Inhibitors of Arachidonate Lipoxygenase from Defatted Perilla Seed

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The effects of phenolic compounds isolated from defatted perilla (*Perilla frutescens* var. *japonica* Hara) seed on arachidonate 12-lipoxygenase (12-LO) and 5-lipoxygenase (5-LO) activities were investigated using rat platelets and polymorphonuclear leukocytes. The ethyl acetate layer obtained by partitioning the ethanolic extract of defatted perilla seed proved to have significant inhibitory activity against lipoxygenases. Potent inhibitory principles in the ethyl acetate layer were isolated and identified as luteolin, chrysoeriol, and rosemarinic acid and its methyl ester. The most effective inhibitor of lipoxygenases was luteolin (IC_{50} = 20 and 102 nM for 12-LO and 5-LO, respectively). These results indicate that defatted perilla seed, now an industrial waste product, may be developed into a useful source of an agent that prevents allergic hyper-reactivity and inflammatory responses due to its antilipoxygenase activity.

Keywords: *Arachidonic acid; lipoxygenase; perilla seed; luteolin*

INTRODUCTION

It is widely accepted that diets rich in saturated fatty acids (S) increase plasma cholesterol levels, while those rich in polyunsaturated fatty acids (P) decrease plasma cholesterol levels. According to a recent investigation, saturated, monounsaturated, and linoleic acids tend to accumulate in the human body, in contrast to $\omega - 3$ fatty acids, which are catabolized (Ishihara et al., 1995). Moreover, Okuyama (1992a,b) reported that a high $\omega - 3/\omega - 6$ ratio in diets and not a high P/S ratio assist in the prevention of cholesterol-associated thrombotic diseases, carcinogenesis, and allergic hyper-reactivity.

These findings suggest that the demand for dietary oil rich in $\omega - 3$ fatty acids will increase soon to balance the present trend of consuming too much dietary oil rich in $\omega - 6$ fatty acids such as linoleic acid. Perilla seed contains α-linolenate ($\omega - 3$) rich oil; however, the production of perilla seed oil results in a large amount of hexane-extracted residue as well, and thus a method that utilizes defatted perilla seed must be developed.

In this paper, we describe a method for the isolation of compounds that inhibit 12-lipoxygenase (12-LO) and 5-lipoxygenase (5-LO) activity using an assay-guided fractionation. Arachidonic acid is metabolized by 12-LO to yield 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), which is a bioregulator of atherosclerotic processes (Nakao et al., 1982) and tumor metastasis (Tang and Honn, 1994). 5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), the product of 5-LO, induces allergies as well as inflammatory responses (Ford-Hutchinson et al., 1980; Morris et al., 1980). Therefore, the specific inhibitors of lipoxygenases have the potential to regulate or prevent these diseases.

We evaluated compounds isolated from the ethanolic extract of defatted perilla seed by monitoring their effects on arachidonic acid metabolism, to establish a use for this industrial waste.

MATERIALS AND METHODS

Materials. Perilla (*Perilla frutescens* var. *japonica* Hara) seed was purchased from Kanematsu Co., Ltd. [1-^{14}C]Arachidonic acid (57 mCi/mmol) was obtained from Dupont/NEN (Boston, MA). Authentic 5-HETE was obtained from Cascade Biochem Ltd. (Reading, U.K.). Precoated silica gel sheets were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

Isolation and Identification of the Active Components from Perilla Seed (Figure 1). Perilla seed was extracted with hot hexane, and the residue was extracted by refluxing with ethanol. The concentrated ethanolic extract was partitioned between hexane and 80% aqueous methanol. The 80% aqueous methanolic layer was evaporated to dryness and then partitioned between ethyl acetate and water. The ethyl acetate layer, which showed the strongest inhibitory activity against 12-LO, was subjected to SiO_2 column chromatography ($CHCl_3$/MeOH, 10:1) to give three active fractions and an inactive fraction.

Fraction 1 was further purified on SiO_2 column chromatography ($CHCl_3$/MeOH, 20:1) and was crystallized from $CHCl_3$ to yield chrysoeriol.

Fraction 2 was subjected to SiO_2 column chromatography ($CHCl_3$/MeOH, 10:1) to give crude luteolin. After the removal of methanol-insoluble impurities and evaporation of the solvent, the residue was crystallized from $CHCl_3$/MeOH (15:1) to give pure luteolin.

