Evidence for Glycosylation of the High Molecular Weight Glutenin Subunits 2, 7, 8, and 12 from Chinese Spring and TAM 105 Wheats[1]

K. A. TILLEY,[2] G. L. LOOKHART,[3] R. C. HOSENEY,[4] and T. P. MAWHINNEY[5]

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ABSTRACT

High molecular weight glutenin subunits (HMW-GS) of wheat, obtained by a modification of the method of Burnouf and Bietz (1989), were characterized by isoelectric focusing, lectin binding, and gas chromatography-mass spectroscopy. The purification method involved a dimethyl sulfoxide extraction of flour, followed by reduction and alkylation of the proteins. The extracted subunits were separated on, and excised from, sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. These subunits, when analyzed by reversed phase high-performance liquid chromatography, eluted at approximately 45% acetonitrile, indicating that, under these conditions, they were more hydrophobic (~30%) than previously reported (Burnouf and Bietz 1989, Wieser and Belitz 1990). The purified HMW-GS were reelectrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis minigels and silver-stained. A single band for each subunit provided an indication of the purity of the subunit. Further characterization of the purified HMW-GS revealed that the proteins were glycosylated. Lectin-binding analyses showed that the terminal carbohydrate moiety of these glycoproteins was mannose. Gas chromatography-mass spectroscopy analyses confirmed the presence of mannose in the total glutenin preparations as well as in each of the individual purified HMW-GS. Gas chromatography-mass spectroscopy analyses also detected glucose and *N*-acetyl glucosamine in the individual purified HMW-GS.

A number of studies have shown that a significant relationship exists between the presence or absence of some of the high molecular weight glutenin subunits (HMW-GS) and the breadmaking potential of wheat cultivars (Branlard and Dardevet 1985, Lawrence et al 1987, Lorenz et al 1987, Payne et al 1979). Over the past several years, it has been the goal of a number of researchers to develop the best method of purifying glutenin in order to further analyze the individual components and determine their effect on the quality of the finished product (Orth and Bushuk 1973, Wasik and Bushuk 1974, Bietz and Wall 1975, Curioni et al 1989, Khan et al 1989).

For many years, the major solvent used in the preparation of wheat glutenin has been dilute acetic acid, which was originally proposed by Osborne (1907). This particular procedure has two major disadvantages. The most critical is that a large fraction of the flour protein is insoluble in acetic acid. A second disadvantage is that this method yields a glutenin fraction that appears to be highly contaminated with gliadin, albumin, and globulin proteins (Chen and Bushuk 1970). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) (Burnouf and Bietz 1989) methods have been developed to identify the individual HMW-GS.

The goals of this research were to develop methods for extracting and purifying HMW-GS and to begin characterizing them to help understand their structure-function relationships.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were reagent grade or better and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The glycan detection and differentiation kits were obtained from Boehringer Mannheim (Indianapolis, IN). Water was purified by reverse osmosis followed by activated carbon filtration and deionization.

Wheat Samples

The cultivar Chinese Spring (1983 crop at the University of California, Davis, CA) was obtained from D. Kasarda, USDA-ARS, Western Regional Research Center, Albany, CA. The cultivar TAM 105 was obtained from the National Small Grains Collection, USDA-ARS, Small Grains Germplasm Research Facility (Aberdeen, ID).

Extraction of HMW-GS

A modification of the dimethyl sulfoxide method of Burnouf and Bietz (1989) was used. Fifty milligrams of flour were extracted twice with 6 ml of 100% dimethyl sulfoxide for 30 min each at room temperature (25°C). The extracts were centrifuged at 5,000 × *g*. The supernatant was discarded after each extraction. The remaining pellets were washed once with 6 ml of 70% ethanol. The proteins were reduced by resuspension in 1 ml of 0.05*M* Tris-HCL-8*M* urea + 5% β-mercaptoethanol, pH 7.5, for 2 hr at room temperature. The reduced proteins were alkylated by reacting for 2 hrs at room temperature with 60 µl of 4-vinylpyridine (Sigma Chemical Co., St. Louis, MO), which was used directly when obtained as a colorless liquid or redistilled before use if not.

