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Specificity of an antibody to a subunit of high-molecular-weight storage protein from wheat seed and its reaction with other cereal storage proteins (prolamins)

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Abstract. An antiserum to subunit 2 from the high-molecular-weight (HMW) subunits of the glutenin fraction of *Triticum aestivum* cv. Highbury was shown to react with related subunits from other cultivars of wheat. The reaction was measured quantitatively by laser nephelometry in polyethylene glycol phosphate-buffered saline after dissolving the HMW fraction in 0.1 M acetic acid; urea used to dissolve the HMW prolamins inhibited the reaction, in some cases at the low concentration of 0.06 M. A study of the comparative reactions of other cereal prolamins was made. 'D' hordein, the homologous HMW protein of barley, showed less reaction, which was more inhibited by urea than the wheat subunits. Some ω-gliadins from the wheat cultivars Chinese Spring and Cheyenne reacted more strongly than the injected fraction and there was less inhibition by urea. A-, β- and γ_3-gliadins of wheat also reacted with the antiserum while a secalin of rye of M_r 40000 gave a weak reaction.

Key words: Cereal storage protein – Prolamin – Storage protein antibody – *Triticum* (prolamin).

Introduction

Wheat gluten is the viscoelastic proteinaceous mass which remains when dough is washed to remove starch and proteins soluble in water (albumins) and dilute saline (globulins). It is a mixture of proteins which are usually classified into two groups on the basis of whether they are present as monomers (gliadins) or aggregates stabilized by covalent disulphide bonds (glutenins). Both groups can be defined as prolamins in that the individual polypeptides, although not necessarily the aggregates, are soluble in mixtures of alcohols (ethanol, propan-1-ol, propan-2-ol) and water and are rich in proline and glutamine.

Gliadins are classified into four groups on the basis of their electrophoretic mobility at low pH. The slowest, the ω-gliadins, form a structurally distinct group and are characterized by a low content of sulphur amino acids. They have therefore been called S-poor prolamins. The faster, α, β and γ-gliadins have a varying degree of structural relationship to each other. They are also related genetically, and probably structurally, to the major group of glutenin proteins which have relative molecular masses (M_rs) in the range 40000–44000 and have been called low-molecular-weight subunits of glutenin or aggregated gliadin. Together the α, β and γ-gliadins and the aggregated gliadins form a second group of prolamins which have been called S-rich because of their high content of cysteine (1–3 mol%). The third group of prolamins, the high molecular weight (HMW) subunits of glutenin, have M_rs of 90000–144000 by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and are characterized by a high content of glycine. Cultivars of wheat contain between four and six HMW subunits, and Payne et al. (1981a) have shown that the presence of certain of these is associated with good baking quality in the progeny of crosses between different wheat varieties.

Immunochemical studies of cereal prolamins have so far been limited because of technical difficulties posed by their unusual solubility properties, their low antigenicity and the difficulty of preparing pure components to raise antibodies. Festenstein and Hay (1982) have recently overcome the first two problems by developing a sensitive, laser

Abbreviations: HMW = high molecular weight; PAGE = polyacrylamide-gel electrophoresis; PBS = phosphate-buffered saline; PE = pyridylethylated; SDS = sodium dodecyl sulphate

nephelometric assay while improved procedures have facilitated the preparation of pure components (Khan and Bushuk 1979; Field et al. 1982). In the present paper we describe the preparation of an antiserum to a purified HMW subunit of glutenin and use this to compare the structural relationships of gliadins, HMW subunits of glutenin and structurally related proteins of barley and rye.

Materials and methods

Plant material

Milled whole grain of field and greenhouse-grown plants was used. The seeds were from plants grown at Rothamsted, except for maize (Dr. K. Hoseney, Manhattan, Kan., USA), pearl millet (Dr. M. Freeling, Berkeley, Cal., USA), rice (Tropical Products Institute, London, UK), sorghum and the wheat cultivars Cheyenne and Chinese Spring (Dr. D.D. Kasarda, Western Regional Research Center, U.S. Department of Agriculture, Berkeley, Cal., USA).

