Purification of a Novel Phospholipase A2 from Bovine Seminal Plasma*

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Phospholipases A2 are enzymes believed to play important roles in numerous physiological systems including sperm cell maturation. Relatively little work has, however, been devoted to study these enzymes in seminal plasma. We therefore undertook the purification and characterization of this enzyme from bovine seminal plasma. After a 330-fold purification, an activity corresponding to a protein of 100 kDa was identified by gel filtration. SDS-polyacrylamide gel electrophoresis analysis of the purified fraction revealed the presence of a 60-kDa band that comigrated with the activity during ion-exchange and gel filtration chromatography as well as polyacrylamide gel electrophoresis. The enzyme possessed a pH optimum around pH 6.5 and was calcium-dependent. Using isolectric focusing, its isoelectric point was determined to be 5.6 ± 0.07. The enzymatic activity was resistant to p-bromophenacyl bromide, but was sensitive to gossypol and dithiothreitol. The enzyme was 2 orders of magnitude more active toward micelles formed with deoxycholate than with Triton X-100. Slight differences in the specificity toward head groups and/or sn-2-side chains were found in both assay systems. The enzyme was acid-labile and did not display affinity for heparin. It would therefore appear that the phospholipase A2 form isolated from bovine seminal plasma is of a novel type.

Phospholipases A2 (PLA2) are ubiquitous enzymes capable of hydrolyzing the sn-2-position of phospholipids. Most PLA2 characterized to date belong to either one of two main groups: high and low molecular mass PLA2 (1, 2). High molecular mass PLA2, also called cytoplasmic PLA2 (cPLA2), are 85-kDa proteins found in the cytoplasm of several cell types (3–6). They are specific for arachidonic acid (6) and possess limited lysophospholipase (7, 8) and phospholipase A1 (9) activities. Low molecular mass PLA2 (sPLA2) form a family of homologous enzymes with molecular masses ranging from 14 to 20 kDa that are found in several secretory fluids as well as in the cytoplasm of various cell types (1, 2, 10). PLA2 are believed to be important regulatory enzymes in numerous physiological systems such as inflammation, membrane remodeling, and cell signalization (11). Several PLA2 that do not belong to either category have also been identified in various tissues and organisms (12–19).

In the reproductive system, PLA2 are widely accepted to play a major role in the late maturational events of spermatozoa, particularly in the acrosomal reaction (20–23). The acrosomal reaction is a multification process that permits the release of hydrolytic enzymes, which are required for spermatozoa to penetrate the acellular layers surrounding the oocyte (24).

Although several studies have been undertaken to characterize the PLA2 present in the spermatozoa and seminal plasma of various species (25–30), only the enzyme from human seminal plasma has been purified to homogeneity and sequenced (31) so as to conclusively assign it to a particular PLA2 group. The enzyme was found to be a 14-kDa protein, identical to the synovial enzyme (32), suggesting the same might be true of other mammalian species.

In bovine seminal secretions, the enzyme was partially purified, but was not characterized enough to assign it to a particular PLA2 group (30). To determine the exact type(s) of PLA2 present in bovine seminal plasma and to assess the generality of the occurrence of sPLA2 in mammalian seminal plasma, we purified and characterized the major PLA2 activity from bovine seminal plasma.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sephacryl S-300, butyl-Sepharose Fast Flow, and Q-Sepharose Fast Flow were purchased from Pharmacia Biotech (Baie d’Urfé, Québec, Canada). Electrophoresis reagents (including ampholytes) were obtained from Bio-Rad. Heparin, gossypol, and P-bromophenacyl bromide were from Sigma. Phosphatidylcholine (PC) (t-α1-palmitoyl-2-[3H]choline (specific activity of 55.6 mCi/mmol) and t-α1-palmitoyl-2-[3H]arachidonyl (specific activity of 52.6 mCi/mmol)) and phosphatidylethanolamine (PE) (t-α1-palmitoyl-2-[3H]arachidonyl (specific activity of 55.6 mCi/mmol)) were obtained from New England Nuclear (Mississauga, Ontario, Canada). The scintillation fluid (Universol) was purchased from ICN (Montreal). Aluminum-backed silica gel TLC plates were from Whatman (Maidstone, United Kingdom). Recombinant PLA2 (porcine pancreatic and Crotalus atrox) were from Sigma. Dialysis membranes were from Spectrum Medical Industries, Inc. (Houston, TX). Ultrafiltration membranes were from Amicon, Inc. (Beverly, MA). All other chemicals used were of analytical grade and were purchased from commercial suppliers.

