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Isolation of Two Anti-inflammatory and One Pro-inflammatory Polyunsaturated Fatty Acids from the Brown Seaweed *Undaria pinnatifida*

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Two anti-inflammatory ω-3 polyunsaturated fatty acids (PUFAs) of stearidonic acid (SA) and eicosapentaenoic acid (EPA) and one pro-inflammatory ω-6 PUFA of arachidonic acid (AA) were isolated from the edible brown seaweed *Undaria pinnatifida*. SA was active against mouse ear inflammation induced by phorbol myristate acetate, with IC_{50} values of 160, 314, and 235 μg per ear for edema, erythema, and blood flow, respectively. EPA was also active against edema, erythema, and blood flow, with IC_{50} values of 230, 462, and 236 μg per ear, respectively. Although AA at low concentrations showed anti-inflammatory activities when measured 10 h later, AA doses of more than 243 μg per ear induced inflammatory symptoms 1 h later. Mature thalli generally had larger amounts of PUFAs than young thalli. The algal blade contained more ω-3 PUFAs than were found in other parts, while the holdfast contained extremely high amounts of AA. Late-season thalli showed increased amounts of PUFAs, especially AA.

KEYWORDS: Anti-inflammation; arachidonic acid; eicosapentaenoic acid; Phaeophyta; stearidonic acid; *Undaria pinnatifida*

INTRODUCTION

A number of seaweed species are consumed as food in various regions of the world. *Undaria pinnatifida* (Harvey) Suringer, commonly known as miyok in Korea and wakame in Japan, is an edible annual brown seaweed, growing up to 2 m long. In 2006, Korean aquaculture produced 305,000 tons (wet wt) (*1*). *U. pinnatifida* is popularly known as a health food among East Asian people. Almost all Korean women, even those who have migrated to other countries, consume miyok soup during a month-long postnatal period, as traditional belief holds that it helps the postnatal recovery, cleans the blood, and increases breast milk production. It has been used traditionally to treat fever, urination problems, lumps, and swelling (*2*). This seaweed is also used as an herbal medicine in China to treat urinary diseases, dropsy, stomach ailments, hemorrhoids, and fistulas (*3*).

Apart from its traditional uses, *U. pinnatifida* has been reported to alter uterine contraction (*4*) and hepatic fatty acid oxidation (*5*). In addition, *U. pinnatifida* inhibits eicosanoid production in MC/9 mouse mast cells (*6*); has a preventive effect on cerebrovascular diseases (*7*); exhibits antitumor (*8*), antiviral (*9*), and antihypertensive activities (*10*); and shows an anti-obesity effect (*11*).

In our previous study (*12*), a methanol extract of *U. pinnatifida* was demonstrated to display potent anti-inflammatory activities against phorbol myristate acetate (PMA)-induced mouse ear inflammation. In an attempt to identify the anti-inflammatory compounds from the seaweed, we isolated two polyunsaturated fatty acids (PUFAs) of stearidonic acid (SA) and eicosapentaenoic acid (EPA) that showed inhibitory effects against the inflammatory symptoms of edema, erythema, and blood flow. We also isolated one pro-inflammatory compound of arachidonic acid (AA) from *U. pinnatifida* thalli. By identifying large amounts of these anti/pro-inflammatory PUFAs from *U. pinnatifida*, we support claims that the seaweed has been used in health care and indigenous medicine as a remedy for inflammation-related symptoms.

MATERIALS AND METHODS

Algal Material. The brown seaweed *Undaria pinnatifida* (Harvey) Suringar (northern type) was harvested from Kijang aquaculture farm, Korea, in January 2005 and January 2006, with the voucher specimen deposited in our laboratory (Y. K. Hong). For convenience, the seaweed tissue was completely dried for 1 week at room temperature, and then ground to powder for 5 min using a coffee grinder. The powder was stored at −20 °C until use.

