In vivo synthesis of complex *N*-glycans by expression of human *N*-acetylglucosaminyltransferase I in the filamentous fungus *Trichoderma reesei*

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Abstract The human *N*-acetylglucosaminyltransferase I gene was introduced in the genome of *Trichoderma reesei* strain VTT-D-80133. Expression was studied after induction from the cellobiohydrolase I promoter. Successful in vivo transfer of GlcNAc was demonstrated by analyzing the neutral *N*-glycans which were synthesized on cellobiohydrolase I. Final proof of the formation of $GlcNAcMan_5GlcNAc_2$ was obtained by NMR analysis.

Key words: Cellobiohydrolase I; Fungus; *N*-acetylglucosaminyltransferase I; *Trichoderma reesei*

1. Introduction

Filamentous fungi are known as saprophytic organisms which secrete high amounts of sugar polymer, degrading enzymes in order to obtain the necessary carbon sources for their growth. Because of their high secretion capacity, they are attractive potential host organisms for the production of glycoproteins with therapeutic importance. At present, several attempts have been made to obtain high amounts of heterologous proteins in fungal species such as *Aspergillus*, *Trichoderma* and *Neurospora* [1–3]. The presence, however, of high-mannose type glycans hampers their real application. An antigenic response is often elicited for which certain substituents such as terminal galactoses or α-1,3-mannoses are held responsible [4]. In addition, terminal mannose residues are recognized by lectins present in the mammalian body. Consequently, the recombinant glycoproteins are rapidly cleared from the blood [5]. To prevent such clearing, the *N*-glycans should have terminal sialic acid residues. Several methods can be designed and tested to obtain fungally produced proteins that can be sialylated easily. As reported before, it is possible to convert at least a fraction of the fungal *N*-glycans to the mammalian type by in vitro mimicking of the mammalian synthesis pathway [6]. The next step in this effort is to introduce the genes of mammalian glycosyltransferases in the genome of filamentous fungi to express them intracellularly so that the number of necessary in vitro steps remains limited. The key enzyme to the complex oligosaccharide synthesis is *N*-acetylglucosaminyltransferase I (GlcNAc-TI: EC 2.4.1.101), which incorporates *N*-acetylglucosamine residues to α-1,3-linked mannose of the core oligosaccharide $Man_5GlcNAc_2$. Introduction of the rabbit GlcNAc-TI gene in the genome of *Aspergillus nidulans* has already been demonstrated [7]. GlcNAc-TI activity was detected intracellularly, but there was no evidence of in vivo transfer of GlcNAc residues to fungal glycans, nor was it possible to precisely define the subcellular location of the enzyme. Here, we studied the in vivo expression of human GlcNAc-TI in the filamentous fungus *Trichoderma reesei*.

2. Materials and methods

2.1. Strain, media and growth conditions

T. reesei strain VTT-D-80133 [8] was obtained from Primalco (Helsinki, Finland) and served as expression host. A minimal non-selective growth medium was used [9]. Glucose was added as a carbon source to the medium to maximize the biomass. The fungal cells were grown in 6 l medium for 7 days at 28°C and at 250 rpm. The cells were collected after centrifugation and transferred to lactose containing medium to induce the simultaneous expression of cellobiohydrolase I (CBHI) and GlcNAc-TI. After 7 days of induction, the cells were separated from the extracellular medium by centrifugation and filtration.