Prolamin samples

Barley hordeins were prepared from *Hordeum vulgare* L. cv. Julia as described by Shewry et al. (1978, 1980) for 'B' and 'C' hordeins and as described by Kreis et al. (1984) for 'D' hordein.

Rye secalins were prepared from *Secale cereale* L. cv. Rheidol as described by Shewry et al. (1982) for γ-secalins of M_r 75000 and 40000 and by Kasarda et al. (1983) for ω-secalin.

Wheat prolamins were prepared as described by Kasarda et al. (1983) for ω-gliadins from *Triticum aestivum* L. cvs. Cheyenne, Chinese Spring, Maris Butler and by Field et al. (1982) and Shewry et al. (1984a) for HMW subunits of glutenin from *T. aestivum* L. cvs. Brigand, Copain, Highbury and Maris Butler. A-, β- and γ_3-gliadins from *T. aestivum* L. cv. Scout 66 were gifts from Dr. D.D. Kasarda, Western Regional Research Center, U.S. Department of Agriculture, Berkeley, Calif. USA.

Preparation of total prolamins

Total prolamins from maize (*Zea mays* L.), pearl millet (*Pennisetum americanum* L.) and sorghum (*Sorghum bicolor* L.) were prepared as follows: meal was defatted with water-saturated butanol, then extracted with 1 M NaCl (3×5 ml g^{-1} for 1 h at 20° C), washed with water, then extracted with 50% (v/v) *n*-propanol-2% (v/v) 2-mercaptoethanol (for sorghum 60% (v/v) tertiary butanol-2% (v/v) 2-mercaptoethanol was used). The prolamins were precipitated from the bulked supernatants by the addition of 2 vols. of 1.5 M aqueous NaCl followed by standing overnight at 4° C. They were removed by centrifugation and lyophilized. The amino-acid compositions and gel electrophoretograms of the preparations are given by Bright and Shewry (1983).

Total prolamins from oats (*Avena sativa* L.) and rice (*Oryza sativa* L.) were extracted as above, but with 10 ml solvent g^{-1} meal.

Electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was as described by Shewry et al. (1978).

Table 1. $A^{1\,cm}_{280\,cm}$ values for wheat PE HMW subunits and barley PE 'D' hordein dissolved in 6 M urea-PBS and 0.1 M acetic acid (1 mg ml^{-1})

Cultivar	Chromosomal control	Subunit	Solvent	
			Urea-PBS (6 M)	Acetic acid (0.1 M)
Wheat				
Brigand	$1B_x$	6	1.11	1.05
Copain	$1B_x$	7	0.87	1.04
Highbury	$1D_x$	2	1.12	0.92
Maris Butler	$1D_x$	2	0.67	0.82
Brigand	$1D_y$	12	0.82	0.80
Barley			0.89	0.98

Protein modification

Some subunits were reduced and pyridylethylated (Friedman et al. 1970) to prevent re-formation of disulphide bonds. These are referred to as PE proteins.

Preparation of antiserum

The antiserum was prepared in a rabbit by Dr. D.A. Govier. Three mg of PE HMW subunit 2 from wheat (cv. Highbury) was stirred with 1 ml 1% Triton X-100 for 18 h and the mixture injected in incomplete Freunds adjuvant. A second injection of 3 mg protein suspended in 1 ml phosphate-buffered saline (PBS) was given seven weeks later. The antiserum used was that collected two weeks after the booster injection.

Immunodiffusion

Immunodiffusion was carried out in 1% agar in the nephelometric PBS buffer containing 4% polyethylene glycol and allowed to proceed for 1–2 days at room temperature.

