JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant and Free Radical Scavenging Properties of Marennine, a Blue-Green Polyphenolic Pigment from the Diatom *Haslea ostrearia* (Gaillon/Bory) Simonsen Responsible for the Natural Greening of Cultured Oysters

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Among microalgae, the marine diatom *Haslea ostrearia* has the distinctive feature of synthesizing and releasing, into the surrounding environment, a blue-green polyphenolic pigment called marennine. The oyster-breeding industry commonly makes use of this natural phenomenon for the greening of oysters grown in the ponds of the French Atlantic coast. This article reports the in vitro antioxidant properties of pure marennine. Two kinds of evaluation systems were adopted to test the antioxidative activity of marennine: antioxidant capacity assays (β-carotene and thymidine protection assays and iron reducing power assay) and free radical scavenging assays (DPPH$^{\bullet}$, $O_2^{\bullet -}$, and HO$^{\bullet}$). In almost all cases, marennine exhibited significantly higher antioxidative and free radical scavenging activities than natural and synthetic antioxidants commonly used in food, as shown by comparing median effective concentration (EC_{50}) values, for each test independently. This medium molecular weight polyphenol (around 10 kDa) from microalgae is thus a potentially useful natural antioxidant. Because of its blue-coloring property and water solubility, it could also be used as a natural food-coloring additive.

KEYWORDS: Antioxidative activity; *Haslea ostrearia*; marennine; oyster greening; pigment; polyphenol; radical scavenging activity

INTRODUCTION

Antioxidants are important industrial additives mainly used to avoid the deterioration of oxidizable products, such as food, drugs, and cosmetics (*1–3*). They are also reported to be good health-promoting agents (*4*). Natural phenolic compounds, mainly flavonoids and anthocyanins, are recognized showing good antioxidant activity (*5–11*), and it has been reported that these compounds could prevent, for instance, vascular diseases (*12–14*), inflammatory processes (*15, 16*), and cancers (*17–19*). As a result of these observations, there has been an increase in recent years in investigations using these natural phenolic antioxidants as nutraceuticals and pharmaceuticals (*20–23*). Many of these studies have been performed with compounds extracted and purified from terrestrial plants, but researchers have also focused on the antioxidative properties of bioactive molecules from marine organisms (*24, 25*). Among them, seaweed polyphenols have been intensively studied, so much so that the antiradical and antioxidative capacities of these

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10.1021/jf073187n CCC: $40.75
Published on Web 07/16/2008

compounds are well-documented (*26–33*). However, phenolics from marine microalgae remain poorly investigated although promising antioxidant activities have been found both in water-soluble and lipophilic extracts (*34–37*).

Haslea ostrearia (*38*) is a marine diatom distributed worldwide and characteristically growing in the oyster ponds of the French Atlantic coast. It has the unique properties, among Bacillariophycae, of synthesizing and releasing into the external environment a blue-green pigment, named "marennine", responsible for the greening of oyster gills (*39*). This phenomenon is of particular economic interest because the oysters so colored have a higher market value. The complete molecular structure of marennine has not yet been fully elucidated, but Pouvreau et al. (*40*) have recently demonstrated that it is a nonhydrolyzable phenolic compound lacking any link to a polypeptide, glycoside, or transition metal but displaying a molecular skeleton different from that of flavonoids and anthocyanins. IMn, the intracellular form of marennine (10 751 Da molecular weight; absorbance maxima at 247 and 672 nm), accumulates in acidic vesicular complexes (*40, 41*), mainly in the apical regions of the cell (*42*). EMn, the extracellular form of marennine (9893 Da molecular weight; absorbance maxima at 247, 322, and 677 nm), exhibits slightly different physicochemical properties from those of IMn (*40*), suggesting that the pigment undergoes molecular rearrangements during excretion. Little is known about the biological role of marennine, but it has been established that it is not involved in the photosynthetic process (*43*).

The aim of the present study was to investigate the antioxidative capacities of IMn and EMn. These were analyzed using six tests, $O_2^{\cdot -}$ (superoxide radical), DPPH$^{\cdot}$ (diphenylpicrylhydrazyl radical) and HO$^{\cdot}$ (hydroxyl radical) scavenging assays, β-carotene and dThd (thymidine) protection assays, and an iron(III) reducing power assay, which are based on different end points. Our results are discussed with respect to the potential use of marennine as a natural antioxidant and coloring additive in food products.

MATERIALS AND METHODS

Algal Production and Marennine Purification. The study was carried out using an axenic strain of *Haslea ostrearia* (NCC-148.7) isolated in Bourgneuf Bay (southeast coast of Brittany, France) and kept alive by NCC (Nantes Culture Collection registered as WDCM856). Intra- and extracellular marennine (IMn and EMn) were purified from crude cellular extracts and blue-colored cell-free culture medium, respectively, by a three-step semipreparative method, including extraction in NH_4HCO_3 buffer, 30 and 3 kDa ultrafiltration processes, and anion exchange chromatography. This is detailed in a procedure described in Pouvreau et al. (*44*)

Chemicals. Acetylthiocholine, β-carotene, BSA, DPPH, DTNB, EIA buffer, hypoxanthine, linoleic acid, NBT, TBA, TCA, Tween 20, Tween 40, and xanthine oxidase were purchased from Sigma Chemical. Apigenin, BHA, ferrozine, quercetin, thymidine, and Trolox were supplied by Aldrich, EDTA by Fisher Chemical, ammonium ferrous sulfate ($Fe[NH_4]_2[SO_4]_2$), ferrous sulfate ($FeSO_4$), potassium dihydrogen phosphate (KH_2PO_4), potassium ferricyanide ($K_3Fe[CN]_6$), and disodium hydrogen phosphate (Na_2HPO_4) by Merck, ferric chloride ($FeCl_3$) and hydrogen peroxide 33% w/v (H_2O_2) by Panreac Quimica. Allopurinol, ascorbic acid, deoxyribose, TRIS-HCl, and xanthine were purchased from ACROS Organics. The antithymidine monoclonal antibody was obtained by the CEA, as described in a previous published work (*45*), and plate coated with polyclonal goat antimouse antibody from Jackson ImmunoResearch Laboratories Inc.

