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Partial Purification and Characterization of PAF Acetylhydrolase in Human Amniotic Fluid

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Platelet-activating factor (PAF) acetylhydrolase, which removes the acetyl moiety at the *sn*-2 position, has been found in human amniotic fluid. We purified this enzyme by ammonium sulfate precipitation, and sequential use of DEAE-Sepharose CL-6B, hydroxyapatite, chelating-Sepharose, and Mono Q column chromatographies. This enzyme exhibited broad pH optima and was unaffected by EDTA. Partially purified enzyme had a molecular weight of approximately 34 kDa on SDS-PAGE. In addition, the enzyme activity was inhibited by either diisopropylfluorophosphate(DFP) or *p*-bromophenacylbromide (*p*-BPB), suggesting that this enzyme possesses active serine and histidine residues. The enzyme showed similar activity towards PAF and oxidatively modified phosphatidylcholine, but didn't hydrolyze phosphatidylcholine or phosphatidylethanolamine with a long chain fatty acyl group at *sn*-2 position.

Key words: PAF-acetylhydrolase, Human amniotic fluid, Oxidized phospholipid

INTRODUCTION

Platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a polar phospholipid autacoid which is synthesized on stimulation of various cell types. PAF activates platelets, various inflammation associated cells and endothelial cells at concentrations as low as 10^{-10} M and increased hypotention, vascular permeability, platelet activation, constracted smooth muscle and occurred edema (Hanahan *et al.*, 1986; Snyder *et al.*, 1987; Braquet *et al.*, 1987). PAF has been identified in human plasma (Carmelo *et al.*, 1984), saliva (Cox *et al.*, 1981)and amniotic fluid (Billah *et al.*, 1983), which suggests that it has physiological and/or pathological functions. Its inactivation is catalyzed by a specific acetylhydolase, which removes the acetyl group at position of the glycerol backbone, to produce the biological inactive lyso-PAF and this enzyme has been widely distributed in mammalian blood, blood cells and tissues (Farr *et al.*, 1980; Blank *et al.*, 1981; Stafforini *et al.*, 1987). It has been reported that PAF acetylhydrolase is divided into intracellular and extracellular (plasma) form. The cytosolic enzyme may help regulate PAF production (e.g. in differentiated macrphages), whereas the plasma form is believed to regulate baseline circulating PAF levels and may be critical in resolving inflammation. PAF acetylhydrolase from human plasma has been purified and well characetrized (Stremler *et al.*, 1991). This enzyme has been an apparent molecular mass of 43 kDa, dose not require calcium ions, and is mainly associated with lipoproteins in plasma. Karasawa *et al.* purified a single polypeptide with molecular weight of 63 kDa from peritoneal fluid during endotoxin schock induced by *E. coli* and suggested that this enzyme might be plasma type (Karasawa *et al.*, 1994; Hattori *et al.*, 1993). Intracellular acetylhydrolase is a heterotrimeric complex composed of 45, 30, and 29 kDa subunits. The 45 kDa subunit, which is not essential for the catalytic activity, exhibits striking homology (99%) with a protein encoded by the causal gene (*LIS-1*) for Miller-Dicker lissencephaly, a human brain malformation manisfested by a smooth cerebral surface and abnormal neural migration (Hattori *et al.*, 1993).

PAF acetylhydrolase activity of rabbit plasma decreased for 15 days in the early pregnacy and the activity was very low before parturition but the activity was recovered after parturition. It was speculated that decrease of acetylhydrolase activity in plasma during pregnancy increase concentration of PAF in plasma and then make an easy action to conctaction of uterus (Satoh *et al.*, 1988). The concentration of PAF was increased in human amniotic membrane tissue after pains. Jonston *et al.* reported that when hormonal re-

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gulation of PAF acetylhydrolase levels in plasma of rats of the latter stage of pregnancy wasn't influenced by progesterone but affected by estrogen and dexametasone. These results showed that PAF acetylhydrolase played an important role in parturition (Katsuhiko *et al.*, 1992). PAF in amniotic fluid seemed to act on amniotic membrane, promote the production of prostaglandin (PGF_2) and be important to help parturition. In this study, we here reported the purification and characterization of PAF acetylhydrolase from human amniotic fluid.

