Glycoconjugate Journal (1996) **13:** 107–113

Identification of a GDP-Fuc:Galβ1-3GalNAc-R (Fuc to Gal) α1-2 fucosyltransferase and a GDP-Fuc:Galβ1-4GlcNAc (Fuc to GlcNAc) α1-3 fucosyltransferase in connective tissue of the snail *Lymnaea stagnalis*

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Received 28 June 1995, accepted 26 July 1995

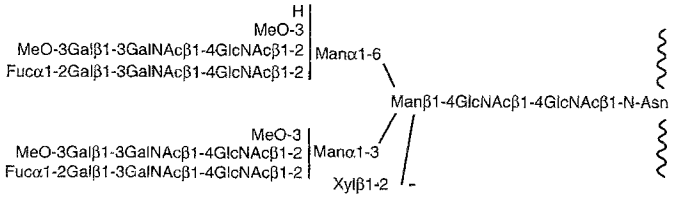
Connective tissue of the freshwater pulmonate *Lymnaea stagnalis* was shown to contain fucosyltransferase activity capable of transferring fucose from GDP-Fuc in α1-2 linkage to terminal Gal of type 3 (Galβ1-3GalNAc) acceptors, and in α1-3 linkage to GlcNAc of type 2 (Galβ1-4GlcNAc) acceptors. The α1-2 fucosyltransferase was active with Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ (K_m = 12 mM, V_{max} = 1.3 mU ml^{-1}) and Galβ1-3GalNAc (K_m = 20 mM, V_{max} = 2.1 mU ml^{-1}), whereas the α1-3 fucosyltransferase was active with Galβ1-4GlcNAc (K_m = 23 mM, V_{max} = 1.1 mU ml^{-1}). The products formed from Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ and Galβ1-4GlcNAc were purified by high performance liquid chromatography, and identified by 500 MHz ^{1}H-NMR spectroscopy and methylation analysis to be Fucα1-2Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ and Galβ1-4(Fucα1-3)GlcNAc, respectively. Competition experiments suggest that the two fucosyltransferase activities are due to two distinct enzymes.

Keywords: α1-2 fucosyltransferase, α1-3 fucosyltransferase, haemocyanin, *Lymnaea stagnalis*, glycoprotein

Abbreviations: α2Fuc-T, α1-2 fucosyltransferase; α3Fuc-T, α1-3 fucosyltransferase; MeO-3Man, 3-*O*-methyl-D-mannose; MeO-3Gal, 3-*O*-methyl-D-galactose.

Introduction

The N-linked oligosaccharides of haemocyanin from the snail *Lymnaea stagnalis* comprise an unusual series of xylose-containing structures [1, 2] which can be summarized as follows:



In our studies on the biosynthesis of the antennae of the N-glycans of this glycoprotein, we have previously described β1-3 galactosyltransferase [3], β1-4 *N*-acetylgalactosaminyltransferase [4], β1-2 *N*-acetylglucosaminyltransferases I and II [5], and β1-2 xylosyltransferase activities [5]. Since haemocyanin is biosynthesized in the pore cells of connective tissue, microsomal suspensions of this tissue have been used as a source for these enzyme activities.

An interesting feature of the glycosylation of haemocyanin is the occurrence of carbohydrate chains terminating in the type 3 blood group H determinant Fucα1-

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2Galβ1-3GalNAc. In humans the determinant Fucα1-2Gal-R is synthesized by two distinct α1-2 fucosyltransferases (α2Fuc-T), the blood group H-enzyme [6–11] and the secretory type enzyme (Se-enzyme) [12, 13]. However, detailed studies on lower animals have not yet been published. We now report the presence of α2Fuc-T activity in connective tissue of the snail *L. stagnalis*. Surprisingly, we also detected in connective tissue microsomes the presence of an α1-3 fucosyltransferase (α3Fuc-T) activity capable of forming the Lewis X determinant Galβ1-4(Fucα1-3)GlcNAc.

Materials and methods

MATERIALS

Connective tissue from mantle edges (a gift from Dr M. de Jong-Brink, Vrije Universiteit Amsterdam, The Netherlands) was dissected from laboratory bred, adult specimens of *Lymnaea stagnalis*, fed lettuce *ad libitum*, and kept at 20 °C under carefully controlled conditions of photoperiod (12 h light–12 h dark). GDP-[^{14}C(U)]-L-Fuc (specific activity 200 Ci mol^{-1}) was obtained from New England Nuclear and was diluted as needed with non-radioactive GDP-L-Fuc (Oxford Glycosystems). Bovine epididymis α-fucosidase was obtained from Oxford Glycosystems.

