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Isolation of the O-glycosidically linked oligosaccharides obtained by alkaline borohydride degradation from oviducal mucins of the toad *Bufo bufo*

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Abstract

A combination of normal-phase high-performance liquid chromatography (HPLC) on amino-bonded silica and reversed-phase HPLC on octadecylsilica has been used to separate the reduced oligosaccharides produced by alkaline borohydride degradation of oviducal mucins obtained from the jelly coat of *Bufo bufo*. The former technique provides suitable separation on the basis of molecular size, while the latter method offers selectivity for stereoisomers. Thirty-four compounds, ranging in size from a trisaccharide to a dodecaoligosaccharide, have been isolated preparatively using a Supelcosyl LC-NH_2 normal-phase column eluted with aqueous acetonitrile and a Zorbax ODS reversed-phase column eluted with water.

Keywords: Alkaline borohydride degradation; Oligosaccharides

1. Introduction

Amphibian eggs are surrounded by a vitelline envelope and several structurally and chemically distinct jelly coats which are synthesized in specific regions of the oviduct and are deposited on the egg as it passes through these regions after its release from the ovary [1,2].

These jelly coats are not a simple barrier for a fertilizing sperm but are obligatory participants in the process of sperm–egg fusion, as dejellied eggs cannot be fertilized. Many functions have been attributed to the egg jelly components such as sperm binding, sperm capacitation, induction of the sperm acrosome reaction, prevention of species cross-fertilization, the blocking of polyspermy and provision of a protective environment for the developing embryo [3–13].

Mucin type glycoproteins have been shown to be the major components of this coat and implicated in important roles in fertilization [14–16]. The question of how these glycoproteins are involved in any function during fertilization and early development is important but has remained unanswered clearly. Of particular interest are several proposals suggesting the role of sugar chains as sperm receptors, due to the action of glycosidases or glycosyltransferases [17–19]. Previous studies have shown that the mucins possess species-specific glycanic chains [20–22], that should support the observed species-specificity of gamete interaction. The structural analysis of these components should be useful to study the mechanism of jelly action in fertilization in more detail.

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Mucins are glycoproteins containing several hundreds of carbohydrate chains attached to the peptide by O-glycosidic linkages between N-acetylgalactosamine and a hydroxylated amino acid (serine or threonine). More than 80% of the molecular mass of mucins may consist of O-glycans, thus most of the serine and threonine residues are glycosylated. O-linked oligosaccharides are usually released from the protein core by alkali-mediated β-elimination. In the alkaline β-elimination procedure, the presence of a strong reducing agent (sodium borohydride) is mandated to minimize the peeling reaction or other undesirable side reactions caused by the alkaline media. Thus the products obtained by this procedure are always reduced, the carbohydrate residue at the reducing end of the oligosaccharide being converted to the corresponding sugar alcohol [23]. The O-linked oligosaccharides released may carry a multitude of different structures. On one single glycoprotein not only the terminal sugar moieties can vary considerably but several different core structures have been reported on the same glycoprotein. The O-glycan may contain from one up to 20 monosaccharide units. The mucin oligosaccharides are extremely polydisperse, with regard to size diversity, numbers of isomers and variable acidic characters due to the presence of sialyl and sulfate residues.

After release from the protein, isolation and characterization of the O-linked oligosaccharides present a significant analytical challenge. O-Glycans may be purified by ion-exchange resins to separate acidic and neutral fractions, by paper chromatography, gel permeation, thin-layer chromatography. These techniques are not efficient enough to resolve the heterogeneity of these oligosaccharides. In order to isolate the various O-glycan structures only the most advanced separation techniques will be able to resolve completely all the structures released from glycoproteins with mucin-type carbohydrate chains.

The anion-exchange chromatography of oligosaccharides at elevated pH coupled with the very sensitive pulsed amperometric detection of carbohydrates has become a widely used method for the analysis of complex mixtures of saccharides [24–27]. The separations were sensitive to sugar composition, molecular size and linkage of the monosaccharide units. Reddy and Bush [28] demonstrated the applicability of this method to several oligosaccharide–alditols.

A number of methods for high-performance liquid chromatography (HPLC) separations of oligosaccharides have been reviewed by Honda [29] and by Daniel [30]. Normal-phase HPLC using amino bonded silica gel and reversed-phase HPLC using alkyl bonded silica gel have been the two most common methods to fractionate oligosaccharides utilizing ultraviolet absorbance for detection. The normal-phase method is sensitive mainly to oligosaccharide chain length. Reversed-phase HPLC which depends on hydrophobic bonding between the sample and the stationary phase, has been applied to complex oligosaccharides. Hydrophobic bonding of highly polar molecules such as carbohydrates to the alkyl groups of C_{18} stationary phases is weak. This nature of chromatography requires the presence of hydrophobic groups such as NAc-, deoxy-, –OMe or –OAc groups for longer retention time and better separation.

