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Identification of a novel UDP-Gal:GalNAcβ1-4GlcNAc-R β1-3-galactosyltransferase in the connective tissue of the snail *Lymnaea stagnalis*

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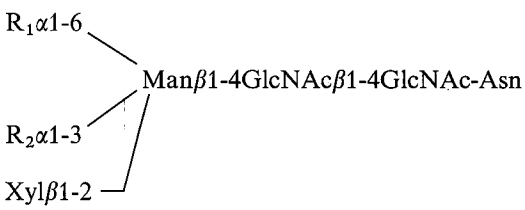
Connective tissue of the freshwater pulmonate *Lymnaea stagnalis* was shown to contain galactosyltransferase activity capable of transferring Gal from UDP-Gal in β1-3 linkage to terminal GalNAc of GalNAcβ1-4GlcNAc-R [R = β1-2Manα1-O(CH_2)$_8$COOMe, β1-OMe, or α,β1-OH]. Using GalNAcβ1-4GlcNAcβ1-2Manα-1-O(CH_2)$_8$COOMe as substrate, the enzyme showed an absolute requirement for Mn^{2+} with an optimum Mn^{2+} concentration between 12.5 mM and 25 mM. The divalent cations Mg^{2+}, Ca^{2+}, Ba^{2+} and Cd^{2+} at 12.5 mM could not substitute for Mn^{2+}. The galactosyltransferase activity was independent of the concentration of Triton X-100, and no activation effect was found. The enzyme was active with GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH_2)$_8$COOMe (V_{max} 140 nmol · h^{-1} · mg protein^{-1}; K_m 1.02 mM), GalNAcβ1-4GlcNAc (V_{max} 105 nmol · h^{-1} · mg protein^{-1}; K_m 0.99 mM), and GalNAcβ1-4GlcNAcβ1-OMe (V_{max} 108 nmol · h^{-1} · mg protein^{-1}; K_m 1.33 mM). The products formed from GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH_2)$_8$COOMe and GalNAcβ1-4GlcNAcβ1-OMe were purified by high performance liquid chromatography, and identified by 500-MHz ^{1}H-NMR spectroscopy to be Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH_2)$_8$COOMe and Galβ1-3GalNAcβ1-4GlcNAcβ1-OMe, respectively. The enzyme was inactive towards GlcNAc, GalNAcβ1-3Galα1-OMe, GalNAcα1-OC_6H_5, GalNAcα1–ovine-submaxillary-mucin, lactose and *N*-acetyllactosamine. This novel UDP-Gal:GalNAcβ1-4GlcNAc-R β1-3-galactosyltransferase is believed to be involved in the biosynthesis of the hemocyanin glycans of *L. stagnalis*.

One of the most conspicuous cell types in the connective tissue (CT) of gastropods and bivalves is the pore cell. Several different functions have been attributed to these cells; one of these being the production and secretion of blood pigments into the hemolymph. It has been suggested that the pore cells of the freshwater snail *Lymnaea stagnalis* synthesize and store hemocyanin [1], a high-molecular-mass copper-containing oxygen-transport protein, which is freely dissolved in the hemolymph. Hemocyanin is a glycoprotein with a carbohydrate content of 3% (by mass), containing L-fucose, D-xylose, 3-*O*-methyl-D-mannose (3-OMe-Man), 3-*O*-methyl-D-galactose (3-OMe-Gal), D-mannose, D-galactose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine as sugar constituents [2]. In previous studies, the primary structures of the hemocyanin glycans of *L. stagnalis* have been identified [3, 4], affording a series of novel N-linked oligosaccharides:



R_1 = Man, 3-OMe-Man, 3-OMe-Galβ1-3GalNAcβ1-4GlcNAcβ1-2Man or Fucα1-2Galβ1-3GalNAcβ1-4GlcNAcβ1-2Man. R_2 = 3-OMe-Man, 3-OMe-Galβ1-3GalNAcβ1-4GlcNAcβ1-2Man or Fucα1-2Galβ1-3Gal-NAcβ1-4GlcNAcβ1-2Man.

As part of our program on the structural analysis, organic synthesis, biosynthesis, and conformational analysis of xylose-containing N-linked carbohydrate chains, we present evidence for a novel galactosyltransferase from the connective tissue of *L. stagnalis*. This galactosyltransferase activity transfers Gal from UDP-Gal in β1-3-linkage to the terminal GalNAc of GalNAcβ1-4GlcNAc-R, and is presumably involved in the biosynthesis of the antennae of the hemocyanin glycan. The novel activity has been called UDP-Gal:GalNAcβ1-4GlcNAc-R β1-3-galactosyltransferase (β3Gal-T).

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Abbreviations. CT, connective tissue; 3-OMe-Man, 3-*O*-methyl-D-mannose; 3-OMe-Gal, 3-*O*-methyl-D-galactose; Gal-T, galactosyltransferase; β3Gal-T, β1-3-galactosyltransferase; β4Gal-T, β1-4-galactosyltransferase; OSM, ovine submaxillary mucin; 2D, two-dimensional; HOHAHA, homonuclear Hartmann-Hahn; Fuc, fucose.