For the six tests, the control mixture contained osmosed water instead of marennine (absorbance A_c). A sample mixture without DPPH, enzyme, H_2O_2, $FeSO_4$, β-carotene, or $FeCl_3$ (absorbance A_{s*}) was prepared for each marennine concentration to take into account the contribution of the pigment to the absorbance of a sample mixture (A_s) and to ensure that any change in the absorbance spectrum of marennine during the incubation period did not interfere with absorbance measurements. Each experiment was conducted independently with two or three replicates of each sample concentration.

DPPH Radical Scavenging Assay. The DPPH$^{\cdot}$ (2,2-diphenyl-1-picrylhydrazyl) scavenging assay of IMn and EMn was carried out according to the method adapted from Morales and Jimenez-Perez (*46*). The sample mixture consisted of 1 mL of freshly prepared DPPH$^{\cdot}$ (0.19 mmol·L^{-1} in 95% ethanol) and 200 μL of aqueous marennine solution ([IMn] and [EMn] ranging from 30 to 6×10^2 μmol·L^{-1}). The solutions were then incubated for 1 h at 25 °C with vigorous shaking and centrifuged for 10 min at 10 000*g*. Aliquots (300 μL) of supernatant were placed in a 96-well microplate, and absorbance (A) was recorded at 520 nm using a UV−visible microplate reader (SpectraMax Plus^{384}).

The scavenging effect (SE) of marennine, expressed as the percentage of scavenged DPPH$^{\cdot}$, was calculated from the following equation:

$$SE_{\%} = 100 \times [A_c - (A_s - A_{s*})]/A_c$$

Ascorbic acid, BHA, and Trolox were used for comparison.

Superoxide Anion Scavenging Assay. The superoxide anion ($O_2^{\cdot -}$) scavenging assay of IMn and EMn was carried out according to the method adapted from Vivas et al. (*47*). The sample mixture (305 μL) consisted of 200 μL of TRIS-HCl buffer (50 mmol·L^{-1}, pH 7.4), 60 μL of hypoxanthine (5 mmol·L^{-1}), 30 μL of NBT (NitroBlue tetrazolium, 0.33 mmol·L^{-1}), and 15 μL of marennine ([IMn] and [EMn] ranging from 25 to 10^3 μmol·L^{-1}). The solutions were then placed in a 96-well microplate, and after incubation at 25 °C for 10 min, the reaction was started by adding 30 μL of xanthine oxidase (16.5×10^{-3} U in TRIS-HCl buffer 50 mmol·L^{-1}, pH 7.4); the absorbance (A) was monitored for 6 min at 560 nm to determine the rate of NBT reduction, using a UV−visible microplate reader (SpectraMax Plus^{384}).

The scavenging effect (SE) of marennine, expressed as the percentage of scavenged $O_2^{\cdot -}$, was calculated from the following equation:

$$SE_{\%} = 100 \times (b_c - b_s)/b_c$$

where b_c and b_s are the linear regression coefficients of the curves A_{560} versus time, related to the absorbance increase of the control mixture and of the sample mixture, respectively. Ascorbic acid was used for comparison.

To rule out the possibility that marennine could directly inhibit the xanthine oxidase activity, assays were performed using the same procedure as the superoxide anion scavenging test but with xanthine (5 mmol. L^{-1}) instead of hypoxanthine as substrate and TRIS-HCl buffer (50 mmol·L^{-1}, pH 7.4) instead of NBT. The rate of formation of the reaction product, uric acid, was monitored at 295 nm over a 6 min period. The results were expressed as the percentage inhibition of uric acid formation with respect to the reaction mixture without marennine. Allopurinol (0.1 mmol·L^{-1}) was used as a positive control.

Hydroxyl Radical Scavenging Assay. The hydroxyl radical scavenging assay of IMn and EMn was performed according to the method adapted from Chung et al. (*48*). The Fenton reaction mixture (150 μL) was prepared by adding the reactants in the following order: 50 μL of $FeSO_4 \cdot 7H_2O$ (20 mmol·L^{-1}), 50 μL of EDTA (20 mmol·L^{-1}), and 50 μL of 2-deoxyribose (20 mmol·L^{-1}). This was then supplemented by 300 μL of sodium−potassium phosphate buffer (100 mmol·L^{-1}, pH 7.4) and 50 μL of marennine ([IMn] and [EMn] ranging from 70 to 2.1×10^3 μmol·L^{-1}). Thereafter, 50 μL of H_2O_2 (20 mmol·L^{-1}) was added, and the sample mixture was incubated at 37 °C for 2 h. After incubation, 250 μL of 2.8% TCA (trichloroacetic acid) and 250 μL of 1% TBA (thiobarbituric acid) were mixed and placed immediately in a water bath for 10 min at 100 °C. After cooling on ice, the mixture was centrifuged at 10 000*g* for 5 min, and the absorbance (A) was measured at 532 nm using a UV−visible spectrophotometer (Perkin-Elmer Instrument Lambda 25). Cuvettes of 1 cm path length were used.

The scavenging effect (SE) of marennine, expressed as the percentage of scavenged HO$^{\cdot}$, was calculated as previously described. Trolox was used for comparison.

To rule out the possibility that marennine could interact with the Fenton reaction, tests were performed to ensure that IMn and EMn did not act as iron(II) chelators. According to the method adapted from Dinis et al. (*49*), the sample mixture (160 μL), consisting of 20 μL of aqueous marennine solution ([IMn] and [EMn] ranging from 10 to 10^3 μmol·L^{-1}), 120 μL of osmosed water, and 20 μL of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.2 mmol·L^{-1}), was incubated for 5 min at 20 °C. The reaction was then initiated by adding 40 μL of ferrozine (3.5 mmol·L^{-1}), and the mixture was placed in a 96-well microplate. After a 10 min resting period, the absorbance (A) was recorded at 560 nm using a UV−visible microplate reader (BIO-TEK Instruments EL800). The chelation capacity (CC) of marennine, expressed as the percentage of the absorbance of the control, was calculated as preciously described. EDTA was used for comparison.