This paper reports the separation of 34 oligosaccharide–alditols obtained by alkaline borohydride treatment of oviducal mucins of *Bufo bufo* by two different HPLC techniques: partition chromatography on primary amine-bonded silica and reversed-phase chromatography. The structures of some minor compounds has not been analysed. Some general rules for the effects of particular structural features on the chromatographic behavior of oligosaccharides are also discussed.

2. Experimental

2.1. Sampling of jelly coat mucus

Eggs from *Bufo bufo* were obtained from natural spawnings in nature. The jelly coat material was lyophilized.

2.2. Isolation of oligosaccharide–alditols

O-linked oligosaccharides were released from the crude material by alkaline borohydride treatment in 1 *M* $NaBH_4$, 0.1 *M* NaOH at 37°C for 48 h. The reaction was stopped by the addition of Dowex 50x8 (mesh 25–50, Bio-Rad, H^+ form) at 4°C. The solution was filtered on glass wool, adjusted to pH 6.5 and then concentrated in a rotatory evaporator at 25°C. Borate salts were removed by repeated evapo-

ration with methanol. The resulting sample was purified by gel permeation on a Bio-Gel P2 (45–90 μm; Bio-Rad Labs., Richmond, CA, USA) column (108×2.3 cm I.D.) eluted with deionised water at a flow-rate of 22 ml/h. Fractions (5 ml) were collected and the absorbance was measured at 206 nm (LKB 2138 Uvicord S, Bromma, Sweden). The fractions were revealed with orcinol–sulphuric acid reagent [31] on silica gel plates (pre-coated silica gel 60; Merck, Darmstadt, Germany.). The carbohydrate-containing fractions were pooled and concentrated in a rotary evaporator at 25°C. Peptide material was removed on a column (37×1 cm I.D.) of Dowex 50x2 (mesh 200–400; Bio-Rad, H^+ form) eluted with water. Oligosaccharide–alditols were applied on a column (37×1 cm I.D.) of Dowex 1x2 (OH^- form). After washing with 500 ml water, oligosaccharide–alditols were eluted with a discontinuous gradient (50, 100 and 200 m*M*) of pyridine–acetic acid buffer. The fractions namely ''50 m*M*'', ''100 m*M*'' and ''200 m*M*'' (500 ml) were concentrated in a rotary evaporator at 25°C and further desalted by gel permeation on a Bio-Gel P2 (45–90 μm; Bio-Rad Labs.) column (90×2.3 cm I.D.) eluted with deionised water at a flow-rate of 14 ml/h. Fractions (4 ml) were collected and the absorbance measured at 206 nm and for hexose by orcinol/sulfuric acid coloration. The carbohydrate-containing fractions were pooled and lyophilized.

2.3. HPLC separation of fraction 100 mM on primary amine-bonded silica

HPLC was performed on a 5-μm primary amine-bonded silica (Supelcosyl LC-NH_2, 250×4.6 mm I.D.; Supelco, Bellefonte, PA, USA) with a Spectra-Physics Model 8700 liquid chromatograph (San Jose, CA, USA) equipped with an LDC variable-wavelength detector (SpectraMonitor D, Milton Roy, Riviera Beach, FL, USA) connected to a Spectra-Physics Model 4100 computing integrator. A 1 mg sample of fraction 100 m*M* was dissolved in 100 μl starting buffer. The column was equilibrated with the initial solvent [acetonitrile–water containing 3 m*M* potassium phosphate, (75:25, v/v)]. Aliquots (80 μl) were injected onto the column. After the injection, isocratic conditions were applied for 15 min with the initial solvent, followed by a linear gradient to acetonitrile–water containing 3 m*M* potassium phosphate (70:30, v/v) for 45 min. The flow-rate was 1.0 ml/min and the absorbance was measured at 206 nm.

2.4. HPLC separation of fraction 200 mM-II on primary amine-bonded silica

HPLC was performed on a 5-μm primary amine-bonded silica (Supelcosyl LC-NH_2, 250×4.6 mm I.D.; Supelco). A 1-mg sample of fraction 200 m*M*-II was dissolved in 100 μl starting buffer. The column was equilibrated with the initial solvent [(acetonitrile–water containing 3 m*M* potassium phosphate, (75:25, v/v)]. Aliquots (80 μl) were injected onto the column. After the injection, isocratic conditions were applied for 5 min, followed by a linear gradient to acetonitrile–water containing 3 m*M* potassium phosphate (65:35, v/v) for 55 min and then a linear gradient to acetonitrile–water containing 3 m*M* potassium phosphate (62:38, v/v) for 10 min. The flow-rate was 1.0 ml/min and the absorbance was measured at 206 nm.

