Eur. J. Biochem. 227, 175–185 (1995)
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Identification of a novel UDP-GalNAc:GlcNAcβ-*R* β1–4 *N*-acetylgalactosaminyltransferase from the albumen gland and connective tissue of the snail *Lymnaea stagnalis*

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(Received 5 September/24 October 1994) – EJB 94 1327/5

Both the albumen gland, one of the female accessory sex glands, and connective tissue of the freshwater snail *Lymnaea stagnalis* contain *N*-acetylgalactosaminyltransferase activity, capable of transferring GalNAc from UDP-GalNAc in β1–4 linkage to the terminal GlcNAc residue of GlcNAcβ-*R*. The albumin gland enzyme was partially purified by affinity chromatography on UDP-hexanolamine–Sepharose 4B. Using GlcNAcβ1–2Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc or GlcNAcβ1-OMe as substrates, the enzyme showed an absolute requirement for Mn^{2+} with an optimum concentration of 12.5–50 mM. The optimal pH was approximately pH 7.0. The enzyme activity was independent of the Triton X-100 concentration in the range 0.25–2.5%, and no activation effect was found. The more labile connective tissue microsomal enzyme, subjected to the same optimization procedure, gave comparable results. Both enzyme activities have similar substrate specificities towards GlcNAc or GlcNAcβ1-OMe, and towards oligosaccharides or glycopeptides with a non-reducing terminal β-GlcNAc unit, but cannot act on GlcNAcα1-OMe. Saccharides with non-reducing terminal Gal or GalNAc residues, and free GalNAc, Gal or Glc residues are not acceptors. Product analysis was carried out for albumen gland *N*-acetylgalactosaminyltransferase and four acceptors having GlcNAcβ1-*R* as the terminal non-reducing unit, and for connective tissue *N*-acetylgalactosaminyltransferase with GlcNAcβ1-OMe as acceptor. In all instances, products with GalNAc β1–4-linked to GlcNAc were obtained, showing that the connective tissue and the albumen gland activities are probably from one enzyme. This enzyme activity can be identified as UDP-GalNAc:GlcNAcβ-*R* β1–4 *N*-acetylgalactosaminyltransferase, and is probably involved in the biosynthesis of *N*,*N*′-diacetyllactosediamine-containing glycoproteins, like hemocyanin, in the snail *L. stagnalis*.

Keywords. Glycoprotein; *Lymnaea stagnalis*; β1–4 *N*-acetylgalactosaminyltransferase.

Glycoproteins containing *N*-acetylgalactosamine β-linked to *N*-acetylglucosamine (*N*,*N*′-diacetyllactosediamine [1]), instead of the more ubiquitous *N*-acetyllactosamine sequence, are being reported with increasing frequency [2]. However, the number of studies on the biosynthesis of *N*-glycans of the *N*,*N*′-diacetyllactosediamine type is still limited. So far, two β1–4-*N*-acetylgalactosaminyltransferase (β4GalNAc-T) activities, incorporating GalNAc in a β1–4 linkage to GlcNAc, have been described, namely a PXR/K tripeptide motif-dependent [3] and a PXR/K tripeptide motif-independent β4GalNAc-T [4]. Furthermore, two different β4GalNAc-T enzymes have been characterized which both transfer GalNAc in β1–4 linkage to the Gal residue of NeuAcα2–3Galβ-*R*. One of these enzymes forms the GalNAcβ1–4[NeuAcα2–3]Galβ-*R* unit found in blood group Sd^a antigens [5–12] and the other converts ganglioside G_{M1} into G_{M2} [7, 13–17]. In addition, two β-glucuronide β4GalNAc-T enzymes are known, which are involved in the synthesis of chondroitin sulfate [18]. A β1–3 GalNAc-T has also been described that transfers GalNAc in β1–3 linkage to the Gal residue of Galβ1–4GlcNAc/Glc to form blood group P antigen-like structures, GalNAcβ1–3Galβ1–4GlcNAc/Glc [19].

Hemocyanin of the freshwater snail *Lymnaea stagnalis* contains diantennary N-linked glycans having the antennary elements Fucα1–2Galβ1–3GalNAcβ1–4GlcNAcβ1–2*R* and 3OMe-Galβ1–3GalNAcβ1–4GlcNAcβ1–2*R* [20, 21]. This glycoprotein is synthesized and glycosylated in the pore cells of the connective tissue. In a previous study, we reported a connec-

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Abbreviations. 3OMe-Man, 3-*O*-methyl-D-mannose; 3OMe-Gal, 3-*O*-methyl-D-galactose; β4GalNAc-T, β1–4 *N*-acetylgalactosaminyltransferase; COSY, correlation spectroscopy; DQF-COSY, two-dimensional double-quantum filtered ^1H-^1H correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn; HPAEC, high-pH anion-exchange chromatography; MLEV, M. Levitt; NOESY, nuclear Overhauser enhancement spectroscopy; PAD, pulsed amperometric detection; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy.

Enzymes. β-D-Galactoside galactohydrolase (EC 3.2.1.23); acylneuraminyl hydrolase (EC 3.2.1.18).

Table 1. Acceptor specificity and kinetic parameters of connective tissue and albumen gland β4GalNAc-T with various acceptors. The acceptor specifities of the β4GalNAc-T enzymes were determined at acceptor concentrations of 1 mM.

Acceptors	Number	β4GalNAc-T				
		CT	AG			
				K_m	V_{max}	Kinetic efficiency (V_m/K_m)
		%		mM	mU/ml	mU · ml^{-1} · mM^{-1}
GlcNAcβ1−2Manα1−6 Manβ1−4GlcNAcβ1−4GlcNAc-Asn GlcNAcβ1−2Manα1−3	1	61	88	1.06	33.4	31.5
GlcNAcβ1−2Manα1−6 Manβ1−4GlcNAcβ1−4GlcNAc GlcNAcβ1−2Manα1−3	2	100[a]	100[b]	0.98	32.3	33.0
GlcNAcβ1−2Manα1−6 Manβ1−4GlcNAc Galβ1−4GlcNAcβ1−2Manα1−3	3	55	81	1.29	46.4	36.0
GlcNAcβ1−2Manα1−6 Man GlcNAcβ1−2Manα1−3	4	–	–	1.14	18.1	15.9
GlcNAcβ1−2Manα1−6 GlcNAcβ1−4-Man GlcNAcβ1−2Manα1−3	5	33	39	7.80	51.4	6.6
GlcNAcβ1−6 Man GlcNAcβ1−2	6	–	–	1.14	48.3	42.4
GlcNAcβ1−4 Man-ol GlcNAcβ1−2	7	–	–	1.68	39.8	23.7
GlcNAcβ1-OMe	8	76	70	1.81	25	13.8
GlcNAc	9	18	20			
GlcNAcα1-OMe	10	0	0			
GlcNAcβ1−4GlcNAc	11	81	87			
GlcNAcβ1−3Galβ1-OMe	12	41	52			
GalNAcβ1−4GlcNAcβ1-OMe	13	0	0			
GalNAcβ1−3Galα1-OMe	14	0	0			
GalNAcα1-phenyl	15	0	0			
Galβ1−4Glc	16	0	0			
Galβ1−4GlcNAc	17	0	0			
GalNAc	18	0	2			
Gal	19	0	0			
Glc	20	0	0			

