Phospholipase A₂ from bovine seminal plasma is a platelet-activating factor acetylhydrolase

Sebastien SOUBEYRAND^{*†}, Claude LAZURE[‡] and Puttaswamy MANJUNATH^{*†1}

^{*}Guy-Bernier Research Centre, 5415 Boulevard de L'Assomption, Montreal, Québec, Canada H1T 2M4, [†]Departments of Medicine and of Biochemistry, University of Montreal, C.P. 6128, Succursale centre-ville, Montreal, Québec, Canada H3C 3J7, and [‡]Neuropeptides Structure and Metabolism Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Québec, Canada H2W 1R7

The major phospholipase A₂ activity from bovine seminal plasma was recently purified [Soubeyrand, Khadir, Brindle and Manjunath (1997) *J. Biol. Chem.* **272**, 222–227]. We here show that the 60 kDa enzyme is in fact a platelet-activating factor acetylhydrolase (PAF-AH). Sequences of the N-terminus as well as of internal fragments showed 100% identity with the cDNA-deduced sequences of bovine plasma PAF-AH. The enzyme has kinetic properties similar to those of the human serum PAF-AH. Although capable of hydrolysing long-chained phosphatidylcholine, it displayed a highly preferential activity towards PAF.

The enzyme activity towards phosphatidylcholine, but not PAF, was Ca²⁺-dependent. Biochemical characterization revealed that the enzyme is extensively N-glycosylated and that it exists predominantly as a dimer in solution. Western blot analysis revealed that the enzyme is highly heterogeneous in charge, with a maximal distribution at an isoelectric point of approx. 5.7. The enzyme was expressed exclusively in the seminal vesicles and the ampulla. No association of the enzyme with either epididymal or ejaculated spermatozoa could be detected.

INTRODUCTION

Phospholipases A₂ (PLA₂) are ubiquitous enzymes that specifically cleave the fatty acid at the *sn*-2 position of phospholipids. This common enzymic property hides several structurally distinct enzymes [1]. Platelet-activating factor acetylhydrolases (PAF-AHs) can be classified as PLA₂ specific for phospholipids bearing short-chain *sn*-2 fatty acids [2]. PAF is an important mediator that is involved in numerous physiological systems [3]. It acts via a specific receptor [4] and is a well established activator of the immune system. By hydrolysing the acetyl group in PAF, PAF-AH inactivates this bioactive lipid.

As with the 'classical' PLA₂, different PAF-AH forms have been identified so far [2]. The plasma form is a highly conserved [5] 44 kDa protein that displays a reversible association with low-density and high-density lipoproteins [6]. Two cellular forms have so far been characterized. A heterotrimer consisting of three subunits of molecular masses 29, 30 and 45 kDa has been isolated from bovine brain and characterized [7,8]. Several isoforms of that enzyme are known to exist [9]. Erythrocytes contain another form of cell-associated PAF-AH that is believed to consist of two 25 kDa subunits [2,10].

The presence and function of PAF in the male and female reproductive systems have been a subject of interest for several years. PAF-like compounds have been identified in bovine and human spermatozoa [11,12]. Except for human seminal plasma, which does not seem to contain PAF [12], no data are available about the seminal plasma of other species. In the female genital tract, PAF has been identified in the secretions of several species [13–17]. PAF is thought to be implicated in a wide variety of events such as ovulation [18], zygote implantation [19], fertilization [20–22], sperm motility [23–25] and maturation [11].

PAF-AH has been detected in the seminal plasma of several species [26], including human [27] and bovine [28]. The work was preliminary and no work has been devoted to characterizing the enzyme responsible at the molecular level. We report here that the enzyme responsible for most of the PLA₂ activity in bovine seminal plasma (BSP) is a PAF-AH of the plasma type with some distinguishing features.

