Re-examination of the Glycosylation of High M_r Subunits of Wheat Glutenin

S. S. J. Bollecker,[†] K.-P. Kaiser,[‡] P. Köhler,[§] H. Wieser,[§] and J. D. Schofield*,[†]

Department of Food Science and Technology, The University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, United Kingdom, Institut für Lebensmittelchemie der Technischen Universität München, Lichtenbergstrasse 4, D-85748 Garching, Germany, and Deutsche Forschungsanstalt für Lebensmittelchemie and Kurt Hess Institut für Mehl und Eiweissforschung, Lichtenbergstrasse 4, D-85748 Garching, Germany

SDS−PAGE-separated high M_r wheat glutenin subunits stained positively in the DIG-glycan procedure, suggesting that they were glycosylated, but control experiments indicated that these were probably false positives. Lack of reaction with concanavalin A indicated the absence of terminal mannose, glucose, or *N*-acetylglucosamine. GC/MS analysis indicated that A-PAGE-prepared subunits contained glucose, mannose, xylose, and galactose. Because gel regions containing no protein contained similar amounts of those sugars, they were most likely unbound contaminants. RP-HPLC-purified subunits from cv. Apollo wheat contained variable amounts of glucose only, probably representing starch contamination. RP-HPLC-purified subunits 1Ax1 and 1Bx7 contained no GC/MS-detectable sugars even though the procedure was sensitive enough to detect 1 sugar residue per 40 subunit molecules. Mapping of tryptic or chymotryptic peptides, before and after deglycosylation, and GC/MS analysis of isolated tryptic peptides provided no evidence for glycosylation of high M_r subunits. It appears unlikely that high M_r wheat glutenin subunits are glycosylated.

Keywords: *Wheat; glutenin; glycosylation; subunits*

INTRODUCTION

The high M_r subunits of glutenin play an important role in the structure and functionality of wheat gluten (Tatham et al., 1990; Schofield, 1994). Variations in gluten and dough properties from different wheat varieties can be accounted for to a large extent in terms of different combinations of high M_r glutenin subunits (Payne et al., 1979) and in terms of their amount (Shewry et al., 1992). However, little is known about the actual structure/function mechanisms. In view of their important impact on functionality, the possibility that these proteins might be glycosylated (Tilley et al., 1993; Tilley and Schofield, 1994; Tilley, 1996) has raised much interest, but also controversy, in the cereal science community over the past few years.

Use of the DIG-Glycan Detection Kit (Boehringer-Mannheim, Mannheim, Germany) procedure performed on proteins immobilized on blots, which involves periodate oxidation followed by reaction with a digoxigenin-substituted hydrazide and subsequent labeling with anti-digoxigenin antibodies tagged with alkaline phosphatase, has been an important method in the attempt to demonstrate the presence of the glycan units (Tilley et al., 1993). Additionally, labeling with specific lectins has been used in the attempt to identify the monosaccharides involved. In this manner, evidence was obtained suggesting that high M_r glutenin subunits 1Dx2, 1Bx7, 1By8, and 1Dy12 of cvs. Chinese Spring and TAM 105 contained covalently bound mannose and *N*-acetylglucosamine (Tilley et al., 1993).

Experiments involving deglycosylation with specific enzymes and β-elimination provided further evidence to suggest that these carbohydrates might be linked O-glycosidically to serine and/or threonine in the repetitive domains of the polypeptides, rather than N-glycosidically linked (Tilley and Schofield, 1994). The absence from all the high M_r subunit amino acid sequences published to date (Halford et al., 1987) of sequences that are typical of the sites at which N-glycosidically linked glycans are attached to proteins makes the occurrence of N-glycosidically linked glycans in high M_r subunits unlikely (Tilley and Schofield, 1994).

Another approach, based on the analysis of purified, alkylated, high M_r glutenin subunits by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry and electrospray injection mass spectrometry (ESI-MS) (Hickman et al., 1995) showed that the determined molecular weights were very similar to those deduced from gene sequences. In contrast to the results of Tilley et al. (1993), those observations indicated that the subunits could not be extensively glycosylated, although the possibility of glycosylation in the order of, say, one monosaccharide residue per subunit could not be excluded.

Subsequently, Tilley (1996) reported the identification of covalently linked mannose within the structure of the 1Dx2 subunit isolated from cv. Chinese Spring. On the other hand, Roels and Delcour (1996) reported that they were unable to detect monosaccharides associated with

* Author to whom correspondence should be addressed (fax +44 118 9316607).
[†] The University of Reading.
[‡] Universität München.
[§] Deutsche Forschungsanstalt für Lebensmittelchemie and Kurt Hess Institut für Mehl und Eiweissforschung.

10.1021/jf980280q CCC: $15.00
Published on Web 12/03/1998

Table 1. Sources of High M_r Subunits Isolated on a Preparative Scale and Purification Method(s) Used

wheat cultivar	high M_r subunits present	purification method	
		RP-HPLC	A-PAGE
Gabo double-null	1Ax1	yes	no
Galatea	1Bx7	yes	no
Apollo	1Dx2, 1Bx6, 1By8, 1Dy12	yes	yes
Monopol	1Ax1, 1Dx5, 1Bx7, 1By9, 1Dy10	no	yes
Rektor	1Dx5, 1Bx7, 1By9, 1Dy10	blotting only	blotting only
CWRS	1Ax2*, 1Dx5, 1By7, 1Dy10	no	yes

purified high M_r subunits by direct chemical analysis, although they, like Tilley et al. (1993), did obtain positive reactions with the DIG-Glycan Detection procedure. Roels and Delcour (1996) concluded that it is very unlikely that high M_r glutenin subunits are glycosylated.

In the research reported here, we have attempted to provide further evidence to clarify whether high M_r subunits are glycosylated using direct analytical approaches. High M_r glutenin subunits were purified and the constituent monosaccharides were characterized and determined directly by analysis of alditol acetate and trimethylsilyl ether derivatives of the constituent monosaccharides by gas−liquid chromatography and mass spectrometry (GC/MS). Chemical and enzymic deglycosylation and identification of the location of the site(s) of glycosylation by peptide mapping have also been attempted.

MATERIALS AND METHODS

Purification of High M_r Glutenin Subunits. *Starting Materials and Selective Extraction and Precipitation Procedures.* In one set of experiments, wheat lines expressing only one high M_r glutenin subunit were used as the starting material to simplify the purification. Two Bühler-milled flour samples, one containing subunit 1Bx7 (cv. Galatea) and one containing subunit 1Ax1 (cv. Gabo double-null; Lawrence et al., 1988), were supplied by Dr. P. I. Payne (PBI Cambridge Ltd., U.K.) and Drs. F. MacRitchie and R. Gupta (CSIRO Grain Quality Research Laboratory, North Ryde, and CSIRO Division of Plant Industry, Canberra, Australia), respectively. In a second set of experiments, glutens were prepared from flours of cvs. Apollo, Monopol, and Rektor and of Canada Western Red Spring (CWRS, a commercial mixture of cultivars) wheat by mixing a dough and washing the glutens manually in excess water. The subunits present in the various samples are shown in Table 1.

Whether using flour or gluten as starting material, fractions containing high M_r glutenin subunits were prepared using the selective extraction and precipitation procedure of Marchylo et al. (1989). In the case of the flours, from which subunits 1Ax1 and 1Bx7 were purified, the samples were extracted first with 2% (w/v) Triton X-114, 0.1 M KCl, and 0.05 M Tris-HCl, pH 7.8, to remove low M_r proteins (Marion et al., 1994) and with 70% (v/v) ethanol to remove gliadins. In the case of glutens, starch and gliadins were removed by extraction with 90% (v/v) dimethyl sulfoxide (DMSO) (Burnouf and Bietz, 1989), and the residue was further extracted for 1 h with water at 40 °C. In both cases, glutenin subunits were then extracted with 50% (v/v) propan-1-ol and 0.05 M dithioerythritol, and high M_r subunits were precipitated by raising the propan-1-ol concentration to 60% (v/v). The precipitated high M_r subunits were dialyzed against deionized water and freeze-dried. In some experiments, subunits were alkylated with 4-vinylpyridine (Friedman et al., 1970), whereas in others the subunits were reduced but not alkylated.