Galβ1-4GlcNAcβ1-2Manα1-O$(CH_2)_7CH_3$ was a gift from Dr J.A.L.M. van Dorst (Bijvoet Center, Department of Bio-Organic Chemistry). GalNAcβ1-4GlcNAcβ1-OMe was a gift of Dr J.G.M. van der Ven (Bijvoet Center, Department of Bio-Organic Chemistry). Galβ1-3GalNAc, Galβ1-OMe, and Galβ1-4GlcNAc were obtained from Sigma, and Galα1-OMe was from Koch Light. The purity of the acceptors was checked by 300 MHz ^{1}H-NMR spectroscopy. Column chromatography of synthetic intermediates of **3** and **7** (see below) was performed on Kieselgel 60 (Merck, <230 mesh) and fractions were monitored by TLC on Kieselgel 60 F_{254} (Merck). Detection was effected by charring with aqueous 50% sulfuric acid after examination under UV light. In the work-up procedures, washings were carried out three times with appropriate quantities of water or aqueous 5% sodium hydrogencarbonate, unless indicated otherwise. Evaporations were conducted under reduced pressure at 40 °C. All solvents were distilled from drying agents.

ALLYL 2-ACETAMIDO-2-DEOXY-3-*O*-β-D-GALACTOPYRANOSYL-β-D-GLUCOPYRANOSIDE(3)

A solution of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl trichloroacetimidate (405 mg, 0.82 mmol) and allyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (200 mg, 0.45 mmol) in 10 ml dichloromethane, containing powdered 4 Å molecular sieves (200 mg), was stirred for 1 h under argon. Trimethylsilyl triflate (4.1 μl, 23 μmol) was added, and the mixture was stirred for 30 min at room temperature, when TLC (hexane:ethylacetate, 6:4 v/v) showed the disappearance of the acceptor and the formation of a new product. Then, triethylamine was added until neutral pH, and the mixture was diluted with dichloromethane (50 ml), filtered through Celite, washed with water, dried ($MgSO_4$), and concentrated. Column chromatography (hexane:ethylacetate, 6:4 v/v) of the residue gave the disaccharide derivative **1** as a white foam (272 mg, 76%), R_F 0.36. A solution of **1** (116 mg, 0.15 mmol) in dichloromethane:water (9:1 v/v) (10 ml) was treated with trifluoroacetic acid (0.17 ml, 2.21 mmol) for 1 h, when TLC (dichloromethane:acetone, 3:1 v/v) showed a complete debenzylidenation. The mixture was diluted with dichloromethane (50 ml), and washed with aqueous 5% sodium hydrogencarbonate (3 × 20 ml) and water, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (dichloromethane:acetone, 3:1 v/v) to yield **2** as a glass (84 mg, 81%), R_F 0.63. Compound **2** (70 mg, 0.10 mmol), was dissolved in 33% methylamine in ethanol (10 ml), was stirred for 48 h at room temperature. After concentration of the solution, the residue was dissolved in methanol (5 ml), and acetic anhydride (0.2 ml) was added. The solution was stirred for 4 h at 0 °C and then concentrated. The residue was purified by Bio-Gel P-2 exclusion chromatography to yield, after lyophilization, **3** as a white solid (35 mg, 83%). ^{1}H-NMR data ($CDCl_3$): δ 2.022 (s, 3 H, NAc), 4.30–4.37 (m, 2 H, $OCH_2CH{=}CH_2$), 4.419 (d, 1 H, H-1′), 4.603 (d, 1 H, H-1), 5.23–5.34 (m, 2 H, $OCH_2CH{=}CH_2$), 5.84–5.97 (m, 1 H, $OCH_2CH{=}CH_2$), $J_{1',2'}$ 7.6, $J_{1,2}$ 8.0 Hz. FAB-MS data: *m/z* 446 $[M + Na]^+$; *m/z* 424 $[M + H]^+$; *m/z* 366 $[M + H - HOAll]^+$.