SDS-PAGE of Extracted Material

A sample buffer solution containing 2% SDS, 10% β-mercaptoethanol, and 20% of a solution of bromophenol blue (0.2%) in glycerol was added to the protein preparation (1:1) immediately after extraction, reduction, and alkylation. The resulting solution was loaded onto a 12% total acrylamide SDS-PAGE gel with 1.35% bis-acrylamide cross-linking (stacking gel 3.83% total acrylamide with 1.35% cross-linking). Electrophoresis was performed at 100 V of constant voltage for 16 hr using the discontinuous method of Laemmli (1970) and Lookhart et al (1993) on a Hoefer SE600 system (16-cm × 18-cm × 1.5-mm gel).

Visualization of Protein Bands

After electrophoresis, the gels were immediately placed in 0.3*M* copper chloride ($CuCl_2$) for 5 min until the bands were readily visible (Lee et al 1987). The background was stained a blue-green color, and the proteins appeared as clear bands. The sensitivity of this staining technique was reported by Lee et al (1987) as being greater than the sensitivity of Coomassie Blue staining. The proteins were reversibly fixed within the gel so that quan-

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[2]Graduate student and research assistant, Kansas State University, Department of Grain Science and Industry, Manhattan.

[3]Research chemist, USDA-ARS, U.S. Grain Marketing Research Laboratory, Manhattan, KS, and adjunct professor, Kansas State University, Department of Grain Science and Industry, Manhattan.

[4]Professor, Kansas State University, Department of Grain Science and Industry, Manhattan, KS.

[5]Director, Experiment Station Chemical Laboratories, University of Missouri-Columbia.

titative recovery was possible. The proteins themselves were not altered as a result of the staining procedure (Lee et al 1987). The gel portion containing each band was excised with a razor blade and destained at 60°C with a 0.25*M* Tris (hydroxymethyl) amino-methane (Tris) solution, pH 9, containing 0.25*M* ethylenediaminetetraacetic acid until completely clear.

Electroelution of Proteins

Proteins were eluted from the excised, destained gel pieces for 5 hr with a model 422 Electro-Eluter (Bio-Rad Laboratories, Richmond, CA) at 10 mV per tube with the protein elution buffer (25 m*M* Tris base, 192 m*M* glycine, and 0.1% SDS). (At this time, restaining of the eluted gel pieces with Coomassie Blue indicated that no protein was left in the gel pieces.)

HPLC of Purified Subunits

HPLC was performed on resolubilized, lyophilized material from the eluter with a Hewlett Packard model 1090 chromatograph using a Vydac (The Separations Group, Hesperia, CA) C18 column (catalog number 218TP52) at 45°C and a flow rate of 0.2 ml/min. The binary solvent system used water and acetonitrile (each with 0.0667%, v/v, trifluoroacetic acid). The multistep linear gradient was continuous from 34% acetonitrile at time 0 to 60% acetonitrile at 40 min to 85% acetonitrile at 43 min. It was held at 85% acetonitrile for 2 min, then returned to initial conditions of 34% acetonitrile. For verification of components, only the center third of each peak was collected to reduce overlap and impurities.

Lyophilization

The protein solutions from either the electroeluted gel pieces or the collected HPLC fractions were frozen and lyophilized to dryness.

SDS-PAGE Minigels

The lyophilized (purified) HMW-GS were checked for purity using the Mini-Protean II Slab Cell (Bio-Rad). The same gel solutions used for the preparatory gel were used for the minigels. The time for resolving the bands was 45 min, at which time the dye front reached the gel anode. The technique of Blum et al (1987) was used to silver-stain the minigels.

