Glycoconj J (2008) 25:903–915
DOI 10.1007/s10719-008-9139-3

A single step method for purification of sulfated oligosaccharides

Estelle Garenaux · Shin-Yi Yu · Doina Florea · Gérard Strecker · Kay-Hooi Khoo · Yann Guérardel

Received: 31 January 2008 / Revised: 22 April 2008 / Accepted: 23 April 2008 / Published online: 6 July 2008

Abstract Purifying and analysing sulfated oligosaccharides by mass spectrometry often constitutes a challenge. We present here a single step method to isolate sulfated compounds from a complex mixture of neutral and acidic oligosaccharide–alditols. The strategy relies on the exclusion of sulfated molecules from strong cation exchange resin. By testing a wide range of reduced mucin type O-glycans isolated from different biological sources, we demonstrate that this method permits, without prior chemical modification, the specific purification of sulfate-containing oligosaccharides present in any quantity from very complex mixtures of molecules.

Keywords Sulfated oligosaccharides · Purification · Mass spectrometry · Mucins

Abbreviations

dHex	deoxyHexose
DHB	2,5-dihydroxybenzoic acid
ESI	ElectroSpray Ionization
HPLC	High Performance Liquid Chromatography
Kdn	2-keto-3-deoxynononic acid
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
MS	Mass Spectrometry
Man	Mannose
S	Sulfate group
TLC	Thin Layer Chromatography

Electronic supplementary material The online version of this article (doi:10.1007/s10719-008-9139-3) contains supplementary material, which is available to authorized users.

E. Garenaux · D. Florea · G. Strecker · Y. Guérardel (✉)
Unité de Glycobiologie Structurale et Fonctionnelle,
CNRS UMR 8576, USTL,
59655 Villeneuve d'Ascq, France
e-mail: yann.guerardel@univ-lille1.fr

S.-Y. Yu · K.-H. Khoo
Institute of Biological Chemistry, Academia Sinica,
Nankang,
Taipei 11529, Taiwan

S.-Y. Yu · K.-H. Khoo
Institute of Biochemical Sciences, National Taiwan University,
Taipei 106, Taiwan

Introduction

Sulfation is a common modification of protein glycans. It is present at a very high prevalence on high molecular weight glycosaminoglycans bearing proteoglycans, whose physico-chemical and biological functions are directly dependent on their number and positions. It is also incorporated in numerous smaller protein glycans, where it may play a crucial role in biological functions of glycoproteins. In humans, it mainly substitutes lactosamine-containing glycans either as GlcNAc-6-sulfate or Gal-3-sulfate [1 3]. Sulfate groups are observed on N-glycans substituted proteins such as thyroglobulin [4], carbonic anhydrase VI [5] and Tamm-Horsfall [6], as well as on O-glycans of ovarian cystadenoma glycoproteins [7]. Unusual sulfations may also occur on very specific epitopes such as HNK1 (SO_4–3-GlcAβ1-4Galβ1-4G1cNAc-R) on myelin-associated glycoproteins [8]. Several glycoconjugates have been shown to play important biological roles mediated by specific recognition of their sulfate moiety by receptors. This is the case with luteinizing hormone receptor on endothelial cells that is highly specific for the SO_4–4-GalNAcβ1-4G1cNAc motifs

present on its N-glycans and is responsible for the rapid clearance of the luteinizing hormone from circulation [9]. Similarly, the oligosaccharide ligand for L-selectin was shown to be the 6′sulfo-sialyl Lex on a mucin-like molecule, termed GIyCAM-1 (for glycosylated cell adhesion molecule-1), during the homing of leukocytes to sites of inflammation [10].

Other than proteoglycans, mucin type glycoproteins are the main carriers of sulfated glycans in humans. Indeed, sulfate groups have been observed in most excreted mucin-containing mucus, including salivary [11], tracheo bronchial [12] and intestinal mucus [13]. Mucins are the major protein component of the mucus protecting the epithelia. They are highly glycosylated macromolecules characterized by the presence of a dense and highly diverse O-glycosylation, linked to the protein core through serines and threonines, that may represent up to 80% of the total weight of mucins. O-glycans contribute to both the physical and chemical protections of sensitive epithelia, such as bronchial epithelia, by providing the rheologic properties of mucus. Furthermore, the extraordinary structural diversity of O-glycosylation generates a wide range of carbohydrate based epitopes that are believed to supply attachment sites for microorganisms, which can be trapped and expelled during the continuous renewal of the mucus layer. By providing competing receptors for cell-surface glycoconjugates, mucins may trap bacteria and make them less successful in their attempts to colonize the epithelium. Thus, the array of oligosaccharides expressed on the mucins of an individual may play a key role in governing the susceptibility to infection [14].

The sulfation status of mucin type O-glycosylation, among other modifications of glycosylation patterns, may be modified in pathological conditions. Such modifications may occur during numerous pathologies affecting airways such as cystic fibrosis or chronic bronchitis. Modifications of sulfation and sialylation patterns were reported for airway mucins prepared from patients suffering from cystic fibrosis or from severe chronic bronchitis [15]. It was suggested that a strong inflammatory reaction, generates an increase in mucin sialylation and an hyperexpression of sialyl-Lewis x that may be responsible for the specific lung colonization by *P. aeruginosa* of cystic fibrosis patients. Indeed, the use of purified sulfated O-glycans coupled to synthetic polymers gave good lines of evidence to directly correlate the increased sulfation of CF mucins and the increased affinity of *P. aeruginosa* to CF mucus [16]. Accordingly, the flagellar cap protein, FliD, from PAO1 strain demonstrated a clear association with sulfosialyl-Lewis x, as well as with other Lewis x derivatives [17].

Structural analysis of sulfated compounds is impaired by the difficulty to purify them from complex mixtures of mucin-type O-glycans. Indeed, anion exchange chromatography cannot easily discriminate sulfated compounds from other acidic compounds such as sialic acid and uronic acid containing O-glycans, which renders subsequent analyses by chromatographic or spectroscopic methods difficult. An elegant method for separating sialylated from sulfated mucin type O-glycans in mixture based on on-column carboxy-methylation of sialic acids was previously proposed [18]. The conversion of sialic acids to methyl esters after collection of neutral glycans permitted to elute them from anion exchanger independently of the sulfated compounds. Although very efficient, this method involves the chemical modification of sialic acids which are then lost for possible later use in biological tests. Furthermore, the necessity to use fairly large amounts of harmful iodomethane and the high concentration of pyridinium acetate for elution of sulfated oligosaccharides is not compatible with possible large scale purification of sulfated glycans from natural sources. Here we propose an alternative procedure aimed to purifying intact sulfated oligosaccharides in batch from complex mixtures of neutral and acidic O-linked glycans. It is based on the charge repulsiveness between sulfate groups of carbohydrates and strong cation exchanger gel and enables an easy one step purification of all sulfate containing glycans.

Material and methods

Preparation of mucins

Amphibian egg-jelly coats were extracted from intact eggs into Dulbecco's phosphate buffered saline (Sigma) containing 10 mM EDTA, 1 mM PMSF and 0.5% 2-mercaptoethanol at 4°C overnight. The mixture was centrifuged and the supernatant was then dialysed for 72 h against water and finally freeze dried. Tracheobronchial mucins were isolated from a CF patient and were a kind gift of Prof. Philippe Roussel. They were prepared according to published methods [2].

Isolation of oligosaccharide–alditols

As starting materials we used mucins from egg-jelly of *Rana temporaria* (1 g), *Pleurodeles waltl* (200 mg) and human tracheobronchial mucins (less than 100 μg). Samples were submitted to reductive β-elimination for 72 h at 37°C in 100 mM NaOH containing 1 M $NaBH_4$. The reaction was stopped by the addition of DOWEX 50× 8 (25–50 mesh, H^+ form; Sigma-Aldrich) at 4°C until pH 6.5 is reached. After filtration on glass wool and evaporation to dryness, boric acid was eliminated by repetitive distillation as its methyl ester in the presence of methanol. The material was submitted to a first cationic exchange chromatography on DOWEX 50×2 (200–400 mesh, H^+

form) in order to remove residual peptides. Total eluate from the first DOWEX 50×2 column (five column volumes) was concentrated and applied to a second Dowex 50×2 column (200–400 mesh, H^+ form) to purify sulfated oligosaccharides.

