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Analysis of β -cryptoxanthin from yellow pigmented marine bacterium *Erythrobacter* sp. kj5

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Abstract. The objectives of this study were to isolate and analyze β -cryptoxanthin from yellow pigmented marine bacterium *Erythrobacter* sp. KJ5. The pigments from *Erythrobacter* sp. KJ5 were extracted from cells with methanol:acetone (7:3, v/v). β -cryptoxanthin standard was isolated from citrus fruit peel using 100% acetone and purified by high-performance liquid chromatography (HPLC) using a C30 column. The existence of β -cryptoxanthin in *Erythrobacter* sp. KJ5 was determined based on spectral properties and co-chromatography analyses after adding the standard β -cryptoxanthin. The co-chromatography results showed that peak 5 at retention time 33.32 min sharply spiked without changing maximum wavelengths ($\lambda_{\max}$) at 453 and 480 nm after adding the standard β -cryptoxanthin. These results likely conclude that peak 5 was β -cryptoxanthin.

Keywords: carotenoid, β -cryptoxanthin, *Erythrobacter* sp. KJ5, co-chromatography, HPLC

1. Introduction

The utilization natural pigments from marine organism should be consider the sustainability marine resources. Marine microorganism such as bacteria could become a fine alternative as source of natural pigment from marine and not damaging marine ecosystem. Another advanted is they easy to be cultured in laboratory. At least, there are 7 kinds of pigment that can be found from marine bacteria. There are prodiginines, carotenoids, violacein, phenazine compound, quinones, tambjamines, melanins [1]. Among those pigments, carotenoids have attractive research object for researcher due to their biological activity that useful for human life.

Carotenoids found in plants, animals, and microorganisms (bacteria and microalgae), play a critical role in the photosynthetic process to collect light energy in the visible region and to protect against photo-oxidation [2]. Carotenoids are consisted of 40-carbon atom to form 8-isoprene and have yellow, orange, and red color [3]. In addition, carotenoids have been reported to have significant value to support human health as pro-vitamin A [4], antioxidant [5], antibacterial [6], protecting from photooxidation and photoreceptor cells [7]. β -carotene is one of the important carotenoids that has function for preventing cancer and antioxidant [8]. Marine bacteria have been known to produce carotenoids, i.e. astaxanthin from *Agrobacterium aurantiacum* [1]. Novel marine bacteria from *Flavobacteriaceae* produce sproxanthin as a secondary metabolite and has function as antioxidant [9]. Another marine bacteria



from genus *Erythrobacter* have been known to produce carotenoids i.e *E. longus* and *E. litoralis* [10] [11].

Erythrobacter sp. KJ5 is a yellow aerobic marine bacterium isolated from a hard coral *Acropora nasuta* [12]. Previous study showed that zeaxanthin and β -carotene were synthesized by this bacterium [13]. In the biosynthetic pathway of main carotenoids, zeaxanthin is known to be produced from β -carotene via β -cryptoxanthin. Therefore, there is a possibility that *Erythrobacter* sp. KJ5 is capable of synthesize β -cryptoxanthin. However, the existence of β -cryptoxanthin has not been identified yet. In this study, we have successfully identified β -cryptoxanthin from *Erythrobacter* sp. KJ5 by co-chromatography using high-performance liquid chromatography (HPLC) and β -cryptoxanthin standard which isolated from citrus fruit peel. Here, we describe the presence of β -cryptoxanthin in *Erythrobacter* sp. KJ5.

2. Research Method

2.1. Materials

Materials and chemicals used in this study were marine bacterium *Erythrobacter* sp. KJ5, NaCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Na_2SO_4 , KCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, ferric citrate, NaHCO_3 , yeast extract, peptone, casamino acid, glycerol, NaOH, acetone, sodium ascorbate, CaCO_3 , methanol, hexane, acetonitrile, ethanol, pyridine, acetic acid, and distilled water.

