Structural Characterization of the Carbohydrate Portion of a Glycopeptide from Wheat Gluten[1]

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ABSTRACT

A carbohydrate-containing fraction, possibly a glycopeptide, was isolated from a pronase digest of wheat gluten and purified by gel filtration and ion-exchange chromatography. The molecular weight of glycopeptide I was estimated to be around 23,000. The methylation analysis of the carbohydrate portion of the glycopeptide indicated that it contained three sugar components—xylose, arabinose, and galactose—and had a highly branched structure. The structure of the carbohydrate portion of this glycopeptide was similar to that of a galactoarabinoxylan type of polysaccharide.

Many authors have found that wheat gluten contains small amounts of carbohydrates (Kasarda et al 1971, Inouye et al 1974, Khan and Bushuk 1979), but few of these carbohydrates have been characterized. McMaster and Bushuk (1983) studied the proportion and composition of carbohydrates in the alcohol-soluble and the alcohol-insoluble fractions of a gluten preparation. They found that the alcohol-soluble fraction contained 0.6% (w/w) carbohydrate, which was 55.5% galactose, 20.7% glucose, 16.1% arabinose, 6.2% mannose, and 1.2% xylose, and that the alcohol-insoluble fraction contained 17.0% carbohydrate, which was 97% glucose with only trace amounts of arabinose, xylose, galactose, and mannose. Graveland et al (1979) reported the presence of six glycoprotein fractions in wheat flour. These fractions accounted for 70% of wheat endosperm protein and appeared to be highly glycosylated, containing up to 40% carbohydrate in wheat flour fractions. These authors did not present any evidence for a covalent linkage between carbohydrate and protein. D'Appolonia and Gilles (1971) isolated the pentosans from the gluten fraction of wheat flour and found that all of the gluten extracted from different hard red spring wheat flours, durum semolinas, and soft wheat flours contained pentosan material. The pentosan material was fractionated by diethylaminoethylcellulose chromatography into five fractions. Fraction 1 was essentially an arabinoxylan with only small amounts of protein. The pentosans associated with gluten were similar to the pentosans extracted from flour. The presence of heat-stable lectins, which are carbohydrate-binding glycoproteins, in wheat gluten proteins was established by Concon et al (1983) using a modified hemagglutination assay. Lectin activity was found in both gliadin and acid-soluble glutenin fractions of gluten.

In a preceding article (Chen et al 1992), wheat gluten protein was separated into three carbohydrate-containing fractions by using sodium dodecyl sulfate (SDS) solution. The carbohydrate components of the SDS-soluble–70% ethanol-soluble fractions were associated with the high molecular weight fraction obtained by gel filtration chromatography on Sephadex G-200 and Sephacryl S-400. SDS-polyacrylamide gel electrophoresis of the three protein fractions showed that the carbohydrate was associated with all of the protein subunits with molecular weight greater than 32,000. It was found that the carbohydrate and protein in wheat gluten were eluted coincidentally from gel filtration and comigrated on SDS-polyacrylamide gel electrophoresis. These observations support the hypothesis that the carbohydrate and protein in the gluten fractions may have been covalently linked. However, additional evidence for the existence of a covalent protein-carbohydrate linkage needs to be demonstrated by the isolation of the glycopeptides containing the linkage region. Therefore, the purpose of this study was to isolate glycoprotein from wheat gluten and to characterize the structure of the carbohydrate portion of the glycoprotein.

MATERIALS AND METHODS

Materials

Gluten was prepared from the defatted flour of the hard red spring wheat variety Wheaton according to the procedure reported by Chen et al (1992).

Biochemicals used in this study were purchased from Sigma Chemical Company, St. Louis, MO, and Aldrich Chemical Company, Milwaukee, WI. Chemicals, reagents, and solvents were obtained from American Scientific Products (McGaw Park, IL) and were analytical grade. Gel filtration media were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and Bio-Rad Laboratory, Richmond, CA.

Defatting and α-Amylase Digestion of Wheat Gluten

Gluten (500 g) was dispersed in a mixture of methanol and chloroform (2:1, v/v, 3,000 ml) with stirring for 3 hr and then filtered through a glass microfiber filter. The residue was again dispersed in a mixture of water, methanol, and chloroform (3:8:4, v/v) with stirring for 1 hr and filtered as before. The residue was further washed with four volumes of absolute ethanol and freeze-dried.

Defatted gluten was treated with α-amylase according to Chen et al (1992).

