Glycoconjugate J (1986) 3: 45—53

Isolation and Structural Studies on a Galactofuranosyl-containing Glycopeptide from *Ascobolus furfuraceus*

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Received October 15 1985/January 7, 1986.

Key words: galactofuranosyl-containing glycopeptide, Ascobolus furfuraceus

A galactofuranosyl-containing glycopeptide has been isolated from mycelium of *Ascobolus furfuraceus* by extraction with water. The glycoconjugate was purified by DEAE-cellulose chromatography followed by gel filtration. A molecular weight of about 20 000 was determined by the latter method using standard dextrans. Neutral sugars accounted for 94.5% of the glycopeptide and were characterized as mannose, galactose, and glucose. Glucosamine was estimated colorimetrically (1.8%). The molar ratio of Man:Gal:Glc:GlcNH$_2$ was 68:32:16:2. A trace amount of total phosphorus (0.2%) was found. The predominant amino acids were threonine and serine. The peptide moiety was labeled with [^{14}C]formaldehyde and the elution of radioactivity was coincident with sugar on gel filtration in the presence of sodium dodecyl sulfate. The peak of radioactivity was retarded on release of galactose by mild acid hydrolysis. These results confirm the sugar-peptide linkage.

Ascobolus furfuraceus is a coprophilous fungus with cellulolytic activity [1]. It accumulates a large amount of glycogen when grown on glucose [2] or microcrystalline cellulose [1]. A maximum glycogen content of 28% of the cell dry weight was found after 4-6 days of growth on glucose which then decreased rapidly to less than 5% on day 13. At this time a galactofuranosyl-containing glycopeptide was isolated. Galactomannans with galactose in the furanose configuration have been obtained from other fungi [3, 4]. A 5-*O*-β-D-galactofuranosyl-containing exocellular glycopeptide from *Penicillium charlesii* has been well characterized [5-8] and a peptidogalactomannan from *Neurospora crassa* was recently reported [9, 10].

In the present paper we describe the purification of, and structural studies on a peptidogalactomannan from *Ascobolus furfuraceus*.

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Materials and Methods

Isolation and Purification of the Glycopeptide

Cultures were obtained from single ascospores of *Ascobolus furfuraceus* strain C-233 as previously described [2]. Cultures of *A. furfuraceus* were filtered after 13-14 days. Lyophilized mycelia (14.5 g) were suspended in boiling water (320 ml) and after 5 min at 100°C they were homogenized in a Potter homogenizer. The residue, collected by centrifugation, was again homogenized with water (150 ml) at room temperature. A precipitate was obtained by addition of four volumes of ethanol to the combined aqueous extracts. The material (A) was suspended in water (0.1 g/ml) and an insoluble residue was removed by centrifugation. The solution (5 ml) was loaded on to a column (2 × 19 cm) of DEAE-cellulose (Merck, Darmstadt, W. Germany), pre-equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. The column was eluted first with 70 ml of the buffer (Fraction I) and then with 40 ml 0.1 M NaCl in the same buffer (Fraction II, Fig. 1). The unadsorbed fraction (I) represented approximately 66% of the total sugar of precipitate A. Aliquots of fraction I (20 mg) were chromatographed on a 1.2 × 85 cm column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden). Pre-equilibration of the column and elution was made with 0.3 M pyridine-acetate buffer, pH 6.5. The opalescent excluded fraction (Fraction Ia) consisted of glycogen. The included fraction (Fraction Ib, Fig. 2) was evaporated under reduced pressure (20-25°C) and the residue was dissolved in water and then reprecipitated with five volumes of ethanol. The glycopeptide thus obtained was collected by centrifugation (4500 × *g*, 30 min.).

Compositional Analysis

Total carbohydrate was determined by the phenol-sulfuric acid method with galactose and mannose (2:3) as standard [11]. Peptide was analysed by the procedure of Lowry *et al.* [12] with bovine serum albumin (BDH, Poole, England) as standard. Phosphate was estimated by a method adapted from Bartlett [13]. Hexosamines were estimated by a modification of the Elson-Morgan method [14] after acid hydrolysis of a 3 mg sample with 4 N HCl at 100°C for 6 h under nitrogen.

