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Purification and Partial Immuno-Characterization of Boar Sperm Proteinase Sperminogen

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Abstract : Polyclonal antibody of the boar sperminogen was used to characterize the boar sperm proteinase sperminogen. Boar sperminogen was purified from the acid extracts of the washed epididymal spermatozoa by gel filtration through a Sephadex G-100 column, followed by preparative SDS-PAGE. The sperminogen band was sliced out and was eluted from the gel matrix. The purified sperminogen was used to produce the polyclonal antibody of the boar sperminogen. When characterized on a Western blot, the final preparation of sperminogen appeared as a homogenous protein with a molecular weight of 32 kDa. The relative migration of sperminogen was distinctly different from the major components of the proacrosin-acrosin system as well as all the observable proacrosin activation by-products detected on the Western blot. The sperminogen antibody, however, cross-reacted with the proacrosin-acrosin system.
Key word : acrosin, proacrosin, sperminogen

Since sperm binding to oocyte and sperm penetration of the zona pellucida were markedly reduced by various trypsin inhibitors *in vitro* (Zaneveld *et al.*, 1973), acrosomal trypsin-like enzymes were considered to play an important role(s) in the fertilization processes.

Proacrosin-acrosin system is a well-known sperm proteinase that has trypsin-like specificity (for review, see Polakoski and Siegel, 1986), and was believed to be responsible for the sperm penetration by the proteolysis of the glycoprotein matrix of the egg zona pellucida (Polakoski and Parrish, 1977; Polakoski and Siegel, 1986). Therefore, most of the research on sperm proteinases were focused on the proacrosin-acrosin system. However, Baba *et al.* (1994) reported that the sperm produced from the mice carrying a mutated acrosin gene could penetrate the oocyte zona pellucida and effect fertilization.

Sperminogen is the another acrosomal proteinase which has trypsin-like specificity. Sperminogen was originally reported from the acid extracts of human sperm by Siegel *et al.* (1987), with marked differences in enzyme activation kinetics from that of proacrosin-acrosin system. As a novel enzyme, sperminogen is believed to be an enzymatically inactive zymogen and turns into spermin by autoactivation. However, through N-terminus peptide sequencing, Cechova *et al.* (1990) reported that the sperminogen is an activation by-product of proacrosin. Even though there is a controversy about the novelty of this enzyme as such, since most of the research on sperm proteinases has been focused on the proacrosin-acrosin system based upon the belief that the proacrosin-acrosin system would be the main proteinase for the fertilization processes, the newly discovered sperminogen could not draw much attention.

We employed the boar system to study this enzyme. The porcine species is a praticularly useful model system with which to study this enzyme since large amounts of the sperminogen were readily availabe. Furthermore, since most of the proacrosin-acrosin system has been studied extensively using boar, it is relatively easy to compare these two trypsin-like proteinase systems. Here we report the presence of sperminogen in boar spermatozoa as described in human sperm, and that the sperminogen antibody cross-reacts with proacrosin-acrosin sytem.

Materials and Methods

Materials

Fresh boar testes were collected from Shinwon Meat Co. (Suwon, Korea). Ham's buffer was purchased from GIBCO Laboratories (Grand Island, USA). Benzamidine, 2-(N-morpholino) ethanesulfonic acid (MES) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, USA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, HRP color develop-

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ment reagent, Tween-20 and electrophoresis materials were purchased from Bio-Rad (Richmond, CA). Nitrocellulose membranes were from MSI (Westboro, MA). All other materials were purchased in molecular biology grade from Sigma Chemical Co. (St. Louis, MO).

Purification of boar sperminogen

Porcine epididymides were dissected from freshly excised tissue and the spermatozoa were flushed with Ham's buffer (pH 7.4) containing 50 mM benzamidine. Spermatozoa were washed via centrifugation through 11% ficoll containing 50 mM benzamidine at 26,000×g for 30 min. The washed spermatozoa were resuspended in 0.01 M HCl and adjusted to pH 4.0 with concentrated HCl. After the resuspended spermatozoa were incubated at room termperature for 1 h, the suspensions were centrifuged at 10,000×g for 20 min in a microcentrifuge. The supernatant was saved and gel filtered according to Polakoski and Parrish (1977) on a 1.6×98 cm column containing Sepahdex G-100 superfine resin at pH 3.0. The fractions containing the sperminogen were pooled, lyophilized, and resuspended in SDS buffer (0.125 M Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, pH 3.0). The sample was electrophoresed in a preparative 12.5% polyacrylamide gel with a discontinuous Tris-borate buffer system (upper buffer; 40 mM boric acid, 40 mM Tris-HCl, pH 8.64, 0.1% SDS, lower buffer; 0.43 M Tris-HCl, pH 9.8) at a constant current of 20 mA. The gel was stained with 0.25% Coomassie brilliant blue R, destained with 50% methanol, and rehydrated with distilled water. The Coomassie stained sperminogen band was excised and fragmented into small pieces and elctroeluted according to Yi *et al.* (1985) with minor modifications. The elution was performed in Tris-borate buffer system containing 0.1% SDS overnight followed by an additional overnight elution in Tris-borate upper chamber buffer containing 0.01 % SDS at 4°C with a constant current of 2 mA/tube. The eluent was collected, estimated for the amount of protein by Lowry *et al.* (1951), and kept at -20°C until used.