EXPERIMENTAL

Materials

Unlabelled PAF and phosphatidylcholine (PC) were from Sigma. The Low Molecular Weight Calibration kit was from Pharmacia Biotech (Baie d'Urfée, Québec, Canada). Electrophoresis reagents (including Ampholytes) were obtained from Bio-Rad. Endoproteinase Glu-C (V8 protease), Glycan Detection Kit and Peptide-N-glycosidase F (PNGase F) were obtained from Boehringer Mannheim (Laval, Québec, Canada). PC (1- α -1-palmitoyl-2-[¹⁴C]arachidonyl-glycerol-3-phosphorylcholine; specific radioactivity 52.6 mCi/mmol) and PAF (1-hexadecyl-2-[³H]acetyl-*sn*-glycerol 3-phosphorylcholine; specific radioactivity 7 Ci/mmol) were purchased from New England Nuclear (Mississauga, Ontario, Canada). Western Blot Chemiluminescence Reagent was from DuPont (Boston, MA, U.S.A.). The scintillation fluid (Universol) was purchased from ICN (Montreal, Québec, Canada). Aluminium-backed silica gel TLC plates were from Whatman (Maidstone, Kent, U.K.). PVDF membranes (Immobilon P) were obtained from Millipore (Mississauga, Ontario, Canada). All other chemicals used were of analytical grade and were purchased from commercial suppliers. Bovine semen was a gift from the Centre d'Insémination Artificiel du Québec (St-Hyacinthe, Québec, Canada).

Abbreviations used: BSP, bovine seminal plasma; PAF, platelet-activating factor; PAF-AH, platelet-activating factor acetylhydrolase; PC, phosphatidylcholine; PLA₂, phospholipase A₂; PNGase F, peptide-N-glycosidase F.

¹ To whom correspondence should be addressed.

Bovine seminal PLA₂ was purified as described previously [29]. The enzyme was preserved at -20°C in 25 % (v/v) glycerol and 2 M NaCl.

Protein sequencing

For sequencing, the purified protein (in solution) was spotted directly onto a trifluoroacetic acid-treated glass fibre filter to which was added 30 μl of a Polybrene solution (1.5 mg/ml in 50 mM NaCl). Sequencing was initiated by a double coupling step and performed with an Applied Biosystems gas-phase sequencer (Model 470A updated to 475A specifications) directly coupled to an Applied Biosystems phenylthiohydantoin analyser (Model 120A) as previously reported, with *N*-methylpiperidine as coupling base [30]. For internal peptide sequencing, the purified protein was dissolved in 3 μl of 0.1 M NH_4HCO_3 /1 % (w/v) SDS (pH 8) to a final concentration of 14 $\mu\text{g}/\mu\text{l}$. After 10 min of boiling, 1 μl of endoproteinase Glu-C (1 $\mu\text{g}/\mu\text{l}$) was added and proteolysis was performed for 24 h at 22°C . The digest was then subjected to SDS/PAGE and electrotransferred to PVDF. After the membrane had been stained with Ponceau Red, selected bands were cut out and sliced into small pieces before introduction into the sequencer cartridge underneath a trifluoroacetic acid-treated glass fibre filter to which 30 μl of Polybrene was added. Three major protein bands were analysed in that fashion yielding the N-terminal sequence as well as an internal sequence.

PAF-AH assay

The assay used was adapted from existing assays [9,31]. Labelled PAF was adjusted with unlabelled PAF to yield a final concentration of 100 μM (40000 d.p.m./40 μl) in 0.1 M Hepes/5 mM EDTA (pH 7.2) (Hepes buffer). The suspension was sonicated with a probe for 2 min (50 W) before use. A typical reaction mixture consisted of 10 μl of enzyme-containing solution and 40 μl of PAF suspension. The reaction was stopped after 30 min at 37°C by the addition of 500 μl of chloroform/methanol (4:1, v/v) and 50 μl of water. A 100 μl sample of the upper phase was then transferred to scintillation vials and scintillation cocktail was added. Radioactivity was then determined in a β -counter.

