

Characterization of the Carbohydrates of Nonreduced Glutenin Fractionated by Multistacking SDS-PAGE from Two Hard Red Spring Wheat Flours¹

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ABSTRACT

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Native (nonreduced) glutenin aggregates of two hard red spring wheat flours (Len of good quality and line 205 of poor quality) containing the same high molecular weight glutenin subunits (2*, 7+9, and 5+10) were investigated for the possibility of glycosylation. Glutenins isolated by pH-precipitation were separated and purified under nonreducing conditions into five different molecular weight species by multistacking SDS-PAGE and by transfer to polyvinylidene difluoride (PVDF) membranes by electroblotting. Carbohydrate compositions of the total glutenin fraction and of the different molecular weight glutenin species separated on the stacking gels (4–12% acrylamide) were determined. More total carbohydrates were found in the total glutenin of the line 205 flour (2.33%) than in that of the Len flour (1.87%), with glucose and xylose contributing to the greater total amount in line 205. However, the total glutenin of Len had approximately twice as much of the total of arabinose, mannose, and galactose as line 205 had. After purification by electrophoresis, smaller

amounts of monosaccharides (glucose, xylose, arabinose, galactose, and mannose) in the different molecular species were detected. After electroblotting to PVDF membrane to increase purification, no arabinoxylans were found. Following extraction of glutenin from membranes, the β -elimination procedure in mild base under reducing conditions was used. In that procedure, the mono- or oligosaccharide side chains are released from the protein core, and the sugars originally involved in the protein-sugar linkage are reduced to the sugar alcohol. After derivatization (with trimethylsilyl), samples were analyzed by gas chromatography and mass spectrometry. Glucose, galactose, and a small amount of mannitol were found in most of the different glutenin aggregates, except that mannitol was not found in the 8% stacking gel glutenin fraction. The content of mannitol was greater in the higher molecular weight glutenin species at the 4 and 6% origins. These results support the hypothesis that the carbohydrates and protein in the glutenin macropolymer may be covalently linked.

Glutenin has been identified by many researchers as the component most responsible for differences in breadmaking quality among wheat cultivars. Especially during the last decade, following the work of Payne and coworkers (1979, 1981) and Payne (1987), who related the presence or absence of certain high molecular weight (HMW) glutenin subunits to breadmaking quality differences of British wheats, many other researchers focused their research on this subject (Ng and Bushuk 1988, Khan et al 1989, Lukow et al 1989, Wrigley et al 1992). Their results indicated that HMW glutenin subunit combinations could not always explain many differences in baking quality and other factors must be responsible. Studies showed that one of these factors could be the aggregative tendency of glutenin with nonprotein constituents such as carbohydrate and lipids (Bekes et al 1983, McMaster and Bushuk 1983, Zawistowska et al 1985). It is generally accepted that nonprotein components play an important role in the functionality of wheat proteins during the dough-making process. However, the relationship between structure and function of the glutenin complex in breadmaking is still not fully understood. Little research has been done on native glutenin and its interactive properties because of its large size and difficult solubility in solvents. Native glutenin actually exists as a series of large polymers. These are, in fact, cross-linked via intermolecular disulfide bonds to form multipolymers with molecular weights ranging from 100,000 to well into the millions. Recently, Khan and Huckle (1992) devised a multistacking SDS-PAGE procedure to fractionate native glutenin into a number of differently sized aggregates. Huang and Khan (1997) further characterized the HMW glutenin subunit of these aggregates. To investigate these native glutenin fractions and their possible interactions with carbohydrates or other constituents seems to be very important in determining how the interactions (if any) contribute to breadmaking performance.

Early studies of glycoproteins were limited mostly to animal sources, with which they had considerable success (Lindberg 1972, Honda and Suzuki 1984, Takemoto et al 1985), leaving the plant glycoproteins unexplored for many years. Recently, interest has increased in the structure, function, and biosynthesis of plant glycoproteins, especially the nature of covalent linkage between protein and carbohydrates moieties (Gleesen 1988, Tilley et al 1993, Hounsell 1994, Tilley and Schofield 1994). Carbohydrate in glycoproteins varies from <1% to >85% of the dry weight of these molecules (Kornfeld and Kornfeld 1976).

