NMR evidence for a novel asparagine-linked oligosaccharide on cellobiohydrolase I from *Trichoderma reesei* RUTC 30

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Abstract The primary structure of a novel phosphate-containing oligosaccharide, isolated from *T. reesei* cellobiohydrolase I, was determined by NMR techniques. The new compound has the same structure as $GlcMan_7GlcNAc_2$, but it is extended by one α-mannopyranosyl unit (Man-P) through a phosphate link. Three different heteronuclear (^{31}P-^{1}H) NMR techniques were used to prove that the phosphate links the glycosidic site of Man-P with C-6 of unit Man-B. The presence of mannoses linked through a phosphate diester resembles glycosyl synthesis in yeast.

Key words: Cellobiohydrolase I; Fungus; Mannose 6-phosphate; NMR; *Trichoderma reesei* RUTC 30

1. Introduction

The protein glycosylation pathway [1,2] still holds many questions in the case of lower eukaryotes. Thus, while the characteristics of some carbohydrate chains, isolated from glycoproteins of certain filamentous fungi such as *Aspergillus* and *Trichoderma*, resemble those found in yeast, others resemble those encountered in mammalians [3,4].

In an attempt to shed some light on the glycosylation in filamentous fungi, we have studied the predominant *N*-glycans from cellobiohydrolase I (CBH I), secreted from *Trichoderma reesei* RUTC 30 [5]. One of the compounds found is a novel phosphate-containing oligosaccharide, which, to the best of our knowledge, has not been reported before.

We present here NMR evidence for the primary structure of this compound, Man-P$GlcMan_7GlcNAc_2$, referred to here for brevity as compound **1**.

2. Materials and methods

2.1. Isolation of CBH I phosphate-containing oligosaccharide

N-linked oligosaccharides were isolated from pure *T. reesei* CBH I [5], using PNGase F. About 0.5 mg of the studied oligosaccharide was obtained in a pure form after Biogel P4 gel filtration according to Kobata [6]. It eluted as a mixture with small amounts of other uncharacterized oligosaccharides, close after the void. A second round of Biogel P4 gel filtration gave satisfying resolution of size fractionation and compound **1** was analyzed by NMR.

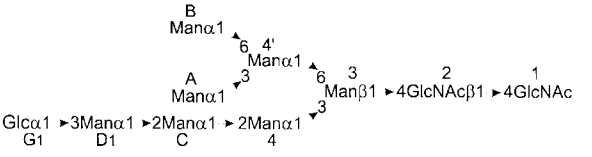
2.2. NMR experiments

About 0.5 mg of compound *1* was twice deuterium exchanged in 99.98% D_2O and transferred to a 5 mm Wilmad tube. All the NMR spectra were measured on a Varian UNITY-500 spectrometer operating at 499.693 MHz for ^{1}H-NMR and at 202.276 MHz for ^{31}P-NMR. All the spectra were run at 33°C. The sample was not spun. ^{1}H-NMR spectra were referenced to the methyl line of free acetate as recommended by Van Halbeek (δ = 1.91) [7] and 75% H_3PO_4 in a capillary was used as an external reference for ^{31}P-NMR spectra. Standard Varian software version vnmr 5.1 was used throughout. Some standard pulse sequences were modified slightly. All experiments (including 1D TOCSY [8,9], DQF-COSY [10], (edited) GHSQC [11], GHMQC [12], GMQFCOSY [13], and DEPT [14]) were performed in a 5 mm 'inverse detection' probe (with typical values for 90° pulses of 7.4 μs for ^{1}H pulses and 14.0 μs for ^{13}C pulses) equipped with pulsed magnetic field gradient coils. Gradient pulses were produced by Performa II PFG source (Varian), shaped pulses were generated by a waveform generator (Varian) using the shaped pulses calculated by the Pandora box program. Further experimental details are indicated in the figure legend of particular spectra.

3. Results

There are 11 resonances (nine integrating for 1 proton and two integrating for 0.5 proton each) in the anomeric region of the ^{1}H-NMR spectrum (Fig. 1). The two multiplets with 0.5 H integrals are centered at δ = 5.18 and δ = 4.68. The latter, which is hidden under the water peak in the spectrum shown, is detected by heteronuclear 2D spectroscopy or by ^{1}H-NMR spectra measured at 5°C.

With the exception of a doublet of doublets at δ = 5.44 (J = 1.7 and 8.1 Hz), all these resonances have the same chemical shifts, the same integration and the same splittings as found in the spectra of the recently studied compound $GlcMan_7GlcNAc_2$ [5] (compound **2**).



Compound *2*

This indicates that the basic structure of $GlcMan_7GlcNAc_2$ is extended by one carbohydrate unit in compound **1**. The results of phase-sensitive COSY and 1D-TOCSY experiments which provided assignments of the resonances of H-2 and H-3 protons of all the monosaccharide units (Table 1), are in agreement with this assumption.