PLA₂ assay

PLA₂ activity was assayed as described previously [29], with slight modifications. Briefly, radiolabelled PC (2000 c.p.m./40 μl ; 6.8 μM) was evaporated under nitrogen and resuspended in Hepes buffer containing 5 mM sodium deoxycholate. The substrate solution was vortex-mixed intermittently for 20 min. Substrate solution (40 μl) was added to each assay tube. After 30 min at 37°C , the lipids were extracted by the addition of chloroform/methanol (2:1, v/v) containing unlabelled arachidonic acid and 4 M KCl. The lower phase was applied to a TLC silica plate (Whatman), which was then developed in light petroleum (boiling range $35\text{--}60^{\circ}\text{C}$)/ether/acetic acid (85:15:1, by vol.). Arachidonic acid was revealed with iodine and the stained spots were cut into scintillation vials. Scintillation fluid was then added and the emitted β -radioactivity evaluated.

Protein estimation

Protein content was determined by the method of Bradford [32].

Liposome binding assays

PC liposomes (2 mM stock) were formed by sonicating PC in 0.1 M Hepes for 2 min. The liposomes were recovered by

centrifugation (30 min, 100000 g) and resuspended in 0.1 M Hepes. PC liposomes (1 mM) were incubated with PAF-AH (0.7 ng per tube) for 15 min and tubes were then centrifuged (30 min, 100000 g). The supernatant was assayed for PAF-AH activity.

Deglycosylation

The freeze-dried enzyme (130 μg) was dissolved in 10 μl of 40 mM Na_2HPO_4 /50 mM EDTA (pH 7.4). After the addition of 3 μl of 5 % (w/v) SDS/10 % (v/v) 2-mercaptoethanol, the solution was boiled for 10 min. PNGase F (3 μl ; 0.2 unit/ μl) was then added and glycolysis was performed at 22°C for 16 h. Samples (2 μg) of treated or untreated PAF-AH were then subjected, in duplicate, to SDS/PAGE; one half was transferred to nitrocellulose and processed for sugar detection as indicated by the manufacturer, whereas the second half was stained directly with Coomassie Blue [33].

Antiserum generation

New Zealand rabbits were injected hypodermically with 100 μg of purified bovine seminal PLA₂ dissolved in PBS and complemented with 1 vol. of Freund's complete adjuvant. Rabbits were then injected every 4 weeks with 50 μg of the same antigen in incomplete adjuvant. Bleedings were performed 10 days after each injection. Antiserum from the second boost was used for the present study. Before use, the antiserum was purified by affinity chromatography on Protein A-Sepharose (Pharmacia Biotech).

Western blotting

Immunodection buffer consisting of PBS, 0.05 % (v/v) Tween-20 and 0.1 % BSA was used throughout the procedure. PVDF membrane was saturated for 1 h before the addition of the anti-PAF-AH antibody (1:5000 dilution) in the same buffer. After 1 h, washes were performed (three times, for 5 min each) and lactoperoxidase-coupled anti-rabbit antibody (1:10000 dilution) was incubated with the membrane for 1 h. Membranes were then washed and development was achieved with the enhanced chemiluminescence system as described by the supplier. Controls in the absence of primary or secondary antibody were also performed.

Affinity cross-linking

PAF-AH (10 μl ; 7 ng/ μl) or ovalbumin (10 μl ; 100 ng/ μl) was incubated with 2 μl of 12 mM bis(sulphosuccinimidyl) suberate for 1 h at 22°C . The reaction was terminated by the addition of 5 μl of 1 M Tris. Proteins were then subjected to electrophoresis, transferred to PVDF and processed for Western blotting.

SDS/PAGE/two-dimensional PAGE

SDS/PAGE was performed essentially as described by Laemmli [34]. Two-dimensional PAGE was performed as described previously [35]. After electrophoresis the gel rod was incubated for 15 min in reducing denaturation sample buffer for SDS/PAGE and electrophoresis was performed on a 15 % gel. Proteins were revealed with Coomassie Brilliant Blue R-250.