**Phospholipase A2 Assay**

Enzymatic activity was assayed using sn-2-radiolabeled 2-arachidonyl-PE unless specified otherwise. The substrate (20,000 cpm/tube, 1.7 μM) was evaporated under nitrogen and resuspended in buffer A (50 mM
Purification of Bovine Seminal PLA₂

RESULTS

Purification of Bovine Seminal PLA₂

Seminal plasma was first passed through a butyl-Sepharose resin (Fig. 1a). Extensive washing (14 column volumes) was required to remove all the weakly adsorbed proteins. The urea-

FIG. 1. Purification scheme. a, butyl-Sepharose chromatographic pattern; b, Sephacryl S-300 chromatographic pattern; c, Q-Sepharose chromatographic pattern. Chromatography was performed as described under "Experimental Procedures." The fractions under the bar were pooled and assayed for purity as indicated in Table I. Where appropriate, the gradients used are indicated. The arrow in a indicates the point of addition of the 5 M urea buffer. In b, the approximate void volume of the Sephacryl S-300 column is indicated by the arrow. ●, absorbance; ○, PLA₂ activity.

TABLE I
Purification summary

| Step | Activity | Protein | Specific activity | Yield | Purification |
|------|----------|---------|------------------|-------|--------------|
|       | units    | mg      | units/mg         | %     | -fold        |
| Seminal plasma | 24,000 | 730 | 33 | 100 | 1            |
| Butyl-Sepharose FF (I) | 28,000 | 6.6 | 4,200 | 120 | 130          |
| Sephacryl S-300 (II) | 32,000 | 3.9 | 8,200 | 130 | 250          |
| Q-Sepharose FF (III) | 11,000 | 1.0 | 11,000 | 45 | 330          |
Purification of Bovine Seminal PLA₂

**Characterization of Bovine Seminal PLA₂**

**The Purified Enzyme Behaves as a 60-kDa Protein on SDS-PAGE**—The active fractions from the successive purification steps were analyzed by SDS-PAGE (Fig. 2) under reducing conditions. After a single purification step (Fraction I; lane 3), a main component at 60 kDa is visible. This component persists throughout until the end of the purification procedure, where it is the only major band detectable by Coomassie Blue staining (Fraction III; lane 3).

**The 60-kDa Band Is Responsible for the Activity**—Fraction III was subjected to PAGE. Measurement of the activity eluted from the gel slices revealed that it was recovered at a position corresponding to the protein (Fig. 3).

**Calcium Requirement and pH Optimum**—In a manner similar to most phospholipases characterized thus far, the enzyme was calcium-dependent and was maximally active at a concentration of 2 mM calcium (Fig. 4a), while analysis of the pH dependence of the activity revealed a single activity maximum at pH 6.5 (Fig. 4b).

**Sensitivity of Bovine Seminal PLA₂ to Known PLA₂ Inhibitors**—Purified PLA₂ was resistant to pBPB, whereas the two positive controls, porcine pancreatic and C. atrox PLA₂, were inhibited (Fig. 5a). Seminal PLA₂ was inhibited by gossypol at inhibitor concentrations higher than those required to inhibit crotal PLA₂, but similar to those required to inhibit the porcine pancreatic enzyme (Fig. 5b). The porcine enzyme and seminal PLA₂ also shared similar sensitivities to the thiol reagent DTT (Fig. 5c); the sensitivity of the crotal enzyme toward DTT was not investigated in this study.

**Determination of the Enzyme pI**—To determine the pI of PLA₂, isoelectric focusing of a partially purified enzyme was performed (Fig. 6). The gel rod was cut into 24 pieces, which were then eluted in H₂O. The supernatants were assayed for PLA₂ activity, and their pH was measured. Several (n = 8) such experiments revealed a single activity peak at pH 5.6 ± 0.07 (mean ± S.E.). Typical activity recoveries on the order of 10–20% were obtained. The true recovery is expected to be higher since the Triton X-100 concentration in the supernatants (−0.001% final concentration) inhibited the activity of a partially purified fraction by −50% (data not shown).

