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Structural characterization of the N-glycans of gp273, the ligand for sperm-egg interaction in the mollusc bivalve *Unio elongatulus*

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Gp273, a glycoprotein of the egg extracellular coats of the mollusc bivalve *Unio elongatulus*, is the ligand molecule for sperm-egg interaction during fertilization. In this study we have analyzed the N-glycans from gp273. N-glycans were enzymatically released by PNGase F digestion and their structures were elucidated by normal phase HPLC profiling of the 2-aminobenzamide-labeled N-glycans, MALDI-TOF mass spectrometry and ^{1}H NMR spectroscopy. The combined data revealed that the N-glycans of gp273 consist of $Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$. In *Unio*, the presence of noncomplex-type N-glycans parallels the inefficacy of these glycans in the ligand function. Their role in the protection of the polypeptide chain from proteolytic attack is suggested by the electrophoretic patterns obtained after enzymatic digestion of the native and the N-deglycosylated protein. These results are discussed in the light of the evolution of the recognition and adhesion properties of oligosaccharide chains in the fertilization process.

***Keywords: Unio elongatulus*, fertilization process, sperm-egg interaction, glycoprotein, N-glycans, mannose rich oligosaccharides**

Introduction

There is increasing evidence that the carbohydrate chains of glycoproteins from the egg extracellular coats of both invertebrates and vertebrates act as ligands in the first step of sperm-egg recognition. This is, in turn, the trigger of the acrosome reaction [1]. However, because of the limited availability of oocytes and the structural complexity of carbohydrate chains, detailed structural characterization of the bioactive oligosaccharides is still a challenging problem.

In frogs and mammals, the vertebrate groups most extensively studied [2–4], both N- and O-linked carbohydrate chains have been correlated with the recognition process at the onset of fertilization and, in some cases, it has been suggested that both types of chains contribute to this function [5]. In invertebrates, knowledge of sperm-egg recognition is limited to sea urchins [3] and the mollusc bivalve *Unio elongatulus* [6]. In sea urchins, unlike in mammals, the molecule responsible for triggering the acrosome reaction is located in the jelly coat, external to the vitelline coat and the ligand is a fucose-sulfate polymer [7]. In *Unio*, the vitelline coat, like the mammalian zona pellucida, mediates recognition and binding of sperm. Recognition, however, is restricted to an area at the vegetal pole, called "the crater area", because at its center the vitelline coat protrudes in a tronco conical structure that looks like a crater [8]. Immunofluorescence studies have shown that one of the two glycoproteins constituting the egg coat (gp273) is prevalently present in this area whereas the other (gp180) is the main component of the rest of the vitelline coat [9]. This suggested that gp273 has a role in sperm recognition, and in fact, an *in vitro* binding study confirmed that it is the ligand molecule and induces the acrosome reaction in sperm [10]. Gp273 was also shown to contain N- and O-linked oligosaccharide chains [11]. Their removal and use in competition binding assays demonstrated that O- but not N-linked oligosaccharides were able to compete with binding of gp273 to immobilized sperm [10]. In order to obtain more information on the bioactivity of the carbohydrates from gp273, we have initiated the structural analysis of its glycans. In this paper we report the first step of this investigation: the elucidation of the N-linked oligosaccharides from gp273 by means

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of different chromatographic, spectroscopic and spectrometric analyses. The relationship between the type of chains and their involvement in recognition is discussed. Furthermore, the role of the N-glycans in protecting gp273 from proteolytic digestion is also investigated.

Materials and methods

Gp273 purification

Specimens of *Unio elongatulus* were collected in a small lake near Siena (Italy). Eggs were aspirated from the gonads of living animals and 40 mg gp273 was purified from the eggs as previously described [12].

