JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Isolation and Characterization of a Phenolic Antioxidant from the Pacific Oyster (*Crassostrea gigas*)

Mitsugu Watanabe,[†,‡,§] Hirotoshi Fuda,[†,∥] Shigeki Jin,[†,∥] Toshihiro Sakurai,[∥] Futaba Ohkawa,[∥] Shu-Ping Hui,[∥] Seiji Takeda,[∥] Takayuki Watanabe,[§] Takao Koike,[‡] and Hitoshi Chiba*[,∥]

[‡]Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan

[§]Watanabe Oyster Laboratory Company Limited, 490-3, Shimo-ongata-cho, Hachioji, Tokyo 190-0154, Japan

[∥]Faculty of Health Sciences, Hokkaido University, Kita-12, Nishi-5, Kita-ku, Sapporo 060-0812, Japan

ABSTRACT: Using an oxygen radical absorbance capacity (ORAC) assay, antioxidant activity was detected in the ethanol extract of the Pacific oyster, which was purified by sequential extraction with organic solvents. The ethyl acetate fraction showed the strongest antioxidant activity and was further purified, yielding a single compound [as assessed by thin-layer chromatography (TLC) and reverse-phase high-performance liquid chromatography (HPLC)]. This compound was identified as 3,5-dihydroxy-4-methoxybenzyl alcohol on the basis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR), heteronuclear multiple-bond correlation (HMBC), and electrospray ionization−mass spectrometry (ESI−MS) spectral analyses, a conclusion that was confirmed by chemical synthesis. The concentration of the compound was 6.7 mg/100 g of whole oyster meat wet weight. This amphiphilic antioxidant retarded the copper-mediated oxidation of low-density lipoproteins (LDLs) and the generation of thiobarbituric acid reactive substances. Furthermore, the compound showed substantial antioxidant activity using the ORAC and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays compared to natural antioxidants. Although the same compound was previously found in brown algae, its presence in other organisms and antioxidant activity are reported here for the first time.

KEYWORDS: *LDL, low-density lipoproteins, ORAC, DPPH, reactive oxygen species, ROS, polyphenol*

INTRODUCTION

Growing evidence indicates that oxidative stress contributes to the degradation of biological molecules and that antioxidants may prevent or retard the progression of oxidation-related diseases, such as cardiovascular diseases,[1] inflammatory diseases,[2] cancers,[3] and neurological disorders, including Alzheimer's disease,[4] Huntington's disease,[5] and Parkinson's disease.[6] Natural phenolic compounds, such as flavonoids and anthocyanins, are known to have high antioxidant activity,[7] with some used as nutraceuticals and pharmaceuticals.[8] They are mostly extracted and purified from terrestrial plants, and marine organisms have not been extensively surveyed.[9] Recent studies have focused on antioxidants from marine organisms, where crude extracts rather than isolated antioxidants were generally investigated. A few studies have reported isolated and chemically identified phenolic antioxidants from natural marine organisms, such as marine sponges.[10]

The Pacific oyster (*Crassostrea gigas*) is a marine bivalve originating from Japan, where it has been farmed since the 1600s. It is the most popular and industrially important oyster in the world because it is easy to grow and spread and is environmentally tolerant.[11] However, relatively few studies have described antioxidants from oysters, and many of them have focused on antioxidant enzymes or proteins, such as superoxide dismutase,[12] catalase,[13] glutathione peroxidase,[13] glutathione *S*-transferase 3,[14] metallothionein 2,[15] and peroxiredoxin 6.[16] A few studies have described small-molecule antioxidants from oysters, including ascorbic acid,[17] α-tocopherol,[18] glutathione,[19] and carotenoids.[20]

In this study, a novel antioxidant with a polyphenolic structure was identified in the Pacific oyster, and its antioxidant activity was evaluated against copper-mediated oxidation of low-density lipoproteins (LDLs) and by an oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The results show the potential utility of this compound in protecting against reactive oxygen species (ROS).

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade or better. The following compounds were obtained commercially: hexane, chloroform, ethyl acetate, butyl alcohol, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), DPPH, L-ascorbic acid, chlorogenic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan); Trolox and fluorescein sodium salt (Sigma-Aldrich Co., St. Louis, MO); and thin-layer chromatography (TLC) plates (silica gel 60 F_{254}) (Merck Chemicals, Tokyo, Japan).

Apparatus. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured at 500 and 126 MHz with an AMX-500 instrument (Bruker, Rheinstetten, Germany). Mass spectrometry (MS) data were obtained by electrospray ionization−time-of-flight−mass spectrometry (ESI−TOF−MS) with a JMS-T100CS mass spectrometer (JEOL, Tokyo, Japan). ESI−TOF−MS was performed with the following settings: needle voltage, 2.0 kV; orifice voltage, 45 V; desolvation temperature, 80 °C; sample flow rate, 10 μL/min; and solvent, water/acetonitrile (1:1). Ultraviolet (UV) spectra were measured using a V-530 instrument (JASCO, Tokyo, Japan).