***β*-Carotene Protection Assay.** The protective effect of IMn and EMn against the oxidization of β-carotene was evaluated according to the method adapted from Miller (*50*). The emulsion, consisting of 2 mg of crystalline β-carotene, 20 μL of linoleic acid, and 200 μL of commercial surfactant Tween 40 (polyoxyethylenesorbitan monopalmitate), was made in 10 mL of reagent grade chloroform. The solvent was then evaporated at 40 °C for 90 min under vacuum, using a rotary evaporator. The resulting viscous oil was immediately mixed with 50 mL of O_2-saturated osmosed water and vigorously shaken for 2 min. Five milliliters of each of these aqueous emulsions was added to 200 μL of marennine ([IMn] and [EMn] ranging from 10 to 5×10^2 μmol·L^{-1}) in test tubes. A zero-time reading was taken at 470 nm for each reaction mixture immediately after addition of the emulsion to a marennine solution. The tubes were then incubated at 50 °C and exposed to daylight in a rotary shaker. At desired intervals (30, 60, and 120 min), aliquots (300 μL) of each solution were placed in a 96-well microplate for subsequent absorbance (A) readings at 470 nm, using a UV−visible microplate reader (SpectraMax Plus^{384}).

The effect of marennine on β-carotene bleaching kinetics was expressed by plotting A_{470} versus incubation time. The absorbance value of the emulsion without β-carotene was subtracted from the absorbance value of the emulsion containing both β-carotene and marennine.

The protective effect (PE) of marennine, expressed as the percentage of β-carotene protection against bleaching, was calculated from the following equation:

$$PE_{\%} = 100 \times \{[(A_{c60} - A_{c0}) - (A_{s60} - A_{s0})]/(A_{c60} - A_{c0})\}$$

where A_c is the absorbance of the sample emulsion, for 0 and 60 min of incubation time. BHA was used for comparison.

Thymidine Protection Assay. The protective effect of IMn and EMn against the oxidization of dThd (thymidine) was carried out according to the method of Meunier et al. (*51*). The sample mixture (150 μL), consisting of 75 μL of dThd (0.015 mmol·L^{-1} in potassium phosphate buffer 50 mmol·L^{-1}, pH 7.4) mixed with 75 μL of aqueous marennine sample ([IMn] and [EMn] ranging from 25 to 10^2 μmol·L^{-1}), was placed in a 96-well quartz microplate. The plate was irradiated for 135 min at 340 Gy in a γ-irradiator (cis bio International IBL 637, equipped with a ^{137}Cs source). Aliquots (25 μL) of the irradiated solution were quenched by the addition of 25 μL of EIA buffer (100 mmol·L^{-1} potassium phosphate buffer pH 7.4, 0.1% bovine serum albumin, 150 mmol·L^{-1} NaCl, and 0.1% NaN_3) and transferred to a second plate (Maxisorb-Nunc) coated with polyclonal goat antimouse antibody. This mixture was then supplemented with 50 μL of thymidine−acetylcholinesterase conjugate (dThd−AchE), prepared according to Pradelles et al. (*52*), and 50 μL of specific monoclonal antithymidine antibody in EIA buffer. After incubation for 2 h at 20 °C, the plate was washed three times with 100 mmol·L^{-1} potassium phosphate buffer pH 7.4 supplemented by 0.05% (v/v) Tween 20 (polyoxyethylenesorbitan monolaurate), and Ellman's reagent (0.25 mmol·L^{-1} DTNB [5,5′-dithiobis-2-nitrobenzoic acid]), 76 mmol·L^{-1} acetylthiocholine, 1.45 mol·L^{-1} NaCl, and potassium phosphate buffer 10 mmol·L^{-1}, pH 7.4) were added. The absorbance (A), related to the dThd−AchE activity, was measured at 415 nm, using a UV−visible microplate reader (Labsystems, Multiskan Bichromatic).

The protective effect (PE) of marennine, expressed as the percentage of dThd protection against γ-radiolysis oxidization, was calculated from the following equation:

$$PE_{\%} = 100 \times ([dThd]_{antiox} - [dThd]_{ox})/([dThd]_0 - [dThd]_{ox})$$

where $[dThd]_{antiox}$ and $[dThd]_{ox}$ are the concentrations of thymidine in the presence and absence of marennine, respectively, and $[dThd]_0$ is the initial concentration of thymidine. Thymidine quantification was achieved using a calibration curve (fitted using a linear log−logit transformation) obtained with pure thymidine. Three flavonoids, apigenin, quercetin, and dihydroxy-methoxyflavone, were used for comparison.

Iron(III) to Iron(II) Reducing Capacity Assay. The iron reducing capacity of IMn and EMn was assessed according to the method adapted from Oyaizu (*53*) and described by Yen and Chen (*54*). The sample mixture (600 μL), consisting of 100 μL of aqueous marennine solution ([IMn] and [EMn] ranging from 10 to 5×10^3 μmol·L^{-1}), 250 μL of potassium phosphate buffer (200 mmol·L^{-1}, pH 6.5) and 250 μL of $K_3Fe(CN)_6$ (30 mmol·L^{-1}), was incubated for 20 min at 50 °C. After cooling rapidly, 250 μL of TCA (0.6 mol·L^{-1}) was added, and the mixture was shaken vigorously before being centrifuged at 10 000g for 10 min. A 20 μL aliquot of supernatant was then placed in a 96-well microplate and supplemented by 180 μL of osmosed water and 20 μL of $FeCl_3 \cdot 6H_2O$ (3.7 mmol·L^{-1}). After incubation at room temperature for 15 min, the absorbance (A) was recorded at 700 nm using a UV−visible microplate reader (BIO-TEK Instruments EL800).

The iron reducing power of marennine was related to the absorbance of the sample mixture at 700 nm. The higher the absorbance of the sample mixture was, the better the reducing capacity of the sample was recognized. The absorbance value at 700 nm (A_{700}) corresponded to

$$A_{700} = (A_s - A_{s*}) - A_c$$

Ascorbic acid, BHA, and Trolox were used for comparison.

