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Inhibitory effects of brown algal phlorotannins on secretory phospholipase A_2s, lipoxygenases and cyclooxygenases

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Abstract

The inhibitory effects of brown algal phlorotannins on secretory phospholipase A_2s (sPLA$_2$s), lipoxygenases (LOXs) and cyclooxygenases (COXs) were determined with an *in vitro* assay. Oligomers of phloroglucinol; eckol (a trimer), phlorofucofuroeckol A (a pentamer), dieckol (a hexamer) and 8,8′-bieckol (a hexamer) isolated from the brown alga *Eisenia bicyclis* had pronounced inhibitory effects on sPLA$_2$ from porcine pancreas and bee venom (IC_{50} 100–200 μM). The phlorotannins inhibited LOX activity more effectively than the well-known LOX inhibitors; resveratrol and epigallocatechin gallate. 8,8′-Bieckol, the strongest LOX inhibitor in this study, inhibited soybean LOX and 5-LOX with IC_{50} values of 38 and 24 μM, respectively. Negligible or very weak effects of the phlorotannins on COX-1 and COX-2 were found, except for an inhibitory effect of dieckol on COX-1 (74.7%) and of eckol on COX-2 (43.2%) at 100 μM.

Introduction

Phospholipase A_2s (PLA$_2$s) (EC. 3.1.1.4) are enzymes that specifically catalyze the hydrolysis of the esters at the *sn*-2 position of phospholipids to produce free fatty acids and lysophospholipids (Dennis 1994). The release of arachidonic acid from membrane phospholipids by PLA$_2$ is believed to be a key step in the control of eicosanoid production within the cell. PLA$_2$s have been divided into two main groups: a 14 kDa secretory enzyme (sPLA$_2$) and a 85 kDa cytosolic enzyme (cPLA$_2$). High levels of sPLA$_2$ are known to be present in synovial fluids, articular cartilage and blood from patients with rheumatic diseases (Bomalaski and Clark 1993). Lipoxygenases (LOXs) are involved in the biosynthesis of various bioregulators, which are closely related to the pathogenesis of allergies, atherosclerosis and some cancers (Spector et al. 1988). 5-Lipoxygenase (5-LOX) (EC.1.13.11.34) catalyzes the first step in the oxygenation of arachidonic acid, thus leading to the production of biologically active compounds such as leukotrienes and 5-hydroxyeicosatetraenoic acid (Yamamoto 1992). The peptidoleukotrienes (leukotriene C_4, leukotriene D_4 and leukotriene E_4) are powerful spasmogens, which have been implicated in inflammatory and allergic responses. Cyclooxygenases (COXs) (EC.1.14.99.1) are known to contain cyclooxygenase and peroxidase activities (William et al. 1996). Although COX-1 is constitutively expressed in a variety of cells and is involved in normal cellular homeostasis, COX-2 is an inducible form of COX and is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions. An inhibitor of these enzymes may be useful as a therapeutic drug for the treatment of inflammatory diseases, atherosclerosis and cancer.

Recent studies have focused on the role of dietary factors such as phenolic compounds or polyphenols

(Koshihara et al. 1984; Kohyama et al. 1997; Yang et al. 1999) in the prevention of significant diseases including cancer, coronary heart diseases and allergies. Previously, we isolated the phlorotannins; eckol (a phloroglucinol trimer), phlorofucofuroeckol A (a pentamer), dieckol and 8,8′-bieckol (hexamers) from the brown alga *Eisenia bicyclis* and reported their antioxidant activity (Nakamura et al. 1996) and their inhibitions of glycosidase (Shibata et al. 2002a) and hyaluronidase (Shibata et al. 2002b). Phlorotannins, which are only known in brown algae, are polymers of phloroglucinol (Ragan and Glombitza 1986). Although terrestrial polyphenols, flavonoids and gallic acids are known to have several bioactive functions (Hollman and Katan 1999), the bioactivities of phlorotannins are obscure. In Japan, *E. bicyclis* is a common brown alga, which has been utilized from ancient times as food and industrial materials of alginic acid. We describe the effects of phlorotannins from *E. bicyclis* on $sPLA_2$s, soybean LOX, 5-LOX, COX-1 and COX-2 in this report.

Materials and methods

Materials

The brown alga *Eisenia bicyclis* (Kjellman) Setchell was collected from the coast of the Itoshima Peninsula (33°37′ N, 130°10′ E) in Fukuoka Prefecture, Japan. The alga was washed with filtered seawater, air-dried, and pulverized. The algal powder was stored at −40 °C until use.

