Pergamon

Tetrahedron Letters 39 (1998) 9777–9780

TETRAHEDRON LETTERS

Unusual Nonreducing Sugar GlcNAcβ(1↔1)Manβ Formation by β-*N*-Acetylhexosaminidase from *Aspergillus oryzae*[1]

Vladimír Křen*, Eva Rajnochová, Zdenka Huňková, Jana Dvořáková and Petr Sedmera

Institute of Microbiology, Academy of Sciences of the Czech Republic, Laboratory of Biotransformation, Vídeňská 1083, CZ 142 20 Prague 4, Czech Republic

Received 10 August 1998; accepted 12 October 1998

Abstract: β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* catalyzes the transfer of *N*-acetylglucosamine moiety from *p*NP-β-GlcNAc to mannose giving major product GlcNAcβ(1↔1)Manβ (**1a**), and GlcNAcβ(1→3)Manα/β (**2a**) and GlcNAcβ(1→6)Manα/β (**3a**) as two minor products. © 1998 Elsevier Science Ltd. All rights reserved.

β-*N*-Acetylhexosaminidase (E.C. 3.2.1.52) from *Aspergillus oryzae* is a good tool in enzymatic synthesis of oligosaccharides.[2-4] It is able to transfer β-GlcNAc and β-GalNAc moieties in comparable yields to both glycosidic[2,3b] and non-glycosidic substrates.[3a] The regioselectivity towards monosaccharides is influenced by their anomeric substitution. This enzyme has been commercially available from Sigma since 1997.

β-GlcNAc bound to Man residues occurs frequently in various glycoproteins especially in multiantennary *N*-glycans. Recently, an enzymatic synthesis of GlcNAcβ(1→2)Man and GlcNAcβ(1→6)Man using reversed glycosylation with β-*N*-acetylhexosaminidase from *Bacillus circulans* was described.[5] To extend the scope of applications of the *A. oryzae* enzyme and to prepare biologically useful oligosaccharides we have chosen mannose as the acceptor for transglycosylation reactions.

No transglycosidation product of methyl-α-mannoside was detected with various β-*N*-acetyl-hexosaminidases (from *Aspergillus oryzae, A. terreus, A. tamarii,*[4] *Penicillium oxalicum,*[6] jack beans (Sigma) and bovine kidneys (Sigma)) and *p*-nitrophenyl-β-*O*-*N*-acetylglucosaminide (*p*NP-β-GlcNAc) as a donor. However, the same reactions with mannose yielded two to four disaccharides. Further investigation was continued with enzyme from *Aspergillus oryzae* that gave the best yields of transglycosylation products.[7]

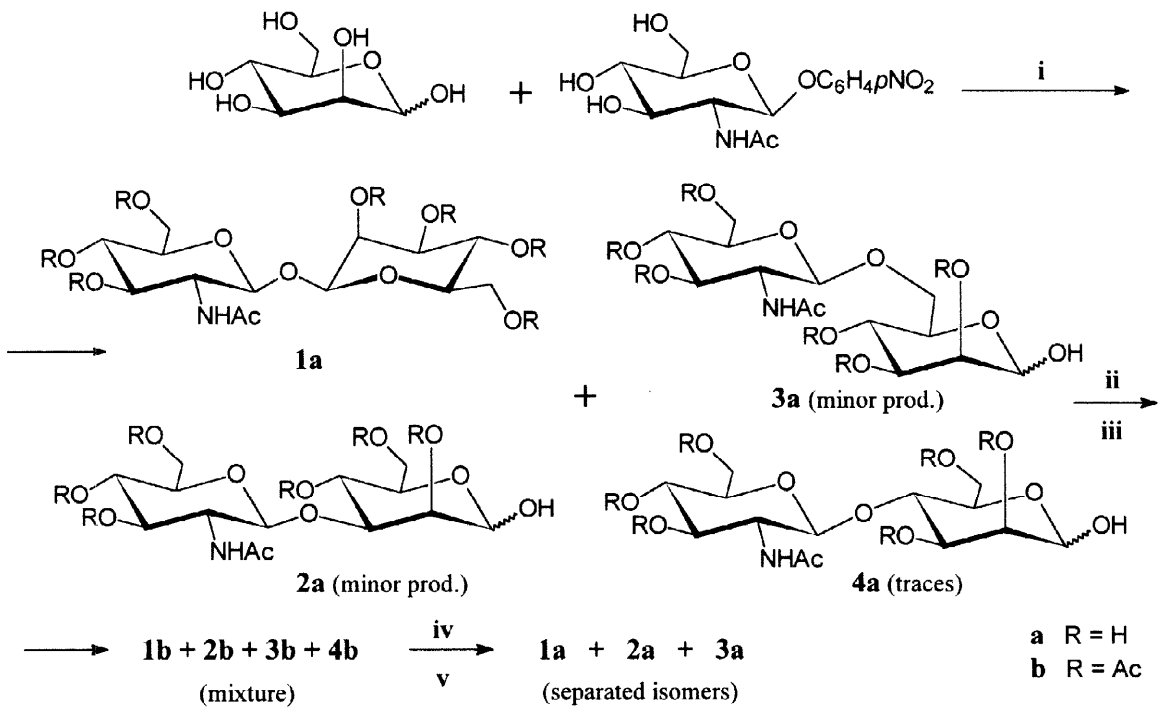
The reaction mixture was subjected to gel filtration and the disaccharide fraction was purified by semipreparative HPLC.[8] NMR spectroscopy suggested two major components: a non-reducing sugar GlcNAcβ(1↔1)Manβ (**1a**) and a disaccharide(s) GlcNAcβ(1→x)Man α/β.