[a] 100% activity for 2 and connective tissue enzyme corresponds to 1.14 mU/ml.
[b] 100% activity for 2 and albumen gland enzyme corresponds to 11.7 mU/ml.

tive tissue β1−3 galactosyltransferase involved in the biosynthesis of the antennae [22]. In this study, we will focus on a novel β4GalNAc-T found in the same species; this enzyme is also presumed to play a role in the glycosylation of hemocyanin. Although in a preliminary tissue survey (unpublished results) β4GalNAc-T activity was detected in connective tissue, a higher GlcNAc-*R* GalNAc-T activity was found in the albumen gland of the snail. This albumen gland, one of the female accessory sex glands, secretes the perivitelline fluid which contains mainly galactogen and surrounds the egg [23]. The β4GalNAc-T enzymes from connective tissue and albumen gland appear to have similar substrate specificities, probably due to one enzyme, defined as an UDP-GalNAc:GlcNAcβ-*R* β1−4 *N*-acetylgalactosaminyltransferase (β4GalNAc-T).

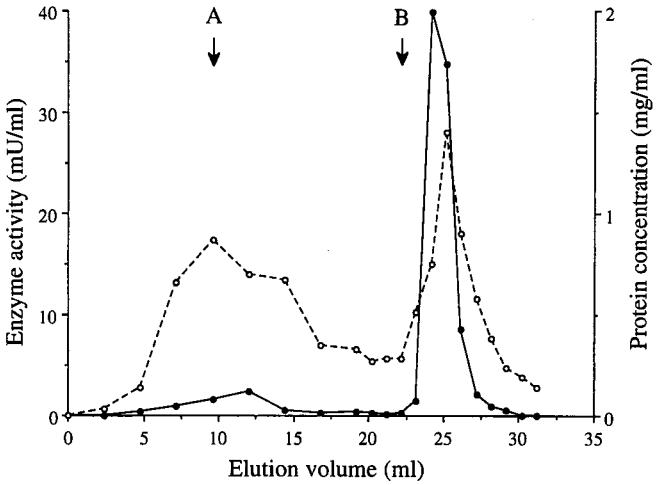


Fig. 1. Affinity chromatography purification of albumen gland β4GalNAc-T on UDP-hexanolamine–Sepharose 4B. A Triton X-100 extract of snail albumen glands was used. The column was washed with equilibration buffer (at A) and then with buffer A, contaning 1.0 M NaCl and 5 mM UMP (at B). For further details, see the Experimental Procedures section. Fractions were collected and assayed for β4GalNAc-T activity (●) and protein (○).

EXPERIMENTAL PROCEDURES

Materials. Connective tissue from mantle edges and albumen glands was dissected from laboratory bred, adult specimens of *L. stagnalis*, fed lettuce *ad libitum*, and kept at 20°C under carefully controlled photoperiod conditions (12-h light/12-h dark). UDP-[1-^{14}C]-*N*-acetyl-D-galactosamine (specific activity 45–55 Ci/mol) and UDP-D-[U-^{14}C]galactose (specific activity 272.8 Ci/mol) were obtained from New England Nuclear, and diluted as needed by non-radioactive UDP-D-GalNAc or UDP-D-Gal (Sigma).

Oligosaccharides and glycopeptides tested with β4GalNAc-T. The structures of all acceptors with connective tissue or albumen gland β4GalNAc-T are summarized in Table 1. Compound 1 was prepared from human fibrinogen [24] and compound 2 from bovine fibrinogen [25]. Compound 3 was a gift of Dr G. Strecker (Université des Sciences et Techniques de Lille Flandres-Artois, Laboratoire de Chimie Biologique, Villeneuve d'Ascq, France), compounds 4–7 were gifts of Dr J. Lönngren (Stockholm University, Stockholm, Sweden), and compound 13 was a gift of Dr J. G. M. van der Ven (Bijvoet Center, Department of Bio-Organic Chemistry). Compounds 8–11 and 16–18 were obtained from Sigma, compounds 12 and 14 from Sockerbolaget, compound 15 from Koch Light, compound 19 from Baker, and compound 20 from Serva. The purity of the various acceptors was checked by 300-MHz ^{1}H-NMR spectroscopy. Human serotransferrin was obtained from Sigma, and human chorionic gonadotropin was a gift from Dr B. C. Nisula (Department of Health and Human services, National Institute of Child Health and Human Development). Both glycoproteins were desialylated with *Vibrio cholerae* neuraminidase (Boehringer Mannheim) and degalactosylated with jack bean β-galactosidase (Oxford Glycosystems), as described by the manufacturers. The quantity of the listed acceptors was determined by monosaccharide analysis.

Protein determination. Protein determinations were carried out using bovine serum albumin as a standard [26].

Monosaccharide analysis. Monosaccharide analysis was performed by GLC on a capillary SE-30 fused-silica column (25 m×0.32 mm, Pierce) using a Varian Aerograph model 3700 gas chromatograph. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis, *N*-(re)acetylation, and trimethylsilylation [27].

Partial purification of enzymes. All procedures were carried out at 4°C. Albumen glands (7 g) were homogenized in 12 ml 10 mM sodium cacodylate, pH 7.0, using a Polytron apparatus, and the homogenates were centrifuged for 60 min at 100000×*g*. The membrane pellet was extracted for 60 min with 7 ml 10 mM sodium cacodylate, pH 7.0, containing 0.5% (by vol.) Triton X-100 and 10 mM $MnCl_2$, and the suspension was centrifuged for 60 min at 20000×*g*. The supenatant (6.8 ml) was applied to a column (10 cm×0.8 cm) of UDP-hexanolamine–Sepharose 4B (7 μmol ligand/ml gel), equilibrated in 10 mM sodium cacodylate, pH 7.0, containing 0.1% (by vol.) Triton X-100 and 10 mM $MnCl_2$. After washing with the equilibration buffer, the column was left overnight in 10 mM sodium cacodylate, pH 6.5, containing 0.1% (by vol.) Triton X-100, 1 M NaCl and 5 mM UMP (buffer A), and eluted with three bed-volumes buffer A. Fractions of 0.3 ml were collected and assayed for protein content and GalNAc-T activity using 30 mM GlcNAc as acceptor. Fractions containing GalNAc-T activity were pooled and dialyzed for 16 h against 10 mM sodium cacodylate, pH 7.0, containing 0.1% (by vol.) Triton X-100, 10 mM $MnCl_2$, and 0.02% (mass/vol.) sodium azide. The enzyme preparation (3.1 ml), containing 3.2 mU and 1.86 mg protein/ml was stored at 4°C until use (1 U enzyme activity is defined as the amount of enzyme catalyzing the transfer of 1 μmol GalNAc/min using the standard incubation system and GlcNAc as an acceptor at 1 mM). To prepare a connective tissue microsomal suspension, connective tissue (5 mg) was homogenized in 10 ml 10 mM sodium cacodylate, pH 7.0, using a Polytron apparatus. After centrifugation of the homogenate for 60 min at 100000×*g*, the membrane pellet was resuspended in 5 ml 0.25 M sucrose with a Potter-Elvehjem homogenizer, and centrifuged again. The resulting pellet was resuspended in 2.5 ml 0.25 M sucrose and stored at 4°C until use.