Received: September 22, 2011
Revised: December 15, 2011
Accepted: December 19, 2011
Published: December 19, 2011

Separation of Oyster Extracts. After oyster meat (3 kg) was boiled for 10 min in water, the meat was removed and the resulting broth (1 L) was precipitated through the addition of 70% ethanol. A supernatant was collected after centrifugation at 10 000 rpm for 10 min, and an aliquot of the supernatant (100 mL) was concentrated *in vacuo* until a sufficient amount of alcohol had evaporated. After concentration, the residue was dissolved in distilled water and extracted twice with hexane. The water layer was then extracted with chloroform, ethyl acetate, and butyl alcohol (in that order). The ethyl acetate extracts were separated into 11 fractions by TLC [SiO_2; elution with ethyl acetate/chloroform (2:1)] with the help of UV absorbance. The R_f values for the different fractions were as follows: 1st fraction, 0.92−1.0; 2nd fraction, 0.86−0.92; 3rd fraction, 0.77−0.86; 4th fraction, 0.69−0.77; 5th fraction, 0.56−0.69; 6th fraction, 0.44−0.56; 7th fraction, 0.32−0.44; 8th fraction, 0.24−0.32; 9th fraction, 0.17−0.24; 10th fraction, 0.09−0.17; and 11th fraction, 0.0−0.09.

ORAC Assay. The ORAC of isolated and synthetic antioxidants was evaluated according to the modified ORAC assay described by Ou et al.[21] A mixture consisting of 20 μL of sample or Trolox in 75 mM phosphate buffer (pH 7.4) containing 200 μL of 9.44×10^{-8} M fluorescein solution and 75 μL of 3.17×10^{-2} M AAPH solution was incubated at 37 °C for 1.5 h in a Wallac 1420 ARVO Mx plate reader (PerkinElmer, Tokyo, Japan) or a FP-6500 spectrofluorometer (JASCO). The fluorescence intensity was measured every 10 s, with excitation at 493 nm and emission at 515 nm.

To measure ORAC values, 75 mM phosphate buffer was used as a blank and 6.25, 12.5, 25, and 50 μM Trolox solutions were applied as standards. One ORAC unit was the net protection area provided by 1 μM Trolox (final concentration). Sample ORAC values were calculated on the basis of a Trolox standard curve. The relative ORAC value (Trolox equivalents) was calculated as follows: relative ORAC value = molarity of Trolox/molarity of the sample.

Isolation of the Antioxidant. The 6th TLC fraction of the ethyl acetate extracts was purified by high-performance liquid chromatography (HPLC) with a CAPCELL PAC C18 reversed-phase column [inner diameter, 250 × 4.6 mm; SHISEIDO, Tokyo, Japan; elution with acetonitrile/water (5:95); temperature, 30 °C; flow rate, 1.0 mL/min; and detection, UV at 270 nm]. The antioxidant was detected at a t_R of 9.5 min, with a yield of 3.0 mg from 160 mL of the extracted oyster solution. The isolated antioxidant yielded the following spectral data: ^{1}H NMR (500 MHz, acetone-d_6) δ_H: 7.82 (2H, br s, phenolic−OH), 6.40 (2H, s, H-2,6), 4.42 (2H, s, H-1′), 3.94 (1H, br s, −OH), 3.79 (3H, s, −OMe). ^{13}C NMR (126 MHz, acetone-d_6) δ_C: 151.1 (C-3,5), 139.4 (C-1), 135.1 (C-4), 106.5 (C-2,6), 64.5 (C-1′), 60.6 (−OMe). ESI−TOF−MS, m/z 153.05451 $[M - OH]^+$ (calculated for $C_8H_8O_3$, 153.05517), 171.06911 $[M + H]^+$ (calculated for $C_8H_{11}O_4$, 171.06573). UV (ethanol), λ_{max} 270 nm.

