Immunological Distinction Between x-Type and y-Type High Molecular Weight Glutenin Subunits

A. CURIONI,[1] A. DAL BELIN PERUFFO,[1] G. PRESSI,[1] and N. E. POGNA[2]

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ABSTRACT

Polyclonal antisera were raised against four high molecular weight glutenin subunits purified by sodium dodecyl sulfate electroendosmotic preparative electrophoresis from the wheat cultivar Clara 2. Immunoblotting with total protein extracts and gliadins from several wheat cultivars revealed that the antisera to x-subunits 1, 5, and 7 bound only to high molecular weight glutenin subunits, whereas the antiserum to y-subunit 12 also reacted with α-, β- and γ-gliadins. The antisera to subunits 1 and 5 reacted more strongly with x-subunits than with y-subunits, and the antiserum to subunit 12 showed more reaction with y-subunits. Comparison of amino acid sequences suggests that some antigenic determinants typical of the y-type and x-type subunits lie in the N-terminus and in the central repetitive domain of the protein, respectively.

Biochemical studies have shown that wheat gluten consists of a complex of highly aggregated proteins called prolamins. Among the prolamins, the proteins able to form intermolecular disulfide bonds are indicated as glutenins, and those that cannot, as gliadins. When fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the glutenin polymers separate into about 20 different subunits that fall into two distinct groups: high molecular weight (HMW) subunits and low molecular weight (LMW) subunits (Huebner and Wall 1976).

The biochemistry, genetics, and molecular biology of HMW subunits of glutenin have been widely described (see Payne 1987 and Shewry et al 1989 for reviews). Wheat cultivars contain between three and five HMW glutenin subunits coded by genes at three homoeologous loci; the subunits coded at each locus were subdivided into x- and y-types on the basis of their M_rs on SDS gels (Payne et al 1981). Certain of these subunits were found to be associated with good viscoelastic properties of gluten (Payne 1983).

Immunological studies proved to be a very useful tool to determine structural relationships among cereal storage proteins thought to be genetically related. However, the studies found some limitations due to the low solubility of prolamins in aqueous solvents and the difficulty of preparing pure components (particularly HMW and LMW glutenin subunits) to be used as immunogens (see Skerritt 1988 for a review). Recently, these problems have been overcome by a new preparative electrophoretic procedure that allows preparation of pure HMW glutenin subunits (Curioni et al 1989), and by the development of immunoblotting techniques that do not require antigens to be soluble in aqueous media.

In this article we report on a study of the immunological relationships among wheat storage proteins by using polyclonal antibodies raised against four HMW subunits of glutenin purified from an Italian bread wheat cultivar. The main purpose was to

[1]Dipartimento Biotecnologie Agrarie, Università di Padova, Via Gradenigo 6, 35131 Padova, Italy.
[2]Istituto Sperimentale per la Ceralicoltura, Via Molino 3, 20079 S. Angelo Lodigiano, Italy.

compare the antigenic properties of the x-type and y-type subunits of glutenin, which have recently been found to have different primary structures (Anderson and Greene 1989).

MATERIALS AND METHODS

Plant Material

Milled whole grain and flours of the bread cultivars used in the study were obtained from plants grown at the Istituto Sperimentale per la Ceralicoltura, S. Angelo Lodigiano, Italy.

Preparation of Pure HMW Glutenin Subunits

HMW glutenin subunits 1, 5, 7, and 12 (nomenclature by Payne and Lawrence 1983) were purified from the Italian bread wheat cultivar Clara 2 by SDS-electroendosmotic preparative electrophoresis (SDS-EPE) as previously described (Curioni et al 1989). Pure subunits were kept frozen in the elution buffer, in which trace amounts of SDS were present.

Preparation of Antisera

Antisera were raised in rabbits by injecting subcutaneously 0.075 mg of each purified subunit in the SDS-EPE elution buffer diluted with 1 volume of complete Freund's adjuvant. Two other injections, each 0.13 mg of protein, were administered at seven-day intervals; one week after the last injection, the rabbits were bled. Coagulated bloods were centrifuged at 3,000 $\times$ *g* for 10 min. Sera were kept frozen at −20°C in 0.1-ml aliquots.