Pituitary gland β4GalNAc-T and β1–4 galactosyltransferase were purified and assayed according to Smith and Baenziger [3].

***N*-acetylgalactosaminyltransferase assay.** The standard incubation mixture contained, in a total volume of 20 μl, 0.2 mM compound 2, 0.25 mM UDP-[^{14}C]GalNAc (2500–5000 dpm/nmol), 0.125 M Mes, pH 7.0, 0.5% (by vol.) Triton X-100, 40 mM $MnCl_2$, 5 mM ATP, and partially purified albumen gland GalNAc-T (16 μU, 9.3 μg protein) or connective tissue GalNAc-T microsomal suspension (1.02 μU, 10 μg protein). Incubations were conducted for 60–120 min at 37°C, and reactions were terminated by adding 0.5 ml 20 mM ice-cold sodium borate containing 2 mM EDTA. The samples were loaded onto Pasteur pipette columns containing 0.5 ml AG 1-X8 (acetate form, 100–200 mesh; Bio-Rad), and neutral carbohydrates (radioactive GalNAc, radioactive product, substrate) were eluted with 0.5 ml water. Solutions were mixed with 4 ml Emulsifier Safe (Packard 6013389), and analyzed for radioactivity. Control incubations in the absence of exogenous acceptor were carried out routinely. All assays were performed at least in duplicate, and were corrected for incorporation in the absence of exogenous acceptor.

Column chromatography. Amino adsorption HPLC was carried out using a Perkin-Elmer series 3 liquid chromatograph, equipped with a Rheodyne injection valve and a 10-μm Lichrosorb-NH_2 column (25 cm×4.6 mm, Chrompack). Elutions were performed with water/acetonitrile [20:80 or 30:70 (by vol.)], at flow rates of 1 or 2 ml/min. Fractionations were monitored at 205 nm, and aliquots of 1-ml fractions were analyzed by measuring the radioactivity using scintillation counting.

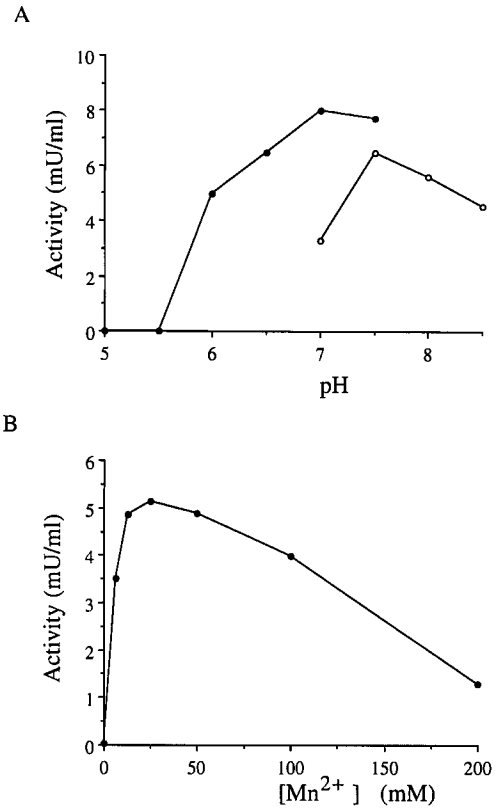


Fig. 2. Optimization of snail albumen gland *N*-acetylgalactosaminyltransferase using compound 2 as acceptor and UDP-[^{14}C]GalNAc as donor with respect to (A) pH and (B) $MnCl_2$ concentration. In the pH optimization assay, 0.5 M Mes (●) or 0.5 M Tricine (○) was added depending on the pH tested.

High-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [28] was carried out on a Dionex LC system consisting of a Dionex Bio LC quaternary gradient module and a PAD 2 detector, using a CarboPac PA1 column (25 cm×4 mm, Dionex). Elutions were performed with a concentration gradient of 30–150 mM NaOAc in 0.1 M NaOH for 25 min, at a flow rate of 4 ml/min. Detection was carried out with a gold electrode and triple-pulsed amperometry, comprising the following pulse potentials and durations: E_1 = 0.05 V (300 ms); E_2 = 0.65 V (60 ms); E_3 = −0.95 V (180 ms). Aliquots of 1-ml fractions were assayed for radioactivity by scintillation counting.

^{1}H-NMR spectroscopy. Prior to ^{1}H-NMR analysis, carbohydrates were exchanged twice in ^2H_2O (99.9 atom/100 atom ^{2}H, MSD Isotopes) with intermediate lyophilization. Finally, samples were dissolved in 99.96% ^2H_2O (MSD Isotopes). ^{1}H-NMR spectra were recorded at 300, 500, or 600 MHz on a Bruker AC-300 (Department of Organic Chemistry, Utrecht University), AMX-500, AMX-600 (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or AM-600 (NSR Center, SON NMR facility, University of Nijmegen, The Netherlands) spectrometers, at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225) [29]. Typically, one-dimensional spectra were recorded with a spectral width of 5000 Hz at 500 MHz or 6000 Hz at 600 MHz, collecting 16–500 free induction decays of 8K or 16K complex data points. Suppression of HO^2H was achieved by applying the WEFT pulse sequence as described previously [30]. The resolution of the one-dimensional spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomial function when necessary.

Two-dimensional nuclear Overhauser enhancement spectroscopy (two-dimensional NOESY) was carried out with a 250-ms mixing time. Two-dimensional homonuclear Hartmann-Hahn (two-dimensional HOHAHA) measurements [31] were performed using a MLEV-17 mixing sequence of 80 ms. For the two-dimensional double-quantum filtered ^{1}H-^{1}H correlation spectroscopy (DQF-COSY), two-dimensional NOESY, and two-dimensional HOHAHA spectra 512 measurements of 2K data points were recorded. The spectral width was 4032 Hz or 4500 Hz in each dimension.

Two-dimensional rotating-frame nuclear Overhauser enhancement spectroscopy (two-dimensional ROESY) was carried out using a spin-lock mixing pulse of 250 ms at a field strength corresponding to a 90° pulse-width of 100–110 µs. The carrier frequency was placed at the left side of the spectrum at δ 5.9 in order to minimise HOHAHA-type magnetisation transfer. The spectral width was 5500 Hz in each dimension, and 512 experiments of 4K data points were recorded.

The DQF-COSY, NOESY, HOHAHA, and ROESY experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation. The HO^2H signal was suppressed by presaturation during 1.0 s. Two-dimensional NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University).

