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Amino Acid Composition Analysis of the 32 kDa Sperminogen

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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 32 kDa sperminogen band was sliced out from the preparative SDS-PAGE and 32 kDa sperminogen was eluted from the gel matrix. The purified 32 kDa sperminogen was subjected to amino acid composition analysis. The amino acid composition of the 32 kDa boar sperminogen showed significant differences from that of either boar proacrosin or β-acrosin, which signifies that 32 kDa sperminogen might not be a breakdown product of proacrosin-acrosin system and that the 32 kDa sperminogen is a different protein from proacrosin-acrosin system.

Keywords: Acrosin, Amino acid composition, Proacrosin, Spermatozoa, Sperminogen.

Introduction

Trypsin-like proteases in acrosome were regarded to play an important role(s) in the fertilization processes. This is confirmed by the facts that various trypsin inhibitors reduced the sperm binding and penetration activities to oocyte *in vitro* (Zaneveld *et al.*, 1973).

The proacrosin-acrosin system is the most abundant sperm protease that has trypsin-like specificity (for review, see Polakoski and Siegel, 1986). Most of the research on sperm proteases were focused on the proacrosin-acrosin system, since it is believed that this enzyme system might 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). 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. This implies that the proacrosin-acrosin system is not the sole protease that is responsible for the sperm penetration of the oocyte zona pellucida.

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

The porcine species is a particularly useful model system with which to study sperm proteases since large amounts of proteases, such as proacrosin-acrosin system and sperminogen, were readily available. Furthermore, studying sperminogen using porcine species gives much added advantages because most of proacrosin-acrosin system has been studied extensively in porcine species; therefore, it is relatively easy to compare these two typsin-like protease systems. Here we report the amino acid composition of the purified 32 kDa sperminogen is different from that of either proacrosin or 35 kDa β-acrosin using boar spermatozoa.

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 was obtained from Sigma Chemical Co. (St. Louis, USA). Electrophoresis materials were purchased from Bio-Rad (Richmond, USA). All other materials were purchased in molecular biology grade from Sigma Chemical Co.

Purification of boar sperminogen Boar spermatozoa were obtained from freshly excised epididymises by flushing with

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Hams buffer (pH 7.4) containing 50 mM benzamidine. Spermatozoa were precipitated via centrifugation through 11% ficoll containing 50 mM benzamidine at 26,000 × g for 30 min. The acid-soluble fraction of precipitated spermatozoa were then extracted by resuspending spermatozoa in 0.01 M HCl and adjusting to pH 4.0 with concentrated HCl. After incubating at room temperature 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 Huh and Yi (1999) on a 1.6 × 98 cm column containing Sephadex G-100 superfine resin at pH 3.0. The fractions showing the sperminogen activity 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. Following electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R, destained with 50% methanol, and rehydrated with distilled water. The 32 kDa sperminogen band from the Coomassie-stained preparative polyacrylamide gel was excised, fragmented into small pieces, and electroeluted according to Yi *et al.* (1992) with minor modifications. The elution was performed in Tris-borate buffer system containing 0.1% SDS overnight followed by an additional overnight elution in a 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.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 Huh and Yi (1999), respectively. Each fraction of the column-fractionated eluents was 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 restainted with silver nitrate. The gelatin SDS-gels were electrophoresed same as SDS-PAGE. However, following electrophoresis, gelatin gels were soaked in 2.5% Triton X-100 for 30 min, washed extensively with distilled water, and incubated in a 0.1 M Tris buffer (pH 8.0) overnight at 37°C. The gels were then stained with 0.1% Amido Black.

Amino acid analysis An aliquot of the 32 kDa sperminogen purified by preparative SDS-PAGE was hydrolysed in 6 M HCl for 24, 48, and 72 h at 110°C *in vacuo*. The hydrolysates were analyzed on HPLC (Waters Associates, Inc., Milford, USA) with a model 840 control with data reduction capabilities. The amino acids were detected by post-column derivatization with o-pthasaldehyde and continuous infusion of hypochlorite to detect prolines.