Preparation of Total Prolamins

Total reduced protein extracts were prepared by suspending 100 mg of flour or meal in 2.5 ml of 25 m*M* Tris; 192 m*M* glycine, pH 8.3, containing 2.5% (v/v) 2-mercaptoethanol; 2.5% (w/v) SDS; and 10% (w/v) glycerol. After stirring for 16 hr at 37°C, the suspension was clarified by centrifugation, and the supernatant was used for SDS-PAGE. Gliadin extracts were prepared by suspending 100 mg of flour in 0.3 ml of 70% aqueous ethanol for 1 hr at room temperature. The extracts were then centrifuged and the supernatants diluted with 3 volumes of 60% glycerol.

Electrophoresis

SDS-PAGE was done according to Laemmli (1970) in a Mini-Protean II cell (Bio-Rad). Composition of the resolving gel was T = 10.3% and C = 3%. Twelve μl of total prolamins was fractionated at 50 mA of constant current until the tracking dye (bromophenol blue) reached the bottom of the gel.

Acidic electrophoresis (A-PAGE) of the gliadin extracts was performed at pH 3.1 in a Pharmacia GE IV apparatus as described by Maier and Wagner (1980). Gliadin extract (15 μl) was fractionated at 100 V until the tracking dye (methyl violet) ran off the gel.

Two-dimensional electrophoresis (A-PAGE $\times$ SDS-PAGE) was performed as described by Payne et al (1982), in a Bio-Protean I apparatus (Bio-Rad). After the acidic separation, gel strips were equilibrated by treatment with 0.5 *M* Tris HCl, pH 7.4, containing 4% (w/v) SDS, 20% (w/v) glycerol, and 3% (v/v) 2-mercaptoethanol for 1 hr at 37°C.

Gels were used for Western blotting or stained according to Koenig et al (1970) and destained with 7% acetic acid and 7% methanol.

Immunoblotting

Protein blotting was performed in a Bio-Rad semidry transfer cell using polyvinylidene difluoride (PVDF) sheets (Immobilon-P, Millipore Corp., Bedford, MA).

One- and two-dimensional SDS gels were transferred between two double layers of 3 MM chromatography paper (Whatman) wetted in a blotting buffer, pH 9.2, containing 48 m*M* Tris, 39 m*M* glycine, 0.04% (w/v) SDS, 10% (v/v) methanol (HPLC grade) in distilled water, and electroblotted at 15 V for 50 min. Before the transfer, gels and membranes were equilibrated in the same buffer for 20 min. Immediately after the electrophoretic run, acidic gels were blotted at 15 V for 30 min in 187 m*M* of acetic acid adjusted to pH 3.1 with solid glycine, with the buffer-equilibrated membrane placed cathodically. Proteins were recognized by staining the membranes with 0.2% (w/v) Ponceau S (Sigma) in 3% (w/v) of trichloracetic acid for 30 sec and destaining with water.

Immunological tests were performed as described by Hoyer-Hansen et al (1985), with appropriate dilutions of the antisera. Antirabbit Ig peroxidase conjugate and 3-amino-9-ethylcarbazole were used as secondary antibody and peroxidase substrate, respectively. The reacting proteins appeared as red bands on a pale yellow background.