Tissue preparation

Each tissue was homogenized with several passes of a Dounce homogenizer in an extraction buffer (10 ml/g of tissue) consisting of 50 mM Tris/HCl, 2 mM EDTA, 10 μM leupeptin, 5 mM

benzamidine and 4 μ g/ml of DNase I, pH 7.4. After centrifugation, 1 ml of supernatant was precipitated with 9 vol. of acetone for 48 h at -20°C . The precipitate was recovered by centrifugation, dried under N_2 and weighed. The pellet was partially resuspended (2 mg/ml) in 50 mM NH_4HCO_3 , pH 8.5, for 3 h at 4°C . After removal of the undissolved portion of the pellet, the supernatant was used for Western blot analysis. For serum preparation, serum was isolated by centrifugation and precipitated with 9 vol. of 95 % (v/v) ethanol for 1 h at 4°C . The pellet was then used for this study. Bovine spermatozoa were obtained by four consecutive 350 g centrifugation/wash cycles of retroperfused (epididymal) sperm or semen (ejaculated): twice in PBS, BSA 0.1 % and twice in PBS at 22°C . The sperm were then directly resuspended in SDS sample buffer before electrophoresis.

RESULTS

Protein sequencing

To characterize better the purified enzyme responsible for most of the PLA_2 activity present in bovine semen, the primary structure of the enzyme was first determined. The N-terminal sequence of the native enzyme and of three fragments (46, 33 and 18 kDa) generated by endoproteinase Glu-C were determined. The sequences of the native protein and the 46 and 33 kDa fragments were identical (Table 1, fragment 1), whereas the 18 kDa polypeptide showed a different sequence (Table 1,

Table 2 Ca^{2+} dependence of PAF-AH and PLA_2 activities

PC micelles were prepared as described in the Materials and methods section. For PAF micelles, preparation was identical except that PC was replaced by labelled PAF diluted with unlabelled PAF to give identical final lipid concentrations (6.8 μM). The amounts of enzyme used were 35 ng for PLA_2 assays and 0.7 ng for PAF-AH assays. The final reaction mixture contained no EDTA but 100 μM EGTA or 1 mM CaCl_2 . Results are expressed as pmol of substrate hydrolysed in 30 min, and are shown as means $\pm$ S.E.M. for three experiments.

Addition	PLA_2 activity (pmol/30 min)	PAF-AH activity (pmol/30 min)
1 mM CaCl_2	2.3 ± 0.6	6.4 ± 0.3
100 μM EGTA	0.2 ± 0.1	3.6 ± 0.1

fragment 2). Both sequences showed 100 % identity with predicted sequences from bovine PAF-AH cDNA (Table 1) [5].

Preferential cleavage of PAF

The relative activities of the enzyme towards PC and PAF were then compared by using the micellar substrate that was used for enzyme purification. As shown in Table 2, the enzyme displayed

Table 1 Peptidic sequences of bovine seminal PLA_2

The N-termini of three endoproteinase Glu-C digested fragments (18, 33 and 46 kDa) as well as the native protein (60 kDa) were partially sequenced. Numbers represent the recovered yield in pmoles of each residue after correcting for background in the case of the native protein and without correction for the 3 fragments.

Polypeptide mass	Sequences
60 kDa	Phe-Asp-Trp-Gln-Asp-Leu-Asn-Pro-Val-Ala-Tyr-Ile-Glu
	54 16 24 27 13 26 46 32 34 39 38 38 15
46 kDa	Phe-Asp-Trp-Gln-Asp-Leu-Asn-Pro-Val-Ala-Tyr-Ile-Glu-Ser-Pro-Ala
	7.0 3.1 0.3 3.2 6.9 6.1 3.7 3.5 2.7 4.1 3.2 1.8 2.2 1.1 1.2 3.6
33 kDa	Phe-Asp-Xaa-Gln-Asp-Leu-Asn-Pro-Val-Ala-Tyr-Ile-Glu-Xaa-Pro-Xaa
	11 11 7.0 5.7 8.7 6.2 3.4 3.9 5.3 3.8 2.2 1.8 1.7
18 kDa	Phe-Asp-Val-Glu-Gln-Leu-Lys-Asp-Ser-Ile-Asp-Xaa-Asp-Lys
	5.0 4.0 4.2 5.0 3.8 3.1 1.0 0.8 0.4 1.1 0.8 0.4 0.4

Figure 1 Preferential hydrolysis of PAF

PAF-AH (2.4 ng) activity was assayed in the presence of increasing concentrations of either PAF or PC. The phospholipid competitors were prepared by probe sonication. The reaction was stopped after 30 min and processed as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three experiments.

