

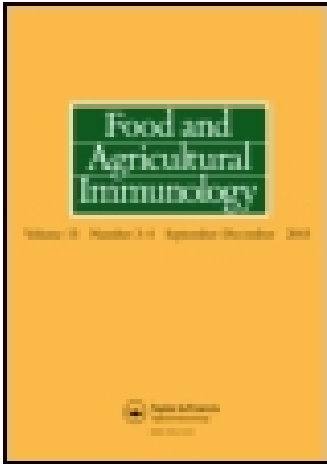
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Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

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Published online: 16 Sep 2008.

To cite this article: A. Stevenson, P. K. McCarthy & M. Griffin (1994) Polyclonal antisera against unheated and heated common wheat specific gamma and omega gliadins for the detection of adulteration of durum wheat and durum wheat products with common wheats, Food and Agricultural Immunology, 6:4, 435-442

To link to this article: <http://dx.doi.org/10.1080/09540109409354855>

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Polyclonal Antisera against Unheated and Heated Common Wheat Specific Gamma and Omega Gliadins for the Detection of Adulteration of Durum Wheat and Durum Wheat Products with Common Wheats

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(Received for publication 3 September 1993)

Common wheat (Triticum aestivum L) specific gamma (γ) and omega (ω) gliadins from native (unheated) and heat-processed (pasta) samples have been purified by reverse-phase high-pressure liquid chromatography and used to raise polyclonal antisera in New Zealand White rabbits. The sera have been examined in both the crude and affinity-purified (against Durum flodur gliadins) states, for reactivity and specificity against gliadins from unheated and heat-processed common wheats. Further, the antisera have been examined for cross-reactivity against Durum wheat (Triticum durum Desf.) and pastas and for their potential as a means of detecting the adulteration of Durum wheats and Durum wheat pasta products with common wheat species (T. aestivum). The efficacy of the antisera was evaluated using two different immunoassay formats, enzyme-linked immunosorbant assay (ELISA) and immunoblotting.

Keywords: γ -Gliadin, ω -gliadin, Durum wheat, wheat, adulteration

INTRODUCTION

Italian and Spanish law states that pasta products must be manufactured only from Durum wheat (*Triticum durum* Desf.) and prohibits the sale of pasta products containing common wheat (*Triticum aestivum* L.), highlighting the need for specific methods which can rapidly and reliably identify the type of wheat used to make the pasta.

Proteins in wheat are largely comprised of the alcohol-soluble storage proteins (prolamins), gliadins (soluble in 70%, (v/v), aqueous ethanol) and glutenins (soluble in propanol and acetic acid) along with the aqueous-soluble albumins.

Immunologically, these proteins have been extensively characterized using a range of

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specific polyclonal and monoclonal antibodies, and many common features have been shown to exist between similar groups of proteins within different wheat species.

Gliadin proteins of wheat can be categorized into two broad groups on the basis of being either rich in sulphur (HMM gliadins, α -, β - and γ -gliadins) or sulphur deficient (ω -gliadins) or three discrete groups (α/β , γ and ω) on the basis of N-terminal sequencing studies. All gliadins have high concentrations of proline and glutamic acid, with the latter present mainly as glutamine. The molecular weight of the gliadin components are in the range 30–40 kDa for the α -, β - and γ -gliadins, about 60 kDa for the ω -gliadins and up to 100 kDa for the HMM gliadins.

The molecular structure of the gliadins is thought to comprise a globular conformation with a low proportion of α -helices. Disulphide bonds are intramolecular and hydrophobic interactions occur between different subunits. Gliadins show varying levels of surface hydrophobicity within their subunits, with low levels being found in the ω -gliadins and high levels in the γ -gliadins; α - and β -gliadins are more heterogeneous, with some proteins having high, and others low, hydrophobicities.

The differences in the gliadin contents of common wheats compared with Durum wheats (Resmini, 1968; Dyssele *et al.*, 1986) provides a possible immunochemical means of differentiating between these wheat species. However, the cross-reactivity shown between these proteins when antibodies have been raised to native purified proteins severely hampers this approach (Festenstein *et al.*, 1987). In the following preliminary study, antisera were raised to both native and heat-treated ω - and γ -gliadins which are specific to common wheat. The ability of these antisera to detect the presence of common wheat flour in high-temperature dried pastas made predominantly from Durum wheat is reported.

MATERIALS AND METHODS

Materials

Chemicals used for electrophoresis were of Electran grade and those used for buffers and immunochemical determinations were of AnalaR grade. All other chemicals were of general-purpose reagent grade. All organic solvents used in conjunction with reverse-phase high-pressure liquid chromatography (RP-HPLC) were of HiPerSolv grade.

