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Identification of a UDP-Gal:β-galactoside β1→3-galactosyltransferase in the albumen gland of the snail *Lymnaea stagnalis*

David H. Joziasse, Hanny C.M. Damen, Marijke de Jong-Brink*, Hommo T. Edzes+ and Dirk H. Van den Eijnden

*Departments of Medical Chemistry, *Biology and Physical and Theoretical Chemistry, Vrije Universiteit, PO Box 7161, NL-1007 MC Amsterdam, The Netherlands*

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Detergent extracts of the albumen gland of the snail *Lymnaea stagnalis* contain an enzyme activity that transfers Gal from UDP-Gal to acceptor substrates with terminal non-reducing β-galactose residues. The products formed with lactose (Galβ1→4Glc) as the acceptor were characterized by HPLC, and subjected to 400-MHz ^{1}H-NMR and methylation analysis. The main product was shown to have the structure Galβ1→3Galβ1→4Glc. Therefore, the galactosyltransferase can be identified as a UDP-Gal:β-galactoside β1→3-galactosyltransferase. In view of its linkage and acceptor specificity, the enzyme may be essential to the biosynthesis of galactogen, the main polysaccharide produced by albumen glands of *L. stagnalis*.

Galactogen; β1→3-Galactosyltransferase; *(Lymnaea stagnalis)*

1. INTRODUCTION

The albumen gland of the pulmonate gastropod *Lymnaea stagnalis* is one of the female accessory sex glands. The gland secretes perivitelline fluid around fertilized eggs. In addition to various proteins, this fluid contains galactogen (galactan), a polymer of β1→3- and β1→6-linked D-galactose residues [1]. The latter compound constitutes the main source of energy for developing embryos as well as for newly hatched snails [2]. Egg production, and also galactogen synthesis, are strongly dependent on a number of external factors such as temperature, photoperiod, and the availability of food [3–5]. The galactogen content of the albumen gland may vary greatly under the influence of these stimuli and during the egg-laying process [6]. Galactogen synthesis has been shown to be stimulated by the gonadotropic hormones DBH (dorsal body hormone) and CDCH (caudo-dorsal cell hormone) [3]. The albumen glands, therefore, constitute a promising model system for study of the regulation of glycoconjugate biosynthesis. Furthermore, this system may yield additional information on the relation between synthesis and secretion of glycoconjugates.

It is not known whether one of the steps in galactogen biosynthesis is under direct control of (one of) the hormones mentioned. A suitable candidate for regulation is the elongation of nascent galactogen chains with galactose. This elongation oc-

Correspondence address: D.H. Joziasse, Dept of Medical Chemistry, Vrije Universiteit, PO Box 7161, NL-1007 MC Amsterdam, The Netherlands

Abbreviations: OSM, ovine submaxillary mucin; PSM, porcine submaxillary mucin; HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectrometry; cer, ceramide; *p*Nph, *p*-nitrophenol; GalNAc, *N*-acetylgalactosamine; Fuc, L-fucose (all sugars are of the D configuration unless noted otherwise)

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curs in the Golgi apparatus, presumably through the action of $\beta 1 \rightarrow 3$- and $\beta 1 \rightarrow 6$-galactosyltransferases. Alternatively, only one β-galactosyltransferase, together with a transglycosylase ('branching enzyme'), is sufficient for galactogen biosynthesis.

Here we report on an albumen gland galactosyltransferase that is presumably involved in the synthesis of galactogen. Extracts of albumen glands were examined for enzymes catalyzing the transfer of D-galactose from UDP-Gal to various acceptors of known structure. The structure of the product obtained with lactose as acceptor was analyzed in detail.

2. EXPERIMENTAL

2.1. *Materials*

Albumen glands were dissected from laboratory-bred, adult specimens of *L. stagnalis*, kept at 20°C under carefully controlled conditions of photoperiod (12 h light–12 h dark). The snails were fed lettuce ad libitum. Dissected glands were stored at −20°C until processing.