a very strong preference for PAF over PC. A 35 ng sample of enzyme hydrolysed approx. 50 % less PC than the amount of PAF hydrolysed by 0.7 ng of PAF-AH. In that particular assay system the presence of Ca^{2+} ions was essential for PC hydrolysis but not for PAF hydrolysis, suggesting that the mechanisms of hydrolysis used towards each of these substrates differ. The efficiencies of the bovine seminal PAF-AH towards PAF and PC were compared with a liposomal assay system (Figure 1). Even when presented with a 20-fold excess of PC, PAF hydrolysis was not affected, suggesting that not only was cleavage inefficient, but so was binding to PC. In contrast, when unlabelled PAF was used, competition with an IC_{50} of approx. $80 \mu\text{M}$ was observed.

Binding capability of PAF-AH to PC

To investigate whether binding to PC, rather than cleavage, was the reason behind inefficient PC hydrolysis, liposome binding experiments were performed. These experiments were made possible by the very low specific activity of PAF-AH towards liposomal PC that allowed only minimal PC hydrolysis during the course of this experiment. After centrifugation, no significant difference in PAF-AH content between liposome-containing solutions [5.6 ± 0.02 (S.E.M.) $\mu\text{mol}/\text{min}$ per mg] or liposome-free solutions ($6.3 \pm 0.32 \mu\text{mol}/\text{min}$ per mg) was detected, confirming that the enzyme shows very little PC-binding capacity; inclusion of Ca^{2+} ions in the medium did not promote binding (results not shown).

Kinetic properties

The kinetic properties of the purified enzyme were then studied. With $80 \mu\text{M}$ of PAF, linear kinetics were observed over 30 min for concentrations of enzymes below 3 ng per tube (60 ng/ml) (Figure 2A). The enzyme showed saturation kinetics at substrate concentrations above $50 \mu\text{M}$ (Figure 2B). With an Eadie-Hofstee representation of the data, the apparent K_m of the enzyme towards PAF was determined as $20 \pm 2.6 \mu\text{M}$ (mean $\pm$ S.D.).

Figure 2 Kinetic characterization of bovine seminal PAF-AH

The indicated final quantities of PAF-AH were incubated with PAF and assayed as described in the Materials and methods section. (A) Time course assay; (B) substrate saturation studies. Results are means $\pm$ S.E.M. for three experiments.

Electrophoretic characterization

Antiserum was first generated against the purified protein. In preliminary studies the antibody immunoprecipitated over 90 % of the PAF-AH activity (results not shown), indicating that it recognized PAF-AH. After electrophoresis and transfer to a PVDF membrane, seminal plasma was probed with the anti-(PAF-AH) antibody (Figure 3). The antibody was quite specific because a single band was detected after Western blotting. The band was slightly diffuse in the second dimension (SDS/PAGE) but very diffuse in the first dimension (isoelectric focusing). The maximal intensity was centred on pI 5.2–6.1, although substantial trailing was observed. The wide pI range suggested the presence of extensive glycosylation. Similar results were obtained by staining the pure protein with Coomassie Blue (results not shown).

Glycosylation studies

Potential N-linked sugars were removed by PNGase F treatment and the resulting protein was resolved by SDS/PAGE, trans-

Figure 3 Western blot analysis of bovine PAF-AH in BSP

BSP (1 μ l) was adjusted to 8 M urea and 2% (w/v) ampholytes and subjected to isoelectric focusing (22 $^{\circ}$ C, 8500 V h). The gel rods (0.3 cm $\times$ 13 cm) consisted of 4% (w/v) polyacrylamide, 2% (w/v) ampholytes (pH range 3–10), 2% (v/v) Triton X-100 and 8 M urea. SDS/PAGE was performed on a 15% (w/v) gel. Proteins were then transferred to a PVDF membrane, probed with anti-PAF-AH antibody and revealed by chemiluminescence. The migration positions and the molecular masses (in kDa) of the protein standards are indicated at the left.

ferred to a nitrocellulose membrane and probed for sugars with a detection kit specific for sugars (Figure 4B); a duplicate gel was stained with Coomassie Blue (Figure 4A). PNGase F treatment of PAF-AH resulted in a major band at 47 kDa as detected by Coomassie staining, which was not revealed by the sugar detection kit (Figures 4A and 4B, lanes 4), whereas the native enzyme showed a very rapid and strong signal, consistent with extensive glycosylation. The higher molecular mass of the bovine seminal enzyme than that of the human serum enzyme seems therefore to be mostly due to extensive (approx. 13 kDa) N-glycosylation.

