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Analytical techniques for detecting food allergens

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7.1 Introduction

Analytical techniques are available to detect very low concentrations of allergenic proteins within complex mixtures. These have potential for use in the food industry in a number of ways:

- Quality control of food manufacturing processes – detection of accidental cross- or carry-over contamination due to shared areas, staff or equipment.
- Confirmation of accidental exposure to an individual.
- Detection of residual allergenicity following processing to reduce biological activity.

There are several considerations to be made when designing or implementing analytical assays:

- Nature of the allergenic molecules
- Detection limits required
- Sensitivity and specificity of the assay
- Sampling and extraction procedure.

The aim of this chapter is to outline the methods available, and to outline the limitations and benefits of such assays, but not to give a step-by-step guide to bench-top techniques. The chapter begins with a brief description of the type of molecules that we need to detect. The detection limits required have been indicated in Chapters 3 and 4 where the extreme sensitivity of some individuals to minute quantities of proteins has been described and the threshold doses discussed. It is apparent that for certain foods, such as nuts and peanuts, as little as 45 mg (Hourihane *et al*. 1997) must be detected in a meal in order to avoid

risk of anaphylaxis. This level of sensitivity must therefore be achieved with the assays. Protocols for sampling are given in Chapters 3 and 4. Sampling procedure could greatly compromise the efficiency of detection, especially if the contamination is likely to be particulate and intermittent. Enzyme-linked immunosorbent assay (ELISA) is an aqueous system, and allergens must be in aqueous form for analysis. Poor extraction and recovery of the allergen could compromise an otherwise adequate assay. All these factors and steps must be considered when implementing allergen detection assays.

7.2 The physical and chemical nature of food allergens

7.2.1 Foods that commonly cause allergy

Foods that can give rise to allergic reactions in susceptible individuals appear to be diverse in nature. However, although reactions to many different foods have been described in individual case reports, the list of *common* causal agents is relatively short. This has led researchers to postulate that there may be certain features characteristic of food allergens. Common causes of allergy are milk, egg, peanut, tree nuts, fish, shellfish, soy and citrus fruits for populations in the UK and the USA. The list can vary for different countries; for example, Mediterranean countries such as Italy have a high incidence of sensitivity to olives, and in Japan even sensitivity to birds' nest soup has been described.

To be capable of inducing an allergic reaction a food must contain substances that are immunogenic, and give rise to allergic sensitisation. This results in the production of IgE antibodies in preference to IgG and T cells of the Th2 phenotype rather than the Th1 phenotype. On subsequent exposures the molecule must be able to cross the mucosal barrier and cross-link IgE on effector cells, causing degranulation and release of the chemical messengers that produce allergic symptoms. The molecule must therefore bear more than one IgE binding site. The majority of described allergens are protein in nature, though carbohydrates/sugar moieties may also cause symptoms as they certainly bind IgE. Carbohydrate epitopes may be responsible for cross-reactivity between plant species (Blanco *et al*. 1999, Caballero and Martin-Esteban 1998). Lipids (fats and oils) do not provoke a specific immune response and so are not causal for allergic reactions. Current allergen detection techniques and diagnostic assays focus on the protein components.

7.2.2 The basic structure of proteins

The building blocks of proteins are amino acids, bound together in a linear fashion by covalent peptide bonds. Each protein has a precise length and amino acid sequence dictated or transcribed by messenger RNA that in turn is translated from the DNA. Once made the protein may be modified, or chopped into smaller pieces, or carbohydrate, lipid or phosphate moieties may be added by the action of enzymes within a plant or animal cell. The linear sequence of

amino acids is termed the *primary structure* of the protein. Proteins are rarely linear in the native form but form distinctive three-dimensional structures. This is due to chemical interactions between amino acids in close proximity, causing the chain to form twists that force it into spirals, termed alpha-helixes, and sharp bends resulting in so-called beta-pleated sheets. These basic forms are part of the *secondary structure* of the protein. The arrangement of these secondary structures in relation to each other gives rise to the *tertiary structure*. This results from non-covalent interactions between the different regions of the same protein or polypeptide molecule. In addition many functional proteins consist of aggregates of two or more polypeptide chains, that are homogeneous or heterogeneous. This is termed the *quaternary structure*. The three-dimensional shape and chemical nature of the amino acid backbone and additional groups contribute to the functional and antibody-binding properties of the molecule.