MATERIALS AND METHODS

Preparation of enzyme sources

Amniotic fluid was obtained from nomal pregnant women by amniotomy or natural delivery from Patima hospital. Cells and debris were removed from amniotic fluid by centrifugation condition immediately at 4°C, and then stored at -20°C until used.

Materials

1-*O*-Hexadecyl-2-[^{3}H]acetyl-sn-glycero-3-phosphocholine ([^{3}H]acetyl-PAF) were purchased from Du Pont-NEW England Nuclear. DEAE-Sepharose CL-6B, chelating-Sepharose, and Mono Q HR5/5 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and hydroxyapatite was from Biochemical Industry Co., Ltd., (Japan). Bovine serum albumin, phenylmethylsulfonylfluoride, iodoacetamide, dithiothreitol, CHAPS, chymotrypsin were purchased from Sigma Chem. Co.. (USA)

Assay of PAF acetylhydrolase activity

The standard incubation system for the assaying of PAF acetylhydrolase comprised 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 pM of [^{3}H-acetyl] PAF, and the sample in a total volume of 250 μl. After 30 min at 37°C the reaction was stopped by adding 2.5 ml of chloroform/methanol (4:1) and 250 μl of water. Aliquots (600 μl) of the upper phase were used for radioactivity measurement to determine the amount of acetate liberated.

Preparation of labeled substrates

1-Acyl-2-[1-14C] linoleoyl-*sn*-glycero-3-phosphocholine was synthesized as discribed previously (Arai *et al.*, 1985)

Preparation of oxidized phosphatidylcholine

Oxidized phospholipid was prepared as described previously (Shimojo *et al.*, 1974). Briefly, phosphatidylcholine (4 μmole) in 1 ml of 90% acetate was mixed with 2 ml of an oxidation solution (24 mM KMnO 4, 20 mM $NaIO_4$), and then the mixture was stirred for 2 h at room temperature. The oxidized phosphatidylcholine was separated with a silica gel plate, and the band corresponding oxidezed phosphatidylcholine was scrapped off and extracted with chloroform.

Protein determination

Protein determination was measured with a Pierce protein assay kit (Kaushal *et al.*, 1991). Bovime serum albumin served as the protein standard.

Purification procedure

The cell-free supernatent was brought to 60% saturation by adding solid ammonium sulfate, stirred for 30 min, and then centrifuged at 10,000×g for 30 min. The precipitate was dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol buffer and this was used as enzyme sources and then dialyzed against the same buffer at 4°C for 24 h.

DEAE-Sepharose CL-6B column chromatography

The dialyzate was loaded onto an anionic exchange column DEAE-Sepharose CL-6B column previously equilibrated with 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol. The column was washed with the same buffer, and then the protein was eluted with a linear gradient of NaCl (0~1 M) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol buffer.

Hydroxyapatite column chromatography

The active fractions from DEAE-Sepharose CL-6B column were dialyzed against 10 mM KH_2PO_4 (pH 6.8), 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS and then was applied to hydroxyapatite column previously equilibrated with 10 mM KH_2PO_4 (pH 6.8), 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS. The column was washed with the same buffer, and then the protein was eluted with a linear gradient of KH_2PO_4 (pH 6.8) (10~800 mM) with 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS.

Cu^{2+}-chelating Sepharose column chromatography

The active fractions from hydroxyapatite column were dialyzed against 0.5 M NaCl, 20 mM Tris-HCl (pH 9), 10% glycerol, 0.1% CHAPS and was applied to a column containing chelateing-Sepharose (0.7×5 cm). The gel was immobilized with Cu^{2+} by the addition of 1.5 ml of $CuSO_4$ (5 mg/ml), and subsequently equilibrated with 0.5 M NaCl, 20 mM Tris-HCl (pH 9), 10% glycerol, 0.1% CHAPS. The column was washed with the same buffer.

