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# Boar spermadhesins AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins

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Trypsin-like inhibitors secreted by the male accessory sex glands have been identified in the seminal plasma of every mammalian species so far investigated. They bind to acceptor molecules on the anterior part of ejaculated sperm, and are thought to play a role in the capacitation of spermatozoa stabilizing zona pellucida binding sites during sperm uterine passage, and then dissociating to allow sperm–egg's zona pellucida interaction. Here we report the identification of acrosin inhibitor acceptor molecules isolated from boar seminal plasma. These proteins, termed AQN-1 and AWN, belong to the recently described spermadhesin protein family, whose members have been implicated in sperm–zona pellucida recognition events. Thus, members of the spermadhesin family, although not possessing detectable enzymatic activity, show features of serine proteinases, and may be involved in both sperm capacitation and sperm–egg recognition and binding events.

Boar acrosin inhibitor acceptor; AQN-1; AWN; Spermadhesin; Sperm capacitation

## 1. INTRODUCTION

Low molecular mass (6–12 kDa) trypsin-like proteinase inhibitors present in the male accessory glands secretions were first isolated by Händle and co-workers in 1965 [1] and have been found in the seminal plasma of every mammalian species so far investigated [2]. The functions of these seminal plasma inhibitors have not been conclusively established. They may protect the epithelia of both the male and the female reproductive tracts, as well as the intact spermatozoa therein, from the proteolytic attack of enzymes released from occasional damaged spermatozoa [3,4]. The seminal plasma inhibitors, once bound to acceptor molecules on the anterior portion of the sperm head, are also thought to play a pivotal role in sperm capacitation [5,6]. The most extensive investigation on trypsin-like proteinase inhibitors of seminal plasma origin has been carried out in the mouse. In this species, a 6.4 kDa acrosin and trypsin, but not chymotrypsin, kallikrein, thrombin or plasmin inhibitor [7] binds to a 15 kDa acceptor protein on the anterior portion of ejaculated spermatozoa which possesses mouse zona pellucida binding site(s) [8–14]. The seminal plasma inhibitor blocks the binding of sperm to its homologous zona pellucida. After 4 h in the utero however, few sperm showed evidence of the inhibitor [9]. The current working hypothesis is that the proteinase inhibitor could stabilize functional zona pellucida binding sites during sperm uterine passage and then dissociate allowing sperm binding to the oocyte's extracellular coat. Thus, the mouse seminal inhibitor-binding component may function in two important aspects of fertilization: capacitation and zona binding. Similarly, in the boar and in guinea-pig, several lines of evidence indicate that a trypsin-like receptor on spermatozoa is involved in zona pellucida binding [15–17].

In a previous paper [18] we have reported the isolation and complete primary structure of three isoforms of a Kazal-type boar sperm-associated acrosin inhibitor. Here, we have concentrated on the identification of acceptor molecules for this inhibitor. Our results show that two members of the recently characterized spermadhesin protein family, AQN-1 [19] and AWN [20], have this binding activity. This implies that some spermadhesins, besides having a role as sperm–zona pellucida recognition molecules, may be also involved in boar sperm capacitation. Binding of the inhibitor did not block the interaction of AQN-1 or AWN with zona pellucida.

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## 2. MATERIALS AND METHODS

Sperm-associated acrosin inhibitors were isolated as described [18,21]. For biotinylation, 0.5 mg of purified acrosin inhibitor, or alternatively soybean trypsin inhibitor (Sigma), were dissolved at a concentration of 1 mg/ml in 0.2 M sodium bicarbonate buffer, pH 8.8, 10 μl of a 4 mg/ml solution of *N*-hydroxysuccinimide biotin (Sigma) in *N*-dimethylformamide were added under stirring, and the reaction mixture was left for 1 h at room temperature and in the dark. Thereafter the reaction was stopped by addition of 50 μl of 1 M ammonium

chloride, pH 6.0. The biotinylated acrosin inhibitor was isolated by reverse-phase HPLC using a Nucleosil C-18 analytical column (25 × 0.4 cm, 5 μm particle size) eluting at 1 ml/min with a linear gradient of 0.1% (v/v) trifluoroacetic acid in (A) water and (B) acetonitrile, first isocratically (10% B) for 5 min followed by up to 50% B in 40 min. The fractions containing acrosin inhibitor, which eluted at about 34% B, were lyophilized. Isolation of the biotinylated soybean trypsin inhibitor was done by size exclusion chromatography on a Sephadex G-25 (50 × 0.5 cm) column equilibrated in, and eluted with, 50 mM Tris-HCl, pH 7.5.

