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PURIFICATION AND CHARACTERIZATION OF A 38-kDa PROTEIN, sp38, WITH ZONA PELLUCIDA-BINDING PROPERTY FROM PORCINE EPIDIDYMAL SPERM

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Summary: A 38-kDa protein, sp38, was purified from the detergent extract of porcine epididymal sperm. Sp38 showed zona pellucida-binding properties similar to those of proacrosin. These two proteins specifically bound to the 90-kDa glycoprotein form of the zona pellucida components in a calcium-dependent manner. The binding of sp38 to the zona pellucida glycoprotein was inhibited by proacrosin. These findings suggest that the two proteins competitively interact with the zona pellucida during the early stage of fertilization.

In the process of mammalian fertilization the sperm binds to and penetrates through the egg extracellular matrix, the zona pellucida (ZP), before fusing with the egg plasma membrane. In mice, ZP3, one of the three components of the ZP, binds to plasma membrane-intact sperm cells to introduce signals for acrosome reaction by aggregating ZP3-receptors on the sperm cells (1,2). Galactosyl transferase (3,4) or 56-kDa galactose binding protein (5) may be involved in this "primary binding". Once the acrosome reaction occures, a molecule on the inner acrosomal membrane of murine sperm binds to ZP2 (6). The binding at this stage is called "secondary binding". In boars, proacrosin, a zymogen of serine protease acrosin (EC 3.4.21.10) localized in the sperm acrosome, exhibits a ZP-binding activity even after inactivation of the protease activity (7), suggesting the presence of a ZP-binding domain apart from the proteolytically active domain. The sulfated carbohydrate moieties of the ZP are believed to be the binding site for proacrosin because the binding is inhibited by dextran sulfate or fucoidan

Abbreviations: ZP, zona pellucida. p-AB, p-aminobenzamidine. PVDF, polyvinilidenedifuluoride.

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(8,9). In this study, we purified a 38-kDa protein, termed sp38, from boar epididymal sperm with ZP-binding properties similar to those of proacrosin. The binding of sp38 to the ZP was competitive with proacrosin. Partial structural data of sp38 are also described.

Materials and Methods

Purification of sp38 from boar sperm Proteins were extracted from porcine cauda epididymal sperm (2.5ml packed cells) as described (7) and subjected to gel filtration column chromatography following the method described for the purification of proacrosin (10) except that a Bio Gel P-60 column (2.5 x 70 cm) was used instead of a Sephadex G-200 column. The fractions with the ZP-binding activity (10mg protein) were pooled, dialyzed against 7M urea adjusted to pH3 with HCl (7M urea, pH3) and subjected to cation exchange HPLC with a TSK gel SP-5PW (7.5 x 75 mm, Tosoh) column equilibrated with 7M urea, 0.2M NaCl, pH3. Proteins were eluted by a linear gradient elution of 0.2-1M NaCl in 7M urea, pH3, in 200 min at room temperature. The flow rate was 0.5ml/min and 1ml fractions were collected. The fractions with the ZP-binding activity were pooled (1.2mg protein) and subjected to reverse phase HPLC with a Bakerbond Wide Pore Butyl 5μm (4.6 x 250 mm, J. T. Baker). Proteins were eluted by a gradient elution of acetonitrile in 0.1% trifluoroacetic acid from 0 to 38% during the first 5min then to 43% during the next 120 min at a flow rate of 0.5ml/min with collection of 1ml fractions.