Acrylamide, *N*, *N*-methylenebisacrylamide, glycine, trichloroacetic acid, ferrous sulphate, ascorbic acid, urea, sodium dodecyl sulphate, *N*, *N*, *N'*, *N'*-tetramethylenediamine, ammonium persulphate, glycerol, bromophenol blue, Coomassie brilliant blue R-250, acetic acid, methanol, ethanolamine, dimethyl sulphoxide, sodium chloride, potassium chloride, sodium phosphate, dibasic sodium phosphate, dibasic potassium phosphate, methanol, acetonitrile and trifluoroacetic acid (Spectrosol) were obtained from BDH Ltd, Poole, Dorset, UK.

Lubrol PX, hydrogen peroxide, pyronine-Y, protein molecular weight standards, Freund's complete and incomplete adjuvants, dithiothreitol, sodium acetate, sodium azide, polyoxyethylene sorbitan monolaurate, ponceau S red, 3,3',5,5'-tetramethyl benzidine, cyanogen bromide-activated Sepharose 4B and anti-rabbit IgG horseradish peroxidase (HRP) conjugate were obtained from Sigma Chemical Co., Poole, Dorset, UK.

2-Chloroethanol was obtained from the Aldrich Chemical Co., Gillingham, Dorset, UK.

Marvel dried skimmed milk powder was obtained from Cadbury Premier Brand (UK), Bournville, Birmingham, UK.

Repel Silane (dimethylchlorosilane 2% w/v) in 1,1,1-trichloroethane was obtained from LKB Producter AB, Bromma, Sweden.

Wheat and pasta samples were supplied by Pasta Foods, Great Yarmouth, Norfolk, UK; The Laboratory of the Government Chemist, Middlesex, UK; Barilla, Parma, Italy; and Laboratoire de Technologie des Cereales, INRA, Montpellier, France.

Gliadin Extraction Procedures

All wheat and pasta samples were ground in a coffee bean grinder (Moulinex, France) for 2 min.

Extraction for RP-HPLC purification of antigen. Ground sample (1 g) was mixed with 4 ml of 70% (v/v) aqueous ethanol containing 1% (w/v) dithiothreitol in 20 ml capped polythene vials. Extraction was performed at room temperature with vigorous shaking on a flask shaker (Gallenkamp, Loughborough, Leicestershire, UK) for 16 h.

Extracted solutions were clarified by centrifugation at $12\,000 \times g$ for 10 min in a bench-top microfuge (Jouan, St Nazaire, France) and filtered through a $0.45\ \mu\text{m}$ GV filter (Millipore, UK). After filtration, 1 ml aliquots were reduced to 30% volume in a centrifugal evaporator (Joun, St Nazaire, France) for approximately 60 min, then resuspended to 50% of their original volume with ethanol containing 1% (w/v) dithiothreitol and vortexed for 2 min before being clarified by centrifugation (MSE 21; $12\,000 \times g$ for 1 min). The protein content of each solution was estimated using the A_{280}/A_{260} spectrophotometric method.

Extraction for enzyme-linked immunosorbent assay (ELISA). Ground sample (100 mg) was mixed with 500 μl of 70% (v/v) aqueous ethanol containing 1% (w/v) dithiothreitol in a capped microfuge tube and extracted with vigorous shaking on a flask shaker (Gallenkamp) for 16 h. Following extraction, samples were clarified by centrifugation in a microfuge (Beckman; $12\,000 \times g$ for 3 min) and the supernatant was removed from the sample pellet. The protein concentration of each sample was then estimated by the A_{280}/A_{260} method.

Extraction for acid polyacrylamide gel electrophoresis (acid PAGE). Ground sample (250 mg) was mixed with 1000 μl of sample buffer (25 ml of 2-chloroethanol; 50 mg of pyronine-Y 30 g sucrose in 100 ml of distilled water) in a capped microfuge tube and extracted with vigorous shaking on a flask shaker (Gallenkamp) for 16 h. After extraction, the sample was clarified by centrifugation in a microfuge ($12\,000 \times g$ for 10 min) and the supernatant was removed from the sample pellet.

The RP-HPLC Purification of Soft Wheat Specific Gliadins

RP-HPLC was performed according to the method of McCarthy (1990a) with modifications.

Separations were performed using a Waters DELTA PAK semi-preparative column (C^{18} , 300 A pore diameter; $7.8 \times 300\ \text{mm}$, $15\ \mu$ particle size) at 40°C using a Tecom C-100 water circulator (Technique, Cambridge, UK). Specific soft wheat gliadin peaks were collected into plastic tubes following their detection by an LKB Uvicord S11 single-beam ultraviolet detector (LKB Producter AB, Bromma, Sweden). Following their collection, purified specific peaks were frozen to -70°C and then freeze-dried to a powder. The purity of the freeze-dried samples was checked by acid-PAGE (Draper, 1987).

