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Full Paper

The cyanobacterial UV-absorbing pigment scytonemin displays radical-scavenging activity

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Scytonemin is a 544-Da hydrophobic pigment that can absorb UV-A radiation. It is present in cyanobacterial sheaths and is thought to function as a UV protectant. In this study, scytonemin was purified from the terrestrial cyanobacterium *Nostoc commune*, and its radical-scavenging activity was characterized. The purified scytonemin quenched an organic radical in vitro and accounted for up to 10% of the total activity of an ethanol extract of *N. commune*. These results suggest that the extracellular UV-absorbing pigment scytonemin has multiple roles, functioning as a UV sunscreen and an antioxidant relevant to anhydrobiosis in *N. commune*.

Key Words——anhydrobiosis; environmental adaptation; *Nostoc commune*; terrestrial cyanobacterium; UV-A

Introduction

Desiccated organisms show no metabolic activity and are able to rapidly resume metabolism upon rehydration—a phenomenon termed "anhydrobiosis" (Billi and Potts, 2002; Clegg, 2001; Crowe, 2002; Crowe et al., 1998; Potts, 2001). The terrestrial cyanobacterium *Nostoc commune* can retain viability for over 100 years upon desiccation (Cameron, 1962; Lipman, 1941). Thus, *N. commune* is considered an anhydrobiotic microorganism with oxygenic photosynthetic capabilities that does not form akinetes (spores) (Potts, 2000). The mechanism of its extreme desiccation tolerance is thought to involve extracellular polysaccharide (EPS) production (Helm et al., 2000; Hill et al., 1994, 1997; Morsy et al., 2008; Sakamoto et al., 2011; Tamaru et al., 2005; Wright et al., 2005), trehalose accumulation (Sakamoto et al., 2009; Yoshida and Sakamoto, 2009), the regulation of photosynthesis, and protection from UV radiation.

In natural habitats, *N. commune* forms visible colonies in which cellular filaments are embeded within the extracellular matrix (Sakamoto et al., 2011; Tamaru et al., 2005; Wright et al., 2005). *N. commune* colonies are naturally subjected to regular cycles of desiccation and wetting. As the water content decreases during desiccation, photosynthetic activity decreases, and no activity is detected in desiccated colonies (Sakamoto et al., 2009). Upon rehydration, photosynthetic activity

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recovers rapidly (Sakamoto et al., 2009; Satoh et al., 2002; Scherer et al., 1984; Tamaru et al., 2005). The regulation of photosynthetic electron transport in response to the availability of water in cells is important to prevent light-induced damage by suppressing reactive oxygen species production under stress conditions (Fukuda et al., 2008; Hirai et al., 2004); however, the mechanisms controlling photosynthesis remain unknown.

Scytonemin is a 544-Da hydrophobic pigment that can absorb UV-A radiation in the range of 320 to 400 nm (Proteau et al., 1993) and occurs exclusively in cyanobacterial sheaths (Garcia-Pichel and Castenholz, 1991). A gene cluster responsible for the production of scytonemin has been identified in *Nostoc punctiforme* ATCC 29113 (Soule et al., 2007) and its biosynthetic pathway investigated to assign the functions of the gene products (Balskus and Walsh, 2008, 2009). The predicted structures of the gene products suggest that the assembly of scytonemin occurs in the periplasmic space (Soule at al., 2007). Scytonemin is synthesized in response to UV-A irradiation (Dillon et al., 2002; Fleming and Castenholz, 2007; Garcia-Pichel and Castenholz, 1991; Soule et al., 2007), consistent with the increase in the level of transcription of the scytonemin biosynthetic gene cluster (Sorrels et al., 2009; Soule et al., 2009). Moreover, scytonemin-producing cyanobacteria are typically found in the upper layers of microbial mat communities, which are exposed to high levels of solar irradiance (Balskus et al., 2011; Büdel et al., 1997). Thus, the extracellular pigment scytonemin is thought to have a protective role against harmful UV irradiation, allowing organisms to adapt to harsh habitats (Castenholz and Garcia-Pichel, 2000; Garcia-Pichel et al., 1992). In addition to its UV protecting ability, scytonemin shows anti-inflammatory and anti-proliferative activities without chemical toxicity, implying biomedical applications (Stevenson, 2002a, b).

