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THE PHOSPHOLIPASE A_2 OF HUMAN SPERMATOZOA; PURIFICATION AND PARTIAL SEQUENCE

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In view of its proposed key role in the acrosome reaction, phospholipase A_2 has been isolated and purified from human spermatozoa. Following SDS-PAGE, a single major band was obtained with an estimated molecular mass of 16.7 kDa. Sequence analysis of the N-terminal portion of the molecule revealed the identity of the first 19 amino acids to be YNYQFGLMIVITKGHFAMV. From this partial analysis it is evident that the phospholipase A_2 of human spermatozoa represents a new sequence. Of interest is the location of glutamine-4, phenylalanine-5, methionine-8 and isoleucine-9; this sequence appears to be highly conserved throughout evolution. © 1992 Academic Press, Inc.

The purification and characterization of phospholipase A_2 (PLA_2) from a variety of mammalian sources has been the subject of several reports (for review, 1). In a recent report, Davidson and Dennis (2) have compared the known sequences of some forty forms of PLA_2. Approximately 120 amino acids form a single peptide chain that includes 10-14 cysteine residues in disulfide pairs. Up to the present, only two human PLA_2's have been completely sequenced. One from the pancreas, which shows a high homology to the PLA_2's of old-world cobra venom while the second, obtained from platelets and synovial fluid (3) bears a high similarity to the enzyme isolated from the venoms of new- and old- world serpents such as the

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diamondback rattlesnake. In addition, the crystalline structure and the mechanism of action of bee venom PLA_2 have also been reported (4-6). A general review article of this class of enzyme has also been published recently (7).

Our interest in this enzyme arises from its postulated role in reproduction via the activation of the acrosome reaction of mammalian spermatozoa, an exocytotic event. Previous reports have shown that PLA_2 activity regulates exocytic events in neutrophils, platelets, mast cells, adrenocortical cells (8) macrophages (9) and pancreatic cells (10). The association of PLA_2 activity with the acrosome reaction of the sea urchin spermatozoa was originally reported by Conway and Metz (11). In the same organism, the cortical granule reaction, another exocytotic event, is also reported to be regulated by PLA_2 activity (12). Finally, the association of PLA_2 activity with the acrosome reaction of mammalian spermatozoa has also been demonstrated (13-16).

PLA_2 is also secreted by the seminal vesicles and prostate gland and is found in the seminal plasma of several animal species with the highest activity being found in human seminal plasma (17). The probable function of this fraction of PLA_2 is to provide precursor for the biosynthesis of seminal fluid prostaglandins (18,19). Using the radiation inactivation technique, our laboratory has reported the presence of two forms of PLA_2 in human semen (20) where a heat-sensitive form was found to be associated with spermatozoa. We have also reported the activation of the PLA_2 of human spermatozoa by proteases, including acrosin (21). While the existence of an inactive precursor form of the enzyme ($proPLA_2$) has been reported in a variety of tissues and fluids (22-25) its presence in human spermatozoa has only recently been demonstrated by Antaki et al (26).

Very recently, Takayama et al (27) have reported the partial sequence of a PLA_2 of 14 kDa obtained from human seminal plasma. The amino acid sequence of the first 20 residues of the N-terminal portion was found to be identical to the human synovial fluid enzyme (28).

PLA_2 is believed to play a key role in the acrosome reaction via the enzymatic hydrolysis of unsaturated fatty acids linked to membrane phospholipids overlying the acrosome of mammalian spermatozoa. Moreover, certain studies have shown that PLA_2 inhibitors are capable of inhibiting the acrosome reaction in vitro. The products generated by PLA_2 activity, namely the lysolipids and unsaturated fatty acids, can either stimulate or induce the acrosome reaction of mammalian spermatozoa (13,29-33). Furthermore, the accumulation of lysophospholipids has been reported to occur following the in vitro incubation of human (34) rat (35) and guinea pig spermatozoa (36,37).

The key role proposed for PLA_2 in the acrosome reaction has prompted us to purify and characterize this enzyme from human spermatozoa. An obvious follow-up to this purification will be the production of specific antibodies as well as the determination of the complete amino acid sequence of sperm PLA_2.

