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Acrosin shows zona and fucose binding, novel properties for a serine proteinase

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The major fucose-binding protein of 53 kDa from boar spermatozoa was isolated to apparent homogeneity using a two-step procedure including high-performance gel filtration and reversed-phase chromatography. The N-terminal sequence of the protein revealed that it is identical with the sperm proteinase acrosin. By means of a solid-phase zona-binding assay based on the avidin-biotin system it was demonstrated that acrosin also interacts strongly with porcine zona pellucida. Thus, the acrosin molecule combines specific proteolytic activity with zona- and carbohydrate-affinity properties, i.e. previously unrecognized properties of a serine proteinase. It seems likely that this special affinity of acrosin directs the proteolytic activity to its structural target in the in vivo situation.

Zona binding; Fucose binding; Acrosin; Sperm-zona interaction; (Boar spermatozoon)

1. INTRODUCTION

A crucial step during fertilization is the penetration of the oocyte zona pellucida by the sperm cell. Limited proteolysis of the glycoprotein matrix of the zona by the acrosomal proteinase acrosin is believed to contribute to sperm penetration [1–3]. A prerequisite to penetration and in the end effect for successful fertilization is the attachment and species-specific binding of spermatozoa to the zona pellucida [4]. Since several sugars have been shown to inhibit sperm-zona binding it seems likely that gamete adhesion and recognition involves carbohydrate-protein interactions [5]. Thus, it has been demonstrated that fucoidan, an algal sulfated L-fucose polymer, and to a minor extent fucose efficiently inhibit the binding of spermatozoa to the zona pellucida in several mammalian species [6]. L-Fucose in the same conformation as fucoidan has been suggested to be part of the recognition signal between mammalian gametes [7]. Recently, a major fucose-binding protein of 53 kDa which might be involved in gamete interaction in the pig was identified and localized in boar spermatozoa [8–10]. Here, evidence is presented that the major fucose-binding protein of boar spermatozoa is identical with the sperm proteinase acrosin and that it also binds in a specific manner to isolated zona pellucida.

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Abbreviations: HPLC, high-performance liquid chromatography; FPLC, fast-protein liquid chromatography

2. EXPERIMENTAL

2.1. *Preparation of spermatozoa*

Freshly ejaculated boar spermatozoa were washed three times with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM benzamidine to remove seminal plasma, pelleted and frozen at −20°C.

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For extraction the spermatozoa were resuspended in 20 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 30 mM octylglucopyranoside (Sigma), 1 mM PMSF, 0.1 mM benzamidine and incubated overnight at 4°C. After centrifugation (50000 × *g*, 10 min) the clear supernatant was dialysed against 7 M urea, 1 M HCOOH.

2.2. *Isolation of the major fucose-binding protein*

The sperm extract (5–10 mg protein in 0.3 ml) was successively passed over a Superose 12 HR 10/30 column (LKB-Pharmacia) equilibrated with 7 M urea, 1 M HCOOH using an FPLC system (LKB-Pharmacia). The eluted fractions were screened for fucose-binding activity using a modified enzyme-linked lectin assay [8]. The activity-containing fractions were pooled, concentrated and then subjected to HPLC for further purification. The crude fucose-binding protein (3 mg) was injected onto a Hipore RP 318 column (250 × 10 mm; Biorad) and eluted with a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at 25°C. The eluted fractions were screened for fucose-binding activity by means of the modified enzyme-linked lectin assay. Protein components were recovered by lyophilization. Protein concentration was determined semi-quantitatively in microtiter plates by means of the dye-binding protein assay [11].