Production of boar sperminogen antibody

The immunization and antiserum preparation for the boar sperminogen were performed according to Siegel *et al.* (1987) and Yi and Polakoski (1992). A female New Zealand white rabbit was inoculated intradermally on day 0 and two more times at 2-week intervals with the highly purified boar sperminogen. Each inoculum was prepared by emulsifying the equal volumes of the sperminogen solution (equivalent to 50 μg of the protein) and Freund's incomplete adjuvant prior to inoculation. On the 9th day following the last inoculation, the blood was drawn from the rabbit's marginal ear vein. The blood was allowed to coagulate for 1 h at room temperature and overnight at 4°C. The serum was then obtained by centrifugation at 10,000×g for 10 min to separate the serum from the clotted blood cells. The antiserum was partially purified by precipitation with 40% ammonium sulfate at 4°C for 1 h. The precipitates were collected by centrifugation and the resulting pellet was resuspended in a half volume of 10 mM Tris-HCl at pH 7.5. The sample was then dialysed against 2 liters of the same buffer to remove the salts.

SDS-PAGE

Both discontinuous SDS-PAGE and gelatin SDS-PAGE of acid extracts of spermatozoa were performed at 4°C on 0.8-mm thick slab gels according to Laemmli (1970) and Siegel and Polakoski (1985), respectively. The samples were electrophoresed in an analytical polyacrylamide gel containing 0.1% SDS with 12.5% separating gel and 5% stacking gel at a constant current of 20 mA until the dye front reached the bottom of the gel. Following electrophoresis, the SDS-gels were stained with Coomassie brilliant blue R, and destained, then restained with silver nitrate. The gelatin SDS-gels were soaked in 2.5% Triton X-100 for 30 min, washed extensively with distilled water, and incubated in 0.1 M Tris buffer (pH 8.0) overnight at 37°C. The gels were then stained with 0.1% Amido Black.

Western blotting

Immuno-blotting was performed according to Towbin *et al.* (1979) with minor modifications. Following SDS-PAGE, the gels were blotted onto nitrocellulose membranes using a MES (25 mM MES, 200 mM glycine, pH 6.1) buffer with a constant current of 30 mA for overnight and then 60 mA for 1 h. The nitrocellulose membrane was washed for 5 min with TBS (200 mM Tris-HCl, 150 mM NaCl, pH 7.5) buffer and air dried. The membrane was then blocked with 3% BSA (w/v) in TBS buffer for 1 h to reduce the possible non-specific binding of antibodies. This was followed by a 5 min wash with T-TBS (0.5% Tween 20 in TBS buffer). The membrane was incubated with the antibody solution (90 μl of partially purified sperminogen antibody in 90 ml of TBS containing 1% BSA) for 2 h on a rocking platform. After being further washed with T-TBS twice and with TBS once for 5 min each, the membrane was incubated with HRP conjugated secondary antibody solution consisting of 30 μl of goat anti-rabbit IgG in TBS containing 1% BSA for 1 h on a rocking paltform. Finally, the membrane was washed twice with T-TBS for 5 min each and TBS for 5 min and incubated with HRP color development reagent until the color intensity of the bands was achieved. The membrane was then

washed with distilled water and air-dried in a light-tight container.

Results

Gel filtration of the acid extracts of boar spermatozoa

The Sephadex G-100 elution profile of acid extracts of boar spermatozoa resulted in two major protein peaks (Fig. 1). Even though there was no initial measurable benzoyl arginine ethyl ester (BzArgOEt) hydrolysis activity from any of the fractions, two distinct hydrolysis peaks were observed when the samples were first activated at pH 8.0 for 4 h (Fig. 1). SDS-PAGE analysis of the fractions in two hydrolysis peaks revealed that the first peak contained both a proacrosin doublet and proacrosin binding proteins, while the second peak displayed prominent bands of sperminogen (Fig. 2). Gelatin SDS-PAGE analysis for the proteinase activities showed that the proacrosin doublet and three protein bands of sperminogen between 32-35 kDa in the second peak were proteinase-active (Fig. 3), matching the fractions that displayed the hydrolysis activities shown in Fig. 1.

