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Isolation, partial characterization and biological activity of mannosyl glycopeptides from seminal plasma

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Affinity chromatography on Concanavalin-A Sepharose, followed by gel filtration and hydrophobic interaction chromatography, permits the isolation of low molecular weight N-glycosidically linked oligomannosidic glycopeptides (MGp) from the autoproteolysis products of human seminal plasma. The monosaccharide composition of MGp showed only mannose, *N*-acetylglucosamine and a small amount of galactose. Structural studies were carried out by methylation analysis and chromium trioxide oxidation, and results were consistent with the structures accepted for high-mannose *N*-glycans. MGp was capable of inhibiting the sperm acrosomal exocytosis mediated by sperm-surface receptors. These data suggest that MGp act as a ''decapacitation'' factor preventing premature sperm exocytosis.

***Keywords*: glycoconjugate, carbohydrate, sperm, exocytosis, human, semen**

Introduction

Mammalian fertilization involves specific recognition between complementary carbohydrate-protein on the surface of gametes [1, 2]. Before binding to the oocyte, sperm must undergo a capacitation process, which consists of physiological modifications during their passage through the female reproductive tract. The binding of the sperm cell to glycoproteins from the egg's coat triggers a physiological acrosomal exocytosis (acrosome reaction, AR). The AR of the fertilizing human spermatozoon has to be precisely timed with regard to the sperm-zona pellucida interaction [3]. Thus, a premature AR renders spermatozoa incapable of fertilizing the oocyte.

Seminal plasma participates in the control of sperm integrity, and the antifertility activity of seminal plasma components has been described for several mammals [4–6]. Human seminal plasma (HSP), being an epithelial secretion, contains a mixture of serum-type and mucin-type glycoproteins. The glycoproteins are proteolysed in ejaculated seminal plasma [7] producing a very complex mixture of glycoprotein fragments together with peptides and even free amino acids [8, 9].

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In human fertilization, mannosyl residues have been postulated to play an important role. Human sperm incubated with mannose are unable to bind and fertilize homologue oocytes [10, 11] and the expression of mannose receptors on the sperm surface is related to their fertilizing capacity [12]. The clustering of binding sites for mannose and *N*-acetylglucosamine on the sperm surface is involved in the induction of AR [13], presenting a positive correlation with zona pellucida-binding capacity [14].

The present report describes a sequential chromatographic procedure for the isolation of oligomannosidic glycopeptides from the autoproteolysis products of human seminal plasma, their structural characterization and their effect on sperm acrosomal exocytosis.

Materials and Methods

Reagents

Concanavalin-A Sepharose CL4B, Sephacryl S-200, Phenyl Sepharose CL6B and Sephadex G-15 were from Pharmacia-LKB (Uppsala, Sweden). Bovine serum albumin, p-aminophenyl-α-D-mannopyranoside, progesterone, mannan, fucoidin, and α-mannosidase from jack beans (21 units mg protein) were from Sigma (St. Louis, USA). All chemicals used were analytical grade from Merck (Darmstadt, Germany). The neoglycoprotein albumin, bovine-p-aminophenyl-α-D-mannopyranoside (BSA-Man) was

synthesized as previously described [13]. Human seminal plasma, was prepared from pooled semen from healthy donors as described elsewhere [15].

Affinity chromatography on Concanavalin-A Sepharose

Human seminal plasma was dissolved in 10 mM sodium phosphate buffer pH 7.2 and loaded on a column (20 × 3.5 cm) of Concanavalin-A Sepharose 4B. Elution was performed at room temperature with the following buffers: 10 mM sodium phosphate pH 7.2, 100 mM sodium borate buffer pH 6.0, and 10 mM sodium phosphate pH 7.2 containing 2% α-methyl-D-glucopyranoside. Fractions were collected at 4 °C. Carbohydrate was monitored by the phenol sulphuric acid reaction [16] and proteins were determined by absorbance at 280 nm. Only those fraction eluted with the last buffer (fraction M) were pooled and used in the present study. Fraction M was freeze-dried and stored at − 20 °C until used.