There is a long-standing controversy concerning glycosylation of glutenin proteins. Some researchers (Donovan and Baldo 1987, Chen et al 1992, Tilley et al 1993, Tilley and Schofield 1994) suggested that the glutenin proteins may be glycosylated. However in her last report, Tilley (1997) provided some evidence for *O*-linked mannose associated with the highly purified HMW glutenin subunit 1Dx2 of Chinese Spring. In contrast, Roels and Delcour (1996a,b) have made statements about the nonglycoprotein nature of HMW glutenin subunits of wheat, although their work does not exclude the possibility of the presence of GlcNAc residue, which could be only one possible evidence for *O*-linked glycoprotein. Also, Bollecker and Schofield (1996) reported conflicting results for glycosylation of HMW glutenin subunits depending on the method of detection used. Therefore, more research is still needed to establish whether a covalent linkage exists between carbohydrate and HMW glutenin subunits. It is still hypothesized that the carbohydrate moieties attached to the glutenin proteins may play a major role in influencing differences in breadmaking quality among wheat cultivars.

The purpose of this research was to obtain information on the nature of the carbohydrates associated with the HMW native glutenin macropolymer of two hard red spring (HRS) wheat genotypes fractionated into five different molecular weight species by multistacking SDS-PAGE: Two HRS flours containing the same high molecular weight subunit composition but of different breadmaking quality were used for comparison.

MATERIALS AND METHODS

Two genotypes of HRS wheat were used for this study: Len, which has a good breadmaking quality, and line 205, which has a weak dough mixing quality. These two genotypes had the same HMW glutenin subunits composition (2*, 7+9, and 5+10) based on the nomenclature of Payne (1987).

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Protein Extraction and Fractionation of Glutenin by Multistacking SDS-Gel Electrophoresis

The AUC solvent (0.1M acetic acid, 3M urea, and 0.01M cetyltrimethyl ammonium bromide) of Meredith and Wren (1966) was used for extraction of protein from flour. Before extraction, a gluten ball was formed and washed thoroughly under a stream of distilled water. The formation of a gluten ball provided a relatively easy method to remove starchy and nonstarchy polysaccharides as well as water-soluble proteins. The glutenin fraction was obtained by pH (6.4) precipitation according to the procedure of Orth and Bushuk (1973). To increase the purity of glutenin (making it free of gliadin and other low molecular weight impurities), the freeze-dried samples were solubilized by stirring overnight in a Tris-HCL sample buffer (pH 6.8) containing 2% SDS, loaded (on an equal protein basis) onto multistacking gels (3-mm thickness; 4, 6, 8, 10, and 12% stacking and 14% resolving gels) as described by Khan and Huckle (1992), and electrophoresed overnight at 40 mA (for two gels). Nonreduced glutenins or glutenin polymers were separated, based on their size and migrations, along the stacking gels of different concentrations of acrylamide. The glutenin species at the origins of the multistacking gels were used for carbohydrate analyses.

Extraction of Proteins from the Stacking Gels

The gel origins (4, 6, 8, 10, and 12%) were cut, and the glutenin protein from each origin was eluted with an equal volume of sample buffer without reducing agent (62.5 mM Tris/HCL, pH 6.8, 2% SDS) by stirring for 2 hr at 60°C and stirring overnight at room temperature. Samples were dialyzed against distilled water for three days with frequent changes of water, freeze-dried, and analyzed for sugar content.

Estimation of Neutral Sugars as Their Alditol Acetates

Estimating neutral sugars as their alditol acetates gives a quantitative analysis of the neutral sugar, but not of amino sugars or sialic acid of glycoproteins (Beeley, 1985). In general, the glycoprotein is hydrolyzed; the monosaccharides are reduced to alditols; and the alditol acetates (one peak for each sugar in the glycoprotein) are quantitated. Protein samples (1–1.5 mg) were hydrolyzed in 2M trifluoroacetic acid for 90 min at 121°C (Merkle and Poppe 1994). The alditol acetate derivatives were prepared as described by Blakeney et al (1983). After hydrolysis, samples were dried under a stream of nitrogen, and sugar residues were dissolved in 100 μ L of 1M NH_4OH . After adding 0.5 mL of dimethyl sulfoxide containing 10 mg of NaBH_4 (sodium borohydride), the mixture was incubated at 40°C for 90 min. The excess borohydride was destroyed by adding 100 μ L of concentrated glacial acetic acid, and 100 μ L of anhydrous 1-methylimidazole was added, followed by 0.5 mL of acetic anhydride. Acetylation was complete in 10 min at room temperature. Then 4 mL of water was added to destroy unreacted acetic anhydride, followed by addition of 1 mL of dichloromethane. The permethylated alditol acetates were partitioned into the organic phase by gentle shaking for a few minutes. The dichloromethane phase was pipetted into a new vial, and the aqueous phase reextracted with another 1 mL of dichloromethane. The combined phases were evaporated in a stream of nitrogen, and derivatives were dissolved in acetone. The alditol acetate derivatives were analyzed by gas chromatography and mass spectrometry (GS-MS), using a Hewlett-Packard system with a Supelco SP2380 column. Separation conditions were: temperature increased from 100 to 230°C at a rate of 30°C/0.5 min and from 230 to 250°C at 4°C/min; finally held at 250°C for 8 min. Sugars were identified and quantified by comparison to standards and their mass spectra. *Myo*-inositol was used as an internal standard.