Enzymes. UDP-Gal:GalNAcα1-R β1-3-galactosyltransferase (EC 2.4.1.122); UDP-Gal:GlcNAc-R β1-4-galactosyltransferase (EC 2.4.1.90; EC 2.4.1.38).

EXPERIMENTAL PROCEDURES

Materials

Connective tissue from mantle edges and albumen glands (female accessory sex glands) were dissected from laboratory bred, adult specimens of *L. stagnalis*, fed lettuce *ad libitum*, and kept at 20°C under carefully controlled conditions of photoperiod (12 h light/12 h dark). Porcine submaxillary gland microsome suspension, containing UDP-Gal:GalNAcα1-R β3Gal-T activity [5], was a gift from Prof. Dr D. H. van den Eijnden (Department of Medical Chemistry, Free University, Amsterdam). UDP-Gal:GlcNAc-R β1-4-galactosyltransferase (β4Gal-T) from human milk was supplied by Boehringer Mannheim. UDP–D-[U-^{14}C]galactose (specific activity 272.8 Ci/mol) was obtained from New England Nuclear, and diluted as needed by non-radioactive UDP–D-Gal (Sigma). Sep-Pak C_{18} columns were supplied by Waters Associates.

GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe [6] (a gift from Dr O. Hindsgaul, Department of Chemistry, University of Alberta, Edmonton, Canada), GalNAcβ1-4GlcNAcβ1-OMe (J. G. M. van der Ven, unpublished results), GalNAcβ1-4GlcNAc (a gift from Prof. Dr K. L. Matta, Buffalo, USA), GalNAcβ1-3Galα1-OMe (Sockerbolaget), Galβ1-4Glc (Sigma), Galβ1-4GlcNAc (Sigma), GalNAcα1-OC_6H_5 (Koch Light), and GalNAcα1–ovine-submaxillary-mucin (OSM; a gift from Prof. Dr D. H. van den Eijnden) were used as substrates.

Protein determination

Protein determination was carried out according to [7] using bovine serum albumin as a standard.

Preparation of snail enzyme homogenates

All procedures were carried out at 4°C. Dissected CT and albumen glands were homogenized in Hepes-buffered saline, pH 7.8 (30 mM NaCl, 1.5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM Hepes, 8 mM $NaHCO_3$ and 2.5 mM NaOH) using a Sonifier B-12 apparatus (Branson Sonic Power Company), and the homogenates were centrifuged for 15 min at 18000 rpm. The membrane pellets were homogenized in 0.25 M sucrose with a Potter-Elvehjem homogenizer and stored at −20°C.

Galactosyltransferase assays

The standard incubation mixture contained, in a total volume of 20 µl, 0.5 mM GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe, 0.25 mM UDP-[^{14}C]Gal (4400–5000 dpm/nmol), 0.125 M Tricine (pH 7.5), 1% (by vol.) Triton X-100, 20 mM $MnCl_2$, 5 mM ATP and snail CT homogenate (10 µg protein). Incubations were carried out 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 then loaded onto Pasteur pipette columns containing 0.5 ml AG 1-X8 (acetate form, 100–200 µm mesh; Bio-Rad), and neutral carbohydrates (radioactive Gal, radioactive product, substrate) were eluted with 0.5 ml water, mixed with 4 ml Aqua-luma (Lumac), and radioactivity measured. 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.

Substrate specificity

GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe, GalNAcβ1-4GlcNAc, GalNAcβ1-4GlcNAcβ1-OMe, GalNAcα1-OC_6H_5, GalNAcα1-OSM, GalNAcβ1-3Galα1-OMe, Galβ1-4Glc, Galβ1-4GlcNAc and GlcNAc were incubated with UDP-[^{14}C]Gal in the presence of snail CT homogenate, snail albumen-gland homogenate, porcine submaxillary-gland microsomes, or human milk β4Gal-T. For all incubations, the standard galactosyltransferase assay conditions were used.

Incubations on a preparative scale with snail CT homogenate

GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe as substrate. The incubation mixture containing, in a total volume of 750 µl, 0.6 µmol substrate, 12 µmol $MnCl_2$, 0.8% (by vol.) Triton X-100, 75 µmol Tricine (pH 7.5), 3 µmol ATP, 0.6 µmol UDP-[^{14}C]Gal (492 dpm/nmol) and snail CT homogenate (0.6 mg protein), was kept for 10 h at 37°C. The reaction was stopped by freezing, and the sample was loaded onto a freshly conditioned Sep-Pak C_{18} column [8]. After washing with five 5-ml portions of water, the column was eluted with two 5-ml portions of methanol. The first 5-ml methanol fraction, containing all radioactivity, was evaporated using a stream of nitrogen, and the residue was fractionated by HPLC on a column (250 mm × 4.6 mm) of RP-C_{18} (Chrompack) using water/acetonitrile (80:20, by vol.) as eluent. The flow rate was 1 ml/min. The elution pattern was monitored at 205 nm, and aliquots of the 1-ml fractions were analyzed by scintillation counting.