2.2. Construction of a GlcNAc-TI expression vector

The encoding region of the human GlcNAc-TI gene was amplified and cloned into a pUC18 vector [6]. To facilitate further cloning work, the GlcNAc-TI gene was subcloned between the *Bam*HI and *Hin*dIII sites of pGEM11Zf(+) (Promega Biotec, Madison, WI, USA). The *T. reesei* expression vector pEN208 [10] was first modified for cloning of the GlcNAc-TI gene. To that end, 5′-TCGGCC-3′ was changed by site-specific mutagenesis at the end of the CBHI signal sequence to 5′-TCGCGA-3′, the latter being an *Nru*I site. The plasmid was then cut by *Nru*I and *Bcl*I and the *Bcl*I site was blunted using T4 polymerase. Self-ligation was prevented by dephosphorylation using calf intestine alkaline phosphatase. The GlcNAc-TI gene was recovered from pGEM11Zf(+) after *Hin*dII digestion and ligated into pEN208. The nucleotide sequence 5′-TCGGACCTCGAG*GGATCC*TCTAGAGTCTCCAGGATG-3′ consists of a *Nru*I/*Hin*dII ligation site up to the GlcNAc-TI start codon, as well as of a *Bam*HI restriction site (italicized). To remove the CBHI signal sequence, a partial *Mst*I digestion was performed. With another preparation of the same plasmid, the *Bam*HI site close to the start codon was cut and then blunted by T4 polymerase. After *Nsi*I digestion, the GlcNAc-TI gene was recovered and then ligated in blunted *Mst*I- and *Nsi*I-digested pEN208 to result in pENhGNTI1.

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2.3. Transformation of T. reesei VTT-D-80133

Transformation of the fungal cells was obtained after protoplasting of the cells [9]. p3SR2 was co-transformed in order to allow selection of transformants that were able to grow on minimal medium with acetamide as the only nitrogen source [10]. Transformants were collected after 7 days of growth. They were submitted to a second and a third round of selection by transferring the mycelium to fresh minimal medium plates containing acetamide as the only nitrogen source. Finally, transformants were sporulated on potato dextrose agar plates, after which spores were resuspended in 0.9% NaCl/20% glycerol to allow storage at −70°C.

2.4. Southern blot analysis

Spores were inoculated in minimal medium [9], to which 0.05% yeast extract, 0.05% peptone and 0.02% corn steep liquor were added. Cells were grown at 30°C for 7 days. Mycelium was precipitated by centrifugation and resuspended into lactose containing minimal medium. After 2 days, cells were collected by centrifugation and pressed between filter paper to remove extracellular liquid as much as possible. Genomic DNA was prepared as described [11]. One sample of DNA (approximately 10 μg) was double-digested with *Eco*47III/*Nsi*I, while another sample was double-digested with *Xba*I/*Nsi*I (reference DNA was from phage lambda digested with *Pst*I). The digests were submitted to gel electrophoresis over a 1% agarose gel. After staining with ethidium bromide to check the quality of the digested DNA, Southern blotting was performed using standard protocols [12]. A probe was prepared by random prime labelling of 100 ng of a GlcNAc-TI gene fragment which was isolated from a mammalian expression plasmid pCAMFhGNTIf1 [6]. The specific activity of the obtained probe was 3.2×10^4 dpm/ng. As a reference, *Pst*I-digested lambda DNA was labelled by random priming and hybridized to its own DNA. The blot was exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA, USA) to detect radioactive signals.

2.5. Lectin screening

Spores (2×10^9) of transformants were inoculated in 200 ml lactose containing minimal medium and grown at 30°C for 8 days. The extracellular medium was collected after precipitating the *T. reesei* cells by centrifugation and filtration. The medium was 30-fold concentrated by ultrafiltration using Microcon-10 devices (Amicon, Danvers, MA, USA). 15 μl was loaded in the wells of a 12.5% polyacrylamide gel to electrophoretically separate denatured proteins. The extracellular proteins were then blotted according to standard procedures. The blot was screened using *Bandeiraea simplicifolia* agglutinin II, which binds specifically to terminal GlcNAc residues [13].

2.6. Production and purification of CBHI

Spores were inoculated and grown in 4 l glucose containing growth medium for 7 days, after which the mycelium was changed to induction medium. After 7 days, the extracellular medium was collected and mycelium was resuspended in 2 l induction medium to allow extra production of CBHI. Purification of CBHI was achieved as described [14,15], the only difference being a repetition of the anion exchange purification step to replace final purification over an affinity column. The protein concentration was determined according to Bradford and using BSA as standard protein.

2.7. Isolation and fractionation of N-glycans

Approximately 250 mg of CBHI was deglycosylated by PNGase F. CBHI was denatured by boiling for 5 min in 50 mM Tris-Cl pH 8.0, 0.5% SDS and 1% β-mercaptoethanol. After addition of NP-40 to 1% final concentration, 100 000 U of PNGase F was added. After 24 h incubation at 37°C, 50 000 extra U was added and incubated for another 24 h. Released *N*-glycans were collected by acetone precipitation and resuspension into 60% methanol [16]. By repeating acetone precipitation, most detergent was removed. The mixture of *N*-glycans was loaded and fractionated over a Biogel P4 column (Bio-Rad, Richmond, CA, USA) [17].

