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Partial Purification and Localization of Platelet-Activating Factor Acetylhydrolase From Bovine Seminal Plasma

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ABSTRACT: Platelet-activating factor (PAF) is a potent lipid mediator that is inactivated by platelet-activating factor acetylhydrolase (PAF-AH). Platelet-activating factor bioactivity has been detected in bovine sperm phospholipids and PAF-AH activity is extraordinarily high in bovine seminal plasma. The purpose of this study was to purify and characterize partially the PAF-AH in bovine seminal plasma. Platelet-activating factor acetylhydrolase was partially purified from bovine seminal plasma using gelatin–agarose and ion-exchange chromatography and nondenaturing polyacrylamide gel electrophoresis (PAGE). Enzyme activity was increased 11-fold over seminal plasma with a yield of 11%. Platelet-activating factor acetylhydrolase activity was eluted from a single band with a R_f of 0.258 from a nondenaturing preparative PAGE gel along with several other proteins of varying molecular weights. Following separation by sodium dodecyl sulfate (SDS)-PAGE under reducing conditions, PAF-AH was identified as a ~60-kD band by western blotting using antiserum directed against human blood PAF-AH. N-terminal sequencing of the ~60 kD band, followed by amino acid-sequence similarity searching, demonstrated a single-sequence match with PAF-AH from bovine blood. Based on western blotting, a ~60-kD band corresponding to PAF-AH was detected in seminal vesicle fluid but not in samples of washed, sonicated sperm or sperm plasma membranes where activity was low (<5% and <0.3%, respectively, of that in seminal plasma), suggesting that seminal plasma PAF-AH does not bind tightly to sperm. Specific PAF-AH activity measured in seminal vesicle fluid was in the lower range of that in seminal plasma. These results demonstrate that PAF-AH activity in bovine seminal plasma is due to PAF-AH secreted by the seminal vesicles with sequence homology to the enzyme in human blood.

Key words: Sperm, semen, phospholipid, peroxidation.

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Platelet-activating factor (PAF) is a potent lipid mediator associated with various pathological states, particularly immune and allergic responses (Lee and Snyder, 1985; Braquet et al, 1987a). Platelet-activating factor acetylhydrolase (PAF-AH) regulates the production and activity of PAF by hydrolyzing acetate from position 2 of PAF (Blank et al, 1980; Lee and Snyder, 1985). The enzyme is widely distributed throughout several cell types, tissues, and biological fluids, and intra- and extracellular forms have been described (Stafforini et al, 1991; Jarvi et al, 1993; Korth et al, 1993; Narahara et al, 1993).

The PAF-AH from human blood has been studied extensively and is reported to be a 43-kD protein that associates with high- and low-density lipoproteins (HDL and LDL) in blood (Stafforini et al, 1987). Because the enzyme also can hydrolyze short- to medium-length *sn*-2 hydrocarbons from peroxidized phospholipids, the association of PAF-AH with blood lipoproteins is thought to be significant in preventing peroxidized forms of HDL and LDL associated with the formation of arthrosclerotic plaques (Stremler et al, 1991; Stafforini et al, 1994). Although PAF-AH functions as a type of unique phospholipase A_2 (PLA_2), several lines of evidence suggest that the plasma PAF-AH is structurally and mechanistically different from other PLA_2s and may instead belong to the class of neutral lipases. Platelet-activating factor acetylhydrolase shares the Ca^{++} independence, substrate interfacial activation, and conserved active site GXSXG motif that is characteristic of the neutral lipases (Tjoelker et al, 1995).

Platelet-activating factor acetylhydrolase activity has been detected in seminal plasma from several species (Letendre et al, 1992; Hough and Parks, 1994). The activity in bovine and stallion seminal plasma is over 50-fold greater than that reported from any other source (Hough and Parks, 1994). Like the human blood PAF-AH, the bovine seminal plasma PAF-AH (bSP PAF-AH) has essential serine and histidine residues at the active site, is Ca^{++} independent, and can hydrolyze synthetic analogs of peroxidized phospholipids (Parks and Hough, 1993; Hough and Parks, 1995). Although it has not been established whether bovine sperm synthesize PAF, we have suggested that, in addition to controlling sperm PAF content, the PAF-AH activity in bovine seminal plasma may regulate the formation of peroxidized sperm-membrane lipids.

The objective of this study was to partially purify the

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PAF-AH activity in bovine seminal plasma, confirm its identity by partial N-terminal amino acid sequencing, and determine its glandular origin.

Materials and Methods

Preparation of Seminal Plasma, Sperm Sonicates, and Sperm Plasma Membranes

Seminal plasma used for purification of PAF-AH was obtained from freshly ejaculated bovine semen provided by Genex, Inc. (Ithaca, New York) within 30 minutes after collection with an artificial vagina. Seminal plasma was recovered by centrifuging semen at 6,000 × *g* for 20 minutes at 5°C. The supernatant was recentrifuged to pellet any remaining sperm. The seminal plasma was then stored at −20°C until used.

For preparation of sperm sonicates and plasma membrane isolation, freshly ejaculated sperm were washed free of seminal plasma by layering aliquots (1 ml) of bovine semen diluted 1:1 with HBS (10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid [Hepes], 0.9% NaCl, 5 mM ethylenediaminotetraacetic acid [EDTA], pH 7.4) over 8 ml of Percoll (Pharmacia, Piscataway, New Jersey, 35% in HBS) and centrifuging at 750 × *g* for 15 minutes. The sperm pellet was then washed once more in HBS (10 ml/wash), and the final pellet was resuspended in 1.0 ml HBS. Sperm concentration was determined using a Coulter Counter model Z_m (Coulter Electronics Inc., Hialeah, Florida). Sperm were diluted with HBS to a final concentration of 500 × 10^6/ml.

For preparation of sperm sonicates, aliquots of washed sperm (1.0 ml) were made with 15 mM sodium azide to inhibit sperm motility and centrifuged at 10,000 × *g* for 5 minutes to pellet sperm. The supernatant was discarded, and the pellets were resuspended in 250 μl ice-cold HBS prior to sonication at 4°C (3 × 15 seconds, setting 2; W350 sonicator with cup horn and flow cell; Heat Systems, Farmingdale, New York). Large particulate material was pelleted by centrifuging sonicates for 5 minutes at 10,000 × *g* at 5°C, and the supernatants were stored at −20°C until used.

