Relation of antigenic structure of cereal proteins to their toxicity in coeliac patients

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1. Unfractionated gliadin and its α-, β-, γ- and ω-gliadin subfractions were used as rabbit immunogens. The antisera were characterized by (1) Ouchterlony double diffusion, (2) binding of ^{125}I-labelled gliadin subfractions, (3) inhibition by several gliadin subfractions of binding between γ-gliadin antiserum and ^{125}I-labelled γ-gliadin.

2. Double diffusion showed identical cross-reactivity between the antisera and the gliadin subfractions with the exception of ω-gliadin. Precipitin lines of partial identity with gliadin were observed against rye secalins and barley hordeins but not oat avenins or maize zeins.

3. Binding was observed between unfractionated ^{125}I-labelled α-, β-, γ- and ω-gliadins and all the antisera. There was binding of ^{125}I-labelled ω-gliadin to the ω-gliadin antiserum but poor binding of ^{125}I-labelled ω-gliadin to unfractionated α-, β- and γ-gliadin antisera. Competitive inhibition of binding between ^{125}I-labelled γ-gliadin and γ-gliadin antiserum diluted 1:250 (v/v) demonstrated similar competition between α-, β- and γ-gliadins and this antiserum but poor competition between ω-gliadin, wheat glutenins, albumins and globulins, rye secalins, barley hordeins and oat avenins.

4. These findings suggest that there is a good correlation between the antigenic structure of gliadin proteins and their toxicity to patients with coeliac disease.

Coeliac disease is a relatively common clinical condition with an overall incidence within the UK of 1 in 1800. The disease is characterized by malabsorption secondary to abnormalities of the small intestine, including enterocyte damage, lymphocyte infiltration and loss of normal villous architecture. It has long been known that these abnormalities improve when wheat gluten is excluded from the diet. The toxic effect of the gluten resides in a group of proteins, the gliadins, which are soluble in ethanol (700 ml/l). These are conventionally subclassified into four groups on the basis of electrophoretic mobility: the α-, β-, γ- and ω-gliadins. Despite earlier reports to the contrary, there is evidence from in vitro and in vivo work that all the subfractions have toxicity (Jos *et al.* 1978, 1982; Ciclitira *et al.* 1984; Howdle *et al.* 1984). Investigation of other cereals has shown that some of these produce clinical toxicity in coeliac patients, while others appear harmless.

We have undertaken a re-examination of the antigenic relationship of the wheat gliadin subfractions, the rye secalins, barley hordeins, oat avenins and maize zeins, to determine whether there is any correlation between clinical toxicity of the proteins and their antigenicity, and to assess methods whereby gliadins might be detected at low concentrations in food.

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METHODS

Preparation of cereal protein fractions

Wheat gliadin and its α-, β-, γ- and ω-gliadin subfractions were derived from the Kolibri strain of wheat by the method of Patey & Evans (1973). Wheat albumins, globulins and glutenins were obtained from a mixture of Timmo and Waggoner wheats as previously described (Ciclitira & Lennox, 1983). Milled rye (var. Rheidol), barley (var. Peniarth), oats (var. Porter) and maize (Prewetts Ltd) flours were twice defatted with *n*-butanol (500 g/l), and ethanol (500 ml/l) extracted (100 g/l) to provide rye secalins, barley hordeins, oat avenins and maize zeins.

Immunization

Either unfractionated gliadin or its α-, β-, γ- or ω-subfractions (100 μg) were dissolved in distilled water and emulsified with 1 ml Freund's complete adjuvant and injected subcutaneously into large White New Zealand rabbits for three consecutive months using the same preparation of immunogen. Because of the poor antibody response observed by Ouchterlony immunodiffusion described below, the quantity of immunogen per injection was increased to 300 μg in 1 ml adjuvant and was given monthly for a further 9 months. The animals were bled 1 week after each immunization. Serum from the different bleeds was stored separately at −20° with sodium azide added to a concentration of 1 g/litre to act as preservative. Sera from the seventh bleeds were used for the experiments described below.