2.5. HPLC separation of fraction 200 mM-I on primary amine-bonded silica

HPLC was performed on a 5-μm primary amine-bonded silica (Supelcosyl LC-NH_2, 250×4.6 mm I.D.; Supelco). A 1-mg sample of fraction 200 m*M*-I was dissolved in 100 μl starting buffer. The column was equilibrated with the initial solvent [acetonitrile–water containing 3 m*M* potassium phosphate, (65:35, v/v)]. A 80-μl aliquot was injected onto the column. After the injection, isocratic conditions were applied for 5 min, followed by a linear gradient to acetonitrile–water containing 3 m*M* potassium phosphate (60:40, v/v) for 40 min, followed by isocratic conditions for 30 min and then a linear gradient to acetonitrile–water containing 3 m*M* potassium phosphate (57:43, v/v) for 15 min. The flow-rate was 1.0 ml/min and the absorbance was measured at 206 nm.

Acetonitrile was evaporated from all collected fractions under a stream of nitrogen and then concentrated in a rotary evaporator at 25°C. All the collected fractions were purified by gel permeation on Sephadex G-10 column (45×0.5 cm I.D., Pharmacia) equilibrated with deionised water at a flow-rate of 6 ml/h, monitored by absorbance at 206 nm (LKB

2138 Uvicord S). Fractions (1 ml) were collected and analyzed for hexose by orcinol/sulfuric acid coloration. The carbohydrate-containing fractions were pooled, concentrated in a rotary evaporator at 25°C and then lyophilized. The primary structures of the oligosaccharide–alditol were established by combining the results of ^{1}H nuclear magnetic resonance (NMR) spectroscopy, methylation analysis and matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) analysis [32,33].

2.6. HPLC separation of fraction 200-I-3 on a Zorbax ODS column

HPLC was performed on a 5-μm Zorbax ODS column (250×4.6 mm I.D.; Life Sciences International). Fraction, namely 200-I-3, from primary amine-bonded silica HPLC was refractionated by C_{18} reversed-phase chromatography. A 1-mg amount of fraction 200-I-3 was dissolved in 100 μl water. The column was equilibrated with water. An 80-μl aliquot was injected onto the column. After injection, isocratic conditions were applied with water for 40 min, followed by a linear gradient to water–acetonitrile (80:20, v/v) for 20 min. The flow-rate was 0.5 ml/min. The oligosaccharide–alditols were detected at 206 nm.

2.7. Carbohydrate analysis

Carbohydrate analysis was performed using gas chromatography (GC) with a silicone OV 101 capillary column (25 m×0.32 mm I.D.). Samples were analysed after methanolysis (0.5 *M* HCl in methanol for 24 h at 80°C) followed by N-reacetylation and trimethylsilylation according to Kamerling et al. [34] modified by Montreuil et al. [35].

3. Results and discussion

Eggs from *Bufo bufo* were obtained from natural spawnings in nature and lyophilized. These eggs (10 g) were submitted to alkaline borohydride degradation to obtain oligosaccharide–alditols. Both neutral and acidic oligosaccharide–alditols were retained on a strong anion ion-exchanger Dowex 1x2 (mesh 200–400; OH^- form). Under these conditions, acidic as well as neutral oligosaccharide–alditols were strongly ionised and retained on the column. They were eluted with 100 m*M* and 200 m*M* pyridine–acetate buffer solution (pH~6). Fraction 200 m*M* was subfractionated into 200 m*M*-I and 200 m*M*-II by gel-permeation on a Bio-Gel P2 column (Fig. 1). The purification of the β-elimination products gave three fractions: 73 mg of fraction 100 m*M*, 126 mg

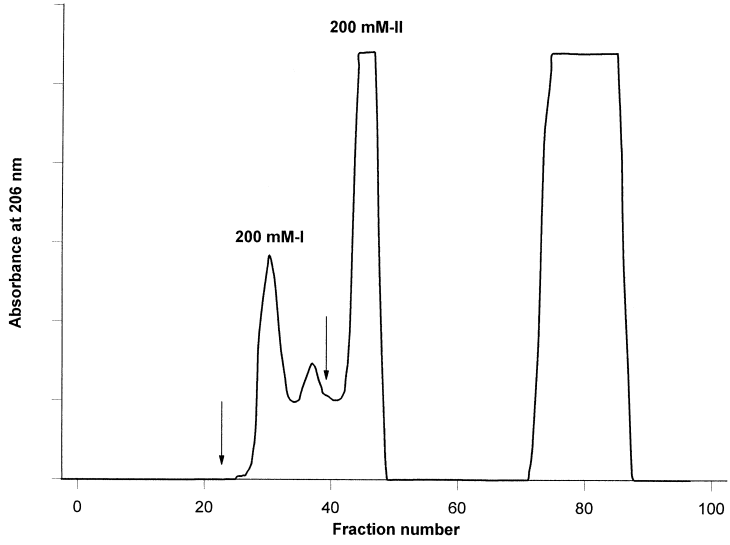

Fig. 1. Gel permeation of oligosaccharide–alditols obtained from fraction 200 m*M* on a Bio-gel P2. Salts were eluted in fractions 70–90. 200 m*M*-I: DP 6 to 12. 200 m*M*-II: DP 3 to 6.