Nephelometry

The Hyland PDQ laser nephelometer (Hyland, Division of Travenol Laboratories, Costa Mesa, Calif., USA) was used as described previously (Festenstein and Hay 1982; Festenstein et al. 1984). Antibody was diluted (usually 1/25) with 4% polyethylene glycol in buffer pH 6.9 which contained 4 g polyethyleneglycol 4000, 0.15 ml Tween 20, 0.85 g NaCl, 0.061 g $NaH_2PO_4 \cdot 2H_2O$, 0.108 g Na_2HPO_4 and 0.05 g sodium azide per 100 ml. The diluted antibody was filtered through a millipore filter (0.22 µm) and 1 ml filtrate placed in tubes for reaction with antigen dissolved in either 6 M urea-PBS or 0.1 M acetic acid. The relative light scattering (RLS) was usually measured after 1 h incubation at room temperature.

Solution of HMW prolamins

These were dissolved in 6 M urea-PBS and also in 0.1 M acetic acid: solutions of 1 mg ml^{-1} were allowed to stand overnight with occasional shaking; the amount dissolved was measured by determining $A^{1\,cm}_{280\,nm}$ for the solution after centrifuging any undissolved material. Table 1 shows $A^{1\,cm}_{280\,nm}$ for solutions of 1 mg ml^{-1}.

Results

Preparation of antiserum. The PE subunit 2 was purified from wheat cv. Highbury by a combina-

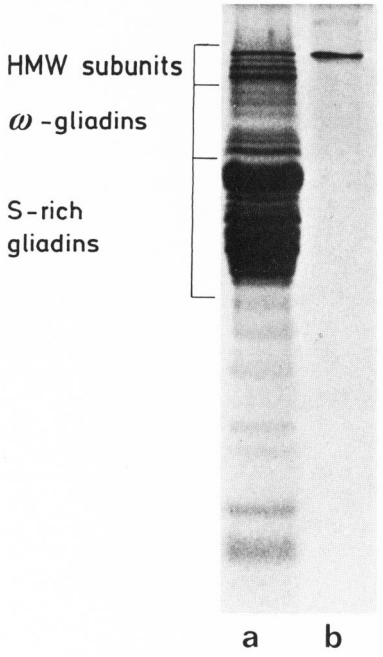

Fig. 1. Analysis by SDS-PAGE of reduced and pyridylethylated components of total prolamin from Highbury wheat. *a* Total prolamin; *b* HMW subunit-2, which was injected to produce the antiserum

tion of gel filtration on Sephacryl S300 and preparative isoelectric focusing in granulated gel (Field et al. 1982). Analysis of the preparation by SDS-PAGE (Fig. 1 b) showed that it was substantially free of other proteins. A 3-mg sample of the preparation was injected initially and a further 3 mg after seven weeks. Antiserum was collected two weeks after the booster injection.

Comparison of the reaction of HMW antiserum with different HMW subunits. Payne et al. (1980, 1981 a, b, 1982) have shown that the HMW subunits are encoded by structural genes on the long arms of the group-1 chromosomes. They have numbered the subunits in approximate order of their mobility on SDS-PAGE. They have further classified the subunits encoded by chromosomes 1B and 1D into high-M_r $1B_x$ and $1D_x$ subunits and low-M_r $1B_y$ and $1D_y$ subunits. European cultivars of wheat always have one subunit each of the $1B_x$, $1D_x$ and $1D_y$ groups and sometimes one $1B_y$ subunit and-or one 1A subunit.

Four different subunits were tested for their reaction with the antiserum raised against subunit 2 ($1D_x$ group). These were the $1B_x$ subunits 6 and 7, $1D_y$ subunit 12, and $1D_x$ subunit 2 itself, the latter purified from two different cultivars. The reactions were carried out after dissolving the subunits in two different solvents, 6 M urea-PBS and 0.1 M acetic acid.