2.2. Methods

2.2.1. Cell culture. Colony clusters of *Erythrobacter* sp. KJ5 cells from agar plate were diluted with 10 ml of Shioi liquid medium [14]. Culture was carried out by shaking aerobically at 100 rpm under room temperature and serially re-cultured every 3–5 days (10 ml, 50 ml, 250 ml). The cells were separated from liquid medium by centrifugation at 10,000 rpm, 4°C for 20 mins and the collected cells were stored at a temperature of –30°C until used.

2.2.2. Pigment extraction. The pigments from *Erythrobacter* sp. KJ5 cells were extracted using a mixture of solvents, methanol:acetone (7:3, v/v) with 1 ml solvent mixture per 0.1 g cells. The extraction was carried out by vortexing for 1 min (repeated 3 times, 1 min vortex, 1 min on ice). The cells were then disrupted by sonication at 60% amplitude in a pulse mode of 10-s on/30-s off for 10 min (QSonica, Newtown, USA). The cell debris were separated by centrifugation at 10,000 rpm for 5 min. The collected supernatant was dried by rotary evaporator (Heidolph Laborota 4010 digital, Germany) at 35°C and 120 rpm. The dried pigment extract was stored at a temperature of –30°C until used.

2.2.3. β -cryptoxanthin extraction and isolation. Citrus fruit peels have been known as a source of β -cryptoxanthin [15]. About 1 g of citrus fruit peel was soaked in a liquid nitrogen, quickly ground using a mortar and subsequently 10 ml acetone was added. The extraction was performed by vortexing for 1 min, then on ice for 1 min and repeated 5 times. Supernatant was separated by centrifugation at 10,000 rpm for 10 min and dried using a rotary evaporator at 35°C and 120 rpm. It is reported that β -cryptoxanthin in crude extract of citrus fruit peel existed as ester form and thus saponification is necessary to obtain free form [16]. The saponification was carried out with 1.7 ml of 60% (w/v) KOH and 8.3 ml crude extract of citrus fruit peel in MeOH. Then, 10 ml diethyl ether were added to the sample solution and stirred at 500 rpm for the following periods of times, 1 h, 2 h, 4 h, and 16 h to obtain the optimum results. Subsequently, saturated NaCl and water were added to the mixture, gently mixed and the upper layer containing β -cryptoxanthin was collected and dried. Isolation and purification of β -cryptoxanthin were carried out by HPLC as described previously [13] using a YMC C30 column (150 x 4.6 mm, 3 μm particle size) with different composition of mobile phase and elution conditions as follows.

Method 1. The gradient elution was performed with 4% H_2O , 81% MeOH, and 15% methyl tert-butyl ether (MTBE) from 1 to 70 min at a flow rate of 1 ml/min and column oven at 30°C.

Method 2. All of parameters used in this method were the same as Method 1, except for composition of mobile phase. The mobile phase consisted of 85% MeOH and 15% MTBE.

Method 3. This method was adopted from Kato et al. (2004) [17]. The gradient elution was carried out with 4% H₂O, 95% MeOH, and 1% MTBE from 1 to 60 min at a flow rate of 1 ml/min.

2.2.4. Co-chromatography analysis. The existence of β -cryptoxanthin in *Erythrobacter* sp. KJ5 was determined by co-chromatography analysis using standard β -cryptoxanthin which was isolated from citrus fruit peel. The standard β -cryptoxanthin was added to the pigment extract of *Erythrobacter* sp. KJ5 and injected to HPLC. Waters C₈ column (150 x 4.6 mm, 3.5 μ m particle size, 100 Å pore size) was used in this analysis according to the method of Zapata et al. (2000) [11]. Mobile phase comprised two solvent mixtures, solvent A (methanol:acetonitrile:pyridine solution (0.25 M, pH 5) = 50:25:25 (v/v/v) and solvent B (methanol:acetonitrile:acetone = 20:60:20 (v/v/v). The gradient elution was carried out with 100% solvent A (min 1–22), 60% solvent A (min 22–28), 5% solvent A (min 28–38), 100% solvent A (min 40–50). The flow rate was 1 ml/min with column oven temperature at 30°C. Carotenoids were detected at λ 450 nm by photodiode array detector.

3. Results

Standard β -cryptoxanthin was obtained from citrus fruit peel by extracting with a solvent mixture, methanol:acetone (7:3, v/v). The crude extract was then injected to analytical HPLC using a YMC C30 column as shown in Figure 1.