Gel filtration chromatography on Sephacryl S-200

Fraction M was dissolved in water and loaded on a column (50 × 1.0 cm) of Sephacryl S-200 HR pre-equilibrated with 10 mM sodium phosphate pH 7.2. Elution was performed with the same buffer and the fractions were collected, monitored and processed as above.

Hydrophobic interaction chromatography

The sample was dissolved in 10 mM phosphate buffer pH 7.2 containing 1 M ammonium sulphate and loaded on a column (14 × 2.0 cm) of Phenyl Sepharose CL-6B. Elution was performed at room temperature with 10 mM phosphate buffer pH 7.2 containing 1 M ammonium sulphate, followed by a linear gradient of 1 → 0 M ammonium sulphate. Fractions were collected and monitored as above. The glycopeptides obtained (MGp) were desalted by gel filtration on Sephadex G-15 and eluted with distilled water.

Acrosome reaction assays

Sperm were separated from seminal plasma and capacitated as described before [13]. After capacitation, sperm (2×10^6 cells per ml) were incubated for 60 min with the indicated concentrations of glycopeptide MGp and then 60 min in the presence or absence of 2 μg ml^{-1} neoglycoprotein BSA-Man or progesterone. Sperm were then washed three times in phosphate buffered saline and plated onto immunofluorescence slides. After the cells had adhered to the surface, the buffer was removed and the slide submerged for 30 seconds in methanol at 4 °C. The acrosome reaction was evaluated using lectin staining with 50 μg ml^{-1} FITC-Pisum sativum agglutinin [17]. Cells were scored using a Zeiss epifluorescence microscope. To test specificity, MGp used in some experiments was digested with α-mannosidase in 100 mM sodium citrate pH 4.5 for 4 h at 37 °C.

Gas-liquid chromatography

Monosaccharide analysis was carried out as described by Reinhold [18], employing the trimethylsialyl derivatives of the corresponding methyl glycosides. A Hewlett-Packard 5840 gas chromatograph was used. The column was a Chromosorb W-HP and the elution was with nitrogen at 29 ml/min. Monosaccharides were obtained by trifluoroacetic acid treatment for 2 h at 121 °C, followed by methanolysis for 16 h at 65 °C.

Determination of the anomeric configuration

Acetylation of glycopeptide was performed in dimethyl formamide. Pyridine was added, with constant stirring, and later acetic anhydride. After 72 h treatment, water was added and the product was extracted with chloroform. The peracetylated derivative was dissolved in anhydrous acetic acid and submitted to oxidation by chromium trioxide as previously described [19]. The oxidized product was extracted with chloroform, washed with water, dried and the chloroform was distilled off.

Methylation analysis

Glycopeptide was dissolved in dimethyl sulphoxide and methylated by the method of Hakomori [20]. The permethylated product was extracted with chloroform and dried. Acetolysis, reduction and acetylation were carried out as described by Stellner [21]. Analysis of the methylated alditol acetates was performed by combined gas-liquid chromatography-mass spectrometry using a glass column (0.3 × 120 cm) of 3% ECNSS-M on Gas Chrom Q (100–200 mesh), programming from 130 °C to 200 °C at a rate of 4 °C/min with helium as a carrier gas (12 ml/min), and a Varian Series 1400 gas chromatograph connected to a Varian MAT CH7A mass spectrometer. Mass spectra were recorded over a mass range of 40–600 atomic mass units, using an ionizing potential of 70 eV; scans were taken every 4 s. The methylated alditol acetates were identified by a combination of gas liquid chromatography retention times (known standards) and mass spectra.

Amino acid analysis

Amino acid analysis was performed as described elsewhere [15], using a Techicon sequential multi-sample amino acid analyzer RM S2, and the amino acids were obtained by peptide hydrolysis with 6 M HCl at 105 °C for 24 h. Amino acids were purified with Amberlite IR-120 before analysis.