RP-HPLC. Freeze-dried subunits 1Ax1 and 1Bx7 were redissolved in 20% (v/v) acetonitrile (ACN) and 0.1% (v/v) trifluoroacetic acid (TFA) and filtered through a 0.2 μm membrane prior to injection onto a Zorbax 300-SB-C8 semi-preparative column (C_8, 30 nm pore size, 5 μm particle size, 9.4 × 250 mm), used in conjunction with a guard column filled with the same material (Jones Chromatography, Hengoed, U.K.). The subunits were chromatographed at room temperature, using a linear gradient of 23−48% (v/v) ACN over 60 min (Marchylo et al., 1989) with a flow rate of 3 mL/min. The column effluent was monitored at 210 nm. The peaks containing the subunits were collected and freeze-dried directly.

High M_r subunit fractions prepared from glutens were redissolved in 50% (v/v) aqueous propan-1-ol and 0.05 M dithioerythritol at 60 °C for 15 min, filtered through a 0.45 μm membrane, and injected onto a Nucleosil 300-5-C_8, column (C_8, 30 nm pore size, 5 μm particle size, 4.6 × 250 mm, Macherey-Nagel, Düren, Germany). RP-HPLC was carried out at 60 °C using a volatile solvent system comprising (A) 15% (v/v) propan-2-ol/0.1% (v/v) TFA and (B) 80% (v/v) ACN/0.1% (v/v) TFA with a flow rate of 0.8 mL/min. The following gradient was used: 0−10 mim, isocratic 12.5% B; 10−25 min, linear 12.5−21% B; 25−45 min, linear 21−22% B; 45−65 min, linear 22−30% B. The column effluent was monitored at 220 nm. The portions of the column effluent corresponding to subunits were collected and dried under a stream of N_2.

Preparative Acid Polyacrylamide Gel Electrophoresis (A-PAGE). Unalkylated high M_r subunits prepared from glutens were purified by preparative-scale A-PAGE using the method of Morel (1994). The zones of interest were cut out and extracted with 50% (v/v) aqueous propan-1-ol. The extracts were then concentrated and desalted by centrifugation with Centricon 30 concentrators, yielding ∼0.1−0.2 mg of subunit per gel.

Indirect Detection of Glycosylation. *SDS−PAGE and Blotting.* Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) was carried out using a modification of the method of Laemmli (1970). For subunits 1Ax1 and 1Bx7 prepared from flour, uniform 10% (w/v) acrylamide minigels were run in a Mini-Protean II slab cell (Bio-Rad, Hemel Hempstead, U.K.). For other subunits prepared from gluten, gels with an exponential gradient from 10 to 13% acrylamide were run in a Pharmacia-LKB Multiphor II (Amersham Pharmacia Biotech, Freiburg, Germany) according to the method of Krause et al. (1988). Alternatively, precast ExcelGel SDS gels (Amersham Pharmacia Biotech, Freiburg, Germany) with a linear gradient of 8−18% acrylamide were used. Aliquots (7−10 μL) of sample solution (protein concentration = 0.5−3.0 mg/mL) were applied to the gels. Electrophoresis was performed at 10 °C for 180 min with a maximal voltage of 600 V, a current of 25 mA, and a maximal power of 25 W. Proteins were visualized by Coomassie Brilliant Blue staining (Westermeier, 1990) or by silver staining (Heukeshoven and Dernick, 1986). The samples were first fractionated by SDS−PAGE and then transferred onto a PVDF membrane. In the case of subunits 1Ax1 and 1Bx7, RP-HPLC-purified polypeptides were examined, whereas for other subunits, high M_r subunit-enriched fractions from the 60% (v/v) propan-1-ol selective precipitation stage (see above) were used.

DIG-Glycan Detection Procedure. The procedure described by Haselbeck and Hösel (1990) was used in initial studies to obtain indirect evidence for glycosylation. The DIG-Glycan Detection Kit (Boehringer Mannheim) was used in these experiments. Two proteins supplied with the kit, that is, transferrin and creatinase (recombinant from *Escherichia coli*), were used as positive and negative controls, respectively, in some experiments, and in others, ovalbumin, ovomucoid, and

Table 2. Sugar Compositions of High M_r Glutenin Subunits and Control Proteins

protein	mol wt	mol of amino acids/mol of protein	sugars reported to be present or sugar standards added and their molar ratios	total reported sugar content or amount of sugar standard added (mol of sugar/1000 mol of amino acids)	sugars detected in this work and their molar ratios	total sugar content found in this work
collagen[a]	3 × 93000	3 × 1000	Gal:Glc[b] (1.2:1.0)	2.9	Gal:Glc (1.5:1.0)	2.1
fetuin[a]	38418	359	Gal:Man:GlcNAc:GalNAc (7.8:5.1:8.3:1.0)	103.4	Gal:Man:GlcNAc:GalNAc (9.2:6.9:7.3:1.0)	93.9
ovalbumin[a]	42750	385	Man:GalNAc (1.8:1.0)	23.0	Man:GalNAc (2.1:1.0)	21.7
subunit 1Ax1	87680	809	no previous data	no previous data	none detected	none detected
subunit 1Ax1 plus sugar standards	87680	809	Man:GlcNAc (1.5:1.0)	2.5	Man:GlcNAc (4.0:1.0)	0.93
subunit 1Bx7	82865	770	Glc:Man:GlcNAc[c] (12.1:1.2:1.0)	162[c]	none detected	none detected
subunit 1Bx7 plus sugar standards	82865	770	Man:GlcNAc (1.0:1.0)	2.6	Man:GlcNAc (2.0:1.0)	0.87

[a] Carbohydrate contents of control proteins taken from following references: Schofield et al. (1971) (collagen), Spiro (1960) (fetuin), and Huang et al. (1970) (ovalbumin). [b] Gal, galactose; Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine. [c] Data for 1Bx7 from cv. Chinese Spring taken from Tilley et al. (1993). Molar ratios of sugars and total sugar content calculated from the data in the same reference.

soybean conglycinin were used as additional positive controls, and carbonic anhydrase B, bovine serum albumin, phosphorylase B, β-galactosidase, and myosin, which were part of the M_r marker mixture, were used as negative controls.

Concanavalin A Binding Procedure. The concanavalin A (Con A) binding method described by Hawkes (1982) was used in attempts to detect glycoproteins containing terminal mannose residues. The PVDF membranes were incubated with Con A and subsequently with horseradish peroxidase (HRP), a glycoprotein that binds to Con A. The resulting conjugate was detected through oxidation of 4-chloro-1-naphthol by HRP. Positive and negative controls were used as described above.

Characterization and Determination of Monosaccharides by Gas−Liquid Chromatography/Mass Spectrometry (GC/MS). The monosaccharides associated with proteins were analyzed directly by GC/MS of their alditol acetate or trimethylsilyl ether derivatives. Well-characterized proteins with various extents of glycosylation were used in some experiments as controls (Table 2). Ovalbumin (chicken egg) and fetuin (fetal bovine serum) were from Sigma; collagen (guinea pig skin) was a gift from Dr. C. A. Shuttleworth (University of Manchester, School of Biological Sciences, U.K.). Additional positive controls involved addition to subunits 1Ax1 and 1Bx7 of both mannose and *N*-acetylglucosamine, calculated to mimic a glycosylation level of 1 residue of each sugar per subunit. At least 5 mg of protein was analyzed in the case of the subunits 1Ax1 and 1Bx7 and the collagen samples, in view of their expected very low levels of glycosylation.

For subunits 1Ax1 and 1Bx7 derived from flour, samples were hydrolyzed with 4 M TFA for 4 h under N_2 at 100 °C in glass tubes fitted with Teflon-faced rubber-lined screw caps. The hydrolysates were then freeze-dried directly. A blank containing the solvents only was also prepared and taken through all of the different steps. Alditol acetate derivatives were prepared essentially as described by Blakeney et al. (1983) from the dry hydrolyzed samples. After being taken up in dichloromethane, some samples were concentrated 4-fold under a flow of nitrogen.