Release and isolation of N-linked carbohydrate chains

N-linked glycan chains were enzymatically released from the protein backbone by PNGase F digestion, according to Van Rooijen et al. [13] with the following modifications. Briefly, purified gp273 was dissolved in 50 mM Tris-HCl, pH 7.2, containing 50 mM EDTA and 1% (v/v) β-mercaptoethanol. Denaturation of the sample was carried out by heating at 100°C for 4 min in the presence of 0.3% (w/v) SDS. After cooling to room temperature, the solution was incubated with recombinant peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) from *Flavobacterium meningosepticum* (Boehringer, Mannheim) (1 U/mg protein) in the presence of 0.6% (w/v) MEGA 10 (decanoyl-N-methylglucamide) for 24 h at room temperature in an end-over-end mixer. After heating at 100°C for 3 min, a fresh aliquot of PNGase F was added and the incubation continued for another 24 h. For some experiments an equivalent amount of gp273 was incubated under the same conditions in the absence of the enzyme. The efficiency of N-deglycosylation was monitored by lectin blotting of the isolated protein with ConA lectin as previously described [11], and by monosaccharide analysis. The mixture of liberated N-glycans was separated by solid phase extraction (SPE) from detergent, protein and salts in a single step, on graphitized carbon columns [14]. Three fractions were collected: fraction 0 was obtained after elution with distilled water, fraction 1 was eluted with 25% acetonitrile and fraction 2 was eluted with 25% acetonitrile containing 0.05% TFA. The fractions were lyophilized and then used for further experiments.

Monosaccharide analysis

Methanolyzed glycan samples (1.0 M methanolic HCl, 24 h, 85°C) were converted into trimethylsilylated derivatives after re-N-acetylation and analyzed by gas chromatography/mass spectrometry according to Kamerling and Vliegenthart [15]. The quantitative analysis was carried out by GC on a capillary EC-1 column (30 m × 0.32 mm, Alltech) using a Chrompack CP 9002 gas chromatograph (temperature program 140–240°C at 4°C/min) and flame-ionization detection. The identification of the monosaccharide derivatives was confirmed by gas chromatography/mass spectrometry on a Fisons Instruments GC 8060/MD 800 system (Interscience) equipped with an AT-1 column (30 m × 0.25 mm, Alltech).

2AB-labeling of N-glycans and HPLC profiling

Purified and dried N-glycans were derivatized with the fluorophore 2-aminobenzamide (2AB) essentially as previously described [16,17]. The 2AB-labeled oligosaccharides were profiled on a GlycoSep-N column (4.6 × 50 mm, Oxford GlycoScience) at 30°C. The HPLC system consisted of a Waters 2690 XE Alliance system. Detection was performed with a Waters 474 fluorescence detector. 2AB-labeled glycans were