Large-scale incubations with partially purified albumen gland GalNAc-T. The generation of products on a preparative scale was performed with the acceptors 1, 2, 5, and 8. The incubation mixture contained 40 mM $MnCl_2$, 0.5% (by vol.) Triton X-100, 125 mM Mes, pH 7.0, 5 mM ATP, 1 mM UDP-[^{14}C]GalNAc (492 dpm/nmol), albumen gland GalNAc-T preparation (0.64 mU, 0.37 mg protein), and acceptor (0.5 mM compound 1 in 800 µl; 0.25 mM compound 2 in 400 µl; 1 mM compound 5 in 1.6 ml; 1.1 mM compound 8 in 800 µl). Each mixture was kept for 24 h at 37 °C, stopped by freezing, diluted with water (0.5 ml), and desalted on a column (50 cm×1 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad), using water as eluent. To remove interfering Triton X-100, the pooled radioactive fractions of each incubation were rinsed over a freshly conditioned Sep-Pak C_{18} column and, after washing with 5 ml water, the total eluates were lyophilized. HPLC on Lichrosorb-NH_2 using water/acetonitrile as eluent was used to fractionate the residues from the incubations with compound 2 [30:70 (by vol.) at a flow rate of 2 ml/min], 5 [30:70 (by vol.) at a flow rate of 1 ml/min], and compound 8 [20:80 (by vol.) at a flow rate of 1 ml/min]. The residue from the incubation with acceptor 1 was fractionated on a column (75 cm×1 cm) of Bio-Gel P-4 (200–400 mesh) using 10 mM NH_4HCO_3 as eluent, at a flow rate of 30 ml/h. Aliquots of the 1-ml fractions were analyzed by measuring the radioactivity using scintillation counting.

Large-scale incubation with connective tissue GalNAc-T and GlcNAcβ1-OMe as substrate. The incubation of compound 8 and a connective tissue GalNAc-T preparation (0.04 mU, 0.4 mg protein) was performed as described for compound 8 using partially purified albumen gland GalNAc-T as enzyme preparation (see above).

RESULTS

Enzyme preparations. Partially purified albumen gland GalNAc-T (purified 100-fold) was obtained by affinity chromatography on UDP-hexanolamine–Sepharose 4B (Fig. 1). Further purification steps were not successful, e.g. on GlcNAc–Sepha-

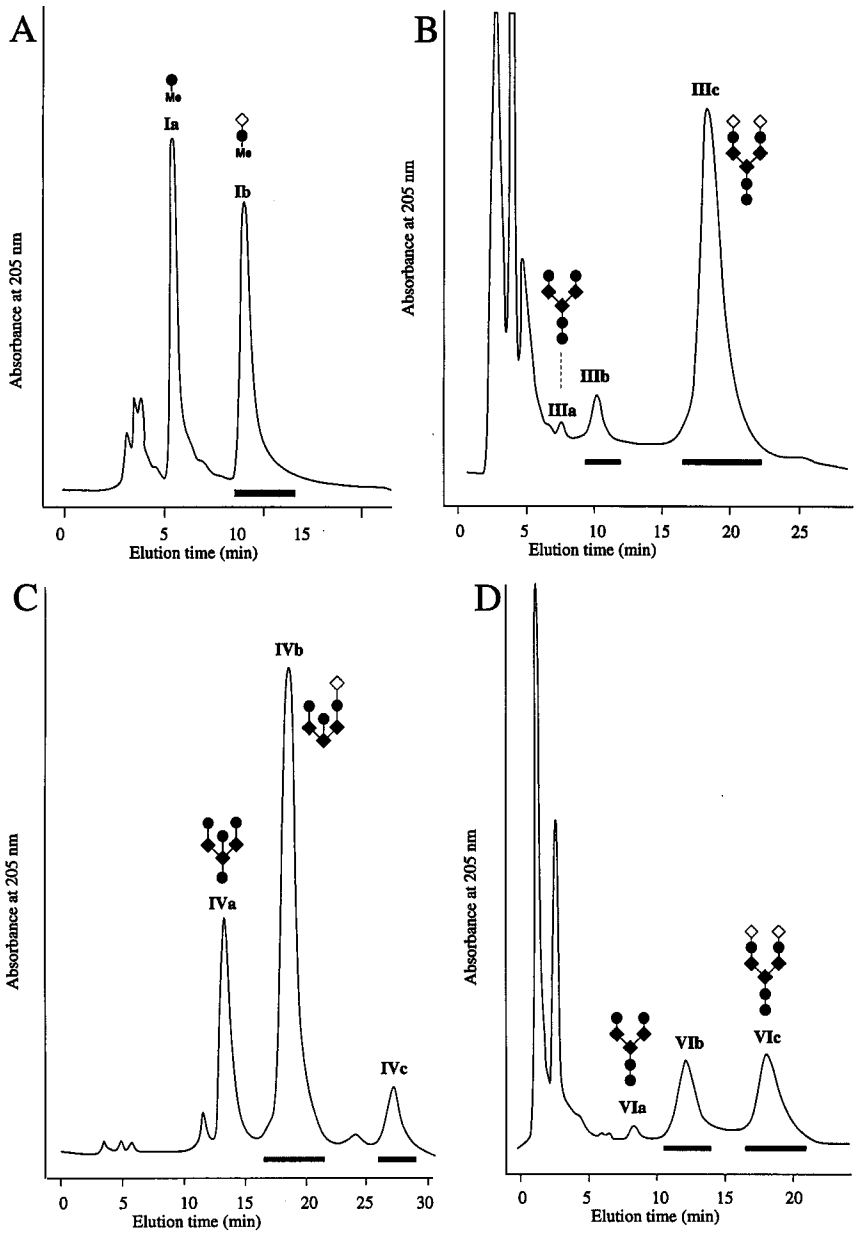


Fig. 3. HPLC of the reaction mixtures obtained by incubation of different acceptors with UDP-[^{14}C]GalNAc and partially purified albumen gland β4GalNAc-T on Lichrosorb-NH$_2$. (A) 24-h incubation of acceptor 8, elution with water/acetonitrile [20:80 (by vol.)] at a flow rate of 1 ml/min; (B) 24-h incubation of acceptor 2, elution with water/acetonitrile [30:70 (by vol.)] at a flow rate of 2 ml/min; (C) 24-h incubation of acceptor 5, elution with water/acetonitrile [30:70 (by vol.)] at a flow rate of 1 ml/min; (D) 2-h incubation of acceptor 2, elution with water/acetonitrile [30:70 (by vol.)] at a flow rate of 2 ml/min. Radioactive fractions are indicated (▬).

rose, mainly because the GalNAc-T enzyme could not be eluted from the column without inactivation. Purification of connective tissue GalNAc-T on UDP-hexanolamine–Sepharose 4B yielded a preparation that lost its activity within 24 h after elution. The connective tissue enzyme was not stable in Triton X-100 extracts (data not shown). Therefore, the experiments with the connective tissue enzyme were performed with a microsomal suspension.