Thin layer chromatography

Elution from cation exchange column was assessed by TLC chromatography. An aliquot of each fraction was run on silica gel 60 thin layer chromatography (Merck) in *n*-butanol/ethanol/Acetic acid/Pyridine/H_2O (10:100:3:10:30). Samples were detected following staining with orcinol reagent and charring.

Permethylation of oligosaccharide–alditols

Glycan samples were permethylated using the NaOH/ dimethyl sulfoxide slurry method [19], with modifications on the subsequent extraction method for sulfate-containing oligosaccharides. Briefly, the reaction mixture was neutralized with 4N TFA before solid phase extraction on a Sep-pack C18 column. Permethylated oligosaccharides were eluted with ACN/ H2O 0.1% TFA (25:75).

Mass spectrometry analysis

Native and permethylated samples were analysed on a Voyager-DE STR MALDI-Tof Mass Spectrometer, (PerSeptive Biosystems, Framingham, MA, USA), equipped with a 337 nm UV laser. Samples were spotted by mixing directly on the target 1 μl of oligosaccharide solution and 1 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/mL dissolved in MeOH/H_2O 0.1% trifluoroacetic acid). For tandem mass spectrometry, native and permethylated oligosaccharides were mixed 1:1 with α-cyano-4-hydrocinnamic acid matrix (in acetonitrile 0.1% trifluoroacetic acid, *v*:*v*) and analysed on a Q-Tof Ultima MALDI instrument (Micromass). The nitrogen UV laser (337 nm wavelength) was operated at a repetition rate of 10 Hz under full power (300 μJ/pulse). MS survey data were manually acquired and the decision to switch over to CID MS/MS acquisition mode for a particular parent ion was made on-the-fly upon examination of the summed spectra. Argon was used as the collision gas with a collision energy manually adjusted (between 50~200 V) to achieve optimum degree of fragmentation for the parent ions under investigation.

Sulfate content analysis

The sulfate content was measured by HPAEC according to [13]. Briefly, sulfate was released by hydrolysis with 1 M HCl (500 μl) for 5 h at 100°C and HCl was evaporated under a stream of nitrogen. The dry residue was dissolved in 200 μl of Milli-Q quality water (Millipore Corp., Milford, MA). 25 μL of this solution were directly injected onto a Dionex BioLC system equipped with an IonPac AS4A column (250×4 mm), an anion micromembrane suppressor, and a CDM 2 conductivity detector. The column was eluted with 0.04 M NaOH at a flow rate of 2 mL/min, and the separated anions were measured by conductivity detection with 30-microsiemens sensitivity. The chromatograms were analysed and integrated with Chromeleon software, version 6.40 (Dionex Corporation). A standard curve was constructed with K_2SO_4 solutions (6.25, 12.5, 25, and 35 μg/mL) to measure sulfate released from oligosaccharide fractions.

Results and discussion

Purification of sulfated compounds

O-linked oligosaccharide alditols were released from several biological sources by reductive β-elimination. For each sample, borates were co-evaporated with methanol and samples were passed on a first Dowex 50×2 cation exchange column (Sigma-Aldrich) to eliminate residual peptides. Columns were eluted with five column volumes of water and total eluates containing all sulfated and neutral oligosaccharides from each sample were freeze-dried. This step was not used to separate glycans, but to remove peptides that are bound on the column. Care was taken to immediately neutralize samples with diluted ammonia after cation exchange chromatography to protect eventual sialic acids from acid hydrolysis. It is noteworthy that eliminating residual peptides on a first column of Dowex 50×2 resin substantially improved the subsequent purification of sulfated oligosaccharides on a second cation exchanger. So, this step was systematically included in the workflow (data not shown).

Three samples were used in the course of the present study: two egg-jelly mucus from the amphibians *R. temporaria* and *P. waltl* and a sample of human tracheo-bronchial mucus. Oligosaccharides from amphibians have been extensively characterized so their glycosylation profiles are already well known and can be used to evaluate the effectiveness of the separation methodology. Total oligosaccharide samples devoid of peptides were directly loaded onto a second 50×2 (200–400 mesh H^+ form) without prior desalting and eluted with water. Column size was adapted depending on the total quantity of carbohydrate for each sample that ranged from about 200 mg for *R. temporaria* to less than 100 μg for tracheo bronchial O-glycans. During the course of the study, an average of 10 mL of resin per mg of carbohydrates was used to obtain

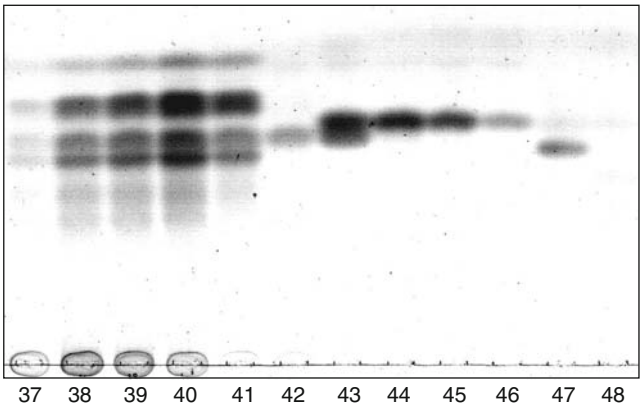


Fig. 1 Fractionation on a strong cation exchange Dowex 50×2 (200–400 mesh H^+ form) chromatography column of total O-glycans released by reductive β-elimination from egg jelly coat of *R. temporaria* eggs, monitored by TLC

a satisfactory resolution of separation between sulfated and non-sulfated glycans. Furthermore, irrespective of column size, columns with highest length to diameter ratios were used in order to maximize the separation efficiency.

O-glycans from *R. temporaria*

About two hundred mg of O-linked oligosaccharides were released and purified from one gram of dried egg jelly coat of the amphibian *R. temporaria*. This species synthesises a very complex mixture of neutral and acidic O-glycans (http://glycobase.univ-lille1.fr/). Acidic properties are conferred by the presence of either sulfate groups, sialic acid (Kdn) or glucuronic acid (GlcA) substituents. Some acidic glycans may be substituted by two different acidic groups. The sheer complexity of this sample appeared as ideal for assessing the selectivity of separation on cation exchange chromatography of different acidic compounds.