In the case of the other subunits derived from gluten, the samples were hydrolyzed with 0.5 M methanolic HCl for 4 h at 90 °C, reacetylated with acetic anhydride, and silylated with a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10:2:1 v/v/v) according to the method of Chambers and Clamp (1971). An internal standard, $^{13}C_6$-glucose, was added to the samples prior to methanolysis.

Alditol acetate derivatives were separated by gas−liquid chromatography (GC) on a polar capillary column [BPX70, 12 m × 0.32 mm i.d., SGE (U.K.) Ltd., Milton Keynes] with a 0.25 μm stationary phase in a Hewlett-Packard HP5890 Series II gas chromatograph equipped with a split−splitless injector. An aliquot (1 μL) of the sample in dichloromethane was injected at 250 °C, with a split ratio of 1:50. Helium was used as carrier gas at a flow rate of 2.9 mL/min. The following temperature gradient was used: 180 °C for 0.5 min, then a programmed rate of 5 °C/min to 250 °C, and 250 °C for 5 min. The gas chromatograph was interfaced with a Hewlett-Packard 5972 mass spectrometer. The electron-impact mass spectra were recorded at 70 eV with an ionization current of 50 μA, a source temperature of 175 °C, and a transfer line temperature of 280 °C. The scan ran from *m*/*z* 400 to 33 (mass-to-charge ratio). The carbohydrate peaks were verified against our prepared sugar standards and spectra from the MS library.

Trimethylsilyl ether (TMS) derivatives were separated by GC using an apolar stationary phase (SE 30) on a 0.12 μm support in a capillary column (60 m × 0.32 mm i.d., J&W Scientific, Folsom, CA) fitted into a Carlo Erba 5300 gas chromatograph (Carlo Erba, Hofheim, Germany) equipped with an on-column injector. An aliquot (0.5−1.0 μL) of the sample in *n*-pentane was injected at 35 °C. Helium was used as carrier gas at a flow rate of 3 mL/min. The following gradient was used: 35 °C for 1 min; then programmed rates of 40 °C/min to 100 °C and 8 °C/min to 300 °C; 300 °C for 15 min. A Finnigan MAT 8230 mass spectrometer (Finnigan MAT, Bremen, Germany) was used as detector. The transfer line was heated at 300 °C. Negatively charged ions were generated by chemical ionization with ammonia at 115 eV using a source temperature of 250 °C, and the scan ran from *m*/*z* 700 to 350. The carbohydrate derivatives were identified by comparison with spectra of sugar standards.

Deglycosylation Studies. Enzymic deglycosylation was performed on tryptic or chymotryptic digests of the alkylated high M_r subunit 1Bx7 from CWRS with *N*-glycosidase F (for N-linked glycosides) and *O*-glycosidase/neuraminidase (for O-linked glycosides) (Thotakura and Bahl, 1987). The RP-HPLC patterns of untreated and glycosidase-treated peptides were then compared.

Subunit 1Bx7 (0.1 mg) was suspended in 0.05 M Tris-HCl, pH 8.0, and 0.05 M $CaCl_2$ (1 mL) containing TLCK-treated bovine α-chymotrypsin (10 μg) and digested for 6 h at 37 °C. For tryptic digestion, the buffer was the same except the $CaCl_2$ concentration was 0.02 M. Hydrolysis was performed with TPCK-treated bovine trypsin (10 μg) for 2 h at 37 °C. The partial hydrolyses were stopped by heating at 100 °C for 10 min. The solutions were injected onto an ODS Hypersil analytical HPLC column (C_{18}, 10 nm pore size, 5 μm particle size, 4.6 × 250 mm; Shandon, Frankfurt, Germany). Chromatography was carried out at 60 °C using a flow rate of 0.8 mL/min and a buffer system comprising (A) 0.01 M triethyl-

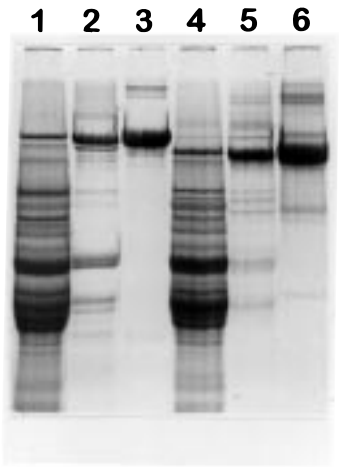

Figure 1. SDS−PAGE of total flour polypeptides and high M_r glutenin subunit preparations from cv. Galatea (lanes 1−3) and cv. Gabo double-null (lanes 4−6) (detection by Coomassie Brilliant Blue staining): (lanes 1 and 4) total flour polypeptides; (lanes 2 and 5) high M_r subunit mixtures obtained by the selective precipitation procedure of Marchylo et al. (1989); (lanes 3 and 6) high M_r subunits subsequently purified by semipreparative RP-HPLC after the selective precipitation procedure.

amine/formic acid, pH 3.5, and (B) 80% (v/v) ACN and 7.65 mM triethylamine/formic acid, pH 3.5. The following gradient was used: 0−60 min, linear, 5−32.5% B; 60−90 min, linear, 32.5−70% B. The column effluent was monitored at 220 nm.

Location of the Glycosylation Site(s). To determine the protein/carbohydrate binding sites, the RP-HPLC-purified, alkylated high M_r glutenin subunit 1Dy12 of cv. Apollo (0.6 mg) was digested with TPCK-treated trypsin in buffer (6 mL) as described above. The peptides were separated by RP-HPLC as described above. Several preparative runs were carried out. Appropriate portions of the effluents containing particular peptides were pooled and the solutions dried under a stream of N_2. Carbohydrates were identified in the tryptic peptides by GC/MS as described above.

Other Analytical Methods. Total carbohydrate was determined colorimetrically using a thymol−sulfuric acid procedure (Kakac and Vejdelek, 1974), and starch was determined by an enzymic procedure as described by the manufacturer (Boehringer-Mannheim, 1983).

RESULTS

Isolation of High M_r Glutenin Subunit Fractions and of Individual High M_r Glutenin Subunits. SDS−PAGE analysis (Figure 1) showed that subunits 1Ax1 and 1Bx7, which were purified from flour by selective extraction and precipitation and RP-HPLC, were free of contamination, apart from some high molecular weight material, which may be attributed to aggregation during the freeze-drying step. The gels were deliberately overloaded with the purified subunits to show the extent of contamination by polypeptides other than high M_r subunits. High M_r subunits were also isolated from gluten on the premise that the gluten washing out procedure might first of all remove large amounts of potentially contaminating carbohydrate, in particular starch. This was considered important especially if levels of glycosylation in the subunits were low as seemed likely on the basis of previous research (Hickman et al., 1995). The gluten preparations were found to contain ∼2.5% (w/w) residual starch, as determined enzymically, which was removed, along with gliadins, by extraction with 90% (v/v) DMSO. No starch was detected in the glutenin residue after DMSO extraction. Colorimetric determination of total carbohydrate in the glutens indicated that they contained ∼4% (w/w) total carbohydrate. The total carbohydrate content of the glutenin residues after DMSO extraction was ∼2.5% (w/w). High M_r subunit-enriched fractions were prepared from the glutenin residues after DMSO extraction, again by a selective precipitation method (Marchylo et al., 1989), and those fractions were found to contain ∼1% (w/w) total carbohydrate.

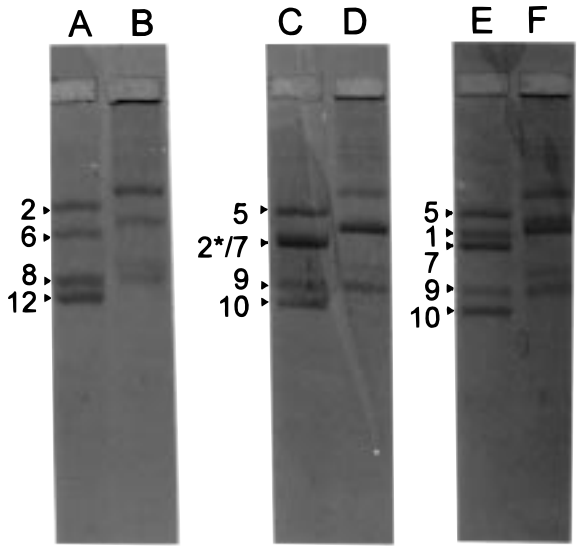

Figure 2. A-PAGE separation of high M_r subunits of glutenin obtained by the selective precipitation procedure of Marchylo et al. (1989) (detection by Coomassie Brilliant Blue staining): (lanes A, C, and E) alkylated subunits; (lanes B, D, and F) reduced but unalkylated subunits. The cultivars or provenances of the wheats were Apollo (lanes A and B), CWRS (lanes C and D), and Monopol (lanes E and F).

