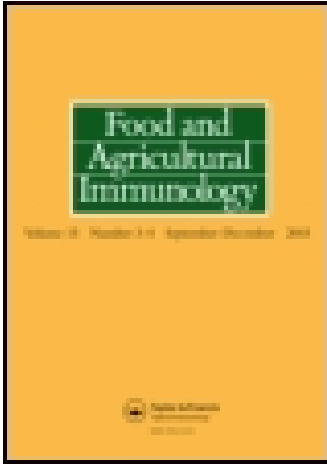




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Production of monoclonal antibodies to gluten proteins and their use in developing tests for gluten quality

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Production of Monoclonal Antibodies to Gluten Proteins and Their Use in Developing Tests for Gluten Quality

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There is a need for rapid, simple tests to give added assurance in gluten quality evaluation which address the needs of plant breeders and the milling and baking industry. As part of fundamental research into gluten protein structure–function relationships, a library of monoclonal antibodies (MAbs) has been developed to various gluten protein fractions, including both high and low molecular weight subunits of glutenin. The difficulties presented by working with insoluble, heterogeneous gluten proteins in raising MAbs of the requisite specificity are discussed. Several of the MAbs have been used to develop rapid immunoassays which are capable of analyzing a single sample in 10 min, and these have been applied to the quantification of total glutenin proteins, and high and low molecular weight subunits of glutenin in flour samples, illustrating the potential of immunotechnology for monitoring flour quality.

Keywords: *Glutens, wheat proteins, monoclonal antibodies*

INTRODUCTION

The fraction of wheat flour known as gluten is generally defined as the proteinaceous mass which remains after washing the starch from a dough. It is remarkable for its properties of elasticity and viscous flow, which enable the manufacture of a diverse range of products from wheat flour. Many of these products require gluten with different physical attributes. In general, a more extensible gluten is required for biscuit manufacture, whereas a stronger gluten is necessary for bread-making. Processing technology, of course, also plays a vital part in dictating gluten quality requirements. The selection of wheat flours with the requisite properties is of great importance to the milling and baking industry. However, the tests currently employed by millers and plant breeders to assess wheat quality are of an empirical nature, and are inadequate for predicting the suitability of wheat flours for a particular end use. Indeed, performing a test-bake, an expensive and time-consuming operation, is still the most reliable method of assessing flour quality.

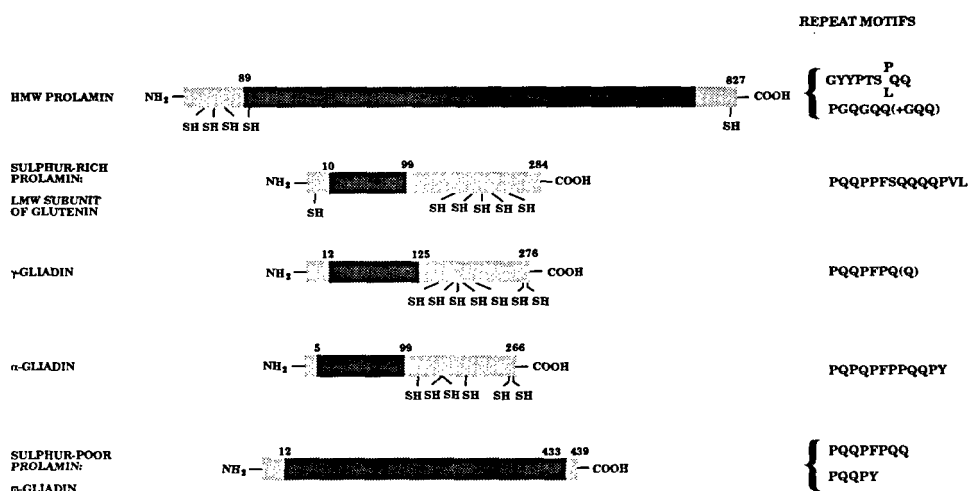


FIG. 1. Structural features of wheat prolamins. The light areas at either end represent the N- and C-terminal domains. The darker central area is the central repetitive domain comprising repeating amino acid sequences of around 5–13 amino acids in length.