Statistical Analysis. For radical scavenging assays, EC_{50}, the concentration of sample needed to scavenge 50% of radical, was determined by plotting $SE_{\%}$ versus sample concentration. For the β-carotene protection assay, EC_{50}, the concentration of sample needed to prevent β-carotene bleaching by 50%, was determined by plotting $PE_{\%}$ versus sample concentration. For the four tests, the EC_{50} value was computed in SigmaPlot 9.0 using a nonlinear regression dose−response model with a four-parameter logistic curve. For the iron(III) to iron(II) reducing capacity assay, EC_{50}, the concentration of sample at which the absorbance at 700 nm was 0.5, was determined by plotting absorbance versus sample concentration (*55*). The EC_{50} value was computed in SigmaPlot 9.0 by interpolation using the linear regression dose−response model.

Statistical analyses were conducted for each test independently with the SigmaPlot 9.0 version software for Windows. One-way analyses of variance procedures were used (ANOVA). Significant difference at $P < 0.05$ between the means of EC_{50} was determined by Dunnett's and Student-Newman-Keul's posthoc multicomparison test (SNK).

RESULTS

DPPH Radical-Scavenging Activity of Marennine. DPPH$^{\cdot}$ is a long-lived nitrogen-centered free radical displaying a deep-purple color ($\lambda_{max} \cong 520$ nm). The assay is based on the ability of a compound to scavenge DPPH$^{\cdot}$. This reaction can be monitored spectrophotometrically because, upon reduction, DPPH$^{\cdot}$ fades to yellow. Thus, compounds able to induce a decrease in absorbance at 520 nm can be looked upon as DPPH$^{\cdot}$ scavengers.

The addition of marennine to DPPH$^{\cdot}$ solution induced a decrease in absorbance at 520 nm. **Figure 1** shows the scavenging effect of different concentrations of IMn and EMn on the DPPH radical. The scavenging activity of marennine rose with increasing concentrations. IMn and EMn reached their maximum activity at 50 μmol·L^{-1} ($SE_{\%} \cong 80$) and 25 μmol·L^{-1} ($SE_{\%} \cong 90$), respectively. Median effective concentrations (EC_{50}) determined from these results are given in **Table 1**. These data revealed that EMn displayed significantly higher DPPH$^{\cdot}$ scavenging ability (SNK; $P < 0.001$) than IMn. In this

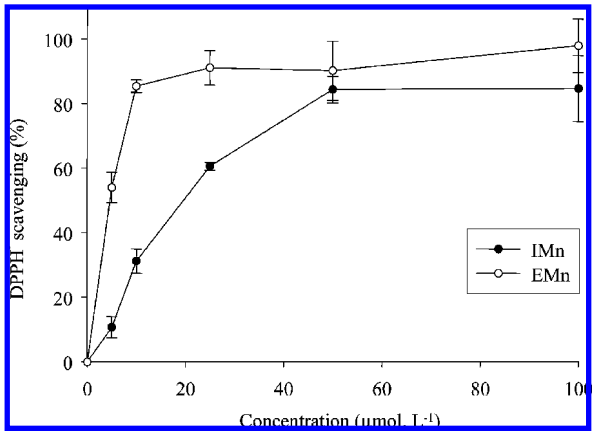

Figure 1. Scavenging effect (SE) of marennine on the DPPH (diphenylpicrylhydrazyl) radical. IMn: intracellular marennine; EMn: extracellular marennine. The concentrations of marennine are expressed as the final concentration in the reaction mixture. The percentage values are means of three replicates ± 95% confidence intervals.

assay, EMn also exhibited scavenging abilities significantly higher (SNK; $0.001 < P < 0.009$) than those of the usual commercial antioxidants, such as ascorbic acid, BHA, and Trolox. IMn was also found significantly more effective (SNK; $P < 0.001$) than ascorbic acid and Trolox at scavenging DPPH$^{\bullet}$ but not BHA (SNK; $P < 0.005$).

Superoxide Radical Scavenging Activity of Marennine. $O_2^{\bullet -}$ (superoxide anion) is a short-lived free radical which is difficult to quantify directly. The assay is based on the capacity of a compound to compete with the reduction of NBT by $O_2^{\bullet -}$, generated by the hypoxanthine/xanthine oxidase system (HX/XO). $O_2^{\bullet -}$ reduces the colorless form of NBT (NBT^{2+}) to blue diformazan (NBT^{+}, $\lambda_{max} \cong 560$ nm). The determination of the mean rate of increase in absorbance at 560 nm over a 1 min period provides a measure of the extent to which a compound is able to inhibit the reduction of NBT by $O_2^{\bullet -}$ and thus to scavenge the superoxide radical.

The addition of marennine to the reaction mixture reduced the increase in absorbance at 560 nm. **Figure 2** shows that the $O_2^{\bullet -}$ scavenging activity of IMn and EMn was dose-dependent. Both IMn and EMn reached their maximum activity at 50 μmol·L^{-1} ($SE_{\%} \cong 100$). Median effective concentrations (EC_{50}) calculated from these data are given in **Table 1**. In this assay, EMn exhibited a scavenging activity significantly higher (Dunnett; $P < 0.05$) than that of IMn. As a commercial antioxidant control, ascorbic acid had a significantly lower scavenging ability (Dunnett; $P < 0.05$) than that of both EMn and IMn.

Control assays ruled out the possibility that marennine directly reduced NBT or inhibited xanthine oxidase activity. Thus, the effect of marennine in the NBT reduction assay could be imputed to its capacity to scavenge superoxide anions.

Hydroxyl Radical-Scavenging Activity of Marennine. HO$^{\bullet}$ is a short-lived free radical widely believed to be produced when hydrogen peroxide reacts with Fe(II) in a Fenton-type reaction. The assay is based on the ability of a compound to scavenge HO$^{\bullet}$. This reaction can be monitored spectrophotometrically because, in the presence of a mixture containing deoxyribose, TCA, and TBA, HO$^{\bullet}$ generated by the Fenton reaction leads to a pinkish TBA acid-reactive adduct ($\lambda_{max} \cong 532$ nm). Thus, compounds able to induce a decrease in absorbance at 532 nm can be considered as HO$^{\bullet}$ scavengers.

