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CAROTENOID SCIENCE



*Proceedings of the 32nd
Annual Meeting on*

Carotenoid Research

*Held in Kumamoto, Japan
15-16 September 2018*



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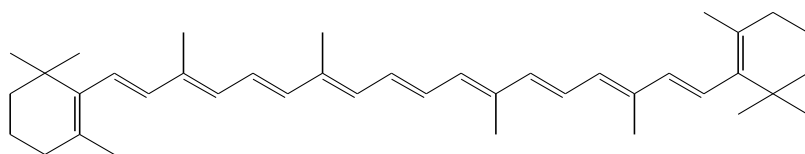
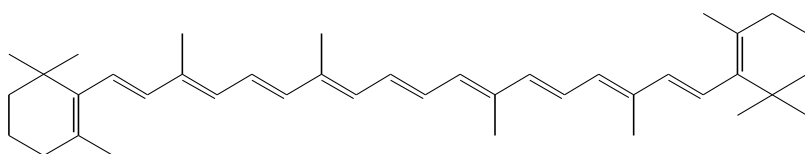
VOLUME 23 (2019)

Japanese Society for Carotenoid Research

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Japanese Society for Carotenoid Research

Preface for the 32nd Annual Meeting

The 32nd Annual Meeting of Japanese Carotenoid Society was held at 100th memorial Hall in Kumamoto University located at Kumamoto City from 15th to 16th of September, 2018. The scientific program of this meeting consisted of five invited lectures by Prof. Koichi Iwata (Gakushuin University), Prof. Kazutoshi Shindo (Japan Women's University), Prof. Sachiko Tsukamoto (Kumamoto University), Prof. Shinsuke Shigeto (Kwansei Gakuin University), and Prof. Mitsuru Sasaki (Kumamoto University) and 17 oral presentations.

In this meeting, 90 participants were attended in total, and scientific topics regarding carotenoids contained a wide variety of research fields on carotenoids such as physics, physical chemistry, biology, molecular biology, pathway engineering, synthetic chemistry, natural products-chemistry, nutrition, and medicine. All presentations and discussions were very attractive and heated for two days including the evening banquet of first day. This meeting would play an important role on promoting the multiple application of carotenoids and supra-disciplinary cooperation among Japanese researchers and workers in the different scientific fields of carotenoids, and Japanese activities on carotenoid science need to be maintained in higher levels.

This meeting was sponsored by following associations and companies to which we must be deeply indebted for their financial supports.

List of sponsors:

Japan Business Association for Carotenoids (JBAC) organized by 21 companies

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Finally, I would like to appreciate our staff members for their assistance during the meeting, and deeply thank all participants at this meeting for making their presentations and discussions very productive and interactive.

We are looking forward to seeing again the regular members and welcoming new attendees in the next 33rd Annual Meeting of Japanese Carotenoid Society, which will be held in Chiba University from 22nd to 23rd of September, 2019.

Daisuke Kosumi

Associate Professor of Kumamoto University

The Meeting Chair of the 32nd Annual Meeting of Japanese Carotenoid Society

Contents

Preface for the 32nd Annual Meeting

Reviews

Raman Spectral Imaging Approach to Carotenoids in Bacterial Biofilms

Shinsuke Shigeto..... 1

Structural Analyses of the Compounds Produced by Acidic Treatment of Epoxycarotenoids (Violaxanthin, 9-*cis*-Violaxanthin, Antheraxanthin, and 9-*cis*-Antheraxanthin), and the Antioxidant Activities of Epoxycarotenoids and the Acid Treatment Products

Kazutoshi Shindo..... 6

Regular Papers

Anomalous Optical Properties of Artificial Short-Polyene Carotenoid

Daisuke Kosumi, Toshiyuki Kusumoto and Hideki Hashimoto..... 9

The Possible Mechanisms of the Chiral Conversion of Astaxanthin in the Prawn

Takashi Maoka..... 15

Accumulation of Euglena-derived carotenoids in egg yolk

Shota Watanabe, Toru Okada, Hideto Ookita, Kyoichi Sakamoto, Kengo Suzuki and Takashi

Maoka..... 19

Proceedings of 32nd Annual Meeting on Carotenoid Research

Analysis on the excited state properties of *cis*- β -apo-8'-carotenal using femtosecond time-resolved absorption spectroscopy

Kota Horiuchi, Chiasa Uragami, Taiki Inoue, Nao Yukihiro, Daisuke Kosumi and Hideki Hashimoto..... 24

Generation of Geometric Isomers of β -Apo-8'-Carotenal and Investigation of Their Optical Properties Using Stark Spectroscopy

Taiki Inoue, Shota Seto, Tomoko Horibe, Kota Horiuchi, Chiasa Uragami and Hideki Hashimoto..... 32

Excitation energy transfer dynamics of phycobilisome from *Thermosynechococcus vulcanus*

Keisuke Kawakami, Hiroki Serikawa, Yuma Hirota, Yuhei O Tahara, Junko Shiomi, Makoto Miyata, Nobuo Kamiya and Daisuke Kosumi..... 37

Photosynthetic megacomplexes by a novel sample preparation method and identification of carotenoids interacting with photosystem II from *Thermosynechococcus vulcanus*

Keisuke Kawakami, Takashi Maoka, Yuhei O Tahara, Junko Shiomi, Makoto Miyata, Nobuo Kamiya and Daisuke Kosumi..... 41

Conversion of solid biomass residue to functional materials and fuels using supercritical fluids and pulsed discharges

Mitsuru Sasaki..... 45

Efficient Energy Transfer in Light-Harvesting Function from an Intra- Molecular Charge Transfer State of Fucoxanthin in Reconstituted LH1 Complex

Masayuki Yoshizawa, Ren Sato, Nao Yukihiro, Chiasa Uragami and Hideki Hashimoto..... 47

Experimental reports**Detailed Chromatographic and Spectral Data of Capsanthone in Paprika Intake Human Plasma and Erythrocytes**

Azusa Nishino, Takashi Ichihara, Kazuhisa Kawamoto and Takashi Maoka..... 50

Abstracts**Study on the Structures and Formation of Marine Natural Products**

Sachiko Tsukamoto..... 54

Conversion of solid biomass residue to functional materials and fuels using supercritical fluids and pulsed discharges

Mitsuru Sasaki..... 56

Towards elucidation of light energy transfer mechanism of the photosynthetic supercomplexes from algaeKeisuke Kawakami, Yuma Hirota, Yuhei Tahara, Junko Shiomi, Takashi Maoka, Susumu Takio,
Makoto Miyata, Nobuo Kamiya and Daisuke Kosumi..... 57**Enhanced Thermal Z-Isomerization of (all-*E*)-Lycopene by Some Foodstuffs**

Masaki Honda, Ryota Takemura and Tetsuya Fukaya..... 59

Analysis of the excited energy-states of *cis*- β -Apo-8'-carotenal using time-resolved absorption spectroscopy

Kota Horiuchi, Chiasa Uragami, Taiki Inoue, Nao Yukihiro and Hideki Hashimoto..... 60

High-yield production of carotenoid in *Escherichia coli* via re-engineered non-mevalonate pathway

Katsuya Minakata, Tashiro Miki, Shigeko Kawai-Noma and Daisuke Umeno..... 61

Comparison of early stages of carotenogenesis among phototrophs

Shinichi Takaichi, Jiro Harada, and Kenjiro Sugiyama..... 62

Light Harvesting Function of LH1 Complex Reconstituted with Fucoxanthin

Masayuki Yoshizawa, Ren Sato, Martin R. Steinel, Nao Yukihiro, Chiasa Urugami
and Hideki Hashimoto..... 63

Relationship between *Oreochromis* fish body coloration and food resources

Kumi Matsui, Koji Mochida, Toshiya Sugiyuchi, A Fukuda, Shingo Fujimoto, Jun Kitano, Asano
Ishikawa, Daniel F. Mokodongan, Ixchel F. Mandagi, Kawilarang W. A. Masengi, Renny K.
Hadiaty and Kazunori Yamahira..... 64

Investigation of Intramolecular Charge-Transfer Character of β -Apo-8'-carotenal using
Stark Absorption Spectroscopy

Taiki Inoue, Shota Seto, Tomoko Horibe, Kota Horiuchi, Chiasa Urugami and Hideki
Hashimoto..... 65

Defect of photosynthetic activity in the *cruC* gene mutant of the green sulfur bacterium
Chlorobaculum tepidum, which lost the ability to synthesize carotenoid glucoside ester

Jiro Harada, Chihiro Azai, Taku Inoue, Shogo Fujimoto, Shinji Masuda and Daisuke Kosumi..... 66

Suppression process of the generation of triplet bacteriochlorophyll *a* by carotenoid in
the LH1 antenna pigment-protein complexes from a purple photosynthetic bacterium
Rhodospirillum rubrum

Hiroki Sato, Nao Yukihiro, Chiasa Urugami, Masazumi Fujiwara, Alastair T. Gardiner,
Richard J. Cogdell and Hideki Hashimoto..... 67

Evaluating the carotenoid-solvent interaction by theoretical simulation of steady state
absorption spectra

Chiasa Urugami, Keisuke Saito, Masayuki Yoshizawa, Péter Molnár and Hideki Hashimoto..... 68

Isolation and functional analysis of carotenoid biosynthesis genes from human gut and probiotic bacteria

Wataru Matsumoto, Haruka Nanaura, Miho Takemura, Takashi Maoka and Norihiko Misawa..... 69

Analysis of Anti-Inflammatory Profile of Siphonaxanthin

Yuki Manabe, Nami Tomonaga and Tatsuya Sugawara..... 71

ζ-Carotene Isomerase (Z-ISO) in Oxygenic Phototrophs

Keisuke Nakazawa, Shinichi Takaichi, Masaharu Yamada and Kenjiro Sugiyama..... 72

Molecular Distortion of Carotenoids and Optical Properties of Their Nanocrystals

Ryuju Suzuki, Kazuma Yasuhara, Satoshi Okada and Shigeru Deguchi..... 73

Origin of Carotenoids in Dragonfly

Takashi Maoka and Naoki Kawase..... 75

Structures of Yellow xanthophylls and Mechanism of Chiral Conversion of Hydroxy group in Astaxanthin in the Prawn

Takashi Maoka, Yuki Kawashima and Mikihiro Takagi..... 76

Raman Spectral Imaging Approach to Carotenoids in Bacterial Biofilms

Shinsuke Shigeto*

Department of Chemistry, School of Science and Technology, Kwansei Gakuin University,
2-1 Gakuen, Sanda, Hyogo 669-1337, Japan.

Abstract: Raman microspectroscopy provides a powerful tool to characterize carotenoids in bacterial biofilms *in situ* and visualize their distributions within biofilms. Here we present our Raman spectral imaging studies on biofilms of *Rhodococcus* sp. SD-74 and pink-pigmented household biofilms (commonly known as pink biofilms). In *Rhodococcus* sp. SD-74 biofilms, a positive correlation is found between carotenoid concentration in the cells and biofilm development, which can be used to estimate the age of biofilms based on carotenoid Raman signatures. In pink biofilms, two types of carotenoid-associated compounds are identified with the use of a multivariate analysis of Raman hyperspectral imaging data. These results clearly show that carotenoids play important roles, such as serving as an antioxidant, in a variety of biofilm systems from single-species to multi-species biofilms and from laboratory-grown biofilms to biofilms in actual environments.

1. Introduction

Biofilms are defined as communities of sessile bacterial cells that are formed on surfaces and interfaces [1]. In most circumstances, bacterial cells occur in biofilms rather than as isolated planktonic cells. Intensive microbiological studies over the past two decades have revealed that bacterial cells inhabiting biofilms often acquire distinct characteristics that are favorable to their survival (e.g., high antibiotic resistance and high stress tolerance) [2,3]. These properties are deemed relevant to chemical heterogeneity within biofilms, but the link between them remains elusive. It is thus highly desirable to develop new methodologies that can look at biofilms with high chemical specificity and in a nondestructive manner.

In this paper, we show that Raman microspectroscopy is an ideal means for nondestructive chemical analysis of biofilms. We demonstrate the method by investigating biofilms of *Rhodococcus* sp. SD-74 [4] and pink biofilms. Many species of the genus *Rhodococcus* are capable of degrading petroleum, so they are promising for bioremediation. *Rhodococcus* sp. SD-74 has also been known to produce a biosurfactant [5]. Pink biofilms are probably one of the most familiar biofilms in our daily life. They typically consist of *Methylobacterium* and *Rhodotorula*, and can be easily found in sinks, bathrooms, and other places in a house as a pink-colored stain [6,7]. In both cases, we observed characteristic features of carotenoid(s) in the biofilm

Raman spectra. With visible light excitation, Raman bands of carotenoids get considerably enhanced owing to the resonance Raman effect and hence can be detected with high sensitivity. We show that in *Rhodococcus* sp. SD-74, carotenoids accumulate as the biofilm grows [4]. Furthermore, by employing Raman imaging coupled with multivariate curve resolution–alternating least-squares (MCR–ALS) analysis [8], we reveal a carotenoid and a carotenoid-like compound that exhibit different spatial distributions within a pink biofilm, suggesting that carotenoids may be used to visualize habitats of different bacterial species within biofilms.

2. Experimental

2.1 Biofilm culture and sampling

Rhodococcus sp. SD-74 was a gift from Prof. Nobuhiko Nomura (University of Tsukuba, Japan). The strain was precultured on a PMY agar plate at 30 °C for 1.5 days. Subsequently, it was maintained at 4 °C. PMY agar medium contained 1.0% (v/v) glycerol, 0.5% (w/v) polypepton, 0.3% yeast extract, 0.3% malt extract, 0.1% KH₂PO₄, and 1.5% agarose. The pH of the medium was adjusted to 7.0 by using KOH. A single colony of *Rhodococcus* sp. SD-74 was harvested from the PMY agar plate and cultured in a tilted glass bottom dish containing 2 mL of tryptic soy broth. *Rhodococcus* sp. SD-74 in the tilted dish was incubated at 30 °C under high-humidity conditions, and biofilms formed at the air–medium interface.

Pink biofilms were collected from near the drainage of the bathroom in a house (house S in Sanda, Hyogo, Japan). The sample was gently rinsed with

* Corresponding author
e-mail: shigeto@kwansei.ac.jp

Milli-Q water and put on a slide glass, followed by sealing with a cover slip and nail polish. No further destructive treatment was done on the sample.

2.2 Raman spectroscopic measurements

A laboratory-built confocal Raman microspectrometer was used to measure space-resolved Raman spectra of *Rhodococcus* sp. SD-74 biofilms, whereas a Horiba Raman microscope system (XploRA Nano) was used for measurements of pink biofilms. The laboratory-built setup has been described in detail previously [8]. It is based on an inverted microscope and the excitation wavelength is 632.8 nm. In contrast, the Horiba setup has an upright microscope and its excitation wavelength is 532 nm.

3. Results and Discussion

3.1 *Rhodococcus* sp. SD-74 biofilms

Figure 1 shows representative Raman spectra of a planktonic cell and an aggregate of cells of *Rhodococcus* sp. SD-74. Whereas Raman bands of proteins are dominant in the planktonic spectrum (**Figure 1**, blue line), the intensities of the Raman bands at 1003, 1156, and 1515 cm^{-1} are markedly enhanced in the aggregate spectrum (**Figure 1**, red line). These Raman bands are characteristic of carotenoids and are unambiguously assigned to the C–CH₃ rocking, C–C stretching, and in-phase C=C stretching, respectively. It comes as no surprise that we observed carotenoid bands in the Raman spectra, because liquid culture containing *Rhodococcus* sp. SD-74 is orange-colored. What is surprising is that those carotenoid bands are much more intense in the aggregate spectrum, although the protein and nucleic acid bands at 1658, 1340, and 781 cm^{-1} are of similar intensity to those in the planktonic spectrum. In the course of observing a bunch of *Rhodococcus* sp. SD-74 aggregates, we came to a hypothesis that the concentration of carotenoids in *Rhodococcus* sp. SD-74 cells increases as their aggregate becomes larger.

To test this hypothesis, we performed a Raman imaging experiment on *Rhodococcus* sp. SD-74 biofilms formed at the air–liquid interface. The result is shown in **Figure 2**. As can be seen from **Figure 2b–d**, the carotenoid concentration markedly increases as the biofilm grows for a longer period, but those of proteins/lipids (**Figure 2e, f**) and nucleic acids (**Figure 2g**) do not change as much. Proteins/lipids were abundant even after 1 day of culture. Furthermore, although there are several sites that give extremely strong carotenoid signals (e.g., white spots in the Raman images of 7- and 9-day-old biofilms), carotenoid accumulation appears to occur widespread in the entire biofilm. The increase in carotenoid concentration is due to carotenoid accumulation and is

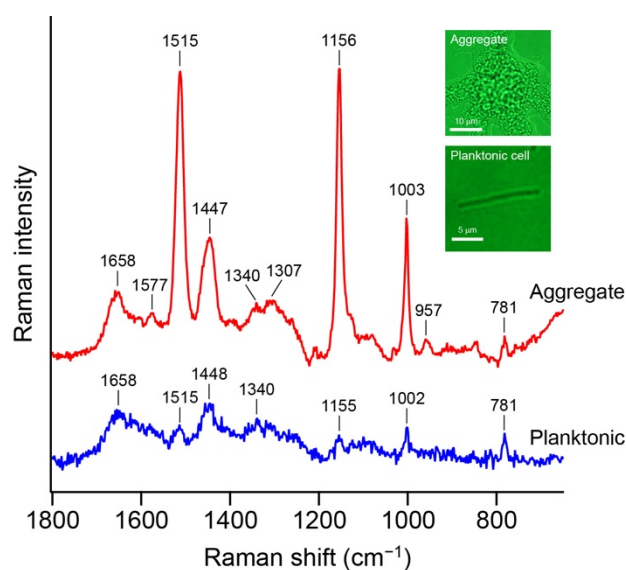


Figure 1. Raman spectra of a planktonic cell (blue) and an aggregate (red) of *Rhodococcus* sp. SD-74. The spectra were taken with excitation laser power of 2.6 mW at the sample point and an exposure time of 60 s. For each spectrum, the background was fitted with an eighth-order polynomial and was subtracted off. Also shown are optical images of a typical planktonic cell (lower image) and an aggregate (upper image) of *Rhodococcus* sp. SD-74. The scale bar measures 5 μm for the lower image and 10 μm for the upper image. Reproduced from ref. 4 with permission (Copyright 2013 American Chemical Society).

not because of the increase in cell density in the probed volume. The positive correlation between the carotenoid Raman intensity and biofilm development found in the present study will enable us to estimate in a noninvasive yet quantitative manner the age of biofilms that consist of carotenoid-containing bacteria like *Rhodococcus* sp. SD-74, which is not possible with dye staining assay or electron microscopy. The biological significance of the carotenoid accumulation needs to be further investigated, but we infer that it is associated with oxidative stress within the biofilm because carotenoids are known to possess an antioxidant ability.

3.2. Pink biofilms

Carotenoids were also detected in pink biofilms. **Figure 3A** shows optical images of part of the pink biofilm sample we collected, and **Figure 3B** displays the background-subtracted Raman spectra measured at three representative points a–c in the enlarged image. The Raman spectrum at point a resembles those of *Rhodococcus* sp. SD-74 biofilms (see **Figure 1**) except for an additional Raman band observed at $\sim 1130 \text{ cm}^{-1}$. This band is more intense than the nearby 1157 cm^{-1} band at points b and c. Although the overall intensity decreases in going from point a to c, the

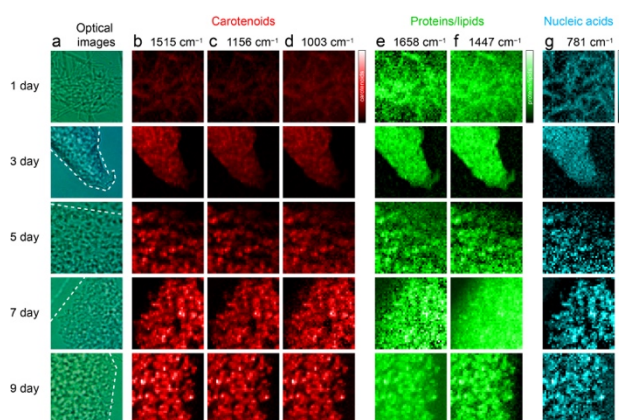


Figure 2. Multimode Raman spectral imaging of *Rhodococcus* sp. SD-74 biofilms after 1, 3, 5, 7, and 9 days of growth. 2.6 mW laser power and a 1 s exposure time per pixel were used. (a) Optical images of the biofilms used for Raman imaging. Dashed lines in the images other than that of 1 day old define the boundary of the biofilm. (b–d) Carotenoid images at the Raman shift of 1515, 1156, and 1003 cm^{-1} . (e,f) Protein/lipid Raman images at 1658 and 1447 cm^{-1} . (g) Nucleic acid Raman images at 781 cm^{-1} . The image size is $16 \times 16 \mu\text{m}^2$ ($41 \times 41 = 1681$ pixels) for all images in panels b–g. The same color scale applies to the Raman images in each column (i.e., each wavenumber). Reproduced from ref. 4 with permission (Copyright 2013 American Chemical Society).

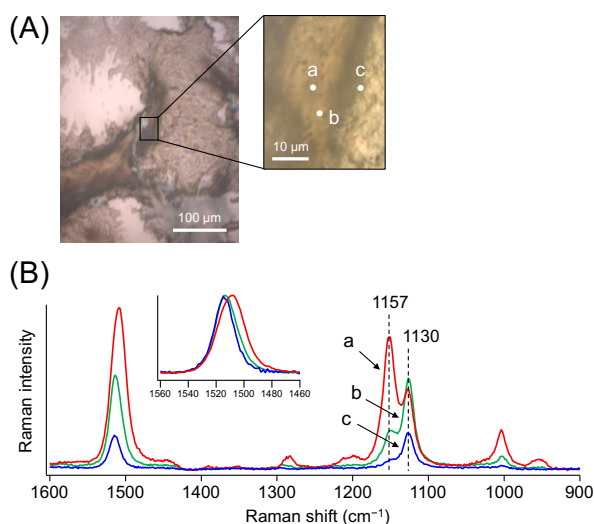


Figure 3. (A) Optical image of part of the pink biofilm we collected from the drainage in a bathroom (left) and its enlarged view (right). In the right image, three selected points (a–c) at which Raman spectra were recorded are indicated. (B) Background-subtracted Raman spectra obtained at points a–c. The inset shows the $\sim 1510 \text{ cm}^{-1}$ band normalized to peak height. The laser power at the sample was 4.4 mW and the acquisition time was 90 s.

spectral patterns of the C=C stretching and C–CH₃ rocking regions remain almost unchanged. The only noticeable change concerns the peak position of the C=C stretch band; as can be seen from the inset of **Figure 3B**, it is lower in Raman shift at point a than at points b and c.

Is the 1130 cm^{-1} band also associated with carotenoids or should it be assigned to another biomolecule? To address this question, we decomposed the Raman spectra of pink biofilms into several component spectra. In this work, we employed MCR–ALS, in which Raman hyperspectral imaging data **A** is approximately factorized into the product of two non-negative, smaller matrices **W** and **H** as $\mathbf{A} \approx \mathbf{WH}$ [9,10] using ALS algorithm such that the Frobenius norm $\|\mathbf{A} - \mathbf{WH}\|^2$ is minimized. Here the columns of **W** represent intrinsic spectra of the underlying components, whereas the rows of **H** represent the spatial profile of each component. The non-negativity imposed during ALS fitting allows us to derive physically interpretable solutions because both concentrations and spectral intensities must be non-negative. We have previously reported successful applications of MCR–ALS Raman imaging to the cell division of fission yeast (*Schizosaccharomyces pombe*) cells [8] and human colon cancer HCT116 cells [11].

Figure 4 displays the results of an MCR–ALS analysis of Raman imaging data in the 800–3100 cm^{-1} region, of the pink biofilm. In the present MCR–ALS analysis, we assumed the number of underlying components to be four by reference to the outcome of singular value decomposition of the hyperspectral data **A**. One of the four components is obviously attributable to the glass substrate, so it is not shown in **Figure 4**. The remaining components are denoted 1–3. The spectrum of component 1 (**Figure 4a**) exhibits Raman bands characteristic of proteins and nucleic acids, including the CH stretch band at $\sim 2900 \text{ cm}^{-1}$, the amide I band at $\sim 1658 \text{ cm}^{-1}$ and the CH bend mode at $\sim 1450 \text{ cm}^{-1}$ [12]. Consistent with **Figure 3B**, the prominent Raman bands, ν_1 – ν_3 , in the spectrum of component 2 (**Figure 4b**) represent carotenoid(s). Minor features between 2000 and 2800 cm^{-1} can be well assigned to overtones and combination bands of ν_1 , ν_2 , and ν_3 .

In addition to component 2, our MCR–ALS analysis reveals component 3, whose spectrum (**Figure 4c**) is very similar to that of component 2 but has an intense Raman band at 1129 cm^{-1} (ν_2') instead of 1157 cm^{-1} (ν_2). The high spectral similarity suggests that component 3 may also be carotenoid or carotenoid-like compound. However, neither the type of carotenoids (i.e., the length of the conjugated chain) nor *cis* isomers [13] can account for a $\sim 30 \text{ cm}^{-1}$ downshift of the C–C stretch frequency. Such a large downshift was indeed observed for

tetrademethyl- β -carotene, which has no methyl group attached to the polyene chain [14,15]. This compound shows the C–C stretch band at 1133 cm^{-1} , very close to our observed peak frequency (1129 cm^{-1}). We thus conclude that in the pink biofilm we have investigated, there exists a polyene compound that is structurally similar to carotenoids, as well as typical carotenoid(s).

The Raman images of components 1–3 derived from our MCR–ALS analysis are presented in **Figure 4d–f**, respectively. Interestingly, the spatial distributions of components 2 and 3 (**Figure 4e, f**) exhibit only small overlap (see the merged image shown in **Figure 4g**). This result seems to indicate that these components are polyene metabolites that were produced by different microorganisms within the pink biofilm. Raman measurements on isolated, pure-cultured microorganisms from the pink biofilm will enable us to examine whether this interpretation is the case or not.

4. Conclusion

We have demonstrated that carotenoids are widely found in bacterial biofilms and that the Raman imaging method is powerful for investigating the carotenoids in biofilms without destroying the biofilm structure. Finally, we point out that the present method is readily applicable to biofilms of any other microorganisms including medically relevant bacteria and photosynthetic bacteria.

Acknowledgments

The author is grateful to his collaborators on the studies presented here: Prof. Nobuhiko Nomura (University of Tsukuba), Dr. Masanori Toyofuku (University of Tsukuba), Yi-Ting Zheng (National Chiao Tung University, Taiwan), and Mai Sasaki (Kwansei Gakuin University). The work on *Rhodococcus* sp. SD-74 biofilm was funded by the Ministry of Science and Technology, Taiwan (grants NSC100-2113-M-009-009-MY2 and NSC100-2738-M-009-002). The work on pink biofilms has been supported by Japan Science and Technology Agency, ERATO (JPMJER1502).

