

Detection of *O*-Glycosidically Linked Mannose Within the Structure of a Highly Purified Glutenin Subunit Isolated from Chinese Spring Wheat¹

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ABSTRACT

A low level of *O*-glycosidically linked D-mannose was detected on the 1Dx2 high M_r glutenin subunit of Chinese Spring wheat. Detection of the *O*-glycosidic linkage was accomplished by subjecting the purified 1Dx2 protein to β -elimination and reduction conditions that resulted in the specific cleavage of *O*-glycosidically linked carbohydrates from the protein backbone and the reduction of the linkage (reducing end) sugars to alditols. Subsequently, acid-catalyzed methanolysis was used to cleave glycosidic linkages on released carbohydrate as well as carbohydrate remaining on the

protein. The repeat units were derivatized with trimethylsilyl and analyzed by gas chromatography and mass spectroscopy (GC-MS). The 1Dx2 high M_r glutenin subunit of Chinese Spring wheat consisted of 1.2% carbohydrate containing D-mannose and D-glucose units. *O*-Glycosidically linked D-mannose residues represented 0.13% of the total mass of the 1Dx2 high M_r glutenin subunit. This represented <1 *O*-glycosidically linked D-mannose residue per protein molecule and provided some initial evidence that there may be various glycoforms of this subunit present in the preparation.

The formation of the large gluten quaternary structure during dough mixing and breadmaking processes is extremely complex and not well characterized. It has been established that the glutenin subunits, a specific subset of the proteins comprising the gluten complex, directly affect dough formation and breadmaking quality. In particular, the breadmaking quality of European wheats is correlated with allelic variation in high M_r glutenin subunit number and composition (Payne et al 1979, 1981; Payne 1987). However, glutenin subunits that have been identified in wheat varieties around the world have not yet exhibited any definitive structural differences that can be directly correlated with their ability to aggregate into the quaternary gluten complex and affect dough formation or breadmaking quality.

Compelling circumstantial evidence indicating that the high M_r glutenins are posttranslationally modified has come from the fact that there are discrepancies in the molecular weights of the high M_r glutenin subunits determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (Hickman et al 1995) and Ferguson plot analysis by capillary electrophoresis (CE) (Werner 1995) compared to the molecular weights predicted from DNA sequences. The data published for the purified glutenin proteins show broad peaks with complex mass spectra (Hickman et al 1995), which is indicative of heterogeneity similar to that caused by glycosylation (Mertz et al 1996). The authors did address this issue and suggested that the mass predictions in their studies could not be considered better than ± 0.5 to 1.0%. The differences in the measured versus the predicted molecular weights of the high M_r glutenin subunits determined by these two techniques varied from as little as 0.2% to as much as 5.9%. It is possible that the fact that the actual molecular weights of the high M_r glutenin subunits are greater than those predicted from the DNA sequences may be accounted for by posttranslational processing and modification of the proteins.

Currently, no confirmed posttranslational modifications of the high M_r glutenin subunits have been reported. However, it has been suggested that the gluten proteins may be glycosylated (Donovan and Baldo 1987, Chen et al 1992, Tilley et al 1993, Lauriere et al 1996), although that hypothesis has never been directly

confirmed or discounted. Tilley et al (1993) reported the purification and characterization of the high M_r glutenin subunits 2, 7, 8, and 12 of the wheat cultivars Chinese Spring and TAM 105 in which D-glucose, D-mannose, and *N*-acetyl-D-glucosamine were detected in each of the purified proteins by several indirect methods as well as by gas chromatography and mass spectroscopy (GC-MS). The initial results were confirmed by Lauriere et al (1996), who also determined that D-xylose was present on the low M_r glutenin subunits isolated from the French cultivar Capitole by the binding of specific anti-xylose antibodies. These antibodies did not bind to the high M_r glutenin proteins, and the authors did not make an attempt to identify the carbohydrates involved in the glycosylation of the high M_r glutenins.

Recently, Roels and Delcour (1996) attempted to disprove the hypothesis that the glutenin subunits were glycosylated. However, those authors could not repeat the indirect carbohydrate staining technique that gave positive results for Tilley et al (1993), Lauriere et al (1996), and Bollecker and Schofield (1996), and they were unable to detect carbohydrate in their glutenins by GC-MS. While Bollecker and Schofield (1996) did obtain positive results with the indirect carbohydrate staining system, they also were unable to detect carbohydrate in their preparations of glutenins by GC-MS. A consistent error in both of these studies was the fact that the glutenins analyzed were not pure. Purification and concentration of the individual proteins is essential to accurately determine the carbohydrate content of each glutenin subunit. Both of these groups were apparently unable to accurately measure carbohydrate in small amounts (<500 μg) of protein, and instead used larger quantities (10 mg) of partially purified protein. It appears that their techniques did not afford the sensitivity levels necessary to detect the carbohydrates in purified glutenin preparations.