Sperm plasma membranes were removed by nitrogen cavitation following equilibration at 650 psi for 10 minutes at 4°C (Parks and Hammerstedt, 1985). Cavitated suspensions (5 × 10^9 total sperm/cavitate) were centrifuged at 800 × *g* for 10 minutes at 5°C, and the supernatants were saved. Pellets were washed by centrifugation in 3 ml of ice-cold HBS, and the supernatants were combined and centrifuged at 6,000 × *g* for 20 minutes at 5°C. The final supernatant containing membrane material was centrifuged at 100,000 × *g* for 2.5 hours at 5°C (Parks et al, 1987). The resulting pellet was resuspended in 200 μl HBS, and total protein was precipitated with absolute ethanol at −20°C prior to lyophilization.

Preparation of Seminal Vesicle Fluid

Reproductive tracts from sexually mature bulls were obtained within 3 hour after slaughter and were held on ice during transport from a regional abattoir. Vesicular glands were excised and rinsed, and fluid was expressed after slicing individual lobes with a scalpel. Samples of vesicular fluid were centrifuged for 5 minutes at 10,000 × *g* at 5°C to pellet any particulate material prior to storage at −20°C.

Assay for PAF-AH Activity

Acetylhydrolase activity was determined by measuring the release of [^{3}H]acetate from PAF according to the method of Stafforini et al (1987b, 1990) as described by Parks and Hough (1993). Briefly, substrate consisting of 100 μM PAF (Sigma P9525, St. Louis, Missouri) supplemented with 1-*O*-[acetyl-^{3}H]-PAF (11.25 μCi/μmol; NET-910, NEN-Dupont, Boston, Massachusetts) was incubated with protein samples diluted in 0.1 M Hepes buffer, pH 7.2, and supplemented with 0.1% bovine serum albumin (BSA) for 30 minutes at 37°C. Reactions were stopped by the addition of acetic acid followed by neutralization with sodium acetate. Labeled acetate was recovered by reverse-phase chromatography on octadecyl silica gel cartridges (Baker, Thomas Scientific, Swedesboro, New Jersey) as before. Eluent (3.0 ml, 0.1 M sodium acetate) was combined with 8.0 ml liquid scintillation fluid (Ultima Gold XR; Packard, Meriden, Connecticut), and the disintegrations per minute (dpm) were determined by liquid scintillation spectrometry after correction for the effect of 0.1 M acetate on counting efficiency. Bovine serum albumin alone was included as a control to ensure that hydrolysis of acetate from PAF was not due to contamination of BSA with PAF-AH.

Purification of PAF-AH

All purification steps were carried out at 25°C unless otherwise specified.

Step 1: Precipitation of Seminal Plasma Proteins—Seminal plasma proteins were precipitated by the dropwise addition of cold (−20°C) absolute ethanol until the ratio of solvent to seminal plasma was 8:1 (v/v). The precipitating mixture was stirred slowly for 2 hours at −20°C. Precipitated material was recovered by centrifuging the mixture at 5,000 × *g* for 15 minutes at −10°C. The supernatant was decanted and the precipitate washed 3× by resuspension and centrifugation in four volumes of −20°C absolute ethanol. The final precipitate was resuspended in four volumes of ice-cold Milli-Q water (Millipore Corp., Bedford, Massachusetts) and frozen at −20°C prior to lyophilization.

Step 2: Gelatin–Agarose Chromatography—Lyophilized, ethanol-precipitated, seminal plasma protein (1,500 mg) was resuspended in PBS (50 mM KPO_4, 0.9% NaCl, pH 7.4, 1 mM EDTA, 250 μM dithiothreitol, aprotinin 3.3 × 10^{-3} trypsin-inhibiting unit [TIU]/ml, 0.02% azide) at a final protein concentration of 15 mg/ml. The protein was loaded onto a gelatin–agarose column (Sigma G-5384, 2.5 × 20 cm) equilibrated with PBS at a flow rate of 0.2 ml/minute. The column was washed with 300 ml PBS, and activity was recovered in the flowthrough.

Step 3: Batch (Diethylamino)ethyl (DEAE) Sepharose Ion-Exchange Chromatography—The flowthrough from step 2 was diluted 3:1 (v/v) in phosphate buffer (10 mM $NaPO_4$, pH 7.2, 250 μM dithiothreitol [DTT], aprotinin 3.3 × 10^{-3} TIU/ml, 0.02% azide) prior to the addition of 100 ml DEAE sepharose (Sigma DCL-6B-100) equilibrated in phosphate buffer. After stirring overnight at 5°C, the slurry was packed into a column (2.5 × 20 cm) and washed with 300 ml phosphate buffer at a flow rate

of 0.5 ml/minute. The column was washed in a stepwise fashion with increasing concentrations of KCl in phosphate buffer, and the active fraction was eluted with 150 ml 0.4-M KCl in phosphate buffer and concentrated by pressure ultrafiltration to a volume of 12 ml.

Step 4: Polyacrylamide Gel Electrophoresis (PAGE)—Nondenaturing (PAGE) and sodium dodecyl sulfate (SDS)-PAGE were performed according to the method of Davis (1964) and Laemmli (1970), respectively. Concentrated protein (7.5 mg) from step 3 was subjected to nondenaturing preparative PAGE (slab, 150 × 150 × 3 mm, 7%). After electrophoresis, a 2.0-cm strip cut from each side of the gel was stained for 30 minutes with 0.1% Coomassie brilliant R-250 in 10% acetic acid/40% methanol. The remainder of the gel was sealed in plastic wrap and stored at 5°C. The gel strips were destained overnight in 7.5% acetic acid/10% methanol and aligned next to the unstained portion of gel to locate areas corresponding to observable protein bands. Sections of gel (1 cm^2) were excised, and proteins were electroeluted into elution buffer (25 mM tris (hydroxymethyl)aminomethane [TRIS]base, 192 mM glycine, pH 8.0). The elution fraction containing PAF-AH activity was subjected to SDS-PAGE (slab, 12%) for western blotting and sequencing.