Isoelectric Focusing

Isoelectric focusing was performed at 5°C and 1,400 V for 3 hr using the Pharmacia Multiphor II system and Ampholine (1 mm) PAGplates, both narrow (pH 4.0–5.0) and wide range (pH 3.5–9.5) (Pharmacia LKB Biotechnology, S-751 82, Uppsala, Sweden). Standard pI markers (isoelectric focusing standards from Bio-Rad Laboratories) were used.

Western Blots

Proteins were transferred to nitrocellulose using the Bio-Rad trans-blot electrophoretic transfer cell. The transfer buffer formulation was: 25 m*M* Tris, 192 m*M* glycine, 20% methanol, and 1% SDS (Towbin et al 1979). The blotting was performed at 400 mA for 1 hr.

Detection of Glycosylation

Glycosylation of proteins was detected by using the procedure of Haselbeck and Hosel (1991) that was issued with the glycan detection kit (Boehringer Mannheim). The kit used periodate oxidation and hydrazide coupling to incorporate the steroid hapten digoxigenin into the carbohydrate portion of a glycoprotein. The labeled glycoproteins were separated on an SDS-PAGE gel and blotted onto a nitrocellulose membrane (western blot). Labeled glycoproteins were detected directly on the blot membrane with an antidigoxigenin antibody conjugated to alkaline phosphatase. Antibody-glycoprotein complexes were visualized colorimetrically.

Determination of Glycosylation Type

The type of glycosylation was determined by using the procedure of Haselbeck and Hosel (1991) that was issued with the glycan differentiation kit (Boehringer Mannheim). The lectins *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinin, *Galanthus nivalis* agglutinin, peanut agglutinin, and *Datura stramonium* agglutinin (Table I) were provided in the kit. The type of glycosylation was determined by the specificities of the lectins as described in Table I.

Glycosyl Composition Analysis

Glycosyl composition analysis of the total glutenin extract was performed at the Complex Carbohydrate Research Center (University of Georgia, Athens, GA) by preparing trimethylsilyl methylglycosides according to the procedure of York et al (1985). The preparation of the trimethylsilyl methylglycosides was also done with *N*-acetylation so that amino sugars would be detected. The samples were methylated in methanolic 1*M* HCl at 80°C for ~18 hr. The solvents were evaporated and *N*-acetylated using acetic anhydride-pyridine-methanol at room temperature for 6 hr. The solvents were again evaporated and trimethylsilyl derivatives were prepared using Tri-Sil reagent (Pierce Chemical Co., Rockford, IL) at 80°C for 30 min. The solvents were again evaporated. The resulting trimethylsilyl methylglycosides were dissolved in hexane and analyzed by gas chromatography-mass spectroscopy (GC-MS) using a Hewlett-Packard system with an SP2330 capillary column (J & W Scientific, Folsom, CA). In addition to the mass spectra, individual glycosyl residues were identified and quantified by comparison to authentic standards. Inositol was added as an internal standard.

Glycosyl composition of each purified HMW-GS was analyzed at the Experimental Station Chemical Laboratories at the University of Missouri-Columbia, Columbia, MO. All solvents for these analyses were purchased from Aldrich Chemical Co. (Milwaukee, WI) and distilled before use. All carbohydrates employed as standards were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL).

Samples were placed into furnaced 13- × 100-mm glass tubes equipped with polytetrafluoroethylene-faced rubber-lined screw caps and hydrolyzed in 1.5 ml of 4.0*N* HCL under a nitrogen atmosphere for 4 hr at 100°C. Samples were dried *in vacuo* in a Savant concentrator and reduced with $NaBH_4$ at 4°C for 5 hr. After acidification of the reactions with acetic acid, the samples were again reduced in volume *in vacuo* in a Savant concentrator. Addition of 4.0 ml of methanol and evaporation under a stream of nitrogen removed borate. Samples were acetylated with 2.5 ml of pyridine-acetic anhydride (1:2, v/v) by constant stirring with a polytetrafluoroethylene stirbar at 70°C for 1 hr. After evaporation under a stream of nitrogen, samples were taken up in 1.5 ml of $CHCl_3$ and sequentially washed against dilute HCl