Even with GC-MS analysis confirming the presence of carbohydrate associated with each individual purified high M_r glutenin subunit (Tilley et al 1993), a direct covalent linkage between any of the carbohydrate and the proteins had not been shown. In the present study, we have identified a covalent linkage between mannose and the glutenin protein backbone. Mannose residues were shown to be *O*-glycosidically linked to the 1Dx2 high M_r glutenin subunit isolated from the cultivar Chinese Spring by exposing these proteins to conditions of β -elimination and reduction before GC-MS analysis. When glycoproteins with carbohydrates covalently linked to their backbones through an *O*-glycosidic linkage were exposed to β -elimination and reduction, the *O*-glycosidic linkage was cleaved and the carbohydrate structure (monosaccharide residue or oligosaccharide chain) was removed from the

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protein (Nakajima and Ballou 1974, Finne et al 1979, Jackson and Tjian 1988, Gentzsch et al 1995). Only those carbohydrate residues that were directly linked to the backbone of the protein (through a serine or threonine residue) were reduced to their alditol forms (e.g., mannose was reduced to mannitol). If other carbohydrates were linked to that reducing carbohydrate (as in a di-mannose or tri-mannose chain), those carbohydrates that were not involved in the direct linkage to the protein backbone were not reduced to their alditol forms. Upon methanolysis, any glycosidic bonds along the carbohydrate chains were cleaved to form the methyl glycosides of the component monosaccharides. All reduced and nonreduced monosaccharides were then derivatized with trimethylsilyl (TMS) ether groups and analyzed by GC-MS.

MATERIALS AND METHODS

Materials

The cultivar Chinese Spring was originally obtained from D. Kasarda, USDA-ARS, Western Regional Research Center, Albany, CA.

Protein Extraction and Purification

High M_r glutenin subunit 1Dx2 from the cultivar Chinese Spring was purified by previously described methods (Tilley et al 1993).

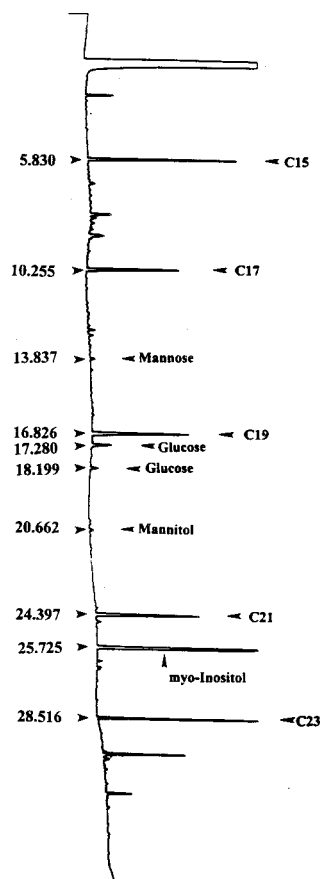


Fig. 1. Gas chromatogram of trimethylsilyl derivatives of the methyl glycosides obtained from high M_r glutenin subunit 1Dx2 after β -elimination, reduction, and methanolysis. Components were detected by mass spectroscopic analysis. Alkanes were added as retention standards (C15, C17, C19, C21, and C23).

Liberation and Reduction of *O*-Linked Mono- and Oligosaccharides by β -elimination and Alkaline-Borohydride Treatment

The method of Finne et al (1979) was modified as follows. A solution (10 μ L) of 1.0M NaBH₄ in 0.05M NaOH (freshly made) was added to ~250 μ g of purified and lyophilized 1Dx2 subunit of Chinese Spring. The mixture was incubated at 45°C for 16 hr. Excess borohydride was destroyed by adding 4N acetic acid dropwise until bubbling ceased. Sodium ions were removed by ion-exchange column chromatography on Dowex AG 50W-X8 (H⁺ form)(1.5 mL of resin/mL of solution). The column was rinsed thoroughly with deionized water before sample application. Column runthrough was collected, and the protein and carbohydrate mixture was eluted with water (5 mL/mL of resin), and the solution was evaporated to dryness on a rotary evaporator at 40°C. Borate ions were removed by the addition of methanol containing 0.1M acetic acid followed by evaporation. This step was repeated three times. Exactly 250 μ g of the resulting material was analyzed for glycosyl composition and all calculations are based upon this final 250- μ g sample.