RESULTS AND DISCUSSION

The Italian wheat cultivar Clara 2 contains HMW glutenin subunits 1, 5, 7 (x-type), and 12 (y-type). The unusual combination of subunits 5 + 12 in this cultivar presumably arose from a recombination event within the *Glu-D1* locus (Pogna et al 1989). HMW glutenin subunits from Clara 2 were purified by SDS-EPE (Fig. 1) and used as immunogens in rabbits. The total protein pattern of Clara 2 was then tested for its reaction with the four antisera. The antisera working dilutions in immunoblotting tests were 1:500 for the antisera raised against subunits 1 and 12 (anti-1 and anti-12, respectively), 1:2,000 for the antiserum to subunit 7 (anti-7), and 1:3,000 for the antiserum to subunit 5 (anti-5). Anti-1 and anti-5 (Fig. 2, lanes A and B, respectively) showed a strong reaction with x-subunits 1, 5, and 7, whereas the reaction with y-subunit 12 was comparatively weak. The reaction with subunit 1 was less strong than those with subunits 5 and 7 because it was difficult to transfer subunit 1 to PVDF sheets, as assessed by Coomassie staining of the gels after blotting. Anti-7 (Fig. 2, lane C) gave very similar reactions with all the HMW glutenin subunits of Clara 2, whereas anti-12 (Fig. 2, lane D) gave a stronger reaction with y-subunit 12 itself than with x-subunits 1, 5, and 7. Moreover, anti-12 recognized some LMW polypeptides (30,000–45,000 mol wt).

The four antisera were then tested against the polypeptide components of the SDS-PAGE patterns of five bread wheat cultivars (Irnerio, Sprint 2, Talent, Demar 4, and Ecrin), which contain the most frequent HMW glutenin subunits in the Italian germ plasm (Pogna et al 1989). The antisera to x-subunits 1 and 5 (Fig. 3) gave stronger reactions with the x-type subunits (1, 2, 2*, 4–7, and 20) than with the y-type subunits (8–10, and 12). Conversely, the antiserum to y-subunit 12 reacted strongly with the y-subunits and gave lower reactions with the x-subunits. Both types of subunits gave similar reactions with anti-7. In addition, the antiserum to subunit 12 confirmed its unique ability to react with LMW polypeptides.

Genetic studies have shown that HMW glutenin subunits are

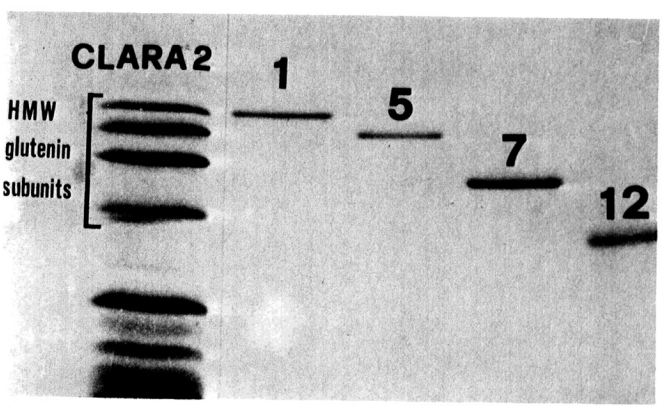

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the high molecular weight glutenin subunits purified by electroendosmotic preparative electrophoresis and used as immunogens. On the left side is the pattern of total reduced proteins from cultivar Clara 2. The subunits are numbered according to Lawrence and Payne (1983).

coded by genes that occur at three compound loci, collectively called *Glu-1,* on the long arms of the group-1 chromosomes (Payne et al 1980). Each *Glu-1* locus contains two tightly linked genes that code for x-type and y-type subunits, respectively (Payne et al 1981). The nucleotide sequences of cloned cDNAs and genes coding for HMW glutenin subunits from a few wheat cultivars reveal a domain structure that comprises nonrepetitive domains at the N- and C-termini, and a central repetitive domain (Thompson et al 1983, 1985; Anderson and Green 1989; Anderson et al 1989). The major differences in the deduced amino acid sequences are between x-type and y-type subunits rather than between homeoallelic subunits encoded by the A, B, and D genomes. In fact, comparison of the amino acid sequences of subunits 2, 2*, 5, and 7 (x-type) with those of 9, 10, and 12 (y-type) shows that the C-terminal domains (42 residues) of both types of subunits are very closely related and differ only in single amino acid substitutions. The N-terminal domains (81–104 residues), however, differ in substitutions and insertions/deletions, with all x-type subunits having an 18-residue deletion, which corresponds to residues 31–48 of the y-type subunits. Also, of the three types of repeats found in the central repetitive domain (i.e., a hexapeptide and two nonapeptides), one of the nonapeptides (consensus sequence: PGQGQQGQQ) is present only in the x-type subunits. These differences in the primary structures may be associated with the different responses observed in the immunological reactions of the two types of subunits.