Dimerization properties

The seminal PAF-AH was previously observed to behave as a 100–110 kDa protein by gel-filtration chromatography [29]. To investigate the possibility of enzyme dimerization, a cross-linking

Figure 4 Glycosylation analysis of seminal PAF-AH

PNGase F-treated PAF-AH (1 μ l) was boiled in reducing sample buffer and subjected to SDS/PAGE [10% (w/v) gel]. After completion of the electrophoresis, one half of the gel was transferred to nitrocellulose that had been treated with a sugar detection kit (Boehringer Mannheim) (B); the other half was directly stained with Coomassie Blue (A). Lane 1, molecular mass standards (top to bottom: 97, 67, 43 and 30 kDa); lanes 2, BSP 30 kDa (positive control); lanes 3, PAF-AH; lanes 4, PNGase F-treated PAF-AH.

Figure 5 Affinity cross-linking of seminal PAF-AH

PAF-AH (70 ng) was treated with (lane 2) or without (lane 1) cross-linker for 1 h at 22 $^{\circ}$ C. Samples were then subjected to SDS/PAGE [7.5% (w/v) gel]. Proteins were transferred to a PVDF membrane and probed with anti-(PAF-AH) antiserum. The migration positions and the molecular masses (in kDa) of the protein standards are indicated at the left.

Figure 6 PAF-AH expression pattern

Proteins obtained from the precipitation of several tissues (4 μ g), serum (40 μ g) and spermatozoa (20 μ g; approx. 10^7 sperm) were subjected to SDS/PAGE, transferred to a PVDF membrane and processed for Western blotting with anti-(PAF-AH) antibodies. (A) Tissues and serum on a 10% (w/v) gel; (B) spermatozoa extracts on a 15% (w/v) gel. The migration positions and the molecular masses (in kDa) of the protein standards are indicated at the left.

experiment was performed (Figure 5). PAF-AH was almost completely cross-linked to form a very diffuse band ranging from 120 to 140 kDa, indicating that in solution the enzyme exists mostly as a dimer. When 30-fold higher concentrations of ovalbumin were treated with the cross-linker no multimers were observed (results not shown), indicating that the dimerization of PAF-AH is specific.

Tissue distribution of PAF-AH

The expression pattern of seminal PAF-AH in various tissues was studied. By means of Western blotting (Figure 6A) the enzyme was found exclusively in the seminal vesicles and the ampulla, indicating that these tissues are responsible for the presence of PAF-AH in seminal plasma. A weak cross-reactivity was observed at approx. 60 kDa in the liver and kidney but the associated band was stainable with Coomassie Blue, indicating that it is a major protein and it is therefore unlikely to represent a PAF-AH. Despite the large quantity of spermatozoa extract loaded, no detectable PAF-AH (i.e. 60 kDa band) was

found associated with either epididymal or ejaculated sperm (Figure 6B), although some other protein bands (16–17, 36–39 and 68 kDa) cross-reacted with the anti-PAF-AH antibodies. Because the 36–39 and 68 kDa bands were detected in both epididymal and ejaculated sperm (results not shown), even in the absence of primary antibody, these bands are probably unrelated to PAF-AH. The 16–17 kDa band, in contrast, exhibits cross-reactivity with the primary antibody as well.

