Structural analysis of the oligosaccharide-alditols released by reductive β -elimination from the jelly coat of *Rana utricularia* eggs

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The O-linked oligosaccharides of the jelly coat surrounding the eggs of *Rana utricularia* were analysed by ^1H -NMR spectroscopy. Comparison of their structures with those characterized from seven other amphibians confirms that the carbohydrate chains of

the jelly coat mucins are markers of the species. The new sequence $\text{GlcNAc}(\beta 1-3)\text{GlcNAc}(\beta 1-6)[\text{Gal}(\beta 1-3)]\text{GalNAc-ol}$ is characteristic of *Rana utricularia*. The presence of blood group A determinants constitutes the main feature of this mucin.

INTRODUCTION

The jelly coats surrounding amphibian eggs play a key role in the process of fertilization by facilitating adherence of the spermatozoa to the egg surface and by acting to block polyspermy [1–5]. Previous studies have shown that such material contains easily accessible sources of carbohydrate [6,7]. Moreover, the abundance of the material and the similarities of their carbohydrate chains with those of some rare human carbohydrate determinants (e.g. Le^x in *Pleurodeles waltl* mucin [6,7]) prompted us to investigate new amphibian species. These first analyses, which were performed on urodeles and anuran species, led to the observation of a species-specific structural diversity which should be used as a basis for taxonomy [8–16]. The present paper describes the O-linked carbohydrate chains of the mucin collected from *Rana utricularia* egg jelly coats.

MATERIALS AND METHODS

Isolation of oligosaccharide-alditols

O-linked oligosaccharides were released from the crude material by alkaline borohydride treatment in 1 M NaBH_4 and 0.1 M NaOH at 37 °C for 48 h. The reaction was stopped by the addition of Dowex 50x8 (25–50 μm mesh; H^+ form) at 4 °C. The solution was filtered, adjusted to pH 6.5 and then concentrated in a rotatory evaporator at 25 °C. Borate salts were removed by repeated evaporation with methanol. The resulting sample was applied to a Bio-Gel P-2 column (1 cm $\times$ 100 cm) and eluted with water at a flow rate of 20 ml/h. Peptide material was removed on Dowex 50x2 (mesh 200–400; H^+ form). Neutral alditols were separated from acidic alditols by fractionation on a column (37 cm $\times$ 1 cm) of Dowex 1x2 (acetate form). Neutral oligosaccharide-alditols were isolated by HPLC on a primary amine-bonded silica column (Supelcosyl LC-NH₂, 4.6 mm $\times$ 250 mm; Supelco Inc., Bellefonte, CA, U.S.A.) using acetonitrile/water at a flow rate of 1 ml/min at room temperature. Oligosaccharide peaks were detected by absorbance at 206 nm. Acetonitrile was evaporated from the collected fractions under a stream of nitrogen prior to lyophilization. The fractions were desalted on a Sephadex G-10 column (45 cm $\times$ 0.5 cm;

Pharmacia) and lyophilized. Four fractions were re-purified by C18 reverse-phase chromatography on a 5 μm Zorbax column (4.6 mm $\times$ 250 mm) run isocratically with water as eluent at a flow rate of 0.5 ml/min at room temperature.

Analytical procedures

Methylation analysis was achieved as described by Ciucanu and Kerek [17].

^1H -NMR experiments were performed on a Bruker ASX 400 WB spectrometer. Chemical shifts are expressed in p.p.m. downfield from internal sodium 4,4'-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ p.p.m. in $^2\text{H}_2\text{O}$ at 25 °C). Two-dimensional homonuclear COSY with simple and double-relay transfer was performed using Bruker standard pulse sequences.

The molecular mass of the oligosaccharides was measured by matrix-assisted laser desorption/ionization (MALDI) on a Vision 2000 time-of-flight (TOF) instrument (Finnigan Mat) equipped with a 337 nm UV laser. The mass spectra were acquired in reflectron mode under a 6 kV accelerating voltage and positive or negative detection.