The oligosaccharides Gal$\beta 1 \rightarrow$3Gal$\beta 1 \rightarrow$4Glc and Gal$\beta 1 \rightarrow$3GlcNAc$\beta 1 \rightarrow$3Gal$\beta 1 \rightarrow$4Glc were kindly donated by Dr M. Messer (University of Sidney, Australia) and Dr V. Ginsburg (NIH, Bethesda, MD), respectively. Fuc$\alpha 1 \rightarrow$2Gal$\beta 1 \rightarrow$3GlcNAc $\beta 1 \rightarrow$3Gal$\beta 1 \rightarrow$4Glc was isolated from human milk according to [7]. Porcine submaxillary asialo/afuco-mucin, ovine submaxillary asialo-mucin and asialo-α_1-acid glycoprotein were prepared as in [8]. Galactogen, prepared from *L. stagnalis* albumen glands, was a kind gift from Mr R. van Elk (Department of Biology, Vrije Universiteit, Amsterdam). Ganglioside G_{M2} was isolated from the brain of a Tay-Sachs patient [9]; lactosylceramide was obtained from Sigma. UDP-[^{3}H]Gal (15.6 Ci/mmol) was purchased from Amersham International (Amersham, England), and diluted with unlabeled UDP-Gal to the desired specific activity. All other chemicals were of the highest grade available from commercial sources.

2.2. *Preparation of enzyme*

The following procedures were carried out at 0–4°C. Albumen glands were homogenized in 10 mM sodium cacodylate buffer at pH 6.5 using a Potter-Elvehjem homogenizer, which yielded a 20% (w/v) homogenate. After addition of Triton X-100 and KCl to concentrations of 0.5% (w/v) and 0.2 M, respectively, the homogenate was stirred for 16 h and then centrifuged at 100 000 × *g* for 90 min. The supernatant, containing the extracted galactosyltransferase(s), was stored at −20°C until use. Porcine submaxillary gland microsomes, as a source of another galactosyltransferase, were prepared as in [10], except that the microsomal pellet was resuspended in 10 mM sodium cacodylate buffer, pH 6.5.

2.3. *Galactosyltransferase assays*

The standard incubation mixture contained 8 μmol sodium cacodylate, 1.6 μmol $MnCl_2$, 0.32 μmol ATP, 0.16 μl Triton X-100, 70 nmol UDP-[^{3}H]Gal (1.41 Ci/mol), 2.4 μmol lactose and enzyme preparation (containing 0.3–0.5 mg protein and 2.0 μmol KCl) in a volume of 80 μl. In acceptor specificity studies the same mixture was used. However, lactose was replaced by the other acceptors studied (table 1) at a concentration of 2 mM (in terms of terminal Gal or GalNAc residues). When glycolipids were tested as acceptors, 0.4 μl Triton X-100 was added to the reaction mixture. Incubations were conducted for 1 h at 37°C. The incorporation of [^{3}H]Gal into oligosaccharides, glycoproteins or glycolipids was determined by previously described procedures [8,11,12].

2.4. *Product identification*

The standard incubation mixture was scaled up 25-fold and incubated for 17 h at 37°C. The products were separated from UDP-[^{3}H]Gal by ion exchange on a column of Dowex 1-X8 (Cl^- form), and fractionated on a calibrated column of Bio-Gel P-4 as described in the legend to fig.1. The major product was characterized by HPLC [16], and identified by 400-MHz ^{1}H-NMR and methylation analysis as in [13].

2.5. *Analytical methods*

Protein was assayed according to Peterson [14]. Hexoses were quantified using the phenol-sulfuric acid assay [15].

3. RESULTS

Using lactose as an acceptor we were able to

detect galactosyltransferase activity in albumen gland homogenates. The enzyme activity was optimal in cacodylate buffer at pH 6.5, had an absolute requirement for Mn^{2+} and was slightly stimulated by Triton X-100 at concentrations up to 0.2% (w/v). Enzyme activity could be solubilized by treatment of albumen gland homogenate with 0.5% Triton X-100 in the presence of 0.2 M KCl.