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Isolation of Anti- and Pro-inflammatory Compounds. To isolate the anti- and pro-inflammatory compounds from *U. pinnatifida* thalli, the algal powder (1.0 kg) was extracted three times with 17.5 L acetonitrile, and the crude extract was evaporated under vacuum to give a dark brown residue (4.8 g). The acetonitrile extract was chromatographed on a silica gel column (70−230 mesh, 22 g, Ø 4.5 cm × 40 cm) and successively eluted with 90 mL each of *n*-hexane, methylene chloride, acetonitrile, and methanol. The active methylene chloride eluent (1.9 g) was dried and dissolved in 4.75 mL of methanol for RP-HPLC. Each 300-μL (120 mg) aliquot was separated on a C18 column (10 mm i.d. × 25 cm) (Ultrasphere; Beckman Coulter, Fullerton, CA). The analysis was performed on a Waters 600 gradient liquid chromatograph (Waters, Milford, MA) monitored at 213 nm. The mobile phase consisted of two solvent systems: acetonitrile with 0.1% TFA and distilled water with 0.1% TFA. Elution was performed with a linear gradient of 0 to 100% v/v acetonitrile over 33 min for compound ATD-2 (stearidonic acid: SA), and with 100% v/v acetonitrile over 40 min for compounds ATD-4 (eicosapentanoic acid: EPA) and ATD-9 (arachidonic acid: AA), at a flow rate of 2 mL/min. Each eluted compound was dried under a stream of nitrogen gas.

Analytical Methods. The purified compounds were analyzed on a GC−MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and compared to the spectral data from the database. EIMS and HR-FABMS data were obtained from a JMS-700 spectrometer (JEOL, Tokyo, Japan) and a JMS HX 110 Tandem mass spectrometer (JEOL), respectively. The 1-D NMR (^{1}H, ^{13}C, and DEPT) and 2-D NMR (HMQC, HMBC, and COSY) spectra were taken on a JNM-ECP 400 NMR spectrometer (JEOL), using methanol-*d* (CD_3OD) for ATD-2, ATD-4, and ATD-9. The structures of the purified compounds were identified and confirmed to be identical to the spectral data in Fu et al. (*13*).

Inflammatory Bioassays. BALB/c mice (8−10 weeks old; 20−25 g body weight) were used for inflammatory assays. The animals were housed at 24 ± 1 °C on a 12-h light/dark cycle, with free access to food and water. Animal experiments were performed in accordance with the U.S. Institutional Animal Care and Use Committee Guidelines and national regulations concerning animal experiments, clinical studies, and biodiversity rights. Various concentrations of the purified compounds and indomethacin as a reference were prepared in 10 μL of 100% ethanol and applied topically to the whole inner side of the mouse ear. Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO; 0.2 μg in 10 μL of acetone) was applied topically to the same side of the ear 30 min later to allow absorption of anti-inflammatory compounds. Ear edema (swelling) was measured 10 h after the PMA application, using a spring-loaded micrometer (Mitutoyo Corp., Tokyo, Japan). The edema value was expressed as $(S_{10} - S_0)/S_0$, where S_{10} is the ear thickness 10 h after PMA application and S_0 is the ear thickness at 0 h. The edema value was 0.81 ± 0.04 with the ethanol vehicle. Ear erythema (redness) was determined at 10 h, using digital photography adjusted to balance white and Photoshop 7.0 (Adobe, San Jose, CA) to measure the magenta value. The erythema value was expressed as $(R_{10} - R_0)/R_0$, where R_{10} is ear redness 10 h after PMA application and R_0 is ear redness at 0 h. Local blood flow in the mouse ear was measured using laser speckle flowgraphy (Inflameter LFG-1; SoftCare, Fukuoka, Japan). The ear skin was scanned by moving the laser beam over the inner surface of the mouse ear. The distance between the scanner and ear surface was 0.5 cm. Blood flow was analyzed for a skin area 5 mm in diameter, after the method of Lee et al. (*14*). Blood flow was calculated as $(B_{10} - B_0)/B_0$, where B_{10} is blood flow 10 h following PMA application and B_0 is blood flow at 0 h.