Testing of antisera

Ouchterlony double diffusion. The antisera were examined against unfractionated gliadin, each of its subfractions, rye secalins, barley hordeins, oat avenins and maize zeins. The antisera were used undiluted and diluted 1: 9 (v/v) with phosphate buffered saline (PBS; g/l: NaCl 8, KCl 0·2, Na_2HPO_4 1·15, KH_2PO_4 0·2). The cereal extracts were dissolved in PBS (100 μg/ml). Normal rabbit serum and PBS served as negative controls.

Binding of iodinated antigens

α-, β-, γ- or ω-Gliadins or unfractionated gliadin served as antigens for further characterization of the antisera. Each fraction was labelled with ^{125}I by a modification of the method described by Hunter & Greenwood (1962) and Ciclitira & Lennox (1983). A 10 μg portion of each protein dissolved in 25 μl distilled water was iodinated with 500 μCi ^{125}I. Distilled water served as the solvent for the protein and chloramine T during iodination, to avoid the precipitation of wheat proteins in salt solutions. Desalting was undertaken on columns which consisted of Pasteur pipettes and contained 2 ml Sephadex G-50. Distilled water served as the eluent and 100-μl fractions were collected. The 600–1200 μl fractions contained the iodinated antigens which were pooled for each gliadin subfraction and diluted in PBS with (g/l): bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex) 20, Nonidet P40 (BDH, Poole, Dorset) 5 and sodium azide (BDH) 1 (PBS/BSA).

Dilution curves for each antiserum were obtained not only against its corresponding ^{125}I-labelled antigen but also against the other gliadin fractions by the method previously described (Ciclitira & Lennox, 1983). Competitive binding to the antiserum against γ-gliadin was assessed by inhibition of binding to ^{125}I-labelled γ-gliadin, using the other gliadin fractions, wheat glutenins, albumins and globulins, rye secalins, barley hordeins and oat avenins.

Table 1. *Precipitin lines between rabbit antisera to α-, β-, γ-, ω-gliadin and unfractionated gliadin, and cereal-protein fractions*

Fractions used to raise antisera... / Antigens	Unfractionated gliadin	α-Gliadin	β-Gliadin	γ-Gliadin	ω-Gliadin	Normal rabbit serum
Unfractionated gliadin	+	+	+	+	+	−
α-Gliadin	+	+	+	+	+	−
β-Gliadin	+	+	+	+	+	−
γ-Gliadin	+	+	+	+	+	−
ω-Gliadin	+	+	+	+	+	−
Alcoholic extracts:						
Rye (secalins)	+	+	+	+	+	−
Barley (hordeins)	+	+	+	+	−	−
Oats (avenins)	−	−	−	−	−	−
Maize (zeins)	−	−	−	−	−	−

+, Present; −, absent.

RESULTS

Identification of gliadin subfractions. The electrophoretic character of the gliadin subfractions by aluminium lactate starch gel electrophoresis compared with an extract of a single grain of Maris Huntsman, a standard control, is shown in Plate 1.

Ouchterlony double diffusion. The results of cross-reaction between the undiluted antisera and each of the fractions is shown in Table 1. When the antisera were diluted 1:10 (v/v) the precipitin lines were less easily seen and no other lines were observed. The only gliadin subfraction which did not share two precipitin lines with the other subfractions was ω-gliadin which shared one line but not the other when examined against antisera raised against α-, β-, γ- or unfractionated gliadins (Plate 2). Serum from the rabbit immunized with ω-gliadin gave one weak precipitin line in the Ouchterlony plates against each of the gliadin subfractions. Rye secalins gave two precipitin lines which were shared with all the gliadin subfractions except ω-gliadin, where only one precipitin line was shared against antisera raised against α-, β-, γ- and unfractionated gliadins. Rye secalins gave one precipitin line against the antiserum to ω-gliadin. Barley hordeins gave two lines against antiserum to α-, β-, γ- and unfractionated gliadins which were shared with all the gliadin subfractions except ω-gliadin, where only one precipitin line was shared. No precipitin line was observed between barley hordeins and the anti-ω-gliadin antiserum. No precipitin lines were observed between either oat avenins or maize zeins and any of the antisera.