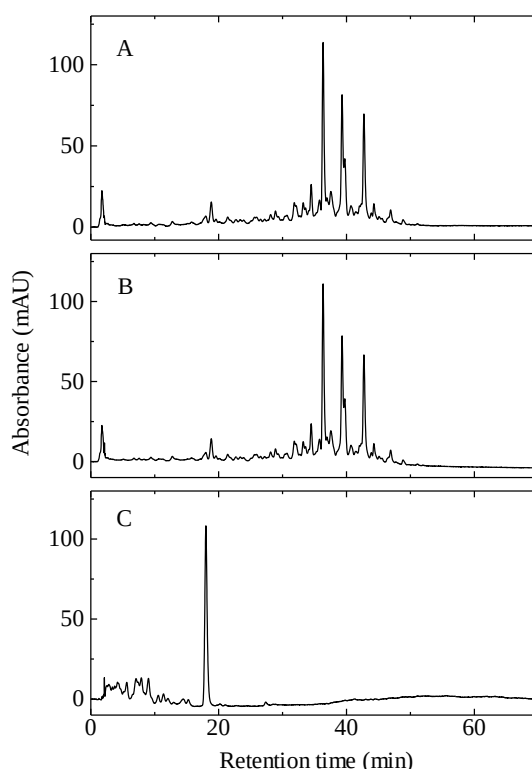


Figure 1. HPLC chromatograms of citrus fruit peel crude extract. All analyses were done by Method 1. (A) without saponification; (B) after saponification for 1 h; (C) after saponification for 16 h.

Figure 1A shows that in the crude extract without saponification, three dominant peaks appeared at retention time 36–43 min, whereas there was almost no β -cryptoxanthin peak. A similar pattern of HPLC chromatogram was obtained in the sample of 1-h saponification (Figure 1 B). In the sample after 16-h saponification, however, three dominant peaks disappeared and β -cryptoxanthin was clearly separated

at retention time 19.2 min (Figure 1C) in accordance with that of standard β -cryptoxanthin. In the former two cases, β -cryptoxanthin might be ester form and longer saponification is needed to break ester moiety of the β -cryptoxanthin. As can be seen from Figure 1C, free form of β -cryptoxanthin was successfully obtained by 16-h period of saponification. To examine the resolution of β -cryptoxanthin in the separation among three HPLC Methods employed (Figure 2), a structurally similar compound, phytofluene ($\lambda_{\max}$ 332, 348, 367 nm) was used as an indicator together with β -cryptoxanthin ($\lambda_{\max}$ 451, 478 nm).