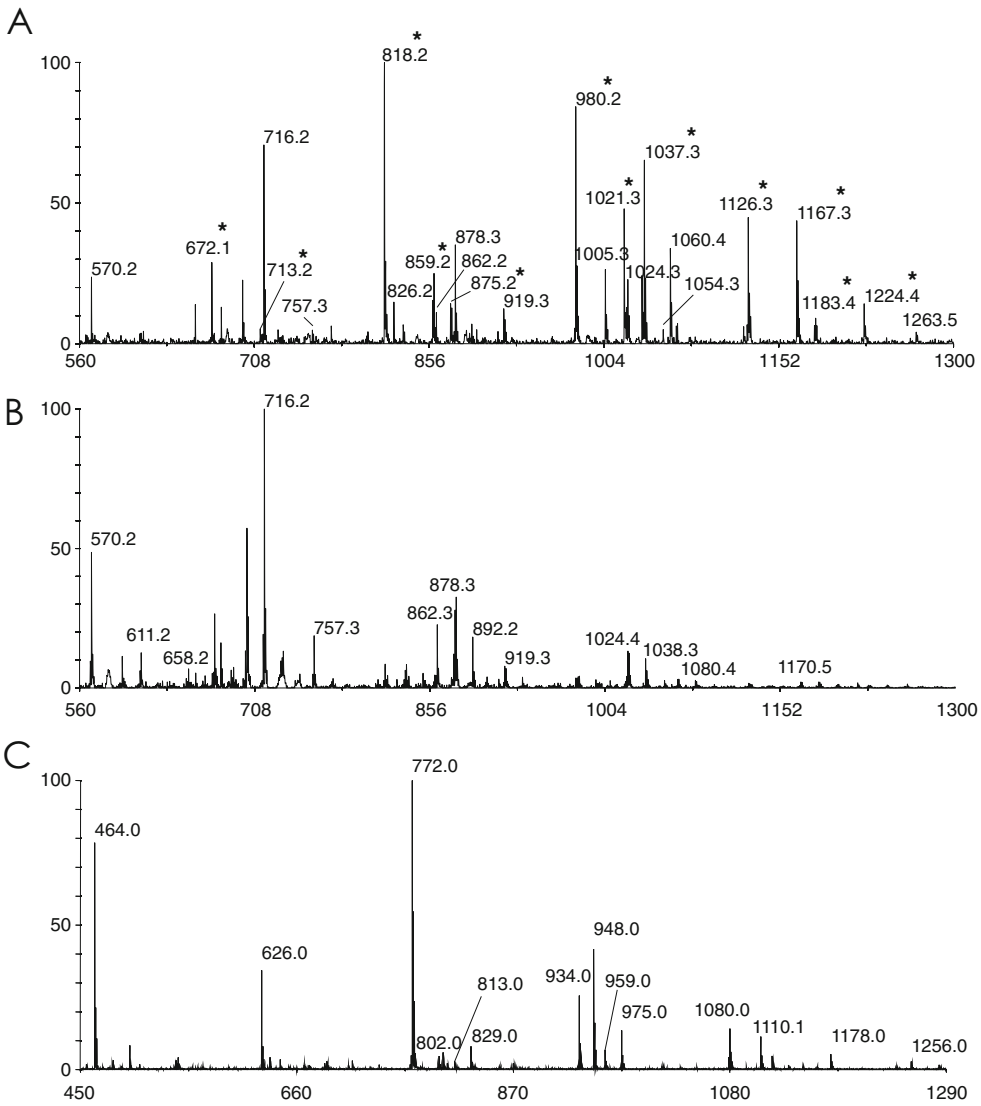
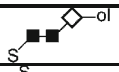
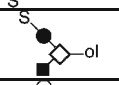
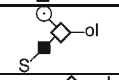
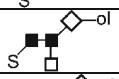
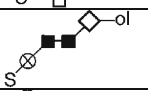
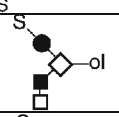
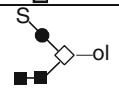
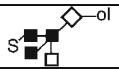
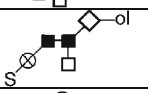
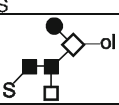
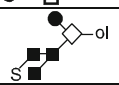
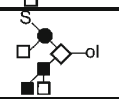
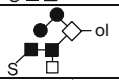
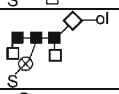
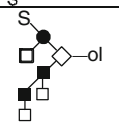


Fig. 2 Mass spectrometry analysis of glycans from *R. temporaria* released by reductive β-elimination. Positive reflectron mode MALDI-TOF spectrum of native **a** excluded fraction and **b** included fraction on a strong cation exchange chromatography. Asterisks labelled *m/z* values correspond to $[M+2Na–H]^+$ molecular ions of sulfated oligosaccharides, the others to $[M+Na]^+$ molecular ions of desulfated neutral oligosaccharides (in **a**) or genuine neutral oligosaccharides (in **b**). **c** negative reflectron mode MALDI-TOF spectrum of native excluded fraction; all *m/z* values correspond to $[M–H]^-$ molecular ions of sulfated oligosaccharides. For predicted compositions, see Tables 1 and 2

Table 1 Assignment of major oligosaccharides released from *Rana temporaria* mucins observed by MALDI-TOF MS in excluded fraction, in positive mode as (*a*) $[M+2Na–H]^+$ adducts and (*b*) negative modes as $[M–H]^-$ adducts. Individual structures were inferred from previous studies [20, 21, 22, unpublished results] and confirmed by ES-MS/MS fragmentation

(A) Excluded fraction.

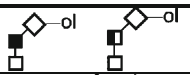
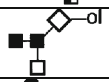
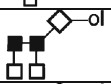
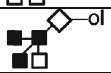
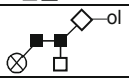
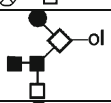
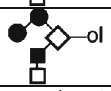
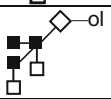
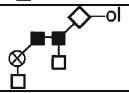
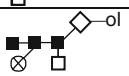
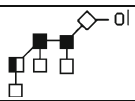
m/z a	m/z b	Calculated compositions	Structures
672.1	626.0	S Hex_2 HexNAc-ol	
713.2	667.0	S HexNAc Hex HexNAc-ol	
760.2	714.0	S Kdn Hex HexNAc-ol	
818.2	772.0	S dHex Hex_2 HexNAc-ol	
848.2	802.0	S HexA Hex_2 HexNAc-ol	
859.2	813.0	S dHex HexNAc Hex HexNAc-ol	
875.2	829.0	S HexNAc Hex_2 HexNAc-ol	
980.2	934.0	S dHex Hex_3 HexNAc-ol	
	948.0	S dHex HexA Hex_2-HexNAc-ol	
1021.2	975.0	S deHex HexNAcHex_2 HexNAc-ol	
1037.3		S HexNAc Hex_3 HexNAc-ol	
1126.3	1080.0	S $dHex_2$ Hex_3 HexNAc-ol	
1167.3	1121.1	S $dHex_2$ HexNAc Hex_2 HexNAc-ol	
1183.4		S dHex HexNAc Hex_3 HexNAc-ol	
1224.4	1178.0	S dHex $HexNAc_2$ Hex_2 HexNAc-ol	
1302.4	1256.0	S $dHex_2$ HexA Hex_3 HexNAc-ol	
1313.5		S $dHex_3$ HexNAc Hex_2 HexNAc-ol	

Structures are depicted according to the nomenclature of Kamerling and Vliegenthart [25]:◇—ol, GalNAc-ol;◆, β-GalNAc;  , *empty diamond*; α-GalNAc; *filled circle*, β-GlcNAc; *left-half-filled circle*, α-GlcNAc; *filled square*, β-Gal; *left-half-filled square*, α-Gal; *empty circle with center dot*, α-Kdn; *empty square*, α-Fuc; *empty circle with x*, β-GlcA

Total glycan sample was loaded on a Dowex 50×2 column (200–400 mesh, H + form; 150×2.5 cm) and was eluted with water at 0.2 ml/min; 2 ml fractions were collected. Considering the large quantity of carbohydrates, the elution of carbohydrate was monitored by orcinol-sulfuric colorimetric reaction after migration of all fractions on Thin Layer Chromatography (TLC). This visual method allowed an easy discrimination of different oligosaccharidic fractions according to their migration. As shown on Fig. 1, a carbohydrate rich fraction was first excluded from the column in tubes 37 to 42, which corresponds to the dead volume of the column. This excluded material exhibited a very heterogeneous pattern of migration on TLC, establishing that it is composed of a complex mixture of

Table 2 Assignment of major oligosaccharides released from *Rana temporaria* mucins observed by MALDI-TOF MS in included fraction in positive mode as $[M+Na]^+$ adducts. Individual structures were inferred from previous studies [20, 21, 22, unpublished results] and confirmed by ES-MS/MS fragmentation