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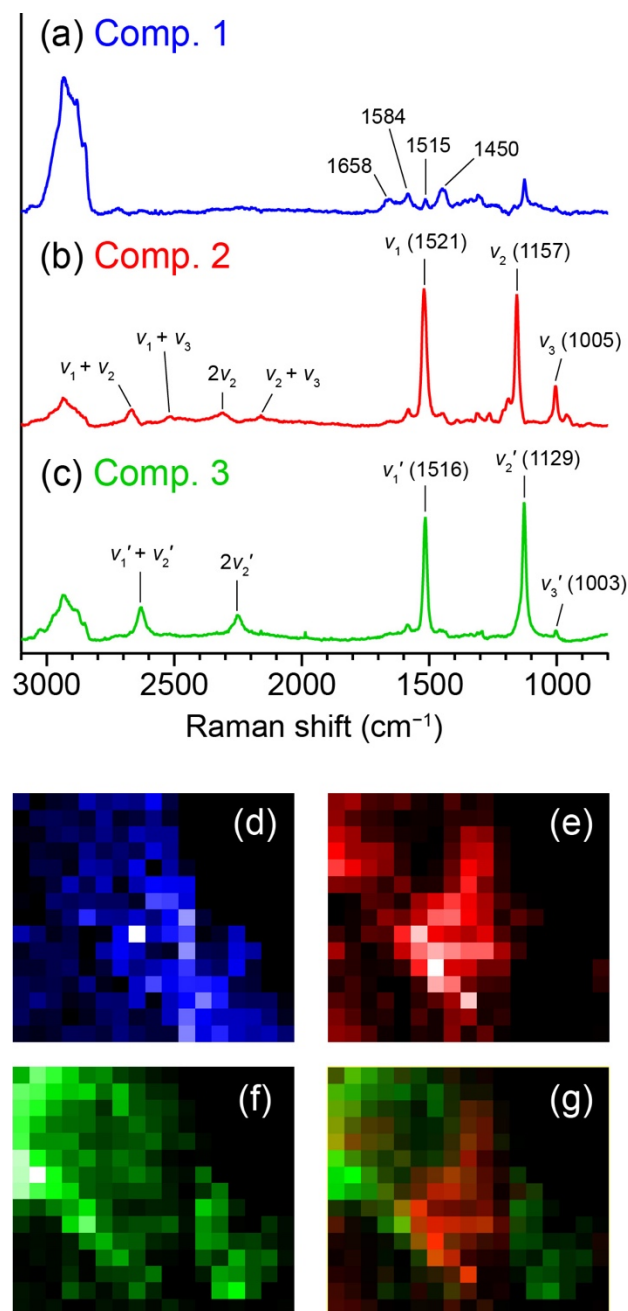


Figure 4. Intrinsic spectra (a–c) and Raman images (d–f) of components 1–3 derived from the MCR–ALS analysis of the Raman hyperspectral imaging data of the pink biofilm we collected in a bathroom. The Raman spectrum at each point was recorded with 4.4 mW laser power and 20 s acquisition time. The image in g represents a merged image of e and f. The size of images d–g is $28 \times 32\text{ }\mu\text{m}^2$ ($15 \times 17 = 255$ pixels).

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Structural Analyses of the Compounds Produced by Acidic Treatment of Epoxycarotenoids (Violaxanthin, 9-*cis*-Violaxanthin, Antheraxanthin, and 9-*cis*-Antheraxanthin), and the Antioxidant Activities of Epoxycarotenoids and the Acid Treatment Products

Kazutoshi Shindo

Department of Food and Nutrition, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

Abstract: Epoxycarotenoids (violaxanthin, 9-*cis*-violaxanthin, antheraxanthin, and 9-*cis*-antheraxanthin (major epoxycarotenoids in fruit)) were purified from some fruits and converted to other carotenoids under acidic conditions. The resulting carotenoid structures were then analyzed in detail. Not only violaxanthin but also 9-*cis*-violaxanthin were found to be converted to (8*S*,8'*S*), (8*S*,8'*R*), and (8*R*,8'*R*)-auroxanthin at an approximate ratio of 4:6:1. Similarly, both antheraxanthin and 9-*cis*-antheraxanthin were found to be converted to (8*R*) and (8*S*)-mutatoxanthin at an approximate ratio of 3:2. Antioxidant activities of epoxycarotenoids and their acid treatment products were examined. They possessed potent lipid peroxidation inhibitory and weak $^1\text{O}_2$ quenching activities.

1. Introduction

Some acylated epoxycarotenoids are contained in fruits a lot, while their changes after ingestions and the antioxidant activities of them have not been studied so far mainly due to their unstable physical properties.

In this study, we prepared pure major epoxycarotenoids (violaxanthin [1], 9-*cis*-violaxanthin [2], antheraxanthin [3], and 9-*cis*-antheraxanthin [4]) (**Figure 1**) from mango, permission fruits, and nable,

and performed acid treatment on them to understand their changes in stomach.

We also examined lipid peroxidation inhibitory (inhibition of rat brain homogenate peroxidation that was initiated by Fe^{2+} - Fe^{3+} - O_2 complex and progressed by lipid peroxides derived radicals [5]) and $^1\text{O}_2$ quenching (inhibition of methylene blue-sensitized linoleic acid photo-oxidation [5]) activities of epoxycarotenoids and their acid treatment products.

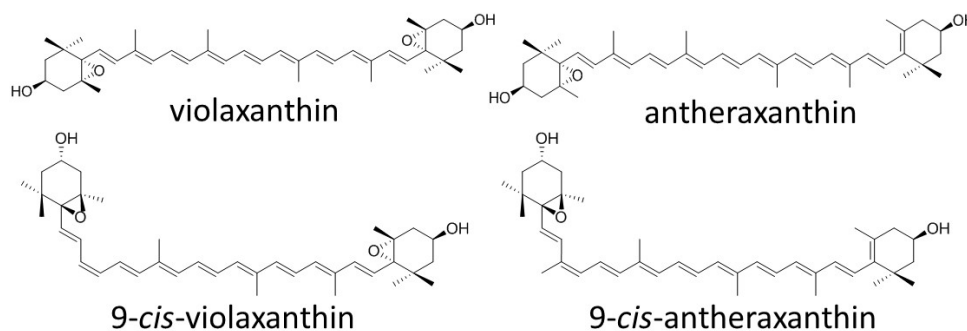


Figure 1. The Structures of epoxycarotenoids

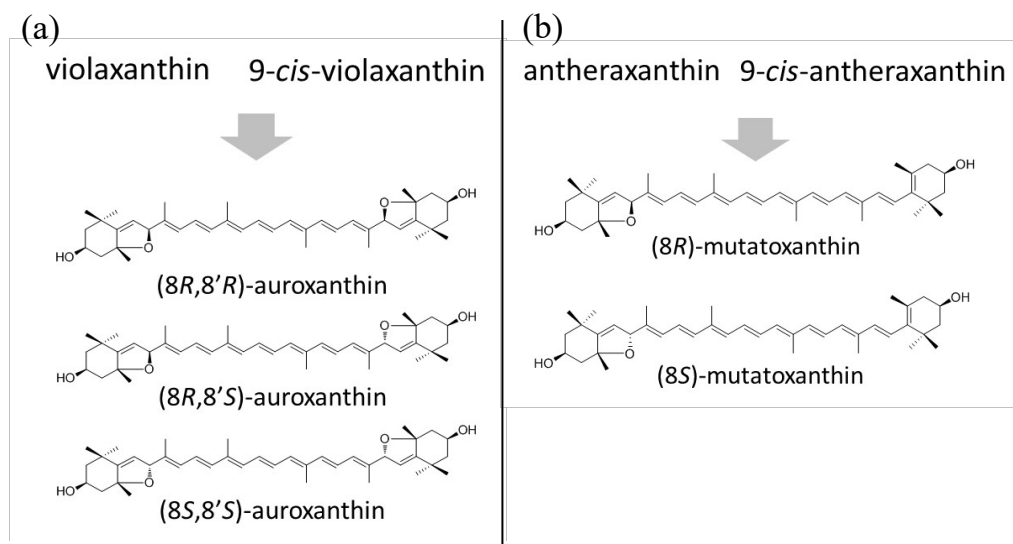


Figure 2. Acid treatment products of violaxanthin and 9-*cis*-violaxanthin (a), and antheraxanthin and 9-*cis*-antheraxanthin (b).

2. Results and Discussions

To obtain the acid conversion products of violaxanthin and 9-*cis*-violaxanthin, each carotenoid was dissolved in 0.1 M HCl in 50% EtOH, and stirred for 10 min at room temperature. The solution was partitioned between EtOAc and H₂O, and EtOAc layers containing the converted products were analysed by silica gel HPLC (Cosmosil 5SL) using hexane: EtOAc (1:1) as the solvent. For both violaxanthin and 9-*cis*-violaxanthin, HPLC analysis showed three peaks with similar UV-Vis absorbance at 17.0 min, 20.5 min, and 23.5 min, and the peak area ratio was approximately 4:6:1. The three peaks derived from violaxanthin and 9-*cis*-violaxanthin were proved to be identical by co-chromatography of the

acid conversion products of violaxanthin and 9-*cis*-violaxanthin (the identities of the three peaks were further substantiated by ¹H NMR spectra). The peaks at 17.0 min, 20.5 min, and 23.5 min were identified to be (8*S*,8'*S*)-auroxanthin, (8*R*,8'*S*)-auroxanthin, and (8*R*,8'*R*)-auroxanthin, respectively [6]. (**Figure 2 (a)**)

Similarly, the products of antheraxanthin and 9-*cis*-antheraxanthin after acid conversion were analysed by silica gel HPLC. The products from each carotenoid were confirmed to be same compounds ((8*R*)-mutatoxanthin and (8*S*)-mutatoxanthin (3:2) [7]) (**Figure 2 (b)**).

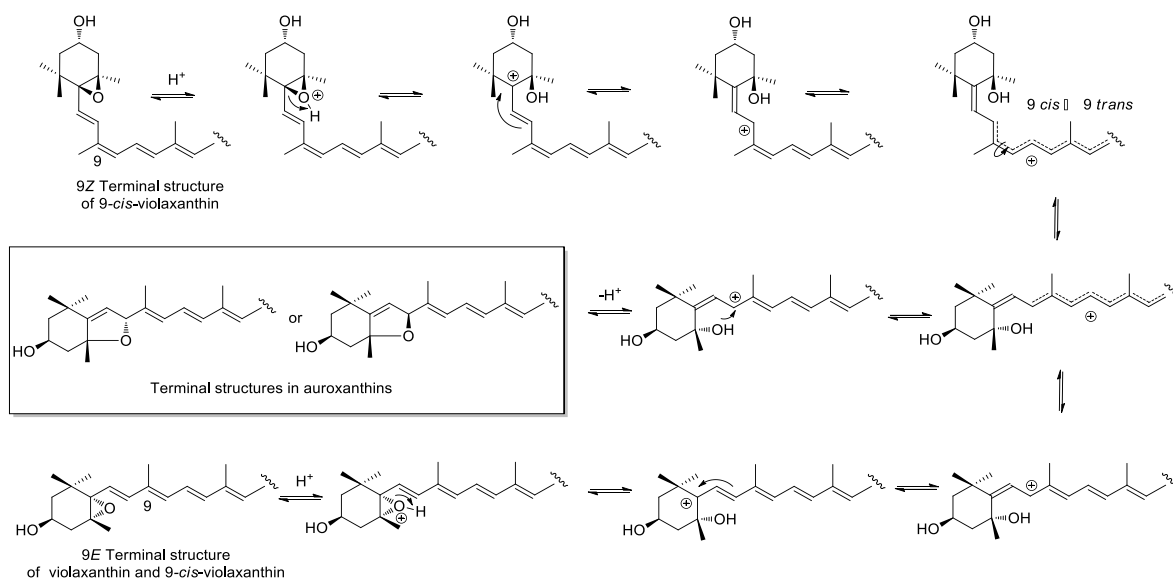


Figure 3. Plausible reaction mechanism for the conversion of violaxanthin and 9-*cis*-violaxanthin to auroxanthins.

A plausible reaction mechanism for the conversion of violaxanthin and 9-*cis*-violaxanthin to auroxanthins is shown in **Figure 3**. The cation generated at epoxide oxygen by protonation conjugates to the olefin structure in the intermediate. Then, the 9-*cis* olefin structure isomerizes to more stable 9-*trans* olefin.

We measured the lipid peroxidation inhibitory and $^1\text{O}_2$ quenching activities of epoxycarotenoids and their acid treatment products. They showed potent lipid peroxidation inhibitory activities (IC_{50} 0.43 – 2.1 μM) (β -carotene IC_{50} 72 μM) and moderate $^1\text{O}_2$ quenching activities (IC_{50} 9.8 – 100 μM) (astaxanthin IC_{50} 1.2 μM).

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Anomalous Optical Properties of Artificial Short-Polyene Carotenoid

D. Kosumi^{1*}, T. Kusumoto² and H. Hashimoto³

¹ *Institute of Pulsed Power Science, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto, 860-8555 Japan*

² *Photonics for Material Processing, The Graduate School for the Creation of New Photonics Industries, 1955-1, Kurematsu-cho, Nishi-ku, Hamamatsu, Shizuoka, 431-1202, JAPAN*

³ *Department of Applied Chemistry for Environment, Faculty of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, 669-1337, Japan.*

*Corresponding Author. E-mail:kosumi@kumamoto-u.ac.jp (DK)

Abstract: Carotenoids possessing a carbonyl group along their polyene backbone exhibit unique excited state properties due to the occurrence of intramolecular charge transfer (ICT) in the excited state. In fact, the ICT characteristics of naturally-occurring carbonyl carotenoids play an essential role in the highly efficient energy transfer that proceeds in aquatic photosynthetic antenna systems. In the present study, we synthesized the short-chain polyene compound 2-(all-*trans*-retinylidene)-indan-1,3-dione with three conjugated double bonds, denoted as C15Ind and performed femtosecond pump-probe spectroscopy on C15Ind in acetone and *n*-hexane. The excited state properties of this compound in a polar solvent acetone and in a nonpolar solvent *n*-hexane demonstrated an ICT character based on the observations of the stimulated emission due to the coupled S₁/ICT state. Furthermore, an additional excited state was found to be located below the S₁/ICT state of C15Ind in acetone.

1. Introduction

In the primary photosynthetic processes, carotenoids bound to photosynthetic antenna pigment-protein complexes absorb sunlight in the blue-green region of the spectrum and the energy is rapidly and efficiently transferred to nearby (bacterio)chlorophylls [1,2]. This transferred energy is eventually converted to electro-chemical energy in reaction centers. In addition, carotenoids protect biological system against photo-destruction caused by excess amount of light exposure [2,3].

The excited states of carotenoids are traditionally described by a three level electronic system (S₀, S₁, and S₂) [4]. The optically-allowed S₂ (1¹B_u⁺) state of carotenoids is generated by a one-photon transition from the S₀ ground state (1¹A_g⁻), and rapidly decays to the lower-lying dark S₁ (2¹A_g⁻) state within 200 fs depending on the length of conjugation [1,2,5,6]. In contrast, non-radiative relaxation from S₁ to S₀ is comparatively slow, occurring on the scale of ps~ns [1]. The excitation energy transfer from the carotenoid S₁ state to (bacterio)chlorophyll in a photosynthetic pigment-protein complex is often inefficient depending on how the optically forbidden S₁ state is involved in the excitation energy-transfer process [1,2]. However, carotenoids containing a carbonyl group along their conjugated polyene backbone, such as fucoxanthin and peridinin, generate an excited state in which intramolecular charge transfer (ICT) occurs just below S₁ in polar environments, leading to the generation of a strongly coupled S₁/ICT state [7]. The S₀-S₁/ICT transition dipole moment of carbonyl carotenoids is increased due to this charge transfer character, and efficient energy transfer from carbonyl carotenoids to

chlorophyll via the S₁/ICT state is realized in light-harvesting photosynthetic antennae [8-12].

The excited state dynamics of carbonyl carotenoids both in solution and bound to pigment-protein complexes have been investigated in detail, using various ultrafast spectroscopic techniques [7-23],

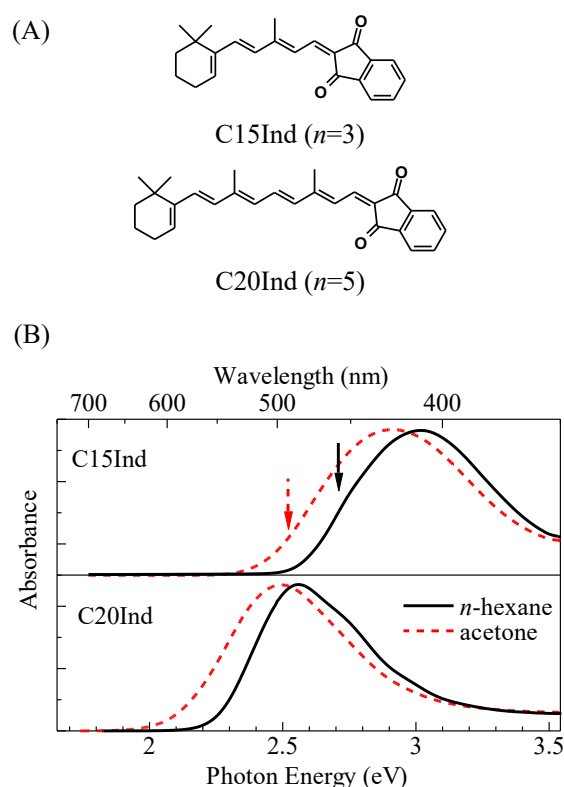


Figure 1. (A) The chemical structures of C15Ind and C20Ind. (B) Steady-state absorption spectra of C15Ind and C20Ind in *n*-hexane and acetone.

and the results have suggested that the ICT character depends on the specific chemical structure of the carotenoid, *e.g.* the location of the carbonyl group position, the asymmetrical position of the carbonyl group within the asymmetrical molecular framework [24-27], and the conjugation length [13,15,28-30]. We previously synthesized a unique compound 2-(all-*trans*-retinylidene)-indan-1,3-dione with five conjugated double bonds *n*, denoted as C20Ind and clarified its photophysical properties by femtosecond and sub-microsecond pump-probe spectroscopy [31,32]. Although C20Ind has two carbonyl groups within the indan group as shown in **Figure 1(A)**, its excited state signatures did not exhibit a pronounced ICT character. In the present study, we synthesized a short polyene compound C15Ind with three conjugated double bonds (*n*=3) as shown in **Figure 1(A)**. It has been reported that carbonyl carotenoids with a short polyene has a strong ICT character [13,15,28-30]. Thus, characteristic spectral signatures is expected such as stimulated emission and transient absorption due to the ICT character of C15Ind. Steady-state absorption and fs pump-probe spectroscopic analyses with 100 fs time-resolution [19,21] were subsequently performed on C15Ind.

2. Experimental

The femtosecond pump-probe spectroscopic measurements based on a mode-locked Ti:Sapphire regenerative amplifier (Spectra Physics, Hurricane-X) have been described in detail elsewhere [19,33]. Visible excitation pulses were obtained from an optical parametric amplifier (Spectra Physics, OPA-800CF) and white light continuum probe pulses, generated using a 2.0 mm sapphire plate, were detected by a 1024ch photo-diode array through a spectrometer (Acton Research, SP275i). The excitation pulses were

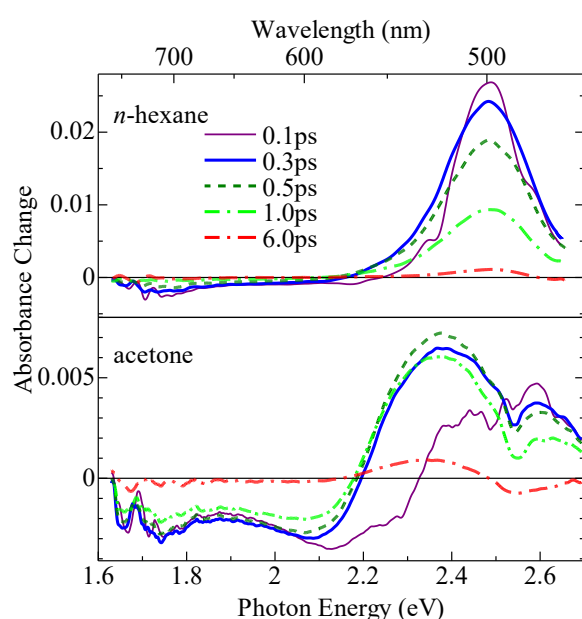


Figure 2. Photo-induced absorption spectra of C15Ind in *n*-hexane and acetone recorded at different delay times.

modulated at 500 Hz by an optical chopper and the data output was synchronized with a laser repetition rate of 1 kHz [33]. Excitation energies were set to 2.67 eV (465 nm) for C15Ind in *n*-hexane and 2.53 eV (490 nm) for that in acetone, and the intensity of each pulse was set to 20 nJ/pulse. The relative polarization between the excitation and probe pulses was set to be magic angle of 54.7°. The instrumental response function and the precise zero time delay were determined by the cross-correlation between excitation and probe pulses. After a chirp compensation, the uncertainty in the zero time delay was less than 10 fs.

The short-chain polyene C15Ind was synthesized by the similar procedure of 2-(all-*trans*-retinylidene)-indan-1,3-dione (C20Ind) [31,34,35]. The samples was dissolved in acetone or *n*-hexane and circulated in a 1.0 mm optical path length flow cell. The absorbance of all the samples solution was adjusted to be 0.5 at the S₂ absorption band. All the measurements were performed at room temperature.

3. Results and discussion

Figure 1(B) shows steady-state absorption spectra of C15Ind in acetone and *n*-hexane together with those of C20Ind. The absorption bands in these spectra correspond to the S₀-S₂ one-photon transition. The spectra of C15Ind and C20Ind acquired in acetone shift to lower-energy and exhibit extended low-energy tail compared to those obtained in *n*-hexane. Previous investigations have proposed that the asymmetric broadening of carbonyl carotenoids in polar solvents can be attributed to both inhomogeneous broadening and variation of the ground and/or excited state conformations (inhomogeneous distribution model) [7,14,17,21,36-39].

C15Ind has three conjugated double bonds in its polyene backbone and a β -ionone ring including a conjugated double bond. Further, the conjugation of C15Ind extended onto carbonyl groups in 1,3-indandione. The 0-0 transition energy of S₂ of C15Ind in *n*-hexane was determined to be 2.74 eV,

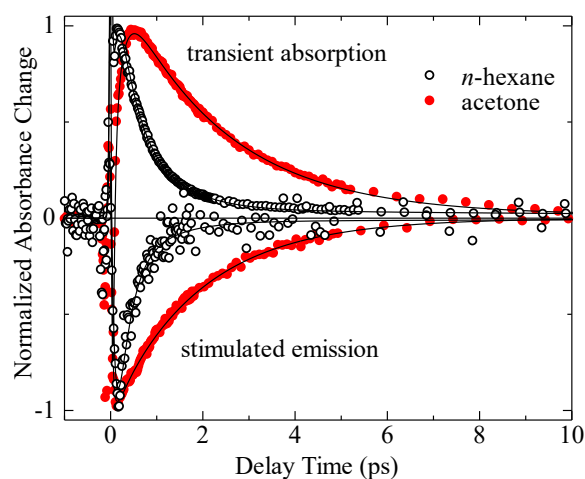


Figure 3. Kinetic traces of transient signals of C15Ind in *n*-hexane and acetone.

corresponding to the 0-0 transition energy of S_2 for open chain carotenoids with an effective conjugated double bonds n_{eff} of 7.7 [40]. Thus, 1,3-indandione much effects on an extension of π -electron delocalization.

The photo-induced absorption spectra of C15Ind in *n*-hexane and acetone are shown in **Figure 2**, recorded at various delay times after excitation at 2.67 eV (465 nm) and 2.53 eV (490 nm). The observed transient absorption signals in the 2.2 to 2.6 eV (480 to 560 nm) region are similar to the S_1 - S_n transition, where S_n is a higher lying $n^1B_u^+$ state [41]. C15Ind did not generate a pronounced polarity-induced ICT-like absorption band, even though their transient absorption spectra acquired in acetone exhibit broader features and lower energy shift compared to those in *n*-hexane, indicating the greater degree of conformational disorder in the electronic excited state. In addition, a pronounced negative absorbance change appears below 2.2 eV (560 nm) in the photo-induced absorption spectra in acetone. Negative absorbance changes in the visible-IR region have been assigned to the stimulated emission from the strongly coupled S_1 /ICT state of carbonyl carotenoids in polar environments [14]. Interestingly, the negative changes due to the stimulated emission were also observed even in a nonpolar solvent *n*-hexane, while their amplitudes in *n*-hexane were much smaller than those in acetone. Thus, the onset of this stimulated emission implies that C15Ind exhibits ICT characteristics both in polar and nonpolar environments. It has been reported that fucoxanthin homologs having short polyene chains exhibit an ICT character even in a nonpolar solvent [30,42]. Naturally-occurring carbonyl carotenoids, such as fucoxanthin and peridinin, do not have an effective electron donor [17], so electron density of the excited state may be transferred from π -electrons in the polyene backbone to the carbonyl group [43,44]. As a result, a larger perturbation of the π -electron distribution is formed in the rest of the polyene chain.

This effect can be enhanced in shorter polyenes and can facilitate generation of the well-stabilized ICT state located much lower than S_1 , realizing a strong coupling of the S_1 and ICT states, even in a nonpolar solvent.

Figure 3 presents the kinetic traces of the photo-induced absorption of C15Ind in *n*-hexane and acetone probed at 2.67 eV (465 nm) and 2.43 eV (510 nm) for transient absorption and at 1.94 eV (640 nm) and 2.07 eV (600 nm) for stimulated emission. These traces were globally analyzed using exponential components for the rise and decay phases convoluted with the instrument response function, assuming a Gaussian temporal profile. Three (in *n*-hexane) or four (in acetone) exponential components were required to properly fit the data. In *n*-hexane these signals are assignable to the S_1 /ICT state, since the transient absorption and stimulated emission signals exhibit the same decay kinetics with a lifetime of 700 fs. It has been reported that the S_1 lifetimes of noncarbonyl carotenoids with $n=3$ exceeded nanoseconds [5,45]. Therefore, the obtained S_1 /ICT lifetime of 700 fs demonstrates a strong ICT character of C15Ind even in a nonpolar solvent.

Although polarity-induced ICT-like absorption bands are not observed in the photo-induced absorption spectra in **Figure 2**, the kinetic traces of these transient absorption bands exhibit a pronounced solvent polarity effect. Interestingly, **Figure 3** demonstrates that the decay of the S_1 /ICT state of C15Ind in *n*-hexane is much faster than that in acetone. It has been generally accepted that the S_1 /ICT decay of carbonyl carotenoids is facilitated in polar environments due to a strong coupling between S_1 and ICT state [7,14]. Even though an ICT state of carbonyl carotenoids can exist in a nonpolar solvent [30,42], an ICT state is stabilized in a polar solvent, facilitating a coupling between S_1 and ICT. Therefore, the decay of the S_1 /ICT state of carbonyl carotenoids in polar solvent should be faster than that in a nonpolar solvent. However, the observed kinetics in this study show opposite behavior. A similar solvent dependence of the kinetic traces has been

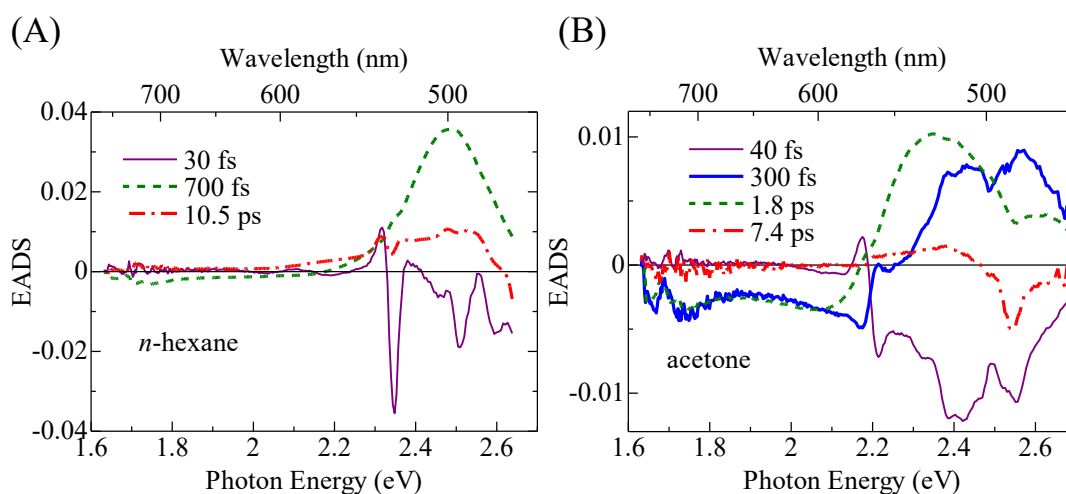


Figure 4. Evolution-associated difference spectra of C15Ind in (A) *n*-hexane and (B) acetone obtained by a global analysis.

reported in C20Ind [31]. The solvent dependence of the excited state dynamics for C20Ind was explained by a consideration of $^1n\pi^*$ state rather than an ICT state.

To assist in visualizing the excited states dynamics of C15Ind, the evolution-associated difference spectra (EADS) obtained by the global analysis are presented in **Figure 4**. The first EADS plot in each solvent exhibits negative signal corresponding to the ground state bleaching and the stimulated emission from S_2 . Thus, the first EADS are assigned to the short-lived S_2 state, which have ultrashort lifetimes (30 and 40 fs). The third EADS for C15Ind in *n*-hexane and the fourth EADS in acetone exhibit transient absorption bands with small amplitudes and their spectral peaks locate at higher energy compared with the transient absorption bands plotted by the broken lines. In addition, these EADS do not have negative changes due to the stimulated emission of the S_1 /ICT state. Therefore, these EADS are tentatively assigned to the previously reported S^* -like state [46-49].

