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Identification of a Major PAF Acetylhydrolase in Human Serum/Plasma as a 43 kDa Glycoprotein Containing About 9 kDa Asparagine-Conjugated Sugar Chain(s)[1]

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Platelet-activating factor (PAF) acetylhydrolase from human serum/plasma was identified on a polyvinylidene difluoride (PVDF) membrane by electroblotting proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme activity was detected in the 43 kDa region on the membrane as a decrease in the β-radioluminescence of [^{3}H]acetyl-PAF or by the convenient method for determining PAF acetylhydrolase activity (the TCA precipitation method). The enzyme activity on treatment with *N*-glycosidase F shifted to the 34 kDa region on the PVDF membrane. On the other hand, only one band was observed, corresponding to a molecular mass of 53 kDa, on analysis by SDS-PAGE with silver staining. Treatment of the 53 kDa protein with *N*-glycosidase F changed its molecular mass to 43 kDa (protein A). The NH_2-terminal 32 amino acid sequence of protein A completely corresponds to that of the heterogenous enzyme with 54 amino acids deleted from the NH_2 terminus reported by Tjoelker *et al.* (*Nature* 374, 549-553, 1995). Even after trypsin treatment of the *N*-glycosidase F-digested enzyme, its PAF-AH activity remained in the 34 kDa region, but the contaminating protein A disappeared, on the PVDF membrane. In addition, the majority of serum PAF-AH was retained on a *Sambucus sieboldiana* agglutinin (SSA)-agarose column and was eluted with the hapten sugar, lactose. These results indicate that PAF acetylhydrolase consisting of a 34 kDa protein and about 9 kDa asparagine-conjugated sugar chain(s) is a major enzyme in human serum/plasma.

Key words: glycoprotein, human serum/plasma, platelet-activating factor (PAF), platelet-activating factor acetylhydrolase.

Platelet-activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) acetylhydrolase (PAF-AH) catalyzes the hydrolysis of the acetyl ester bound at the *sn*-2 position of PAF to yield biologically inactive lyso-PAF. A family of individually distinct PAF-AHs exists. The latter are classified as extracellular and intracellular PAF-AHs, and furthermore membrane-bound and cytosol PAF-AHs (*1, 2*). Serum/plasma PAF-AH is considered to be indispensable for control of the level of circulating PAF, which exhibits a wide spectrum of biological activity at very low concentrations (in the range of 10^{-10} to 10^{-11} M) in diverse pathophysiological processes. For example, since the probability of the occurrence of PAF-AH deficiency is significantly higher in certain groups, such as severely asthmatic children (11.4%) and individuals with hornet-sting anamnesis (13%), than in healthy Japanese adults (3.8%), an abnormality in the level of blood PAF due to a deficiency or low activity of serum PAF-AH appears to cause more severe symptoms (*3*).

Tjoelker *et al.* have purified PAF-AH from human plasma low density lipoprotein by sequential column chromatography on DEAE-Sepharose Fast Flow, Blue Sepharose Fast Flow, and Cu^{2+}-charged chelating Sepharose, and cloned the plasma PAF-AH cDNA using a plasmid complementary DNA library derived from messenger RNA of monocyte-derived macrophages, and the combination of the polymerase chain reaction (PCR) and hybridization for the NH_2-terminal sequence (IQVLMAAASFGQTKIP) of the purified protein (M_r 44k) (*4*). The NH_2-terminal sequence of the purified protein has been shown to be heterogenous with an NH_2-terminal deletion (*5*). The recombinant 45 kDa protein hydrolyzes PAF, and the properties of the enzyme, such as its calcium-independence, and the putative active serine in the GXSXG motif characteristic of lipases and esterases, distinguish it from those of other known phospholipase A_2, but its substrate specificity, except for PAF and related phospholipids (*e.g.*, lipase activity), has not yet been reported (*4, 5*). The majority of mammalian lipases (colipase-dependent pan-

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creatic lipase, hepatic lipase, lipoprotein lipase, lysosomal acid lipase, and pancreatic- and milk-bile salt-stimulated lipase) are glycoproteins (*6-11*). In this study, we found that a major PAF-AH in human serum/plasma is also a glycoprotein(s), which is different from the enzyme purified by Tjoelker *et al.* (*4*).