Solid-phase binding assay ZP glycoproteins prepared as described from ovarian oocytes (11) were biotinylated (7). An aliquot of each effluent of sperm proteins was coated in each well of ELISA plates in 0.1M $NaHCO_3$ containing 2mM p-aminobenzamidine (p-AB). After washing with the washing buffer (10mM Tris-HCl, pH 7.4, 0.15M NaCl, 2mM $CaCl_2$, 2mM p-AB, 0.05% Tween-20), each well was incubated with the blocking buffer (5% BSA in the washing buffer without Tween-20) for 1hr at 37°C followed by the incubation with biotinylated ZP proteins diluted into 250 ng/ml with the dilution buffer (2% BSA in the washing buffer) for 1hr at room temperature and the subsequent incubation with streptavidin-peroxidase (ZYMED, 1:1000) at 37°C for 1hr. After the extensive washing and the color development the absorbance at 492nm was determined. In some experiments rabbit antiproacrosin antisera (1:10000) prepared as described (12) and antirabbit IgG, $F(ab')_2$ (CAPPEL, 1:5000) were used as a probe. In other experiments, biotinylated ZP glycoproteins were separated into the components by reverse phase HPLC prior to applying to the solid-phase binding assay. For this purpose ZP glycoproteins (25μg) were dissolved in 7M urea and subjected to a Bakerbond Wide Pore Butyl 5μm. Proteins were eluted by a linear gradient of 0-100% acetonitrile during a 60 min period at room temperature at a flow rate of 0.5ml/min with collection of 1ml fractions. In the competitive inhibition assay, ZP glycoproteins (50μg/ml) were coated in the wells of ELISA plates and ^{125}I-labeled sp38 ($5x10^5$ cpm/well) was incubated in the presence of various concentrations of unlabelled sp38 or proacrosin for 1 hr at room temperature. The radioactive components bound to the ZP was dissolved by 10% SDS following extensive washing and the radioactivity was counted by an auto-γ-counter. The same washing buffer, blocking buffer and dilution buffer as described above were used throughout the binding assay.

Amino acid sequence analysis Three μg of sp38 was subjected to 10% SDS-PAGE, followed by electroblot onto a polyvinilidenedifluoride (PVDF) membrane. *In situ* digestion with *Achromobacter* protease I (Wako) was performed after S-carboxymethylation of the protein as described (13). The peptides released from the membrane were separated by reverse phase HPLC using a μ-Bondasphere 5μC8-300A (2.1x150mm, Waters) column eluted with a linear gradient of 2-50% Solvent B in 40 min at a flow rate of 0.25 ml/min using the following solutions: Solvent A; 0.05% trifluoroacetic acid in water and Solvent B; 0.02% trifluoroacetic acid in 2-propanol/acetonitrile (7/3,

v/v). Amino acid sequence analysis was performed with an Applied Biosystems model 470A gas-phase sequencer. The resultant PTH-amino acids were identified by isocratic HPLC.

Electrophoresis and western blotting SDS-PAGE and electroblot were performed as usual. After blocking nonspecific binding, proteins were probed with ^{125}I-labeled ZP followed by extensive washing and autoradiography with Kodack X-Omat AR, or probed with rabbit antiproacrosin antisera (1:2000) followed by the visualization with peroxidase-antirabbit IgG F(ab')$_2$ (1:1000) and 3,3'-diamino benzidine tetrachloride. The same buffers described for the solid-phase binding assay were used for blocking, dilution and washing.

Other experimental methods ^{125}I-labelling of sp38 and ZP glycoproteins was performed by the chloramine T method.

Results and Discussion

Purification and partial structural analysis of sp38

The detergent extract of porcine cauda epididymal sperm was subjected to a Bio-Gel P-60 column and effluents were examined for the ZP-binding activity by the solid-phase binding assay. Proteins with ZP-binding activity were eluted broadly (not shown). The active fractions were combined and subjected to cation exchange HPLC using a TSK gel SP-5PW column (Fig.1). Two or three peaks of ZP-binding proteins were observed. All fractions containing the binding activity were combined and further purified by reverse phase HPLC on a Bakerbond Wide Pore Buthyl column (Fig.2A). By this column chromatography ZP-binding activity was clearly separated into two peaks (peaks a and b). SDS-PAGE analysis of fractions from No.14 to 24 revealed that 55- and 53-kDa

Fig.1. Cation exchange HPLC of ZP-binding proteins. ZP-binding proteins were subjected to a TSK gel SP-5PW and eluted by a gradient elution of 0.2-1M NaCl. The effluents were examined for ZP-binding by the solid-phase binding assay.