Western Analysis

Aliquots of protein (3.15 μg) containing PAF-AH activity eluted from nondenaturing PAGE were subjected to SDS-PAGE (slab, 6 × 9 cm, 12%) at 30 mA for 1.5 hours. Proteins were then electrotransferred to polyvinylidene difluoride (PVDF) membrane (Immobilion-P, Millipore Corp., Bedford, Massachusetts) for 15 hours at 250 mA in Towbin buffer (25 mM TRIS base, 192 mM glycine, 20% methanol). Rapid immunodetection without blocking was performed according to the Immobilon-P transfer membrane user guide. Briefly, the blotted membrane was dried by soaking in methanol for 10 seconds, followed by air evaporation for 15 minutes. The blot was then incubated for 1 hour at room temperature with rabbit anti-human PAF-AH polyclonal antisera (generously supplied by ICOS Corp., Bothell, Washington) diluted 1:8,000 in blocking buffer (10 mM Na-phosphate, 0.9% NaCl, pH 7.2, 1% BSA, 0.05% Tween-20). Following two 10-second washes in PBS, the blot was incubated for 30 minutes at room temperature with anti-rabbit IgG-alkaline phosphatase conjugate (Sigma A-3687) diluted 1:30,000 in blocking buffer. The blot was washed twice more in PBS and then incubated with 50 ml of 5-bromo-4-chloro-3-indoyl phosphate (0.15 mg/ml) and nitro blue tetrazolium (0.30 mg/ml) in 100 mM TRIS buffer, pH 9.5, with 5 mM $MgCl_2$ (Sigma B5655). After sufficient color development, the reaction was stopped by washing the blot in Milli-Q water.

For western analysis of seminal vesicle fluid, sperm sonicates, and sperm plasma membrane preparations aliquots (20–40 μg) of total protein were electrophoresed and blotted as described above. Positive bands were identified using the rapid immunodetection procedure as before.

Sequencing

Aliquots of protein (10.5 μg) containing PAF-AH activity eluted from nondenaturing PAGE were subjected to SDS-PAGE (slab, 15 × 15 cm, 12%) at 40 mA for 4 hours. Proteins were electrotransferred to PVDF membrane as described above. After staining with Coomassie Blue, the protein band to be sequenced was identified by alignment with a section of blot previously probed by western blot analysis. N-terminal sequencing was carried out on a Procise model 492 Sequencer (New York State Center for Advanced Technology, Cornell University, Ithaca, New York). An internal standard of (Abu-nVal-nLeu-hArg)[5] was used to constantly monitor the intrument performance during the analysis. Amino acid-sequence similarity searches were carried out using the program BLASTP from GenBank®.

Table 1. *Purification of PAF-AH from bovine seminal plasma*

Fraction	Total activity (μmol/minute)	Protein (mg)	Specific activity (nmol/minute/mg)	Purification (-fold)	Yield (%)
Seminal plasma	188	668	282	—	—
ETOH precipitation	188	668	282	—	100
Gelatin–agarose	174	334	521	1.8	92
Batch DEAE	88	60.6	1,460	5.2	47
Preparative PAGE	21	6.85	3,100	11.0	11

PAF-AH, platelet-activating factor acetylhydrolase; ETOH, ethanol; DEAE, (diethylamino)ethyl; PAGE, polyacrylamide gel electrophoresis.

Protein Assay

The protein content of all samples was determined using Bio-Rad protein dye reagent (500-0006, Bio-Red Laboratories, Hercules, California) in a modified Bradford assay with BSA as a standard.

Results

The stability of bovine seminal plasma PAF-AH at room temperature and to repeated freezing and thawing was tested throughout the purification procedure. No change in specific PAF-AH activity was detected after aliquots of raw seminal plasma or of partially purified enzyme were incubated at room temperature for 24 hours or were subjected to five cycles of freezing and thawing (data not shown).

Ethanol-precipitated seminal plasma protein was subjected to gelatin–agarose chromatography to remove gelatin-binding proteins (15–16 kD and 30 kD) that typically constitute up to 50% of the total protein of bSP (Manjunath et al, 1987). The low molecular weight proteins were retained by gelatin–agarose resin, did not possess PAF-AH activity, and resulted in bands at 15 and 16 kD when analyzed by SDS-PAGE (data not shown). The 30-kD fraction did not bind gelatin resin even upon rechromatography of the flowthrough. Greater than 90% of the total PAF-AH activity, along with 50–69% of the total protein originally loaded, was consistently recovered in the flowthrough over 10 separate runs (Table 1).

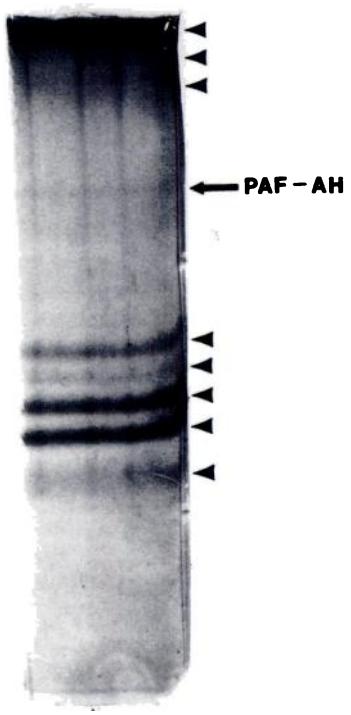

FIG. 1. Nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) of partially purified bovine seminal plasma (bSP) platelet-activating factor acetylhydrolase (PAF-AH). A subsample of enzyme (500 μg protein) that had been purified through the batch (diethylamino) ethyl (DEAE) step was subjected to nondenaturing preparative PAGE (150 × 150 × 1.5 mm, 7%). Individual protein bands on a 2-cm gel strip were visualized by staining with Coomassie Blue. Areas corresponding to observable protein bands were excised, and proteins were electroeluted from the gel slices prior to assay for PAF-AH activity. Protein bands assayed for activity are indicated by arrows. Platelet-activating factor acetylhydrolase activity migrated with an R_f of 0.258.

