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Targeted release and fractionation reveal glucuronylated and sulphated N- and O-glycans in larvae of dipteran insects

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ABSTRACT

Mosquitoes are important vectors of parasitic and viral diseases with *Anopheles gambiae* transmitting malaria and *Aedes aegypti* spreading yellow and Dengue fevers. Using two different approaches (solid-phase extraction and reversed-phase or hydrophilic interaction HPLC fractionation followed by MALDI-TOF MS or permethylation followed by NSI-MS), we examined the N-glycans of both *A. gambiae* and *A. aegypti* larvae and demonstrate the presence of a range of paucimannosidic glycans as well as bi- and tri-antennary glycans, some of which are modified with fucose or with sulphate or glucuronic acid residues; the latter anionic modifications were also found on N-glycans of larvae from another dipteran species (*Drosophila melanogaster*). The sulphate groups are attached primarily to core α-mannose residues (especially the α1,6-linked mannose), whereas the glucuronic acid residues are linked to non-reducing β1,3-galactose. Also, O-glycans were found to possess glucuronic acid and sulphate as well as phosphoethanolamine modifications. The presence of sulphated and glucuronylated N-glycans is a novel feature in dipteran glycomes; these structures have the potential to act as additional anionic glycan ligands involved in parasite interactions with the vector host.

Mosquitoes are important vectors for a range of human and animal pathogens, including the malaria parasite *Plasmodium* [1] and the yellow fever, Dengue, Chikungunya and West Nile viruses [2–5]. As glycans coat both pathogen and host cell surfaces, many interactions between host and pathogen are glycan-mediated. In the case of malaria, attachment of the parasites to the gut and salivary glands of the mosquito vector are dependent on chondroitin and heparin sulphates, respectively [6,7], whereas heparin sulphate is also a 'receptor' for *Plasmodium* sporozoites in the mammalian liver [8]. However, mosquitoes are expected to have a range of cell surface glycoconjugates other than proteoglycans and these may also play roles in parasite transmission.

The basic repertoire of N- and O-glycans in insect species is well known with recent glycomic studies being centred on either the fruit fly *Drosophila melanogaster* or on the recombinant proteins expressed by insect cell lines [9,10]. In general, so-called paucimannosidic N-glycans (with three or fewer mannose residues) with and without fucosylation as well as standard oligomannosidic N-glycans dominate, whereas the O-glycomes primarily consist of mono- and disaccharides. Nevertheless, studies over the past ten years have shown that low levels of more complex N- and O-glycans can be found in insect tissues. Triantennary and sialylated N-glycans have been found [11], whereas glucuronylation is a feature of both O-linked 'mucin-type' and 'O-fucose' oligosaccharides [12,13]. Thus, it is apparent that procedures for analysing glycomes of insects should be 'open' to the possibility of finding more complicated neutral and anionic glycans.

We have developed different schemes suitable for analysing anionic glycans from either protist or insect sources [14,15]. In the former case, solid-phase extraction to separate neutral from anionic glycans was found to be important to detect sulphated N-glycans from *Dictyostelium* by off-line LC-MALDI-TOF MS in their native state [14], whereas the use of phase extraction after permethylation enabled detection by NSI-MS of sialylated and glucuronylated glycans from *Drosophila* as well as sulphated O-glycans from mammalian mucins [15,16]. In the present study, both approaches were employed in the analysis of the N- and O-glycans from the larvae of two mosquito species, the malaria vector *Anopheles gambiae* and the viral vector *Aedes aegypti*; thereby, we reveal a hitherto unknown complexity of the glycomes of both species.

