# THE STRUCTURAL BASIS OF THE DIFFERENT AFFINITIES OF TWO TYPES OF ACIDIC *N*-GLYCOSIDIC GLYCOPEPTIDES FOR CONCANAVALIN A–SEPHAROSE

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## 1. Introduction

Affinity chromatography on lectins covalently bound to Sepharose has proved to be a useful tool for the fractionation and purification of glycopeptides and glycoproteins. The most commonly used lectin for this purpose has been concanavalin A. Studies on the binding of oligosaccharides and glycopeptides to this lectin have indicated that at least two non-substituted or 2-*O*-substituted α-mannosyl residues are required [1]. Recent reports on the fractionation of glycopeptides from various sources on concanavalin A–Sepharose have shown that in addition to the neutral mannose-rich glycopeptides, some acidic *N*-glycosidic glycopeptides are bound by the lectin, whereas others are not [2–5]. Since the carbohydrate composition of both types of acidic glycopeptides is rather similar [3–5], the reason for the difference in affinity is not understood.

The purpose of the present investigation was to study the structural basis of the separation of acidic *N*-glycosidic glycopeptides on concanavalin A–Sepharose. Glycopeptides with known (or partially known) structures were chromatographed on concanavalin A and fractions bound and not bound by the lectin were analyzed by methylation. It was found that glycopeptides possessing two peripheral Neu*N*Ac-Gal-Glc*N*Ac*-branches linked to the core pentasaccharide were bound by the lectin, whereas glycopeptides with three branches were not (the structures of these compounds are shown below). The different behaviour of the glycopeptides is explained by the substitution pattern of their mannose residues.

*Abbreviations*: Neu*N*Ac, *N*-acetylneuraminic acid; Glc*N*Ac, *N*-acetylglucosamine; Gal, galactose; Man, mannose; Asn, asparagine

## 2. Experimental

### 2.1. *Materials*

Glycopeptides were prepared by papain digestion from fetuin (Type II, Sigma), transferrin (Grade II, Sigma) and whole rat brain as described before [6]. The glycopeptides were purified by gel filtration on Sephadex G-25. Peptides were removed by passing the samples through a small column of Dowex 50 WX16 ($H^+$) [7]. The purified glycopeptides were *N*-[$^3$H]acetylated in their peptide moiety with [$^3$H]-acetic anhydride and separated from free radioactivity by gel filtration on Sephadex G-25 [5].

### 2.2. *Affinity chromatography of glycopeptides on concanavalin A-Sepharose*

*N*-[$^3$H]acetylated glycopeptides were fractionated on a column of concanavalin A–Sepharose (1.3 × 8 cm) by stepwise elution with methyl α-D-glucoside as described previously [5]. Acidic glycopeptides not bound by the lectin were eluted with buffer. Acidic glycopeptides bound by the lectin were obtained by elution with 15 mM methyl α-D-glucoside and neutral, mannose-rich glycopeptides with 200 mM methyl α-D-glucoside.

### 2.3. *Analytical methods*

Glycopeptides were methylated with methyl iodide in dimethylsulfoxide in the presence of dimethylsulfinyl carbanion [8]. The permethylated product was degraded as described previously [9,10].

To facilitate the interpretation of the mass spectra of hexose derivatives, $NaBH_4$ was replaced by $NaBD_4$ in the reduction. Otherwise the reduction and acetylation were carried out as described before [9]. Partially methylated alditol acetates were analyzed by gas–liquid chromatography and mass spectrometry. Alditol acetates of neutral sugars were separated on 1% OV-225 at 175°C [11] and the corresponding derivatives of hexosamines on 1% SE-30 at 190°C [12]. The methyl substitution pattern was confirmed in every case by mass spectral analysis [10,13,14]. Mass spectra were recorded with a Varian Mat CH-7 mass spectrometer equipped with a SpectroSystem 100 ms data processing system. The ionization potential was 70 eV and ionization current 300 μA.

## 3. Results

### 3.1. *Affinity chromatography of glycopeptides on concanavalin A-Sepharose*

All of the fetuin glycopeptides passed through the concanavalin A–Sepharose column unbound. In contrast to this, most of transferrin glycopeptides were bound by the lectin and eluted with 15 mM methyl α-D-glucoside (fig.1). No glycopeptides were eluted with 200 mM methyl α-D-glucoside, indicating the absence of neutral, mannose-rich glycopeptides in fetuin and transferrin. Affinity chromatography of rat-brain glycopeptides produced three fractions, the first two being acidic glycopeptides similar to the corresponding fractions of fetuin and transferrin. The third fraction was composed of neutral, mannose-rich glycopeptides [5].