Individual reduced and alkylated high M_r subunits from cv. Apollo were then isolated according to two procedures. The first was by RP-HPLC using the volatile solvent system described above. The identities of the individual subunits were confirmed by amino acid analysis and SDS−PAGE (results not shown). Because the RP-HPLC conditions (pH <2.0, temperature = 60 °C, pressure = 8 MPa, time = 20−60 min) were relatively harsh, the high M_r subunits were also separated by preparative A-PAGE. Both reduced and reduced/alkylated subunits from cvs. Apollo and Monopol and CWRS wheat were generally well separated by A-PAGE, with the exception of subunits 1Ax2* and 1Bx7 of CWRS wheat (Figure 2). Subunits from cv. Apollo were purified by preparative A-PAGE. The zones of the gel containing subunits were cut out and eluted with 50% (v/v) propan-1-ol and desalted by centrifugation on Centricon 30 membranes. Except for subunit 1By8, which contained a small amount of subunit 1Dy12, the individual subunits were pure as determined by SDS−PAGE (Figure 3) and by analytical RP-HPLC (results not shown). The yields were 0.1−0.2 mg of subunit per gel.

Detection of Glycosylation Using the DIG-Glycan Detection Procedure with Polypeptides Immobilized on PVDF Membranes. Tilley et al. (1993) were the first to apply the DIG-Glycan Detection procedure of Haselbeck and Hösel (1990) to investigate the possibility that high M_r glutenin subunits might be glycosylated. They obtained positive results for subunits from cvs. TAM 105 and Chinese Spring, suggesting that the subunits are glycosylated. In the present

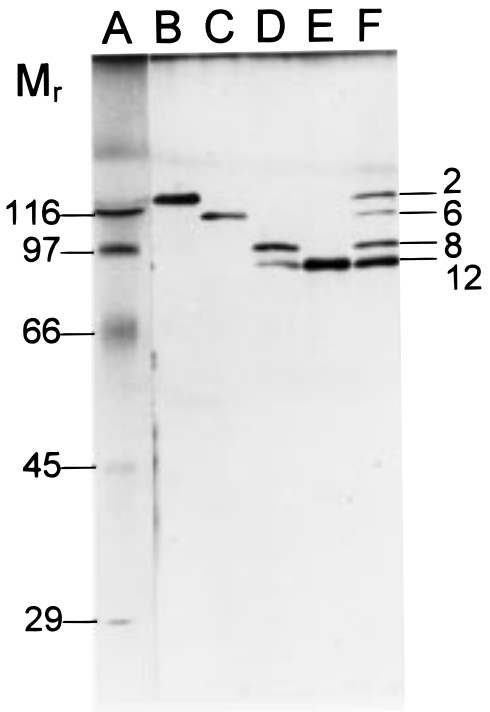

Figure 3. SDS−PAGE of individual alkylated high M_r glutenin subunits obtained by preparative A-PAGE from cv. Apollo (detection by silver staining): (lanes B−E) high M_r subunits purified by preparative A-PAGE; (lane F) high M_r subunits prepared by the selective precipitation procedure of Marchylo et al. (1989) before separation by preparative A-PAGE. Relative molecular mass (M_r, thousands) marker proteins were carbonic anhydrase (29K), ovalbumin (45K), bovine serum albumin (66K), phosphorylase B (97K), and β-galactosidase (116K).

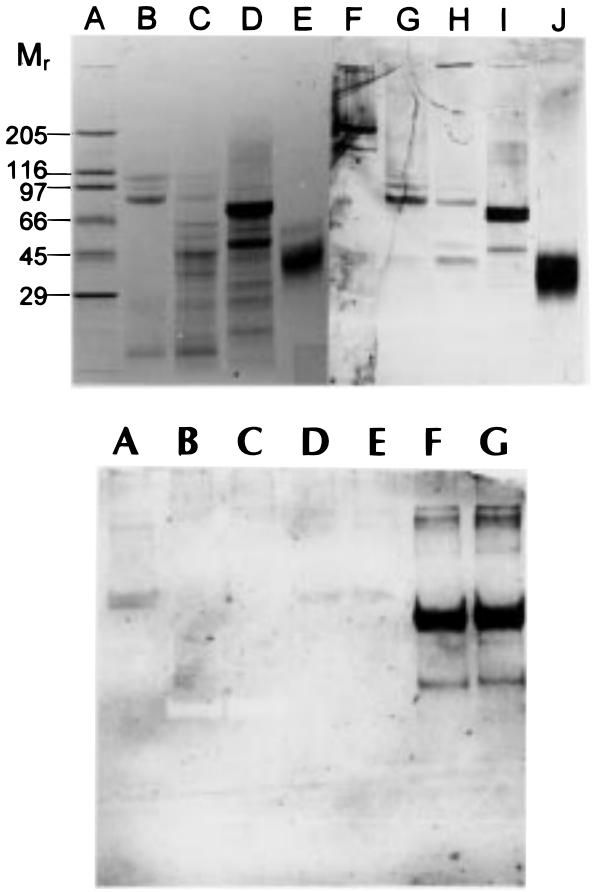

Figure 4. Detection of glycosylation after SDS−PAGE and blotting using the DIG-Glycan Detection Kit. (a, top) (Lanes A−E) protein detection in the SDS−PAGE gel by Coomassie Brilliant Blue staining; (lanes F−J) glycoprotein detection with the DIG-Glycan Detection Kit. M_r values are given in thousands. M_r marker proteins in lanes A and F were carbonic anhydrase (29K), ovalbumin (45K), bovine serum albumin (66K), phosphorylase B (97K), β-galactosidase (116K), and myosin (205K). (Lanes B and G) high M_r subunits of cv. Rektor prepared by the selective precipitation procedure of Marchylo et al. (1989); (lanes C and H) reduced total glutenin of cv. Rektor; (lanes D and I) soybean conglycinin (7S fraction); (lanes E and J) ovomucoid from hen egg white. (b, bottom) Glycoprotein detection with the DIG-Glycan Detection Kit: (lane A) high M_r subunit 1Bx7 reduced and alkylated; (lanes B and C) creatinase; (lanes D and E) high M_r subunit 1Bx7 reduced but not alkylated; (lanes F and G) transferrin.

research, purified subunits 1Ax1 and 1Bx7, derived from flour as the starting material, were analyzed using the DIG-Glycan Detection procedure, as well as high M_r subunit-containing fractions obtained by selective extraction and precipitation and derived from glutens as starting materials. The labeling of the carbohydrate moieties and the subsequent detection with the anti-digoxigenin antibodies were all performed on the PVDF membranes. Although less sensitive, this is the procedure recommended by the manufacturer of the kit (Boehringer Mannheim) as it is less susceptible to interference by contaminants in the sample preparation; additionally, the solubility properties of the high M_r glutenin subunits are not suited to the alternative oxidation and digoxigenin labeling in solution prior to electrophoresis. The positive controls, transferrin, ovalbumin, ovomucoid, and conglycinins, appeared as darkly stained bands (Figure 4). Unglycosylated controls, such as creatinase, were not stained.

Figure 4a shows the results of experiments performed on total high M_r subunit fraction derived from cv. Rektor gluten as starting material by selective extraction and precipitation. All of the subunits gave positive reactions. Identical results were obtained; that is, the high M_r subunits gave positive reactions, with similarly prepared partially purified subunit preparations derived from flours as starting materials (data not shown). Subunits 1Bx7 (Figure 4b) and 1Ax1 (data not shown), purified from flour by RP-HPLC, were also positively stained in the DIG-Glycan Detection procedure. These findings are consistent with those of Tilley et al. (1993). It was noted that some low M_r subunits of glutenin also gave positive staining in the DIG-Glycan Detection procedure (Figure 4a, lane H).