The lack of reliable, simple tests for assessing gluten quality stems primarily from a lack of understanding of which components present in gluten are important in determining visco-elasticity and how the interactions of the gluten components give rise to these physical properties. Investigations into gluten proteins at a molecular level have been fraught with difficulties. Gluten is comprised almost entirely of wheat seed storage proteins, which, by virtue of the high contents of the amino acids glutamine and proline, can be termed prolamins (Shewry & Tatham, 1990). Their unusual solubility properties, coupled with their heterogeneity, have made it difficult to study these proteins by conventional means. Generally, they can be classified on the basis of their sequences and amino acid compositions into the sulphur-poor (S-poor), sulphur-rich (S-rich) and high molecular weight (HMW) prolamins. More traditionally, they have been divided into two groups—the monomeric gliadins and the polymeric glutenins (Figure 1). Gliadins are readily soluble in aqueous alcohol and can be classified on the basis of their electrophoretic mobility at low pH into α -, β - and γ -gliadins, all of which are S-rich and ω -gliadins, which are S-poor. S-rich gliadins have molecular weights in the range 36–44 kDa, while S-poor gliadins are larger, with molecular weights in the range 44–78 kDa, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Glutenins are generally only soluble under reducing conditions and the reduced polypeptides may be separated into two groups by SDS-PAGE: the S-rich, low molecular weight (LMW, 36–44 kDa) and high molecular weight (HMW, 95–136 kDa) subunits of glutenin. The 50 or so prolamin polypeptides are highly homologous, a feature which has made the purification of individual components difficult. One unique structural feature is the central repetitive domain (Figure 1) made up of a number of motifs ranging from three to nine amino acids in length. Secondary structure prediction algorithms suggest that these repetitive regions may adopt regularly repeated β -turns, and a model has been proposed based on spectroscopic and hydrodynamic studies in which the central domains form a loose spiral (Shewry & Tatham, 1990).

Antibody methods of analysis have been used to great effect in studying food proteins and also for quality assurance testing, particularly in the areas of speciation and adulteration. Indeed, the first enzyme-linked immunosorbent assay (ELISA) for food analysis to receive official first action approval from the AOAC was for the detection of soya proteins

(McNeal, 1988). For several years, antibodies have been used to study wheat gluten proteins. A number of problems have been encountered in developing and using such probes because of the lack of solubility of gluten proteins in aqueous buffers and their heterogeneity and structural homology. This paper describes these problems, approaches employed to develop specific antibodies and the application of these antibodies to the analysis of gluten quality.

SOLUBILITY OF GLUTEN PROTEINS

A range of solvents have been used to extract gluten proteins from flours (Mifflin *et al.*, 1983), but generally gliadins are soluble in 70% (v/v) ethanol or 10 mM-acetic acid, while total prolamins (i.e. gliadins plus glutenins) are soluble in 50% (v/v) propan-1-ol, 2% (v/v) acetic acid containing either 2% (v/v) 2-mercaptoethanol or 100 mM-dithiothreitol. Such solvents are clearly not compatible with use in ELISAs. Figure 2 shows the effect of ethanol concentration on the binding of a monoclonal antibody (MAb) (IFRN 0614) raised to wheat glutenins which recognizes S-poor prolamins of wheat, barley and rye (Brett *et al.*, 1990) using an indirect, non-competitive ELISA format. While binding was tolerant to ethanol up to a proportion of 10% (v/v), higher levels of solvent considerably reduced binding activity. Even when ELISAs were set up using 10% (v/v) ethanol, no dose-response curve could be obtained with the intact prolamins C hordein (the S-poor prolamins of barley), although one was observed using a synthetic peptide corresponding to the S-poor repeat motif (Figure 2(b)). It is likely that the C hordein had become aggregated, a process which is promoted by the presence of salt in the buffer employed for ELISA, and this was not overcome by such a relatively low level of ethanol. This aggregation clearly affected its reactivity in an inhibition ELISA, in marked contrast to the reactivity towards the readily soluble synthetic peptide.