Pronase Digestion of Wheat Gluten

α-Amylase-digested, defatted gluten (200 g) was suspended in 4,000 ml of 0.1*M* Tris-HCl buffer, pH 8.0, containing 1 m*M* calcium chloride. A stock solution of pronase (pronase protease, *Streptomyces griseus* 53702, Calbiochem-Behring Co., La Jolla, CA) in the same Tris-HCl buffer was added to the sample (1% by weight of sample), and further additions (0.5% by weight of sample) were made at 24 and 48 hr. The pH was checked and readjusted to pH 8.0 before each addition of enzyme. The suspension was digested at 37°C with gentle shaking, and a few drops of toluene were added to suppress bacterial growth. The rate of digestion was monitored by analyzing aliquots for free amino acids with ninhydrin reagent (Moore and Stein 1954), and the digestion was continued until no further cleavage of peptide bonds occurred.

Gel Filtration Chromatography

The pronase digest of wheat gluten was fractionated on a column (2.5-cm diameter × 70-cm bed height) of Biogel P-6 eluted with 0.1*M* acetic acid solution at a flow rate of 31.8 ml/hr. Fractions of 5.3 ml were collected. Void and total volumes were determined

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by chromatographing bovine serum albumin and tryptophan. Polypeptides in each fraction were monitored by UV absorption at 230 nm. Carbohydrate in the eluate was monitored by the phenol-sulfuric acid method (Dubois et al 1956), and amino sugar in each fraction was monitored by the method of Ohno et al (1985). Amino acid in each fraction was determined by Moore and Stein's method (1954).

The fractions containing carbohydrate and amino sugar were collected, dialyzed, freeze-dried, redissolved in 0.1*M* acetic acid, and further fractionated on a column (2.0-cm diameter × 90-cm bed height) of Sephadex-200 as described previously (Chen et al 1992), except that 0.1*M* acetic acid was used as the eluting solvent.

Ion-Exchange Chromatography

Cation-exchange resin AG-50Wx2 (200–400 mesh) (Bio-Rad Laboratory; please see manufacturer's instructions for use) was converted into the hydroxyl form by stirring with 2*M* sodium hydroxide. The resin then was washed successively with distilled water and 1 m*M* sodium acetate buffer (pH 2.6). A column (1.5 × 100 cm) of this resin was prepared and equilibrated with the sodium acetate buffer for at least two days at room temperature and at a constant flow rate of 27 ml/hr.

The freeze-dried carbohydrate-containing fraction (10 mg) from Sephadex G-200 was dissolved in 1 ml of sodium acetate buffer (pH 2.6, 1 m*M* Na^+) and loaded on the column. The column was first eluted with 560 ml of sodium acetate buffer (1 m*M* Na^+, pH 2.6). Then 1,125 ml of the second solvent, 0.05*M* sodium acetate, pH 6.0, was used to elute the column. Finally the column was eluted with 0.1*M* sodium acetate. Protein in each fraction was monitored by UV absorption at 230 nm. Carbohydrate was determined by the phenol-sulfuric acid method (Dubois et al 1956).

Monosaccharide Analysis

Monosaccharide compositions were determined by gas-liquid chromatography (GLC) analysis (McGinnis 1982) as outlined by Chen et al (1992).

Methylation Analysis

The dried glycopeptide (3 mg) in dry dimethyl sulfoxide (200 μl) was methylated by the method of Parente et al (1985) using lithium methylsulfinyl carbanion and methyl iodide. The methylated sample was hydrolyzed with trifluoroacetic acid (2*M*, 0.3 ml) containing 0.2 mg/ml of myoinositol for 1 hr at 121°C. The sample tube was cooled and placed in a water bath (40°C), and the mixture was evaporated to dryness with a stream of nitrogen.

Reduction and Acetylation of Methylated Sugars

Ammonium hydroxide solution (1*N*, 100 μl) was added to the dry hydrolysate to dissolve sugars, and a freshly prepared solution of dimethyl sulfoxide containing 20 mg/ml of sodium borohydride (0.5 ml) was then added. The mixture was reduced for 1.5 hr at 45–50°C, neutralized with 100 μl of glacial acetic acid, and added to 1-methylimidazole (100 μl). To this mixture 0.5 ml of anhydrous acetic anhydride was added, mixed thoroughly, and allowed to stand for 10 min. Then 1.5 ml of distilled water was added to the mixture, shaken vigorously, and allowed to stand until the vial was cooled to room temperature. Dichloromethane (1 ml) was added and the tube was shaken vigorously for 1–2 min. After phase separation, the lower phase was removed with a Pasteur pipette and stored at −20°C in a screw-capped glass vial before GLC and mass spectrometry.