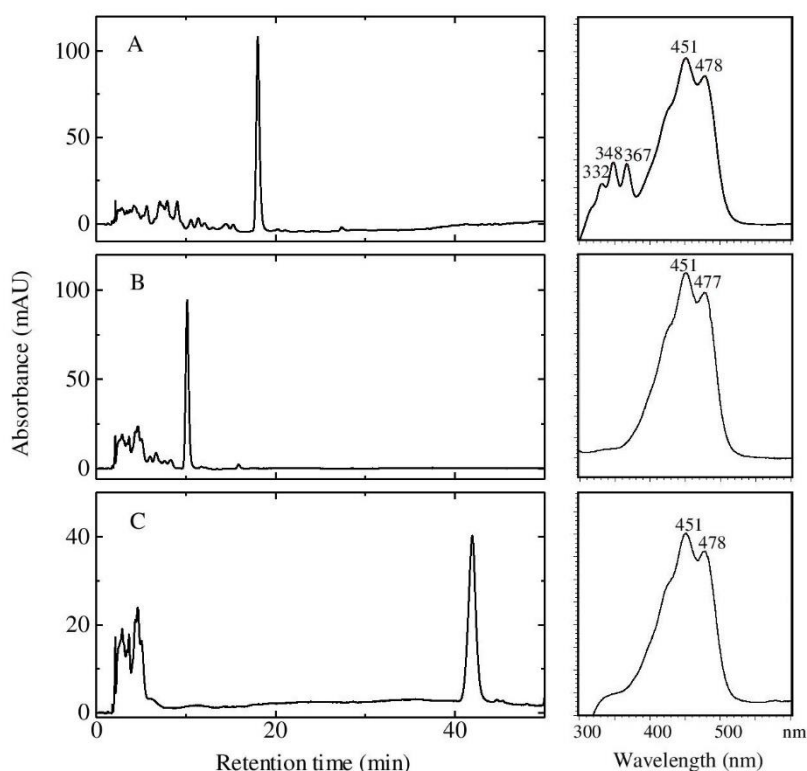


Figure 2. HPLC chromatograms of citrus fruit peel crude extract after saponification for 16 h by different elution Methods (Left). In this study, a mixture of β -cryptoxanthin ($\lambda_{\max}$ 451, 478 nm) and phytofluene ($\lambda_{\max}$ 332, 348, 367 nm) was used. (A) Method 1; (B) Method 2; (C) Method 3. Right: absorption spectra of β -cryptoxanthin peaks in each separation.

In Method 1 (Figure 2A and right spectrum), a single β -cryptoxanthin peak was observed, but its absorption spectrum showed a mixture of phytofluene and β -cryptoxanthin, indicating that these pigments were not completely separated. However, in Method 2 (Figure 2B and right spectrum) and Method 3 (Figure 2C and right spectrum) showed a single peak of β -cryptoxanthin and no absorption peaks of phytofluene. These results indicate that these two Methods could be completely resolved between β -cryptoxanthin and phytofluene. Therefore, β -cryptoxanthin was isolated and purified using Method 2 for further study.

Subsequently, analysis of β -cryptoxanthin in *Erythrobacter* sp. KJ5 was conducted by co-chromatography using a Waters C₈ column. The results of co-chromatography are shown in Figure 3 and summarized in Table 1.

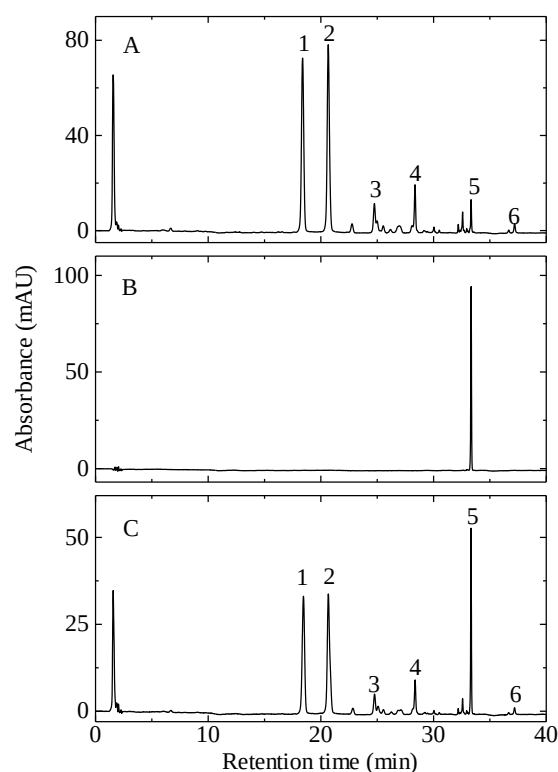
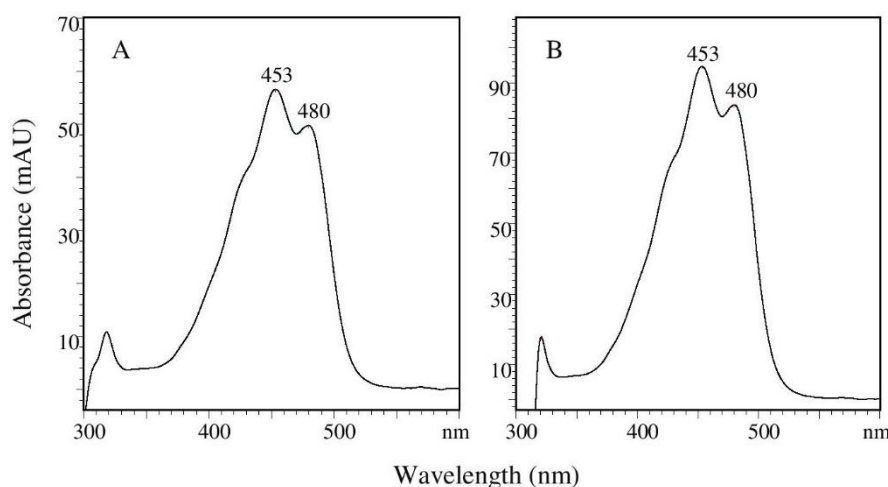