Component sugars were determined after total hydrolysis with 2 M trifluoroacetic acid at 105°C for 3 h in a sealed tube under nitrogen. Acid removal was accomplished by evaporation at 30°C under diminished pressure with repeated addition of water. Mild acid hydrolysis was conducted using 0.05 M trifluoroacetic acid at 100°C for 2 h. Acid was removed as described above.

Chromatographic Methods

Descending paper chromatography was carried out on Whatman no. 1 filter paper. The following solvents were used: A) 1-butanol/pyridine/water, 6/4/3 by vol; B) ethyl acetate/acetic acid/formic acid/water, 18/3/1/4 by vol. Sugars were detected with a) silver nitrate-sodium hydroxide [15]; b) *p*-anisidine hydrochloride [16]. Aminoacids and aminosugars were detected with c) ninhydrin [17]. The ammonium molybdate reagent [18] was used for phosphate.

Radioactivity was determined on paper chromatograms by cutting longitudinal strips (2 cm wide) into 1 cm horizontal sections, which were counted in 3 ml of Bray's scintillation solution [19]. Radioactivity was counted in a Packard Tri-Carb 3003 scintillation counter.

GLC was performed with a Hewlett-Packard 5830 A gas chromatograph, equipped with glass columns packed with 3% ECNSS-M on Gas Chrom Q (100-120 mesh), nitrogen 29 ml/min; T_i 210°C; T_d 210°C; T_c 190°C. Sugars were analysed as alditol acetates [20].

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed in glass tubes (6 × 80 mm) using Tris-borate buffer [21]. Polyacrylamide gels (10%) were prepared as described by Segrest and Jackson [22]. The experiment was performed at 4 mA/tube, for about 2.5 h. Gels were stained for carbohydrate with periodic acid-Schiff reagent [22].

Amino Acid Analysis

A sample (2.4 mg) of the glycopeptide was hydrolysed as recommended [23] to avoid interference by neutral sugars. Analysis was conducted after hydrolysis for 24 h with 6 M HCl at 100-105°C under nitrogen. A Beckman 119 CL amino acid analyser with a Beckman W3 (6 × 220 cm) resin column was used. The elution sequence was done with the following buffers: i) 0.2 N sodium citrate buffer pH 3.25, ii) 0.4 N sodium citrate buffer pH 3.95 and iii) 1.0 N sodium citrate buffer pH 6.4. Samples were dissolved in 50-100 μl of 0.2 N sodium citrate buffer, pH 2.2.

Preparation of ^{14}C-Glycopeptide

The glycopeptide (1.5 mg) was labeled with [^{14}C]-formaldehyde (0.01 mCi, New England Nuclear, Boston, MA, USA) by the method of Rice and Means [24]. Albumin (1.0 mg) and glycogen (1.7 mg) were treated in the same way.

Mild Periodate Oxidation

Periodate oxidation was performed by treating the glycopeptide (2.4 mg) with 0.05 M $NaIO_4$ (0.9 ml) in water for 30 min at room temperature in the dark. The excess of oxidant was decomposed after acidification with 1 N sulfuric acid (0.6 ml) by addition of 0.5 M sodium arsenite (0.6 ml). The mixture was dialysed in a sealed tube and the formaldehyde produced was determined in the dialysate by the chromotropic acid method [25]. Formaldehyde generated by oxidizing a known quantity of mannitol with periodate was used as reference.

Another sample of glycopeptide (1.8 mg) was dissolved in 0.8 ml 0.05 M sodium phosphate buffer pH 7.2 and oxidized with 0.05 M sodium metaperiodate for 30 min in the dark. The oxidation was stopped by addition of ethylene glycol. After evaporation (20-25°C), the residue was dissolved in water (0.5 ml) and reduced with NaB^3H_4. After 20 min, excess unlabeled sodium borohydride was added and the reduction was allowed to continue for another 20 min. The solution was acidified to pH 5 with acetic acid, and borate was eliminated by repeated evaporation with successive additions of methanol.

