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Characterization of Le^x, Le^y and A Le^y antigen determinants in KDN-containing *O*-linked glycan chains from *Pleurodeles waltlii* jelly coat eggs

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Novel acidic oligosaccharides were isolated in large amounts by reductive alkaline treatment of the jelly coat of *Pleurodeles waltlii* (Michah) eggs. The oligosaccharides were found to contain the newly described KDN as acidic monosaccharide and possess either the Le^x, Le^y and A Le^y antigenic determinants. Occurrence of Le^x and Le^y determinants previously recognized as tumor-associated antigen (TAA) demonstrates that mucins of lower animals may represent a rich and easily available source for preparing TAA. Moreover, it reinforces the hypothesis according to which TAA are evolution markers.

Lewis X antigen; Lewis Y antigen; A Lewis Y antigen; *Pleurodeles waltlii*; Jelly coat egg; Mucin

1. INTRODUCTION

Animal mucins represent natural sources for the large scale preparation of *O*-glycosidically linked glycans. In this respect, previous reports on bird [1] and fish mucins [2,3] have demonstrated that these glycoproteins may provide easily available sources of carbohydrate material. In the course of a systematic structural survey of carbohydrates from animal mucins, we describe for first time the primary structure of acidic oligosaccharides released by alkaline borohydride treatment from the jelly coat of the newt *Pleurodeles waltlii*. All of these oligosaccharides were found to contain the recently discovered KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) [4–6] and bear either the Le^x and Le^y antigenic determinants, which have been previously recognized as human tumor associated antigens [7]. The newt egg jelly coat appears to be a valuable source for a large scale preparation of such antigens.

Abbreviations: KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; TAA, tumor-associated antigen.

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2. MATERIALS AND METHODS

2.1. *Fractionation of reduced oligosaccharides*

Eggs from *Pleurodeles waltlii* (Michah) were obtained from natural spawnings in aquariae. The jelly coat egg material was lyophilized and the dry material (160 mg) was submitted to alkaline reductive degradation in 50 mM NaOh containing 1.0 M $NaBH_4$ (10 ml) at 37°C for 24 h. The reaction was stopped by the addition of Dowex 50x8 (25–50 mesh; H^+ form) at 4°C. The solution was filtered, adjusted to pH 5.5 with 0.1 M NaOH and then concentrated. Boric acid was distilled as methyl ester in the presence of methanol. The material was then fractionated on a Bio-Gel P2 column (2 × 50 cm).

2.2. *Analytical procedures*

Oligosaccharide-alditols were isolated by HPLC on primary amine bonded silica (Supelcosil LC-NH_2 4.6 mm × 25 cm, Supelco Inc., Bellefonte USA) using a solution of acetonitrile – 30 mM KH_2PO_4 buffer, pH 5.2 (65:35), with a flow rate of 1 ml/min. Oligosaccharides were detected by UV spectroscopy at 206 nm.

Prior to ^{1}H-NMR spectroscopic analysis, the oligosaccharide-alditol fractions were repeatedly exchanged with D_2O at pD 7, and lyophilized. ^{1}H-NMR spectroscopy was performed on a Bruker AM-400 WB spectrometer. The indicated probe temperature was 27°C. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 27°C).

3. RESULTS AND DISCUSSION

3.1. *Fractionation of reduced oligosaccharides*

Oligosaccharides released from the jelly coat of newt eggs by alkaline borohydride treatment were fractionated as two peaks (I and II) on a Bio-Gel P2 column, and examined by TLC on silica gel. The material