To make the separation and structure elucidation easier, the crude products after the gel filtration step

PII: S0040-4039(98)02171-6

were peracetylated and the anomeric hydroxyls were deprotected (piperidine/THF). Flash chromatography on silica gel neatly separated **1b** from regioisomeric disaccharides **2b** and **3b**. Large dispersion of chemical shifts and observation of N-H protons made full proton assignment possible.[9-12] The anomeric configuration of GlcNAc was β in all cases ($J_{1,2}$ 8.0 - 8.6 Hz). Mannose C-1 in **1a** and in its acetate **1b** has also β configuration (J[^{13}C(1)-H(1)] = 165.3 Hz, ref. [13], NOE between H-1 and H-5). In addition, NOE between both H-1 was also observed. The site of GlcNAc attachment in **2b** and **3b** was inferred from the upfield shift of the involved mannose proton and the downfield shift of the corresponding carbon (bold in footnotes[11,12]). Some impurity signals[11] are consistent with the presence of the (1→4) isomer **4b**.

Thus, mannose acts as an "ambident" acceptor for β-*N*-acetylhexosaminidases from *A. oryzae*. The formation of **1a** is probably the first example of 1-OH glycosidation by glycosidases. Two cases of enzymatic anomeric hydroxyl glycosylation have been already described, however, with glycosyltransferases: α-glucosylation of lactose[14] and β-galactosylation of xylose.[15]



i β-*N*-Acetylhexosaminidase from *Aspergillus oryzae*, **ii** Ac_2O/Py, **iii** 5% piperidine/THF, **iv** flash chromatography/SiO_2, **v** MeONa/MeOH.

Acknowledgements: Supported by the grants from Grant Agency of the Czech Academy of Sciences No. A6020605, from Grant Agency of the Czech Republic No. 203/96/1267, and by Volkswagen Foundation.