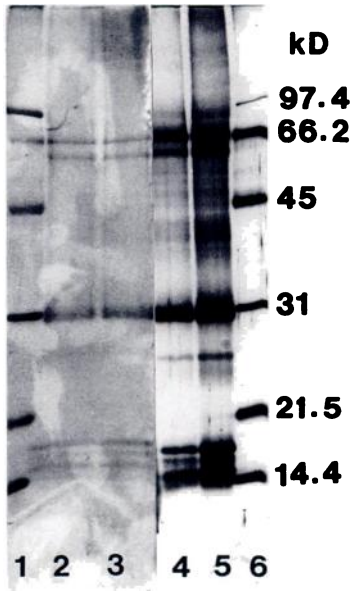

FIG. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of partially purified bovine seminal plasma (bSP) platelet-activating factor acetylhydrolase (PAF-AH). A sample of enzyme that had been purified through the nondenaturing PAGE step was subjected to SDS-PAGE (12%) as described in the text. Individual protein bands were visualized either by staining with Coomassie Blue (**lanes 1–3**) or by silver staining (**lanes 4–6**). **Lanes 1** and **6**: Molecular weight standards; **lanes 2–5**: partially purified bSP PAF-AH.

Using batch chromatography, PAF-AH activity from the gelatin–agarose flowthrough was eluted from DEAE–Sepharose ion exchange resin in a stepwise fashion with increasing concentrations of KCl in phosphate buffer. Activity was always eluted in the 0.4-M KCl step wash. Attempts to further purify PAF-AH by ion exchange were made by diluting the 0.4-M KCl batch elution seven-fold with phosphate buffer and rechromatographing the diluted eluent with a DEAE–Sepharose column (20 × 2.5 cm). Platelet-activating factor acetylhydrolase activity was eluted with 0.4 M KCl as before, but specific activity did not increase (data not shown). In another effort to improve purification, batch ion-exchange chromatography was performed in the presence of 10 mM CHAPS detergent (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, Sigma C5070, Sigma Chemical Co., St. Louis, Missouri) in an effort to solubilize PAF-AH from any protein aggregates. While PAF-AH activity was not affected by the presence of CHAPS in the standard PAF-AH assay, there was no increase in specific PAF-AH activity eluted from DEAE–Sepharose in the presence of CHAPS (data not shown).

Following concentration by ultrafiltration, the batch DEAE eluent was subjected to preparative nondenaturing PAGE. Areas corresponding to protein bands identified by staining with Coomassie Blue were electroeluted and tested for PAF-AH activity (Fig. 1). Activity migrated corresponding to a single band with an R_f of 0.258 (Fig. 1.). No activity was detected in other bands or in the stacking gel.

Platelet-activating factor acetylhydrolase activity electroeluted from a single band on preparative nondenaturing PAGE resulted in several bands of varying molecular weights when analyzed by SDS-PAGE (Fig. 2). Based on western analysis using antisera directed against the human blood plasma PAF-AH, bSP PAF-AH was identified as a single band corresponding to a molecular weight of ~60 kD (Fig. 3). Controls indicated that results from seminal plasma samples were not due to endogenous alkaline phosphatase activity or binding of secondary antibody (Fig. 3, lanes 3, 6). Two bands were detected in human serum at 66 and 54 kD (Fig. 3, lane 8). The 66-kD band was also detected by the secondary antibody in the absence of the primary antiserum (Fig. 3, lane 5).

Partial PAF-AH activity was recovered from the 60-kD band identified as PAF-AH following SDS-PAGE of samples electroeluted from nondenaturing PAGE. Specific ac-

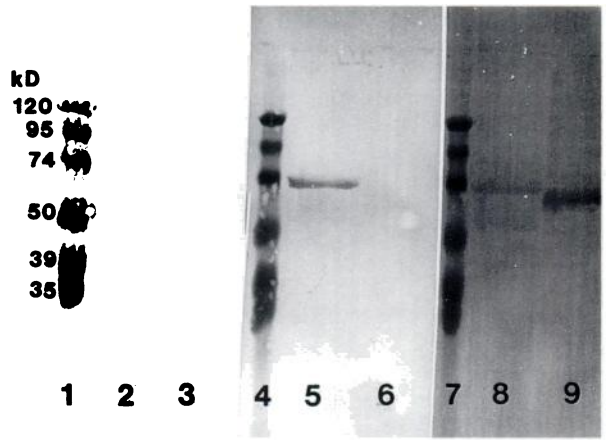

FIG. 3. Western blot of partially purified bovine seminal plasma (bSP) platelet-activating factor acetylhydrolase (PAF-AH) activity. A sample of enzyme that had been purified through the nondenaturing polyacrylamide gel electrophoresis (PAGE) step was subjected to sodium dodecyl sulfate (SDS)-PAGE (12%), blotted onto polyvinylidene difluoride (PVDF) membrane, and probed with antiserum directed against the human blood PAF-AH. Positive bands were visualized using alkaline–phosphatase-conjugated secondary antibody. **Lanes 1, 4, 7**: Prestained molecular weight standards; **lanes 2, 5, 8**: human blood plasma (5 μg); **lanes 3, 6, 9**: partially purified bSP PAF-AH following nondenaturing PAGE (3 μg). **Lanes 2, 3**: Omission of 1° antiserum and 2° antibody. **Lanes 5, 6**: Omission of 1° antiserum.

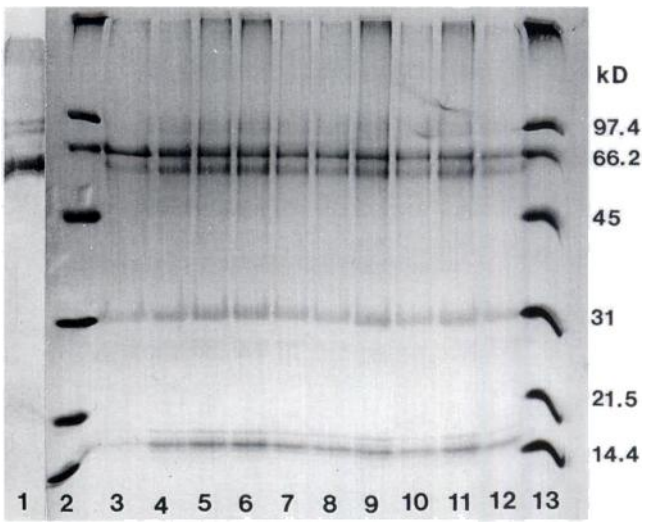

FIG. 4. Blot of partially purified bovine seminal plasma (bSP) platelet-activating factor acetylhydrolase (PAF-AH) activity submitted for sequencing. Samples of enzyme that had been partially purified through the nondenaturing polyacrylamide gel electrophoresis (PAGE) step were subjected to sodium dodecyl sulfate (SDS)-PAGE (150 × 150 × 1.5 mm, 12%) and blotted onto polyvinylidene difluoride (PVDF) membrane. Protein bands were visualized by staining with Coomassie Blue (**lanes 2, 13**: molecular weight standards; **lanes 3–12**: partially purified bSP PAF-AH following nondenaturing PAGE, 3 μg/lane). A strip of membrane was subjected to western analysis (**lane 1**) and used to identify the protein band corresponding to PAF-AH. A band at 60 kD from each of **lanes 3–12** was excised and submitted for N-terminal sequencing.

tivity could not be determined based on the Bradford protein assay.

