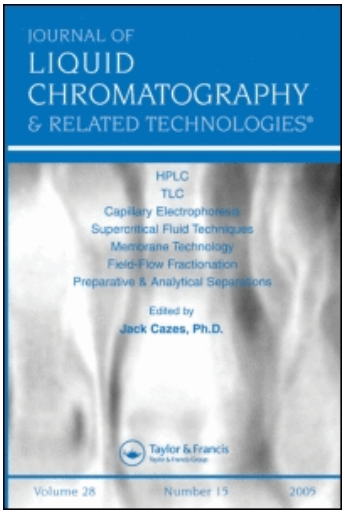

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SEPARATION OF POLAR LIPIDS FROM SOYBEAN OIL AND COTTON SEED OIL BY ONE-STEP HPLC SYSTEM. BIOLOGICAL ACTIVITY OF ISOLATED LIPIDS

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ABSTRACT

Many epidemiological studies suggest that vegetable oils present a protective effect against atherosclerosis, in a preventive level. Although the atherogenic mechanism is not clear, a number of reports indicate that platelet activating factor (PAF) plays a critical role in atherogenesis.

In this study, total lipids of two vegetable oils were separated into polar and neutral lipids. Total lipids, total polar lipids, and total neutral lipids were tested in vitro for their biological activity against washed rabbit platelets, that is their ability to inhibit platelet aggregation induced by PAF, or to cause platelet aggregation. Total polar lipids, which are more potent than total

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neutral lipids, were further fractionated by HPLC and each fraction was tested for its biological activity.

The experimental data give an answer for the preventive protective effect of vegetable oils against atherogenesis.

INTRODUCTION

Atherosclerotic cardiovascular disease is the major cause of mortality and morbidity in developed industrialized countries.(1) It is characterized by irregular scavenger receptor-mediated uptake of plasma oxidized–low density lipoprotein (Ox–LDL) from macrophage and foam cell formation.(2)

The reduction of the risk for coronary heart disease (3) is not proportional to fat restriction. Besides, epidemiological data show that dietary components, such as antioxidants like vitamin E, prevent coronary heart disease.(4)

The only approach seems to be that of primary prevention, and for that the atherogenic mechanism has to be clarified. Although some epidemiological studies correlate plasma cholesterol and saturated fat with the incidence of atherosclerosis (5) and others present unsaturated fatty acids as beneficial constituents (6), atherosclerosis remains an unresolved controversy.

The "response to injury" hypothesis (4) indicates that atherosclerosis is a result of inflammatory response and suggests the endothelial injury as a primary event for the cardiovascular disease.

Platelet Activating Factor (PAF), chemically characterized as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (7), is a mediator in inflammation, immune disorders, and ischemic diseases (8), and is synthesized, released, and metabolized by inflammatory cells, such as macrophages, polymorphonuclear leukocytes (PMNL), and platelets, as well as by endothelial cells. It has also been reported that PAF may be involved in atherogenesis in several ways.(9)

Many studies proclaim that vegetable oils exhibit anti-atherogenic effects (10,11). Thereby, it is worthwhile to investigate if there are compounds in vegetable oils to inhibit PAF action. A positive result from this study would enforce the theory that PAF plays a primary role in atherogenesis.

EXPERIMENTAL

Materials and Reagents

Soybean oil and cotton seed oil were purchased from a local supermarket. All reagents and chemicals were of analytical grade supplied by Merck (Darmstadt, Germany). The solvents used for High Performance Liquid

Chromatography (HPLC) were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards were obtained from Supelco (Bellefonte, PA, USA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described.(7)

Procedure

Lipid Analysis

Soybean oil and cotton seed oil were used as total lipids in the form that they were purchased, and diluted in bovine serum albumin (2.5 mg/mL). Total lipids were separated to polar and neutral lipids with a countercurrent distribution-extraction procedure. Briefly, an amount of vegetable oil is diluted in quadruple volume of petroleum ether (b.p. 40–70°C) and the whole mixture is washed with 87% ethanol several times. Afterwards the combined ethanol phases are washed with petroleum ether several times. Finally, the combined petroleum phases are washed with ethanol. The combined ethanol phases contain polar lipids in a yield of 96%, while the combined petroleum phases contain neutral lipids.(12)

Polar lipids were further fractionated on HPLC. Separation (13) was performed on an HP HPLC Series 1100 liquid chromatography model (Hewlett Packard, Waldbronn, Germany) equipped with a 100 μL loop Rheodyne (7725 i) loop valve injector, a degaser G1322A, a quad gradient pump G1311A, and an HP UV spectrophotometer G1314A as a detection system. The spectrophotometer was connected to a Hewlett Packard (Hewlett Packard, Waldbronn, Germany) model HP-3395 integrator plotter. A normal phase column, Sphereclone 5 μNH_2, 25 cm × 4.6 mm (I.D.), Phenomenex (Hurdsfield Ind. Est., UK) was used. The flow rate was 1 mL/min. The solvent system consisted of an isocratic elution with 100% solvent A (acetonitrile–methanol: 70/30) for 35 min, followed by a linear gradient to 100% solvent B (methanol) in 5 min, a hold for 5 min in 100% B, followed by a linear gradient to 100% solvent C (water) in 5 min, and finally a hold in 100% C for 10 min. For the next injection, a 5 min elution with methanol and a 10 min equilibration with solvent A is required.

