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Fractionation and characterization of boar seminal plasma spermadhesin PSP-II glycoforms reveal the presence of uncommon *N*-acetylgalactosamine-containing N-linked oligosaccharides

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Lectin mapping, carbohydrate analysis and electrospray mass spectrometry of boar seminal plasma PSP-II glycoforms show that its single N-glycosylation site displays a repertoire of carbohydrate structures consisting of the basic pentasaccharide core Manα 1–6[Manα 1–3]Manβ1-4GlcNAcβ1-4GlcNAc with a fucosyl residue α1-6-linked to the innermost *N*-acetylglucosamine residue. Other glycoforms display fucosylated hybrid-type or monoantennary complex-type chains, some of which contain α2-6-linked sialic acid. *N*-acetylgalactosamine, possibly in Galβ1-3GalNAc sequence, is present in most of the PSP-II glycoforms.

***Keywords*: boar seminal plasma, electrospray mass spectrometry, lectin mapping, N-glycoforms, PSP-II, spermadhesin**

***Abbreviations*: PSP-I and PSP-II, porcine seminal plasma proteins I and II; PNGaseF, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase (EC 3.5.1.52) from *Flavobacterium meningosepticum*; ConA, Cannavalia ensiformis (jack bean) agglutinin; GNA, *Galanthus nivalis* (snowdrop) agglutin; SNA, *Sambucus nigra* (elderberry) agglutinin; MAA, *Maackia amurensis* (maakia) agglutinin; PNA, *Arachis hypogaea* (peanut) agglutinin; DSA, *Datura stramonium* (jimson weed) agglutinin; AAA, *Aleuria aurantia* agglutinin**

Introduction

The recognition and initial binding of mammalian spermatozoa to the glycoprotein network surrounding the investing egg, the zona pellucida, are mediated by protein-carbohydrate interactions, and this basic mechanism appears to be conserved throughout the whole evolutive scale [1]. Spermatozoa possess a complement of surface-associated proteins that mediate interactions with glycoconjugates of the zona pellucida of the oocyte at fertilization. Both, integral membrane and peripherally associated sperm lectins have been described in a number of species [2].

Accumulating evidence from studies on porcine fertilization indicates that members of the spermadhesin family with affinity for heparin and zona pellucida carbohydrates might play a prominent role in sperm capacitation and initial gamete interaction [3, 4]. Spermadhesins are a group of seminal plasma proteins which coat the acrosomal cap of the sperm head at ejaculation [5]. They contain 110–133 amino acids, two conserved disulphide bridges, and have 40–60% sequence identity. Spermadhesins are built by a single domain architecture [6], whose three-dimensional structure has not been reported. However, a sequence-pattern search analysis has suggested that spermadhesins are a subgroup of a large family of functionally diverse, often developmentally regulated proteins, which share the CUB domain, a 110-amino acid module with a predicted antiparallel β-barrel topology similar to that of immunoglobulin V domains [7].

PSP-I/PSP-II heterodimer is the major component of boar seminal plasma [8, 9]. Both subunits belong to the spermadhesin protein family [9]. The PSP-I/PSP-II heterodimer is a lectin which contains a binding site for zona pellucida glycoproteins located in PSP-II [9]. We have recently crystallized the PSP-I/PSP-II heterodimer [10].

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This, its carbohydrate-binding characteristics and easiness of isolation, makes this spermadhesin a paradigm for studying protein-carbohydrate interactions involved in gamete recognition.

The two subunits of the spermadhesin PSP-I/PSP-II heterodimer are glycoproteins containing a single oligosaccharide attached to asparagine residues 47 and 98, respectively [9]. The glycosylation site of both PSP-I and PSP-II accommodates different glycan moieties, *ie* displays site heterogeneity. Characterization of these carbohydrate structures is important since it has been shown that glycosylation modulates the ligand-binding capabilities of glycosylated spermadhesins [11, 12]. Furthermore, preliminary analysis of the carbohydrate content of PSP-I and PSP-II showed that both glycoproteins contain *N*-acetylgalactosamine [9], which is a quite uncommon feature of N-linked oligosaccharides [13, 14]. On the other hand, the complete covalent structure would be of help for an accurate interpretation of the electron density map of crystallized PSP-I/PSP-II. Here, we report the fractionation by affinity chromatography of PSP-II glycoforms and their characterization using monosaccharide analysis, lectin mapping, and mass spectrometry.