A series of 1D-TOCSY experiments performed for various mixing times provided the assignments of other multiplets belonging to the additional unit: δ(H-2) = 4.02 (a doublet of

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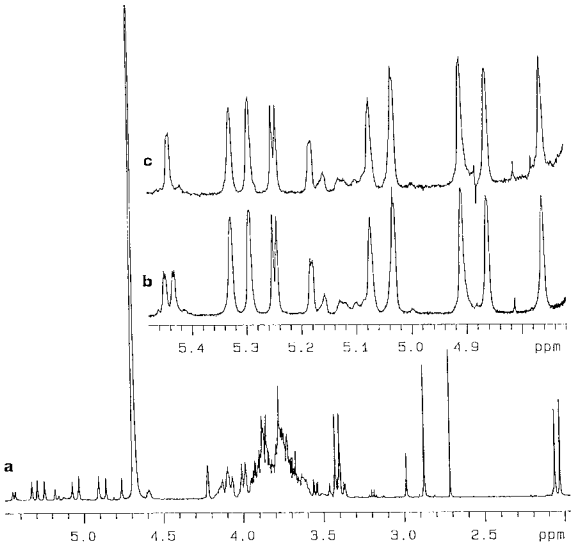


Fig. 1. ^{1}H-NMR spectra of compound *1* in D_2O. (a) Full spectrum. (b) Anomeric part of (a). (c) Same as (b) but measured with {^{31}P} decoupling (spectral width 3100 Hz, acquisition time 4 s, relaxation delay 1 s, FID zero-filled to 32 K data points, no FID weighting, 580 transients accumulated).

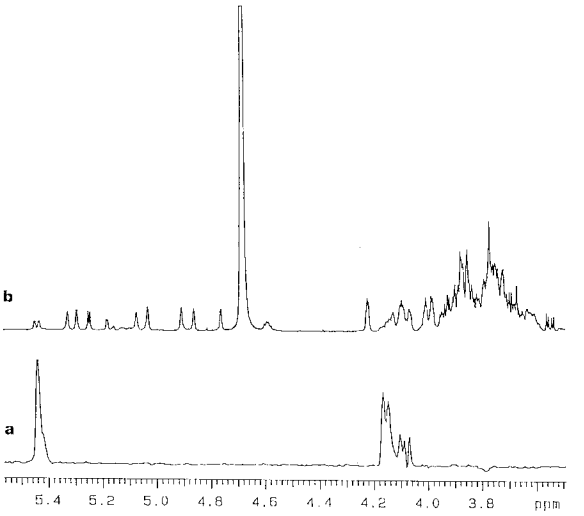


Fig. 2. Trace (a) from GHSQC (^{1}H-^{31}P) spectrum along the detection axis (^{1}H) compared with (b) partial ^{1}H-NMR spectrum of compound **1** (experimental parameters for (a): acquisition time 0.256 s, spectral widths 2000 and 14000 Hz for f_2 and f_1, respectively, 240 transients accumulated for each of 98 increments, shifted sine-bell filtering in both dimensions, data matrix 2048×512, garp {^{31}P} decoupling during acquisition).

triplets, $J(1,2) = 1.7$ Hz, $J(2,3) = 3$ Hz and a further heteronuclear coupling ^{1}H-^{31}P of 3 Hz), δ(H-3) = 3.91 (a doublet of doublets), δ(H-4) = 3.71 (a triplet, two coupling constant of about 9.5 Hz), δ(H-5) = 3.86 (multiplet), δ(H-6A) = 3.84 (a doublet of doublets, $J = 2$ and 12 Hz) and δ(H-6B) = 3.78 (a doublet of doublets, $J = 5$ and 12 Hz). According to these data and according to the coupling constant of the anomeric proton (H-1; $J(1,2) = 1.7$ Hz), the additional monosaccharide unit (δ = 5.44) is a mannose in the α-anomeric form.

The other coupling ($J = 8.1$ Hz) observed on the anomeric multiplet of this α-mannose is a heteronuclear coupling ^{1}H-^{31}P as proved by heteronuclear decoupling (see Fig. 1c) of the single phosphorus multiplet seen in the ^{31}P-NMR spectrum (according to its chemical shift, δ = −1.03, phosphorus is present as a phosphate). As demonstrated by the appropriate trace from ^{1}H-^{31}P correlation shown in Fig. 2, the phosphorus nucleus is also coupled to H-2 and H-6A of a mannose. To determine which mannose unit is involved, an indirect approach had to be taken because severe overlap prohibits direct determination. So, a series of 1D TOCSY experiments each selectively exciting anomeric proton of one mannose unit and employing long mixing times (300 ms) was performed with and without phosphorus decoupling. Quite conclusively, the H-1 proton of mannose-B exhibited a coupling pathway to both H-6 protons (δ(H-6A) = 4.16 and δ(H-6B) = 4.10) of the