Table 2. Reaction of 1 ml diluted wheat cv. Highbury HMW subunit-2 antiserum with 10-µl and 5-µl quantities of PE HMW-subunit solutions in 6 M urea-PBS and 0.1 M acetic acid (0.1 mg ml^{-1})

Wheat cultivar	Subunit	Chromosomal control	Nephelometer reading (% RLS)			
			Volume of subunit solution added			
			10 µl		5 µl	
			Urea-PBS (6 M)	Acetic acid (0.1 M)	Urea-PBS (6 M)	Acetic acid (0.1 M)
Brigand	6	$1B_x$	125	175	140	122
Copain	7	$1B_x$	122	172	117	139
Highbury	2	$1D_x$	154	168	129	129
Maris Butler	2	$1D_x$	113	161	80	124
Brigand	12	$1D_y$	104	160	100	128

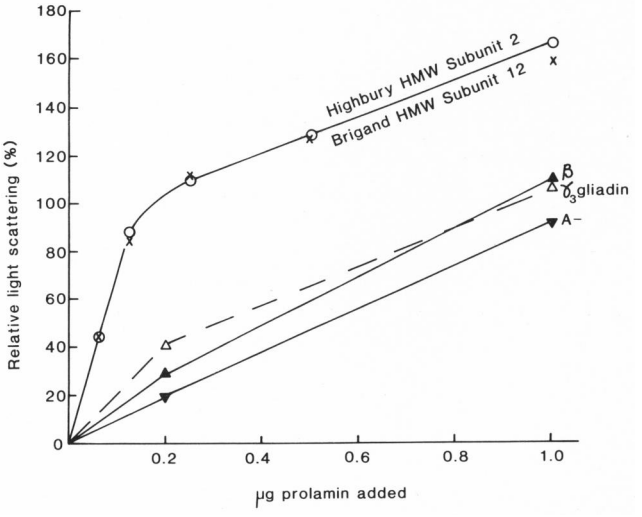

Fig. 2. Reaction of Highbury wheat HMW subunit-2 (○), Brigand wheat HMW subunit-12 (x), and α-(A-) (▼), β-(▲) and γ_3-(△) gliadins with Highbury HMW subunit-2 antiserum (1:25)

The results are given in Table 2. All the subunits reacted similarly to the Highbury subunit 2. When 10 µl of subunit solution was added to 1 ml diluted antiserum, there was a greater reaction when the subunits were dissolved in 0.1 M acetic acid than in 6 M urea-PBS, but there was no difference with 5-µl amounts even though the results with urea were more variable. Thus urea inhibits the reaction at a final concentration of 0.06 M when 10 µl of subunit solution was added, but not at a final concentration of 0.03 M when 5 µl of subunit solution was used.

Figure 2 shows the reaction of subunits 2 and 12 dissolved in 0.1 M acetic acid in the range

Table 3. Reaction of 1 ml diluted wheat cv. Highbury HMW subunit-2 antiserum with barley PE 'D' hordein dissolved in 6 M urea-PBS and in 0.1 M acetic acid

Concentration of 'D' hordein (mg ml^{-1})	Volume added (µl)	Nephelometer reading (%RLS)	
		Urea-PBS (6 M)	Acetic acid (0.1 M)
0.25	10	31	134
	5	44	116
0.125	10	16	114
	5	24	49

0.06–1 µg. The reactions for the two subunits are very similar and show two regions of approximate linearity, from 0 to 0.125 µg and 0.25 to 1.0 µg. The latter part of the curve indicates an inhibitory effect of increasing antigen concentration, possibly due to aggregation of the molecules masking the immunological binding sites.

The homologous HMW prolamin of barley, 'D' hordein, gave a much lower reaction (Fig. 2, Table 3), which was more inhibited by the presence of urea, than the wheat subunits.