TABLE I
Description of Lectin Type and Specificity

Type	Specificity	Reaction with HMW-GS[a]
Sambucus nigra agglutinin	Sialic acid linked alpha (2-6) to galactose or galactosamine	Negative
Maackia amurensis agglutinin	Sialic acid terminally linked alpha (2-3) to galactose	Negative
Galanthus nivalis agglutinin	Mannose terminally linked	Positive
Peanut agglutinin	Galactose beta (1-3) linked to *N*-acetelygalactosamine	Negative
Datura stramonium agglutinin	Galactose beta (1-4) linked to *N*-acetylgalactosamine	Negative

[a] High molecular weight glutenin subunits.

and $NaHCO_3$ and dried as previously described (Mawhinney et al 1980, Mawhinney 1986). For these studies, gas-liquid chromatographic separation of the alditol and hexaminitol acetates were performed on a gas-liquid chromatograph (model 3700 Varian, Sugarland, TX) equipped with a Quadrex (New Haven, CT) 25-m × 0.25-mm i.d. fused silica capillary column with a bonded 0.25 μm OV-17 stationary phase, a split-splitless injector, and a dual-flame ionization detector. With a split ratio of 1:30, a 1 μl aliquot of alditol acetate sample in $CHCl_3$ was injected at 170°C for an initial hold of 4 min and then programmed at 6°C/min to 250°C with a He flow rate of 1.5 cm^3/min.

Mass spectra for all carbohydrate derivatives were obtained and verified against standards on a Kratos MS50S mass spectrometer interfaced with a Carlo Erba model 4160 gas chromatograph. Mass spectra were recorded at 70 eV with an ionization current of 50 μA, a source temperature of 250°C, and a transfer temperature 218°C.

RESULTS

Glutenin proteins from the cultivars Chinese Spring and TAM 105 were extracted, reduced, alkylated, and purified by the modified method of Burnouf and Bietz (1989). The HPLC profiles of the purified HMW-GS of Chinese Spring and TAM 105 after electrophoretic purification are shown in Figure 1. Both cultivars possessed subunits 2, 7, 8, and 12 according to the nomenclature of Payne et al (1981). The HMW-GS for both cultivars eluted at about 45% acetonitrile, which indicates that under those conditions they were more hydrophobic than previously reported (Burnouf and Bietz 1989, Wieser and Belitz 1990). SDS was present in the electrophoresis of these proteins and is difficult to remove from proteins; therefore, its presence would increase the overall hydrophobicity and decrease the differences in hydrophobicities among the subunits.

