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A Two-site Enzyme-linked Immunosorbent Assay for Wheat Gliadins

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A two site enzyme-linked immunosorbent assay for wheat gliadins has been developed. Polyclonal antibodies from chicken eggs were used as the capture agent adsorbed to the surface of the microtitration plate. Murine monoclonal antibodies bound to captured gliadin were detected by addition of enzyme-labelled, species-specific antibodies. The assay was developed for the purposes of screening monoclonal antibodies specific for conformational epitopes on gliadins. The properties of a panel of 6 anti-gliadin antibodies in the assay were assessed and compared to properties exhibited under conditions where conformational epitopes were less likely to be present. Three of the monoclonal antibodies appeared to be specific for conformational epitopes.

INTRODUCTION

Wheat is the most widely grown cereal in the world. In addition to providing a range of nutrients, it also possesses unique technological properties which allow the production of a variety of different processed foodstuffs such as bread, biscuits and pasta. Many wheat cultivars are available, some of which produce flour better suited to biscuit making, others to bread making, and of all the wheat species, *Triticum durum* is by far the best for pasta production. On removing the water-soluble components of wheat flour, an insoluble proteinaceous mass remains which is termed gluten.

It is the alcohol soluble proteins (known collectively as prolamins) in gluten which are believed to be primarily responsible for its viscoelastic properties. The precise nature of these properties is critically important in dictating whether a particular flour will be more suited to one process rather than another. The prolamins have been extensively studied, and can be categorized into the gliadin fraction and the glutenin fraction based on their solubility in various solvents (Milfin *et al.*, 1983).

The way in which these proteins contribute at the molecular level to the functional properties of different wheat flours is only poorly understood at the present time. However, there is some evidence that the conformations adopted by these proteins

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play an important role in dictating the functional properties of wheat gluten (Tatham *et al.*, 1985).

The solubility characteristics of wheat prolamins, coupled with their heterogeneity makes the study of their conformation using conventional biophysical techniques such as X-ray crystallography difficult, if not impossible. Monoclonal antibody technology offers an alternative means to study conformational aspects of cereal storage proteins, exploiting the ability of antibodies to recognize secondary and tertiary structural elements, in addition to the primary sequence of a protein (Berzofsky, 1985).

In order to select monoclonal antibodies able to recognize the spatial (as opposed to sequential) organization of residues, the screening assay must be chosen with some care. Appropriate antibodies are more likely to be identified if appropriate recognition is possible. It has been established that direct adsorption of protein on to the surface of microtitration plates causes considerable loss of native structures (Friguet *et al.*, 1984; Kilshaw *et al.*, 1986).

Such an approach (a direct assay) would not be an efficient way of screening antibodies able to recognize structural features. We have, therefore, developed a two-site indirect enzyme-linked immunosorbent assay (ELISA) for wheat gliadins. Chicken antibodies against gliadin are adsorbed to the microtitration plate surface as capture antibodies, which are able to retain adsorbed gliadin without disrupting conformational epitopes. The performance of a panel of monoclonal anti-gliadin antibodies in the indirect two-site assay is assessed and compared to that in the direct assay.

MATERIALS AND METHODS

Tris-hydroxymethylaminomethane, dextran sulphate (sodium salt) and horseradish peroxidase (HRP) labelled anti-mouse IgM antibodies were purchased from Sigma (London) Chemical Company, UK; Freund's complete and incomplete adjuvants were obtained from Difco, UK; horseradish peroxidase (HRP) labelled anti-chicken IgG antibody was obtained from Guildhay Antisera, Guildford, UK; substrate based on tetramethylbenzidine was bought from Cambridge Life Sciences (CLS), Cambridge, UK; and microtitration plates (Nunc Immunoplate I) were obtained from Gibco Europe Ltd., UK. All other chemicals were of the highest quality available.

Protein Fractions

The various protein fractions used in this study were the gift of A. Tatham and P. Shewry, AFRC Rothamsted Experimental Station, Harpenden, UK. In order to prepare total gliadins, wheat flour (cv. Avalon, 50 g) was defatted with chloroform (2 × 500 ml) and dry n-butanol (2 × 250 ml) prior to drying in air for 48 h. Non-prolamins were extracted twice with 0.5 M-NaCl (2 × 500 ml) by stirring at room temperature for 2 h then centrifuged and the supernatant discarded. The pellet was washed with distilled water to remove residual salt and then extracted with 70% (v/v) aqueous ethanol (3 × 250 ml) for 2 h at 4°C. After centrifugation the supernatant was dialysed against 1% (v/v) acetic acid and freeze-dried. The extracted protein was fractionated by gel permeation chromatography on Sephacryl S300 (2.2 × 90 cm column) in 6 M-guanidine hydrochloride to separate the monomeric gliadins. This preparation was termed 'total gliadins'.

