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PLATELET ACTIVATING FACTOR AND LYSO-PHOSPHATIDYLCHOLINES FROM STRAWBERRY

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Key Word Index—*Fragaria ananassa*; Rosaceae; strawberry; phospholipids; ether lipids; platelet activating factor (PAF); lyso-PAF.

Abstract—Lipids from strawberry fruits, leaves, achenes and pollen were separated into classes by TLC, purified by HPLC and tested for biological activity. A lipid fraction from fruits with the same chromatographic behaviour as authentic platelet activating factor (PAF) showed identical biological activity, namely, dose-dependent aggregation of washed rabbit platelets, inhibition of aggregation by CV 3988, platelet desensitization to PAF and vice versa, and loss of activity by alkaline hydrolysis and recovery of activity by reacetylation. The presence of PAF was confirmed by FAB mass spectrometry. Lyso-phosphatidylcholines, including lyso-PAF, were also found in all the plant parts tested.

INTRODUCTION

Platelet activating factor (PAF) with the chemical structure of a 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine was initially described as a product of IgE-sensitized basophils and as a factor that activates blood platelets. It is well established now that PAF is a potent biological mediator, synthesized by a variety of animal cells [1–3]. Besides the initially recognized ability to cause platelet activation, PAF was subsequently shown to induce aggregation, degranulation and chemotaxis of neutrophils, bronchoconstriction, increased vascular permeabiltiy, hypotension, decreased cardiac output and altered liver metabolism [4–6]. It appears to function both in normal and pathological events, mediating responses particularly in inflammation and allergy [2]. The presence of PAF in microorganisms has been reported [7,8], together with the finding that cell suspension cultures of different plants are capable of incorporating exogenous alkylglycerols into PAF and other biologically active ether lipids [9]. A PAF-like compound has also been isolated from thylakoids of pea leaves [10], but the presence of PAF in plants in general is not well established as yet.

The presence of PAF in plants causing an allergenic action has not been thoroughly investigated. Strawberry is well known to be responsible for allergic symptoms, but there is no valid clinical evidence for possible allergenic constituents. In this paper, we report the isolation from strawberry fruits of a phospholipid identified as PAF, together with the isolation of lyso-phosphatidylcholines (lyso-PCs) from all parts of the strawberry plant.

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RESULTS AND DISCUSSION

Lipid extraction from strawberry fruits yielded 0.1–0.3% total lipids based on wet fruit weight. The variation observed was due to the heterogeneity of the fruit population regarding maturity and size.

Total lipids were separated by preparative TLC into eight fractions (PL-1–PL-8) and all fractions were tested for their ability to induce platelet aggregation or inhibit platelet aggregation induced by 2.5×10^{-10}M PAF, using washed rabbit platelets (Table 1). Lipid fractions PL-1, PL-2 and PL-4 to PL-8 inhibited platelet aggregation, but the inhibitory effect was not further investigated. Glycosylglycerides were present in some fractions (e.g. digalactosyl diacylglycerols in fraction PL-7). Only fraction PL-3, with an R_f value corresponding to authentic PAF, induced dose-dependent platelet aggregation (Fig. 1). Platelet aggregation was similar to that of PAF, that is, being rapid, reversible at low doses and irreversible at higher ones. Results were essentially the same when platelets were preincubated with creatine phosphate–creatine phosphate kinase (data not shown). Fraction PL-3 was then subjected to HPLC for further purification. The HPLC fraction with an R_t corresponding to authentic PAF and eluting between standard sphingomyelin and lyso-PAF (data not shown) was collected and tested on washed rabbit platelets with essentially the

Table 1. Biological activity of strawberry fruit lipids on washed rabbit platelets

Fractions	(%) Aggregation†	(%) Inhibition‡
PL-1	—	1.1
PL-2	—	54.3
PL-3*	114.3	100
PL-4	—	—
PL-5	—	22
PL-6	—	100
PL-7	—	94.2

The results shown are the means of five or six experiments.

*Fraction PL-3 dissolved in 600 μl EtOH; all other fractions were dissolved in 200 μl EtOH.

†Aggregation calculated as a percentage of aggregation induced by 2.5×10^{-10}M PAF.

‡Inhibition calculated as % decrease of aggregation induced by 2.5×10^{-10}M PAF.

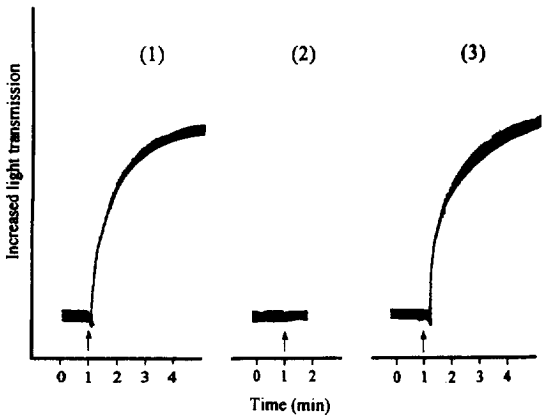

Fig. 2. Effect of alkaline hydrolysis and reacetylation on activity of fraction PL-3. Curves represent platelet aggregation induced (1) before hydrolysis, (2) after alkaline hydrolysis and (3) after reacetylation (arrows indicate time of addition of samples).