Results

Purification of MGp

The isolation of mannose-containing glycopeptides from human seminal plasma was carried out by liquid chromatography. Concanavalin-A Sepharose chromatography yielded

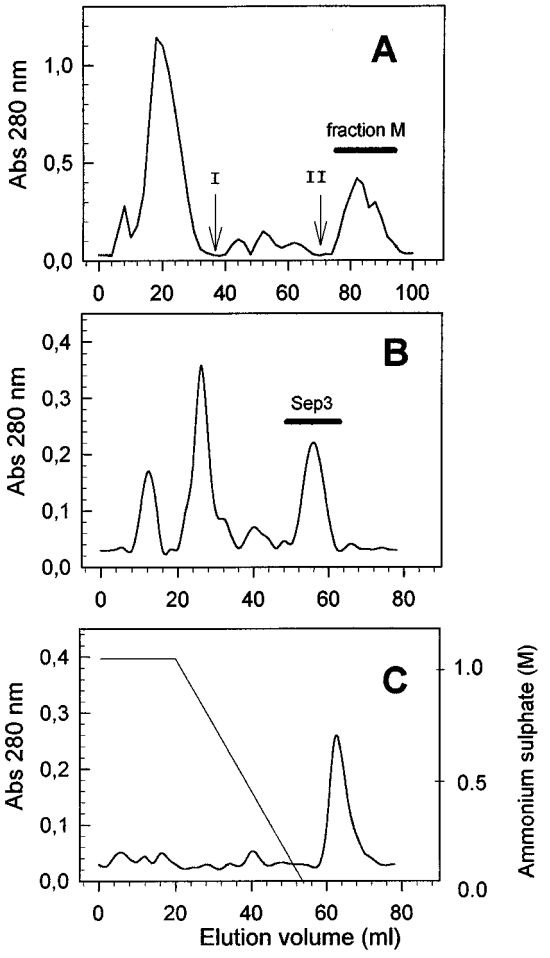

Figure 1. Isolation of mannose-containing glycopeptides. Human seminal plasma was submitted to sequential chromatography on Concanavalin-A Sepharose 4B (A), Sephacryl S-200 HR (B), and Phenyl Sepharose CL6B (C) as described in Materials and Methods. Arrows indicate the changes of eluting buffers to 100 mM borate pH 6.0 (I), and then to 10 mM phosphate pH 7.2 containing 2% *a*-methyl-D-glycopyranoside (II).

non-interacting fractions eluted with phosphate and borate buffers, followed by a glycopeptide fraction (fraction M) which specifically bound to the lectin (Figure 1A). The fraction M was processed as described in Materials and Methods and submitted to gel filtration chromatography on Sephacryl S-200, eluting as three major peaks (Figure 1B).

The effect of mannose-containing glycoconjugate fractions from HSP on sperm exocytosis was assessed using an *in vitro* AR assay, as described in Materials and Methods. The fraction M from concanavalin-A Sepharose and the fraction 3 (Sep3) from Sephacryl were capable of significant inhibition of the AR (Table 1).

Sep3 was then submitted to hydrophobic interaction chromatography on Phenyl Sepharose CL-4B. It is noteworthy that only one peak coincident for protein and carbohydrate was eluted (Figure 1C). Further elimination of salts,

Table 1. Effect of mannose-containing glycoconjugate fractions on the sperm acrosome reaction

Fraction[a]	*% AR (spontaneous)*	*% AR (induced)*
Control	7.7 ± 2.1	26.3 ± 2.1
ConA	8.7 ± 3.0	9.3 ± 1.5*
Sep1	9.7 ± 2.9	21.6 ± 4.0
Sep2	14.0 ± 6.1	26.7 ± 3.0
Sep3	9.0 ± 2.6	12.0 ± 1.0*
Phe	7.2 ± 2.1	11.0 ± 1.7*

Capacitated sperm were incubated for 60 min with 100 μg ml^{-1} of each fraction. Then fresh medium was added to "spontaneous" samples, and NGP (2 μg ml^{-1} final concentration) was add to "induced" samples.
[a]Control, no seminal protein was added; ConA, fraction specifically bound to Concanavalin-A; Sep1, Sep2, Sep3, fractions from Sephacryl S-200 chromatography; Phe, fraction from Phenyl Sepharose.
*Significantly different from control ($p < 0.05$ by Student's t-test)