As a consequence of these difficulties, the characterization of antibodies to cereal prolamins has been carried out using non-competitive ELISAs, performing antibody titration curves in parallel using a control protein (usually the immunogen) and different purified protein fractions. The percentage cross-reactivity can then be calculated using the ratio of absorbance values obtained for the test protein to that of the control protein at saturating antibody concentrations. However, it has been suggested that this ELISA format can overestimate cross-reactivity (Schwab & Bossard, 1992), a problem observed with ELISAs used to characterize MAbs to soya storage proteins (Plumb *et al.*, 1995). Artefactual cross-reactivity may also be compounded by the fact that it is very difficult to prepare, for example, a γ -gliadin preparation free from contamination with related prolamins. ELISA is a sensitive technique, and, not surprisingly, MAbs have been found to detect contamination of purified proteins at levels of less than 5% (Mills *et al.*, 1994a). Another factor that can influence MAb-binding characteristics is the effect that adsorbing proteins onto microtitration plates has on protein structure (Fruiget *et al.*, 1984), and the problems of knowing whether a representative proportion of polypeptides become adsorbed when using mixtures of polypeptides to coat microtitration plates (Skerritt & Martinuzzi, 1988).

Similar problems can be encountered when using immunoblotting procedures to characterize MAbs. Non-reproducible transfer of gluten polypeptides due to solubility problems can make the interpretation of immunoblots difficult. This methodology, unlike the ELISA, cannot give an assessment of the degree of cross-reactivity, although it can give an indication of the contamination of purified gluten fractions.

Generally, the lack of solubility of gluten proteins in buffers compatible with antibodies, their tendency to form macromolecular aggregates and the lack of purified individual polypeptides makes the use of any one of these techniques alone, somewhat unsatisfactory as a means of characterizing MAbs. However, some degree of certainty as to the binding characteristics of a MAb can be determined using a combination of techniques.

