* 1994; 49:906–9.
- Rodrigo M^aJ, Morell F, Helm RM et al. Identification and partial characterization of the soybean dust allergens involved in the Barcelona asthma epidemics. *J Allergy Clin Immunol* 1990; 85:778–84.
- Swanson MC, Li JTC, Wentz-Murtha PE et al. Source of the aeroallergen of soybean dust: a low molecular mass glycopeptide from the soybean tela. *J Allergy Clin Immunol* 1991; 87:783–8.
- Bousquet J. In vivo methods for study of allergy: skin tests, techniques and interpretation. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, eds. *Allergy principles and practice*. CV Mosby, St Louis, 1988:419–36.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Beynon RJ. Initial planning. In: Harris ELV, Angal S, eds. *Protein purification methods: a practical approach*. IRL Press, Oxford, 1989:1–64.
- Berzofsky JA, Berkower IJ. Immunogenicity and antigen structure. In: Paul WE, ed. *Fundamental immunology*. 2nd edn. Raven Press, New York, 1989:169–208.