7.2.3 Molecular characteristics of common allergens

The majority of allergens described are protein in nature with or without carbohydrate moieties (glycosylated), with a molecular weight ranging between 10–100 kDa. Most proteins in foods can be immunogenic and provoke production of specific antibody, mainly IgG, in individuals with or without an atopic tendency. Only a limited range of proteins is commonly associated with the production of IgE in the atopic individual, and is considered allergenic. Protein molecules that initiate immune responses are commonly over 7000 daltons in size (Roitt *et al*. 1998). No common molecular motif for allergens has been described, but they do have some properties in common. Allergens, particularly those that lead to persistent allergies, are thought to be resistant to digestion (Astwood *et al*. 1996, Becker 1997), the rationale being that this results in persistence in the body and stimulation of the immune system. There are certain fruit allergens, which may be unstable, even being degraded by enzymes released in the fruit by crushing (Bjorksten *et al*. 1980). Many allergens have enzymatic ability (Bufe 1998) so function in addition to stability may be related to allergenicity. Commonly a food will contain more than one allergenic protein, such as beta-lactoglobulin, lactoferrin and the caseins of cow's milk, and ovomucoid, ovalbumin and lysozyme of egg, indicating that the context as well as molecular structure must be important.

7.2.4 Techniques for identifying allergens and quantifying allergenicity

A number of techniques have been used to identify allergenic proteins, most being based on the principle of:

- Solubilising/extracting proteins
- Isolation of protein fractions
- Determining IgE binding ability of each fraction
- Characterisation of the protein/glycoprotein and larger-scale purification.

The techniques most often used in the current literature for allergen identification are:

- Separation on a gel such as SDS PAGE followed by Western blotting and immuno-labelling
- Separation by chromatography (often High Performance Liquid Chromatography) followed by ELISA.

Using ELISA or Western blotting, quantitative or semi-quantitative data on the binding of serum IgE to specific proteins can be calculated for individual patients. Generalisations on allergenicity of specific proteins in a food are made by assessing the proportion of affected individuals that have elevated IgE to that protein. These methods cannot predict the degree of symptoms that may be produced on exposure to each individual protein or the outcome of introducing novel foods into a community.

7.3 Principles of food allergen detection techniques

The choice of assay has a great effect on the sensitivity and specificity. There are some foods where the sensitivity is paramount whereas specificity is not, such as detection of protein in oils extracted from allergenic seeds. In most other situations proteins will normally be present in the food and specificity without sacrificing sensitivity is required.

7.3.1 Protein detection

A number of assays have been developed to quantitate proteins in solution. All are susceptible to interference by other compounds that may be present. The Bradford method is widely used, but the BCA method is more robust. However, the latter is sensitive to interference from reducing sugars. These assays give us an approximation of the quantity of protein present but not whether these proteins are allergens or not. They are, however, useful for the estimation of residual protein in, for example, oils extracted from seeds where the source material is known to be allergenic.

The Bradford Method (Bradford 1976)

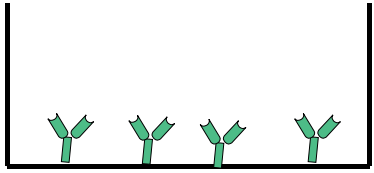
This assay makes use of the acidic dye, Coomassie Brilliant Blue G-250, which binds to any basic and aromatic amino acids present on the polypeptide molecule. This changes the colour of the dye from brownish (absorbance at 465 nm) to blue (absorbance at 595 nm). The colour change is recorded using a spectrophotometer at wavelength 595 nm and the results are read from a standard curve generated from a protein of known concentration. A good description of the technique is provided in Rosenberg (1996), and the detection limit of the assay is approximately 200–1400 μg/ml. Reagents are available from Sigma-Aldrich.