Table 1
Carbohydrate compositions of fractions obtained by Biogel P2 chromatography of oligosaccharides released by alkaline borohydride treatment from oviducal mucins of *Bufo bufo*

Fraction	Molar ratio[a]					
	Fuc	Gal	GalNAc	GlcNAc	A.S.	GalNAc-ol
100 m*M*	1.1	1.7	0.9	0.3	0	1
200 m*M*-I	1.5	2.9	1.1	0.6	0.5	1
200 m*M*-II	0.8	1.5	0.8	0.1	0.7	1

[a] The molar ratio of GalNAcol was taken as 1.
GalNAc: N-Acetylgalactosamine.
A.S.: Sialic acid.

of fraction 200 m*M*-I and 96 mg of fraction 200 m*M*-II. The carbohydrate composition of these fractions was determined by GC (Table 1). These three fractions possesses a molar carbohydrate composition in accordance with the structure of O-glycosidically linked oligosaccharides: a high content of N-acetylgalactosaminitol and no mannose residues.

The fraction 100 m*M* from the Dowex 1x2 chromatography of oligosaccharide–alditols was subjected to partition chromatography on a primary amine-bonded silica. The effective separation of eight fractions was obtained in 60 min (Fig. 2). The structures of the oligosaccharide–alditols of this fraction are summarized in Fig. 3. The structures belong to core types 1 and 2, i.e., Gal(β1-3)GalNAc-ol or Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol, respectively. The oligosaccharide–alditol 100-4 can be considered as resulting from the peeling of compound 100-7.

The fraction 200 m*M*-I from Bio-Gel P2 chromatography of oligosaccharide–alditols 200 m*M* was subjected to HPLC using acetonitrile–water containing 3 m*M* potassium phosphate as a solvent and primary amine-bonded silica. Fifteen fractions were obtained (Fig. 4). The assembly of peaks denoted 3 in Fig. 4, was subfractionated into three fractions, 200-I-3a to 200-I-3c, by C_{18} reversed-phase chromatography (Fig. 5). The fraction 200-I-10 contains

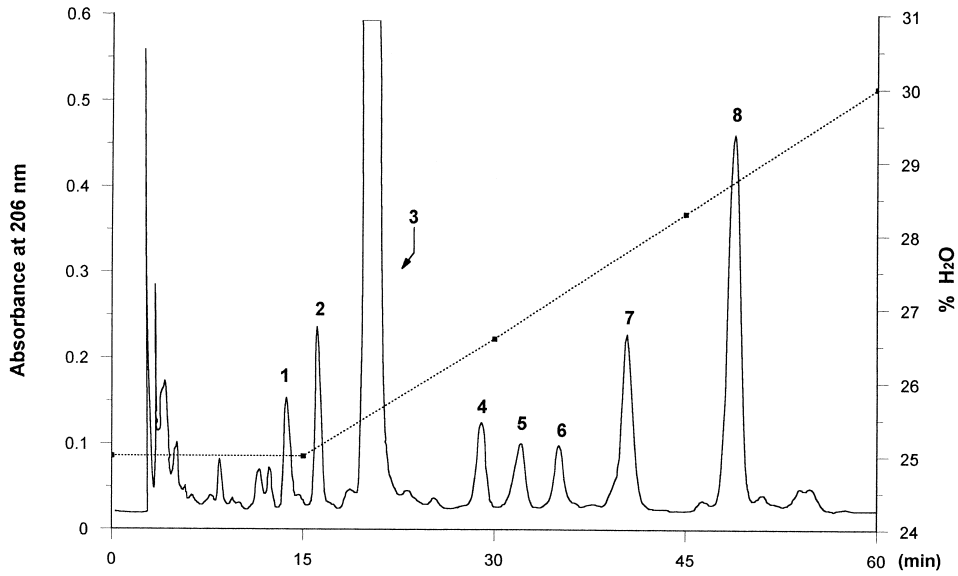

Fig. 2. HPLC elution profile of oligosaccharide–alditols from the fraction 100 m*M*. Samples were chromatographed on a column (250×4.6 mm I.D.) of primary amine-bonded silica (Supelcosyl LC-NH_2; Supelco) using a gradient elution with acetonitrile–water containing 3 m*M* potassium phosphate, as depicted on the profile. Flow-rate was 1.0 ml/min.