Mycosporine-like amino acids (MAAs) are water-soluble pigments that absorb specific UV-B radiation in the range of 280 to 320 nm, and structurally distinct MAAs have been found in taxonomically diverse organisms (Carreto and Carignan, 2011). Recently, two novel glycosylated MAAs were found in *N. commune* (Matsui et al., 2011). An MAA with an absorption maximum at 335 nm was identified as a pentose-bound porphyra-334 derivative with a molecular mass of 478 Da. The other identified MAA has double absorption maxima at 312 and 340 nm and a molecular mass of 1,050 Da. Its unique structure consists of two distinct chromophores, 3-aminocyclohexen-1-one and 1,3-diaminocyclohexene, and two pentose and hexose sugars (Matsui et al., 2011). Given that it contains both scytonemin and MAAs, *N. commune* is thought to be adapted to terrestrial environments that receive high levels of solar radiation.

Desiccated *N. commune* exhibits extreme longevity (Lipman, 1941), suggesting the involvement of antioxidants in the protection of biomolecules from oxidation. It is known that Fe-superoxide dismutase (SodF) is the third most abundant water-soluble protein in *N. commune* (Shirkey et al., 2000). We have characterized low-molecular-weight antioxidants in *N. commune* and found potent radical-scavenging activity associated with the 1,050-Da MAA, suggesting that this novel glycosylated MAA has multiple roles, functioning as a UV protectant and an antioxidant relevant to anhydrobiosis in this unique organism (Matsui et al., 2011). It has been reported that the UV-A absorbing pigment scytonemin has antioxidant activity (Takamatsu et al., 2003), but the study focused primarily on the qualitative characterization of novel antioxidants in marine natural products. Herein, we report the direct demonstration of the antioxidant activity of scytonemin purified from the cyanobacterium *N. commune*.

Materials and Methods

Microorganisms. Colonies of the terrestrial cyanobacterium *Nostoc commune* growing naturally in the field were collected from the Kakuma Campus of Kanazawa University (N 36.547187, E 136.70537). *N. commune* colonies were harvested, washed with tap water to remove soil, air-dried, and stored at room temperature until use. At least three genotypes of *N. commune* that can be differentiated based on differences in their 16S rRNA genes are known to be present on our campus (Arima et al., 2012). Because no obvious differences in their appearance can be observed by eye, we did not determine the genotype of the starting material for the scytonemin purification. There are two types of *N. commune* characterized by the different MAAs that they express; one has a 1,050-Da MAA, and the other has a 478-Da MAA (Matsui et al., 2011). Ethanol extracts were prepared from both MAA types of *N. commune* and were characterized by HPLC analysis as described below.

