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Characterization of AWN-1 glycosylated isoforms helps define the zona pellucida and serine proteinase inhibitor-binding region on boar spermadhesins

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Spermadhesin AWN-1 (14 kDa) belongs to a recently described family of boar sperm surface-associated proteins. AWN-1 is a multifunctional protein which possesses heparin-, serine proteinase inhibitor-, and zona pellucida glycoprotein-binding capability. Therefore it has been implicated in sperm capacitation and sperm-oocyte attachment. Here, we report the characterization of 22–25 kDa isoforms of AWN-1 isolated by heparin-affinity chromatography, which fail to bind to zona pellucida glycoproteins or serine proteinase inhibitors. Our results show that the structure of the high and low molecular mass AWN-1 forms differ in that the former is N-glycosylated at Asp^{50} and truncated at the C-terminus. The inability of the glycosylated AWN-1 molecules to bind ligands is due solely to the presence of the oligosaccharide moieties, however. This indicates that glycosylation of AWN-1 may modulate its ligand-binding capabilities. On the other hand, the effect of glycosylation on ligand-binding suggests that both the zona pellucida- and the serine proteinase inhibitor binding domain(s) may be located around the glycosylation point.

Boar sperm protein; Zona pellucida-binding protein; Trypsin inhibitor-binding protein; Spermadhesin; AWN; Glycosylation

1. INTRODUCTION

Species-specific sperm–egg recognition and binding is mediated by a series of interactions between complementary molecules located at the surface of homologous gametes [1]. In mammals, this biological activity involves a carbohydrate-recognition mechanism between proteins located on the sperm's acrosomal cap and saccharide chains of the oocyte's extracellular matrix called zona pellucida [2]. A wide variety of mammalian sperm antigens involved in binding to the zona pellucida have been described in a number of species [2,3]. However, neither are the structural data available to compare apparently similar molecules from different species, nor has the relative contribution of different zona pellucida-binding molecules to the process of sperm–egg interaction been evaluated.

AWN-1 (14 kDa) belongs to a boar sperm-associated zona pellucida-binding protein family termed 'spermadhesin' [2,4–6]. It is a multi-functional protein which combines within the same molecule zona pellucida glycoprotein- [7], heparin- [8], and serine proteinase inhibitor-binding [9] capabilities. This indicates that spermadhesin AWN may play a role in at least two important aspects of pig fertilization, i.e. sperm capacitation by heparin-like glycosaminoglycans present in the female sexual tract, and primary receptor for oligosaccharide moieties attached to oocyte zona pellucida glycoproteins. The molecular features of the AWN-1 structure responsible for its diverse ligand-recognition abilities remain unknown, however.

In a previous paper [8], we identified a heparin-binding protein the N-terminal sequence of which is identical to that of AWN-1 but which possesses a higher molecular mass (22–25 kDa) and which fails to bind zona pellucida glycoproteins and soybean trypsin inhibitor. Here, we show that this AWN-like molecule is actually a mixture of single-point glycosylated isoforms of AWN-1 (G-AWN-1) at Asp^{50}. Moreover, glycosylation appears to be the sole cause of the inability of G-AWN to bind both zona pellucida glycoproteins and soybean trypsin inhibitor. These data indicate that glycosylation may modulate the ligand-binding activity of spermadhesin isoforms. In addition, our results suggest that the ligand-binding pocket for zona pellucida oligosaccharide(s) and serine proteinase inhibitors may (partially) overlap within the region where the glycosylation site is located.

2. MATERIALS AND METHODS

Isolation of boar seminal plasma heparin-binding proteins was done by affinity chromatography followed by reverse-phase HPLC as described [8].

SDS-polyacrylamide gel electrophoresis was performed according to [10].

Polyclonal monospecific anti-AWN-1 antibodies were raised in

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chicken and the IgG fraction was purified from the egg's yolk as described [11].

For peptide mapping, the isolated proteins (2 mg/ml in 100 mM Tris-HCl, 0.5 M guanidine hydrochloride, pH 8.0) were incubated at 37°C overnight with tosylphenylalanine chlormethane-treated trypsin (sequencing grade; Boehringer-Mannheim) at an enzyme/substrate ratio of 1:100 (w/w). Enzymatic digestion mixtures were lyophilized and the tryptic peptides isolated by reverse-phase HPLC on a Lichrospher RP-100 (Merck) C_{18} column (25 × 0.4 cm, 5 μm particle size) eluting at 1 ml/min with a gradient of 0.1% trifluoroacetic acid in (A) water and (B) acetonitrile, first isocratically (5% B) for 5 min followed by 5–50% B for 90 min, and 50–70% for 20 min. Peptides were detected at 220 nm and manually collected for further analysis.