Fraction 3 was purified by column chromatography on Sephadex LH-20 (MeOH) followed by SiO_2 ($CHCl_3$/MeOH/H_2O, 7:3:0.5) to give rosemarinic acid methyl ester and crude rosemarinic acid. Further purification was carried out using high-performance liquid chromatography (HPLC) to give pure rosemarinic acid. The following conditions were used for purification: solvent, MeOH/H_2O, 1:1, containing 0.1% trifluoroacetic acid; column, Deverosil ODS(10 × 250 mm, Nomurakagaku); flow rate, 2.5 mL/min; room temperature; detection at 254 nm.

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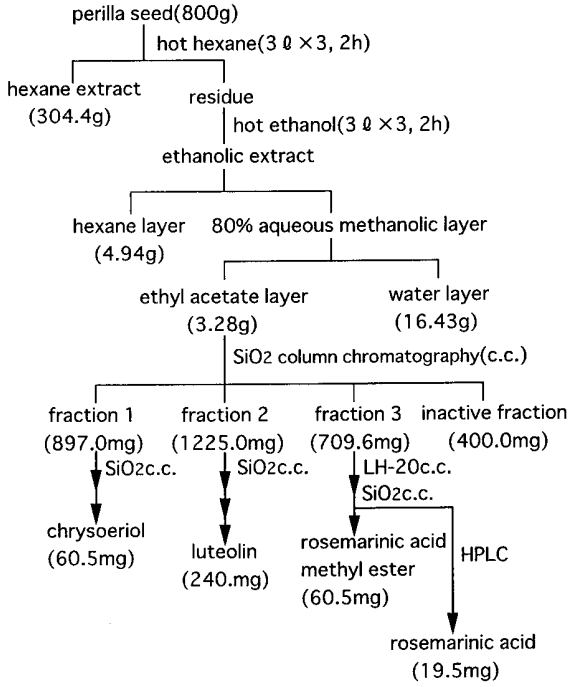

Figure 1. Extraction and isolation of active compounds from defatted perilla seed.

^{1}H NMR and ^{13}C NMR spectra of isolated compounds were measured in DMSO-d_6 with tetramethylsilane as the internal standard, on a JEOL alfa-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C).

Assay for Platelet 12-LO Activity. Blood was collected from male Wistar rats (200−300 g). Washed platelets were prepared by differential centrifugation as reported previously (Sekiya and Okuda, 1982). Experimental conditions for the assay were the same as those used by Sekiya et al. (1982). Tris/saline buffer (25 mM Tris, 130 mM NaCl, pH 7.4) containing 1 mM EDTA was used as the solvent in the reaction. Sonicated platelets (130 mL, 2 mg of protein/mL) were preincubated at 37 °C for 5 min with 20 mL of the testing solution. Fifty milliliters of [1-^{14}C]arachidonic acid (0.05 μCi) was then added, and the mixture was incubated at 37 °C for 5 min. The reaction was terminated by adding 200 mL of 0.5 N formic acid. The products were then applied quantitatively to silica gel sheets and developed with $CHCl_3$/MeOH/AcOH/H_2O (90:8:1:0.8, v/v). Authentic 12-HETE was identified by gas chromatography/mass spectrometry (Sekiya and Okuda, 1982). The radioactive metabolites were detected and quantified using a model BAS 1000 laser imaging system (Fujix, Tokyo, Japan). 12-LO activity was measured by the formation of 12-HETE (R_f = 0.50) and expressed as percent of control.

Assay for PMNL 5-LO Activity. Peritoneal polymorphonuclear leukocytes (PMNL) were prepared from male Wistar rats injected intraperitoneally with 5% glycogen (20 mL/kg). The washed cells were homogenized and centrifuged (100000*g*) at 4 °C for 1 h. The supernatant was used as the crude enzyme (2 mg of protein/mL). Potassium phosphate buffer (50 mM, pH 7.4) was used as the solvent in the reaction. The testing solution (20 mL) was preincubated with 130 mL of the enzyme solution containing 3 mM $CaCl_2$ and 2 mM ATP at 37 °C for 5 min, followed by the addition of [1-^{14}C]arachidonic acid. After incubation at 37 °C for 5 min, the products were subjected to thin-layer chromatography and developed with ether/petroleum ether/acetic acid (50:50:1, v/v) at 4 °C (Vanderhoek et al., 1980). The radioactive metabolites were quantified as described above, and 5-LO activity was measured by the formation of 5-HETE (R_f = 0.38).