Total prolamins were extracted from milled grain of oats (cv. Goodland) and rye (cv. Frontier) by stirring with 50% (v/v) aqueous propan-1-ol containing 2% (v/v) 2-mercaptoethanol, after prior defatting with water-saturated butanol and extraction of salt-soluble proteins with 1.0 M-NaCl. The supernatants were filtered and the prolamins precipitated by the addition of 2 volumes of 1.5 M-NaCl and allowing to stand overnight at 4°C. The precipitated protein was dissolved in 8 M-urea, dialysed exhaus-

tively against water and lyophilized. Prolamins were extracted from milled seed of barley (cv. Sundance) using the same procedure but with 50% (v/v) aqueous propan-1-ol as a solvent.

Fractions containing total α -gliadins, total γ -gliadins, total aggregated gliadins, fast ω -gliadins and slow ω -gliadins were purified from wheat (cv. Maris Butler) as described previously (Shewry *et al.*, 1983; Tatham & Shewry, 1985).

Mouse Anti-Gliadin Monoclonal Antibodies

Anti-gliadin monoclonal antibodies IFRN 033–038 were raised in mice (E. N. C. Mills and M. R. A. Morgan, unpublished data), essentially as follows. Balb/c mice were immunized by intraperitoneal injection with total gliadins cv. Avalon, emulsified in Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (all subsequent injections) and mixed with saline (7:3, v:v). A final booster injection was given 4 days prior to sacrifice. Spleen cells were isolated from the mouse and fused with the mouse myeloma line X63 Ag8653 (Flow Laboratories, UK), in the presence of polyethylene glycol following the method of Galfré and Milstein (1981). Culture supernatants were screened using the direct ELISA described below for determining chicken antibody titres. Cell-lines secreting anti-gliadin antibodies were cloned by limiting dilution using rat erythrocytes as feeder cells. The specificity of each of these monoclonal antibodies was characterized with respect to different gliadin and glutenin fractions, and prolamins from different species of cereal. Characterization was achieved using a combination of direct immunoassay and immunoblotting (E. N. C. Mills & M. R. A. Morgan, unpublished data).

In short, IFRN 033 was found to be specific for the ω -gliadins, with some cross-reactivity against γ -gliadins. It also recognized prolamins from oat and barley. Monoclonal antibody IFRN 038 bound exclusively to the α -gliadins, and recognized some polypeptides present in oat and barley prolamins. IFRN 035, 036 and 037 were of a much broader specificity, all recognizing (to different extents) the various gliadin fractions tested as well as prolamins from rye, barley and oat.

For use in developing the two-site assay, monoclonal antibodies were used either as culture supernatants, or had been concentrated by 50% ammonium sulphate precipitation followed by dialysis against 0.1 M-phosphate-buffered saline, pH 7.4 containing 0.05% (w/v) thiomersal. Monoclonal antibodies in both forms were stored in aliquots at -20°C until required and once thawed they were stored at 4°C .

Chicken Polyclonal Antibody Production

Day-old chicks were raised on a wheat-free diet. Prior to coming into lay the chickens were immunized using conventional methods. Each hen received, intra-muscularly, 100 μg wheat gliadins emulsified in complete Freund's adjuvant and sterile saline (7:3, v:v; 1×0.2 ml per leg). In the subsequent monthly inoculations incomplete Freund's adjuvant was used (7:3, v:v; 1×0.2 ml per leg). Eggs were collected daily for two weeks, collection commencing fourteen days from immunization. This proved to be a sufficient length of time to account for the delay in transfer of IgG from the hen to the egg. The eggs used in this study were collected after the 4th and 5th booster.