Figure 3. Analysis of existence of β -cryptoxanthin in *Erythrobacter* sp. KJ5 by co-chromatography using a Waters C₈ column. (A) HPLC chromatogram of *Erythrobacter* sp. KJ5 crude pigment extract; (B) HPLC chromatogram of standard β -cryptoxanthin isolated from citrus fruit peel; (C) HPLC co-chromatogram using a mixture of standard β -cryptoxanthin and *Erythrobacter* sp. KJ5 pigment extract.

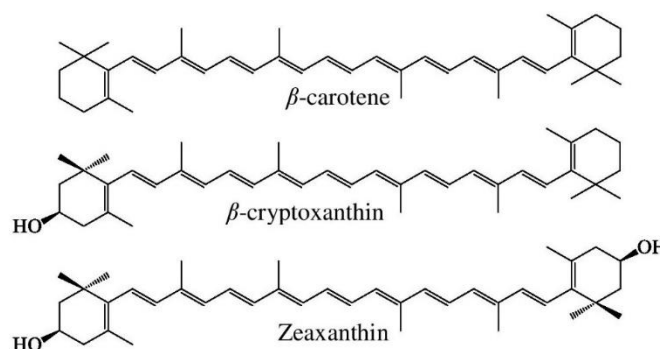
Figure 3A shows the separation of carotenoids in the pigment extract from *Erythrobacter* sp. KJ5. Based on the previous study [13], peak 4 and peak 6 were zeaxanthin and β -carotene, respectively. Zeaxanthin is more polar than β -carotene so that it appears earlier than β -carotene. Figure 3B is the chromatogram of standard β -cryptoxanthin isolated from citrus fruit peel. A single peak at retention time of 33.32 min is shown to be β -cryptoxanthin. As can be seen from Figure 3C, co-chromatography results showed that peak 5 spiked significantly after adding standard β -cryptoxanthin. Before adding, area of peak 5 in the pigment extract (Figure 3A) and standard β -cryptoxanthin (Figure 3B) were 80,812 and 446,429, respectively (See Table 1). After adding the standard β -cryptoxanthin to the pigment extract (Figure 3C), area of peak 5 was increased to 517,828 that corresponds to almost sum of the both peak areas of the pigment extract and standard β -cryptoxanthin. Moreover, the both peak 5 and standard β -cryptoxanthin had the same spectral properties in maximum wavelength ($\lambda_{\max}$) at 453 and 480 nm (Table 1 and Figure 4). These results likely conclude that peak 5 is β -cryptoxanthin.

Table 1. Summary of co-chromatography analysis of β -cryptoxanthin in *Erythrobacter* sp. KJ5

Sample	Peak	$\lambda_{\max}$ (nm)	Retention time (min)	Peak area
<i>Erythrobacter</i> sp. KJ5 pigment extract	5	453, 480	33.32	80,812
β -cryptoxanthin standard	-	453, 480	33.32	446,429
<i>Erythrobacter</i> sp. KJ5 pigment extract + β -cryptoxanthin standard	5	453, 480	33.32	517,828

**Figure 4.** Absorption spectra of carotenoid peak 5 in *Erythrobacter* sp. KJ5 (A) and standard β -cryptoxanthin isolated from citrus fruit peel (B). Both have the same $\lambda_{\max}$ at 453 and 480 nm.