Synthesis of 3,5-Dihydroxy-4-Methoxybenzyl Alcohol. The antioxidant was synthesized from methyl gallate by a two-step reaction, as described previously.[22] The compound obtained after the first step, methyl 3,5-dihydroxy-4-methoxybenzoate, yielded the following spectral data: ^{1}H NMR (500 MHz, methanol-d_4) δ_H: 7.01 (2H, s, H-2,6), 3.85 (3H, s, −OMe), 3.82 (3H, s, −OMe). ^{13}C NMR (126 MHz, methanol-d_4) δ_C: 168.5 (−C═O), 151.7 (C-3,5), 141.2 (C-1), 126.5 (C-4), 110.1 (C-2,6), 60.7 (−OMe), 52.5 (−OMe). ESI−MS, m/z 199 $[M + H]^+$, 197 $[M - H]^-$. The target compound obtained after the second step, 3,5-dihydroxy-4-methoxybenzyl alcohol, yielded the following spectral data: ^{1}H NMR (500 MHz, methanol-d_4) δ_H: 6.36 (2H, s, H-2,6), 4.39 (2H, s, H-1′), 3.76 (3H, s, −OMe). ^{13}C NMR (126 MHz, methanol-d_4) δ_C: 151.6 (C-3,5), 138.7 (C-1), 136.0 (C-4), 107.3 (C-2,6), 65.1 (C-1′), 60.8 (−OMe).

DPPH Assay. The relative DPPH value was determined using a modified method by Brand-Williams et al.[23] A mixture consisting of 100 μL of sample or Trolox in 75 mM phosphate buffer (pH 7.4) containing 900 μL of 75 mM phosphate buffer (pH 7.4) and 1 mL of 300 μM DPPH solution in 100% ethanol was incubated at room temperature in the dark for 30 min. The decrease in absorbance of the resulting solution was measured at 517 nm using a V-530 UV−vis spectrophotometer (JASCO).

To measure DPPH values, 75 mM phosphate buffer was used as a blank and 25, 50, and 100 μM Trolox solutions were applied as standards. Sample DPPH values were calculated on the basis of a Trolox standard curve. The relative DPPH value (Trolox equivalents) was calculated as follows: relative DPPH value = molarity of Trolox/molarity of the sample.

Copper-Mediated Oxidation of LDL. The LDL fraction was isolated from human serum by sequential ultracentrifugation, as described previously.[24] Briefly, ultracentrifugation was performed using an Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA) with a MLN-80 near-vertical tube rotor (Beckman Coulter). Serum (2 mL) was adjusted with potassium bromide (KBr) solution to d = 1.019 kg/L and then centrifuged at 40 000 rpm for 20 h at 4 °C. After the removal of the upper fraction (2.5 mL) containing d < 1.019 lipoproteins (chylomicrons, very low-density lipoproteins, and intermediate-density lipoproteins), the lower fraction was adjusted with KBr solution to d = 1.063 kg/L and centrifuged again at 50 000 rpm for 18 h at 4 °C. The upper fraction (2.5 mL) containing LDL was recovered, and its protein concentration was measured by a modified Lowry method. Lipoprotein separation was confirmed by polyacrylamide gel electrophoresis (LipoPhor; Jokoh Co., Ltd., Tokyo, Japan) (data not shown).

To prepare copper-oxidized LDL (oxLDL), the isolated LDL fraction was dialyzed against phosphate-buffered saline (PBS) for 16 h at 4 °C. The LDL was adjusted to a final protein concentration of 0.35 mg/mL. The synthesized antioxidant was then added at a concentration of 0−540 μM, and oxidization initiated through incubation at 37 °C for 0−6 h in the presence of 3.2 μM copper sulfate ($CuSO_4$). The resulting solution was immediately subjected to measurement of thiobarbituric acid reactive substances (TBARS). Briefly, the reactive solution was added to the reagent mixture in the TBARS kit and then boiled for 1 h at 100 °C. After cooling on ice, the resulting solution was centrifuged at 12 000 rpm for 10 min at room temperature to remove the aggregates. The fluorescence intensity of the supernatant was measured using a FP-6500 spectrofluorometer, with excitation at 530 nm and emission at 550 nm.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD) and then analyzed by the t test using Prism 5.03 (GraphPad, San Diego, CA). The significance level was set to $p < 0.05$.

RESULTS

Isolation of the Antioxidant. The 70% ethanol oyster extract was further separated by sequential liquid−liquid extractions using hexane, chloroform, ethyl acetate, and butyl alcohol (Figure 1). Each separated extract was tested for antioxidant activity by a modified ORAC method. As shown in Figure 1, the ethyl acetate extract exhibited the strongest antioxidant activity. It was fractionated further by TLC and then analyzed by the ORAC assay (Figure 2). The 6th and 11th fractions exhibited the strongest antioxidant activity. The 6th fraction contained one compound, and the 11th fraction contained three compounds. The weight of the 11th fraction was 3.2 times higher than that of the 6th fraction. The compound of the 6th TLC fraction, which exhibited the strongest antioxidant activity, was further purified by HPLC, yielding a single peak. The concentration of the antioxidant, as tentatively nominated as E6, was determined by normal-phase HPLC to be approximately 6.7 mg/100 g of whole oyster meat wet weight.

