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Design and development of immunoassays for detection of proteins

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Abstract

Immunoassay methodology is currently the method of choice for the quantitative and semi-quantitative detection of many types of proteins in complex mixtures. A combination of sensitivity, specificity and cost-effectiveness in terms of analytical performance are allied to a diverse array of assay formats suitable for laboratory and field use. In the present article, the problems of setting up immunoassays for novel food proteins, and the questions that need to be answered if a successful outcome is to be achieved, are discussed. Speculation on future developments in immunochemistry leads to the conclusion that antibody technology will play an important role in detection of novel proteins from genetically modified organisms. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Historical background

Genetically modified organisms have one defining characteristic- an altered genome leading to production of unique protein(s). The problem of detection is, therefore, straight-forward in that one detects either the altered gene(s) or the novel protein(s). The present paper will examine the background to practicable analysis of proteins based on immunoassay procedures.

The ability to analyse specific protein has been (and in some cases, remains) problematic unless the protein has a unique functionality (such as might be possessed by an enzyme) or physical property (such as a spectroscopic characteristic bestowed by a non-protein component). Even so it was usually necessary to submit the analytical sample to extensive, time-consuming and non-routine pre-purification prior to analysis. It is not so long ago that bioassays based on whole animal responses were the only option available; pregnancy testing using toads and urine samples is more recent than generally realised! In 1959, the modern era of immunoassays was initiated when the use of high affinity antibodies against the hormone insulin in a high through-put, sensitive and specific in vitro test was described (Yalow & Berson, 1959), replacing the established bioassay procedure with a quantum leap forward in analytical potential. The new technique revolutionised research and, subsequently, routine analysis in endocrinology and clinical science. These advances have now been applied across the whole range of analytical science, including applications in agriculture and food, based on the unique combination of robustness and simplicity combined with high performance in terms of sensitivity, specificity and cost-effectiveness (based on equipment and reagent needs, sample work-up and through-put, and staffing costs).

Subsequent developments of greatest significance in immunochemistry focus on two key areas – antibody production and assay formats. The immune system of the higher animals has the ability to produce an enormous diversity of response, making itself capable of interacting with (and protecting itself against) the diversity of molecular and cellular threats. Each of these antibodies has a different structure, and each antibody of different structure is produced by a different line, or clone, of cells. The antibodies used by Yalow and Berson, and which remain key reagents for the immunochemist, were polyclonal antibodies, a mixed population of antibodies each capable of interacting with the target in a different way as determined by their different structures. In 1975, a procedure was described that allowed isolation and large scale production of individual antibodies of identical structure and reactivity, each the product of a single clone of cells and known as a monoclonal antibody (Kohler & Milstein, 1975). The ability to produce monoclonal antibodies has made possible world-wide distribution of key

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analytical reagents in (theoretically, at least) unlimited amounts, isolation of individual antibodies with unique reactivities, and the potential for novel assay formats. More recently, further advances in antibody production hold the tantalising prospect of making the whole process an in vitro one (McCafferty, Griffiths, Winter & Chiswell, 1990), extending the repertoire of possible antibody reactivities and even allowing design and manipulation of binding sites to improve specificity and affinity.

Immunoassay has been widely adopted as the analytical method of choice for several reasons, but the availability of a variety of robust and simple assay formats is a particularly important factor. The use of non-isotopic methods is now the standard procedure. The use of monoclonal antibodies enhances the potential of two-site assays (see later) which have particular relevance for detection of protein analytes (Miles & Hales, 1968). Immunoassays can be in quantitative or semi-quantitative forms. In this connection it is important to note that immunoassays have always been used in research as quantitative assays, and that the availability of semi-quantitative assays has been a response to routine user requirements. The AOAC has given Official First Action Approval to quantitative immunoassays even for trace analytes (Patey, Sharman & Gilbert, 1992). Semi-quantitative immunoassays come in a wide variety of formats. Particularly popular are dipstick procedures, often based on lateral flow devices, which provide not only simplicity but the potential for 'fail-safe' indicators of correct assay procedure.

2. Antibody interactions with proteins

It is instructive in thinking about detection of proteins with antibodies to understand how antibodies interact with protein targets in order to gain a better perspective on both the possibilities and the limitations. Whilst it is, in our experience, undoubtedly easier to raise antibodies against protein targets than, say, a pesticide, it is also true that there are considerable difficulties and pitfalls in raising antibodies against specific parts of a protein or in applying the assays to highly diverse, processed food material.