The results of purification of PAF acetylhydrolase from human serum are shown in Table I. Serum PAF-AH was purified essentially by the method of Stafforini *et al.* (*12*), and PAF-AH activity was determined by the TCA precipitation method (*3*). Low density lipoprotein and very low density lipoprotein were precipitated from human serum, obtained from healthy donors, by treatment with sodium phosphotungstate and $MgCl_2$ by the method of Burstein *et al.* (*13*). The precipitate was dissolved in 10% citrate and then dialyzed against 10 mM potassium phosphate buffer (pH 6.8). The low density lipoprotein preparation was suspended in the same volume of the initially used serum in 10 mM potassium phosphate buffer (pH 6.8) containing 0.1% Tween 20, 10 μM butylated hydroxytoluene (BHT), and 0.02% NaN_3, and then stirred gently for 2 h. Then PAF-AH was highly purified by sequential column chromatographies on Q-Sepharose FF, Q-Sepharose FF again, Mono-Q HR, Superdex 200 pg, concanavalin A (Con A)-agarose, and *Sambucus sieboldiana* agglutinin (SSA)-agarose. Through these procedures, the enzyme was purified about 400,000-fold in a 5% yield (Table I), and only a single protein band was observed, corresponding to a molecular mass of 53 kDa, on silver staining after SDS-PAGE (Fig. 1A, lane 2). The enzyme activity of PAF-AH blotted onto a PVDF membrane was detected as a decrease in the radioactivity of membrane-bound [^{3}H]acetyl-PAF resulting from the hydrolysis by PAF-AH, as previously reported (*14*). Treatment of the enzyme preparation with 0.05% SDS for 1 min at 50°C for SDS-PAGE did not reduce the PAF-AH activity; the remaining activity was 97.3±4.1%. Surprisingly, the highest activity was near the 43 kDa region (Fig. 1A, lane 3). Next, we examined the influence of *N*-glycosidase F (which removes *N*-linked sugar moieties) on the molecular size of the PAF-AH protein. The resulting molecular mass of serum PAF-AH was 34 kDa (Fig. 1B, lane 4), and that of the contaminating protein was 43 kDa (Fig. 1B, lane 2). In addition, when the blotted PVDF membrane was cut at 2-mm intervals with a razor blade and the PAF-AH activity in each section was determined by the TCA precipitation method (*14*), high enzyme activity was

TABLE I. **Partial purification of PAF-AH from human serum.** PAF-AH activity was measured by the TCA precipitation method using 1-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine as a substrate (*1*).

Procedure applied	Protein (mg)	PAF-AH activity [nmol/min (U)]	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Serum (1,000 ml)	88,580	54,440	0.61	1	100
LDL particles	760	37,400	49	79	68.7
Solubilized LDL particles	760	30,600	40	65	56.2
Q-Sepharose	230	28,560	125	203	52.5
Re-Q-Sepharose	50.3	29,940	595	968	55.0
Mono-Q HR	6.6	14,960	2,270	3,690	27.5
Superdex 200 pg	0.4	7,940	20,380	33,160	14.6
ConA-agarose	0.2	5,720	40,670	66,170	10.5
SSA-agarose	0.01	2,560	240,000	400,000	4.7