Polyclonal Antibody Production

The immunization procedure was that outlined by Hudson and Hay (1980), initially using 300 μg of gliadins followed by three booster injections of 200 μg , using New Zealand White rabbits injected subcutaneously into three separate sites. A control rabbit was similarly immunized with 5 mM-acetic acid in the appropriate Freund's adjuvant.

The immunoglobulin G (IgG) fraction of immune and non-immune antisera obtained from rabbits was purified as described by Johnstone and Thorpe (1987).

Preparation of Soft Wheat Immunospecific Anti-gliadin Antisera

The procedure involved the coupling of total gliadins obtained from *Durum flodur* to

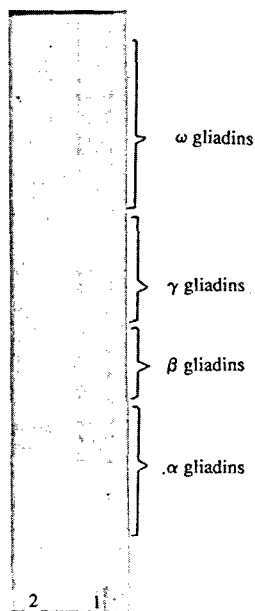


FIG. 1. Acid-PAGE separation profile of the total gliadins of soft wheat (Avalon) (lane 1) and *D. flodur* (lane 2).

cyanogen bromide-activated Sepharose 4B following the method of Johnstone and Thorpe (1987).

The antibody solution (albumin removed) was allowed to react with the affinity support at 4°C overnight, with end-over-end rotation. After this, the *D. flodur* affinity-purified antiserum was separated from the gel matrix and retained for analysis by ELISA and immunoblotting (see later).

ELISA

ELISAs performed were all of the heterologous, non-competitive, solid-phase format, in 96-well polystyrene microtitre plates, using a modification of the method of Engvall and Perlmann (1972). All the tests were performed in triplicate.

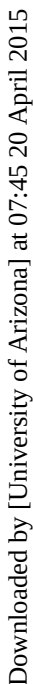
Bound gliadins (1 µg) were first reacted with the primary anti-gliadin antisera and the primary antibody then reacted with goat anti-rabbit IgG conjugated with HRP. The amount of HRP was then determined using tetramethyl benzidine substrate and the amount of colour development quantitated by absorbance at 450 nm in a multiscan microplate reader.

Protein Immunoblot Analysis

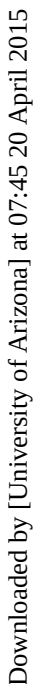
Total gliadin extracts containing approximately 2 mg ml⁻¹ protein, in 25% (v/v) 2-chloroethanol (see gliadin extraction for acid-PAGE) were separated by acid-PAGE using the method outlined by Draper (1987). Immunoprobings of blots with anti-gliadin antisera was carried out as previously described (McCarthy *et al.*, 1991).

RESULTS

The acid-PAGE profiles (Figure 1) of the total gliadin proteins of common (soft) wheat (Figure 1, lane 1) and Durum wheat (Figure 1, lane 2), are well established and differences



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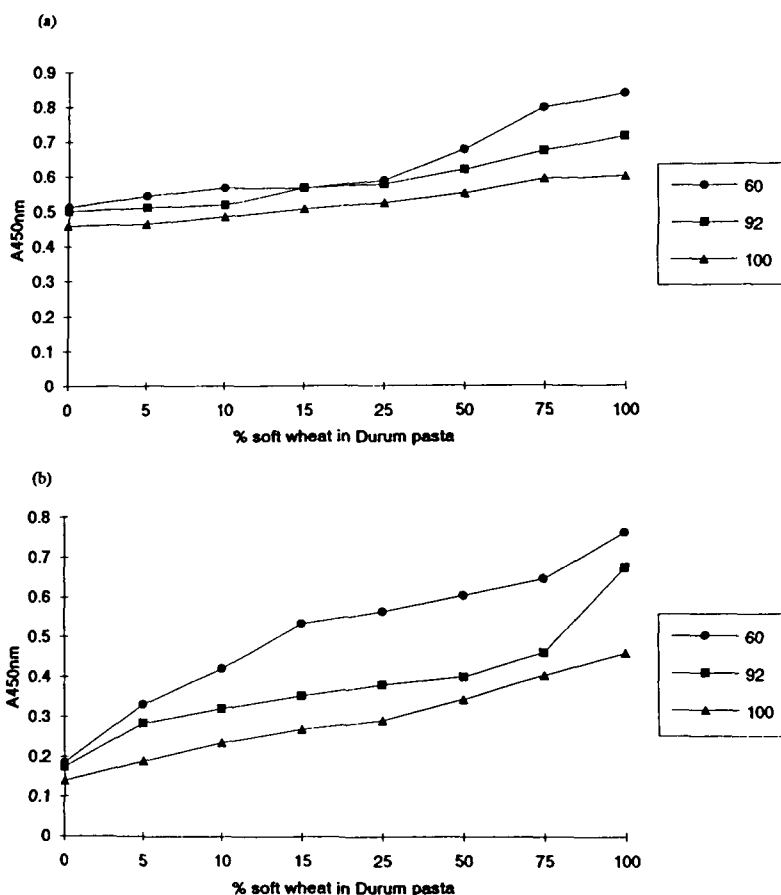