Purification of scytonemin. Dry colonies of *N. com-*

mune (5 g) were ground using an electric mortar and pestle at 4°C. The *N. commune* powder was suspended in acetonitrile (300 ml), and scytonemin was extracted with stirring at room temperature for 1 h. The extract was filtered with a Buchner funnel type sintered-glass filter and dried under reduced pressure with a rotary evaporator. The solid matter in the flask was collected and suspended in petroleum benzine (2 ml). Petroleum-benzine-insoluble matter was collected by a centrifugation at 21,500 × *g* for 5 min at 4°C, and the pellet was washed with petroleum benzine until the supernatant became colorless. The pellet was dissolved in dimethyl sulfoxide (DMSO, 1.5 ml), and an equal amount (1.5 ml) of acetonitrile/acetic acid (99.8 : 0.2, v/v) was added. After centrifugation at 21,500 × *g* for 5 min at 4°C to remove the insoluble matter, the supernatant was filtered through a 0.20-μm syringe filter (Minisart RC 15, Sartorius Stedim) and injected into an HPLC system equipped with a reverse-phase column (IRICA C18, 20 × 250 mm). The mobile phase was acetonitrile/acetic acid (99.8 : 0.2, v/v) at a flow rate of 3 ml·min^{-1}. The A_{380} was monitored with a Hitachi L-4200 UV-VIS detector. The fraction containing scytonemin was recovered and stored at −30°C overnight to precipitate the scytonemin. After centrifugation at 21,500 × *g* for 5 min at 4°C, the pellet was dissolved in DMSO. The final product of scytonemin was analyzed by HPLC with a reverse-phase column (Wakosil 5C18, 4.6 mm × 250 mm; Wako, Osaka, Japan) using 100% methanol at a flow rate of 1 ml·min^{-1} as the mobile phase. The A_{380} was detected with a UV-VIS detector. The scytonemin concentration was determined from the A_{380} using the extinction coefficient of 112.6 L·g^{-1}·cm^{-1} (Garcia-Pichel et al., 1992). Scytonemin was reduced by adding an equal molar amount of L-ascorbate. UV-VIS spectra were recorded with a Hitachi U-2800 spectrophotometer.

MALDI-TOF MS analysis. MALDI-TOF MS analysis was performed at the Division of Functional Genomics, Advanced Science Research Center, Kanazawa University, with a tandem mass spectrometer (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA) using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

NMR analysis. NMR spectra of scytonemin in $(CD_3)_2SO$ were recorded with a JOEL ECA600 spectrometer at the Research Institute for Instrumental Analysis at Kanazawa University. 3-(Trimethylsilyl)propanoic acid (TMP) was used as an internal NMR standard.

Measurement of the Trolox equivalent antioxidant capacity (TEAC). The radical-scavenging activity was measured using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate in a colorimetric assay (Re et al., 1999). Methanol was used as a solvent for the reaction mixtures. Decolorization was monitored spectrophotometrically by measuring the A_{734} for 1 h. The electron spin resonance (ESR) signals of ABTS or 2,2-diphenyl-1-picrylhydrazyl (DPPH) were recorded with a free radical monitor (JOEL JES-FR30EX). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard.

Chromatographic separation of scytonemin and radical-scavenging activity in ethanol extracts. N. commune powder (200 mg) containing the 1,050-Da MAA was suspended in ethanol (5 ml) and extracted at 5°C with stirring overnight in the dark. After centrifugation at 2,300 × *g* for 5 min at 4°C, the supernatant was concentrated with a centrifugal concentrator (VC-360, TAITEC) and centrifuged again at 2,300 × *g* for 5 min at 4°C. The supernatant was filtered through a syringe-driven Millex-GV filter (0.22 μm, PVDF, hydrophilic, Millipore). The concentrated ethanol extract containing 364 nmol TEAC was injected into an HPLC system equipped with a Hitachi L-6200 pump, a L-4200 UV-VIS detector, and a reverse-phase column (Wakosil-II 5C18 AR, 4.6 mm × 250 mm; Wako, Osaka, Japan). The mobile phase changed stepwise from 60% ethanol for the initial 3 min, to 75% ethanol for 14 min, to 90% ethanol for 14 min, and to 100% methanol for the final 31 min. The flow rate was constant at 0.5 ml·min^{-1}. Fractions of 1 ml were collected every 2 min. Scytonemin was detected based on the A_{380} and identified based on its UV-absorption spectrum. The radical-scavenging activity was measured using the ABTS decolorization assay. The fraction containing scytonemin was recovered and concentrated with a centrifugal concentrator (VC-360, TAITEC). The concentrated scytonemin fraction containing 19 nmol TEAC was injected into the HPLC system again and fractionated under conditions identical to those for the first chromatographic run.