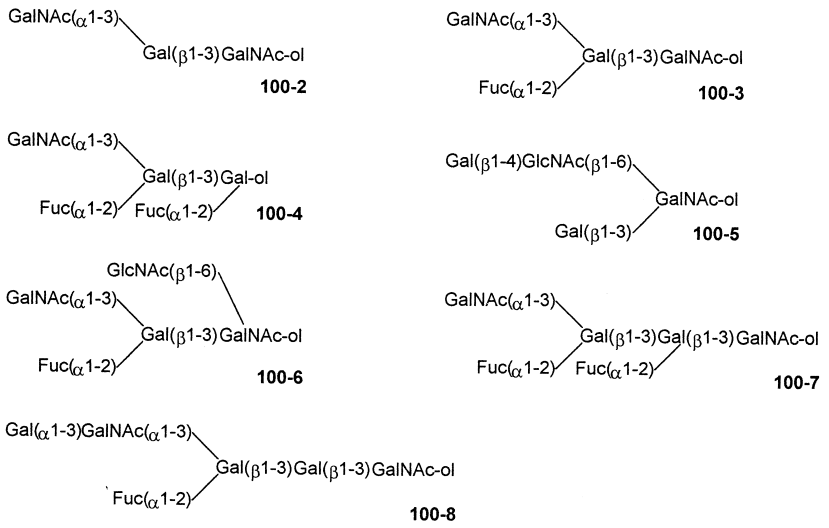

Fig. 3. Structures of oligosaccharide–alditols isolated from the fraction 100 m*M* [32].

three compounds, namely 200-I-10a, 200-I-10b and 200-I-10c. The fractions 200-I-11 and 200-I-13 contain two compounds namely 200-I-11a, 200-I-11b and 200-I-13a, 200-I-13b, respectively. The structures of seventeen fractions are summarized in Fig. 6. The sequence Gal(β1-3)Gal(β1-3)GalNAc, which has been observed in different amphibian mucins [21] is also present in *Bufo bufo*, but a new type of sequence – the attachment of a β-1,6-linked N-acetylglucosamine (GlcNAc) to galactose (Gal) residue instead of N-acetylgalactosaminitol (GalNAc-ol) – constitutes another feature of this mucin. This

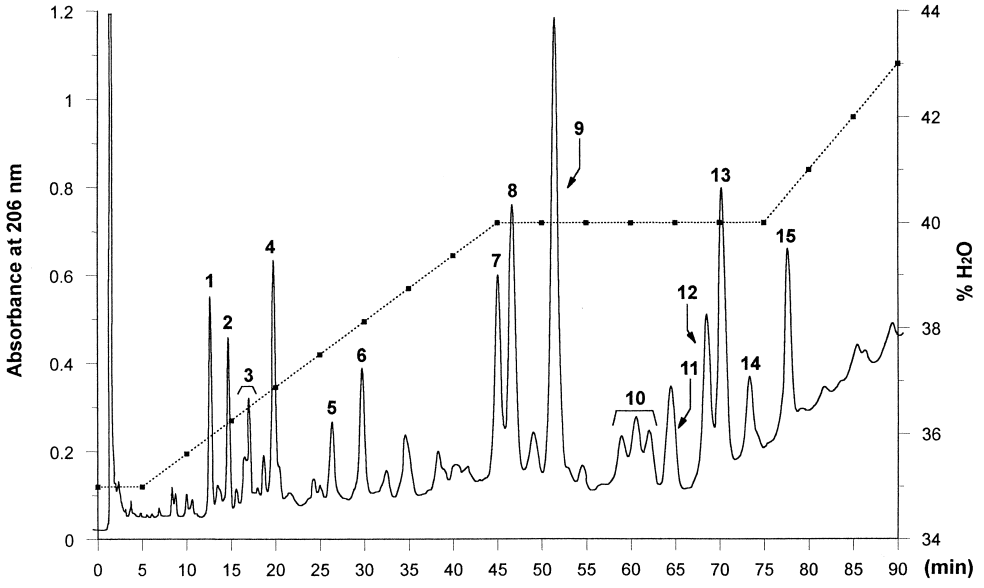

Fig. 4. HPLC elution profile of oligosaccharide–alditols from the fraction 200 m*M*-I. Samples were chromatographed on a column (250×4.6 mm I.D.) of primary amine-bonded silica (Supelcosyl LC-NH_2) using a gradient elution with acetonitrile–water containing 3 m*M* potassium phosphate, as depicted on the profile. Flow-rate was 1.0 ml/min.