2.8. Analysis of CBHI N-glycans by fluorophore-assisted carbohydrate electrophoresis (FACE)

A small fraction of the *N*-glycan mixture and samples of the fractionated oligosaccharides were fluorescently labelled with 8-amino-1,3,6-naphthalene trisulfonic acid (ANTS) or aminoacridone and submitted to FACE analysis [18]. Electrofluorograms were recorded with a LumiImager F1 (Boehringer Mannheim, Mannheim, Germany). Commercial high-mannose *N*-glycans from bovine pancreas ribonuclease B (Oxford Glycosystems, Reading, UK) served as references. In addition, an oligo-glucose ladder was prepared by hydrolyzing dextrane [17].

2.9. NMR analysis

Approximately 250 μg of oligosaccharides was dissolved in 0.7 ml deuterium oxide (Aldrich Chemical, Milwaukee, WI, USA), without internal reference. The ^{1}H-NMR spectrum was measured on a UNITY-500 spectrometer, operating at 499.478 MHz (Varian Analytical Instruments, San Fernando, CA, USA). The experiment was performed with a 5 mm inverse detection probe equipped with pulsed magnetic field gradient coils. The standard Varian software vnmr version 6.1 was used throughout. The spectrum was run at 27°C and referenced to the value of the high-field methyl proton line of sodium acetate (δ 1.91) by applying the value of the high-field methyl proton line of the GlcNAc acetates (δ 2.03). The ^{1}H-90° pulses were 5.5 μs. Presaturation was chosen in order to suppress the water peak, with a pulse of 2 ms and a power of 2 dB. An exponential weighting function lb = 0.75 was used.

3. Results

3.1. Transformation of GlcNAc-TI

In order to express human GlcNAc-TI in *T. reesei*, the corresponding gene was amplified and cloned in pEN208. After random integration in the genome of this filamentous fungus, the CBHI promoter was induced after depletion of glucose in the growth medium and by adding an inducing carbon source such as lactose. Several acetamidase-positive transformants were obtained. Successful integration of pENhGNTI1 was analyzed by Southern blotting. After hybridization with a GlcNAc-TI gene fragment to *Eco*47III/*Nsi*I and with *Xba*I/*Nsi*I-digested genomic DNA of the transformants, expected bands of 1230 bp and 1350 bp, respectivily, were detected (result not shown). Southern blotting confirmed the presence of at least one intact expression cassette for GlcNAc-TI.

3.2. Lectin screening

After blotting extracellular proteins to a nitrocellulose membrane, screening with *B. simplicifolia* II lectin resulted in detection of transformants which apparently synthesized

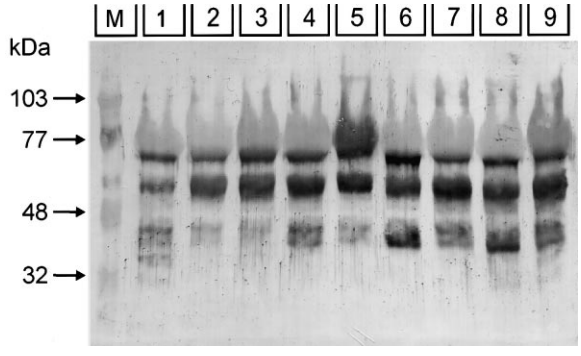

Fig. 1. Lectin screening. Comparable amounts of extracellular proteins from different transformants were submitted to PAGE, blotted and screened with a GlcNAc-specific lectin of *B. simplicifolia*. Proteins from non-transformed *T. reesei* strain VTT-D-80133 were screened (lane 1). Lanes 2–9 contain secreted glycoproteins from different transformants. The transformant T6 produced CBHI which bound lectin better (lane 5) than CBHI of most other transformants. M, low molecular mass marker proteins.