Properties of albumen gland *N*-acetylgalactosaminyltransferase. Detailed studies were carried out using UDP-[^{14}C]GalNAc as donor and compound 2 as acceptor with partially purified albumen gland GalNAc-T as enzyme source. The radioactive product formation was found to be proportional with time over a period of 4 h and with an enzyme concentration up to at least 5.4 mg albumen gland protein/ml (data not shown). The pH optimum for the GalNAc-T activity was found to be approximately pH 7.0 (Fig. 2A), and the enzyme showed an absolute requirement for Mn^{2+} with an optimum concentration of 12.5–50 mM (Fig. 2B). The divalent cations Mg^{2+}, Ca^{2+}, Ba^{2+} and Cd^{2+} at 12.5 mM could not substitute for Mn^{2+}. The albumen gland GalNAc-T activity was independent of the Triton X-100 concentration in the range 0.25–2.5%, and no activation effect was found (data not shown).

The optimization procedure was repeated for the GalNAc-T activity in the connective tissue microsomal suspension, and for the partially purified albumen gland GalNAc-T, in both cases using compound 8 as an acceptor. No differences were found

Table 2. Relevant ^{1}H-NMR chemical shifts of structural-reporter groups of constituent monosaccharides for a series of acceptors and products involved in enzymic studies focused on snail albumen gland and connective tissue β4GalNAc-T. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 27°C. In the case of data accurate to two decimal places, the δ values are obtained from two-dimensional experiments, n.d., not determined. Compounds are represented by short-hand symbolic notation: ◇, GalNAc; ●, GlcNAc; ◆, Man; *R*, peptide. For numbering of the residues see Fig.4.

Residue	Reporter group	Chemical shift in					
		8/Ia/Va	Ib/Vb	1	IIa	2	IIIc
		Me	Me	3 6 R	R		
		ppm					
GlcNAc-1	H1α					5.187	5.191
	H1(β)			5.095	5.058	4.697	n.d.
	NAc			2.005	2.006	2.037	2.040
GlcNAc-2	$H1_α$					4.613	n.d.
	$H1_{(β)}$			4.616	4.615	4.604	n.d.
	NAc			2.079	2.078	2.078	2.082
Man-3	H2			4.247	4.238	4.249	4.245
Man-4	H1			5.118	5.108	5.118	5.109
	H2			4.187	4.172	4.188	4.178
Man-4′	H1			4.919	4.912	4.918	4.916
	NAc			4.107	4.092	4.107	4.098
GlcNAc-5	H1	4.445	4.438	4.556	4.552	4.556	4.555
	H2	2.035	2.027	2.052	2.042	2.051	2.045
GlcNAc-5′	H1			4.556	4.558	4.556	4.560
	NAc			2.052	2.037	2.051	2.040
GlcNAc-5/5′	H2	3.688	3.72	3.701	3.73	n.d.	n.d.
	H3	3.531	3.69	3.52	3.69	n.d.	n.d.
	H4	3.438	3.63	3.55	3.64	n.d.	n.d.
	H5	3.456	3.52	3.45	3.49	n.d.	n.d.
	H6	3.917	3.863	3.92	3.82	n.d.	n.d.
	H6′	3.747	3.68	3.76	n.d.	n.d.	n.d.
GalNAc-GN	H1		4.517		4.512		4.514
	NAc		2.070		2.063		2.066
GalNAc-GN′	H1				4.520		4.522
	NAc				2.070		2.073
GalNAc-GN/GN′	H2		3.928		3.93		n.d.
	H3		3.733		3.76		n.d.
	H4		3.940		3.94		n.d.

between acceptors 2 and 8 using the albumen gland enzyme nor between the albumen gland and connective tissue enzymes using compound 8 as acceptor (data not shown).

The substrate specificities of the albumen gland and connective tissue β4GalNAc-T enzymes towards several acceptors under the optimized conditions are summarized in Table 1. All effective acceptors (1–8, 11, and 12) have a non-reducing terminal β-GlcNAc residue, whereas ineffective acceptors have GlcNAc with an α-linkage (10) or comprise saccharides with non-reducing terminal Gal or GalNAc residues (13–17). The free monosaccharides GalNAc, Gal or Glc (18–20) were ineffective acceptors, whereas free GlcNAc (9) is an effective acceptor. The substrate specificity and preparative scale incubations (see below) of the albumen gland and connective tissue β4GalNAc-T enzymes suggest that both activities are from one enzyme. Even though the connective tissue enzyme showed a different stability in the Triton X-100 extract, the albumen gland GalNAc-T and the connective tissue GalNAc-T will be discussed as one enzyme activity.

Product identification using albumen gland *N*-acetylgalactosaminyltransferase. *GlcNAcβ1-OMe (8) as substrate:* Compound 8 was incubated with UDP-[^{14}C]GalNAc and partially purified albumen gland GalNAc-T for 24 h at 37°C. The mixture was fractionated by HPLC on Lichrosorb-NH_2 yielding a non-radioactive fraction Ia and a radioactive fraction Ib (Fig. 3A). Both fractions were analyzed by 500-MHz ^{1}H-NMR spectroscopy, and the relevant ^{1}H-NMR data are summarized in Table 2. The ^{1}H-NMR spectrum of Ia corresponds with the spectrum of unmodified substrate 8, whereas the ^{1}H-NMR