GalNAcβ1-4GlcNAcβ1-OMe as substrate. The incubation mixture containing, in a total volume of 600 µl, 0.6 µmol substrate, 12 µmol $MnCl_2$, 1.0% (by vol.) Triton X-100, 75 µmol Tricine (pH 7.5), 3 µmol ATP, 0.6 µmol UDP-[^{14}C]Gal (492 dpm/nmol) and snail CT homogenate (0.6 mg protein), was kept for 24 h at 37°C. The reaction was terminated by freezing, and after adding 2 ml water, the mixture was passed over a 10-ml column of AG 1-X8 (acetate form, 100–200 µm mesh). The resin was washed with 20 ml water, and the total eluate was lyophilized. The residue was taken up in water, and desalted on a column (50 cm × 1 cm) of Bio-Gel P-2 (100–200 µm mesh, Bio-Rad) using water as eluent. To remove interfering Triton X-100, the pooled radioactive fractions were rinsed over a freshly conditioned Sep-Pak C_{18} column. After washing with 5 ml water, the total eluate was lyophilized, and the residue was fractionated by HPLC on a column (250 mm × 4.6 mm) of Lichrosorb-10-NH_2 (Chrompack) using water/acetonitrile (25:75, by vol.) as eluent. The flow rate was 1 ml/min. The elution pattern was monitored at 205 nm, and aliquots of the 1-ml fractions were analyzed by scintillation counting.

500-MHz ^{1}H-NMR spectroscopy

Carbohydrates were exchanged twice in ^2H_2O (99.9% ^{2}H/0.1% ^{1}H, MSD Isotopes) with intermediate lyophilization. ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) operating at 500 MHz at a probe temperature of 27°C. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone (δ = 2.225 ppm) [9]. Two-di-

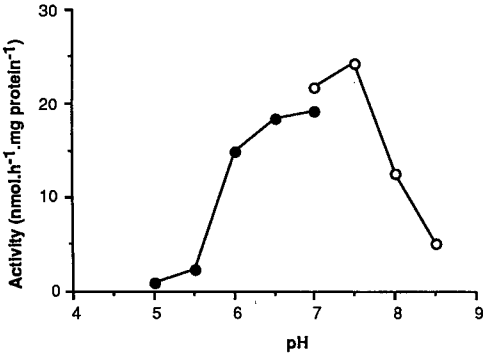

Fig. 1. *Optimal pH of snail CT galactosyltransferase activity using GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe as acceptor and UDP-[^{14}C]Gal as donor.* In the assay 0.5 M Mes (●) or 0.5 M Tricine (○) was added depending on the pH tested

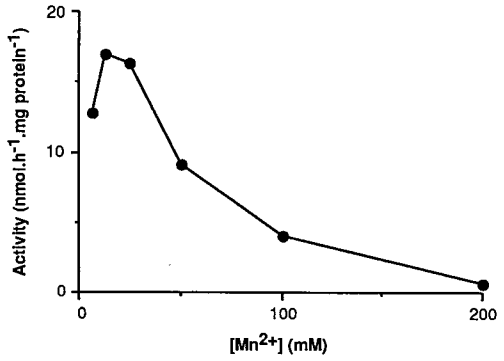

Fig. 2. *Optimal concentration of Mn^{2+} using GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe as acceptor and UDP-[^{14}C]Gal as donor in the presence of snail CT homogenate.* Different concentrations of $MnCl_2$ were added to the standard assay mixture

mensional homonuclear Hartmann-Hahn (2D HOHAHA) spectra [10] were obtained with a M. Levitt 17 mixing time of 120 ms.

RESULTS

Properties of CT galactosyltransferase

Detailed studies were carried out using UDP-[^{14}C]Gal as donor and GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe as acceptor with *L. stagnalis* CT homogenate as a crude enzyme source. The radioactive product formation was found to be proportional to time over a period of 4 h and to an enzyme concentration of up to at least 6 mg CT protein/ml (data not shown). The pH optimum for the glycosyltransferase (Gal-T) activity was found to be around 7.5 (Fig. 1), and the enzyme showed an absolute requirement for Mn^{2+} with an optimum concentration between 12.5 mM and 25 mM (Fig. 2). The divalent cations Mg^{2+}, Ca^{2+}, Ba^{2+} and Cd^{2+} at 12.5 mM could not substitute for Mn^{2+}. When compared with Mn^{2+}, only Cd^{2+} showed a small influence on the enzyme activity (10% of activity with Mn^{2+}). The snail CT Gal-T activity was independent of the concentration of Triton X-100, in the range 0 – 2.5% (data not shown), and no activation effect was found.