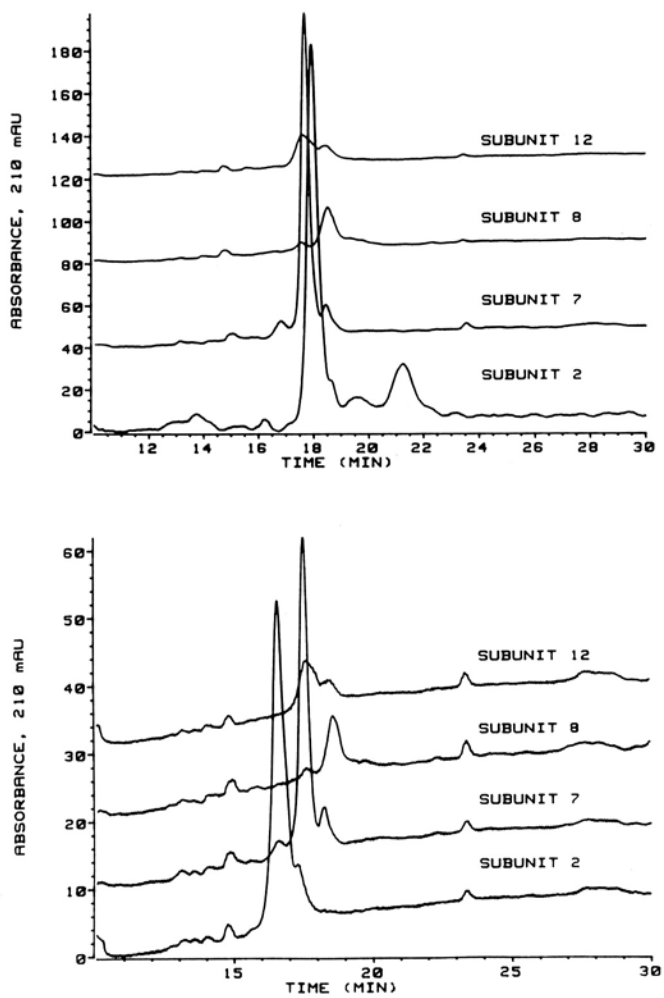

Fig. 1. High-performance liquid chromatography profiles of Chinese Spring (top) and TAM 105 (bottom). High molecular weight glutenin subunits 2, 7, 8, and 12 separated on, and excised from, sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.

The HPLC peaks for the subunits of Chinese Spring and TAM 105 (Fig. 1) eluted within the same 3-min range (16–19 min). Each pair of identically numbered subunits (e.g., 2 for Chinese Spring and 2 for TAM 105) exhibited similar, but not identical, elution times (hydrophobicities). The HPLC peak for a purified protein consistently eluted within a 0.05-min range for a series of injections over a period of one month or more.

Each HPLC peak was collected individually, separated on an SDS-PAGE minigel, and silver-stained. The silver-stained minigels of the purified subunits of Chinese Spring and TAM 105 are shown in Figure 2. Lanes A and F contained the total protein extracts of Chinese Spring and TAM 105, respectively. They were reduced and alkylated according to the method of Burnouf and Bietz (1989). Silver-staining caused some proteins to appear bright yellow, depending on the nature of the proteins (Dunbar 1987). The HMW-GS and some other proteins shown in Figure 2 were bright yellow (negatively stained). Lanes B–E and G–J contain subunits 2, 7, 8, and 12 for Chinese Spring and TAM 105, respectively. The single HMW-GS bands observed on the overloaded silver-stained SDS-PAGE minigels (Fig. 2) were evidence of individual subunits.

The purified individual HMW-GS were further characterized by isoelectric focusing. Preliminary screening used a broad-range PAGplate, pH 3.5–9.5. Multiple bands were seen only in the pH 4–6 range (data not shown). Narrow-range PAGplates (pH 4.0–5.0) were used to further characterize the components. Identical sets of multiple bands were detected within the pI 4.15–5.85 range (Fig. 3) for each of the purified HMW-GS preparations. Standard pI markers were used to define the pH range. The dark, stained area at the bottom of the gel (Fig. 3) indicated the edge of the cathode strip. This data is in agreement with the results of Brown and Flavell (1981), Holt et al (1981), and Curioni et al (1990), which showed each HMW-GS that appeared as a single polypeptide in one-dimensional SDS-PAGE separated into two or more components during isoelectric focusing. Brown and Flavell (1981) reported pIs between 4 and 7 for total glutenin extracts, which is agreement with our data on purified glutenin subunits.