Since the antisera raised against x-subunits 1 and 5 reacted more strongly with the x-subunits than with the y-subunits, this implies that the y-subunits lack some of the epitopes present in the x-subunits. The differential epitopes probably lie in the central repetitive domain of the x-subunits and may involve the nonapeptide repeat PGQGQQGQQ. On the other hand, anti-12 recognized well all the y-subunits tested and gave lesser reactions with the x-subunits, suggesting the presence of antigenic determinants typical of the y-subunits. This aspect will be considered later.

The antiserum to subunit 7 reacted similarly with both types of subunits, which was different from the reactions of other antisera. Recently, Anderson and Green (1989) showed that the central repetitive domain of subunit 7 from wheat cultivar Cheyenne is rather peculiar in having only four PGQGQQGQQ repeats, compared with 16–22 in the homeoallelic subunits. If these repeats play a key role in determining the antigenic properties of the x-type subunits, then it is likely that their frequency may affect the proportion of related antibodies in antiserum.

The anti-12 serum reacted strongly with LMW polypeptides. To determine the identity of these LMW polypeptides, prolamins from cultivar Clara 2 were extracted with 70% ethanol and fractionated by A-PAGE at pH 3.1. After acidic electroblotting to a PVDF membrane, the immunological reaction with anti-12 serum showed only a diffuse red color in the anodal (upper) region of the gel (data not shown). When proteins in the acidic gels were reduced with 3% (v/v) 2-mercaptoethanol in the blotting buffer, pH 9.2, and transferred to a PVDF membrane, the antiserum to subunit 12 bound to two groups of bands, corresponding in mobility to β- and γ-gliadins, whereas α-gliadins showed a very faint reaction (data not shown).

Two-dimensional fractionation (A-PAGE × SDS-PAGE) was then performed to assess the precise identity of the cross-reacting gliadins in cultivar Clara 2 (Fig. 4). The ω-gliadins did not show any reaction with anti-12, whereas strong reactions were observed for γ-gliadins and β-gliadins coded by 1A, 1B, 1D, and 6B chromosomes. The α- and β-gliadins coded by chromosome 6D and the α-(A)-gliadins coded by chromosome 6A gave low reactions. The peculiar antigenic behavior of anti-12 confirms that subunit 12 contains antigenic determinants that are absent in the x-subunits tested, which is consistent with stronger reactions of anti-12 with y-subunits than with x-subunits.

Gliadins reacted with anti-12 only after treatment with a reducing agent, suggesting that the region containing the epitopes shared with subunit 12 is located between two cysteine residues involved in an intrachain disulfide bond. The α/β and γ-types of gliadins show three regions of unique sequence designated A, B, and C (Kreis et al 1985), which are related to sequences present in the N-terminal domain (A and B regions) and C-terminal domain (region C) of HMW glutenin subunits. The 18-residue