Methods

Isolation of boar seminal plasma PSP-II

The PSP-I/PSP-II heterodimer was isolated from the non-heparin-binding fraction of boar seminal plasma by gel-filtration chromatography on Sephadex G-50 (Pharmacia, Sweden) as described [9]. PSP-II was purified from the heterodimer by reverse-phase HPLC on a Lichrocart (Merck, Germany) preparative (250 × 10 mm, 10 μm particle size) column eluted at 3 ml min^{-1} with a mixture of 0.1% trifluoroacetic acid in water (A) and acetonitrile (B), isocratically (25% B) for 5 min, followed by 25–35% B for 10 min, and 35–60% B for 75 min. Fractions containing PSP-II (eluted with 45–48% B) were pooled, dialysed against MilliQ water, and lyophilized. The purity of the preparation was assessed by SDS-(15%)polyacrylamide gel electrophoresis [15], N-terminal sequencing (done with an Applied Biosystems 473A sequencer), and amino acid analysis (using a Pharmacia Alpha Plus analyser after sample hydrolysis with 6 N HCl for 24 h at 110°).

Fractionation of PSP-II glycoforms

For fractionation of glycoforms, 40 mg of PSP-II (10 mg ml^{-1} in 10 mM Tris/HCl, 150 mM NaCl, pH 7.8, TBS) were chromatographed on a ConA-Sepharose column (11 × 1.65 cm, 14 mg lectin per ml, Sigma, USA). After washing with TBS, bound PSP-II glycoforms were eluted at 15 ml h^{-1} with a 230 ml gradient of α-methyl-D- glucopyranoside (0–50 mM) and then isocratically with 0.2 M α-methyl-D-glucopyranoside. The eluate was monitored at 280 nm and 7.5 ml fractions were collected. Samples were concentrated by ultrafiltration using Centriprep-10 (Amicon, USA) and stored at − 20° until use.

Enzymatic deglycosylation

Deglycosylation with O-glycanase and PNGaseF (Boehringer Mannheim, Germany) was done as in [16]. Native and glycanase-treated proteins, recovered by precipitation with cold methanol [11], were analysed by SDS-polyacrylamide gel electrophoresis, and electrotransferred onto nitrocellulose sheets [17]. Desialylation of blotted glycoproteins was done by treatment with 50 mM H_2SO_4 for 90 min at 80 °C.

Lectin mapping

Blots of native, deglycosylated, and desialylated proteins were stained with the following digoxigenin-labelled lectins (Boehringer Mannheim, Germany) [18], whose primary binding specificity is indicated in parentheses: GNA [Manα(1-2,-3, or -6)-Man]; SNA [Neu5Acα2-6-Gal]; MAA [Neu5Acα2-3-Gal]; PNA [Galβ1-3-GalNAc]; DSA [Galβ1-4-GlcNAc]; and AAA [Fucα1-6-GlcNAc].

Monosaccharide analysis

For amino sugar and neutral sugar analyses the samples were hydrolysed with 4 N HCl for 4 h or 2 N HCl for 2 h, respectively, at 110°C. For sialic acid determination, the samples were hydrolysed with 0.2 N trifluoroacetic acid for 1 h at 80 °C. After drying the hydrolysates in a SpeedVac (Savant, Germany), monosaccharides were resolved on a CarboPac PA1 column (4 × 250 mm) eluting at 1 ml min^{-1} isocratically with either 16 mM NaOH (for amino and neutral sugars) or with 20 mM NaOH in 60 mM sodium acetate (for sialic acids), and analysed using a Dionex DX-300 analyser equipped with pulsed amperometric detector and the AI-450 chromatographic software [19].

Mass spectrometry

Molecular masses were determined by electrospray ionization mass spectrometry using a Sciex API-III LC/MS/MS time-of-flight triple quadropole instrument. Molecular masses of the oligosaccharides were calculated by subtracting 12643 [9] from the experimentally determined masses of the PSP-II glycoforms.

