

# *O*-Mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in *Saccharomyces cerevisiae*

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A glycosylated form of recombinant human insulin-like growth factor I (IGF-I) expressed in *Saccharomyces cerevisiae* was shown to contain mannose as the only carbohydrate constituent. All oligosaccharide chains of the glycoprotein could be released by mild alkaline treatment, and separated from the protein by gel-permeation chromatography on Bio-Gel P-2. The structures of these *O*-linked carbohydrate chains were determined by 500-MHz <sup>1</sup>H-NMR spectroscopy, affording the disaccharide Man $\alpha$ 1-2Man as the major component and the tetrasaccharide Man $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man as a minor component. Reference oligosaccharides were prepared from mannoproteins released from the cell wall of *S. cerevisiae* X2180 ( $\alpha$ -wild type). In addition to previously reported structures, ranging from mannose to mannotetraose, the pentasaccharide Man $\alpha$ 1-3Man $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man was identified in the cell wall mannoprotein.

Insulin-like growth factor I; Recombinant glycoprotein; Carbohydrate, *O*-linked; (Human, *Saccharomyces cerevisiae*)

## 1. INTRODUCTION

There is great interest in the yeast *Saccharomyces cerevisiae* as a host for the biotechnological production of recombinant human (glyco)proteins, since it is relatively easy for one to manipulate this eukaryotic organism which can be cultured in large amounts [1-3]. Especially for recombinant glycoproteins with a pharmacological application, knowledge of the exact structure of the carbohydrate chains is required. Glycosylation may influence the secretion efficiency [4], biological activity [4,5] and stability of the protein [6]. Furthermore, yeast-specific glycans on a human protein may introduce new immunological determinants. The glycosylation machinery in yeast can produce both *N*- and *O*-linked glycans [2]. *N*-linked glycans from yeast possess the same core structure as is found in human glycoproteins, but yeast *N*-glycans often contain highly branched

mannans. In contrast to most other eukaryotic organisms the *O*-glycosidic carbohydrate chains in yeast are linked via mannose to serine or threonine [7]. However, despite the rapid development in yeast gene-cloning research, there are no reports about the detailed structure of the glycans present on human proteins expressed in yeast [1].

Here, we report on the carbohydrate structures present in a glycosylated form of recombinant human insulin-like growth factor I (IGF-I or somatomedin C) expressed in *S. cerevisiae*. Human serum IGF-I is not glycosylated, but the recombinant human IGF-I from yeast can be separated into both an unglycosylated and a glycosylated form (Gellerfors, P., personal communication).

## 2. MATERIALS AND METHODS

### 2.1. Insulin-like growth factor I

The recombinant glycosylated human IGF-I preparation used here was provided by Dr P. Gellerfors (Kabi, Stockholm, Sweden). The concentration of IGF-I was determined by radioreceptor assay using placental membrane, the specific activity being 15000 U/mg protein.

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### 2.2. Isolation of yeast cell wall mannoprotein

Reference oligosaccharides were prepared from yeast cell wall mannoprotein, solubilized from the cell wall of *S. cerevisiae* X2180 ( $\alpha$ -wild type) by boiling in the presence of 2% (w/w) SDS, according to [8].

### 2.3. Liberation and isolation of O-linked carbohydrate chains

The O-linked carbohydrate chains were released by alkaline treatment as described [7]. Recombinant IGF-I (16 mg) was dissolved in 16 ml of 0.1 M NaOH and incubated for 18 h at 21°C. After neutralization with 2 M acetic acid and lyophilization, the released oligosaccharides were separated from the protein by gel filtration on a column (1.2 × 50 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) using water as eluent at a flow rate of 6 ml/h. The carbohydrate-positive fractions (orcinol/sulfuric acid spot test) were pooled and desalted on a column (1.2 × 8 cm) containing equal layers of AG1 X-2 (acetate-form, 200–400 mesh, Bio-Rad) and AG 50W (H<sup>+</sup>-form) with water as eluent.