(B) Included fraction

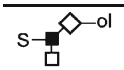
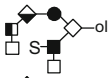
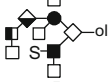
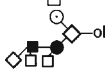
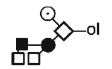
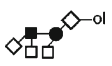
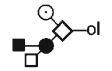
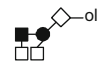
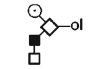
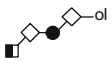
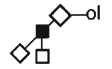
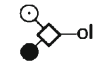
m/z	Calculated compositions	Structures
554	dHex Hex HexNAc-ol	
570	Hex_2 HexNAc-ol	
611	Hex HexNAc HexNAc-ol	
658	Kdn Hex HexNAc-ol	
716	dHex Hex_2 HexNAc-ol	
757	dHex HexNAc Hex HexNAc-ol	
862	$dHex_2$ Hex_2 HexNAc-ol	
878	dHex Hex_3 HexNAc-ol	
892	dHex HexA Hex_2 HexNAc-ol	
919	dHex Hex_2 HexNAc HexNAc-ol	
960	dHex Hex $HexNAc_2$ HexNAc-ol	
1024	$dHex_2Hex_3$ HexNAc-ol	
1038	$dHex_2HexAHex_2$ HexNAc-ol	
1054	dHex HexA Hex_3 HexNAc-ol	
1170	$dHex_3$ Hex_3 HexNAc-ol	

Structures are depicted according to the nomenclature of Kamerling and Vliegenthart [25]: ◇—ol, GalNAc-ol; ◆, β-GalNAc;  , *empty diamond*; α-GalNAc; *filled circle*, β-GlcNAc; *left-half-filled circle*, α-GlcNAc; *filled square*, β-Gal; *left-half-filled square*, α-Gal; *empty circle with center dot*, α-Kdn; *empty square*, α-Fuc; *empty circle with x*, β-GlcA

oligosaccharides of different sizes. Then, retarded carbohydrates were eluted from tube 43 onward. Orcinol positive fractions (tubes 43 to 76) were all pooled as a single included fraction for analysis. Excluded and included material show very distinct chromatographic patterns establishing that both fractions essentially contain different oligosaccharides.

The oligosaccharide content of each fraction was screened by MALDI-TOF MS in both positive and negative modes. Monosaccharide compositions of individual signals were calculated according to the observed *m*/*z* values and the nature of each compound was inferred from previously described oligosaccharides isolated from *R. temporaria* egg jelly coat (20, 21, 22, unpublished results) and confirmed by ESI-MS/MS fragmentation of most oligosaccharides (data not shown). As expected, MS profiles in positive mode of excluded and included fractions are different (Fig. 2). The included fraction is characterized by a complex pattern of $[M+Na]^+$ signals attributed to neutral and acidic oligosaccharides as presented on Tables 1 and 2. Identification of these oligosaccharides established that the acidic glycans are substituted either by Kdn or by GlcA, but never by sulfate group. In contrast, the profile of excluded fraction was characterized by the presence of $[M+2Na–H]^+$ signals attributed to the sulfate containing oligosaccharides previously described in *R. temporaria* jelly coat. This assignment was confirmed by acquiring MS spectrum in negative mode, which produced a series of $[M–H]^-$ ions of composition identical to their $[M+2Na–H]^+$ counterparts. It is noteworthy that none of the signals attributed to sulfated glycans could be observed in the included fraction. Positive mode MS spectrum of excluded fraction additionally shows

Table 3 Elution profile of oligosaccharide–alditols from *Pleurodeles waltl* mucin after cationic exchange column assessed by positive mode MALDI-TOF MS of each fraction 26 to 45 as (*a*) $[M+2Na–H]^+$ and (*b*) $[M+Na]^+$ adducts

	a	b	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
	656		+	+	+	+	+	+	+	+	+	+	+									
	1370		+	+	+	+	+	+	+	+	+											
	1516		+	+	+	+	+	+	+	+												
		1356										+	+	+	+	+	+	+	+	+		
		1210										+	+	+	+	+	+	+	+	+		
		1153															+	+				
		1106								+	+	+	+	+	+	+	+	+	+	+		
		960									+	+	+	+	+	+	+	+	+	+	+	
		1007																+	+	+		
		903													+	+	+	+	+	+	+	
		804														+	+					
		814												+	+		+	+	+	+		
		757													+	+	+	+	+	+		
		699																			+	+

Individual structures were inferred from previous studies [23, 24] and confirmed by ES-MS/MS fragmentation

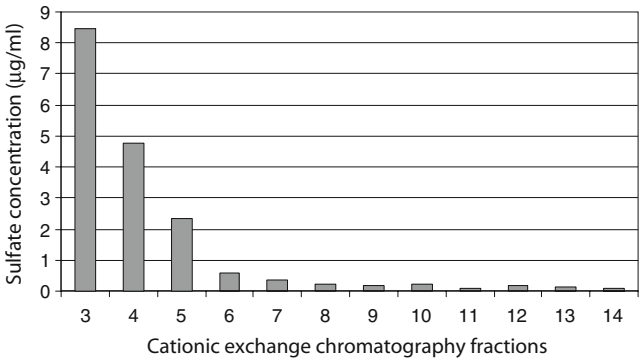


Fig. 3 Sulfate content of oligosaccharide fractions released from human tracheobronchial mucin after separation on strong cation exchange chromatography

numerous $[M+Na]^+$ ions attributed to non-sulfated oligosaccharides that have already been observed in the included fraction. As will be demonstrated in the next section, these ions resulted from a laser-induced desulfation process. Indeed, most of the $[M+2Na–H]^+$ major signals were accompanied by a signal at 102 mass units lower, corresponding to loss of a sodium sulfite moiety, *e.g.* *m*/*z* 672/570, 818/716, 859/757 and 980/878.

These data demonstrated that strong cation exchange chromatography enables a complete exclusion of sulfate containing oligosaccharides from the column. We hypothesise that repulsion forces between sulfate groups substituting the oligosaccharides and sulfonic acid functional groups attached to the styrene divinylbenzene resin prevent sulfated glycans to penetrate copolymer lattice and exclude them from the column, in contrast to other glycans, which are retarded in the polymer. Optimal separation between excluded and included fractions are obtained when using highest porosity (2% crosslinkage) and lowest particle size (200–400 mesh) resin, which maximizes the retardation of included fraction. So, Dowex 50×2 200×400 mesh (Sigma-Aldrich) and AG 50×2 200×400 mesh (Bio-Rad) appeared as the most suitable resins. The presence of non sulfated GlcA and Kdn containing oligosaccharides in the included fraction established that carboxylated functions are not excluded from the resin, which permits an easy separation between acidic oligosaccharides exclusively based on the presence of sulfate groups.

O-glycans from *P. waltl*

In a second step, we assessed the usefulness of the described method for purifying minute amounts of sulfated oligosaccharides from very complex mixture of non-sulfated glycans. Contrarily to the amphibian *R. temporaria* that synthesises large amounts of sulfated oligosaccharides, oviductal mucins of *P. waltl* are almost exclusively substituted by neutral and sialylated oligosaccharides, as previously established [23, 24]. However, careful re-evaluation of the oligosaccharidic content of *P. waltl* permitted the isolation, by multidimensional HPLC, of single sulfated glycans as a very minor component, establishing the presence of small amounts of sulfate substitutions (Supplementary Fig. 1; unpublished data).