The spermadhesins AQN-1, AQN-2, AQN-3, AWN-1 and AWN-2 were isolated by reverse-phase HPLC as previously described [20,22]. SDS-gel electrophoresis was done according to [23].

Electroblotting onto nitrocellulose sheets was performed as in [24]. After blocking, the blots were incubated for 2 h at room temperature with either 2.5 μg/ml biotinylated pig zona pellucida [25], 3.7 μg/ml biotinylated acrosin inhibitor, or 0.8 μg/ml biotinylated soybean trypsin inhibitor. These values corresponded to the concentrations which produced maximum binding of the ligands to isolated spermadhesins in an ELISA-like assay (not shown). Thereafter, the blots were washed, incubated for 1 h with streptavidin peroxidase (1:1000, v/v), washed with 20 mM Tris-HCl, 0.5 M 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 $H_2O_2$.

For zona pellucida-binding inhibition experiments, the blots were first incubated in the presence of 3.7 μg/ml acrosin inhibitor or 0.1 mg/ml soybean trypsin inhibitor. To test the possibility that zona pellucida could displace the bound acrosin inhibitor, blots were first incubated with biotinylated inhibitor, washed, and then incubated with 2.5 μg/ml zona pellucida prior to incubation with streptavidin peroxidase. Capacitation of spermatozoa was done as described [19].

The topography of AWN on boar sperm was studied by indirect fluorescence microscopy. Briefly, 10 μl of the capacitated spermatozoa suspension ($10^6$/ml) were spread on slides, air-dried, fixed for 15 min in methanol, incubated with PBS buffer containing 5% (w/v) BSA for 2 h at 37°C followed by incubation with a 0.1 mg/ml solution of biotinylated acrosin inhibitor in PBS/BSA buffer. After washing with PBS/ BSA buffer the sample was incubated for 2 h at 37°C with FITC-labeled streptavidin (Calbiochem) (1:100, v/v) and washed exhaustively with PBS/BSA buffer. Finally, 10 μl of PBS/glycerol (1:9, v/v) were added and the sample observed under a fluorescence microscope (Zeiss, 100 × 10 magnification).

To detect possible protease activity of isolated AQN-1 and AWN, the protease substrate gel tablet kit supplied by Bio-Rad was used. To mimic its natural medium, the sample buffer was 20 mM phosphate, 150 mM NaCl, pH 7.4 in which an identical volume of boar seminal plasma was dialyzed for 1 h in a 3 kDa cut-off dialysis membrane.

## 3. RESULTS AND DISCUSSION

Boar seminal plasma contains an 8 kDa acrosin inhibitor which binds tightly to ejaculated spermatozoa [18]. In a number of mammalian species sperm-associated proteinase inhibitor acceptor molecules play a role in both sperm capacitation [5,6] and zona pellucida binding [8–17]. Recently, we have characterized several boar sperm carbohydrate- and zona pellucida-binding proteins [19–22], and have coined the term spermadhesin to collectively denominate them. Spermadhesins are low molecular mass (12–16 kDa) boar seminal plasma proteins, which become coated to the sperm head surface upon ejaculation. Five members of this protein family have been structurally and functionally characterized: AQN-1 [19,22], AQN-2 [22], AQN-3 [26], AWN-1 and AWN-2 [20]. All of them, except AQN-2, possess zona pellucida binding activity, and have been, thus, implicated in sperm–egg recognition and binding events.

Here we have tested the capability of isolated spermadhesins to bind serine proteinase inhibitors, and have studied the topographical localization of the sperm-associated boar acrosin inhibitor-acceptor molecule complexes by fluorescence microscopy.

Figure 1 shows that the inhibitor–acceptor complex is located on the anterior part of the sperm head. This is the compartment where spermadhesins were located [19,27], suggesting that members of this protein family could be implicated in the binding of the inhibitor to the sperm surface. Indeed, when the isolated, electroblotted spermadhesins were probed, AQN-1, AWN-1 and AWN-2 possessed the ability to bind the acrosin inhibitor (Fig. 2). AWN-2 contains an acetylated N-terminus but an otherwise identical polypeptide chain as AWN-1 [20,27], and will be considered here, therefore, as a single chemical unit, which we will designate AWN. Our result implies that some, but not all, of the zona pellucida-binding spermadhesins may function as sperm-