Electroblotting of Native Glutenin to PVDF Membrane

Electroblotting of protein from gels to a membrane sheet is a simple, generic approach for the purification and removal of con-

taminants from protein (Weizhandler et al 1993, Rosenberg 1996). The polyvinylidene difluoride (PVDF) (Bio-Rad) membrane was chosen because of its high protein binding capacity and inertness to most solvents (Matsudaira 1989).

After electrophoresis, the gels were washed in distilled water and equilibrated for 30 min in the Towbin transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3). During this time, a PVDF membrane was rinsed with 100% methanol and soaked in transfer buffer. The stacking gels were cut horizontally and covered by PDVF membrane. The native proteins were transferred onto the membrane using a constant current (400 mA for 3 hr) in a transfer unit (trans-blot electrophoretic transfer cell with plate electrodes, Bio-Rad) which was cooled to 4°C by a super cooling coil connected to a refrigerated recirculating bath. After electroblotting, the membrane was washed in deionized water. After drying, the membrane was cut and put in a test tube, and proteins were extracted by stirring with 40% acetonitrile in a water bath at 37°C for 3 hr. Then the supernatant was removed by Pasteur pipet to a new tube, and samples were stirred at 50°C for 30 min using 40% acetonitrile with 0.05% trifluoroacetic acid, according to Matsudaira (1989).

The supernatants were combined, and the solution was evaporated to dryness on a rotary evaporator. Deionized water was added and the solution once more evaporated to dryness. Residue representing HMW native (nonreduced) glutenin aggregates was checked for transfer recovery of blotted protein after elution from the membrane as follows: samples were dissolved in SDS-PAGE sample buffer containing a reducing agent (1% dithiothreitol) and loaded onto a Mini Protean II electrophoresis unit (Bio-Rad) with 14% acrylamide concentration in the separation gel. The electrophoretic separation was complete in 50 min. After staining with Coomassie Brilliant Blue G-250, typical subunit patterns for reduced glutenins were observed, indicating successful transfer by electroblotting. Then residues were subjected to a β -elimination procedure.

β -Elimination Procedure

The β -elimination procedure of Finne et al (1979) was used under mild alkaline conditions in the presence of sodium borohydride as modified by Tilley (1997).

Estimation of Methyl Glycosides

The most widely adopted method for analysis of neutral and amino sugars is gas-liquid chromatography of derivatives of the sugars released by methanolysis. Methanolysis liberates the methyl glycosides of monosaccharides in high yield and with little destruction (Beeley, 1985). Volatile derivatives of the glycosides are obtained by formation of trimethylsilyl derivatives. All of the neutral sugars, amino sugars, and sialic acids can be obtained in a single analysis. One disadvantage is that some monosaccharides give rise to two or more peaks. After β -elimination, dried samples were treated with 0.5 mL of 1.0M anhydrous methanolic HCL for 18 hr at 80°C in the heating block. The methanolic HCL was evaporated by drying at 40°C under a stream of nitrogen. In the end, methanol was added twice (0.2 mL) and evaporated to dryness. Samples were *N*-acetylated (in consideration of amino sugar detection) by adding 200 μ L of methanol, 40 μ L of pyridine, and 40 μ L of acetic anhydride and incubated at room temperature for 6 hr. The solvent and excess acetylating reagents were evaporated under nitrogen at 40°C. Derivatization by trimethylsilylation was done according to Merkle and Poppe (1994). To silylate, 0.2 mL of the Tri-Sil reagent (Pierce, Rockford, IL) was added and incubated at 80°C for 40 min. After cooling to room temperature, the reagents were evaporated under a gentle stream of nitrogen. The residues were dissolved in hexane and analyzed by GS-MS using a Hewlett Packard system with an HP-1 capillary column (12 m \times 0.2 mm \times 0.33 μ m film thickness) in the splitless mode. The separation program was as follows: temperature was increased from 140 to 180°C at a rate of 2°C/min and finally held at 180°C for 6 min. Standards for

mannitol, galactitol, *N*-acetylglucosaminitol, and *N*-acetylglactosaminitol were prepared according to Tilley (1997). In addition to the retention time, the identities of peaks were confirmed by comparison of their mass spectra with those obtained for standards. *Myo*-inositol was used as an internal standard.