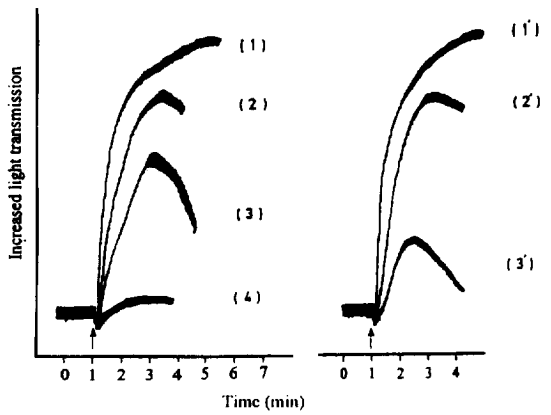

Fig. 1. Dose-dependent platelet aggregation induced by TLC fraction PL-3 in comparison with standard PAF. Curve (1) represents aggregation induced by PL-3 dissolved in 600 μl EtOH (initial concentration). Curves (2)–(4) represent dilutions of 1:10, 1:50 and 1:100 of the initial concentration, respectively: (1) 1×10^{-9}M PAF, (2) 2.5×10^{-10}M PAF and (3) 5×10^{-11}M PAF (arrows indicate time of addition of samples).

same results (Fig. 1). Sphingomyelin was used as a standard (even though it is not present in plants) for the exact location of the PAF band. Furthermore, CV 3988, a specific PAF inhibitor, caused inhibition of PL-3 induced platelet aggregation, indicating that PL-3 activated platelets through the same receptors as PAF.

PL-3, subjected to base treatment, lost its biological activity [Figs 2(1) and (2)], but, after reacetylation, activity was restored [Fig. 2(3)], suggesting the presence of an active acetyl group in the compound.

In desensitization studies, when platelets were preincubated with PAF 1×10^{-6}M in Ca^{2+}-free buffer, they were not aggregated by PL-3 and vice versa, platelets treated with PL-3 were not aggregated by PAF. These cross-desensitization tests confirmed that PL-3 and authentic PAF have the same receptors on rabbit platelets.

It was thus evident that PL-3 was a PAF-like compound sharing common characteristics with PAF: (a) R_f on TLC and R_t by HPLC; (b) both caused dose-dependent platelet aggregation, which was inhibited by CV-3988; (c) both were inactivated after alkaline hydrolysis and the activity was recovered after reacetylation; and (d) platelets desensitized to PAF were not aggregated by this fraction and vice versa. These characteristics are commonly used for PAF identification [11, 12].

Finally, FAB mass spectral data were consistent with the presence of a small amount of 16:0 PAF and 18: 0 PAF along with larger amounts of lyso-PC. Except for the peak at *m/z* 184 (typical of phosphocholine-containing molecules), representing the polar head group, there were also peaks representative for PAF 16:0 ($[M - H]^+$ *m/z* 524) and for PAF 18:0 ($[M - H]^+$ *m/z* 552 and $[MH - 42]^+$ *m/z* 510). The presence of acyl-analogues of PAF 16:0 (*m/z* 496 and 538) and lyso-PC (*m/z* 104 and 258) was also indicated.

For quantification of PAF found in strawberry fruits, the washed rabbit platelet aggregation assay was used, together with a chemical assay (phosphorus determination). For the aggregation assay, a calibration curve with known quantities of authentic PAF (16:0) was constructed and the concentration of strawberry PAF was calculated assuming a 1:1 aggregation of synthetic to strawberry PAF. In this way, the amount of PAF was estimated to be 40 pg g^{-1} fruit. The phosphorus assay gave a much higher estimate of total phospholipids in the fraction, but this is consistent with the presence of much less active acyl-analogues of PAF together with PAF itself.

Among the rest of the preparative TLC lipid fractions, PL-2 corresponding to lyso-PCs was also examined. This fraction inhibited platelet aggregation induced by 2.5×10^{-10}M PAF (Table 1). It has been already reported that lyso-phospholipids are potent inhibitors of PAF-induced platelet aggregation [13]. PL-2 was further

purified by HPLC and eluted at a R_f corresponding to authentic lyso-PAF. After acetylation, PL-2 induced aggregation to platelets. PL-2 contained lyso-PAF and lyso-PC as confirmed by a FAB mass spectrum, which revealed the presence of 16:0 and 18:0 lyso-PAF, together with the corresponding acyl-analogues. In this spectrum the major peaks of phosphocholine (*m/z* 184) and of the choline moiety (*m/z* 104) were observed, together with an ion at *m/z* 224 corresponding to 1-propene-3-phosphocholine. Finally, there was an ion at *m/z* 258, which was formed by the loss of an alkyl or acyl group along with the transfer of a proton to the remaining glycerophosphocholine. There were also fragments at *m/z* 496 for 16:0 acyl-lyso-PC and *m/z* 522 for 18:1 acyl-lyso-PC.