Results and Discussion

Purification and identification of scytonemin

Scytonemin was purified from field-isolated, naturally growing colonies of the terrestrial cyanobacterium

Nostoc commune (Table 1). The purified scytonemin had a characteristic UV absorption spectrum with an absorption peak at 380 nm, and its reduced form also had a characteristic UV-VIS absorption spectrum (Fig. S1: See electronic data in J-STAGE). These absorption spectra were identical to those reported previously (Garcia-Pichel and Catenholz, 1991; Squier et al., 2004). The purified scytonemin showed a single elution peak when analyzed by HPLC (Fig. S2: See electronic data in J-STAGE), indicating high purity. The molecular weight was 544 and its predicted molecular formula was $C_{36}H_{20}N_2O_4$, as determined by MALDI-TOF MS analysis, which were identical to those of the oxidized form of scytonemin. NMR spectra of the purified scytonemin were identical to those reported previously (Table S1: See electronical data in J-STAGE). These results confirm the chemical structure of scytonemin from *N. commune* (Fig. S1; Proteau et al., 1993; Squier et al., 2004).

Using our methods, scytonemin was efficiently extracted and purified with a high yield without degradation (Table 1; Fig. S2). Scytonemin is a major pigment and accounts for 0.04% of the dry weight of field-isolated naturally growing colonies (Sakamoto et al., 2011). Based on this scytonemin content, the estimated amount of scytonemin in 5 g of *N. commune* powder was 2 mg. At the initial step of our purification, 2.8 mg of scytonemin was extracted from 5 g of starting material (Table 1), suggesting a high efficiency of the extraction with acetonitrile. At the final step of our purification, scytonemin was precipitated at −30°C (Table 1; Fig. S1). The resulting yellow-brown scytonemin pellet was not soluble in acetonitrile or chloroform, although it was soluble in DMSO. Thus, we used DMSO to dissolve the final product. This change of its solubility

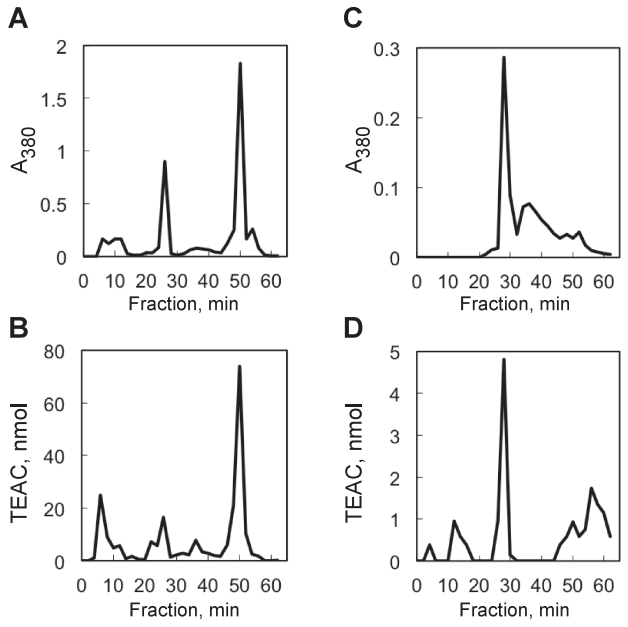

Fig. 1. Characterization of scytonemin and antioxidants in an ethanol extract of *Nostoc commune*.

The ethanol extract of *N. commune* containing 364 nmol TEAC was injected into an HPLC system and fractionated with a reverse-phase column (A and B). Scytonemin and the radical-scavenging activity co-eluted in the fraction at 26 min. The scytonemin fraction containing 19 nmol TEAC was recovered and injected again into the same HPLC system (C and D). Scytonemin and the radical-scavenging activity co-eluted in the same fraction at 28 min. Scytonemin was detected based on the A_{380} and identified based on its UV-absorption spectrum (A and C). The radical-scavenging activity was measured by the ABTS decolorization assay (B and D). The data from a single experiment using *N. commune* with the 1,050-Da MAA are shown, but similar experiments were repeated independently at least three times with the other type of *N. commune* containing a different MAA. Identical results were obtained in all cases.