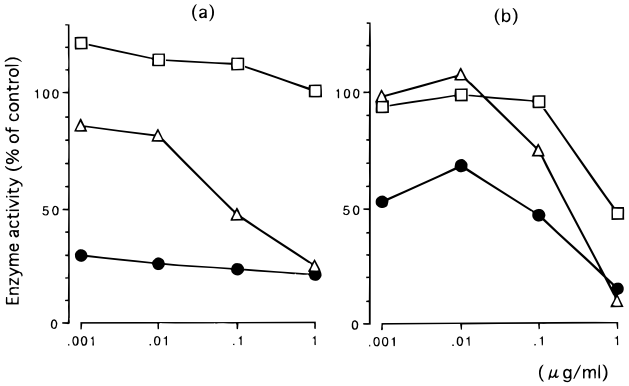

Figure 2. Effect of hexane layer (□), ethyl acetate layer (●), and water layer (△) on platelet 12-LO (a) and PMNL 5-LO (b) activities. Rat platelets were incubated with [1-^{14}C]arachidonic acid in the presence of sample for 5 min at 37 °C. 12-LO activity was measured by 12-HETE formation and expressed as percent of control. 5-LO activity was measured by 5-HETE formation, using PMNL incubated with the substrate in the presence of sample. Values were obtained from a single experiment.

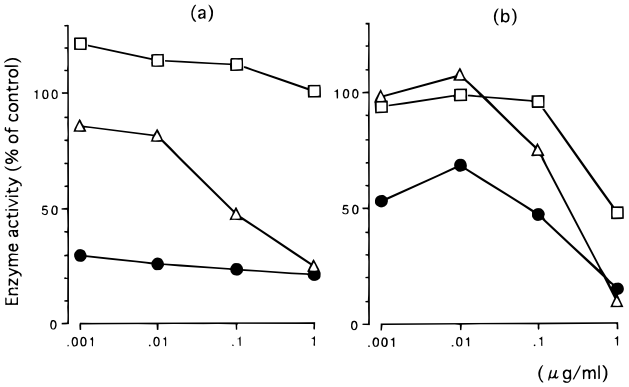

Figure 3. Effect of fractionated ethyl acetate layer by silica gel column chromatography on platelet 12-LO (a) and PMNL 5-LO (b) activities. 12- and 5-LO activities of fraction 1 (□), fraction 2 (●), and fraction 3 (△) were assayed as described in Figure 1. Values were obtained from a single experiment.

RESULTS AND DISCUSSION

Isolation of Active Compounds. The ethyl acetate layer obtained by partitioning the ethanolic extract of defatted perilla seed showed significant inhibitory activity against lipoxygenases as indicated in Figure 2. After fractionation on SiO_2 column chromatography, strong lipoxygenase inhibitory activity was found in fraction 2, whereas fractions 1 and 3 exhibited weak lipoxygenase inhibitory activity (Figure 3). The major components of these fractions were purified by column chromatography and/or crystallization and identified as luteolin, chrysoeriol, rosemarinic acid, and rosemarinic acid methyl ester by comparison of ^{1}H NMR and ^{13}C NMR spectral data with literature values (Berghöfer and Hölzl, 1987; Wagner and Chari, 1976; Kohda et al., 1989). The chemical structures of the isolated compounds are shown in Figure 4.

Inhibitory Activity of Isolated Compounds against Lipoxygenase. As indicated in Figures 5 and 6, luteolin, chrysoeriol, rosemarinic acid, and rosemarinic acid methyl ester inhibit 12- and 5-LO activities in a dose-dependent manner.

The IC_{50} values of luteolin were 20 nM (12-LO) and 102 nM (5-LO), while those of chrysoeriol, which has a

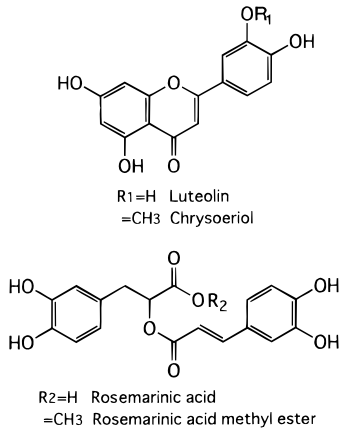

Figure 4. Structures of active components from defatted perilla seed.