2.3. *Modified enzyme-linked lectin assay*

Screening for carbohydrate-binding proteins, i.e. lectin-like proteins, was performed as described [8]. Briefly, aliquots of the effluents were first adjusted to neutrality with an equivalent volume of 1 M Tris and then immobilized on microelisa plates (Dynatec) in coating buffer (0.05 M Na_2CO_3, $NaHCO_3$, pH 9.6; overnight, 4°C). After extensive washing the immobilized proteins were incubated with 100 μl fucosylperoxidase (2 μg/ml; IBF) in washing buffer (10 mM Tris-HCl, pH 7.4, 0.85% NaCl, 0.05% Tween 20, 5 mg/ml bovine serum albumin containing 3 mM $CaCl_2$) in the presence of 0.2 M α-methyl-D-mannoside (Fluka) to avoid unspecific binding [8]. Binding of the fucose residues, covalently linked to the peroxidase, could be directly visualized by enzymatic amplification with *o*-phenylenediamine (0.2%) and H_2O_2 (0.02%) in substrate buffer (0.024 M citric acid, 0.051 M NaH_2PO_4, pH 5.0; 100 μl/well). The enzyme reaction was stopped after 30 min with 4.5 M H_2SO_4 (50 μl/well). The absorbance was determined at 492 nm. The specificity was checked by inhibition of the binding reaction with fucoidan (1 mg/ml; Sigma).

2.4. *Biotinylation of zona pellucida proteins*

Isolated porcine zona pellucida [12] was resuspended in 0.2 M $NaHCO_3$, pH 9.0, and solubilized by heating at 72°C for 20 min. After centrifugation the clear supernatant was adjusted to a protein concentration of 0.5–1 mg/ml and reacted with 0.1 mg *N*-hydroxysuccinimido-biotin (Sigma) in 25 μl dimethylformamide for 15–30 min at 25°C. The reaction was stopped by addition of 1 M NH_4Cl, pH 6.0 (15 μl/ml). The biotinylated zona pellucida was immediately dialysed against 20 mM Tris-HCl, pH 7.8, 0.4 M NaCl, and stored at −20°C.

2.5. *Solid-phase zona-binding assay*

The zona-binding activity of sperm proteins was screened by means of the biotin-avidin system [13]. Aliquots of the effluent were immobilized on microelisa plates as described above. After washing the immobilized proteins were first incubated with 100 μl biotinylated zona pellucida in washing buffer (1:8000) in the presence of 3 mM $CaCl_2$ for 2 h at 37°C and subsequent to additional washing steps treated with 100 μl streptavidin-peroxidase (1:5000 in washing buffer; Calbiochem) for 60 min at 37°C. Following washing the enzyme reaction was developed as described in section 2.3. The specificity of binding was determined by inhibition with fucoidan (1 mg/ml). Unspecific binding of streptavidin-peroxidase was evaluated by omitting the binding reaction with biotinylated zona pellucida.

2.6. *Electrophoresis*

SDS-PAGE was performed in 0.1% SDS on 7.5–20% polyacrylamide slab gels as in [8]. After electrophoresis the proteins were transblotted to nitrocellulose [14]. For identification of fucose-binding activity the sheets were stained with fucosylperoxidase as described [8].

2.7. *Amino acid sequence analysis*

Sequence determination was performed by the Edman degradation method in a prototype

spinning-cup sequenator [15]. Phenylthiohydantoins were identified by HPLC in an isocratic system [16].

3. RESULTS AND DISCUSSION

A fucose-binding protein has recently been identified and localized in boar spermatozoa using fucosylperoxidase as a standard carbohydrate ligand [8–10]. In intact spermatozoa fucose binding is concentrated at the plasma membrane covering the rostral tip of the sperm head. After ionophore-induced acrosome reaction fucose-binding sites become exposed over the entire acrosomal cap. Ultrathin section of acrosome-reacted spermatozoa labelled with a fucosylperoxidase-colloidal gold complex showed a preferential labelling of the fuzzy material adhering to the outer acrosomal membrane whereas the newly exposed inner acrosomal membrane shows only moderate labelling. It could be shown that predominantly a protein with an apparent molecular mass of 53 kDa and, to some extent, a low-molecular-mass protein (17 kDa) are responsible for the fucose-binding activity in boar spermatozoa. There exists evidence suggesting that these proteins may be involved in the sperm-zona interaction in the pig [10,17].