The results of substrate specificity studies using snail CT as enzyme source are shown in Fig. 3. Effective substrates were GalNAcβ1 - 4GlcNAcβ1 - 2Manα1 - O(CH$_2$)$_8$COOMe (V_{max} 140 nmol · h^{-1} · mg protein^{-1}; K_m 1.02 mM), GalNAcβ1-4GlcNAc (V_{max} 105 nmol · h^{-1} · mg protein^{-1}; K_m 0.99 mM), and GalNAcβ1-4GlcNAcβ1-OMe (V_{max} 108 nmol · h^{-1} · mg protein^{-1}; K_m 1.33 mM), all containing terminal non-reducing GalNAc β1-4-linked to GlcNAc (GalNAcβ1-4GlcNAc-R). Ineffective substrates were GlcNAc, GalNAcβ1-3Galα1-OMe, GalNAcα1-OC$_6$H$_5$, GalNAcα1-OSM, lactose and *N*-acetyllactosamine.

Product identification using CT galactosyltransferase

The effective substrates GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe and GalNAcβ1-4GlcNAcβ1-OMe were used in large-scale experiments, in order to identify the radioactive products formed.

(a) The oligosaccharide mixture obtained by incubation of GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe with UDP-[^{14}C]Gal and snail CT was separated by HPLC on RP-C$_{18}$ yielding two radioactive (Fig. 4, Ia and Ib) and two non-radioactive (Fig. 4, Ic and Id) fractions. The four fractions were analyzed by ^{1}H-NMR spectroscopy, and relevant ^{1}H-NMR data are summarized in Table 1.

The NMR spectrum (Fig. 5A) of the non-radioactive fraction Ic (Fig. 4) corresponds with that of the unmodified substrate GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe [6], characterized by the H-1 signals of Man (δ 4.854 ppm), GlcNAc (δ 4.561 ppm, virtual coupling), and GalNAc (δ 4.517 ppm), the NAc singlets of GlcNAc and GalNAc (δ 2.045 ppm and 2.068 ppm, respectively), and the spacer related COOMe (δ 3.688 ppm) and CH$_2$COO (δ 2.387 ppm) resonances.

Radioactive fraction Ib represents the galactosylated spacer-containing product. Its ^{1}H-NMR spectrum (Fig. 5B) shows in the anomeric region four signals at δ 4.853 ppm ($J_{1,2}$ = 0 Hz), δ 4.575 ppm ($J_{1,2}$ = 8.3 Hz), δ 4.560 ppm ($J_{1,2}$ = 7 Hz, virtual coupling) and δ 4.450 ppm ($J_{1,2}$ = 7.7 Hz). Furthermore, a typical broad doublet is observed outside the bulk resonance at the downfield position of δ 4.193 ppm. Compared to the NMR data of the substrate, the values at δ 4.853 ppm and δ 4.560 ppm can be attributed to Man H-1 and GlcNAc H-1, respectively. In a 2D HOHAHA experiment, it could be demonstrated that the H-1 signal at δ 4.575 ppm and the broad doublet at δ 4.193 ppm belong to the same monosaccharide residue. This set of structural-reporter groups is similar to that found earlier for the GalNAc residue as part of the hemocyanin structural element 3-OMe-Galβ1-3GalNAcβ1-4GlcNAcβ1-2ManαR (GalNAc H-1, δ 4.573 – 4.583 ppm; GalNAc H-4, δ 4.183 – 4.185 ppm) [4], showing that the incubation product contains a C-3-substituted GalNAc residue. Taking into account the remaining anomeric signal at δ 4.450 ppm, which has to originate from β-linked Gal, the structure of the radioactive incubation product is Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe. Comparison of the value of the Gal H-1 signal in Ib (δ 4.450 ppm) with the 3-OMe-Gal H-1 signal (δ 4.461 – 4.463 ppm) in the hemocyanin structural element shows a similar chemical shift effect of 3-*O*-methylation, as observed earlier going from β-Gal to 3-OMe-β-Gal ($\Delta\delta$ + 0.008) [4].

The ^{1}H-NMR spectrum of radioactive fraction Ia was interpreted on the basis of the NMR data of Ib and Ic. It contains a mixture of saponified Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe and GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe in the same ratio as that found for Ib and Ic (1:4). In a separate experiment, it could be demonstrated that the loss of the methyl ester group

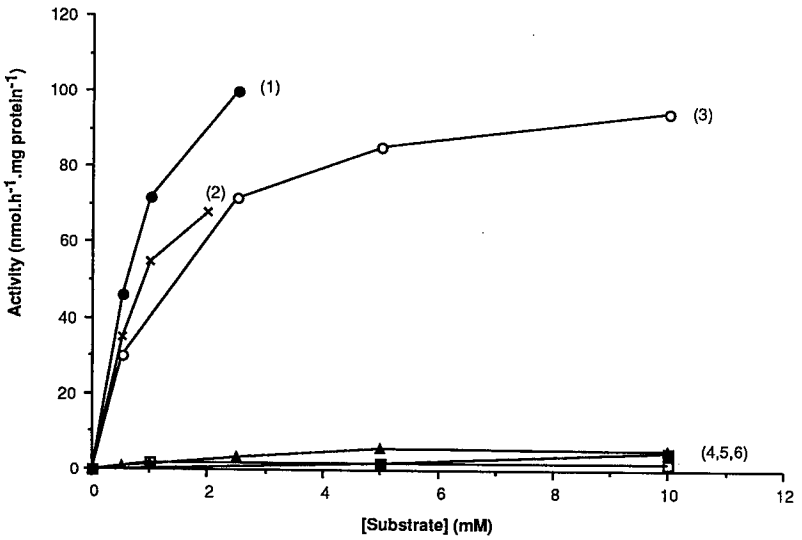

Fig. 3. *Substrate specificity of snail CT galactosyltransferase.* Incubations were carried out using the standard assay. (●) GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe (1); (x) GalNAcβ1-4GlcNAc (2); (○) GalNAcβ1-4GlcNAcβ1-OMe (3); (▲) GalNAcα1-OC_6H_5 (4); (■) GalNAcα1-OSM (5); (□) GalNAcβ1-3Galα1-OMe (6)