$sPLA_2$ (porcine pancreas), LOX (soybean), linoleic acid and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $sPLA_2$ (bee venom), 5-LOX (human recombinant), COX-1, 2 (ovine) and their inhibitor screening kit were purchased from Cayman chemical Co. (Ann Arbor, MI, USA). Catechin and epigallocatechin gallate (EGCG) were kindly donated by Kurita Water Ind. (Kanagawa, Japan).

Extraction and purification of phlorotannins

Phlorotannins were extracted from the algal powder according to the method described by Nakamura et al. (1996). Each of the phlorotannins in the crude extracts was purified on a column of Wakogel C-300HG (1.5 cm i.d. × 150 cm, Wako Pure Chemical Ind., Osaka, Japan) with $CHCl_3$-MeOH-water (80:20:2, v/v) as the eluent. Purity of the phlorotannins was checked by thin-layer chromatography (TLC) (Nakamura et al. 1991, 1996) and HPLC. TLC plates (Silica Gel 60 F_{254}, 0.25 mm, Merck Co., Darmstadt, Germany) were developed with $CHCl_3$-MeOH-water-acetic acid (65:25:4:3, v/v). 50% H_2SO_4 and paprika pigment were used as detecting agents (Nakamura et al. 1991, 1996). HPLC analysis was carried out with an HPLC system (L-7100 pump and L-7420 UV detector, Hitachi Co., Tokyo, Japan) and an Inertsil ODS-3 column (6 mm i.d. × 150 mm, GL Science Co., Tokyo, Japan). Elution was performed at a flow-rate of 1.0 mL min^{-1} with a linear gradient from 30% to 100% MeOH for 20 min, and followed by 20 min with 100% MeOH. The UV detector was set at 290 nm.

Assay of enzyme activity

Secretory phospholipase A_2s

The substrate for $sPLA_2$ was the 1,2-dithio analog of diheptanoyl phosphatidylcholine (Cayman Chemical Co., Ann Arbor, MI, USA) (Hendrickson et al. 1983; Reynolds et al. 1992). Bovine pancreas $sPLA_2$, bee venom $sPLA_2$ and the substrate were dissolved separately in 25 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, 100 mM KCl, 0.3 mM Triton X-100, and 1 mg mL^{-1} BSA. Ten μL of $sPLA_2$ (10 units) was added to 200 μL of the substrate (1.66 mM). After incubation at room temperature, the thiols released by the $sPLA_2$ were detected by adding 10 μL of 5,5′-dithio-*bis*-(2-nitrobenzoic acid) and measuring absorbance at 414 nm. Polyphenols were dissolved in 5 μL of dimethyl sulfoxide (DMSO). In the control test, 5 μL of DMSO was used instead of the polyphenol solution.

Soybean lipoxygenase

Soybean LOX activity was measured by the spectrophotometric method of Tappel et al. (1952). Polyphenols were dissolved in 20 μL of MeOH, and the solution was mixed with 2 mL of a 0.2 M borate buffer at pH 9.0. The increase in absorbance at 234 nm was measured and compared with that in the control test.

5-lipoxygenase

5-LOX activity was determined by measuring 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), according to the method of Zhang et al. (1993). Polyphenols were dissolved in 10 μL of DMSO. The reaction mixture was centrifuged (15,000

