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Anti-inflammatory property of biomaterial carotenoids production by *Rhodobacter sphaeroides* WL-APD911

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Abstract. A *Rhodobacter sphaeroides* WL-APD911 with the capability of producing the end products of neurosporene and ξ-carotene, rather than the normal end products of spheroidene, was isolated from hundreds of strains by using chemical mutagenesis. The strain WL-APD911 grew well in LB medium, and the colored carotenoids were produced within 3 days of incubation at 30℃. The Rs-M of colored carotenoid extracts obtained from strain WL-APD911 by using methanol extraction inhibited the NO production and iNOS expression by LPS-induced RAW 264.7 cells. This probability of reason for the Rs-M as the best source was to prompt the anti-inflammation and anti-oxidation in nutraceutical products. Finally, the carotenoid extracts (Rs-M) with neurosporene and ξ-carotene was called lycogene to be a newly biomaterial.

Introduction

Carotenoids with unsaturated hydrocarbons are important natural pigments produced by many microorganisms and plants. They are the most diverse and widely distributed in nature, and are used commercially as food colorants, animal feed supplements, and cosmetic and pharmaceutical applications [1]. Carotenoids have some biological functions such as antioxidants, anticarcinogenic agents, prevention of chronic diseases, vision, and cellular growth and development [2].

In *Rodobacter sphaeroides*, the synthesis of carotenoid the first step is the condensation of two molecules of geranylgeranyl pyrophosphate to yield phytoene. Phytoene is colorless and incapable of photoprotection, and is followed by subsequent a series of desaturations to convert into colored carotenoids by phytoene desaturase (CrtI) [3]. The *R. sphaeroides* CrtI catalyses three desaturations, producing neurosporene. This neurosporene is further modifications catalysed by the CrtC/D/F/A enzyme to produce the ends of the molecule in the spheroidene pathways [4].

The aim of this work is to produce neurosporene and its derivatives by mutation in c*rtC* gene of *R. sphaeroides*, rather than the normal end products of spheroidene. These mutants were produced by random chemical mutagenesis [5]. Although carotenoids are widely used in pharmacogical application, neurosporene has not yet been evaluated its anti-inflammatory properties. This present study is desigend to investigate the effect of the methanol extract on potential inhibition of NO production and inducible nitric-oxide synthase (iNOS) expression in mouse macrophage cells (RAW 264.7) induced with LPS [6].

Materials and Methods

Bacterial strain, Media and chemicals. *Rhodobacter sphaeroides* WL-APD911 was isolated from mutants by using chemical mutagenesis and deposited in the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. The strain was grown at 30℃ on agar plate of Luria-Bertani (LB) medium. Dulbecco's modified Eagle's medium, FBS, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Polyclonal anti- iNOS was purchased from BD Biosciences. NTG and LPS (lipopolysaccharide) were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of carotenoid extracts. The *Rhodobacter sphaeroides* WL-APD911 was harvested and washed with saline buffer; then it was extracted twice in the dark with methanol at room temperature. The methanol extract was filtered and removed the solvents under reduced pressure in a rotary evaporator to yield dried crude total extracts. For in vitro experiments, the dried extract was dissolved to 100 mg/mL with DMSO and stored at −20 ◦C until use.

Identification and Characterization of carotenoid extracts. Dried carotenoid extracts were dissolved in methanol and methyl tertbutyl ether in the ratio of 50:50 (v/v), which was the starting equilibration buffer. High-performance liquid chromatography (Agilent Technologies, Waldborm, Germany) connection with electrospary ionization-triple stage quadrupole mass spectrometer (Sciex API 4000, Applied Biosystems, Foster City, CA, USA) was used. Carotenoids were separated on YMC column (4.6 × 250 mm, 5 µm; YMC, CT99S05-2546WT).

Cell culture. Murine RAW264.7 macrophages obtained from A.T.C.C. (Manassas, VA, U.S.A.) were grown at 37 ◦C in 5% CO_2 using Dulbecco's modified Eagle's medium containing 10% (v/v) FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. When examining the effects of drug, cells were treated with vehicle (DMSO) as a control.