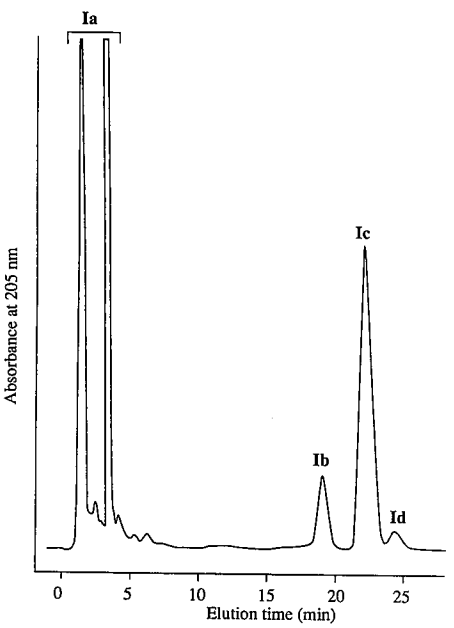

Fig. 4. *HPLC of the oligosaccharide mixture obtained by incubation of GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe with UDP-[^{14}C]Gal and snail CT galactosyltransferase on RP-C_{18}.* The column was eluted with water/acetonitrile (80:20, by vol.) at a flow rate of 1 ml/min. In fraction Ia, the intense peak with the smallest elution time represents the solvent peak

is time dependent (data not shown), and probably due to the presence of an esterase in the crude enzyme preparation. ^{1}H-NMR analysis of fraction Id shows a major and a minor component. The minor component is the substrate, and stems from overlap with fraction Ic. The NMR parameters of the major component indicate the spacer-containing oligosaccharide GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe, missing terminal GalNAc (Table 1). The joint values of the H-1 and NAc signals of GlcNAc fit those of terminal GlcNAc residues in antennary N-linked carbohydrate chains [9]. The origin of this compound is not clear, but can be due to an *N*-acetylgalactosaminidase activity in the crude enzyme preparation.

(b) The oligosaccharide mixture obtained by incubation of GalNAcβ1-4GlcNAcβ1-OMe with UDP-[^{14}C]Gal and snail CT was separated by HPLC on Lichrosorb-10-NH_2 yielding mainly two fractions, denoted IIa and IIb (Fig. 6). Both fractions were analyzed by ^{1}H-NMR spectroscopy and relevant ^{1}H-NMR data are included in Table 1. The NMR spectrum of the non-radioactive fraction IIa corresponds with that of the starting substrate GalNAcβ1-4GlcNAcβ1-OMe, characterized by the typical NMR parameters for GlcNAc (H-1, δ 4.438 ppm; NAc, δ 2.027 ppm; OMe, δ 3.498 ppm) and GalNAc (H-1, δ 4.517 ppm; H-4, δ 3.941 ppm; NAc, δ 2.070 ppm; Fig. 7A). In the NMR spectrum of radioactive fraction IIb (Fig. 7B), the same typical set of structural reporters for GalNAc (H-1, δ 4.577 ppm; H-4, δ 4.192 ppm; NAc, δ 2.055 ppm) and Gal (H-1, δ 4.450 ppm) is observed as in the spacer-containing incubation product, discussed above, indicating the galactosylated incubation product to be Galβ1-3GalNAcβ1-4GlcNAcβ1-OMe.

Substrate specificity in relation to enzyme source

In the previous sections, it has been demonstrated that the connective tissue of the snail *L. stagnalis* contains a specific UDP-Gal:GalNAcβ1-4GlcNAc-R β3Gal-T. As has been shown earlier, the ineffective compounds GalNAcα1-OC_6H_5 and GalNAcα1-OSM are effective substrates for UDP-Gal:GalNAcα1-R β3Gal-T from porcine submaxillary gland microsomes [5]. To establish the difference in substrate specificity between these β3Gal-T species, GalNAcβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_8$COOMe, GalNAcβ1-4GlcNAc, GalNAcβ1-3Galα1-OMe, GalNAcα1-OC_6H_5, and GalNAcα1-OSM were incubated with UDP-[^{14}C]Gal in the presence of porcine submaxillary-gland microsome preparation. As is evident from Fig. 8, this preparation shows the expected β3Gal-T activity towards GalNAcα1-OC_6H_5 (V_{max} 21 nmol · h^{-1} · mg $protein^{-1}$; K_m 1.31 mM) and GalNAcα1-