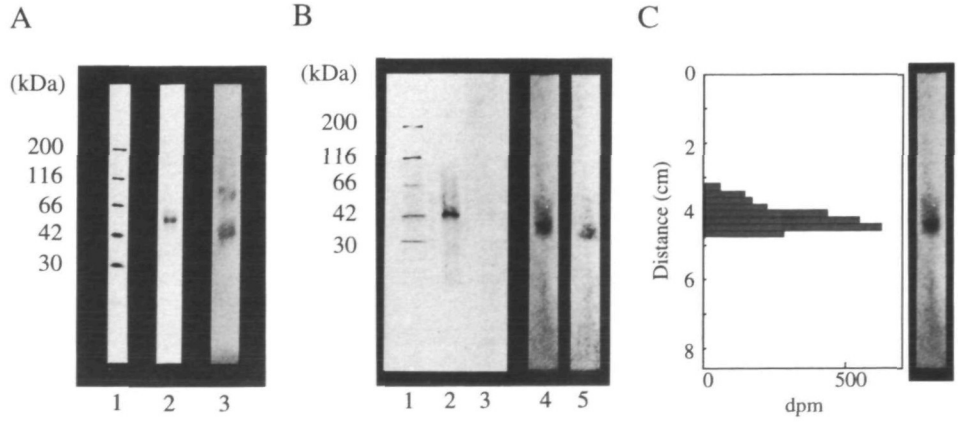

Fig. 1. **Detection of PAF-AH activity on a PVDF membrane.** (A) PAF-AH activity of the enzyme highly purified by SSA-agarose column chromatography. Lanes 1 and 2 show SDS-PAGE analysis on a 2-15% acrylamide gradient gel, which was stained with silver. Lane 1 shows molecular mass markers and lane 2 the highly purified fraction eluted from a SSA-agarose column. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane (lane 3). Then the PVDF membrane was immersed in 5×10^{-6} M [^{3}H]acetyl-PAF at 0°C for 3 h. After washing with 0.1 M Tris/HCl buffer (pH 7.5), the membrane was incubated at 37°C for 20 h. The radioactivity of [^{3}H]acetyl-PAF, which was measured as the intensity on photocounting using an ultrahigh-sensitivity TV camera system, decreased in the area of the membrane where the enzyme reaction occurred, since the [^{3}H]acetate released was removed from the PVDF membrane by washing with the buffer. (B) Effects of *N*-glycosidase F and trypsin on serum PAF-AH activity. The active fractions (30 U) from an SSA-agarose column were incubated with *N*-glycosidase F from *Flavobacterium minigoseptcum* (2.1 U) in buffer A for 74 h at 37°C, and then further incubated without (lanes 2 and 4) and with (lanes 3 and 5) TPCK-trypsin (160 U) for 17 h at 37°C. The samples (10 U) were separated by SDS-PAGE on a 2-15% acrylamide gradient gel and then electroblotted onto a PVDF membrane. Lanes 1, 2, and 3 were stained with colloidal gold. Lane 1 shows molecular mass markers. Lanes 4 and 5 show the PAF-AH activity. (C) Detection of PAF-AH activity by the TCA precipitation method. A blotted PVDF membrane (Fig. 1B, lane 4) was cut at intervals of 2 mm. The blotted proteins in each section were incubated with 100 μl of 5×10^{-9} M [^{3}H]acetyl-PAF suspended in 100 mM Tris/HCl- buffered saline (pH 7.5) containing 0.1% BSA and 2 mM EDTA for 20 h, and then the PAF-AH activity was determined by the TCA precipitation method.

detected at 4.0 to 4.8 cm from the origin. The PAF-AH activity determined by the TCA precipitation method (Fig. 1C) coincided with that detected by the current method (Fig. 1B, lane 4). Even when we purified PAF-AH from plasma, we obtained the same result; the molecular mass of plasma PAF-AH was near 43 kDa (40–50 kDa) before *N*-glycosidase F digestion and 34 kDa after *N*-glycosidase F digestion (data not shown). The fact that the 34 kDa protein was serum/plasma PAF-AH is in contrast with the previous observation that the molecular mass of so-called plasma PAF-AH containing no sugar chain was 44 kDa (*4*).