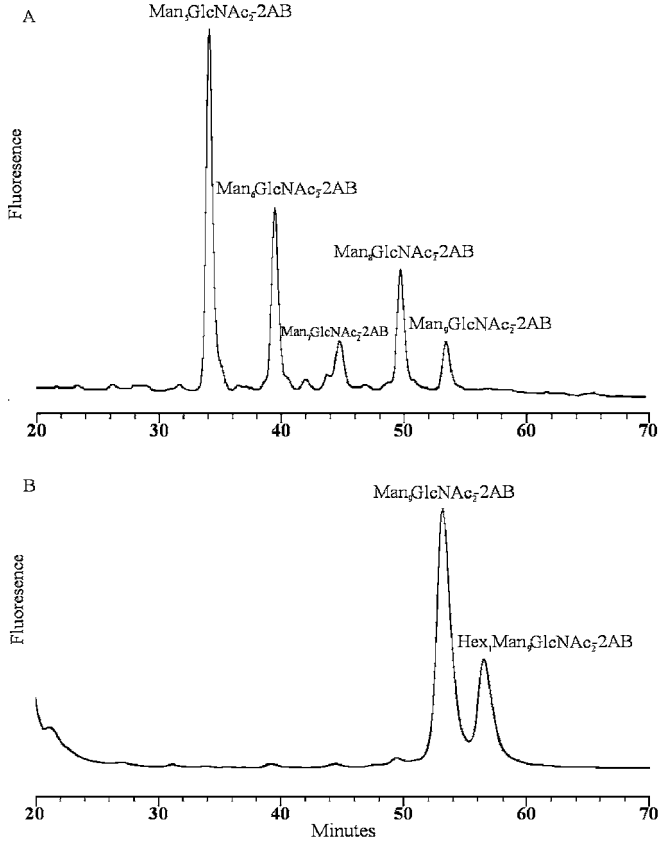

Figure 1. (A) Elution profile of 2AB-labeled oligomannose series from Ribonuclease B: $Man_5GlcNAc_2$-2AB (6.12 GU), $Man_6GlcNAc_2$-2AB (7.01 GU), $Man_7GlcNAc_2$-2AB (7.79 GU), $Man_8GlcNAc_2$-2AB (8.82 GU), $Man_9GlcNAc_2$-2AB (9.57 GU). (B) Relevant part of the normal phase HPLC chromatogram of the 2AB-labeled N-glycans derived from gp273. The column was eluted using the following gradient conditions: solvent A was 50 mM ammonium formate, pH 4.4, and solvent B was acetonitrile. Initial conditions were 35% A at a flow rate of 0.4 ml/min, followed by a linear gradient of 35–58% A over 92 min, followed by 58–100% A over the next 3 min. The flow rate was then increased to 1.0 ml/min over the next 2 min and the column washed in 100% A for 5 min before being reequilibrated in 35% A.

eluted using the gradient conditions described by Guile et al. [18], with slight modifications as depicted in the legend of Figure 1. The profiles were standardized in glucose unit values (GU) using a known dextran hydrolysate. The GU values obtained for the gp273 N-glycans were then compared with those obtained for known 2AB-labeled oligomannose-type structures released from RNase B [17].

MALDI-TOF mass spectrometry

Positive-ion mode Matrix-Assisted Laser Desorption Ionization (MALDI-TOF) mass spectrometric analysis of 2AB-labeled oligosaccharides was performed on a Voyager-DE (PerSeptive Biosystems) instrument operating at an accelerating voltage of 24 kV (grid voltage 93.0%, ion guide wire voltage 0.01%) and equipped with a VSL-337ND-N_2 laser. The sample was dissolved in water and, subsequently it was mixed on the target plate with 2,5-dihydroxybenzoic acid (10 mg/ml in H_2O) at a ratio of 1:3. Linear mass scans were recorded over 3,000 Da by using a pulse delay time of 90 ns. Recorded data were processed by using GRAMS/386 software (v. 3.04, Galactic Industries, Salem NH) [19].

Exoglycosidase digestion

Purified 2AB-labeled oligosaccharides were incubated with α-mannosidase (from Jack Bean Meal; Glyko, Oxford, UK) according to Li and Spector [20]. The sample was then purified on a SPE column and profiled by normal phase HPLC.

^{1}H NMR spectroscopy

Prior to analysis, samples were repeatedly exchanged with D_2O (99.9% D Cambridge Isotopes Ltd.) with intermediate lyophilization and finally dissolved in 500 μl D_2O. ^{1}H NMR spectra were recorded on a Bruker DRX 500 instrument (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at the probe temperature of 300 K. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225) [21]. HOD signal suppression was achieved by applying a water-eliminated Fourier transform pulse sequence in 1D experiments and by presaturation for 1 s in 2D experiments. 2D TOCSY spectra were recorded using MLEV-17 mixing sequences with effective spin-lock times of 100 ms [22,23]. Spectra were processed on Silicon Graphics IRIS workstations (Indigo 2 and O2) by using XINSP2 software (Bijvoet Center, Department of Bio-Organic Chemistry).

Peptide mapping

Peptide mapping after limited proteolysis of native and N-deglycosylated gp273 with Proteinase K (from *Tritirachium albium*, Merck, Darmstadt, Germany) was performed according to Cleveland et al. [24] as previously described [12]. In-gel digestion was performed on Coomassie blue stained gp273 spots essentially as described by Shevchenko et al. [25] using modified trypsin (Promega, Madison, WI, USA). After proteolysis, the gel pieces were centrifuged, supernatant collected and further peptides extracted by one change of 20 mM