ALLYL 2-ACETAMIDO-2-DEOXY-3-O-β-D-GALACTOPYRANOSYL-β-D-GALACTOPYRANOSIDE (7)

Compound **2** (150 mg, 0.22 mmol) was dissolved in pyridine (10 ml), and *tert*-butyldimethylsilyl chloride (98 mg, 0.65 mmol) was added. The reaction was stirred for 6 h at room temperature, when TLC (dichloromethane:acetone, 9:1 v/v) showed the disappearance of **2** and the formation of a new substance. The solution was concentrated, and co-concentrated with toluene (3 × 20 ml), ethanol (3 × 20 ml), and dichloromethane (3 × 20 ml). The residue was purified by column chromatography (dichloromethane:acetone, 9:1 v/v) to yield **4**, isolated as a glass (175 mg, 85%), R_F 0.58. A solution of **4** (140 mg, 0.16 mmol) in dichloromethane (7 ml) was treated with pyridine (123 μl, 1.55 mmol) and trifluoromethanesulfonic anhydride (104 μl, 0.62 mmol) at 0 °C for 2.5 h, when TLC (dichloromethane:acetone, 97:3 v/v) showed the formation of a higher moving compound. The mixture was diluted with ethylacetate (150 ml), washed with aqueous 5% sodium hydrogencarbonate and water, dried ($MgSO_4$), and concentrated.

The residue was taken up in *N,N*-dimethylformamide (4 ml), and tetrabutylammonium acetate (230 mg, 0.78 mmol) was added. The brown solution was stirred for 30 min, when TLC (dichloromethane:ethylacetate, 9:1 v/v) showed the formation of a new compound. The mixture was diluted with ethylacetate (100 ml) and washed with aqueous 5% sodium chloride. The aqueous phase was extracted with ethylacetate (3 × 20 ml), and the combined organic layers were dried ($MgSO_4$), and concentrated. Column chromatography (dichloromethane:acetone, 97:3 v/v) of the residue gave **5**, isolated as a white foam (102 mg, 75%), R_F 0.24. A solution of **5** (100 mg, 0.12 mmol) in acetonitrile:water (9:1 v/v) (10 ml), was treated with *p*-toluenesulfonic acid (114 mg, 0.60 mmol) for 45 min, when TLC (dichloromethane:acetone, 9:1 v/v) analysis showed the formation of one lower moving spot. The mixture was diluted with dichloromethane (100 ml), and washed with aqueous 5% sodium hydrogencarbonate (3 × 25 ml) and water, dried ($MgSO_4$), and concentrated. Column chromatography (dichloromethane:acetone, 9:1 v/v) of the residue gave **6**, isolated as a glass (82 mg, 91%), R_F 0.33. Compound **6** (70 mg, 0.10 mmol), dissolved in 33% methylamine in ethanol (10 ml) was stirred for 56 h at room temperature. Then the solution was concentrated, and the residue was dissolved in methanol (5 ml), subsequently acetic anhydride (0.2 ml) was added. The solution was stirred for 4 h at 0 °C and then concentrated. The residue was purified by Bio-Gel P-2 exclusion chromatography to yield, after lyophilization, **7** as a white solid (28 mg, 67%). ^{1}H-NMR data ($CDCl_3$): δ 2.021 (s, 3 H, NAc), 4.32–4.38 (m, 2 H, $OCH_2CH{=}CH_2$), 4.437 (d, 1 H, H-1′), 4.559 (d, 1 H, H-1), 5.25–5.35 (m, 2 H, $OCH_2CH{=}CH_2$), 5.85–5.98 (m, 1 H, $OCH_2CH{=}CH_2$), $J_{1',2'}$ 7.6, $J_{1,2}$ 8.5 Hz. FAB-MS data: *m/z* 446 $[M + Na]^+$; *m/z* 424 $[M + H]^+$; *m/z* 366 $[M + H - HOAll]^+$.

PREPARATION OF CONNECTIVE TISSUE MICROSOMAL SUSPENSION

All procedures were carried out at 4 °C. Dissected connective tissue (5 mg) was homogenized in 10 ml 10 mM sodium cacodylate buffer (pH 7.0) using a Polytron apparatus and the homogenate was centrifuged for 60 min at 100 000 × **g**. The pellet was resuspended in 5 ml 0.25 M sucrose with a Potter-Elvehjem homogenizer and re-centrifuged. The resulting pellet was resuspended in 2.5 ml 0.25 M sucrose and stored at 4 °C until use.