Total O-glycans released by reductive β-elimination from 200 mg of *P. waltl* were separated on a Dowex 50×

Table 4 Assignment of major human tracheobronchial oligosaccharides observed in excluded fraction 3 as $[M–H]^-$adduct by negative mode MALDI-TOF MS and included fraction 12 as $[M+Na]^+$ adduct by positive mode MALDI-TOF MS

m/*z*	Calculated compositions
Excluded fraction 3 as $[M–H]^-$adduct by negative mode MALDI-TOF MS	
813	S dHex HexNAc Hex HexNAc-ol
829	S HexNAc Hex_2 HexNAc-ol
975	S dHex HexNAcHex_2 HexNAc-ol
1040	NeuAc HexNAc Hex_2 HexNAc-ol
1121	S $dHex_2$ HexNAc Hex_2 HexNAc-ol
1137	S dHex HexNAc Hex_3 HexNAc-ol
1178	S dHex $HexNAc_2$ Hex_2 HexNAc-ol
1186	NeuAc dHex HexNAc Hex_2 HexNAc-ol
1283	S $dHex_2$ HexNAc Hex_3 HexNAc-ol
1340	S dHex $HexNAc_2$ Hex_3 HexNAc-ol
1486	S $dHex_2$ $HexNAc_2$ Hex_3 HexNAc-ol
1632	S $dHex_3HexNAc_2Hex_3$ HexNAc-ol
1648	S $dHex_2HexNAc_2Hex_4$ HexNAc-ol
1794	S $dHex_3HexNAc_2Hex_4$ HexNAc-ol
1851	S $dHex_2HexNAc_3Hex_4$ HexNAc-ol
1997	S $dHex_3HexNAc_3Hex_4$ HexNAc-ol
Included fraction 12 as $[M+Na]^+$ adduct by positive mode MALDI-TOF MS	
757	dHex HexNAc Hex HexNAc-ol
773	HexNAc Hex_2 HexNAc-ol
862	$dHex_2$ Hex_2 HexNAc-ol
878	dHex Hex_3 HexNAc-ol
919	dHex HexNAc Hex_2 HexNAc-ol
935	HexNAc Hex_3 HexNAc-ol
976	$HexNAc_2$ Hex_2 HexNAc-ol
1024	$dHex_2$ Hex_3 HexNAc-ol
1065	$dHex_2$ HexNAc Hex_2 HexNAc-ol
1081	dHex HexNAc Hex_3 HexNAc-ol
1122	dHex $HexNAc_2$ Hex_2 HexNAc-ol
1138	$HexNAc_2$ Hex_3 HexNAc-ol
1211	$dHex_3$ HexNAc Hex_2 HexNAc-ol
1227	$dHex_2$ HexNAc Hex_3 HexNAc-ol
1243	dHex HexNAc Hex_4 HexNAc-ol
1284	dHex $HexNAc_2$ Hex_3 HexNAc-ol
1373	$dHex_3$ HexNAc Hex_3 HexNAc-ol
1389	$dHex_2$ HexNAc Hex_4 HexNAc-ol
1430	$dHex_2$ $HexNAc_2$ Hex_3 HexNAc-ol
1446	dHex $HexNAc_2$ Hex_4 HexNAc-ol
1576	$dHex_3$ $HexNAc_2$ Hex_3 HexNAc-ol
1592	$dHex_2$ $HexNAc_2$ Hex_4 HexNAc-ol
1649	dHex $HexNAc_3$ Hex_4 HexNAc-ol

2 column (200–400 mesh, H^+ form; 50×2 cm) under similar conditions to those above and collected as 2 mL fractions. Elution profile of oligosaccharides was directly assessed by MALDI-TOF MS analysis of each eluted fraction. As shown in Table 3, three oligosaccharides observed as signals at *m/z* 656, 1370 and 1516 are clearly excluded from the cation exchange column in fractions 26 to 34, whereas most other compounds are eluted from fraction 34 to fraction 45. Signals at *m/z* 656, 1370 and 1516 observed in the excluded fractions were attributed to $[M+2Na–H]^+$ adducts of $SO_3deHex_1Hex_1HexNAc_1$-ol, $SO_3deHex_2Hex_2HexNAc_3$-ol and $SO_3deHex_3Hex_2HexNAc_3$-ol, respectively. ^1H NMR analysis of the pooled excluded fraction (data not shown) established the exact

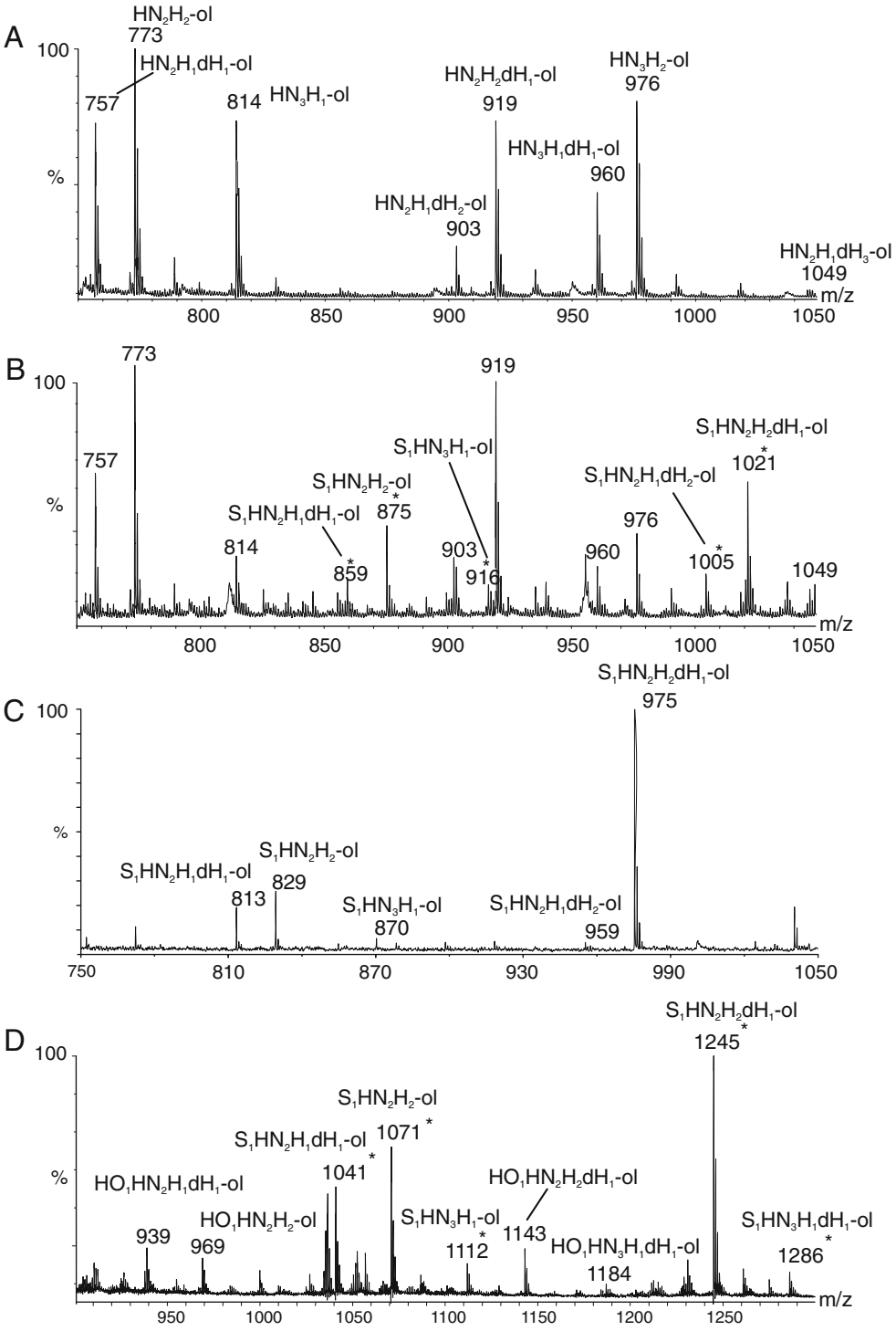


Fig. 4 Details of mass spectrometry analyses of O-glycans released from human bronchial mucins. Positive-mode MALDI-TOF spectra of **a** the native neutral fraction, **b** the native sulfated fraction and **d** the permethylated sulfated fraction. **c** Negative mode MALDI-TOF spectra of the native sulfated fraction. Asterisks labelled *m/z* values correspond to disodiated ions of sulfated oligosaccharides. For predicted compositions, see Table 4