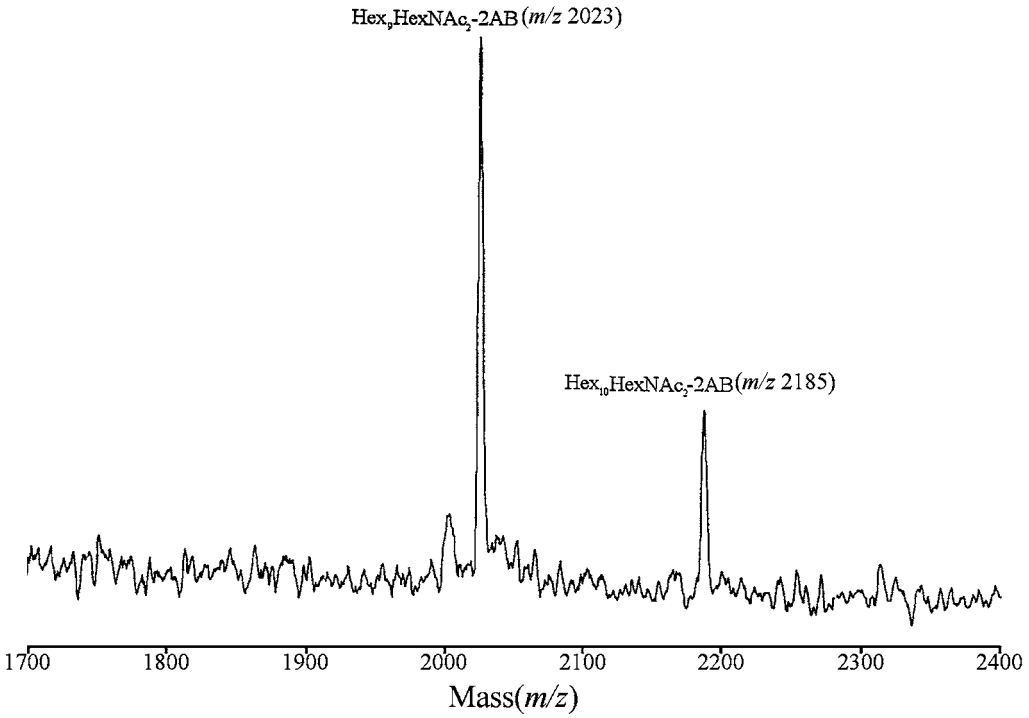

Figure 2. MALDI-TOF mass spectrum showing the two peaks corresponding to the main species constituting the N-glycans of gp273.

NH_4CO_3 and three changes of 5% formic acid in 50% CH_3CN. The pooled supernatant was dryed in a speedvac until desired volume reached and peptides resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide mini-slab running gels under denaturating conditions, according to Laemmli's procedures [26]. The polypeptide bands were visualized by silver staining.

Results

Monosaccharide analysis of gp273 revealed the presence of fucose (Fuc), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc), *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc), and the carbohydrate content was found to be around 3.5% by mass. N-linked glycans were released from purified gp273 by PNGase F digestion and the resulting mixture was separated into a neutral and an acidic fraction by SPE. Monosaccharide analysis of the two fractions revealed that no carbohydrates were present in the acidic fraction, whereas the neutral fraction contained Xyl, Man, Glc and GlcNAc. Over a range of analyses, the molar ratio of Man and GlcNAc approximated 9:2, thus suggesting the presence of oligomannose-type carbohydrate chains. Xyl was present in variable trace amounts and thus considered as a contaminant. The Glc content varied over the different analyses and could be significantly reduced but not removed by extended dialysis of gp273 prior to monosaccharide analysis. As a consequence, this could reflect the presence of Glc as constituent of the carbohydrate chains of gp273. Further analyses were performed on the neutral fraction.

HPLC profiling and MALDI-TOF mass spectrometric analysis

The released oligosaccharides were derivatized by 2AB and profiled by HPLC. The chromatogram (Figure 1B) revealed the presence of two major peaks at GU 9.55 and GU 10.22, respectively, in relative amounts of 7:3 (67.2% versus 32.8%). From comparison with GU values obtained for the oligomannose-type structures ($Man_5GlcNAc_2$-2AB to $Man_9GlcNAc_2$-2AB) from RNase B (Figure 1A), the peak eluting at GU 9.55 was tentatively assigned to $Man_9GlcNAc_2$-2AB. The peak eluting at GU 10.22 corresponded to a larger structure, probably containing one additional monosaccharide, and was tentatively assigned to $Hex_1Man_9GlcNAc_2$-2AB.

The MALDI-TOF mass spectrometric analysis of the carbohydrate-containing fraction showed two peaks at *m/z* 2023 and *m/z* 2185 $(M+Na)^+$ corresponding to pseudomolecular ions of $Hex_9HexNAc_2$-2AB and $Hex_{10}HexNAc_2$-2AB, respectively (Figure 2). These results corroborated those from HPLC profiling.