Amino acid and amino sugar analyses were done with a Pharmacia-LKB Alpha Plus analyzer after hydrolysis at 110°C in 6 M HCl for 24 h and 4 M HCl for 4 h, respectively. N-Terminal sequence analyses were performed using an Applied Biosystems 473A sequencer.

For enzymatic deglycosylation, 0.5 mg of isolated protein (2 mg/ml in 50 mM phosphate, pH 7.0) was incubated overnight at 37°C with 10 U of *N*-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (Boehringer-Mannheim).

Electroblotting onto nitrocellulose sheets was done following [12]. To test the ligand-binding activities of electroblotted proteins (intact or deglycosylated), the blots were blocked with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% (w/v) BSA, and incubated for 1 h at 37°C with either (A) anti-AWN-1 antibody (1:1,000 dilution), washed and incubated for 1 h at 37°C with a 1:300 (v/v) dilution of biotinylated rabbit anti-chicken IgG antibody [4], (B) 2.5 μg/ml biotinylated pig zona pellucida [13], or (C) 15 μg/ml biotinylated soybean trypsin inhibitor (Sigma) [9]. After extensive washing, the blots were incubated for 1 h at 37°C with streptavidin–peroxidase (1:1,000), washed with 20 mM Tris-HCl, 500 mM NaCl, pH 7.4, and finally developed with this buffer containing 20% methanol, 1 mg/ml 4-chloro-1-naphtol (Bio-Rad), and 15 μl H_2O_2.

3. RESULTS AND DISCUSSION

3.1. *Biochemical characterization of the AWN-related 22–25 kDa protein*

To ascertain the degree of structural relationship between the boar seminal plasma 22–25 kDa heparin-binding protein and AWN-1, we investigated their chemical composition, immunological reactivity towards monospecific polyclonal chicken anti-AWN-1 antibodies, and peptide maps.

The amino acid composition of both proteins was almost identical, and amino sugar analysis revealed that only the 22–25 kDa protein contained glucosamine (3.25 mol/100 mol amino acids) (data not shown). To investigate whether N-glycosylation was the sole difference between the two proteins, the 22–25 kDa protein was enzymatically deglycosylated with PNGase F. Strikingly, the apparent molecular mass of the deglycosylated protein was around 2 kDa lower then that of native AWN-1 (Fig. 1, lane e). This was not due to a protease activity in the PNGaseF preparation since in a parallel experiment, the endoglycosidase did not cleave isolated AWN-1 (Fig. 1, lane c). On the other hand, the three proteins were identified with anti-AWN-1 antibodies, indicating that they are closely immunological related (Fig. 2).

To further characterize the structural differences between AWN-1 and the deglycosylated AWN-related

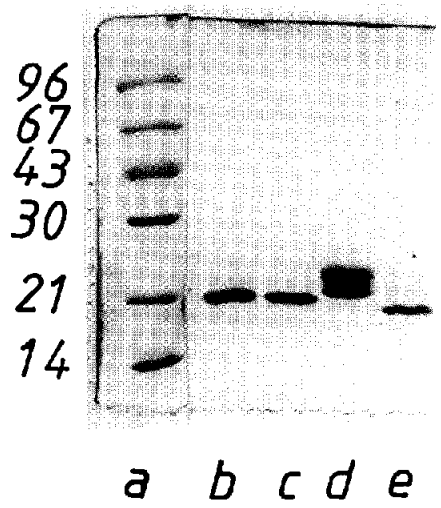

Fig. 1. Non-reduced SDS-polyacrylamide (10–25%) gel electrophoresis of boar spermadhesin AWN (lane c) and the 22–25 kDa heparin-binding AWN-related protein (lane d). Lanes b and e, the same samples as in lanes c and d, respectively, after treatment with PNGaseF. Lane a, molecular mass standards: from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. The numbers at the left indicate molecular masses in kDa.

protein, both were cleaved with trypsin and their tryptic peptide maps analyzed by reverse-phase HPLC (Fig. 3). The only difference found was the presence of an extra peak (labeled 3 in Fig. 3A) in the AWN-1 digest. Amino acid analysis of this fragment showed that its composition was (in mol/peptide): 2 Thr, 2 Glu/Gln, 1 Ala, 2 Ile, and 1 Lys. This clearly indicated that peptide 3 corresponded to the C-terminal AWN polypeptide stretch 123–130 [4]. On the other hand, the amino acid composition of the common peaks failed to show any differences between corresponding pairs. Therefore, we conclude that the 22–25 kDa protein contains a mixture of N-glycosylated isoforms of AWN 1–122.