DISCUSSION

On sequencing of the enzyme, it became clear that the enzyme responsible for most of the PLA_2 activity present in the seminal enzyme was a PAF-AH. The notion that bovine seminal PLA_2 is a PAF-AH is surprising. Numerous studies on PAF-AH support the view that the enzyme is inactive towards phospholipids bearing long-chain fatty acids in the *sn*-2 position [7,36,37]. The enzyme was, however, purified to homogeneity on the basis of its activity towards micelles composed of labelled arachidonoyl-phosphatidylethanolamine and deoxycholate, a substrate that would not be expected to be cleaved by a PAF-AH. This discrepancy can be explained by a combination of factors: first, the higher specific activity of the substrate used than is commonly used for PAF-AH assays, permitting the detection of lower hydrolysing activities; secondly, the high concentrations of PAF-AH in bovine semen (approx. 0.5 mg/ml) that allowed its detection throughout the purification of very low hydrolytic activities. In comparison, bovine and human serum contained PAF-AH-specific activities two orders of magnitude lower (results not shown). It is unclear whether the low PLA_2 activity expressed by the seminal PAF-AH is associated with any physiological roles. Despite its low specific activity as a PLA_2 , the high concentration of the enzyme in bovine semen leaves open the possibility that *in vivo* the enzyme might function as a non-specific PLA_2 .

Whereas the PAF-AH activity was, as reported previously, Ca^{2+} -independent [26,38], Ca^{2+} ions did stimulate the activity relative to the presence of EGTA; PLA_2 activity was strictly Ca^{2+} -dependent. The Ca^{2+} -dependence suggests that the enzyme shares towards long-chain phospholipids a catalytic mechanism similar to that of the other Ca^{2+} -dependent phospholipases such as the low-molecular-mass PLA_2 [39]. In support of this notion, the strong pH dependence of the PLA_2 activity [29] contrasts with its pH insensitivity towards PAF [40]. Alternatively it could, like cytoplasmic PLA_2 , require Ca^{2+} ions only to associate with PAF-free membranes [41], although this is unlikely because the inclusion of Ca^{2+} ions did not permit a detectable association with PC liposomes.

Overall the kinetic properties resemble those of the human serum enzyme. Both enzymes shared similar saturation kinetics above 50 μM PAF [36] and apparent K_m values (20 μM compared with 14 μM) [42]. Differences were mostly limited to structural levels. Extensive N-glycosylation seems to account for the higher molecular mass of the enzyme relative to the human serum enzyme (60 kDa compared with 44 kDa) [40,42] because after PNGase F treatment a single major band at 47 kDa was obtained on SDS/PAGE. High glycosylation could also be responsible for the lack of recognition of the serum form by the anti-(PAF-AH) antibody (Figure 6A). Alternatively, the absence of cross-reactivity could be attributed to the relatively low PAF-AH concentration in serum.

Bovine seminal PAF-AH contains two potential N-glycosylation sites (Asn-Xaa-Ser/Thr) located near its C-terminus (Asn-421 and Asn-432). These sites are, however, not conserved in dog, mouse or chicken [5] and the human enzyme does not

seem to be glycosylated [40], arguing against a pivotal role of N-glycosylation in the functionality of the enzyme. Glycosylation might be required for the dimerization properties of the enzyme. It also remains to be determined whether or not dimerization is linked to the enzyme active state. The molecular mass of the seminal enzyme is similar to that of the enzyme purified from guinea-pig peritoneal fluid after endotoxin shock [43]. The seminal PAF-AH, however, forms a dimer, whereas, on the basis of gel-filtration results, the guinea-pig enzyme does not. Whether these enzymes share more biochemical properties, such as high glycosylation levels, will need to be clarified.

Despite possessing identical predicted sequences (Table 1), the bovine seminal N-terminal sequence was shifted by 21 amino acid residues relative to the cDNA-inferred N-terminal sequence [5]. A post-transcriptional cleavage of the enzyme between Pro-21 and Phe-22 could account for this discrepancy. N-terminal sequencing of the human plasma enzyme [40] revealed a similar shift relative to the predicted N-terminal sequence, suggesting that a common processing pathway might be shared by both enzymes.