The Bicinhoninic Acid Method (Smith et al. *1985)*
When a protein is placed in an alkaline system containing Cu^{2+}, a coloured complex forms between the peptide bonds of the protein and the copper atoms. Bicinhoninic acid forms a complex with cuprous ion (Cu^{1+}) in an alkaline environment, resulting in a stable, highly coloured chromophore with an absorbance maximum at 562 nm. The sensitivity of the assay is approximately 0.5–10 μg/ml. See Rosenberg (1996) for a description of the method.

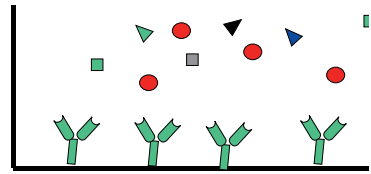
7.3.2 Detection of specific proteins – the immunoassay

The most commonly used technique for quantification of allergenic or antigenic substances is the enzyme-linked immunosorbent assay (ELISA). ELISA has the advantage over radioimmunoassay (RIA) of being more cost-effective and, with modern techniques, not compromising sensitivity. The specificity of all immunoassays is in part dependent on the efficiency of the capture and detector antibodies. Once optimised and standardised the ELISA is relatively economical, and large numbers of samples can be analysed on each test run. The assay is carried out in standard plastic 96 well plates designed for use in ELISA. The wide use of such plates has led to a variety of plate washing and reading systems being available. The sensitivity of the antibodies in forming a complex with the protein is paramount for the sensitivity and specificity of the assay. The sensitivity of the basic assay may be further increased by using indirect labelling or amplification techniques. In non-competitive assays all the constituents are in excess, apart from the protein to be detected. The optimum quantities of each constituent are determined by preliminary experiments. An alternative is the inhibition ELISA, also highly sensitive, but this technique is susceptible to non-specific interactions.

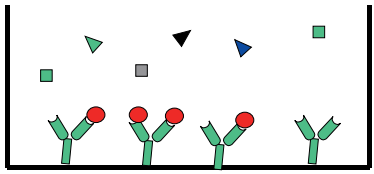
Sandwich enzyme-linked immunosorbent assay
The two-site or sandwich ELISA is ideal for detecting proteins in complex mixtures (Fig. 7.1). The antibodies may be from monoclonal or polyclonal origin. Monoclonal antibodies are most often used for capture, since polyclonal antibodies with wider specificity may theoretically mask the binding site for the monoclonal antibody. It is important that the capture antibody does not interact directly with any of the subsequent assay stages, or vice versa, as this leads to abnormally high background values that reduce sensitivity. In the majority of assays antibodies from different animal species are used to avoid this. An assay may utilise polyclonal antibodies both capture and detector stages. Monoclonal antibodies may be used for both capture and detector stages. In this situation the assay designer usually ensures that the capture and detector antibodies are directed against different parts of the molecule (so-called two-site assays) to avoid competition or interference between the antibodies. The example given in Fig. 7.1 is a direct assay where the enzyme label is directly conjugated to the antibody. Amplification steps are described below.



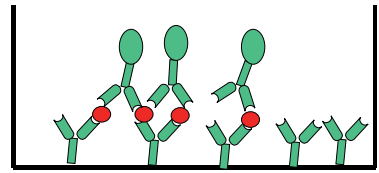
(a) Protein, in this case 'capture' antibody, is incubated in the well, usually of alkaline pH, and small quantities become absorbed onto the plastic surface.