The second EADS for C15Ind in *n*-hexane is assignable to the S_1 /ICT state because the spectrum has both the transient absorption and stimulated emission signals. The second and third EADS for C15Ind in acetone have the similar spectral features, showing both the positive and negative changes, while the transient absorption band of the second EADS locates at higher energy than the third EADS. The third EADS with a time constant of 1.8 ps contributed mainly to the decay kinetics of the transient absorption and stimulated emission shown in **Figure 3**. To clarify the origin of the second and third EADS, a comparison of the kinetic traces probed at 2.61 eV (475 nm) and 2.43 eV (510 nm) is shown in **Figure 5**. The transient absorption kinetics probed at 2.43 eV (510 nm) rises with the decay of the transient absorption at 2.61 eV (475 nm). This invokes that the second EADS originates from a vibrationally hot state [22,30]. However, a hot transient absorption band generally becomes narrower and shifts to higher energy during vibrational relaxation. Thus, the assignment of the second EADS to a vibrationally

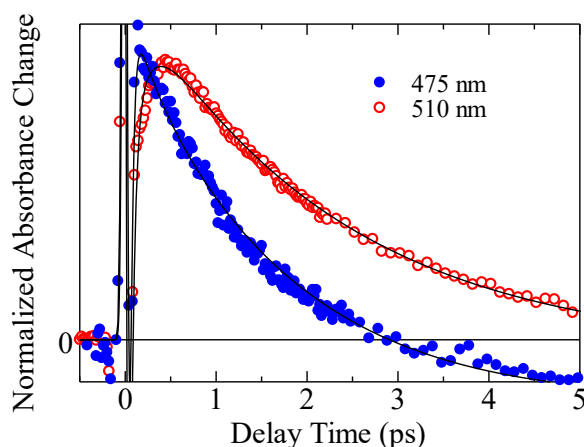


Figure 5. A comparison of the kinetic traces of transient signals of C15Ind in acetone probed at 2.61 eV (475 nm) and 2.43 eV (510 nm).

hot state may be excluded. The observation for C15Ind in acetone implies a participation of an additional electronic excited state located below the optically allowed S_2 , rather than a consideration of a vibrational hot state. Both the second and third EADS exhibits a pronounced negative signal due to the stimulated emission. Therefore, a tentatively postulated intermediate state also has an ICT character.

Finally we discuss the solvent dependence of the excited states dynamics of C15Ind. **Figure 4** exhibits that the transient absorption and stimulated emission decays of C15Ind in *n*-hexane are much faster than those in acetone. In addition, the S_1 /ICT transient absorption of C15Ind in *n*-hexane locates lower energy than that in acetone. This observation is an opposite behavior to solvent dependence of the S_1 /ICT transient absorption observed in various carbonyl carotenoids [7,14,30,50,51]. On the other hand, the second EADS of C15Ind in acetone has the higher energy transient absorption band and the shorter lifetime than those of the S_1 /ICT state in *n*-hexane. Therefore, it is likely that the second EADS of C15Ind in acetone is assigned to the S_1 /ICT state. On a basis of this assumption, the solvent dependence of the excited state dynamics of C15Ind observed in this study agrees with the previous works.

The excited states dynamics of C15Ind in *n*-hexane and acetone are summarized in **Figure 6**. It is still not clear what the third EADS of C15Ind in acetone originates from. The third EADS exhibits similar spectrum to the second EADS assigned tentatively to the S_1 /ICT state, while the transient absorption shifts to higher energy compared with the second EADS. A possible interpretation is a consideration of $^1n\pi^*$ state as postulated for excited state dynamics of C20Ind [31]. In C20Ind, the $^1n\pi^*$ state was assumed to be located just above the S_2 state and this idea was supported by a quantum-chemical calculation. The S_2 energy of C15Ind is higher than that of C20Ind as shown in **Figure 4 (B)**. Thus, the $^1n\pi^*$ state can be located lower energy than the S_1 /ICT state if the energy of the $^1n\pi^*$ state does not depend on *n*. Indeed, the presence of the $^1n\pi^*$ state between the S_1 and S_0 states has been reported in retinal with *n*=5 and a carbonyl group

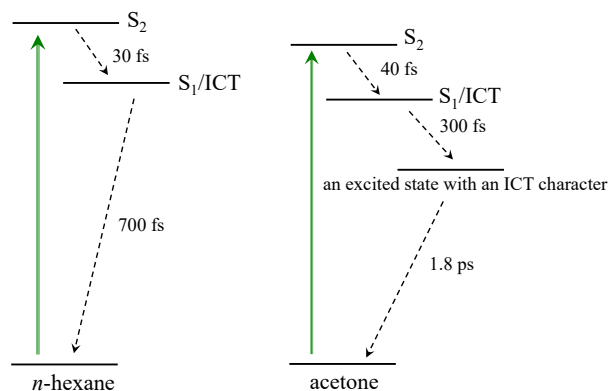


Figure 6. Schematic representations of the excited state dynamics of C15Ind in *n*-hexane and acetone.

[52,53]. However, it should be noted that the third EADS for acetone also has the negative change due to the stimulated emission. It is not clarified whether the $^1n\pi^*$ state has an ICT-like character or not. Nevertheless, the third EADS originates from an excited state with an ICT-like character.

4. Conclusions

In this study, we have investigated the ultrafast excited state dynamics of C15Ind in *n*-hexane and acetone by means of femtosecond pump-probe spectroscopic measurements. The results demonstrated that C15Ind exhibits an ICT character even in a nonpolar solvent on a basis of the observation of the stimulated emission due to the S_1 /ICT state. In *n*-hexane, the excited state dynamics of C15Ind can be explained by a three state model: $S_2 \rightarrow S_1/\text{ICT} \rightarrow S_0$. On the other hand, an additional excited state participates in the excited state dynamics of C15Ind in acetone and locates below the S_1 /ICT state.

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The Possible Mechanisms of the Chiral Conversion of Astaxanthin in the Prawn

Takashi Maoka*

Research Institute for Production Development, 15 Shimogamo-morimoto-cho, Sakyo-ku,
Kyoto 606-0805, Japan *E-mail: maoka@mbox.kyoto-inet.or.jp.

Abstract: The chiral conversion of astaxanthin was observed in the (3*S*,3'*S*)-astaxanthin administration experiment of the prawn *Penaeus japonicus*. Isoastaxanthin (4,4'-dihydroxy- ϵ,ϵ -carotene-3,3'-dione **1**), which was first reported by Schiedt et al. (1991, 1993), was isolated and completely characterized by UV-VIS, MS, ¹H-NMR, and CD spectral data. Isoastaxanthin was assumed to be a key intermediate of chiral conversion of 3 (3')-hydroxy group of astaxanthin in the prawn. The possible mechanisms for the chiral conversion of astaxanthin through isoastaxanthin in the prawn were discussed.

Keywords: chiral conversion, astaxanthin, isoastaxanthin,

1. Introduction

It was reported that astaxanthin in Penaeidae shrimps was presented as almost racemic mixture [1-3]. Furthermore, racemization of astaxanthin was observed in the prawn *Penaeus japonicus* by a feeding experiment using [³H]-labeled astaxanthin by Schiedt et al. [2-3]. As the results, [³H]-labeled (3*S*,3'*S*)-astaxanthin was converted to (3*R*,3'*R*), *meso*, and (3*S*,3'*S*)-isomers at an approximate ratio of 1:2:1 in this prawn [2-3]. Similarly, we also observed the chiral conversion of administrated (3*S*,3'*S*)-astaxanthin in the prawn *Penaeus japonicus* [4]. Furthermore, a novel carotenoid, isoastaxanthin (4,4'-dihydroxy- ϵ,ϵ -carotene-3,3'-dione **1**), was isolated from (3*S*,3'*S*)-astaxanthin administrated prawn. This carotenoid has a unique 3-keto-4-enol ϵ -end group in the molecule and was assumed to be a key intermediate of chiral conversion of astaxanthin. The present report describes the isolation and characterization of isoastaxanthin from the prawn and discusses the possible mechanisms for chiral conversion of 3 (3')-hydroxy group of astaxanthin in the prawn.

2. Experimentals

2.1 Feeding Experiment

Sixteen specimens of prawns, average body weight 3.6 g, were kept in each 200 L tank. The prawns were fed to satiation with experimental food one time a day for 8 weeks. HIGASHIMARU's based feed was used for control diet. PANAFERD-AX (JXTG Nippon Oil & Energy Corporation) was used as pigmentation source. The experimental diets containing 100 ppm of (3*S*,3'*S*)-astaxanthin.

2.2 Carotenoid Extraction, Isolation, and Identification

Details of the extraction, isolation, identification, and quantification of carotenoids in the prawn were described in the previous literature [4]. The UV-VIS

spectra were recorded with a Hitachi U-2001 spectrophotometer in ether. The MS spectra were obtained by Waters Xevo G2S Q TOF mass spectrometer (Waters Corporation, Milford, CT, USA). The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z* 100 to 1,500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120°C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured with a quadrupole-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V. The ¹H NMR (500 MHz) spectrum was measured with a Varian UNITY INOVA 500 spectrometer in CDCl₃ with TMS as an internal standard. The CD spectrum was recorded in Et₂O at room temperature with a Jasco J-500C spectropolarimeter.

Isoastaxanthin (1): UV-VIS: 419, 439, 468 nm. ESI TOF MS: *m/z* 619.3768 (M+Na⁺, calcd. for C₄₀H₅₂O₄Na, 619.3763), *m/z* 597.3937 (M+H⁺, calcd. for C₄₀H₅₃O₄, 597.3944). Product ions of ESI TOF MS/MS from M+H⁺: *m/z* 579 (M+H-H₂O), 561 (M+H-2H₂O), 523, 445, 377, 225, 211, 197, 153, 139, 125. ¹H NMR δ (CDCl₃): 0.97 (6H, s, CH₃-17, 17'), 1.10 (6H, s, CH₃-16, 16'), 1.84 (6H, s, CH₃-18, 18'), 1.92 (6H, s, CH₃-19, 19'), 1.97 (6H, s, H-20, 20'), 2.22 (2H, d, *J*=17 Hz, H-2, 2'*ax*), 2.53 (2H, d, *J*=17 Hz, H-2, 2'*eq*), 2.64 (2H, d, *J*=9 Hz, H-6, 6'), 5.55 (2H, dd, *J*=15.5, 9.5, H-7, 7'), 6.05 (2H, br.s, OH-4,4'), 6.17 (2H, d, *J*=11 Hz, H-10, 10'), 6.21 (1H, d, *J*=15 Hz, H-8, 8'), 6.27 (1H, m, H-14, 14'), 6.38 (2H, d, *J*=15 Hz, H-12, 12'), 6.60 (2H, dd, *J*=15, 11 Hz, H-11, 11'), 6.65 (2H, m, H-15, H-15'). CD λ ($\Delta\epsilon$): 235 (0), 256 (+20.3), 268 (0), 285 (-20.4), 360 (0).

3. Results and Discussion

3.1 Chiral Conversion of Administered (3*S*,3'*S*)-Astaxanthin in the Prawn

The amounts of (3*R*,3'*R*)-, (*meso*)-,

(3*S*,3'*S*)-astaxanthin, and isoastaxanthin (**1**) in the control group and 100 ppm (3*S*,3'*S*)-astaxanthin administered group in the carapace of the prawn are shown in **Table 1**. Not only the amount of (3*S*,3'*S*)-astaxanthin but also those of (*meso*)- and (3*S*,3'*S*)-astaxanthin were increased by the administration of 100 ppm (3*S*,3'*S*)-astaxanthin. This indicated that a part of (3*S*,3'*S*)-astaxanthin ingested by the prawn was converted to (*meso*)- and (3*R*,3'*R*)-astaxanthin, as reported by Schiedt et al [2,3]. Furthermore, the amount of isoastaxanthin (**1**) was also increased by the administration of 100 ppm (3*S*,3'*S*)-astaxanthin. Schiedt et al. reported the presence of isoastaxanthin in the prawn in 1991 [2] and 1993 [3]. However, detailed spectral data on isoastaxanthin have not been reported yet [5-7]. In the present investigation, structure of isoastaxanthin was completely characterized by UV-VIS, MS, MS/MS, ¹H NMR, and CD spectral data (**Figure 1** and Experimentals). These spectral data were agreement with the structure of (6*S*,6'*S*)-4,4'-dihydroxy- ϵ,ϵ -carotene-3,3'-dione for isoastaxanthin (**1**) [5-7]. This is the first report of the characterization and demonstration of spectral data of isoastaxanthin (**1**).

3.2 Possible mechanisms of Chiral Conversion of Astaxanthin through Isoastaxanthin (**1**) in the Prawn

Chiral conversion at the 3 (3')-hydroxy group in astaxanthin could be explained by the presence of isoastaxanthin (**1**), having a 3-keto-4-hydroxy- ϵ -end group, as an intermediate. Namely, the 3 (3')-hydroxy group in astaxanthin was oxidized once to a carbonyl group with double-bond translation from C5-C6 (C5'-C6') to C4-C5 (C4'-C5') to form (**1**). Then, the 3-keto-4-hydroxy- ϵ -end group in isoastaxanthin (**1**) was reversibly converted to the 3-hydroxy-4-keto- β -end group to form astaxanthin. Another possible mechanism of the chiral conversion of astaxanthin is keto-enol tautomerization of the 3 (3')-hydroxy group, as shown in **Figure 2**. Through these conversions, the chirality of the 3 (3')-hydroxy group of astaxanthin could be converted. In the present experiment, the amount of (*meso*)-astaxanthin was also increased. Chiral conversion of 3'-hydroxy group in (3*S*,3'*S*)-astaxanthin to (3*S*,3'*R*: *meso*)-astaxanthin could be explained the presence of semi-isoastaxanthin (3,4'-dihydroxy- β,ϵ -carotene-4,3'-dione **2**) as intermediate (**Figure 2**). In the present investigation, semi-isoastaxanthin could not be detected. This compound might be presented as very minor carotenoid in the prawn.

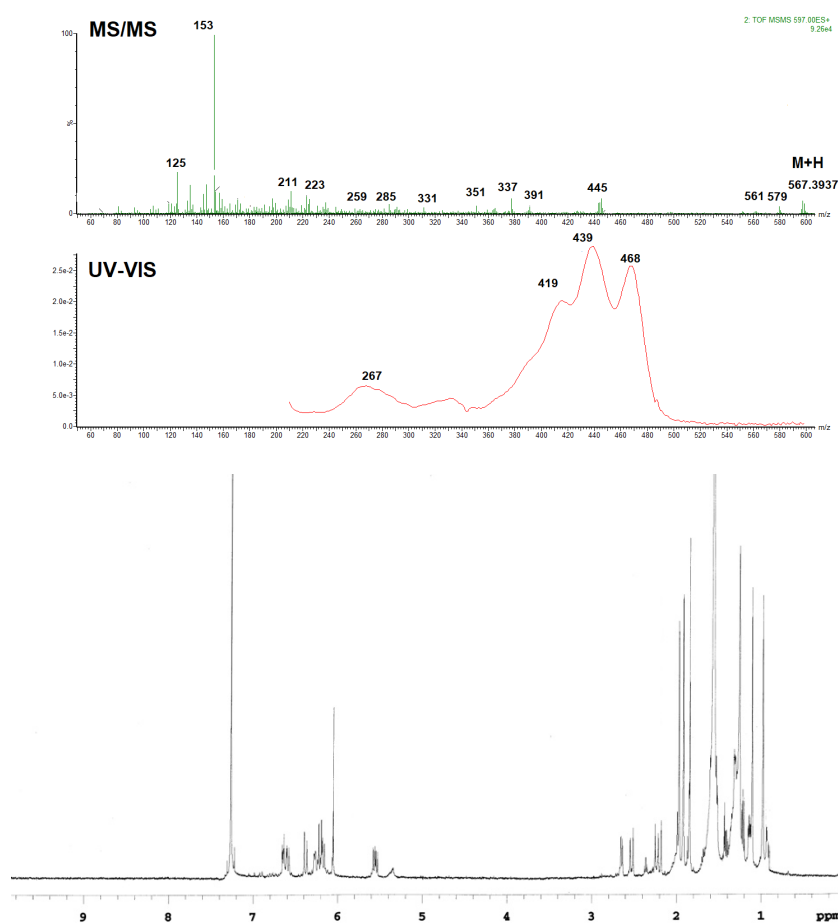
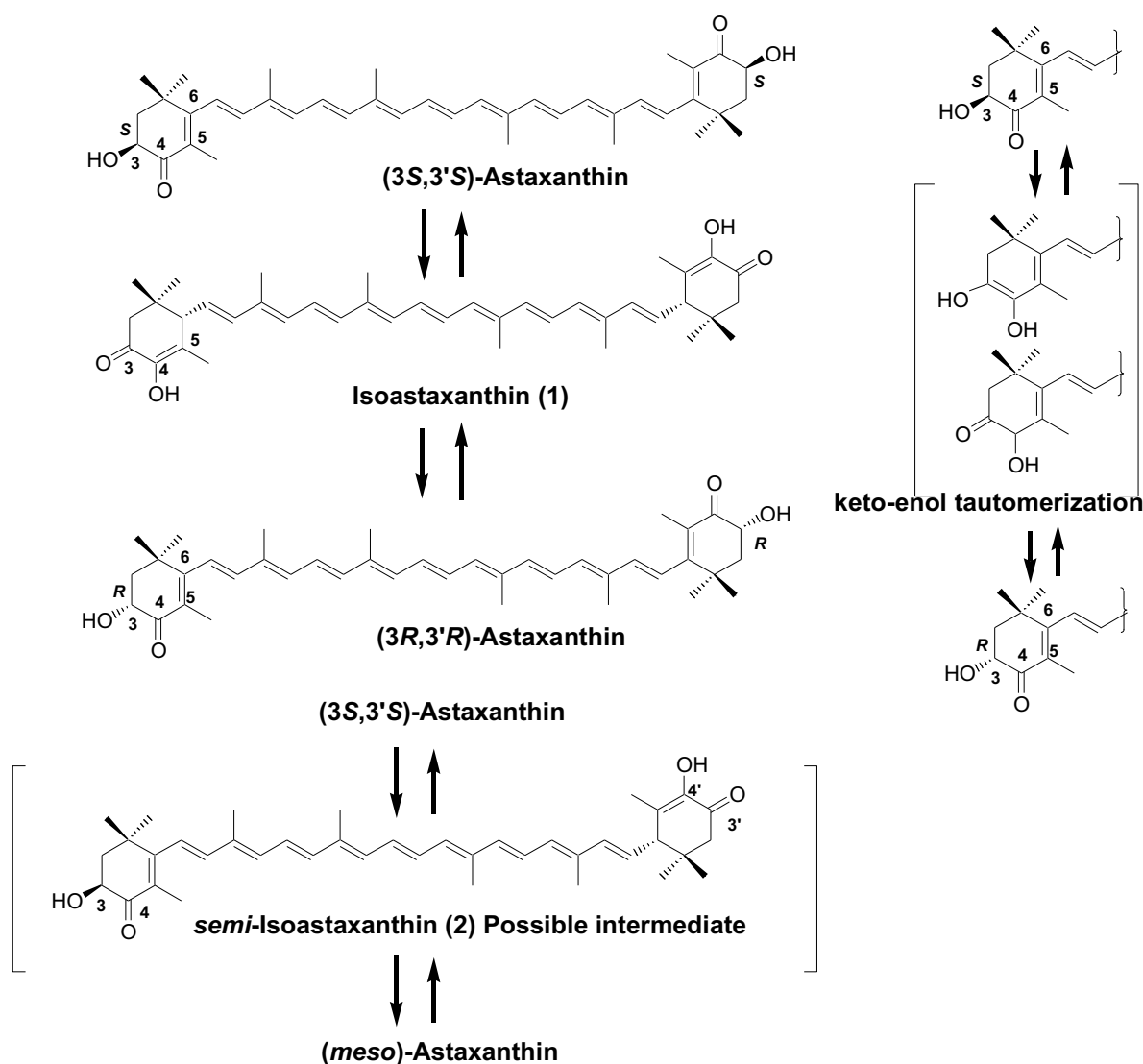


Figure 1. ESI-TOF MS/MS, UV-VIS, and ¹H-NMR (in CDCl₃) of isoastaxanthin (**1**).

Table 1. Content ($\mu\text{g/g}$) of optical isomers of astaxanthin and isoastaxanthin (**1**) in the carapace of control group and 100 ppm astaxanthin administration group

Exp. Group	(3 <i>R</i> ,3' <i>R</i>)-Asta.	(<i>meso</i>)-Asta.	(3 <i>S</i> ,3' <i>S</i>)-Asta.	Isoastaxanthin
Control	7.7	15.4	7.7	1.2
(3 <i>S</i> ,3' <i>S</i>)-Asta 100 ppm	13.8	34.7	53.3	3.0

Cont: control group, (3*S*,3'*S*)-Asta 100 ppm: contained 100 ppm (3*S*,3'*S*)-astaxanthin to control diets.

**Figure 2.** Possible mechanisms of the chiral conversion of astaxanthin in the prawn.

4. Conclusion

A novel carotenoid, isoastaxanthin (**1**) was isolated from the prawn. This carotenoid has a unique 3-keto-4-enol ε -end group in the molecule. Chiral conversion of astaxanthin could be explained presence of isoastaxanthin (**1**) as an intermediate. Until now, chiral conversion of astaxanthin has been reported only in Penaeidae shrimps [2-4]. Detailed stereochemical investigation of carotenoid will reveal the chiral conversion of astaxanthin in other animals.

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Accumulation of Euglena-derived carotenoids in egg yolk

Shota Watanabe¹, Toru Okada², Hideto Ookita³, Kyoichi Sakamoto³, Kengo Suzuki⁴, Takashi Maoka^{5*}

¹*Kitasato University Graduate School of Veterinary Science, Towada city, Aomori 034-8628, Japan.*

²*Aska animal health Co., Ltd. Shibaura, Minato-ku, Tokyo 108-0023, Japan*

³*Ehime Research Institute of Agriculture, Forestry and Fisheries Livestock Research Center Research Institute of Poultry Saijo-city, Ehime 799-1316, Japan*

⁴*Euglena Co., Ltd. Shiba Minato-ku, Tokyo 108-0014, Japan.*

⁵*Research Institute for Production Development, Sakyo-ku, Kyoto 606-0805, Japan*

*Corresponding author e-mail maoka@mbox.kyoto-inet.or.jp

Abstract: This study evaluated egg production, egg yolk color score and carotenoids in laying hens fed diets containing replacement Euglena to clarify the influence of Euglena feeding on egg production rate and egg quality. In this experiment, Boris Brown laying hens (616 days old of age) were randomly housed into 4 groups with 2 replicates per group (two birds per cage, 7 cages per replicate) and fed with 4 experimental diets. The control diet (17.0%CP, 2,850kcal/kgME) and the control diet supplemented with 1%, 2%, and 5% Euglena. The feeding trial lasted 28 days after the acclimatization. There was no difference in feed intake amount and egg production rate among the treatment groups, although color score of egg yolk showed tendency to increase in hens fed with Euglena. In addition, diatoxanthin and aroxanthin, two types of carotenoids, were detected from egg yolk from hens fed Euglena. We measured the inhibitory effect of the detected diatoxanthin and aroxanthin on the lipid peroxidation effect, and confirmed that those carotenoids have the same inhibitory effect as lutein. This is the first report showing accumulation of carotenoids with triple bonds, diatoxanthin and aroxanthin, suggesting that the laying hen may possibly use and accumulate carotenoids with acetylenic bonds.

Keywords: egg yolk, carotenoid, Euglena (*Euglena gracilis*), diatoxanthin, aroxanthin

1. Introduction

Algae are rich in proteins, amino acids, as well as bioactive compounds, and they can be useful resources for commercial use, such as livestock feed. Chlorella and Spirulina, kinds of microalgae containing abundant proteins and vitamins have been widely used as food materials and feed materials [1,2]. Chlorella or spirulina added to feed for laying hens affect their growth performance, total cholesterol, and LDL-cholesterol of laying hens, resulting in change in egg yolk color [3,4].

Euglena (*Euglena gracilis*), one of microalgae has also long been researched like Chlorella and Spirulina. Euglena, a kind of unicellular eukaryotic organism, carrying both plant and animal properties; It perform photosynthesis by chloroplasts and swim by flagella and cell contraction (**Figure 1**). Likely because of this nature, Euglena contains many nutrients such as proteins, amino acids, vitamins and minerals [5-7]. Euglena have the outer cell membrane which are mainly composed of protein unlike plants. Thus, digestibility of Euglena protein reaches 93.1% by rats' test [8]. Due to its abundant nutrients and high digestibility, research on Euglena has been carried out not only in the food and medical fields, but also in the field of feed [9,10].

Previous studies reported that feeding with Euglena improve protein digestibility in ruminant animals [11], and accumulation of taurine in muscle of broilers [12]. It is not reported, however, whether Euglena ingestion have the influence on egg laying hen, and nutritional components in their eggs. Thus, in this study, we evaluated the effect of Euglena supplementation on egg production results and egg quality in Boris Brown laying hens.

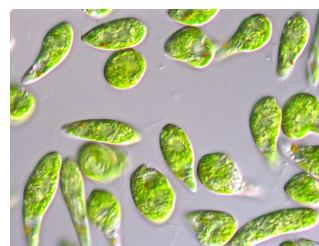


Figure 1. A micrograph of *Euglena gracilis*

2. Materials and Methods

2.1 Feeding tests on laying hens, and sampling eggs

Euglena, powder with 100% purity was obtained from Euglena Co. Ltd., Japan. Feeding tests were conducted at Ehime Research Institute of Agriculture, Forestry and Fisheries Livestock Research Center Research Institute of Poultry (Ehime, Japan). 616 days

old of age Boris Browns laying hens were divided into 4 groups with 2 replicates per group (two hens per cage, 7 cages per replicate) and diets containing 1%, 2% and 5% of *Euglena* powder were added to the control feed, and the prepared feeds were fed ad libitum for 28 days after acclimation. For the control diet, a commercially available laying hens feed (CP 17% or more, ME 2,850 kcal / kg or more, low carotenoid) was used. During the experiment, feed ingestion, egg production rate, egg yolk color score and carotenoid in egg yolk were measured. Feed intake and egg production rate were averaged in the replicating section, and five egg yolks were mixed into one and used as a measurement sample.

2.2 Analysis of carotenoids in *Euglena* powder and egg yolk

Carotenoids were extracted with acetone from *Euglena* powder. In the case of egg yolk, carotenoids were extracted with acetone from frozen egg yolk. Then carotenoids were transferred to n-hexane-ether (1:1) by addition of water. Individual carotenoids were separated by silica gel column chromatography and preparative HPLC with silica gel acetone:n-hexane (2:8) as mobile phase. Identification of individual carotenoids were carried out on UV-VIS, ESI TOF MS and ¹H-NMR spectral data. UV-VIS and ESI MS TOF spectra were recorded with Waters Xevo G2S Q TOF mass spectrometer with ESI positive ion mode equipped with Acquity photo diode detector. ¹H-NMR spectra were recorded with Varian UNITY INOVA at 500 MHz in CDCl₃.

2.3 Antioxidative activity for lipid peroxidation

According to the methods described by Terao [13, 14], carotenoids were dissolved in EtOH at a concentration of 2 mM (final concentration of 167 μM in the reaction mixture). The sample solution, 0.1 mL, was added to 1 mL of 0.1M methyl linolate solution [n-hexane/2-propano (1/1, vol/vol)], and the solution was incubated at 37°C for 5 min. As a control, EtOH alone was used instead of the sample solution. The oxidation reaction was then performed by incubation with air at 37°C. At regular intervals, the oxidation reaction products, methyl linolate hydroperoxides, were quantified by HPLC. HPLC was performed with a Hitachi L-6000 intelligent pump and an L-4250 UV-VIS detector. The following HPLC conditions were employed for the quantitative analysis of methyl linolate hydroperoxides: column Lichrosorb Si 100 5 μm (4.6i.d. × 250 nm) (Merck); solvent system: 2-propanol/n-hexane (1/99, vol/vol); flow rate: 1 mL/min; and detection: 235 nm.

3. Results

3.1 Effects of *Euglena* powder intake on egg production rates and egg yolk color score

Figures 2, 3, and 4 show the rates of feed intake, egg production results, and egg yolk color score in each

group. Feed intake and egg production rates were not affected by *Euglena*, but the egg yolk color score showed a tendency to rise depending on the addition rate of *Euglena* and the coefficient of determination was $R^2 = 0.99$ (Figure 5).