3.1. *Substrate specificity studies*

Detergent extracts prepared from *L. stagnalis* albumen glands were incubated, in the presence of UDP-[^{3}H]Gal, with various oligosaccharides, asialo-glycoproteins and glycolipids as potential acceptor substrates for galactosyltransferase (table 1). With oligosaccharides it appeared that the presence of a terminal β-Gal residue was a minimal requirement for activity. Very little, if any, transfer of galactose was observed with α-galactosides. Compounds with β-Gal in a 1→4 linkage were better substrates than those having β-Gal in a 1→6 or 1→3 linkage. Lactose was found to be the best ac-

Table 1

Acceptor specificity of *Lymnea stagnalis* albumen gland galactosyltransferase

Acceptor	Relative rate of galactosylation (%)
Oligosaccharides	
1. Galβ1→4Glc (lactose)	100
2. Galβ1→4GlcNAc (*N*-acetyllactosamine)	48
3. Galβ1→6GlcNAc	4
4. Galβ1→3Galβ1→4Glc	33
5. Galβ1→3GlcNAcβ1→3Galβ1→4Glc (lacto-*N*-tetraose)	19
6. Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc	9
7. Galβ1→*O*-Me	21
8. Galβ1→*O*-*p*Nph	9
9. Hydrolytic fragments of galactogen[a]	3–5
10. Gal	3
11. 3-*O*-Me-Gal	2
12. Galα1→*O*-Me	2
13. Galα1→6Glc (melibiose)	<1
14. Galα1→6Glcα1→2βFru (raffinose)	<1
15. GlcNAc	2
16. GalNAc	2
17. GalNAcα1→*O*-*p*Nph	26
Glycolipids	
18. Galβ1→4Glc1→cer (lactosylceramide)	14
19. GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glc→cer (ganglioside G_{M2})	<1
Glycoproteins	
20. GalNAcα1→protein (asialo-OSM)	2
21. GalNAcα1→protein and Galβ1→3GalNAcα→protein (asialo/afuco-PSM)	<1
22. Galβ1→4GlcNAcβ1→R-protein (asialo-α_1-acid glycoprotein)	<1

[a] Fragments prepared by partial hydrolysis of galactogen in trifluoroacetic acid, tested at a concentration of 5 mM galactose residues. The latter are not necessarily in a terminal position

Substrates were incubated together with UDP-[^{3}H]Gal and Triton X-100 extracts of the albumen gland at final concentrations of terminal Gal or GalNAc residues of 2 mM, as described in section 2. Galactosylation rates are expressed as the percentage of that obtained with lactose, which amounted to 0.43 nmol · mg^{-1} protein · h^{-1}

ceptor. Lactosylceramide also showed acceptor properties. Asialo-α_1-acid glycoprotein, however, was a very poor acceptor.

Interestingly, GalNAcα1→*O*-*p*Nph could be galactosylated, although the galactosyltransferase preparation was virtually inactive with the asialomucins. By contrast, porcine submaxillary gland microsomes, which are known to contain an active UDP-Gal:GalNAcα1→R β1→3-galactosyltransferase [10], showed a high activity with both GalNAcα1→*O*-*p*Nph and asialo-OSM. The ratio of activities observed with lactose, asialo-OSM and GalNAcα1→*O*-*p*Nph was 1:15:6 for the porcine enzyme vs 1:0.01:0.3 for the enzyme from *L. stagnalis*.

3.2. *Product identification*

The major product obtained with lactose as the acceptor was analyzed in detail. This product

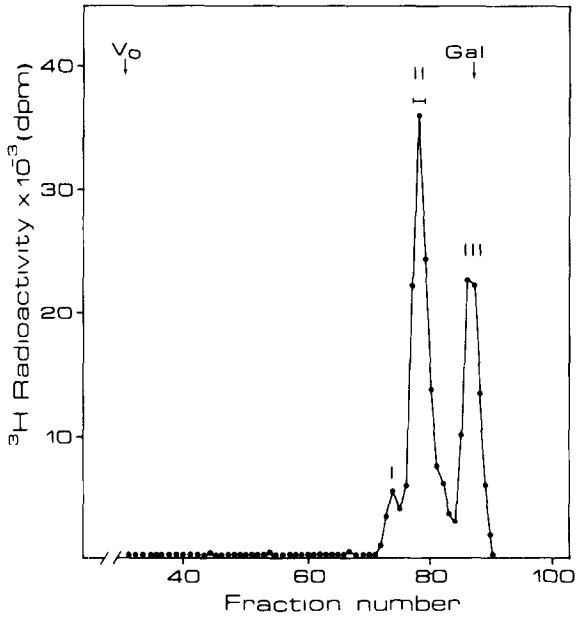

Fig.1. Gel filtration of the products obtained by enzymatic galactosylation of lactose. Lactose was incubated with UDP-[^{3}H]Gal and detergent extract of *L. stagnalis* albumen glands. Products were isolated as described in section 2, and fractionated by gel filtration on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37°C at a flow rate of 15 ml/h. Fractions of 4 ml were collected and assayed for ^{3}H radioactivity (●). The bar indicates the fractions that were pooled.