Unexpectedly, a major contaminating 43 kDa protein devoid of *N*-linked sugar chains co-migrated with the PAF-AH activity on a Superdex 75 column equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 0.15 M NaCl, 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-propane sulfonic acid (CHAPS), and 0.02% NaN_3. Additionally, the behavior of the contaminating 53 kDa protein containing *N*-linked sugar chains on Q-Sepharose, Mono-Q, and immobilized-lectin columns seems to be similar to that of serum PAF-AH. In order to determine whether or not the contaminating 43 kDa protein is an NH_2-terminal amino acid-deleted 45 kDa protein [so-called plasma PAF-AH reported by Tjoelker *et al.* (*4*)], the band of the contaminating 43 kDa protein on a PVDF membrane stained with Coomassie Brilliant Blue was excised and the NH_2-terminal sequences were determined by automated Edman degradation. Two types of NH_2-terminal sequences, A and B, were present in a 1:1 mixture. Surprisingly, except for the fact that the NH_2-terminal amino acid in sequence (A) was alanine instead of serine, 34 and 32 amino acids in NH_2 terminal sequences (A) and (B) completely corresponded to the amino acid sequences comprising Phe-51 and Tyr-84 and Lys-55 and Pro-86 of the so-called plasma PAF-AH reported by Tjoelker *et al.* (*4*), respectively. Furthermore, in order to confirm the possibility that the 34 kDa protein resulted from deletion of amino acids from the NH_2-terminus and COOH-terminus of the so-called plasma PAF-AH reported by Tjoelker *et al.* (*4*), we examined the influence of proteases on the PAF-AH activity and the molecular mass. The highly purified serum PAF-AH (30 U), which was bound to the SSA-agarose column and eluted with lactose, was treated with and without *N*-glycosidase F (2.1 U) for 74 h at 37°C and then incubated with *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) at the concentration of 2 mg/ml for 30 min at 37°C. The PAF-AH activity of the enzyme preparation treated with and without *N*-glycosidase F was not affected by trypsin (103 and 102% of the control, respectively). In addition, even after incubation with proteinase K at the concentration of 2 mg/ml for 30 min at 37°C, the remaining PAF-AH activity of the enzyme preparation treated with and without *N*-glycosidase F was 91 and 86% of the control level, respectively. These observations coincided with the fact that plasma PAF-AH is resistant to trypsin, as reported by Stafforini *et al.* (*15, 16*). Interestingly, as shown in Fig. 1B, the PAF-AH activity after treatment with *N*-glycosidase F and TPCK-trypsin (lane 5) was detected in the 34 kDa region on a PVDF membrane, as after treatment with *N*-glycosidase F and without TPCK-trypsin (lane 4). On the other hand, the 43 kDa protein was not detected after digestion with TPCK-trypsin (Fig. 1B, lanes 2 and 3). These results indicate that the 34 kDa protein does not result from deletion of amino acids from the NH_2-terminus and COOH-terminus of the contaminating 43 kDa protein, that is, the possibility that the so-called PAF-AH reported by Tjoelker *et al.* (*4*) is a major enzyme in human plasma/serum is ruled out.

As the isoelectric point of serum PAF-AH, a value of pH 5.5 was assigned from the isoelectric focusing pattern on Ultrodex. Treatment of the active fraction from Superdex 200 pg with *N*-glycosidase F shifted the isoelectric point to pH 6.0. We confirmed that serum PAF-AH is a glycoprotein by examining its binding affinity to immobilized-lectin columns. The active fractions from a Superdex 200 pg column was applied to a Con A-agarose column (10×10 mm), which was equilibrated with 10 mM phosphate buffer (pH 6.8) containing 0.15 M NaCl, 0.01% Tween 20, 1 μM BHT and 0.02% NaN_3 (buffer A). Contrary to in the case of guinea pig plasma PAF-AH (*17*), most of the enzyme activity (90%) was not adsorbed to this column. The flow-through fraction was then applied to a column of SSA-agarose (10×24 mm), which was equilibrated with buffer A. Seventy percent of the enzyme activity was retained on the SSA column, and eluted with a linear gradient of lactose. The activity was eluted with about 50 mM lactose. When this eluate (230 U) from the SSA column was incubated with sialidase (30 mU) at 37°C for 40 h, substantially all the enzyme activity passed through the SSA column. Typical profiles of elution of the enzyme activity from the SSA column are shown in Fig. 2, A and B. Sixty percent of the PAF-AH activity in the active fractions from Superdex 200 pg was adsorbed to a *Ricinus communis* agglutinin (RCA60)-agarose column equilibrated with buffer A, but after treatment with *N*-glycosidase F, the enzyme activity was not adsorbed to this column. Fifty percent of the PAF-AH activity bound to the RCA60-agarose column was retained on a *Datura stramonium* agglutinin (DSA)-agarose column equilibrated with buffer A. The enzyme activity was eluted with the hapten sugar of each lectin. These observations indicate that the major serum/plasma PAF-AH is a glycoprotein(s) containing heterogenous asparagine-conjugated sugar chain(s) involving sialic acid. Although the *N*-linked carbohydrates of PAF-AH from pooled human serum/plasma were of the complex type and heterogenous, we obtained similar results using human plasma from one subject, indicating that differences in glycosylation may reflect plasma PAF-AH glycoforms (*18*).