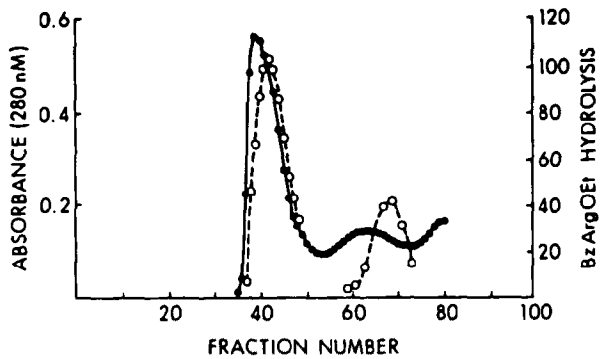

Fig. 1. Gel filtration of acid extracts from boar epididymal spermatozoa through a Sephadex G-100 column at pH 3.0. Fractions of 60 drops were collected for each tube and the absorbance at 280 nm (●—●) was measured. Aliquots of the fractions were assayed for proteinase activity spectrophotometrically after activation at pH 8.0 and expressed as units of Benzoyl arginine ethyl ester (BzArgOEt) hydrolysed $min^{-1}ml^{-1}$ (○—○).

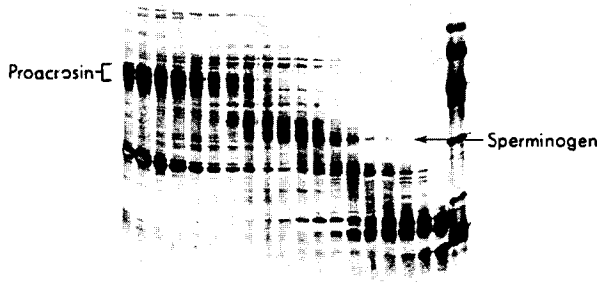

Fig. 2. SDS-PAGE analysis of the Sephadex G-100 fractionated acid extracts from boar epididymal spermatozoa. Fifteen microlitre aliquots of the odd numbered fractions starting at tube 39 were electrophoresed in a 12.5% polyacrylamide gel at a constant current of 20 mA. The gel was stained with Coomassie brilliant blue R, destained and subsequently stained with silver nitrate.

Purification of sperminogen by preparative SDS-PAGE

Among the three putative sperminogen bands, only the 32 kDa sperminogen band was chosen for further purification since this band was shown to be most proteinase-active in gelatin SDS-PAGE analysis (Fig. 3). After the fractions containing the sperminogen bands were pooled and then lyophilized, the samples were electrophoresed on a preparative SDS-PAGE. Three sperminogen bands were stained with Coomassie brilliant blue R (Fig. 4). Even though the 32 kDa form of sperminogen was not as densely stained as 34 kDa form

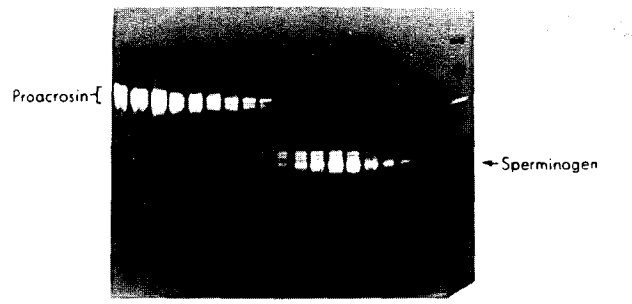

Fig. 3. Gelatin SDS-PAGE analysis of the Sephadex G-100 fractionated acid extracts from boar epididymal spermatozoa. Five microlitres of samples designated in Fig. 2 were analysed in a 12.5% polyacrylamide gel containing 0.1% gelatin in the gel matrix.

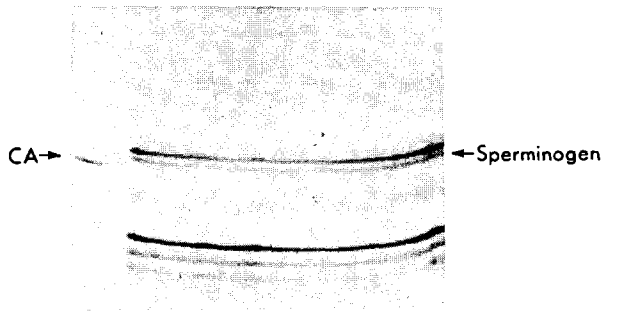

Fig. 4. Preparative SDS-PAGE of the fractions containing the sperminogen. The fractions starting from 61 to 65 were pooled, lyophilized, and resuspended in SDS buffer. After electrophoresis, the gel was stained with Coomassie brilliant blue R, and destained with 50% methanol. The sperminogen band which was excised was marked with arrow and designated as sperminogen. CA denotes carbonic anhydrase which was used as a molecular weight marker.