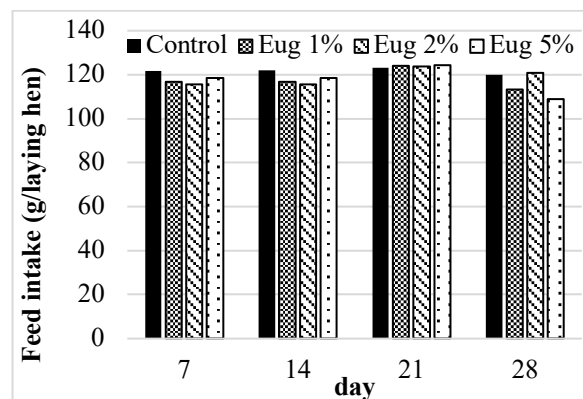


Figure 2. Influence of dietary *euglena* on feed intake in laying hens, Control = control(basal diet); Eug 1% = 99% basal diet+1% *Euglena*; Eug 2% = 98% basal diet+2% *Euglena*; Eug 5% = 95% basal diet+5% *Euglena*.

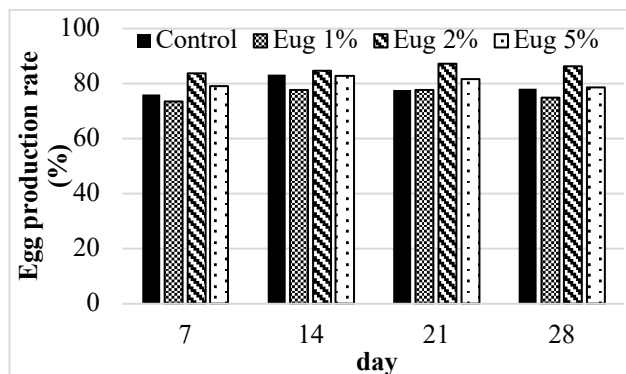


Figure 3. Influence of dietary *euglena* on egg production rate in laying hens, Control = control(basal diet); Eug 1% = 99% basal diet+1% *Euglena*; Eug 2% = 98% basal diet+2% *Euglena*; Eug 5% = 95% basal diet+5% *Euglena*.

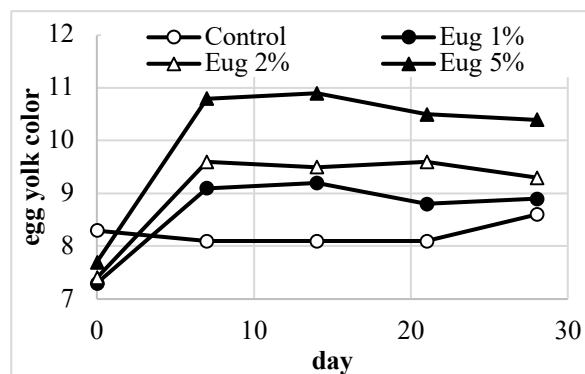


Figure 4. Influence of dietary *euglena* on egg yolk color in laying hens, Control = control(basal diet); Eug 1% = 99% basal diet+1% *Euglena*; Eug 2% = 98% basal diet+2% *Euglena*; Eug 5% = 95% basal diet+5% *Euglena*.

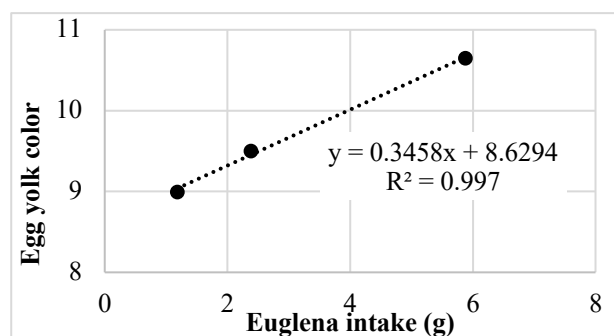


Figure 5. Relation between egg yolk color and euglena intake

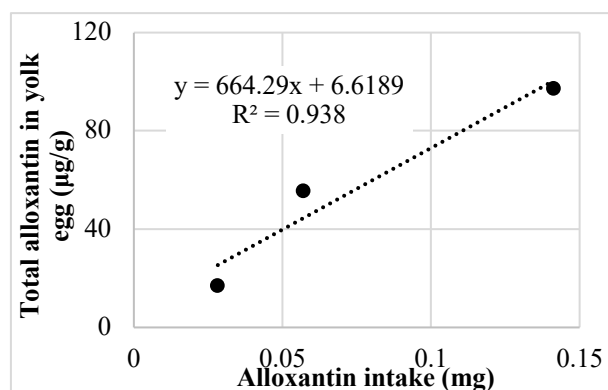


Figure 7. Relation between total alloxanthin in egg yolk and alloxanthin intake

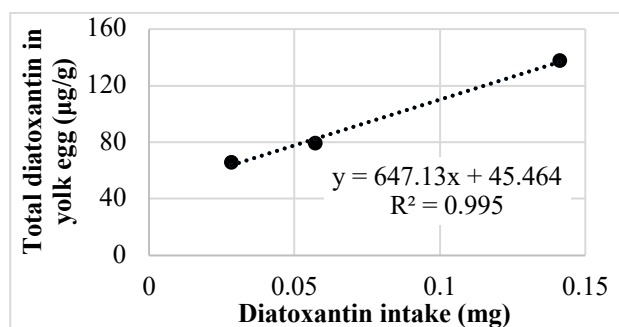


Figure 6. Relation between total diatoxanthin in egg yolk and diatoxanthin intake

3.2 Carotenoids of Euglena and hens yolk

The analysis results of the Euglena powder and the egg from the UV-VIS absorption maximum of the test sample, the molecular ion peak in FAB-MS and ¹H-NMR and ¹³C-NMR are shown in **Table 1**.

We found positive correlation between dose of Euglena in feed and the amounts of diatoxanthin and aroxanthin in egg yolk.

For diatoxanthin, the coefficient of determination was $R^2 = 0.996$ and for alloxanthin, the coefficient of determination was $R^2 = 0.938$ (**Figure 6, 7**). The spectral data were as follows. Diatoxanthin:UV-Vis (in Et₂O) 425, 450, 479 nm, FAB MS m/z 566[M⁺]. Alloxanthin:UV-Vis (in Et₂O) 426, 451, 480 nm, FAB MS m/z 564[M⁺].

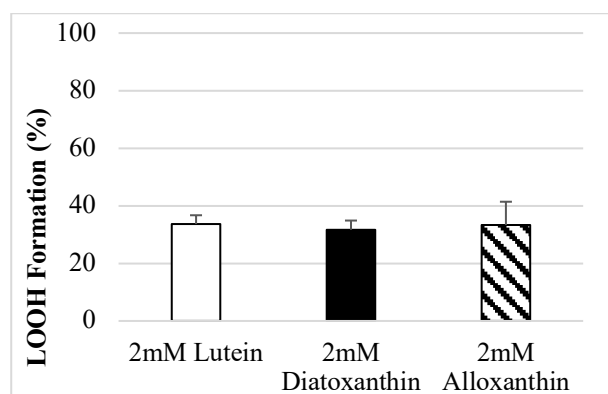


Figure 8. Lipid peroxidation inhibition effects of carotenoids

3.3 Antioxidant activity of diatoxanthin and alloxanthin

The antioxidant effect of diatoxanthin and alloxanthin was evaluated based on lipid peroxidation inhibition using AMVN radical. The result showed that the lipid peroxidation inhibitory effect of diatoxanthin and aroxanthin was almost equivalent to lutein (**Figure 8**).

Table 1. Total carotenoid of test yolk egg and

	Control	Euglena 1%	Euglena 2%	Euglena 5%	Euglena 100%
Total Carotenoid (µg/g)	26.3	24.4	26.5	25	100
Composition(%)					
β-Cryptoxanthin	4.7	6.9	8.1	6.9	8.5
3'-Hydroxy-b,e-caroten-3'-one	5.1	7.7	5.4	5.4	
Lutein	42	31.4	29.3	31.1	
Zeaxanthin	28.4	32.9	31.1	27.1	10.4
Diatoxanthin		2.7	3	5.5	24
Alloxanthin		0.7	2.1	3.9	12.3
Others	19.8	17.7	21	20.1	

Control = control(basal diet) = 99% basal diet+1% Euglena = 98% basal diet+2%

Euglena = 95% basal diet+5% Euglena Euglena 100% = 100% Euglena.

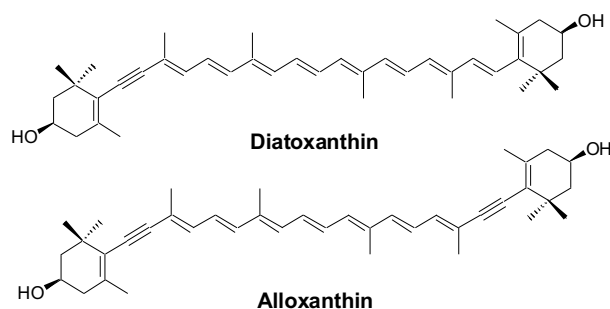


Figure 9. Structural formula of diatoxanthin and alloxanthin

4. Discussion

4.1 Effects of feed intake, egg production rates and egg yolk color score

Feed intake and egg production rates were not affected by the addition rate of *Euglena* (Figures 2, 3, 4), but there was a positive correlation that the egg yolk color score increased with the addition rate of *Euglena* (Figure 5).

In Table 1, it was confirmed that the β -cryptoxanthin content tends to rise due to *Euglena*. Heying *et al.*; (2014) [18] report that the color score of egg yolk rise by feeding β -cryptoxanthin. Therefore, it is presumed that the egg yolk color score rises as the laying hens accumulate β -cryptoxanthin contained in *Euglena*. Carotenoid is a generic term for tetraterpene pigments which are red, orange or yellow widely distributed in microorganisms, plants and animals, and by 2004 more than 750 species have been reported [19].

From the results of the egg yolk color score, MS and NMR analysis of egg yolk fed with *Euglena* powder, it was revealed that diatoxanthin and alloxanthin accumulated in egg yolk by feeding *Euglena*. Thus, it is presumed that carotenoids in *Euglena* were accumulated in egg yolk.

Microorganisms and plants can synthesize carotenoids in the body, while animals cannot by themselves. Therefore, it is considered that all carotenoids present in animal's body are derived from food ingested. It is known that diatoxanthin and alloxanthin accumulate in scallops [20]. Previously, it has been reported that astaxanthin and lutein are accumulated in laying egg yolk [21, 22], although there is no report on the accumulation of diatoxanthin and alloxanthin like scallops, here we revealed that diatoxanthin and aroxanthin are transferred and accumulated to laying egg yolk in laying hens.

4.2 Physiological reaction of diatoxanthin alloxanthin

Unsaturated fatty acids, one of the constituents of biological membranes, become lipid radicals when protons are withdrawn by active oxygen or free radicals, and lipid radicals react with to produce lipid peroxy radicals.

Lipid radicals and lipid peroxy radicals undergo chain-like lipid peroxidation reactions. Carotenoids

show a remarkable inhibitory effect on this lipid peroxidation reaction. The antioxidant effect of diatoxanthin and alloxanthin was evaluated based on lipid peroxidation inhibition using AMVN radical as an indicator. As shown in (Figure 8) that lipid peroxidation inhibitory effect of diatoxanthin and aroxanthin is equivalent to lutein. In addition, Tsushima *et al.*, (1995) [23] measured the anti-carcinogenic promoting activity of diatoxanthin and alloxanthin using Epstein-Barr virus early antigen (EBV-EA) to inhibit Epstein-Virus activation. As the results, both diatoxanthin and alloxanthin showed anti-cancer effects.

In this study, feeding *euglena* to the egg laying chickens could improve the color score of egg and accumulate carotenoid with triple bond, diatoxanthin and alloxanthin in their eggs (Figure 9). This is the first report that diatoxanthin and alloxanthin, having acetylenic bond, could be accumulated in egg yolk. Our finding will be useful for research and of carotenoid metabolism of egg-laying hens and suggests greater attention should be paid to *Euglena* feeding when developing value-added eggs.

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Analysis on the excited state properties of *cis*- β -apo-8'-carotenal using femtosecond time-resolved absorption spectroscopy

Kota Horiuchi¹, Chiasa Uragami¹, Taiki Inoue¹, Nao Yukihira¹, Daisuke Kosumi² and Hideki Hashimoto^{1*}

¹ Department of Applied Chemistry for Environment, School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan.

² Institute of Pulsed Power Science, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto, 860-8555 Japan

Abstract: When carotenoids absorb photons, the excitation energy is efficiently transferred to chlorophyll. Involvement of the intramolecular charge-transfer (ICT) excited state in the excitation energy-transfer (EET) process is one of the factors to enhance the EET efficiently. Carotenoids that have a carbonyl group coupled to its polyene backbone is thought to generate the ICT excited state. In this study, we investigated *cis*-isomers of β -apo-8'-carotenal that have a carbonyl group and nine conjugated C=C double bonds. The mixture of *cis-trans* isomers of β -Apo-8'-carotenal were obtained by photoisomerization of the all-*trans* isomer. Each *cis* isomer was isolated and purified from the isomeric mixture by means of HPLC. We applied femtosecond pump-probe transient absorption spectroscopy to investigate the excited state properties of *cis*- β -Apo-8'-carotenal in *n*-hexane (as a nonpolar solvent), acetone (as a polar and aprotic solvent), and methanol (as a polar and protic solvent). We found that *cis*- β -apo-8'-carotenal (13'-*cis*, 13-*cis*, 9,13'-*cis*, and 9-*cis*) generated the ICT excited state in acetone and methanol. In addition, the transient absorption spectra of the *cis*- β -Apo-8'-carotenal were redshifted than that of the all-*trans* isomer. The results of global and target analysis of the 13-*cis* isomer in any solvents show the shortest lifetime of S₁/ICT state in the *cis*- β -Apo-8'-carotenal.

1. Introduction

Carotenoids have a light-harvesting function in the primary process of photosynthesis. They absorb the energy of sunlight in blue-green spectral region where chlorophylls have scant absorption [1, 2]. The absorbed energy is transferred to chlorophylls. In addition, carotenoids have a photoprotective function that protects photosynthetic organisms from harmful photooxidative processes [3]. To perform this function, 15-*cis* isomer of carotenoid is selectively bound to the reaction center in purple photosynthetic bacteria [4]. When carotenoids absorb photons, they are excited up to the second excited singlet state (S₂ state) from the singlet ground state (S₀ state). The transition from the S₀ state to the first excited singlet state (S₁ state) is forbidden because of symmetry reason [5]. The relaxation pathways from the singlet excited states of carotenoids are key issues to understand the exact mechanisms of excitation energy transfer in photosynthetic light-harvesting. An excited energy-state called intramolecular charge transfer (ICT) excited state is very important to improve the efficiency of excitation energy-transfer from carotenoid to chlorophyll [5]. The energy level of the ICT excited state is postulated to be nearby or coupled to the S₁ state, resulting in a state usually denoted as S₁/ICT state [6-8]. Although a number of studies have

already been carried out to clarify the origin of the ICT excited state, there still remains contradictions for the interpretation [5, 6].

All-*trans*- β -apo-8'-carotenal has a carbonyl group coupled to its polyene backbone and has nine conjugated C=C double bonds (see **Figure 1** for its chemical structure). It is confirmed that all-*trans*- β -apo-8'-carotenal generates the ICT excited state following photoexcitation when it is dissolved in polar organic solvents [5, 6]. The *cis* isomers of β -apo-8'-carotenal: 13'-*cis*, 15-*cis*, 13-*cis*, 9,13'-*cis*, 13,13'-*cis*, 9,13-*cis*, 9-*cis*, and 7-*cis* (see **Figure 2** for their chemical structures) can be obtained by photoisomerization. Chemical structures of the *cis*-isomers isolated using high-performance liquid chromatography (HPLC) were already determined by nuclear magnetic resonance (NMR) spectroscopy [1]. Steady-state absorption spectra of the *cis*-isomers show two characteristic behaviors compared to that of all-*trans*. They show blue-shifted absorption than all-*trans*, and show *cis*-peaks around 330 nm. Therefore, each *cis*-isomer should have its own intrinsic characteristics in terms of its excited

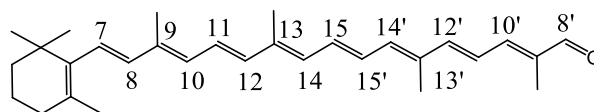


Figure 1. Chemical structure of all-*trans*- β -apo-8'-carotenal.

* Corresponding author
e-mail: hideki-hassy@kwansei.ac.jp

energy states. In this study, we have applied femtosecond time-resolved absorption spectroscopy to the *cis*-isomers of β -apo-8'-carotenal to investigate the relaxation pathways in their excited electronic states.

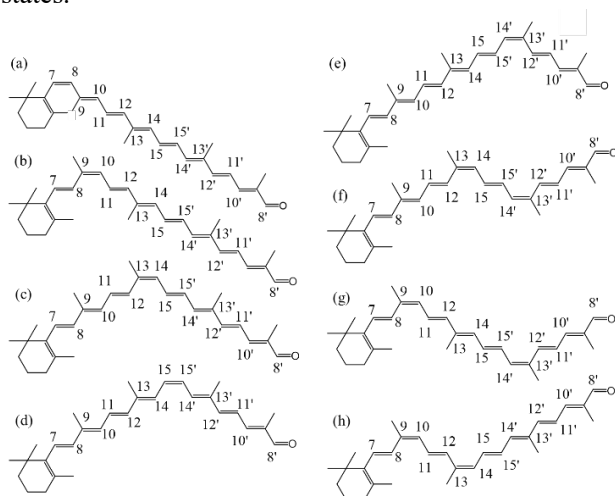


Figure 2. The chemical structure of *cis*- β -Apo-8'-carotenal. (a) 7-*cis*, (b) 9-*cis*, (c) 13-*cis*, (d) 15-*cis*, (e) 13'-*cis*, (f) 13,13'-*cis*, (g) 9,13'-*cis*, (h) 9,13-*cis*.

2. Experimental

2.1 Photoisomerization of all-trans- β -apo-8'-carotenal and isolation and purification of the *cis*-isomers by HPLC

All-*trans*- β -apo-8'-carotenal (purity $\geq 96.0\%$) was purchased from SIGMA and used as it received. Light from W-halogen lamp (50 W) was irradiated for 3 hours to *n*-hexane solution of the all-*trans* isomer to generate the *cis*-isomers using iodine as a photocatalyst. The *cis*-isomers were isolated and purified by the use of a set of HPLC (JASCO PU-4180 and JASCO UV-4075). Condition: Column, Inertsil SIL-100A 7.6×250 mm, $5\ \mu\text{m}$; Eluent, diethyl ether/*n*-hexane 4/96 v/v; Flow rate, 2.5 mL/min; Detection wavelength, 450 nm. In the separation of the 13'-*cis* and 15-*cis* isomers, we used another column (LiChrosorb Si 60, $5\ \mu\text{m}$). The purified *cis*-isomers (13'-*cis*, 13-*cis*, 9,13'-*cis*, and 9-*cis*) were dissolved in three different solvents. *n*-Hexane was chosen as a nonpolar solvent, while methanol and acetone were chosen as polar solvents, although methanol is protic, while acetone is aprotic. Steady-state absorption spectra were recorded using a JASCO V670 spectrophotometer at room temperature.

2.2 Femtosecond time-resolved absorption spectroscopy

Details of the femtosecond time-resolved absorption spectroscopic measurements with a pump-probe technique is described elsewhere [9]. The excitation laser pulse was generated by an optical parametric amplifier (Spectra Physics, OPA-800CF). Each isomer in three different kind of solvents was excited at 490 nm that corresponds to the wavelength region of the 0-0 transition of the $S_0 \rightarrow S_2$ absorption

band. The excitation energy was set to 15 nJ/pulse, and the pulse width was approximately 130 fs. The probe pulse was the white-light continuum that spreads in the wavelength region between 436 to 738 nm and had ~ 100 fs pulse duration. Optical density of each sample solution was set to be 2.3 – 2.8 at the absorption maxima. The sample solution was placed in a quartz cuvette with 2.0 mm optical path-length. During the measurements, the sample solution was continuously stirred using a magnetic stirrer to avoid the degradation of the samples. The purity of the isomers was checked by comparison of the steady-state absorption spectra and HPLC profiles before and after the transient absorption measurements. All the measurements were performed at room temperature (20 – 23 °C). Time-resolved transient absorption spectra were analyzed by singular value decomposition (SVD) that was followed by Global and Target analysis. This analysis was performed using a Glotaran software package [10, 11]

3. Results and Discussions

3.1 HPLC profiles and steady-state absorption spectra of the isomers of β -apo-8'-carotenal

Figure 3 shows the chromatograms of the *cis-trans* isomeric mixture of β -Apo-8'-carotenal. Each peak can be assigned by referring to the results of the previous report [1]. Peak 1 in **Figure 3a** is well separated in the chromatogram recorded with another column (see **Figure 3b**). Hence, we can successfully separate the 13'-*cis* and 15-*cis* isomers.

Table 1 summarizes the assignment of the peaks in the HPLC profile together with the absorption maxima of the isomers used in the transient absorption measurements.

3.2 Femtosecond time-resolved absorption measurements followed by global and target analysis

Figure 6 shows the steady-state absorption spectra of the isomers of β -Apo-8'-carotenal recorded at room temperature. All the *cis* isomers show blue-shifted absorption and *cis*-peak at 331 nm as compared to the all-*trans* isomer. **Figure 4** shows the transient absorption spectra of all-*trans*- β -Apo-8'-carotenal. To avoid interference due to the initial vibrational relaxation in the S_2 state, all the samples were excited at 490 nm (0-0 transition position). Relatively sharp transient absorption band at 550 nm observed in *n*-hexane shows the $S_1 \rightarrow S_n$ transition. A positive shoulder observed around 520 nm in *n*-hexane is ascribable to the S^* state, although its origin is still controversial [12]. In polar solvent, the generation of the ICT excited state can be identified by a broad transient absorption signal from 600 nm to 700 nm as has previously been reported [5].

We applied sequential model as illustrated in **Figure 5**, which is composed of three components, i.e. stimulated Raman and stimulated emission (internal conversion from $S_2 \rightarrow S_1$), vibrational relaxation in the S_1 or S_1 /ICT excited state, and relaxation to the ground

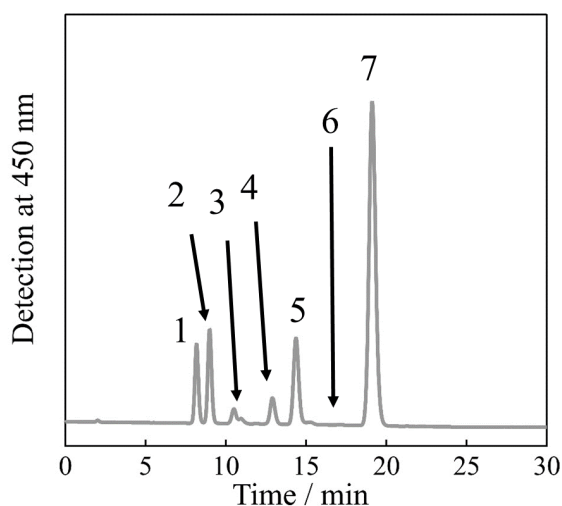


Figure 3a. The HPLC profile detected at 450 nm of the isomeric mixture of β -Apo-8'-carotenal with Inertsil SIL-100A as a fixed phase and diethyl ether/*n*-hexane 4/96 (v/v) as a mobile phase (flow rate, 2.0 mL/min).

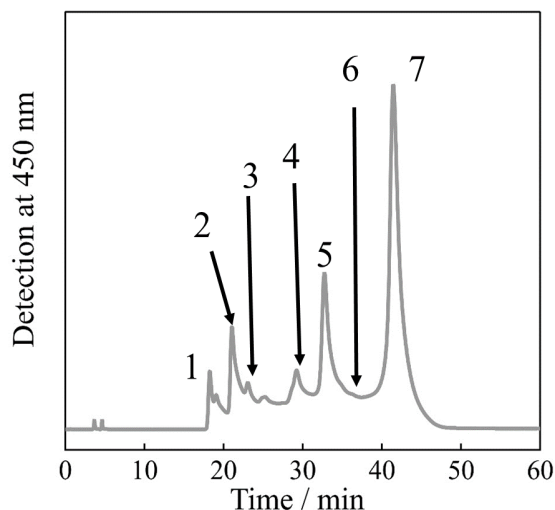


Figure 3b. The HPLC profile detected at 450 nm of the isomeric mixture of β -Apo-8'-carotenal with Lichrosorb Si 60 as a fixed phase and diethyl ether/*n*-hexane 4/96 (v/v) as a mobile phase (flow rate, 2.0 mL/min).

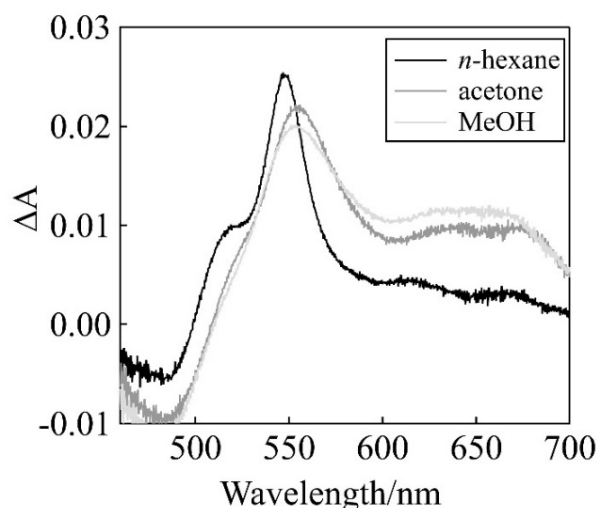


Figure 4. Transient absorption spectra of all-*trans*- β -apo-8'-carotenal in *n*-hexane, acetone, and MeOH measured at 1.0 ps after excitation at 490 nm at room temperature.

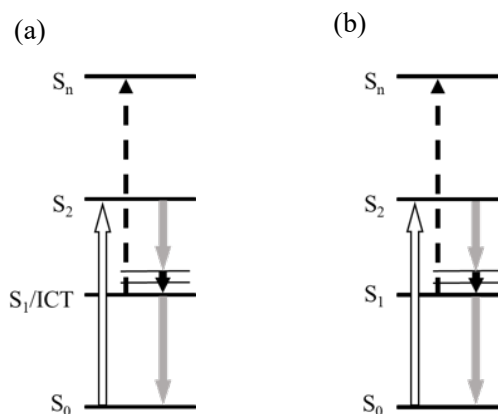


Figure 5. Kinetic model that shows the relaxation of β -apo-8'-carotenal in (a) nonpolar and (b) polar solvents. White arrows show the $S_0 \rightarrow S_2$ transition, broken arrows show transient absorption from S_1 or S_1/ICT state, gray arrows show the internal conversion from the excited state, and black arrows shows the vibrational relaxation.

Table 1. The assignment of the peaks in the chromatogram shown in **Figure 3a** and the wavelengths of the absorption maxima of the isomers of β -Apo-8'-carotenal in *n*-hexane.