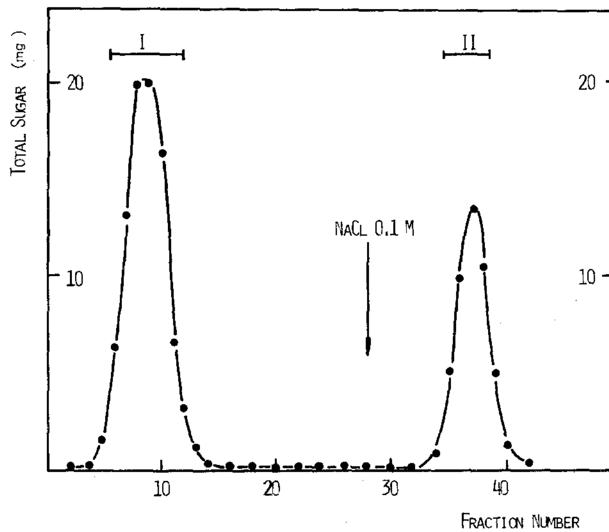

Figure 1. DEAE-Cellulose chromatography of fraction A from the aqueous extract of *A. furfuraceus*. A 570 mg sample of A was dissolved in water (7 ml) and loaded on a column (19 × 2 cm) of DEAE-cellulose equilibrated with 0.01 M Tris-HCl, pH 8.0. The column was eluted with 70 ml of this buffer and then with 40 ml of 0.1 M NaCl (2.5 ml fractions were collected). Fractions were monitored for sugar by the phenol-sulfuric acid procedure.

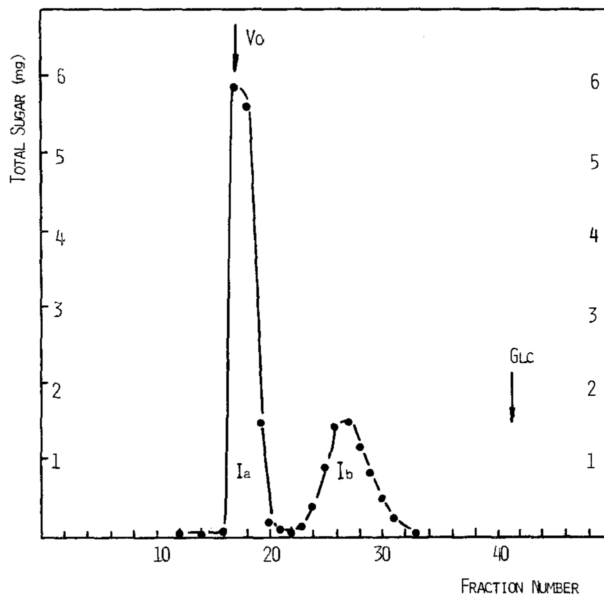

Figure 2. Purification of the glycopeptide by gel filtration chromatography. A 20 mg sample of Fraction I (Fig. 1) was loaded on to a column (85 × 1.2 cm) of Sephacryl S-200 and eluted with 0.3 M pyridine-acetate buffer, pH 6.5. Fractions of 2.5 ml were collected.

The sample was hydrolysed with 1 N HCl (2 ml) for 2 h at 100°C. The solution was evaporated *in vacuo* and the sugars were analysed by paper chromatography using solvent B, scanning for radioactivity by scintillation spectrometry.

Results

Isolation and Purification of the Glycopeptide

Carbohydrate material was extracted with water from mycelia of *A. furfuraceus* and was precipitated by addition of four volumes of ethanol (Fraction A). Examination of crude material (A) by polyacrylamide gel electrophoresis in Tris-borate buffer showed three main components with R_m values of 0, 0.12 and 0.27, respectively, which were stained for carbohydrate with the periodic acid-Schiff reagent. Ion-exchange chromatography of fraction A on DEAE-cellulose afforded two fractions (Fig. 1). Fraction I, which represented 66% of the sugar applied to the column was further chromatographed on Sephacryl S-200 (Fig. 2). Glycogen, previously characterized [2] appeared in the void volume (Ia), separated from the glycopeptide (Ib). The yield was 37 mg from 14 g of lyophilized mycelia. Polyacrylamide gel electrophoresis revealed pure glycopeptide (R_m 0.27). The other two bands which appeared in A, corresponded to glycogen (R_m 0) separated on Sephacryl, and to another peptidogalactomannan (R_m 0.12). In fact, hexose analysis of fraction II from DEAE cellulose (Fig. 1) gave mannose and galactose in a 65:30 molar ratio.