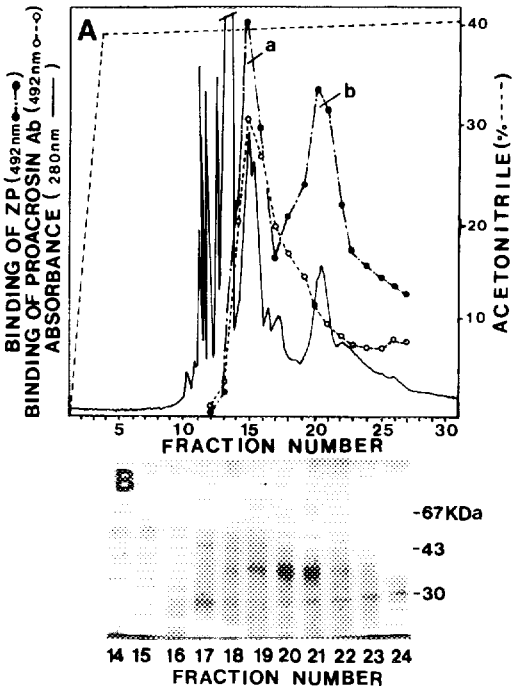

Fig.2. Reverse phase HPLC of ZP-binding proteins (A) and SDS-PAGE of the effluents (B). A: The ZP-binding proteins were subjected to a Bakerbond Wide Pore Butyl column and eluted by acetonitrile increased from 0 to 38% during the first 5 min then to 43% during the next 120 min. The effluents were examined for ZP-binding and antiproacrosin antibody-binding by the solid-phase binding assay. B: Fractions from No.14 to 24 were subjected to 12.5% SDS-PAGE and visualized by silver staining.

proteins were the major proteins in peak a, whereas the major protein in peak b migrated as a 38-kDa protein (Fig.2B), termed sp38. To verify that these proteins corresponded to ZP-binding proteins, Western blot analysis was carried out using ^{125}I-labeled ZP as a probe (Fig.3). Sp38 as well as the 55- and 53-kDa proteins were found to bind to the probe (lane 2 and lane 1 respectively). Also, the 55- and 53-kDa proteins in fraction No.15 were identified as proacrosin due to the immunoreactivity with an antibody against porcine sperm proacrosin (lane 3). Sp38 was not immunoreactive with the same antibody (lane 4), demonstrating that this protein was not a degraded product of proacrosin. Fractions No.20 and 21 were combined and used as a source of sp38. Approximately forty μg of this protein was obtained from 2.5 ml of packed sperm cells. Sp38 was not present in the epididymal fluid, in contrast with the spermadhesin, a family of ZP-binding proteins adsorbed from the outer fluid onto the surface of the sperm (14).

Then partial amino acid sequences of sp38 were determined after electroblotting of the protein to PVDF membrane followed by digestion with Achromobacter protease I. Fig.4 shows the elution pattern of the peptides by

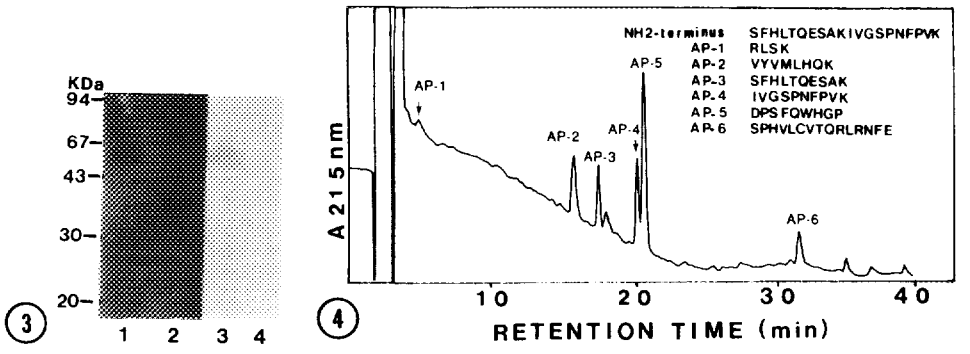

Fig.3. Western blotting of ZP-binding proteins. Aliquots of fractions No.15 (lanes1 and 3) and No.22 (lanes2 and 4) in Fig.2 were subjected to 12.5% SDS-PAGE, electroblotted and probed with ^{125}I-labelled ZP (lanes1 and 2) or antiproacrosin antibody followed by the enzymatic visualization (lanes3 and 4).

Fig.4. Peptide map of sp38 digested with Achromobacter protease I. The peptides released by in situ digestion were separated by reverse phase HPLC using a μ-Bondasphere 5μC8-300A. Amino acid sequences of the peptides (AP-1~AP-6) as well as the NH_2-terminal sequence of intact sp38 were analyzed and the results were inserted in the figure.

reverse phase HPLC. The amino acid sequences of the fragments (AP-1~AP-6) and amino terminal sequence of intact sp38 were also shown in Fig.4. The amino acid sequence of AP-3 was identical with the amino terminal sequence of sp38 and the amino terminus of AP-4 was connectable to carboxyl terminus of AP-3. None of these sequences showed significant homology with known sequences listed in the National Biomedical Research Foundation protein library.

