Sialic acids in gastropods

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Received 14 August 2001; revised 29 September 2001; accepted 11 October 2001

First published online 24 October 2001

Edited by Hans-Dieter Klenk

Abstract The occurrence of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid residues in preparations of the slug *Arion lusitanicus* (Gastropoda) was determined by sodium dodecyl sulphate electrophoresis of the proteins followed by lectin blots stained with the sialic acid specific lectin from *Maackia amurensis*, by the sensitivity of this binding to sialidase from *Clostridium perfringens*, by specific fluorescent labelling of sialic acids with 1,2-diamino-4,5-methylenedioxybenzene, by the determination of the sensitivity to sialate-pyruvate-lyase, by co-migration with standards on high performance anion exchange chromatography with pulsed amperometric detection and by identification of the typical masses in the fragmentation patterns of the trimethylsilyl derivatives after gas chromatography. It is the first time sialic acids are identified in gastropods.

Key words: Sialic acid; *N*-glycolylneuraminic acid; *N*-acetylneuraminic acid; Gastropod; *Arion lusitanicus*

1. Introduction

Sialic acids are a family of more than 40 naturally occurring derivatives of neuraminic acid. They are a common compound of deuterostoma and have been found in microorganisms and viruses generally in terminal position of oligosaccharide chains of glycoproteins and glycolipids on cell surfaces involved in a huge number of adhesion events and selective recognition processes of cells [1–3]. The occurrence of special variants seems to be highly species specific and dependent on the developmental stage.

In protostoma sialic acids have been encountered only occasionally. In insects they have been identified to be present in developing testis of *Galleria mellonella* [4] as well as in α2,8-linkage in a very early developmental stage of *Drosophila melanogaster* [5] and in larvae of the cicada *Philaenus spumarius* [6]. The controversial data on investigations of various cell cultures might reflect that sialylation in insects is at the most a very rare event in some early stages of development and that adult insects lack the ability to sialylate their glycoconjugates [7,8].

Only scattered data exist on the glycosylation abilities of mollusks. For mollusks up to now detailed glycan analysis is restricted to the *N*-glycans of the hemocyanins of the freshwater snail *Lymnea stagnalis* [9,10] and the land snail *Helix pomatia* [11,12]. No negative charged glycans of mollusks have been published. A few hints on sialylation were given by the detection of a small acidic fraction (about 3% of total carbohydrate) in *L. stagnalis* [9] and the observation of an increase of binding sites for peanut agglutinin after sialidase treatment on the surface of satellite glial cells of *H. pomatia* [13].

However, a number of sialic acid specific lectins from gastropod origin have been found so far [14,15] and enzymes from oyster releasing the compound 2-keto-3-deoxynonulosonic acid (KDN) have been described [16,17].

Here we report the determination of sialic acids in skin and viscera preparations of *Arion lusitanicus* (Gastropoda) by staining with sialic acid specific lectins on blots, HPLC analysis after chemical release and specific fluorometric labelling, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and by gas chromatography/mass spectrometry (GC/MS).

2. Materials and methods

2.1. Materials

Slugs were collected by Dr. Manfred Pintar (Department of Zoology, Universität für Bodenkultur, Wien, Austria) and his students in local gardens and were frozen immediately at −80°C. The reversed phase ODS column (4×250 mm, 5 μ) was purchased from Forschungszentrum Seibersdorf, Austria, the CarboPac PA-100 (4×250 mm) and the PA-100 guard columns were from Dionex. The column for GC was a HP-1 (0.25 μm×0.25 mm×30 m) column from Hewlett Packard.

Neuraminidase from *Clostridium perfringens* and sialate-pyruvate-lyase (*N*-acetylneuraminic acid (NeuAc) aldolase, E.C. 4.1.3.3) were from Sigma, peptide:*N*-glycosidase F was obtained from Roche Diagnostics, sodium hydroxide 50% (w/v) solution and sodium acetate were from Baker, the Tri-Sil reagent was from Pierce. The biotinylated lectins from *Maackia amurensis* agglutinin (MAA II) and *Sambucus nigra* agglutinin (SNA), as well as the horseradish peroxidase-conjugated streptavidin were from Vector laboratories. All other materials purchased were of the highest quality available from Merck or Sigma.