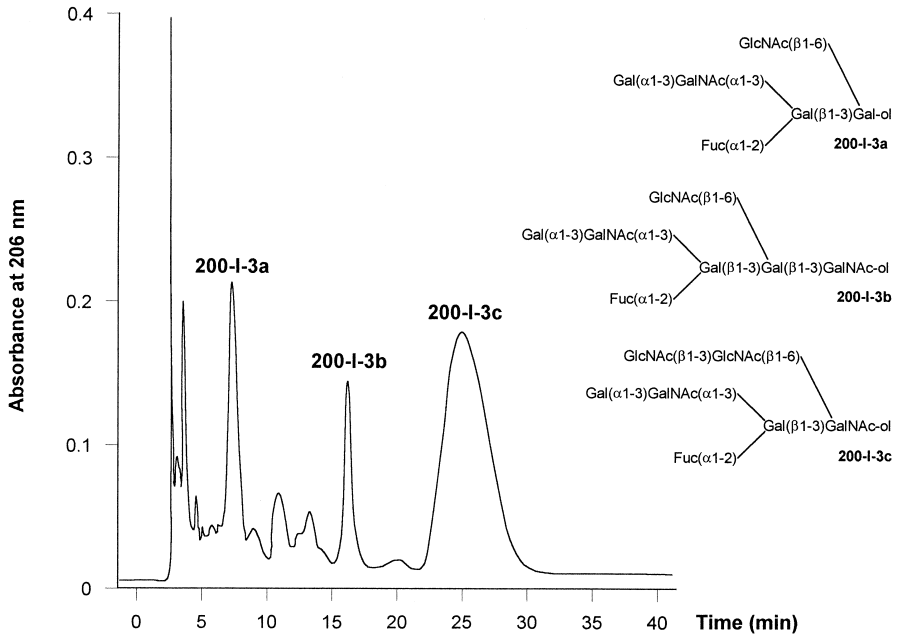

Fig. 5. Further separation of fraction 200-I-3 on a C_{18} reversed-phase 5 μm Zorbax ODS column (250×4.6 mm I.D.). Elution was performed isocratically by water at a flow-rate of 0.5 ml/min.

new structural characteristic is well represented in compounds 200-I-7, 200-I-13b, 200-I-14 and 200-I-15.

The fraction 200 m*M*-II from Bio-Gel P2 chromatography of oligosaccharide–alditols 200 m*M* was subjected to HPLC using acetonitrile–water containing 3 m*M* potassium phosphate as a solvent and primary amine-bonded silica. The effective separation of nine fractions was obtained in 70 min (Fig. 7). The structures of the oligosaccharide–alditols of this fraction are summarized in Fig. 8. This fraction contains both neutral and acidic compounds. Neutral compounds showed a shorter retention time than acidic compounds. Some compounds possess a blood-group A determinant, linked to a type I Gal(β1-3)GalNAc-ol carbohydrate core. Whereas this peripheral blood-group A trisaccharidic sequence definitively blocks the elongation of the carbohydrate chain in human glycoconjugates, the α-GalNAc residue represents for *Bufo bufo* a good acceptor for the transfer of an additional α-Gal unit, owing to the expression of a new α1,3-galactosyltransferase.

HPLC is a powerful tool for the separation of oligosaccharide–alditols [36–39]. The combination of two HPLC steps, i.e., separation on a column of primary amine-bonded silica, followed by a second run on an octadecylsilica has been applied to isolate preparatively 34 compounds. These compounds vary in size from three to twelve sugars. The first column allows the separation of, at least, 32 fractions. Fraction 200-I-3 has been separated into three subfractions by HPLC on a reversed-phase column.

The comparison of the retention times of the oligosaccharide–alditols renders possible the identification of a few structural features responsible for the chromatographic behaviour of these compounds. As already shown by Blanken et al. [40], the retention time on primary amine-bonded silica depend mainly on the number of sugar residues (see Figs. 2 and 3), the presence of a Fuc or a GlcNAc residue, and the presence of a 1→6 linkage. Generally a large number of sugar residues results in an increase in the retention time as is shown in Fig. 2. Moreover, within a series of oligosaccharide–alditols having the same number of sugar residues, the retention time of oligosaccharide–alditols are lower when an additional fucose (Fuc) residue is present (compare 100-7 with 100-8, 200-I-11b with 200-I-12). Such an