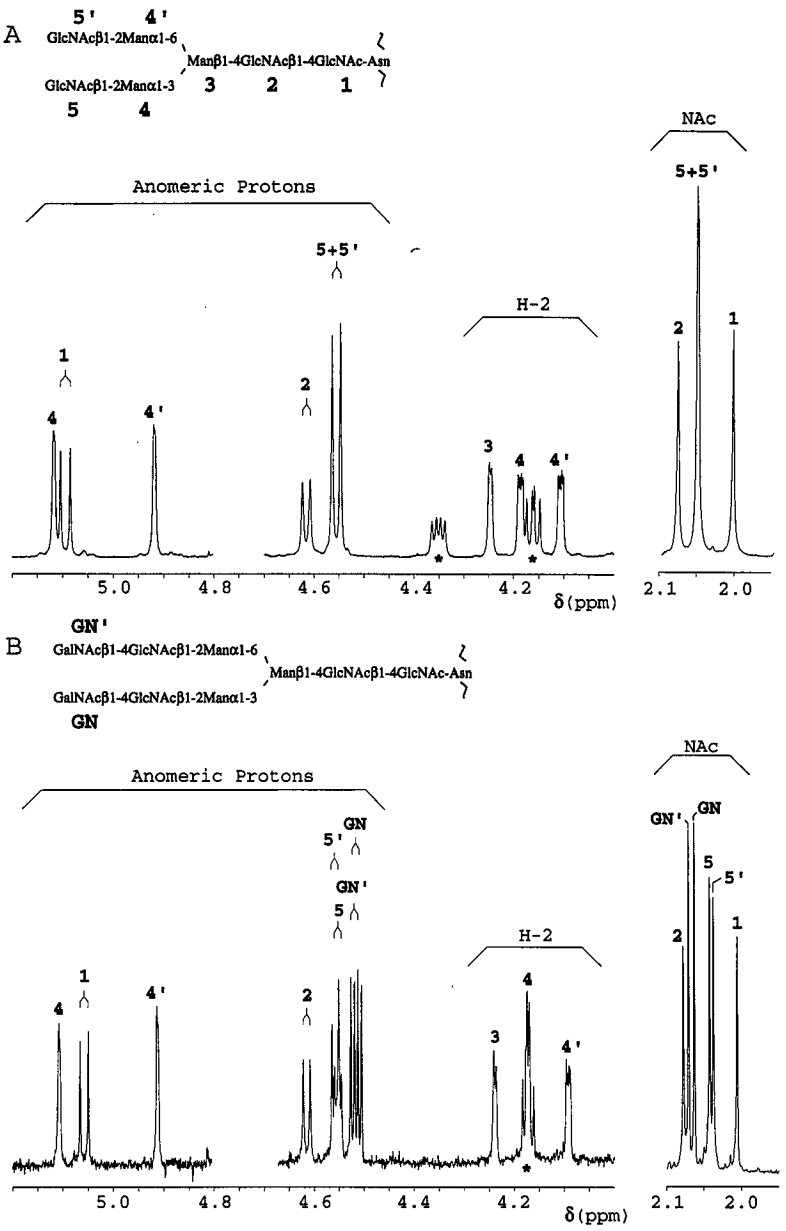


Fig. 4. Structural-reporter-group regions of the resolution-enhanced 600-MHz ^{1}H-NMR spectra of (A) compound 1 and (B) GalNAcβ1–4GlcNAcβ1–2Manα1–6(GalNAcβ1–4GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-glycopeptide in ^2H_2O at 27°C. The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum. *, represents H_α signals of peptide amino acid residues.

spectrum of Ib is identical to that of authentic GalNAcβ1–4GlcNAcβ1-OMe [32]. Two-dimensional COSY, HOHAHA and ROESY measurements resulted in an almost complete assignment of the ^{1}H-NMR spectrum of Ib. The ROESY experiments showed the expected strong interresidual cross-peak between GalNAc H1 and GlcNAc H4.

GlcNAcβ1–2Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4-GlcNAcβ1–4GlcNAcβ1-peptide (1) as substrate: Compound 1 was incubated with UDP-[^{14}C]GalNAc and partially purified albumen gland GalNAc-T for 24 h at 37°C. The mixture was fractionated on Bio-Gel P-4, and one radioactive fraction IIa was collected (data not shown) and analyzed by 600-MHz ^{1}H-NMR spectroscopy (Table 2). Comparison of the ^{1}H-NMR spectra of glycopeptide IIa and acceptor 1 (Fig. 4) shows clearly the introduction of two β-linked GalNAc residues (GalNAc-GN: H1, δ 4.512; NAc, δ 2.063 and GalNAc-GN′: H1, δ 4.520; NAc, δ 2.070). The ^{1}H-NMR structural-reporter-group data of the two GalNAcβ1–4GlcNAcβ1–2Man branches in IIa match completely those of the α1–6 fucosylated reference oligosaccharide N1.3 from urokinase [2], and show that glycopeptide IIa corresponds to GalNAcβ1–4GlcNAcβ1–2Manα1–6(GalNAcβ1–4-GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-peptide. Additional two-dimensional COSY and HOHAHA experiments were performed on both acceptor 1 and IIa in order to generate more detailed ^{1}H assignments with respect to the antennary GlcNAc and GalNAc residues. The assignments of the H1 signals of GlcNAc-5 and GlcNAc-5′ are based on a 500-MHz two-dimensional NOESY experiment showing GlcNAc-5 H1/Man-4 H1 and GlcNAc-5′ H1/Man-4′ H1 cross-peaks.

GlcNAcβ1–2Manα1–6(GlcNAcβ1–2Manα1–3)Manβ1–4-GlcNAcβ1–4GlcNAc (2) as substrate: Incubation of compound

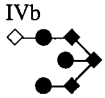
2 with UDP-[^{14}C]GalNAc and partially purified albumen gland GalNAc-T followed by fractionation of the mixture by HPLC on Lichrosorb-NH_2, yielded a non-radioactive fraction IIIa, eluting with acceptor 2, and two radioactive fractions IIIb and IIIc (Fig. 3B). The relevant 500-MHz ^{1}H-NMR data of compound 2 and IIIc are presented in Table 2. The ^{1}H-NMR structural-reporter-group data of oligosaccharide IIIc are comparable to those of glycopeptide IIa, showing that the structure of IIIc is GalNAcβ1−4GlcNAcβ1−2Manα1−6(GalNAcβ1−4GlcNAcβ1−2Manα1−3)Manβ1−4GlcNAcβ1−4GlcNAc. The isolated amounts of IIIb were too low for structure determination, but IIIb eluted with (GalNAcβ1−4)$_{0\text{-}1}$GlcNAcβ1−2Manα1−6[(GalNAcβ 1 − 4)$_{1\text{-}0}$ GlcNAcβ 1 − 2 Manα 1 − 3] Manβ 1 − 4GlcNAcβ1−4GlcNAc, having only one GalNAc residue, as found in the branch specificity study (fraction VIb, see below).

GlcNAcβ1−2Manα1−6(GlcNAcβ1−4) (GlcNAcβ1−2Manα1−3)-Man (5) as substrate: Compound 5 was incubated with UDP-[^{14}C]GalNAc and partially purified albumen gland GalNAc-T, and the mixture was fractionated by HPLC on Lichrosorb-NH_2 yielding a non-radioactive fraction IVa and two radioactive fractions, IVb and IVc (Fig. 3C). Fractions IVa and IVb were analyzed by ^{1}H-NMR spectroscopy, and relevant ^{1}H-NMR data are summarized in Table 3. The included assignments are based on 500-MHz two-dimensional HOHAHA studies and a comparison with the ^{1}H-NMR data of GlcNAcβ1−2Manα1−6(GlcNAcβ1−4)(GlcNAcβ1−2Manα1−3)Manβ1−4GlcNAc [29]. The 500-MHz ^{1}H-NMR spectrum of fraction IVa (Fig. 5A) corresponds with that of substrate 5. The *N*-acetyl region in the ^{1}H-NMR spectrum of IVa showed five singlets (δ 2.052, δ 2.058, δ 2.061, δ 2.063, δ 2.070) which could not be assigned unequivocally due to anomerization effects. Inspection of the 500-MHz ^{1}H-NMR spectrum of IVb (Fig. 5B) showed one extra β-anomeric signal at δ 4.517($J_{1,2}$ 8.4 Hz), suggesting the introduction of one β-linked GalNAc residue: in the *N*-acetyl region, six singlets (δ 2.053, δ 2.056, δ 2.064, δ 2.065, δ 2.068, δ 2.070) occur. Going from acceptor 5 to product IVb, significant chemical shift differences are observed for Man-4′ H2 ($\Delta\delta$-0.016 ppm) and GlcNAc-5′ H2 ($\Delta\delta$+0.04 ppm), H3 ($\Delta\delta$-0.04 ppm), and H4 ($\Delta\delta$+0.18 ppm), indicating that the β-linked GalNAc residue is introduced exclusively at C4 of GlcNAc-5′, yielding the following structure: GalNAcβ1−4GlcNAcβ1−2Manα1−6-(GlcNAcβ1−4)(GlcNAcβ1−2Manα1−3)Man. Further support was obtained from 600-MHz two-dimensional ROESY experiments, showing a strong interresidual cross-peak between GalNAc-GN′ H1 and GlcNAc-5′ H4. The amount of material in fraction IVc was not sufficient for a detailed structure determination, but IVc probably contains a product having two GalNAc residues.