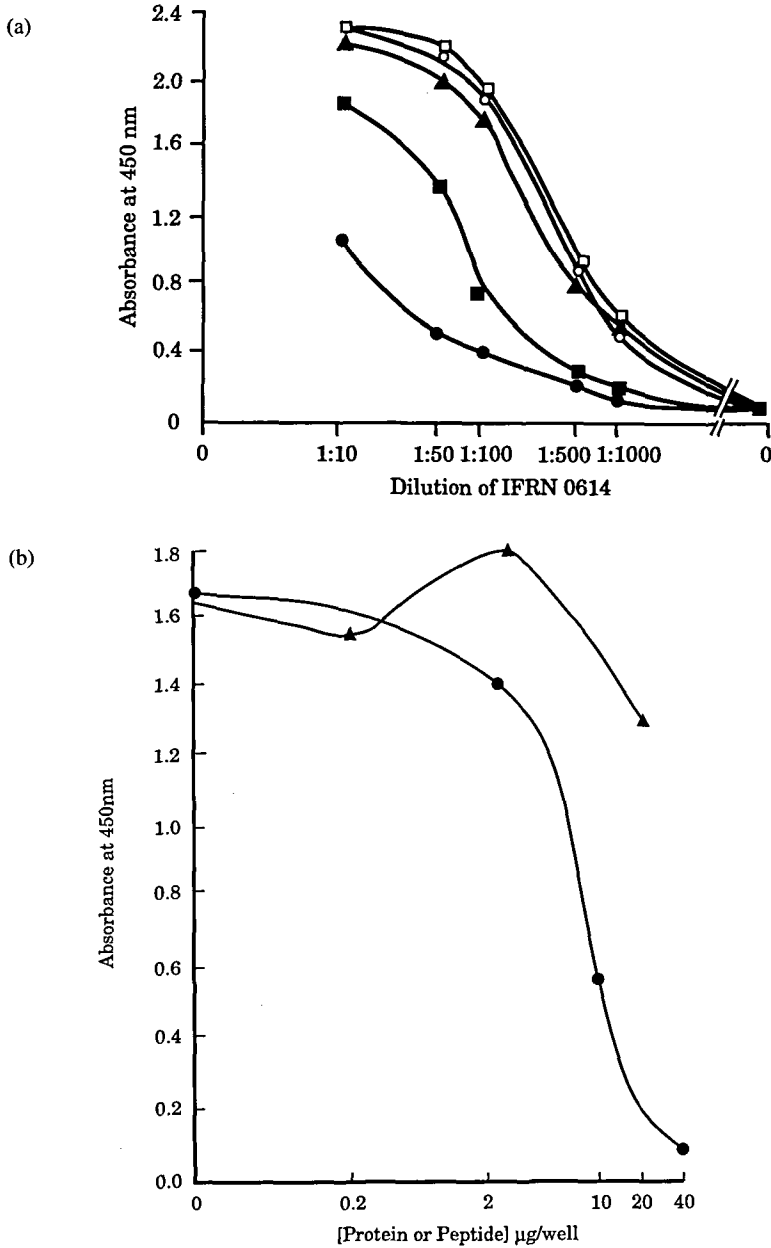


FIG. 2. (a) Effect of ethanol concentration of the binding of MAb IFRN 0614 to the S-poor prolamin of barley, C hordein, as determined by indirect ELISA. Ethanol concentrations: ▲, 0% (v/v); ○, 5% (v/v); □, 10% (v/v); ■, 20% (v/v); ●, 35% (v/v). (b) Dose-response curves obtained for IFRN 0614 in the presence of 10% (v/v) ethanol by indirect ELISA. ▲, C hordein; ●, synthetic peptide PQQFPQQ.

STRATEGIES FOR ANTIBODY PRODUCTION

Some of the earlier work involving the production of polyclonal antisera to gluten proteins gave rise to the suggestion that they were poorly immunogenic (Festenstien *et al.*, 1985).

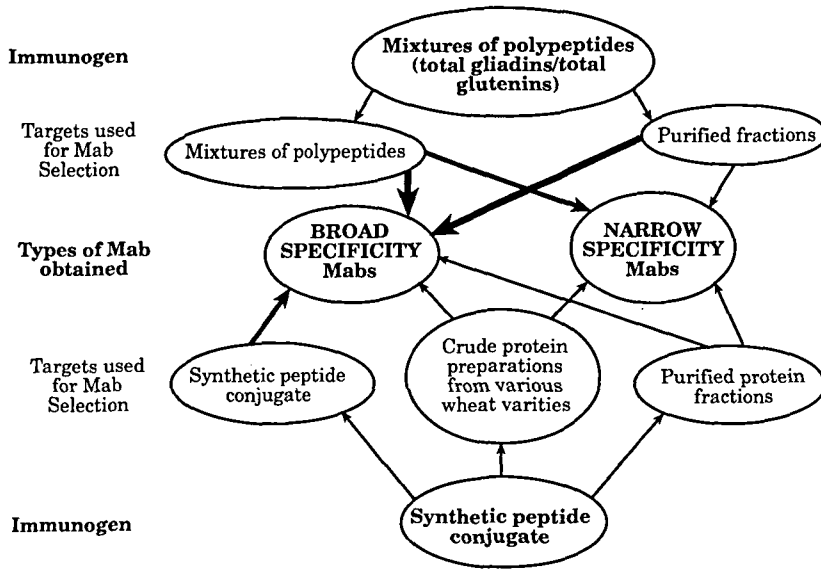


FIG. 3. Strategies for producing MAbs to cereal prolamins.