Statistical Analysis

The statistical analysis system of the SAS Institute (Cary, NC) and Duncan's tests were used to analyze the data of this study.

RESULTS AND DISCUSSION

Carbohydrate Compositions in Glutenin Fractions of Two HRS Flours

Gluten proteins from the two flours (Len and line 205) were extracted with AUC solvent, and the glutenin fraction was obtained by pH precipitation. The sugar composition of each glutenin fraction was determined as its alditol acetate derivative by GS-MS. The carbohydrate contents found in glutenin fractions of Len and experimental line 205 flours are given in Table I. The two cultivars contained five sugar components: arabinose, xylose, mannose, galactose, and glucose. More total carbohydrate was found in the total glutenin fractions of the line 205 flour (2.33%) than in those of the Len flour (1.87%), with glucose and xylose especially contributing to the greater total amount in line 205. However, Len had approximately twice the amount of arabinose, mannose, and galactose as that of line 205 in the total glutenin fraction. After purification by electrophoresis, smaller amounts of glucose and xylose were detected in the glutenin at the five stacking gels. Chen et al (1992) found a lower total sugar content (1.05%) in Len for SDS soluble-70% insoluble gluten fractions after defatting and α -amylase digestion. McMaster and Bushuk (1983) reported a much higher total sugar content in the alcohol-insoluble fraction of Canadian spring wheats, where considerable amount of glucose (97%) and smaller levels of arabinose, xylose, galactose, and mannose were found. For glutenin extraction, Tilley et al (1993) used dimethyl sulfoxide followed by washing with alcohol, reduction, and alkylation. The major sugar found in total glutenin was glucose (28%); however, xylose (\approx 0.40), and mannose (\approx 0.20%) occurred in quantities comparable with our findings. Tilley et al (1993) noted that the high glucose levels were for starch. Considering the fact that glucose was the major sugar found in every gluten preparation, it is likely that the glutenin fraction could be contaminated with starchy polysaccharide or that sugars could be derived from external sources. So far, all glutenin preparations studied contained

TABLE I
Carbohydrate Compositions^a (%) of Total Glutenin Fractions and of Native (Nonreduced) Glutenins of Different Molecular Weights Eluted from Stacking Gels^b from Two Hard Red Spring Wheat Flours by Gas-Liquid Chromatography

Carbohydrates	Total Glutenin Fraction	Glutenin Isolated from Stacking Gel Origins, ^c %				
		4	6	8	10	12
Len						
Arabinose	0.42	0.13a	0.15a	0.06b	0.08b	0.04c
Xylose	0.34	1.14a	0.16a	0.15a	0.10b	0.02c
Mannose	0.26	0.11a	0.10a	trace	0.08b	0.08b
Galactose	0.32	0.15a	0.16a	0.06c	0.10b	0.12b
Glucose	0.53	0.29a	0.31a	0.35a	0.28a	0.25a
Line 205						
Arabinose	0.21	0.10a	0.11a	0.10a	0.12a	0.09a
Xylose	0.71	0.14a	0.12a	0.06b	0.08b	0.06b
Mannose	0.21	0.11a	0.09a	trace	0.06b	0.04b
Galactose	0.17	0.08a	0.08a	0.02b	0.07a	0.08a
Glucose	1.03	0.28a	0.34a	0.27a	0.29a	0.32a

^a Carbohydrates were estimated as their alditol acetates.

^b Gels had acrylamide concentrations of 4–12%.

^c Means with the same letter in line are not significantly different ($\alpha = 0.05$).

a small amount of carbohydrates associated with the glutenin components of gluten. It is believed that the varying amounts of carbohydrates found in glutenin fractions by some researchers depend not only on the extraction and purification procedure for obtaining gluten but also on the wheat cultivar. Recently, Roels and Delcour (1996a,b) reported the presence of only small amounts of glucose in their purified HMW glutenin subunits in some European wheat cultivars, indicating that glutenins may not be glycosylated.