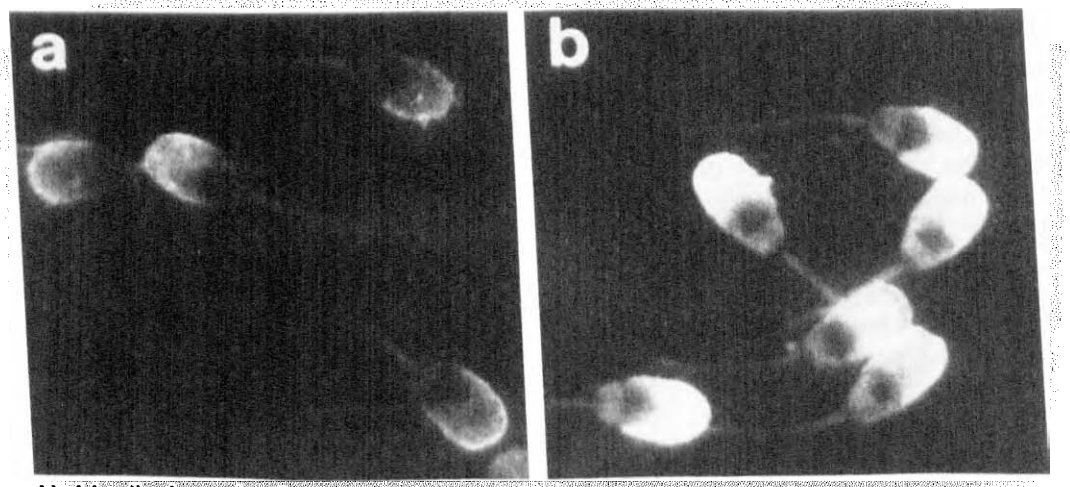


Fig. 1. Topographical localization, using fluorescence microscopy, of biotinylated acrosin inhibitor-acceptor molecules on the surface of (a) non-fixed and (b) fixed boar capacitated spermatozoa. The same pattern was observed using biotinylated soybean trypsin inhibitor.

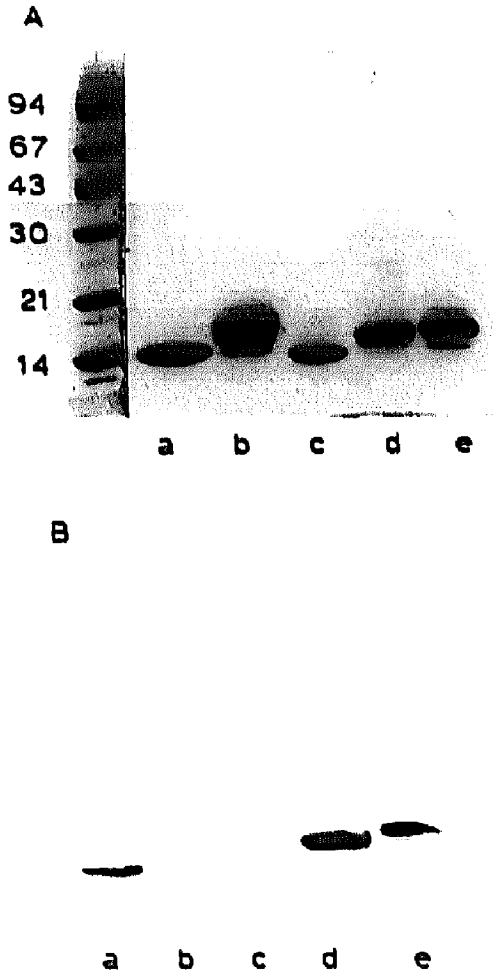


Fig. 2. (A) 10–25% polyacrylamide SDS gel electrophoresis of isolated boar spermadhesins. Lane a–e each contained 5 μg of AQN-1, AQN-2, AQN-3, AWN-1, and AWN-2, respectively. On the left, standard molecular mass markers in kDa. (B) The isolated boar spermadhesins were electrophoresed as in (A), electrotransferred to nitrocellulose sheets and probed for its ability to bind biotinylated Kazal-type acrosin inhibitor. The same result was obtained when the experiment was repeated with biotinylated Kunitz-type soybean trypsin inhibitor.