**Substrate Specificity of Seminal PLA₂**—The substrate specificity was studied in the presence of phospholipid micelles consisting of either PC or PE and deoxycholate or Triton X-100. As summarized in Table II, PLA₂ was 2 orders of magnitude more active toward the deoxycholate-containing substrate than toward the Triton X-100-containing substrate or vesicular substrate (data not shown). In the presence of deoxycholate, the enzyme discriminated between the sn-2-fatty acid as it was less active toward PC carrying linoleoyl (1111 ± 98) than arachi-
donyl (1716 ± 73). For a given sn-2-side chain, no selectivity was observed between PE/deoxycholate- or PC/deoxycholate-containing micelles as both substrates were hydrolyzed at similar rates, suggesting that the enzyme shows little, if any, head group specificity in this assay system. When micelles comprising Triton X-100 were used, however, head group specificities were observed. The ethanolamine phospholipid was cleaved more efficiently than the corresponding choline phospholipid were observed. The ethanolamine phospholipid was cleaved

When micelles consisting of either ethanolamine or choline as head group. The substrate was prepared 20 min in advance and was diluted 10 times in the assay tube to yield the indicated detergent concentrations. The results represent the means ± S.E. of three independent experiments.

| Phospholipid       | Enzymatic activity |
|--------------------|--------------------|
|                    | Deoxycholate (1 mM) | Triton X-100 (0.01%) |
| Arachidonoyl-PE    | 2,000 ± 32         | 56 ± 5.8              |
| Arachidonoyl-PC    | 1,700 ± 73         | 17 ± 1.5              |
| Linoleoyl-PC       | 1,100 ± 98         | 48 ± 1.7              |

**DISCUSSION**

The seminal PLA₂ activity bound specifically to the butyl-Sepharose resin, thus permitting a 130-fold purification in a single step. Choline had to be included throughout this step to prevent the heparin-binding proteins, the main component of bovine seminal plasma (37), from strongly binding to the resin.

Rechromatography of the unadsorbed fraction did not permit further binding of the activity, thus suggesting the presence of another form of PLA₂, which was not further investigated in this study. Chromatography on both gel filtration and ion-exchange resins (Fig. 1, b and c) resulted in activity and protein absorbance patterns that eluted closely together, indicating that the major protein (absorbance at 280 nm) was also responsible for the activity. When analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue, a major 60-kDa band was visible in both chromatographic runs (Fig. 2). Further confirmation that the 60-kDa band was responsible for the activity was obtained by PAGE. Fractions that consisted of eluates of gel slices were assayed for PLA₂ activity, and again, the band intensity and the corresponding enzymatic activity variations matched closely (Fig. 3). Gel filtration revealed that the activity behaved as a 100-kDa protein (Fig. 1b), whereas SDS-PAGE analysis showed a 60-kDa band (Fig. 2). This discrepancy might be attributed to dimerization of the 60-kDa enzyme. This dimer appears stable since moderately stringent conditions (0.1% deoxycholate or 5 M urea) failed to shift the elution position of PLA₂ (data not shown). Since the omission of 2-mercaptoethanol did not change its behavior on SDS-PAGE (data not shown), it appears that the interaction is noncovalent. Consistent with the dimer hypothesis, the enzyme behaves on native PAGE as a much larger protein than bovine serum albumin despite a very similar pI (Fig. 3).

Binding to Q-Sepharose at pH 7.4 (Fig. 1c) as well as isoelectric focusing (Fig. 6) indicate that the enzyme is acidic. In comparison, most mammalian sPLA₂ are neutral to basic proteins, with one notable exception (10). cPLA₂ on the other hand, possess pI values similar to those of the seminal enzyme (Fig. 6) (8, 35). Besides this similarity, however, the seminal enzyme shares little in common with cPLA₂. Using two different assay systems, the seminal enzyme did not show the char-
acteristic specificity for arachidonoylphospholipids found in high molecular mass PLA₂. In the Triton X-100 assay system, the seminal plasma PLA₂ activity toward sn-2-arachidonyl was −3-fold lower than the activity toward linoleyl, whereas cPLA₂, in a similar assay system, displayed a 3-fold higher activity (5). Moreover, while cPLA₂ is inhibited by deoxycholate micelles relative to sonicated vesicles (4), the reverse is observed for the seminal enzyme (data not shown).

The resistance of the enzyme to pBPB supports the view that this enzyme is novel. pBPB inactivates sPLA₂ by alkylating a histidine residue located in the active site of the enzyme (38). It also inactivates cPLA₂ (39) by an unknown mechanism, which is likely to be quite different from sPLA₂ since cPLA₂ does not possess an active-site histidine (3). At the pBPB concentrations is likely to be quite different from sPLA₂ since cPLA₂ does not possess an active-site histidine (3). At the pBPB concentrations used, both enzyme types should be inactivated, and yet, the seminal enzyme is unaffected. As expected, the two PLA₂ controls, the type I porcine pancreatic and the type II Crotalus enzymes, were inactivated (Fig. 5a). The greater resilience of the crotal enzyme is most likely due to its tendency to shield its active site through dimerization (40, 41). This raises the possibility that seminal PLA₂ possesses a histidine or some other susceptible residue in its active site, which would be completely shielded from the environment in the absence of substrate and/or Ca²⁺.