Carbohydrates in Glutenin Aggregates After Their Separation by Multistacking SDS-PAGE

It is of great importance to distinguish between noncovalent binding and the covalent interaction between core protein and polysaccharide chain. If protein and carbohydrates are covalently linked, they will copurify both when the polypeptide chain is in its native state and under denaturation conditions. Comigration of carbohydrates and protein during electrophoresis has been cited as indicative of a covalent linkage between the carbohydrates and protein (Beeley, 1985). Therefore, in the next step of our study, the glutenin fractions from both flours were subjected to SDS-PAGE on the multistacking gels. In that procedure, the native glutenins were separated without being reduced, based on their size and migration along the stacking gels. The purposes of these experiments were to increase the purification of glutenin, to separate it into its various molecular species, and to determine whether the protein and carbohydrates would separate during electrophoresis or migrate as a single component. Following elution of the native (nonreduced) glutenin from the stacking gels (with acrylamide concentrations of 4–12%), the same carbohydrates as in the total glutenin fraction before electrophoresis were found in almost every glutenin species, but in small amounts (Table II). The carbohydrates were distributed among the native (nonreduced) glutenins of different molecular weights. The major monosaccharide detected in the glutenins eluted from the stacking gels was glucose (0.25–0.35% in both flours), with lower amounts of arabinose, xylose, galactose, and mannose at the lowest level. The highest statistically significant ($\alpha = 0.05$) level of sugars was found in aggregates with the largest molecular weight at the 4 and 6% origins of stacking gels. Chen et al (1992) reported the presence of three sugar components—xylose, arabinose, and galactose—in a glutenin fraction after extensive procedures to dislodge associated (noncovalently linked) carbohydrates by subjecting

TABLE II
Carbohydrate Compositions^a (%) of Total Glutenin Fractions and of Native Glutenins (Nonreduced) of Different Molecular Weights Eluted from the Stacking Gels^b from Two Hard Red Spring Wheat Flours After β -Elimination and Gas-Liquid Chromatography

Carbohydrates	Total Glutenin Fraction	Glutenin Isolated from Stacking Gel Origins, ^c %				
		4	6	8	10	12
Len						
Arabinose	0.20	0.09a	0.05b	0.04b	0.05b	0.03b
Xylose	0.37	0.14a	0.15a	0.11b	0.10b	0.03c
Mannose	0.01	0.02a	0.02a	trace	0.01a	0.01a
Galactose	0.20	0.24a	0.23a	0.06b	0.08b	0.05b
Glucose	0.68	0.38a	0.34a	0.27b	0.24b	0.26b
Mannitol	0.05	0.07a	0.04a	NP ^d	0.01b	0.01b
Line 205						
Arabinose	0.45	0.18a	0.11b	0.13b	0.12b	0.08b
Xylose	0.78	0.10a	0.05b	0.03b	0.11a	0.10a
Mannose	0.04	0.003b	trace	NP	0.01a	0.01a
Galactose	0.12	0.08a	0.06a	0.02b	0.07a	trace
Glucose	1.16	0.32ab	0.31ab	0.37a	0.21a	0.32ab
Mannitol	0.007	0.004a	0.005a	NP	0.005a	0.006a

^a Carbohydrates were estimated as their trimethylsilyl methyl glycosides derivatives after β -elimination, reduction, and methanolysis.

^b Gels had acrylamide concentrations of 4–12%.

^c Means with the same letter in line are not significantly different ($\alpha = 0.05$).

^d Not present.

the preparation to pronase digest of the wheat gluten, purification by gel filtration, and ion-exchange chromatography. The fact that carbohydrates and protein comigrated on SDS-PAGE would support the hypothesis that these carbohydrates in the gluten fraction may be covalently linked. However, it can still be argued that sufficient purification was not achieved and that some sugars could be captured by glutenin aggregates during precipitation procedures and strongly associate with them. Beeley (1985) reported that tenacious noncovalent interactions can occur between carbohydrates and lectins, or enzymes, that act on polysaccharide substrates.