The addition of marennine to the reaction mixture induced a decrease in the absorbance at 532 nm. **Figure 3** shows the scavenging effect of different concentrations of IMn and EMn on the hydroxyl radical. The scavenging activity of marennine rose with increasing concentrations. IMn and EMn reached their maximum activity at 100 μmol·L^{-1} ($SE_{\%} \cong 90$) and 35 μmol·L^{-1} ($SE_{\%} \cong 90$), respectively. Median effective concentrations (EC_{50}) calculated from these results are presented in **Table 1**. The statistical analysis revealed that EMn was significantly more effective than IMn (Dunnett; $P < 0.05$) at scavenging HO$^{\bullet}$. In comparison, both IMn and EMn displayed scavenging capacities significantly higher (Dunnett; $P < 0.05$) than those of Trolox, used as an antioxidant control.

Control tests were performed to rule out the possibility that marennine directly interacted with the Fenton reaction by chelating iron(II). Ferrozine can make red-colored complexes ($\lambda_{max} \cong 560$nm) with iron(II). In the presence of chelators, the formation of the complexes is blocked, and as a result, the red color decreases. The chelation power of a compound can be spectrophotometrically monitored by measuring the decrease in absorbance at 560 nm. Therefore, compounds able to induce an absorbance decrease at 560 nm can be considered as iron(II) chelators. The iron(II) chelation capacity of marennine was investigated with various concentrations of IMn and EMn, ranging from 0 to 100 μmol·L^{-1}. No change in absorbance at 560 nm was observed, even with the highest marennine concentrations. Thus, in this assay, both IMn and EMn were unable to chelate iron(II). As a result, the activity of marennine in the assay was due to its ability to scavenge hydroxyl radicals.

Protective Effect of Marennine against β-Carotene Oxidization. Native β-carotene ($\lambda_{max} \cong 470$ nm) is a red-orange pigment for which the chromophore has numerous conjugated double bonds. During the oxidization process, the unsaturated β-carotene molecules lose their double bonds and become colorless. In the reaction mixture, β-carotene is oxidized by the additive effects of light, air, heat, and peroxyl radicals produced by linoleic acid present in the emulsion. The assay measures the ability of a compound to inhibit the extent of β-carotene bleaching due to light, air, heat, and linoleate-free radicals. Thus, compounds able to reduce the decrease in absorbance at 470 nm delay the oxidative degradation of β-carotene and can be considered as antioxidants.

The addition of marennine to the emulsion of native β-carotene reduced the decrease in absorbance at 470 nm. **Figure 4** shows the effect of various concentrations of IMn (**Figure 4A**) and EMn (**Figure 4B**) on the decreasing absorbance of β-carotene at 470 nm over 120 min. Marennine reduced the absorbance decrease in a dose-dependent manner. BHA was used as a positive reference, and antioxidant-free osmosed water was used as a negative control. The mean protective effect of marennine against β-carotene bleaching, determined from absorbance values recorded for an incubation time of 60 min, is shown in **Figure 5**. The antioxidant activity of marennine rose with increasing concentrations. At 20 μmol·L^{-1}, IMn and EMn had a protective effect of ≅ 90% and ≅ 100%, respectively. Median effective concentrations (EC_{50}) calculated from these data are given in **Table 1**. IMn exhibited a significantly lower ability to prevent β-carotene oxidization (SNK; $P < 0.05$) than EMn. Tested under the same experimental conditions, BHA displayed a significantly lower (SNK; $P < 0.05$) protective effect than both IMn and EMn.

Protective Effect of Marennine against Thymidine Oxidization. This procedure is based on the oxidization of dThd by γ-radiolysis under aerobic conditions. The unmodified dThd remaining after irradiation is quantified by using a competitive enzyme immunoassay (EIA). As intact dThd competes with the dThd−enzyme conjugate for antibody binding sites, the decrease

Table 1. EC_{50} Values of Marennine in the Antioxidant Activity Assays Compared with Those of Commercial Antioxidants Used in Food[a]

	EC_{50} value (μmol·L^{-1}) mean ± S.E., (n = 3)				
product	DPPH·[b]	$O_2^{\cdot -}$[b]	HO·[b]	β-carotene[c]	Fe(III)[d]
IMn	15.14^{e} ± 0.99	2.21^{e} ± 0.16	22.39^{e} ± 2.35	9.73^{e} ± 0.98	9.55^{e} ± 0.52
EMn	4.47^{f} ± 0.18	0.85^{f} ± 0.04	9.66^{f} ± 0.43	2.19^{f} ± 0.22	4.29^{f} ± 0.23
AA	34.79^{g} ± 1.46	3.28^{g} ± 0.04	nt	nt	0.40^{g} ± 0.07
BHA	9.57^{h} ± 0.85	nt	nt	27.74^{g} ± 5.67	1.46^{h} ± 0.11
TX	48.73^{i} ± 1.49	nt	805.44^{g} ± 34.10	nt	1.55^{h} ± 0.13

[a] IMn: intracellular marennine; EMn: extracellular marennine; AA: ascorbic acid; BHA: butylated hydroxyanisole; TX: Trolox. nt: not tested. Significant differences at $P < 0.05$ are indicated with different letters (e−i) for each evaluation test independently. [b] EC_{50} value: effective concentration at which 50% of radicals are scavenged. [c] EC_{50} value: effective concentration needed to prevent β-Carotene bleaching by 50%. [d] EC_{50} value: effective concentration at which the absorbance is 0.5.