Incubations on a preparative scale of GlcNAcβ1-OMe (8) with connective tissue GalNAc-T. Compound 8 was incubated with UDP-[^{14}C]GalNAc and connective tissue GalNAc-T for 24 h at 37 °C. The mixture was fractionated by HPLC on Lichrosorb-NH_2, yielding a non-radioactive fraction Va and a radioactive fraction Vb (data not shown). ^{1}H-NMR analysis of both compounds demonstrated that Va is identical to Ia (acceptor GlcNAcβ1-OMe), and Vb to Ib (GalNAcβ1−4GlcNAcβ1-OMe). This means that the connective tissue enzyme, like the albumen gland enzyme, introduces a GalNAc residues with a β1−4 linkage to GlcNAcβ-*R*.

Branch specificity of the albumen gland *N*-acetylgalactosaminyltransferase. To investigate whether β4GalNAc-T shows branch specificity, diantennary compound 2 was incubated for 2 h and for 4 h with UDP-[^{14}C]GalNAc and albumen gland enzyme. Both incubation mixtures were subjected to HPLC on Lichrosorb-NH_2 (Fig. 3D; 2 h), and in each case two major radioactive fractions were observed, VIc eluting with GalNAcβ1−4GlcNAcβ1−2-Manα1−6(GalNAcβ1−4GlcNAcβ1−2Manα1−3)Manβ1−4-GlcNAcβ1−4GlcNAc and VIb eluting between VIa (≡ 2) and VIc. Incubation for 4 h shows a relative increase of the radioactive products VIb and VIc, with a higher amount of VIc, as compared to the 2-h incubation. The ^{1}H-NMR spectrum of VIc was identical to that of IIIc. The intermediate elution time of VIb suggested that this fraction contained

Table 3. Relevant ^{1}H-NMR chemical shifts of structural-reporter groups of constituent monosaccharides for the components IVa and IVb of the incubation mixture of component 5/UDP-[^{14}C]GalNAc/snail albumen gland enzyme. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 27 °C. In the case of data accurate to two decimal places, the δ values are obtained from two-dimensional experiments. For the short-hand symbolic notation see Table 2. For numbering of the residues see Fig. 5.

Residue	Reporter group	Chemical shift in	
		5/IVa 	IVb 
		ppm	
Man-3	H1α	5.126	5.123
	H1β	4.875	4.873
	H2	4.03	4.03
	H3	3.93	3.92
	H4	4.13	4.12
Man-4	H1$_\alpha$	5.100	5.098
	H1$_\beta$	5.079	5.079
	H2	4.243	4.249
	H3	3.90	3.90
	H4	3.49	3.49
	H5	3.68	3.68
Man-4′	H1$_\alpha$	4.988	4.984
	H1$_\beta$	5.024	5.018
	H2	4.182	4.166
	H3	3.82	3.82
	H4	3.50	3.49
	H5	3.63	3.63
GlcNAc-5	H1	4.552	4.550
	H2	3.70	3.72
	H3	3.59	3.60
	H4	3.47	3.47
GlcNAc-5′	H1$_\alpha$	4.558	4.559
	H1$_\beta$	4.593	4.596
	H2	3.68	3.72
	H3	3.56	3.52
	H4	3.45	3.63
GlcNAc-9	H1$_\alpha$	4.496	4.493
	H1$_\beta$	4.491	4.488
	H2	3.70	3.70
	H3	3.57	3.57
	H4	3.270	3.271
	H5	3.45	3.45
GalNAc-GN′	H1		4.517
	H2		3.93
	H3		3.73
	H4		3.93
	H5		3.72

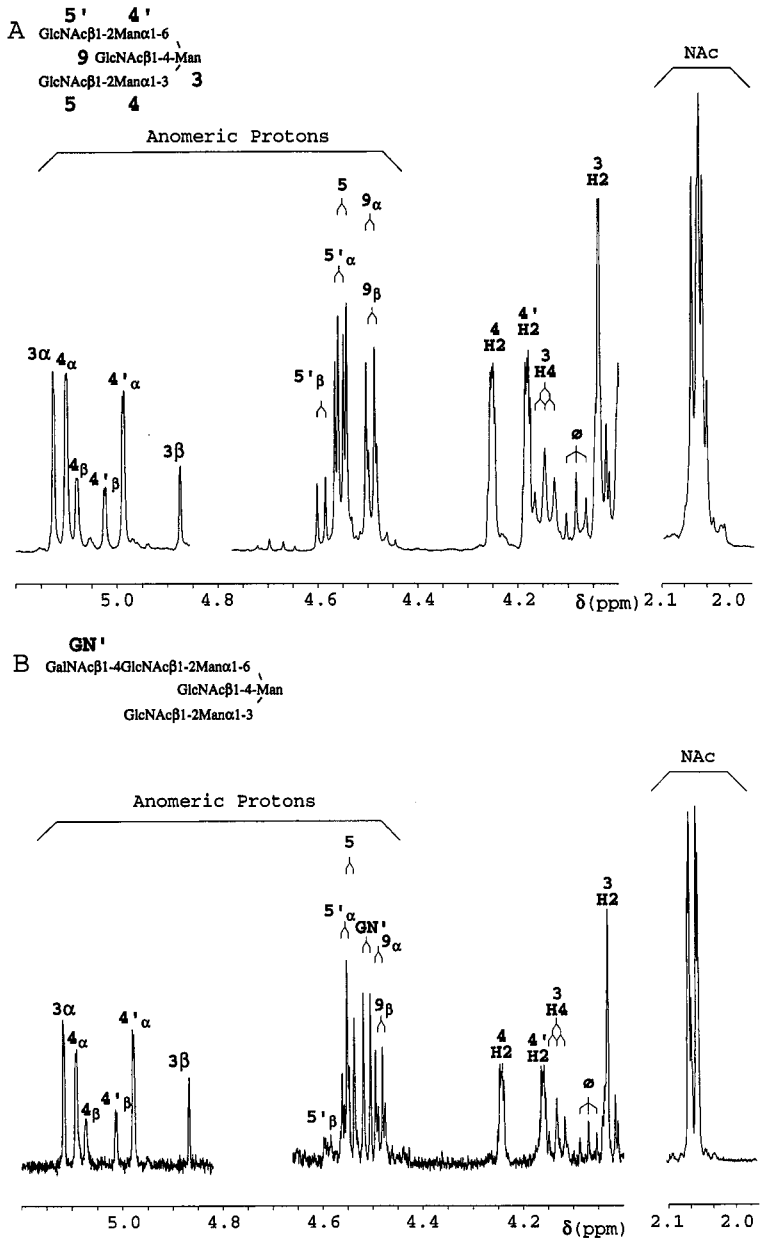


Fig. 5. Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of (A) compound 5 and (B) GalNAcβ1–4GlcNAcβ1–2Manα1–6(GlcNAcβ1–4)(GlcNAcβ1–2Manα1–3)Man in ^2H_2O at 27°C. The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum.

