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Isolation and biochemical characterization of a zona pellucida-binding glycoprotein of boar spermatozoa

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Lectin-like molecules on the sperm surface are implicated in the process of gamete recognition and adhesion. We have isolated and biochemically characterized a 15 kDa glycoprotein from ejaculated boar sperm which possess zona pellucida-binding- and haemagglutinating-activity. The zona/15 kDa protein interaction is inhibited by fucoidan, suggesting that the glycoprotein is one of the sperm components which participate in the initial gamete interaction. N-Terminal sequence analysis of the isolated 15 kDa glycoprotein showed that it may belong to the same sperm/egg recognition-mediating protein family as the sea urchin sperm protein binding.

Boar sperm; Sperm protein; Zona pellucida-binding proteins

1. INTRODUCTION

A crucial step during fertilization is the recognition and binding of the sperm to the homologous oocyte zona pellucida, the glycoprotein network which surrounds all mammalian eggs [1]. It is believed that specific adhesion molecules or receptors located at the limiting surfaces are involved in the initial gamete interaction [2,3]. In mouse, the most studied model species in fertilization, the zona pellucida glycoprotein 3 (ZP3) serves as a primary receptor for intact sperm [4] via *O*-linked oligosaccharide chains containing an essential galactose residue at their non-reducing ends [5]. Similarly, in the pig, sperm receptor activity has been reported to reside on a zona glycoprotein of M_r 55 000 [6]. On the other hand, bindin, a M_r 24 000 protein located in the sea urchin sperm acrosome granule, which binds to an egg vitelline membrane glycoprotein receptor, is the best-characterized sperm receptor-binding protein [7,8]. Putative high- and low-molecular weight sperm zona pellucida-binding proteins have been recognized in various species by: (i) incubating Western blots of sperm proteins with labelled zona proteins [9–14]; (ii) with the help of specific polyclonal and monoclonal antisera which inhibit sperm-zona binding [15–17]; or (iii) by using crosslinking reagents [18,19]. Recently, we and others showed that boar sperm acrosin (M_r 53–55 000) possess zona-binding site(s) [20,21] associated with the N-terminal peptide of its heavy chain [14]. In the present work we have searched for low molecular weight boar sperm zona-binding proteins. A 15 kDa protein showing the required biological activity was isolated and biochemically characterized. Its N-terminal sequence, and its biological function, show analogy to the sea urchin sperm receptor, termed bindin, suggesting that both sperm proteins may belong to the same evolutionary-related family.

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2. MATERIALS AND METHODS

Ejaculated boar spermatozoa were collected, washed and extracted as previously described [22]. The extract was centrifuged (1 h at 20 000 × *g*) and the supernatant fractionated on a Superose-12 preparative column (Pharmacia, Sweden) eluted with 1 M formic acid containing 7 M urea. The fractions were neutralized with 1 M Tris, diluted 1:1 (v/v) with 50 mM $Na_2CO_3/NaHCO_3$, pH 9.6, immobilized on ELISA plates, and tested for zona-binding activity [20]. Positive fractions were pooled and fractionated on a FPLC Pro-RPC (Pharmacia, preparative grade) column eluting at 3 ml/min with a linear gradient of (A) water/TFA 0.1% and (B) acetonitrile/0.1% TFA (0–60% B in 180 min). Fractions containing zona-binding protein(s) were chromatographed on a HPLC RP-100 Lichrospher (Merck) C18 column (25 × 0.4 cm, 5 μm particle size) eluting at 1 ml/min with a linear gradient of 0.1% TFA in (A) water and (B) acetonitrile (isocratically (20% B) for 5 min, followed by 20–38% B in 15 min, and 38–46% B in 32 min).

SDS-gel electrophoresis was done according to [23].

Electroblotting onto nitrocellulose sheets was performed following [24]. After blocking, the blots were incubated for 2 h at 37°C with biotinylated zona pellucida [20], washed, incubated for 1 h at 37°C with strepavidin peroxidase (1:1000 (v/v)), washed with 20 mM Tris, 0.5 M NaCl, pH 7.4, and finally developed with this buffer containing 20% methanol, 1 mg/ml 4-chloro-1-naphthol (Bio-Rad), and 15 μl

H_2O_2. For inhibition experiments, the blots were incubated in the presence of 1 mg/ml fucoidan.

Amino acid analyses were done with a Biotronik analyzer after hydrolysis at 110°C in 6 M HCl for 24 h.

Hexosamines were analyzed after sample hydrolysis at 110°C in 4 M HCl for 4 h as their dansyl-derivates [25].

N-Terminal sequence analyses were effected in a prototype spinning cup sequenator as described [26].

Reduction and alkylation was done as described [27].