Carbohydrate Content of Glutenin from Different Isolation Procedures

Recently, Tilley (1997) detected glucose and *O*-glycosidically linked mannose in the 1Dx2 high molecular weight glutenin subunit of Chinese Spring wheat. In contrast, Roels and Delcour (1996a) found only small amounts of glucose in the purified HMW glutenin subunits from European wheat cultivars and concluded that the HMW glutenin subunits are not glycoproteins. The different results in these two studies may have resulted from differences in purification procedures, wheat cultivars, and methods of analysis for carbohydrates. We used the procedure of Roels and Delcour (1996a) to obtain partially purified HMW glutenins. The goal was to discriminate between two preparations of glutenin. The glutenin fraction prepared from Len flour according to the procedure of these authors (by removal of glycolipids and arabinogalactan-peptides) contained 0.05% arabinose, 0.03% xylose, 0.10% mannose, 0.12% galactose, and 0.39% glucose. Smaller amounts of sugars, especially arabinose and xylose, were found in glutenin samples purified according to Roels and Delcour (1996a) than in the glutenin fraction obtained by pH precipitation (Table I). However, much higher amounts of mannose (10 times more) were detected in cultivar Len than were reported by Roels and Delcour (1996a) for cultivar Rector, which they used in their experiment. Considering the fact that, after a β -elimination procedure, a small amount of mannitol was also found, we cannot exclude the possibility that the wheat cultivar itself is responsible for these differences. Furthermore, the fact that these researchers were able to detect only glucose in 1 mg of purified HMW glutenin subunits was most likely due to the fact that they used high amounts (30 μ g) of internal standard for monosaccharide analysis by gas chromatography. We believe this large quantity of standard could mask the possibility of detection of very low levels of carbohydrates in a small amount of glutenin. In our experiments, we used 1 mg of inositol standard to 0.8–1.5 mg of samples to obtain the sensitivity level necessary for carbohydrate detection.

TABLE III

Carbohydrate Compositions^a (%) of Native Glutenins of Different Molecular Weights Separated on Stacking Gels,^b Electroblooded to Polyvinylidene Difluoride Membranes, and Eluted with Tris/HCl/SDS Buffer

Carbohydrates	Glutenin Isolated from Stacking Gel Origins ^c (%)				
	4	6	8	10	12
LEN					
Mannose	0.03a	0.04a	0.005b	0.003b	0.005b
Galactose	0.14a	0.17a	0.13a	0.11a	0.11a
Glucose	0.69a	0.48a	0.25b	0.20b	0.28b
Mannitol	0.08a	0.06a	trace	0.02b	0.01b
FLOUR 205					
Mannose	0.01a	NP ^d	NP	trace	0.003b
Galactose	0.09a	0.11a	0.07a	0.07a	0.06a
Glucose	0.62a	0.24b	0.27b	0.20b	0.18b
Mannitol	0.01a	0.01a	NP	0.004b	NP

^a Carbohydrates were estimated as their trimethylsilyl derivatives of the methyl glycosides were obtained after β -elimination, reduction, and methanolysis.

^b Gels had acrylamide concentrations of 4–12%.

^c Means with the same letter in line are not significantly different ($\alpha = 0.05$).

^d Not present.

Evidence for *O*-Linked Glycoprotein in HMW Glutenin

It is known that carbohydrates could be attached to the protein via *O*- or *N*-glycosidic linkages. Some methods are available for cleavage of protein-carbohydrate linkages to release oligosaccharides from glycoproteins. Of these methods, the most widely employed has been the alkali β -elimination of *O*-glycosyl GalNAc-Ser/Thr linkage, which often produces good yields of oligosaccharides. To determine the linkage by which mannose could be attached to the backbone of the protein, Tilley (1997) used the fact that *O*-glycosidic linkages are sensitive to β -elimination procedure in mild base under reducing conditions (Jackson and Tijan, 1988). Five sugars are known to form *O*-glycosidic linkages with the side chain of serine or threonine (Beeley, 1985). Finne et al (1979) observed in their study on the glycoprotein carbohydrate chains of the rat brain proteoglycan that, in addition to oligosaccharides containing N-acetylgalactosaminitol, oligosaccharides terminated with mannitol were also produced by mild alkaline borohydride treatment (terminal sugar moieties are those that reside at the terminus of an oligosaccharide chain). In that procedure, the oligosaccharide unit is released with the linkage sugar as the terminal residue and an unsaturated amino acid derivative is formed. If the alkaline elimination is done in the presence of NaBH₄, the linkage sugars or amino sugars originally involved in the protein-sugar linkage are concomitantly reduced to the corresponding sugar alcohol (e.g., mannose to mannitol, galactose to galactitol). This minimizes alkaline degradation of the oligosaccharide by the "peeling reaction" and allows identification of the linkage sugar. Upon methanolysis, all glycosidic bonds along the carbohydrate chains are cleaved to form the methylglycosides of the component monosaccharides (released carbohydrates as well as carbohydrates remaining in the protein). All reduced and nonreduced monosaccharides are then derivatized and analyzed by GC-MS.