Quantification of Anti- and Pro-inflammatory Compounds. To measure the amounts of anti- and pro-inflammatory compounds in *U. pinnatifida*, the thalli were completely dried in shade at room temperature for a week and then ground for 5 min to powder. The powder (0.4 g) was extracted with 8 mL of dichloromethane on a rotator for 1 h at 30 rpm. After centrifugation at 2000g for 5 min, 4 mL of the clean supernatant was evaporated to 5 mg/mL for RP-HPLC. Each 100-μL aliquot was separated on an Ultrasphere C18 column, using the same isolation procedure as that for SA, EPA, and AA. Each isolated compound was reconfirmed by ^{1}H and ^{13}C NMR. The amount of each compound was assessed by measuring the dimensions of HPLC peaks, using the standard curve of each pure compound.

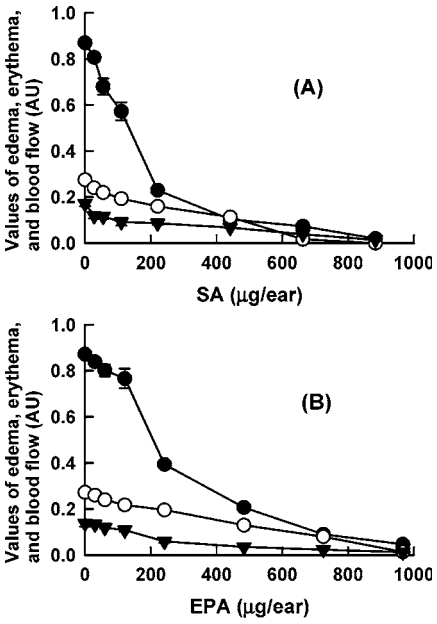

Figure 1. Anti-inflammatory activities of stearidonic acid (SA) and eicosapentaenoic acid (EPA) isolated from *U. pinnatifida*. Edema (●), erythema (○), and blood flow (▼) on mouse ear were measured with different concentrations of SA (A) and EPA (B) per ear. The indomethacin as a positive control yielded IC_{50} of 90, 172, and 179 μg/ear for edema, erythema, and blood flow, respectively. Values represent the mean ± SE ($n \geq 5$).

Statistical Analysis. The experiments were replicated at least seven times for each independent assay, and the highest and lowest values were discarded. The mean values of the indices were compared to the control using Student's *t*-test.

RESULTS

Identification of Compounds. Of the two anti-inflammatories, the ATD-2 compound was eluted at 98% (on 32.4 min) and the ATD-4 compound at 100% (on 33.6 min) acetonitrile by RP-HPLC. They appeared as oily compounds, weighing 8.4 mg and 9.2 mg, respectively, and yielding 8.4×10^{-4}% and 9.2×10^{-4}%, respectively, from the seaweed powder. A pro-inflammatory ATD-9 was eluted at 100% (on 35.5 min) acetonitrile, as an oily compound weighing 19.3 mg, which amounted to a 1.9×10^{-3}% yield. The GC−MS analyses of these compounds lead to the tentative identification of three alkenoic acids using the library of the GC−MS. These were considered as more than 90% similar to octadeca-tetraenoic acid, eicosa-pentaenoic acid, and eicosa-tetraenoic acid, respectively. The molecular composition of ATD-2 is $C_{18}H_{28}O_2$ on the basis of HR-FABMS data (negative mode, $[M - H]^-$ at *m*/*z* 275.2011), which indicated that ATD-2 contained five double-bond equivalents, comprising four carbon−carbon double bonds and one carbonyl carbon. The ^{1}H NMR spectrum revealed the presence of a methyl proton at δ_H 0.96 (H-18), eight methylene protons, and eight methine proton signals. From the ^{13}C NMR spectrum, we observed one carbonyl carbon (C-1), one methyl carbon at δ_C 14.6 (C-18), eight methylene carbons, and eight methine carbons. From the COSY spectrum, we determined the first double-bond position from the terminal methyl carbon (C-18). The terminal methyl proton (H-18), methylene proton (H-17), and methine proton (H-16) showed a series of COSY correlations with each other, demonstrating that the first double