Despite this resistance, some common structural features between pancreatic and seminal PLA₂ are suggested by the inhibition patterns of DTT and gossypol. The pancreatic enzyme is inhibited by gossypol at concentrations very close to those required to inhibit the porcine enzyme (Fig. 5b). Although the precise structural modifications induced by gossypol are unknown, the similar concentrations required to inhibit pancreatic PLA₂ and the seminal enzyme suggest some common structural elements. This resemblance appears to be quite specific as the inhibition pattern of the crotal enzyme, which shares strong structural homologies with the pancreatic enzyme (1, 2), is completely different. The shared DTT sensitivities (Fig. 5c) further support the view that common features between mammalian sPLA₂ and seminal PLA₂ exist. Biochemical characterization revealed that seminal PLA₂ shows catalytic properties common to most sPLA₂ identified so far: the enzyme is Ca²⁺-dependent (Fig. 4a) and is optimally active in the neutral to alkaline pH range (Fig. 4b) (42).

The substrate selectivity profile of purified PLA₂ is also reminiscent of mammalian sPLA₂ (43, 44). For instance, these enzymes are activated by the introduction of negative charges (as with deoxycholate versus Triton X-100) in the lipid substrate, most likely due to the accumulation of positive charges near the phospholipid-binding site (45). In the absence of deoxycholate, for a given acyl side chain, they are more active toward the anionic phospholipid PE than toward the zwitterionic phospholipid PC (43).

Beside these catalytic similarities, major structural differences appear to exist between these enzymes. For instance, mammalian sPLA₂ are low molecular mass (14–20 kDa) and mostly basic proteins, whereas the seminal enzyme possesses a 60-kDa mass and an acidic pl. Pancreatic PLA₂ and the human seminal/synovial enzyme demonstrate affinity for heparin (46–48), while bovine seminal PLA₂ does not (data not shown). Moreover, sPLA₂ are resistant to acidic conditions as relatively good recoveries are routinely obtained following chromatography performed under acidic conditions (49–51), whereas the major PLA₂ activity found in seminal plasma is acid-labile (data not shown).

The seminal enzyme displays a specific activity (under suboptimal conditions) of ~0.01 μmol/min/mg, which is rather low compared with that of low molecular mass PLA₂ (for instance, ~40 and 1500 μmol/min/mg for bovine pancreatic and Naja naja venom PLA₂, respectively) or with that of cPLA₂ (~0.6 μmol/min/mg) (4). The activity range of these well-characterized PLA₂ thus covers 5 orders of magnitude. The resistance of seminal PLA₂ to pBPB (Fig. 5a) might indicate that it acts via a different, less efficient catalytic mechanism than the established enzymes. The lower catalytic efficiency of bovine seminal PLA₂ could be required for its proper function in seminal plasma. Alternatively, it could possess some yet undetermined advantages over other types of PLA₂ that would render it better suited to the particularity of the bovine reproductive physiology.

These results differ significantly from those reported previously concerning bovine (30) or human (28, 29, 31, 47, 52) seminal plasma PLA₂. The major human seminal plasma PLA₂ has been found to be identical to the synovial enzyme (31, 32). A minor form that was not recognized by the anti-synovial PLA₂ antibody was also reported (31). In the bovine species, the preliminary characterization of the enzyme published previously (30) did not permit any definitive conclusions to be drawn as to the nature of the seminal enzyme. Two different enzymatic activities were partially purified from seminal vesicle secretions. SDS-PAGE of the most purified fraction showed a doublet migrating as 14–16-kDa proteins. This enzyme may represent a minor PLA₂ form. The human prostate enzyme has also been partially purified and characterized (53). Overall, its biochemical properties appear to be quite distinct from those of bovine seminal plasma PLA₂.

The activities found in bovine, ram, and porcine seminal plasma amount to ~1, 10, and 0.03%, respectively, of the human seminal plasma PLA₂ activity (29), suggesting that qualitative differences might exist between the PLA₂ types found in these species. The structural characterization of the enzyme that is currently underway should reveal the reasons behind these differences.

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