The sample was dissolved in water at a concentration of 100 pmol/ μl . A 2 μl sample of the analyte solution was mixed with an equal volume of the matrix solution on to the target and then allowed to crystallize at room temperature. We used 2,5-dihydroxybenzoic acid (10 mg/ml; dissolved in water/ethanol, 4:1, v/v) for the neutral or monoacidic fractions. External calibration was performed using an angiotensin I standard purchased from Sigma (mass 1296.7 Da). Between 10 and 15 shots were accumulated for each spectrum.

RESULTS

HPLC separation of oligosaccharide-alditols

The HPLC elution profile of neutral oligosaccharide-alditols on Supelcosyl LC-NH₂ is shown in Figure 1. Fractions 4, 7 and 8 were further subfractionated by reverse-phase HPLC on Zorbax ODC C18 (Figure 2). The acidic fraction eluted from Dowex 1x2

Abbreviations used: Kdn, 2-oxo-3-deoxy-D-glycero-D-galactonolonic acid; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight.

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Figure 1 Separation by HPLC of neutral oligosaccharide-alditols released from the egg jelly coat of *Rana utricularia*

Samples were chromatographed on a column (4.6 mm $\times$ 250 mm) of primary amine-bonded silica (Supelcosyl LC-NH₂; Supelco Inc.) using gradient elution with acetonitrile/water. The flow rate was 1 ml/min. Absorbance was measured at 206 nm.

Figure 2 Repurification of fractions 4 (A), 7 (B) and 8 (C)

A C18 reverse-phase 5 μ m Zorbax column (4.6 mm $\times$ 250 mm) was used. Elution was performed isocratically with water.

was analysed on Supelcosyl LC-NH₂, using acetonitrile/30 mM KH₂PO₄. A major peak was obtained (fraction 9), while minor components remain to be further fractionated. The oligosaccharides were described in such a way as to assign the NMR spectra according to their increasing complexity.

Structural determination of oligosaccharide-alditols

Compounds **2a**, **2b**, **3** and **6**

The chemical shifts of the structural group of these compounds (see Figure 7) are identical with those observed for oligosaccharide-alditols described previously [18,19].

Compound **5**

Compound **5** (Table 1) is composed of GalNAc-ol, Gal, Fuc and GalNAc in the molar proportions 1:1:1:1. The chemical shifts of the anomeric protons of Gal, Fuc and GalNAc exactly match those of the carbohydrate units present in the tetra-oligosaccharide-alditol Fuc(α 1-2)[GalNAc(α 1-3)]Gal(β 1-3)GalNAc-ol described by Dua et al. [20].

Compound **4b**

This compound (Table 1) is a trisaccharide-alditol composed of Gal-ol, Fuc and GalNAc in the molar proportions 1:1:1. Methylation analysis (Table 2) indicated the Gal-ol to be O-2-

Table 1 ¹H-NMR chemical shifts of the oligosaccharide-alditols **4b**, **5**, **7b** and **9**

The monosaccharides are represented by the symbolic notation: ◇-ol, GalNAc-ol; ■, β-Gal; ●, β-GlcNAc; □, α-Fuc; ◇, α-GalNAc; n.d., not determined. The linkage position is specified by the direction of the connecting bars as follows:

		Chemical shifts (p.p.m.) in:			
Residue	Reporter	5	9	4b	7b
GalNAc-ol	H-1	3.810	3.790	—	—
	H-1 < >	3.780	3.790	—	—
	H-2	4.298	4.236	—	—
	H-3	4.095	4.072	—	—
	H-4	3.600	3.611	—	—
	H-5	4.118	4.183	—	—
	H-6	3.676	3.925	—	—
	H-6 < >	3.650	3.674	—	—
	NAc	2.045	2.045	—	—
Gal-ol	H-1	—	—	3.971	4.009
	H-2	—	—	4.146	4.168
	H-3	—	—	4.018	4.178
	H-4	—	—	3.769	3.945
	H-5	—	—	3.768	4.207
	H-6	—	—	3.659	3.693
Gal β-3	H-1	4.710	4.713	—	4.732
	H-2	3.890	3.910	—	3.969
	H-3	4.017	4.016	—	4.014
	H-4	4.224	4.209	—	4.204
	H-5	n.d.	n.d.	—	n.d.
GalNAc α1-3	H-1	5.190	5.182	5.115	5.162
	H-2	4.246	4.254	4.218	4.248
	H-3	3.927	3.925	3.925	3.857
	H-4	4.020	4.020	4.025	4.012
	H-5	4.155	4.168	4.141	4.163
	H-6	3.78	3.768	3.880	3.772
	H-6 < >	n.d.	n.d.	3.865	n.d.
	NAc	2.049	2.050	2.064	2.041
GlcNAc-6-S	H-1	—	4.590	—	—
	H-2	—	3.737	—	—
	H-3	—	n.d.	—	—
	H-4	—	n.d.	—	—
	H-5	—	n.d.	—	—
	H-6	—	4.369	—	—
	H-6 < >	—	4.235	—	—
	NAc	—	2.054	—	—
Fuc α1-2 F, FI	H-1	5.389	5.384	5.106	5.035
	H-2	3.800	3.797	3.802	3.824
	H-3	n.d.	n.d.	3.887	3.750
	H-4	n.d.	n.d.	n.d.	3.836
	H-5	4.339	n.d.	4.210	4.038
	CH ₃	1.235	1.233	1.201	1.218
Fuc α1-2 F, FII	H-1	—	—	—	5.435
	H-2	—	—	—	3.806
	H-3	—	—	—	n.d.
	H-4	—	—	—	3.740
	H-5	—	—	—	4.452
	CH ₁	—	—	—	1.235

Table 2 Molar proportions of monosaccharide methyl ethers present in the methanolysates of the permethylated oligosaccharide-alditols

A plus sign (+) indicates that the compound was present but was not quantified.

Methyl ether	Amount in oligosaccharide-alditol (mol)			
	4a	4b	7b	8b
1,4,5,6-Me ₄ -GalN(Me)Ac-ol	+	—	—	—
2,3,4,6-Me ₄ -Gal	1.0	—	—	1.0
2,4,6-Me ₃ -Gal	0.9	—	—	—
1,4,5,6-Me ₄ -Gal-ol	—	1.0	1.0	—
4,6-Me ₂ -Gal	—	—	0.7	—
3,4, 6-Me ₃ -GalNMeAc	—	0.8	0.9	—
2,3,4-Me ₃ -Fuc	—	0.7	1.7	—
1,4,5-Me ₃ -GalN(Me)Ac-ol	—	—	—	+
4,6-Me ₂ -GlcNMeAc	—	—	—	0.9
3,4,6-Me ₃ -GlcNMeAc	—	—	—	0.8

Figure 3 COSY spectrum of compound 7b

and O-3-substituted. Therefore compound 4b has the following structure (Scheme 1):

Scheme 1 **Figure 4** COSY spectrum of compound 9

This compound must be considered to be the result of a peeling reaction of compound 5.

Compound 7b

This compound, which contains GalNAc, Gal, Fuc and Gal-ol in the molar proportions 1:1:2:1, also results from the peeling reaction of an oligosaccharide that has not been isolated during the fractionation. The ¹H-NMR spectrum (Figure 3 and Table 1) indicates the presence of the trisaccharidic sequence Fuc(α1-2)[GalNAc(α1-3)]Gal, previously described for compound 5, as indicated by the relevant H-1 resonances of α-Gal, α-GalNAc and α-Fuc (Table 1). According to the results of the methylation analysis (Table 2), the Gal-ol unit is O-2- and O-3-substituted. The original, non-degraded, oligosaccharide can be considered as an extension of compound 4a (see below), with two Fuc and one GalNAc units, which provides the following sequence (Scheme 2):

Scheme 2

Table 3 ^1H -NMR chemical shifts of the oligosaccharide-alditols **4a**, **8a** and **8b**