The antibody was extracted from the egg yolk using a variation of the dextran sulphate precipitation method developed by Jensenius *et al.* (1981). After separating the yolk from the white, the vitelline membrane was cut open and the yolk poured out. The contents were diluted with four volumes of Tris-buffered saline (TBS; 0.14 M-NaCl, 0.01 M-Tris hydroxymethylaminomethane/HCl, pH 7.3) and centrifuged at 2000 rpm for 10 minutes at room temperature. The sediment was discarded. To the supernatant 10% (w/v) dextran sulphate (average molecular weight 500 000) in TBS was added at 0.3 ml/ml of original yolk, followed by addition of 1 M- CaCl_2 .

(0.75 ml/ml of original yolk). The solution was incubated for 30 minutes at room temperature after which it was centrifuged as before. The supernatant was poured off, thiomersal added to 0.05% (w/v) and the solution stored at 4°C.

Determination of Chicken Antibody Titres

(a) *Plate Coating.* Microtitration plates were coated with total gliadins diluted from a stock solution (2.0 mg/ml in 10 mM-acetic acid) to 1 µg/ml in 0.05 M-carbonate/bicarbonate buffer, pH 9.6. Microtitration plate wells were filled with 0.3 ml of this solution and incubated overnight at 4°C. Plates were then washed three times with distilled water using a Titertek Microplate Washer 120 (Flow Laboratories Ltd., UK). Excess moisture was shaken out and, after allowing to dry at room temperature, plates were stored at -20°C until required.

(b) *ELISA Procedure.* Plates were first washed three times in 0.1 M-phosphate-buffered saline, pH 7.4, containing 0.05% (v:v) Tween 20 (PBSTween). Chicken antibody samples were diluted in PBSTween, and 0.2 ml/well of each dilution added in triplicate to the gliadin coated plates. After incubating overnight at 4°C, plates were washed 5 times in PBSTween and 0.2 ml of anti-chicken IgG-HRP conjugate, diluted 1:1000 (v:v) in PBSTween, was added to each well. Plates were then incubated for 3 h at 37°C. After a further 5-cycle wash in PBSTween, 0.2 ml of HRP substrate was added to each well and the plates incubated for approximately 30 mins at 37°C. The reaction was then stopped with 50 µl/well of 2 M-sulphuric acid and the absorbance of the wells determined at 450 nm using a Titertek Multiskan MCC plate reader (Flow Laboratories Ltd., UK).

Two-Site Assay for Gliadin

The chicken antibody used as the capture antibody in the assay was diluted in TBS, and 0.3 ml added to each well. After incubating overnight at 4°C, plates were washed three times in TBS. All chicken antibody-coated plates were freshly prepared for each assay. Stock solutions of gliadins (2 mg/ml in 10 mM-acetic acid), were diluted in TBS and 0.2 ml added per well. After incubating for 3 h at 37°C plates were again washed three times in TBS, and 0.2 ml/well added of anti-gliadin monoclonal antibody diluted 1:50 or 1:100 (v:v) in TBS. After an overnight incubation at 4°C with mouse antibody, the plates were again washed three times with TBS, and 0.2 ml/well of anti-mouse IgM-HRP conjugate (diluted 1:1000, v:v, in PBSTween) added. The assay was then performed as for determination of chicken antibody titres.

RESULTS

Figure 1 shows a typical series of hen egg antibody titration curves obtained on a microtitration plate coated with total gliadins, and is representative of the eggs collected between 16–22 days after first immunizing the hens. An inhibitory effect was observed when using neat egg antibody, but this was removed on diluting the preparation in buffer.

Using the chicken antibody titres as a guide, a two-site assay was then set up using the chicken antibody diluted in TBS 1:20–1:50 (v:v), depending on the preparation, to coat the microtitration plates. Standard curves were set up with 6 different anti-gliadin monoclonal antibodies (Mabs), and are shown in Figure 2. Only 3 of the Mabs bound to the gliadin in the two-site assay, namely 033, 036 and 037. The standard curves obtained for each of these Mabs had slightly different limits of detection, 037 giving the least sensitive assay with a detection limit of 5.0 µg gliadin/ml. The curve for 036