Carbohydrate analysis of the glutenin aggregates extracted from the stacking gels after their exposure to β -elimination procedure and reducing conditions is shown in Table II and their separation by GS-MS in Fig. 1. A very low amount of mannitol was found in almost all of the glutenin aggregates except at the 8% origin. Simultaneously, smaller amounts of mannose were detected, but other carbohydrates remained at almost the same level as before β -elimination. The aggregates with the largest molecular weights at the 4 and 6% origins of stacking gels had a statistically ($\alpha = 0.05$) higher level of mannitol than those at the 10 and 12% origin in Len flour (Table III). Only a very small amount of mannitol was detected in all glutenin aggregates in flour 205. Mannose and mannitol were not present in the medium molecular weight glutenin polymers, that is, at the 8% origin of stacking gels in both Len and

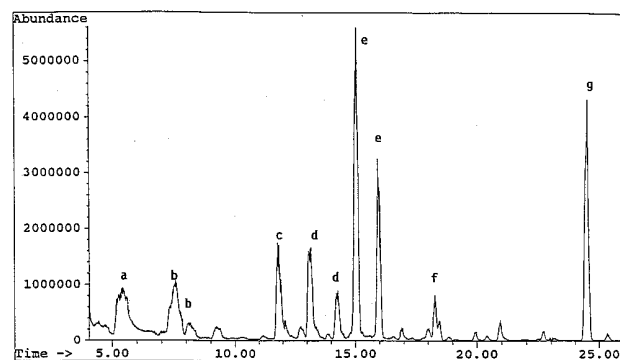


Fig. 1. Gas-liquid chromatogram of the trimethylsilyl methyl glycoside derivatives of: arabinose (a), xylose (b), mannose (c), galactose (d), glucose (e), mannitol (f), *myo*-inositol (internal standard) (g). Sample was separated on an HP-1 capillary column. Program: temperature increased from 140 to 180°C at a rate of 2°C/min and held at 180°C for 6 min.

line 205 flours. It is believed that different β -elimination conditions must be applied for every glycopeptide considered. So far, the conditions used in our study presumably were able to release only mannose from the backbone of the protein, and other sugars may not have been cleaved under these conditions. However, their existence may be demonstrated by alkali hydrolysis under more vigorous conditions. For example, Muir and Lee (1970) used more concentrated NaOH and a longer incubation time to release galactose from glycoprotein in the collagen of earthworm cuticle.

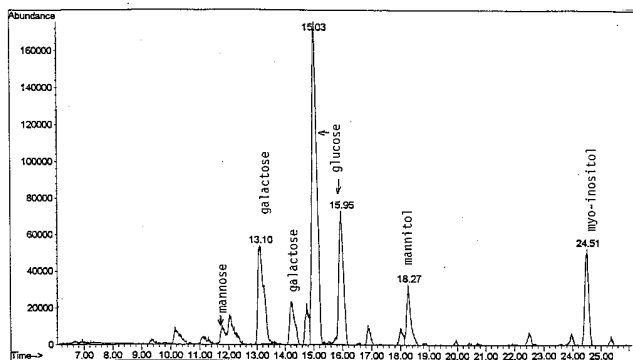


Fig. 2. Gas-liquid chromatogram of the trimethylsilyl methyl glycoside derivatives of glucose, galactose, mannose, mannitol, and *myo*-inositol (internal standard) in the glutenin fraction of Len flour after electrophoresis, blotting, β -elimination, reduction, and methanolysis. Sample was separated on HP-1 capillary column. Program: temperature was increased from 140 to 180°C at a rate of 2°C/min and held at 180°C for 6 min. Helium was used as the carrier gas at a flow rate of 0.42 mL/min.

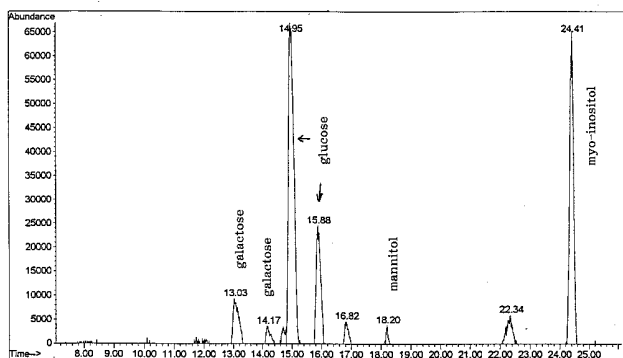


Fig. 3. Gas-liquid chromatogram of the trimethylsilyl methyl glycoside derivatives of galactose, glucose, mannitol, and *myo*-inositol (internal standard) in the glutenin fraction of line 205 flour after electrophoresis, blotting, β -elimination, reduction, and methanolysis. Sample was separated as indicated in Fig. 2.