MATERIALS AND METHODS

Purification procedure

A pool of human semen, obtained from the fertility clinic and stored at -80°C was used as starting material. After thawing at 4°C, the sample was diluted in an equal volume of 0.05 M Tris buffer, pH 7.8 and 0.9% NaCl and 100 mM benzamidine HCl. The diluted mixture was centrifuged for 60 minutes at 1300 g at 4°C. The pellet was retained and washed twice with the dilution medium and recentrifuged at 1300 g for 10 minutes at 4°C. The pellet of spermatozoa was resuspended and an aliquot was counted to yield a total of 36.5 X 10^9 spermatozoa. The medium was again removed by centrifugation at 1300 g for 10 minutes at 4°C. The spermatozoa were then extracted with 1% trifluoroacetic acid (TFA) in 40% isopropanol; 250 X 10^6 spermatozoa/ml of extraction medium. This mixture was subjected to ultrasound treatment 4 X 30 sec bursts at 60 W at 4°C. The sample was then centrifuged at 20,000 g at 4°C for 20 minutes. The isopropanol was evaporated and the sample was then lyophilized. The residue was redissolved in a minimum volume of 1% TFA and applied to 3 X 6 ml hydrophobic columns; SEP-PAK-WP Butyl C_4 (Baker). The columns were eluted with an increasing gradient of isopropanol. The following fractions were collected; 1) 9 ml 20% isopropanol: 1% TFA; 2) 18 ml 30% isopropanol: 1% TFA; 3) 18 ml 35% isopropanol: 1% TFA; 4) 9 ml 40% isopropanol: 1% TFA and finally 5) 9 ml 100% isopropanol. The major peak of PLA_2 activity was eluted in fraction 3. Enzyme activity was assayed according to the method of Guerette (21) using ^{3}H-phosphatidylethanolamine as substrate.The isopropanol was evaporated and the extract was then lyophilized.The residue was redissolved in 0.02 M ammonium acetate buffer, pH 5.0, and applied to a 2 gm cation exchange column (Waters Accell-CM). The column was developed with an increasing molarity of ammonium acetate buffer, pH 5.6 at room temperature.Two fractions of 10 ml each were collected; 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 M ammonium acetate. The enzyme was eluted in the 0.8 - 1.0 M fractions which were applied to a preparative 15% SDS-PAGE according

to the method of Laemmli (38). The gel was developed using the copper-staining method of Lee et al (39). The stained gel along with the molecular weight standards of Bio-Rad are shown in Figure 1. The single band was electroeluted using the Bio-Rad system (model 422 Electro-Eluter); 25 mM Tris buffer containing 192 mM glycine. A membrane with a 3500 molecular weight cut-off was used overnight at 10 mA per tube. The protein was eluted and dialyzed twice using a membrane of 5000 molecular weight cut-off in 2 liters of 0.05% ammonium carbonate. Following lyophilisation, an aliquot of the purified fraction was forwarded for sequence analysis. Approximately 300 pmoles of protein was subjected to N-terminal sequencing on an Applied Biosystems 477A/120 A pulsed-liquid sequenator.

RESULTS AND DISCUSSION

Table I lists the various purification procedures including the specific activity of the enzyme following each step. A purification of 116-fold, prior to SDS-PAGE, was achieved starting from the initial crude acid extract. The purified enzyme was also located using polyclonal antibodies obtained from rabbits immunized with the purified PLA_2 followed by the addition of a second antibody (anti-IgG) coupled to horse radish peroxidase.

Figure 1 illustrates the protein pattern obtained with the Bio-Rad standard proteins along with the band obtained with the enzyme preparation. An average molecular mass of 16.7 kDa was estimated following the electrophoresis of four samples of the purified enzyme. Of the many PLA_2's that have been purified, their molecular masses have ranged from 12 to 18 kDa. Figure 2 illustrates the N-terminal sequence of the first 19 amino acids along with a comparison of this sequence with several of those PLA_2's that have been characterized from a variety of biological sources. The following sequence was obtained; YNYQFGLMIVITKGHFAMV. Of the large number of PLA_2's that have been sequenced to date from a wide variety of mammalian

Table I

Purification of PLA_2 of Human Spermatozoa

Purification step	pmol substrate deacylated/hour	µg protein /100µL	Specific activity	Purification factor
Crude extract	57.6	434	133	1
Butyl C_4 Column	191.6	549	349	2.6
Accell CM column	206.4	13.4	15,402	116

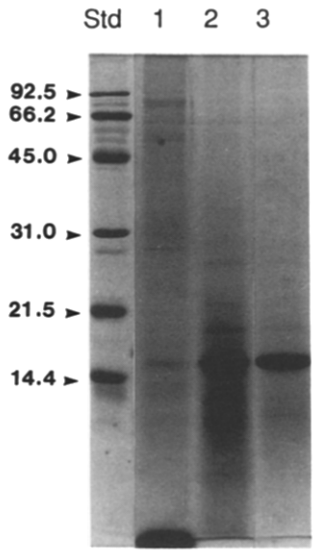

Figure 1. Electrophoretic Patterns of Standard Proteins and Sperm Proteins.