Glycosyl Analysis

TMS derivatives of sugars and amino sugars were prepared as previously described (York et al 1985, Merkle and Poppe 1994). Methyl glycosides of sugars were prepared by methanolysis in 1M HCl in methanol. After removal of methanol and hydrochloric acid, the glycosides were converted to TMS derivatives. In the case of 2-amino sugars, their methyl glycosides were *N*-acetylated, then converted to TMS ethers. Standards for mannitol, *N*-acetylglucosaminitol, and *N*-acetylgalactosaminitol were prepared as follows. Authentic D-mannose, 2-*N*-acetylglucosamine, and 2-*N*-acetylgalactosamine (100 μ g each) were added separately to vials containing 20 μ g of myo-inositol. To each vial was added 100 μ L of 10 mg/mL of NaBH₄ in 1M NH₄OH, and the solution was allowed to stand for 4 hr. Glacial acetic acid was added dropwise until effervescence stopped, and the solution was evaporated to dryness by a stream of N₂. The residue was dissolved in 250 μ L of a 10% solution of HOAc/MeOH and evaporated to dryness over N₂. This step was repeated five times. The residue was dissolved in 250 μ L of MeOH and evaporated to dryness under N₂. This step was repeated five times. The residue was then subjected to methanolysis and TMS derivatization in parallel with the protein samples, as described above. GC analysis of the TMS methyl glycosides was performed on a Hewlett Packard 5890 GC

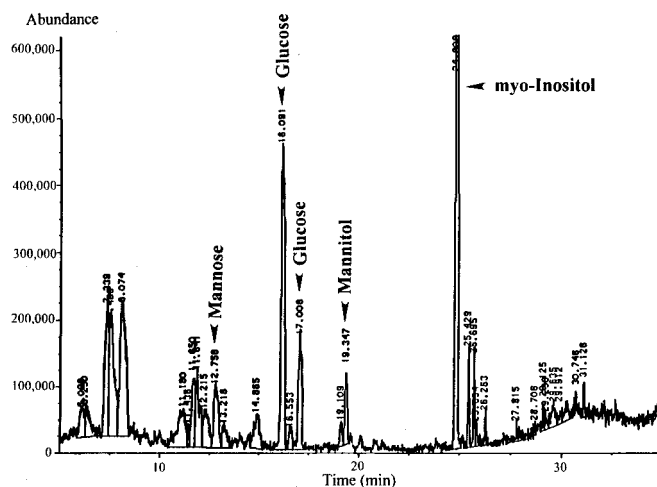


Fig. 2. Gas chromatogram and mass spectrum of trimethylsilyl derivatives of methyl glycosides obtained from high M_r glutenin subunit 1Dx2 after β -elimination, reduction, and methanolysis. Retention times of reference standards are designated as glucose, mannose, and mannitol. Myo-inositol was added as an internal standard.

using a Supelco DB1 fused silica capillary column. GC-MS analysis was performed using a Hewlett Packard 5890 GC coupled to a mass selective detector (5970 MSD). Inositol was added to the sample before derivatization as an internal standard, and a number of *n*-alkanes were coinjected as retention standards for the GC analysis. Monosaccharides were identified by their retention times in comparison to standards; the carbohydrate character of these was authenticated by mass spectra.

RESULTS AND DISCUSSION

After exposure to β -elimination and reduction conditions, the entire preparation of 1Dx2 glutenin subunit was derivatized and analyzed by GC. Mannose, glucose, and mannitol were detected (Fig. 1). These carbohydrates were identified based upon their retention times in comparison to alkane retention standards. In the com-

bined GC-MS analyses, these monosaccharides were identified by their retention times in comparison to the internal standard, myo-inositol, and the carbohydrate character of the monosaccharides was authenticated by their mass spectra (Figs. 2 and 3).