Haemagglutination assays were done incubating 0.1 ml of a 5 mg/ml solution (or successive 1:1 (v/v) dilutions) of the isolated 15 kDa sperm protein in 0.9% w/v NaCl with 0.1 ml of washed erythrocytes (rabbit or human A1, A2, 0, or B type). Erythroagglutination was observed under a light microscope.

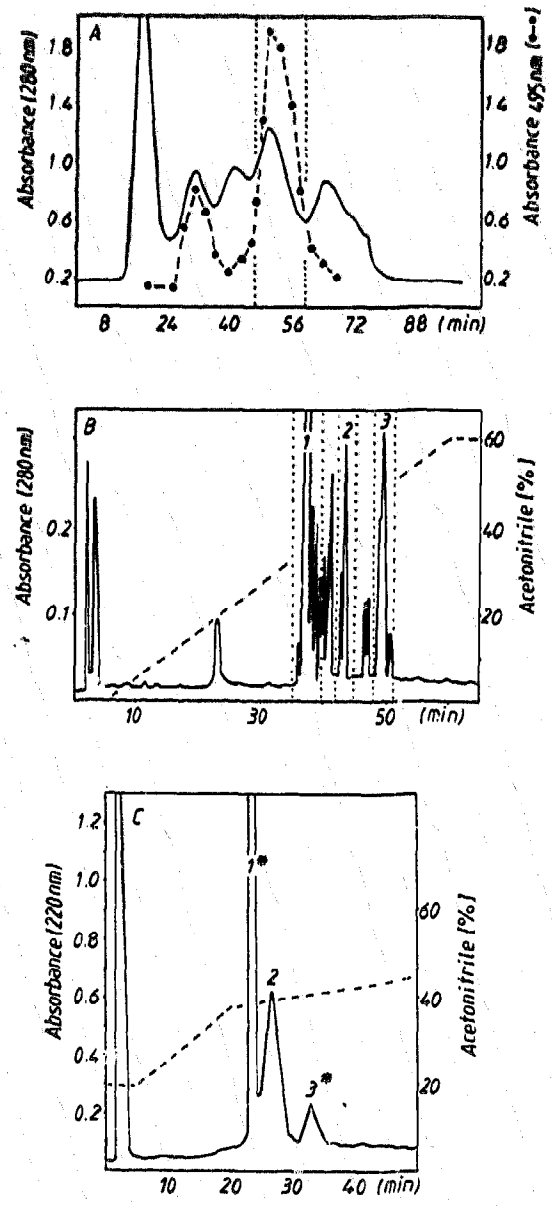

Fig. 1. (A) Fractionation of acid-extracted boar sperm proteins on a Superose 12 column. (•—•) indicate zona pellucida-binding activity. The fractions between vertical broken lines, containing low molecular weight proteins, were pooled and further purified. (B) Desalting and initial purification by reverse-phase FPLC of the components from the protein pool isolated in (A). (C) Purification by reverse-phase HPLC of the proteins contained in fraction 1 from B. (*) denotes zona pellucida-binding proteins.

3. RESULTS AND DISCUSSION

3.1. *Isolation and biological characterization of the 15 kDa sperm protein*

Fractionation of acid-extracted boar sperm proteins yielded a number of unidentified zona pellucida-binding proteins which we are continuing to characterize. In this report, however, only the major, in terms of abundance, FPLC-fraction containing zona-binding activity (fraction 1 from Fig. 1B) was further purified (Fig. 1C). Three components, two of them containing zona pellucida-binding activity, could be separated (Fig. 1C). Our purification procedure yielded several hundreds micrograms of the major protein (peak 1, Fig. 1C) per 100 mg of total acid-extracted sperm proteins.

SDS-gel electrophoresis of the isolated major zona pellucida-binding protein shows a single band migrating as 15 kDa (Fig. 2). Its mobility did not change after