### 3.2. *Carbohydrate linkages*

In order to study the substitution of the sugar residues, glycopeptide fractions obtained by affinity chromatography on concanavalin A were subjected to methylation analysis. Methylation products of fetuin were identified as alditol acetate derivatives of 2,3,4,6-tetra-*O*-methyl galactose, 2,4,6-tri-*O*-methyl-galactose, 2,3,4-tri-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannose, 3,6-di-*O*-methylmannose and 2,4-di-*O*-methyl mannose (fig.2). The only methylation product of glucosamine found was 3,6-di-*O*-methyl-glucosamine.

The results are in agreement with the partial

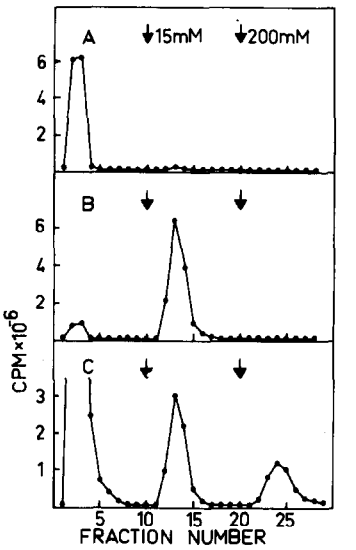


Fig.1. Fractionation of glycopeptides on a column of concanavalin A–Sepharose (1.3 × 8 cm). Glycopeptides were dissolved in 8 ml of 5 mM sodium acetate buffer (pH 5.2) containing $CaCl_2$, $MgCl_2$ and $MnCl_2$ (1 mM each) and applied to the column equilibrated with the same buffer. Elution was carried out first with 5 mM sodium acetate buffer (pH 5.2) containing 0.1 M NaCl and the divalent cations and then with 15 mM and 200 mM methyl α-D-glycoside in the starting buffer. (A) Glycopeptides from fetuin, (B) glycopeptides from transferrin and (C) glycopeptides from rat brain.

structure of fetuin proposed by Bayard [17]. The fetuin glycopeptides are composed of the core pentasaccharide mannotriosido-di-*N*-acetylchitobiose, to which three periferal Neu*N*Ac-Gal-Glc*N*Ac- branches

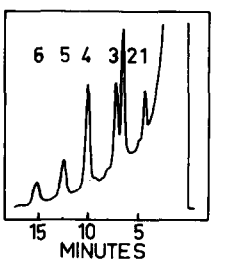


Fig.2. Gas–liquid chromatographic separation of partially methylated alditol acetates. Conditions: 1% OV-225, 175°C. Peaks: (1) 2,3,4,6-tetra-*O*-methylgalactitol; (2) 3,4,6-tri-*O*-methylmannitol; (3) 2,4,6-tri-*O*-methyl-galactitol; (4) 2,3,4-tri-*O*-methylgalactitol; (5) 3,6-di-*O*-methylmannitol and (6) 2,4-di-*O*-methylmannitol.

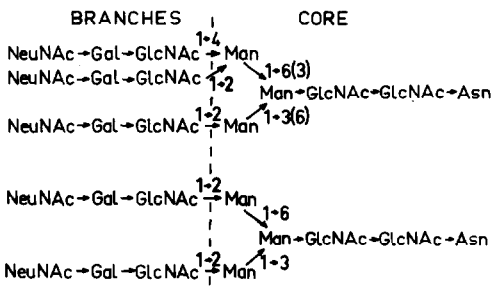


Fig.3. Proposed structures of the carbohydrate chains of fetuin [15] (on the top) and transferrin [17] (at the bottom). The substitution pattern of the mannose residues suggested in the present work is indicated.

are linked. However, the site of attachment of the branches to the core has not previously been elucidated. The methylation products indicate that two of the branches are attached by a 1→2-linkage to the α-mannose residues and the third by a 1→4-linkage to one of the 2-*O*-substituted α-mannose residues of the core pentasaccharide (fig.3).