Peak number	Isomer	$S_0 \rightarrow S_2$ 0-0 transition	$S_0 \rightarrow S_2$ 0-1 transition	$S_0 \rightarrow S_2$ 0-2 transition	<i>Cis</i> -peak
1	13'- <i>cis</i>	470 nm	447 nm	427 nm	331 nm
2	13- <i>cis</i>	473 nm	448 nm	430 nm	331 nm
3	9,13'- <i>cis</i>	468 nm	444 nm	426 nm	331 nm
5	9- <i>cis</i>	478 nm	451 nm	431 nm	331 nm
7	all- <i>trans</i>	481 nm	455 nm	434 nm	—

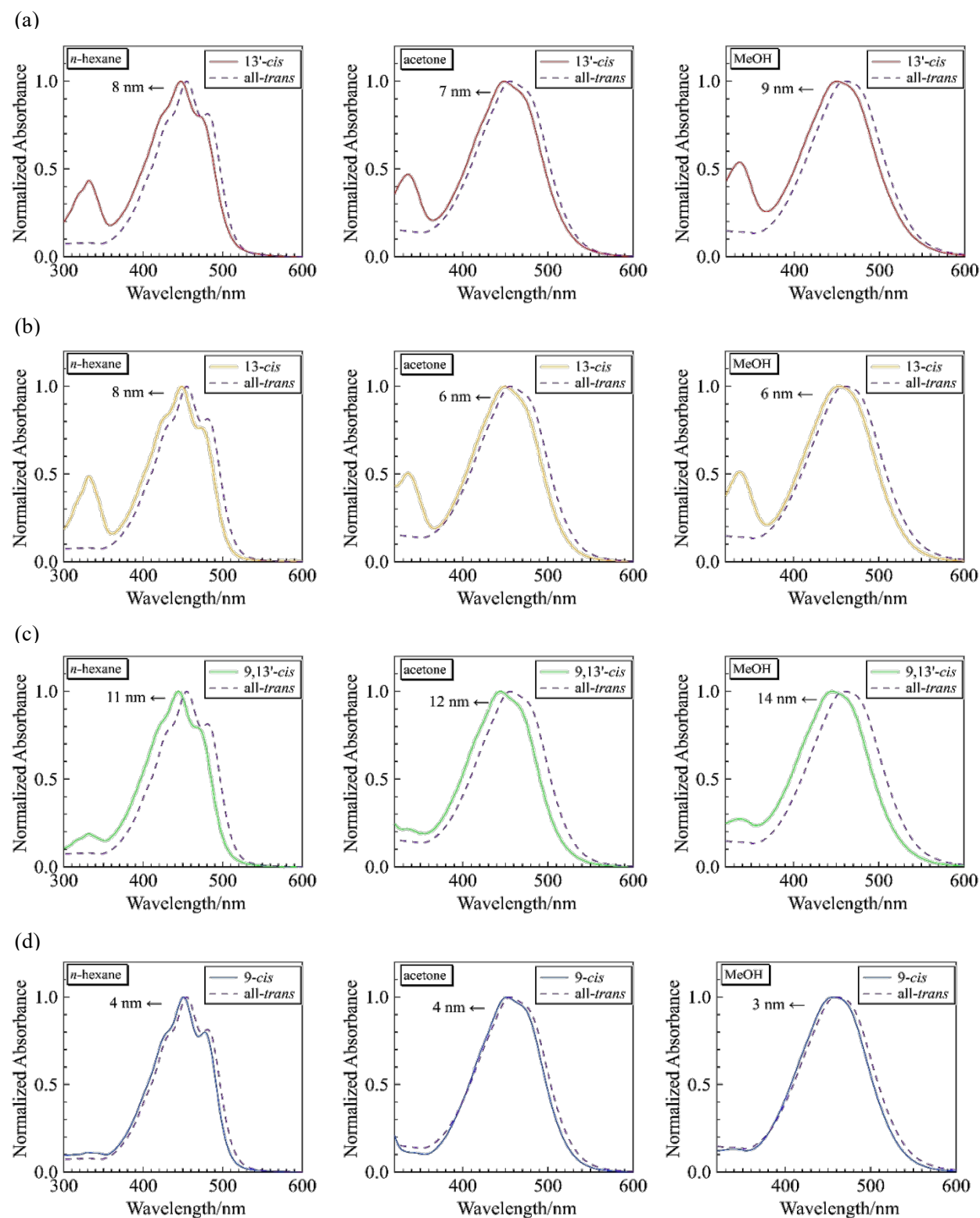


Figure 6. Steady-state absorption spectra of the (a) 13'-cis (solid line), (b) 13-cis (solid line), (c) 9,13'-cis (solid line), and (d) 9-cis (solid line) isomers together with those of the all-trans isomer (broken line) of β -apo-8'-carotenal in *n*-hexane, acetone, and methanol recorded at room temperature. The arrows show the wavelength shift of the absorption maxima with reference to all-trans.

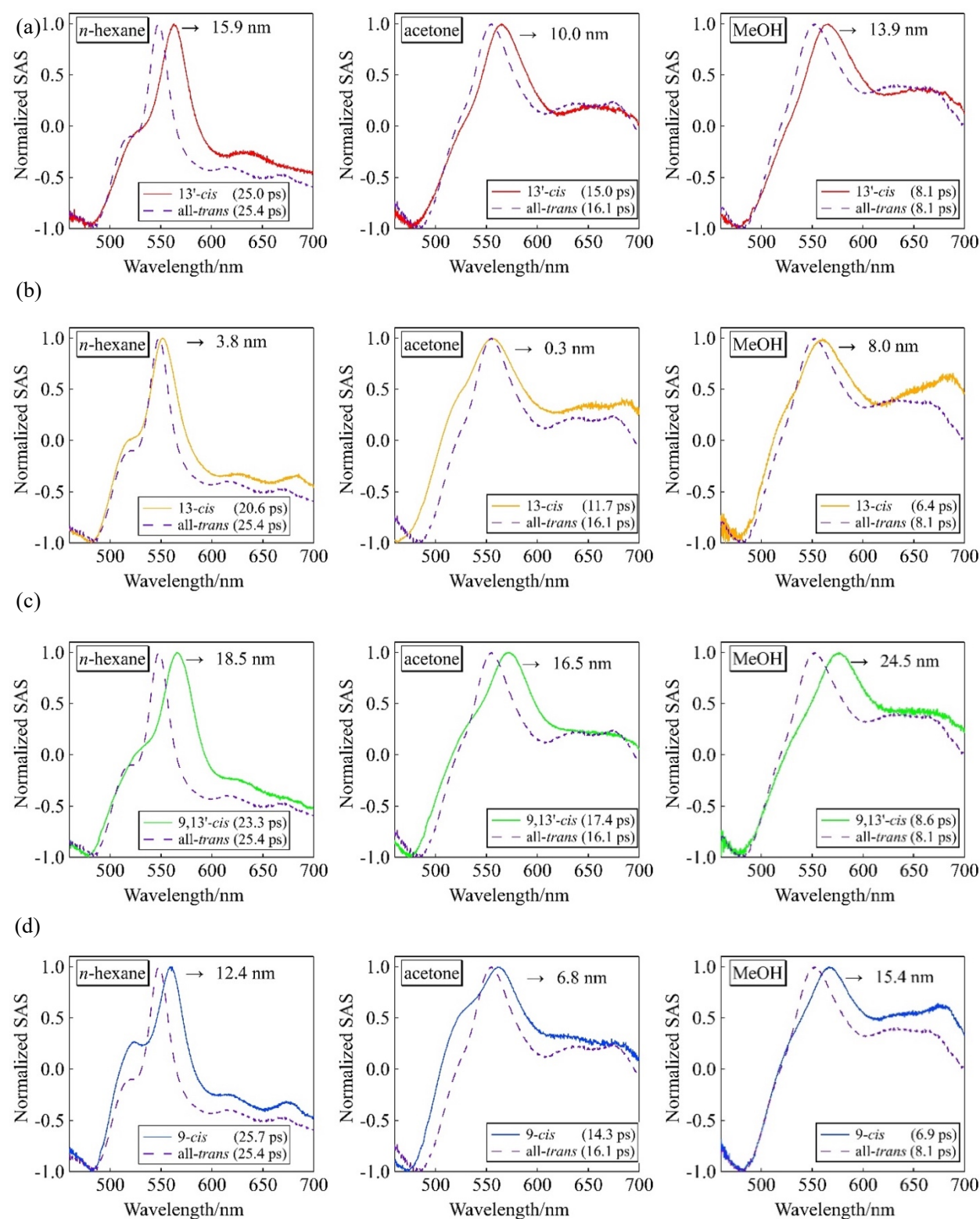


Figure 7. Normalized species associated spectra (SAS) of the S_1/ICT state generated from the (a) 13'-cis (solid line), (b) 13-cis (solid line), (c) 9,13'-cis (solid line), and (d) 9-cis (solid line) isomers together with those of the all-trans isomer (broken line) of β -apo-8'-carotenal in *n*-hexane, acetone, and methanol. The arrows show the wavelength shift with reference to the all-trans isomer.

state. **Figure 7** shows the species associated spectra (SAS) obtained using global and target analysis of the whole set of time-resolved absorption spectra of the isomers of β -apo-8'-carotenal in three different solvents. It is known that the polarity of the solvent affects the lifetime of the S_1 /ICT state of carbonyl carotenoids [13, 14]. In fact, the S_1 /ICT lifetime is in the order, methanol < acetone < *n*-hexane for all the isomers. The results of all the *cis*-isomers in polar solvents (acetone and methanol) show broad transient absorption bands between 600 nm to 700 nm wavelength region. This is a good indication that the *cis*-isomers also generate the ICT excited state, since similar transient absorption band is already found in all-*trans*. It is also noteworthy that the SAS of all the *cis*-isomers are redshifted compared to that of the all-*trans* in any solvents. The lifetimes of the S_1 (in *n*-hexane) or S_1 /ICT (in acetone or MeOH) species generated from the 13'-*cis*, 9,13'-*cis* and 9-*cis* isomers are similar to those of the all-*trans*, while those from the 13-*cis* isomer show marked decrease in any solvents. It is reported that the S_1 lifetime of carotenoids has a relationship with the energy gap between the S_1 and S_0 states, which can be described by the equation shown below.

$$k_{ic}(S_1 \rightarrow S_0) = \frac{C^2(2\pi)^{1/2}}{\hbar(\Delta E \hbar \omega_M)^{1/2}} \exp\left(-\gamma \frac{\Delta E}{\hbar \omega_M}\right),$$

where $k_{ic}(S_1 \rightarrow S_0)$ is the internal conversion rate of the radiationless deactivation from the S_1 state, which corresponds to the reciprocal of the S_1 lifetime. ΔE is energy difference between the S_1 and S_0 states. According to this equation, it is clear that the shorter the $S_1 \rightarrow S_0$ lifetime, the smaller the energy difference. If we apply the same idea to the case of the 13-*cis* isomer of β -Apo-8'-carotenal, we can assume that the energy difference between the S_1 or S_1 /ICT and S_0 states of the 13-*cis* isomer is the smallest among all the isomers examined in this study. The lifetimes of S_1 or S_1 /ICT species of isomeric β -apo-8'-carotenal are summarized in **Table 2**.

Spectral features of the transient absorption bands generated from the isomers are summarized as follow.

Table 2. The lifetimes of the S_1 or S_1 /ICT species of isomeric β -apo-8'-carotenal determined by global and target analysis.

Isomer	<i>n</i> -hexane	acetone	MeOH
all- <i>trans</i>	25.4 ps	16.1 ps	8.1 ps
13'- <i>cis</i>	25.0 ps	15.0 ps	8.1 ps
13- <i>cis</i>	20.6 ps	11.7 ps	6.4 ps
9,13'- <i>cis</i>	23.3 ps	17.4 ps	8.6 ps
9- <i>cis</i>	25.7 ps	14.3 ps	6.9 ps

n-Hexane solution

Despite the non-polarity of the solvent, all the isomers show small ICT-like transient absorption bands in the 600 – 700 nm wavelength region. The all-*trans*, 13-*cis*, 9,13'-*cis*, and 9-*cis* isomers show two peaks at 618 and 671 nm, 630 and 685 nm, 630 and 675 nm, and 619 and 675 nm, respectively, but 13'-*cis* shows only one peak in this wavelength region. These two small ICT-like absorption bands of the 13'-*cis*, 13-*cis*, and 9,13'-*cis* are red-shifted compared to those of the all-*trans* isomer. The $S_1 \rightarrow S_n$ absorption band around 563 nm of the 13'-*cis* and 9,13'-*cis* are also red-shifted from that of the all-*trans* isomer. It should be noted that the 9-*cis* isomer shows intense S^* -like absorption feature at 522 nm.

Acetone solution

The 13-*cis* isomer shows two intense ICT absorption bands at 653 and 689 nm. The ICT absorption band of the 13'-*cis*, 13-*cis*, and 9,13'-*cis* are red-shifted from that of the all-*trans* isomer. The $S_1 \rightarrow S_n$ absorption band at 573 nm of the 9,13'-*cis* isomer is also red-shifted from that of the all-*trans* isomer. The 13-*cis* and 9-*cis* isomers show marked S^* -like absorption band around 523 nm. The bleaching signals of all the *cis*-isomers are blue-shifted from that of the all-*trans* isomer. This is because the $S_0 \rightarrow S_2$ absorption bands of the *cis*-isomers are always blue-shifted from that of the all-*trans* isomer.

Methanol solution

The 13-*cis* and 9-*cis* isomers show highly intense ICT absorption bands at 685 and 677 nm. The ICT absorption band of the 13-*cis* isomer shows marked red-shift compared to that of the all-*trans* isomer. The marked red-shift of the $S_1 \rightarrow S_n$ absorption band from the all-*trans* isomer can also be seen for the 9,13'-*cis* isomer.

The wavelength of S_1 or S_1 /ICT $\rightarrow S_n$ absorption bands and the wavelength shift with reference to the all-*trans* isomer are summarized in **Table 3**.

4. Conclusion

The 13'-*cis*, 13-*cis*, 9,13'-*cis*, and 9-*cis*- β -apo-8'-carotenal were isolated and purified with an Inertsil SIL-100A column. We obtained the transient absorption spectra of the *cis*-isomers dissolved in *n*-hexane, acetone, and methanol excited at 490 nm. Species associated spectra (SAS) were determined using global and target analysis. The lifetime of S_1 or S_1 /ICT state was in the order; methanol < acetone < *n*-hexane for all the isomers. The S_1 or S_1 /ICT lifetime of the 13-*cis* isomer was the shortest among all the isomers examined. We assume that the energy difference between the S_1 or S_1 /ICT and S_0 states of the 13-*cis* isomer is the smallest based on the consideration using the energy gap law.

Table 3. The wavelength of S_1 or $S_1/ICT \rightarrow S_n$ absorption band of β -Apo-8'-carotenal. The parenthesis indicates the wavelength shift with reference to the all-*trans* isomer.

Isomer	<i>n</i> -hexane	acetone	MeOH
all- <i>trans</i>	547.4 nm (–)	555.1 nm (–)	551.8 nm (–)
13'- <i>cis</i>	563.0 nm (+ 15.9 nm)	565.1 nm (+ 10.0 nm)	565.7 nm (+ 13.9 nm)
13- <i>cis</i>	551.2 nm (+ 3.8 nm)	555.4 nm (+ 0.3 nm)	559.8 nm (+ 8.0 nm)
9,13'- <i>cis</i>	565.7 nm (+ 18.5 nm)	571.6 nm (+ 16.5 nm)	576.3 nm (+ 24.5 nm)
9- <i>cis</i>	559.8 nm (+ 12.4 nm)	561.9 nm (+ 6.8 nm)	567.2 nm (+ 15.4 nm)

The SAS of β -Apo-8'-carotenal show three characteristic features of transient absorption bands: (1) S^* -like transient absorption band around 520 nm. (2) $S_1 \rightarrow S_n$ transient absorption band around 550 nm. (3) ICT transient absorption band around 600 – 700 nm. The intensity of the S^* -like absorption bands are enhanced in 13-*cis* isomer (in acetone) and 9-*cis* isomer (in *n*-hexane and acetone). The $S_1 \rightarrow S_n$ absorption bands of the *cis*-isomer are redshifted from those of the all-*trans* isomer in any solvents. Especially, those of 9,13'-*cis* isomer are the most redshifted. We are planning to investigate whether this large redshift is common to other di-*cis* isomers. The intensities of the ICT absorption bands were in the order of methanol > acetone > *n*-hexane. Despite the non-polarity of the solvent, the 13-*cis*, 9,13'-*cis*, and 9-*cis* isomers show two small peaks and the 13'-*cis* isomer shows one small peak in the ICT absorption region in *n*-hexane. In aprotic solvent acetone, the all-*trans* and 13-*cis* isomers show two peaks in this spectral region. In protic solvent methanol, the 13-*cis* and 9-*cis* isomers show highly intense ICT absorption bands. In all three solvents the ICT absorption bands of the 13'-*cis*, 13-*cis*, and 9,13'-*cis* are redshifted from those of the all-*trans* isomer.

As the future perspective, we are planning to investigate the transient absorption spectra of the other *cis*-isomers (15-*cis*, 13,13-*cis*, 9,13-*cis*, 7-*cis*) in *n*-hexane, acetone, and methanol. Also, quantum chemical calculations will be performed to interpret the transient absorption data.

Acknowledgements

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Generation of Geometric Isomers of β -Apo-8'-Carotenal and Investigation of Their Optical Properties Using Stark Spectroscopy

Taiki Inoue¹, Shota Seto², Tomoko Horibe², Kota Horiuchi¹, Chiasa Uragami¹ and Hideki Hashimoto^{1*}

¹ School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1338, Japan.

² Fuji Chemical Industries Co. Ltd., 55 Yokohoenji, Kamiichi-cho, Nakaniikawa, Toyama 930-0397, Japan.

Abstract: β -apo-8'-carotenal is a carotenoid that contains an aldehyde group attached to its conjugated polyene system. It is expected to have an ICT (Intramolecular Charge-Transfer) character in the excited state. In this study, we generated various kinds of geometric isomers of β -apo-8'-carotenal via photoisomerization of all-*trans* isomer. All-*trans*, 9-*cis*, and 13-*cis* isomers were separated and purified using HPLC. Investigation of their electronic states including the static dipole-moment change upon photoexcitation ($\Delta\mu$), which indicates the strength of ICT character, was attempted by analyzing spectra obtained from Stark (electroabsorption) spectroscopy. As a result, we have determined that there is high possibility that difference in geometric structure affect more on the direction of $\Delta\mu$ with respect to the transition dipolemoment, rather than its magnitude.

1. Introduction

Carotenoids are natural pigments that serve important roles in the primary process of natural photosynthesis. They absorb blue to green region of sunlight, and transfer the captured energy to surrounding chlorophylls. The intramolecular charge-transfer (ICT) excited energy-state plays an important role in realizing highly efficient energy transfer from carotenoid to chlorophyll. It is claimed that the large S_1 /ICT- S_0 transition dipole moment enables carotenoid to efficiently transfer the energy to chlorophyll [1]. The strength of ICT character can be estimated by measuring the magnitude of static dipole-moment change upon photoexcitation ($|\Delta\mu|$) and this value is obtained from the analysis of Stark spectra.

The relationship between the functional group in a carotenoid and its ICT character has been studied [2,3]. Keto-carbonyl group, allene moiety, and ylidenebutenolide ring are considered to be important functional groups that contribute to increase the strength of ICT character [2,3]. For example, peridinin, which contains all of these functional groups listed above, is known to have the highest $|\Delta\mu|$ in all carotenoids, which is reported to be more than 20 Debye [3].

On the other hand, the relationship between the geometric structure of a carotenoid and its ICT character has not yet been studied much. In this research, we have measured Stark absorption spectra of all-*trans*, 9-*cis*, and 13-*cis* isomers of β -apo-8'-carotenal (Figure 1) to investigate this particular relationship.

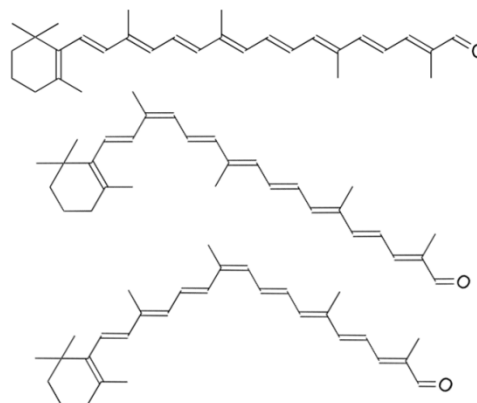


Figure 1. Chemical structure of all-*trans* (up), 9-*cis* (middle), and 13-*cis* (down) β -apo-8'-carotenal.

2. Experimental

2.1 Preparation of the isomers of β -apo-8'-carotenal

All-*trans*- β -apo-8'-carotenal was purchased from SIGMA-Aldrich and dissolved in *n*-hexane. Iodine was added to the solution as a catalyst and the solution was irradiated with light from W-halogen lamp to conduct photo-isomerization. The isomers were separated and purified using HPLC (Column: Inertsil SIL 100A 5 μ m, Flow rate: 2.0 mL/min, Eluent: 4% diethylether/hexane (v/v), Temperature: 23 $^{\circ}$ C). After the purification, the solvent was evaporated and the samples were stored in an inert atmosphere at -30° C until use.

2.3 Preparation of the sample cell for Stark absorption measurements

Comb-shaped aluminum electrodes were vapor-deposited on the surface of glass substrates. The distances between two electrodes were determined to be between 40 to 50 μ m. Each isomer was dissolved in

* Corresponding author
e-mail: hideki-hassy@kwansei.ac.jp

acetone, and diluted until its maximum absorbance to be between OD 70 to 100, respectively. Also, 10% methylmethacrylate polymer (PMMA, Wako Pure Chemical Industries Ltd.)/acetone (w/v) solution was prepared. Each isomer solution and PMMA solution was mixed with the ratio of 1:1. The mixed solution was dropped on top of the electrodes and dried under ambient condition.

2.3 Steady-state absorption measurements

Steady-state absorption spectra of the samples installed in the Stark absorption cells were recorded using a JASCO V-670 UV-vis spectrophotometer at room temperature.

2.4 Stark absorption measurements

The block diagram of the measurement apparatus is shown in **Figure 2**. The details of the set-up of Stark absorption measurements were described elsewhere [4, 5]. 150-W Xenon arc-lamp (Hamamatsu, C7535) was used as a light source. Linearly-polarized monochromatic light was generated by passing through a monochromator (ZOLIX, Omni-λ150) and a polarizing plate. It was irradiated to the sample cell, and the transmitted light intensity was detected by a silicon photodiode detector (Hamamatsu, S1336-8BQ). During the measurement, external electric field was applied between the electrodes in the sample cell. 491 Hz sinusoidal AC signal was produced using a function generator (NF, E-1201A). This signal was amplified using a bi-polar power amplifier (NF, 4305) and applied to the electrodes. The electric field between electrodes was varied from 40 to 50 kV/cm, depending on the distance between them. The $2f$ component of the changes of the transmitted light intensity (ΔI) induced by the external electric field was selectively amplified by using a dual-phase lock-in amplifier (NF, 5610B). On the other hand, the DC component of the detected signal was measured by a digital multimeter (Fluke, 45). The absorbance change (ΔA) caused by an external electric field was calculated using the following equation.

$$\Delta A = -\log\left(\frac{I + \Delta I}{I}\right).$$

Here, I is the transmitted light intensity.

The Stark absorption measurement was conducted in the wavelength region from 400 to 650 nm. The angle between the electric field vector of the incident light and that of the externally applied electric field was set to 0° , 30° , 54.7° , 70° , and 90° . By measuring spectra with different polarization angles, we could determine the angle ($\theta_{\Delta\mu}$) between the transition dipole moment vector ($\mathbf{M}$) and $\Delta\mu$.

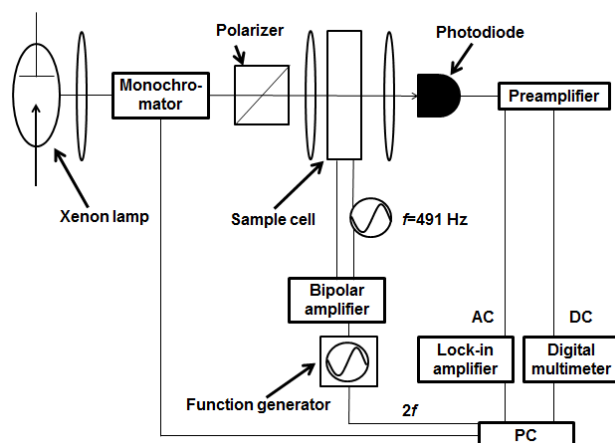


Figure 2. Schematic drawing of the set-up of Stark absorption spectroscopy.

2.5 Analysis of Stark absorption spectra

Liptay's equation (a) was used to analyze the Stark absorption spectra [6]. This equation shows that the magnitude of field induced absorbance change ($\Delta A(\nu)$) is represented by a linear combination of zeroth-, first-, and second-order derivatives of the steady-state absorption spectrum.

$$\Delta A(\nu) = \left[A_\chi \cdot A(\nu) + B_\chi \cdot \frac{\nu}{15h} \cdot \frac{d(A(\nu)/\nu)}{d\nu} + C_\chi \cdot \frac{\nu}{30h^2} \cdot \frac{d^2(A(\nu)/\nu)}{d\nu^2} \right] \cdot (fE)^2 \quad (a)$$

Here, χ is the angle between the direction of external electric field and the direction of polarization of the transmitted light, h is Planck's constant, ν is wavenumber of light, and E is external electric field, respectively. f is a local electric field correction factor, and here, we assume that $f \approx 1.1$ for PMMA [7]. A_χ , B_χ , and C_χ can be expressed as follows.

$$A_\chi = \frac{D}{3} + (3 \cos^2 \chi - 1) \cdot \frac{E}{30} \quad (b)$$

$$B_\chi = 5F + (3 \cos^2 \chi - 1) \cdot G \quad (c)$$

$$C_\chi = 5H + (3 \cos^2 \chi - 1) \cdot I \quad (d)$$

$$D = \frac{1}{|\mathbf{M}|^2} \sum_{ij} (X_{ii}X_{jj} + M_i Y_{ijj}) \quad (e)$$

$$E = \frac{3}{|\mathbf{M}|^2} \sum_{ij} (X_{ij}^2 + X_{ij}X_{ji} + M_i Y_{jij} + M_i Y_{jji})$$

$$-2D \quad (f)$$

$$F = \frac{1}{2} \text{Tr}(\Delta\alpha) + \frac{2}{|\mathbf{M}|^2} \left(\sum_{ij} M_i X_{ij} \right) \cdot \Delta\boldsymbol{\mu} \quad (\text{g})$$

$$G = \frac{3}{2} \mathbf{m} \cdot \Delta\alpha \cdot \mathbf{m} - \frac{1}{2} \text{Tr}(\Delta\alpha) + \frac{3}{2} \cdot \frac{2}{|\mathbf{M}|^2} \left[\sum_{ij} (M_i X_{ji} + M_i X_{jj}) - 3 \left(\sum_{ij} M_i X_{ij} \right) \cdot \Delta\boldsymbol{\mu} \right] \quad (\text{h})$$

$$H = |\Delta\boldsymbol{\mu}|^2 \quad (\text{i})$$

$$I = 3 \cdot (\mathbf{m} \cdot \Delta\boldsymbol{\mu})^2 - |\Delta\boldsymbol{\mu}|^2 \quad (\text{j})$$

Here, $\mathbf{M}$ represents the transition dipole moment vector, $\mathbf{m}$ represents the unit vector of $\mathbf{M}$, X represents the polarizability tensor of transition dipole moment, Y represents the hyperpolarizability tensor of transition dipole moment, $\Delta\alpha$ represents the changes of polarizability upon photoexcitation, and $\Delta\boldsymbol{\mu}$ represents the changes of static dipole moment upon photoexcitation, respectively.

3. Results and Discussion

Figure 3 shows the result of HPLC analysis after the isomerization of β -apo-8'-carotenal. Identification of each peak was done by previous study [8].

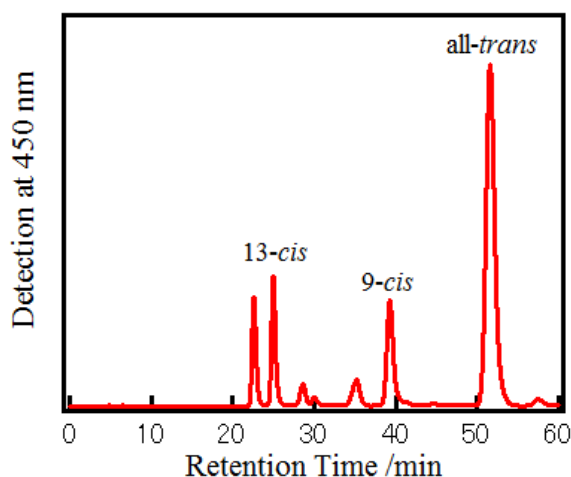


Figure 3. HPLC after photo-isomerization. Peaks belong to all-*trans*, 9-*cis*, and 13-*cis* isomers are labeled for clarity.

Figure 4 shows the normalized steady-state absorption spectrum of all-*trans*- β -apo-8'-carotenal measured at room temperature. The observed spectrum could be decomposed into 6 Gaussian profiles. Here, Band 1, which is the band with the lowest energy, is considered to be an absorption band of S_0 to S_2 /ICT transition. The other bands are considered to be absorption bands of electronic transitions from S_0 to different vibrational levels inherent to S_2 .