Results and discussion

Fractionation of PSP-II glycoforms and lectin mapping

Six major fractions of spermadhesin PSP-II glycoforms (G), termed G1–G6, were separated by affinity chromatography on ConA-Sepharose (Figure 1A). Each fraction showed a distinct protein profile, as revealed by SDS-polyacrylamide

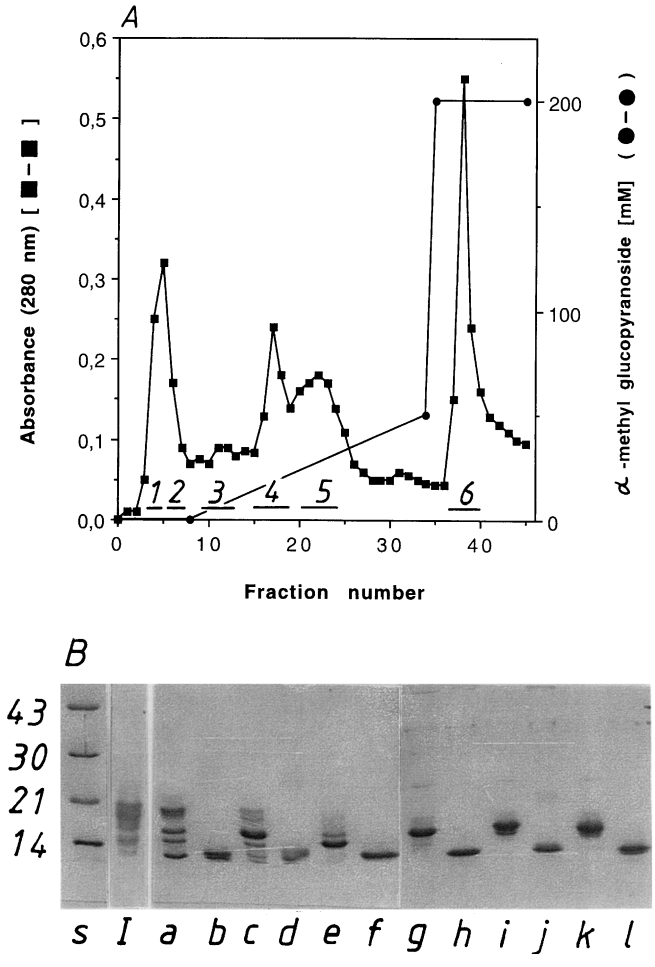


Figure 1. (A) Fractionation of PSP-II glycoforms by affinity chromatography on ConA-Sepharose. The bars indicate pooled fractions herein referred to as G1–G6. (B) SDS-polyacrylamide gel electrophoresis of PSP-II glycoforms G1–G6, before (lanes a,c,e,g,i, and k, respectively), and after deglycosylation with PNGaseF (lanes b,d,f,h,j, and l, respectively). Lane I, initial PSP-II sample. Lane s, molecular mass standards, from top to bottom, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, whose molecular masses are indicated on the left.

gel electrophoresis (Figure 1B, lanes a,c,e,g,i,k). G1, which accounted for 5.3% of the loaded protein, eluted in the flow-through fraction and was composed of four major bands designated G1.1, G1.2, G1.3, and G1.4 in order of decreasing apparent molecular mass (Figure 1B, lane a), whereas G2–G6 were predominantly composed of a major band (Figure 1B, lanes c,e,g,i, and k, respectively). G1.2 was apparently the same band as the major component of G2 (10.3% of total PSP-II), which eluted in a slightly retarded position with the non-bound material (Figure 1A). G1.3 showed a similar electrophoretic mobility as the major band of G3. G3 accounted for 3% of the loaded protein. PSP-II glycoforms G4 and G5 were eluted with 18 and 30 mM α-methyl-D-glucopyranoside, respectively, and contained 15% and 27.3% of total PSP-II. Fraction G6 eluted isocratically with 0.2 M α-methyl-D-glucopyranoside and accounted for 21.5% of PSP-II glycoforms. Treatment of G1–G6 with PNGaseF yielded the same electrophoretic band with apparent molecular mass of 12.5 kDa (Figure 1B, lanes b,d,f,h,j,l). Deglycosylated G1 migrated as a doublet, which may correspond to the previously reported mixture of native PSP-II (residues 1–116, $M + H^+ = 12643$ Da) and a truncated form encompassing residues 2–115 ($M + H^+ = 12410$ Da) [9].