REFERENCES AND NOTES

1. Presented at 9th European Carbohydrate Symposium Eurocarb9, Utrecht, June 6-11 1997, Netherlands.

2. (a) Singh, S.; Packwood, J.; Samuel, C.J.; Critchley, P.; Crout, D.H.G. *Carbohydr. Res.* **1995**, *279*, 293-305. (b) Crout, D.H.G.; Howarth, O.W.; Singh, S.; Swoboda, B.E.P.; Critchley P.; Gibson, W.T. *J. Chem. Soc., Chem. Commun.* **1991**, 1550-1551.
3. (a) Křen, V.; Ščigelová, M.; Přikrylová, V.; Havlíček V.; Sedmera, P. *Biocatalysis* **1994**, *10*, 181-193. (b) Rajnochová, E.; Dvořáková, J.; Huňková, Z.; Křen V. *Biotechnol. Lett.* **1997**, *19*, 869-872.
4. Huňková, Z.; Křen, V.; Ščigelová, M.; Weignerová, L.; Scheel, O.; Thiem, J. *Biotechnol. Lett.* **1996**, *18*, 725-730.
5. Fujimoto, H.; Isomura, M.; Miyazaki, T.; Matsuo, I.; Walton, R.; Sakakibara, T.; Ajisaka, K. *Glycoconjugate J.* **1997**, *14*, 75-80.
6. Kadowaki, S.; Saskiawan, I.; Watanabe, Y.; Yamamoto, K.; Bunno, M.; Ichihara, Y.; Kumagai, H. *J. Ferm. Bioeng.* **1997**, *83*, 341-345.
7. Procedure: Mannose (7.2 g, 40 mmol) and *p*NP-β-GlcNAc (130 mg, 0.38 mmol) were dissolved by heating in McIlvain buffer (10 ml, 0.1 M, pH 5.0). After cooling, β-*N*-acetylhexosaminidase from *A. oryzae* (10 U, ammonium sulphate precipitate)[3b] was added and the mixture was incubated with stirring at 37 °C. After 20 min., another portion of *p*NP-β-GlcNAc (100 mg, 0.3 mmol) was added and the reaction was terminated after 4 hours by boiling (10 min.). The reaction mixture was diluted with H_2O and liberated *p*-nitrophenol was removed by extraction with diethyl ether (2 x 10 ml). The rest of the solvent was removed by short heating and the last traces of *p*-nitrophenol and unreacted *p*NP-β-GlcNAc were removed by passing the mixture through small column of polystyrene resin SM-2 (BioRad, USA) followed by washing with H_2O. The mixture was then fractionated by gel filtration through a BioGel P4 column (90 × 103 cm) being eluted with water. The disaccharidic fraction (56.5 mg, 24.6 %) was acetylated with pyridine/Ac_2O. Mixtures of the acetates were partially deacetylated with piperidine in THF (5 %, 5 ml) at 0 °C for 15 hours. Acetates **1b**, **2b** and **3b** were isolated by flash chromatography on silica gel (Merck 40 - 60 μm) eluted with $CHCl_3$ with 2 % (v/v) MeOH. Zemplén deacetylation yielded pure **1a** (4.2 mg, 1.8 %) $[\alpha]_D^{23}$ = + 3.5 (*c* = 0.59, H_2O), and minor amounts of **2a** and **3a**.
8. Semipreparatory HPLC was carried out on a Waters high performance liquid chromatograph equipped with solvent delivery system 6000A, universal injector U6K and with refractometric detector ALC 202; GL-PACK LiChrosorb NH_2-5 column (250 x 10 mm) (GL Sciences Inc., USA); the mobile phase was acetonitrile - water (78 : 22, v/v) at a flow rate 5.0 ml/min, temperature 50 °C; retention times were 12.86 and 14.63 min for **1a** and **2a**, respectively.
9. ^{1}H NMR (400 MHz, D_2O, 25 °C) data of **1a** (GlcNAc moiety denoted by primed numbers in all following cases) δ 2.046 (3 H, s, Ac), 3.369 (1 H, ddd, *J* = 10.0, 6.8, 2.1, H-5'), 3.451 (1 H, dd, *J* = 9.8, 9.8, H-3'), 3.473 (1 H, ddd, *J* = 9.7,5.5,2.1, H-5), 3.532 (1 H, dd, *J* = 9.7, 9.6, H-4), 3.577 (1 H, dd, *J* = 10.0, 9.8, H-4'), 3.648 (1 H, dd, *J* = 9.6, 3.3, H-3), 3.713 (1 H, dd, *J* = 12.1, 6.8, H-6'u), 3.740 (1 H, dd, *J* = 12.1, 5.5, H-6u), 3.757 (1 H, dd, *J* = 9.8, 8.6, H-2'), 3.916 (1 H, dd, *J* = 12.1, 2.0, H-6d), 3.933 (1 H, dd, *J* = 12.1, 2.1, H-6'd), 3.984 (1 H, dd, *J* = 3.3, 1.6, H-2), 4.907 (1 H, d, *J* = 8.6, H-1'), 4.986 (1 H, d, *J* = 1.6, H-1). ^{13}C NMR (100 MHz, D_2O, 25 °C) data of **1a** δ 25.09 q (CH_3CO), 58.09 d (C-2'), 63.49 t (C-6'),

64.06 t (C-6), 69.72 d (C-4), 72.66 d (C-4'), 73.40 d (C-2), 75.57 d (C-3), 76.78 d (C-3'), 78.94 d (C-5), 79.34 d (C-5'), 99.57 d (C-1), 100.64 d (C-1'), 177.92 s (C=O).