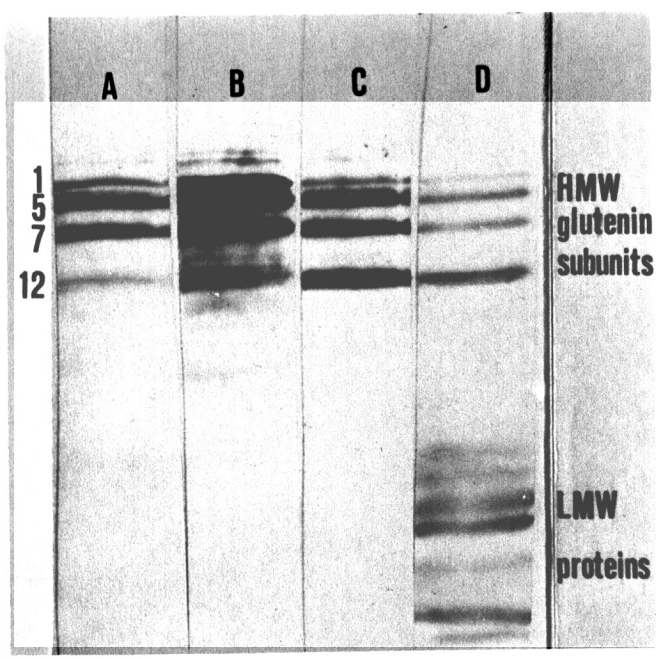

Fig. 2. Immunoblot of total reduced proteins from cultivar Clara 2 with antisera raised against four high molecular weight subunits of glutenin. **A,** antiserum to subunit 1 (anti-1); **B,** anti-5; **C,** anti-7; **D,** anti-12.

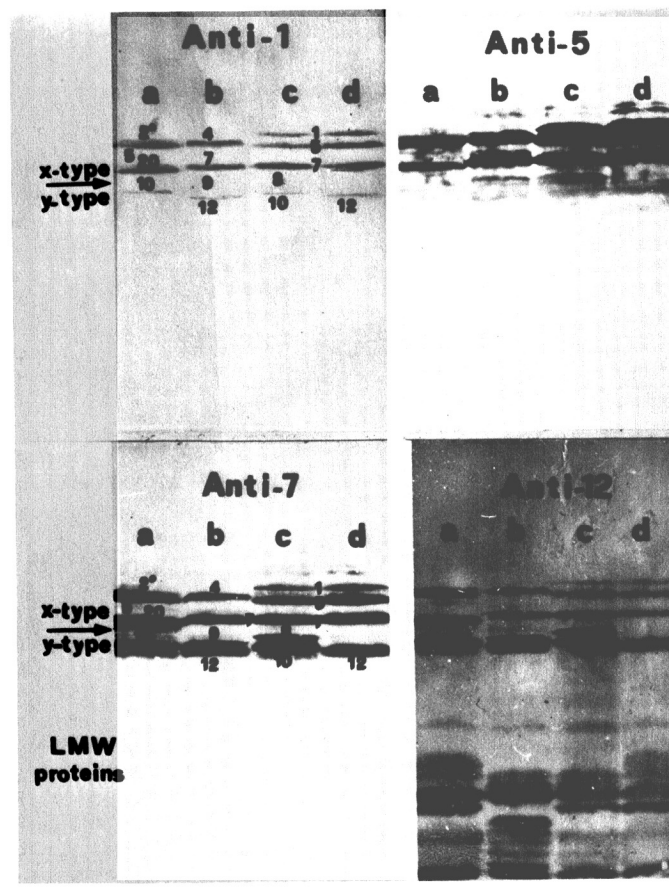

Fig. 3. Immunoblots with anti-1, anti-5, anti-7, and anti-12 of total reduced proteins from the following cultivars: **a,** Sprint 2; **b,** Talent; **c,** Irnerio, and **d,** Clara 2.

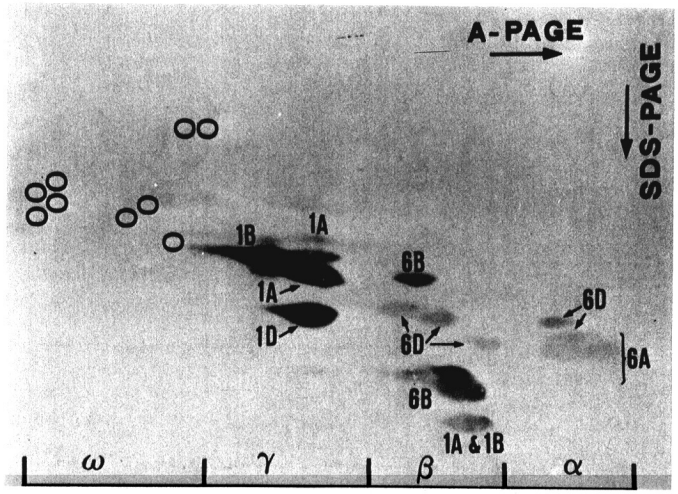

Fig. 4. Immunoblot with anti-12 of gliadins fractionated by two-dimensional electrophoresis A-polyacrylamide gel electrophoresis × sodium dodecyl sulfate-polyacrylamide gel electrophoresis from cultivar Clara 2. Circles = map positions of ω-gliadins coded by the group-1 chromosomes.