The normal class of antibody employed in an immunoassay is an IgG molecule (molecular weight about 160 000), a glyco-protein with two identical binding sites able to recognise the target with high affinity. The area of a protein target recognised (the epitope) seems to be that occupied by around 10–15 amino acids, some of which might only be involved in low affinity recognition around the fringes of the binding site. A polyclonal antibody preparation would contain antibodies recognising different parts of the protein; a monoclonal antibody only one (and a comparatively small one) unless the protein had more than one site capable of recognition by the antibody.

The epitope can be made up of amino acids sequential in the primary sequence of the protein. Such a sequence is known as a continuous epitope. Alternatively, the epitope might be made up from amino acids distant in the primary sequence but brought together by the forces of secondary and tertiary structure. Such a recognition site is known as a discontinuous epitope. It is clear that disruption of secondary and tertiary structure will alter (perhaps abolish) antibody recognition of a discontinuous epitope. It is possible that denaturation of a protein could also alter recognition of a continuous epitope, depending on the nature of the peptide.

In any given conformation of a protein and with any particular assay format there could be antibodies specific for that protein that do not bind to the protein not because the epitope is not present, but because the epitope is hidden and not available for antibody recognition.

Assay format can have profound effects on whether an epitope is available for binding by antibody. The method of presentation of the protein to the antibody can itself cause conformational change (such as has been observed when immobilising certain proteins directly to the surface of a plastic surface; Friguet, Djavadi-Ohaniance & Goldberg, 1984), or can result in the 'hiding' of an epitope making antibody recognition and binding impossible for steric reasons. Even mild procedures, such as the use of an antibody to capture a protein, can cause conformational change or hide epitopes.

3. Antibody interactions with food proteins

In the previous section, the molecular interactions of antibodies with proteins in a general sense was considered. What are the particular problems generated by proteins associated with food materials and characteristic of genetically modified organisms? The major assumption is that the characteristic protein has been identified and is available in amounts sufficient for antibody production, for assay development and for use as analytical standards. An understanding of how the protein behaves during food production and processing can be helpful, particularly if it is desired to apply the analytical determination at all points in the food chain right up to consumption. Thus, is the protein modified post-translation and is it always present in a homogeneous form? Does processing, including possible thermal and enzymic treatments, give rise to peptide fragments derived from the original protein and is it necessary to include or exclude these in the analytical determination? How much sequence homology is shared by the novel protein and other proteins normally present, and how much is there a relationship with proteins and peptides present in other food materials? The more knowledge that is available

before development begins, the more one is fore-warned of possible problems in development.

In looking at problems associated with the unique nature of the protein target, there can be insurmountable difficulties if an identical protein is present elsewhere. However, if there are only proteins with less than 100% sequence homology, then the difficulties can be overcome by use of antibodies against unique, characteristic peptide sequences.

Processing of food can lead to extensive changes in conformation of proteins. As described earlier, such changes can cause abolition of antibody recognition in extreme cases and reduced recognition in others. A typical example has been provided by the detection of soya protein in food by immunoassay, something very difficult to do by any alternative method of analysis (McNeal, 1988). The immunoassay procedure works extremely well for detection in all food types, and can be used quantitatively on non-processed materials and on processed foods where the exact nature of the soya ingredient is known, allowing use of appropriate standards. However, where such information is unavailable (which is the normal situation for the analyst) then quantitative information has been difficult to obtain. The problem can be overcome if antibodies directed against processing-stable epitopes can be identified and used. In this laboratory we have explored just such an approach for soya (Huang, Brett, Mills & Morgan, 1997). We have produced a monoclonal antibody against a continuous epitope from soya conglycinin that is stable to extremes of heating, as evidenced by observations on the thermal behaviour of a synthetic peptide corresponding to the sequence identified as the epitope (Huang, Mills, Carter, Plumb & Morgan, 1998a). The antibody can be incorporated in an immunoassay able to quantify soya in processed food using only simple extraction procedures (Huang, Mills & Morgan, 1998b).

4. What kind of antibody?

The antibody preparation(s) used in an immunoassay must have the desired properties of affinity and specificity, must be available in appropriate amounts, and, if necessary, must be able to be purified and labeled/derivatised as necessary (in which form it must be stable) for the immunoassay format desired. Given these properties, the nature of the antibody is irrelevant. A monoclonal antibody does not have inherently superior properties to a polyclonal antibody preparation, and it should be noted that polyclonal antibodies can be obtained in very large amounts if necessary and widely disseminated. Polyclonal antibodies can often exhibit much better specificity than monoclonal antibodies, though production of antibodies against particular peptides (rather than generally against whole proteins) might be better achieved by the monoclonal antibody route. The key factor could well be that some immunoassay formats are much better suited to monoclonal antibody use. Indeed, two-site assays (see below), which have superior properties to competitive assays, work better with at least one monoclonal antibody involved. Some of the very rapid immunoassays (such as the dipstick assays) consume very large amounts of antibody, amounts unlikely to be realistically satisfied by polyclonal antibody preparations.