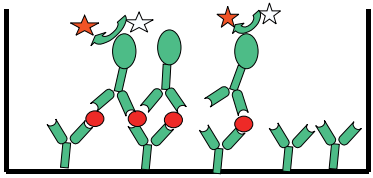
(b) The protein solution to be tested is added to the plate at the appropriate dilution in duplicate or triplicate.



(c) The capture antibody binds the specific protein, anchoring it to the plate surface. Any unbound proteins will be washed off the plate and discarded.



(d) A 'detector' antibody specific for the protein is then used to link an enzyme label to the protein.



(e) The enzyme then acts on a colourless substrate to form a coloured product that is quantitated using a specialised optical density plate reader. The generation of standard curves using known antigen concentrations allows for the accurate estimation in weight/volume.

Fig. 7.1 The enzyme-linked immunosorbent assay (ELISA) – antigen detection by direct sandwich.

Choice of antibody

The assay specificity and to some extent its sensitivity are primarily linked to the efficiency of the antibodies used. Polyclonal antibodies are cheaper to prepare than monoclonal; both types of antibody should be purified to reduce non-specific interactions, and in the case of polyclonal antibodies they may also need to be affinity purified. Polyclonal antibody preparations contain a heterogeneous mix of antibodies directed against any number of epitopes on the protein surface. This gives the assay an advantage if food processing results in some epitopes being denatured or masked, but increases the likelihood of non-specific interactions between the antibodies and unrelated proteins. Monoclonal antibody preparations have a specificity directed against one epitope; this may increase

specificity greatly if the epitope is not present on unrelated proteins. The assay may prove to be less versatile if the epitope is more susceptible to denaturing than the allergenic epitopes.

Amplification systems

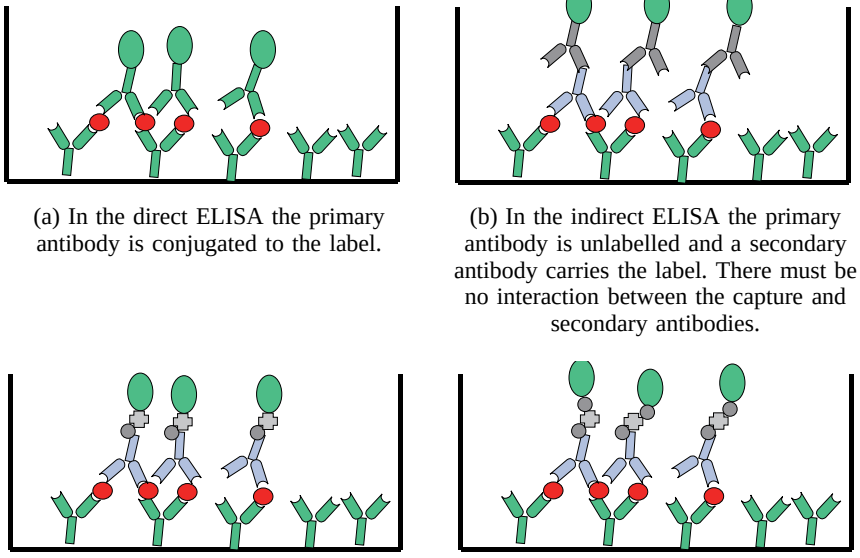
The detector antibody may be conjugated directly to the enzyme label – direct assay. Or various amplification steps may be used:

- Conjugation of the detector antibody to biotin, a compound that binds to avidin with a great affinity and specificity, thus amplifying the signal – indirect biotinylated assay
- The detector antibody, for example IgG from rabbit, may be unlabelled, and subsequently detected using a third enzyme-labelled antibody specific for the detector antibody – indirect assay.

See Fig. 7.2.

Substrates

Substrates of choice in traditional assays gave a coloured product or chromogen, but more recently fluorescent or luminescent substrates are being used. The



(a) In the direct ELISA the primary antibody is conjugated to the label.