The monosaccharides are represented by the symbolic notation: $\diamond$ -ol, GalNAc-ol; $\blacksquare$, β -Gal, $\bullet$, β -GlcNAc. n.d., not determined. The linkage position is specified by the direction of the connecting bars as follows:

		Chemical shifts (p.p.m.) in:		
Residue	Reporter	4a	8a	8b
GalNAc-ol I	H-1	3.811	3.799	3.784
	H-1 < >	3.749	3.775	3.728
	H-2	4.394	4.391	4.388
	H-3	4.089	4.070	4.058
	H-4	3.519	3.453	3.467
	H-5	4.206	4.274	4.265
	H-6	3.682	3.922	3.917
	H-6 < >	3.633	3.683	3.659
	NAc	2.048	2.068	2.064
Gal β 1-3 II	H-1	4.539	4.519	4.464
	H-2	3.733	3.718	3.555
	H-3	3.831	3.814	3.674
	H-4	4.186	4.174	3.900
	H-5	3.734	3.725	n.d.
Gal β 1-3 III	H-1	4.687	4.673	—
	H-2	3.432	3.419	—
	H-3	3.533	3.525	—
	H-4	3.752	3.75	—
	H-5	3.427	3.425	—
GlcNAc β 1-6 II < >	H-1	—	4.539	4.490
	H-2	—	3.701	3.787
	H-3	—	3.464	3.731
	H-4	—	n.d.	3.516
	H-5	—	n.d.	3.469
	H-6	—	3.929	3.920
	H-6 < >	—	3.743	3.740
GlcNAc β 1-3 III < >	NAc	—	2.068	2.084
	H-1	—	—	4.582
	H-2	—	—	3.669
	H-3	—	—	3.583
	H-4	—	—	3.473
	H-5	—	—	3.469
	H-6	—	—	3.920
	H-6 < >	—	—	3.740
	NAc	—	—	2.016

Compound 9

Compound **9** consists of GlcNAc, GalNAc, Fuc, Gal and GalNAc-ol in the molar proportions 1:1:1:1:1, and also contains a sulphate or phosphate group, as indicated by the pseudo-molecular ion ($M - \text{H} + \text{Na}$) $^+$ at m/z 1039 in a MALDI-TOF analysis of the native oligosaccharide-alditol. The H-6 and H-6' atom resonances of GlcNAc are deshielded at 4.369 and 4.235 p.p.m. respectively, and the absence of an additional coupling with ^{31}P confirms the presence of a sulphate group attached at O-6 of this monosaccharide unit (Figure 4 and Table 1). The core type 2 {namely GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc-ol} is defined by the H-6 and H-6' signals of GalNAc-ol resonating at 3.925 and 3.674 p.p.m. respectively. The chemical shifts of the

anomeric protons of Fuc, GalNAc and Gal point to the occurrence of a blood group A determinant [20]. Compound **9** is therefore (Scheme 3):

Scheme 3

Figure 5 COSY spectrum of compound 8a

Compound 4a

This consists of GalNAc-ol and Gal in the molar proportions 1:2. The core type 1 is defined by the H-2 and H-5 signals of GalNAc-ol resonating at 4.394 and 4.206 p.p.m. respectively (Table 3). The Gal III unit is terminal, as indicated by its H-2, H-3 and H-4 signals at 3.432, 3.533 and 3.752 p.p.m. respectively. Methylation analysis (Table 2) indicated an O-3-substituted Gal II unit. These results provide the structure Gal(β1-3)Gal(β1-3)GalNAc-ol.

Compound 8a

Compound 8a consists of Gal, GlcNAc and GalNAc-ol in the molar proportions 2:1:1. The H-2 and H-5 signals of GalNAc-ol, observed at 4.391 and 4.274 p.p.m. respectively, indicate the presence of the core type 2 (Figure 5 and Table 3). By comparison with the spectrum of compound 9, the sequence Gal(β1-3)Gal(β1-3) appears to be present. Therefore the structure of compound 8a has been established as (Scheme 4):