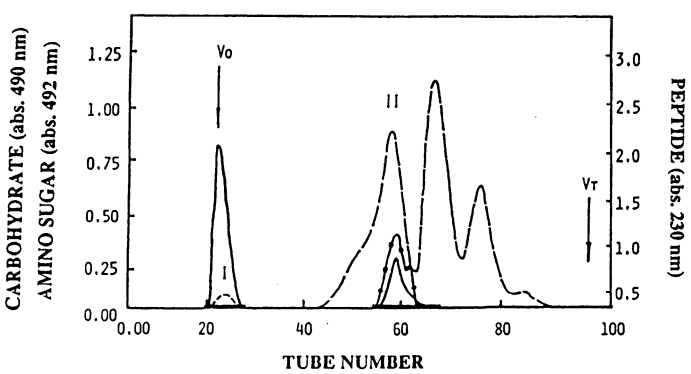

Fig. 1. Elution profile form gel filtration chromatography on Bio-Gel P-6 of pronase digest of glycoprotein of wheat gluten. Peptide, - - -; protein, – – –; carbohydrate, ———.

GLC and Mass Spectrometry

Acetylated alditols of methylated sugars were separated on a SP 2340 silicon capillary column (Supelco, Bellefonte, PA; 30 m × 0.2 mm i.d.) in a Hewlett-Packard 5840A gas chromatograph (Hewlett-Packard, Palo Alto, CA) linked to an INCOS 50 mass spectrometer (Finnigan MAT, San Jose, CA). An SGE on-column injector (SGE PTY, Austin, TX) was used. High-purity helium was used as the carrier gas. The oven temperature was maintained for 2 min at 100°C following injection and then raised at 3°C/min to 240°C. The oven temperature was maintained at 240°C for 10 min. The mass spectra were recorded at an inlet temperature of 240°C and an ionizing potential of 70 eV. The temperature of the ion source was 180°C. The mass spectrometer was connected to a Data General computer (Finnigan MAT) using INCOS 50 series software (Finnigan MAT). The alditol acetates of partially methylated sugars were identified by comparison of their retention times on GLC with partially methylated sugar standards and by comparison of their mass spectra with those recorded in the literature (Bjorndal et al 1967, Jansson et al 1976). The partially methylated sugar standards were made by the method of Kuhn (Ovodov and Evtushenko 1973).

RESULTS AND DISCUSSION

The wheat gluten from the defatted flour used in this study was subjected to additional defatting by extracting with the chloroform-methanol mixture. The purpose of this defatting step was to remove more lipid and glycolipid from wheat gluten. The difference between the gluten weight before and after defatting was 40 g, indicating that 8% lipid material was removed by the additional defatting step. To remove starch associated with gluten, the wheat gluten was digested with α-amylase. It was observed after 25 hr of digestion that no more reducing sugars were released by α-amylase action on the wheat gluten and that most of the starch was completely digested by the enzyme. It was possible that some starch remained in the gluten matrix and was inaccessible to enzymatic digestion.

Pronase was chosen to nonselectively cleave and disrupt the protein component of wheat gluten after removal of lipid and glycolipid. During the first 16 hr of digestion, the amount of amino acid released increased rapidly and linearly. After 16 hr, the rate of digestion appeared to decrease. After the second addition of enzyme at 24 hr, a small amount of amino acid was released and the incubation was extended for 72 hr. After 96 hr, the digestion curve showed no further cleavage of peptide bonds. The hydrolysis of the wheat gluten by enzyme released 9.8 g of carbohydrate into the Tris-HCl buffer. This represented a recovery of 81% of the total carbohydrates in defatted and amylase-digested gluten. No further carbohydrate was removed by washing the residue with Tris-HCl buffer. Thus, it may be concluded that treatment of wheat gluten with pronase released a major proportion of the carbohydrate component present in wheat gluten. This result indicated that the carbohydrate was strongly held in the gluten complex because it was released only after enzymatic hydrolysis of the protein.