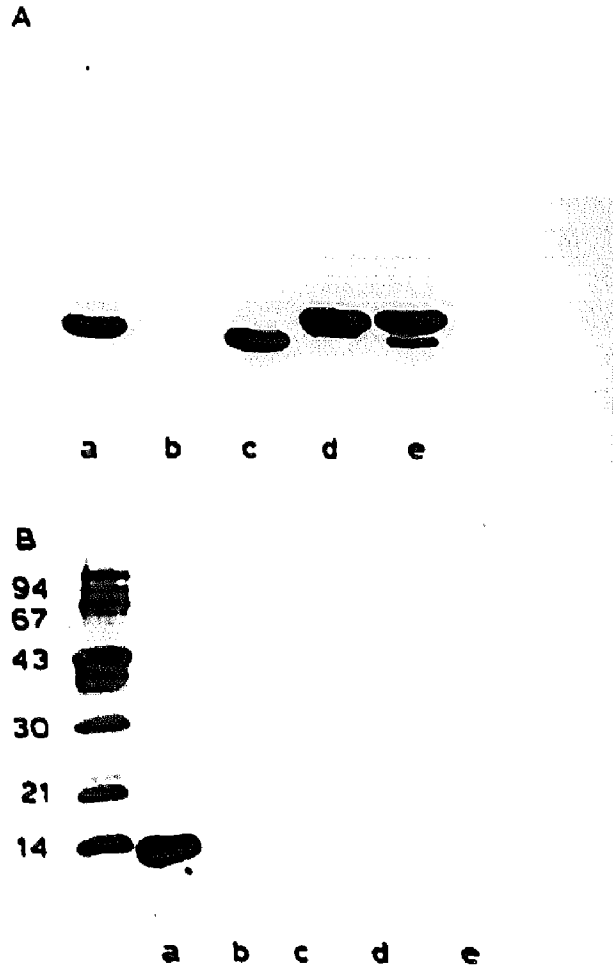


Fig. 3. Analysis of the ability of the acrosin inhibitor to block the binding of solubilized zona pellucida to isolated spermadhesins. In (A) a blot containing a replica of the SDS gel shown in Fig. 2A was first incubated with acrosin inhibitor followed by biotinylated pig's zona pellucida. In (B) a similar blot was first incubated with biotinylated acrosin inhibitor and then with native zona pellucida.

associated proteinase inhibitor acceptor molecules. Since in a number of mammalian species seminal plasma proteinase inhibitor-binding components, which show similar sperm compartimentalization and zona pellucida-binding activity as AQN-1 and AWN, act as extrinsic regulatory capacitation factors (see Introduction), our data point out that the boar spermadhesins AQN-1 and AWN may play a dual role in fertilization, both as capacitation factors and as sperm adhesion molecules.

Interestingly, AQN-1 shares 50% sequence identity with AQN-3 [19]. Proteins with such high primary structure conservation often show tertiary structures that can be superimposed with a root-mean-square deviation of less than 1 Å [28]. Thus, we believe that the tertiary structures of AQN-1 and AQN-3 may show a high overall similarity. However, since AQN-3 failed to bind the acrosin inhibitor, the fine ligand-binding specificity among different members of the boar spermadhesin protein family has not been evolutionarily conserved.

AQN-1 and AWN bind both boar seminal plasma acrosin inhibitor and soybean trypsin inhibitor in a mutually exclusive manner (Fig. 2). These inhibitors contain a similar proteinase-binding loop, but are otherwise structurally unrelated. Therefore, we hypothesize that both inhibitors may utilize the protease-binding site for interaction with its sperm acceptor molecule. No enzymatic activity was associated with AQN-1 or AWN, however. Thus, although these inhibitor acceptor molecules may share some features with trypsin-like enzymes, their binding sites must be different from the active sites of serine proteinases.

Although spermadhesins AQN-1 and AWN functionally resemble the 15 kDa mouse sperm proteinase inhibitor acceptor protein [7–14], the relationship be-

tween them remains to be established. Contrary to what happens with the mouse 15 kDa acceptor molecule, the binding of the boar seminal plasma acrosin inhibitor to the electroblotted boar spermadhesins did not inhibit the subsequent binding of solubilized pig's biotinylated zona pellucida (Fig. 3A). This may indicate that either the binding sites for the acrosin inhibitor and zona pellucida reside within different, and mutually non-exclusive, protein binding domains within the spermadhesin structure, or that the binding of the second ligand competed and displaced the first one. To distinguish between these two possibilities, a blot containing the individual spermadhesins was first incubated with biotinylated acrosin inhibitor and then with native solubilized zona pellucida. Figure 3B shows that only AQN-1 retained bound inhibitor after interaction with solubilized zona pellucida. Thus, we may conclude that in AQN-1 different binding sites for both ligands may exist, and that there is no steric interference between the binding of zona pellucida and the acrosin inhibitor. In the case of AWN, we interpret that interaction of solubilized zona pellucida with the AWN-inhibitor complex somehow alters its conformation and displaces the bound inhibitor. Whether zona pellucida and the proteinase inhibitor compete for the same, or for a structurally related, binding site can, however, not be deduced from our experiments.