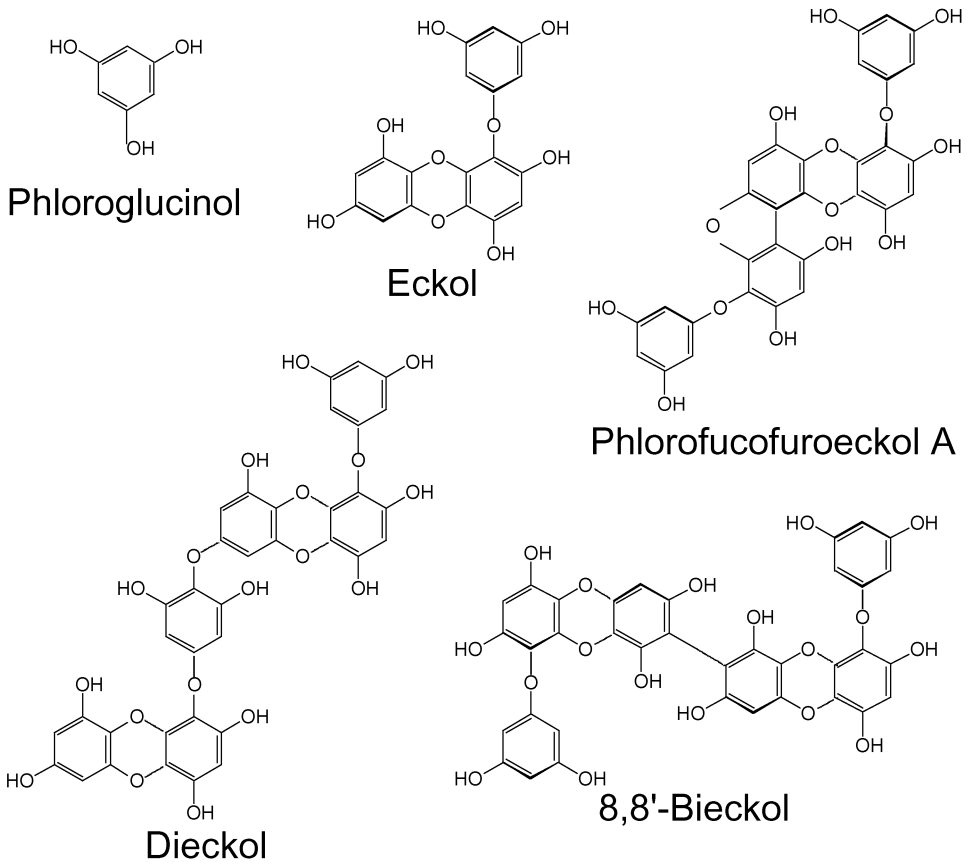

Figure 1. Structures of phlorotannins from the brown alga *Eisenia bicyclis*.

rpm for 5 min), the supernatant was processed by HPLC (Inertsil ODS-2 column, 4.6 mm i.d. × 250 mm, GL Science Co., Tokyo, Japan; MeOH:H_2O:acetic acid = 80:20:0.01, v/v; flow rate, 1.6 mL min^{-1}), and the effluent was monitored at 233 nm. Fluorene (Wako Pure Chem. Ind.) was used as the internal standard. The production of 5-HPETE was compared with that from the control test.

Cyclooxygenase-1 and cyclooxygenase-2

Activities of both ovine COX-1 and COX-2 were determined with the COX inhibitor assay screening kit (Cayman Chemical Co., Ann Arbor, MI, USA). Prostaglandin F_2, which is produced in the COXs reaction, was quantified *via* enzyme immunoassay with a specific antibody that binds to major prostaglandin compounds. Polyphenols were dissolved in 10 µL of DMSO and diluted with 10 µL of 0.1 M Tris-HCl buffer at pH 8.0 containing 5 mM EDTA and 2 mM phenol. In the control test, 10 µL of DMSO was used instead of the polyphenol solution.

Three terrestrial polyphenols (resveratrol, catechin and EGCG) were used as positive controls. All reagents used in this experiment were of analytical grade.

Results

Detection and isolation of phlorotannins

The crude extract, which comprised 3% of the algal powder, contained phloroglucinol (0.9%), an unidentified phloroglucinol tetramer (4.4%), eckol (7.5%), phlorofucofuroeckol A (21.9%), dieckol (23.4%), 8,8′-bieckol (24.6%) and other compounds (17.3%). These compounds were purified by silicic acid column chromatography. The purity of each oligomer in this experiment (Figure 1) was more than 90%.