2.2. Preparation of the samples

Thawed slugs were washed to remove the extraneous mucous components and dissected into three fractions; the skin and inner organs (viscera) were lyophilised separately, while the intestinal tract was removed.

For electrophoreses and lectin blots the lyophilised material was homogenised in 0.05 M Tris/HCl buffer, pH 7.5 and centrifuged at 1000×*g* for 15 min to remove insoluble material.

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Abbreviations: DMB, 1,2-diamino-4,5-methylenedioxybenzene; GC/MS, gas chromatography/mass spectrometry; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; KDN, 2-keto-3-deoxynonulosonic acid; MAA, *Maackia amurensis* agglutinin; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; SNA, *Sambucus nigra* agglutinin

0014-5793/01/$20.00
PII: S0014-5793(01)03024-1

For HPAEC-PAD, GC/MS analysis and the 1,2-diamino-4,5-methylenedioxybenzene (DMB) method 1 g of the lyophilised material was homogenised with 30 ml of 4 M propionic acid and sialic acids were released by incubation at 80°C for 4 h [18]. The samples were cooled, ultrafiltrated (10 kDa cut-off, Amicon YM 10, Millipore, USA) and washed with water. The retained material was adjusted with HCl to attain a final concentration of 0.1 M and incubated for 1 h at 80°C for a complete release of the sialic acids from any kind of glycoconjugate and ultrafiltrated as above. All filtrates were lyophilised, dissolved in a small amount of water and purified by a cation (Dowex AG 50 W×8) and an anion (Dowex AG 1×8) exchange chromatographic step as previously described [19]. Adsorbed sialic acids were eluted from the latter resin with 2 M formic acid. The sialic acid-containing eluate was lyophilised.

2.3. Electrophoresis and lectin blots

The skin and viscera preparations were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a Bio-Rad Mini-Protean II Cell with gels containing 12.5% acrylamide and 1% bisacrylamide and electroblotted onto nitrocellulose [20]. The nitrocellulose sheets were stained either with amido black or were blocked with bovine serum albumin 1% in buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and subsequently incubated for 2 h with the biotinylated lectins diluted 1:200 in buffer. After washing the nitrocellulose membrane three times with buffer, bound lectins were detected by horseradish peroxidase-conjugated streptavidin, followed by colour detection using 4-chloro-1-naphthol as substrate [21].

Aliquots were incubated with sialidase from *Cl. perfringens* (100 μU of enzyme in 100 mM sodium acetate, pH 5.0) or *N*-glycosidase F (100 μU of enzyme in 50 mM Tris/HCl, pH 8.5) for 20 h at 37°C prior to electrophoresis.

2.4. DMB derivatisation and separation on HPLC

15.75 mg of DMB, 697 μl of 2-mercaptoethanol, 17.1 mg of $Na_2S_2O_5$ were dissolved in water to a final volume of 10 ml and stored into 100 ul aliquots at −20°C. Dry slug-derived samples were incubated in 40 μl 25 mM H_2SO_4 for 30 min at 80°C. 20 μl of this mixture was added to a 100 μl aliquot of the reagent and heated for 2.5 h at 60°C. The incubation was stopped on ice. After derivatisation, 10 μl of the sample was applied on a reversed phase ODS column and eluted isocratically in methanol:acetonitrile:water = 7:9:84 (v/v/v) at a flow rate of 1 ml/min. The derivatised sialic acids were detected with a flow-through fluorometer (emission and excitation wavelengths 448 nm and 373 nm respectively) [22]. The column was calibrated using authentic NeuAc, *N*-glycolylneuraminic acid (NeuGc) and KDN derivatised as described above. To check sensitivity to lyase dry samples were dissolved in 30 μl of 50 mM Tris/HCl buffer pH 7.5, and incubated with 25 mU lyase in the presence of 1 mM NADH for 1 h at 37°C prior to DMB derivatisation.