N-terminal sequencing of the partially purified 60-kD protein (identified in Fig. 4 by Coomassie Blue staining) resulted in a single amino acid determination for 12 of the 25 total residues sequenced. Two or three amino acids were identified for each of the remaining 13 residues, indicating heterogeneity of the sample (Table 2). Sequence similarity searches using the 12 unequivocal amino acids revealed a single match with bovine blood plasma PAF -AH (GenBank™ accession no. U34247). Where two or three amino acids were identified per residue sequenced, the amino acid with the greatest mass detected at each position directly matched the sequence in bovine blood for 12 of the 13 residues analyzed (Table 2). Sequence alignment showed that the N-terminal sequence of seminal plasma PAF-AH matched a sequence that began at residue no. 22 of the bovine blood plasma sequence (Table 2).

Platelet-activating factor acetylhydrolase in seminal vesicle fluid from each of four bulls was detected as a single band at 60 kD, based on western analysis (Fig. 5, lanes 5–8). No bands were detected based on labeling of secondary antibody only (Fig. 5, lanes 1–4). Platelet-activating factor acetylhydrolase activity was measured in seminal vesicle fluid from the same four bulls using the standard assay for PAF-AH (described previously) and was 44.5 ± 17.2 nmol/mg/minute. Platelet-activating factor acetylhydrolase activity measured in sonicates of whole sperm was 5.88 ± 2.4 nmol/mg/minute (n = 4) and in sperm plasma membrane preparations was 0.403 ± 0.12 nmol/mg/minute (n = 3).

Table 2. *Amino acid sequence alignment of PAF-AH from bovine blood and seminal plasma**

	22																							46
Blood	F	D	W	Q	D	L	N	P	V	A	Y	I	E	S	P	A	W	V	S	K	I	Q	A	L	M
	1								10															25
Seminal plasma	F	D	I	Q	D	L	N	P	V	A	Y	I	E	S	P	A	D	V	S	K	I	Q	A	L	M
	L	E	R	P	A	P	K	E	K	N			G					L			Y			
	G		E				E														L			

PAF-AH, platelet-activating factor acetylhydrolase.

* PAF-AH purified from bovine seminal plasma was subjected to N-terminal sequencing for 25 cycles. Results gave a single amino acid determination for 12 residues. For each of the remaining 13 residues, 2–3 amino acids were detected. The amino acids with greatest mass at each position are presented in the seminal plasma sequence and other amino acids detected are listed directly below. A sequence similarity search using sequence results from residue 11 through 25 gave a single match with PAF-AH from bovine blood (GenBank accession no. U34247).

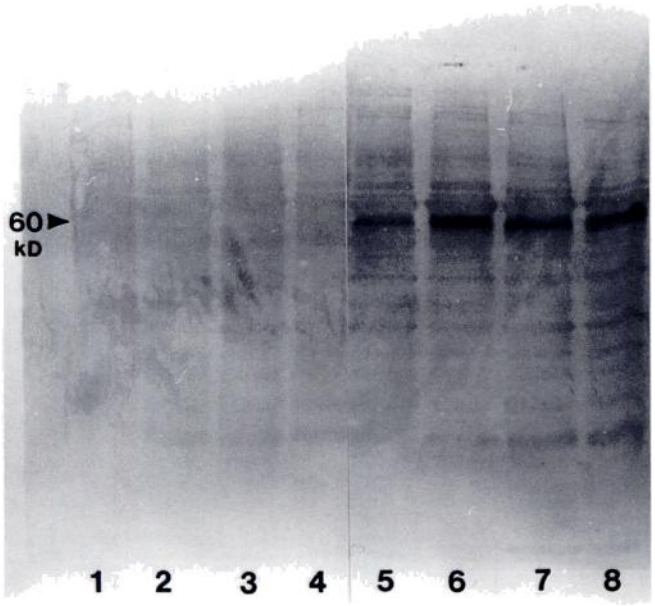

FIG. 5. Western blot of platelet-activating factor acetylhydrolase (PAF-AH) in seminal vesicle fluid. Aliquots of total protein (20 μg) of seminal vesicle fluid obtained from each of four bulls (**lanes 1–4** and **5–8**) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%), blotted onto polyvinylidene difluoride (PVDF) membrane, and probed with antiserum (**lanes 5–8**) directed against the human blood PAF-AH. Positive bands were visualized using alkaline–phosphatase-conjugated secondary antibody. **Lanes 1–4.** Omission of 1° antiserum.

When aliquots of total protein from sperm plasma membrane samples were analyzed by western blotting, no band at 60 kD corresponding to seminal plasma PAF-AH could be detected (Fig. 6, lanes 3, 4). However, a single band was detected at ~78 kD that was not due to endogenous alkaline phosphatase activity or labeling of secondary antibody (Fig. 6, lanes 3, 4). No positive bands could be detected on western blots of total protein from whole-sperm sonicates (not shown).