The total mass of carbohydrate (2.9 μ g) detected in the sample represented 1.2% of the mass (250 μ g) of the purified 1Dx2 high M_r glutenin subunit sample isolated from Chinese Spring. The total glycosyl composition is shown in Table I. Glucose represented 0.88%, mannose 0.19%, and mannitol 0.13% of the total mass of the glycoprotein. Mannitol was present in the sample, indicating that mannose had been directly *O*-glycosidically linked to the backbone of the 1Dx2 glutenin subunit. The other carbohydrates found in their nonreduced forms may or may not have been linked to the *O*-glycosidically linked mannose residues by α -1,4 glycosidic bonds. It is not possible to determine that with this particular method. This method could only confirm those carbohydrates that were directly linked to the protein backbone, and no information was provided regarding whether only single residues of mannose were bound to the protein or whether oligosaccharide chains (with mannose as the linkage sugar) were present.

The amount of mannitol (0.13%) detected indicates that there are very few *O*-linked mannose residues present on the 1Dx2 high M_r glutenin subunit of Chinese Spring and, hence, very few glycosylation sites. Only those carbohydrates that were reduced to their alditol forms were considered when calculating the number of glycosylation sites because they are the only carbohydrates that were confirmed as covalently linked to the protein backbone. With mannitol representing only 0.13% of the total mass of the 1Dx2 subunit, and the total mass of the 1Dx2 subunit predicted from the DNA sequence being 88,979 Da (Anderson and Greene 1989), only 116 Da of the entire mass (0.13% of 88,979 Da) of each 1Dx2 high M_r glutenin subunit is represented by *O*-linked mannose. This does not equal enough mass for even one *O*-linked mannose residue per protein molecule. A single mannose residue covalently bound to the protein would have resulted in a mass increase of 162 Da, which is 0.18% of 88,979 Da. From this data, it appears that some of the 1Dx2 glutenin subunits in this study were not glycosylated. This indicates that partial glycosylation of the glutenin subunits may occur. This possibility is corroborated by the apparent heterogeneity in the peaks representing the purified high M_r glutenin subunits analyzed by MALDI-MS (Hickman et al 1995).

This data in which ~1.2% carbohydrate is associated with the high M_r glutenin subunit 1Dx2 corresponds well to the reports of Werner (1995) and Hickman et al (1995) who indicate discrepancies of 0.2–5.9% between the predicted M_r of the glutenin proteins derived from DNA sequences and the molecular weights determined by Ferguson plot analysis by CE and MALDI mass spectrometry respectively. The fact that there are differences between the predicted and the actual molecular masses of these proteins indicates that some form of posttranslational processing of the glutenins may occur. The discrepancies in actual molecular weights of the proteins from their predicted molecular weights allow for only a few covalently bound carbohydrate residues. In particular, the 1Dx2 glutenin subunit isolated from the cultivar Scout 66 indicated a difference in actual molecular weight from its predicted molecular weight of 1,800 Da (Werner 1995). This