Structural Analyses. The ^{1}H NMR spectrum of E6 in acetone-d_6 showed a simple signal pattern comprising of five singlet signals. The signal at δ = 3.79 ppm was characteristic of a methoxy group connected to a double-bonded carbon, as found in carbonyl, vinylene, and aromatic groups. The two broad signals at δ = 7.82 and 3.94 ppm were derived from hydroxy groups, and the signal at δ = 6.40 ppm was derived from a vinylene or aromatic group. In the ^{13}C NMR spectrum,

dx.doi.org/10.1021/jf2038532 | J. Agric.Food Chem. 2012, 60, 830−835

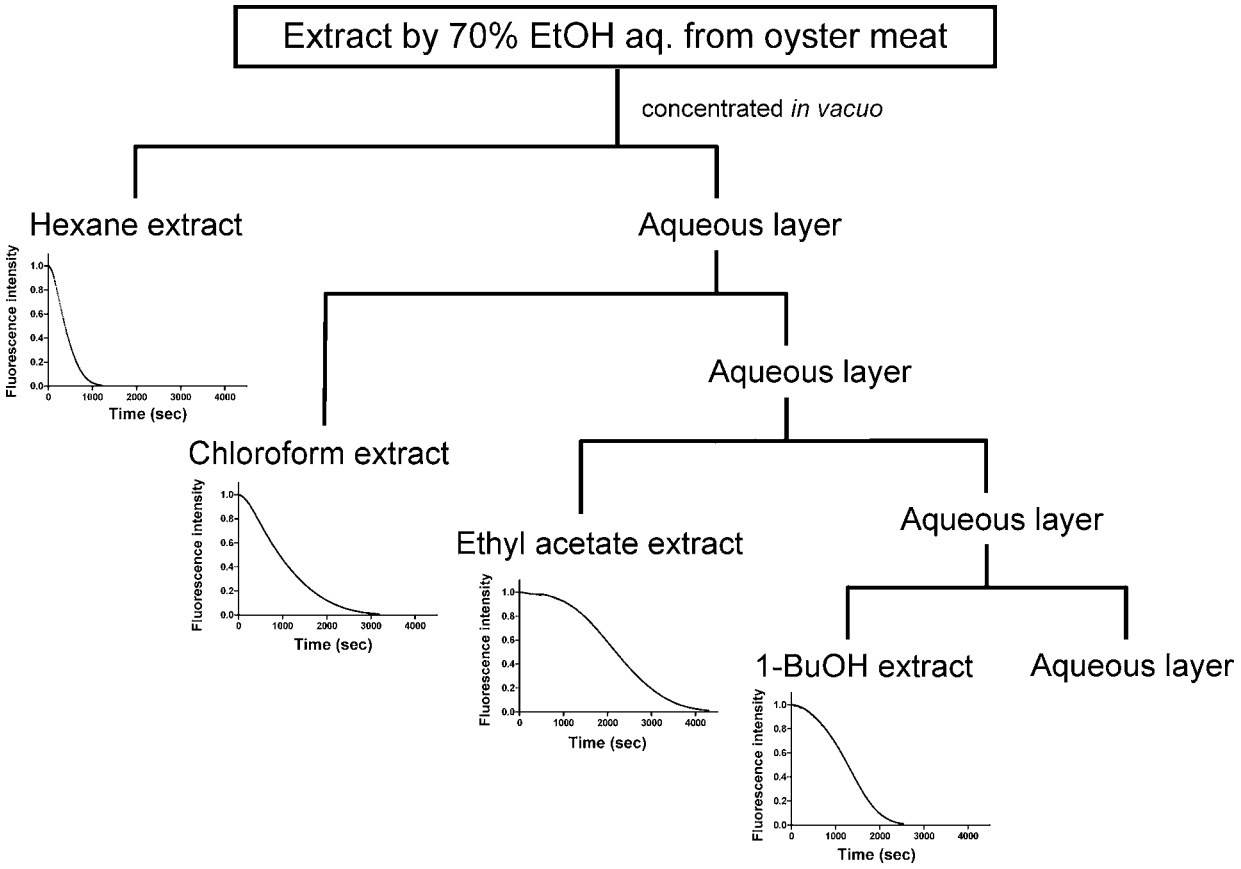

Figure 1. Scheme for the extraction of the antioxidant, together with antioxidant assay data. Antioxidant activity is represented as the length the fluorescence intensity at the beginning of the assay that was maintained. A longer time indicates stronger activity.

the four signals at δ = 106−151 ppm suggested the presence of an aromatic group, a conclusion supported by the local maximum UV absorption of 270 nm. Furthermore, the aromatic group was suggested to have a symmetrical structure because only four signals were observed. Accordingly, the heteronuclear multiple-quantum coherence (HMQC) measurement indicated a 4-substituted benzene and a hydroxymethyl group, suggesting the structure of E6 as 3,5-dihydroxy-4-methoxybenzyl alcohol (Figure 3). The spectral data and the chromatographic behaviors of E6 corresponded exactly to those for the synthetic compound.