Results

Gel filtration of the acid extracts of boar spermatozoa

The protein concentration of each eluted fraction of the acid extracts of boar spermatozoa through Sephadex G-100 resulted in two major protein peaks (Fig. 1). Protease activities were analyzed for the fractions belonging to two protein peaks. Even though there was no initial measurable hydrolytic activity of bezoyl arginine ethyl ester (BzArgOet), two distinct hydrolysis peaks were observed at similar fractions with the two protein peaks when the eluents were first activated at pH 8.0 for 4 h (Fig. 1). SDS-PAGE analysis of the fractions showing hydrolytic activity revealed that the first peak contained a 53~55 kDa proacrosin doublet and 28~29 kDa proacrosin binding proteins (Yi *et al.*, 1992), while the second peak displayed prominent bands of 32~34 kDa with minor proteins in the low molecular weight region (Fig. 2). The immuno-characterization of the 32-34 kDa band with anti-

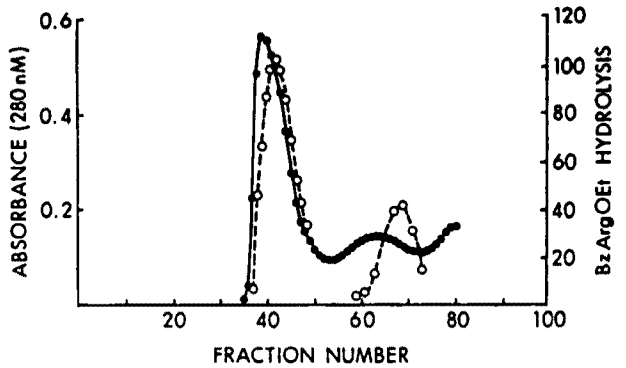

Fig. 1. Gel filtration profile of the acid extracts from boar epididymal spermatozoa through a Sephadex G-100 column at pH 3.0. Sixty drops were collected for each fraction. Protein concentration of each fraction was measured by the absorbance at 280 nm (●-●). Protease activity was assayed spectrophotometrically after each fraction was activated at pH 8.0. Protease activity is expressed as units of Benzoyl arginine ethyl ester (BzArgOEt) hydrolyzed $min^{-1}ml^{-1}$ (○-○).

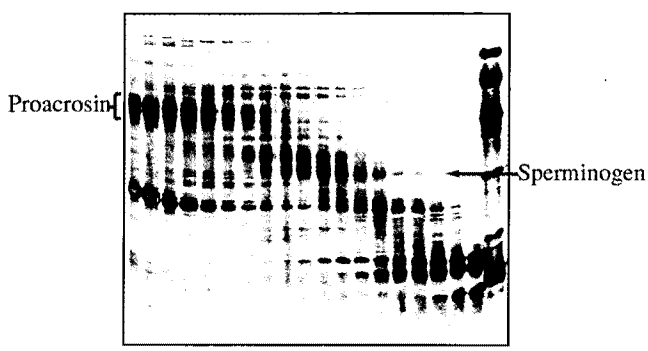

Fig. 2. SDS-PAGE analysis of the Sephadex G-100 fractionated acid extracts from boar epididymal spermatozoa. Fifteen microlitre aliquots of the odd-numbered fractions starting at tube 39 were electrophoresed in a 12.5% polyacrylamide gel at a constant current of 20 mA. The gel was stained with Coomassie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

Fig. 3. Gelatin SDS-PAGE analysis of the Sephadex G-100 fractionated acid extracts from boar epididymal spermatozoa. Five microlitres of samples used in Fig. 2 were analyzed in a 12.5% polyacrylamide gel containing 0.1% gelatin in the gel matrix. Following electrophoresis, protease activity was analyzed as stated in methods.

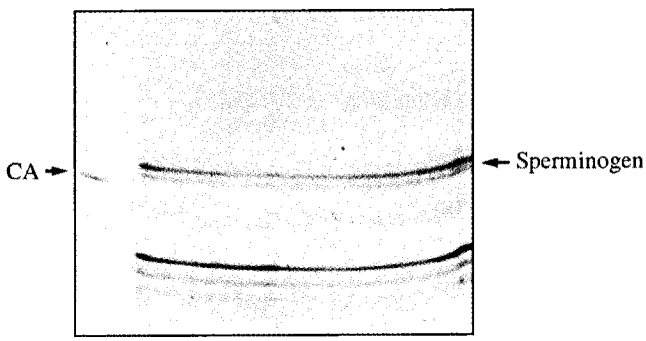

Fig. 4. Preparative SDS-PAGE of the fractions containing the sperminogen. The fractions showing the strongest protease activity (fractions from 61 to 65) were pooled, lyophilized, and resuspended in SDS buffer. Electrophoresis was performed same as described for analytical SDS-PAGE. The gel was stained with Coomassie brilliant blue R, destained with 50% methanol, and rehydrated for effective protein elution from the gel matrix. The protein band marked with an arrow and designated as sperminogen is the excised 32 kDa sperminogen band. CA denotes carbonic anhydrase which was used as a molecular weight marker.

sperminogen Ab has demonstrated that these bands represent boar sperminogen (data not shown). When the same fractions were analyzed for protease activity by gelatin SDS-PAGE, only the proacrosin doublet in the first peak and sperminogen triplet in the second peak were protease-active (Fig. 3). This demonstrates that the fractions displayed in the hydrolytic activities shown in Fig. 1 are also protease-active in gelatin gel. The molecular weights of sperminogen were estimated to be 32-34 kDa from the SDS-PAGE and gelatin SDS-PAGE analyses.