Based on our data, we propose that AQN-1 and AWN may function in a similar, but not overlapping way in spermatozoa capacitation. Recently, Aarons et al. [29] have shown that immunoaggregation of a proteinase inhibitor bound to the murine sperm head was sufficient to trigger the acrosome reaction. Whether interaction of the sperm surface-associated AQN-1/inhibitor complexes with the repeating structure of the oocyte's zona pellucida network induces its clustering and initiates thereby the exocytosis of the acrosome content will be the subject of future investigation.

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## REFERENCES

[1] Händle, H., Fritz, H., Trautschold, I. and Werle, E. (1965) Hoppe-Seyler's Z. Physiol. Chem. 343, 185–188.
[2] Falase, E.A.O., Storey, B.T. and Teuscher, C. (1991) Molec. Reprod. Develop. 29, 29–39.
[3] McRorie, R.A. and Williams, W.L. (1974) Annu. Rev. Biochem. 43, 777–803.
[4] Fritz, H., Schiessler, H., Schill, W.B., Tschesche, H., Heimburger, N. and Wallner, O. (1975) in: Proteases and Biological Control (Reich, E., Rifkin, D. and Shaw, E., eds.) pp. 737–766, Cold Spring Harbor Laboratory, New York.
[5] Zaneveld, L.J.D., Srivastava, P.N. and Williams, W.L. (1969) J. Reprod. Fertil. 20, 337–339.
[6] Schill, W.B., Heimburger, N., Schiessler, H., Stolla, R. and Fritz, H. (1975) Hoppe-Seyler's Physiol. Chem. 356, 1473–1476.
[7] Poirier, G.R. and Jackson, J. (1981) Gamete Res. 4, 555–569.
[8] Nicholson, N., Irwin, M. and Poirier, G.R. (1983) J. Exp. Zool. 225, 481–487.
[9] Irwin, M., Nicholson, N., Haywood, J.T. and Poirier, G.R. (1983) Biol. Reprod. 28, 1202–1206.
[10] Aarons, D., Speake, L.J. and Poirier, G.R. (1984) Biol. Reprod. 31, 811–817.
[11] Poirier, G.R., Robinson, R., Richardson, R., Hinds, K. and Clayton, D. (1986) Gamete Res. 14, 235–243.
[12] Robinson, R., Richardson, R., Hinds, K., Clayton, D. and Poirier, G.R. (1987) Gamete Res. 16, 217–228.
[13] Boettger, H., Richardson, R., Free, D., Rushing, S. and Poirier, G.R. (1989) J. Exp. Zool. 249, 90–98.
[14] Benau, D.A. and Storey, B.T. (1987) Biol. Reprod. 36, 282–292.
[15] Saling, P.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6231–6235.
[16] Jones, R., Brown, C.R. and Lancaster, R.T. (1988) Development 102, 781–792.
[17] Jones, R. and Williams, R.M. (1990) Development 109, 41–50.
[18] Jonáková, V., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R. and Töpfer-Petersen, E. (1992) FEBS Lett. 297, 147–150.
[19] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R. and Töpfer-Petersen, E. (1992) Eur. J. Biochem. (in press).
[20] Sanz, L., Calvete, J.J., Schäfer, W., Mann, K. and Töpfer-Petersen, E. (1992) Biochim. Biophys. Acta (in press).
[21] Jonáková, V., Čechová, D., Töpfer-Petersen, E., Calvete, J.J. and Veselský, L. (1991) Biomed. Biochim. Acta 50, 691–695.
[22] Jonáková, V., Sanz, L., Calvete, J.J., Henschen, A., Čechová, D. and Töpfer-Petersen, E. (1991) FEBS Lett. 280, 183–186.
[23] Laemmli, U.K. (170) Nature 227, 680–685.
[24] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[25] Töpfer-Petersen, E. and Henschen, A. (1988) Biol. Chem. Hoppe-Seyler 369, 69–75.
[26] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R. and Töpfer-Petersen, E. (1991) FEBS Lett. 291, 33–36.
[27] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R., Amselgruber, W., Sinowatz, F., Ehrhard, M. and Töpfer-Petersen, E. (1992) FEBS Lett. (submitted).
[28] Doolittle, R.F. (ed.) Methods Enzymol. 183, Academic Press, San Diego.
[29] Aarons, D., Boettgertong, H., Holt, G. and Poirier, G.R. (1991) Mol. Reprod. Dev. 30, 258–264.