Discussion

Platelet-activating factor acetylhydrolase was purified from seminal plasma using gelatin–agarose and ion-exchange chromatography and nondenaturing PAGE. Specific activity of PAF-AH was not affected by ethanol precipitation, a step included to remove the significant lipid fraction of seminal plasma prior to column chromatography. Low molecular weight proteins (15–16 kD), constituting nearly half the total seminal plasma protein, were removed using gelatin–agarose resin and were presumed to be the bovine seminal proteins (BSP-A1, -A2, and -A3) previously described by Manjunath et al (1987). This resulted in an approximate two-fold increase in specific activity (Table 1). Although the 30-kD fraction of bSP (BSP 30 kD) apparently also binds gelatin resin (Manjunath et

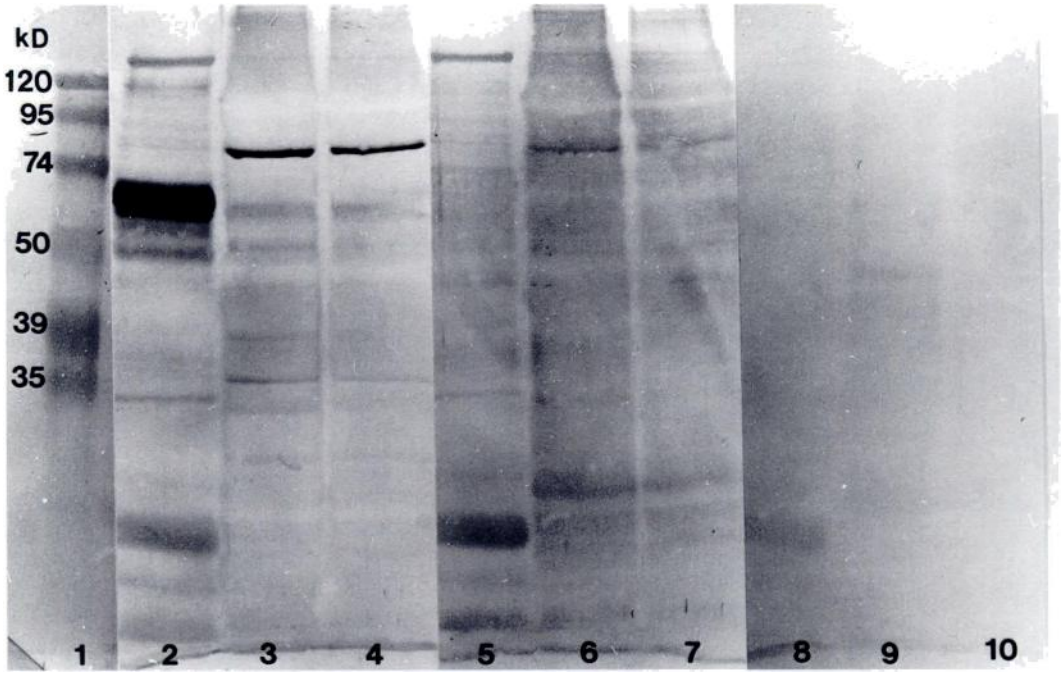

FIG. 6. Western blot of samples of bovine sperm plasma (bSP) membrane. Aliquots of total protein (40 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%), blotted onto polyvinylidene difluoride (PVDF) membrane, and probed with antiserum directed against the human blood platelet-activating factor acetylhydrolase (PAF-AH). Positive bands were visualized using alkaline–phosphatase-conjugated secondary antibody. Dilutions of primary antiserum and secondary antibody were 1:1,000 and 1:7,500, respectively. **Lane 1**: Prestained molecular weight standards; **lanes 2, 5, 8**: bovine seminal plasma (20 μg); **lanes 3, 4, 6, 7, 9, 10**: sperm plasma membrane; **lanes 5, 6, 7**: omission of 1° antiserum; **lanes 8, 9, 10**: omission of 1° antiserum and 2° antibody.

al, 1987), proteins corresponding to this molecular weight were not retained by gelatin resin in our procedure, even after rechromatography on freshly equilibrated resin. As determined by SDS-PAGE, this 30-kD fraction was the major contaminating factor remaining after the final purification step (Fig. 2). It has since been determined that BSP-30 kD does not bind commercially prepared gelatin resin consistently but does bind custom-made resin prepared with gelatin isolated from calf skin (P. Manjunath, personal communication).

Purification to homogeneity of PAF-AH was complicated due to the apparent association of PAF-AH activity with an aggregate of proteins throughout the purification procedure. As a result, no step yielded more than a threefold purification (Table 1). Several proteins of widely differing molecular weights consistently coeluted with PAF-AH activity on gelatin–agarose, ion-exchange, gel filtration, and hydrophobic interaction chromatography (data not shown) and were recovered together in a single band along with PAF-AH activity on nondenaturing PAGE (Fig. 1). The lower molecular weight bands (Fig. 2) likely represent BSP-A1, -A2, and -A3 that were not completely removed by gelatin–agarose chromatography. Bovine seminal plasma proteins tend to form large aggregates that can be separated only by treatment with citrate or low pH (Manjunath and Sairam, 1987; Hameed et al, 1991; A-Somai et al, 1994), suggesting that purification results may be due, in part, to this phenomenon. Because seminal plasma PAF-AH is inactivated at low pH (Parks and Hough, 1993), this treatment was not included in the protocol. CHAPS and Tween-20 were not effective in separating PAF-AH activity from protein aggregates during ion-exchange or gel-filtration chromatography. Finally, attempts to purify PAF-AH by gel filtration under complete denaturing conditions using guanidine–HCl were unsuccessful as PAF-AH activity could not be recovered upon renaturation. Following nondenaturing preparative PAGE, the final specific activity achieved was 3,100 nmol/mg/minute, representing a purification factor of 11.0 with a yield of 11%. This falls within the range of specific activity reported for PAF-AH purified from mouse and human blood, 380 and 9,463 nmol/mg/minute, respectively (Tsaoussis and Vakirtzi-Lemonias 1994; Stafforini et al, 1987b). Since our final preparation contained partially purified material, it is anticipated that the specific activity of pure bSP PAF-AH would more closely approximate that reported for PAF-AH from human blood.

Although we did not sequence the entire seminal plasma PAF-AH, the high degree of homology with the single match returned by a GenBank® sequence similarity search strongly suggests that the protein we have identified is PAF-AH and that its sequence is very similar to the enzyme from bovine blood. The seminal plasma enzyme appears to lack the 22 amino acid hydrophobic signal peptide associated with the blood form (Tjoelker et al, 1995). Based on sequencing, we were unable to identify the N-terminus as a single amino acid; however, this is not surprising, given that Tjoelker et al (1995) have reported detecting extensive N-terminal heterogeneity in several preparations of purified human plasma PAF-AH. While the PAF-AH purified from human blood was reported to be 43 kD (Stafforini et al, 1987b), we recognized the human PAF-AH as a 54-kD protein based on western analysis. Differences in the molecular weight of PAF-AH in human blood are likely due to differences in glycosylation and tendency toward degradation at the N-terminus during purification (L. Tjoelker, personal communication).