FIG. 4. ELISA using antisera raised against heat-treated (60°C, 92°C and 100°C) soft wheat specific γ -gliadins for the detection of soft wheat adulteration of Durum wheat pastas processed at 60°C, 78/92°C and 100°C. (a) Using IgG-enriched serum. (b) Using IgG-enriched and affinity-purified (with *D. flodur* gliadins) serum. (●) 60°C processing; (■) 92°C processing; (▲) 100°C processing.

heat-processed ω - and pooled (equal amounts) 60°C, 78/92°C and 100°C heat-treated γ -gliadins, purified by RP-HPLC from common wheat flour and pastas respectively.

Antisera against ω -Gliadins

Antisera raised against both heated and unheated omega gliadins in the IgG purified state were found to react very similarly with gliadin extracts from common wheat and Durum wheat pastas in the ELISA. Figure 3 shows antiserum raised against ω -gliadin purified from pasta dried at 100°C, assayed by ELISA against common (soft) wheat adulterated Durum pastas dried at 60°C, 78/92°C and 100°C. Only when the common (soft) wheat content of the pasta reached a concentration (m/m) of 60% was any significant difference in A_{450 nm} observed. All visible signs of reactivity towards either common or Durum wheat pastas were lost (data not shown) following affinity purification of each serum using total gliadin extracts from *D. flodur* when assayed against the same sample extracts as previously.

Antisera against γ -Gliadins

Antisera raised against common wheat specific γ -gliadins obtained from common wheat flour failed to differentiate between common wheat and Durum wheat pasta gliadin extracts in the IgG purified state when assayed in the ELISA (data not shown). However, upon affinity purification with total gliadins from *D. flodur*, it was found that the serum showed a greater specificity for common wheat pasta gliadin extracts compared with Durum wheat pasta gliadins, as determined in the ELISA of gliadin extracts from common wheat adulterated Durum wheat pastas dried at temperatures between 60 and 100°C (data not shown).

Similarly, antisera raised against pooled 60°C, 78/92°C and 100°C γ -gliadins purified from heat-treated pastas when assayed by ELISA in the IgG purified state against common wheat adulterated Durum pastas were also found to cross-react strongly with Durum gliadins, although the reaction of this antisera proved to be greater with common (soft) wheat gliadins (Figure 4(a)). When this serum was affinity-purified against *D. flodur* gliadins, it showed a significant improvement in specificity towards common (soft) wheat gliadin extracts when assayed against the same samples. A standard curve showing the change in A_{450} nm against soft wheat adulteration is shown in Figure 4(b).

DISCUSSION

Polyclonal antisera (IgG purified), raised to electrophoretically slow-moving ω -gliadins purified from unheated common wheat flour and from high-temperature dried pastas, showed extensive cross-reaction against gliadins extracted from Durum wheat pastas in the ELISA. The total reactivity of this serum was lost, even towards common (soft) wheat pastas, after extensive affinity purification of the serum with Durum wheat gliadins. This indicates that all the antibodies raised against the original immunogen recognized the gliadins within Durum wheat, resulting in all the antibodies being bound to the affinity column and removed from the serum. This result is not totally unexpected, due to the extensive sequence homology that is known to exist between the gliadins of both common and Durum varieties, and confirms the results of other work using monoclonal antibodies (Skerritt & Hill, 1990).

In contrast, when antibodies were raised to common wheat specific γ -gliadins from heat-treated pastas purified by RP-HPLC, affinity purification against Durum gliadins did not remove total reactivity of this serum to common wheat gliadins. This confirms the original observations using Western blotting (McCarthy *et al.*, 1991) and indicates that the serum must contain a significant proportion of antibodies specific to epitopes present only on the surface of common wheat specific γ -gliadins.

The finding that this antisera is still active against common wheat adulterated Durum pastas dried at 100°C indicates the potential of this method as a means of defining the purity of Durum wheat pastas.

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