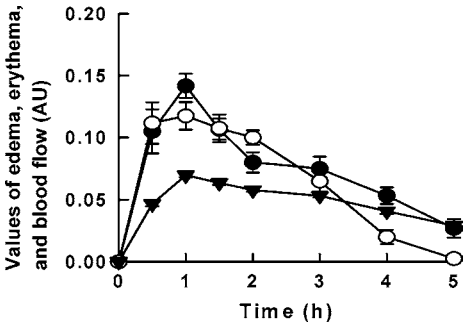

Figure 2. Pro-inflammatory activities of arachidonic acid (AA) on mouse ear. Edema (●), erythema (○), and blood flow (▼) were measured with 100 mM AA. Values represent the mean ± SE ($n \geq 5$).

Table 1. Amount of Stearidonic Acid (SA), Eicosapentaenoic Acid (EPA), and Arachidonic Acid (AA) Extracted from Different Parts of *U. pinnatifida* Thalli[a]

	mature thalli			young thalli		
	SA	EPA	AA	SA	EPA	AA
injured blade	329 ± 4	218 ± 4	447 ± 4	200 ± 4	132 ± 4	551 ± 9
blade	730 ± 12	433 ± 19	930 ± 19	673 ± 13	365 ± 6	744 ± 16
midrib	211 ± 7	204 ± 6	154 ± 9	122 ± 4	79 ± 4	762 ± 11
sporophyll	0 ± 0	36 ± 4	261 ± 9	ND	ND	ND
holdfast	11 ± 0	72 ± 4	2394 ± 12	50 ± 4	200 ± 4	1263 ± 4

[a] Data are microgram amounts of the mean ± SE ($n \geq 5$) from 1 g dry weight of thalli. ND = not determined.

bond was at the third position. From these spectral data, we identified ATD-2 as octadeca-6,9,12,15-tetraenoic acid (C18:4 ω-3), stearidonic acid (SA), or moroctic acid. We identified the molecular composition of ATD-4 as $C_{20}H_{30}O_2$ from the HR-FABMS (negative mode, $[M - H]^-$ at *m/z* 301.2168), which indicated that ATD-4 contained six double-bond equivalents, comprising five carbon−carbon double bonds and one carbonyl carbon. The ^{1}H NMR spectrum revealed the presence of a methyl proton at δ_H 0.90 (H-20), eight methylene protons, and ten methine protons. The ^{13}C NMR spectrum revealed one carbonyl carbon at δ_C 174.9 (C-1), one methyl carbon at δ_C 15.0 (H-20), eight methylene carbons, and ten methine carbons. From these data, ATD-4 was identified as eicosa-5,8,11,14,17-pentaenoic acid (EPA) (C20:5 ω-3), or timnodonic acid. The molecular composition of ATD-9 is $C_{20}H_{32}O_2$ based on the HR-FABMS (negative mode, $[M - H]^-$ at *m/z* 303.2324). These data indicated that ATD-9 contained five double-bond equivalents, comprising four carbon−carbon double bonds and one carbonyl carbon. The ^{1}H NMR spectrum revealed the presence of a methyl proton at δ_H 0.83 (H-20), ten methylene protons, and eight methine proton signals. From the ^{13}C NMR spectrum, we observed one carbonyl carbon (C-1), one methyl carbon at δ_C 14.8 (C-20), eight methylene carbons, and eight methine carbons. From the COSY and HMBC spectra, we determined the first double-bond position from the terminal methyl carbon (C-20). The terminal methyl proton (H-20) showed a COSY correlation to H-19 and a HMBC correlation to C-18, and the proton-attached C-18 (H-18) showed a HMBC correlation to C-17. Two methylene protons (H-16, H-17) and a methine proton (H-15) showed a series of COSY correlations with each other, demonstrating that the first double bond was at the sixth position. From these spectral data, ATD-9 was identified as eicosa-5,8,11,14-tetraenoic acid (C20:4 ω-6), or arachidonic acid (AA).