1. Experimental procedures

1.1. Biological material

A. gambiae (Keele) were reared and maintained in an environmental chamber at 27 °C with a relative humidity ≥80% and a 12 h light/dark

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cycle. Eggs were hatched in distilled water, transferred to plastic pans and larvae were fed on a slurry of ground cat food (Purina cat chow). After 5 to 6 days of feeding, L3–L4 instar larvae were collected by centrifugation and stored at −80 °C. *Aedes aegypti* (Rockefeller) larvae were reared, dependent on the source laboratory either using a slurry of Vitakraft Premium VITA 'Flockenfutter für alle Zierfische' (22 102) or as for *A. gambiae*. *Drosophila* larvae were prepared by standard cultivation techniques. In order to determine whether any of the glycans detected in the mosquito larval samples originated from the food source, N-glycans were prepared from the cat food and the fish food and were analysed by NSI-MS and off-line MALDI-TOF MS respectively; thereby fragmentation patterns and elution times could be compared in order to check for any potential contaminating glycans in the larval samples.

1.2. N-glycan purification and MALDI-TOF/TOF MS analysis

Frozen larvae (approximately 1.5–2.0 g wet weight) were boiled in deionised water for 10 min before grinding in a mortar. Thereafter, the slurry was made up to a total volume of 10 mL prior to the addition of formic acid (up to 5% (v/v)) and 1 mg porcine pepsin (Sigma-Aldrich). After proteolysis, cation exchange and gel filtration chromatography were performed as previously described [17] and the N-glycans released using PNGase F (recombinant; Roche) as well as by subsequent PNGase A (from almonds; Roche) digestion of remaining glycopeptides. De-N-glycosylated glycopeptides were gel filtrated and subject to β-elimination prior to LC–MS of native O-glycans (see below, see section 1.7). The N-glycans were then subject to nonporous graphitized carbon (NPGC) chromatography for separation into neutral and anionic fractions [14]. As required, neutral N-glycans were further purified by a second solid-phase extraction using a Lichroprep RP18 cartridge column (25–40 μm; Merck). Pyridylamination was performed [17,18] and the fluorescently-labelled N-glycans were fractionated by HPLC using either an Ascentis® Express RP-Amide column (150 × 4.6 mm, 2.7 μm; Supelco) with a gradient of 0.3% methanol per minute at a flow rate of 0.8 mL/min, or an IonPac AS11 column (HIAX, Dionex) as described previously [14,19]. The control pyridylaminated triantennary N-glycan from foetal calf serum (the major component being fetuin) was purified by RP-HPLC as part of a previous study [17]. Collected fractions were lyophilized and reconstituted in water and analysed by MALDI-TOF/TOF MS in positive and negative ion modes (Bruker Autoflex Speed or Bruker UltrafleXtreme) with 6-aza-2-thiothymine (ATT) as matrix. The approximately 5500 MS and MS/MS spectra generated in this study were initially processed using the manufacturer's software (Bruker Daltonics FlexAnalysis 3.3.80) and then manually interpreted. Theoretical masses were calculated using the software GlycoWorkbench 2.0. The qualitative/semi-quantitative estimation of glycan amounts (from '+++' through 'trace') is based on HPLC fluorescent peak intensities; in case of multiple glycans in an HPLC fraction, a subestimation on the basis of MALDI-TOF MS data was made.

1.3. Exoglycosidase treatments

Further analysis of whole N-glycome pools or of selected HPLC fractions (see the Results section) by MALDI-TOF MS was performed after treatment overnight with either β-galactosidase (either *Xanthomonas manihotis* β1,3-galactosidase from NEB or recombinant *Aspergillus niger* lacA β1,4-galactosidase prepared in-house [20]), α-fucosidases (bovine kidney from Sigma-Aldrich or almond α1,3-specific from Prozyme), α-mannosidases (jack bean from Sigma, *Xanthomonas* α1,2/3-specific from NEB or *Xanthomonas* α1,6-specific from NEB), *Escherichia coli* β-glucuronidase (the kind gift of Megazyme; ultrafiltrated to remove some impurities before use) or β-hexosaminidase (either jack bean β-hexosaminidase from Sigma, β1,3/4-specific *Streptomyces* chitinase from NEB or recombinant *Apis mellifera* FDL β1,2-*N*-acetylglucosaminidase; prepared in-house [21]) in 25 mM ammonium acetate, pH 5.0 (pH 7.0 in the case of β-glucuronidase), at 37 °C overnight (three hours at 30 °C in the case of FDL, which under the conditions removes specifically the N-acetylglucosamine linked to the core α1,3-mannose). Desulphation was performed by solvolysis as described below; a previously-studied sulphated N-glycan from *Dictyostelium* [14] was used as a positive control. Generally, chemically or enzymatically treated glycans were analysed by MALDI-TOF MS without further purification.