structure of its major component as a core 1 based sulfated trisaccharide Fuc(α1–2)[SO_3(4)]Gal(β1–3)GalNAc-ol, in agreement with the presence of a signal at *m/z* 656 in MS analysis. Signals at *m/z* 1370 and 1516 were identified as very minor components whose structures were tentatively assigned to sulfated trisaccharide extended from GalNAc-ol C6 with Fuc(α1–2)GalNAc(β1–4)GlcNAc(β1- and Fuc (α1–2) GalNAc(β1–4)[Fuc(α1–3)]GlcNAc(β1-branches, respectively. It is noteworthy that all sulfated oligosaccharides are excluded in the same fractions, irrespective of their molecular weights, which demonstrates that their elution volume is exclusively dependent on the presence of sulfate group. Signals observed in included fractions were all attributed to $[M+Na]^+$ adducts of neutral and sialylated oligosaccharides whose structures were assigned based on previous work (Strecker 1992 a and b; unpublished work) and in accordance with their calculated compositions. In contrast to the sulfated compounds, the elution volumes of included compounds are dependent on their molecular weight, which demonstrates that the cation exchanger resin acts as a molecular sieve for neutral and sialylated oligosaccharides. These data established that the cation exchange chromatography is useful for purifying minor sulfated oligosaccharides among a complex mixture of non-sulfated compounds.

Human tracheobronchial mucins

In a last step, we validated the method by analysing minute amounts of oligosaccharides isolated from human tracheobronchial mucins. Less than 100 μg of reduced O-glycans released from purified tracheobronchial mucins by reductive β-elimination were loaded on 150 μL of DOWEX 50× 2 resin (200–400 mesh, H^+ form) packed in a 200 μL automatic pipette cone. Column was eluted with water and 25 μL fractions were collected. Elution of sulfated oligosaccharides was monitored by assessing sulfate concentration on a Dionex BioLC system fitted with an IonPac AS4A column and a conductivity detector. As expected,

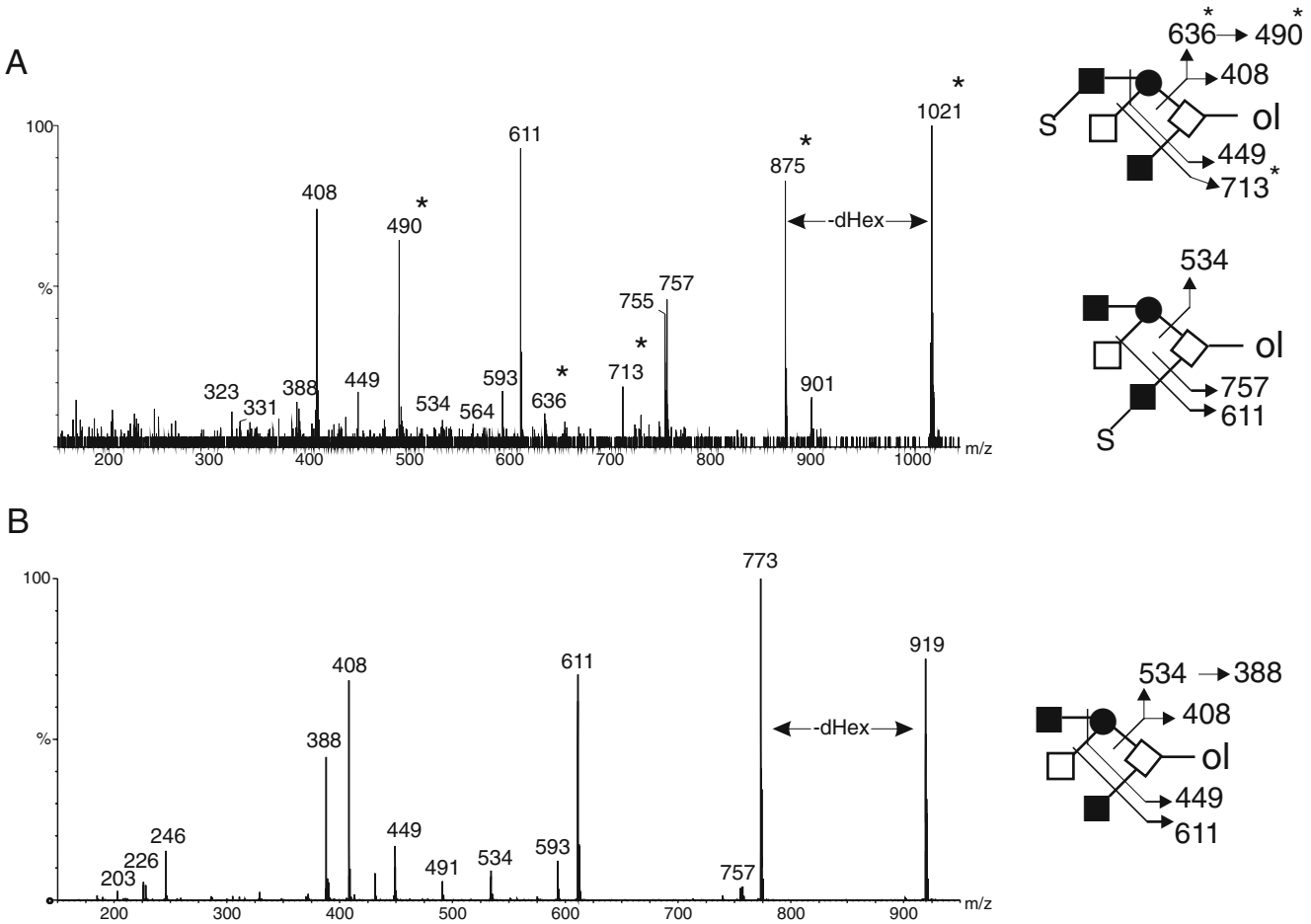


Fig. 5 Collision induced MALDI-Q-TOF fragmentation spectra of **a** $[M+2Na–H]^+$ parent ion at *m/z* 1021 of sulfated oligosaccharide $SO_3Fuc_1Gal_2GlcNAc_1GalNAc$-ol and **b** $[M+Na]^+$ parent ion at *m/z* 919 of on-target desulfated oligosaccharide $Fuc_1Gal_2GlcNAc_1Gal$ NAc-ol isolated from human bronchial mucin. Symbols are depicted in legend from Tables 1 and 2. *Asterisks* labelled *m/z* values correspond to $[M+2Na–H]^+$ molecular ions of the sulfated oligosaccharides. Fragmentation pattern of sulfated oligosaccharides demonstrate the presence of two different isobaric structures in mixture that only differ by the position of sulfation either on the upper or lower branch

sulfate concentration is maximal in fractions 3, 4 and 5, which correspond to the dead volume of the column (Fig. 3). As observed by MALDI-TOF MS, sulfate containing fractions are composed of a very complex mixture of almost exclusively sulfated oligosaccharides (Table 4). No neutral oligosaccharide was detected in sulfate containing fractions, but two minor sialylated oligosaccharides were observed (Table 4). Despite the low bed volume of resin, sulfated and non sulfated molecules were unexpectedly well resolved. Oligosaccharides were detected up to fraction 12, which is completely devoid of sulfated oligosaccharides and exclusively composed of neutral compounds (Table 4).