Exoglycosidase digestion and ^{1}H NMR spectroscopic analysis

In order to establish the nature (probably a Glc residue) and the branch position of the unknown hexose residue in the

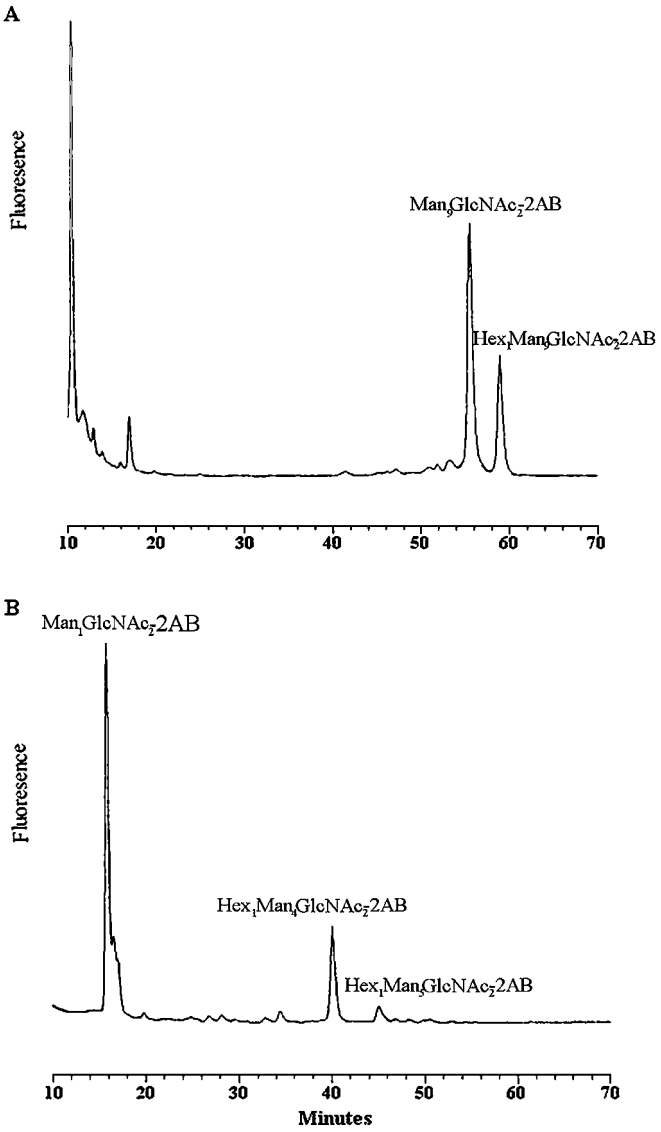

Figure 3. Relevant part of the normal phase HPLC profile of 2AB-labeled N-glycans of gp273 after α-mannosidase digestion (B), compared to the profile without α-mannosidase digestion (A).

$Hex_1Man_9GlcNAc_2$ structure, an α-mannosidase digestion was performed and the resulting HPLC profile (Figure 3B) revealed that the two original structures (Figure 3A) had been digested giving rise to three new peaks corresponding to GU 2.43 (68.7%), GU 6.80 (25.9%) and GU 7.63 (5.4%), respectively.

The peak at GU 2.43 could be identified as $Man_1GlcNAc_2$-2AB by comparison to literature [18] and most likely originated from the $Man_9GlcNAc_2$-2AB (GU 9.55). The remaining peaks at GU 6.80 and GU 7.63 should therefore result from the $Hex_1Man_9GlcNAc_2$-2AB (GU 10.22) and revealed that the unknown hexose residue is not mannose. Taking into account the preferential cleavage of the enzyme (α1-2 > α1-3 > α1-6)