The expanding literature on the generation of libraries of MAbs to gluten polypeptides has exploded this myth. It seems likely that the difficulties encountered in raising antibodies stems from the limitations of the techniques used in the past to detect the desired antibody responses in immunized animals. The ELISAs currently employed are considerably more sensitive and are able to address the solubility problems of gluten proteins more effectively than the immunodiffusion methods used previously (Skerritt, 1987).

Figure 3 shows the main strategies employed in this study to produce MAbs to gluten proteins using either mixtures of polypeptides or synthetic peptides based on gluten protein sequences as immunogens. Hybridomas secreting antibody of the desired specificity have been selected using ELISAs employing either one or several of the following: mixtures of proteins; purified proteins; crude gluten protein preparations from wheat varieties known to differ in their protein composition; and synthetic peptide conjugates. Irrespective of the strategy employed, a preponderance of MAbs have been generated which are broadly reactive, although specific antibodies have also been obtained. Thus, in one fusion, using a mixture of polypeptides (a total glutenin fraction of wheat cv. Avalon) as both the immunogen and selection agent, 27 hybridomas were obtained. Three were found to be specific for single polypeptides, two recognizing LMW subunits of glutenin (IFRN 0067; Brett *et al.*, 1993; 0628, Mills *et al.*, 1993) and one binding to a trypsin/ α -amylase inhibitor type protein (Kauffman *et al.*, 1994). The remainder of the MAbs were found to be broadly reactive, recognizing a number of gluten polypeptides, although such MAbs may be highly specific for the sequence recognized. For example, two MAbs (IFRN 0061 and 0614) developed in this fusion bound to gliadins and glutenins, but recognized the S-poor prolamins especially well. Although they were broadly reactive, these MAbs were found to be specific for the S-poor repeat motif PQQPFPQ (Brett *et al.*, 1990), parts of which are present in other prolamins such as γ -gliadins (cf. Figure 1).

When a synthetic peptide, GSVTCPPQQV, conjugated to bovine serum albumin (BSA) was used as the immunogen, around 30 hybridomas were identified which produced antibody that bound to the peptide conjugated to bovine thyroglobulin. Three antibodies bound to BSA and five recognized the peptide conjugate but not prolamins. The remaining 22 antibodies bound to prolamins, 10 of which were able to discriminate between varieties

on the basis of their 1A *x*-type HMW subunits. Two of the latter group of MAbs, IFRN 1601 and 1602, have been characterized in detail and have been found to recognize 1A and 1D *x*-type HMW subunits of glutenin with only a low level of cross-reactivity (ca. 10%) with *y*-type HMW subunits (Mills *et al.*, 1994a).

The broad reactivity of many MAbs raised to gluten proteins results from their specificity for epitopes lying within the repetitive domain. Such reactivity is to be expected when the epitopes are present in multiple copies within the repetitive domains and show close homologies between different prolamins types (cf. Figure 1). It also appears that the repetitive domains are immunodominant, possibly as a consequence of being rich in β -turns, a structural feature thought to be involved in the immunodominance of other proteins (Dyson *et al.*, 1985). Thus, a polyclonal antiserum to C hordein appeared to be practically monospecific in terms of its recognition pattern, binding almost entirely to the repetitive domain (Festenstein *et al.*, 1987). It is also possible to generate MAbs to discrete epitopes in gluten proteins, which, therefore, recognize a single polypeptide or a group of closely related polypeptides, although the yield of such MAbs in any single fusion is low. Synthetic peptide immunogens probably offer the most effective means of maximizing the yield of MAbs to the non-repetitive regions, although care must be taken that hybridomas are selected which produce antibodies able to recognize both the peptide and its cognate protein. Indeed, such has been the experience of others raising polyclonal antisera to unique N-terminal sequences of gluten proteins (Denery-Papini *et al.*, 1994).

APPLICATION OF ANTIBODIES TO THE ANALYSIS OF WHEAT FLOURS

Using both broadly reactive and highly specific MAbs, a battery of immunologically based tests are currently being developed to address the issues of reliability and speed which are required for gluten quality assurance testing. A filter-plate ELISA format has been devised (Mills *et al.*, 1993) which addresses the need for speed, ensuring that a single sample can be analyzed in less than 20 min. To date, probes have been produced which are suitable for determining total gluten and HMW and LMW subunits of glutenin.