5. Strategy for detection of proteins from novel foods by immunoassay

The following is an outline of our current views on how one might go about setting up an immunoassay for detection of proteins from novel foods by immunoassay.

(i) *Analytical target*. The uniqueness of the protein must be considered, firstly in the novel food itself and secondly in food materials in general. If necessary, specific peptide sequences would have to be identified, synthesized and used as markers and standards in the assay. The target need not be the material used for antibody production.

(ii) *Immunogen*. The selection of the immunogen, the material used to generate the antibodies in vivo, requires great care. For polyclonal antibody production, as much extraneous material as possible should be eliminated, i.e. non-specific peptide sequences and impurities. It is possible to use negative affinity chromatography to remove undesirable antibodies from the population at a later stage, but this should be avoided if possible. Positive purification of desirable antibodies can often be problematic and is rarely used. For production of monoclonal antibodies, purity can be much less of a problem since it is feasible to select out the desired antibodies during the screening process. However, since the availability of such screening usually implies the availability of purified material anyway, then the economy is difficult to justify. The selection process employed during screening should be given considerable thought at an early stage. The screening process should be as exclusive as possible, and should reflect thoughts on how the final assay should be carried out. Ideally, the screening process would be identical to the final assay format, and would include ways of looking at matrix effects, assessments of sensitivity and specificity, and the ability to perform under particular conditions of, for example, temperature and time- but this is rarely possible for practical reasons.

If it is desired to raise antibodies to a particular peptide sequence, then selection of immunogen is more problematic. The major difficulty to be overcome is that the antibodies to the peptide recognise the peptide very well but fail to recognise the peptide sequence as

present in the parent protein. The cause of this phenomenon usually relates to extensive structural mobility of the peptide related to the more limited structures adopted when anchored within the protein. In addition, further complications are caused by the need to regard the peptide as a hapten and the consequent requirement to conjugate the peptide to a carrier immunogen in order to stimulate antibody production. Experience has evolved our thinking so that (for monoclonal antibody production at least) we are inclined to use intact protein as an immunogen, and to use the peptide to select the appropriate monoclonal antibodies. In this way we believe that we have a greater probability of selecting the desired antibodies specific for a particular peptide sequence within the structure of the complete protein.

(iii) *Immunoassay format*. As indicated previously, it is advisable to give early consideration to ideas on final assay format as things like choice of immunogen and type and properties of the antibody sought could be affected. Almost always, a two-site assay is preferable to a competitive assay. In a competitive assay, the unknown analyte and a given amount of marker analyte compete for a limited number of antibody binding sites. The amount of marker analyte that ends up antibody-bound is determined by how much unknown is present, and the unknown can be quantified by reference to the behaviour of standards.

Thus,

$$2\mathrm{Ab} + \mathrm{An}^{*} + \mathrm{An} \leftrightarrow \mathrm{AbAn}^{*} + \mathrm{AbAn}$$

where Ab is antibody, An* is labelled analyte, and An is the unknown analyte. In a two-site immunoassay – also known as a reagent excess assay or sandwich assay – an excess of the first antibody (which is normally immobilised to a solid surface and which is commonly referred to as the capture antibody) is allowed to capture the analyte present in a sample. An excess of second antibody (usually labelled and commonly referred to as the detector antibody) is allowed to react with and 'label' the captured analyte. Again, quantification can be achieved by comparison to the behaviour of standards.

$$\text{First stage}: n\mathrm{Ab} + \mathrm{An} \rightarrow \mathrm{AbAn} + (n-1)\mathrm{Ab}$$

$$\text{Second stage}: n\mathrm{Ab}^{*} + \mathrm{AbAn} \rightarrow \mathrm{AbAnAb}^{*}$$

where Ab is the capture antibody, An the analyte, and Ab* the labelled second antibody.

The use of the two-site assay is the procedure of choice (provided of course that the analyte protein is big enough to allow binding of two antibodies at the same time) because the sensitivity of the assay is not limited by the affinity of the antibodies since it is a reagent excess assay, because the combination of antibodies can increase specificity, and because the format is well-suited to dipstick-type procedures.