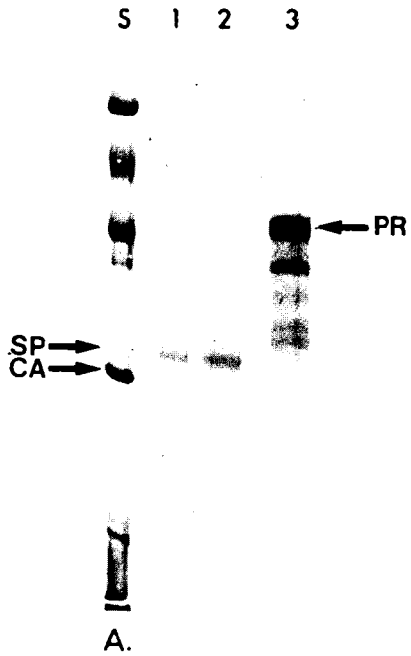

Fig. 5. Western blot analysis of sperminogen and proacrosin with its activation by-products. S denotes standard molecular weight marker. Among the markers used, fifth from top of the blot marked as CA is carbonic anhydrase. Lane 1 was loaded with 10 μg of purified sperminogen and lane 2 was loaded with 20 μg of sperminogen. Lane 3 was loaded with approximately 100 μg of purified proacrosin with its activation by-products. SP denotes sperminogen and PR denotes proacrosin.

with Coomassie brilliant blue R, the proteinase activity estimated by the gelatin hydrolysis activity was much stronger for the 32 kDa form than the 34 kDa form of the sperminogen (Fig. 3). The 32 kDa form of sperminogen band was excised by a razor blade from the gel and was electroeluted. The eluted sample was analysed by analytical SDS-PAGE and was shown as a single 32 kDa band (data not shown).

Immuno-characterization of the sperminogen and proacrosin by the sperminogen antibody

Partially purified sperminogen antibody was used to analyse the acid extracts of boar spermatozoa. When analysed on a Western blot, purified sperminogen was clearly detected by the partially purified sperminogen antibody (Fig. 5, lanes 1 and 2). The same sample, however, was not detected with the preimmune serum on a Western blot (data not shown). The sperminogen antibody was then checked for the cross-reactivity with proacrosin. For this experiment, proacrosin was purified by the method same as described for sperminogen. The purified proacrosin was then allowed to partially activate by itself at 4℃ in SDS buffer. When this sample was analysed by the sperminogen antibody on a Western blot, the sperminogen antibody detected proacrosin and most of its activation by-products (Fig. 5, lane 3).

Discussion

Prior investigations of sperm proteinases have been mainly devoted to the well-characterized component of the proacrosin-acrosin system. As shown in Fig. 3, proacrosin-acrosin system is the most prominent proteinase found in mammalian sperm; therefore, it is believed that proacrosin-acrosin system is the major proteinase which is responsible for the sperm penetration of the plasma membrane of oocyte. However, it was reported that the sperms produced by the mouse whose proacrosin gene was knocked out could fertilize the egg and produced its offsprings (Baba *et al.*, 1994), which strongly implies that the proacrosin-acrosin system is not an absolutely requirement for the sperm to penetrate the egg. If the proacrosin-acrosin system is not responsible for the sperm penetration, some other proteinase should be responsible for that role. It can be guessed that sperminogen is a good candidate for that role since the sperminogen is the most prominent trypsin-like proteinase found in sperm other than proacrosin-acrosin system. As noted previously, sperminogen was reported as a new proteinase from human sperm, which has marked differences in enzyme activation kinetics from that of proacrosin-acrosin system. Cechova *et al.* (1990), however, reported that sperminogen was the activation by-product of proacrosin from N-terminus sequencing. These contradicting results were not verified by other research groups until now whether the sperminogen is an activation by-products of proacrosin or not. The immunocharacterization results reported here support the Cechova's conclusion. However, one conflicting result to conclude that the sperminogen is an activation by-products of proacrosin is when the proacrosin was allowed to activate by itself *in vitro* and then was screened with sperminogen antibody, protein band which matches the molecular weight of the sperminogen was not detected (Fig. 5 land 3). The lack of protein band which matches the sperminogen from the activation by-products of proacrosin can be due to the fact that proacrosin was activated *in vitro* instead of being activated *in vivo*. However, since the molecular weight range in SDS-gel where sperminogen is detected is so crowded with many proteins with similar molecular weights, we cannot exclude the possibility that the sperminogen preparations can have minor contamination of proacrosin activation by-products. It is premature and, therefore, should be cautious to conclude that the sperminogen is an activation by-products of proacrosin only with these data even though the immuno-characterization results reported here

clearly showed that there is a strong cross-reactivity between sperminogen and proacrosin immunologically.

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