Table 1. Purification of scytonemin from *Nostoc commune*[a].

Step	Volume (ml)	Scytonemin concentration[b] ($mg \cdot L^{-1}$)	Scytonemin amount (mg)	Yield (%)
Acetonitrile extract	375	7.5	2.8	100
Petroleum-benzine washed pellet[c]	2.5	930	2.3	82
Reverse-phase HPLC	37.5	38.4	1.4	51
Scytonemin pellet	—	—	1.0	36

[a] Dry colonies of *N. commune* (5 g) were used as the starting material, and scytonemin was purified as described in MATERIALS AND METHODS. [b] The scytonemin concentration was determined using an absorption coefficient of 112.6 $L \cdot g^{-1} \cdot cm^{-1}$(Garcia-Pichel et al., 1992). [c]Solubilized in dimethyl sulfoxide/acetonitrile/acetic acid (50 : 49.9 : 0.1, v/v).

may suggest the difference in its forms in vivo and in vitro. It has been reported that scytonemin occurs in the extracellular matrix and binds to the WspA protein, a major extracellular matrix protein of *N. commune* (Wright et al., 2005). When extracted, scytonemin molecules may interact each other to precipitate in a solution because of its planar molecular structure (Fig. S1).

Radical-scavenging activity of scytonemin

Table 2 shows the radical-scavenging activity detected in the purified scytonemin from *N. commune*. Scytonemin showed ABTS radical-scavenging activity when the decolorization of ABTS radicals was monitored. During the time course experiments, the decolorization of ABTS radicals increased as the incubation time increased from 10 min to 2 h (Fig. S3: See electronic data in J-STAGE), suggesting that scytonemin is a slow-acting radical scavenger. When the decrease in the number of ABTS radicals by ESR was directly monitored, scytonemin showed higher activity than did Trolox (Table 2). Trolox, which is a common standard in this type of assay, is a vitamin E derivative and is known to be a fast-acting scavenger (Fig. S3). The DPPH radical is known to have selectivity for scavengers with strong radical-scavenging activity, and scytonemin was incapable of scavenging DPPH radicals (Table 2). These results indicate that scytonemin is a slow-acting radical scavenger that reacts with an ABTS organic radical in vitro. It has been reported that scytonemin synthesis is enhanced by oxidative stress (Dillon et al., 2002) and by periodic desiccation (Fleming and Castenholz, 2007) in the presence of UV-A irradiation, consistent with the multiple roles of scytonemin (i.e., shielding the cells from UV irradiation and scavenging free radicals).

Table 2. Radical-scavenging activity in scytonemin[a].

Assay	Colorimetry[b]	ESR[c]	
Substrate	ABTS	ABTS	DPPH
		IC_{50} (μM)	
Trolox[d]	300	167	93
Scytonemin	567	36	—

[a] Radical-scavenging activity was measured using ABTS or DPPH as an organic radical source. [b] The decolorization of ABTS was monitored with a spectrophotometer for 1 h. [c] The ESR signals were monitored with a free radical monitor (Joel JES-FR30EX). [d] Trolox was used as a standard. —, not detected. IC_{50} (50% inhibitory concentration) values are shown.