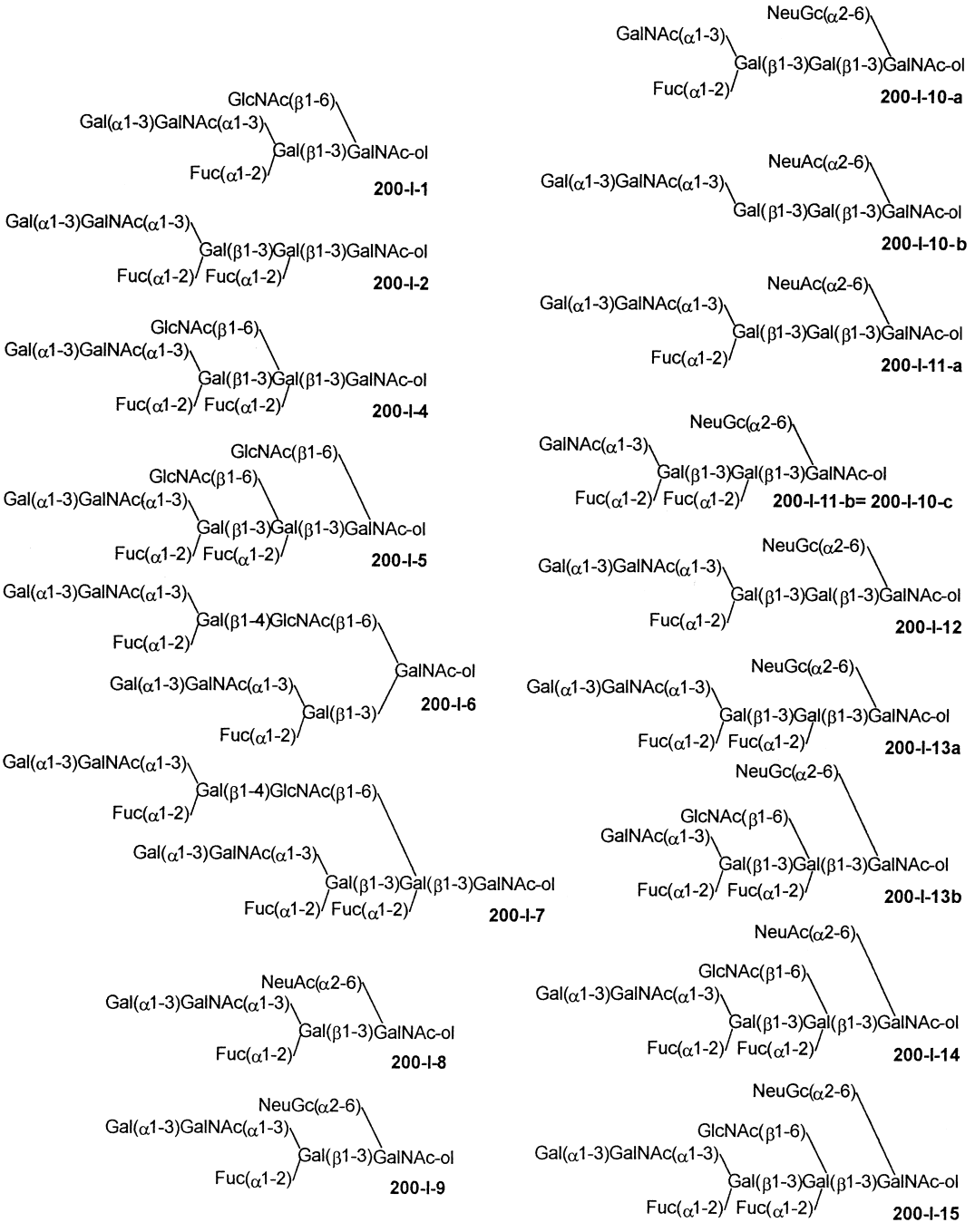

Fig. 6. Structures of oligosaccharide–alditols isolated from the fraction 200 m*M*-I [33].

observation has been also reported for separations using high-performance anion exchange chromatography (HPAEC) [41–43]. Within a series of reducing oligosaccharides, a β(1→6) linkage usually induces a higher retention time as compared to other types of linkage [32]. Blanken et al. [40] explained this

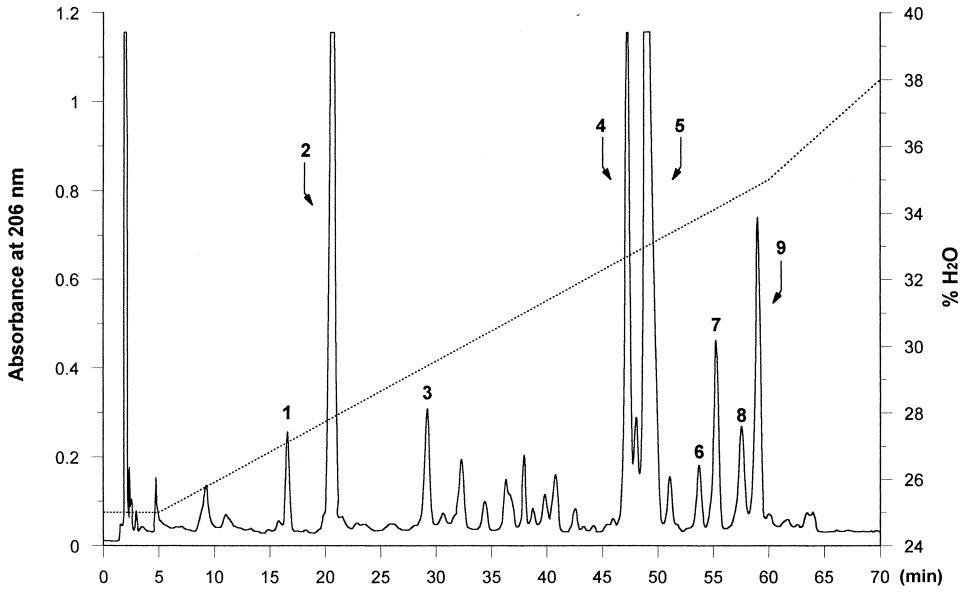

Fig. 7. HPLC elution profile of oligosaccharide–alditols from the fraction 200 m*M*-II. Samples were chromatographed on a column (250×4.6 mm I.D.) of primary amine-bonded silica (Supelcosyl LC-NH_2) using a gradient elution with acetonitrile–water containing 3 m*M* potassium phosphate, as depicted on the profile. Flow-rate was 1.0 ml/min.