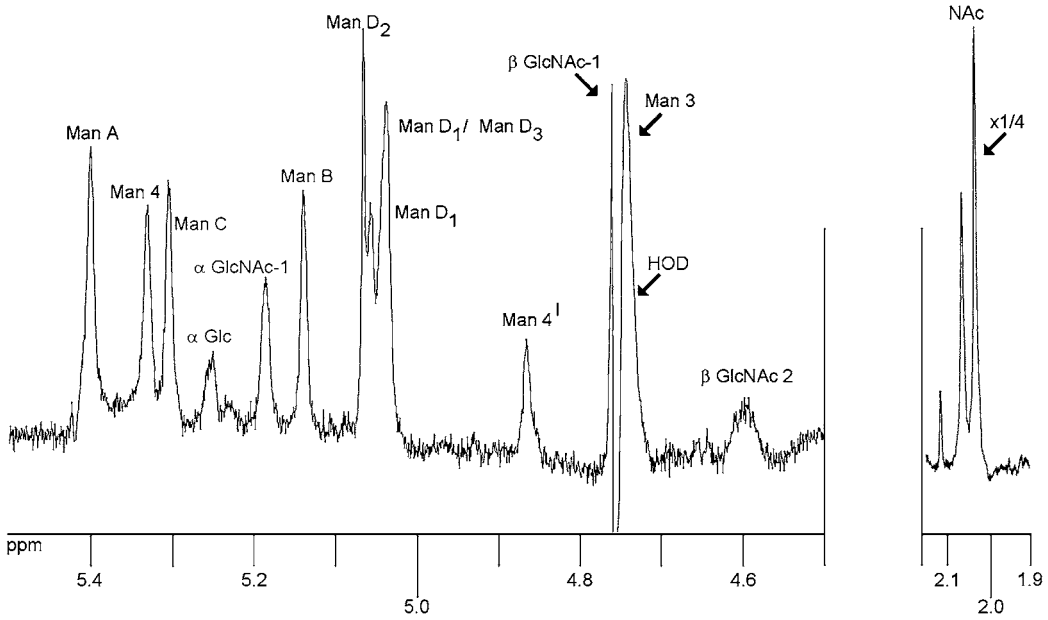

Figure 4. The anomeric and NAc region of the ^{1}H NMR spectrum of N-glycans released from gp273 in D_2O at 300 K. Each H1 signal has been assigned and confirmed by 2D TOCSY. The relative intensity of the NAc region was decreased by a factor 4.

and the influence of steric hindrance due to the end-capping monosaccharide, the structures were tentatively assigned to $Hex_1Man_4GlcNAc_2$-2AB and $Hex_1Man_5GlcNAc_2$-2AB, respectively, by comparison to the GU values for $Man_5GlcNAc_2$-2AB (GU 6.12) and $Man_6GlcNAc_2$-2AB (GU 7.01). The $Hex_1Man_4GlcNAc_2$-2AB structure would represent a linear structure with the unknown hexose located at the non-reducing terminus, thus preventing digestion of this branch and the $Hex_1Man_5GlcNAc_2$-2AB would result from incomplete digestion, indicating one α-1,6-linked Man residue still present, positioning the unknown hexose residue at the non-reducing terminus of the α-1,3-linked branch.

The 1D ^{1}H NMR spectrum of the purified N-glycan fraction (Figure 4) revealed the typical reporter group signals as

Table 1. ^{1}H-NMR chemical shifts (δ ppm) of the two assigned N-glycan structures in D_2O at 300 K, compared to those reported in literature

		Chemical shift			
Reporter group	*Residue*	*$Man_9GlcNAc_2$*	*$Glc_1Man_9GlcNAc_2$*	*$Man_9GlcNAc_2$ [27]*	*$Glc_1Man_9GlcNAc_2$ [28]*
H-1	GlcNAc-1 α	5.188	5.188	5.187	–
	β	n.d.	n.d.	n.d.	–
	GlcNAc-2α/β	4.60	4.60	4.603	4.63
	Man-3	n.d.	n.d.	n.d.	n.d.
	Man-4	5.346	5.346	5.334	5.337
	Man-4'	4.871	4.871	4.868	4.867
	Man-A	5.404	5.404	5.403	5.401
	Man-B	5.145	5.145	5.142	5.140
	Man-C	5.310	5.310	5.308	5.306
	Man-D_1	5.045	5.040	5.045	5.038
	Man-D_2	5.059	5.059	5.061	5.054
	Man-D_3	5.040	5.040	5.040	5.038
	α-Glc	–	5.256	–	5.256
NAc	GlcNAc-1	2.037	2.037	2.038	–
	GlcNAc-2	2.068	2.068	2.068	2.068

n.d. means not determined.