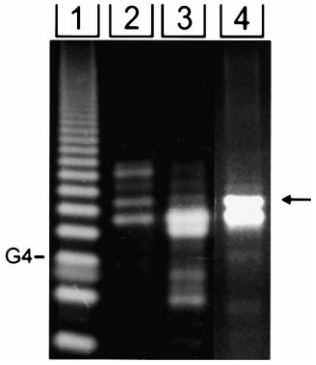

Fig. 2. FACE analysis of CBHI *N*-glycans synthesized by *T. reesei* VTT-D-80133 before and after transformation with the human GlcNAc-TI gene. After enzymatic release of *N*-glycans from CBHI, the complete mixture of *N*-glycans was fluorescently labelled with ANTS and electrophoretically separated. The resulting glycan patterns of transformed (lane 4) versus non-transformed *T. reesei* (lane 3) were compared. Upward shifts of *N*-glycans from the transformant strain, corresponding to the addition of one sugar monomer, were observed. One compound migrates to a position which is expected for $GlcNAcMan_5GlcNAc_2$ (arrow). An oligo-Glc standard ladder served as reference (lane 1). G4 indicates the position of a glucose tetramer. $Man_{(5-9)}GlcNAc_2$ glycans from bovine ribonuclease B (lane 2) are additional references.

extra terminal GlcNAc residues (Fig. 1). Enhanced binding to some proteins (presumably EGI and/or CBHII and some other unidentified proteins) of the transformants was observed. One transformant, designated as T6, produced CBHI which bound lectin better than CBHI of most other transformants and was therefore chosen for large scale production and purification of CBHI to allow a detailed analysis of *N*-glycans.

3.3. Analysis of N-glycans by FACE

ANTS-labelled glycan mixtures from CBHI from the non-transformed VTT-D-80133 strain and from transformant T6 were submitted to electrophoresis. A slower migration behavior was observed with *N*-glycans from the transformed strain (Fig. 2). A fraction of the oligosaccharides of the transformed strain migrated to a position between Glc_6 and Glc_7 of the oligoglucose standard ladder, which is the expected position of $GlcNAcMan_5GlcNAc_2$. A fraction of the *N*-glycans from the non-transformed strain, which migrated to the position of Glc_5, was not detected anymore in the *N*-glycan pattern of the transformant T6. This is in accordance with an expected upward shift corresponding to addition of one extra sugar monomer. The fact that more than one compound shifted was interpreted as a possibility that GlcNAc-TI used other acceptors besides the expected $Man_5GlcNAc_2$.

3.4. Fractionation and purification of CBHI N-glycans

To prepare glycans for NMR analysis, fractionation and purification by gel filtration were performed. As previously observed with *N*-glycans from *T. reesei* strain RUTC 30 [15], only two pure compounds were obtained, their elution positions corresponding to those expected for $Man_5GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$. The other glycans eluted at or close after the void and were only partially fractionated, rendering their characterization by NMR impossible.

3.5. Mass spectrometry

Spectrograms of purified and fractionated *N*-glycans showed that the molecular mass of the compounds analyzed was 1235 Da and 1438 Da, which corresponds to the molecular mass of $Man_5GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$, respectively.

3.6. ^{1}H-NMR data of compound 1 identified as $Man_5GlcNAc_2$

The anomeric region of the ^{1}H-NMR spectrum (δ 4.4–5.4) of compound 1 shows six resonances (besides an impurity at δ 4.816). There is a doublet at δ 5.184 (integrating for 0.4 protons), two collapsing singlets at δ 5.094 (integrating for two protons), three enlarged singlets at δ 4.776, δ 4.868 and δ 4.904, each integrating for one proton and a disturbed doublet at δ 4.587 ascribed to resonance for the glycosidic proton of GlcNAc-2 (integrating for one proton). The presence of five enlarged singlets points to the presence of five mannose units.