Interestingly, in the experiments with subunits derived from glutens as starting materials, high amounts of unglycosylated control proteins were found to give faint positive reactions. Therefore, $\sim^1/_{15}$ of the amount of polypeptide that gave false positive results was used when the glutenin subunits were tested to minimize the possibility of false positive results with the subunits. Moreover, in experiments with subunits 1Ax1 and 1Bx7, when the periodate oxidation step was omitted, transferrin, and, more notably, subunit 1Bx7 again reacted positively (results not shown), indicating presumably that the digoxigenin-substituted hydrazide reacted with non-carbohydrate-derived groups present on the polypeptides; subunit 1Ax1 was not tested in this manner. This phenomenon has been observed previously (Roels and Delcour, 1996) and could account for false positive results with this DIG-Glycan Detection method.

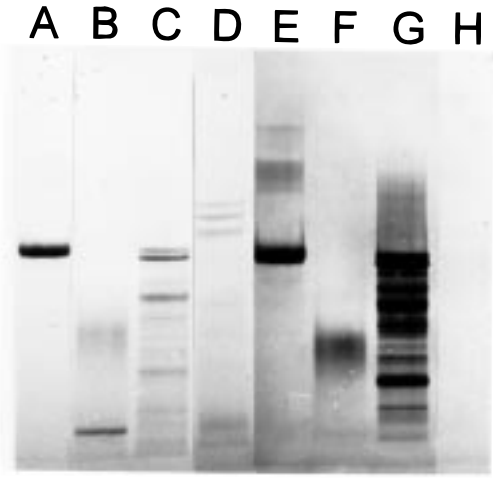

Figure 5. Detection of glycosylation after SDS−PAGE and blotting using the Con A method according to Hawkes (1982): (lanes A−D) protein detection in the SDS−PAGE gel by Coomassie Brilliant Blue staining; (lanes E−H) glycoprotein detection using Con A; (lanes A and E) transferrin; (lanes B and F) ovomucoid from hen egg white; (lanes C and G) soybean conglycinin (7S fraction); (lanes D and H) total high M_r glutenin subunits from cv. Rektor prepared by the selective precipitation procedure of Marchylo et al. (1989).

Detection of Glycosylation by the Concanavalin A Method. The Con A procedure described by Hawkes (1982) was applied to high M_r subunit mixtures obtained by the selective precipitation method of Marchylo et al. (1989) as well as to positive and negative control proteins. All of the positive proteins were stained strongly, whereas all of the negative controls and the high M_r subunits of glutenin were unstained (Figure 5). Because Con A has a high affinity for terminal mannose residues and a much lower specificity for glucose and *N*-acetylglucosamine, it could be concluded that the high M_r subunits examined here did not contain mannose, glucose, or *N*-acetylglucosamine as terminal sugars.

Detection of Glycosylation by Direct Analysis of Monosaccharides by GC/MS. The results obtained using the DIG-Glycan Detection procedure were generally consistent with those of Tilley et al. (1993) and appeared to confirm that high M_r glutenin subunits are glycosylated. Attempts were subsequently made to confirm these preliminary observations by direct chemical analysis of the monosaccharides involved.

High M_r glutenin subunits of cv. Apollo, isolated using gluten as starting material by selective extraction/precipitation followed by either RP-HPLC or A-PAGE, were subjected to methanolysis, a method in which methanolic HCl is used to release the monosaccharides. After methanolysis, the sample is acetylated with acetic anhydride because the *N*-acetyl groups of *N*-acetylhexosamines, if present, are removed by methanolysis. TMS derivatives of the sugars were then prepared. Qualitative examination of the chromatograms for the separation of TMS derivatives by GC/MS showed that glucose was present in all of the cv. Apollo subunits isolated by RP-HPLC or A-PAGE. Mannose, xylose, and galactose were detected in all of the subunits purified by A-PAGE but only in some of those isolated by RP-HPLC. Hexosamines were not detected. This is an important observation because in many glycoproteins the site of glycosylation occurs through Asn-*N*-acetylglucosamine or Ser/Thr-*N*-acetylgalactosamine linkages (Sharon and Lis, 1981). Tilley and Schofield (1994) had previously obtained indirect evidence that high M_r subunits might contain covalently bound hexosamine. The absence of hexosamines but the presence of mannose, xylose, and galactose in partially purified subunits or of glucose in purified subunits, respectively, agrees with findings reported elsewhere (Roels and Delcour, 1996).

Quantitative determination of the sugars associated with subunits 1Dx2, 1Bx6, 1By8, and 1Dy12 of cv. Apollo isolated by A-PAGE using $^{13}C_6$-glucose as internal standard was not possible because the areas of the glucose peaks in all samples exceeded the linear range of the detector. On the basis of the highest possible area within the linear range, minimal monosaccharide contents of 10% were calculated for the high M_r subunits. Such high amounts of carbohydrate can be explained only by the presence of free carbohydrates in the samples. This was confirmed by the analysis of gel slices in which protein was not detected. In this case almost the same amounts of sugars were found.

Consequently, further experiments with cv. Apollo subunits were conducted on RP-HPLC-purified polypeptides only. Monosaccharides associated with high M_r subunits from cv. Apollo were released by methanolysis and quantified by isotope dilution analysis using $^{13}C_6$-glucose as internal standard. In this case, only glucose, in amounts of 0.10% (w/w) (subunit 1Dx2), 0.19% (w/w) (subunit 1Bx6), 0.17% (w/w) (subunit 1By8), and 0.24% (w/w) (subunit 1Dy12), was identified, which is in agreement with previously reported results (Roels and Delcour, 1996). Assuming a molecular weight of 80 000 for high M_r glutenin subunits, these experiments indicate that only one monosaccharide residue at most could be linked to each subunit. However, it should also be noted that glucose is a relatively uncommon monosaccharide constituent of glycoproteins and that, because of the very large proportion of starch in wheat flour, contamination with glucans derived from starch is a real possibility. It is also worth noting that the cv. Apollo subunits analyzed here were purified more extensively (gluten isolated by aqueous washing from flour, DMSO extraction of *gluten* to remove starch, gliadin, and other low M_r proteins, selective precipitation with propan-1-ol of high M_r subunits, and separation of individual subunits by RP-HPLC) than the subunits that have been analyzed in some other work [extraction of *flour* first with DMSO then with 70% (v/v) ethanol and separation of individual subunits present in the residue by preparative SDS−PAGE (Tilley et al., 1993; Tilley, 1997)]. This probably accounts for the considerably lower levels of glucose (0.1−0.24% w/w) found to be associated with the cv. Apollo subunits and the absence of mannose in those subunits in the present work than were found to be associated with subunit 1Dx2 (0.88% w/w glucose and a total of 0.32% w/w mannose) from cv. Chinese Spring (Tilley, 1997).

In experiments with RP-HPLC-purified subunits 1Ax1 and 1Bx7, the samples were hydrolyzed using 4 M TFA, alditol acetate derivatives were prepared, and the derivatives were analyzed by GC/MS. A number of proteins, for which the amounts and nature of the carbohydrate components are well established (Table 2), were used as controls. The collagen sample was of particular interest as its level of glycosylation is very close to that which would be present in glutenin subunits if they were glycosylated at levels of about ∼1−2 sugar residues per subunit (∼2−2.5 sugar resi-

dues per 1000 amino acid residues). Thus, if glycosylation could be detected and quantified for the collagen sample, even very low levels of glycosylation should also be detectable and quantifiable in the glutenin subunits. The sugar standards (arabinose, galactose, glucose, mannose, xylose, *N*-acetylglucosamine, *N*-acetylgalactosamine) were all resolved as single peaks, and their mass spectra matched those in the MS library, when available.