Specific activity of iodination

The percentage Lowry positive protein for each immunogen, the ^{125}I specific activity and approximate quantity of protein per 25 μl portion of each of the iodinated fractions tested in the binding studies, is shown in Table 2.

Binding of ^{125}I-labelled subfraction to the antisera

Similar binding curves between the antisera and the iodinated gliadin subfractions were observed, with the exception of ^{125}I-labelled ω-gliadin for which the antibody titres were much lower than with the other iodinated fractions with antisera against α-, β-, γ- and unfractionated gliadins. An example of this is shown in Fig. 1. This is further shown in Table 3 where the dilution of each antiserum which bound 25% of the counts of each iodinated antigen is presented.

Table 2. *Percentage Lowry positive protein in each immunogen, the ^{125}I specific activity of the iodinated subfractions and approximate quantity of protein per 25 μl portions of each of the iodinated fractions tested in the binding studies*

	α-Gliadin	β-Gliadin	γ-Gliadin	ω-Gliadin	Unfractionated gliadin
Lowry positive protein in gliadin subfractions which served as immunogens (%)	88	62	83	59	76
Protein in iodinated gliadin fractions (μCi/g) ($\times 10^6$)	12·4	4·4	6·6	4·6	7·2
Approximate quantity (ng) of protein per 25 μl iodinated gliadin fraction in binding studies	0·2	0·8	0·4	0·8	0·2

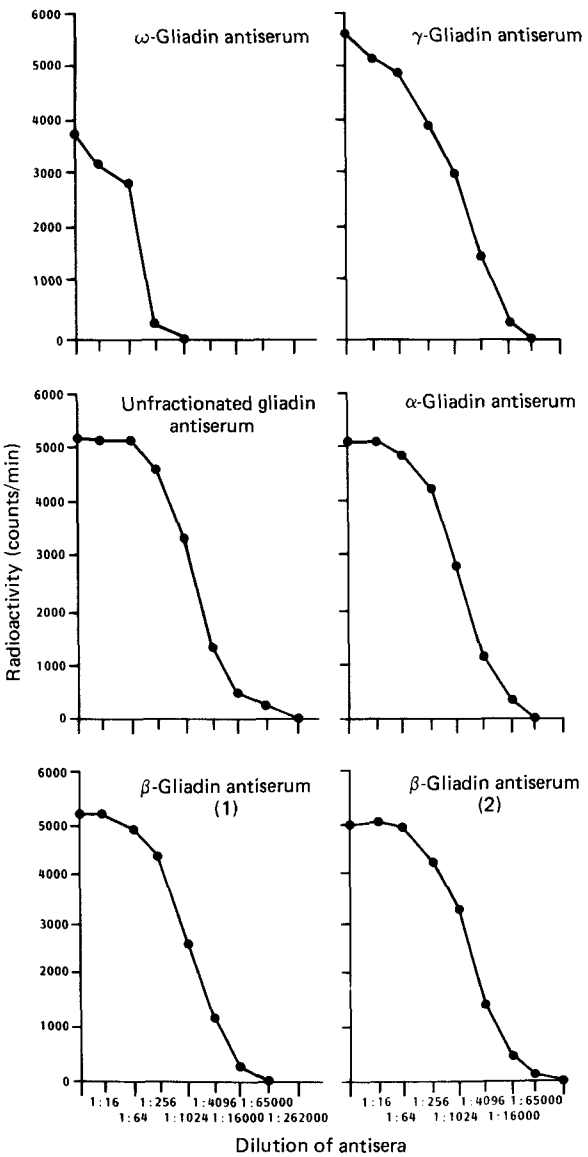


Fig. 1. For caption, see opposite page.

