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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 ^{1}H-NMR spectroscopy, affording the disaccharide Manα1-2Man as the major component and the tetrasaccharide Manα1-3Manα1-2Manα1-2Man as a minor component. Reference oligosaccharides were prepared from mannoproteins released from the cell wall of *S. cerevisiae* X2180 (α-wild type). In addition to previously reported structures, ranging from mannose to mannotetraose, the pentasaccharide Manα1-3Manα1-3Manα1-2Manα1-2Man 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).

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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 (α-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^+-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**), Manα1-2Man (**M2**), Manα1-2Manα1-2Man (**M3**) and Manα1-3Manα1-2Man α1-2Man (**M4**) [7], a carbohydrate-positive fraction was obtained with a larger retention volume on HPLC (Lichrosorb-NH_2) than **M4**. The anomeric region of the 500-MHz ^{1}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 Manα1-3Manα1-3Manα1-2Manα1-2Man (**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_2 10 μm 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).

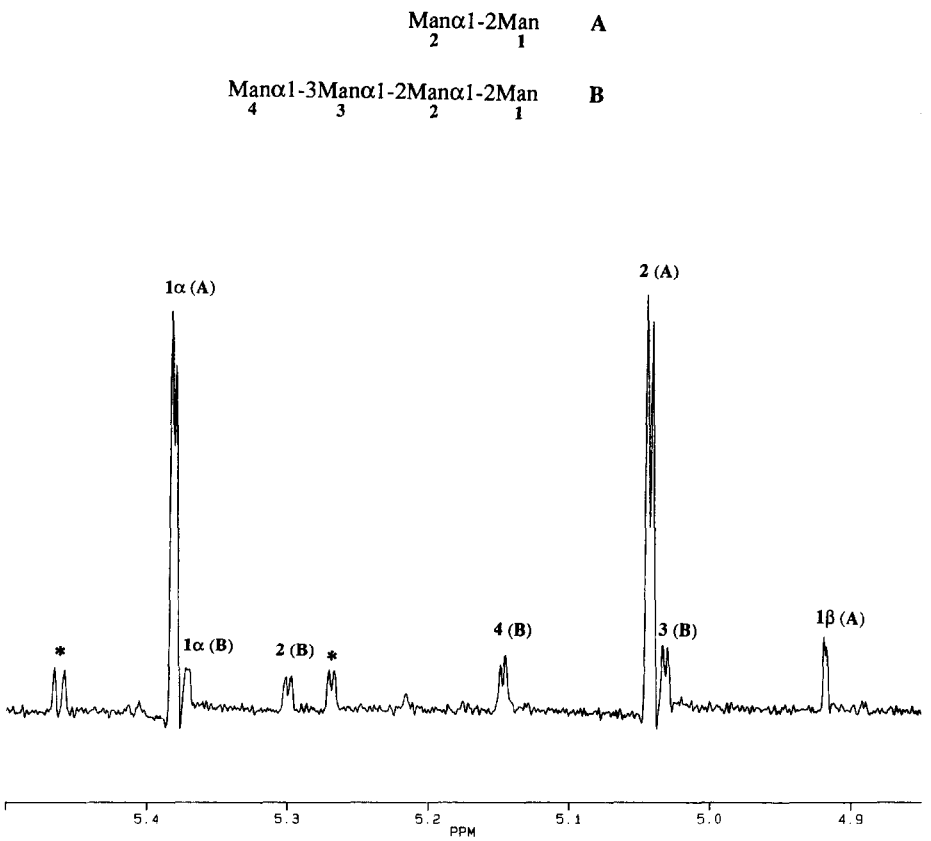

Fig.1. Anomeric region of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the pool of oligosaccharides obtained from recombinant human IGF-I. The low-intensity signals designated by the asterisks at δ 5.462 ppm ($J_{1,2}$ = 3.7 Hz) and δ 5.269 ppm ($J_{1,2}$ = 1.5 Hz) probably belong to an as yet 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 $NaBH_4$ in water (1 ml) for 14 h at room temperature. The reaction was terminated by the addition of AG 50W (H^+-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 ^{1}H-NMR spectroscopy*

Carbohydrate samples were repeatedly exchanged in ^2H_2O (finally using 99.96 atom% ^{2}H, Aldrich) with intermediate lyophilization. Resolution-enhanced 500-MHz ^{1}H-NMR spectra were recorded in ^2H_2O at 27°C on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University). Chemical shifts (δ) are given relative to internal acetone (δ = 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 ^{1}H-^{1}H correlation spectrum (DQF-COSY) [13] 350 measurements of 2K data points were performed. The 90° ^{1}H pulse width was 32.6 μs and the residual HO^{2}H signal was suppressed by presaturation for 1.0 s.

Table 1

^{1}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 group	Residue[a]	A	B	M2	M3	M4	M5
H-1	1α	5.381	5.371	5.380	5.370	5.371	5.371
	1β	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
	5	–	–	–	–	–	5.137
H-2	1α	3.96	3.96	n.d.	3.960	n.d.	n.d.
	1β	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.
	4	–	4.07	–	–	4.068	n.d.
H-3	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.