The radical-scavenging activity associated with scytonemin was examined in an ethanol extract of *N. commune* by HPLC analysis (Fig. 1). The elution peak of oxidized scytonemin was identified according to its characteristic UV-absorption spectrum (Fig. S1). The radical-scavenging activity eluted together with scytonemin at 26 min, and this scytonemin-associated activity accounted for approximately 10% of the total recovered activity (Fig. 1AB). The scytonemin fraction in the first chromatographic run was recovered and refractionated using the same HPLC system (Fig. 1CD). Scytonemin and the radical-scavenging activity eluted together in the same fraction in the second chromatographic run, indicating the association of scytonemin and the radical-scavenging activity. The remainder of the radical-scavenging activity in the ethanol extract was eluted in the fractions between 46 and 54 min (Fig. 1AB) and was associated with chlorophyll and carotenoids, which are known antioxidants (El-Agamey et al., 2004; Lanfer-Marquez et al., 2005) present in these cells. Scytonemin is thought to be a main hydrophobic antioxidant functioning in the extracellular matrix; thus the level of the scytonemin-associated radical-scavenging activity should not be underestimated. However, its role in vivo remains to be examined in future studies.

There are two types of *N. commune* characterized by different MAAs; one expresses a 1,050-Da MAA, and the other expresses a 478-Da MAA (Matsui et al., 2011). Consistent with these types of MAAs, different water-soluble antioxidants are found in these different types of *N. commune* (Matsui et al., 2011). An ethanol extract was prepared from *N. commune* colonies expressing the 478-Da MAA and was examined by the same HPLC assay to compare the ethanol-soluble antioxidants. No difference was found in terms of the elution profiles of the pigments or the radical-scavenging activities (data not shown), suggesting that the compositions of ethanol-soluble antioxidants are similar in these types of *N. commune* even though the water-soluble antioxidant compositions are different (Matsui et al., 2011).

The MAAs in *N. commune* are hydrophilic UV-B absorbing pigments with radical-scavenging activities (Matsui et al., 2011) and are components of the extra-

cellular matrix (Wright et al., 2005). It is noteworthy that both types of UV-absorbing pigments—scytonemin and MAAs—are extracellular antioxidants in *N. commune*. Under desiccated conditions, both hydrophilic and hydrophobic antioxidants along with the extracellular polysaccharides cover the cells to protect them from oxidation. Supposedly, scytonemin is required for survival in terrestrial environments and for sustaining viability in a desiccated state for over 100 years; however, the mechanisms of action for the radical-scavenging abilities and the ecophysiological roles of scytonemin in the terrestrial cyanobacterium *N. commune* remain to be elucidated in future studies.

Concluding Remarks

Scytonemin has multiple roles and functions as a UV protectant and as an antioxidant relevant to anhydrobiosis in the terrestrial cyanobacterium *Nostoc commune*. Scytonemin may be a useful secondary metabolite from cyanobacteria to use as a sunscreen and/or a preservative.

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Supplementary Materials

Fig. S1. Scytonemin.

(A) The pellet of purified scytonemin observed with a microscope. (B) Chemical structures of scytonemin and its reduced form. (C) UV-VIS absorption spectra of scytonemin and its reduced form in acetonitrile. The purified scytonemin was reduced with L-ascorbate.

Fig. S2. HPLC analysis of scytonemin.

The purified scytonemin of *Nostoc commune* was analyzed by HPLC with a reverse-phase column (Wakosil 5C18, 4.6 mm × 250 mm; Wako, Osaka, Japan) using 100% methanol at a flow rate of 1 ml·min^{-1} as the mobile phase and was detected by the A_{380}. Scytonemin was eluted at 4.5 min.

Fig. S3. Time-course of ABTS radical scavenging in scytonemin.

The reduction of the A_{734} was monitored during the incubation at room temperature. Trolox (0.1 mM) and L-ascorbate (0.2 mM), which are fast-acting radical scavengers, and β-carotene (1 mM), which is a slow-acting radical scavenger, were used for comparison. The experiment was performed in triplicate, and the averages ± SD are shown.

Table S1. NMR analysis of scytonemin.

Chemical shifts (ppm) for the purified scytonemin in dimethyl sulfoxide (DMSO) are compared with those reported in *N,N*-dimethylformamide (DMF) (Proteau et al, 1993). Coupling constants (Hz) are shown in parentheses.

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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