Table 1
Assigned ^{1}H-NMR chemical shifts of compound **1**[a]

Unit[b]	H-1	H-2	H-3
GlcNAc-1α	5.18	3.87	3.88
GlcNAc-1β	4.69	3.68	[c]
GlcNAc-2	4.59	3.78	3.77
Man-3	4.76	4.22	3.71
Man-4	5.33	4.09	4.01
Man-C	5.29	4.10	3.95
Man-D1	5.03	4.22	3.93
Glc-G1	5.25	3.55	3.76
Man-4′	4.86	4.13	3.90
Man-A	5.07	4.06	3.89
Man-B[d]	4.91	3.98	3.84
Man-P[e]	5.44	4.01	3.91

[a]Anomeric proton (H-1) chemical shifts are accurate within less than ±0.01 ppm, other proton shifts are estimates either from 1D TOCSY spectra or phase-sensitive double-quantum filtered COSY spectra, their precision being estimated at ±0.02 ppm.
[b]For monosaccharide unit labelling see the formula; proton atoms in the units are labelled in the standard way.
[c]Not determined.
[d]δ(H-4) = 3.14, δ(H-5) = 3.80, δ(H-6A) = 4.16, δ(H-6B) = 4.10.
[e]δ(H-4) = 3.71, δ(H-5) = 3.86, δ(H-6A) = 3.84, δ(H-6B) = 3.78.

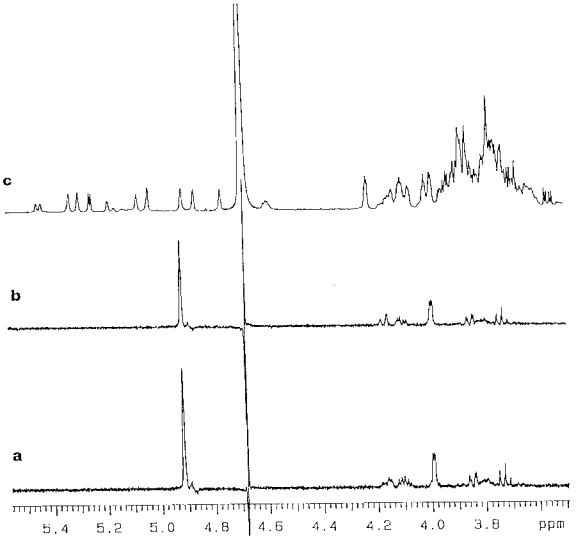
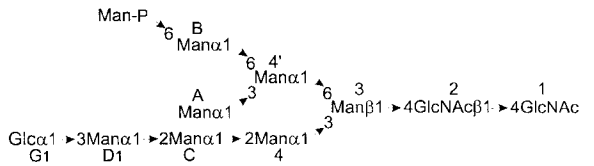


Fig. 3. Selective (1D) TOCSY spectra (a) measured without {^{31}P} decoupling and (b) with {^{31}P} decoupling compared with (c) conventional ^{1}H-NMR spectrum (note the difference between (a) and (b) in the region around δ = 4.1) (Experimental conditions for (a,b): line at δ = 4.91 was excited by a full Gaussian 90° pulse 182 ms long, trim pulses of 2 ms preceded and followed the MLEV-17 spin-lock of mixing time 300 ms, spectral width 3100 Hz, acquisition time 2 s, no weighting used, 5000 transients accumulated.)

same mannose unit and multiplets of both H-6 protons were affected by phosphorus decoupling (see Fig. 3).

Accordingly, compound **1** has the following structure (the labels of monosaccharide units shown are used throughout this text):



Compound *1*

4. Discussion

The characterized compound *1* is unusual in several aspects:

First, it is surprising to find one glucose residue in the studied oligosaccharide from *T. reesei* CBH I, the major secreted glycoprotein. Apparently, the third glucose residue had not been removed from the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ by glucosidase II. It was observed that secretion of *T. reesei* RUTC30 was not affected by this lack of trimming. This is different from mammalian cells, where inefficient glucosidase trimming has a negative effect on secretion of certain glycoproteins, since terminal glucose residues are intracellularly recognized, leading to retention of glycoproteins in the endoplasmic reticulum by a set of lectin-like chaperones [15].

While trimming by glucosidase II seemed to be inefficient or inhibited, trimming by α-1,2-mannosidase was not. This was suggested by the removal of two α-1,2-linked mannoses from the analyzed compound, before or during addition of the mannose phosphate group.

Finally, the presence of phosphate in diester linkage, with mannose as a terminal residue is typical of fungal glycosyl synthesis [16]. This again suggests a difference from the secretion system of mammalian cells, where phosphate in diester linkage has *N*-acetylglucosamine as the terminal sugar residue [17].

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