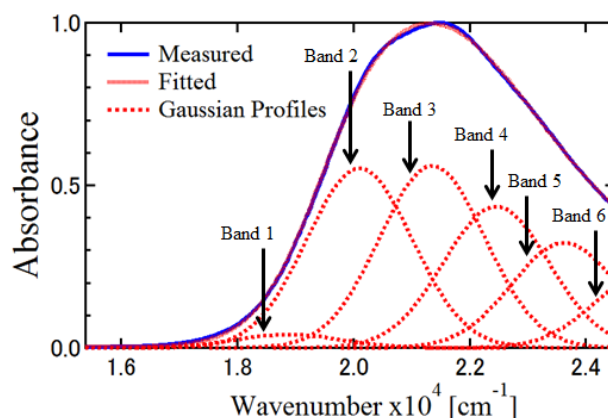


Figure 4. Normalized steady-state absorption spectrum and the result of spectral fitting of all-*trans*- β -apo-8'-carotenal measured at room temperature.

Figure 5 shows the normalized steady-state absorption spectra of the all-*trans*, 9-*cis*, and 13-*cis* isomers of β -apo-8'-carotenal, and their Stark absorption spectra with spectral fittings. From the fittings, we could determine C_χ values against different polarization angles (χ). Band 1 should have the significant contribution of the ICT state, so we investigated its angular dependence of C_χ based on equation (d). The angular dependence of C_χ of all-*trans* isomer is shown in **Figure 6**.

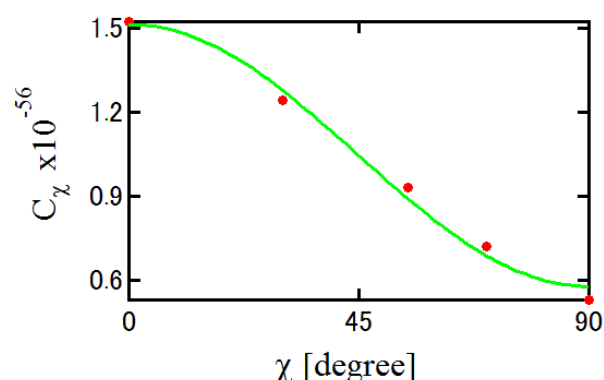


Figure 6. The angular dependence of C_χ of Band 1 in the all-*trans* isomer together with the result of fitting.

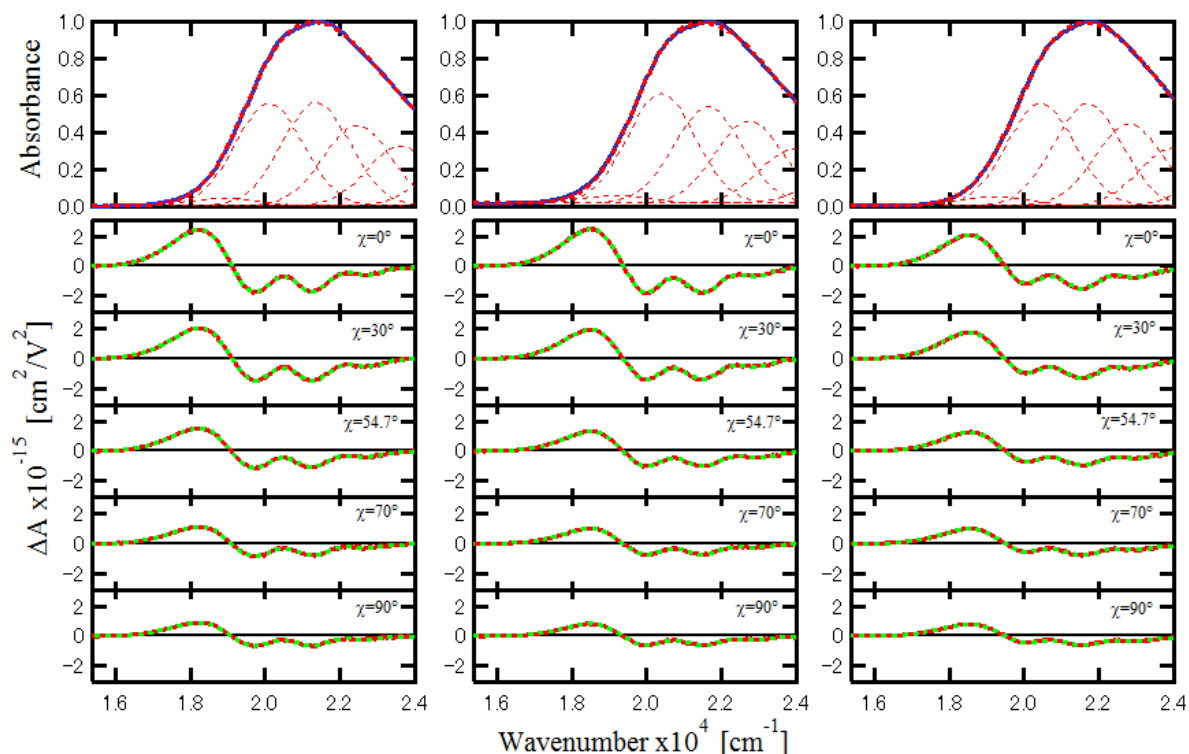


Figure 5. Normalized absorption spectra (top) and Stark absorption spectra measured at different χ of all-*trans* (left), 9-*cis* (middle), and 13-*cis* (right) isomers in PMMA at room temperature. Solid lines are the measured spectra and dotted lines are the fittings. Stark absorption spectra fittings were conducted based on equation (a).

From the fitting, we determined the value of $|\Delta\mu|$ to be 13.0 ± 0.3 Debye with $\theta_{\Delta\mu}$ of $17 \pm 7^\circ$. The same analysis was conducted for the 9-*cis* and 13-*cis* isomers. As a result, we determined $|\Delta\mu| = 12.3 \pm 0.5$ Debye and $\theta_{\Delta\mu} = 18 \pm 10^\circ$ for 9-*cis*, and $|\Delta\mu| = 12.0 \pm 0.4$ Debye and $\theta_{\Delta\mu} = 31 \pm 7^\circ$ for 13-*cis*. Illustration of **M** and $\Delta\mu$ of the all-*trans*, 9-*cis*, and 13-*cis* isomers are shown in **Figure 7**. Oxygen atom in aldehyde group is an electron acceptor, therefore, we assumed the arrow of $\Delta\mu$ points toward it.

All the above results exemplify that the geometric structure affects both $|\Delta\mu|$ and $\theta_{\Delta\mu}$. We can also find that there is a possibility that $|\Delta\mu|$ increases as $\theta_{\Delta\mu}$ approaches 0° . In the previous report, $|\Delta\mu|$ and $\theta_{\Delta\mu}$ of the peridinin and its analogue, which does not have allene moiety, were compared. The difference of $|\Delta\mu|$ was more than 4 Debye, but the difference of $\theta_{\Delta\mu}$ was only 7° [9] among the peridinin and its analogues. Therefore, it can be suggested that the geometric structure affects more on $\theta_{\Delta\mu}$ compared to $|\Delta\mu|$ and functional groups affect more on $|\Delta\mu|$ compared to $\theta_{\Delta\mu}$.

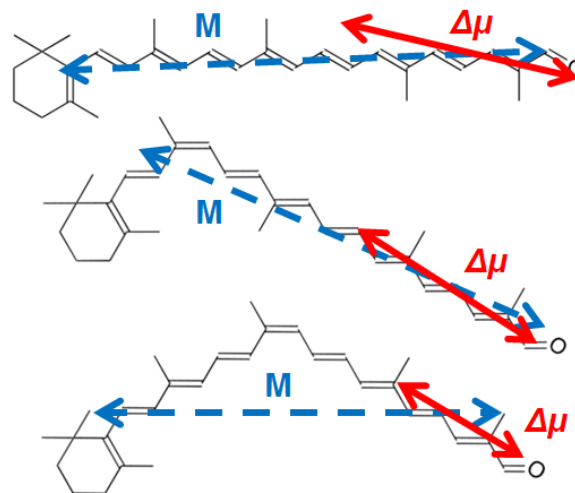


Figure 7. Illustration of **M** and $\Delta\mu$ of all-*trans* (up), 9-*cis* (middle), and 13-*cis* (down).

4. Conclusion

Steady-state and Stark absorption spectra of the all-*trans*, 9-*cis*, and 13-*cis* isomers of β -apo-8'-carotenal were recorded at room temperature. They were analyzed to determine the $|\Delta\mu|$ and $\theta_{\Delta\mu}$ values. We found that the difference in geometric structure causes a significant difference on $\theta_{\Delta\mu}$ but not much on $|\Delta\mu|$. We are going to investigate the ICT characters of

the other geometric isomers to have a good support of this idea. Attempt to measure the Stark absorption in liquid nitrogen temperature to obtain more accurate data with well resolved vibrational structures of absorption is now under progress.

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Excitation energy transfer dynamics of phycobilisome from *Thermosynechococcus vulcanus*

Keisuke Kawakami^{1*}, Hiroki Serikawa², Yuma Hirota², Yuhei O Tahara³, Junko Shiomi³, Makoto Miyata³, Nobuo Kamiya¹ and Daisuke Kosumi^{4*}

¹The OCU Advanced Research Institute for Natural Science & Technology (OCARINA), Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka City, Osaka, Japan.

²Department of Science, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto, 860-8555 Japan.

³Department of Chemistry, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585, Japan.

⁴Institute of Pulsed Power Science, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto, 860-8555 Japan.

Abstract: Cyanobacterial photosynthetic systems capture sunlight using pigment-protein megacomplexes, phycobilisomes (PBS), and the energy is subsequently transferred to water splitting protein complex, photosystem II (PSII), to produce electrochemical energy. In the present study, we isolated intact PBS from a thermophilic cyanobacterium, *Thermosynechococcus vulcanus*, and performed picosecond time-resolved fluorescence spectroscopy on the isolated PBS to reveal excitation energy transfer dynamics in PBS. The fluorescence kinetics excited at 570 nm, which corresponds to the phycocyanin (PC) absorption band, demonstrated excitation energy transfer from the PC to the allophycocyanin (APC) core.

1. Introduction

Plants and algae absorb sunlight energy and split water molecules, releasing oxygen molecules necessary for the growth and development of aerobic organisms. The amount of solar radiation, which is one of the key requirements for photosynthesis, always changes considerably. When photosynthetic organisms undergo photoinhibition under high light irradiation conditions, they regulate the dissipation of excess excitation energy using light-harvesting antenna proteins [1,2]. Phycobilisome (PBS) is one of the pigment-protein megacomplexes that efficiently capture light and supply energy to the photosynthetic membrane proteins (photosystem I and II; PSI and PSII) in order to convert light energy to chemical potential. PBS is mainly composed of a number of allophycocyanin (APC) and phycocyanin (PC) pigment-protein complexes, as shown in **Figure 1** and absorb light in the spectral region ranging from 500 nm to 700 nm [3-5]. The detailed crystal structures of the isolated PC and APC protein complexes have been reported [6] and their photophysical properties have been clarified by various spectroscopic methods [7-9]. However, it has not been elucidated in detailed how PBS and PSII (and PSI) interact to each other [4].

In the present study, we isolated intact PBS from a thermophilic cyanobacterium, *Thermosynechococcus*

vulcanus (*T. vulcanus*), and performed picosecond (ps) time-resolved fluorescence spectroscopy on the isolated PBS to reveal energy transfer dynamics.

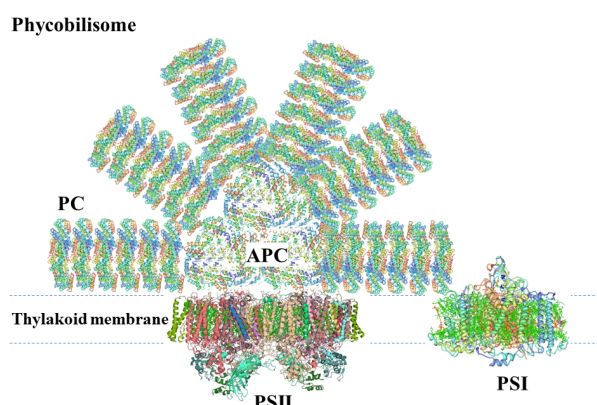


Figure 1. A schematic supercomplex model of PBS-PSII-PSI association.

2. Experimental

2.1 Sample preparation

T. vulcanus cells (5.0 L flasks × 6 pieces) were cultured in phosphate medium [10,11]. Obtained cells were disrupted by lysozyme treatment, a freeze-thawing method, and solubilized with 2.0% (w/v) Triton X-100 at a chlorophyll concentration of 1.0 mgChl/mL for 30 min on ice under dark conditions. Subsequently, the samples were centrifuged (38,600 × g, 90 min, 4 °C) to remove the unsolubilized cells, and the obtained supernatants were subjected to trehalose

* Corresponding author

e-mail: keikawa@ocarina.osaka-cu.ac.jp (K.K.),
kosumi@kumamoto-u.ac.jp (D. K.)

density gradient ultracentrifugation ($\sim 100,000 \times g$, 15 h, 4 °C).

2.2 Negative-staining electron microscopy

For measurement of negative-staining electron microscopy (EM), the isolated intact PBS was placed on a carbon-coated grid. The sample was stained for 1 min using 16% (w/v) ammonium molybdate, air-dried, and observed under a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) which was equipped by a FastScan-F214 (T) charge-coupled-device camera (TVIPS, Gauting, Germany), at 80 kV.

2.3 Time-resolved fluorescence measurement

The ps time-resolved fluorescence measurements were performed by a mode-locked Ti:Sapphire regenerative amplifier (Spectra Physics, Spitfire-Pro) and a time-correlated single photon counting (TCSPC) system. The visible excitation pulse at 570 nm was obtained from an optical parametric amplifier (Spectra Physics, TOPAS-C). The fluorescence of the sample at the right angle to the excitation beam was detected by a single photon avalanche diode (MPD, PD-050-CTD) through a spectrometer (Acton Research, SP275i). The detected photon signals were taken by the TCSPC module (Becker & Hickles, SPC130EM) [12]. Relative polarization between the excitation pulse and the detected fluorescence was set to a magic angle of 54.7°. The instrumental response function of the system was more than 20 ps. The spectral resolution of the spectrometer was 0.5 nm and fluorescence kinetics were measured with a 10-nm step. The sample was packed in a static cuvette with a 10-mm optical length. All optical measurements were performed at room temperature.

3. Results and Discussion

An intact PBS band was confirmed in the middle of the gradient tube, and its overall structure could be observed by negative-staining EM (Figure 2). The length of the long axis of PBS was about 700 Å, and its size was similar to that of PBS from a red alga *Griffithsia pacifica* [13]. Since PBS from cyanobacteria does not contain phycoerythrin (PE) like red algae, its structure is composed of a high amount of PC. Also, PC is easy to dissociate from PBS because the interaction between PCs in PBS is very weak, and it could be observed from the negative-staining EM.

Figure 3 shows the steady-state absorption spectrum of the isolated PBS. The absorption band peaked at 620 nm, which is due to the transition from the ground state to the lowest-lying singlet excited state

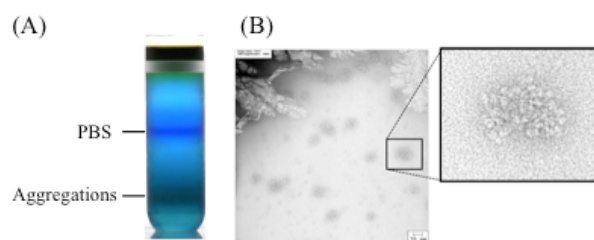


Figure 2. (A) PBS isolated using trehalose density ultracentrifugation. (B) Electron micrograph of PBS. The enlarged view on the right shows PBS.

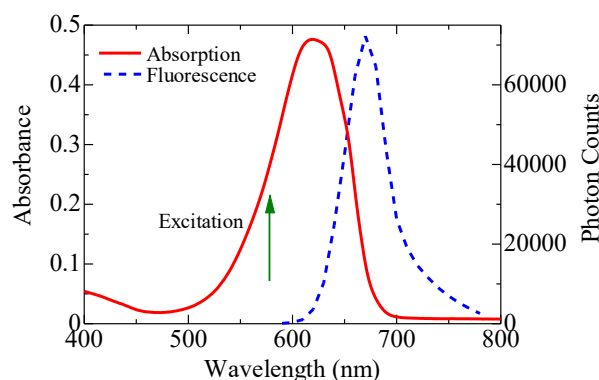


Figure 3. The steady-state absorption and time-integrated fluorescence spectra of the isolated PBS at room temperature. An arrow denotes excitation wavelength for time-resolved spectroscopy.

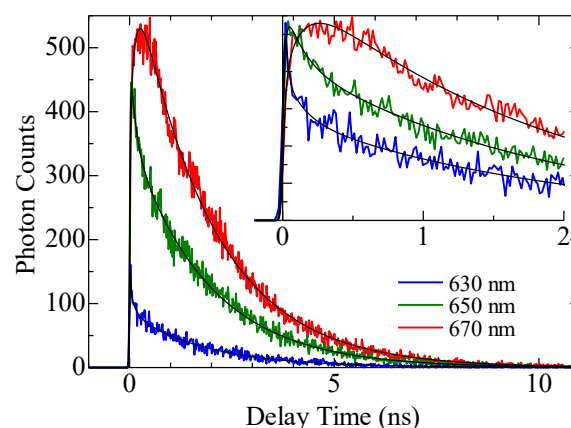


Figure 4. Kinetic traces of fluorescence given by excitation at 570 nm. The thin solid lines are the best-fit curves.

of phycocyanobilin bound to PBS. Even though PBS from *T. vulcanus* consists of the PC and APC protein complexes, the absorption bands due to PC and APC are indistinguishable from each other because of high inhomogeneity at room temperature. Nevertheless, it has been reported that PC protein complexes absorb shorter wavelengths of light than APC [14]. Therefore, to observe PC→APC energy transfer dynamics, we selected the excitation wavelength to be 570 nm, which corresponds to the blue spectrum of the PC absorption band.

The kinetic traces of the fluorescence from the

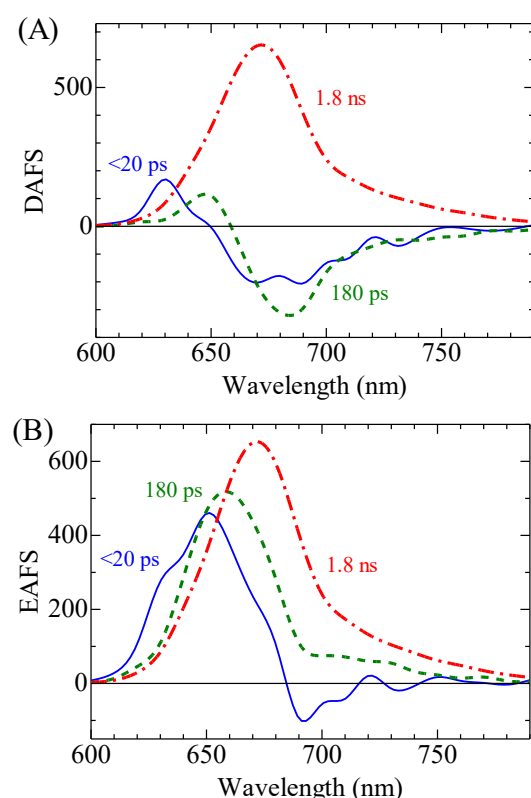


Figure 5. (A) Decay-associated fluorescence spectra and (B) Evolution-associated fluorescence spectra of the isolated PBS excited at 570 nm.

isolated PBS after excitation at 570 nm are represented in **Figure 4**. The solid lines are the best-fit curves for the rise and decay phases convoluted with the instrument response function assuming a Gaussian temporal profile. We analyzed the obtained kinetics traces globally, and then three exponential decay/rise components were required in order to fit the kinetics traces fully. Time-constants of each exponential component were determined to be <20 ps, 180 ps, and 1.8 ns. The obtained shortest time-constant is less than the instrument response function of 20 ps; therefore, we could not precisely determine the time-constant.

Time-integrated fluorescence signals at each wavelength are plotted in **Figure 3**, which corresponds to steady-state fluorescence spectra. The observed fluorescence spectrum has a narrower feature than the absorption spectrum. The peak of the isolated PBS fluorescence was located at 670 nm, which was consistent with the fluorescence spectrum of APC [15–19]. The results demonstrate that PC→APC energy transfer was observed after excitation of PBS at 570 nm.

To clarify detailed energy transfer dynamics in PBS, we show the decay-associated fluorescence spectra (DAFS) of the isolated PBS excited at 570 nm in **Figure 5(A)**. The longest decay DAFS of 1.8 ns was obtained as a positive phase in all spectral regions. Therefore, the component was assigned to APC acting as a terminal emitter in PBS as well as the core-membrane linker (L_{CM}) [18]. Conversely, the first and

second DAFS exhibit both negative and positive phases. Therefore, the components appear as rises at shorter detected wavelengths and as decays at longer wavelengths in the kinetic traces as shown in **Figure 4**.

The evolution-associated fluorescence spectra (EAFS) of the isolated PBS excited at 570 nm shown in **Figure 5(B)** were calculated based on a sequential irreversible model: $(<20 \text{ ps})^{-1} \rightarrow (180 \text{ ps})^{-1} \rightarrow (1.8 \text{ ns})^{-1}$. The third EAFS with the longest decay time-constant of 1.8 ns with a peak at 670 nm and is consistent with previously reported fluorescence spectra of isolated APC complexes [9–13]. The first and second EAFS had fluorescence peaks at 650 nm and 660 nm, respectively. It has been reported that a PC→APC energy transfer takes place within a 50–500 ps timescale [9], which implies that the second EAFS is associated with a PC site interacting with APC. However, the previously reported fluorescence spectra of the isolated PC have been observed at a shorter wavelength (~645 nm) than that of the second EAFS under room temperature [14,15]. The result suggests that the second EAFS obtained in the present study is associated with APC and the corresponding time-constant of 180 ps should be assigned to the energy transfer time within the APC core. The first EAFS was observed at a peak wavelength of 650 nm. Therefore, the first EAFS potentially originates from PC fluorescence.

Liu *et al.* have reported PC→PC energy transfer with 340 ps in isolated PC and PC→APC energy transfer with 430 ps in isolated PBS at 77 K [15]. The energy transfer time-constants have also been reported to be 6 and 77 ps at 77 K for PBS in an intact cell from cyanobacteria *Synechocystis* sp. PCC 6803 [20]. Based on a comparison between the previously reported fluorescence spectra and EAFS obtained in the present study, the first and second EAFS seem to be assigned to PC→APC and APC→APC energy transfer, respectively. However, it should be noted that the PC fluorescence wavelength in PBS could become longer than that of the isolated PC because of strong interactions among PC proteins and between PC and APC proteins. Therefore, we cannot exclude a possibility that the first and second EAFS originate from PC→PC and PC→APC energy transfer, respectively.

4. Conclusions

In the present study, we obtained isolated PBS from a thermophilic cyanobacterium, *T. vulcanus*, observed its overall structure by negative-staining EM, and performed picosecond time-resolved fluorescence spectroscopy on the isolated PBS to reveal excitation energy transfer dynamics in an intact PBS. The fluorescence kinetics measurements excited at 570 nm and a global analysis suggest the PC→APC energy transfer takes place within a picosecond timescale. The results obtained in this study may assist to understand energy transport between PBS and photosystems.

Acknowledgements

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Photosynthetic megacomplexes by a novel sample preparation method and identification of carotenoids interacting with photosystem II from *Thermosynechococcus vulcanus*

Keisuke Kawakami^{1*}, Takashi Maoka², Yuhei O Tahara³, Junko Shiomi³, Makoto Miyata³, Nobuo Kamiya¹ and Daisuke Kosumi^{4*}

¹ The OCU Advanced Research Institute for Natural Science & Technology (OCARINA), Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka City, Osaka, Japan.

² Research Institute for Production Development, Kyoto, 606-0805 Japan.

³ Department of Chemistry, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585, Japan.

⁴ Institute of Pulsed Power Science, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto, 860-8555 Japan.

Abstract: Thermophilic cyanobacteria have photosynthetic megacomplexes in which photosynthetic membrane proteins (photosystem I and II) and phycobilisomes which is one of the light-harvesting antenna proteins interacted, and they contain numerous carotenoids to provide photoprotection through the dissipation of excess absorbed light energy. However, the intact megacomplexes have not been prepared in previous studies, and the type and number of carotenoids interacting with the megacomplexes are yet to be identified. Although eleven β -carotenes and one β -cryptoxanthin have been identified in the crystal structure of photosystem II, *T. vulcanus* cell contains carotenoids, such as β -carotene, β -cryptoxanthin, zeaxanthin, caloxanthin, nostoxanthin, and 2-Hydroxymyxol 2'-fucoside. There are no reports that carotenoids other than β -carotene and β -cryptoxanthin interact and function with PSII. In the present study, we prepared the intact megacomplexes by a novel sample preparation method and performed pigment analysis of carotenoids-enriched PSII to determine carotenoids that potentially interact with PSII.

1. Introduction

Oxygen-evolving photosystem II (PSII) absorbs sunlight energy and splits water molecules, in turn releasing molecular oxygen. The components of PSII from thermophilic cyanobacteria include four large membrane-spanning subunits (D1, D2, CP43, and CP47), 13 low-molecular-weight membrane-spanning subunits (PsbE, PsbF, PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbT, PsbX, PsbY, PsbZ, and Psb30), and three extrinsic proteins (PsbO, PsbU, and PsbV). In addition, PSII contains a large number of cofactors such as chlorophylls (Chls), carotenoids, and lipids [1]. Phycobilisomes (PBS), one of the light-harvesting antenna proteins, is interacted with PSII in the thylakoid membranes and they are formed a megacomplex. In addition, eleven β -carotenes and one β -cryptoxanthin have been identified in the PSII monomer [2], whereas only β -carotenes have been identified in photosystem I (PSI) from cyanobacteria [3,4]. Zeaxanthin, antheraxanthin, and violaxanthin, which are involved in a photoprotection mechanism

(xanthophyll cycle) in higher plants, have been identified in the light-harvesting complex (LHCII) of land plants [5-8], however, these carotenoids are yet to be identified within PSII and PSI. Carotenoids are one of the essential pigments for the growth and development of higher plants and algae, facilitating the dissipation of excess excitation energy [9]. Pigment analysis of *Thermosynechococcus elongatus* (*T. elongatus*), a close relative of *T. vulcanus*, has revealed that *T. elongatus* cells contain β -carotene, β -cryptoxanthin, zeaxanthin, caloxanthin, nostoxanthin, and 2-Hydroxymyxol 2'-fucoside [10,11]. However, the interaction sites in PSII for carotenoids other than β -carotene and β -cryptoxanthin are yet to be elucidated. To understand photoprotection mechanism of algae, it is important to prepare the intact megacomplexes and identify the composition of carotenoids localized around PSII and their interaction sites. In the present study, the intact megacomplexes and carotenoids-enriched PSII (Car-PSII) were prepared from *T. vulcanus* using a novel sample preparation method and a pigment analysis of Car-PSII was performed.

* Corresponding author

e-mail: keikawa@ocarina.osaka-cu.ac.jp

kosumi@kumamoto-u.ac.jp

2. Experimental

2.1 Quick-freeze deep – etch replica Electron Microscopy

Cells were collected by centrifugation and washed twice with a phosphate buffer. The cell suspension was placed on mica flake and frozen at liquid helium temperature using a CryoPress (Valiant Instruments, St. Louis, MO). The cells were rotary-shadowed using platinum at an angle of 20°, and backed with carbon in a JFDV freeze-etching device (JEOL Ltd, Akishima, Japan). Replicas were floated off the mica by slow immersion along the surface of full-strength hydrofluoric acid, cleaned with a commercial bleach, rinsed in water, and picked up onto 400-mesh copper grids. Replica specimens were observed using a transmission electron microscope at 80 kV.

2.2 Sample preparation

A thermophilic cyanobacterium, *T. vulcanus*, was used to prepare PSII. The thylakoid membranes obtained by cell disruption were solubilized with 1.0% (w/v) digitonin or 2.0% (w/v) n-dodecyl- β -D-maltoside (β -DDM) at a concentration of 1.0 mgChl/ml for 30 min in the dark. After precipitating the unsolubilized samples by centrifugation, the supernatants were loaded onto a trehalose density gradient and the gradients were ultracentrifuged for 15 h at $\sim 100,000 \times g$ and at 4 °C.

2.3 Absorption spectroscopy

Absorption spectra of the prepared samples were measured using a UV-Visible (UV-VIS) spectrophotometer (UV-2600; Shimadzu Co., Kyoto, Japan) at room temperature.