Homogeneity of the glycopeptide was confirmed by gel filtration on Sephacryl S-200 and Sepharose (not shown) under dissociating conditions (0.1 M sodium phosphate buffer, pH 7.0 in 0.1% sodium dodecyl sulphate). The molecular weight of the glycopeptide was estimated to be 20 000 by comparison with standard dextrans.

Compositional Analysis

Neutral sugars accounted for 94.5% of the glycopeptide as determined by the phenol-sulfuric acid method [11]. Mannose, galactose and glucose were identified by paper chromatography (solvent A, reagents a and b). Glucosamine and other ninhydrin-positive compounds were also detected. The same sugars as the acetyl alditol derivatives were characterized and quantitatively analysed by GLC. The molar ratio of mannose:galactose:glucose was 41:19:10. The molar proportions varied when glycopeptides from different cultures were analysed. The extreme values found for the mannose:galactose ratio were 44:10 and 1:2. Glucose was always a minor component and in some samples only traces were detected.

Analysis by the method of Lowry *et al.* [12] gave 1.2% protein. Total phosphorus [13] was 0.2% and glucosamine, analysed by a modification of the Elson-Morgan method [14] was 1.8%. These values agree with the presence of 1.3 mol of phosphorus and two units of the aminosugar per mol of glycopeptide, assuming a molecular weight of 20 000 as determined by gel filtration.

To confirm the covalent linkage between the carbohydrate and the peptide, the glycopeptide was labelled with [^{14}C]-formaldehyde. In order to check the specificity of the reaction, albumin and glycogen were labeled in the same way. The radioactive pro-

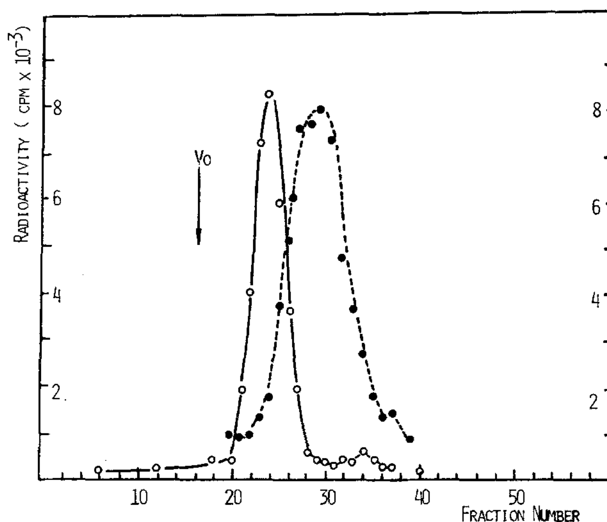

Figure 3. Gel filtration on Sephacryl S-200 of the [^{14}C] labeled glycopeptide (○; 51 × 10^3 cpm) and of the labeled sample after mild acid treatment (●; 60 × 10^3 cpm). The column (77.5 × 1.2 cm) was eluted with 0.3 M pyridine-acetate buffer, pH 6.8 and 2.5 ml fractions were collected.

ducts had 175 000 cpm/mg; 248 000 cpm/mg and 4 900 cpm/mg respectively. The elution volume of the ^{14}C-glycopeptide on Sephacryl S-200 was the same as for the original sample (not shown).

Mild Acid Hydrolysis of the Glycopeptide

Treatment of the glycopeptide with 0.05 N trifluoroacetic acid for 2 h at 100°C released only galactose as shown by paper chromatography. A sample of the ^{14}C-glycopeptide was degraded by dilute acid in the same way. After this treatment the elution of radioactivity on the Sephacryl column was retarded due to the decrease of the molecular weight of the oligosaccharide chain (Fig. 3).

Periodate Oxidation

Mild periodate oxidation cleaves the primary hydroxyl group of hexofuranoses unsubstituted at C-5 and C-6. The amount of formaldehyde determined corresponded to 80-90% of the galactose present. Sequential mild oxidation and [^3H] borohydride reduction, followed by acid hydrolysis, gave radioactive arabinose as shown by paper chromatography.

Amino Acid Composition

The amino acid composition of the glycopeptide is given in Table 1. Serine and threonine are the predominant amino acids. In addition, there is a relatively high proportion of aspartic and glutamic acids, proline, glycine and alanine.