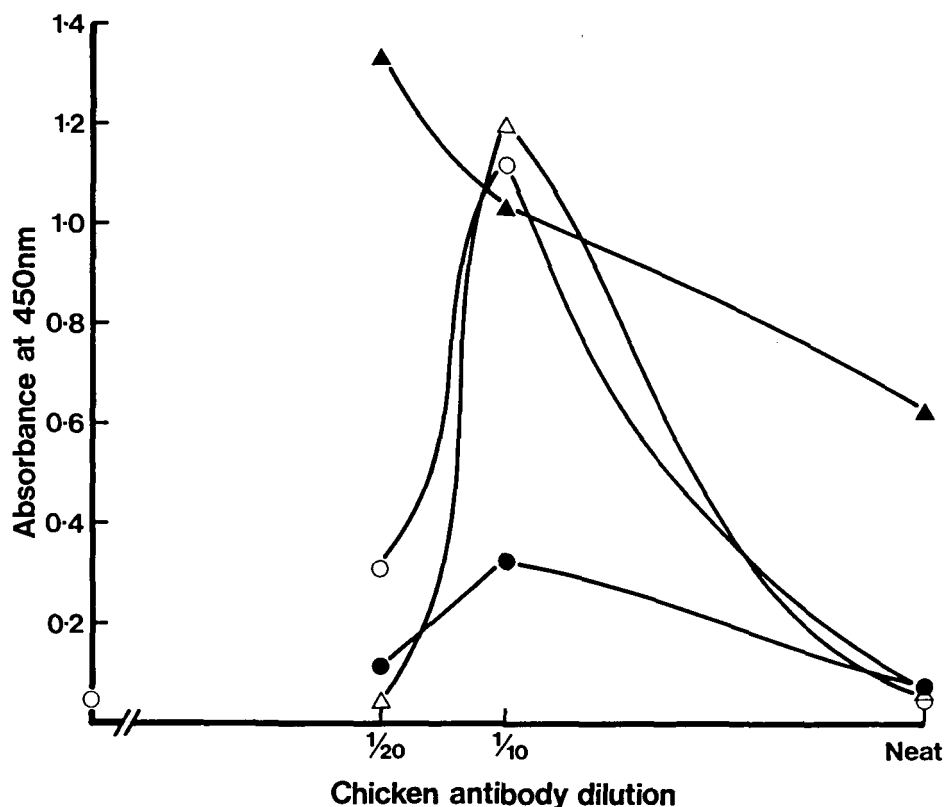


FIG. 1. Chicken antibody preparation dilution curves using an ELISA against adsorbed gliadins. Curves are shown for 4 different eggs laid by hen H400, collected 15 (●-●), 17 (Δ-Δ), 20 (○-○) and 21 (▲-▲) days after giving a booster injection.

is slightly more sensitive with a detection limit of $2.0 \mu\text{g/ml}$, whilst 033 gave the lowest limit, $0.1 \mu\text{g/ml}$.

The cross-reactivities of 033, 036 and 037 with different cereal prolamins and purified gliadin fractions were then determined, and the results are given in Table 1. In relation to different cereals, 033 was the most specific Mab, with very low binding to rye prolamins, and slightly higher cross-reactivity with barley and oat prolamins. Both 036 and 037 bound to the other cereal prolamins, 036 showing a pattern of binding similar to that of 033, with barley and oat prolamins being recognized equally well by 036, and rye prolamins least of all. By contrast, 037 bound to oat prolamins quite well, with a cross-reactivity of 61%, followed by the prolamins of rye (53%) and then barley (41%).

TABLE 1. Cross-reactions of Monoclonal Antibodies 033, 036 and 037 in the 2-Site Assay.

Monoclonal Antibody	Total Gliadins	Rye Prolamins	Barley Prolamins	Oat Prolamins	α -gliadin	γ -gliadin	fast ω -gliadin	slow ω -gliadin
033	100	12	39	35	111	149	0	0
036	100	31	42	44	35	103	0	0
037	100	53	41	61	58	137	0	0

Note: Cross-reactions are expressed as the ratio (as a percentage) of the optical density given by the protein under test at $20 \mu\text{g/ml}$, to that given by total gliadins at the same point.