(b) In the indirect ELISA the primary antibody is unlabelled and a secondary antibody carries the label. There must be no interaction between the capture and secondary antibodies.

(c) In the biotinylated assay use is made of the strong specific interaction between biotin and streptavidin. The primary antibody is conjugated to biotin, and the avidin molecules are conjugated to the enzyme label.

(d) An adaptation of the biotinylated assay in which the enzyme is conjugated with biotin and avidin forms a bridge between the enzyme and the antibody.

Fig. 7.2 Amplification of the ELISA.

coloured product had the advantage of being cheap and requiring fairly inexpensive optical density plate readers. Fluorescent or luminescent assays have the advantage of increased sensitivity, but the disadvantage of higher backgrounds, and expensive costs for the small laboratory or institution.

Standardisation
Reagents and procedures must be standardised in order to produce reliable results. Calibration and the appropriate quality controls ensure that the results can be compared between assay runs and between laboratories. Use of a single standard or reference preparation worldwide (such as that of the National Institute for Biological Standards and Control) allows universal comparison of results. However, at the time of this book going to press, none are available for food allergens, so appropriately stored in-house or secondary standards must be used. Assays must be validated for the types of samples to be processed. It is not sufficient to determine by experiment that an assay has a sensitivity of so many μg/ml for peanut in flour and then assume that it would have the same sensitivity for peanut contamination of chocolate. The sample matrix or composition may affect the assay, giving artificially high or low readout values. Spiking a pure preparation of known allergen with the analyte-free food will reveal what these effects are.

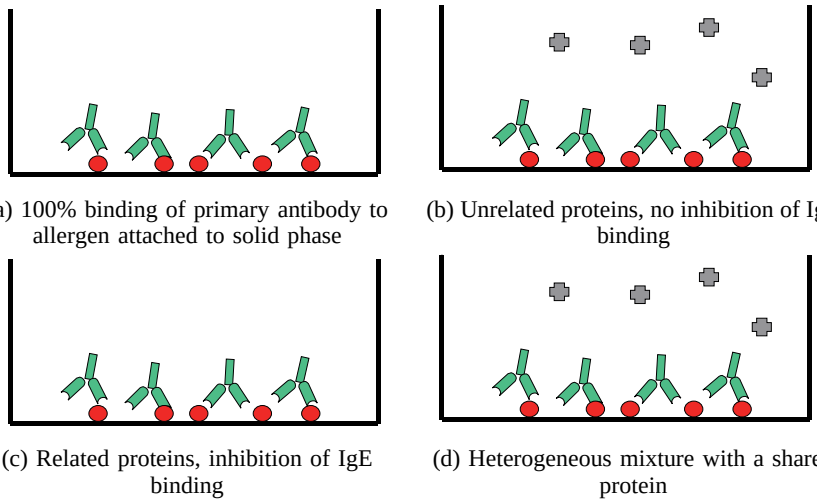
7.3.3 Comparison of allergen contents of different foods or food sources

ELISA with a standard curve provides information on quantities of specific allergens. Sometimes it is necessary to compare one extract with another to determine if the allergens present are the same (homologous) or different (heterologous). This is useful for determining if a particular treatment reduces or increases the allergenicity of a particular food source, for example whether hydrolysis has removed cow's milk allergens from infant formula. It is particularly useful for determining if a food causing an allergic reaction was contaminated or contains cross-reacting proteins with another known allergen, e.g. a food containing hazelnuts that caused a reaction in an individual with peanut allergy. Figure 7.3 shows a schematic representation of an ELISA inhibition to determine the similarity of two allergenic food sources. This assay may also be adapted to provide quantitative data where the percentage inhibition obtained with dilutions of a homologous antigen to the one bound to the plate is used as the standard curve. In this type of assay mouse monoclonal or rabbit antibodies are used rather than patient sera.