Immunoblotting analysis. After stimulation, cells were rinsed twice with ice-cold PBS, and 100 µl of cell lysis buffer (20 mM Tris/HCl, pH 7.5/125 mM NaCl/1% Triton X-100/1 mM $MgCl_2$/25 mM β-glycerophosphate/ 50 mM NaF/100 µM Na_3 VO_4/1 mM PMSF/10 µg/ml leupeptin/10 µg/ml aprotinin) was then added to each plate. Protein was denatured in SDS, electrophoresed on SDS/polyacrylamide (10% gel), and transferred on to nitrocellulose membrane. Non-specific binding was blocked with TBST (50 mM Tris/HCl, pH 7.5/150 mM NaCl/0.1% Tween 20) containing 5% (w/v) non-fat milk for 1 h at room temperature. After incubation with the appropriate first antibodies, membranes were washed three times with TBST. The secondary antibody was incubated for 1 h. After three washes with TBST, the protein bands were detected with the ECLR reagent.

Nitrite measurement. Cells were cultured in 24-well plates in 500 µl of culture medium until confluence. They were treated with LPS in the absence or presence of extracts for 24 h, and then the culture media were collected. Nitrite was measured by adding 100 µl of Griess reagent (1%sulphanilamide and 0.1% naphthylethylenediamide in 5% (v/v) phosphoric acid) to 100 µl samples of the culture medium. The absorbance at 550 nm (OD_{550}) was measured using a microplate reader, and the nitrite concentration was calculated by comparing with the OD_{550} produced using standard solutions of sodium nitrite in the culture medium.

Statistical evaluation. Values are expressed as means± S.E.M. for at least three experiments, which were performed in duplicate. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a $P<0.05$ was considered to be statistically significant.

Results and Discussion

Isolation and properties of a *crtC* mutant of *Rhodobacter sphaeroides* WL-APD911. A mutant strain capable of producing colored carotenoids, compared to the phentype of wild type with colorless pigments, was isolated from among hundreds of strains by using chemical mutagent. The isolated mutant was identified as *R. sphaeroides* WL-APD911, and its *crtC* was sequenced and conferred in comparison with the original gene of wild type (data not shown).

Identification and characterization of Rs-M. To find out whether or not lycopene production in strain WL-APD911, the methanol extracts (Rs-M) were identified by HPLC analysis with lycopene standard. As showed in Fig.1, the profile of HPLC traces showed the Rs-M without lycopene. HPLC analysis demonstrated the carotenoid extracts were neurosporene and ξ-carotene in Rs-M of a *crtC* mutant of WL-APD911 was shown fig. 2. The *crtC* mutant resulted in the synthesis of mainly neurosporene. This produced carotenoid extracts named as lycogene.

Rs-M inhibited LPS-induced nitric oxide production from RAW 264.7 cells. To investigate the inhibitiory effect of Rs-M on LPS-induced NO production in cultured RAW 264.7 cells, the cells were treated with increasing concentrations of Rs-M resulted to a dose-dependent reduction of NO

production. As showed in Fig. 3, the maximal inhibition of 60 % at 100 μg/mL of Rs-M on the LPS-induced RAW 264.7 cells. Using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as an index of mitochondria activity, Rs-M incubation at concentrations up to 100 μg/mL for 24 h did not cause cell toxicity (data not shown).

Rs-M inhibited LPS-induced iNOS protein expression. Furthermore, the expression of iNOS protein in response to treat with LPS (100 ng/mL) was examined in Rs-M -pretreat RAW264.7 cell by immunoblotting using an anti-iNOS antibody. Apparently, the iNOS protein is significantly reduced according to increase the concentration of Rs-M. As showed in Fig. 4, the maximal inhibition at 100 μg/mL of Rs-M on the LPS-induced RAW 264.7 cells.

Conclusions

The purpose of this work was to isolate a *crtC* mutant of *R. sphaeroides*, and the isolated mutant was identified as *R. sphaeroides* WL-APD911, which produced the colored carotenoids. The Rs-M of colored carotenoid extracts obtained from *R. sphaeroides* WL-APD911 by using methanol solvent were identified the characters of neurosporene and ξ -carotene, but without lycopene, and the mixtures were named as lycogene. Subsequently, the anti-inflammatory property of Rs-M was examined by using the LPS-induced RAW 264.7 cells. The results of this study have showed that NO production and iNOS expression were suppressed apparently in the 100 μg/mL of Rs-M. Thus we have suggested an important role for lycogene in human health, cosmetic and nutraceutical development.