[a] For numbering of monosaccharide residues, see fig.1

n.d., not determined. Chemical shifts are given in ^2H_2O at 300 K relative to internal acetone (δ 2.225 ppm)

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 ^{1}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 ^{1}H-NMR spectrum of the mixture of IGF-I oligosaccharides is depicted in fig.1. The major component turned out to be Manα1-2Man (compound **A**), reflected by two about equally intense H-1 signals at δ 5.380 ppm ($J_{1,2}$ = 1.5 Hz) and δ 5.042 ppm ($J_{1,2}$ = 1.5 Hz), and having corresponding H-2 signals at δ 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 reported for the α1-3, α1-4, and α1-6-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 ^{1}H-NMR spectrum of the mixture of oligosaccharides.

In addition to the disaccharide, small amounts of Manα1-3Manα1-2Manα1-2Man (compound **B**) could be identified. The most indicative structural reporter group signals for this tetrasaccharide are the H-1 signals at δ 5.147 and 5.032 ppm, being characteristic for the terminal α1-3-linked Man-**4** and the subterminal 3-substituted α1-2-linked Man-**3**, respectively. From the HOHAHA experiment the corresponding H-2 signals were assigned to δ 4.07 ppm (Man-**4**) and δ 4.23 ppm (Man-**3**). The signal at δ 5.371 ppm is assigned to H-1 of the α-anomer of Man-**1**, with the H-2 signal at δ 3.79 ppm, whereas the H-1 of Man-**2** resonates at δ 5.297 ppm, with the H-2 signal at δ 4.11 ppm (cf. **M4**, table 1). The relatively high intensity of the signal at δ 5.147 ppm, as compared to the signal at

δ 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 Manα1-2Manα1-2Man can occur in IGF-I.

4. DISCUSSION

Here it is shown that Manα1-2Man is the predominant oligosaccharide in a glycosylated form of human recombinant IGF-I produced in *S. cerevisiae*. As a minor constituent Manα1-3Man α1-2Manα1-2Man 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 Manα1-3Man α1-3Manα1-2Manα1-2Man, 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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REFERENCES

[1] Kukuruzinska, M.A., Bergh, M.L.E. and Jackson, B.J. (1987) Annu. Rev. Biochem. 56, 915–944.
[2] Tanner, W. and Lehle, L. (1987) Biochim. Biophys. Acta 906, 81–99.
[3] Hinnen, A., Heim, J. and Meyhack, B. (1988) Yeast 4, S26.
[4] Gill, G.S., Zaworski, P.G., Marotti, K.R. and Rehberg, E.F. (1988) Yeast 4, S148.
[5] Moonen, P., Mermod, J.-J., Ernst, J.F., Hirschi, M. and DeLamarter, J.F. (1987) Proc. Natl. Acad. Sci. USA 84, 4428–4431.
[6] Van Arsdell, J.N., Kwok, S., Schweikart, V.L., Ladner, M.B., Gelfand, D.H. and Innis, M.A. (1987) Bio/Technology 5, 60–64.
[7] Nakajima, T. and Ballou, C.E. (1974) J. Biol. Chem. 249, 7679–7684.
[8] Valentin, E., Herrero, E., Pastor, F.I.J. and Sentandreu, R. (1984) J. Gen. Microbiol. 130, 1419–1428.
[9] Cohen, R.E. and Ballou, C.E. (1980) Biochemistry 19, 4345–4358.
[10] Kamerling, J.P. and Vliegenthart, J.F.G. (1982) Cell Biol. Monogr. 10, 92–125.
[11] Vliegenthart, J.F.G., Dorland, L. and Van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. 41, 209–374.
[12] Bax, A. and Davis, D.G. (1985) J. Magn. Res. 65, 355–360.
[13] Marion, D. and Wütrich, K. (1985) Biochem. Biophys. Res. Commun. 117, 967–974.
[14] Van Halbeek, H., Dorland, L., Veldink, G.A., Vliegenthart, J.F.G., Michalski, J.-C., Montreuil, J., Strecker, G. and Hull, W.E. (1980) FEBS Lett. 121, 65–70.
[15] Kobata, A. (1984) in: Biology of Carbohydrates, vol.2 (Ginsburg, V. and Robbins, P.W. eds) pp.87–161, Wiley, New York.
[16] Zopf, D.A., Tsai, C.-M. and Ginsburg, V. (1978) Arch. Biochem. Biophys. 185, 61–71.
[17] Ballou, C.E., Lipke, P.N. and Raschke, W.C. (1974) J. Bacteriol. 117, 461–467.
[18] Rinderknecht, E. and Humbel, R.E. (1978) J. Biol. Chem. 253, 2769–2776.
[19] Lehle, L. and Bause, E. (1984) Biochim. Biophys. Acta 799, 246–251.
[20] Fiat, A.M., Jollés, J., Aubert, J.P., Loucheux-Lefebvre, M.H. and Jollés, P. (1980) Eur. J. Biochem. 111, 333–339.
[21] Marshall, R.D. (1972) Annu. Rev. Biochem. 41, 673–702.