On the basis of methylation analysis it was established that the major part of galactose was 6-*O*-substituted. In addition, 3-*O*-substituted and a small amount of terminal galactose were also found. This is in disagreement with the results of Spiro [16], who has suggested from periodate oxidation studies that all galactose is 3-*O*-substituted.

In contrast to fetuin, not a trace of 3,6-di-*O*-methylmannose was found in the methylation products of transferrin (and the amount of 2,4,6-tri-*O*-methylgalactose was very small). The other methylation products were the same as those of fetuin. This suggests that transferrin glycopeptides bound by concanavalin A contain only two peripheral branches, which is in keeping with the proposed structure [17] (fig.3).

Methylation products of mannose derived from rat-brain glycopeptides not bound by concanavalin A were the same as those of fetuin, and the methylation products of mannose derived from acidic glycopeptides bound by the lectin were the same as those of transferrin. These findings indicate similarity in structure between brain glycopeptides and the corresponding two model glycopeptides.

## 4. Discussion

In the present work, the structural basis of the interaction of acidic *N*-glycoside glycopeptides with concanavalin A was investigated. This was established by chromatography of model glycopeptides with known (transferrin) and partially known (fetuin) structures on concanavalin A–Sepharose.

Fetuin and transferrin are composed of a core pentasaccharide mannotriosido-di-*N*-acetylchitobiose (fig.3). In transferrin glycopeptides two peripheral Neu*N*Ac-Gal-Glc*N*Ac- branches are attached by 1→2-linkages to the two α-mannose residues of the core pentasaccharide [17]. Fetuin glycopeptides have a similar structure, with the difference that they contain three peripheral branches [15]. Since the site of attachment of the branches to the core was not known, this was studied by methylation analysis. The results of methylation analysis of transferrin and fetuin glycopeptides are in agreement with the structures described above and suggest that in fetuin glycopeptides two of the three branches are attached by a 1→2-linkage to the α-mannose residues and the third branch by a 1→4-linkage to one of the 2-*O*-substituted α-mannose residues of the core pentasaccharide.

Montreuil et al. have shown that in the spatial model of glycopeptides the core pentasaccharide occurs as a compact core, from which the peripheral Neu*N*Ac-Gal-Glc*N*Ac- branches stick out like antennae [18]. Glycopeptides having two or three peripheral branches are therefore called 'biantennary' and 'triantennary' glycopeptides. It is possible that the structure and number of these branches may be of importance in determining the biological specificity of the carbohydrate chains of glycoproteins.

The present study shows that glycopeptides with a biantennary structure are bound by concanavalin A, whereas glycopeptides with a triantennary structure are not. This is in keeping with the results of Ogata et al. [1]. They have suggested that at least two 2-*O*-substituted α-mannose residues are required for the interaction of glycopeptides with concanavalin A. Glycopeptides with a biantennary structure contain two 2-*O*-substituted α-mannose residues and are bound by the lectin. Glycopeptides with a triantennary structure contain only one 2-*O*-substituted α-mannose residue and are therefore not recognized by con-

canavalin A. However, glycopeptides containing two 2-*O*-substituted α-mannose residues interact only weakly with concanavalin A, since they are eluted with only 15 mM methyl α-D-glucoside. Glycopeptides composed of only mannose and *N*-acetylglucosamine [5] are more strongly bound by the lectin and need a much higher methyl α-D-glucoside concentration for their elution (fig.1). This indicates that the structural requirements for maximal binding to concanavalin A are more complex than in the model of Ogata et al. [1].

It is generally agreed that transformed cells have on their surface an increased amount of glycopeptides of large molecular size [18,19]. Recent observations indicate that these glycopeptides are not bound by concanavalin A, whereas the smaller glycopeptides of the same cells with a similar sugar composition are bound by the lectin [20]. We therefore suggest that transformed cells have an increased amount of triantennary glycopeptides on their surface. This might be due to altered activity of the glucosaminyl transferases responsible for the substitution of the mannosyl residues on the core pentasaccharide.

The results of the methylation analysis of the acidic *N*-glycosidic glycopeptides derived from whole rat brain suggest similarity in structure to the two model compounds. The presence of two types of acidic *N*-glycosidic glycopeptides containing either two or three peripheral branches seems therefore to be a common feature in biological samples. Affinity chromatography on concanavalin A–Sepharose offers a valuable method for the isolation of these glycopeptides.

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