Bioactivity_of_Carotenoid_from _Bacterial_Symbiont_Sinularia

by Peerj Simbion

Sun protection and antibacterial activities of carotenoids from soft coral *Sinularia* sp. symbiotic bacteria from Panjang Island, North Java Sea

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Abstract

Secondary metabolites are used by various organisms as defense mechanisms to survive environmental stress, such as the carotenoids secreted by soft coral and other marine organisms. This study analyzed the ability of the carotenoids from an isolated bacterial symbiont from soft coral *Sinularia* sp. collected from Panjang Island, North Java Sea to act as a sun protector as well as to inhibit the growth of multidrug-resistant (MDR) bacteria. The bacteria were isolated from soft coral *Sinularia* sp. and the carotenoid content was determined, showing that 19.PP.Sc.13 possessed the highest carotenoid content, so was selected for further analysis. The carotenoids extracted from this soft coral *Sinularia* sp. bacterial symbiont possessed biological activities, including significant antioxidant, sun protection, and antibacterial activities against MRSA. This study provides empírical evidence to further investigate the de novo biological production of secondary metabolites via bacterial symbiont and its benefit for the pharmaceutical industry.

Keywords: carotenoids, bacterial symbiont, *Sinularia* sp., antioxidant, antibacterial, sun protection, multidrug-resistant

Introduction

Carotenoids are potent nonessential antioxidants and fat-soluble pigments characterized by their yellow-to-orange color (Stahl and Sies, 2003). They consist of long-chain isoprene with one or more conjugated double bonds (Fiedor and Burda, 2014) and since they cannot be synthesized in humans, the carotenoids must be obtained from an exogenous source, such as terrestrial and marine organisms. Carotenoids from marine organisms including seaweed, microalgae, marine animals, hard corals, and soft corals have a unique chemical structure and physiochemical characteristics. Soft coral is enriched with secondary metabolites, including a unique class of carotenoids but the harvesting of coral could potentially damage the environment, so one potential strategy is by exploring symbiotic bacteria associated with soft corals since these symbionts produce secondary metabolites similar to their host (J. Grant Burgess *et al*., 2003; Murti *et al*., 2016)Kusmita *et al*., 2017; Kusmita, Nuryadi, *et al*., 2021). Many carotenoids produced from soft coral symbiotic bacteria possess antioxidant (Kusmita *et al*., 2017; Mutiara *et al*., 2017; Kusmita, Nuryadi, *et al*., 2021), anti-aging (Kusmita, Mutmainah, *et al*., 2021), and antibacterial activities (Murti *et al*., 2016; Kusmita, Nuryadi, *et al*., 2021). Therefore, this study isolated symbiotic bacteria associated with *Sinularia* sp. and analyzed their sun protection and antibacterial activities.

MATERIAL AND METHODS

Sampling

Samples of soft coral *Sinularia* sp. were collected from Panjang Island by scuba divers at a depth of approximately 2 m (**Figure 1**) and placed in a plastic container containing sterile seawater in a coolbox (Nugraheni *et al*., 2010; Kusmita *et al*., 2017). Images of the *Sinularia* sp. samples were taken *in situ* and at the surface using a Canon S50 underwater camera.

Figure 1. Sampling location

Isolation of bacterial symbionts from soft corals

Bacterial symbionts were isolated by the distribution method (Radjasa, *et al*, 2009). Briefly, soft corals were rinsed using sterile seawater and were cut into pieces, then serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) and 1 ml of the sample was plated onto Zobell 2216E agar. The Petri dishes were incubated at 30ºC for 1-2 days and the colored bacterial colonies on the agar surface were separated by a streak method to obtain pure bacterial strains (Burgess *et al*. 2003; Radjasa *et al*. 2009; Murti *et al*. 2016).

Pigment extraction and total carotenoid content

The carotenoid-containing bacteria were extracted in methanol. The pigment-containing fraction was filtered, evaporated, and dried under N_2 (Nugraheni *et al*., 2010; Kusmita *et al*., 2017) before the total carotenoid content was determined using a UV-Vis spectrophotometer at a wavelength of 470 nm. Total carotenoids were calculated using the Gross equation (1990) as follows:

$$\mu\text{g carotenoid/g} = \frac{A \times V \times 10^6}{A_{1\,cm}^{1\%} \times 100 \times G}$$