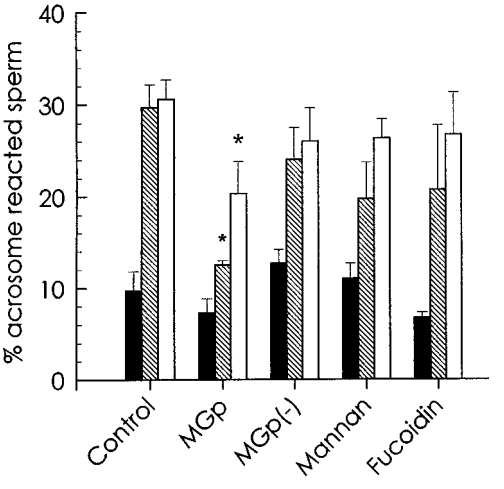

Figure 2. Effect of MGp on the sperm acrosome reaction. Sperm were incubated for 60 min with 50 μg ml^{-1} glycopeptide (MGp), 50 μg ml^{-1} mannosidase-treated glycopeptide (MGp(−)), 500 μg ml^{-1} mannan, 500 μg ml^{-1} fucoidin, or fresh medium as control. Then, cells were incubated for an additional 60 min in the absence (■) or presence of 2 μg ml^{-1} BSA-Man (□) or 2 μg ml progesterone (□). Bars are the mean ± SEM of five experiments. *$p < 0.05$ by Student's t-test.

free amino acids and low molecular weight peptides was achieved by gel filtration chromatography on Sephadex G-15. The final glycopeptide fraction (MGp) was obtained in 1.7% yield of the total HSP, and contained only carbohydrate (47%) and peptide (53%).

Biological activity of MGp

The effect of isolated mannose-containing glycopeptides on the sperm acrosome reaction was investigated. The glycopeptide MGp inhibits the sperm exocytosis induced by both progesterone and neoglycoprotein (Figure 2). As shown in

Figure 2, spontaneous AR was also decreased by incubation with MGp. To test the specificity of the inhibitory effect, MGp was pre-incubated with α-mannosidase before the incubation with sperm cells. The mannosidase-treated MGp was not capable of inhibiting the sperm AR (Figure 2). The effects of mannan and fucoidin were also tested. Although both polysaccharides caused a decrease in the percentage of AR, the differences from the control were not significant (Figure 2).

Structural analysis of MGp oligosaccharides

The monosaccharide and amino acid average composition of MGp is given in Table 2. The oligosaccharides are constituted only by mannose and *N*-acetylglucosamine with a small amount of galactose. The amino acid analysis showed seven different amino acids. The amount of aspartic acid was similar to that of serine plus threonine (Table 2).

Oxidation of the oligosaccharide moiety of MGp with chromium trioxide/acetic acid showed that 84–90% of the mannose units are linked through α-D-glycosidic linkages while the rest have the anomeric carbon atom with β-D-configuration. These results indicated that there is one β-D-mannose unit for every six to eight α-D-mannose residues. No GlcNAc was detected after the treatment confirming that it is present in the β-D-configuration.

Methylation analysis of the glycopeptide MGp was carried out. Hydrolysis of the permethylated derivative and characterization of the partially methylated neutral sugars by gas liquid chromatography-mass spectrometry showed the presence of major amounts of 2,3,4,6-tetra-*O*-methylmannose together with 3,4,6-tri-*O*-methylmannose and 2,4-di-*O*-methylmannose which correspond to a molar ratio of 3.1: 1.8: 2.2 (Table 3). Minor amounts of 2,3,6-tri-*O*-methylmannose, 3,4-di-*O*-methylmannose and 2,3-di-*O*-methylmannose were also detected.

Table 2. Monosaccharide and amino acid composition of the glycopeptide MGp.

Residue	*mole %*
Monosaccharide	
Mannose	74.9
Galactose	2.2
N-Acetylglucosamine	22.8
Amino acid	
Ala	14.1
Asp	18.8
Glu	23.1
Gly	16.7
Pro	8.1
Ser	12.4
Thr	6.8

Table 3. Methylation analysis of the glycopeptide MGp

Residue	*mole %*
2,3,4,6-tetra-*O*-methylmannose	35.8
3,4,6-tri-*O*-methylmannose	21.3
2,4-di-*O*-methylmannose	25.5
2,3,6-tri-*O*-methylmannose	1.8
3,4-di-*O*-methylmannose	6.3
2,3-di-*O*-methylmannose	9.2

Discussion

Affinity chromatography of Concanavalin-A Sepharose 4B of HSP and some antigenic fractions isolated from it showed that HSP is a complex mixture of glycoprotein/glycopeptides with different percentages of carbohydrates [22]. The procedure was first used to isolate the fractions containing the main glycoprotein fragments which do not interact specifically with Con-A and were separated according to non-specific ionic exchange and hydrophobic interactions with the lectin [8]. All the attempts to isolate pure glycoproteins and glycopeptides were in general difficult since the formation of molecular aggregates prevents the separation of protein material and low molecular weight fragments [8].