Mono Q HPLC column chromatography

The active fractions from Cu^{2+}-chelating Sepharose column were pooled and dialyzed against 10 mM KH_2PO_4 (pH 6.8), 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS for 24 h. The dialyzate was loaded onto Mono Q HR 5/5 column equilibrated with the same buffer and the protein was eluted in with linear gradient of NaCl (0~1 M) in 10 mM KH_2PO_4, 10% glycerol. The chromatography was performed at the flow rate of 0.5 ml/min and 0.5 ml fractions were collected.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Laemmeli was followed. The sample was boiled with Laemmeli sampling buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue) at 100°C for 3 min. SDS-PAGE was carried out, using 12.5% acrylamide gel. The proteins were visualized by silver staining using a Silver Staining II Kit (Wako Pure Chemical Industries, Japan).

RESULTS

Purification of PAF acetylhydrolase

We found the PAF acetylhydrolase activity in the human amniotic fluid. Table I summurize the result of typical partial purification of PAF acetylhydrolase. The overall purification from the amniotic fluid was apporimately 240 fold. The specific activity of the final preparation against PAF was 72 pmol/min/mg. When this sample was applied to DEAE-Sepharose CL-6B column, the activity was eluted from the column with about 0.5 M NaCl (Fig. 1). Moreover, when the active fractions from DEAE-Sepharose column were applipied to hydroxyapatite column, enzyme activity was eluted from the column with about 300 mM KH_2PO_4 (Fig. 2). The active fractions from hydroxyapatite column were dialyzed against 0.5 M NaCl, 20 mM Tris-HCl (pH 9), 10% glycerol, 0.1% CHAPS and were applied to Cu^{2+}-chelating Sepharose column chromatograpy (Fig. 3.). The enzyme activity passed through the column. The active fractions from Cu^{2+}-chelating Sepharose column were pooled

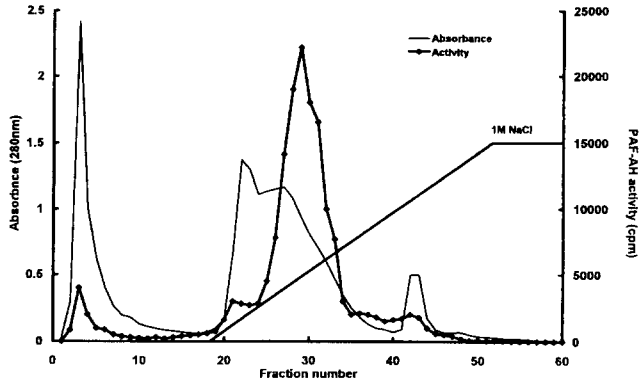

Fig. 1. Elution profile of PAF acetylhydrolase on DEAE-Sepharose CL-6B column. Crude amniotic fluid was applied to a DEAE-Sepharose CL-6B column previously equilibrated with 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol. The column was washed with the same buffer, and then the protein (—) was eluted with a linear gradient of NaCl (0~1 M) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol buffer. The PAF acetylhydrolase activity (◆) was eluted from the column with about 0.5 M NaCl. Protein (—) was measured as the absorbance at 280nm, and the enzyme activity (◆) was measured as described under "MATERIALS AND METHODS".