Molecular profiling

The genomic DNA of the selected bacterial strain was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the manufacturer's protocol. The PCR mixture contained 1 μl of genomic DNA, 1 μl of 20 pmol 27F primer, 1 μl of 20 pmol 1492R primer, 12.5 μl of My Taq Red Mix 2x (Bioline), and 9.5 μl of ddH_2O. The PCR cycles were initial denaturation at 95°C for 1 min, followed by 35 cycles of 95 °C for 15 s, 52°C for 15 s, and 68°C for 45 s. The quality of the PCR product was checked using 1% agarose gel before sequencing. The sequence was input into GenBank (Altschul *et al*., 1997; Radjasa *et al*., 2013) and aligned with 35 sequences according to the MUSCLE algorithm. A maximum likelihood phylogenetic tree based on the 16S rRNA gene was constructed for phylogenetic analyses using the GTR+G+I model with 1000 bootstrap replications using MEGA 7.0 (Kumar, Stecher & Tamura, 2016). The sequence was deposited to GenBank under accession number LC537902.

Antioxidant activity

The extract was dissolved in methanol and serially diluted. The blank was a methanol solution, while the sample solution consisted of 4 mL DPPH plus 1 mL extract. The blank and samples were incubated for 30 minutes in the dark and the absorbance was measured at a wavelength of 517 nm using a single UV-visible spectrophotometer Shimadzu 1240 to calculate the antioxidant activity:

$$\%\ \text{Inhibitory} = \frac{[DPPH]_0 - [DPPH]_s}{[DPPH]_0} \text{X}100\ \%$$

Where, $[DPPH]_0$ = Initial DPPH concentration, $[DPPH]_s$ = Final DPPH concentration remaining.

Determination of the Sun Protection Factor (SPF)

The sun protection was assessed *in vitro* with a UV-Vis spectrophotometer at a wavelength of 290-320 nm. The average absorption was set at intervals of 5 nm and the carotenoid extracts were used at the IC_{50} by antioxidant activity. The SPF value was calculated using the Mansyur equation (Gonzalez *et al*., 2007; Pelizzo *et al*., 2012; Liandhajani *et al*., 2013) and categorized according to Table 1:

$$SPF = CV \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda)$$

Where, CF = correction factor, EE = Spectrum of erythema effect, I = spectrum of the sun's intensity, and Abs = Absorbance of the sample.

Table 1. Prediction of SPF

Determination of % transmission of erythema and pigmentation

The % transmission of erythema was determined by the absorption of the carotenoid extract at 292−372 nm every 5 nm (Abdassah *et al*., 2015). Based on the absorption value (A) obtained, the transmission (T) was calculated by the formula:

$$A = - \log T$$

Transmission of erythema (Te) calculated by the formula:

$$Te = T \times Fe$$

Where Fe is the erythema flux value at wavelength 290-320 nm and Fp is the value of pigmentation flux at wavelength 320-375 nm (Balsam & Saragin, 1972). The amount of erythema flux that is passed on by sunscreen (Ee) is calculated by the formula:

$$\%\ \text{transmission of erythema (Te)} = \frac{Ee}{\sum Fe} = \frac{\sum(T \times Fe)}{\sum Fe}$$

$$\%\ \text{transmision of pigmentation (Tp)} = \frac{Ep}{\sum Fp} = \frac{\sum(T \times Fp)}{\sum Fp}$$

%Te and Tp can determine the sunscreen category in **Table 2.**

Table 2. The category of sunscreen activity

Antibacterial activity

Antibacterial activity was assessed based on the previous agar diffusion method (Radjasa, Salasia, *et al*., 2007; Murti & Radjasa, 2012). A dilution series of the carotenoid extract (4%, 6%, and 8% m/v) was prepared and plated onto MSA or EMB agar. Ciprofloxacin 0.05% (b/v) served as the positive control. The microbial plate was then incubated at 37ºC for 24 h and the growth inhibition zones were measured.

RESULTS AND DISCUSSION

Sinularia sp. samples (Figure 2) were collected from Panjang Island, Jepara Regency, Central Java, Indonesia (Figure 1) on January 19, 2019. Six bacterial isolates were obtained (Table 1) but only four isolates contained carotenoids as indicated by the yellow/orange color (Figure 3) and were subjected to further analyses.

Figure 2. *Sinularia sp*.

Table 3. Bacterial isolates from *Sinularia* sp. 19.PP.Sc.1

Figure 3. Carotenoid producing bacteria (a) 19.PP.Sc.13, (b) 19.PP.Sc.14, (c) 19.PP.Sc.15, and (d) 19 PP.Sc.16

The total carotenoid content of the four bacterial isolates was determined (Figure 4) showing that 19.PP.Sc.13 had the highest content. The spectrum of the carotenoid extract from the symbiont bacteria 19.PP.Sc.13 is presented in Figure 5, showing two peaks at 487.90 nm and 458.50 nm, indicating a carotenoid compound with an absorption peak between 300−600 nm (Rodriguez-Amaya & Kimura, 2004).