(peak II in fig.1) migrated like a hexotriose on a calibrated Bio-Gel P-4 column. The fractions containing this product were pooled as indicated by the bar and lyophilized. HPLC showed that this material appeared to consist of greater than 85% of one radioactive compound. It was eluted in the position of a hexotriose without a 1→6 linkage [16]. This material was then investigated by 400-MHz ^{1}H-NMR and methylation analysis. The relevant parts of the ^{1}H-NMR spectrum of the product hexotriose are shown in fig.2. In table 2 the chemical shift values and coupling constants of the various structural-reporter groups are compared for lactose, the labeled trisaccharide product, and authentic Galβ1→3Galβ1→4Glc. Compared to lactose, an additional H-1 signal is observed at δ 4.616, and an H-4 signal at δ 3.924. These signals are due to the addition of a galactose residue, and indicate a β-configuration for this sugar. Furthermore, the H-1 and H-2 signals of glucose do not change, but in contrast, H-1 and H-4 signals of the Galβ1→4 are shifted downfield. This indicates that the additional galactose became linked to the Gal, and not the Glc residue of lactose. Moreover, the fact that the H-4 signal of Galβ1→4 is affected is

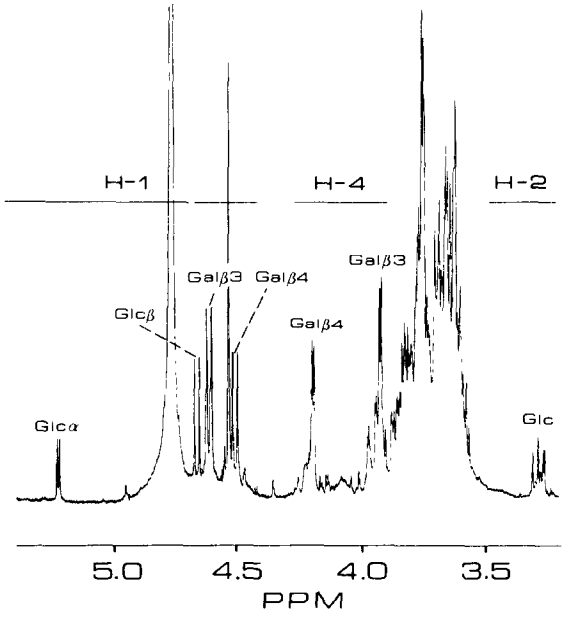

Fig.2. 400-MHz ^{1}H-NMR spectrum of the trisaccharide product formed in an incubation of lactose and UDP-[^{3}H]Gal with albumen gland galactosyltransferase.

Table 2

400-MHz ^{1}H-NMR parameters of lactose (Galβ1→4Glc), the galactosyltransferase product, and Galβ1→3Galβ1→4Glc

Residue	Proton	Chemical shift (coupling constant)		
		Galβ1→4Glc	Product formed with lactose	Galβ1→3Galβ1→4Glc
Glc	αH-1	5.223 (3.7)	5.224 (3.8)	5.225 (3.8)
	βH-1	4.664 (8.0)	4.666 (7.9)	4.666 (8.0)
	H-2	3.286 (8.6)[a]	3.286 (8.6)[a]	3.287 (8.5)[a]
Galβ1→4	H-1	4.451 (7.9)	4.511 (7.8)	4.511 (7.8)
	H-4	3.926 (3.3)[b]	4.196 (3.4)[b]	4.195 (3.2)[b]
Galβ1→3	H-1	-	4.616 (7.5)	4.615 (7.6)
	H-4	-	3.924 (3.3)[b]	3.922 (3.4)[b]

[a] Coupling constant represents $J_{2,3}$ value
[b] Coupling constant represents $J_{3,4}$ value

Chemical shifts: in ppm; coupling constants: Hz

indicative of a substitution at C-3 [17]. Also, the unusual downfield position of the H-1 signal of the added Gal is typical of the presence of a Galβ1→3Gal sequence [17]. The NMR spectrum of the product is largely identical to that of authentic Galβ1→3Galβ1→4Glc [13].