GlcNAcβ1−2Manα1−6(GlcNAcβ1−2Manα1−3)Manβ1−4-GlcNAcβ1−4GlcNAc bearing one extra β1−4-linked GalNAc. Further conclusions with respect to branch location could be drawn on the basis of a comparison of the *N*-acetyl regions in the ^{1}H-NMR spectra of VIb, 2, and IIIc (see also Table 2). For VIb, singlets of equal intensity are observed for terminal GlcNAc-5 (δ 2.051), substituted GlcNAc-5 (δ 2.046), terminal GlcNAc-5′ (δ 2.051), substituted GlcNAc-5′ (δ 2.039), terminal GalNAc-GN (δ 2.067), and terminal GalNAc-GN′ (δ 2.074) indicating the presence of equal amounts of GalNAcβ1−4GlcNAcβ1−2Manα1−6(GlcNAcβ1−2Manα-1−3)Manβ1−4GlcNAcβ1−4GlcNAc and GlcNAcβ1−2-Manα1−6(GalNAcβ1−4GlcNAcβ1−2Manα1−3)Manβ1−4GlcNAcβ1−4GlcNAc. These findings demonstrate that albumen gland β4GalNAc-T shows no branch specificity towards compound 2. It should be noted that a further separation of fraction VIb by HPAEC on CarboPac PA-1 could not be achieved.

Incubation studies with desialylated-degalactosylated human serotransferrin and human chorionic gonadotropin. Partially purified albumen gland β4GalNAc-T was shown to be active towards both desialylated-degalactosylated human serotransferrin and desialylated-degalactosylated human chorionic gonadotropin. However, no β1−4 galactosyltransferase activity was found towards either of these protein substrates with snail albumen gland extracts. It should be noted that also in snail connective tissue no β1−4 galactosyltransferase activity has been detected [22]. For comparison, an enzyme homogenate from bovine pituitary glands was also tested for β4GalNAc-T and β1−4 galactosyltransferase activities towards desialylated-dega-

lactosylated human serotransferrin and desialylated-degalactosylated human chorionic gonadotropin according to a previous method [3]. Following the published protocol [3], the pituitary β1−4 galactosyltransferase activities towards the protein substrates were comparable with data from previous studies. However we were unable to reproduce the results with respect to pituitary β4GalNAc-T. Nevertheless, since snail albumen gland β4GalNAc-T was effective towards both protein acceptors and towards monosaccharide acceptors, whereas pituitary β4GalNAc-T has been reported to show a preference for the human chorionic gonadotropin acceptor over the human serotransferrin acceptor [3], it is likely that the pituitary enzyme and the snail enzyme are different.

DISCUSSION

Connective tissue and albumen gland of the snail *L. stagnalis* contain a UDP-GalNAc:GlcNAcβ-*R* β1−4 *N*-acetylgalactosaminyltransferase capable of transferring *N*-acetylgalactosamine from UDP-GalNAc to GlcNAcβ-*R*. Based on the products formed and the substrate specificity found, it is likely that the connective tissue and albumen gland β4GalNAc-T activities are due to a single enzyme species present in both tissues. The results of a branch specificity study of the partially purified albumen gland β4GalNAc-T showed no preference for the Manα1−3 or Manα1−6 branches of the diantennary compound 2. Earlier, a distinct branch specificity for pituitary β4GalNAc-T has been suggested [33], since the GalNAcβ1−4GlcNAc-*R* sequence was found almost exclusively in the Manα1−3 branch, whereas the Galβ1−4GlcNAc-*R* sequence was found mainly in the Manα1−6 branch of glycan structures on human lutropin. The glycosylation pattern of this glycohormone cannot be explained from the branch specificity of β1−4 galactosyltransferase, as this enzyme prefers the Manα1−3 branch [34, 35]. Therefore, the preferential occurrence of GalNAc at the Manα1−3 branch of pituitary hormone oligosaccharides might be due to the fact that the pituitary gland β4GalNAc-T seems to recognize also the PXR/K peptide motif in the glycoprotein hormones [3]. The finding of GalNAc exclusively in the Manα1−6 branch in the case of acceptor 5 is probably due to steric hindrance by the intersecting GlcNAc residue coupled at the βMan unit [36].

The snail β4GalNAc-T can be distinguished from the pituitary gland β4GalNAc-T by the fact that the snail enzyme is capable of transferring GalNAc to monosaccharide and oligosaccharide acceptors as well as to glycoproteins like human serotransferrin, which do not have the PXR/K-specific tripeptide motif, necessary for activity with the PXR/K-specific GalNAc-transferase [3, 4]. Our results also suggest that the snail β4GalNAc-T is different from the transferrin-specific β4GalNAc-T [4], because the snail β4GalNAc-T can transfer GalNAc effectively to desialylated-degalactosylated human serotransferrin and desialylated-degalactosylated human chorionic gonadotropin. The snail β4GalNAc-T must also be different from the β4GalNAc-T enzymes that transfer GalNAc in β1−4 linkage to the Gal residue of NeuAcα2−3Galβ-*R*, since these enzymes cannot act on asialo substrates [5-15].

Experiments using connective tissue or albumen gland as enzyme sources, UDP-Gal or UDP-GalNAc as donors, and GlcNAcβ1-OMe as acceptor, showed that the snail β4GalNAc-T activity cannot be due to the promiscuous action of a galactosyltransferase using UDP-GalNAc as a donor, as previously described for β1−4 galactosyltransferase from bovine milk [37] and human blood group B α1−3 galactosyltransferase [38]. Moreover, the two β3Gal-T enzymes that have been described in albumen gland [39] and connective tissue [22] do not show activity with acceptors containing βGlcNAc as non-reducing terminal monosaccharide, and do not transfer GalNAc.

The β4GalNAc-T activity in connective tissue is probably involved in the biosynthesis of the N-glycans of hemocyanin since connective tissue is the location where hemocyanin is synthesized and glycosylated [22, 40]. The function of the β4GalNAc-T activity in albumen glands is not known, but it is tempting to speculate that this enzyme controls the synthesis of *N,N'*-diacetyllactosediamine-containing oligosaccharide chains on albumen gland glycoproteins. Recently, an enzyme activity similar to the snail GalNAc-T activity has been described in cercariae of *Trichobilharzia ocellata* for which the parasite *L. stagnalis* is the intermediate host [41].

In conclusion, in this study a novel β4GalNAc-T has been described which shows a broad specificity towards acceptors having non-reducing terminal βGlcNAc residues in saccharides and glycoproteins. Even GlcNAc can serve as an acceptor.

This study was supported by the Netherlands Foundation for Chemical Research (SON/NWO). We thank Dr J. G. M. van der Ven, Dr G. Strecker, and Dr J. Lönngren for their generous gifts of (bio)chemical materials.

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