The reference saccharides were released from 405 mg yeast cell wall mannoprotein in 80 ml of 0.1 M NaOH. In addition to

the previously reported structures Man (M1), Man $\alpha$ 1-2Man (M2), Man $\alpha$ 1-2Man $\alpha$ 1-2Man (M3) and Man $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man (M4) [7], a carbohydrate-positive fraction was obtained with a larger retention volume on HPLC (Lichrosorb-NH<sub>2</sub>) than M4. The anomeric region of the 500-MHz <sup>1</sup>H-NMR spectrum of M5 showed five signals of similar intensity. By comparing the chemical shift values of the anomeric protons with those from reference compounds [9], the oligosaccharide was identified as the pentasaccharide Man $\alpha$ 1-3Man $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man (M5).

### 2.4. HPLC

The oligosaccharide mixture obtained from *S. cerevisiae* cell wall mannoprotein was fractionated on a Kratos Spectroflow 400 HPLC system (Kratos Analytical) using a Lichrosorb-NH<sub>2</sub> 10  $\mu$ m column (0.46 × 25 cm, Chrompack). Prior to injection, samples were dissolved in water (Milli-Q quality). Carbohydrates were eluted isocratically with acetonitrile/water (80:20, v/v) at a flow rate of 2 ml/min and monitored at 195 nm with a Spectroflow 783 absorbance detector (Kratos Analytical).

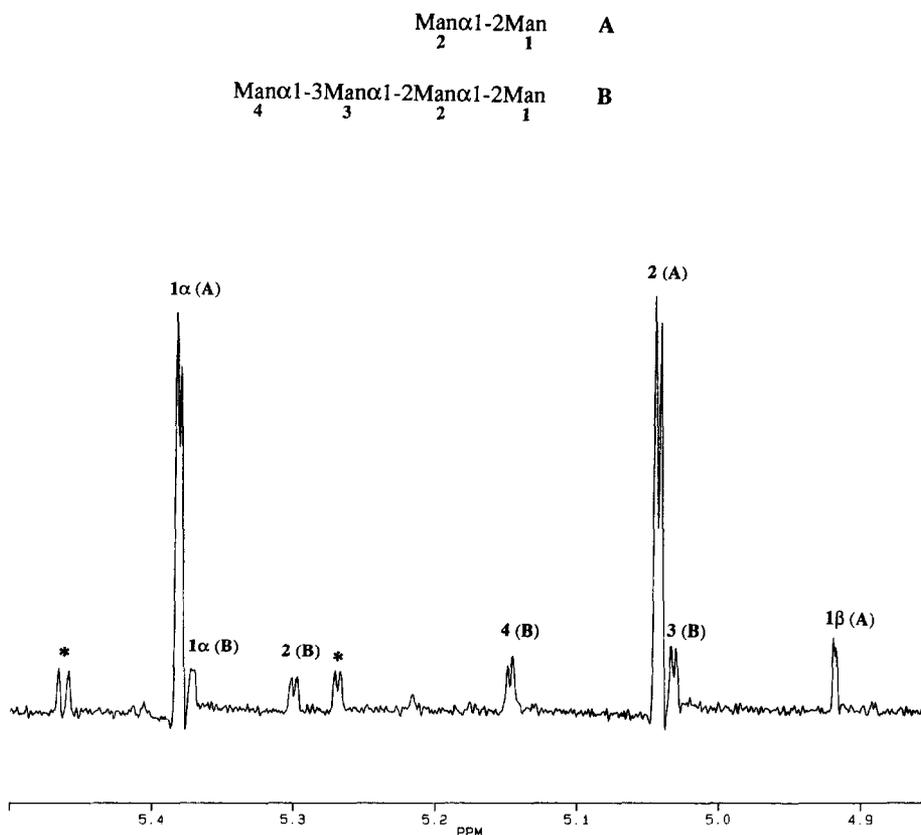


Fig.1. Anomeric region of the resolution-enhanced 500-MHz <sup>1</sup>H-NMR spectrum of the pool of oligosaccharides obtained from recombinant human IGF-I. The low-intensity signals designated by the asterisks at  $\delta$  5.462 ppm ( $J_{1,2} = 3.7$  Hz) and  $\delta$  5.269 ppm ( $J_{1,2} = 1.5$  Hz) probably belong to an as yet unidentified carbohydrate. However, the available amount of IGF-I was too low to permit further analysis of the minor compounds.