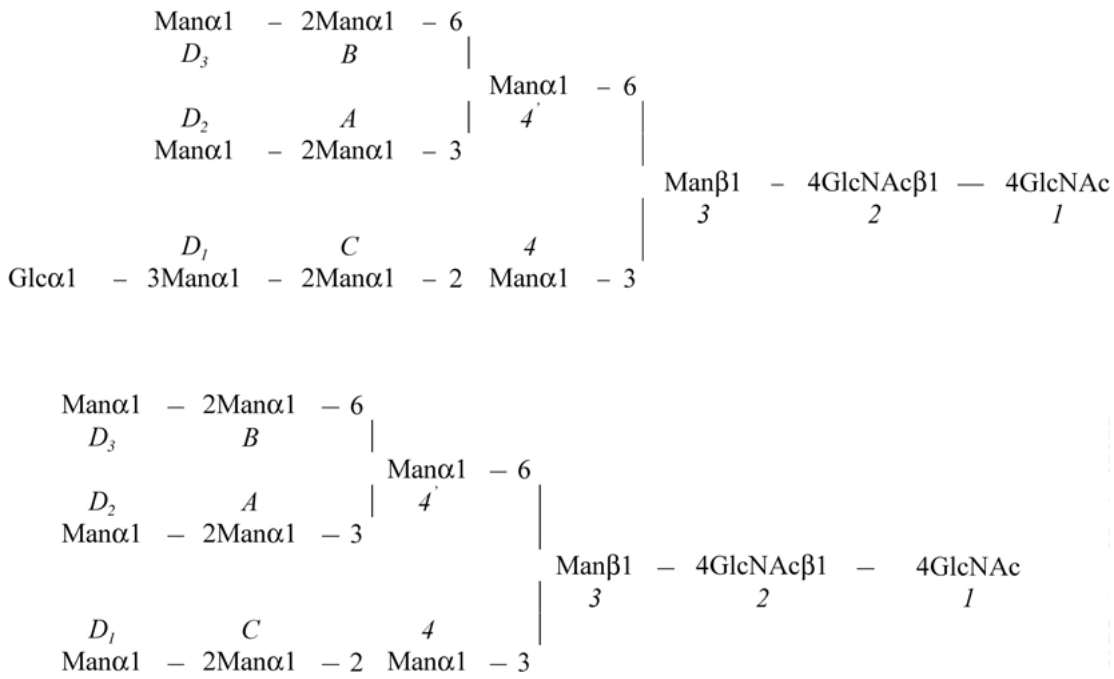

Figure 5. Primary sturctures of the two N-glycan types of gp273.

observed for oligomannose-type structures [27,28] and are summarized in Table 1. Based on the number and intensities of the anomeric signals, the main constituent of the N-linked glycans from gp273 could be identified as $Man_9GlcNAc_2$. In addition to the anomeric signals stemming from $Man_9GlcNAc_2$, an additional anomeric doublet was observed at δ 5.256, but with a lower intensity than the other anomeric signals. This doublet could be assigned to an α-linked Glc residue, based on literature data [28] and permitted the identification of the unknown hexose residue as Glc and of $Glc_1Man_9GlcNAc_2$ as the other main constituent of N-linked carbohydrate chains in gp273 (Figure 5). In the 2D TOCSY spectrum (data not shown), the upfield chemical shift for the H2 proton of Man-D_1, when compared to the non-substituted residue, confirmed the Glc(α1-3)Man-D_1 structure.

Peptide mapping

Since the N-glycans do not appear to be directly involved in the recognition mechanism, we verified whether they could participate in the protection of gp273 against protease attack. The same amounts of native and N-deglycosylated gp273 were separated by gel electrophoresis, and the band corresponding to the (glyco)protein excized and digested with Proteinase K or with modified trypsin. The extent of proteolysis was then assessed by SDS-PAGE. The peptide maps obtained revealed a greater susceptibility of the N-deglycosylated peptides with respect to native gp273 to both the treatments. As shown in Figure 6, after 15 min of trypsin treatment the peptide map of native protein consists of three intense bands with apparent MW of 28, 13 and 11 kDa and a lot of minor smearing components running from 50 to 7 kDa, whereas that of the deglycosylated protein completely lacks the smearing components probably reduced to peptides running with the dye front. Only in this case it shows the three intense bands. These also appear sharper than those of the native protein. These results indicate that the N-deglycosylated protein is more susceptible to protease digestion than the native protein.