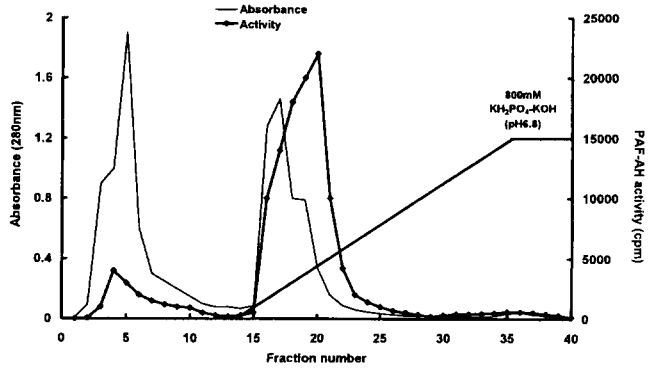

Fig. 2. Elution profile of PAF acetylhydrolase on hydroxyapatite column. The active fractions from DEAE-Sepharose CL-6B column were dialyzed against 10 mM KH_2PO_4 (pH 6.8), 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS and then the dialyzate was applied to hydroxyapatite column previously equilibrated with the same buffer. The column was washed with the same buffer, and then the protein (—) was eluted with a linear gradient of KH_2PO_4 (pH 6.8) (10~800 mM) with 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% CHAPS. The enzyme activity (◆) was eluted from the column with about 300 mM KH_2PO_4.

Table I. Summary of the purification of PAF acetylhydrolase from human amniotic fluid

Purification steps	Proein (mg)	Total activity (pmol/min)	Specific activity (pmol/min/mg)	Purification fold	Recovery (%)
Crude	1,120	343	0.31	1	100
60% $(NH_4)_2SO_4$	900	314	0.35	1.1	91.4
DEAE-Sepharose	180	294	1.63	5.3	85.6
Hydroxyapatite	2.82	129	45.6	149	37.5
Chelating-Sepharose	0.5	30	60	193	8.7
Mono Q HPLC	0.064	4.6	72	235	0.013

and dialyzed, the dialyzate was loaded on Mono Q anion HPLC column. The activity was detected in fractions containing 0.5 M NaCl (Fig. 4.). Although we failed to purify the acetylhydrolase in human amniotic fluid to near homogeneity overall purification procedure. In this step, PAF acetylhydrolase was purifed about 240 fold, and the recovery was 0.013%, the specific acitivity of final preparation was 72 pmol/min/mg (Table I). From the SDS-PAGE pattern of the preparation of at final step, the molecular weights of this enzyme was approximately 34 kDa (Fig. 5).

Substrate specificity

The ability of the crude PAF acetylhydrolase to attack phospholipids other than PAF was investigated (Fig. 6). The enzyme had little or no activity against phosphatidylcholine or phosphatidylethanolamine with two long acyl chains. In contrast, PAF acetylhdyrolase could hydrolyze phosphatidylcholine that had oxidatively fragmented acyl chain at the sn-2 position was approximately 40% as efficient a substrate as PAF. This result clearly demonstrate that the acetylhydrolase is distinct from phospholipase A_2, which utilize long chain diacyl phospholipids as substrate.

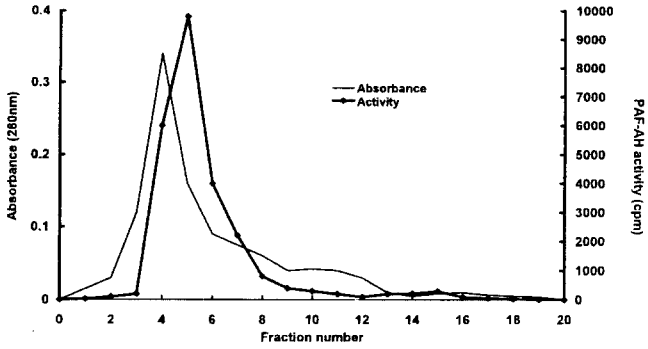

Fig. 3. Elution profile of PAF acetylhydrolase on Cu^{2+}-chelating sepharose column. The active fractions from hydroxyapatite column were dialyzed against 0.5 M NaCl, 20 mM Tris-HCl (pH 9), 10% glycerol, 0.1% CHAPS and the dialyzate was applied to Cu^{2+}-chelating Sepharose column chromatograpy. The enzyme activity (◆) passed through the column.