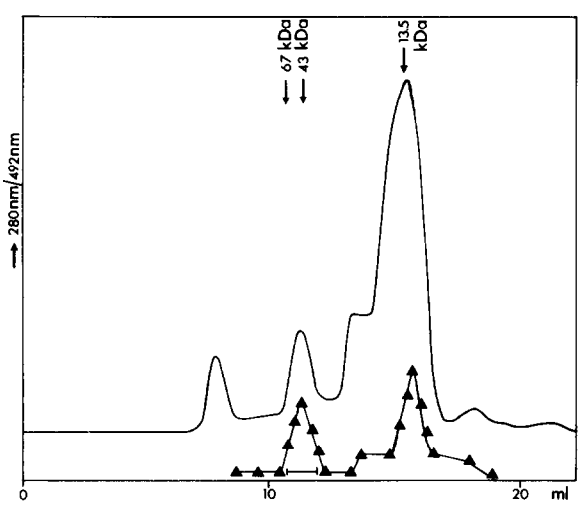

Fig.1. Chromatography of the sperm extract on a Superose 12 HR10/30 column in 1 M HCOOH, 7 M urea. Flow rate, 0.5 ml/min. (——) A_{280}; (▲—▲) A_{492}, fucose-binding activity. Pooled fractions indicated by horizontal bar.

In order to study the nature and properties of the major fucose-binding protein in more detail it was first isolated. For this purpose a two-step procedure including high-performance gel filtration (fig.1) and reversed-phase chromatography (fig.2a) was used. The presence of fucose-binding activity was conveniently monitored by the modified enzyme-linked lectin assay. A fucose-binding component was eluted from the reversed-phase HPLC column at a concentration of 42–44% acetonitrile. On SDS gel electrophoresis the HPLC component gave rise to a single band of 53 kDa which showed fucose-binding activity when tested with fucosylperoxidase after transblotting to nitrocellulose (fig.3a). This binding was

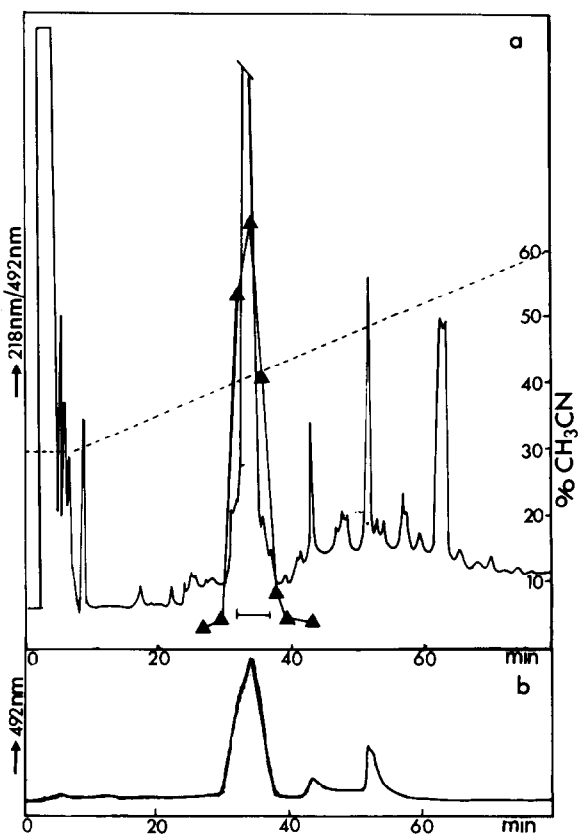

Fig.2. (a) Chromatography of the crude fucose-binding protein on a reversed-phase Hipore HP 318 column (250 × 10 mm). Flow rate, 3 ml/min. (——) A_{218}; (▲—▲) A_{492}, fucose-binding activity; (---) acetonitrile gradient in 0.1% (v/v) CF_3COOH. Pooled fractions indicated by horizontal bar. (b) Determination of the zona-binding activity by means of the solid-phase zona-binding assay.