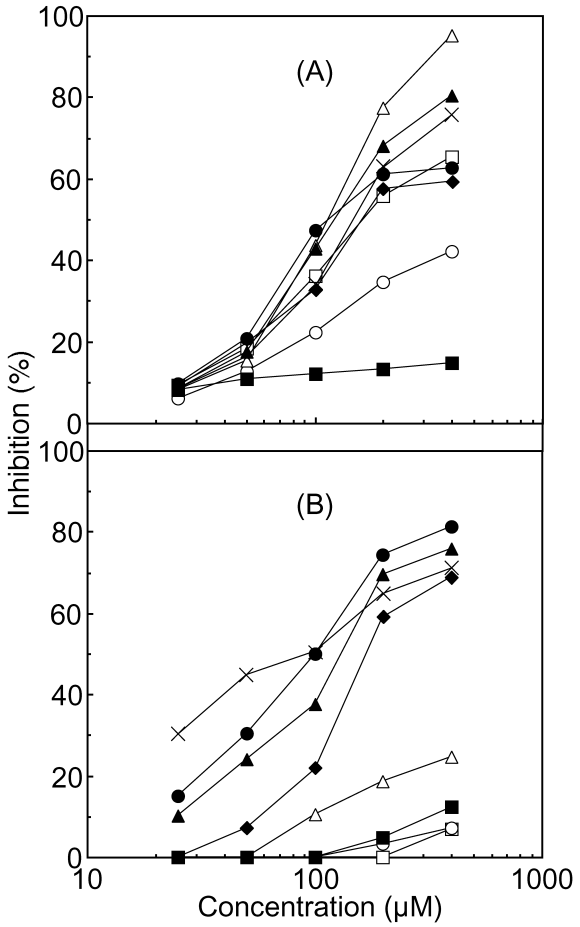

Figure 2. Dose-dependent inhibition of $sPLA_2$s by brown algal phlorotannins and terrestrial polyphenols. (A): porcine pancreas $sPLA_2$. (B): bee venom $sPLA_2$. Symbols indicate: phloroglucinol (filled squares), eckol (filled circles), phlorofucofuroeckol A (filled triangles), dieckol (crosses), 8,8′-bieckol (filled lozenges), resveratrol (open squares), catechin (open circles), and EGCG (open triangles). Analytical data are presented as the mean of three determinations.

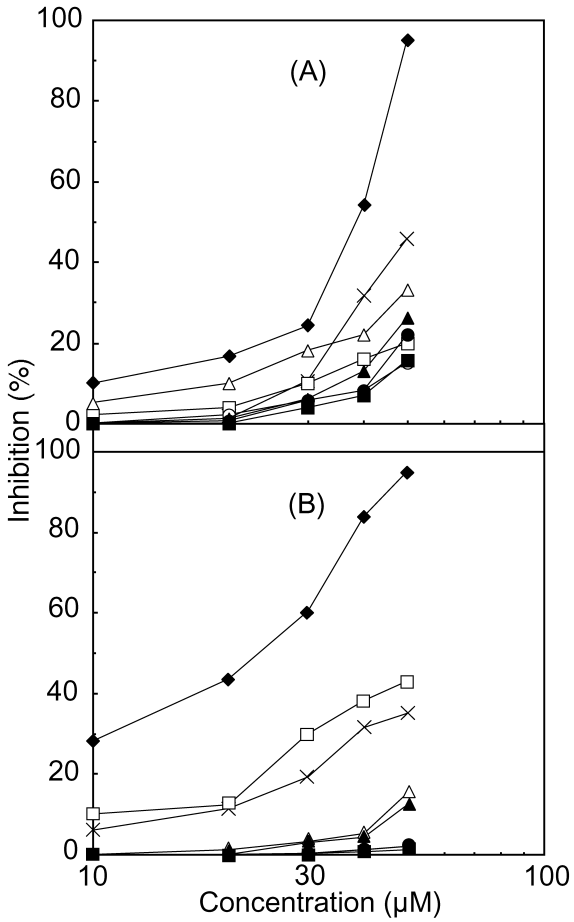

Figure 3. Dose-dependent inhibition of soybean LOX and 5-LOX (human recombinant) by brown algal phlorotannins and terrestrial polyphenols. (A): soybean LOX. (B): 5-LOX (human recombinant). Symbols indicate: phloroglucinol (filled squares), eckol (filled circles), phlorofucofuroeckol A (filled triangles), dieckol (crosses), 8,8′-bieckol (filled lozenges), resveratrol (open squares), catechin (open circles), and EGCG (open triangles). Analytical data are presented as the mean of three determinations.

Inhibition of enzymes by phlorotannins

Secretory phospholipase A_2s

The five phlorotannins, purified from *E. bicyclis*, were tested for inhibitory effects on both porcine pancreas $sPLA_2$ and bee venom $sPLA_2$. The phlorotannins and terrestrial polyphenols, except for catechin and phloroglucinol, were dose-dependent inhibitors of porcine pancreas $sPLA_2$ activity (Figure 2). The half-maximal inhibition (IC_{50}) values of eckol, phlorofucofuroeckol A, dieckol, 8,8′-bieckol, resveratrol and EGCG were 120, 130,160, 180, 170 and 110 μM, respectively. In the case of the bee venom $sPLA_2$ (Figure 2B), eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were more active than resveratrol, catechin and EGCG. In particular, the inhibitory effect of dieckol on $sPLA_2$ from bee venom showed a lasting tendency and even a concentration of 25 μM led to more than 30% inhibition. Resveratrol and EGCG had negligible or little inhibitory effect on bee venom $sPLA_2$, in contrast to porcine pancreas $sPLA_2$. IC_{50} values of eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were 100, 150, 90 and 180 μM, respectively.