Biological Activity

Total lipids, total neutral lipids, and total polar lipids, as well as purified fractions of polar lipids from HPLC separation, were collected and tested for biological activity. PAF and the examined samples were dissolved in 2.5 mg bovine serum albumin (BSA) per mL saline. Thrombin was dissolved in saline. Various concentrations of the examined sample were added into the aggregometer cuvette and the aggregation induced by the sample was studied in a Chronolog

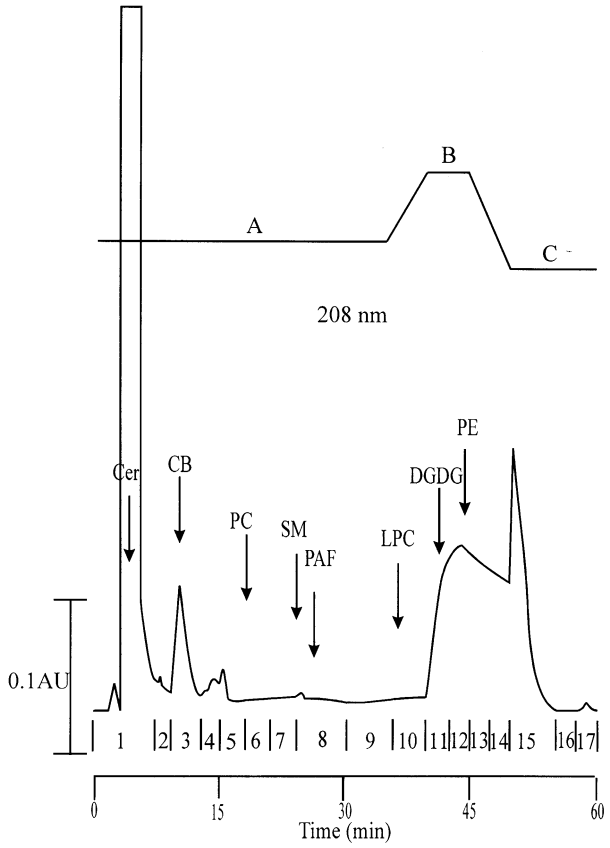

Figure 1. HPLC separation of polar lipids from soybean oil on adsorption column with a stepped gradient elution system as shown in figure. Cer, Ceramides; CB, Cerebrosides; PC, Phosphatidylcholine; SM, Sphingomyeline; PAF, Platelet-Activating Factor; DGDG, Digalactosyldiglycerides; LPC, Lysophosphatidylcholine; PE, Phosphatidylethanolamine.

(Havertown, PA) aggregometer coupled to an Omniscribe recorder (Houston, TX) (7). The platelet aggregation induced by PAF (1×10^{-10} M, final concentration) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the examined sample (7). Consequently, the plot of percent inhibition (ranging from 20% to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample, which inhibited 50% PAF-induced aggregation, is calculated. This value is defined as IC_{50} namely, inhibitory concentration for 50% inhibition. The IC_{50} values are expressed as μL of

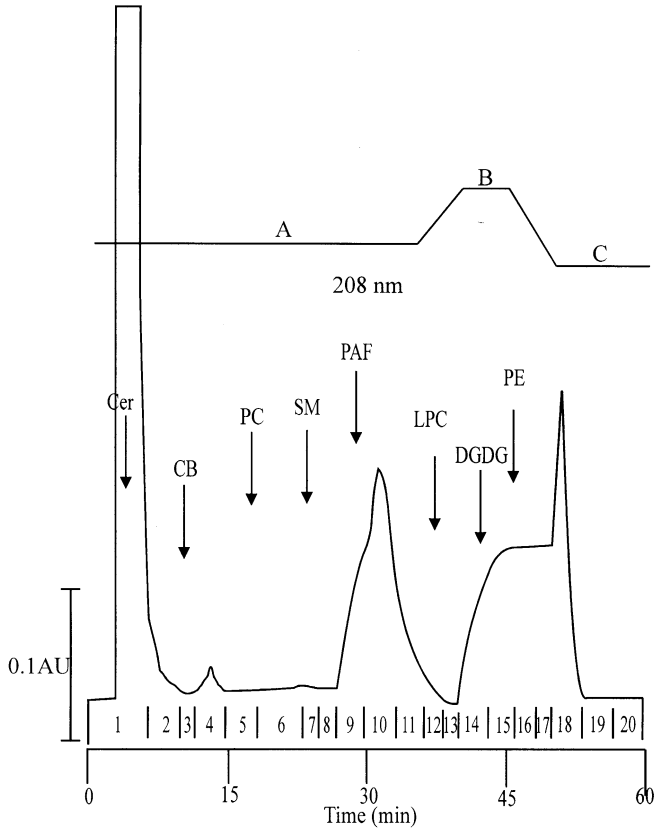

Figure 2. HPLC separation of polar lipids from cotton seed oil on adsorption column with a stepped gradient elution system as shown in figure. Cer, Ceramides; CB, Cerebrosides; PC, Phosphatidylcholine; SM, Sphingomyeline; PAF, Platelet-Activating Factor; DGDG, Digalactosyldiglycerides; LPC, Lysophosphatidylcholine; PE, Phosphatidylethanolamine.