FUCOSYLTRANSFERASE ASSAY

The standard incubation mixture contained in a total volume of 20 μl: 10 mM Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$, 0.25 mM GDP-[^{14}C]Fuc (5000–10 000 dpm nmol^{-1}), 0.125 M sodium cacodylate buffer (pH 7.0), 0.5% (by vol) Triton X-100, 40 mM $MnCl_2$, 5 mM ATP, and connective tissue microsomal suspension (2.5 μU, 10 μg protein; one unit of enzyme activity is defined as the amount of enzyme catalysing the transfer of 1 μmol Fuc per min using the standard incubation system and Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ as an acceptor at a concentration of 10 mM). Incubations were performed 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 mesh; Bio-Rad), and neutral carbohydrates (radioactive Fuc, radioactive product, and substrate) were eluted with 0.5 ml water. After mixing with 4 ml Emulsifier Safe (Packard 6013389) the solutions were scintillation counted 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.

DETERMINATION OF KINETIC PARAMETERS

Kinetic values were determined for type 3 (Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ and Galβ1-3GalNAc) and type 2 (Galβ1-4GlcNAc) acceptors assuming that they served as acceptors only for α2Fuc-T and α3Fuc-T, respectively. The K_m values were evaluated from the initial rate data using Lineweaver-Burk plots. Intercepts were calculated by the least squares method.

LARGE SCALE INCUBATIONS

(i) Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ as substrate

The incubation mixture contained in a total volume of 400 μl: 40 mM $MnCl_2$, 0.5% (by vol) Triton X-100, 125 mM sodium cacodylate buffer (pH 7.0), 5 mM ATP, 1 mM GDP-[^{14}C]Fuc (500 dpm nmol^{-1}), snail connective tissue microsomal suspension (0.05 mU, 0.2 mg protein), and 5 mM Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$. The mixture was kept for 24 h at 37 °C, and after freezing and thawing was diluted with water (0.5 ml) and passed over a 10 ml column of AG 1-X8 (acetate form, 100–200 mesh). The resin was washed with 20 ml water, and the total eluate was lyophilized. The residue was taken up in water, and the solution was desalted on a column (50 × 1 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad) using water as eluent. After lyophilization, interfering Triton X-100 was removed by passing the pooled radioactive fractions over a freshly conditioned Sep-Pak C_{18} column (Waters Associates), and after washing with 5 ml water, the total eluate was lyophilized. The residue was taken up in water and fractionated by HPLC on a 10 μm Lichrosorb-NH_2 column (25 cm × 4.6 mm, Chrompack) using water:acetonitrile (15:85 v/v) as eluent at a flow rate of 1 ml min^{-1}. The elution pattern was monitored at 205 nm and aliquots

of the 1 ml fractions were analysed by scintillation counting.

(ii) Galβ1-4GlcNAc as substrate

Incubation with Galβ1-4GlcNAc (10 mM) was carried out and processed as described for Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$. After rinsing over Sep-Pak C_{18}, a radioactive 5 ml water fraction was isolated, concentrated, and fractionated by HPLC, using water:acetonitrile (15:85 v/v) as eluent. The flow rate was 1 ml min^{-1}.

DIGESTION WITH α-FUCOSIDASE

Oligosaccharides were incubated for 24 h at 37 °C with bovine epididymis α-fucosidase, according to the manufacturer's instructions. After stopping the incubation by boiling for 1 min, the mixture was fractionated on a column (50 × 1 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad) using water as eluent. Fractions of 1 ml were analysed for radioactivity by scintillation counting.

PROTEIN DETERMINATION

Protein concentration was determined according to [14] using bovine serum albumin as a standard.

METHYLATION ANALYSIS

Oligosaccharides were permethylated [15], and then converted into a mixture of partially methylated alditol acetates by hydrolysis with 2 M trifluoroacetic acid (1 h, 120 °C), reduction with NaB^2H_4 and acetylation with acetic anhydride (3 h, 120 °C) [16]. Partially methylated alditol acetates were analysed by GLC-MS [17] using a Fisons MD800 mass spectrometer (electron energy, 70 eV) coupled with a Fisons GC8060 gas chromatograph that was equipped with a DB-1 capillary column (30 m × 0.32 mm, J&W Scientific).

FAST ATOM BOMBARDMENT MASS SPECTROMETRY

FAB mass spectrometry was performed on a JEOL JMS-SX/SX 102A tandem mass spectrometer, operated at 10 kV accelerating voltage, equipped with a JEOL MS-FAB 10 D FAB gun operated at 10 mA emission current, producing a beam of 6 keV xenon atoms and using glycerol as the matrix. Data acquisition and processing was accomplished using JEOL Complement software.