Table 1. *Relevant ^{1}H-NMR chemical shifts of structural-reporter groups of constituent monosaccharides for components Ia—Id of the incubation mixture GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe/UDP-[^{14}C]Gal/snail CT homogenate, and for components IIa and IIb of the incubation mixture GalNAcβ1-4GlcNAcβ1-OMe/UDP-[^{14}C]Gal/snail CT homogenate*
Chemical shifts are given downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to acetone, δ 2.225 ppm) in ^2H_2O at 27°C. n.d., not determined

Residue	Reporter group	Chemical shift in					
		Ia	Ib	Ic	Id	IIa	IIb
		ppm					
Man	H-1	4.853	4.853	4.854	4.857	–	–
	H-2	4.033	4.030	4.032	4.046	–	–
GlcNAc	H-1	4.561	4.560	4.561	4.556	4.438	4.439
	NAc	2.046	2.047	2.045	2.053	2.027	2.028
	OMe	–	–	–	–	3.498	3.499
GalNAc	H-1	4.519/4.578	4.575	4.517	–	4.517	4.577
	H-4	3.941/4.192	4.193	3.943	–	3.941	4.192
	NAc	2.068/2.053	2.053	2.068	–	2.070	2.055
Gal	H-1	4.449	4.450	–	–	–	4.450
	H-4	n.d.	3.910	–	–	–	3.910
Spacer	CH$_2$COO[a]	2.165	2.387	2.387	2.387	–	–
	COOMe	–	3.688	3.688	3.688	–	–

[a] Saponification of the methyl ester group in GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe also shows a shift from δ 2.387 ppm to 2.165 ppm

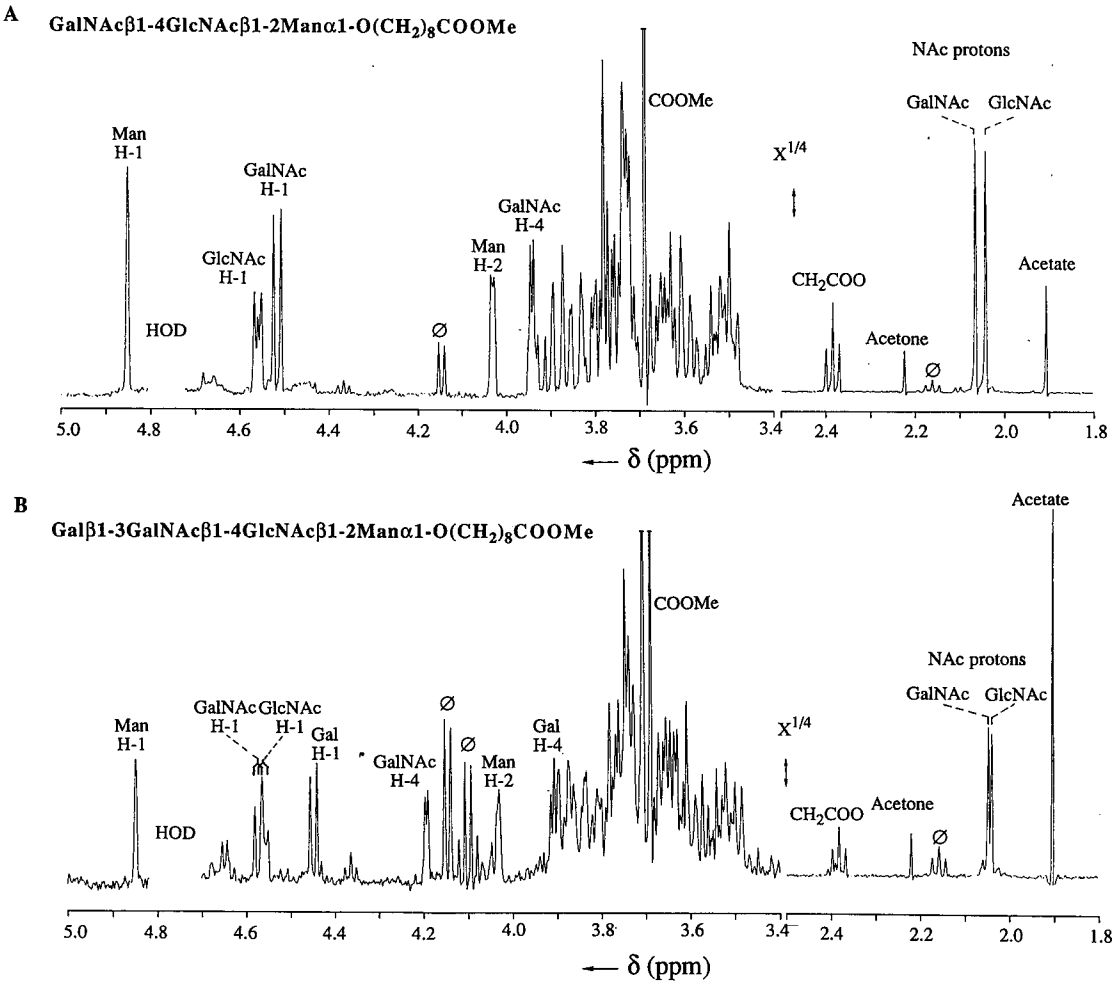

Fig. 5. *Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of (A) GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe and (B) Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe in ^2H_2O at 27°C.* The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum. Ø, non-carbohydrate HPLC contaminant