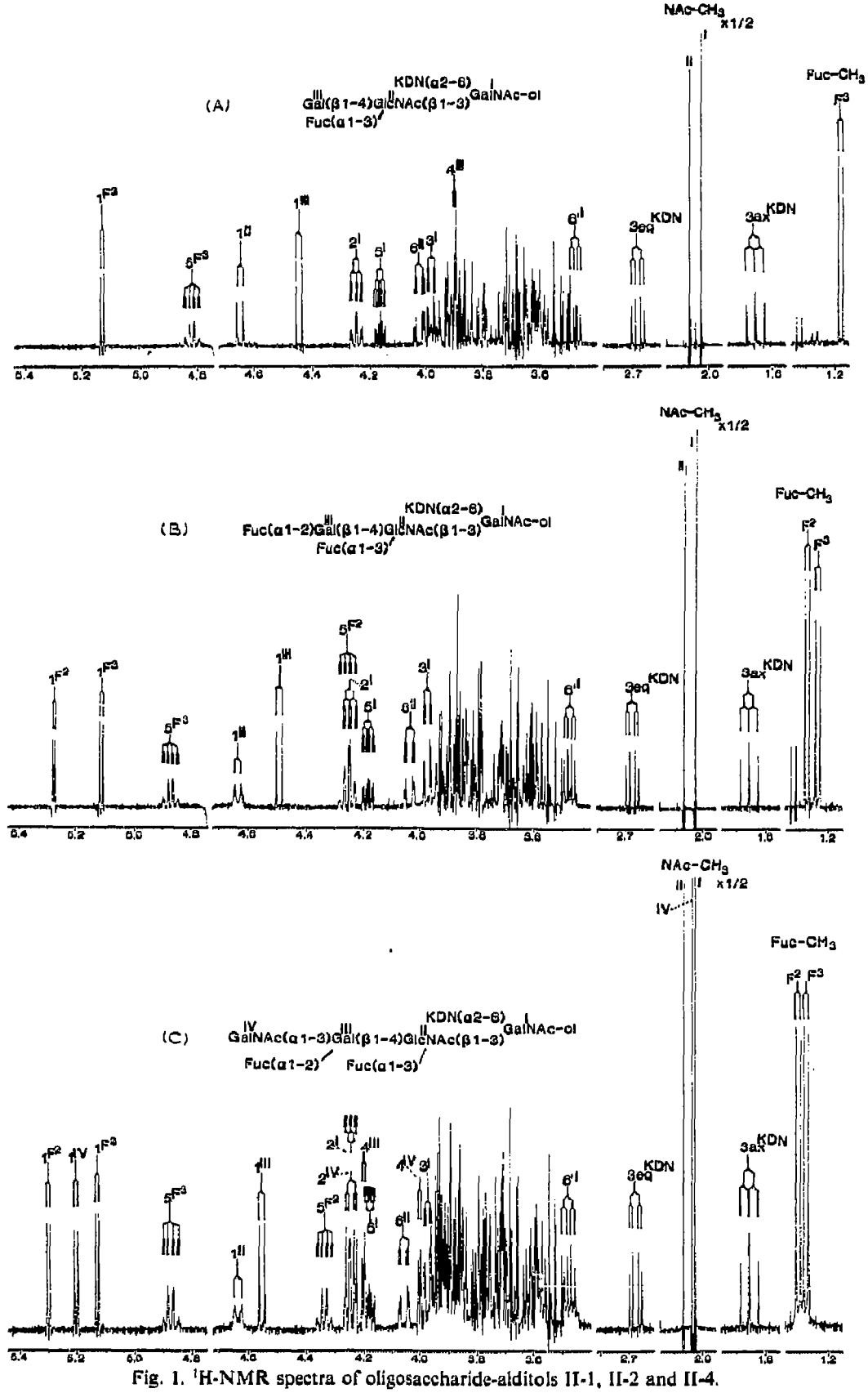


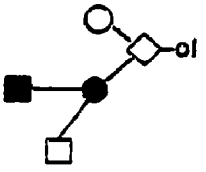
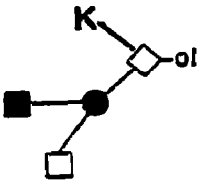
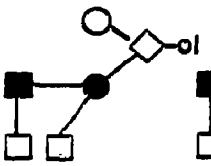
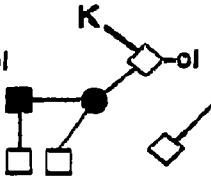
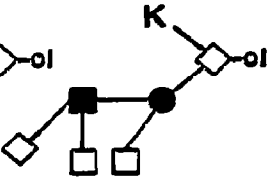
Fig. 1. ^{1}H-NMR spectra of oligosaccharide-alditols II-1, II-2 and II-4.

in peak I did not migrate and was not further analyzed. Analysis of peak II by TLC revealed the presence of four components (II-1 to II-4) which were further isolated by preparative HPLC as described in section 2.

Table I

^{1}H-chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharide-alditols released from the jelly coat of *Pleurodeles waltlii* eggs.

Symbolic notation: ◇, GalNAc; ●, GlcNAc; ■, Gal; □, Fuc; ○, NeuAc; K, KDN.

Residue	Reporter Group	Chemical shift in compound				
		8b1[8]	II-1	9b2[8]	II-2	II-4
						
GalNAc-ol	H-2	4.247	4.244	4.250	4.247	4.246
	H-3	3.984	3.978	3.976	3.971	3.970
	H-5	4.179	4.161	4.198	4.184	4.180
	H-6'	3.489	3.474	3.48	3.474	3.474
	NAc	2.025	2.023	2.026	2.024	2.024
GalNAc^3	H-1	-	-	-	-	5.198
	H-2	-	-	-	-	4.248
	H-4	-	-	-	-	3.998
	NAc	-	-	-	-	2.035
GlcNAc^3	H-1	4.66	4.645	4.635	4.631	4.634
	H-6	4.021	4.020	N.D.	4.034	4.053
	NAc	2.066	2.066	2.069	2.065	2.066
Gal^4	H-1	4.444	4.444	4.493	4.491	4.552
	H-4	N.D.	3.902	N.D.	3.871	4.200
Fuc^2	H-1	-	-	5.276	5.276	5.297
	H-5	-	-	4.258	4.256	4.336
	CH_3	-	-	1.274	1.273	1.303
Fuc^3	H-1	5.132	5.131	5.113	5.113	5.128
	H-5	N.D.	4.818	4.874	4.871	4.871
	CH_3	1.177	1.176	1.238	1.236	1.274
NeuAc^6	H-3ax	1.698	1.652	1.699	1.655	1.654
or	H-3eq	2.732	2.676	2.735	2.675	2.674
KDN^6	NAc	2.034	-	2.033	-	-

3–6 mg of each component were isolated starting from 160 mg of crude material. Compound II-3 was a mixture of two oligosaccharide-alditols, the structure of which was not investigated.