Characterization of sp38 as a ZP-binding protein

The specificity of the binding of sp38 to the ZP was verified by the solid-phase binding assay since the addition of 100 times excess of unlabeled ZP completely inhibited the binding between sp38 and biotin-labeled ZP. Moreover, the binding was inhibited effectively by dextran sulfate with a molecular range of 5000-7000 (ID_{50}=1μg/ml) and poorly by keratan sulfate from bovine cornea (ID_{50}=0.5mg/ml). Porcine ZP is composed of three sulfated glycoprotein families, including 90-kDa, 55-kDaα and 55-kDaß families, each of which contains glycoproteins with a common peptide and heterogeneous sugar chain (15). When biotin-labeled ZP was subjected to reverse phase HPLC, most of the 90-kDa form was separated from the 55-kDaα and 55-kDaß families (Fig.5A,B). The solid-phase binding assay demonstrated that both sp38 and proacrosin bound specifically with the 90-kDa ZP glycoprotein (Fig.5A). This result is consistent with the fact that the 90-kDa form is more susceptible to acrosin proteolysis than other forms (16). Interestingly, in the absence of the Ca^{2+} ion, the binding of the ZP to sp38 as well as that to proacrosin was abolished. All of these findings indicate the similarity in ZP-binding

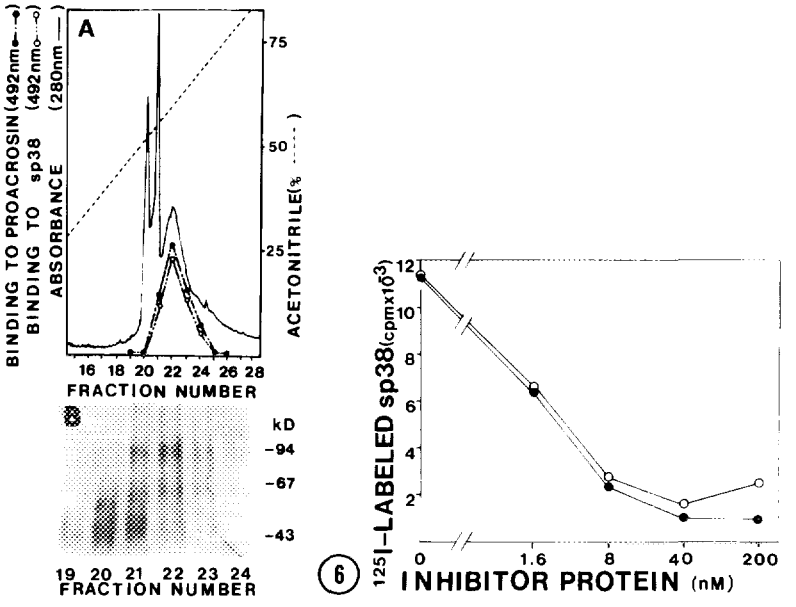

Fig.5. Reverse phase HPLC of biotin-labelled ZP (A) and SDS-PAGE of the effluents (B). A: Biotin-labelled ZP glycoproteins were subjected to Bakerbond Wide Pore Butyl column and eluted by a gradient elution of 0-100% acetonitrile in 60 min. The effluents were examined for the binding activity to sp38 or to proacrosin coated in the wells of ELISA plates. B: Fractions from No.19 to 24 in (A) were subjected to 10% SDS-PAGE followed by electroblotting and enzymatic staining.

Fig.6. Competitive inhibition assay of proacrosin in the binding between sp38 and ZP. ^{125}I-labelled sp38 was incubated with ZP glycoproteins coated in the wells of ELISA plates in the presence of various concentrations of unlabelled sp38 (●—●) or proacrosin (○—○). The radioactivity bound to the ZP was dissolved with 10% SDS and counted.

properties between sp38 and proacrosin, suggesting that the two proteins bound to the same binding site of the 90-kDa form of the ZP. This possibility was verified since the binding of ^{125}I-labeled sp38 to ZP glycoproteins was inhibited by the addition of proacrosin as effectively as sp38 itself (Fig.6). Although whether sp38 or proacrosin binds to the sulfated carbohydrate moieties of 90-kDa ZP glycoprotein or not is still uncertain, because keratan sulfate which has sulfated carbohydrate structures similar to those of the ZP (17) inhibited the binding only poorly, sp38 and proacrosin were found to compete with each other in the binding to the 90-kDa glycoprotein of the ZP. The interaction mechanism of these two proteins in the course of fertilization remains to be studied.

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