STD: SDS-PAGE pattern of Bio-Rad standard proteins of low molecular weight.
Phosphorylase B, 92.5; Bovine Serum Albumin, 66.2; Ovalbumin, 45.0; Carbonic Anhydrase, 31.0; Soya bean trypsin inhibitor, 21.5; and lactalbumin 14.4 kDa, respectively.

Lane 1: Crude extract
Lane 2: Protein Pattern following Butyl C_4 colum
Lane 3: Protein pattern following Accell CM column

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Y	N	Y	Q	F	G	L	M	I	V	I	T	K	G	H	F	A	M	V	
2	N	L	Y	Q	F	K	N	M	I	V	C	A	G	T	-	-	R	P	W	I
3	N	L	Y	Q	F	K	N	M	I	K	C	T	V	P	S	-	R	S	W	W
4	N	L	Y	Q	F	K	N	M	I	H	C	T	V	P	N	-	R	P	W	W
5	N	L	Y	Q	F	K	N	M	I	H	C	T	V	P	S	-	R	P	W	W
6	N	L	Y	Q	F	K	N	M	I	H	C	T	V	P	S	-	R	P	W	W
7	N	L	Y	Q	F	K	N	M	I	K	C	T	V	P	S	-	R	S	W	L
8	N	L	Y	Q	F	K	N	M	I	Q	C	T	V	P	N	-	R	S	W	W
9	A	L	W	Q	F	R	S	M	I	K	C	A	I	P	G	S	H	P	L	M
10	A	V	W	Q	F	R	K	M	I	K	C	V	I	P	G	S	D	P	F	L
11	N	L	V	N	F	H	R	M	I	K	L	T	T	G	K	E	A	A	L	S

Figure 2. Comparison of Amino Acid Sequence of Various PhospholipasesA_2.

1. Human spermatozoa; 2. Bungarus multicinctus; 3. Hemachatus hemachatus; 4. Naja melanoleuca; 5. Naja mosambica; 6. Naja nigricollis; 7. Naja naja oxiana; 8. Naja naja kaouthia; 9. Porcine pancreas; 10. Human pancreas; 11. Human seminal plasma, platelets and synovial fluid.

and reptilean sources (1), the PLA_2 of human spermatozoa represents a new sequence. Of interest is the location of glutamine-4, phenylalamine-5, methionine-8 and isoleucine-9; this sequence appears to be highly conserved throughout evolution.

The partial sequence of the 14 kDa secreted form of the PLA_2 present in seminal plasma as reported by Takayama et al (27) is quite different from the PLA_2 associated with the spermatozoa as reported here. Those positions that are common however, are phenylalanine-5, methionine-8, isoleucine-9, threonine-12, glycine-14, and alanine-17. Although the first 19 amino acid sequence of the sperm enzyme shares homology with several Group I PLA_2's, it differs by the absence of a half-cystine at position-11, a common feature of the Group I enzymes.