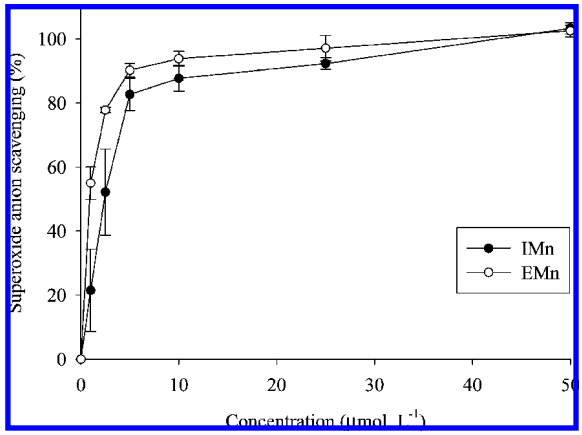

Figure 2. Scavenging effect (SE) of marennine on the superoxide radical. IMn: intracellular marennine; EMn: extracellular marennine. The concentrations of marennine are expressed as the final concentration in the reaction mixture. The percentage values are means of three replicates ± 95% confidence intervals.

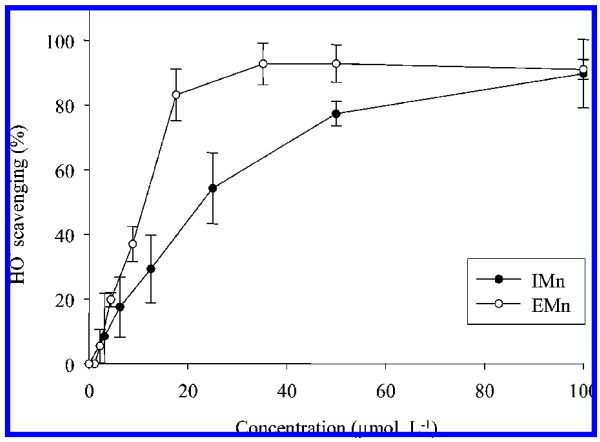

Figure 3. Scavenging effect (SE) of marennine on the hydroxyl radical. IMn: intracellular marennine; EMn: extracellular marennine. The concentrations of marennine are expressed as the final concentration in the reaction mixture. The percentage values are means of three replicates ± 95% confidence intervals.

in antibody-bound-enzyme activity, which generates a yellow-colored reaction product ($\lambda_{max} \cong 415$ nm), is related to the protective effect of the tested compound.

The presence of marennine induced efficient thymidine protection when submitted to γ-radiolysis. The mean protective effect of marennine against dThd oxidization is presented in **Figure 6**. The antioxidant activity of marennine rose with increasing concentrations. At 50 μmol·L^{-1}, the protective effect was ≅ 50% for IMn and ≅ 30% for EMn. Under the same experimental conditions, at 50 μmol·L^{-1}, three polyphenols,

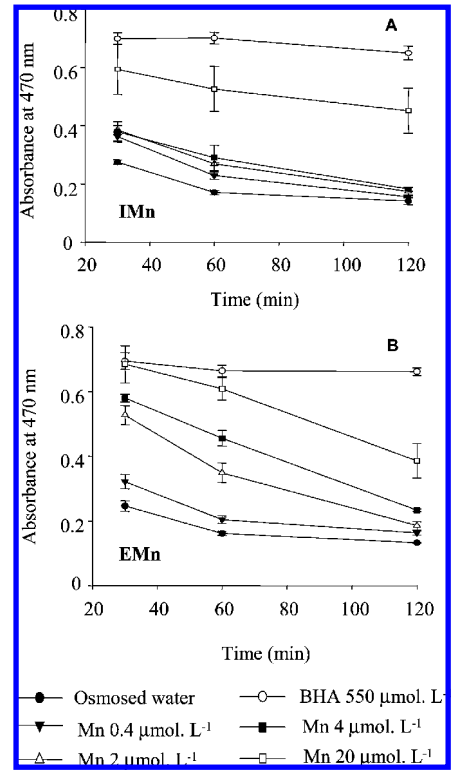

Figure 4. Decay curves of absorbance at 470 nm related to the protective effect of marennine against β-carotene bleaching: (**A**) intracellular marennine (IMn); (**B**) extracellular marennine (EMn). The concentrations of marennine are expressed as the final concentration in the reaction mixture. Absorbance values are means of three replicates ± 95% confidence intervals.

apigenin, quercetin, and 4′,5-dihydroxy-7-methoxyflavone, used as positive control counterparts, displayed a maximum protective effect of 47, 34, and 8%, respectively.

Iron(III) Reducing Power of Marennine. The evaluation of the reducing power is based on the reduction of hexaferricyanide (Fe(III)) to hexaferrocyanide (Fe(II)). At low pH, the yellow-colored ferrous form reacts with the ferric chloride to generate Prussian Blue, which develops an intense blue color ($\lambda_{max} \cong 700$ nm). The concentration of Fe(II) can be monitored by measuring the formation of Prussian Blue at 700 nm. Therefore, compounds able to induce an increase in absorbance at 700 nm can be considered as iron reductants and potential antioxidants.

Figure 7 depicts the reducing effect of marennine on iron(III). The reducing power of marennine increased in a dose-dependent manner. IMn and EMn reached their maximum reducing power at 50 μmol·L^{-1} ($\Delta A_{700} \cong 1.1$) and 20 μmol·L^{-1} ($\Delta A_{700} \cong 1.4$), respectively. Median effective concentrations (EC_{50}) determined from these results are exhibited in **Table 1**. These data

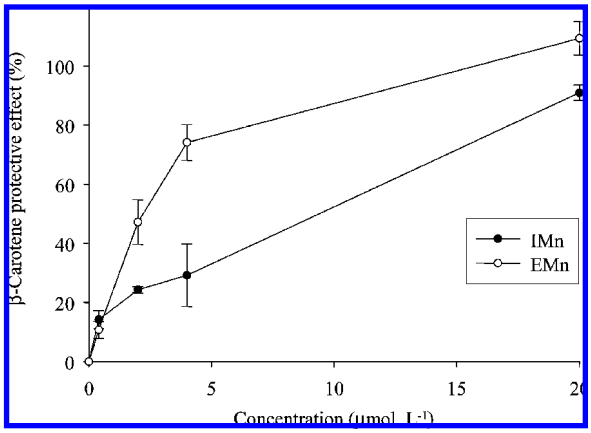

Figure 5. Dose−response curves for the protective effect (PE) of marennine against β-carotene bleaching (after 60 min of incubation). IMn: intracellular marennine; EMn: extracellular marennine. The concentrations of marennine are expressed as the final concentration in the reaction mixture. The percentage values are means of three replicates ± 95% confidence intervals.