Table 1. Effect of brown algal phlorotannins and terrestrial polyphenols against COX-1 and COX-2. Each value indicates an inhibition ratio (%) at 100 μM. All the analytical data in the table are presented as means of three determinations. ND; not detected.

Compound(s)	Inhibition (%)	
Polyphenols	COX-1	COX-2
Phloroglucinol	43.1	23.1
Eckol	52.4	43.2
Phlorofucofuroeckol A	19.9	ND
Dieckol	74.7	ND
8,8′-Bieckol	61.0	ND
Resveratrol	98.8	ND
Catechin	19.3	ND
Epigallocatechin gallate	46.0	35.1

Soybean lipoxygenase and 5-lipoxygenase

8,8′-Bieckol was the most effective inhibitor of soybean LOX and showed more than 90% inhibition at 50 μM (Figure 3A). The IC_{50} value of 8,8′-bieckol against the soybean LOX was 38 μM. Dieckol was more active than resveratrol and EGCG and caused 50% inhibition at 50 μM (Figure 3A). Further study was carried out using 5-LOX (human recombinant). Among the five phlorotannins of *E. bicyclis* tested, only 8,8′-bieckol had a pronounced inhibitory effect on the 5-LOX (IC_{50}: 24 μM) and was more effective than resveratrol or EGCG (Figure 3B).

Cyclooxygenase-1 and cyclooxygenase-2

Terrestrial polyphenols and phlorotannins were examined for their inhibitory effects on COX-1 and COX-2 (Table 1). Resveratrol is well known to be a COX-1 inhibitor (Jang et al. 1997) and showed 98.8% inhibition of COX-1 at 100 μM in this study. The phlorotannins had negligible or little inhibitory effect on either COX-1 or COX-2, except for an inhibitory effect of dieckol on COX-1 (74.7%). However, it is interesting that eckol showed 43.2% inhibition of COX-2 at 100 μM.

Discussion

This is the first study demonstrating an inhibitory effect of brown algal phlorotannins on $sPLA_2$s, soybean LOX, 5-LOX and COXs. Inhibitors of these enzymes could become leading compounds in the development of new nonsteroidal anti-inflammatory drugs. In recent studies, dietary factors such as polyphenols, resveratrol and catechins are known to have inhibition effect of these enzymes. Compared with terrestrial polyphenols, the phlorotannin oligomers; dieckol and 8,8′-bieckol showed pronounced inhibition of $sPLA_2$s and LOXs. $sPLA_2$s have been divided into groups I, II, III, V and X (Dennis 1997). The groups are 14 kDa proteins, and have a common active domain, His48, and a Ca^{2+} loop. Group I $sPLA_2$ (porcine pancreas and bee venom) inhibitors; dieckol and 8,8′-bieckol may act on groups X (macrophage) and II $sPLA_2$s, which are inflammation-induced enzymes. Nordihydroguiaretic acid, a natural product, inhibits the activities of soybean LOX and 5-LOX (Yasumoto et al. 1970; Komoda et al. 1995). The terrestrial polyphenols resveratrol, catechin and EGCG are known to inhibit not only soybean LOX (Tamagawa et al. 1999; Fan and Matthesis 2001) but also 5-LOX and COX (Maccarrone et al. 1999; Hong et al. 2001). There is homology in the amino acid sequences of the active sites of soybean LOX and 5-LOX (Shibata et al. 1987; Funk et al. 1989). Therefore, it may be possible for soybean LOX inhibitors to act on 5-LOX. It is worth noting that 8,8′-bieckol inhibited soybean LOX activity more effectively than did resveratrol and EGCG, and it specifically inhibited 5-LOX (human recombinant). COXs catalyze the conversion of arachidonic acid to prostaglandin H_2. In particular, inducible COX-2 is believed to be the target enzyme for inflammatory activity. Although dieckol and eckol had inhibitory effect on COX-1 and COX-2, respectively, other phlorotannins had negligible or little inhibitory effect on either COX-1 or COX-2.

Recently, we reported the inhibitory effect of the phlorotannins on hyaluronidase (Shibata et al. 2002b). Hyaluronidase is known to be involved in allergic effects and inflammation. The inhibitory effect of the phlorotannins was much stronger than that of anti-allergic drugs such as disodium chromoglycate. The results obtained in the previous and present studies suggest that phlorotannins have a potential as anti-inflammatory drugs and that the brown alga *E. bicyclis* may be a useful foodstuff with an anti-inflammatory activity.

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