The monosaccharides present in the control proteins are shown in Table 2. They were found to be as expected on the basis of results reported previously in the literature, in terms of the identities of the sugars present (as confirmed by the MS data) and their molar ratios. In particular, the monosaccharides associated with collagen were easily identified and quantified. The recoveries of sugars that were added deliberately to the subunit samples were rather low. This may have been due to the fact that free sugars were present throughout the hydrolysis period and thus were susceptible to degradation, whereas sugars in the glycoproteins first of all had to be released from glycosidic linkage by hydrolysis, making glycosidically linked sugars in the control glycoproteins less susceptible to degradation. Such a hypothesis would be consistent with the high ratio of mannose to *N*-acetylglucosamine in the sugars recovered after deliberate addition to the glutenin subunits because hexosamines are well-known as being more susceptible to degradation during hydrolysis than hexoses. Despite the rather low recoveries of sugars added to the glutenin subunits during hydrolysis, those sugars, which were added at levels to simulate a level of glycosylation of ~1 residue of sugar per molecule of subunit, were easily detected and quantified. In fact, it could be calculated that a level of glycosylation as low as 1 sugar residue per 40 molecules of subunit would have been detectable under our experimental conditions.

Hydrolysis of the subunits 1Ax1 and 1Bx7, in amounts greater than those that had been used for the other proteins, also failed to reveal the presence of sugars (results not shown). Particular attention was paid to the areas of the chromatogram where mannose and *N*-acetylglucosamine would have been present, as evidence for the presence of those sugars in high M_r subunits had been presented in other studies (Tilley et al., 1993; Tilley and Schofield, 1994). Analysis of selectively extracted and precipitated high M_r subunit 1Bx7 prior to RP-HPLC purification and of a similarly prepared glutenin sample from wheat cv. Laura (results not shown) did, however, show the presence of high levels of glucose contaminating these fairly crude preparations, as also shown by Roels and Delcour (1996). We speculate that our observation that sugars were absent from the RP-HPLC-purified 1Ax1 and 1Bx7 subunits, whereas very small amounts of sugar were associated with the cv. Apollo subunits, which were also prepared using RP-HPLC as a final purification step, might be accounted for by the use of a new RP-HPLC column in the preparation of subunits 1Ax1 and 1Bx7. The column had, therefore, not been exposed previously to extracts of wheat flour, whereas the use of an older column that had been used for fractionating wheat extracts may have resulted in slight carry-over of contaminating glucan from previous experiments.

Detection of Glycosylation Using Enzymic Deglycosylation. Another approach that was used in trying to establish whether high M_r subunits are glycosylated was to use enzymic deglycosylation and to determine its effects on the properties of the residual polypeptide moieties. The enzymes *N*-glycosidase F and *O*-glycosidase/neuraminidase, which release N- and O-linked glycans, respectively, were used, and, because they act more efficiently with low molecular weight, soluble substrates, tryptic or chymotryptic digests of high M_r glutenin subunits were used, rather than the intact polypeptides. RP-HPLC chromatograms of untreated and glycosidase-treated digests were then compared. The carbohydrate-containing peptides may be identified by a change in their retention times after cleavage of the glycan moiety. However, the peptide patterns were identical with or without deglycosylation (results not shown), thus providing no evidence for the presence of a peptide-linked glycan.

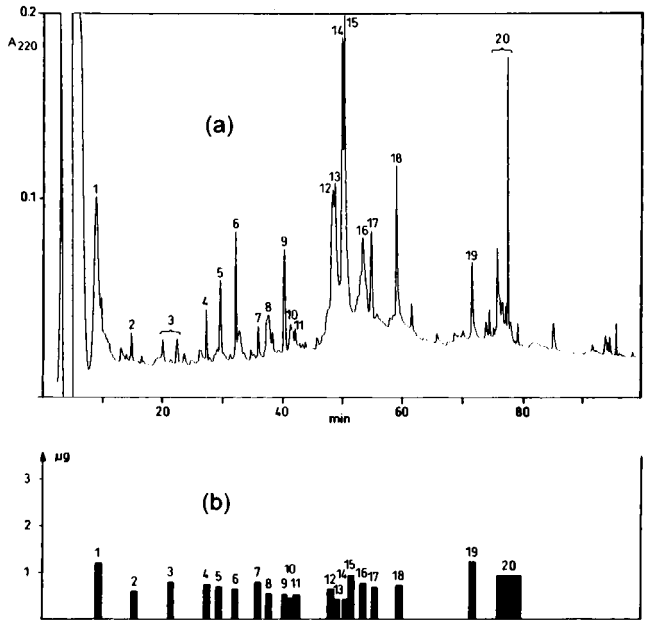

Figure 6. RP-HPLC profile of tryptic peptides of subunit 1Dy12 from cv. Apollo (a) and their associated monosaccharide contents (b).

In a further experiment, single tryptic peptides from cv. Apollo subunit 1Dy12 (total carbohydrate content 0.24%, w/w) were isolated by RP-HPLC and analyzed directly for the presence of monosaccharides by GC/MS of trimethylsilyl ether derivatives. Glucose was found in all of the peptides together with small amounts of mannose and xylose; they were evenly distributed over the whole elution profile (Figure 6). Neither *N*-acetylglucosamine nor *N*-acetylgalactosamine was ever present. It is noteworthy that the column effluent corresponding to regions of the chromatograms that did not contain peptides was found to contain 27, 24, and 84% of the total glucose, mannose, and xylose, respectively. Therefore, sugars seemed to be eluted continuously as contaminants, rather than in specific association with one or more of the peptides derived from the subunit, and again this suggests the possibility of carry-over from previous experiments.

GENERAL DISCUSSION

The possible contribution to functionality resulting from an association of carbohydrates with the gluten proteins has been an incentive for a number of investigations. Older studies, such as those published by Graveland et al. (1979) and McMaster and Bushuk

(1983), have described the isolation of carbohydrate-rich fractions from gluten. However, after analysis of the constituent monosaccharides (chiefly arabinose, xylose, and glucose), these carbohydrates were suggested to derive probably from pentosans or starch. It was also emphasized that they formed a stable complex with the protein moiety (especially of glutenin-type) and that their amounts varied with the purification procedure; no evidence for covalent linkages was ever presented. Chen et al. (1992a) provided more compelling evidence for a close association by showing that sugars were coincident with the proteins after size exclusion chromatography or SDS−PAGE. These authors (Chen et al., 1992b) also attempted to show whether the association was covalent or not by preparing carbohydrate-containing peptides from gluten, but the results were inconclusive.

Concentrating on single polypeptides, and consistent with previously reported research (Tilley et al., 1993), the results of our experiments in which the presence of carbohydrate was determined indirectly by the DIG-Glycan Detection procedure led us initially to believe that high M_r glutenin subunits were glycosylated. However, a number of well-known factors can give rise to false positive results when using this technique.

Comigration of carbohydrate moieties, whether in the form of glycans/oligosaccharides or glycopeptides, with the polypeptides of interest during gel electrophoresis can be a source of contamination, as shown by our A-PAGE results. Additionally, we have indicated in the present study that the digoxigenin-substituted hydrazide is able to react with non-carbohydrate-derived groups present on the polypeptides. This phenomenon has also been described by Roels and Delcour (1996). Moreover, the same authors observed positive reactions when using subunits expressed in *E. coli* and postulated they were more likely due to intrinsic properties of the proteins rather than sample contamination. The DIG-Glycan Detection method cannot be relied upon as an absolute means of determining whether gluten proteins are glycosylated.

Attempts to detect glycosylation under mild conditions with the lectin, Con A, showed that it did not bind to the high M_r subunits. Because Con A has high affinity for terminal mannose residues and lower affinity for glucose and *N*-acetylglucosamine, those results indicate that those sugars are not present in the high M_r subunits and to that extent provide further evidence for the lack of glycosylation of those subunits. However, because of its specificity, Con A binding is not suitable for providing evidence of glycosylation with other types of sugars.

In the present work, direct analysis of sugars associated with RP-HPLC-purified subunits 1Dx2, 1Bx6, 1By8, and 1Dy12 of cv. Apollo by GC/MS of trimethylsilyl ether derivatives showed the presence of glucose, in amounts of only one sugar residue per subunit at most for subunit 1Dy12, which is consistent with results reported elsewhere (Roels and Delcour, 1996). In fact, most of the subunits contained glucose at levels below this, but, because high M_r subunits are derived from flour (starch content of ~80% w/w), the presence of glucose should be regarded with considerable caution because contamination with starch or starch-derived saccharides is a strong possibility.