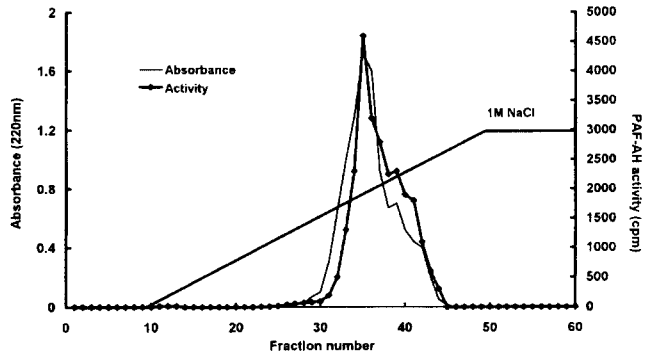

Fig. 4. Elution profile of PAF acetylhydrolase on Mono Q HPLC column. The active fractions from Cu^{2+}-chelating Sepharose column were pooled and dialyzed, the dialyzate was loaded on Mono Q anion HPLC column. The activity (◆) was eluted in fractions containing 0.5 M NaCl.

Effect of pH

Partially purified PAF acetylhydrolase from human amniotic fluid was incubated from pH 4 to 11 at 37°C

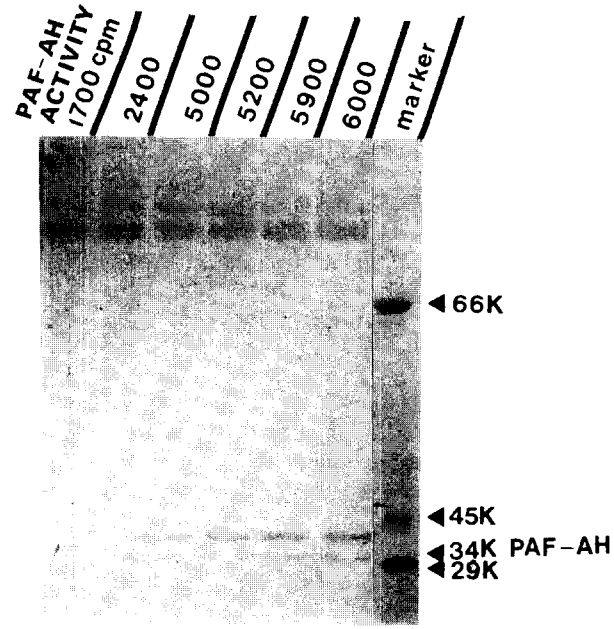

Fig. 5. SDS-PAGE. SDS-PAGE was carried out as described under "MATERIALS AND METHOD". The samples are the active fractions from Mono Q HPLC column. Molecular weight standards: bovine serum albumin (66 K), ovalbumin (45 K), carbonic anhydrase (29 K).

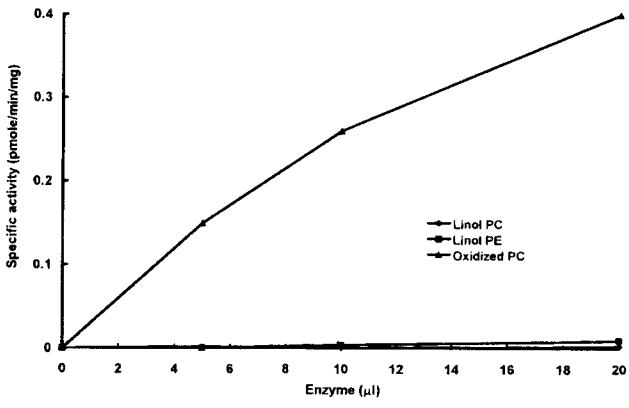

Fig. 6. Hydrolyzing activities of PAF acetylhydrolase in amniotic fluid toward various phospholipids. The crude enzyme (20 μl) was incubated with radio-labeled substrate. The enzyme had little or no activity against phosphatidylcholine (◆) or phosphatidylethanolamine (■) with two long acyl chains. In contrast, PAF acetylhdyrolase could hydrolyze artificially oxidized phosphatidylcholine (▲) was approximately 40% as efficient a substrate as PAF.