### 2.5. Monosaccharide analysis

Samples were subjected to the methanolysis procedure and analyzed by GLC on CP-Sil 5, as described [10].

### 2.6. Reduction of oligosaccharides

Part of the oligosaccharide mixture obtained from IGF-I was reduced with NaBH<sub>4</sub> in water (1 ml) for 14 h at room temperature. The reaction was terminated by the addition of AG 50W (H<sup>+</sup>-form) and the cation-exchange resin was removed by filtration. Boric acid was removed by co-evaporation with 1% (v/v) acetic acid in methanol.

### 2.7. 500-MHz <sup>1</sup>H-NMR spectroscopy

Carbohydrate samples were repeatedly exchanged in <sup>2</sup>H<sub>2</sub>O (finally using 99.96 atom% <sup>2</sup>H, Aldrich) with intermediate lyophilization. Resolution-enhanced 500-MHz <sup>1</sup>H-NMR spectra were recorded in <sup>2</sup>H<sub>2</sub>O at 27°C on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University). Chemical shifts ( $\delta$ ) are given relative to internal acetone ( $\delta$  = 2.225 ppm) [11]. The two-dimensional homonuclear Hartmann-Hahn (HOHAHA) spectrum was obtained using a 100 ms MLEV-17 mixing sequence [12], recording 512 measurements of 2K data points with 16 scans per  $t_1$  value. For the two-dimensional double-quantum filtered <sup>1</sup>H-<sup>1</sup>H correlation spectrum (DQF-COSY) [13] 350 measurements of 2K data points were performed. The 90° <sup>1</sup>H pulse width was 32.6  $\mu$ s and the residual HO<sup>2</sup>H signal was suppressed by presaturation for 1.0 s.

## 3. RESULTS

Sugar analysis of glycosylated recombinant human IGF-I revealed only mannose, and the total carbohydrate content was about 8% (w/w). After alkaline treatment of IGF-I, the pool of released oligosaccharides was isolated by gel-permeation chromatography on Bio-Gel P-2 (not shown), desalted, and analyzed by 500-MHz <sup>1</sup>H-NMR spectroscopy. As has been shown before, the chemical shifts of the H-1 and H-2 atoms of manno-oligosaccharides are characteristic of both the type of linkage and the sequence of the constituent monosaccharides [9,14]. The anomeric region of the <sup>1</sup>H-NMR spectrum of the mixture of IGF-I oligosaccharides is depicted in fig.1. The major component turned out to be Man $\alpha$ 1-2Man (compound A), reflected by two about equally intense H-1 signals at  $\delta$  5.380 ppm ( $J_{1,2}$  = 1.5 Hz) and  $\delta$  5.042 ppm ( $J_{1,2}$  = 1.5 Hz), and having corresponding H-2 signals at  $\delta$  3.96 and 4.073 ppm, stemming from the reducing and nonreducing Man residues, respectively. The structural reporter groups match completely those obtained for the reference M2, isolated from the cell wall mannoprotein of *S. cerevisiae* (see table 1), but are different from those

Table 1

<sup>1</sup>H chemical shifts of structural reporter group protons of the constituent monosaccharides for the oligosaccharides A and B derived from recombinant human IGF-I together with those for reference compounds M2–M5, prepared from yeast cell wall mannoprotein

Reporter Residue <sup>a</sup> group		A	B	M2	M3	M4	M5
H-1	1 $\alpha$	5.381	5.371	5.380	5.370	5.371	5.371
	1 $\beta$	4.918	n.d.	4.918	n.d.	n.d.	n.d.
	2	5.042	5.297	5.042	5.299	5.297	5.296
	3	–	5.032	–	5.044	5.033	5.036
	4	–	5.147	–	–	5.142	5.121
H-2	5	–	–	–	–	–	5.137
	1 $\alpha$	3.96	3.96	n.d.	3.960	n.d.	n.d.
	1 $\beta$	4.073	n.d.	n.d.	n.d.	n.d.	n.d.
	2	4.073	4.11	4.073	4.109	4.11	n.d.
	3	–	4.23	–	4.065	4.221	n.d.
H-3	4	–	4.07	–	–	4.068	n.d.
	1	3.69	n.d.	n.d.	n.d.	n.d.	n.d.
	2	3.86	n.d.	n.d.	n.d.	n.d.	n.d.