10. ^{1}H NMR (400 MHz, $CDCl_3$, 30 °C) data of **1b** δ 1.965, 1.994, 2.024, 2.030, 2.062, 2.115, 2.133, 2.143 (all s, each 3 H, 8×Ac), 3.677 (1 H, m, *J* = 9.7, 5.9, 2.7, H-5), 3.683 (1 H, ddd, *J* = 9.6, 4.6, 2.7, H-5'), 4.029 (1 H, ddd, *J* = 10.4, 9.3, 8.4, H-2'), 4.141 (1 H, dd, *J* = 12.3, 2.7, H-6'u), 4.143 (1 H, dd, *J* = 12.2, 2.7, H-6u), 4.239 (1 H, dd, *J* = 12.3, 4.6, H-6'd), 4.369 (1 H, dd, *J* = 12.2, 5.9, H-6d), 4.796 (1 H, d, *J* = 8.4, H-1'), 4.965 (1 H, d, *J* = 1.3, H-1), 5.061 (1 H, dd, *J* = 9.9, 3.3, H-3), 5.070 (1 H, dd, *J* = 9.6, 9.2, H-4'), 5.154 (1 H, dd, *J* = 10.4, 9.2, H-3'), 5.207 (1 H, dd, *J* = 9.9, 9.7, H-4), 5.488 (1 H, dd, *J* = 3.3, 1.3, H-2), 5.535 (1H, d, *J* = 9.3, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 30 °C) data of **1b** δ 20.53 q (2 C), 20.58 q, 20.62 q, 20.67 q, 20.75 q, 20.84 q, 23.15 q, (8×CH_3CO), 53.59 d (C-2'), 61.89 t (C-6'), 62.45 t (C-6), 66.00 d (C-4), 68.11 d (C-2), 68.24 d (C-4'), 70.61 d (C-3), 72.25 d (C-3'), 72.37 d (C-5'), 72.96 d (C-5), 94.76 d (C-1), 97.41 d (C-1'), 169.19 s, 169.61 s, 169.88 s, 169.95 s, 170.12 s, 170.66 s, 170.70 s, 170.86 s (8×CH_3CO).

11. ^{1}H NMR (400 MHz, CD_3Cl, 30 °C) data of **2b** [α/β 2:1] δ 1.937, 2.008, 2.016, 2.055, 2.072, 2.103, 2.157 (all s, each 3 H, 7×Ac), 3.710 (1 H, ddd, *J* = 10.2, 4.7, 2.4, H-5'α), 3.713 (1 H, dd, *J* = 8.5, 8.0, H-2'β), 3.759 (1 H, dd, *J* = 10.3, 8.3, H-2'α), 4.086 (1 H, dd, *J* = 12.2, 4.7, H-6'uα), 4.170 (1 H, dd, *J* = 12.4, 1.2, H-6uα), 4.181 (1H, 1-OHα), 4.200 (1 H, ddd, *J* = 10.0, 6.5, 1.2, H-5α), 4.217 (1 H, dd, *J* = 12.4, 6.5, H-6dα), **4.262** (1 H, dd, *J* = 9.6, 3.4, H-3α), 4.336 (1 H, dd, *J* = 12.2, 4.7, H-6'dα), 4.739 (1 H, d, *J* = 8.3, H-1'α), 4.779 (1 H, d, *J* = 8.8, H-1'β), 5.037 (1 H, dd, *J* = 10.8, 9.6, H-4'β), 5.075 (1 H, dd, *J* = 10.3, 9.2, H-3'α), 5.161 (1 H, dd, *J* = 10.0, 9.6, H-4α), 5.236 (1 H, d, *J* = 1.8, H-1α), 5.244 (1 H, dd, *J* = 10.2, 9.2, H-4'α), 5.285 (1 H, dd, *J* = 3.4, 1.8, H-2α), 5.325 (1 H, dd, *J* = 10.8, 8.5, H-3'β), 5.616 (1H, d, *J* = 8.4, NH'α), 5.821 (1H, d, *J* = 8.5, NH'β). ^{13}C NMR (100 MHz, $CDCl_3$, 30 °C) data of **2b** δ 20.4 q, 20.6 q, 20.7 q, 20.8 q, 20.9 q (7*×CH_3CO), 54.83 d (C-2'α), 61.95 t (C-6'α), 62.71 t (C-6α), 66.36 d (C-4α), 68.32 d (C-3'α), 68.98 d (C-2α), 69.12 d (C-5α), 71.96 d (C-5'α), 73.05 d (C-4'α), **74.04** d (C-3α), 90.96 d (C-1), 92.57 d (C-1α), 98.66 d (C-1'α). * Signal overlaps. ^{1}H NMR of **4b** - present as a minor impurity (under 10 %) in the sample of **2b** - δ 3.867 (1H, dd, *J* = 9.5, 9.5, H-4α), 5.449 (1H, dd, *J* = 9.5, 3.3, H-3α).