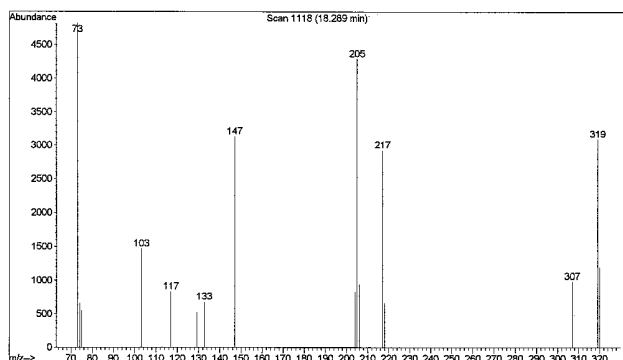


Fig. 4. Mass spectra of the trimethylsilyl methyl glycoside derivative of mannitol.

Electroblotting of Nonreduced Glutenin from Stacking Gel Origins

To increase the purity of the analyzed protein, we decided to transfer the native glutenins from the stacking gels to PVDF membranes by electroblotting. According to Weizhandler et al (1993) and Rosenberg (1996), electroblotting of protein from gels to membrane is a simple method for removal of contaminants from protein. Figures 2 and 3 show the separation of the trimethylsilyl derivatives of carbohydrates obtained from glutenin fractions of Len and line 205 flours, respectively, after blotting, β -elimination, reduction, and methanolysis. Figure 4 shows the mass spectrum of mannitol after the same treatments. By using the same procedures for carbohydrate analysis after β -elimination under reducing conditions as we had used under nonreducing conditions, we found glucose, galactose, and a small quantity of mannitol in some glutenin aggregates, with the larger amounts in the larger molecular weight polymers. Higher levels of galactose and mannitol were found in Len, the better breadmaking quality flour, than the line 205 flour (Table III). No other nonstarchy polysaccharides, nor arabinoxylans or amino sugars, were detected. These results indicate that the blotting procedure increases purification of the glutenin fraction. So far, we were not able to find galactitol as a result of β -elimination and reduction of the galactose released from the backbone of the protein. Whether or not this carbohydrate and the protein components are linked to each other remains to be studied. The alkali-sensitive *O*-glycosidic linkages (included xylose, GlcNAc, galactose or mannose, and serine and threonine) are readily split under relatively mild conditions. However, it should be recognized that the rate of cleavage of protein-carbohydrate linkages of a particular type in alkali can be greatly influenced by the nature of the peptide moiety and the structure of the oligosaccharide (Beeley 1985). It is known that more than one type of protein-carbohydrates linkage can occur in a single glycoprotein molecule.

SUMMARY AND CONCLUSION

The native (nonreduced) glutenin fractions obtained from the flours of two HRS wheats, Len and line 205, by pH precipitation contained the same sugars (glucose, xylose, arabinose, galactose, and mannose) but in different quantities. Comparing our data with those of other researchers, we concluded that the different values for sugars in glutenin fractions may have resulted not only from differences in purification but also from differences in wheat cultivars and methods of analysis. After separation and purification of glutenins under nonreducing conditions by multistacking gel electrophoresis and by transfer to PVDF membranes by electroblotting, the β -elimination procedure in mild base under reducing conditions was performed. Besides glucose and galactose, a small amount of mannitol was found in some glutenin aggregates in the two flours examined. This verifies that carbohydrates associated with the glutenin fraction were nonstarchy in nature. It also supports the evidence of Tilley (1997) that mannose can be involved in the glycosylation of the gluten protein and can be covalently bound to serine and threonine, forming the *O*-linked carbohydrates. Our findings are also in agreement with the results of Hickman et al (1995) on the molecular weight of HMW glutenin subunits determined by mass spectrometry. These researchers argued against extensive glycosylation of HMW glutenins, but they did not exclude the possibility of single sugar residues being present in some subunits. The central question of how glycosylation contributes to glycoprotein function still remains unclear, although for *O*-linked sugars, there is evidence for participation in biological phenomena such as cell-cell interaction (Paulson 1989). Since the higher molecular weight glutenin species at the 4 and 6% multistacking origins contained higher amounts of mannitol, it may be speculated that this sugar participates as a linking element with the large glutenin aggregates in forming the backbone of the gluten structure in dough. The fact that no mannitol was detected at the 8% origin may point to the possibility that all glutenin species may not be glycosylated.

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