The elution profile of the wheat gluten pronase digest from the Bio-Gel P-6 column is given in Figure 1. Two carbohydrate peaks were eluted and labeled as glycopeptides I and II according to the order of elution. Glycopeptide I eluted at the void volume of the column. Glycopeptide I contained 93% of the total carbohydrate, and no amino sugar was found in this fraction. Glycopeptide II was eluted in the included volume of the column, and

it contained 7% of the total carbohydrate and a small amount of amino sugar. Analysis of the pronase enzyme itself on Bio-Gel P-6 indicated that glycoprotein subunits from the enzyme were eluted in the same volume as glycopeptide II. Thus, glycopeptide II could come from pronase itself. In this study, however, the structure of the carbohydrate portion of the major glycopeptide in wheat gluten, glycopeptide I, was further characterized using gel-filtration and ion-exchange chromatography.

Glycopeptide I was subjected to further chromatography before the study of its structure. Glycopeptide I eluted as a single peak on the Sephadex G-200 column (Fig. 2). Its molecular weight was estimated to be around 23,000. This glycopeptide also eluted as a single peak on an ion-exchange column of AG 50x2 (Fig. 3). These results demonstrated that glycopeptide I was relatively low in molecular weight and was homogeneous in size and charge.

Glycopeptide I was 95% polysaccharide and contained three sugar components: xylose, arabinose, and galactose. Glucose and mannose were present only in trace amounts. The sugar composition of glycopeptide I was similar to that of the pentosans associated with gluten (D'Appolonia and Gilles 1971). The ratio of xylose to arabinose to galactose was 1:3.7:2.6. The sugar composition of glycopeptide I suggested that its carbohydrate portion was a galactoarabinoxylan polysaccharide.

Six sugar derivatives resulted from the methylation analysis of glycopeptide I (Table I and Fig. 4). These derivatives were the methylated glycitol peracetates of 2,3,5-tri-*O*-ethylarabinose, 2,3-di-*O*-methylarabinose, 2,3-di-*O*-methylxylose, xylose, 2,4-di-*O*-methylgalactose, and 2-monomethylgalactose. The carbohydrate portion of the glycopeptide had a highly branched structure as indicated by the high proportions of nonreducing end-groups, i.e., 2,3,5-tri-*O*-methylarabinose. This was confirmed by the high proportion of branching points, reflected by the derivatives of 2,4-di-*O*-methylgalactose, 2-*O*-methylgalactose, and xylose. Characterization of 2,3-di-*O*-methylxylose suggested that the xylopyranosyl residues were linked with other sugar residues through *O*-1 and *O*-4. The presence of doubly substituted xylose also has been reported in arabinoxylans from wheat (Medcalf and Gilles 1968), rice endosperm (Shibuya et al 1983), sorghum husk (Woolard and Bathbone 1976), corn bran (Medcalf 1985), and rice bran (Shibuya and Iwasaki 1985). Arabinose occurred mainly as terminal residues but also was linked through *O*-1 and *O*-5. These minor structural features are commonly encountered in side terminal arabinosyl groups in some gramineae xylans. Arabinofuranose residues were found to be present in short side chains in the xylans from oat stem and bamboo leaves (Wilkie and Woo 1977). Identification of 2,4-di-*O*-methylgalactose and 2-*O*-methylgalactose showed that the galactopyranosyl residues were involved in the branch points of the polysaccharide.

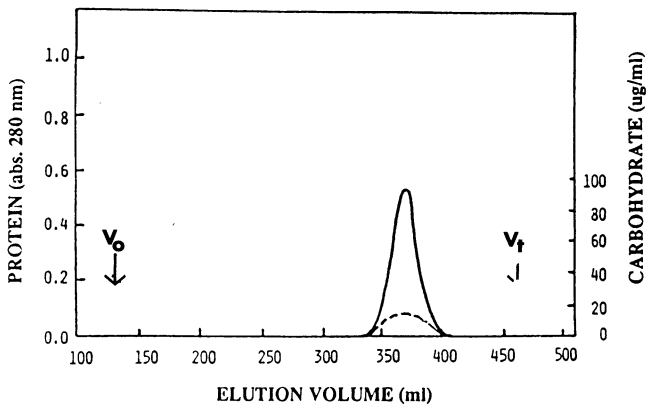

Fig. 2. Elution profile of gel filtration chromatography of glycopeptide I of wheat gluten on Sephadex G-200. Protein, – – –; carbohydrate, ———.