Substitution of the β1→4-linked Gal residue at the 3-position was confirmed by methylation analysis. After reduction of the product, methylation, methanolysis, and acetylation, the partially methylated sugar derivatives were subjected to GC/MS. Selected ion monitoring helped in identifying sugar derivatives. We detected 1,2,3,5,6-penta-*O*-methyl-4-*O*-acetylglucitol (due to a reducing 4-substituted Glc), both anomers of 2,4,6-tri-*O*-methyl-3-*O*-acetyl-1-*O*-methylgalactoside (due to 3-substituted Gal), and those of 2,3,4,6-tetra-*O*-methyl-1-*O*-methylgalactoside (derived from Gal at the non-reducing terminus), in the proportion of 0.21 : 1.0 : 0.68.

4. DISCUSSION

Triton X-100 extracts of *L. stagnalis* albumen glands contain a galactosyltransferase capable of transferring Gal from UDP-Gal to lactose (Galβ1→4Glc). The product that was formed migrated on HPLC as well as on gel filtration columns as a hexotriose. Its ^{1}H-NMR spectrum was virtually identical to that of authentic Galβ1→3Galβ1→4Glc, and the transfer of Gal in a 1→3 linkage to Galβ1→4 was confirmed by methylation analysis. The enzyme activity involved can, therefore, be identified as a UDP-Gal:β-galactoside β1→3-galactosyltransferase. Acceptor specificity studies indicated that the enzyme was most active with lactose, lactosylceramide, and oligosaccharides containing a non-reducing terminal β-linked Gal residue. The fact that almost no galactose was transferred to GlcNAc, Galβ1→4GlcNAcβ1→R-protein, or ganglioside G_{M2} distinguishes the albumen gland enzyme from the galactosyltransferases involved in the synthesis of Galβ1→4GlcNAc [11], Galα1→3Galβ1→4GlcNAcβ1→R-protein [11], and ganglioside G_{M1} [18], respectively. Interestingly, both the snail enzyme and the β1→3-galactosyltransferase from porcine submaxillary gland act on lactose and GalNAcα1→*O*-*p*Nph. The preference of the snail enzyme for lactose over GalNAcα1→*O*-*p*Nph, in addition to its very low activity with asialo-OSM, however, clearly distinguishes it from the mammalian enzyme involved in mucin biosynthesis [19].

In the urine of patients with early myoclonic epileptic encephalopathy three oligosaccharides containing Galβ1→3Gal linkages have been detected [20]. A β1→3-galactosyltransferase that might be involved in the biosynthesis of such compounds has been detected recently in human kidney [21]. The kidney enzyme transferred Gal in a β1→3 linkage to the Gal residue in Galβ1→4Glc,

as well as to that in Galβ1→4Glc-ceramide. Since, in this respect, the acceptor specificity of the enzyme is similar to that of the snail galactosyltransferase, it will be of interest to compare the properties of both enzymes in more detail.

Galβ1→3Galβ1→4Glc, the product formed from lactose by the snail galactosyltransferase, has been described as being the major oligosaccharide in the milk of marsupials [22]. So far this structure has not been found in the body fluids of other mammals. It is possible that in mammary glands of marsupials a galactosyltransferase occurs with a specificity very similar to that of the snail enzyme.

Studies are in progress to identify the role of the *L. stagnalis* β1→3-galactosyltransferase in galactogen biosynthesis. Our enzyme preparation showed only relatively low rates of galactose transfer to galactogen preparations purified from albumen glands. This may be due to the fact that the albumen glands, used here as the enzyme source, had not been selected for being active in galactogen synthesis at the time of their dissection. Alternatively, galactogen biosynthesis may require an assay system in which cells are not disrupted. Purification of the β1→3-galactosyltransferase, and subsequent preparation of antibodies against the protein, may contribute to a better understanding of the function of this enzyme in galactogen synthesis, and of the possible hormonal regulation of its activity.

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