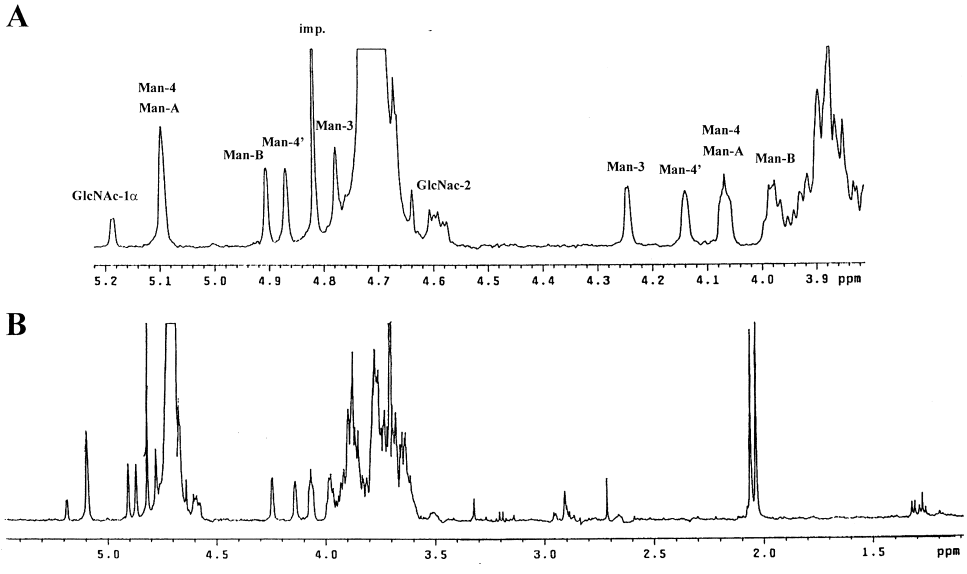

Fig. 3. (A) ^{1}H-NMR spectrum at 500 MHz (without solvent suppression) of $Man_5GlcNAc_2$ (compound 1) in D_2O solution. (B) Extension of the region δ 4.50–5.20.

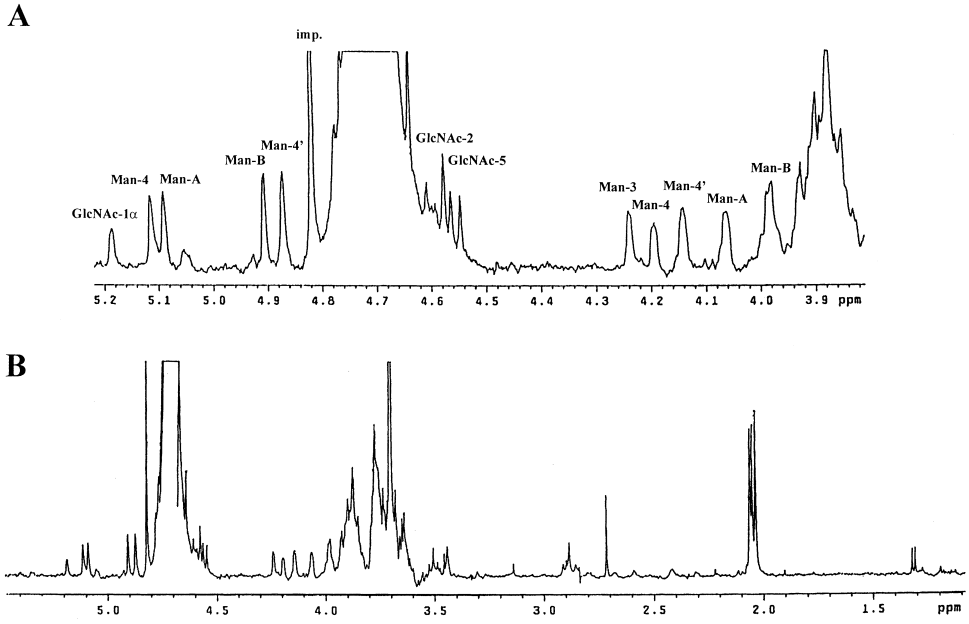

Fig. 4. (A) ^{1}H-NMR spectrum at 500 MHz (without solvent suppression) of $GlcNAcMan_5GlcNAc_2$ (compound 2) in D_2O solution. (B) Extension of the regions δ 4.50–5.20 (resonance of the glycosidic and anomeric protons) and δ 3.80–4.30 (resonance of the H-2 protons of mannose residues).