Acetylation of fraction PL-2 (lyso-PC and lyso-PAF) resulted in the formation of PAF from lyso-PAF. This PAF was then estimated using the biological assay. According to its biological activity, lyso-PAF content was estimated to be 80 pg g^{-1} fruit. Total phospholipids of PL-2 were also estimated by phosphorus determination and the results obtained again revealed the presence of lyso-acyl-analogues.

Strawberry fruits thus contain a PAF together with its inactive precursor, although it is not possible at present to connect these findings with the allergenic action. PAF activity was independent of the degree of fruit maturity (data not shown). The PAF precursor, lyso-PAF, which can also be a PAF metabolite, is an intermediate in the formation of several other lipid-mediators of the inflammatory response.

Besides strawberry fruits, leaves, achenes and flower pollen were also examined. Achenes were collected from mature fruits after peeling and drying on paper for a few days. Pollen was collected from strawberry plants during the course of anthesis. Lipids from the above plant parts were extracted, fractionated, purified and subjected to the same biological tests and chemical treatments as fruits. It was evident that a fraction containing lyso-PAF was present in all plant parts. Fraction PL-3 did not cause platelet aggregation, indicating that a PAF-like compound was not present in strawberry leaves, achenes or pollen, although the possibility of the presence of very small amounts of PAF in these parts cannot be excluded.

EXPERIMENTAL

Plant material. Strawberry plants (*Fragaria ananassa* cv. Duglas) were collected from Vioryl greenhouses and extracted immediately after collection. Pollen was also collected from strawberry flowers of the same species.

Extraction and analysis of lipids. Total lipids were extracted from the tissue essentially according to ref. [14], after homogenization of fruits or leaves in an Omnimixer or after sonication of pollen. The $CHCl_3$ extracts were submitted to prep. TLC on Silica gel G plates (0.5 mm, 20 × 20 cm, *ca* 40 mg lipids on each plate) using $CHCl_3$–MeOH–H_2O (65:35:7) as solvent system. Authentic phosphatidylcholine, lyso-phosphatidylcholine and sphingomyelin were used as reference compounds. Lipid classes on TLC plates were detected by exposure to I_2 vapour, and the appropriate bands were extracted and resubmitted to a second TLC and/or HPLC analysis for further purification. For determination of phospholipids, a colorimetric assay for P was used [15].

HPLC. A cation-exchange column 5 cm × 4.6 mm i.d. (Partisil 10 SCX Whatman) was used eluting with AcN–MeOH–H_2O (60:30:7) at 1.5 ml min^{-1} (240 kg cm^{-2}) with UV detection at 206 nm [16].

Biological assay. Washed rabbit platelets were prepd essentialy as previously described [17] and resuspended in a Ca^{2+}-free Tyrode–gelatin buffer, pH 6.5 (1.25×10^9 plts ml^{-1}). Lipid frs were dissolved in 200–500 μl EtOH, unless otherwise indicated. PAF and/or inhibitor activity was tested by adding 5 μl of the appropriate lipid fr. to 500 μl of platelet suspension (2.5×10^8 plts ml^{-1}) in Tyrode–gelatin buffer (pH 7.2) containing 1.3 mM Ca^{2+}, with constant stirring at 37°. After 1 min, PAF ($1–2.5 \times 10^{-10}$M) was added [18]. Platelet aggregation was measured by the change in light transmission monitored by a Chronolog aggregometer.

Desensitization test. Washed rabbit platelets (1 ml) in Ca^{2+}-free Tyrode–gelatin buffer, pH 6.5 (1.25×10^9 plts ml^{-1}) were incubated with 1×10^{-6}M PAF for 30 min at room temp. Platelets were then centrifuged (1000 *g* for 15 min), washed, resuspended in Tyrode–gelatin buffer (pH 6.5) and used for bioassay.

Alkaline hydrolysis-acetylation. Lipid frs with biological activity were subjected to base treatment with 0.5 N methanolic KOH (1 hr at room temp.) and acetylated with Ac_2O in the presence of $HClO_4$ [19]. Lipids were then extracted and used for bioassays and other treatments.

FAB-MS. An Ion Tech saddle field atom gun was used with Xe at 9 kV. The ion source temp. was *ca* 70° and the accelerating voltage 3 kV. Samples were dissolved in $CHCl_3$–MeOH (1:1) and a few μl were applied to the copper probe tip. Thioglycerol (2 μl) was then added and mixed with the sample [20].

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