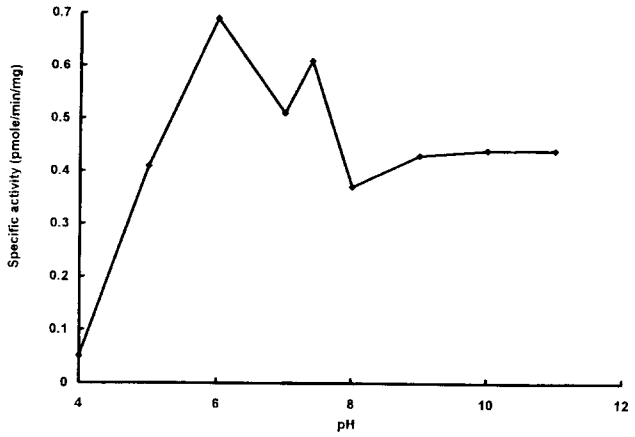

Fig. 7. Effect of pH on PAF acetylhydrolase. The partially purifed enzyme (20 μl) and 20 nmole of [^{3}H-acetyl]PAF were incubated for 30 min at 37°C in the presence of 5 mM EDTA. The buffers used were 100 mM sodium acetate-acetic acid range from pH 4 to 5, 100 mM Tris-maleic acid buffer from 5 to 6, 100 mM Tris-HCl buffer pH 6 to 9, 100 mM glycine-NaOH buffer from pH 9 to 11.

(Fig. 7.). PAF acetylhdyrolase exhibited pH optima in the weak acid to alkaline region. At values below pH 4 the enzyme was inactivated but at higher pH values, up to 11, the enzyme activity still remained. It has been reported that the opti

Effect of various chemical reagent

Various compounds were tested for their effects on the activity of the partially purified enzyme (Table II). Both a cofactor of phospholipase A_2, Ca^{2+}, and a chelating agent, EDTA had no effect upon the enzyme activity. *p*-Bromophenacylbromide (*p*-BPB), which has been shown to block various phospholipase A_2 activities by derivatizing the histidine residue(s) at their active sites, inhibited the activity of the enzyme about 30% at 5 mM. This result suggest that amniotic fluid PAF acetylhydolase might be different from plasma type PAF acetylhydrolase. Phenylmethylsulfonylfluoride (PMSF) and diisopropyl fluorophosphate (DFP), which have been shown to block by derivatizing the serine residue(s) at their active sites, inhibited the activity of the enzyme about 40%, 95% at 5 mM, respectively. On the other hand, NaF, acting as phosphatase inhibitor, and dithiothreitol, hydrolyzing disulfide bond and changing conformation of protein, did not affect enzyme activity. Idoacetamide, an inhibitor of enzymes that need free sulfydryl groups for their activities, could not inhibit the activity of enzyme. It these results suggested that activie site of PAF acetylhydrolase in human amniotic fluid might be serine residue(s) and histidine residue(s).

DISCUSSION

Tabel II. The effects of various chemical reagents on PAF acetylhydrolase

Chemical reagents (final concentration 5 mM)	Inhibition %
EDTA	00
$CaCl_2$	00
p-BPB	30
DFP	95
PMSF	60
NaF	00
Iodoacetamide	10
DTT	00

Each compound was incubated with the partially purifed acetylhydrolase for 15 min at 37°C and then the enzyme reactions was started by adding the substrate.