The monosaccharide composition of MGp indicated that it contains *N*-glycosidic oligomannosidic type oligosaccharide chains [23]; a small amount of galactose was also detected suggesting the presence of about 5% of an *N*-acetyllactosaminic structure. If an *N*-acetyllactosaminic unit is discounted, the molar ratio Man:GlcNAc is 3.6 which would correspond to about seven residues of mannose for every two residues of GlcNAc and to an average molecular weight of 1540. The percentage of peptide suggests that the average molecular weight of the glycopeptide is about 3300, in agreement with the fact that it is eluted at the total volume in the gel filtration on Sephacryl S-200 and at the void volume on Sephadex G-15. Neutral N-glycosidically linked glycopeptides with similar composition and molecular size have been characterized in brains from adult albino Wistar rats [24] and IgM [25].

Structural studies were carried out on glycopeptides MGp by methylation analysis and chromium trioxide oxidation. Analysis of the partially methylated neutral sugars revealed the presence of terminal non-reducing mannose, 2-*O*-substituted mannose and 3, 6-di-*O*-substituted mannose which constitute 82.5 mole %. The relative percentages of tetra- and tri-methyl derivatives agree with the general basic structure accepted for high-mannose type N-glycans (Figure 3). The determination of the anomeric configuration confirmed the expected α-D-glycosidic linkages; the low percentage of β-D-configuration would correspond to the mannose residue linked to the di-*N*-acetylchitobiose unit in the

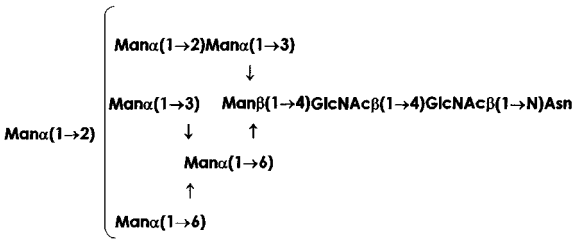

Figure 3. Proposed structure for MGp core oligosaccharides.

inner core. Based on the data obtained the basic primary structure for the core of the MGp glycan is proposed as indicated in Figure 3, where the third terminal mannose residue could be linked to any of the subterminal mannose units. This structure resembles the oligomannosidic-type structure conventionally assigned as M9, present in calf thyroglobulin A, CHO cell glycoproteins, bovine lactotransferin, soy bean agglutinin, human IgD, Newcastle virus and scorpion hemocyanin [26].

The specific structures of complex oligosaccharide are responsible for their properties as carriers of significant biological information and cellular recognition [27]. The importance of carbohydrates in reproductive physiology has been reported. Gamete interaction is mediated by protein-carbohydrate recognition, and clustering of sperm receptors to the zona pellucida oligosaccharides has been proposed as the mechanism of sperm exocytosis induction [1, 2]. Mannose receptors are present on the sperm surface [12] and their occupancy mediates the physiological mechanism of AR [13]. We have described here that high-mannose glycopeptides of low MW inhibit the sperm AR, confirming that carbohydrates must be linked to a macromolecule to induce the sperm exocytosis [11]. This information suggests a physiological role for MGp in fertilization, as follows: (a) glycoproteins from seminal plasma are proteolysed after ejaculation producing high-mannose glycopeptides; (b) these low MW fragments bind specifically to the mannose receptor on the sperm surface hindering an earlier AR; (c) during sperm passage through the female reproductive tract MGp is removed from the mannose receptors; and (d) the unoccupied receptors bind the zona pellucida glycoproteins. In conclusion, the results obtained indicate that mannosyl glycopeptides from seminal plasma act as protective factors against a premature exocytosis mediated by sperm-surface receptors.

Acknowledgments

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