1.4. Glycan permethylation and NSI-MS analysis

Permethylated N- and O-glycans from insect larvae were prepared as described [15]. Frozen larvae were homogenised in ice-cold 50% (v/v) aqueous methanol and delipidated with chloroform/methanol/water (4:8:3, v/v/v). Insoluble proteins were precipitated by centrifugation and the resulting pellet was washed with acetone to produce a fine protein powder, a portion of which was subject to trypsinisation. Tryptic peptides were purified on C18 cartridges (Baker C18) and digested with either PNGase F (Prozyme) or PNGase A (Calbiochem) prior to another round of C18 chromatography to separate released N-glycans from residual glycopeptides. Separately, 2–3 mg of protein powder were subject to reductive β-elimination and released oligosaccharide alditols were purified on a C18 cartridge column as for the N-glycans. Permethylation of enzymatically- or chemically-released glycans was performed using iodomethane in a suspension of sodium hydroxide in dimethyl sulphoxide [22]. The permethylated glycans were then treated with water/dichloromethane (DCM; 1:1) to separate non-sulphated and sulphated glycans by phase partition; the lower organic phase contained non-sulphated permethylated glycans, whereas the upper aqueous phase contained sulphated glycans and both pools were subject to solid-phase extraction on C18 cartridges [16].

1.5. Solvolysis and re-permethylation of glycans with deuterated methyl iodide (CD_3I)

Sulphated glycans were dissolved in 100 μL of 50 mM methanolic HCl (Supelco) and hydrolyzed for 4 h at room temperature [16,23,24]. After drying under a gentle N_2 stream, resulting neutral glycans were re-permethylated with deuterated methyl iodide (CD_3I; Sigma-Aldrich) as described above (section 1.4). The lower organic (DCM) phase was extensively washed with water and dried under a N_2 stream prior to analysis with a nanospray ionisation mass spectrometer (NSI-MS^n; Thermo Fisher Scientific) in positive ion mode [12].

1.6. Glycan analysis by Nanospray Ionisation Mass Spectrometry (NSI-MS^n)

For MS analysis of non-sulphated glycans in positive ion mode, permethylated glycans were dissolved in 50 μL of 1 mM sodium hydroxide in 50% (v/v) aqueous methanol for subsequent infusion. For MS analysis of sulphated glycans, permethylated glycans were reconstituted in 50 μL of methanol/2-propanol/1-propanol/13 mM aqueous ammonium acetate (16:3:3:2 by volume) for infusion and analysed in negative ion mode. Samples were infused directly into a linear ion trap mass spectrometer (LTQ-Orbitrap Discovery; Thermo Fisher Scientific) using a nanoelectrospray source at a syringe flow rate of 0.40 to 0.60 μl/min and a capillary temperature set to 210 °C. Automated acquisition of MS/MS fragmentation (at 35–50% collision energy) was obtained using the total ion mapping (TIM) functionality of the XCalibur instrument control software (version 2.0, Thermo Scientific). As described by Aoki and Tiemeyer [15], in TIM analysis the *m/z* range from 200 to 2000 was scanned in successive 2.8 mass unit windows with a window-to-window overlap of 0.8 mass units. For subsequent manual MS/MS and MS^n analyses by collision-induced dissociation (CID), normalised collision energy of 35% was applied. As internal calibration,