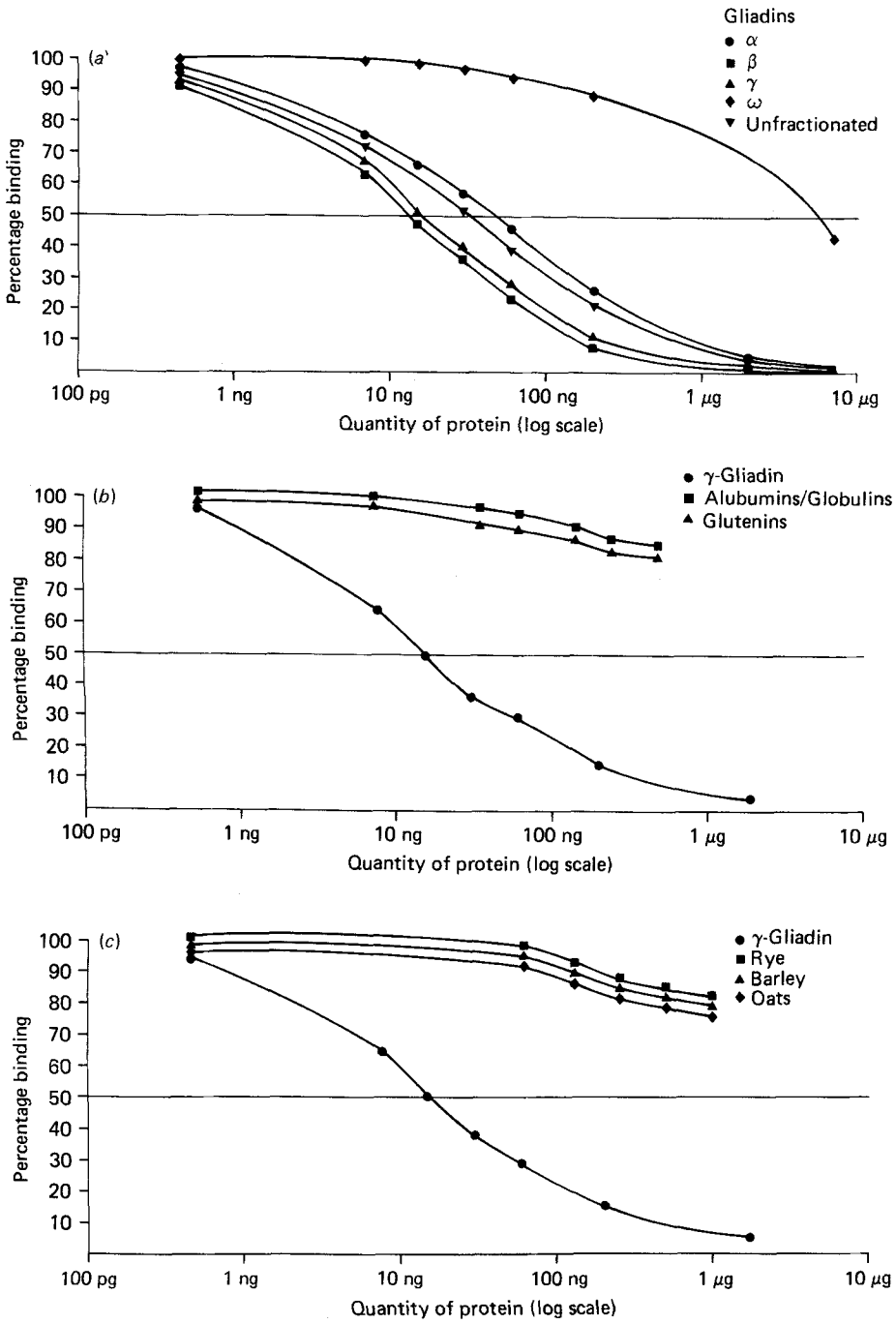


Fig. 2. Radioimmunoassay cross-reactivity curves (antibody dilution 1:250) for (*a*) unfractionated gliadin and its subfractions, (*b*) wheat glutenins, albumins and globulins, (*c*) ethanol extracts of rye, barley and oats.

Fig. 1. Radioactivity (counts/min) bound by serial dilutions of the seventh bleed of rabbit antiserum raised against α-, β-, γ- and ω-gliadins and unfractionated gliadin, and ^{125}I-labelled γ-gliadin ($7{\cdot}1 \times 10^3$ counts/min). Two rabbits were immunized against β-gliadin.