7.3.4 Extraction

The steps required are as follows

- Sampling
- Grinding and/or homogenising



(a) 100% binding of primary antibody to allergen attached to solid phase

(b) Unrelated proteins, no inhibition of IgE binding

(c) Related proteins, inhibition of IgE binding

(d) Heterogeneous mixture with a shared protein

Fig. 7.3 Schematic representation of ELISA inhibition to determine the similarity of two allergenic food sources.

- Concentration and/or extraction into suitable buffer
- Removal of particulate matter.

The efficiency of the extraction procedure will vary for individual allergens and for different food matrixes. The expected recovery should be estimated by experiment in all circumstances.

Dry powders and cereals

Many food sources will be dry or semi-dry. In order to achieve adequate extraction the matrix must be broken down. This can usually be achieved by grinding, using a warring blender. Matrixes such as chocolate may be most easily treated by liquefying by heating and then extracted as a liquid using a warm buffer. Most allergens are water soluble and so can be extracted directly into the assay buffer. Common methods employ mixing (using a rotary shaker) the ground food with phosphate-buffered saline overnight at 4ºC. Once extracted, particulate material is removed by sedimentation with or without centrifugation and filtration where necessary.

Liquids

Liquids must of course be homogenised by mixing. It may be necessary to concentrate the sample. Common techniques involve dialysis to exchange buffers and/or remove low molecular weight contamination, followed by freeze-drying to concentrate. Proteins may also be concentrated by virtue of size using an Amicon filtration unit, or a Sephadex G25 column.

Oils

As the majority of allergens investigated are water soluble, the oil can simply be shaken overnight with an equal volume of aqueous assay buffer. The oil/aqueous layers are then separated by cold centrifugation and the aqueous layer decanted. Concentration can then be employed as above. Alternatively detergents may be used to extract the proteins, but these may interfere with the subsequent assays.

7.4 Processing and effects on allergenicity

7.4.1 Food processing

Some foods are encountered unprocessed, such as allergens from fruit and nuts. Foods such as cereals, egg and fish are, however, more commonly processed, and as mentioned earlier proteins vary in their stability. In some cases food processing practices have been developed in order to reduce allergenicity. Only the allergens from fresh fruits and vegetables are very unstable, such as apple allergen (Bjorksten *et al.* 1980), and are inactive after mild heating or even mashing. The majority show varying degrees of resistance to processing, the extreme being shrimp allergen that may remain active even in steam droplets.

7.4.2 Heat treatment and cooking

As a general rule heat decreases the allergenicity of proteins, and heat in the presence of moisture even more so, but this biological activity is rarely removed. Allergenicity of whole wheat flour or purified gluten is only reduced and not eliminated by heating up to 120^{o}C for up to one hour (Varjonen *et al*. 1996, Sutton *et al*. 1982). Heating rice glutelin and globulin fractions also reduces IgE binding ability by 40-70% (Shibasaki *et al*. 1979), but the food remains allergenic. Peanut and nut allergens are resistant to heating and even roasting.

7.4.3 Hydrolysis

Hydrolysed casein and whey infant feeding formulas have been developed with the aim of reducing symptoms of milk allergy in infants. However, allergic reactions have occurred in some infants fed with these formulas, so tests have been developed to estimate residual activity. Hydrolysis is aimed at destroying the allergenic epitopes by cleaving the protein molecules into peptide fragments. Some are extensively hydrolysed and filtered, and it is becoming apparent that only these reduce the risk of atopic sensitisation.

7.5 Summary

At present only a limited range of detection kits are available commercially. As this is a rapidly expanding field the current manufacturers have not been listed.

Instead it is hoped that the reader will be equipped with an understanding of the techniques involved and what criteria should be specified for the assay. The following questions should be raised when considering the use of the various methods:

- What are the detection limits required?
- How sensitive is the assay under the conditions in which you wish to use it?
- How specific is the assay for the matrix in which you are trying to detect the allergen?
- What is the percentage recovery of your extraction procedure?

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