Figure 4. Total carotenoid content

Figure 5. The spectrum of carotenoids from the bacteria symbiont 19.PP.Sc.13 extract

The molecular profiling revealed that the 16 bacterial rDNA sequence of 19.PP.Sc.13 was 1500 bp (Figure 6, Table 3) and 100% homologous to *Virgibacillus* sp strain CARE V34 (Table 4).

Figure 6. The 16 bacterial rDNA sequence of 19.PP.Sc.13 was 1500 bp

Table 4. Nucleotide sequence of symbiotic bacterium 19.PP.Sc.13

Table 5. Results of the BLAST Homology Search for Symbiotic Bacteria 19.PP.Sc.13

The BLAST results were confirmed by the phylogenetic tree created using MEGA version 7.0 (Molecular Evolutionary Genetics Analysis) software (Figure 7). The phylogenetic tree is useful for showing the kinship of each species based on the inter-molecular characteristics (Felix *et al*., 2011).

Figure 7. Bacterial phylogenetic tree of 19.PP.Sc.13

According to Heyndrickx *et al*. (1998), *Virgibacillus* sp. is a gram-positive bacterium that belongs to the Kingdom *Bacteria*, Phylum *Firmicutes*, Class *Bacilli*, Order Bacillales, Family Bacillaceae and Genus *Virgibacillus*. This anaerobic bacterium often forms chains and small cream/yellowish-white colonies on Tryptic Soy Agar media and requires the nutrients pantothenic acid, thiamine, biotin, and amino acids to grow optimally at 37°C.

Regarding antioxidant activity, the carotenoids extracted from 19.PP.Sc.13 had an IC_{50} of 506 ppm, slightly lower than that of the β-carotene standard (510 ppm) according to the DPPH assay (Figure 8).

Figure 8. Antioxidant activity of the carotenoid extract of the symbiont bacteria 19.PP.Sc.13 and β-carotene as the positive control.

Kusmita *et al*. (2017) reported that the carotenoid extracts of soft coral symbionts originating from Karimunjawa had the highest antioxidant activity compared to carotenoids from other types of bacteria symbionts.

Assessment of the sun protection of the IC_{50} concentration (506 ppm) of the carotenoid extract categorized the SPF as extra protection (Table 6), indicating that it belonged to the UVA sunblock category (Cumpelik, 1972; (Abdassah *et al*., 2015).

Table 6. The assessment of the sun protection of the 19.PP.Sc.13 carotenoid extract

The antibacterial activity results demonstrated that the carotenoid pigment extract had a greater inhibitory effect on MRSA bacteria than MDR E.*coli* bacteria, indicating that the carotenoid pigment extract of 19 PP.Sc 1.3 is more effective against gram-positive bacteria (Table 7). Many factors can affect antibacterial action, including concentration, number of bacteria, bacterial species, presence of organic matter, temperature, and environmental pH (Rotua Silitonga, Nursyirwani and Effendi, 2020). According to (Salni, Marisa and Mukti, 2011), gram-positive bacteria have a cell wall containing polysaccharides (teichoic acid) with more peptidoglycan and less lipid content, thus the cell walls of gram-positive bacteria are more polar. The carotenoid pigment extracted in a polar solvent, methanol, easily penetrates the polar peptidoglycan layer, thus inhibiting the growth of gram-positive bacteria. Carotenoids can also react with porins (transmembrane proteins) on the outer membrane of the bacterial cell wall, forming strong polymer bonds and destroying the porin, thereby reducing the bacterial cell wall permeability to nutrients and inhibiting bacterial growth (Evans and Cowan, 2016).

Table 7. Antibacterial activity of the carotenoid extracted for the bacterial symbiont 19.PP.Sc.13

Figure 9. Antibacterial activity of the carotenoid extracts of symbiotic bacteria 19.PP.Sc.13 against MDR *E. coli* (a) and MRSA (b) bacteria

CONCLUSION

The carotenoids extracted from a soft coral *Sinularia* sp. bacterial symbiont possess biological activities, including significant antioxidant and sun protection activities, as well as growth inhibition of MDR bacteria. This study provides empírical evidence for further investigation of the application of de novo biological fabrication of secondary metabolites via bacteria.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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