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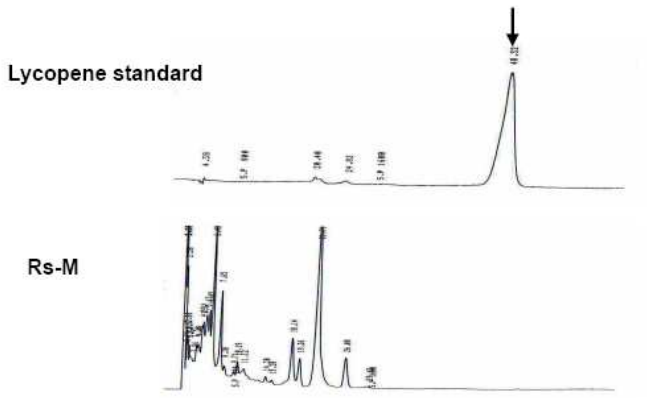

Fig. 1 HPLC elution profile of lycopene standard and Rs-M produced in *R. sphaeroides* WL-APD911. An arrow indicated the retention time of lycopene standard.

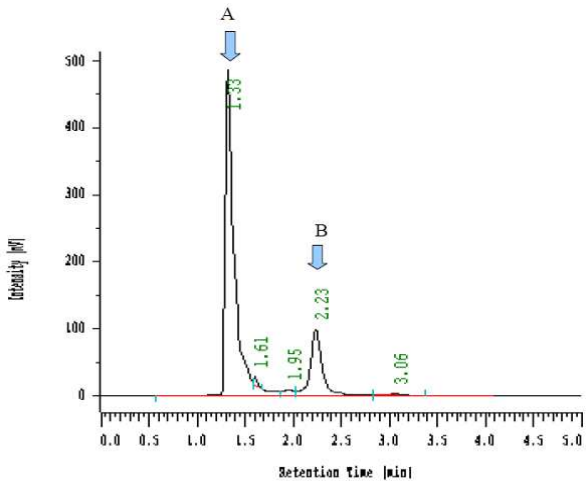

Fig. 2 Profile of characteristic of the Rs-M. A and B denoted neurosporene and ξ -carotene, respectively.

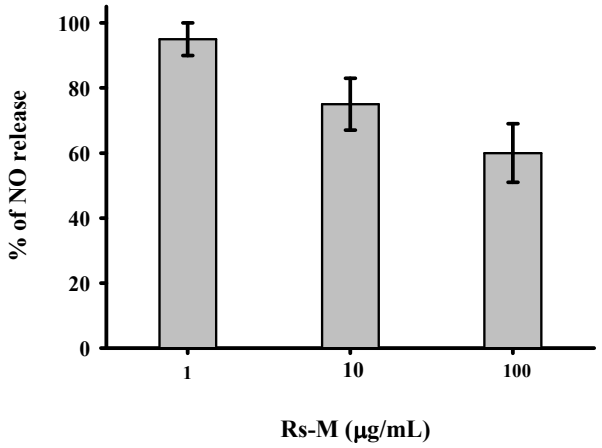

Fig. 3 Effect of Rs-M on NO production. RAW 264.7 macrophage cells were treated with LPS (100 ng/mL) and various concentration of Rs-M with lycogene mixtures for 24 h. The amount of nitrite in supernatant from each treatment group was measured by using Griess reagent.

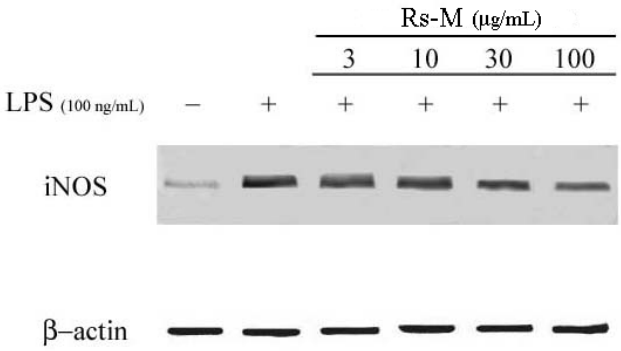

Fig. 4. Effect of Rs-M on inhibition of LPS-induced iNOS protein expression. RAW 264.7 macrophage cells were treated with LPS (100 ng/mL) and various concentration of Rs-M with lycogene mixtures for 24 h. The detection of β-action was done in the same blot as a loading control.

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