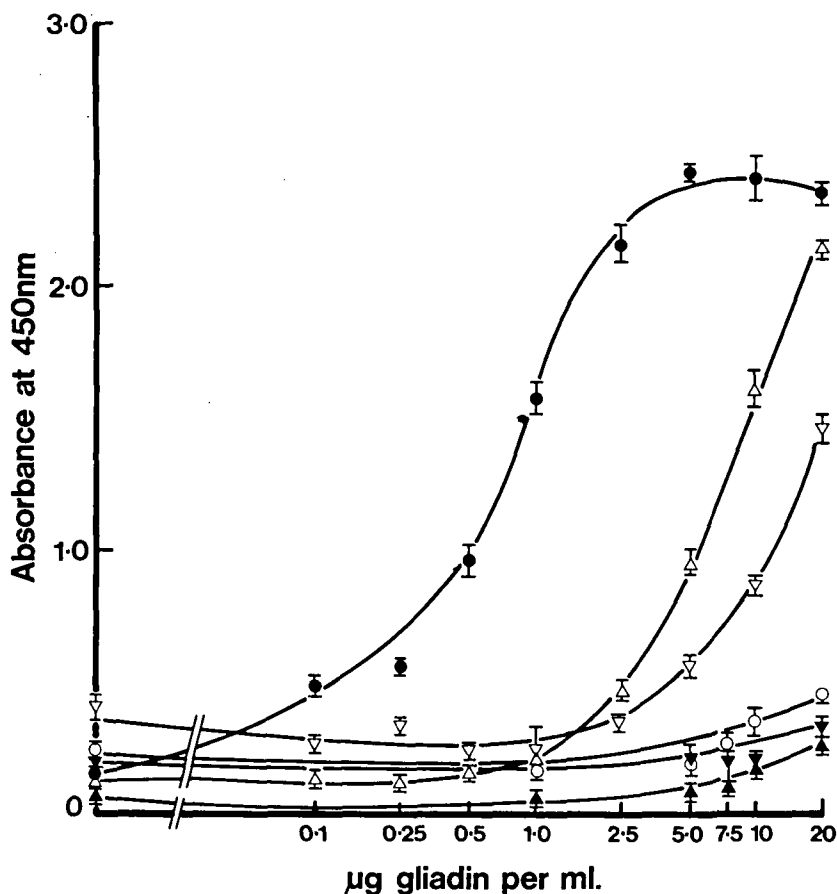


FIG. 2. Standard curves for 6 monoclonal antibodies raised to gliadins, using the two-site assay. Monoclonal antibodies were as follows: IFRN 033 (●-●); 036 (Δ-Δ); 037 (▽-▽); 035 (○-○); 034 (▼-▼); 038 (▲-▲).

With respect to purified gliadin fractions, none of these Mabs recognized either fast or slow ω -gliadins. High cross-reactions against both α - and γ -gliadins were observed, 033 and 037 recognizing the latter best of all with binding at well over 100%. Antibody 033 gave the highest binding to the α -gliadin fraction, followed by 037 and 036.

DISCUSSION

Both chickens used for the production of polyclonal antibodies responded well to immunization with wheat gliadins, and the antibody could be obtained from the egg yolks with ease using the method of Jensenius *et al.* (1981). The lack of binding of neat chicken antibody to gliadin-coated plates may be the result of interference from either residual egg-yolk components, such as lipid, or residual levels of dextran sulphate and calcium carried over from the antibody preparation procedure.

Once the chicken polyclonal antibody preparation had been successfully obtained, we proceeded to optimize the conditions for its use as a capture antibody in a two-site assay. When the conventional 0.05 M-carbonate/bicarbonate buffer was used for

coating the microtitration plates, very inconsistent results were obtained. On diluting the chicken antibody in this buffer, a white precipitate was formed which adhered to the plate and remained throughout the assay. When the substrate was added, the precipitate turned dark brown, which made reading the absorbance of the microtitration plate well contents impossible.

Similar results were observed when using PBS as the coating buffer. The precise origin of the brown precipitate is unclear, but may result from the formation of insoluble carbonate and phosphate salts of egg-components still present in the antibody preparation. Any residual calcium from the clean-up procedure would also lead to formation of calcium carbonate or phosphate but those salts are unlikely to stick to the plates or react with the substrate.

Moudallal *et al.* (1984) who used chicken antibodies as a capture antibody in an ELISA tobacco mosaic virus did not report having these difficulties. The problem was overcome by using TBS as the buffer for coating, and for plate washing in all but the last step. By using TBS, it was found that the chicken antibody preparations could be used at dilutions of 1:20 or 1:50 (v:v) for coating as the capture antibody, the precise dilutions depending upon the preparation used.