Effect on Copper-Mediated Oxidation of LDL. To evaluate the influence of E6 on LDL oxidation, the lag time was estimated from the fast increase in the TBARS level. As shown in Figure 4, the lag time was prolonged in a dose-dependent manner by concentrations of the synthetic compound in the range of 180−540 μM.

Antioxidant Activity of E6 Using ORAC and DPPH Assays. As shown in Table 1, both the relative ORAC value and the relative DPPH value for E6 were similar to that for the synthetic compound, although a small discrepancy possibly because of the impurity of E6 was observed. The relative ORAC for E6 was higher than those for Trolox and L-ascorbic acid and lower than that for chlorogenic acid, and the relative DPPH value of E6 was lower than that for chlorogenic acid.

DISCUSSION

3,5-Dihydroxy-4-methoxybenzyl alcohol was first isolated from a brown alga (*Leathesia nana*),[25] but its biological function, such as antioxidant activity, has not been previously described. Additionally, this compound was only described as a synthetic intermediate in the synthesis of the phenolic compounds (±)-galanthamion,[22] gadusol,[26,27] and 4-methoxycoumarin[28] but was not investigated biologically in these studies either.

In the present study, the relative ORAC value for 3,5-dihydroxy-4-methoxybenzyl alcohol was 1.24 for the isolated compound (E6) and 1.47 μmol of TE/μmol for the synthetic compound. Also, the DPPH values for isolated and synthetic compounds were 1.55 and 1.78 μmol of TE/μmol, respectively. We think that the latter is more accurate than the former considering the possible impurity of E6. Basically, the ORAC measures a fluorescent signal from a probe that decreases or is "quenched" in the presence of a radical generator, AAPH. The addition of an antioxidant absorbs the general radical, thereby allowing for the fluorescent signal to persist.[21] On the other hand, the stable radical of DPPH absorbs at 515 nm. When DPPH was reduced by an antioxidant, the absorption disappears.[23] Hence, the antioxidant activity of 3,5-dihydroxy-4-methoxybenzyl alcohol depends upon its radical scavenging action.

5-Dihydroxy-4-methoxybenzyl alcohol showed a dramatic antioxidant effect against the copper-mediated oxidation of LDL. Oxidation of LDL is one of the key events in the pathogenesis of atherosclerosis. LDL is known to infiltrate arterial walls and to be oxidized to oxLDL in vascular cells. The oxLDL can be taken up by macrophages to form foam cells and develop atherosclerosis.[1] LDL contains many endogenous antioxidants, including α-tocopherol, β-carotene, and retinyl esters. These lipophilic antioxidants are mainly derived from the diet and are known to localize to the LDL core.[29] The major natural antioxidants found in LDL are α-tocopherol, with a reported