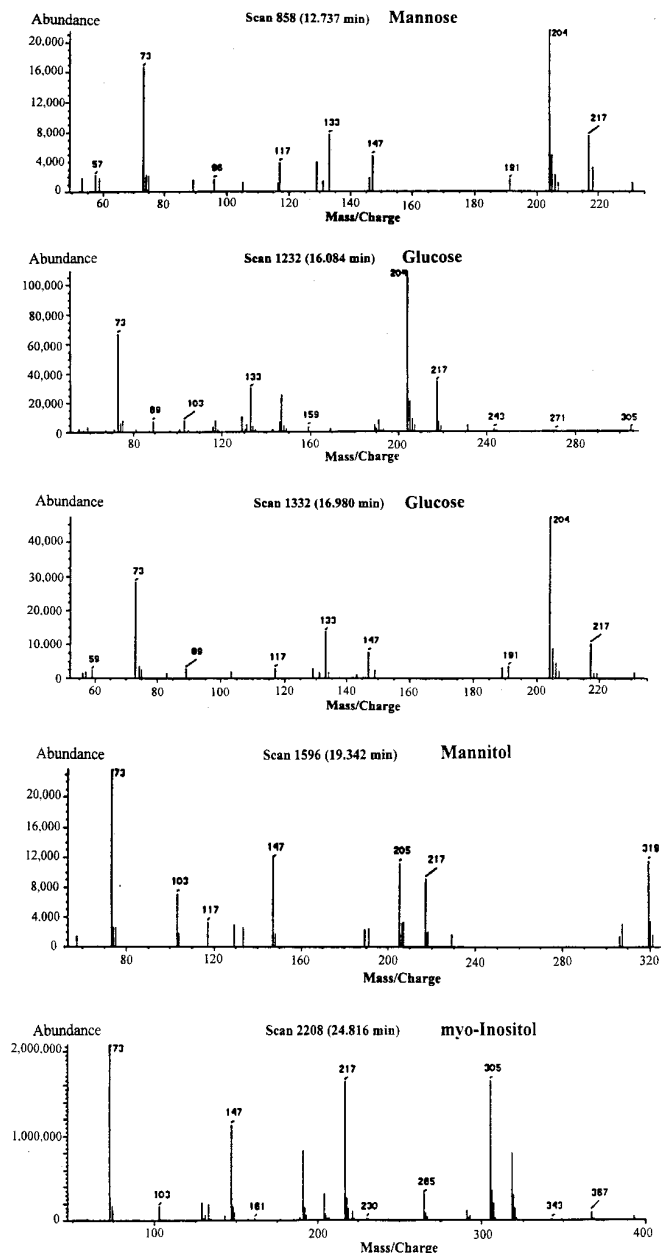


Fig. 3. Total ion chromatogram of trimethylsilyl derivatives of carbohydrates detected in gas chromatographic and mass spectroscopic analysis of trimethylsilyl derivatives of methyl glycosides obtained from high M_r glutenin subunit 1Dx2 after β -elimination, reduction, and methanolysis.

TABLE I
Composition of Carbohydrates Isolated from the 1Dx2 High M_r Glutenin Subunit of Chinese Spring Wheat

Glycosyl Residue ^a	% Total Carbohydrate	% Total Protein Mass
Glucose	73.2	0.88
Mannose	16.1	0.19
Mannitol	10.6	0.13

^a Methyl glycosides (trimethylsilyl derivatives) obtained from the high M_r glutenin subunit 1Dx2 from Chinese Spring after β -elimination, reduction, and methanolysis.

LITERATURE CITED

represented a difference of 2.06% in the total mass of the protein and allowed for ~11 carbohydrate residues per protein molecule. Hickman et al (1995) found only a 936 Da mass difference in glutenin subunit 1Dx2 isolated from the cultivar Chinese Spring, which represented a 1.1% mass discrepancy similar to the 1.2% carbohydrate associated with the 1Dx2 subunit of Chinese Spring isolated in this study. The mass discrepancies in both of these studies allow for a maximum of only five to six carbohydrate residues being present per protein molecule. It is also important to consider the various possible glycoforms that may exist and acknowledge that some of the glutenin protein molecules may not be glycosylated, while others are glycosylated at varying levels. It is likely that the amount of glycosylation will vary from cultivar to cultivar and even within the actual source of the wheat sample due to potential environmental influences on glycosylation during the period of time the wheat plant is producing these storage proteins. It is also likely that there may be other modification and processing of the glutenin proteins that have yet to be described which may also account for some of the discrepancies in molecular weights of these proteins.

It is unknown whether glycosylation of the high M_r glutenin proteins plays any role in the build-up of the aggregated gluten quaternary structure or whether it relates to the dough formation and breadmaking processes in any way. Certainly, glycosylation of this nature may play a role in the processing of the glutenin proteins in the cell. Recombinant barley α -amylase has been shown to be glycosylated with an *O*-linked di-mannose structure on the *C*-terminal portion of the protein (Andersen et al 1994). The authors suggested that this glycosylation may aid in protecting the enzyme from *C*-terminal degradation caused by the abundance of carboxypeptidases present in barley seed. Whether this mechanism is employed by wheat is unknown.

The actual sites of glycosylation of the high M_r glutenin proteins have yet to be determined, however, the confirmation of the first known posttranslational modification of the high M_r glutenin proteins is a significant step forward in furthering the understanding of the structure of the proteins and possibly will provide a mechanism for following the processing of these proteins through the cell and into the protein bodies of the wheat kernel.

CONCLUSIONS

With this direct evidence of *O*-linked mannose associated with the high M_r glutenin subunit 1Dx2 of Chinese Spring, the theory that some of the high M_r glutenin subunits of wheat are glycosylated has been reinforced. It appears that the level of glycosylation is quite low, and it may be that partial glycosylation of the glutenins occurs. It is also unknown whether all subfractions of the high M_r glutenin subunits are glycosylated or not, and whether or not this phenomenon varies from cultivar to cultivar or is influenced by environmental conditions. These important considerations remain to be determined.

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