Analysis of sulfated glycans by MALDI-MS/MS

As mentioned above, monosulfated oligosaccharides crystallized in DHB matrix are observed as $[M+2Na-H]^+$ molecular ions in positive-mode MALDI-TOF MS spectra. As an example, the spectrum of a sulfated fraction of human bronchial O-glycans show five molecular ion signals at *m/z* 859, 875, 916, 1005 and 1021, corresponding to $[M+2Na-H]^+$ of sulfated oligosaccharides (Fig. 4b). Assignment was confirmed by observing their $[M-H]^-$ molecular ions at *m/z* 813, 829, 870, 959 and 975 in negative-mode (Fig. 4c). Furthermore, a series of monosodiated ions at *m/z* 773, 814, 903, 919, 960 and 976 were clearly observed in positive-mode (Fig. 4b), which correspond to the neutral equivalents of all sulfated oligosaccharides having loss a sodium sulfite moiety. All these signals were also observed in the neutral fraction of O-glycans (Fig. 4a), which suggests that they originate either from a contamination of the sulfated fraction by neutral oligosaccharides or from a partial desulfation of oligosaccharides during MALDI-TOF analysis. Positive-mode MALDI-TOF analysis of the permethylated derivatives of

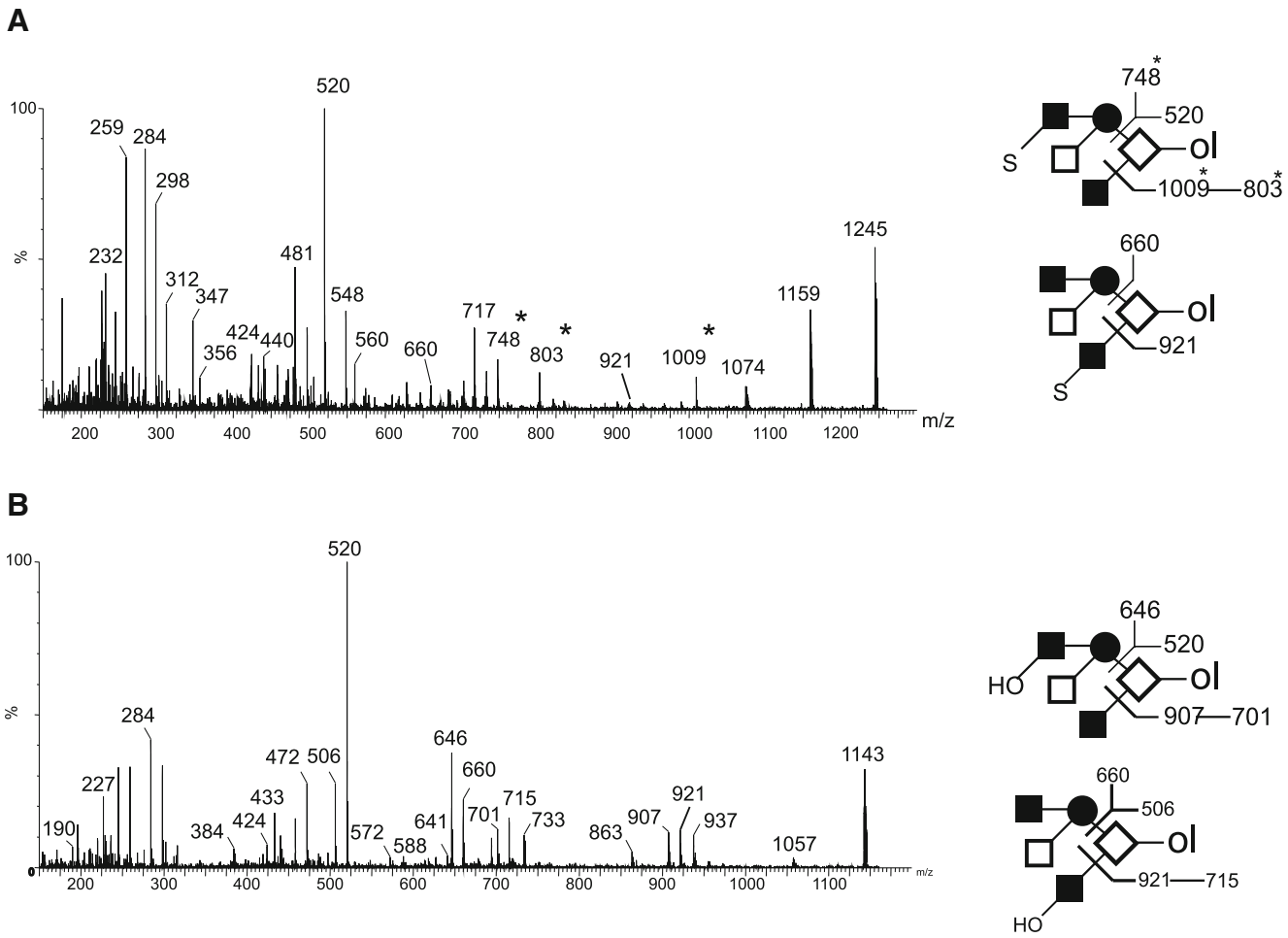


Fig. 6 Collision induced MALDI-Q-TOF fragmentation spectra of **a** $[M+2Na-H]^+$ parent ion at *m/z* 1245 of sulfated oligosaccharide $SO_3Fuc_1Gal_2GlcNAc_1GalNAc$-ol permethylated derivative and **b** $[M+Na]^+$ parent ion at *m/z* 1143 of on-target desulfated oligosaccharide $Fuc_1Gal_2GlcNAc_1GalNAc$-ol permethylated derivative isolated from human bronchial mucin. Fragmentation patterns of both parent ions demonstrate the presence of two different isobaric structures in mixture that only differ by the position of sulfation either on the upper or lower branch

sulfated fraction showed an identical set of sulfated oligosaccharides as $[M+2Na–H]^+$ at *m/z* 1041, 1071, 1112, 1245 and 1286 (Fig. 4d), each accompanied by signals corresponding to loss of sodium sulphite (*m/z* 939, 969, 1010, 1143 and 1184), as described above. These are the neutral equivalents of permethylated sulfated oligosaccharides in which a $–CH_3$ group has been replaced by a hydroxyl group, originating from an on-target desulfation process and not from genuine unsulfated molecules.

The detection of different sets of di- and mono-sodiated adducts enables an easy distinction of sulfated and non sulfated components in positive-mode MALDI-TOF MS spectra. This principle can be extended to MS/MS analyses of sulfated oligosaccharides that generate both mono- and disodiated ions in a single MS/MS spectrum, depending on the presence of sulfate group in the fragment ions. Indeed, as shown in the MS/MS spectrum of sulfated oligosaccharides (Fig. 5a and 6a), sulfated fragment ions, marked by an asterisk, are all characterized by being disodiated. As an example, fragmentation of parent ion at *m/z* 1021 (Fig. 5a) established the presence of a sulfo-Lewis x containing core 2 O-glycans $SO_3Fuc_1Gal_2GlcNAc_1GalNAc$-ol. Although many non-sulfated fragment ions are observed on the spectra, we believe that they do not originate from a secondary fragmentation induced desulfation, as demonstrated by the total absence of ion at *m/z* 773 potentially resulting from desulfation of primary fragment ion at *m/z* 875. This particular ion is however clearly observed in the MS/MS spectrum of the parent ion at *m/z* 919 that originates from the primary on-target desulfation of the sulfated oligosaccharide. A comparison of the MS/MS spectra of the permethylated derivatives of $SO_3Fuc_1Gal_2GlcNAc_1GalNAc$-ol at *m/z* 1245 with its desulfation product at *m/z* 1143 confirmed that all sulfate containing disodiated fragment ions are replaced by monosodiated fragment ions with a free hydroxyl group. Fragmentation of permethylated derivatives also confirmed the absence of desulfation during collision induced fragmentation.