Platelet-activating factor acetylhydrolase activity in seminal plasma is contributed to largely by the seminal vesicles as PAF-AH was recognized as a 60-kD band using western analysis, and specific PAF-AH activity in seminal vesicle fluid was about half that of the activity originally reported in seminal plasma (Parks and Hough, 1993) and within the lower range of activity detected more recently in a sample of bulls (Parks et al, 1996). The difference in activity may be accounted for, at least partially, by proteolysis prior to collection of glandular fluid or by contribution from other accessory sex glands. However, a contribution from the prostate or bulbo-urethral glands must be relatively minor, because a sufficient volume of fluid could not be expressed from these tissues to determine PAF-AH activity.

Although PAF-AH activity was detected in sonicates of washed sperm and in plasma membrane preparations, activity was much lower than in seminal plasma, which is consistent with our previous finding that over 99% of total activity can be removed from sperm by washing (Parks and Hough, 1993). The activity detected in sperm sonicates is within the range of that reported previously for ejaculated sperm (Parks and Hough, 1993). That PAF-AH activity in plasma membrane preparations was <7% of that in whole-sperm sonicates suggests that seminal plasma PAF-AH is not selectively localized in or associated tightly with the sperm plasma membrane. This is supported by our inability to detect PAF-AH as a 60-kD band in plasma membrane samples by western blotting. In addition, PAF-AH could not be localized on sperm following immunocytochemical staining using primary antiserum directed against human PAF-AH, since no difference could be detected in staining patterns between primary antiserum and nonimmune serum.

Whether the ~78-kD band identified by western analysis of plasma membrane protein accounts for PAF-AH activity has not been determined but may represent an endogenous form of PAF-AH (Muguruma and Johnston, 1997). We have previously detected only very low levels of PAF-AH activity in epididymal sperm (Parks and

Hough, 1993) and have not determined if activity is associated with the plasma membrane.

These studies demonstrate that bSP PAF-AH activity is contributed by the seminal vesicles and can be largely removed from sperm by washing. Although PAF-AH activity is measurable in sonicates of ejaculated sperm and in isolated sperm plasma membranes, the 60-kD PAF-AH from seminal plasma could not be detected in these samples by western analysis using the methods described in this study or by immunocytochemical staining of whole sperm. That seminal plasma PAF-AH does not appear to associate tightly with sperm suggests that PAF-AH may be most important during the period when sperm are bathed in seminal plasma from the time of ejaculation through transport in the vagina and cervix. The association of PAF-AH with sperm during early transport in the female may be of importance in preventing the accumulation of PAF or biologically active lipid species resulting from membrane lipid peroxidation, which in turn could prevent premature capacitation or the acrosome reaction.

The outer leaflet of the plasma membrane overlying the apical ridge and acrosomal region is largely enriched in ether-linked docosahexanoyl and/or pentanoyl choline phosphatides (CP) with 2-acyl moieties (Selivonchick et al, 1980). These features indicate that sperm CP can serve as a substrate pool for PAF production that could be regulated by seminal plasma PAF-AH at the sperm surface. Although we have been unable to demonstrate PAF synthesis by bovine sperm, PAF receptors identified in the uterus and oviduct (Montrucchio et al, 1987; Yang et al, 1992; Zhu et al, 1992) and indirect evidence for sperm receptors (Ricker et al, 1989; Krausz et al, 1994; Luconi et al, 1995) suggest a potential role for sperm-derived PAF in sperm physiology.

Extensive work on substrate preference of the PAF-AH associated with serum lipoproteins from humans has shown that PAF-AH can effectively hydrolyze fragmented fatty acyl chains from position 2 of peroxidized phospholipids (Stremler et al, 1991). We have recently shown that the PAF-AH from seminal plasma recognizes and hydrolyzes synthetic analogs of these short acyl-chain subtrates (Hough and Parks, 1995). Tanaka et al (1993) have shown that peroxidative fragmentation of docosahexanoyl CP, the predominant molecular species in bovine sperm, results in potent, biologically active short-chain lipid species that act through the PAF receptor. Because sperm CP is highly susceptible to lipid peroxidation due to the high degree of unsaturation of the *sn*-2 fatty acyl chain, seminal plasma PAF-AH associated with the sperm plasma membrane may function to regulate the formation of fragmented sperm lipid species and thereby regulate their structural and/or biological effects. Like other forms of PAF-AH, the bSP PAF-AH is Ca^{++} independant, does not require activation, and does not hydrolyze unoxidized phospholipids (Parks and Hough, 1993). Thus, it appears that like the blood PAF-AH, seminal plasma PAF-AH may function as a fully active scavenger of peroxidized and/or biologically active lipid species (Stremler et al, 1991). de Lamirande and Gagnon (1993) have suggested that the sperm hyperactivation and capacitation *in vivo* may occur through a process of controlled lipid peroxidation. As sperm are transported through the female tract, loss of seminal plasma PAF-AH could play a permissive role in effecting controlled lipid peroxidation.

Soubeyrand et al (1997) have recently purified a 60-kD protein from bSP based on Ca^{++}-dependent PLA_2 activity against phosphatidylethanolamine. Partial sequencing revealed a match with PAF-AH from bovine blood (Manjunath, personal communication), and the purified protein had high specific activity for PAF-AH as determined in our routine assay for PAF-AH activity.

The specific relationship between seminal plasma PAF-AH and sperm relative to regulation of sperm-lipid peroxidation and generation of biologically active lipid species is under investigation.

References

Al-Somai N, Vishwanath R, Molan PC, Shannon P. Anionic and cationic components from protein aggregates in bovine seminal plasma and their effects on sperm motility. *Molec Reprod Dev* 1994;39:328–336.

Blank ML, Lee T, Fitzgerald V, Snyder F. A specific acetylhydrolase for 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). *J Biol Chem* 1980;256(1):175–178.

Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev* 1987;39:97–133.

Davis BJ. Disc electrophoresis: II. Method and application to human serum proteins. *Ann NY Acad Sci* 1964;121:404–427.

de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic Biol Med* 1993; 14:157–166.

Hameed I, Molan PC, Shannon P. Nature of the large antibacterial proteins in bovine seminal plasma formed by reversible aggregation. *NZ J Agric Res* 1991;34:467–470.

Hough SR, Parks JE. Platelet-activating factor acetylhydrolase activity in seminal plasma from the bull, stallion, rabbit, and rooster. *Biol Reprod* 1994;50:912–916.