Lectin mapping of blotted PSP-II glycoforms revealed different binding patterns for the different fractions (Figure 2, Table 1). On the other hand, after PNGaseF treatment no reactivity was observed with any of the lectins tested, indicating that deglycosylation was quantitative. Similarly, G1.4 did not interact with any of the lectins, suggesting that this component corresponded to non-glycosylated PSP-II. O-glycanase-treated PSP-II displayed the same lectin-binding capability as the native protein (data not shown), in agreement with previous results indicating that PSP-II contains a single N-glycosylation site [9].

Galanthus nivalis agglutinin (GNA) interacted strongly with the major and minor components of G5 and G6 (designated G5.1 and G5.2, and G6.1 and G6.2, respectively; see Figure 2B, lanes 5 and 6, and Table 1), and more weakly with glycoform G1.1 (Figure 2B, lane 1) and minor components of G2 and G3 (Figure 2B, lanes 2 and 3; Table 1). This lectin, which is specific for terminal mannose α1-2, -3, or -6 linked to mannose and does not react with complex-type chains [20], indicates the presence of high-mannose or hybrid-type structures in these PSP-II glycoforms.

PSP-II glycoforms G1.1, G4, G5.1, and G6.1 interacted with *Sambucus nigra* (elderberry) agglutinin (Figure 2C, lanes 1, 4, 5, and 6, respectively, and Table 1), which is specific for sialic acid α2-6-linked to galactose [21]. None of the glycoforms were recognized by MAA, the *Maackia amurensis* (maakia) agglutinin with specificity for α2-3-linked sialic acid [22], indicating that the sialylated PSP-II glycoforms contain only the α2-6 isomer.

Most PSP-II glycoforms gave a positive reaction with AAA (Figure 2D, Table 1), the *Aleuria aurantia* lectin, which shows a strong preference for fucosyl residues α1-6-linked to the proximal *N*-acetylglucosamine moiety [23].

Except for G1.1 and some minor components of G2 and G3, none of the PSP-II glycoforms displayed binding to *Datura stramonium* (jimson weed) agglutinin (DSA) Figs. 2E and 2F, Table 1), which is specific for the Galβ1-4-GlcNAc termini of complex oligosaccharides [24]. Desialylation of blotted glycoproteins by mild acid hydrolysis did not modify the DSA binding pattern (compare Figure 2E and F), indicating the absence of sialylated Galβ1-4-GlcNAc sequences.

Arachis hypogaea (peanut) agglutinin (PNA) bound to various degrees to all PSP-II glycoforms with exception of G3 (Figure 2G, Table 1). This indicated the presence of the