The role of the PAF-AH in seminal plasma is unknown. The bovine sperm is known to be rich in a PC analogue that possesses an ether linkage in position 1 [44,45]. It has previously been proposed that these phospholipids could serve as a pool of precursor that, pending oxidation, could be converted to short-chain bioactive PAF-like compounds that would then be cleavable by a PAF-AH [36,46]. Seminal PAF-AH might then function as a 'scavenger-like' enzyme by inactivating this generated PAF-like material either at the sperm surface or in the seminal plasma. However, this is unlikely because BSP contains 20–40 mg/ml choline phospholipid-binding proteins, the so-called BSP proteins [47]. These proteins would be expected to sequester any PAF or PAF-like compounds produced, thereby preventing PAF-AH action on its substrate. Indeed, we have shown recently that the PLA_2 activity of PAF-AH towards a limiting quantity of substrate is completely inhibited by the BSP proteins unless interactions between BSP proteins and PC are prevented [48]. Hence PLA_2 and probably PAF-AH activities are probably minimal in BSP.

As determined by Western blot analysis, no PAF-AH was associated with either ejaculated or epididymal sperm. The presence of the primary antibody did, however, enhance the 16–17 kDa signal, indicating that it probably shares important antigenic determinants with PAF-AH. Controls (after Protein A-Sepharose treatment or performed in the absence of secondary antibodies) indicated that it is not an IgG-like protein and that it does not possess any endogenous peroxidase activity (results not shown). This band is unlikely to represent a seminal PAF-AH fragment for several reasons. First, bovine spermatozoa contain a very weak endogenous PAF-AH activity (results not shown) and hence, presumably, very little PAF-AH, making it unlikely that a hypothetical fragment would generate a signal as intense as that detected. Secondly, the enzyme is very stable to proteolysis *in vitro* by either endoproteinase V8 or trypsin (results not shown). Thirdly, a comparable signal was not detected in any other tissue, suggesting that it is derived from spermatozoa rather than the seminal vesicles and the ampulla. Lastly, no 60 kDa band was ever detected in different spermatozoa preparations, making it unlikely that the enzyme is hydrolysed completely each time. The nature of that band is currently under investigation.

The inability of PAF-AH to interact with sperm seems to contradict a previous study indicating that PAF-AH bound to spermatozoa [28]. In that study, however, spermatozoa were washed only twice (diluted 10-fold each time) and subjected to a

Percoll gradient before assay. Under these conditions, because PAF-AH activity is very high in BSP, the final spermatozoa-containing fraction could still contain a significant amount of PAF-AH. We cannot, however, completely exclude the possibility that some PAF-AH, below detection level, does interact with spermatozoa. The amount bound would, however, be extremely low compared with the amount in the seminal fluid (approx. 0.5 mg/ml protein). The presence of high concentrations of PAF-AH in BSP argues for another, important, physiological function.

Initial investigations into the role of PAF-AH in reproduction yielded interesting, albeit physiologically arguable, results. The addition of physiological concentrations of purified PAF-AH to washed ejaculated spermatozoa resulted in an immediate and marked stimulation of sperm motility (results not shown). The addition of seminal plasma prevented this effect, suggesting that PAF-AH, *in vivo*, does not demonstrate this ability. Prolonged incubation of spermatozoa with purified PAF-AH stimulated capacitation *in vitro* as well, but the lack of affinity between the two again casts doubt on the relevance of these experiments *in vivo* (results not shown). Therefore some interactions *in vitro* between spermatozoa and PAF-AH are possible, but such interactions are unlikely to occur *in vivo*.

Instead we propose that the physiological function of PAF-AH lies in the female tract itself. The enzyme might function as an immunosuppressant in the female tract by hydrolysing locally produced PAF. The cervix epithelium might, in a manner similar to that of the intestine in newborns [49], specifically internalize PAF-AH into the uterine vascularization. The enzyme concentration in seminal plasma is so high that it could conceivably result in the immunosuppression of the whole uterus. PAF-AH might thereby contribute to reducing or affecting the inflammatory response occurring after mating [50,51].

In summary, the results presented here represents the first biochemical characterization of a serum-type PAF-AH purified to homogeneity as well as the first characterization of a pure PAF-AH from a reproductive system. Bovine seminal PAF-AH is a dimer that is kinetically and structurally similar to the human serum enzyme, although heavily glycosylated. Clarification of the role of PAF-AH in reproductive biology will require further studies. Because of the abundance of PAF-AH in BSP and its relative ease of purification [29], this system is also advantageous for the isolation of pure PAF-AH for biochemical studies.

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