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REFERENCES

1. Waite, M. (1987) The Phospholipases, In: Handbook of Lipid Research (Hanahan D.J. Ed.) Plenum Press, N.Y.
2. Davidson, F.F., Dennis, E.S. (1990) J. Molec. Evol. 1, 228-238.
3. Wong, P.Y-K, Dennis, E.A. (1990) In: Phospholipase A_2: Role and Function in Inflammation. p 1-199, Plenum Press, New York.
4. White, S.P., Scott, D.L., Otwinowski, Z., Gelb, M.H. and Sigler, P.B. (1990) Science 250, 1560-1563.
5. Scott, D.L., Otwinowski, Z., Gelb, M.H. and Sigler, P.B. (1990) Science 250, 1563-1566.
6. Scott, D.L., White, S.P., Otwinowski, Z., Yuan, W., Gelb, M.H. and Sigler, P.B. (1990) Science 250, 1541-1546.
7. Mansbach, C.M. (1990) Gastroenterology 98, 1369-1382.
8. Rubin, R.R. (1982). In: Calcium and Cellular Secretion, Plenum Press, N.Y., p. 179-196.
9. Wightman, P.D., Humes, J.L., Davies, P., Bonney, P.J. (1981) Biochem. J. 195, 427-433.
10. Laychok, S.G. (1982) Cell Calcium 3, 43-54.
11. Conway, A.F. and Metz, C.B. (1976) J. Exp. Zool. 198, 39-48.
12. Ferguson, J.E. and Shen, S.S. (1984) Gamete Res. 9, 329-338.
13. Fleming, A.D. and Yanagimachi, R. (1981) Gamete Res. 4, 253-273.
14. Ono, K., Yanagimachi, R. and Huang, T.T.F. (1982) Dev. Growth Differ. 24, 305-310.
15. Llanos, M.N., Lui, C.W. and Meizel, S. (1982) J. Exp. Zool. 231, 107-117.
16. Langlais, J. and Roberts, K.D. (1985) Gamete Res. 12, 183-224.

17. Kunze, H., Nahas, N. and Wurl, M. (1974) Biochim. Biophys. Acta 348, 34-44.
18. Kunze, H. and Bohn, E. (1978) Adv. Prosta. Thromboxane Res. 3, 159-165.
19. Kunze, H. (1981) Fortsch. Med. 99, 1609-1611.
20. Antaki, P., Langlais, J., Ross, P., Guerette, P. and Roberts, K.D. (1988) Gamete Res. 19, 305-314.
21. Guérette, P., Langlais, J., Antaki, P., Chapdelaine, A. and Roberts, K.D. (1988) Gamete Res. 19, 203-214.
22. Etienne, J., Paysant, M., Gruber, A. and Polonovski, J. (1969) Bull. Soc. Chim. Biol. 51, 709-716.
23. Grataroli, R., De Caro, A., Guy, P., Amic., J. and Figarella, C. (1981) Biochimie 63, 677-684.
24. Paysant, M., Bitran, M., Etienne, J. and Polonovski, J. (1969) Bull. Soc. Chim. Biol. 51, 863-873.
25. Nieuwenhuizen, W., Steenburgh, P. and de Haas, G.H. (1973) Europ. J. Biochem. 40, 1-7.
26. Antaki, P., Guérette, P., Chapdelaine, A. and Roberts, K.D. (1989) Biol. Reprod. 41, 241-246.
27. Takayama, K., Hara, S., Kudo, I., Inoue, K. (1991) Biochem. Biophys. Res. Comm., 178, 1505-1511.
28. Kraner, R.M., Hession, C., Johansen, B., Hayes G., McGray, P., Chow, E.P., Tizard, R., Pepinsky, R.B. (1989) J. Biol. Chem., 264, 5768-5775.
29. Gabara, B., Gledhill, B.C., Croce, C.M., Cesarini, J.P. and Koprowski, H. (1973) Proc. Soc. Exp. Biol. Med. 143, 1120-1124.
30. Langlais, J., Plante, L., Bleau, G., Chapdelaine, A. and Roberts, K.D. (1982) Fertil. Steril. 38, 135-136.
31. Ohzu, E. and Yanagimachi, R. (1982) J. Exp. Zool. 224, 259-263.
32. Meizel, S. and Turner, K.O. (1983) FEBS Lett. 161, 315-318.
33. Fleming, A.D. and Yanagimachi, R. (1984) J. Exp. Zool. 229, 485-489.
34. Soupart, P., Anderson, M.L., Albert, D.H., Coniglio, J.G. and Repp, J.E. (1979) Fertil. Steril. 32, 450-454.
35. Davis, B.K., Byrne, R. and Hungund, B. (1979) Biochim. Biophys. Acta 558, 257-266.
36. Elliot, M. and Higgins, J.A. (1983) Cell. Biol. Int. Rep. 7, 1091-1096.
37. Yanagimachi, R. and Suzuki, F. (1985) Gamete Res. 11, 29-40.
38. Laemmli, U.K. (1990) Nature (London) 227, 680-685.
39. Lee, C., Levin, A. and Branton, D. (1987) Anal. Biochem. 166, 308-312.