The finding that glucose was present in subunits purified by A-PAGE and, in some cases, mannose, xylose, and galactose were present also, must be treated with the same caution, especially because regions of the gels that did not contain polypeptides were, nevertheless, found to contain the same sugars in the same amounts. The importance of the method of purification has been highlighted previously (Roels and Delcour, 1996), when arabinose, xylose, mannose, glucose, and galactose were detected in partially purified subunits of cv. Rektor, but only glucose was detected after further purification involving ion-exchange and RP-HPLC chromatographic steps.

Direct analysis of the monosaccharides associated with RP-HPLC-purified subunits 1Ax1 and 1Bx7 by GC/MS of alditol acetate derivatives performed in the present study showed that no sugars were present. Furthermore, experiments aimed at performing deglycosylation and also at identifying the site(s) of glycosylation on RP-HPLC-purified material gave negative results and provided further evidence that any sugars detected were present as contaminants that were not covalently bound to the glutenin subunits.

This is in contrast to conclusions reached by Tilley (1997), who provided evidence purporting to show the presence of low levels of mannose linked *O*-glycosidically to high M_r glutenin subunit 1Dx2 isolated from cv. Chinese Spring. This was based on the detection of mannitol after a β-elimination reaction was performed in the presence of sodium borohydride on the subunit that had been prepared by selective extraction and preparative electrophoresis. Less than one residue of mannitol was detected per molecule of high M_r subunit, indicating that glycosylation was substoichiometric, if, indeed, the mannitol detected was actually derived from mannose that was O-glycosidically linked to the high M_r subunit and not to a contaminant. The results presented here, and those obtained by Roels and Delcour (1996), indicate that this is a very real possibility and that such contamination is difficult to remove by differential extraction and preparative electrophoresis as used by Tilley (1997). Furthermore, Tilley (1997) found ~7 times more glucose than mannitol and also slightly more mannose than mannitol after methanolysis of the entire products of β-elimination of high M_r subunit 1Dx2. Those levels of sugars (equivalent to ~6 mol of sugar per subunit or ~7.5 mol of sugar per 1000 mol of amino acids) are considerably less than the levels reported previously by Tilley et al. (1993) to be associated with high M_r subunits purified in the same way. In that paper, subunit 1Bx7 from cv. Chinese Spring was reported to contain glucose, mannose, and *N*-acetylglucosamine in molar ratios of about 12:1:1 and the total sugar content was ~162 mol of sugar per 1000 mol of amino acids [values calculated from data presented by Tilley et al. (1993)]. As noted above, the presence of glucose at levels considerably above those of mannitol, together with the variability in the levels of glucose associated with some preparations of purified high M_r subunits in the present and previous studies (Tilley et al., 1993; Roels and Delcour, 1996; Tilley, 1997), is a cause for considerable concern in that glucose is an uncommon sugar component of glycoproteins [although it does occur in some, such as collagen (Schofield et al., 1971)] and in view of the very high levels of glucan (starch) and lower, but still significant, levels of glucomannan that are present in wheat flour.

In fact, the evidence for mannose actually being linked to the high M_r subunit analyzed (1Dx2; Tilley,

1997) is incomplete. There is no reason to doubt that O-glycosidically linked mannose was detected, but the O-glycosylation site on the polypeptide, that is, the sequence of amino acids around the serine or threonine residue to which the mannose was linked, was not characterized and, therefore, was not identified as part of the known amino acid sequence of the high M_r subunit. Thus, the conclusion that the serine or threonine residue to which the mannose was linked formed part of the structure of high M_r subunit 1Dx2 is not supported by the data presented. It seems most likely, in fact, that the sugars associated with the subunits analyzed by Tilley et al. (1993) and Tilley (1997) were present in contaminating non-gluten oligo- or polysaccharides or glycoproteins.

Tilley (1997) commented on the work of Roels and Delcour (1996) and some of the present work, which was published in a preliminary form (Bollecker and Schofield, 1996), suggesting that the analytical techniques used were insufficiently sensitive to detect the levels of glycosylation claimed by Tilley (1997) and implying that the conclusion that the high M_r subunits are unlikely to be glycosylated was erroneous because the subunit preparations examined in both of those studies were not pure. Neither criticism withstands close scrutiny. We have carried out careful control experiments in the present work, both with well-characterized glycoproteins, especially with skin collagen, which is glycosylated at levels similar to those claimed for high M_r subunits by Tilley (1997), and with synthetic mixtures of high M_r subunits and sugars added to the subunit preparations at levels intended to simulate the *minimum* levels of O-glycosylation claimed by Tilley (1997), that is, one residue of glycosidically linked mannose per molecule of high M_r subunit. In both cases, the GC/MS method used was able to detect and quantify the sugars easily, and both the nature and the amounts of sugar determined were very similar to those expected (Table 2). In fact, it could be calculated that the sugar analysis procedure would have been capable of detecting as low as 1 mol of sugar/40 mol of high M_r subunit. Lack of sensitivity of the analytical method used here patently cannot be an explanation of the failure to detect glycosylation of the high M_r subunits.

The assertion that the 1Ax1 and 1Bx7 high M_r subunits examined here were not completely pure (Tilley, 1997) is to some extent valid in that minor bands, particularly some that were of higher M_r than the high M_r subunits, were present when the subunits were deliberately overloaded during analysis by SDS−PAGE (Figure 1) and that minor bands were also detected by Roels and Delcour (1996) in their high M_r subunit preparations. This is not uncommon with RP-HPLC-purified high M_r subunits. Because neither the parent flour nor the high M_r subunit-enriched fraction that was applied to the RP-HPLC column contained those minor bands, however, we attributed their presence possibly to aggregation of the high M_r subunits occurring during freeze-drying. Regardless of whether this interpretation is correct, given that our analytical method could detect 1 mol of sugar/40 mol of high M_r subunit, the level of contamination of our high M_r subunit preparations with unglycosylated proteins or other non-carbohydrate material would have had to have been at such unrealistically high levels (at least 40-fold greater than the level of high M_r subunits) as to make untenable the possibility of contamination an explanation for our failure to detect glycosylation. In fact, contamination is much more likely to be a problem when one attempts to determine whether a protein is glycosylated by analyzing very small amounts of protein using very sensitive techniques, as was the case in Tilley's work (Tilley, 1997). Under such circumstances, incomplete removal of oligo- and polysaccharides not covalently linked to the protein and/or contamination of glassware/plasticware used during preparation of the protein and processing during analysis, for example, by dust from the atmosphere and/or by adsorption of contaminants onto glass surfaces during previous use, are real possibilities; contamination of buffers, solvents, reagents, etc., may further exacerbate such problems. These are well-known problems in carrying out protein chemistry work at high levels of sensitivity.

Considering the evidence presented to date, the case for even low, substoichiometric levels of glycosylation of high M_r subunits of glutenin is far from being proven. On the basis of the research presented here, and that presented by Roels and Delcour (1996), it appears very unlikely, in fact, that high M_r glutenin subunits are glycosylated, and certainly not at levels that would be envisaged as having a significant effect on the functionality of glutenin in baking.

ABBREVIATIONS USED

ACN, acetonitrile; A-PAGE, polyacrylamide gel electrophoresis at acid pH; cv., cultivar; Con A, concanavalin A; CWRS, Canada Western Red Spring; DMSO, dimethyl sulfoxide; GC/MS, gas−liquid chromatography/mass spectrometry; M_r, relative molecular mass; PVDF, poly(vinylidene difluoride); RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TLCK, *N*-tosyl-L-lysine-chloromethyl ketone; TMS, trimethylsilyl ether; TPCK, *N*-tosyl-L-phenylalanine-chloromethyl ketone.

ACKNOWLEDGMENT

We thank Mrs. D. Schweiger-Recknagel, Mrs. A. Wiesner, Mr. C. J. Humphrey, and Mr. A. Dodson for their excellent technical assistance and also Drs. P. I. Payne, F. MacRitchie, and R. Gupta for supplying flour samples and Dr. C. A. Shuttleworth for providing the collagen sample.

LITERATURE CITED

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharides analysis. *Carbohydr. Res.* **1983**, *113*, 291−299.

Boehringer-Mannheim. *UV-Test combination for the determination of native starch in food and other materials*, Boehringer-Mannheim, Mannheim, Germany, 1983.