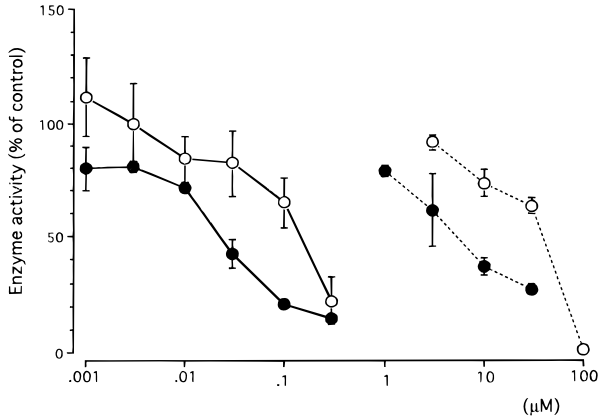

Figure 5. Effects of luteolin and chrysoeriol on arachidonate 12- and 5-LO: 12-LO activity (●), 5-LO activity (○), luteolin (—), chrysoeriol (· · ·). Assay for enzyme activity is described in Figure 1. Values are means ± SD of three replications.

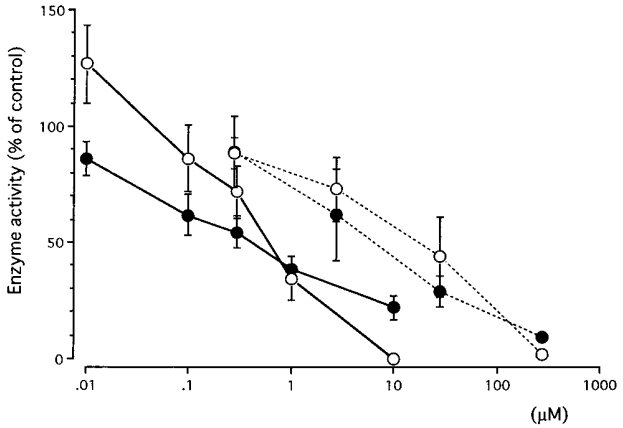

Figure 6. Effects of rosemarinic acid and its methyl ester on arachidonate 12- and 5-LO: 12-LO activity (●), 5-LO activity (○), rosemarinic acid (· · ·), rosemarinic acid methyl ester (—). Enzyme activities were measured as described in Figure 1. Values are means ± SD of three replications.

methoxy group at the 3′-position, were 5.9 mM (12-LO) and 38.9 mM (5-LO). These two compounds inhibited 12-LO activity more effectively than 5-LO activity. Although Robak et al. (1988) reported that a free hydroxyl group at the 3′-position is unimportant for the inhibition of lipoxygenase activity, our results suggest that the absence of a hydroxyl group at the 3′-position of the flavonoid B-ring reduces antilipoxygenase activity.

Table 1. IC_{50} Values for Inhibition of Lipoxygense Activities by Phenolic Compounds

compound	IC_{50} (mM) 12-LO	5-LO
luteolin	0.02	0.1
chrysoeriol	5.9	38.9
rosemarinic acid methyl ester	0.4	0.6
rosmarinic acid	6.4	6.2
2-(3,4-dihydroxyphenyl)ethanol[a]	4.2	13.0
caffeic acid[a]	5.1	72.0

[a] Kohyama et al. (1997).

The inhibitory activity of rosemarinic acid against lipoxygenase was weaker than that of its methyl ester. The hydrophilicity of the carboxyl group may decrease the inhibitory activity.

Table 1 shows the IC_{50} values of phenolic compounds isolated from perilla seed as well as other chemicals reported having anti-lipoxygenase activity (Kohyama et al., 1997). Lipoxygenase inhibitory activity of luteolin was >100 times stronger than that of 2-(3,4-dihydroxyphenyl)ethanol, which was isolated from olive.

12-HETE and 5-HETE as Lipid Mediators. 12-HETE is an important factor in the genesis of atherosclerosis (Nakao et al., 1982) and in tumor metastasis (Tang and Honn, 1994). Morris et al. (1980) reported the slow-reacting substance of anaphylaxis (SRS-A) and the chemotactic substance leukotriene B were arachidonic acid metabolites formed via the 5-LO pathway. These reports suggest that selective inhibitors of 12-LO and 5-LO are capable of preventing these diseases.

Our observations suggest that the ethanolic extract of defatted perilla seed contains a large quantity of luteolin and has the potential to play a role in the regulation of atherosclerotic processes, tumor metastasis, and allergic diseases.

ABBREVIATIONS USED

5-LO, 5-lipoxygenase; 12-LO, 12-lipoxygenase; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; PMNL, polymorphonuclear leukocytes.

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