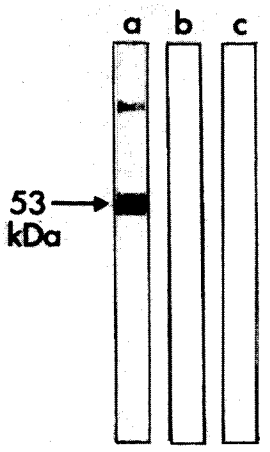

Fig.3. (a) Detection of fucose binding after SDS-PAGE and electrophoretic transfer to nitrocellulose with fucosylperoxidase (50 μg/ml). (b) Control experiment in the presence of fucoidan (1 mg/ml). (c) Control experiment with non-fucosylated peroxidase (50 μg/ml).

completely inhibited by the presence of fucoidan (fig.3b) indicating the specificity of the interaction. The ability of the fucose-binding protein to bind to the zona pellucida was examined by means of a solid-phase zona-binding assay utilizing biotinylated heat-solubilized porcine zona pellucida. It could be demonstrated that the HPLC component also has a strong affinity to the zona glycoproteins (fig.2b).

Amino acid sequence analysis of the HPLC component by Edman degradation indicated that the fucose-binding protein contains two polypeptide chains as two different amino acid residues could be assigned to most positions. It was then observed that these amino acids are identical with those at the N-terminus of boar acrosin [18,19], a protein with two amino-termini due to the presence of a light and heavy chain linked by disulfide bridges. Because of this identity two contiguous N-terminal sequences could also be established for the fucose-binding protein (fig.4). The presence of only one amino acid residue in positions 3, 6, 9 and 10 was then easily explained by the facts that glycosylated Asn and underivatized Cys will not give rise to identifiable products and that Pro occupies the same position in both peptide chains, respectively.

The biological function of acrosin has for many

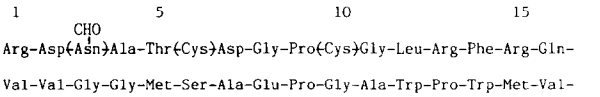

Fig.4. Amino-terminal amino acid sequences in boar sperm fucose-binding protein, identical with those in boar acrosin [18,19]. Residues in parentheses were not identified in the present analysis but previously established in acrosin.

years been the subject of debate. Novel evidence for its role in sperm penetration of the oocyte zona pellucida has recently been presented [1–3]. In this communication, it has been demonstrated that acrosin is identical with the major spermatozoal fucose-binding protein which also shows zona binding and which can be employed as a potent inhibitor of sperm binding to the zona [17]. It may therefore be suggested that acrosin participates in the sperm-egg interaction by means of its fucose-binding sites. Recently, O'Rand et al. [20] described a model for sperm penetration of the oocyte zona pellucida where alternating cycles of binding to and release from the zona together with a forward motility of the spermatozoon would be required for the penetration. In the light of our present findings it seems clear that acrosin could be instrumental in both the binding and release reactions. Thus, first the acrosome-reacted spermatozoon binds to the oocyte zona pellucida via the fucose-binding sites of acrosin and then the spermatozoon is released by limited acrosinolysis of the zona glycoproteins; subsequently these steps are repeated until the spermatozoon has completed penetration. Like several highly specific serine proteinases, e.g. those involved in blood coagulation and fibrinolysis, acrosin combines the proteolytic property which identifies a suitable substrate at the level of the peptide bond and the affinity property which recognizes the appropriate target at the level of the specific protein. The affinity of acrosin may be described as lectin-like, as it binds fucose-containing carbohydrates, certain natural glycoproteins and artificial carbohydrate-protein complexes. Acrosin seems to be the first serine proteinase showing this type of affinity.

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