Reaction of HMW antiserum with S-rich prolamins. The reactions of α-(A-), β- and γ_3-gliadins dissolved in 6 M urea-PBS are shown in Fig. 2. These gave lower reactions than the HMW subunits, similar to that of 'D' hordein over the concentration range 0 to 0.25 µg. Preliminary sequence analyses of reduced HMW subunits of wheat indicate some homology with α-, β- and γ-gliadins (Bietz and Wall 1980).

The 40000-M_r γ-secalins of rye, which are homologous with the γ_3 gliadin of wheat, gave a low reaction (33% RLS for 2 µg). The 75000-M_r γ-secalins of rye, a group which are probably derived from the 40000-M_r γ-secalins (Shewry et al. 1984b), and the 'B' hordeins of barley did not react, using 1-µg quantities.

Reaction of HMW antiserum with S-poor prolamins. Kasarda et al. (1983) have classified the S-poor ω-gliadins of hexaploid *T. aestivum* into three types on the basis of their N-terminal amino-acid sequences. One type, called the ARE variant because its N-terminal sequence is NH_2-Ala-Arg-Glu-, was proposed to be the ancestral type because of the presence of related types in diploid *T. monococcum*, rye and barley. The SRL (NH_2-Ser-Arg-Leu) variant differed from the ancestral type in the deletion of eight residues at the N-terminus and was present in a single component

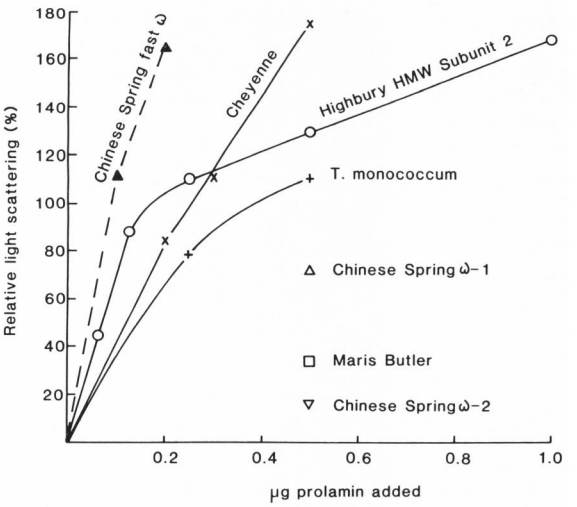

Fig. 3. Reaction of ω-gliadins from Cheyenne (x), *T. monococcum* (+) and Maris Butler (□) and fractions of Chinese Spring ω-gliadin: ω-1 (△), ω-2 (▽), fast ω (▲) with Highbury wheat HMW subunit-2 antiserum (1:25), compared with HMW subunit-2 (○)

with fast mobility during electrophoresis at pH 3.2. The third variant, the KEL (NH_2-Lys-Glu-Leu), was present in components with slow electrophoretic mobilities and differed from the ancestral type in substitutions affecting five of the first eight residues.

Five ω-gliadin fractions dissolved in 6 M urea-PBS were tested with the antiserum (Fig. 3). Electrophoretic analyses of these preparations have been presented previously (Festenstein et al. 1984). The ω-gliadin from Cheyenne was of the SRL type (personal communication by P.R. Shewry, E.J.-L. Lew and D.D. Kasarda, Western Regional Center, Berkeley, CA 94710, USA). The Chinese Spring fast ω-gliadin had similar mobilities on SDS-PAGE and on electrophoresis at pH 3.2 to the Cheyenne ω-gliadin (Festenstein et al. 1984), and was probably also of the SRL type. The Chinese Spring fast ω-gliadin particularly, reacted more strongly with the antiserum than did subunit 2 itself (Fig. 3).

The *T. monococcum* ω-gliadin (sequence type unknown) reacted less strongly as did the Chinese Spring ω-1 gliadin (KEL type (Kasarda et al. 1983)). The Chinese Spring ω-2 gliadin (ARE type (Kasarda et al. 1983)) and the Maris Butler ω-gliadin (similar mobility to Chinese Spring ω-2) both gave very weak reactions.