In this report, we provide the first information on the physical properties of human amniotic fluid PAF acetylhydrolase. We partially purified PAF acetylhydrolase from human amniotic fluid by sequential use of DEAE-Sepharose CL-6B, hydroxyapatite, chelating-Sepharose and Mono Q HPLC column chromatographies. At final step, PAF acetylhydrolase was purified about 240 fold and its specific activity was 72 pmol/min/mg. PAF acetylhydrolase in human amniotic fluid hydrolyzed the acetyl ester of PAF but not the long chain fatty acyl ester of phospholipids. We identified a monomeric polypeptide with a molecular weight of approximately 34 kDa on 12.5% SDS-PAGE, which differs from that of other known acetylhydrolases. Stafforini *et al.* purified human plasma PAF acetylhydrolase and determined its molecular weight to be 43 kDa (Stafforini *et al.*, 1987) and they also purified human erythrocyte acetylhydrolase with molecular weight of 25 kDa (Stafforini *et al.*, 1993). Karasawa *et al.* purified acetylhydrolase of guinea pig peritoneal fluid with molecular weight of 63 kDa (Karasawa *et al.*, 1994). Hattori *et al.* reported the purification of PAF acetylhydrolase from bovine brain cytosol, and demonstrated that the native enzyme consisted of subunits with molecular weights of 45, 30 and 29 kDa (Hattori *et al.*, 1993). These purified enzymes had similar biochemical properties notwithstanding the difference in molecular weight. This partial purified enzyme was Ca^{2+}-independent and did not hydrolyze the ester bond of phospholipids with a long chain fatty acyl group at the *sn*-2 position. First, this partially purified enzyme is distinguished with phospholipase A_2, which require a divalent cation, usually Ca^{2+}, and which shows broad range for fatty acids esterified at the sn-2 position. Second, this enzyme was inhibited by PMSF, as other purified acetylhydrolase, indicating that the active center of this enzyme is a serine residue.

PAF acetylhydrolase can be grouped into two, plasma and intracellular type. Blank *et al.* reported that

plasma type had a higher molecular weight than intracellular type and was resistant to protease, whereas the intracellular acitivity was sensitive (Blank *et al.*, 1983). PAF acetylhydrolase purified from guinea pig peritoneal fluid after *E. coli* LPS administration was resistant to *p*-BPB, NaF and trypsin, like human plsama acetylhydrolase (Karasawa *et al.*, 1994). PAF acetylhydrolase purified from bovine brain cytosol, including intracelullar type, was inhibited by p-BPB and DFP but was not inhibited by iodoacetamide and it was essential for the optimal activity to need reducing agent (Mitsuharu *et al.*, 1993). PAF acetylhydrolase purified from human amniotic fluid was relatively sensitive to *p*-BPB and the origin of this enzyme might be different from plasma type. PAF-acetylhydrolase from human plasma was associated with lipoprotein and was not affected by protease, but the activity of this enzyme from human amniotic fluid was decreased by chymotrypsin. In the presence of 0.4% chymotrypsin, partially purified amniotic fluid enzyme activity was decreased about 80%, but plasma enzyme activity was decreased about 10% (Data not shown).

It should be mentioned here that like several PAF acetylhydrolases, acetylhydrolase from human amniotic fluid can hydrolyze oxidatively modified phospholipid. Oxidative degradation of phospholipids results in the formation of biologically active products (Itabe *et al.*, 1988), and these phospholipids are involved in some pathological states including disorganization of membrane structure. PAF acetylhydrolase in human amniotic fluid dose-dependently hydrolyzed artificially oxidized phosphatidylcholine as a substrate. It is suggested that PAF acetylhdyrolase could hydrolyze the oxidative lipids of biomembrane, lyso derivatives with long chain fatty acyl group were reacylated and then the properties of biomembrane could be recovered. Recently, McIntyre et al. demonstrated that the PAF-like bioactivity of a certain type of oxidatively fragmented phosphatidylcholine, suggesting a proinflammatory action of these lipids. It has generally been assumed that (per)oxidized fatty acyl chains in phosphoglyceride may be preferentially hydrolyzed in vivo. Phospholipase A_2 has been regarded as a good candidate for their hydrolysis, since in parallel with phospholipid degradation, the accumulation of lysophospholipids was often observed after the oxidative tissue damage (Bazan *et al.*, 1970; Chien *et al.*, 1978; Edgar *et al.*, 1982; Glende *et al.*, 1986). However, the biochemical properties of the enzyme(s) that scavenge (per)oxidized phospholipids remain totally unknown. Intracellular PAF acetylhydrolase, which is constitutively active and selectively hydrolyzes oxidized phospholipids, may participated in the removal of such toxic phospholipids. The function of PAF acetylhydrolase in the amniotic fluid remains unknown. To understand the physiological role of PAF acetylhydrolase during the labor, futher studies must be performed to clarify the origin and regulation of PAF acetylhydrolase.

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