Total Gluten

Three broadly reactive MAbs were assessed for their suitability for quantifying gluten protein present in flour. Figure 4 shows the calibration curves obtained for IFRN 0610, 0065, and 0312 using a filter-plate ELISA. IFRN 0610 gave the most sensitive and reproducible standard curve, detecting as little as 5 ng gluten protein ml⁻¹.

LMW Subunits of Glutenin

A number of LMW gluten-specific MAbs have been obtained. The best characterized of these (IFRN 0067) showed binding which was related to baking quality (Brett *et al.*, 1993). Using 0067 in the filter-plate ELISA, it was found that levels of the LMW subunit recognized by this antibody were reduced in sulphur-deficient wheat grown under glass (Mills *et al.*, 1994b).

HMW Subunits of Glutenin

As described above, two MAbs, IFRN 1601 and 1602, have been developed using a synthetic peptide immunogen. They were specific for the 1A and 1D encoded *x*-type HMW subunits (Mills *et al.*, 1994a). Using IFRN 1602 in the filter-plate ELISA, it was found that the HMW subunit content of wheat increased when the plant was grown under sulphur-deficient conditions (Mills *et al.*, 1994b).

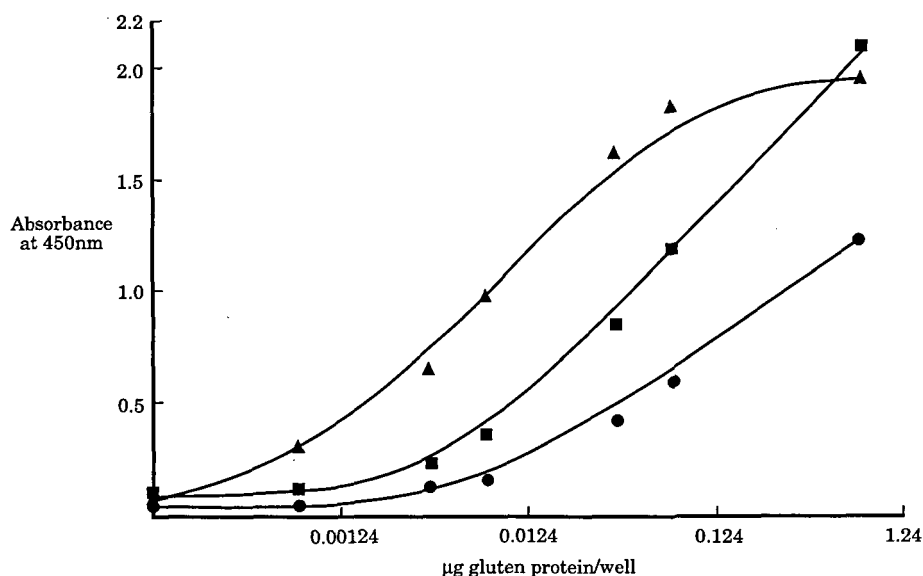


FIG. 4. Gluten protein calibration curves obtained for three bread specificity MABs using a filter-plate ELISA: ▲, IFRN 0610; ■, IFRN 0065; ●, IFRN 0312.

CONCLUSION

Immunological methods can be used to quantify both total gluten proteins and individual components. The value of the immunological approach will increase as MAB probes of different specificities become available (for example, development of an MAB which could quantify total HMW subunits). The technology not only offers a means of gluten quality assurance testing, but also provides a tool for investigating the effects of agronomic practices on the synthesis of gluten proteins, and hence how compositional changes may account for some of the environmental factors that affect gluten quality. In addition, antibody technology enables an increased understanding of the relationships between gluten polypeptides and the functional properties of wheat doughs and gluten, which are important for underpinning the development and control of processing technologies.

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