Chemical structure elucidation of the carotenoids in the extremely radiation-resistant bacterium *Deinococcus* *radiodurans* R1

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Abstract

The main carotenoid in the extremely radiation-resistant bacterium *Deinococcus radiodurans* is deinoxanthin. In this paper, based on UV-Vis, MS/MS spectra as well as the chemical properties, the other main two carotenoids in this bacterium were identified as ((all-E)-1'-hydroxy-3',4'-didehydro-1',2'-dihydro- β,ψ -carotene-4-one-1), and ((all-E)-1'-hydroxy-3',4'-didehydro-1',2',2,3-quahydro- β,ψ -carotene-4-one-1), respectively. The fully structure elucidation for the carotenoids should help to gain a better understanding of the radiation-resistant mechanisms in this remarkable bacterium.

Keywords: Radiation-resistant; *Deinococcus radiodurans*; Carotenoids: Free radicals

29 Introduction

30 *Deinococcus radiodurans* is a gram-positive red-pigmented,
 31 nonsporeforming, nonmotile spherical bacterium that was originally identified
 32 as a contaminant of canned meat irradiated with a sterilizing dose of γ
 33 radiation. *D. radiodurans* is a polyextremophile, showing remarkable
 34 resistance to a range of several damage caused by ionizing radiation,
 35 desiccation, UV radiation, a number of agents and conditions that damage
 36 DNA, or electrophilic mutagens. This bacterium is the most radiation-resistant
 37 organism described to date; exponentially growing cells are 200 times as
 38 resistant to ionizing radiation and 20 times as resistant to UV irradiation as
 39 *Escherichia coli*.

40 Carotenoids represent one of the most widely distributed and structurally
 41 diverse classes of natural pigments, producing colors ranging from light yellow
 42 through orange to deep red. At least 600 different carotenoids are synthesized
 43 naturally, performing important functions in photosynthesis, nutrition and
 44 protecting against oxidative damage. The instability of the carotenoids to heat,
 45 light and air make isolation, identification and quantitation of these natural
 46 pigments unusually challenging. Many carotenoids exhibit provitamin A activity,
 47 others might function as significant dietary antioxidants, and other beneficial
 48 effects of carotenoids that are currently under investigation include inhibition of
 49 carcinogenesis, enhancement of the immune response and prevention of
 50 cardiovascular disease. Recent transcriptome analysis revealed that
 51 *Deinococcus radiodurans* efficiently coordinate their recovery from ionizing
 52 radiation through a complex network of DNA repair and metabolic pathway
 53 switching. However, the additional discovery of numerous irradiation response
 54 genes has provided new targets for the identification of genes primarily crucial
 55 to radiation resistance. Although such investigations provide useful insights
 56 into the mechanisms underlying radiation resistance, a more detailed empirical
 57 explanation of why *D. radiodurans* is so radiation resistant is still needed.

Further research based on alternative genetic and biochemical approaches should help to gain a better understanding of the mechanisms involved in DNA repair

The main carotenoids pigment of *Deinococcus radiodurans* has been identified as deinoxanthin ((all-E)-2,1'-dihydroxy-3',4'-didehydro-1',2'-dihydro- β,ψ -carotene-4-one-1) by Lemee et al. however, the chemical structures of other carotenoids in this bacterium have not been reported, The present work is to deal with the determination of the chemical structures of the red pigments in *D. radiodurans*.

Materials and methods

Chemicals and reagents

Acetonitrile, dichloromethane and methanol (HPLC grade) was obtained from Fisher (Fair Lawn, NJ, USA). Acetone was purchased from Merck (Darmstadt, Germany). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and samples solutions.

Bacteria strain and growth conditions

The R1 strain of *Deinococcus radiodurans* was obtained from CCTCC (China Center for Type Culture Collection, Wuhan, China) was grown in TGY media (0.5% tryptone, 0.3% yeast extract, 0.1% glucose, pH 7.2) with aeration on rotary shakers or on TGY plates (TGY broth supplemented with 1.5% agar) at 30 °C.

Extraction of carotenoids from *D. radiodurans*

After 48 hours of incubation at 30 °C under aerobic conditions and shaking (250 rpm), the culture was harvested by centrifugation (4000 rpm, 20 min), after washing three times with deionized and sterilized water, the cell pellet was mixed with a 20 ml mixture of cold acetone and methanol (3:2, v/v) with 0.1% BHT(buthlated hydroxytoluene) as antioxidant, blended in a mechanical

blender for 20 minutes, and then centrifuged (6000 rpm, 20 min at 4 °C). The extraction was repeated for three times until the residue was devoid of any color and washing were colorless. The extracts were combined and concentrated under vacuum in a rotary evaporator to dryness in the dark at 30 °C. The extract was transferred quantitatively to a 10 ml volumetric flask using portions of 2 ml of mobile phase for the sequent analysis.

HPLC and LC/MS/MS analysis of carotenoids in *D. radiodurans*

The HPLC analysis was performed on an Agilent 1100 high-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) equipped with a diode-assay detector (DAD) covering the range of 190–600 nm. Separation was carried out on an Agilent reverse-phased ODS C₁₈ column (5 µm, 4.6 mm × 250 mm I.D.) maintained at 25 °C. A gradient consisting of solvent A: 85% acetonitrile, 10% dichloromethane and 5% methanol; solvent B: water, 0-12 min 100% A; 12-35 min 85% A was used. The mobile phase consisting of acetonitrile-dichloromethane-methanol (85:10:5, v/v/v) was used for the isocratic elution. The flow rate was 1.0 mL min⁻¹ and the UV-visible DAD detection was monitored at 470 nm.