^{1}H-NMR SPECTROSCOPY

Prior to ^{1}H-NMR analysis, carbohydrates were exchanged twice in ^2H_2O (99.9 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 MHz with a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University) or at 500 MHz with a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University), operating at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm by reference to acetone (δ 2.225) [18].

Results

PROPERTIES OF CONNECTIVE TISSUE FUCOSYLTRANSFERASE ACTIVITY WITH Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ AS ACCEPTOR

Fucosyltransferase activity was studied using GDP-[^{14}C]Fuc and Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ as substrates and connective tissue microsomal suspension as enzyme source. Radioactive product formation was proportional to time of incubation over a period of 2 h, and to an enzyme concentration up to at least 5 mg connective tissue protein per ml (data not shown). The pH optimum for the Fuc-T activity was found to be about 7.5 (Fig. 1A), and the enzyme was stimulated by Mn^{2+} with an optimum concentration at about 20 mM (Fig. 1B). The Fuc-T

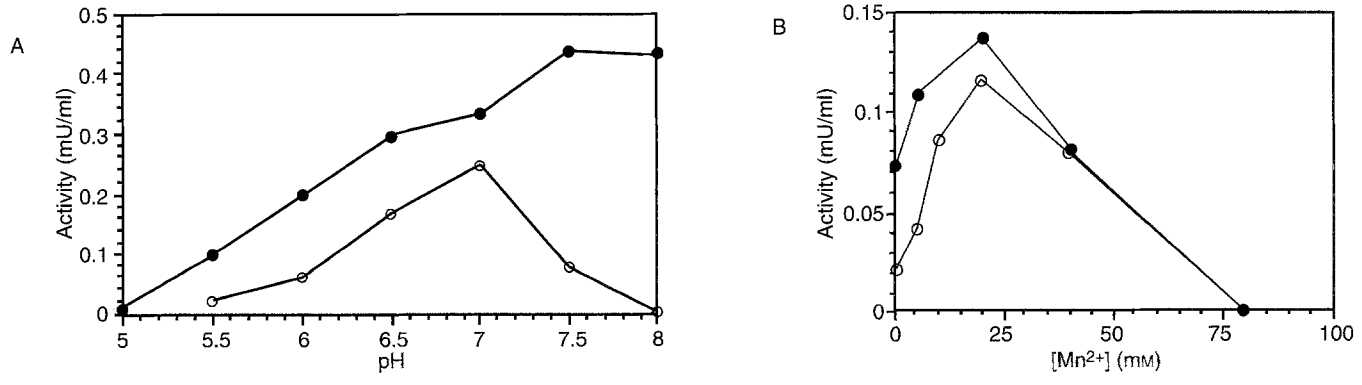

Figure 1. Optimization of snail connective tissue fucosyltransferase activity using Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ (●) or Galβ1-4GlcNAc (○) as acceptor and GDP-[^{14}C]Fuc as donor with respect to (A) pH and (B) $MnCl_2$ concentration. In the pH optimization assay 0.5 M sodium cacodylate buffer was added depending on the pH tested.

activity was independent of the Triton X-100 concentration over the range of 0.25–1% and no activation effect was found (data not shown).

SUBSTRATE SPECIFICITY OF CONNECTIVE TISSUE FUCOSYLTRANSFERASE ACTIVITIES

The ability of connective tissue extracts to transfer Fuc to various acceptors is shown in Table 1. Changing the penultimate monosaccharide residue from GalNAc into GlcNAc, e.g. from Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ to Galβ1-3GlcNAcβ1-$OCH_2CH{=}CH_2$, results in a 94% reduction in activity. However, enzyme activity is increased when the β1-3-linkage between Gal and GlcNAc is replaced by a β1-4-linkage (substrate Galβ1-4GlcNAc). To determine whether these two activities are due to a single enzyme or to two distinct fucosyltransferases, a competition experiment was carried out using Galβ1-3GalNAc and Galβ1-4GlcNAc as acceptors (Table 2). The close agreement between the experimentally measured activities and the calculated values for two distinct enzymes clearly demonstrates that the fucosylation of these two acceptors is catalysed by two different enzymes identified as α2Fuc-T and α3Fuc-T (see product identification, below). Optimization of the α3Fuc-T was carried out using GDP-[^{14}C]Fuc and Galβ1-4GlcNAc as substrates and connective tissue microsomal suspension as enzyme source (Fig. 1).