Conclusion

The present data established the use of strong cation exchange resin as a reliable method for specifically purifying sulfated oligosaccharides from a very complex mixture of neutral, sialylated and sulfated components. The purification method, which is exclusively based on the presence of sulfate groups, is selective enough to extract very minor sulfated oligosaccharides. Although not exemplified in the present report, this method also permits to purify disulfated glycans which are co-eluted with monosulfated glycans (unpublished data). Furthermore, this method may be adapted for any quantity of purified glycans from the microgram scale up to at least hundreds of milligram. Indeed, we demonstrated that it may be used for large scale purification simply by adapting bed resin size, collection and detection procedures. It does not require any chemical modification of oligosaccharides of interest and thus can be used in the context of analysis of biologically relevant molecules. Furthermore, contrary to classical protocols of purification of acidic oligosaccharides based on anion exchange chromatography, sulfated molecules are eluted under salt free conditions, which permits an omission of a tricky desalting step.

References

1. Degroote, S., Ducourouble, M.P., Roussel, P., Lamblin, G.: Sequential biosynthesis of sulfated and/or sialylated Lewis x determinants by transferases of the human bronchial mucosa. Glycobiology **9**, 1199–1211 (1999)
2. Degroote, S., Maes, E., Humbert, P., Delmotte, P., Lamblin, G., Roussel, P.: Sulfated oligosaccharides isolated from the respiratory mucins of a secretor patient suffering from chronic bronchitis. Biochimie **85**, 369–379 (2003)
3. Lamblin, G., Degroote, S., Perini, J-M., Delmotte, P., Scharfman, A., Davril, M., Lo-Guidice, J-M., Houdret, N., Dumur, V., Klein, A., Roussel, P.: Human airway mucin glycosylation: a combinatory of carbohydrate determinants which vary in cystic fibrosis. Glycoconj. J. **18**, 661–684 (2001)
4. Spiro, R.G., Bhoyroo, V.D.: Occurrence of sulfate in the asparagine-linked complex carbohydrate units of thyroglobulin-identification and localization of galactose-3-sulfate and *N*-acetylglucosamine-6-sulfate residues in the human and calf proteins. J. Biol. Chem. **263**, 14351–14358 (1988)
5. Hooper, L.V., Beranek, M.C., Manzella, S.M., Baenziger, J.U.: Differential expression of GaINAc-4-sulfotransferase and GaINAc-transferase results in distinct glycoforms of carbonic anhydrase VI in parotid and submaxillary glands. J. Biol. Chem. **270**, 5985–5993 (1995)
6. Hård, K., Van Zadelhoff, C., Moonen, P., Kamerhing, J.P., Vliegenthart, J.F.C.: The Asn-linked carbohydrate chains of human Tamm-Horssfal glycoprotein of one male-novel sulfated and novel *N*-acetylgalactosamine containing N-linked carbohydrate chains. Eur. J. Biochem. **209**, 895–915 (1992)
7. Yuen, C.-T., Lawson, A.M., Chai, W., Larkin, M., Stoll, M.S., Stuart, A.C., Sullivan, F.X., Ahem, T.J., Feizi, T.: Novel sulfated ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among O-linked ohigosaccharides on an ovarian cystadenoma glycoprotein. Biochemistry **31**, 9126–9131 (1992)
8. Burger, D., Simon, M., Perruisseau, G., Steck, A.J.: The epitope (s) recognized by HNK-1 antibody and IgM paraprotein in neuropathy is present on several N-linked oligosaccharide structures on human P0 and myelin-associated glycoprotein. J. Neurochem. **54**, 1569–1575 (1990)
9. Fiete, D., Srivastava, V., Hindsgaul, O., Baenziger, J.U.: A hepatic reticuloendothelial cell receptor specific for $S0_4$–4GalNAcb1,4GlcNAcb that mediates rapid clearance of lutropin. Cell **67**, 1103–1110 (1991)
10. Hemmerich, S., Leffler, H., Rosen, S.D.: Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. J. Biol. Chem. **270**, 12035–12047 (1995)

11. Thomsson, K.A., Schulz, B.L., Packer, N.H., Karlsson, N.G.: MUC5B glycosylation in human saliva reflects blood group and secretor status. Glycobiology **15**, 791–804 (2005)
12. Lo-Guidice, J.M., Wieruszeski, J.M., Lemoine, J., Verbert, A., Roussel, P., Lamblin, G.: Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. J. Biol. Chem. **269**, 18794–18813 (1994)
13. Robbe, C., Capon, C., Maes, E., Rousset, M., Zweibaum, A., Zanetta, J.P., Michalski, J.C.: Evidence of regio-specific glycosylation in human intestinal mucins: presence of an acidic gradient along the intestinal tract. J. Biol. Chem. **278**, 46337–46348 (2003)
14. Thornton, D.J., Sheehan, J.K.: From mucins to mucus: toward a more coherent understanding of this essential barrier. Proc. Am. Thorac. Soc. **1**, 54–61 (2004)
15. Davril, M., Degroote, S., Humbert, P., Galabert, C., Dumur, V., Lafitte, J.J., Lamblin, G., Roussel, P.: The sialylation of bronchial mucins secreted by patients suffering from cystic fibrosis or from chronic bronchitis is related to the severity of airway infection. Glycobiology **9**, 311–321 (1999)
16. Scharfman, A., Delmotte, P., Beau, J., Lamblin, G., Roussel, P., Mazurier, J.: Sialyl-Le(x) and sulfo-sialyl-Le(x) determinants are receptors for *P. aeruginosa*. Glycoconj J. **17**, 735–740 (2000)
17. Scharfman, A., Arora, S.K., Delmotte, P., Van Brussel, E., Mazurier, J., Ramphal, R., Roussel, P.: Recognition of Lewis x derivatives present on mucins by flagellar components of *Pseudomonas aeruginosa*. Infect. Immun. **69**, 5243–5248 (2001)
18. Karlsson, N.G., Karlsson, H., Hansson, G.C.: Strategy for the investigation of O-linked oligosaccharides from mucins based on the separation into neutral, sialic acid- and sulfate-containing species. Glycoconj. J. **12**, 69–76 (1995)
19. Ciucanu, I., Kerek, F.: A simple and rapid method for the permethylation of carbohydrates. Carbohyd. Res. **131**, 209–217 (1984)
20. Maes, E., Florea, D., Delplace, F., Lemoine, J., Plancke, Y., Strecker, G.: Structural analysis of the oligosaccharide–alditols released by reductive b-elimination from oviducal mucins of *Rana temporaria*. Glycoconj. J. **14**, 127–146 (1997)
21. Florea, D., Maes, E., Strecker, G.: Primary structure of seven sulfated oligosaccharide–alditols released by reductive b-elimination from oviducal mucins of *Rana temporaria*. Characterization of the sequence HSO_3(3)GlcA(b1–3)Gal. Carbohydr. Res. **302**, 179–189 (1997)
22. Coppin, A., Maes, E., Morelle, W., Strecker, G.: Structural analysis of 13 neutral oligosaccharide–alditols released by reductive b-elimination from oviducal mucins of *Rana temporaria*. Eur J Biochem. **266**, 94–104 (1999)
23. Strecker, G., Wieruszeski, J.M., Michalski, J.C., Alonso, C., Boilly, B., Montreuil, J.: Characterization of Le(x), Le(y) and A Le(y) antigen determinants in KDN-containing O-linked glycan chains from *Pleurodeles waltl* jelly coat eggs. FEBS Lett. **298**, 39–43 (1992)
24. Strecker, G., Wieruszeski, J.M., Michalski, J.C., Montreuil, J.: ^{1}H- and ^{13}C-n.m.r. spectroscopy of 2-oxo-3-deoxy-D-glycero-D-galactononulosonic acid-containing oligosaccharide–alditols bearing Lewis X, Lewis Y and A-Lewis Y determinants isolated from the jelly coat of *Pleurodeles waltl* eggs. Biochem. J. **287**, 905–909 (1992)
25. Kamerling, J.P., Vliegenthart, J.F.G.: High-resolution ^{1}H-nuclear magnetic resonance spectroscopy of oligosaccharide–alditols released from mucin-type O-glycoproteins. Biol. Magn. Res. **10**, 1–288 (1992)