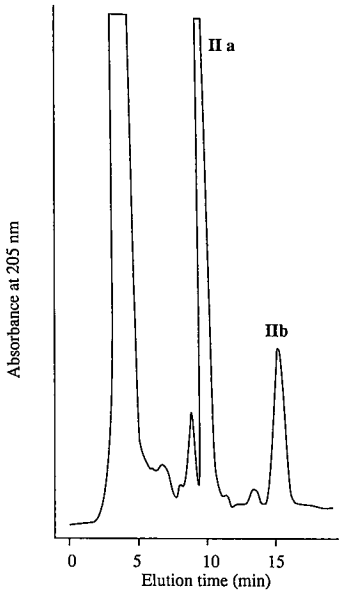

Fig. 6. *HPLC of the oligosaccharide mixture obtained by incubation of GalNAcβ1-4GlcNAcβ1-OMe with UDP-[^{14}C]Gal and snail CT galactosyltransferase on Lichrosorb-10-NH$_2$.* The column was eluted with water/acetonitrile (25:75, by vol.) at a flow rate of 1 ml/min

OSM (V_{max} 177 nmol · h^{-1} · mg protein^{-1}; K_m 3.12 mM) [5]. However, the other three oligosaccharides are ineffective substrates, indicating clearly the difference in substrate specificity between the two transferases.

A UDP-Gal:β-galactoside β3Gal-T activity with a high specificity for lactose and *N*-acetyllactosamine has been described in the albumen glands of *L. stagnalis* [11]. Lactose, *N*-acetyllactosamine, GalNAcβ1-4GlcNAcβ1-2Manα1-O(CH$_2$)$_8$COOMe, and GalNAcβ1-4GlcNAc were each incubated with UDP-[^{14}C]Gal in the presence of a crude albumen gland preparation. All four compounds were effective substrates (data not shown). In view of the ineffectiveness of lactose and *N*-acetyllactosamine as substrates using a CT preparation (see above), the albumen gland seems to contain two different β3Gal-T activities.

The substrate specificity of β4Gal-T from human milk towards Glc yielding lactose (in the presence of α-lactalbumin), and GlcNAc to form *N*-acetyllactosamine (in the absence of α-lactalbumin) has been well established [12]. As mentioned above, snail CT was ineffective in transferring Gal to GlcNAc. Incubation studies using GlcNAc or GalNAcβ1-4GlcNAcβ1-OMe with UDP-[^{14}C]Gal in the presence of β4Gal-T demonstrated no activity for the disaccharide, indicating that human milk β4Gal-T is different from snail CT β3Gal-T.