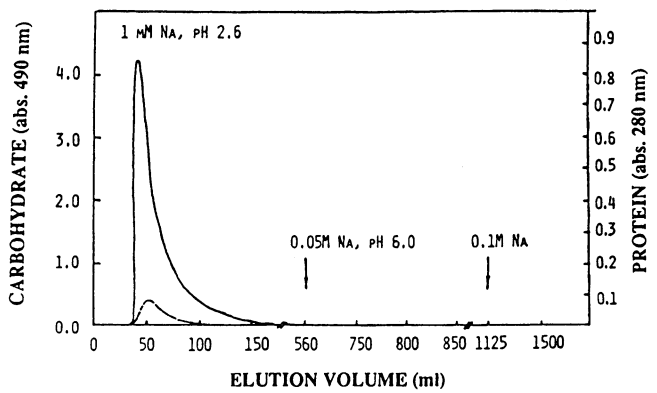

Fig. 3. Elution profile of gel filtration chromatography of glycopeptide I of wheat gluten on an ion-exchange Ag 50x2 column. Protein, – – –; carbohydrate, ———.

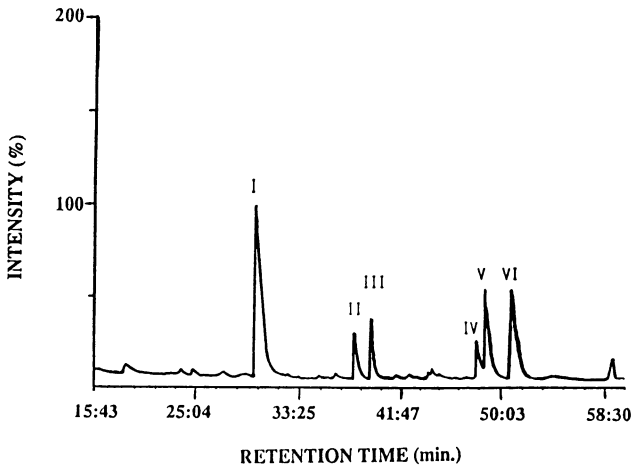

Fig. 4. Gas chromatography of alditol acetates of methylated sugars formed in methylation analysis of glycopeptide I of wheat gluten. Peak I, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-D-arabitol; peak II, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-D-xylitol; peak III, 1,5,4-tri-*O*-acetyl-2,3-di-*O*-methyl-D-arabitol; peak IV, xylitol pentaacetate; peak V, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol; peak VI, 1,3,4,5,6-penta-*O*-acetyl-2-mono-*O*-methyl-D-galactitol.

TABLE I
Methylation Analysis of Glycopeptide I of Wheat Gluten

Components	Retention Time (min)	Mode of Linkage
1,4-Diacetyl-2,3,5-tri-*O*-methylarabitol	30.01	Araf[a]-(1-
1,4,5-Triacetyl-2,3-di-*O*-methylarabitol	39.20	-5) Araf (1-
1,4,5-Triacetyl-2,3-di-*O*-methylxylitol	37.59	-4) Xylp[b](1-
Xylitol pentaacetate	48.20	-3,4) Xylp (1,2-
1,3,5,6-Tetracetyl-2,4-di-*O*-methylgalactitol	49.05	-3,6) Galp[c] (1-
1,3,4,5,6-Pentaacetyl-2-mono-*O*-methylgalactitol	51.09	-3,6) Galp (1,4-

[a] Araf = Arabinofuranose.
[b] Xylp = Xylopyranose.
[c] Galp = Galactopyranose.

CONCLUSIONS

The gluten preparation of Chen et al (1992), obtained by exhaustive fractionation procedures to dissociate starchy polysaccharides from gluten, was used in this study. To further dislodge any tightly associated carbohydrate components that may not have been accessible in previous purification efforts, the gluten preparation was subjected to proteolytic digestion to hydrolyze the gluten proteins and release any noncovalently linked carbohydrates. The proteolytic digest then was subjected to gel-filtration and ion-exchange chromatography to separate away any dissociated carbohydrate from protein on the basis of size and charge differences. A fraction containing peptide and carbohydrate, possibly a glycoprotein, was isolated from the chromatographic procedures. Characterization of the methylated fraction by mass spectrometry showed that it contained three sugar components—xylose, arabinose, and galactose—and had a highly branched structure. These results provided good evidence for a covalent linkage of carbohydrates to gluten proteins. However, additional, more conclusive evidence is needed to establish a covalent linkage.

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