Anti- and Pro-inflammatory Activities. Purified compounds of SA and EPA were tested for anti-inflammatory activities against the PMA-induced mouse ear inflammation symptoms of edema, erythema, and blood flow. The inhibitory effects of different concentrations of SA and EPA topically applied to mouse ears were dose-dependent. The SA concentrations producing 50% inhibition (IC_{50}) were 160, 314, and 235 μg per ear for edema, erythema, and blood flow, respectively (**Figure 1A**). The EPA concentrations producing IC_{50} were 230, 462, and 236 μg per ear for edema, erythema, and blood flow, respectively (**Figure 1B**). The topical application of indomethacin as a positive control significantly decreased the PMA-induced inflammation and yielded IC_{50} of 90, 172, and 179 μg per ear for edema, erythema, and blood flow, respectively. Thus, pure SA and EPA showed almost half the anti-inflammatory activity of indomethacin. Additionally, when purified AA without PMA was topically applied to mouse ears, edema, erythema, and blood flow reached maximal values 1 h later (**Figure 2**). Low AA concentrations showed anti-inflammatory activities against PMA-induced edema, erythema, and blood flow when measured 10 h later (data not shown). Concentrations >243 μg per ear demonstrated pro-inflammation effects.

Amount of Anti- and Pro-inflammatory Compounds in Thalli. We examined the amounts of SA, EPA, and AA in different parts of *U. pinnatifida* thalli. A mature thallus consists of a pinnately divided blade with midrib, a mature undulated sporophyll, and a fibrous holdfast. The blade part contained the highest amounts of ω-3 PUFAs: SA and EPA (**Table 1**). The mature sporophyll had very low amounts of SA, EPA, and AA, whereas holdfasts from mature and young thalli contained extremely high amounts of ω-6 AA, with 2.4 and 1.3 mg, respectively, per 1 g dry powder. Mature thalli (~1 m in height) generally had higher amounts of SA, EPA, and AA than young thalli (~20 cm in height). Additionally, four representative local types of *U. pinnatifida* from the east (Kijang), west (Taean), south (Wando) seas, and Japan (Sanriku) sea were cultured at Kijang aquaculture farm. The amounts of SA, EPA, and AA from mature blades of each local type were measured at different collection times during the harvest season. The Taean and Sanriku types generally had more SA, EPA, and AA (**Table 2**). The Taean cultivar showed relatively high amounts of ω-3 PUFAs (SA and EPA) compared to ω-6 AA at the 11 January harvest. Thalli collected late in the season had higher amounts

Table 2. Amount of Stearidonic Acid (SA), Eicosapentaenoic Acid (EPA), and Arachidonic Acid (AA) from Blades of Different Local Types of *U. pinnatifida*[a]

	harvested on 11 Jan. 2006			harvested on 18 Jan. 2006			harvested on 26 Jan. 2006		
local types	SA	EPA	AA	SA	EPA	AA	SA	EPA	AA
Kijang, Korea	712 ± 7	411 ± 4	873 ± 7	1227 ± 7	733 ± 7	1699 ± 4	712 ± 7	569 ± 6	1528 ± 25
Taean, Korea	973 ± 4	1062 ± 22	819 ± 4	1943 ± 6	1306 ± 4	2504 ± 7	1173 ± 9	758 ± 7	1696 ± 12
Wando, Korea	261 ± 7	229 ± 9	623 ± 11	1055 ± 4	948 ± 7	969 ± 7	1692 ± 9	1084 ± 12	2097 ± 13
Sanriku, Japan	1231 ± 9	991 ± 13	1617 ± 4	1767 ± 9	1374 ± 6	2751 ± 7	1012 ± 9	737 ± 9	1953 ± 11