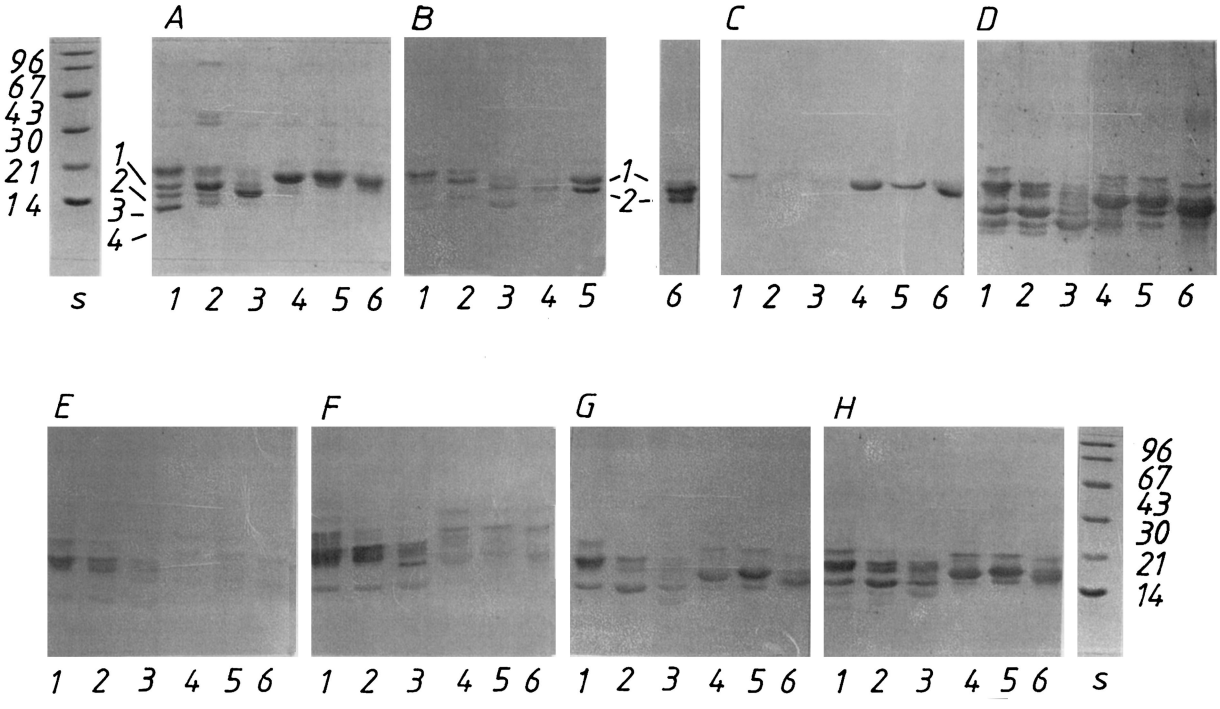


Figure 2. Electrophoretic (A) and lectin mapping analysis using lectins GNA (B), SNA (C), AAA (D), DSA before and after desialylation (E and F, respectively), and PNA before and after desialylation (G and H, respectively) of PSP-II glycoforms G1–G6 (lanes 1–6, respectively). Lane s, molecular mass standards, from top to bottom, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, whose molecular masses are indicated on the left.

Table 1. Lectin binding patterns and monosaccharide composition of PSP-II glycoforms (G) fractionated by affinity chromatography on ConA-Sepharose (Figure 1). Only major glycoforms in fractions G1–G6 of Figure 2A are listed. −, ±, and +, ++, +++, indicate negative, weak and positive reaction of increasing intensity, respectively.

	Lectins						*Monosaccharide ratio* (mol:mol)				
	MAA	GNA	SNA	AAA	PNA	DSA	Fuc:	Gal:	GalNAc:	Man:	GlcNAc
G1.1	−	+	+	+++	++	+					
G1.2	−	−	−	+++	+	−					
G1.3	−	−	−	++	−	−					
G1.4	−	−	−	−	−	−					
G2	−	−	−	+++	+	−	0.6	1	1	3	2.6
G3	−	±	−	+	−	−	0.7	−	−	3.2	2.1
G4	−	−	+++	++	+	−	0.6	0.8	1.1	3.8	4
G5.1	−	+++	+++	++	+++	−	0.9	1	0.9	5	2.8
G5.2	−	+++	−	+	±	−					
G6.1	−	+++	+++	+++	+	−	1.1	0.7	1	5	3.1
G6.2	−	+++	−	±	−	−					

Galβ1-3-GalNAc sequence [25]. Desialylation significantly improved binding of PNA to G4 and minor glycoforms of G3 (compare lanes 3 and 4 of Figure 2G and H), indicating that the Galβ1-3-GalNAc sequence in these glycoproteins may be substituted with sialic acid.

Mass spectrometry and monosaccharide analysis

Further characterization of the carbohydrate chains of isolated PSP-II glycoforms was attempted by determining the molecular mass and carbohydrate composition of G2–G6. Mass spectrometric analysis showed single ions in G2