As usual for reducing oligosaccharides ending with GlcNAc-1 β(1-4) linked to GlcNAc-2, the spectrum is a superposition of the two anomeric forms in a proportion of approximately 2/1 (β/α). The resonance at δ 5.184, viz. integration 0.4 protons and J(1,2) being 3.22 Hz, must be ascribed to the resonance for H-1 of the α-form. The corresponding resonance for the anomeric proton of the β-form is, as expected, hidden under the HDO peak [15]. At δ 2.034 and δ 2.060, the two singlets for the methyl resonances of the *N*-acetyl groups of the two GlcNAc residues are found.

A β-Man residue Man-3 is (1-4)-linked to GlcNAc-2 and is characterized by the chemical shifts of H-1 and H-2. The chemical shift of H-2 of Man-3 at δ 4.242 points to a disubstitution at C-3 and C-6 by α-Man-4 and α-Man-6. The resonance of H-1 is found at δ 4.776 [19]. The chemical shifts of the glycosidic protons of the residues Man-A and Man-B at δ 5.094 and δ 4.904, respectively, show that they are terminal [20]. For residue Man-4, the chemical shift of the resonance for H-1 is 5.094, which also points to a terminal position, the reference value being δ 5.108 [21]. These data support the structure of $Man_5GlcNAc_2$ (Fig. 3). The chemical shifts previously found for $GlcMan_7GlcNAc_2$ agree with the experimental data obtained for this structure. For NMR, we assume that the substitution is β(1,2), the β-D connection being verified by the value of 8.5 Hz of the vicinal coupling constant J(1,2) of residue 5.

3.7. ^{1}H-NMR data of compound 2 identified as $GlcNAcMan_5GlcNAc_2$

The spectrum obtained with presaturation of the HDO peak was studied (Fig. 4). Eight resonances, each integrating for one proton, can be distinguished. There are five enlarged singlets, pointing to the fact that this compound contains five mannose residues. There is a doublet at δ 5.183 with J(1,2) = 2.5 Hz, integrating for 0.4 protons and ascribed to the δ-form of the reducing GlcNAc-1. At δ 4.59, there is a disturbed doublet integrating for one proton, assigned to H-1 of the reducing GlcNAc-2. Finally, at δ 4.558, there is a doublet, integrating for one proton with a coupling constant J(1,2) = 8.5 Hz, pointing to a β-form of the third GlcNAc residue. The presence of a supplementary GlcNAc is confirmed by inspection of methyl resonances of the NAc groups in the region δ 2.00–2.10, where three singlets, each integrat-

	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6
H-1						
GlcNAc-1α	5.184	5.183	5.181	—	—	—
GlcNAc-1β	—	—	4.689	—	—	—
GlcNAc-2	4.587	~4.59	4.588	—	—	—
Man-3	4.776	4.771	4.762	4.765	4.779	4.77
Man-4	5.094	5.109	5.333	5.123	5.122	5.121
Man-4'	4.868	4.868	4.865	4.930	4.920	4.876
Man-A	5.094	4.067	5.089	—		5.079
Man-B	4.904	4.900	4.902	—		4.911
GlcNAc-5	—	4.558	—	4.582	4.579	4.576
H-2						
Man-3	4.242	4.235	4.218	4.259	4.253	4.256
Man-4	~4.067	~4.192	4.026	4.193	4.193	4.202
Man-4'	4.139	4.138	4.135	4.108	4.0	4.146
Man-A	~4.067	~4.002	4.058			4.049
Man-B	3.978	3.974	3.978			3.98
NAc						
CH_3CO	2.034	2.055	2.03			
CH_3CO	2.060	2.030	2.05			
CH_3CO	—	2.045	—			

Fig. 5. ^{1}H-NMR chemical shifts of compounds 1 and 2 compared with reference compounds: $GlcMan_7GlcNAc_2$ (compound 3 [15]), a biantennary non-sialylated complex (compound 4 [19]), a non-sialylated hybrid (compound 5 [19]) and a sialylated hybrid (compound 6 [19]).

ing for three protons, are found. As the compound is hydrolyzed from the peptide chain, GlcNAc-1 must show mutarotation, so that the spectrum must be a superposition of the two structures. At δ 5.183, with an integration of 0.4 protons, the resonance of the anomeric proton of the α-form is found. That of the β-form is expected to occur at the same frequency of the HDO peak.