Whichever format is used the sample preparation should be minimal and limited if at all possible to solubilisation of the analyte from the food matrix. If the antibody screening process was correctly designed and implemented then simple sample preparation should already be assured.

Should the assay be quantitative or semi-quantitative? The ease and simplicity with which semi-quantitative procedures can be applied is a highly attractive option, readily applied away from the specialised laboratory, requiring minimal interpretation or skill in use. In our view, however, the quantitative immunoassay has been unwisely neglected by food analysts. Immunoassays can be processed in a batch-wise manner, giving quantitative information. This takes longer, of course, than a single, semi-quantitative determination though the average time per sample might be very similar. Given that one of the advantages of immunoassay ought to be the potential for handling large sample numbers, and given that carrying out proper sampling procedures could generate more samples, then single sample analysis might be construed as false economy. In addition, the ability to generate quantitative data and to analyse the same sample at different dilutions can yield valuable information that would be missed by semi-quantitative procedures. The number of food companies that seem happy to contract-out analytical services and lose contact with the process of result generation means that large amounts of valuable information about those products is disappearing!

(iv) *Assay validation*. Analysis of food can be a complex issue because of the large diversity of matrices that can be encountered, a number possibly unequaled in other fields. The range puts a particular strain on analysts and on assay validation. In addition to all the normal procedures (behaviour of standards, specificity, recovery, absence of matrix effects, performance against other methods, performance in other laboratories), then performance and experience over time become important; no method can be tested against every conceivable food matrix. Immunoassay does have the advantage that larger numbers of samples can be processed in a shorter time to make assay validation a more complete procedure than is possible with some alternative procedures, but there is still no substitute for long-term experience and extensive data to call on.

(v) *Confirmatory method*? Given the difficulties of analysing proteins outlined earlier and the lack of robust, widely available alternatives to immunoassays, it might be thought that the need for confirmatory methods might be a problem. This is not the case provided new antibodies can be identified. No two antibody preparations are identical in their behaviour, and similarly no two analytes will react in the same way with an antibody. Consequently it is possible to use different, quantitative immunoassays based on different antibodies to generate

statistically significant data on the identity of analytes. This can be used to quantify cross-reacting material if present (Karu, Lin, Breiman, Muldoon & Hsu, 1994) or to confirm that a positive finding is indeed positive for the analyte in question.

(vi) *Sampling*. Though sampling is not the subject of the present paper, it should be emphasised that the best of analytical procedures will only be as good as the sampling procedure used to provide the sample(s) to be analysed. One of the benefits of immunoassays is that it should not be the limiting factor if the sampling procedure employed under particular conditions generates large sample numbers. In addition, the use of immunoassays can help generate the information required to validate new sampling plans in a cost-effective manner.

6. Future potential of immunoassay for detection of novel foods from GMOs

As described earlier, immunoassays have come to be seen as the analytical method of choice for robust, specific, sensitive and cost-effective detection of proteins in complex mixtures.

We do not feel that sensitivity is a particular issue in immunoassay development at this time. It is not too difficult to make immunoassays of the desired sensitivity from antibodies generated with modern methods, and there are many methods described in the literature for obtaining immunoassay sensitivities down to extreme levels such as 600 molecules, for example (Harris, Yolken, Kroken & Hsu, 1979) if so desired. Indeed, extreme sensitivity can be associated with new and difficult analytical problems associated with contamination, particularly where (as with food and agricultural matrices) the analytical samples are available in bulk.

The rate-limiting steps in immunoassay development and application are the need for widespread availability of appropriate antibodies and standards, the difficulties of producing antibodies to particular sequences from a protein, and the inability to generate on demand antibodies capable of reacting normally at extremes of pH or in high concentrations of salt or solvent. These are areas where the application of recombinant antibody technology should bring considerable benefits as it becomes possible to more easily select antibodies with rare properties and to manipulate the properties of antibodies already available. As understanding of food materials and food processing is increased, so it will become possible to understand the changes undergone by biopolymers subjected to thermal and other denaturing forces.

It is difficult to imagine how immunoassay methods could be made any easier or foolproof to perform and interpret, but one area where advances are being made is in the combination of antibody methods with instrumental techniques. In addition to the 'hyphenated' methods such as immunoassay-mass spectrometry, considerable advances are now being made in real-time observations of antibody binding to target molecules – the biosensors that have been sought for so long. Instruments using optical methods for detection of molecular interactions are now being used for analysis of real samples (Elliott, Hellenäs, Sternesjö & van der Gaag, 1997). Whilst there is still some way to go before the disposable biosensor based on antibodies becomes available, these are significant advances, and provide much optimism for yet further progress.

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