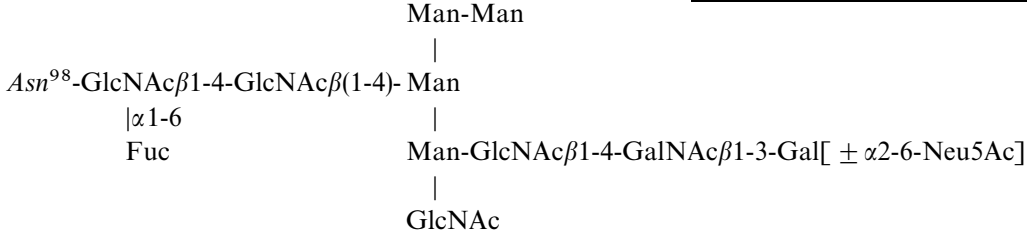
and G3 with molecular masses 14095 ± 15 Da and 13683 ± 5 Da, respectively. On the other hand, G4, G5, and G6 displayed two major ions of molecular masses 14612 ± 2 Da and 14907 ± 2 Da, 14733 ± 3 Da and 14787 ± 6 Da, and 14215 ± 13 Da and 14855 ± 11 Da, respectively. Taking into account the molecular mass of 12643 Da for non-glycosylated PSP-II (polypeptide chain and 4 disulphide bridges [9]), these data indicate the presence of oligosaccharide chains ranging from 1040–2264 Da.

Compositional analysis confirmed the presence of galactosamine in all PSP-II fractions, with the only exception of G3 where neither galactosamine nor galactose were detected. Fucose was detected in all samples, and *N*-acetylneuraminic acid was detected in G4, G5, ad G6. Fuc:Gal:GalNAc:Man:GlcNAc ratios are shown in Table 1.

On the carbohydrate structures of PSP-II glycoforms

The results from the present study are fully consistent with the existence of multiple PSP-II glycoforms which contain a single N-linked oligosaccharide chain with an unusual structure. On the basis of monosaccharide analysis, lectin mapping, and mass spectrometry, it can be concluded that the oligosaccharide chain in fraction G3 may consist of the basic pentasaccharide core Manα1-6[Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc with a fucosyl residue α1-6-linked to the innermost *N*-acetylglucosamine residue, while other glycoforms may display fucosylated hybrid-type (G4, G5.2, and G6) or monoantennary complex-type (G2 and G5.1) chains, some of which may contain α2-6-linked sialic acid. The monosaccharide composition and lectin binding patterns unequivocally reveal the presence of *N*-acetylgalactosamine in most of the hybrid- and complex-type chains, possibly in the Galβ1-3GalNAc sequence.

The two species found in G4 by mass spectrometry (14907 and 14612 ± 2 Da), may correspond to the same carbohydrate structure differing in their sialic acid content (292 Da). A possible carbohydrate structure compatible with our data is:



The calculated molecular mass of the proposed non-sialylated G4 PSP-II glycoform is 14613 Da. Our working hypothesis is that the site heterogeneity of PSP-II may be due to the attachment of an oligosaccharide chain out of a collection of structures related to that proposed for G4. However, it must be clearly stated that the carbohydrate structures proposed here are only tentative and must be verified by structure elucidation methods. ^{1}H-NMR studies have been initiated to determine the actual structure of the oligosaccharides of major PSP-II glycoforms.

Although the presence of GalNAc in di-, tri-, and tetra-antennary complex-type and hybrid-type N-linked carbohydrate chains has been documented, in most of these structures it occurs in a GalNAcβ1-4GlcNAc or, more rarely, GalNAcβ1-4Gal sequence [26–30]. A Galβ1-3GalNAc sequence has also been reported in a collection of N-linked oligosaccharides from hemocyanin of the snail *Lymnaea stagnalis* [31], as well as in the GPI anchor of porcine and human renal membrane dipeptidase [32]. The present study provides another example of a glycoprotein displaying a repertoire of GalNAc-containing N-linked oligosaccharides.

Glycoforms of boar seminal plasma proteins AQN-3, PSP-I, and AWN-1 of the spermadhesin family have been reported [9, 11, 12, 16]. None of them, however, contain carbohydrate structures similar to those predicted here for PSP-II. Glycosylation appears to modify the aggregation state and ligand binding capabilities of spermadhesins [11, 12, 16, 33]. In particular, glycosylation of asparagine-50 prevents zona pellucida and serine proteinase inhibitor binding to AQN-3, AWN-1, and PSP-I. On the other hand, PSP-II is unique among its protein family because glycosylation of asparagine-98 does not impair its binding activities [9]. Furthermore, PSP-II glycoforms recruit certain PSP-I glycoforms into heterodimers [9]. Thus, the occurrence of uncommon carbohydrate chains in PSP-II opens the question of their possible involvement in modulating the biological activities and heterodimer formation properties of this glycoprotein.

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