3.2. *Structure determination of the oligosaccharide-alditols*

3.2.1. Structure of compound II-1.

The main characteristics of the NMR spectrum of compound II-1 (Fig. 1) are the signals relative to an α-1,3-linked fucose (δ H-1 = 5.131 ppm; δ H-5 = 4.818 ppm; δ CH_3 = 1.176 ppm) and the doublets at δ = 4.444 and 4.645 ppm which correspond to the anomeric protons of Gal and GlcNAc residues involved in the Le^x determinant. In agreement with Van Halbeek et al. [8], we also conclude that the sequence Gal(β1-4) [Fuc(α-3)]GlcNAc is attached to the C-3 atom of GalNAc-ol residue. Indeed, these characteristic structural reporter groups have been previously examined for the monosialyl oligosaccharide so-called '8b1' isolated from respiratory mucus of patients suffering from bronchiectasis [8] or cystic fibrosis [9]. The upfield shifted H-6′ resonance of GalNAc-ol (δ H-6′ = 3.474 ppm) confirms the C-6 substitution of the hexitol with sialic acid. Nevertheless, the absence of NeuAc acetamido signal in the region 2.030–2.040 ppm, and the shift of the H-3ax and H-3eq signals at δ = 1.652 and δ = 2.676 ppm, supported the evidence that a residue of deamino-neuraminic acid (KDN) was attached to the C-6 atom of GalNAc-ol, as previously reported in *O*-linked glycan chains of rainbow trout egg vitelline enveloppe [4–6]. Further evidence was also provided by analysis of ^{1}H-NMR spectrum of free deamino-neuraminic acid released by mild acid hydrolysis, which confirmed the characterization of 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid in newt egg jelly coat when compared with previously published results [5].

Thus, the structure of compound II-1 has been established as:

Gal(β1-4) [Fuc(α1-3)]GlcNAc(β1-3)[KDN(α26)]-GalNAc-ol

3.2.2. Structure of compound II-2.

The ^{1}H-NMR spectrum of compound II-2 (Fig. 1) indicates the presence of two Fuc residues involved in the Le^y determinant attached to the C-3 atom of GalNAc-ol. Indeed, the Fuc^2, Fuc^3, Gal^4 and $GlcNAc^3$ H-1 resonances are absolutely superposable to those described for compound 9b2 [8], the structure of which is reported in Table I. As for compound II-1, a residue of KDN is linked to the C-6 atom of GalNAc-ol. Thus, compound II-2 was readily identified as:

Fuc (α1-2)Gal(β1-4) [Fuc(α1-3)]GlcNAc (β1-3)-[KDN(α2-6)]GalNAc-ol

3.2.3. Structure of compound II-4.

Compound II-4 contains two Fuc residues α-1,2- and α-1,3-linked to an *n*-acetyllactosamine unit (Le^y determinant). The presence of an additional GalNAc (α1-3) residue was verified by the observation of the H-1 (δ = 5.198 ppm) and NAc resonance (δ = 2.035 ppm), as well as the analysis methylation, which confirmed the GalNAc to be in terminal non-reducing position. The H-4 resonances of Gal III and GalNAc IV residues were determined by double relay COSY experiments (Table I). Thus, the structure of compound II-4 has been established as:

GalNAc(α1-3) [Fuc(α1-2)]Gal(β1-4)[Fuc(α13)]-GlcNAc(β1-3) [KDN(α2-6)]GalNAc-ol

4. DISCUSSION

The present work represents the second report of the occurrence of KDN-containing *o*-linked glycan chains in nature, first one described in rainbow trout eggs [4–6]. Moreover, the jelly coat of newt eggs is particularly rich in Le^x and Le^y determinants. As the expression of Le^y antigen in human colonic cancer and polyps has diagnostic and prognostic value [7], the presence of large quantity of the antigen in such available material as newt eggs makes possible the large scale preparation of reference compounds. In addition, the A Le^y antigen determinant has been here isolated for the first time, after previous immunological characterization in human pyloric cells and Brünner's glands [11].

In conclusion, the jelly coat of newt eggs is mainly constituted of mucin type carbohydrate exhibiting structural determinants related to human tumor-associated antigens, which may be also considered as markers of evolution.

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