dx.doi.org/10.1021/jf2038532 | J. Agric.Food Chem. 2012, 60, 830−835

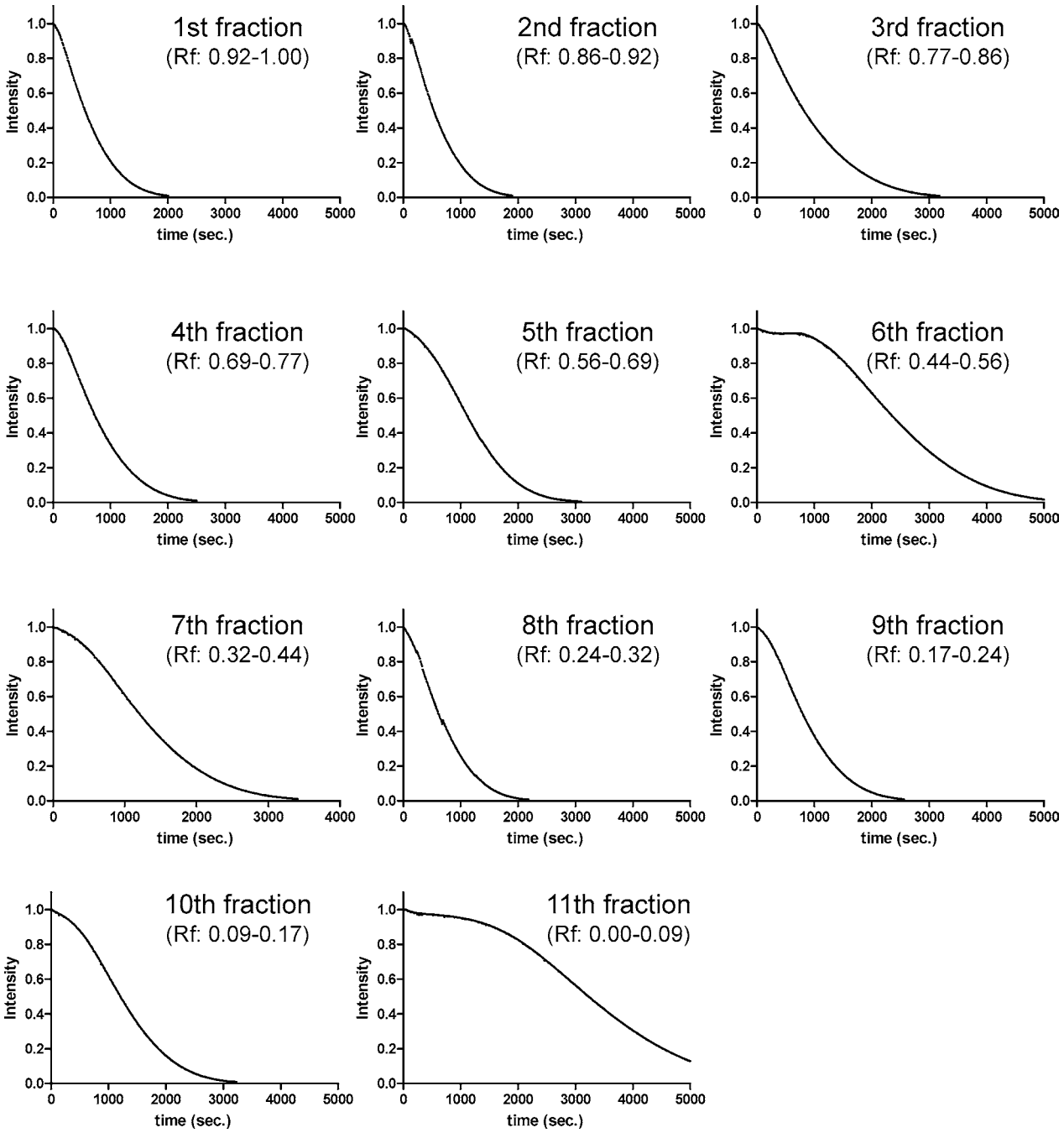

Figure 2. Antioxidant assay of the TLC fractions. TLC and the antioxidant assay are described in the Materials and Methods.

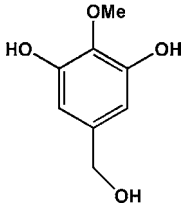

Figure 3. Structure of 3,5-dihydroxy-4-methoxybenzyl alcohol.

concentration of 11.58 nmol/mg of LDL protein, and β-carotene, with a lower concentration.[1,30] The concentration of 5-dihydroxy-4-methoxybenzyl alcohol in LDL, when ingested orally, remains to be elucidated.

3,5-Dihydroxy-4-methoxybenzyl alcohol belongs to a group of polyphenols with more than two hydroxy groups attached to a benzene ring;[31] this group of polyphenols is widely distributed in vegetables, fruits, nuts, and beverages.[7] Polyphenols can largely be divided into flavonoids and phenolic acids according to the number of benzene rings,[32] and because 3,5-dihydroxy-4-methoxybenzyl alcohol carries only a single benzene ring, it is a phenolic acid.

According to our estimate, the content of 3,5-dihydroxy-4-methoxybenzyl alcohol is approximately 6.7 mg/100 g of whole oyster meat wet weight. Previous reports of non-enzymatic antioxidants in oysters have been limited to α-tocopherol, whose concentration was reported to be 0.624 mg/100 g of whole oyster meat wet weight, much lower than that of the

dx.doi.org/10.1021/jf2038532 | J. Agric.Food Chem. 2012, 60, 830–835

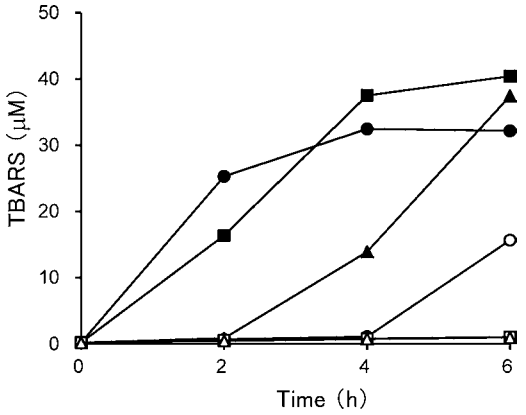

Figure 4. Inhibition of copper-mediated LDL oxidation by 3,5-dihydroxy-4-methoxybenzyl alcohol. The LDL fraction was oxidized by incubating with copper sulfate for 0−6 h at 37 °C in the presence of 0−540 μM of the antioxidant and was measured for TBARS. Antioxidant concentration: (●) 0 μM, (■) 180 μM, (▲) 270 μM, (○) 360 μM, (□) 450 μM, and (△) 540 μM. Each symbol represents three independent experiments, all of which produced similar results.