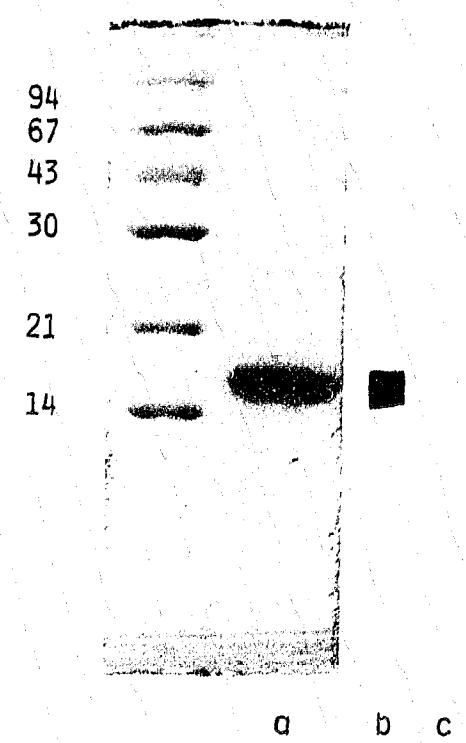

Fig. 2. (Lane a) Coomassie blue-stained SDS-polyacrylamide (10–25%) gel electrophoresis of the isolated 15 kDa protein (peak 1 from Fig. 1C). (lane b) Binding of biotinylated zona pellucida to the electroblotted 15 kDa protein. (Lane c) Incubation of the immobilized 15 kDa with zona pellucida in the presence of fucoidan. In the left lane, molecular weight markers: lysozyme, 14 K; soybean trypsin inhibitor, 21 K; carbonic anhydrase, 30 K; ovalbumin, 43 K; bovine serum albumin, 67 K; and phosphorylase *b*, 94 K.

Fig. 3. Alignment of the N-terminal sequences of the 15 kDa boar sperm protein and of the sea urchin sperm acrosome granule protein bindin. The amino acid residues of bindin and of the 15 kDa boar protein which cannot be aligned are shown raised and lowered of their respective sequences.

reduction, indicating that the protein may contain a single polypeptide chain.

The 15 kDa protein binds biotinylated zona pellucida (Fig. 2), this interaction being inhibited by the sulphated heteropolysaccharide fucoidan (Fig. 2). Fucoidan has been shown to block fertilization in a wide variety of species, including rabbit [18], guinea pig]12,28,29], boar [30], mouse [31], human [28], brown algae [32] and sea urchin [7]. The hypothesis has been born that the initial sperm/egg interaction is mediated by a lectin-like protein on the sperm surface recognizing fucose- and sulphate-containing structures of the zona pellucida. The 15 kDa boar glycoprotein agglutinates rabbit or human erythrocytes (data not shown), meeting the classical requirements of a lectin. This strengthens our conclusion that this sperm protein is actually one of the components responsible for the initial gamete interaction.

3.2. *Biochemical characterization of the 15 kDa glycoprotein*

N-Terminal sequence analysis of the isolated 15 kDa glycoprotein yielded a single sequence:
A Q N K G P H K C G G V L R N Y S G R I S T Y E G P K T D C I V...

This sequence shows a weak analogy to the sea urchin sperm protein bindin [33] (Fig. 3). Moreover, internal amino acid sequence data from of the 15 kDa protein indicate that the analogy to bindin is not restricted to the N-terminus (Sanz et al., unpublished results). The other two protein peaks in Fig. 1C were analyzed (data not shown) and the following sequences were found: peak 2 (17 kDa) - A Q N K G S D D C G G V ; peak 3 (15 kDa) - A Q N K G P D D C G G V. Recently, Moos et al. [13] have identified a 17 kDa boar sperm zona pellucida-binding protein with the N-terminal sequence: A Q N L P X R F L X P A I. It is tempting to speculate that boar sperms possess a family of analogous low molecular weight proteins some of which have a mediating role in the sperm/egg interaction, as has been identified in the rabbit [18], and were also shown to be present in human and mouse spermatozoa [10,16].

Amino acid analysis of the 15 kDa protein (Table I) shows that it contains mostly polar residues, its serine and glycine content unusually being high. The protein

Table I

Amino acid and amino sugar composition of the 15 kDa glycoprotein

Residue	Mol/100 mol amino acids
Asx	8.90
Thr	4.81
Ser	10.53
Glx	6.38
Pro	4.92
Gly	13.84
Ala	7.42
Cys*	3.23
Val	4.28
Met	N.D.
Ile	5.42
Leu	8.22
Tyr	7.65
Phe	2.51
Lys	6.49
His	1.93
Arg	3.44
Glcn	3.53
Galn	N.D.

(*) determined as the ethyl pyridyl derivative of cysteine. N.D. = not detected.

does not contain any methionine residue. When the 15 kDa protein was treated with 4-vinylpyridine under denaturing, but non-reducing, conditions, no cysteine residue could be alkylated, whereas after reduction and alkylation 4 mol of ethylpyridylcysteine/mol protein was found. This may indicate that the 15 kDa glycoprotein contains two disulphide bridges within its structure.

Amino-sugar analysis of the 15 kDa protein showed that it contained 4 mol glucosamine/mol glycoprotein, but did not contain galactosamine, indicating that it will only contain *N*-glycosydically-linked sugar chain(s).

Further studies are in progress in order to elucidate the biophysical constants governing the interaction of this protein with the zona pellucida component(s), the complete primary structure of this biologically relevant glycoprotein, its origin and its compartmentalization in the sperm. Identification of the component(s) in the zona pellucida acting as receptor(s), as well as the region of the sperm glycoprotein containing the complementary activity, is a challenge for future investigations.

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