Table 1. Amino acid composition of the glycopeptide from *A. furfuraceus.*

Amino acid	a	b
Lysine	3.3	0.2
Histidine	1.5	0.1
Arginine	2.1	0.2
Aspartate	9.4	0.7
Threonine	22.5	1.6
Serine	14.7	1.0
Glutamate	9.9	0.7
Proline	9.2	0.6
Glycine	8.9	0.6
Alanine	8.4	0.6
Valine	4.5	0.3
Isoleucine	1.4	0.1
Leucine	3.1	0.2
Tyrosine	t[c]	t
Phenylalanine	1.1	0.1

[a] Mol per 100 mol of total amino acids.
[b] Amino acid residues per mol of glycopeptide based on a molecular weight of 20000.
[c] Trace amounts.

Discussion

Aqueous extraction of cells of *A. furfuraceus* yielded a mixture of soluble peptidogalactomannan and glycogen. The soluble glycopeptide could not be detected in a culture after 6-8 days of growth, when the glycogen content was at its maximum [2]. However, the glycopeptide could be obtained in a low yield (0.2% of the dry cells) from 13 day old mycelia.

The purity of the glycopeptide was proved by gel filtration under dissociating conditions and by gel electrophoresis. Mannose and galactose are the main component monosaccharides with a smaller amount of glucose also present. Evidence for the furanose configuration of most of the galactose was obtained from a) its release by mild acid hydrolysis; b) the determination of formaldehyde after short term periodate oxidation; c) the identification of arabinose, following hydrolysis of the sequentially periodate oxidized, borohydride reduced product.

A variation in the ratio of sugars was observed when glycopeptides from different cultures were analysed. Different percentages of component sugars were also reported for the galactofuranosyl-containing exocellular glycopeptide from *Penicillium charlesii*. The action of an exo-β-D-galactofuranosidase [26] was considered responsible for the heterogeneity of the glycopeptides. This possibility has not yet been investigated for *A. furfuraceus*. Quantification of glucosamine gave approximately 2 mol of the aminosugar per mol of the glycopeptide. This value suggested the presence of an *N*-glycosidic linkage through chitobiose to the peptide. This was confirmed by

hydrolysis with endo-β-*N*-acetylglucosaminidase [27] after removal of the galactofuranosyl units; also *O*-linkages through mannose were identified (unpublished results). The protein percentage by the Lowry method would be underestimated due to the low content of tyrosine in the sample, since the color developed in this reaction strongly depends on the relative proportion of phenolic groups and peptide bonds [12]. A peptide content of approximately 4% was calculated by difference to 100% of the glycopeptide components. According to this value the peptide would comprise 10 aminoacid residues. The presence of fractional quantities and trace amounts of some aminoacids indicate that the peptide is heterogeneous. The aminoacid composition is similar to that of other fungal glycopeptides [6, 10, 28-30].

The peptide moiety of the glycopeptide was labeled with [^{14}C]-formaldehyde. The fact that the label was coincident with the sugar on gel filtration in the presence of sodium dodecyl sulphate indicates firm association between the peptide and the oligosaccharide chain. The modification of the elution pattern for the radioactivity on mild acid degradation of the sugar chain further confirms the sugar-peptide linkage.

The galactofuranosyl-containing glycopeptide of *A. furfuraceus* is another example of this type of structure in fungi. In general, the peptide is a minor component, thus the exocellular glycopeptide from *Peniillium charlesii*, which is well characterized, has about 10% peptide [6]. A similar percentage was determined in the peptidogalactomannan from *Cladosporium werneckii* [28]. It is possible that more fungal galactomannans are originally linked to a peptide moiety which is lost during the extraction or purification steps. Thus, the galactomannans from *Aspergillus niger* [31] and other Aspergillus species [32], as well as those from *Sporothrix schenckii* and *Ceratocystis stemoceras* [33], were extracted with hot alkali, a procedure that cleaves sugar-peptide linkages. On the other hand, the galactomannan extract from *Cordyceps cicadae* was subjected to pronase digestion in one of the purification steps [34]. It is likely that the water extraction used for the isolation of the glycopeptide from *A. furfuraceus* does not extract all the glycoconjugate from the cell, but has the advantage of preserving the molecule for further studies of its structure.

Acknowledgements

This investigation received financial support from CONICET (Consejo Nacional de Investigaciones Cientificas y Técnicas).

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