It is clear from standard curves in Figure 2 that the panel of Mabs investigated can be categorized into those that do recognize gliadin in the two-site assay (033, 036, 037) and those that do not (034, 035, 038). All six of these antibodies were originally identified using a screening procedure based on binding to adsorbed gliadin. In such an assay it is likely that, as a result of the interaction between the protein and plate surface, the protein will become unfolded to some degree (Fruiget *et al.*, 1984). As a consequence, parts of the primary sequence may be exposed which would not otherwise be available for antibody binding, and in addition only the most stable of conformational epitopes might remain. Thus the Mabs identified by screening in this manner are more likely to be those directed towards primary structure and stable features of secondary structure.

Such an observation would seem to be borne out by the ability of all these antibodies to recognize gliadins denatured after electrophoresis and immunoblotting procedures (Mills and Morgan, unpublished data). In a two-site ELISA, where the protein is held in position by interactions with a capture antibody, the structure of the proteins would not be expected to be distorted. The inability of 034, 035 and 038 to bind to total gliadins in the two-site assay suggests that some degree of unfolding is necessary to allow these antibodies to bind. Conversely, the epitopes recognized by 033, 036 and 037 must be available in both types of assay. This in turn suggests that these epitopes might be on the surface of the gliadin polypeptides, whilst those of 034, 035 and 038 are buried when binding is examined under the conditions of the two-site assay. Such a phenomenon has been observed by several workers (Fruiget *et al.*, 1984; Vaidya *et al.*, 1985) and serves to emphasize the importance of the type of presentation method used in screening for monoclonal antibodies in dictating the types of antibody eventually obtained.

The different limits of detection observed for 033, 036 and 037 in the two-site assay might reflect the differing affinities of these antibodies for their respective epitopes, but the number of epitopes presented by the captured proteins could also be a contributory factor. All three assays are less sensitive than the two-site assays of Windemann *et al.* (1982), Troncone *et al.* (1986) and Ayob *et al.* (1988). The reason for this may be that these assays employed polyclonal antisera throughout and were probably of broader specificity in terms of the gliadin sub-fractions recognized. Thus greater signal generation would be obtained for a given gliadin standard. In the present case, the Mabs 033, 036 and 037 recognize far fewer epitopes on the captured polypeptides. Of course, monoclonal antibodies demonstrating broader specificity than in the present case will also be able to generate bigger signals. Thus Freedman *et*

al. (1987), were able to demonstrate a sensitive gliadin assay using a combination of polyclonal and monoclonal antibodies.

It is interesting to note that the cereal species cross-reactivity of 036 and 037 in the two-site assay is broadly the same as that observed against adsorbed gliadin in the direct assay. However, 033 exhibited much lower cross-reactions than in the direct assay, particularly with barley and oat prolamins. This altered cross-reactivity pattern is also observed with 033 against gliadin fractions. Using an ELISA based on binding to adsorbed protein and immunoblotting procedures, 033 had previously been shown to be highly specific for ω -gliadins, binding to only two components representing the fast and slow forms of ω -gliadin. However, in the two-site assay, no binding to ω -gliadins was observed at all, but a very high cross-reaction with α -gliadins was seen. 036 and 037 also showed slightly different cross-reactivity in relation to gliadin sub-fractions, in that they bound to the γ -gliadins better in the two-site assay than they did to the adsorbed γ -gliadin. These altered cross-reactions, particularly for 033, are difficult to explain, but they do indicate how differently the protein is presented in the two types of assay.

Of course the conformations taken up by the wheat gliadins in the two-site assay may not bear any resemblance to structures found *in vivo* or in a food matrix such as bread dough. Many other workers have used monoclonal antibodies to study protein conformation but the proteins studied are normally soluble in aqueous buffers, and two-site assays have been used in order to retain a 'native' conformation. The concept of 'native' is difficult to apply to cereal prolamins, which require the presence of organic solvents (such as ethanol or propanol) as well as reductant for complete solubility, and are found *in vivo* in the dehydrated protein bodies of mature wheat endosperm. To what extent the conformation of these proteins differs in a two-site assay from that found in the environment of a bread dough is a matter for speculation. There is some evidence that β -turn structures, which have been proposed by Tatham *et al.*, (1985) to play a central role in gluten elasticity, may be very stable (Dyson *et al.*, 1985) and may well be present intact, along with other stable structural motifs, under the conditions employed in this two-site assay.

Work is in progress to utilize the assay described for identifying other monoclonal antibodies capable of recognizing conformational epitopes on wheat prolamins and to use such antibodies to investigate the structures of these cereal proteins in food matrices such as bread.

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