1. HMW x-subunit 5	L----------RD
	30 40 50
2. HMW y-subunit 12	LAGRLPWSTGLQMRCCQQLRD
3. LMW glutenin subunit	QMLQQSSCHVMQQQCCQQLLQ
4. α-(A) gliadin	QVLQQSTYQLLQELCCQHLWQ
5. α/ß gliadin	QVLQQSTYQPLQQLCCQQLWQ
6. γ-gliadin	MIWPQSDCQVMRQQCCQQLAQ
7. γ-secalin	KIFPQSECQVHQQQCCQQLAQ
8. B-hordein	QMLQQSSCHVLQQQCCQQLPQ
9. Maize glutelin-2	TPYCSPQCQSLRQQCCQQLRQ

Fig. 5. The amino acid sequence of the N-terminal region of high molecular weight glutenin subunit 12 (y-type), which corresponds to the 18 residue deletion of subunit 5 (x-type). Arrow = the first amino acid of the region B described by Kreis at al (1985). The amino acid sequences of homologous regions present in gliadins, γ-secalin, B-hordein, and maize glutelin-2 are listed on the right. The highly conserved pentapeptide CCQQL is underlined. (References: 1, Anderson et al 1989; 2, Thompson et al 1985; 3, Bartels and Thompson 1983; 4, Anderson et al 1984; 5, Sumner-Smith et al 1985; 6, Bartels et al 1986; 7, Kreis et al 1985; 8, Forde et al 1985; 9, Prat et al 1987.)

deletion present in the N-terminus of the x-subunits includes the first eight residues of region B. This eight-residue sequence is highly conserved and can be found in the central part of α-(A)-gliadins, α/β-gliadins, γ-gliadins, LMW glutenin subunits, B-hordein, γ-secalin, and maize glutelin-2; also, it contains the essentially unchanged pentapeptide CCQQL (Fig. 5). In the A type of α-gliadin, a substitution of histidine for glutamine occurs at position 4 of the pentapeptide (Anderson et al 1984, Kasarda et al 1984).

These findings suggest that one of the epitopes recognized by the anti-12 lies within the B region and includes the highly conserved pentapeptide. This assumption is consistent with recent studies of monoclonal antibodies that react to gliadins, which suggested the presence of antigenic sites in the Pro-poor domains of α/β-gliadins, γ-gliadins, hordein, and LMW subunits of glutenin (Freedman et al 1988). Previous studies with polyclonal antisera (Festenstein et al 1987) also showed that the A-gliadins are antigenetically distinct from other prolamins. The poor reaction given by the A-gliadins from cultivar Clara confirms this immunological distinctness.

The anti-12 gave no reaction with ω-gliadins. This is in accordance with the absence of cysteine residues and of sequences homologous to the B region in this gliadin group (Shewry et al 1986). The immunological distinctness of ω-gliadins also has been demonstrated by several authors (see Skerritt 1988 for a review and Freedman et al 1988).

Our results differ from those obtained by Festenstein et al (1985), who used an antiserum raised against the HMW x-subunit 2 of glutenin. This antiserum gave identical reactions with y-subunit 12 and x-subunit 2. A-, β,- and γ-gliadins as well as some ω-gliadins also reacted with the antiserum. Since the antigenicity depends on the three-dimensional structure of the protein, these contrasting results may be partly due to the conformational changes of the immunogens caused by the different procedures used for protein purification and preparation of antisera.

In conclusion, the results reported and discussed here indicate that the x-type and y-type of HMW glutenin subunits are related immunologically to each other but also have specific antigenic determinants. Moreover, y-subunit 12, but not the x-type subunits, has close immunochemical relationships with α-, β-, and γ-gliadins.

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