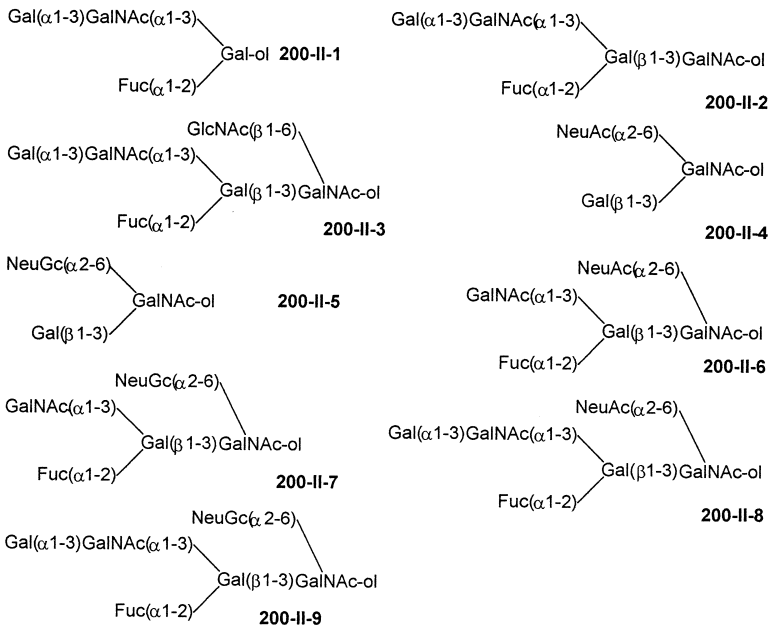

Fig. 8. Structures of oligosaccharide–alditols from the fraction 200 m*M*-II [32].

phenomenon by the flexibility of the linkages, allowing an increased interaction of hydroxyl groups with the matrix. In the present series of oligosaccharides, this phenomenon was also observed (compare 100-3 with 100-5, 200-I-3b, 200-I-3c with 200-I-2).

This study also pointed to the presence of both N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc), instead of Kdn which is largely distributed in most of amphibian species. The elution sequence of the sialyl–oligosaccharide–alditols seems to be essentially related to size (Figs. 4 and 6). Our results indicate that a NeuAc $\alpha(2\rightarrow6)$ residue is associated with a decreased retention time as compared to the NeuGc $\alpha(2\rightarrow6)$ residue (compare 200-II-4 with 200-II-5, 200-II-6 with 200- II-7, 200-II-8 with 200-II-9, 200-I-8 with 200-I-9 and 200-I-14 with 200-I-15). Because the molecular basis for the retention time of carbohydrates on primary amine-bonded silica is probably hydrogen bonding between the hydroxyl groups of the sugar and the amine groups of the stationary phase [44,45] we can conclude that the presence of an acetamido group instead of a hydroxyl group at the 5-position of an acidic sugar is responsible for the reduced retention.

The comparison of the retention times of oligosaccharide–alditols separated on an octadecyl-bonded column is also interesting. As already shown by Dua et al. [46], the elution of compounds with the Fuc(α1-2)Gal(β1-3)GalNAc-ol element is strongly retarded (see retention times of 200-I-3b, 200-I-3c with 200-I-3a). The elution order on C_{18} columns depends on the hydrophobicity of the oligosaccharide which is mainly due to the methyl or acetamido groups of the sugar units [47] and their accessibility [48]. The relatively large peak width of compound 200-I-3c might be due to these groups of the sugar units (one methyl group and four acetamido groups).

For the fractionation of oligosaccharide–alditols, normal-phase HPLC on amino-bonded silica is a very efficient method, but not sufficient when the number of sugar residues becomes large. The separation on amino-bonded silica had to be complemented by a second step on an octadecylsilica column.

The combination of normal-phase and reversed-phase chromatography allows one to prepare 34 oligosaccharide–alditols from oviducal mucins of the toad *Bufo bufo*. One of the aims of this study was to extend the hypothesis that glycanic chains of amphibian oviducal mucins are highly species-specific. The results obtained for *Bufo bufo* confirm this point. Such a species-specificity of the carbohydrates which constitute more than 60% of the jelly material should be relevant to the species-specific gamete recognition which characterizes most of amphibians. It would also reflect the taxonomic and phylogenetic relationships between species under study. That approach would allow to evaluate the natural diversity of molecules and the evolution of molecules in term of amphibian species.

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