Discussion

The data presented in this paper represent the first rigorous structural investigation of N-glycans of a ligand molecule for sperm-egg interaction in an invertebrate species. The species is *Unio elongatulus*, a freshwater mollusc bivalve with a very peculiar highly polarized egg [8]. In *Unio*, the surface recognizing and binding sperm is, as in mammals, the outer surface of a densely packed fibrous network coat that surrounds the egg. Two main glycoproteins with masses of 273 (gp273) and 180 kDa (gp180) have been shown to account for about 90% of the material dissolved from this egg coat [12]. Their purification and rising of the corresponding antibodies has led to assign the role of ligand to gp273 [10]. Here, we present the results of HPLC profiling, MALDI-TOF and NMR analyses of the N-glycans liberated from purified gp273. Gp273 contains only N-glycans of the high-mannose type with two structures $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$. The exclusive

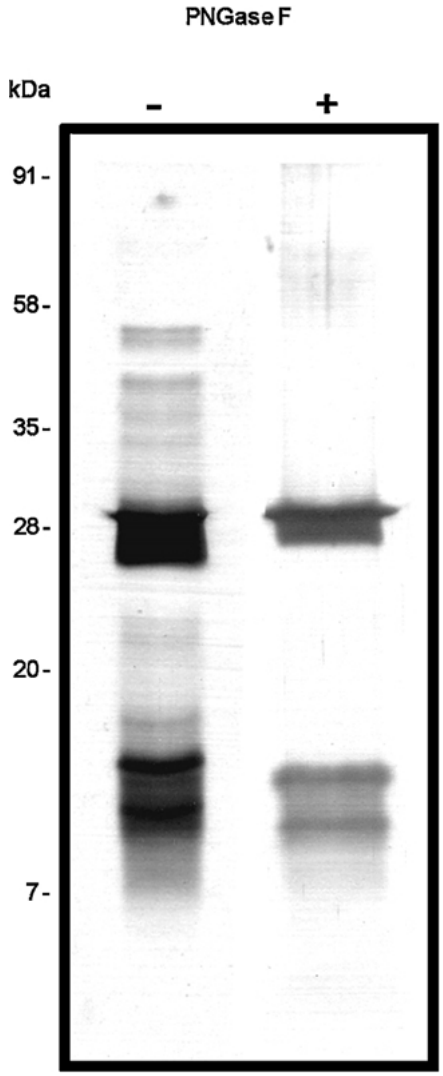

Figure 6. Peptide maps obtained after digestion for 15 min with modified trypsin of native (−) and N-deglycosylated (+) gp273.

presence of these N-linked chains is, at moment, unique in the panorama of N-glycosylation of the egg coat components, since the same types of chains are reported to be absent from the zona pellucida proteins of most of the widely analyzed mammalian species, such as porcine and mouse. In the coat glycoproteins of these species, the N-glycans consist of a fucosylated complex-type core elongated with heterogeneous polylactosamine chains [5,29]. It is interesting to note that in *Unio* the presence of N-glycans without any terminal elaborations parallels the complete inefficacy of these chains in competing with sperm interaction to gp273 [10]. In *Unio*, fucose has been indicated as essential in the ligand function of gp273 since it interferes with its binding to sperm [10], its presence is restricted to the glycopeptide with the ligand activity [30] and it is prevalently present in the crater area [8]. The complete lack of fucosylation reported here for the gp273 N-oligosaccharides confirms that in *Unio* the recognition is only dependent on O-linked oligosaccharides [10]. This finding is in agreement with the evolving view of glycosylation that assign the role of recognition markers to terminal variations of N- or O-linked glycans which are largely viewed as terminal elaborations [31]. The occurrence of Glc-containing oligomannose-type carbohydrate chains as a natural part of gp273 was quite unexpected, since these chains usually are the key intermediates in the biosynthesis and processing of the asparagine-linked chains [32]. Occurrence of similar oligomannose structures with an unusual terminal glucose residue has been already reported in egg-yolk antibody (IgY) from hens eggs [33]. Interestingly, the same type of chain has been also demonstrated in glycoproteins isolated from the ovary of the starfish *Asteria rubens*, where the N-linked chains are of the oligomannose type only [28], and from the egg jelly coat of *Asteria amurensis* [34]. Since the main effort of the biosynthetic pathway of the oocyte is the formation of the coat glycoproteins, it can be hypothesized that the processing of the N-linked chains in these cells is at lower efficiency. Concerning the role of N-glycosylation in gp273 our results leads to the hypothesis that it may serve to protect the protein from protease attack.

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