12. ^{1}H NMR (400 MHz, $CDCl_3$, 30 °C) data of **3b** [α/β 3:1] δ 1.996, 1.998, 2.024, 2.041, 2.080, 2.092, 2.169, (all s, each 3 H, 7×Ac), 3.509 (1 H, dd, *J* = 10.2, 8.2, H-2'β), **3.607** (1 H, dd, *J* = 12.3, 2.6, H-6uα), 3.702 (1 H, ddd, *J* = 10.0, 5.5, 1.6, H-5'α), 3.707 (1 H, ddd, *J* = 10.0, 4.4, 2.3, H-5'β), **3.712** (1 H, dd, *J* = 12.5, 4.4, H-6uβ), 3.763 (1 H, ddd, *J* = 10.5, 8.3, 8.2, H-2'α), **3.915** (1 H, dd, *J* = 12.0, 1.6, H-6dα), 3.954 (1H, d, *J* = 3.4, 1-OHα), **4.003** (1 H, dd, *J* = 12.7, 2.3, H-6dβ), 4.119 (1 H, dd, *J* = 12.5, 2.3, H-6'uβ), 4.131 (1 H, ddd, *J* = 10.3, 5.5, 1.6, H-5α), 4.173 (1 H, dd, *J* = 12.3, 2.6, H-6'uα), 4.214 (1 H, ddd, *J* = 9.9, 4.6, 2.3, H-5β), 4.243 (1 H, dd, *J* = 12.0, 1.6, H-6'dα), 4.293 (1 H, dd, *J* = 12.5, 4.6, H-6'dβ), 4.481 (1 H, d, *J* = 8.2, H-1'β), 4.942 (1 H, d, *J* = 8.3, H-1'α), 5.012 (1 H, dd, *J* = 10.3, 9.9, H-4α), 5.051 (1 H, dd, *J* = 10.2, 10.0, H-3'β), 5.071 (1 H, dd, *J* = 10.0, 9.2, H-4'α), 5.071 (1 H, dd, *J* = 10.0, 10.0, H-4'β), 5.190 (1 H, dd, *J* = 3.3, 1.7, H-1β), 5.229 (1 H, d, *J* = 1.8, H-1α), 5.240 (1 H, dd, *J* = 9.9, 9.9, H-4β), 5.257 (1 H, dd, *J* = 3.5, 1.8, H-2α), 5.310 (1 H, dd, *J* = 10.5, 9.2, H-3'α), 5.320 (1 H, dd, *J* = 3.5, 1.7, H-2β), 5.435 (1 H, dd, *J* = 9.9, 3.5, H-3β), 5.444 (1 H, dd, *J* = 9.9, 3.5, H-3α), 5.607 (1H, d, *J* = 10.9, NH'β), 5.616 (1H, d, *J* = 3.3, 1-OHβ), 5.955 (1H, d, *J* = 8.2, NH'α). ^{13}C NMR (100 MHz, $CDCl_3$, 30 °C) data of **3b** δ 19.77 q, 20.35 q, 20.62 q, 21.10 q, 23.21 q. (7*×CH_3CO), 53.8 d (C-2'α), 62.1 t (C-6'α), **69.5** t (C-6α), 67.8 d (C-4α), 68.4 d (C-4'α), 69.7 d (C-2α), 68.9 d (C-3α), 70.7 d (C-5α), 72.2 d (C-5'α), 72.5 d (C-3'α), 92.5 d (C-1α), 100.8 d (C-1'α). * Signal overlaps.

13. Bock, K.; Pedersen C. *J. Chem. Soc. Perkin Trans. 2* **1974**, 293-297.

14. Kitahata, S.; Hara, K.; Fujita, K.; Nakano, H.; Kuvahara, N.; Koizumi, K. *Biosci. Biotech. Biochem.* **1992**, *56*, 1386-1391.

15. Wiemann, T.; Nishida, Y.; Sinnwell, V.; Thiem J. *J. Org. Chem.* **1994**, *59*, 6744-6747.