Table 1. Acceptor specificity of *Lymnaea stagnalis* connective tissue Fuc-T preparation with various acceptors at concentrations of 10 mM.

Acceptors	*Fuc-T activity (%)*
Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$	100[a]
Galβ1-3GalNAc	80
Galβ1-4GlcNAc	44
Galβ1-4GlcNAcβ1-2Manα1-$O(CH_2)_7CH_3$	27
GalNAcβ1-4GlcNAcβ1-OMe	13
Galβ1-3GlcNAcβ1-$OCH_2CH{=}CH_2$	6
Galβ1-OMe	0
Galα1-OMe	0

[a]100% activity for Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ corresponds to 0.5 mU ml^{-1}.

Table 2. Competition experiments. Enzyme rates were measured as described for the fucosyltransferase assay. Calculated values are obtained for two enzymes (2E) as the sum of the activities for separate incubations with only a single substrate; for one enzyme (1E) theoretical values were calculated according to the following equation [19]: $v = [V_1(S_1/K_1) + V_2(S_2/K_2)]/[1 + S_1/K_1 + S_2/K_2]$, where S_1 and S_2 are the concentrations of the two substrates, V_1 and V_2 are the V_{max} values and K_1 and K_2 are the K_m values. The kinetic values used for the two substrates in the calculations are: Galβ1-3GalNAc (K_m = 20 mM, V_{max} = 2.1 mU ml^{-1}) and Galβ1-4GlcNAc (K_m = 23 mM, V_{max} = 1.1 mU ml^{-1}).

Substrate concentrations (mM)		*Fucosyltransferase activity (mU ml^{-1})*		
Galβ1-3GalNAc	*Galβ1-4GlcNAc*	*Experimental*	*Calculated*	
			2E	*1E*
25	–	1.19		
50	–	1.94		
–	25	0.58		
–	50	1.14		
25	25	1.79	1.77	1.14
50	50	2.98	3.08	1.35

PRODUCT IDENTIFICATION

(i) Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ as substrate

Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ was incubated with GDP-[^{14}C]Fuc and connective tissue extracts for 24 h at 37 °C. After processing of the incubation mixture, the product was fractionated by HPLC on Lichrosorb-NH_2 yielding a non-radioactive fraction **Ia**, coeluting with Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$, and a radioactive fraction **Ib** (Fig. 2A). Methylation analysis of fraction **Ia** showed the presence of terminal Gal*p* (galactopyranoside) and 3-substituted Hex*p*NAc, indicating that fraction **Ia** is the substrate Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$. Methylation analysis of fraction **Ib** demonstrated the occurrence of terminal Fuc*p*, 2-substituted Gal*p*, and 3-substituted Hex*p*NAc, showing that this fraction contained the product Fucl-2Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$. Gel filtration on Bio-Gel P-2 of radioactive compound **Ib** before and after treatment with bovine epididymis α-fucosidase showed that the enzyme releases Fuc in accordance with an α-configuration for the Fuc residue. ^{1}H-NMR analysis demonstrated that the structural-reporter groups of **Ib** (Fuc H1, δ 5.236; H5, δ 4.235; CH_3, δ 1.212; Gal H1, δ 4.602; GalNAc H1, δ 4.374; H4, δ 4.104) are in the same range as those reported for the antennary elements Fucα1-2Galβ1-3GalNAcβ1-R in N-linked chains of haemocyanin from *L. stagnalis* (Fuc H1, δ 5.230; H5, δ 4.208–4.216; CH_3, δ 1.205–1.210; Gal H1, δ 4.611–4.616; GalNAc H1, δ 4.425–4.436; H4, δ 4.103–4.105) [2]. In conclusion, **Ib** corresponds to Fucα1-2Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$. It should be noted that the organic synthesis of the Fucα1-2Galβ1-3GalNAc sequence (as propyl β-glycoside) has also been reported recently [20].