vegetable oil required for 50% inhibition against PAF (1×10^{-10} M, final concentration). IC_{50} values of the various fractions were tested against thrombin (0.025 units/cuvette) activity and the percent inhibition was determined.

RESULTS

Typical profiles of the polar lipids from soybean oil and cotton seed oil separation on an adsorption HPLC column, along with the elution times of

Table 1. Biological Activities of Each Lipid Fraction of Cotton Seed Oil

Lipid Classes	IC_{50} [μL] Against PAF	IC_{50} [μL] Against Thrombin
Total lipids	0.262	
Total neutral lipids	0.088	
Total polar lipids*	75	
Polar lipid fractions		
PL_1	195	100
PL_2	5658	2800
PL_3	2299	1150
PL_4	8970	5550
PL_5	8500	4250
PL_6**	–	–
PL_7	9100	6600
PL_8	16300	8150
PL_9	3700	1850
PL_{10}	10600	5300
PL_{11}	9600	5400
PL_{12}	1000	650
PL_{13}	4500	2250
PL_{14}	12000	6450
PL_{15}	4300	4700
PL_{16}	3400	2360
PL_{17}	5157	5900

*Total polar lipids in a reflected amount of 3375 μL of oil cause platelet aggregation.
**No biological activity.

standard lipids, are shown in Figure 1 and Figure 2, respectively. Seventeen lipid fractions were collected from polar lipid separation of soybean oil and twenty lipid fractions were collected from polar lipid separation of cotton seed oil. These fractions as well as total lipids, total neutral lipids, and total polar lipids were tested for their biological activity. All the HPLC fractions of polar lipids (from both HPLC separations) were tested for their ability to inhibit PAF-induced and/or thrombin-induced washed rabbit platelet aggregation. The results on the biological activities of each fraction are summarized in Table 1 for the cotton seed oil and in Table 2 for the soybean oil.

The majority of the fractions collected from polar lipid HPLC separation of soybean oil exerted biological activity. All of them inhibited PAF-induced aggregation as well as thrombin-induced aggregation, in a dose-dependent manner. All the fractions collected from the polar lipid HPLC separation of cotton seed oil exerted biological activity. The majority of them inhibited PAF as well as

Table 2. Biological Activities of Each Lipid Fraction of Soybean Oil

Lipid Classes	IC_{50} [μL] Against PAF	IC_{50} [μL] Against Thrombin
Total lipids	0.255	
Total neutral lipids	0.092	
Total polar lipids*	62.5	
Polar lipid fractions		
PL_1	2100	1050
PL_2	3758	–
PL_3	32300	–
PL_4	3800	6350
PL_5	32566	65132
PL_6	2700	1800
PL_7	3000	6000
PL_8	2126	1330
PL_9	5000	–
PL_{10}	1300	650
PL_{11}	1900	1260
PL_{12}	16000	8000
PL_{13}	32300	16150
PL_{14}	12900	6450
PL_{15}**	–	–
PL_{16}	24700	12350
PL_{17}	2926	3000
PL_{18}	3400	4000
PL_{19}	7000	3700
PL_{20}	7800	3900

*Total polar lipids in a reflected amount of 4500 μL of oil cause platelet aggregation.
**Shape change.

thrombin-induced aggregation, in a dose-dependent manner. Three of the fractions (PL_2, PL_3, and PL_9) are specific inhibitors of PAF. One of the fractions (PL_{15}) caused shape change of the platelets and it was not studied any further.

DISCUSSION

In this work, we demonstrated the existence of a variety of PAF inhibitors that minimize the biological effects of PAF. The above biologically active compounds of soybean oil and cotton seed oil also inhibited thrombin-induced aggregation. These compounds might belong to the classes of phospholipids and

glycolipids, based on their isolation procedure and their chromatographic behavior.

This HPLC separation is a powerful tool for yielding results that throw further light on the nature of the beneficial effects of vegetable oil consumption, since vegetable oils contain a significant number of lipids with anti-thrombotic as well as anti-atherogenetic action in vitro.

Moreover, the above data may explain why people who are fed on a Mediterranean diet have low incidence of atherosclerosis as a result of receiving many antioxidants and PAF inhibitors from foodstuffs included in a Mediterranean diet.

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