Purification of sperminogen by preparative SDS-PAGE Among the three sperminogen bands, the 32 kDa sperminogen band was chosen for further analysis since this sperminogen band was shown to be most active in proteolysis in the gelatin SDS-PAGE analysis (Fig. 3). As stated in methods, the fractions containing sperminogen were pooled, lyophilized, and electrophoresed on a preparative SDS-PAGE. As expected, three sperminogen bands were stained with Coomassie brilliant blue R (Fig. 4). The 32 kDa sperminogen was not the most densely stained band among the three sperminogen bands; rather, the 34 kDa sperminogen was stained most densely. However, protease activity estimated by the gelatin hydrolysis activity was much stronger for the 32 kDa than the 34 kDa sperminogen (Fig. 3). Therefore, the 32 kDa sperminogen band was excised by a razor blade from the preparative gel and electroeluted. The eluted sample was analyzed by analytical SDS-PAGE and was shown as a single 32 kDa band (data not shown).

Table 1. Amino acid composition of sperminogen β-acrosin and proacrosin

Amino acid	Sperminogen	β-Acrosin	Proacrosin
Asp/Asn	23	21	24
Thr	19	18	23
Ser	28	17	24
Glu/Gln	26	28	43
Pro	15	35	64
Gly	49	33	40
Ala	28	20	28
Cys	5	11	12
Val	16	21	26
Met	1	6	6
Ile	13	17	19
Leu	22	21	28
Tyr	-	9	12
Phe	10	9	12
Lys	24	15	19
His	-	5	5
Arg	14	24	28
Total	245	310	413

Amino acid composition analysis The amino acid composition of the purified 32 kDa sperminogen showed marked differences from that of either proacrosin or 35 kd β-acrosin, especially in the amino acid ratio of lysine and arginine residues of the protein (Table 1).

Discussion

Since the proacrosin-acrosin system is the most abundant protease in spermatozoa, it is thought that proacrosin-acrosin is the major protease which is responsible for the sperm penetration of the zona pellucida of oocyte. Therefore, most of the prior investigations on sperm protease were focused on the proteolytic components of the proacrosin-acrosin system. However, Baba *et al.* (1994) reported that the sperm produced by the mouse, whose proacrosin gene was knocked out, could fertilize an egg and produce offspring. This is a strong implication that the proacrosin-acrosin system may not be the

sole absolute requirement for the sperm penetration to oocyte. Even though we can not completely exclude the involvement of the proacrosin-acrosin system in the fertilization processes, if the sperm produced from the mouse without proacrosin-acrosin system can fetilize oocyte, at least we can speculate that some other proteases may dissolve the egg's vestments. In this regard, sperminogen seems to be a good candidate. Sperminogen is one of the most prominent trypsin-like protease found in spermatozoa other than the proacrosin-acrosin system. Furthrmore, sperminogen has marked differences in enzyme activation kinetics from that of proacrisn-acrosin system. By performing N-terminus sequencing, Cechova *et al.* (1990) reproted that sperminogen was an activation by-product of proacrosin. Also, the immuno-characterization by Yi (1997) supported their conclusion by demonstrating that the sperminogen antibody strongly cross-reacted with proacrosin and the breakdown products of proacrosin when the *in vitro* activated proacrosin was screened with the sperminogen antibody in western blot. These contradicting results, whether sperminogen is an activation by-products of proacrosin or not, can be verified by other biochemical methods. The amino acid composition analysis is one of the useful biochemical methods to solve this controversy. The amino acid composition analysis data of sperminogen reported in this paper, however, support the original finding that sperminogen is a novel protease system, especially when the ratio of the two amino acids, lysine and arginine residues, were compared. As shown in Table 1, the ratios of these two amino acids in the 32 kDa sperminogen (24 Lys and 14 Arg) and the 35 kDa β-acrosin (15 Lys and 24 Arg) are reversed. If 32 kDa sperminogen is the activation by-product of proacrosin, it is highly unlikely that the ratio of lysine and arginine residues is reversed with only a 3 kDa molecular weight difference. This strongly implies that 32 kd sperminogen is a different protein from any breakdown products of proacrosin. However, since there are some conflicting data as stated above, it is premature to decide whether or not the 32 kDa sperminogen is an activation by-product of proacrosin or a novel enzyme system. Therefore, independent peptide sequencing of the 32 kDa sperminogen will be helpful in resolving these differences.

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