The amount of sialic acids was determined by integrating the HPLC peaks in comparison to a NeuAc and a NeuGc standard curve respectively. Whole sugar content was quantified by the orcinol-sulfuric acid method according to [23].

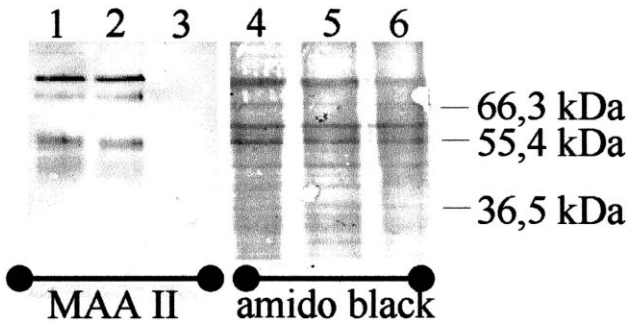

Fig. 1. Western blot of the viscera preparation stained with MAA II (lanes 1–3) or amido black (lanes 4–6); undigested viscera preparation (lanes 1 and 4), digested with *N*-glycanase F (lanes 2 and 5) or sialidase (lanes 3 and 6).

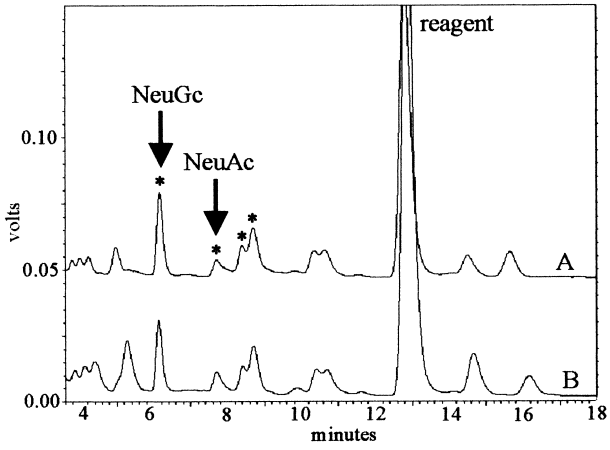

Fig. 2. HPLC pattern of DMB derivatives released by 0.1 M HCl (A) from skin and (B) from viscera. Peaks labelled with asterisks (*) are sensitive to lyase.

2.5. HPAEC-PAD analysis

HPAEC-PAD analyses of negatively charged sugars was carried out on a Dionex DX 500 chromatography system according to [24] with 100 mM NaOH as solvent A and 500 mM NaOAc in 100 mM NaOH as solvent B. Elution was carried out for 2 min with 100% solvent A followed by a linear gradient to 30% solvent B with an increase of 1% solvent B per min at a flow rate of 1 ml/min. NeuAc, NeuGc and KDN were used as standards.

2.6. GC/MS analysis

The sugars were converted into their trimethylsilyl derivatives [25] and GC/MS analysis was performed on a Hewlett Packard HP 6890 GC system coupled to a 5973 mass selective detector with splitless injection. The programme started with an initial temperature of 40°C held for 3 min, then increasing with a rate of 15°C/min to a final temperature of 230°C which was held for 10 min. The peaks were identified by their fragmentation patterns in comparison with a standard derived from fetuin. Identification was supported by automated identification using WILEY library (390 K library, contains 390 000 spectra, Agilent part nr. G1035B).

3. Results

The slug *A. lusitanicus*, which is a commonly occurring slug causing severe damage in European vegetable gardens, has been analysed for the occurrence of sialic acids by four independent methods.