The semipreparative separation of the carotenoids was performed on Waters Delta 600E semi-HPLC equipped with PDA detector, an autosampler, and a fraction collector. A Symmetry PrepTM reverse-phased ODS C₁₈ column (7 µm, 7.8 mm × 300 mm I.D.) was used. The Empower Pro was used for the instrument control and data analysis. The purified major product was identified by reversed phase HPLC with a purity of 97%.

The LC/MS/MS analysis of carotenoids was performed using a Finnigan LCQTM Deca XP MAX ion trap mass spectrometer (Thermo Finnigan, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface (Finnigan), which connected with a Finnigan Surveyor HPLC system equipped with a quaternary pump, a vacuum membrane degasser, a LightPipeTM UV-visible photodiode-array (PDA) detector, an autosampler. Chromatography

was carried out on a column with a 2.1 mm ×5.0 mm I.D. guard column. A gradient consisting of solvent A: 85% acetonitrile, 10% dichloromethane and 5% methanol; solvent B: water, 0-12 min 100% A; 12-35 min 85% A was used. Multiple reaction monitoring (MRM) was used to perform mass spectrometric identification of a unique product ion from a selected molecular ion that can be monitored and quantified even in the midst of a very complicated biological matrix. The column effluent was introduced into the mass spectrometer using an APCI interface in the positive mode. The voltage of source was 6000 V and the capillary voltage was 19 V. Selected $[M+H]^+$ was analyzed by collision-induced dissociation with argon gas, and product ion spectra were recorded.

Results and discussion

HPLC analysis of carotenoids of *D. radiodurans*

The carotenoids isolated from the cell culture of *D. radiodurans* was separated and detected by HPLC with diode array detection at 470 nm. Three main peaks were found in the HPLC chromatogram (Fig. 1). The UV/Vis spectra of peak 1, 3 and 4 were compared with that of deinoxanthin (peak 2), we found the maximum of peak 1 and 4 are almost the same as that of deinoxanthin, indicating that they have very close chemical structure. That is, they may have the same number of the conjugated polyene system. Peak 2 is the cis isomer of deinoxanthin, as they have the same molecular ion M^+ at m/z at 582 and the maxima at 451, 580 and 506 nm from the PDA detection, whereas peak 3 have another UV/Vis spectrum at 270 nm that is the characteristic spectra of the cis isomer of a carotenoids.

HPLC-tandem MS analysis of carotenoids in *D. radiodurans*

Carotenoids of *D. radiodurans* were analyzed with both positive and negative ion modes. They all showed the protonated molecule $[M+H]^+$ in the positive ion mode. In the negative ion mode, their deprotonated molecules

were very weak. Therefore, the positive ion mode was utilized for the carotenoids analysis.

In the MS/MS experiment, For peak 1, the m/z at 565.3 ($[M+H]^+$) corresponding to a molecular formula $C_{40}H_{52}O_2$ produced a cluster of ions at m/z 546, 505, 401 and along with a series of product ions in the mass range of m/z 100-300. Further characteristic fragmentation were observed at m/z 547.2 ($[M+H]^+-18$) indicating the loss of H_2O . For peak 3, the m/z at 583.2 ($[M+H]^+$) corresponding to a molecular formula $C_{40}H_{54}O_3$. Further characteristic fragmentation were observed at m/z 547.2 ($[M+H]^+-18$) indicating the loss of H_2O . For peak 4, the m/z at 567.3 ($[M+H]^+$) corresponding to a molecular formula $C_{40}H_{54}O_2$. Further characteristic fragmentation were observed at m/z 548.3 ($[M+H]^+-18$) indicating the loss of H_2O . Based on the UV-Vis, and MS/MS spectra as well as the chemical properties the two carotenoids were identified as, ((all-E)-1'-hydroxy-3',4'-didehydro-1',2'-dihydro- β,ψ -carotene-4-one-1) for peak 1, ((all-E)-1'-hydroxy-3',4'-didehydro-1',2',2,3-quahydro- β,ψ -carotene-4-one-1) for peak 4, respectively (Fig. 4). The proposed biosynthesis pathway was also shown.

Conclusion

It has been shown that carotenoids appear, at least in part, as a radioprotective agent. Ionizing radiation can induce singlet oxygen and free radicals and carotenoids were initially known as a photo reductone and a singlet oxygen quencher. It has been widely accepted that very strong relationships exist between the chemical structure and antioxidant function of carotenoids. The two main carotenoids besides deinoxanthin were isolated and analyzed by UV-Vis, APCI-MS/MS as well as the chemical properties test to obtain structure information. This study will help to gain a better understanding of the radiation-resistant mechanisms in this remarkable bacterium.

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Figures:

Figure 1. HPLC chromatogram of carotenoids of *D. radiodurans*. Conditions were as described in the “Materials and methods” section.

27 Figure 2. UV-Vis spectra of four peaks from the HPLC chromatogram of
28 carotenoids in *D. radiodurans*.

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34 Figure 3. Mass spectra of the carotenoids in *D. radiodurans*.

wdyq20061201_567ms2_01 #1 RT: 0.00 AV: 1 NL: 2.30E6
T: + c APCI corona Full ms2 567.20@30.00 [155.00-1500.00]

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wdyq20061201_583ms2_03 #1 RT: 0.00 AV: 1 NL: 2.89E4
T: + c APCI corona Full ms2 583.20@40.00 [160.00-1500.00]

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wdyq20061201_565ms2_02 #1 RT: 0.00 AV: 1 NL: 6.87E5
T: + c APCL corona Full ms2 565.20@34.00 [155.00-1500.00]

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Figure 4. The chemical structures of the main carotenoids in *D. radiodurans* and the proposed biosynthesis pathway.