Posttranslational modification of proteins is a common explanation for the multiple bands (Fig. 3) showing slight differences in pI caused by minor charge differences of modifying groups (Dunbar 1987). Glycosylation is one type of posttranslational protein modification. Donovan and Baldo (1987) and Chen et al

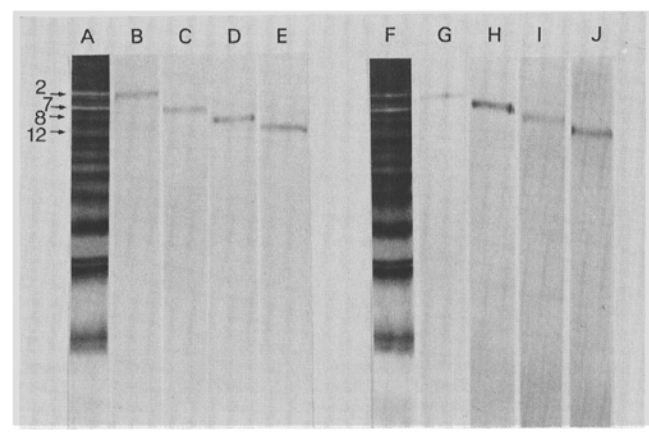

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% total acrylamide, 1.35% cross-linking) minigel of purified subunits of Chinese Spring (Lanes **B–E**) and TAM 105 (Lanes **G–J**) as detected by silver-staining. Total glutenin extracted from Chinese Spring and TAM 105 (Lanes **A** and **F**, respectively).

(1992) showed that some of the wheat storage proteins may be glycosylated. The HMW-GS, as well as some lower molecular weight proteins from the cultivars Chinese Spring and TAM 105, were determined to be glycosylated by using staining gels according to the periodic acid Schiff method (data not shown) and by the glycan detection method (Haselbeck and Hosel 1991). The glycosylated proteins in the total glutenin extracts of Chinese Spring and TAM 105, are shown in Figure 4, lanes A and B, respectively. Differences were apparent in the staining intensities of the HMW-GS in lanes A and B (Fig. 4). The dark staining of protein bands in lanes C–F (Fig. 4) indicates glycosylation of the individual purified HMW-GS of Chinese Spring. Results from the purified subunits of TAM 105 showed similar reactivity (data not shown). The glycan detection kit contained transferrin (1 mg/ml) as a positive glycoprotein control and creatinase (1 mg/ml) as a negative control (nonglycosylated protein). Both controls performed accurately in our laboratory, indicating that the glycan detection kit was an efficient method for glycoprotein detection.

The terminal sugar was determined by using various lectins (Table I) and also was detected visually on western blots of the purified proteins with a positive reaction by alkaline phosphatase. Only *Galanthus nivalis* agglutinin lectin gave a positive response (Table I), which strongly suggests that the terminal sugar on these glycoproteins is mannose. When mannose (100 m*M*) was added along with the *Galanthus nivalis* agglutinin lectin in the incubation solution, it was able to compete out binding of the lectin. Visual examination revealed that the blot, which had been incubated with free mannose in the solution, had essentially no reaction while a similar blot, incubated without free mannose in the solution, developed normally. This indicated that *Galanthus nivalis* agglutinin was binding specifically to mannose on the glycoproteins and was not binding nonspecifically to other sugar moieties.

The extracts of HMW-GS prepared according to the procedures of Burnouf and Bietz (1989) were dialyzed against deionized water and freeze-dried. A portion (250 mg each) of total glutenin material from Chinese Spring and TAM 105 was analyzed at the Complex Carbohydrate Research Center, University of Georgia, Athens, GA, for carbohydrate composition. The sugars found in the total glutenin extracts were glucose, xylose, and mannose (Table II). These data are consistent with the recent report of Chen et al (1992), who found mannose in SDS-insoluble and SDS-soluble gluten fractions. Chen et al (1992) concluded that the nonstarchy carbohydrates may be involved in the glycoprotein structure of gluten.