Lectin blots of skin and viscera preparations after SDS–PAGE were carried out with the sialic acid specific lectins from MAA II (specific for α2,3-linkages), and SNA (specific for α2,6-linkages). Staining with MAA II was observed in both tissue preparations (Fig. 1), whereas SNA gave no significant results.

The MAA II binding could be completely inhibited by treatment with sialidase from *Cl. perfringens*. This enzyme was chosen for its broad specificity. Incubations with *N*-glycanase F revealed in a shift of one band compared with the undigested sample but there was no significant reduction of MAA II staining.

Whole tissue extracts of the slugs as well as ultrafiltrates after release with propionic acid and HCl were analysed by fluorescent labelling with DMB and compared with the retention times of NeuAc, NeuGc and KDN after the same derivatisation procedure (Fig. 2).

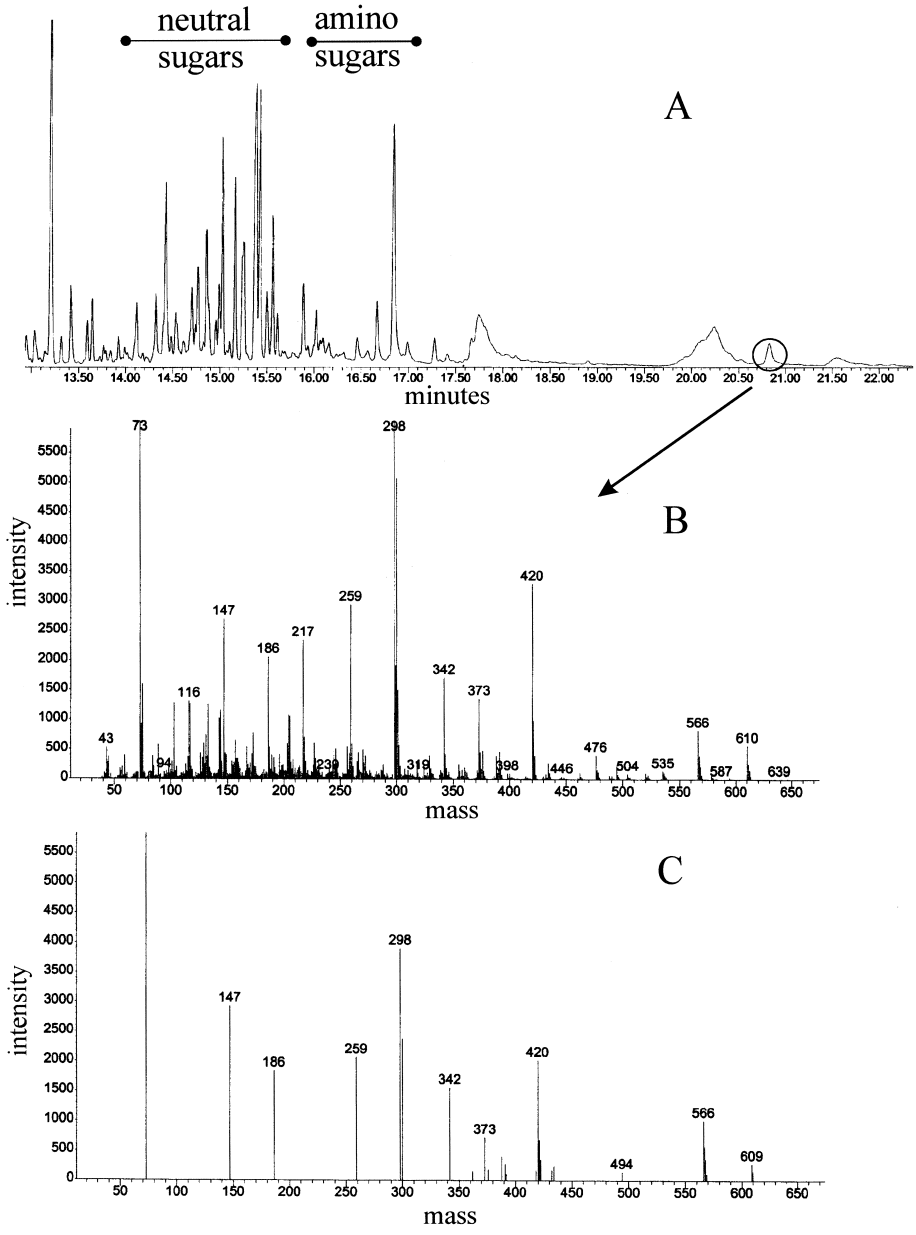

Fig. 3. GC/MS analysis of trimethylsilyl derivatives released by 0.1 M HCl from skin (A). Fragmentation pattern of the circled peak from A (B), fragmentation pattern of sialic acid from WILEY library (C).

In all samples NeuAc, as a minor compound, and NeuGc, as a major compound, could be identified. In order to confirm the identity of the DMB peaks as sialic acids, similar amounts were incubated with lyase prior to derivatisation with DMB. The patterns of the digests showed a complete loss of the small NeuAc peak and a reduction of the area of the NeuGc peak as well as in some other peaks. The different sensitivity of NeuAc and NeuGc to lyase has been described before [26]. No hints of KDN were observed.

The overall sialic acid content in each tissue was calculated by comparison of the HPLC areas with standard curves for NeuAc and NeuGc respectively, and by summation of the sialic acids released by propionic acid and by HCl. Peaks which were sensitive to lyase, but were not identified by co-migration with a standard are not included into this calculation. In both tissues the amount of sialic acids was about 3% of total sugar measured by the orcinol-sulfuric acid method. It has to be noted that most of the sialic acid from viscera could be released by propionic acid whereas the skin preparation was mainly desialylated by HCl.

Furthermore, the existence of NeuAc and NeuGc in acid liberated skin and viscera samples could be confirmed on a HPAEC-PAD system by co-elution with the appropriate standard sugars (data not shown).