2.4 Apparatus for pigment analysis

UV-VIS absorption spectra for pigment analysis were recorded using a Hitachi U-2001 spectrophotometer (Hitachi Field Navigator, Tokyo, Japan) in diethyl ether (Et₂O). Liquid chromatography coupled to mass spectrometry analysis of carotenoids was performed using a Waters Xevo G2S Q ToF mass

spectrometer (Waters Corporation, Milford, CT, USA) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system. Electron-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from m/z 100 to 1,500 with a capillary voltage of 3.2 kV, a cone voltage of 40 eV, and a source temperature of 120°C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. Tandem mass spectrometry (MS/MS) spectra were measured using a Quadrupole TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V using a photodiode array detector (PDA) to record UV-VIS absorption spectra from 200 to 600 nm. An Acquity 1.7 μ m BEH UPLC C18 column (Waters Corporation, Milford, CT, USA) was used as a stationary phase and MeCN:H₂O (85:15) to MeCN:MeOH (65:35) at a flow rate of 0.4 mL/min as a mobile phase in the HPLC system. Proton nuclear magnetic resonance (¹H-NMR) (500 MHz) spectrum was measured with a Varian Unity Inova 500 spectrometer (Varian Corporation, Palo Alto, California USA) in CDCl₃ with trimethylsilyl (TMS) as an internal standard. Preparative HPLC was performed using a Hitachi L-6000 HPLC intelligent pump and Hitachi L-4250 UV-VIS detector (Hitachi Field Navigator, Tokyo, Japan) set at 450 nm. The columns used were 250 $\times$ 4.6 mm i.d., 5 μ m Cosmosil 5SL-II, and Cosmosil 5C18-II (Nacalai Tesque, Kyoto, Japan).

2.5 Extraction and separation of carotenoids

T. vulcanus cells were extracted with acetone at room temperature. The extracts were concentrated to a small volume by evaporation and partitioned between diethyl ether (Et₂O) and saturated saline. Subsequently, the Et₂O layer was evaporated to dryness and subjected to silica gel column chromatography followed by preparative HPLC. Identification of carotenoids was carried out using UV-VIS and ESI-TOF-MS. Myxol and 2-Hydroxy-myxol glucosides were identified using ¹H-NMR after acetylation.

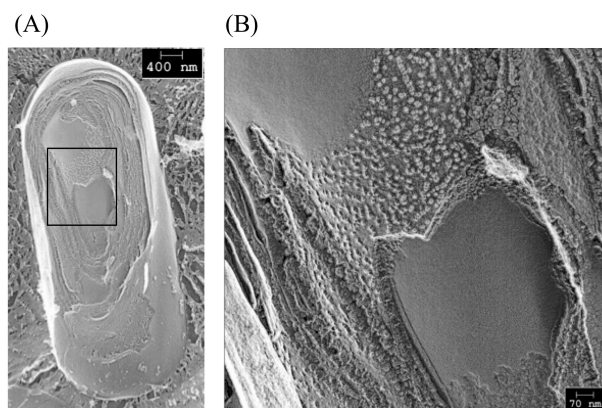


Figure 1. (A) Freeze-fracture deep-etch replica EM of *T. vulcanus* cell. (B) The enlarged view on (A).

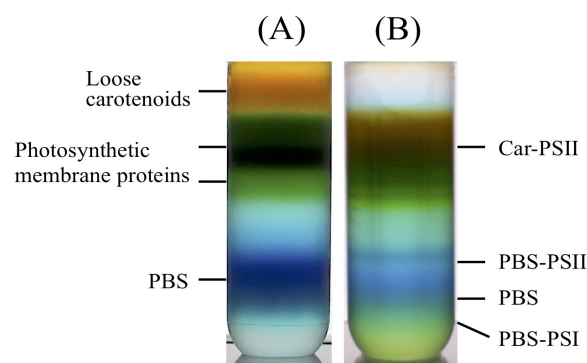


Figure 2. (A) Photosynthetic megacomplexes from *T. vulcanus* treated by β -DDM. (B) Photosynthetic supercomplexes from *T. vulcanus* treated by digitonin.

3. Results and Discussion

3.1 Preparation of photosynthetic megacomplexes and carotenoid-enriched PSII

In order to identify the arrangement of the intact megacomplexes, we performed the freeze-fracture electron microscopy (EM) (**Figure 1**). Quick-freeze deep-etch replica EM is one of the optimal methods for visualizing fragile and rapidly moving structures [12]. A high amount of soluble proteins and membrane proteins are contained in the cell, and PSII and PSI, which are essential for photosynthesis, are embedded in the thylakoid membranes. Although the fracture plane of a *T. vulcanus* cell could be observed by the freeze-fracture EM, we could not identify the arrangement of the intact megacomplexes.

Subsequently, we prepared a variety of the intact megacomplexes using a novel sample preparation method. Particularly, in the solubilization using β -DDM, numerous carotenoids lost from the intact megacomplexes were confirmed in the upper layer of the gradient tube, and PBS fractions were confirmed in the lower layer in the gradient tube (**Figure 2**). Moreover, Car-PSII and the intact megacomplexes bound to PBS (PBS-PSII and PBS-PSI) were prepared by using digitonin. Identification of these megacomplexes was carried out by absorption spectroscopy (**Figure 3**). In the result, we confirmed that the compositions of the photosynthetic membrane proteins prepared using detergents used for solubilization are altered, and that carotenoids and PBS, which are dissociated from the complexes by the DDM-treatment, maintain their interactions with complexes subjected to digitonin-treatment.

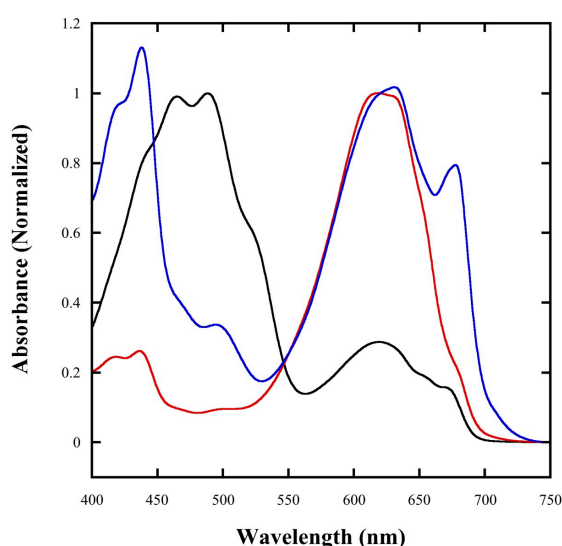


Figure 3. Absorption spectra of photosynthetic megacomplexes and Car-PSII. The spectra of Car-PSII, PBS-PSII, and PBS-PSI are plotted by the black, red, and blue lines, respectively.

3.2 Pigment analysis of Car-PSII

We prepared Car-PSII and analyzed carotenoids that potentially interacted with PSII (**Figure 4, Table 1**). β -carotene, β -cryptoxanthin, zeaxanthin, caloxanthin, nostoxanthin, and 2-Hydroxymycol 2'-fucoside were included in Car-PSII, and the result is consistent with that of *T. elongatus*. However, the composition ratios of the carotenoids were different [10]. In Car-PSII from *T. vulcanus*, the amount of zeaxanthin was the highest (34.9%), followed by 2-Hydroxymycol 2'-fucoside (16.7%), β -carotene (10.3%), caloxanthin (6.7%), β -cryptoxanthin (2.5%), and nostoxanthin (2.3%). Although *cis*-carotenoids were also included in Car-PSII, it has been reported that carotenoids from *T. elongatus* are all *trans*-carotenoids [1,10,11]. Therefore, many of these are potentially isomers arising from photoisomerization during sample treatment. In the future, it would be necessary to measure the amounts of *cis*-carotenoids in Car-PSII in *T. vulcanus* through comprehensive pigment analyses.

Table 1. Carotenoid content in Car-PSII from *T. vulcanus*.

Carotenoid compound	Content (%)
2-Hydroxymycol 2'-fucoside (2-OH)	16.7
Isomer of 2-OH	9.0
Nostoxanthin	2.3
Caloxanthin	34.9
Zeaxanthin	6.7
9- <i>cis</i> -Zeaxanthin	1.8
13- <i>cis</i> -Zeaxanthin	1.5
β -Cryptoxanthin	2.5
<i>cis</i> - β -Cryptoxanthin	1.4
β -Carotene	10.3

4. Conclusion

In the present study, we prepared the intact megacomplexes (PBS-PSII and PBS-PSI) using a novel sample preparation method, in which PBS and PSII (or PSI) interacted, and Car-PSII interacted with carotenoids other than β -carotene and β -cryptoxanthin. A further analysis of the excitation energy transfer dynamics of Car-PSII, PBS-PSII, and PBS-PSI are required.

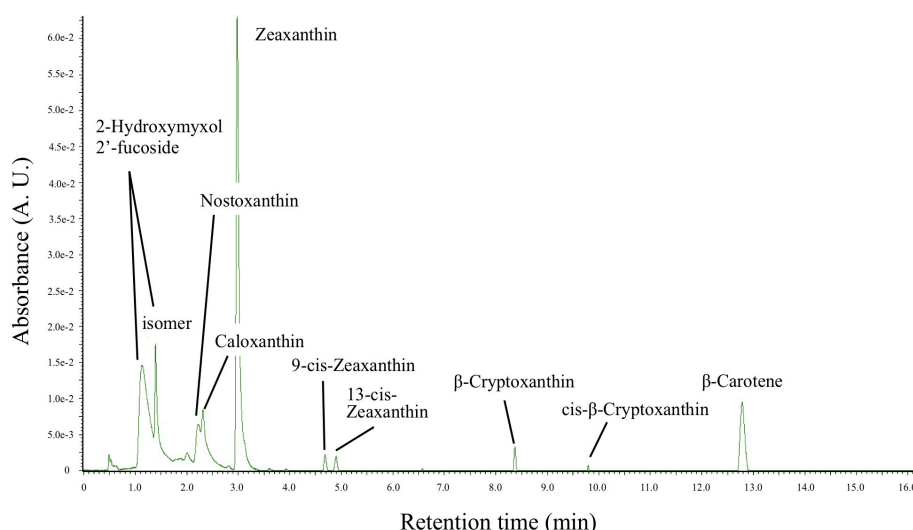


Figure 4. Carotenoid composition in Car-PSII from *T. vulcanus*. Carotenoid content (%) is shown in Table 1.

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Conversion of solid biomass residue to functional materials and fuels using supercritical fluids and pulsed discharges

Mitsuru Sasaki^{1,2}

¹ Institute of Pulsed Power Science, Kumamoto University, ² Faculty of Advanced Science and Technology, Kumamoto University)

1. Preface

The authors have succeeded in efficiently converting unused or residual biomass in useful components by converting molecules into molecular forms (chemical structures and molecular sizes) which functions are expressed, while taking advantage of their properties and chemical structures. We are promoting research aiming to create and improve technologies for effective utilization, as well as for creating high-performance technologies for producing chemical substances. As a technical seed for that purpose, the author is paying attention to supercritical fluid and pulse energy. In this report, through practical examples, supercritical fluid and pulse energy are introduced for the purpose of recovering useful ingredients from solid residue biomass.

2. Example of Functionalization using supercritical fluids

The majority of companies that process large amounts of processing waste generated at food processing sites, are considered a serious problem that puts pressure on the management of the company. From the environmental point of view, massive emissions of solid waste are causing problems such as deterioration of the atmosphere and soil. This, due to the utilization of incineration, landfill, and the leakage of harmful substances to rivers and seawater. By using supercritical carbon dioxide or subcritical water, it is possible to efficiently recover essential oils and low polarity useful substances (carotenoids, flavonoids), polyphenols, proteins, and polysaccharides contained in such solid residue biomass. Reduction of solid residue can also be realized. Here, some examples are listed.

2.1 Recovery of high value added component / functional ingredient from solid residual biomass

Essential oils and low polarity useful substances contained in the pericarp of domestic Japanese citrus can be extracted by using supercritical CO₂. When using Kabosu peel as a raw material, it was possible to obtain a maximum of about 1.5 wt% of Kabos essential oil under supercritical CO₂ conditions of 80 °C, and pressure of 30 MPa. Although the recovery rate of the essential oil is less than about 70%, when compared to the conventional steam distillation method which gives 100%, the supercritical CO₂

extraction method can control the obtained essential oil composition by changing the operating conditions of temperature and pressure. For example, in the supercritical CO₂ extraction method, it is possible to obtain an essential oil having a high proportion of sesquiterpenes and esters at high pressure, whereas the composition is uniquely determined when steam distillation method or cold press method are used. Under these conditions carotenoids can be efficiently extracted. In addition, when extracting flavonoids or polymethoxyflavonoids with a slightly higher polarity, it is possible to extract them by allowing several vol% of water or ethanol to coexist in supercritical CO₂.

2.2 Functionalization by solubilization (reduction in molecular weight) of solid residue biomass

Utilizing rice bran produced at the rice production site as a raw material, hydrolyzing the underlying protein or nucleic acid under subcritical water in the absence of catalyst is able to make it water soluble (lower molecular weight), and produce newly containing amino acid at higher concentration. We are aiming to produce a mechanism to produce functional liquid fertilizer and use it on agricultural field cultivation site. Until now, it was found out that in treatment with distilled water and rice bran as raw material in an autoclave at a temperature of 160 to 200 °C (internal pressure is about 10 to 26 atm) for 30 to 120 minutes, 80% of the protein contained in the solid material can be converted into peptides or amino acids (some of which are converted to ammonia). Elution of minerals (main components: K, Ca, Mg) from raw materials has also occurred, and production of liquid nutritive material with high nutritional value is expected, along with volume reduction of solid residue.

2.3 Functionalization of essential oil extraction residues

The solid residue from which useful components have been removed by supercritical extraction or subcritical water hydrolysis or the like is ordinarily subjected to incineration treatment or landfill treatment, but cellulose skeleton and lignin bridge structure mostly remains. By effectively utilizing this structure, carbonization treatment can produce nano-carbon material, which can be further used as an electrode for fuel cell, high capacity capacitor, or solid

catalyst. The authors prepared carbide by two methods of gas-phase carbonization and hydrothermal carbonization, using dried and pulverized products of fucoxanthin extracted beforehand from unclustered seaweeds as raw materials. Samples were prepared by sulfonating them to increase the degree of oxidation. As a result, it was found that esterification reaction experiment of acetic acid and ethanol was carried out using any of the carbides, and it was found that the produced amount of ethyl acetate was increased compared with the case of no catalyst. Furthermore, the catalyst activity was exhibited. It was confirmed that the sulfonated carbide has higher acidity and is highly active as a solid acid catalyst. However, as compared with existing solid acid catalysts, both the conversion of raw materials and the yield of ethyl acetate are low. We understood that for the improvement of the porosity and acidity of the prepared sulfonated carbide, a more efficient attachment of the functional group to the solid surface is necessary.

3. Example of oligopeptide synthesis using pulse power technology

Oligopeptides are peptides composed of 2 to 10 amino acid residues. These peptides have attracted attention for health food and medicine. In general, peptides are synthesized in organic solvents or enzyme reaction fields, but there are problems such as multi-step process, long synthesis time, and the need of post-treatment. Although it was reported that oligopeptide could be synthesized from glycine in subcritical / supercritical water field, the yield of chain oligopeptide of trimer or higher was less than 0.1%, and the thermally stable dimer, cyclized product (diketopiperazine, DKP) was the main product. The authors conducted pulsed arc discharge for about 5 to 10 minutes in an aqueous solution at room temperature under atmospheric pressure using an aqueous solution of glycine, alanine, or a chain dimer as a raw material. DKP was not produced, however chain dimers to tetramers could be obtained in about several yield (%). In addition, it was found that by using this method (pulse arc discharge), on the DKP aqueous solution resulted in about 30% disappearance of DKP, of which 10 to 15% was converted to amino acids and chain dimers to tetramers.

4. Conclusion

In this report, we outlined how the supercritical fluid and pulse energy can be used in development of the overall utilization technology of solid residual biomass by citing concrete implementation examples. Although both of them are at the laboratory level, the authors are expecting that it can become technology seeds group which realizes both reduction of environmental burden and efficient target substance production compared with the prior art. In the future, I would like to develop this research into a phase that

can be applied industrially while solving various problems for social implementation such as collection, transportation of raw materials, separation and refining of products, storage and application development.

Efficient Energy Transfer in Light-Harvesting Function from an Intra-Molecular Charge Transfer State of Fucoxanthin in Reconstituted LH1 Complex

Masayuki Yoshizawa^{1*}, Ren Sato¹, Nao Yukihiro², Chiasa Urugami² and Hideki Hashimoto²

¹ *Department of Physics, Graduate School of Science, Tohoku University,
6-3 Aramaki-aza-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan.*

² *Department of Applied Chemistry for Environment, School of Science and Technology,
Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1338, Japan.*

Abstract: We have applied femtosecond spectroscopy to light-harvesting complex (LH1) reconstituted with fucoxanthin. Fucoxanthin has an intramolecular charge transfer state (ICT) additional to S_2 and S_1 excited states. Efficient excitation energy transfer from an equilibrium state of S_1 and ICT (S_1 /ICT) in fucoxanthin to bacteriochlorophyll has been observed following the photoexcitation of the S_2 state.

1. Introduction

In primary process of bacterial photosynthesis, carotenoids play two important roles, light harvesting and photo protection [1,2]. Carotenoids harvest blue and green light which bacteriochlorophyll (BChls) cannot absorb. The absorbed energy is transferred to BChl with high efficiency in femtosecond to picosecond time scale.

Ultrafast dynamics in carotenoids have been generally described using three low-lying states (S_0 , S_1 and S_2). The lowest singlet S_1 excited state is optically forbidden because of same symmetry with the S_0 ground state. The S_2 state is the lowest optically allowed excited state. After photoexcitation to the S_2 state, internal conversion to the S_1 state occurs within a few hundred femtoseconds. The S_1 state relaxes to the S_0 ground state in picosecond time scale.

We have reported dynamics of excitation energy transfer (EET) in LH1 complex reconstituted with several carotenoids [3,4]. EET to BChl occurs both from the S_2 and S_1 excited states in carotenoids. However, EET from the S_1 state is less efficient than that from the S_2 state. It might be due to the optically forbidden transition between the S_1 and S_0 states, because EET by simple Förster mechanism requires optically allowed transitions.

Fucoxanthin has a dipole-allowed intra-molecular charge transfer (ICT) state in polar solvents additional to the S_2 and S_1 states [5,6]. The ICT state cannot be directly photoexcited, but it is quickly generated following the photoexcitation to the S_2 state. The relaxation from the S_2 state occurs within a few hundred femtoseconds and the ICT state makes an equilibrium state with the S_1 state, S_1 /ICT. Highly

efficient EET from fucoxanthin has been reported in marine brown alga [7]. Origin of the high efficiency is explained in terms of the dipole allowed transition of the ICT state and strong interaction with chlorophyll due to the energy lower than the S_1 transition [8].

EET from fucoxanthin to BChl has been already reported in LH1 complex reconstituted with fucoxanthin, LH1(Fx) [9]. Fluorescence from BChl occurs following the excitation of the S_2 state of fucoxanthin. Femtosecond spectroscopy shows that lifetime of the S_1 /ICT state in LH1(Fx) is shorter than that of fucoxanthin in buffer. It suggests that efficient energy transfer occurs from the S_1 /ICT state. However, the detailed dynamics has not been well-understood.

In this study, we have investigated EET dynamics in LH1(Fx) by femtosecond absorption spectroscopy using tunable pump pulses and probe with broad wavelength region from visible to near infrared.

2. Experimental

Femtosecond spectroscopy setup was based on an amplified mode-locked Ti:Sapphire laser system operating at 1 kHz [10]. The output pulse (800 nm, 150 fs) was separated to generate pump and probe pulses. Tunable pump pulse was generated by a noncollinear optical parametric amplifier (NOPA) pumped by the second harmonic pulse of the Ti:Sapphire laser. Femtosecond super-continuum was used as probe pulse. Temporal resolution of the system was 80 fs. Relative polarization of the pump and probe pulses was set to the magic angle (54.7°).

The reconstituted LH1(Fx) was prepared as described elsewhere [9,11]. In this study, the solution of LH1(Fx) was dispersed in a poly-vinyl alcohol film on a glass plate. During the femtosecond measurement, the film sample was translated linearly to avoid

* Corresponding author

e-mail: m-yoshizawa@m.tohoku.ac.jp

degradation and the accumulation of any potential photoproducts.

3. Results and Discussion

Stationary absorption spectra in visible region of the LH1(Fx) film and fucoxanthin in methanol are shown in **Figure 1**. The $S_0 \rightarrow S_2$ transition of fucoxanthin appears at 400-500 nm. The Q_x band of BChl in LH1 is observed at 590 nm. Broad base of LH1(Fx) is due to scattering of the film. The LH1(Fx) film has equivalent spectrum with LH1(Fx) in solution [9]. Pump wavelength of transient absorption spectroscopy was 500 nm and 590 nm for resonant excitation of the S_2 state of fucoxanthin and the Q_x state of BChl, respectively.

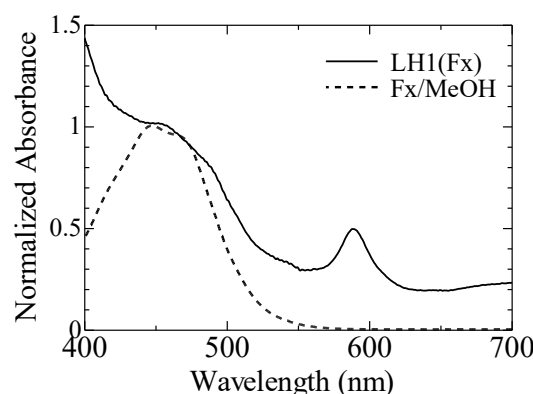


Figure 1. Stationary absorption spectra of LH1(Fx) and fucoxanthin in methanol.

Figure 2(a) shows time-resolved absorbance change spectra following excitation to the Q_x state of BChl. Small negative signal at 590 nm is assigned to bleaching of the Q_x band. Signal around 890 nm is due to bleaching and emission of the Q_y band. The signal has a peak 875 nm at a delay time of 0.0 ps. It is equal to the peak of the stationary absorption. It means that BChl exists in the Q_x state at 0.0 ps and only the bleaching of the Q_y band is observed. The peak shifts to 885 nm at 0.4 ps, because the Q_x state relaxes to the Q_y state and emission from the Q_y state appears. The relaxation time from the Q_x state to the Q_y state is 70 ± 20 fs. The Q_y state remains long time, because reaction center which captures energy from BChl does not exist in the reconstituted LH1(Fx). The lifetime of the Q_y state is 400 ± 20 ps. Signal of fucoxanthin is not observed following the BChl excitation. It means that EET from BChl to fucoxanthin does not occur in LH1(Fx).

Figure 2(b) shows time-resolved absorbance change spectra following excitation to the S_2 state in fucoxanthin. The observed signal can be explained by a model shown in **Figure 3**. At 0.02 ps, transient absorption of the photoexcited S_2 state appears around 900 nm. It decreases quickly with a time constant of 70 ± 40 fs. At the same time, signals due to the S_1 /ICT

state and the Q_y state appear at 500-700 nm and 885 nm, respectively. The visible signal has typical three peaks of the S_1 /ICT state at 550, 630, and 680 nm [5,6,9]. The S_1 /ICT state decreases with a time constant of 67 ± 5 ps. The observation of the Q_y signal following the S_2 excitation means that EET from fucoxanthin to BChl occurs efficiently in the LH1(Fx) film as well as LH1(Fx) in solution [9].

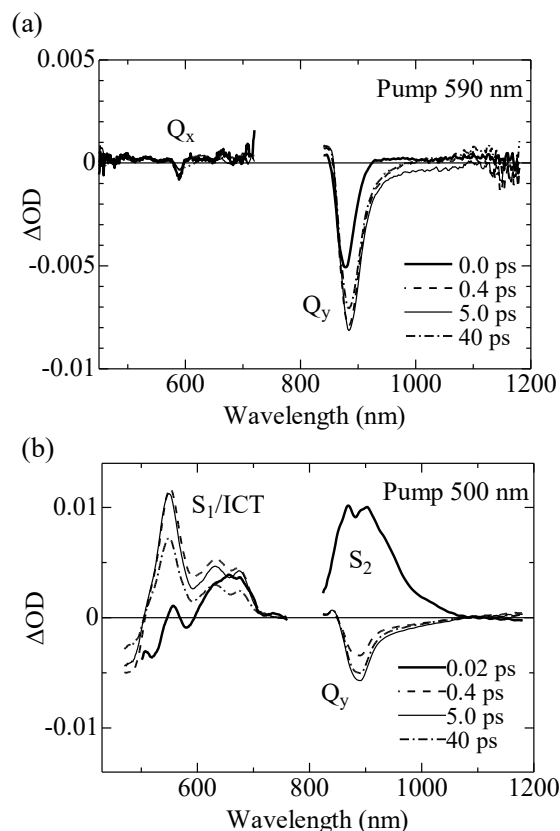


Figure 2 Time-resolved absorbance change of LH1(Fx). (a) BChl Q_x pump and (b) Fx S_2 pump.

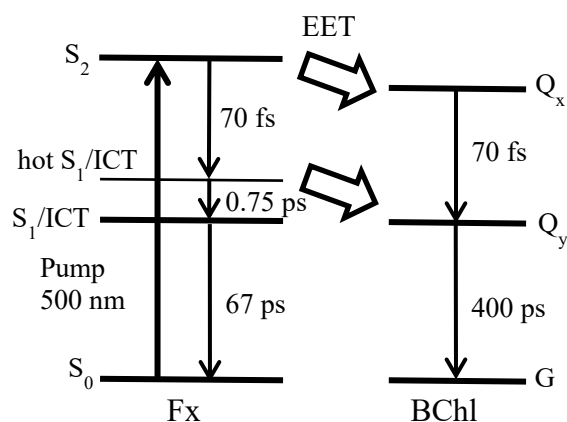


Figure 3 A model of relaxation and energy transfer in LH1(Fx).

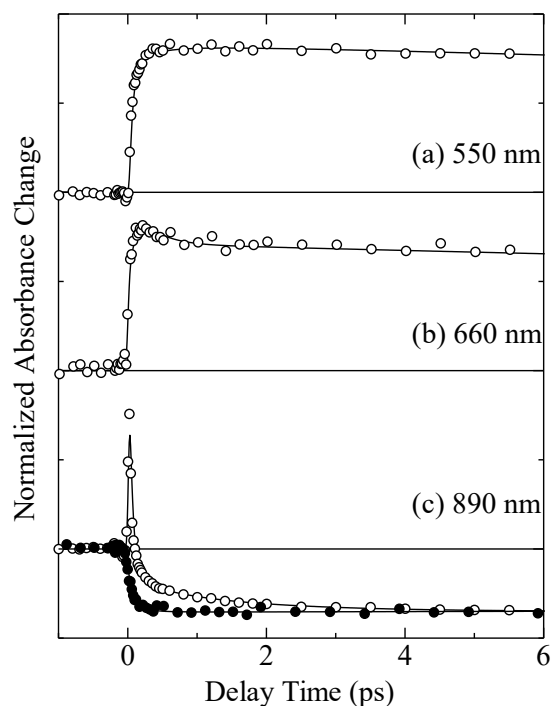


Figure 4 Temporal response of absorbance change in LH1(Fx) at several probe wavelength. Open circles: Fx S_2 pump and solid circles: BChl Q_x pump.

Temporal responses of absorbance change at early delay times are shown in **Figure 4**. Open circles are the signals following the S_2 excitation, while closed circles are the signals following the Q_x excitation. Solid lines are best-fitted curves assuming double or triple exponential responses. The visible signals at 550 nm and 660 nm are assigned to the S_1 /ICT state. Initial fast decay at 660 nm is assigned to vibrational relaxation in the S_1 /ICT state. The time constant obtained at 660 nm is 0.80 ± 0.20 ps. The initial response at 890 nm following the S_2 excitation has two time constants. The shorter one is equal to the decay time of the S_2 state. The other slower one is 0.75 ± 0.25 ps. The faster response is assigned to the decay of the S_2 state and the formation of the Q_y state by EET from the S_2 state through the Q_x state. The slower response is assigned to formation of the Q_y state by EET from the hot S_1 /ICT state, because the time constant is almost equal to the vibrational relaxation of the S_1 /ICT state. However, formation with the S_1 /ICT lifetime of 67 ps cannot be observed. It suggests that EET from the vibrational ground level of the S_1 /ICT state is less effective than that from the hot S_1 /ICT state.