Table 1. Antioxidant Activity of Hydrophilic Compounds Using ORAC and DPPH Assays

antioxidant	relative ORAC value[a] (μmol of TE/μmol)	relative DPPH value[b] (μmol of TE/μmol)
chlorogenic acid	4.57 ± 0.30[c]	2.65 ± 0.20[d]
synthetic compound	1.47 ± 0.40	1.78 ± 0.16
isolated compound	1.24 ± 0.35	1.55 ± 0.09
Trolox	1	1
L-ascorbic acid	0.53 ± 0.13[e,f]	1.45 ± 0.09

[a]Relative ORAC values are expressed as Trolox equivalent (TE) (average ± SD; n = 4).[21] [b]Relative DPPH values are expressed as Trolox equivalent (TE) (average ± SD; n = 4).[23] [c]$p < 0.001$ versus synthetic or isolated compound. [d]$p < 0.001$ versus synthetic or isolated compound. [e]$p < 0.005$ versus synthetic compound. [f]$p < 0.01$ versus isolated compound.

present compound.[18] 3,5-Dihydroxy-4-methoxybenzyl alcohol may be the major non-enzymatic antioxidant in the Pacific oyster and may possibly protect oysters from environmental stress. Once oysters attach to a substrate, such as a rock, at the fully developed larval stage, they cannot move from that place anymore during their lifetime. Therefore, they are more frequently exposed than is generally thought to a variety of natural and anthropogenic stressors, including fluctuations in temperature, salinity, oxygen content, and pollution.

In conclusion, 3,5-dihydroxy-4-methoxybenzyl alcohol identified in the Pacific oyster extract might be a candidate antioxidant for the protection of humans from oxidative stress. Its metabolism and clinical usefulness should be studied further.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-11-706-3698. E-mail: chibahit@med.hokudai.ac.jp.

Author Contributions

[†]These authors contributed equally to this work.

Funding

This work was supported by Sapporo Biocluster "Bio-S", The Regional Innovation Cluster Program, The Ministry of Education, Culture, Sports, Science, and Technology, Japan, and the Foundation for Scientific Research at the Research Institute of Personalized Health Science of Hokkaido Health Sciences University, Japan. Toshihiro Sakurai was supported by a grant from the Japan Society for the Promotion of Science.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; ESI−TOF−MS, electrospray ionization−time-of-flight−mass spectrometry; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; TLC, thin-layer chromatography; DPPH, 2,2-diphenyl-1-picrylhydrazyl

REFERENCES

(1) Stocker, R.; Keaney, J. F. Role of oxidative modifications in atherosclerosis. *Physiol. Rev.* **2004**, *84*, 1381−1478.

(2) Camuesco, D.; Comalada, M.; Rodriguez-Cabezas, M. E.; Nieto, A.; Lorente, M. D.; Concha, A.; Zarzuelo, A.; Galvez, J. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br. J. Pharmacol.* **2004**, *143*, 908−918.

(3) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.

(4) Zhu, X.; Raina, A. K.; Lee, H. G.; Casadesus, G.; Smith, M. A.; Perry, G. Oxidative stress signalling in Alzheimer's disease. *Brain Res.* **2004**, *1000*, 32−39.

(5) Segovia, J.; Perez-Severiano, F. Oxidative damage in Huntington's disease. *Methods Mol. Biol.* **2004**, *277*, 321−334.

(6) Przedborski, S.; Jackson-Lewis, V.; Vila, M.; Wu, D. C.; Teismann, P.; Tieu, K.; Choi, D. K.; Cohen, O. Free radical and nitric oxide toxicity in Parkinson's disease. *Adv. Neurol.* **2003**, *91*, 83−94.

(7) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **1996**, *20*, 933−956.

(8) Dimitrios, B. Sources of natural phenolic antioxidants. *Trends Food Sci. Technol.* **2006**, *17*, 505−512.

(9) Sakata, K. Antioxidants from marine organisms. In *Food and Free Radicals*; Hiramatsu, M., Yoshikawa, T., Inoue, M., Eds.; Springer: Tokyo, Japan, 1997; pp 85−100.

(10) Shahidi, F.; Xhong, Y. Food uses of marine by-products (Part 2). Antioxidants from marine by-products. In *Maximising the Value of Marine By-products*; Shahidi, F., Ed.; Woodhead Publishing: Cambridge, U.K., 2007; pp 397−412.

(11) Food and Agriculture Organization (FAO) of the United Nations. *Pacific Oyster Factsheet*; http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en.