Table 3. *The greatest dilution at which 25 μl of each antiserum gave 25% binding to 5 × 10³ counts/min in 25 μl PBS/BSA of each of the iodinated antigens*

¹²⁵I-labelled gliadin subfraction	Antiserum				
	α-Gliadin	β-Gliadin	γ-Gliadin	ω-Gliadin	Unfractionated gliadin
α	500	500	500	<25%	1000
β	1000	2000	1000	<25%	1000
γ	4000	4000	4000	64	4000
ω	500	100	500	32	250

PBS/BSA, phosphate buffered saline/bovine serum albumin (see p. 40).

Cross reactivity

The antiserum to γ-gliadin was investigated further by competitive binding between this antiserum diluted 1:250 (v/v) and ¹²⁵I-labelled γ-gliadin with gliadin, and its subfractions and ethanol extracts of rye, barley and oats. The amount of Lowry positive protein to inhibit 50% binding between the γ-gliadin antiserum diluted 1:250 (v/v) and ¹²⁵I-labelled γ-gliadin was 45 ng α-gliadin, 14 ng β-gliadin, 15 ng γ-gliadin and 5 μg ω-gliadin (Fig. 2(*a*)). There were low levels of inhibition of binding with wheat glutenins, albumins and globulins (Fig. 2(*b*)), rye secalins, barley hordeins and oat avenins (Fig. 2(*c*)).