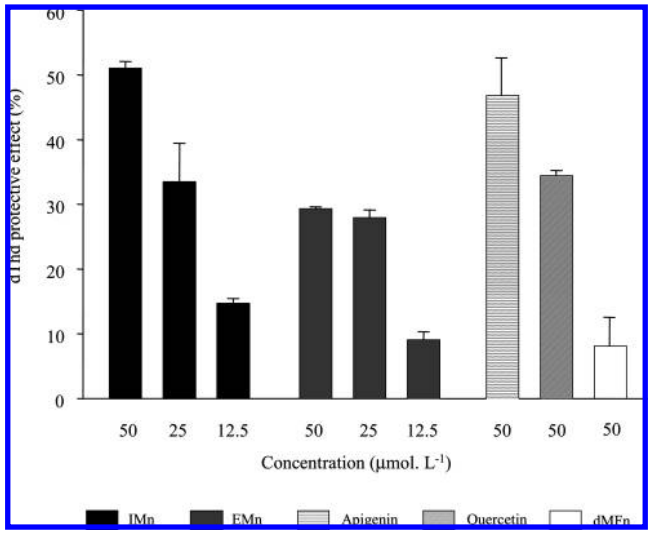

Figure 6. Dose−response protective effect (PE) of marennine against thymidine oxidization. The antioxidant activities of intra- (IMn) and extracellular marennine (EMn) are compared with the activity of apigenin, quercetin, and 4′,5-dihydroxy-7-methoxyflavone (dMFn), used as positive protective agent references. The concentrations of marennine are expressed as the final concentration in the reaction mixture. The percentage values are means ± standard deviation, $n = 2$.

demonstrated that EMn displayed a reducing power significantly higher (SNK; $P < 0.001$) than that of IMn. However, both IMn and EMn were found to have an iron(III) reducing capacity significantly lower (SNK; $P < 0.001$) than that of several commercial antioxidants used as positive counterparts: ascorbic acid, BHA, and Trolox.

DISCUSSION

In this study, pure marennine, a natural blue-green polyphenolic pigment, exhibited in vitro antioxidant and reducing activities toward various types of molecular targets. It was found to protect conjugated double bonds compounds, like β-carotene and thymidine, against in vitro-induced oxidization and to scavenge DPPH, superoxide, and hydroxyl free radicals. Marennine also reduced iron(III) to iron(II).

Free radical scavenging experiments indicated that marennine was a very good radical scavenger. Indeed, both IMn and EMn

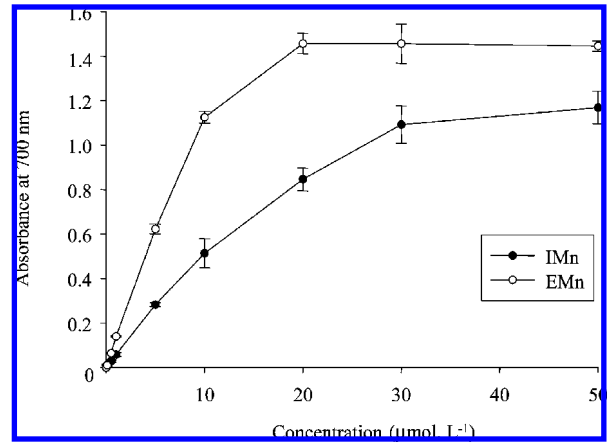

Figure 7. Iron(III) reducing capacity of marennine. IMn: intracellular marennine; EMn: extracellular marennine. The concentrations of marennine are expressed as final concentration in the reaction mixture. The absorbance values are means of three replicates ± 95% confidence intervals.

were more effective, under the test conditions, at scavenging DPPH$^{\bullet}$, $O_2^{\bullet -}$, and HO$^{\bullet}$ than the usual commercial counterparts, such as ascorbic acid, BHA, and Trolox. HO$^{\bullet}$ is a highly reactive radical able to induce the peroxidation of lipids, nucleic acids, and proteins (*56–58*). $O_2^{\bullet -}$ is less reactive, but it may also cause indirect damage by generating HO$^{\bullet}$ and ONOO$^-$ (*59, 60*). Therefore, because of its high capacity to scavenge both $O_2^{\bullet -}$ and HO$^{\bullet}$, marennine could protect biological macromolecules against radical-mediated degradation.

Protection tests showed that marennine prevented oxidization of β-carotene, linoleic acid, and thymidine. The protective effect of IMn and EMn against β-carotene bleaching was better than that of BHA. In the thymidine protection assay, IMn and EMn displayed activities quite similar, or even better, than those of the phenolic compounds generally used as controls for this antioxidant evaluation test (*7*). The peroxidation of unsaturated fatty acids generates highly toxic end products involved in radical chain reactions and leads to great disorders in the metabolism of lipids (*61, 62*). The oxidative degradation of DNA generates thymidine hydroperoxides, which contribute to mutagenesis in aerobic organisms (*63, 64*). The ability of IMn and EMn to inhibit the bleaching of β-carotene suggests that marennine can neutralize the linoleyl-free radicals (LOO$^{\bullet}$) and as a result could be considered as a compound for preventing the oxidization of lipids and their derivatives. Moreover, the protective effect of marennine against the oxidization of thymidine suggests the potential of this pigment as a compound to minimize oxidative damage in DNA.

The reducing power test revealed the ability of marennine to induce the iron(III)−iron(II) transformation. IMn and EMn showed good reducing capacities but slightly lower than those of the commercial antioxidants tested, ascorbic acid, BHA, and Trolox. The extent of oxidization-related damage in cells is believed to be controlled by the potency of antioxidative systems (*65*), and a correlation between an increase in iron(III) reducing activity and a decay in cellular senescence has been demonstrated (*66*). Moreover, the application of exogenous active iron(III) reducing agents has proved effective at inhibiting the degradation of biological macromolecules (*66*). Thus, the iron(III) reducing capacity of marennine may serve as a significant indicator of its potential antioxidant power.