(ii) Galβ1-4GlcNAc as substrate

Incubation of Galβ1-4GlcNAc with GDP-[^{14}C]Fuc and connective tissue extracts was carried out for 24 h at 37 °C. After processing of the incubation mixture, the product was fractionated by HPLC on Lichrosorb-NH_2

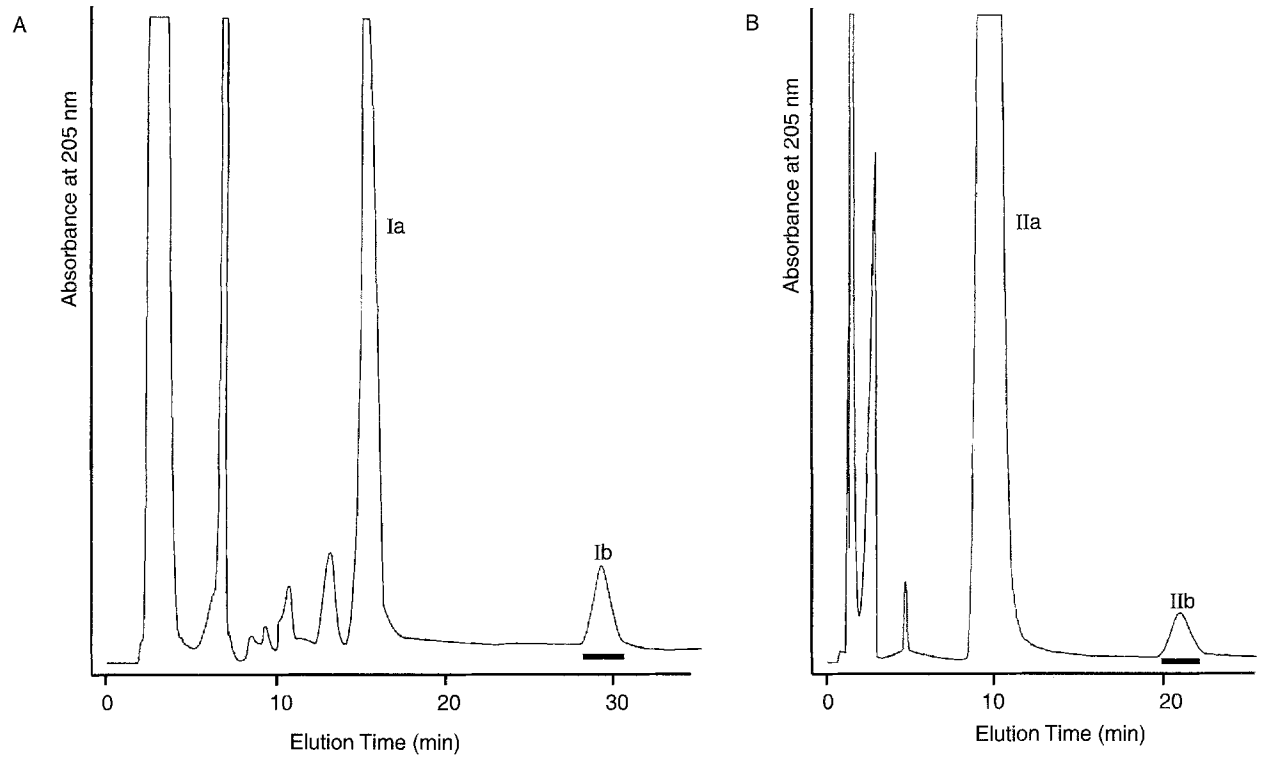

Figure 2. HPLC of the mixtures of oligosaccharides obtained by incubation of different acceptors with GDP-[^{14}C]Fuc and connective tissue microsomes on Lichrosorb-NH_2. (A) 24 h incubation of Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$; (B) 24 h incubation of Galβ1-4GlcNAc. The column was eluted with water:acetonitrile (15:85 v/v) at a flow rate of 1 ml min^{-1}. The bar indicates radioactive fractions.

yielding a non-radioactive fraction **IIa**, coeluting with Galβ1-4GlcNAc, and a radioactive fraction **IIb** (Fig. 2B). ^{1}H-NMR spectroscopy (Gal H1, δ 4.478 (α)/4.474 (β); GlcNAc H1, δ 5.207 (α)/4.725 (β); NAc, δ 2.044) and methylation analysis (terminal Gal*p* and 4-substituted Hex*p*NAc) of fraction **IIa** confirmed its identity as the substrate Galβ1-4GlcNAc. Methylation analysis of fraction **IIb** showed the occurrence of terminal Fuc*p*, terminal Gal*p*, and 3,4-disubstituted Hex*p*NAc residues. Furthermore, the ^{1}H-NMR data of **IIb** match those of reference Galβ1-4[Fucα1-3]GlcNAc [21] (Fuc H1, δ 5.108 (α)/5.100 (β); H5, δ 4.85; CH_3, δ 1.179 (α)/1.174 (β); Gal H1, δ 4.467 (α)/4.456 (β); GlcNAc H1, δ 5.100 (α); NAc, δ 2.033). Thus, connective tissue contains an α1-3 fucosyltransferase capable of forming the Lewis X determinant Galβ1-4[Fucα1-3]GlcNAc.