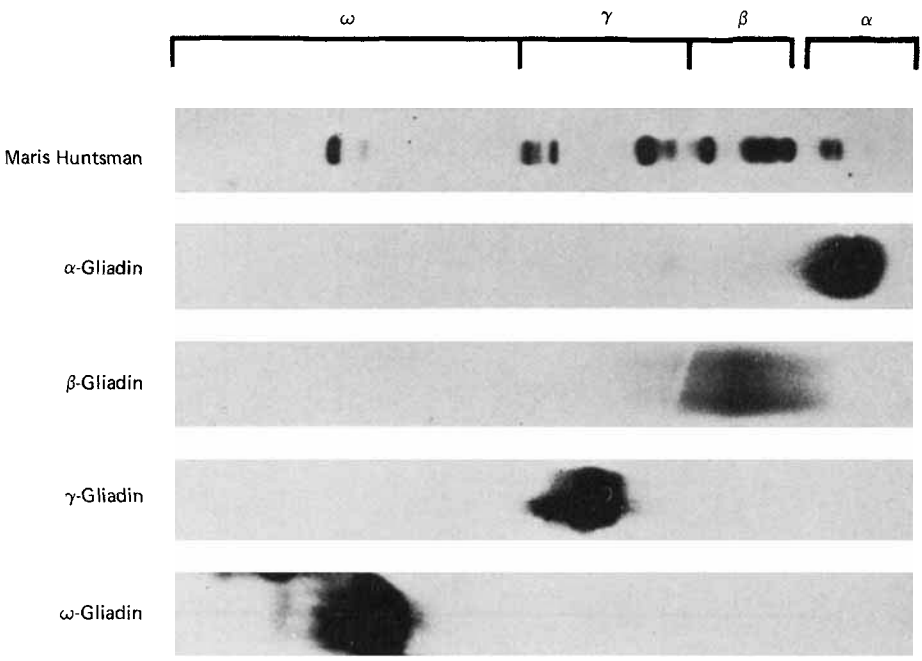
DISCUSSION

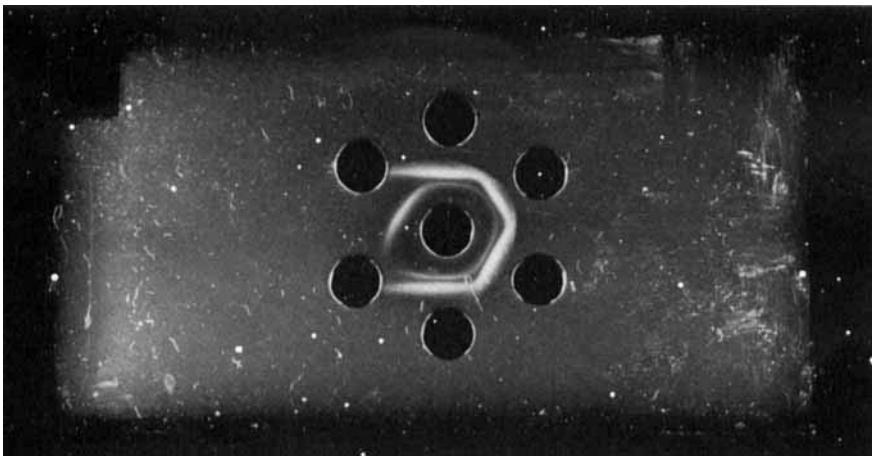
Assessment of the toxicity of a protein for patients with coeliac disease is a matter of considerable difficulty, especially if only small quantities of protein are available, and we have reviewed this problem elsewhere (Ciclitira *et al.* 1984). The most widely accepted evidence of toxicity is still the production of morphological changes in the small bowel by the protein in a patient with coeliac disease whose small intestine has reverted to normal after treatment with a gluten-free diet. On this basis, gliadin and its subfractions α-, β-, γ- and ω-gliadin are toxic (Ciclitira *et al.* 1984), as are secalins and hordeins (Dicke *et al.* 1953; Rubin *et al.* 1962). With regard to oat avenins, opinions have varied over the years but the most recent evidence (Anand *et al.* 1978) suggests that they lack toxicity.

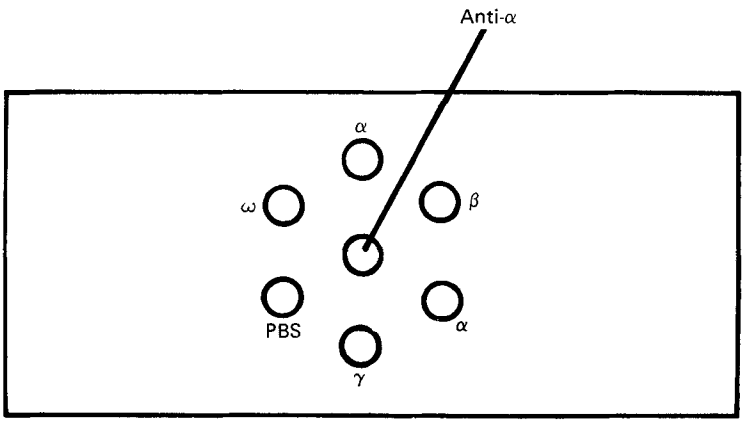
Similarities between gliadins of different groups have previously been shown by peptide maps and partial sequences (Kasarda, 1981), so the existence of antigenic similarities is not surprising. On the basis both of the immunodiffusion findings and radioimmunoassay, the ω-group differs substantially from the others, although it shares some antigenic determinants. The α-, β- and γ-groups appear very similar both in the immunodiffusion and radioimmunoassays.

Of the other cereal proteins examined, rye secalins showed precipitin lines against all the antisera, barley showed precipitin lines against all the antisera except the one raised against ω-gliadin. No precipitin lines were seen between oats and maize and any of the antisera. These findings are in agreement with those of Ewart (1966) who investigated a rabbit antiserum to unfractionated Wichita gliadin with extracts of wheat, rye, barley, oats and maize.

To summarize: shared antigenic determinants can be demonstrated between all gliadin subgroups; the secalins and hordeins appear related although more closely to the α-, β- and γ-gliadins than to ω-gliadin. Avenins and zeins appear unrelated antigenically to the gliadins.







There does, therefore, appear to be a correlation between antigenic structure and toxicity. This does not necessarily imply that damage to the intestine by these proteins is immunologically mediated, although it is consistent with that possibility. Although the antigenic similarities could be coincidental, it seems far more likely that they reflect a common structural feature of the toxic proteins.

The similarity between the gliadin subfractions indicates the feasibility of using a single radioimmunoassay to estimate the whole gliadin content of food.

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EXPLANATION OF PLATES

Plate 1. Electrophoretic patterns of gliadin subfractions used compared with those of a single grain of Maris Huntsman.

Plate 2. Precipitin lines between α-, β-, γ- and ω-gliadin subfractions and antiserum raised against α-gliadin. PBS, phosphate buffered saline.

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