The ability of marennine to generate catalytically active iron(II), by reducing iron(III), also indicates that IMn and EMn

could stimulate the formation of $O_2^{\cdot -}$ and $HO^{\cdot}$, via O_2 or H_2O_2. Therefore, there is the possibility of a pro-oxidant activity of marennine, as it has been demonstrated in other respects for several common antioxidants (*67, 68*). However, in the $HO^{\cdot}$ scavenging test, the absorbance of marennine samples did not exceed 100% of the control, even at a final concentration of 100 μmol·L^{-1}. This result suggests that IMn and EMn possessed, at least in the range of concentration tested, an antioxidant capacity greater than their ability to degrade deoxyribose.

Phenolic compounds are effective antioxidants due to their ability to donate hydrogen from hydroxyl groups and the structural relationships between different parts of their chemical structure (*5, 7, 69*). The pure native form of marennine, prepared as described by Pouvreau et al. (*44*), was used in this study to investigate the antioxidant properties of this pigment, known to have a polyphenolic-related structure but lacking any link to a polypeptide, glycoside, or transition metal. Because of their slightly distinct molecular structures (*40*), both the intra- (IMn) and extracellular (EMn) forms of marennine were tested. The structural analysis of these complex molecules is in progress, and there are cumulative spectroscopic data (NMR, Raman, and IR) that demonstrate unequivocally the presence of hydroxyl and phenyl groups in their molecular skeleton but exhibiting an organization of the aromatic rings different from that of proanthocyanidins (*40, 70*). IMn (MW = 10 751 Da) and EMn (MW = 9893 Da) molecules contain 8−9 and 10−12 phenol equivalents (mol/mol), respectively (*40*). This difference in terms of phenolic ratio could explain why EMn displayed significantly stronger antioxidant activity than IMn in most of the in vitro evaluation tests. In fact, it has been established that the antioxidant power of the polyphenols, and especially the antiradical activity, depends on the location (*7, 69*) and the number of hydroxyl substitutions in the structure (*71, 72*). In the same manner, the greater antioxidant activity recorded for IMn and EMn, compared to that of the usual commercial antioxidants, could be imputed, given its relatively higher molecular weight, to a higher proportion of phenolic and hydroxylic groups in marennine.

Among many bioactive molecules, the polyphenolic compounds are the topic of numerous investigations because of their great potential as antioxidants, so much so that they have also been ascribed health-promoting properties. Indeed, the most active antioxidants belong to the family of phenolic compounds (*73*). Currently, plants and seaweeds are considered as major polyphenol-producing organisms, while microalgae, and more especially marine species, receive little attention. Herein, we report the in vitro antioxidant activities of a natural polyphenolic pigment produced by a marine diatom, *H. ostrearia*, well-known to be responsible for the greening of cultured oysters and as a result present in human consumption. In addition, as regards possible antioxidative protection, it is important to emphasize that the oysters accumulate mainly the excreted form (EMn) of marennine (*70*); thus, it is the strongest antioxidant form of the pigment that is consumed.

In our opinion, the potential use of marennine as a food additive is based on two relevant properties: its natural blue coloration and its antioxidant capacities. Furthermore, it must fulfill three requirements: absence of toxicity, bioavailability, and possibility of production on an industrial scale. The fact that there are not many natural blue colorants commercially available (*74*) constitutes a great advantage for marennine. Pouvreau and co-workers (*40, 70*) have described the main physicochemical characteristics of the pigment, and its colorimetric parameters have been studied and indexed in the CIE *L*a*b** scale, which is recognized for a complete definition of colors (*75*). The pigment is highly water-soluble and nonhydrolyzable. Its blue-green dye is very heat- and light-resistant, and it does not exhibit any significant modification in a pH range from 6 to 8. The apparent antioxidant properties of marennine might be beneficial to the antioxidant system of the human body and also raise the possibility of it being used as a protective agent against the oxidative damage of food products. Of course, further metabolic and toxicological investigations are needed to demonstrate that marennine could be used as a food grade supplement, but no toxicity related to the pigment has ever been reported during all the time marennine-greened oysters have been eaten by humans. Recent studies have also demonstrated the absorbability and the bioavalability of polyphenols in humans (*76, 77*). The industrial production of marennine would require an optimization of the cultivation of *Haslea ostrearia* and the purification of the pigment. In fact, the method proposed by Pouvreau et al. (*44*) includes semipreparative technical procedures, like ultrafiltration through membranes, which could easily be scaled up to a larger production system.

In conclusion, on the basis of all these observations, we think that the blue-green polyphenolic pigment marennine, from the marine diatom *Haslea ostrearia*, may be considered as a novel natural source of antioxidant, as a possible food additive or coloring ingredient.

ABBREVIATIONS USED

AA, ascorbic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DPPH, diphenylpicrylhydrazyl; dThd, thymidine; dMFn, 4′,5-dihydroxy-7-methoxyflavone; DTNB, dithionitrobenzoate; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immuno assay; EMn, extracellular marennine; IMn, intracellular marennine; IR, infrared; MW, molecular weight; NBT, nitroblue tetrazolium; NMR, nuclear magnetic resonance; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TX, Trolox.

ACKNOWLEDGMENT

We are grateful to P. Gaudin (MMS, EA 2160, PML-UFR Sciences, Université de Nantes) for part of the diatom strain cultivation, Dr. V. Turpin and Dr. L. Barillé (MMS, EA 2160, PML-UFR Sciences, Université de Nantes) for helpful discussions about statistical estimations, D. Laroque, A. Chabeaud, and A. Denes (Laboratoire ANTiOX, Université de Bretagne Occidentale) for their kind help during the measurement of antioxidative and radical scavenging activities, and Carol Robins for correcting the English of the manuscript.

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Received for review October 31, 2007. Revised manuscript received April 8, 2008. Accepted May 20, 2008. Part of the research presented here was supported by a Valbiomar European Grant.

JF073187N