Scheme 4

Figure 6 COSY spectrum of compound 8b

Compound 8b

This compound contains Gal, GlcNAc and GalNAc-ol in the molar proportions 1:2:1. The core type 2 is defined by the chemical shifts of the H-2 and H-5 signals of GalNAc-ol (Figure 6 and Table 3). The Gal unit occurs in a terminal position, as deduced from its H-2, H-3 and H-4 atom resonances, and from the methylation analysis (Table 2). The set of H-2, H-3 and H-4 signals of GlcNAc II' and III' are indicative of the O-3-substituted and terminal units respectively. The 1,3 linkage was confirmed by the results of the methylation analysis (Table 2). Structure 8b is therefore (Scheme 5):

Scheme 5

Chemical analysis of compound 1 (Figure 7) indicated the presence of GlcNAc, without any evidence of other carbohydrate material. NMR analysis showed ethylenic protons already ascribed for the component Gal(β1-4)GlcNAc(β1-6)hexenetetrol

Figure 7 Oligosaccharide-alditols released from *Rana utricularia* egg jelly coat

(W. Morelle and G. Strecker, unpublished work). Moreover, the MALDI-TOF spectrum with the ion $(M+Na)^+$ at m/z 374 confirms the presence of this unsaturated hexitol. On the other hand, the characterization of Kdn(α 2-6)hexenotetrol (where Kdn is 2-oxo-3-deoxy-D-glycero-D-galactononulosonic acid) among the products of reductive β -elimination of the oviducal mucins of *Bufo americanus* (W. Morelle and G. Strecker, unpublished work) has proved that this hexitol results from the degradation of GalNAc itself, and not from the degradation of Gal, as might be argued on the basis of the absence of the NAc group.

DISCUSSION

The present study of the jelly coat of *Rana utricularia* eggs has led to the characterization of 12 oligosaccharide-alditols, the structures of which are summarized in Figure 7. These results confirm previous observations concerning the species-specificity of the carbohydrate chains present in amphibian mucins (Figure 8). In contrast with those from other anourens or urodeles, this new mucin is devoid of Kdn or NeuAc, and its anionic properties are connected with the occurrence of sulphate groups. The blood group A determinant described here as a major structural element of the carbohydrate chains is common to several species (*Pleurodeles waltl* [7,9]; *Ambystoma tigrinum* [13]) in which, however, it represents a minor component.

The core Gal(α 1-3)GalNAc-ol is also observed in *Rana temporaria* mucin [16], and the sequence Gal(β 1-3)Gal(β 1-3)GalNAc-ol is characteristic of one subspecies of *Xenopus laevis* [14,15]. The new carbohydrate sequence GlcNAc(β 1-

3)GlcNAc(β 1-6)GalNAc-ol appears to be specific for *Rana utricularia*, and represents the most original feature of the species.

Until now, no experimental results have been obtained that support the hypothesis of carbohydrate-mediated species-specific sperm binding in amphibians, as appears to occur for mammals [21]. It is generally accepted that the biological functions of the amphibian egg jellies include sperm binding, induction of the sperm acrosome reaction, formation of a barrier to sperm penetration, and a block to polyspermy in the case of Anourens [2]. Many reports have shown that coelomic eggs cannot be fertilized, but became capable of fertilization in the presence of the appropriate jelly preparation [3]. Because of the observed species-specificity of jelly carbohydrate chains, it would be tempting to assign to these glycans a gamete recognition function. Nevertheless, it has been demonstrated that mineral factor(s) that were obtained by dialysis and were resistant to heating at 600 °C retained biological activity, showing that the jelly essentially maintains the ionic environment required for successful fertilization [3,5]. The blockade of sperm migration observed in some heterologous jellies has been claimed to be due to an incompatibility of the physical architecture [3]. To extend this point of view, we propose that the observed carbohydrate polymorphism may also help to modulate the biological and physical properties of these mucins.

In addition, previous results have shown a natural polymorphism occurring in two subspecies of *Xenopus laevis* [14,15]. The chemical analysis of these carbohydrate chains could be used to support the taxonomy of amphibians. The present and previous studies have also shown that amphibian egg jellies represent a source of carbohydrates that are involved in human

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