To localize the glycosylated residue(s), the 22–25 kDa was cleaved with trypsin and its tryptic peptide map (Fig. 3B) compared with that of AWN-1 (Fig. 3A). Amino acid analysis of peak 8 (marked with an asterisk in Fig. 3B) showed that it contained the disulphide-bonded peptides, 36–55 and 73–82, and amino sugar determination revealed the presence of glucosamine (not shown). The primary structure of this fragment includes the motif ^{50}Asn-Leu-Ser^{52} [4], a consensus sequence for N-glycosylation. This is the only NXS/T sequence found in AWN-1 [4] and, thus, our results strongly suggest that the observed electrophoretic heterogeneity of the 22–25 kDa isoform of AWN may be due to different glycosylation patterns on a single site.

3.2. *The structural basis of the inability of glycosylated AWN to bind ligands*

To establish whether the failure of the glycosylated AWN-1 isoforms to bind zona pellucida glycoproteins

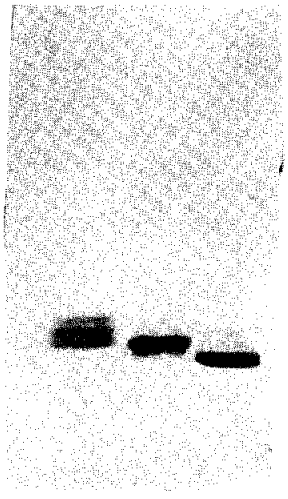

Fig. 2. Detection by immunoblotting analysis of anti-AWN binding activity. Lane a, the 22–25 kDa heparin-binding protein; lane b, boar spermadhesin AWN; lane c, the 22–25 kDa protein after PNGaseF treatment.

and serine proteinase inhibitor was due to the C-terminal truncation or to the presence of an oligosaccharide chain, we analyzed the ligand-binding capabilities of native and PNGaseF-treated 22–25 kDa proteins using Western blot. It was found that the deglycosylated low molecular mass isoform of AWN-1 was able to bind both biotinylated zona pellucida glycoproteins (Fig. 4A) and biotinylated soybean trypsin inhibitor (Fig. 4B). These results indicate that glycosylation may regulate the ligand-binding abilities of AWN-1, and that the C-terminal tail of AWN-1 (residues 123–133) may not be necessary for ligand binding.

It is worth noting that boar spermadhesin AWN-2, which is identical to AWN-1 except that it contains an acetylated N-terminus [7], also has 22–25 kDa Asn^{50}-glycosylated isoforms which show the same ligand-binding spectrum as G-AWN-1 (unpublished observations). Spermadhesins AWN-1 and AWN-2 have been implicated in two important events of sperm function, i.e. in sperm capacitation (as a positive extrinsic factor by its heparin- and serine proteinase inhibitor-binding activities) [8,9] and in sperm–egg binding (as a primary counter-receptor for zona pellucida) [4,7]. Hence, the suggested hypothesis that glycosylation/deglycosylation could represent a mechanism for switching the receptor functions of the AWN isoforms deserves further investigation.

Finally, the different effect of glycosylation on the ligand-binding capabilities of the 22–25 kDa AWN (1 or 2) isoforms indicates that the protein domain involved in heparin binding is different from the zona pellucida- and serine proteinase inhibitor-binding

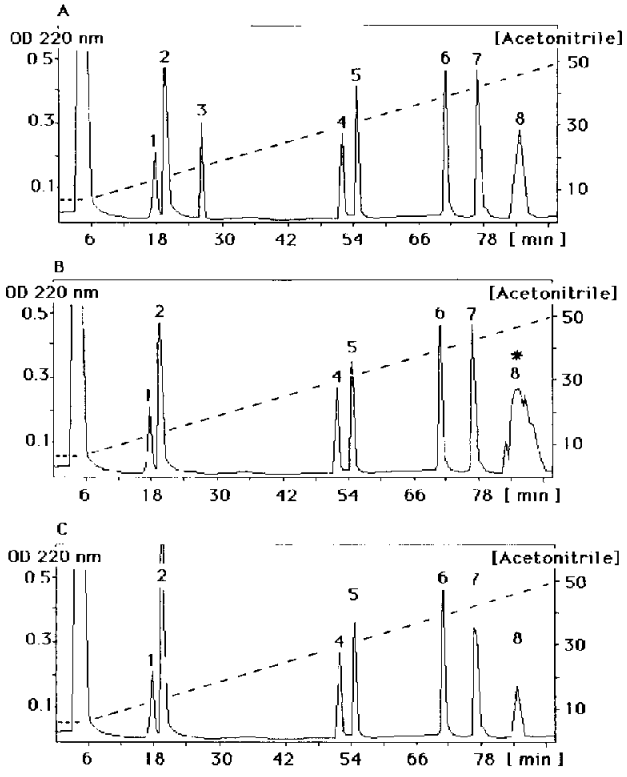

Fig. 3. Comparison of the reverse-phase HPLC tryptic peptide maps of AWN (A), native glycosylated 22–25 kDa protein (B), and the 22–25 kDa protein after enzymatic deglycosylation with PNGaseF (C).