[a] All cultured at Kijang Aquaculture Farm, Korea, and harvested at different times. Data are microgram amounts of the mean ± SE ($n \geq 5$) from 1 g dry weight of blades.

of SA, EPA, and AA, especially the latter. Generally, *U. pinnatifida* gathered early in the harvest season yielded more ω-3 PUFAs than ω-6 PUFA.

DISCUSSION

From the *U. pinnatifida* extract, we isolated two ω-3 PUFAs, SA and EPA, with anti-inflammatory activity and one ω-6 PUFA, AA, with pro-inflammatory activity. The two anti-inflammatory fatty acids reduced edema, erythema, and blood flow potently. The use of this seaweed as a cure for fever, urination problems, lumps, and swelling is recorded in the Oriental medical textbook *Donguibogam*, published in 1613 (*2*). As an herbal medicine in China, *U. pinnatifida* has been used to treat urinary diseases and dropsy (*3*). The seaweed is also known to contain SA, which inhibits leukotriene production in inflammation (*6*). Most of these effects are directly or indirectly related to the anti-inflammatory action of the seaweed. Furthermore, the ω-3 PUFA of SA is reported as a 5-lipoxygenase inhibitor (*15*). EPA also suppresses inflammation and is associated with a reduction in arachidonic acid levels (*16*). Ear inflammations induced by arachidonic acid and ultraviolet-B irradiation were significantly suppressed in mice at a dose of 300 mg EPA per kg body weight per day for 2 weeks (*17*). SA and EPA inhibit UV-induced dermal fibroblasts (*18*), leukocyte−endothelial interactions (*19*), and inflammatory mediator release in blood and splenocytes of mice (*20*). Supplementing with 50−100 μg/mL ω-3 PUFAs reduces the expression and activity of aggrecanases and inflammation-inducible cytokines (interleukin-1α and tumor necrosis factor-α) and cyclooxygenase-2 (*21*). However, they had no effect on constitutively expressed cyclooxygenase-1. Thus, these findings for SA and EPA from *U. pinnatifida* reinforce the claims of the healthcare industry and indigenous medicine that the seaweed can be used as a remedy for inflammation-related symptoms. In addition, the amounts of SA and EPA in *U. pinnatifida* can be used as criteria for quality assessment of the seaweed products and strain improvement. However, this seaweed also contains AA, a pro-inflammatory compound. Serhan (*22*) found that AA is not only a precursor to pro-inflammatory lipid mediators but can also be converted to anti-inflammatory lipid mediators, such as the lipoxins, in the resolution phase. In this study, we also observed anti-inflammatory activities of AA at low concentrations against edema, erythema, and blood flow when measured 10 h later, but not 1 h later.

Eskra et al. (*23*) quantified leukotrienes and hydroxyeicosatetraenoic acids in biological samples using RP-HPLC. We modified the solvent extraction to enhance the purity of PUFAs from *U. pinnatifida* and quantified yields using RP-HPLC. Mature thalli of *U. pinnatifida* contained greater amounts of SA, EPA, and AA than young thalli. Generally, young thalli produce more primary metabolites for fast growth, whereas mature thalli have more mechanisms to modify biocompounds or protect cellular functions (*24*). *U. pinnatifida* is a common brown seaweed along temperate coastal regions of the northeast Pacific, including Korea, Japan, and northern China (*25*). Currently, it occurs in temperate regions worldwide as an invasive species (*26*). We plan to increase the amount of ω-3 PUFAs and reduce ω-6 PUFA by modifying after-harvest processing.

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