Compounds 1 and 2 were compared with four other compounds (Fig. 5). The chemical shifts of the glycosidic protons of the residues Man-3, Man-4′, Man-A and Man-B of compounds 2, 3 and 6 are very close to the corresponding values in the basic compound 1, suggesting that this part of the molecule remained unsubstituted. The value of the chemical shifts of the residues A and B (δ 5.085 for residue A and δ 4.900 for residue B) are typical when they are terminal [20]. For non-terminal residues, a value of δ 5.39–5.40 would be expected for residue A, i.e. more than 0.30 ppm at a higher frequency, and δ 5.14 for residue B, i.e. 0.24 ppm at a higher frequency. Hence, the substitution did not occur on either of both compounds. The values of the chemical shifts of H-1 and H-2 of residue Man-4 are typical in the case of substitution. This is shown by the chemical shifts of these resonances in compounds 4, 5 and 6. The resonance of H-1 of residue Man-4 of compounds 4, 5 and 6 shows a downfield shift of this proton for the corresponding non-substituted residue in compound 1. When only a GlcNAc residue is β(1,2)-substituted on Man-4, instead of *N*-acetyl-lactosamine (compounds 4 and 5) or even an elongated chain by a sialyl residue (compound 6), the downfield shift versus the non-substituted compound 1 is only 0.015 ppm. Although no reference values are available, this small downfield shift points to a substitution [20] of residue Man-4.

Sometimes, inspection of the resonances of H-2 is more conclusive [21]. When residue Man-4 is not substituted (compound 1), a chemical shift of δ 4.067 is found for H-2. In the case of β(1,2) substitution by GlcNAc (compounds 2, 4, 5 and 6), a downfield shift of 0.125–0.135 ppm is observed. Taking into account the increment values previously proposed [20], the present downfield shift for H-2 of Man-4 is diagnostic for substitution. The resonance for H-1 of GlcNAc is found at 0.020 ppm if the unit is not further substituted by a galactose-6 residue (compound 2), a frequency lower than when this residue is further substituted. When the spectral data of compound 2 are used as reference, we conclude that the supplementary resonance at δ 4.558, together with a coupling constant $J(1,2)$ of 8.5 Hz and the presence of a third methyl resonance of a NAc group, suggests the presence of a supplementary GlcNAc. Since the chemical shifts of the glycosidic protons of the residues Man-A and Man-B allow us to conclude that these units are terminal, the third GlcNAc can only be linked to Man-4. The chemical shifts of H-1 and H-2 of Man-4 are in agreement with a β(1-2)-linked GlcNAc to Man-4 (a conserved linkage).

4. Discussion

Mammalian glycosyltransferases were introduced in various yeasts and filamentous fungi and their expression and in vivo transferring activity have been studied [7,22–24]. So far, successful in vivo synthesis of a mammalian type *N*-glycan by a fungus has not been demonstrated. The first enzyme needed to be expressed in order to obtain a rudimentary hybrid *N*-glycan is GlcNAc-TI. The gene of rabbit GlcNAc-TI was introduced in the genome of *A. nidulans*, which led to expression and resulted in a fully active enzyme [7]. However, it was not possible to demonstrate actual transfer of GlcNAc residues to fungal oligosaccharides. An explanation for the inability to demonstrate in vivo transfer of GlcNAc to fungal *N*-glycans might be the choice of the host strain. Chen et al. [25] studied *N*-glycans on invertase secreted by *A. nidulans* and suggested the presence of galactomannans. In addition, it has been shown that there are important strain-dependent differences in oligosaccharide structures [6,26]. While the acceptor substrate $Man_5GlcNAc_2$ may be present in some strains [27–29], it may not be synthesized in others. Hence, it is assumed that the choice of a suitable fungal strain will be important to allow successful and abundant in vivo synthesis of mammalian-like *N*-glycans. Our results suggest that donor and acceptor substrates are present in sufficient concentrations in the secretion apparatus of *T. reesei* to allow in vivo synthesis of hybrid *N*-glycans.

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