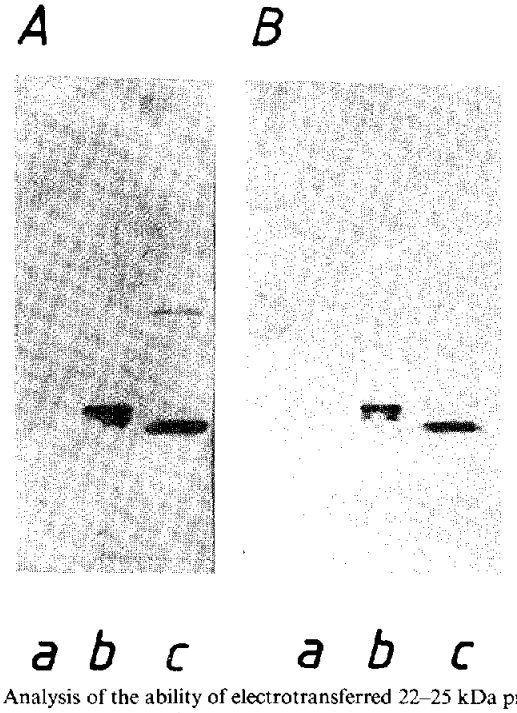

Fig. 4. Analysis of the ability of electrotransferred 22–25 kDa protein (lane a), AWN (lane b) and PNGaseF-treated 22–25 kDa protein (lane c) to bind (A) biotinylated zona pellucida glycoproteins and (B) biotinylated soybean trypsin inhibitor.

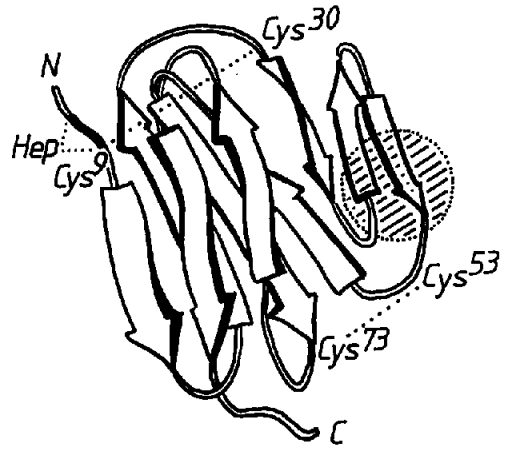

Fig. 5. Molecular model of spermadhesin AWN showing its two disulphide bridges and the position of the glycosylation site (shadowed surface), and the putative heparin-binding region (filled polypeptide stretch labeled 'Hep'). N, N-terminus; C, C-terminus.

site(s). On the other hand, the ligand-binding pocket for zona pellucida and soybean trypsin inhibitor may, at least partially, overlap within an AWN region surrounding the glycosylation site. This latter conclusion agrees with the current hypothesis that the main function of sperm-bound seminal plasma serine proteinase-like inhibitors may be the stabilization, or protection, of sperm membrane sites with specificity for oocyte recognition, and that release of the bound inhibitor during sperm residence in the uterine tract allows sperm to bind to the oocyte's zona pellucida [14,15].

Based on a pattern–search approach, Bork and Beckmann [16] have predicted that AWN (and the other boar spermadhesins [2]) may belong to the CUB-domain superfamily of proteins, the topology of which may resemble that of an immunoglobulin-like antiparallel β barrel domain. Based on this information, and taking the coordinates of the recently solved X-ray structure of the third immunoglobulin-like domain of rat CD4 [17], a schematic model for a spermadhesin molecule is depicted in Fig. 5. Should this model turn out to be right, then our present data indicate that the binding sites for zona pellucida oligosaccharide and soybean trypsin inhibitor may reside within the loop formed between Cys^{30} and Cys^{53}, being β-strands 3–5, the main contributors to the ligand-binding pocket surface. Interestingly, AWN possesses the heparin-binding consensus sequence, ^{3}NRRSRS^{8} (labeled 'Hep' in Fig. 5), which in the AWN molecular model is located at the opposite face of that containing the zona pellucida/inhibitor binding sites. This would be in accordance with the fact that heparin binding to AWN (isoforms 1 or 2) is not affected by glycosylation. Whether this AWN region actually contains the heparin binding site needs, however, further investigation.

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