Carbohydrate analyses of the individual HMW-GS are shown in Table III. Mannose and glucose were found in each of the individual subunits. The amount of mannose in the total glutenin extract was smaller (Table II) than that in the individual HMW-GS (Table III) because the total glutenin extract contained many proteins, not all of which were glycosylated. The high glucose levels were most likely caused by external sources. Glucose is a ubiquitous contaminant and can be introduced from sources such as water, hydrolysis vials, and cornstarch powder from gloves (Weitzhandler et al 1993). Although starch has been found in most gluten preparations (Bushuk et al 1984), it is unlikely that the source of the glucose in these samples was starch. The purification process was designed to reduce interactions of proteins to other components. Starch levels can be minimized through the use of: 1) dimethyl sulfoxide to remove the starch from the protein fraction; 2) 8*M* urea to eliminate H-bonding between any residual starch moieties (amylose-amylopectin) and the protein fraction; and 3) SDS to reduce hydrophobic interactions between components.

In addition, *N*-acetyl glucosamine was found in each of the purified individual glutenin subunits. This sugar is essential for *N*-linked glycosylation. The significance of the variations in levels of carbohydrates in the HMW-GS to breadmaking is unknown. The data presented here make it clear that these proteins are glycosylated with mannose as the terminal sugar.

CONCLUSIONS

The HMW-GS of Chinese Spring and TAM 105 were glycosylated. The terminal sugar moiety was mannose. Further analyses of these glycoproteins are continuing in our laboratory.

TABLE II
Carbohydrates in Total Glutenin[a]

	Cultivar	
Glycosyl Residue	**Chinese Spring**	**TAM 105**
Xylose	0.42	0.45
Mannose	0.29	0.13
Glucose	28.4	27.8

[a] Values represented in percentage of total sample.

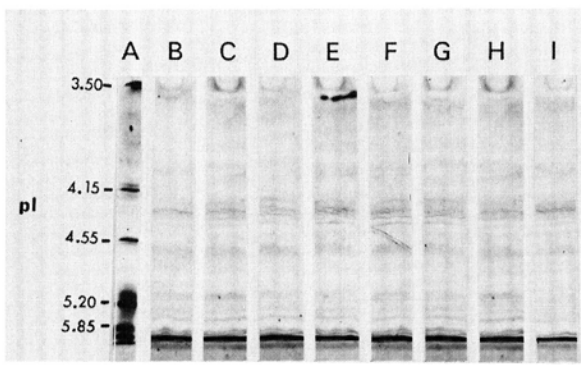

Fig. 3. Protein profiles after isoelectric focusing. Standards (Lane **A**). High molecular weight glutenin subunits 2, 7, 8, and 12 for Chinese Spring (Lanes **B**–**E**) and TAM 105 (Lanes **F**–**I**).

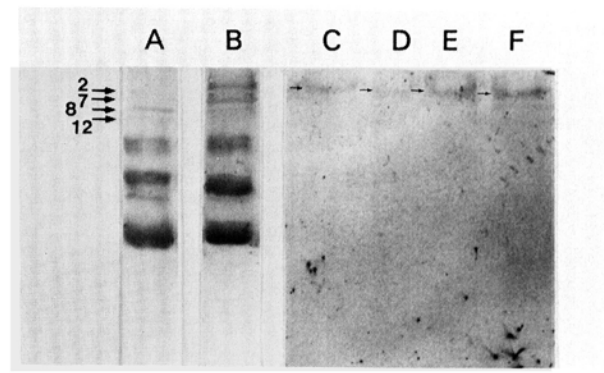

Fig. 4. Western blots of extracted proteins. Total glutenin extracted from Chinese Spring (Lane **A**) and TAM 105 (Lane **B**). Chinese Spring purified glycosylated subunits 2, 7, 8, and 12, (Lanes **C**–**F**, respectively).

TABLE III
Glycosyl Composition of Individual Subunits[a,b]

	C2	T2	C7	T7	C8	T8	C12	T12
Mannose	3.4	3.0	2.2	2.0	1.7	3.7	2.7	3.8
Glucose	24	18	23	15	18	28	13	25
N-acetyl glucosamine	2.4	1.4	1.9	2.9	2.5	3.8	2.5	4.9

[a] Values represented in percentage of total sample.
[b] C = Chinese Spring, T = TAM 105.

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