The Chinese Spring fast ω-gliadin and Highbury HMW subunit 2 gave single confluent lines in an immunodiffusion test indicating the presence of identical immunological determinants. This was confirmed by adding 25 µl of a 1 mg ml^{-1} solution

Table 4. Effect of urea on reaction of 1 ml diluted wheat cv. Highbury HMW subunit-2 antiserum with Highbury PE HMW subunit-2 and with wheat cv. Chinese Spring fast ω-gliadin. The sensitivity of the nephelometer was reduced for this experiment, and the results are not comparable with those in Table 1

Concentration of prolamin in 6 M urea-PBS (mg ml^{-1})	Volume added (µl)	Final concn. of urea (M)	Nephelometer reading (% RLS)	
			Highbury HMW subunit-2	Chinese Spring fast ω-gliadin
0.1	5	0.03	49	157
0.01	50	0.3	1	133

of Chinese Spring fast ω-gliadin to 0.25 ml of antiserum, when the reactions to the Chinese Spring fast ω-gliadin, the Cheyenne ω-gliadin and the Highbury HMW subunit 2 were abolished.

The ω-secalins of rye and 'C' hordeins of barley have N-terminal amino acid sequences related to the ancestral (ARE) type. These did not react with the antiserum, using 1-µg quantities.

Comparative effect of urea on the reaction of the antiserum with HMW subunit 2 and the Chinese Spring fast ω-gliadin. To see if the inhibition by urea of the reaction with the antiserum was general or specific to the HMW subunits, the effect of urea on the reaction with ω-gliadin was also tested. The inclusion of 0.3 M urea completely abolished the reaction of the antiserum with HMW subunit 2, but gave only 15% reduction in the reaction with the Chinese Spring fast ω-gliadin (Table 4). The reaction of the antiserum with the Chinese Spring fast ω-gliadin dissolved in 0.1 M acetic acid was the same as when dissolved in 6 M urea-PBS, when 5- and 10-µl quantities of a 0.01 mg ml^{-1} solution were assayed.

Reaction of the antiserum with other prolamins. Total prolamin fractions (1 µg) from maize, sorghum, pearl millet, oats and rice did not react with the antiserum.

Discussion

The almost identical reactions given by the different HMW subunits of *T. aestivum* are consistent with the previously reported high degree of homology of these components (Shewry et al. 1984a). The poor reaction given by the homologous 'D' hordein of barley is unexpected since complementary-DNA clones related to 'D' hordein and HMW subunits cross-hybridize (Forde et al. 1983), indicating the presence of sequence homology. This result emphasizes the fact that the antigenicity is related to the three-dimensional structure of the protein rather than to the primary structure. When complete amino-acid sequences become available it will be possible to attempt a prediction of the likely antigenic sites on the basis of the relative hydrophilicity of the amino acids, sites of high hydrophilicity being the most usual areas recognized by antibody (Hopp and Woods 1981).

The inhibition of the reactions of the HMW prolamins by low concentrations of urea indicates that their antigenicity is affected by small conformational changes resulting from the chaotropic action of the reagent. Our results showing the inhibition of reaction of HMW wheat subunits with antiserum by urea in a final concentration as low as 0.06 M, indicate that the HMW-subunit molecules are specially sensitive to unfolding or alteration of structure so as to affect their antigenic properties. This is in contrast to the effect on the Chinese Spring fast ω-gliadin where a concentration of 0.3 M showed only a 15% reduction in nephelometer reading but completely abolished the reaction between HMW subunit and antiserum (Table 4).

The antiserum also gave stronger reactions with gliadin fractions from wheat than with homologous prolamins from rye and barley. The only S-rich prolamins from the latter species which reacted were the 40000-M_r γ-secalins of rye. This is not unexpected since these appear to be most closely related to the γ_2 and γ_3 gliadins in their relative molecular masses, amino-acid compositions and N-terminal amino-acid sequences (Shewry et al. 1982).