Analysis of the trimethylsilyl derivatives of the acid liberated compounds on GC/MS showed a small peak, which was identified as sialic acid by the typical masses in its fragmentation pattern in comparison with standards derived from fetuin and was further confirmed in its identity by WILEY library (Fig. 3).

4. Discussion

In the course of this work the occurrence of sialic acid in *A. lusitanicus* was confirmed in all preparations obtained, by four independent methods. NeuAc and NeuGc could be identified. These sugars were found to be linked to glycoproteins and could be easily removed by *Cl. perfringens* sialidase. They were not released in the course of a digestion by *N*-glycosidase F. However, a linkage to *N*-glycans cannot be completely excluded as there are already some structures known which cannot be cleaved by this type of *N*-glycosidase [27].

Labelling with DMB, which has been proven to be a very sensitive and selective method for the determination of sialic acids [19], revealed, besides the expected NeuAc and NeuGc peaks, some further derivatives which were partly also sensitive to lyase. These molecules may be other members of the large sialic acid family. A co-migration with NeuAc and NeuGc standards on HPAEC-PAD and the determination of a peak with the typical mass fragments for sialic acids after the separation of the trimethylsilyl derivatives on GC completed the evidence supporting the presence of these relatively widespread compounds also in the gastropods.

Preliminary experiments on other kinds of snails (water and land species, with and without shell) showed that sialic acids are common for these organisms and occur in various variants and linkages. The identification of these negative charged sugars is currently under investigation.

Acknowledgements: This project was financed by the Austrian Fonds zur wissenschaftlichen Forschung Project number P13928-BIO. We want to thank Agilent Technologies for technical support and the opportunity to use their GC/MS, Dr. Manfred Pintar (Department of Zoology, Universität für Bodenkultur, Wien, Austria) for collecting, identification and classification of the slugs and Dr. Iain Wilson for reading the manuscript. The technical help of Susanna Eglseer is highly appreciated.

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