KINETIC PARAMETERS FOR CONNECTIVE TISSUE α2Fuc-T AND α3Fuc-T

TheF kinetic parameters were as follows: α2Fuc-T using Galβ1-3GalNAcβ1-$OCH_2CH{=}CH_2$ as substrate, K_m = 12 mM, V_{max} = 1.3 mU ml^{-1}, and with Galβ1-3GalNAc, K_m = 20 mM, V_{max} = 2.1 mU ml^{-1}; α3Fuc-T using Galβ1-4GlcNAc as substrate, K_m = 23 mM, V_{max} = 1.1 mU ml^{-1}.

Discussion

Connective tissue of the snail *L. stagnalis* contains two fucosyltransferases, one capable of transferring fucose from GDP-Fuc to Galβ1-3GalNAc (type 3 determinant) and one capable of transferring fucose from GDP-Fuc to Galβ1-4GlcNAc (type 2 determinant). Based on the products formed, these transferases can be defined as GDP-Fuc:Galβ1-3GalNAc (Fuc to Gal) α1-2-fucosyltransferase and GDP-Fuc:Galβ1-4GlcNAc (Fuc to GlcNAc) α1-3 fucosyltransferase. Similar α2Fuc-T and α3-Fuc-T activities have been found by us in the albumen gland of the snail (data not shown).

With respect to α2Fuc-T, the α2Fuc-T from pea epicotyl membranes is specific for xyloglucans and can therefore be distinguished from the snail connective tissue enzymes [22, 23]. The α2Fuc-T from porcine submaxillary glands shows no absolute specificity for a particular penultimate residue nor for the linkage between galactose and the penultimate residue but it is more active towards type 1 (Galβ1-3GlcNAc) and type 3 (Galβ1-3GalNAc) oligosaccharide acceptors than towards type 2 (Galβ1-4GlcNAc) acceptors [6]. The secretory (Se) type α2Fuc-T, like the porcine submaxillary gland enzyme, is active towards type 1 and type 3 acceptors [12]. The blood group H α2Fuc-T transfers fucose

equally well to type 1 as to type 2 acceptors, with type 3 acceptors being less efficient substrates [9, 11]. The substrate specificity of the *L. stagnalis* connective tissue α2Fuc-T is different from the above α2Fuc-Ts in that it prefers type 3 acceptors over type 1 and type 2 acceptors suggesting it is a novel enzyme and is probably involved in the biosynthesis of N-linked oligosaccharide chains on glycoproteins in the snail.

With respect to α3Fuc-T, in humans at least five different α3Fuc-Ts have been described (α3Fuc-T III, IV, V, VI and VII [24–27]). Although they differ in their substrate specificities (reviewed in [28]), they all transfer Fuc with high efficiency from GDP-Fuc to type 2 acceptors (Galβ1-4GlcNAc), forming the Lewis X determinant. Likewise, the *L. stagnalis* α3Fuc-T is able to form the Lewis X determinant. However, to date, neither β1-4 galactosyltransferase activity [3] nor α1-3 fucosylated structures have been identified in *L. stagnalis* connective tissue. The biological role of the snail α3Fuc-T remains to be established. Its existence in the snail connective tissue may suggest that it is responsible for the formation of a not yet found GalNAcβ1-4[Fucα1-3]GlcNAc-R determinant. It has previously been shown that human milk α3/4Fuc-T can convert GalNAcβ1-4GlcNAcβ1-OMe into GalNAcβ1-4[Fucα1-3]GlcNAcβ1-OMe [29]. GalNAcβ1-4GlcNAcβ1-OMe was found to be an acceptor of fucose using snail connective tissue extracts as enzyme source (Table 1) suggesting that a similar α3Fuc-T activity may be present in the snail.

Acknowledgements

This work has been supported by the Netherlands Foundation for Chemical Research (SON/NWO). We thank Drs M. de Jong-Brink, J.A.L.M. van Dorst, J.G.M. van der Ven for their generous gifts of (bio)chemical materials, and Dr B.A. Spronk for recording ^{1}H-NMR spectra. We thank Ms A. van der Kerk-van Hoof for carrying out FAB-MS analyses.

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