The S-poor prolamins varied widely in their strength of reaction, and this appeared to correlate with the different groups recognized by Kasarda et al. (1983) on the basis of their N-terminal amino-acid sequences. Thus the 'ancestral' ARE type of ω-gliadins gave low reactions and the related ω-secalins and 'C' hordeins did not react at all. The strongest reactions, which exceeded even those of the HMW subunits used for immunisation, were given by the fast ω-gliadins of the SRL type. The strong reaction shown by these is analogous to the heteroclitic antibody effect described by Mäkelä (1965) for bacteriophage antisera.

The relative antigenicities of the ω-gliadins are in contrast to those recently reported for the same components using an antiserum raised against 'C' hordein (Festenstein et al. 1984). The results of these two studies are summarized in Table 5. It can be seen that the only ω-gliadin fraction which gave a similar strength of reaction was the ω-2

Table 5. Reactivity of ω-gliadins from various wheat cultivars with barley 'C'-hordein antiserum (Festenstein et al. 1984) and wheat Highbury HMW subunit-2 antiserum. Sequence types are shown in parentheses

Reactivity	Barley 'C'-hordein antiserum			Wheat HMW subunit-2 antiserum		
Strong	Maris Butler	ω	(?)	Chinese Spring	fast ω	(SRL?)
	Chinese Spring	ω-1	(KEL)	Cheyenne	ω	(SRL)
				T. monococcum	ω	(?)
Medium	Chinese Spring	fast ω	(SRL?)	Chinese Spring	ω-1	(KEL)
	Cheyenne	ω	(SRL)			
Weak	*T. monococcum*	ω	(?)	Maris Butler	ω	(?)
	Chinese Spring	ω-2	(ARE)	Chinese Spring	ω-2	(ARE)

of Chinese Spring. The 'C' hordein antiserum also reacted strongly with 'C' hordein itself, but only weakly with ω-secalin. Presumably the two antisera recognize different antigenic determinants on the S-poor prolamins.

Wheat, barley, and rye are classified in the tribe Triticeae of the sub-family Festucoideae. The other cereals tested are classified in either separate tribes of the Festucoideae (oats, rice) or in the subfamily Panicoideae (maize, sorghum, pearl millet). These did not react with the HMW subunit antiserum, although the oat prolamins (avenin) did give a low reaction with the antiserum to 'C' hordein (Festenstein et al. 1984).

Ewart (1977) in his review of the immunochemistry of wheat proteins noted antigenic similarities between glutenin and gliadin, but suggested that this was caused by mutual contamination. These results may have been the consequence of the presence of common antigenic determinants on the gliadins and HMW subunits of glutenin as reported here, but may also have resulted from antigenic determinants on the LMW subunits of glutenin. These components are more like the S-rich gliadins in character (see Field et al. 1983; Shewry et al. 1983), but were not used in the present study because of the difficulty of preparing homogeneous fractions. An interesting result noted by Ewart (1977) was that the reduction of the disulphide bonds of glutenin released a new antigenic determinant. Reduced and alkylated subunits of glutenin were used throughout the present study.

The antigenic relationship between gliadins and glutenins reported here raises the possibility that the latter may have a role in coeliac disease, where patients have circulating antibodies against different wheat protein fractions (Kieffer et al. 1982). Earlier work has shown that the toxicity is due to S-rich prolamins, mainly α-gliadins (see Kasarda 1978) but also β-gliadins and γ-gliadins (Charbonnier et al. 1980). More recently Kieffer et al. (1982) have studied the reactions of gliadins with sera from patients with coeliac disease. A-(α-)gliadin was most reactive, but some activity was shown by β-gliadins, γ-gliadins and even by ω-gliadins. Prolamins from rye, barley and oats, were also reactive but not those from rice or maize. It would be of interest, in the light of the present results, to study the reaction of the HMW subunits of glutenin.

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