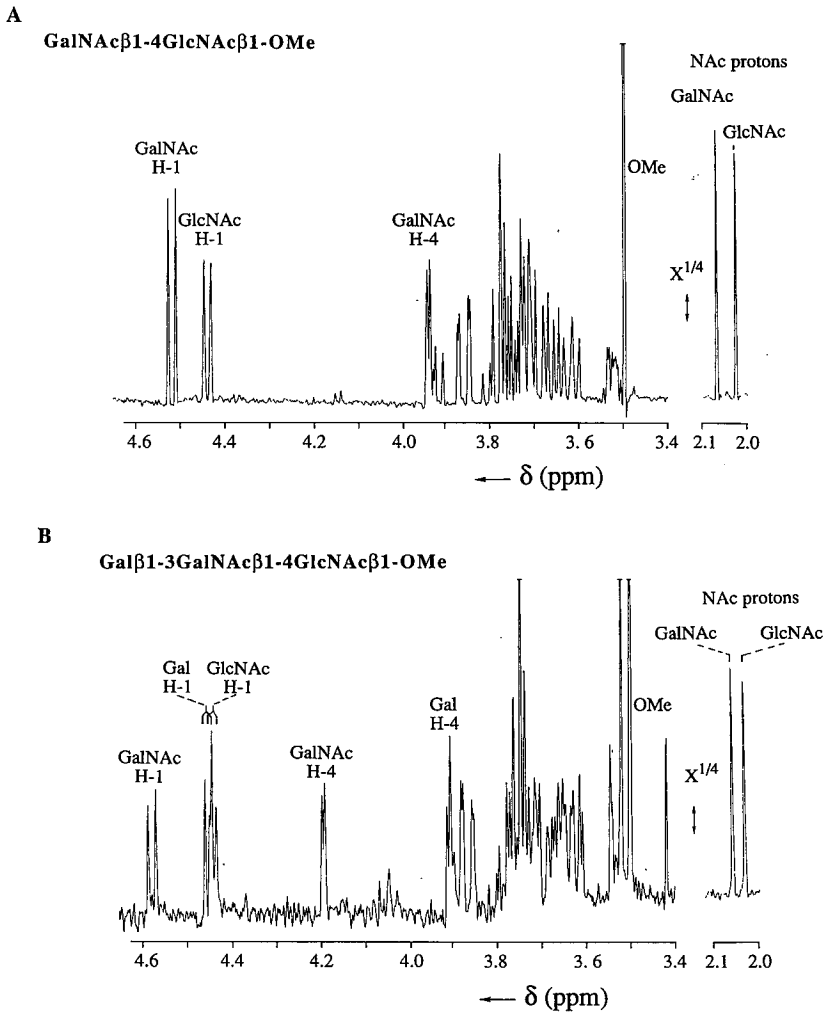

Fig. 7. *Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of (A) GalNAcβ1-4GlcNAcβ1-OMe and (B) Galβ1-3GalNAcβ1-4GlcNAcβ1-OMe in ^2H_2O at 27°C.* The relative scale of the *N*-acetyl region differs from that of other parts of the spectrum

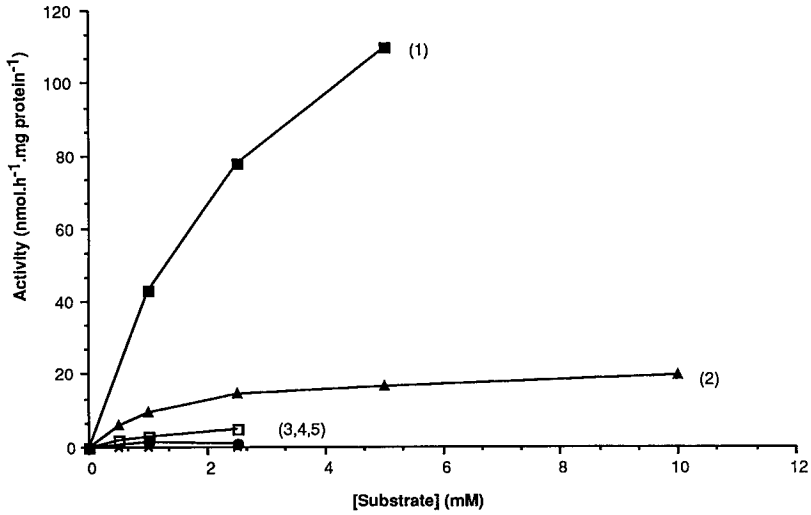

Fig. 8. *Substrate specificity of porcine submaxillary-gland microsome galactosyltransferase.* Incubations were carried out using the standard assay. (■) GalNAcα1-OSM (1); (▲) GalNAcα1-OC_6H_5 (2); (□) GalNAcβ1-3Galα1-OMe (3); (●) GalNAcβ1-4GlcNAcβ1-2Manα1-$O(CH_2)_8COOMe$ (4); (×) GalNAcβ1-4GlcNAc (5)

DISCUSSION

The CT of *L. stagnalis* contains a galactosyltransferase capable of transferring galactose from UDP-Gal to GalNAcβ1-4GlcNAc1-R. The isolated products formed by incubation of GalNAcβ1-4GlcNAcβ1-2Manα1-$O(CH_2)_8COOMe$ and GalNAcβ1-4GlcNAcβ1-OMe have been characterized by ^{1}H-NMR spectroscopy, and shown to be Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-$O(CH_2)_8COOMe$ and Galβ1-3GalNAcβ1-4GlcNAcβ1-OMe. The enzyme activity can therefore be identified as UDP-Gal:GalNAcβ1-4GlcNAc-R β1-3-galactosyltransferase. In view of the substrate specificity, we assume that this enzyme is involved in the biosynthesis of the (→Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1→)-containing carbohydrate chains of the snail hemocyanin. Concerning the specificity of the acceptor, it is not yet clear whether the β1-4 linkage between GalNAc and GlcNAc, or a penultimate GlcNAc residue, or both, are essential for enzyme activity. A series of incubation experiments with several substrates have shown that the novel β3Gal-T in snail CT differs clearly in substrate specificity from the UDP-Gal:GalNAcα-R β3Gal-T occurring in porcine submaxillary-gland microsomes, and from the UDP-Gal:Galβ-R β3Gal-T in the snail albumen glands. On the basis of data obtained from incubations in the presence of albumen gland, it appears that this gland contains both the UDP-Gal:GalNAcβ1-4GlcNAc-R and UDP-Gal:Galβ-R β3Gal-T activities. Finally, the CT Gal-T activity differs from the human milk UDP-Gal:GlcNAc-R β4Gal-T.

The α1-3-galactosyltransferase, involved in the biosynthesis of the blood group B determinant [13], has an absolute requirement for acceptors with a non-reducing terminal Fucα1-2Galβ-R element. The α1-3-galactosyltransferase from calf thymus [14], rabbit bone marrow [15], Ehrlich ascites tumor cells [16, 17] and rabbit stomach mucosa [18] has been shown to be active towards glycoproteins, glycolipids and oligosaccharides having a non-reducing terminal Galβ1-4GlcNAcβ unit. These substrate specificities show that the β3Gal-T described in this paper differs from the above α3Gal-T activities.

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