Hough SR, Parks JE. PAF-acetylhydrolase from bovine seminal plasma utilizes *sn*-2 short-chain choline phospholipids. *Biol Reprod* 1995;52 (Suppl 1):59.

Jarvi K, Langlais J, Gagnon C, Roberts KD. Platelet-activating factor acetylhydrolase in the male reproductive tract: origin and properties. *Int J Androl* 1993;16:121–127.

Korth R, Bidault J, Palmantier R, Benveniste J, Ninio E. Human platelets release a PAF-acether:acetylhydrolase similar to that in plasma. *Lipids* 1993;28(3):193–199.

Krausz CS, Gervasi G, Forti G, Baldi E. Effect of platelet-activating factor on motility and acrosome reaction of human spermatozoa. *Hum Reprod* 1994;9:471–476.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

Lee T-C, Snyder F. Function, metabolism, and regulation of platelet ac-

tivating factor and related ether lipids. In: Kuo JF, ed. *Phospholipids and Cellular Regulation.* Boca Raton, Florida: CRC Press; 1985:1–39.

Letendre ED, Miron P, Roberts KD, Langlais J. Platelet-activating factor acetylhydrolase in human seminal plasma. *Fertil Steril* 1992;57:193–198.

Luconi M, Bonaccorsi L, Krausz C, Gervasi G, Forti G, Baldi E. Stimulation of protein tyrosine phosphorylation by platelet-activating factor and progesterone in human spermatozoa. *Molec Cell Endocrinol* 1995;108:35–42.

Manjunath P, Sairam MR. Purification and biochemical characterization of three major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma. *Biochem J* 1987;241:685–692.

Manjunath P, Sairam MR, Uma J. Purification of four gelatin-binding proteins from bovine seminal plasma by affinity chromatography. *Biosci Rep* 1987;7(3):231–238.

Montrucchio G, Massobrio M, Alloatti G, Roffinello C, Emanuelli G, Benedetto C, Piva S, Vigliani MC, Camussi G, Tetta C. In vitro oxytocic activity of platelet-activating factor on human myometrium. *Adv Prostaglandin, Thromboxane, Leukotriene Res* 1987;17:1129–1132.

Muguruma K, Johnston JM. Metabolism of platelet-activating factor in rat epididymal spermatozoa. *Biol Reprod* 1997;56(2):529–536.

Narahara H, Frenkel RA, Johnston JM. Secretion of platelet-activating factor acetylhydrolase following phorbol–ester-stimulated differentiation of HL-60 cells. *Arch Biochem Biophys* 1993;301(2):275–281.

Parks JE, Arion JW, Foote RH. Lipids of plasma membrane and outer acrosomal membrane from bovine spermatozoa. *Biol Reprod* 1987; 37:1249–1258.

Parks JE, Hammerstedt RH. Developmental changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. *Biol Reprod* 1985;32:653–658.

Parks JE, Hough SR. Platelet-activating factor acetylhydrolase activity in bovine seminal plasma. *J Androl* 1993;14:335–339.

Parks JE, Hough SR, Simkin ME, Kaproth MT. Relationship of PAF-acetylhydrolase in bovine seminal plasma to semen characteristics. *Biol Reprod* 1996;54 (Suppl 1):64.

Ricker DD, Minhas BS, Kumar R, Robertson JL, Dodson MG. The effects of platelet-activating factor on the motility of human spermatozoa. *Fertil Steril* 1989;52:655–658.

Selivonchick DP, Schmid PC, Natarajan V, Schmid HO. Structure and metabolism of phospholipids in bovine epididymal sperm. *Biochim Biophys Acta* 1980;618:242–254.

Soubeyrand S, Khadir A, Brindle Y, Manjunath P. Purification of a novel phospholipase A_2 from bovine seminal plasma. *J Biol Chem* 1997; 272(1):222–227.

Stafforini DM, McIntyre TM, Carter ME, Prescott SM. Human plasma platelet-activating factor acetylhydrolase: association with lipoprotein particles and role in the degradation of platelet-activating factor. *J Biol Chem* 1987a;262(9):4215–4222.

Stafforini DM, McIntyre TM, Prescott SM. Platelet-activating factor acetylhydrolase from human plasma. *Meth Enzymol* 1990;187:344–356.

Stafforini DM, Prescott SM, McIntyre TM. Human plasma platelet-activating factor acetylhydrolase: purification and properties. *J Biol Chem* 1987b;262(9):4223–4230.

Stafforini DM, Prescott SM, Zimmerman GA, McIntyre TM. Platelet-activating factor acetylhydrolase activity in human tissues and blood cells. *Lipids* 1991;26(12):979–985.

Stafforini DM, Zimmerman GM, McIntyre TM, Prescott SM. The plasma PAF-acetylhydrolase prevents oxidative modification of low density lipoprotein. *J Lipid Mediators Cell Signalling* 1994;10:53–56.

Stremler KE, Stafforini DM, Prescott SM, McIntyre TM. Human plasma platelet-activating factor acetylhydrolase can utilize oxidatively fragmented phospholipids as substrates. *J Biol Chem* 1991;266(17): 11095–11103.

Tanaka T, Minamino S, Unezaki H, Tsukatani H, Tokumura A. Formation of platelet-activating factor-like phospholipids by Fe2+/ascorbate/EDTA-induced lipid peroxidation. *Biochim Biophys Acta* 1993;1166: 264–274.

Tjoelker LW, Eberhardt C, Unger J, Trong HL, Zimmerman GA, McIntyre TM, Stafforini DM, Prescott SM, Gray PW. Plasma platelet-activating factor acetylhydrolase is a secreted phospholipase A_2 with a catalytic triad. *J Biol Chem* 1995;270(43):25481–25487.

Tsaoussis V, Vakirtzi-Lemonias C. The mouse plasma PAF acetylhydrolase: I. Characterization and properties. *J Lipid Mediators Cell Signalling* 1994;9:301–315.

Yang YQ, Kudolo GB, Harper MJK. Binding of platelet-activating factor to oviductal membranes during early pregnancy in the rabbit. *J Lipid Mediators* 1992;5:77–96.

Zhu Y, Word RA, Johnston JM. The presence of platelet-activating factor binding sites in human myometrium and their role in uterine contraction. *Am J Obstet Gynecol* 1992;166(4):1222–1227.