Based on these results, peak 5 at retention time 33.32 min was identified as β -cryptoxanthin. In the reverse-phase HPLC chromatogram (Figure 3A), position of peak 5 was located between zeaxanthin (peak 4) and β -carotene (peak 6), indicating that peak 5 is more polar than β -carotene, but slightly non-polar than zeaxanthin, as suggested by their structure. Every these carotenoid species have the same basic structure with nine conjugated double bonds and two cyclic rings at the end group but different in OH number. Zeaxanthin has two atom O, and β -cryptoxanthin has only one atom O, while β -carotene has no atom O. Chemical structure of β -carotene, β -cryptoxanthin, and zeaxanthin is shown in Figure 5.

**Figure 5.** Chemical structure of β -carotene, β -cryptoxanthin, and zeaxanthin. [18]

4. Discussions

Carotenoids are yellow to orange-red pigment functioned as photosynthetic pigments together with chlorophylls differing from other isoprenoids, sterol, prenylquinones, and prenols in plants. Carotenoids can be distinguished into two groups, carotenes that are oxygen-free compound and xanthophylls that contain oxygen on their structure with different forms. β -carotene and α -carotene are carotenes as a precursor for xanthophyll group. Xanthophylls consist of many kinds of carotenoids such as β -cryptoxanthin, lutein, zeaxanthin, antheraxanthin, and violaxanthin [19]. Naturally, several carotenoids from xanthophyll group are occurred as esters with long-chain or medium-chain fatty acids [13]. In plants, carotenoids are present as free and ester forms. β -cryptoxanthin esters are found in papaya and used for human diet. Saponification using 5–10% KOH is a suitable purifying procedure to remove chlorophylls and fats which are not desired in the purified sample. The carotenoid esters from Brazilian Valencia orange were extracted with 100% acetone and saponified using 10% methanolic KOH. β -cryptoxanthin was the main carotenoid that giving orange color in the Brazilian Valencia orange juice [20]. β -carotene, α -carotene, β -cryptoxanthin, and ascorbic acid are belonged to bioactive compounds as provitamin A and vitamin C and found in Japanese and American persimmons [21].

β -cryptoxanthin also can be found in marine organisms, especially in marine bacteria. Several marine bacteria are known to synthesize β -cryptoxanthin. It is reported that *Erythrobacter longus*, the first species from genus *Erythrobacter* isolated from seaweed *Enteromorpha liza*, synthesized C_{40} skeletal carotenoids belong to bicyclic carotenoids such as β -carotene and its hydroxyl derivatives: β -cryptoxanthin, zeaxanthin, caloxanthin, and nostoxanthin [18]. Other *Erythrobacter* species, *Erythrobacter litoralis*, is known to produce bacteriorubixanthinal and erythroanthin sulfate as the major carotenoids [11]. Whereas *Erythrobacter odishensis* synthesized zeaxanthin, erythroanthin, and β -carotene as the major carotenoid [22]. On the other hand, *Thermosynechococcus elongates* strain BP-1 produced β -carotene as the major carotenoid and its hydroxyl derivatives, β -cryptoxanthin, zeaxanthin, caloxanthin and nostoxanthin [23]. β -cryptoxanthin is one of the important carotenoids, because it has function as provitamin A as well as β -carotene. Besides that, β -cryptoxanthin was associated with reducing risk of lung cancer. Also, dietary β -cryptoxanthin is a chemopreventive agent for lung cancer in humans [24].

The results of this study indicated that *Erythrobacter* sp. KJ5 synthesized simple carotenoids with β -carotene as a core structure, i.e. β -carotene derivatives of β -cryptoxanthin and zeaxanthin. Those carotenoids have same structure with nine conjugated double bonds and two cyclic end group but different in OH number and the mobility of the carotenoid decreased with the increasing number of hydroxyl moiety. This suggests a simple biosynthetic pathway involving genes *CrtZ*

5. Conclusions

It is concluded that in *Erythrobacter* sp. KJ5, β -cryptoxanthin was identified as the compound having a retention time at 33.32 min in HPLC chromatogram. The existence of β -cryptoxanthin showed that *Erythrobacter* sp. KJ5 synthesized bicyclic carotenoids with β -carotene as a core structure. Further study on identification of all carotenoids using LC-MS and NMR are now going on to understand the carotenoids biosynthetic pathway in *Erythrobacter* sp. KJ5.

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