(12) Green, T. J.; Dixon, T. J.; Devic, E.; Adlard, R. D.; Barnes, A. C. Differential expression of genes encoding anti-oxidant enzymes in Sydney rock oysters, *Saccostrea glomerata* (Gould) selected for disease resistance. *Fish Shellfish Immunol.* **2009**, *26*, 799−810.

(13) Jo, P. G.; Choi, Y. K.; Choi, C. Y. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2008**, *147*, 460−469.

(14) Chen, J.; Xiao, S.; Deng, Y.; Du, X.; Yu, Z. Cloning of a novel glutathione S-transferase 3 (*GST3*) gene and expressionanalysis in pearl oyster, *Pinctada martensii*. *Fish Shellfish Immunol.* **2011**, *31*, 823−830.

(15) Tanguy, A.; Moraga, D. Cloning and characterization of a gene coding for a novel metallothionein in the Pacific oyster *Crassostrea gigas* (CgMT2): A case of adaptive response to metal-induced stress? *Gene* **2001**, *273*, 123−130.

(16) David, E.; Tanguy, A.; Moraga, D. Peroxiredoxin 6 gene: A new physiological and genetic indicator of multiple environmental stress

response in Pacific oyster *Crassostrea gigas*. *Aquat. Toxicol.* **2007**, *84*, 389−398.
(17) Ogunlei, M.; Okiei, W.; Ofor, E.; Awonuga, O. Determination of the concentration of zinc and vitamin C in oysters and some medical plants used to correct male factor infertility. *J. Nat. Prod.* **2009**, *2*, 89−97.
(18) Watanabe, M. Antioxidants activity of *Crassostrea gigas* meet extract in diabetic mice. *Oyster Res. Inst. News* **2009**, *24*, 31−38.
(19) Ivanina, A. V.; Cherkasov, A. S.; Sokolova, I. M. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. *J. Exp. Biol.* **2008**, *211* (Part 4), 577−586.
(20) Maoka, T.; Fujiwara, Y.; Hashimoto, K.; Akimoto, N. Structure of new carotenoids with a 3,4-dihydroxy-*β*-end group from the oyster *Crassostrea gigas*. *Chem. Pharm. Bull.* **2005**, *53*, 1207−1209.
(21) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.
(22) Node, M.; Kodama, S.; Hamashima, Y.; Katoh, T.; Nishide, K.; Kajimoto, T. Biomimetic synthesis of (±)-galanthamine and asymmetric synthesis of (−)-galanthamine using remote asymmetric induction. *Chem. Pharm. Bull.* **2006**, *54*, 1662−1679.
(23) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free-radical method to evaluate antioxidant activity. *LWT—Food Sci. Technol.* **1995**, *28*, 25−30.
(24) Hirano, T.; Ito, Y.; Yoshino, G. Measurement of small dense low-density lipoprotein particles. *J. Atheroscler. Thromb.* **2005**, *12*, 67−72.
(25) Xiu-li, X.; Xiao, F.; Fu-hang, S.; Jian-gong, S. Isolation and structure elucidation of four compounds from brown alga *Leathesia nana*. *Mar. Sci.* **2004**, *28*, 40−43.
(26) Plack, P. A.; Fraser, N. W.; Grant, P. T.; Middleton, C.; Mitchell, A. I.; Thomson, R. H. Gadusol, an enolic derivative of cyclohexane-1,3-dione present in the roes of cod and other marine fish. Isolation, properties and occurrence compared with ascorbic acid. *Biochem. J.* **1981**, *199*, 741−747.
(27) Grant, P. T.; Plack, P. A.; Thomson, R. H. Gadusol, a metabolite from fish egg. *Tetrahedron Lett.* **1980**, *21*, 4043−4044.
(28) Ahluwalia, V. K.; Kumar, D. Constitution and synthesis of a novel 4-methoxycoumarin from *Platymiscium praecox*. *Indian J. Chem.* **1977**, *15B*, 18−20.
(29) Jessup, W.; Rankin, S. M.; De Whalley, C. V.; Hoult, J. R.; Scott, J.; Leake, D. S. Alpha-tocopherol consumption during low-density-lipoprotein oxidation. *Biochem. J.* **1990**, *265*, 399−405.
(30) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.* **1992**, *13*, 341−390.
(31) Yoshihara, D.; Fujiwara, N.; Suzuki, K. Antioxidants: Benefits and risks for long-term health. *Maturitas* **2010**, *67*, 103−107.
(32) Hollman, P. C.; Cassidy, A.; Comte, B.; Heinonen, M.; Richelle, M.; Richling, E.; Serafini, M.; Scalbert, A.; Sies, H.; Vidry, S. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. *J. Nutr.* **2011**, *141*, 989S−1009S.