Bollecker, S. S. J.; Schofield, J. D. Glycosylation of high M_r subunits of glutenin: A re-examination. In *Gluten '96*; Wrigley, C. W., Ed.; Royal Australian Chemical Institute: Melbourne, 1996; pp 173−176.

Burnouf, T.; Bietz, J. A. Rapid purification of wheat glutenin for reversed-phase high performance liquid chromatography—Comparison of dimethyl-sulfoxide with traditional solvents. *Cereal Chem.* **1989**, *66*, 121−127.

Chambers, R. E.; Clamp, J. R. An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochem. J.* **1971**, *125*, 1009−1018.

Chen, J.; Khan, K.; Shelton, D. R.; D'Appolonia, B. L. Isolation and fractionation of carbohydrate-containing proteins from wheat gluten. *Cereal Chem.* **1992a**, *69*, 475−480.

Chen, J.; Shelton, D. R.; D'Appolonia, B. L.; Khan, K. Structural characterisation of the carbohydrate portion of a glycopeptide from wheat gluten. *Cereal Chem*. **1992b**, *69*, 481−484.

Friedman, M.; Krull, L. H.; Cavins, J. F. The chromatographic determination of cystine and cysteine residues in proteins as S-β-(4-pyridylethyl)cysteine. *J. Biol. Chem*. **1970**, *245*, 3868−3871.

Graveland, A.; Bongers, P.; Bosveld, P. Extraction and fractionation of wheat flour proteins. *J. Sci. Food Agric*. **1979**, *30*, 71−84.

Halford, N. G.; Forde, J.; Anderson, O. D.; Greene, F. C.; Shewry, P. R. The nucleotide and deduced amino acid sequence of an HMW glutenin subunit gene from chromosome 1 of bread wheat (*Triticum aestivum* L.) and comparison with those of genes from chromosomes 1A and 1D. *Theor. Appl. Genet*. **1987**, *75*, 117−126.

Haselbeck, A.; Hösel, W. Description and application of an immunological detection for analysing glycoproteins on blots. *Glycoconjugate J*. **1990**, *7*, 63−74.

Hawkes, R. Identification of Concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. *Anal. Biochem*. **1982**, *123*, 143−146.

Heukeshoven, J.; Dernick, R. Neue Ergebnisse zum Mechanismus der Silberfärbung. In *Elektrophoreseforum '86*; Radola, B. J., Ed.; Institut für Lebensmitteltechnologie und Analytische Chemie, Technische Universtät München: Freising-Weihenstephan, Germany, 1986; pp 22−27.

Hickman, D. R.; Roepstorff, P.; Shewry, P. R.; Tatham, A. S. Molecular weights of high molecular weight subunits of glutenin determined by mass spectrometry. *J. Cereal Sci*. **1995**, *22*, 99−103.

Huang, C. C.; Mayer, H. E.; Montgomery, R. Microheterogeneity and paucidispersity of glycoproteins. Part I. The carbohydrate of chicken ovalbumin. *Carbohydr. Res*. **1970**, *13*, 127−137.

Kakac, B.; Vejdelek, Z. J. Saccharide. In *Handbuch der Photometrischen Analyse Organischer Verbindungen*, Vol. 2; Verlag Chemie: Weinheim, Germany, 1974; pp 827−1001.

Krause, I.; Müller, U.; Belitz, H.-D. Charakterisierung von Weizensorten durch SDS−Polyacrylamidgel-Elektrophorese (SDS−PAGE) und zweidimensionale Elektrophorese (2D-PAGE) der Glutenine. *Z. Lebensm. Unters. Forsch*. **1988**, *186*, 398−406.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lawrence, G. J.; MacRitchie, F.; Wrigley, C. W. Dough and baking quality of wheat lines deficient in glutenin subunits controlled by the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. *J. Cereal Sci*. **1988**, *7*, 109−112.

Marchylo, B. A.; Kruger, J. E.; Hatcher, D. W. Quantitative reversed-phase high performance liquid chromatographic analysis of wheat storage proteins as a potential quality prediction tool. *J. Cereal Sci*. **1989**, *9*, 113−130.

Marion, D.; Nicolas, Y.; Popineau, Y.; Branlard, G.; Landry, J. A new and improved sequential extraction procedure of wheat proteins. In *Wheat Kernel Proteins: Molecular and Functional Aspects*; CNR/Università Degli Studi della Tuscia: S. Martino al Cimino, Italy, 1994; pp 197−199.

McMaster, G. J.; Bushuk, W. Protein-carbohydrate complexes in gluten: fractionation and proximate composition. *J. Cereal Sci*. **1983**, *1*, 171−184.

Morel, M. H. Acid-polyacrylamide gel electrophoresis of wheat glutenins: A new tool for the separation of high- and low molecular weight subunits. *Cereal Chem*. **1994**, *71*, 238−242.

Payne, P. I.; Corfield, K. G.; Blackman, J. A. Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. *Theor. Appl. Genet*. **1979**, *55*, 153−159.

Roels, S. P.; Delcour, J. A. Evidence for the non-glycoprotein nature of high molecular weight glutenin subunits of wheat. *J. Cereal Sci*. **1996**, *24*, 227−239.

Schofield, J. D. Wheat proteins: structure and functionality in milling and bread-making. In *Wheat: Production, Properties and Role in Human Nutrition*; Bushuk, W., Rasper, V., Eds.; Blackie Academic and Professional: Glasgow, Scotland, 1994; pp 23−106.

Schofield, J. D.; Freeman, I. L.; Jackson, D. S. The isolation, and amino acid and carbohydrate composition, of polymeric collagens prepared from various human tissues. *Biochem. J*. **1971**, *124*, 467−473.

Sharon, N.; Lis, H. Glycoproteins: Research booming on long-ignored, ubiquitous compounds. *Chem. Eng. News* **1981**, *59*, 21−44.

Shewry, P. R.; Halford, N. G.; Tatham, A. S. High molecular weight subunits of wheat glutenin. *J. Cereal Sci*. **1992**, *15*, 105−120.

Spiro, R. G. Studies on fetuin, a glycoprotein of fetal serum. 1. Isolation, chemical composition, and physicochemical properties. *J. Biol. Chem*. **1960**, *235*, 2860−2869.

Tatham, A. S.; Shewry, P. R.; Belton, P. S. Structural studies of cereal prolamins, including wheat gluten. *Adv. Cereal Sci. Technol*. **1990**, *10*, 1−78.

Thotakura, N. L.; Bahl, O. P. Enzymatic deglycosylation of glycoproteins. *Methods Enzymol*. **1987**, *138*, 350−359.

Tilley, K. A. Determination of O-linked mannose within the structure of the 1Dx2 high M_r glutenin subunit of Chinese Spring. In *Gluten '96*; Wrigley, C. W., Ed.; Royal Australian Chemical Institute: Melbourne, 1996; pp 154−158.

Tilley, K. A. Detection of O-glycosidically linked mannose within the structure of a highly purified glutenin subunit isolated from Chinese Spring wheat. *Cereal Chem*. **1997**, *74* (4), 371−374.

Tilley, K. A.; Schofield, J. D. Deglycosylation of the high M_r subunits of wheat glutenin by β-*N*-acetylhexosaminidase and the β-elimination reaction: evidence for O-glycosylation. In *Wheat Kernel Proteins: Molecular and Functional Aspects*; CNR/Università Degli Studi della Tuscia: S. Martino al Cimino, Italy, 1994; pp 213−220.

Tilley, K. A.; Lookhart, G. L.; Hoseney, R. C.; Mawhinney, T. P. Evidence for the glycosylation of the high-molecular-weight glutenin subunits 2, 7, 8 and 12 from Chinese Spring and TAM 105 wheats. *Cereal Chem*. **1993**, *70*, 602−606.

Westermeier, R. Coomassie- und Silberfärbung. In *Elektrophorese-Praktikum*; VCH Verlagsgesellschaft: Weinheim, Germany, 1990; pp 180−182.

Received for review March 16, 1998. Revised manuscript received August 24, 1998. Accepted September 1, 1998. Funding from The University of Reading Research Endowment Trust Fund to support this work is gratefully acknowledged.

JF980280Q