There is the question of whether or not the *N*-linked sugar chains of serum/plasma PAF-AH contribute to the enzyme action. Treatment with *N*-glycosidase F did not affect the enzyme activity or the apparent K_m value calculated from a Lineweaver-Burk double reciprocal plot; the apparent K_m value of serum/plasma PAF-AH with *N*-glycosidase F digestion was the same as that of the native enzyme (0.17 mM). Accordingly, a strict carbohydrate structure seems not to be required for serum/plasma PAF-AH activity, whereas the asparagine-conjugated sugar chains of lysosomal acid lipase were essential for its enzyme activity (*11*). On the other hand, although the human transferrin receptor lacking one of its three *N*-linked sugar chains was reported to be easily degraded by proteases in the endoplasmic reticulum (*19*), removal of the *N*-linked sugar chains did not affect its resistance to proteases such as trypsin and proteinase K (Fig. 1B). This

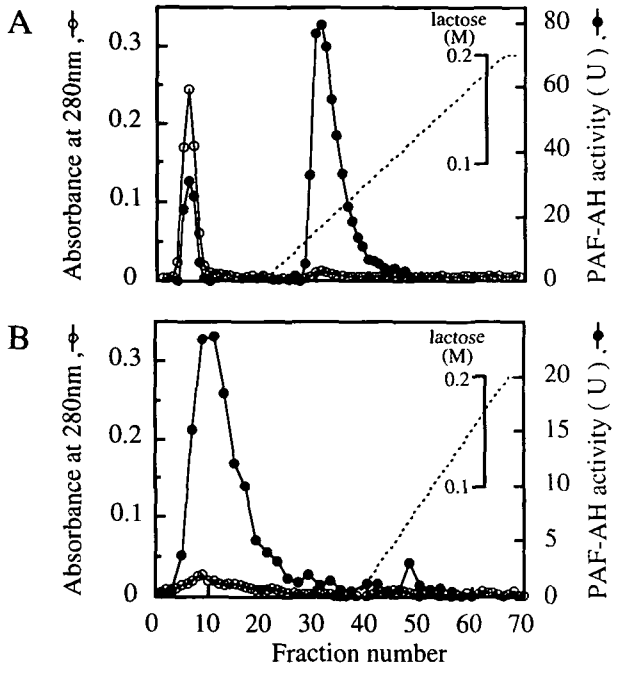

Fig. 2. **Elution profiles of PAF-AH on an SSA-agarose column.** (A) The flow-through fraction from a Con A column (1,700 U) was applied to an SSA-agarose column (10×24 mm) equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 0.15 M NaCl, 0.01% Tween 20, 1 μM BHT, and 0.02% NaN_3 (buffer A). The adsorbed proteins were subsequently eluted with a 25 ml linear gradient of lactose (0 to 0.2 M). Fractions (0.5 ml) were collected. Each fraction was subjected to PAF-AH activity determination. The open circles (○) represent the absorbance at 280 nm and the closed circles (●) PAF-AH activity. (B) The active fraction from the SSA-agarose column (230 U) was dialyzed against buffer A and then incubated with sialidase (30 mU) from *Arthrobacter ureafaciens* at 37°C for 40 h. Then the sample was applied to an SSA-agarose column (10×10 mm). Fractions (0.5 ml) were collected and examined for PAF-AH activity. The open circles (○) represent the absorbance at 280 nm and the closed circles (●) PAF-AH activity.

study showed (1) that the major serum/plasma PAF-AH is a glycoprotein(s) containing about 21% carbohydrates, while little is known about the functional role of the sugar chains, and (2) that the present enzyme activity is powerful enough to hydrolyze PAF, whereas the enzyme was only faintly detected with silver (Fig. 1A, lanes 2 and 3) and colloidal gold (Fig. 1B, lanes 2 and 4). The present serum/plasma PAF-AH may contribute to the degradation of PAF in the blood, which exhibits a wide spectrum of biological activity at very low concentrations in the range of 10^{-10} to 10^{-11} M. In order to elucidate the mechanism of serum/plasma PAF-AH deficiency, we are in the progress of purifying the major serum/plasma PAF-AH and of cloning its cDNA.

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