<sup>a</sup> For numbering of monosaccharide residues, see fig.1

n.d., not determined. Chemical shifts are given in <sup>2</sup>H<sub>2</sub>O at 300 K relative to internal acetone ( $\delta$  2.225 ppm)

reported for the  $\alpha$ 1-3,  $\alpha$ 1-4, and  $\alpha$ 1-6-linked mannosyl-mannoses [9]. The assignments of additional protons for the IGF-I disaccharide, as given in table 1, were made using COSY and HOHAHA experiments. The disaccharide accounts for about 80 mol% of the oligosaccharides present in IGF-I, estimated by integrating the anomeric region of the <sup>1</sup>H-NMR spectrum of the mixture of oligosaccharides.

In addition to the disaccharide, small amounts of Man $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man (compound B) could be identified. The most indicative structural reporter group signals for this tetrasaccharide are the H-1 signals at  $\delta$  5.147 and 5.032 ppm, being characteristic for the terminal  $\alpha$ 1-3-linked Man-4 and the subterminal 3-substituted  $\alpha$ 1-2-linked Man-3, respectively. From the HOHAHA experiment the corresponding H-2 signals were assigned to  $\delta$  4.07 ppm (Man-4) and  $\delta$  4.23 ppm (Man-3). The signal at  $\delta$  5.371 ppm is assigned to H-1 of the  $\alpha$ -anomer of Man-1, with the H-2 signal at  $\delta$  3.79 ppm, whereas the H-1 of Man-2 resonates at  $\delta$  5.297 ppm, with the H-2 signal at  $\delta$  4.11 ppm (cf. M4, table 1). The relatively high intensity of the signal at  $\delta$  5.147 ppm, as compared to the signal at

$\delta$  5.297 ppm, is due to overlap with another, as yet unassigned signal, which was detected by a second cross-peak in the HOHAHA experiment. In the trisaccharide **M3** and the tetrasaccharide **M4** the chemical shift values of the H-1 atoms of Man-1 and Man-2 are the same. However, based on the intensities of the signals it can be concluded that only trace amounts, if any, of the trisaccharide Man $\alpha$ 1-2Man $\alpha$ 1-2Man can occur in IGF-I.

#### 4. DISCUSSION

Here it is shown that Man $\alpha$ 1-2Man is the predominant oligosaccharide in a glycosylated form of human recombinant IGF-I produced in *S. cerevisiae*. As a minor constituent Man $\alpha$ 1-3Man  $\alpha$ 1-2Man $\alpha$ 1-2Man could be identified. Both oligosaccharides are typical yeast *O*-glycans, but it should be noted that the same carbohydrate epitope as in the disaccharide occurs as a terminal sequence in several human *N*-glycoproteins containing oligomannose-type carbohydrate chains [15]. On the other hand, the tetrasaccharide does not normally occur in human glycoproteins and strongly reacting antibodies against this yeast oligosaccharide have been raised in rabbit [16]. The *O*-linked pentasaccharide Man $\alpha$ 1-3Man  $\alpha$ 1-3Man $\alpha$ 1-2Man $\alpha$ 1-2Man, which was found in yeast cell wall mannoprotein, was not found in the recombinant protein, but has previously been isolated from *S. italicus* [17].

In IGF-I there are eight serine or threonine residues [18], being potential *O*-glycosylation sites. It has been shown that a proline residue in the vicinity of a serine or threonine residue markedly enhances the *O*-glycosylation efficiency [19,20], therefore the most likely glycosylation sites in IGF-I are the threonines at positions 4, 29 and 41. The lack of *N*-linked saccharides in IGF-I is explained by the absence of an Asn-X-Ser/Thr sequence, required for *N*-glycosylation [21].

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