EET from the S_1 state has been already observed in LH1 reconstituted with spheroidene, LH1(Sph) [4]. The EET ratio from the S_1 state is about 40 % of that from the S_2 state in LH1(Sph). On the other hand, EET ratio from the S_1 /ICT state in LH1(Fx) is estimated to

be almost equal to that from the S_2 state. It means that the ICT state has more ability of EET to BChl. However, the reported total EET efficiency in LH1(Fx) is 38 % [9]. It is smaller than the total efficiency of 61 % in LH1(Sph). The small efficiency has been explained by existence of unconnected fucoxanthin in the reconstituted sample. Detailed measurement of the transient spectra may reveal the dynamics of the connected and unconnected fucoxanthin and determine the practical EET efficiency in LH1(Fx).

4. Conclusion

The EET dynamics have been investigated in LH1(Fx) by femtosecond absorption spectroscopy. EET to BChl occurs from the S_2 state and the S_1 /ICT state in fucoxanthin. The EET from the S_1 /ICT state is more effective than that from the S_1 state in LH1(Sph). However, there is the unconnected fucoxanthin in the sample used in this study. More detailed study is needed to clarify the advantage of the ICT state for EET in the light-harvesting function.

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Detailed Chromatographic and Spectral Data of Capsanthone in Paprika Intake Human Plasma and Erythrocytes

Azusa Nishino¹, Takashi Ichihara¹, Kazuhisa Kawamoto², and Takashi Maoka^{3*}

¹*Institute of Health Sciences, Ezaki Glico Co., Ltd, 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502, Japan.*

²*Faculty of Human Development and Culuture, Fukushima University, 1 Kanagawa, Fukushima, 960-1296, Japan*

³*Research Institute for Production Development, 15 Shimogamo, Morimoto Cho, Sakyo-ku, Kyoto 606-0805, Japan.*

*Correspondence should be addressed. Tel: +81-75-481-1107, FAX: +81-75-791-7659

E-mail: maoka@mbox.Kyoto-inet.or.jp

Abstract : Detailed chromatographic and ESI TOF MS, MS/MS, UV-VIS spectral data of capsanthone in paprika carotenoid ingested human plasma and erythrocytes are described in this experimental report

1. Introduction

Ripe fruits of paprika is a good dietary source of xanthophylls such as β -cryptoxanthin, zeaxanthin, capsanthin, and capsorubin [1]. Xanthophylls are now receiving increasing attention for their superior anti-oxidation performance and anticipated health-benefits [1]. Especially, characteristic carotenoids of paprika, capsanthin and capsorubin have been reported to show excellent antioxidative activity for lipid peroxidation [2] and suppressing generation of superoxide and nitric oxide [3], uptake peroxynitrite and inhibit nitration of tyrosine [4]. Therefore, we investigated the absorption and accumulation of paprika carotenoid in human plasma and erythrocytes in healthy young subjects [5]. As the results, characteristic carotenoids in paprika, capsanthin, cucurbitaxanthin A, cryptocapsin (**Figure 1**) were detected in both plasma and erythrocytes. Furthermore, capsanthone was detected in both plasma and erythrocytes by LC/MS. This experimental report describes detailed chromatographic and spectral data of capsanthone in paprika carotenoid ingested human plasma and erythrocytes.

2. Experimentals

2.1 HPLC analysis

Quantitative analysis of carotenoids was performed with a normal phase HPLC system detected at 450 nm. The column used was a 250 $\times$ 4.6 mm i.d., 5 μ m Cosmosil SSL-II (Nacalai Tesque, Japan) with

acetone:hexane (2:8) as a solvent at a flow rate of 1.0 mL/min [5]. ESI TOF MS and MS/MS spectra were recorded with a Waters Xevo G2S Q TOF mass spectrometer (Waters Corporation, Milford, CT, USA), equipped with an Acquity UPLC system. The electrospray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from m/z 100 to 1,500, with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120 °C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured with a quadrupole-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V. UV-VIS absorption spectra were recorded from 200 to 600 nm using a photodiode-array detector (PDA). An Acquity 1.7 μ m BEH UPLC C18 column (Waters Corporation) was used as a stationary phase and MeOH as a mobile phase at a flow rate of 0.4 mL/min for the HPLC system, according to the methods described by Maoka and Miki 2014 [6].

2.2 Preparation of Paprika carotenoid Containing Supplement

Paprika GY-1 (Glico Nutrition Co., Ltd, Osaka, Japan) was used for paprika carotenoid supplement. Test drink was prepared by mixing 0.7 g of Paprika GY-1, 3g of Highly Branched Cyclic Dextrin (Glico Nutrition Co., Ltd, Osaka, Japan), 7 g of caster sugar, 0.18 g of [anhydrous citric acid](#), 0.12 g of sodium citrate, 0.005 g of sodium chloride, 0.008 g of

potassium chloride, with 100 ml of water, and adjusted pH 3.5. with 10% torisodium citrate solution. The test drink contained 7 mg of carotenoids. Individual carotenoids content in Paprika GY-1 was as follows; β -carotene (0.90 mg/100mL), β -cryptoxanthin (0.54 mg/100mL), cryptocapsin (0.07 mg/100 mL), cucurbitaxanthin A (0.57 mg/100 mL), zeaxanthin (0.46 mg/100 mL), capsanthin 3,6-epoxide (0.27 mg/100mL), capsanthin (3.27 mg/100mL), capsorbin (0.18 mg/100mL), and other minor carotenoids (0.74 mg/100 mL). Details were described in Nishino et al., 2015 [5].

2.3 Subjects and supplementation trial

The present study was conducted according to the guideline laid down in Declaration of Helsinki, and all procedures involving human subjects were approved by the ethics committees of Fukushima University. The healthy 3 males and 2 females (aged 22.0 ± 1.9), students of Fukushima University were enrolled. Prior to entry, written consent was obtained from each subject. During the 4 weeks trial, the subjects drank 100 mL of paprika carotenoid contained drink twice a day (in the morning and evening) for 4 weeks. Every 10 mL of blood sample was obtained (taken) before, after 2 weeks, and after 4 weeks of ingestion of paprika carotenoid, and after 4 weeks of wash out ingestion, using vacuum blood-collecting vessel with heparin as an anticoagulant. Subjects did not ingest paprika carotenoid drink before 24 hrs. of blood sampling [5].

2.4 Analysis of carotenoids in plasma and erythrocytes

Blood sample was stand for 30 min after sampling at room temperature. Plasma and erythrocytes were

separated by centrifuged (1000 G for 10min at room temperature) from blood. Erythrocytes were washed with phosphate-buffered saline (pH 7.2) 4 times to remove leukocyte. Plasma and serum were stored at -80°C until carotenoid analysis. 2mL of ethanol was added to 1mL of plasma and stirring with vortex. Then carotenoid was extracted with 5mL of ether : hexane (2:8) solution from this mixture with stirring. After standing for 10 min, the upper layer was filtrated by Millipore membrane filter and was evaporated. The residue was dissolved in acetone : hexane (2:8) and was subjected to HPLC. Erythrocyte membrane was broken by repeating freezing and de-freezing for three times. Then 1mL of water was added to de-freezing erythrocytes. After adding 10mL of acetone, the extract was filtrated. Then carotenoid was extracted with ether : hexane (2:8) solution by addition of water using separating funnel. After evaporation of ether : hexane (2:8) solution, the residue was dissolved in acetone : hexane (2:8) and was subjected to HPLC.

3. Results and Discussion

3.1 Identification of capsanthone in human plasma and erythrocytes

Figure 2 shows a normal phase HPLC of carotenoids in plasma (A) and erythrocytes (B). Carotenes (β -carotene was excited as main carotene), β -cryptoxanthin, cryptocapsin, cucurbitaxanthin A, lutein, zeaxanthin, and capsanthin were identified in both plasma and erythrocytes. HPLC of Peak-5 in human plasma is a minor carotenoid (**Figure 1 A**). Therefore, peak 5 in all plasma samples was collected by a normal phase HPLC system. Then this carotenoid was submitted to a reversed phase HPLC equipped with ESI TOF MS and PDA system to obtain MS, MS/MS, and

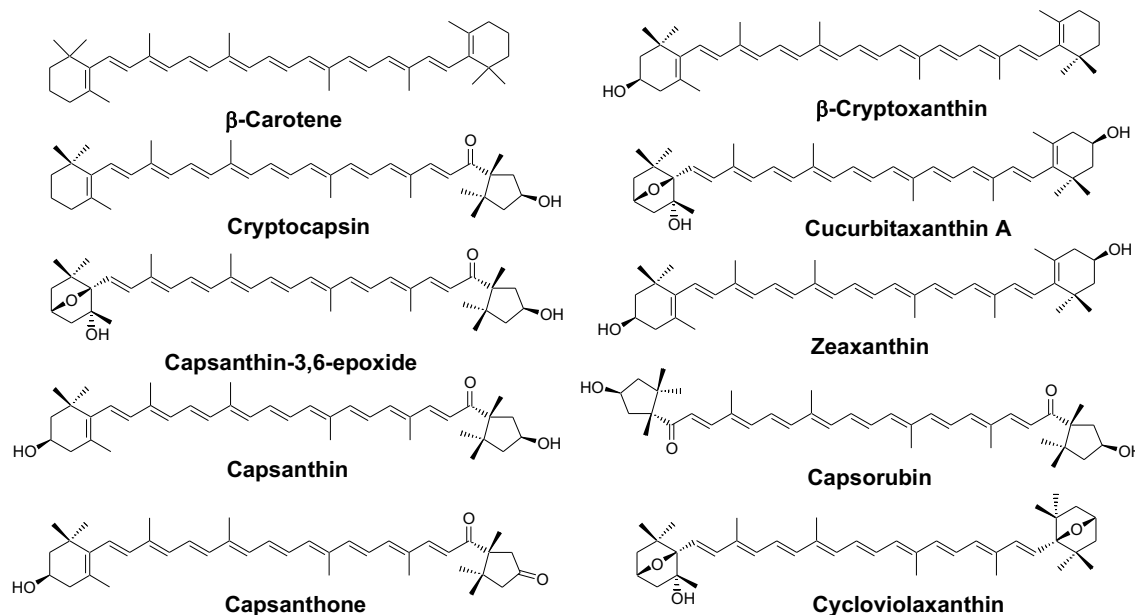


Figure 1. Structure of paprika carotenoids identified in human plasma and erythrocytes.

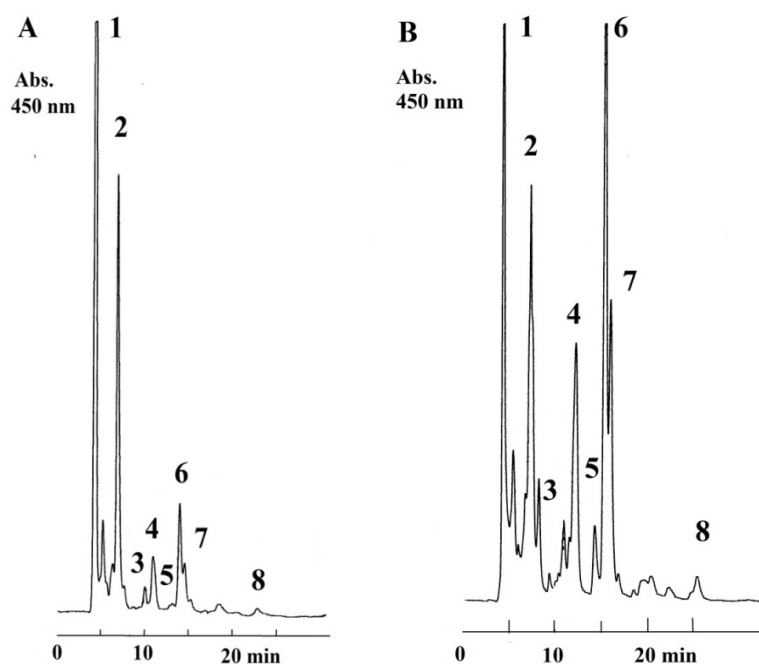


Figure 2. Normal phase HPLC of carotenoids in plasma (A) and erythrocytes (B). Peak identification: Peak-1 Total carotenoids, Peak-2 β -Cryptoxanthin, Peak-3 Cryptocapsin, Peak-4 Cucurbitaxanthin A, Peak-5 Capsanthone, Peak-6 Lutein, Peak-7 Zeaxanthin, Peak-8 Capsanthin. HPLC conditions are described in Material and Methods.

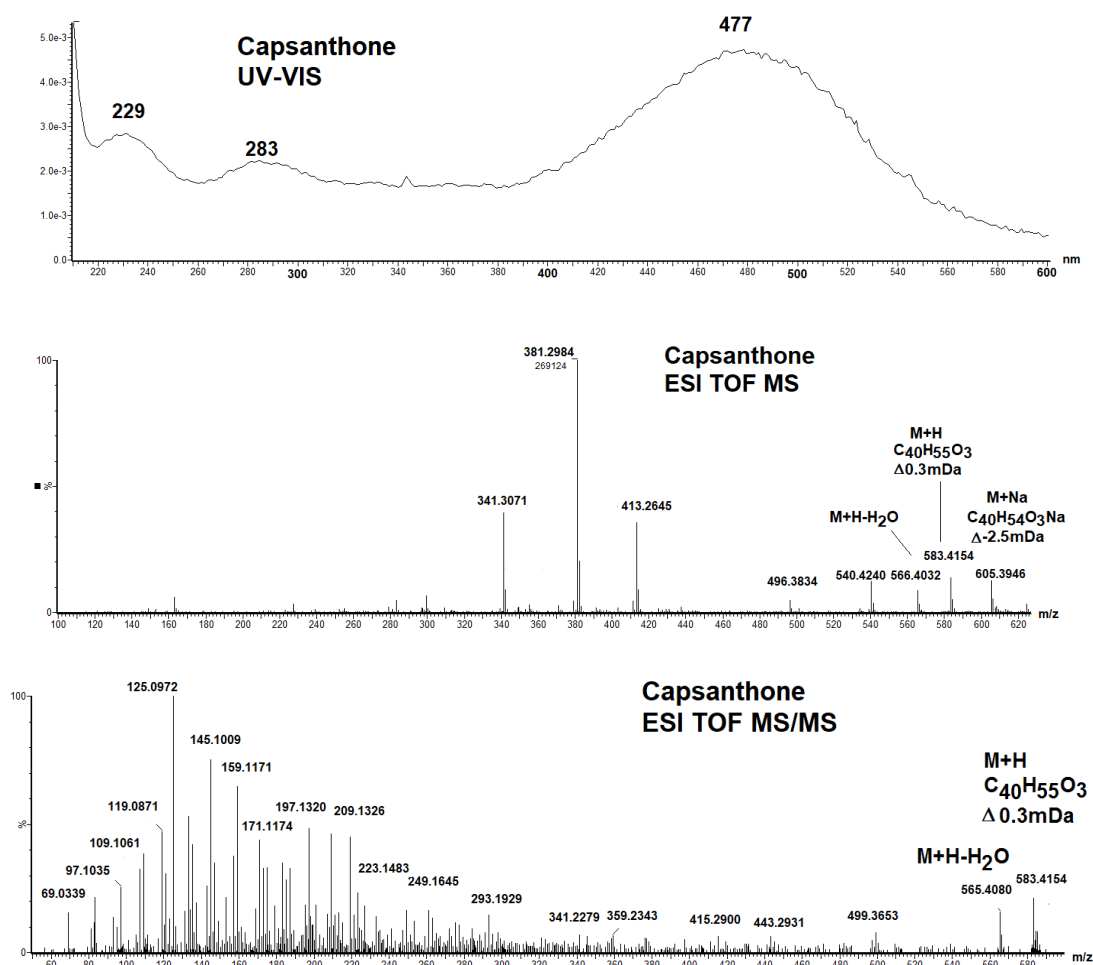


Figure 3. UV-VIS, ESI TOF MS, and ESI TOF MS/MS spectra of capsanthone in human plasma.

UV-VIS spectral data. Carotenoid in peak 5 showed UV-VIS (max) at 477nm (in mobile phase). The molecular formula of this carotenoid was determined to be $C_{40}H_{54}O_3$ (m/z 583.4154 $[M+H]^+$ $C_{40}H_{55}O_3$, Calc 583.4151, m/z 605.3946 $[M+Na]^+$ $C_{40}H_{54}O_3Na$, Calc. 605.3971 by ESI TOF MS as shown in **Figure 3**. MS/MS spectrum of this carotenoid (**Figure 3**) was identical with that of capsanthone. Furthermore, this carotenoid showed the same retention time as that of capsanthone authentic sample. The same result was obtained in the case of peak 5 in erythrocytes. In 2000, Etoh et al reported the presence of capsanthone by HPLC comparison of retention time with authentic sample in human plasma after the ingestion of paprika juice [7]. However, details of spectral data of capsanthone in human plasma were not reported. Therefore, this is the first report of the identification of capsanthone in human plasma and erythrocytes by MS, MS/MS and UV-VIS spectral data.

3.2 Accumulation ratio of capsanthone and capsanthin in human plasma and erythrocytes

In the present investigation, capsanthone, an oxidative product of capsanthin, was detected in both plasma (1.7~2.5% of the total carotenoid) and erythrocytes (8.6~7.1 % of the total carotenoid) after two and four weeks of paprika carotenoid supplementation. Capsanthone was also presented in paprika, but at a very low level (less than 0.5% of the total carotenoid). Therefore, it was assumed that part of ingested capsanthin was oxidatively metabolized to capsanthone in human.

Before supplementation of paprika carotenoid, both capsanthin and capsanthone were not detected in human plasma. After two weeks ingestion of paprika carotenoid, content of capsanthin and capsanthone in plasma were increased to 74.8 ± 33.7 pmol/mL and 39.6 ± 7.4 pmol/mL, respectively. After four weeks ingestion of paprika carotenoid, capsanthin content in plasma was slightly decreased to 70.1 ± 33.7 pmol/mL. On the other hand, capsanthone content in plasma was increased to 54.6 ± 12.6 pmol/mL. Accumulation ratio of capsanthin and capsanthone suggested that part of ingested capsanthin was metabolized to capsanthone in human as shown in **Figure 4**.

In the case of erythrocytes, both capsanthin and capsanthone were not detected in human erythrocytes before supplementation of paprika carotenoid. After two weeks ingestion of paprika carotenoid, content of capsanthin and capsanthone in erythrocytes were increased to 2.1 ± 0.8 pmol/mL and 7.5 ± 3.4 pmol/mL,

respectively. After four weeks ingestion of paprika carotenoid, content of capsanthin and capsorubin in erythrocytes were 3.4 ± 0.7 pmol/mL and 5.7 ± 0.4 pmol/mL, respectively. In erythrocytes, capsanthone was more incorporated than capsanthin. Capsanthone might have more affinity for incorporation to erythrocytes than capsanthin.

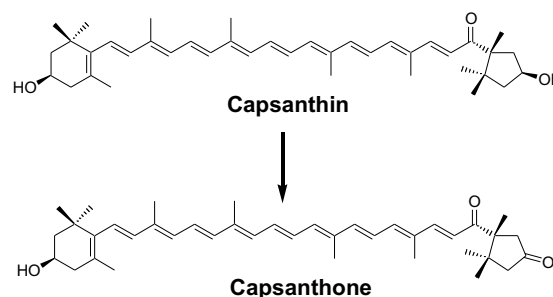


Figure 4. Oxidative metabolic pathway of capsanthin to capsanthone

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Study on the Structures and Formation of Marine Natural Products

Sachiko Tsukamoto^{1*}

¹ Department of Natural Medicines, Graduate School of Pharmaceutical Sciences,
Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.

Abstract: Himeic acid A (**1**), a pyrone derivative, was isolated as the ubiquitin-activating enzyme (E1)-specific inhibitor from the culture of the fungus *Aspergillus japonicus* MF275 along with its congeners, himeic acids B (**2**) and C (**3**). During culture, **1** was gradually converted to the corresponding 4-pyridone derivative **3**. Our study showed that a decrease from pH 6.5 to pH 2 in the culture is essential for production of **1**, while a subsequent increase to pH 5 is necessary for production of **3**. Further, **1** was non-enzymatically converted to **3** by the incorporation of an ammonium nitrogen atom in a pH 5 buffer, and **1** was converted to **2** at a conversion ratio of 50% in MeOH for five days but not in H₂O.

1. Introduction

From ancient times, we have been using plants for medicinal sources. In 1929, penicillin was discovered from the fungus as the first antibiotic, and at the present time marine invertebrates are used for drug development. In our laboratory, we search for the biologically active compounds as drug leads from marine organisms and microorganisms. Here, I show the structures and formations of biologically active second metabolites isolated from marine organisms.

2. Results and Discussions

The ubiquitin-proteasome pathway comprises the ubiquitin system and the proteasome. In the former ubiquitin system, the sequential actions of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), are required for the ubiquitination. Following FDA approval of the proteasome inhibitors for the treatment of relapsed multiple myeloma, the proteasome has become an emerging oncology target. In the ubiquitin-proteasome pathway, E1 activity is essential for the activation of ubiquitin, and therefore E1 inhibitors could also become drug leads for anti-cancer agents.

We screened the extracts of marine invertebrates and fungi collected in our laboratory for inhibition of the formation of the E1-ubiquitin intermediate. Then,

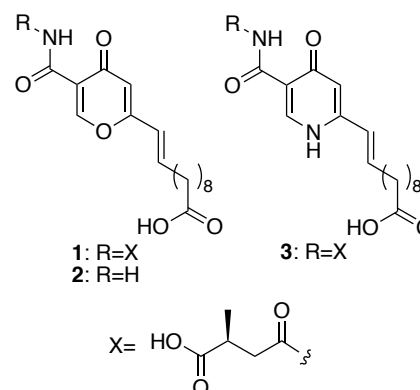


Fig. 1. Structures of himeic acids A–C (**1**–**3**).

we isolated a new 4-pyrone derivative, himeic acid A (**1**) (Fig. 1), as the E1 inhibitor from the culture of a marine-derived *Aspergillus japonicus* MF275 [1]. Although **1** showed 65% inhibition at 50 μ M, its congeners, himeic acids B (**2**) and C (**3**), did not inhibit even at 100 μ M, which suggested that both the pyrone ring and the substituent designated as X in Fig. 1 must be present for E1 inhibition. To study further the direct relationships among **1**–**3** with respect to their production, we explored the time-dependent metabolite profile. Interestingly, we found that one-week fungal culture yielded **1** as a main metabolite along with **3** as a minor metabolite, whereas two-week culture afforded **3** as a main metabolite with a small amount of **1**. These results suggested that **1** was gradually converted to **3** during the culture growth. We recently reported that **1** is a

* Corresponding author

e-mail: sachiko@kumamoto-u.ac.jp

metabolite produced by the PKS–NRPS pathway [2]. However, no gene responsible for the conversion of a pyrone moiety in **1** to a pyridone moiety in **3** was detectable in the *him* gene cluster.

In this study, we found that a decrease from pH 6.5 to pH 2 in the culture is essential for production of **1**, while a subsequent increase to pH 5 is necessary for production of **3** [3]. In addition, we revealed that **1** was non-enzymatically converted to **3** by the incorporation of an ammonium nitrogen atom in a pH 5 buffer, and that **1** was converted to **2** at a ratio of 50% during incubation in MeOH for five days but not in H₂O.

3. Conclusion

We here showed the interesting result that a non-enzymatic reaction was involved in the formation of himeic acid C (**3**). Such reactions occurred in nature consequently may extend the structural diversities of natural products.

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Conversion of solid biomass residue to functional materials and fuels using supercritical fluids and pulsed discharges

Mitsuru Sasaki^{1,2}

¹ Institute of Pulsed Power Science, Kumamoto University, ² Faculty of Advanced Science and Technology, Kumamoto University

The authors have succeeded in efficiently converting unused or residual biomass in useful components by converting molecules into molecular forms (chemical structures and molecular sizes) which functions are expressed, while taking advantage of their properties and chemical structures. We are promoting research aiming to create and improve technologies for effective utilization, as well as for creating high-performance technologies for producing chemical substances. As a technical seed for that purpose, the author is paying attention to supercritical fluid and pulse energy. In this paper, through practical examples, supercritical fluid and pulse energy are introduced for the purpose of recovering useful ingredients from solid residue biomass.

First of all, supercritical carbon dioxide (SC-CO₂) is mainly employed to extract or recover precious and essential oils and functional low-polarity useful compounds such as carotenoids, flavonoids and hydrocarbons from solid residual biomass feedstocks such as various kinds of citrus peels. Although the extraction efficiency (recovery rate) of essential oil on the SC-CO₂ extraction method is not high (about 70%) compared with that of the conventional steam distillation method (almost 100%), the SC-CO₂ extraction method can control the chemical composition of essential oils obtained by manipulating the operating temperature and pressure under the near- and supercritical state of carbon dioxide. As for slightly higher polarity species like flavonoids and polymethoxyflavonoids, it is possible to extract them by allowing several vol% of water or ethanol to coexist in SC-CO₂.

Secondly, protein, lipid, polysaccharides are still remaining in the solid residues obtained by the SC-CO₂ extraction treatment. Hydrolysis in subcritical water (Sub-H₂O) is effective for breaking peptide or glycosidic bond linkages of these dehydration condensation biopolymers to produce bioactive or highly-functionalized oligopeptides and oligosaccharides at temperatures ranging from 100 to 200°C under over-saturated pressure conditions. The solubilized aqueous solutions can be utilized as liquid fertilizer and so forth. On the other hand, as for the extraction and solubilization residues, which obtained after the SC-CO₂ extraction and Sub-H₂O treatment, has the skeletons of cellulose skeleton and lignin bridge structure in them. By effectively utilizing this structure, carbonization treatment can produce nano-sized carbon-based materials, which can be further used as an electrode for fuel cell, high capacity capacitor, or solid catalyst. Recently, we successfully prepared solid acid catalysts by using combination of gas-phase carbonization and hydrothermal ones. This method can be applied for the preparation of marine biomass such as seaweeds.

Finally, we can propose a novel method for decomposing lignin structure in woody biomass feedstocks by the pulsed discharge plasmas under ambient temperature and pressure. Especially, the nano-second pulsed “arc” discharge is effective for the C-C bond breaking in lignin structure due to the locally-high-temperature, high concentration of active species (*e.g.* OH radical and H₂O₂) as well as strong shockwave when discharged.

In summary, I would like to develop a new system that solid biomass waste can perfectly convert to value-added components and industrially applicable functional materials by using sub/supercritical fluids and pulsed discharge techniques. I believe that this system will be applied for the recycling of waste plastics, modification of heavy oil resource, and purification of contaminants in wastewater, so I would like to build a social system that reclaims solid wastes in the world as available resources in future.

Towards elucidation of light energy transfer mechanism of the photosynthetic supercomplexes from algae

Keisuke Kawakami^{1*}, Yuma Hirota², Yuhei Tahara³, Junko Shiomi³, Takashi Maoka⁴, Susumu Takio⁵, Makoto Miyata³, Nobuo Kamiya¹ and Daisuke Kosumi^{6*}

¹ The OCU Advanced Research Institute for Natural Science & Technology (OCARINA), Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka City, Osaka, Japan.

² Department of Physics, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto 860-0855, Japan.

³ Graduate School of Science, Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka City, Osaka, Japan.

⁴ Research Institute for Production Development, 15, Shimogamomorigoto-cho, Sakyo-ku, Kyoto, Japan.

⁵ Center for Marine Environment Studies, Kumamoto University, 39-1, Kurokami 2-chome, Chuo-ku, Kumamoto, Japan.

⁶ Institute of Pulsed Power Science (IPPS), Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto City, Kumamoto, Japan.

Plants and algae absorb sunlight energy and split water molecules, releasing oxygen molecules necessary for the living of aerobic organisms. They perform photoprotection utilizing light-harvesting antenna proteins under high light irradiation. Phycobilisomes (PBS) is one of the light-harvesting antenna proteins that transfer light energy to the photosynthetic membrane proteins (photosystem I and II; PSI and PSII) and the light-harvesting system in algae is controlled by the interaction between PBS and PSII (and PSI). To understand the detailed mechanism of the system, it is necessary to obtain the supercomplexes that interacted with PBS and PSI (and PSII). However, no purification method has been established that can obtain the intact supercomplexes in previous studies. In this study, we established a new preparation method that can purify the PBSs-PSI and PBSs-PSII supercomplexes from a thermophilic cyanobacterium and a red alga and then analyzed the energy transfer from PBS to PSII.

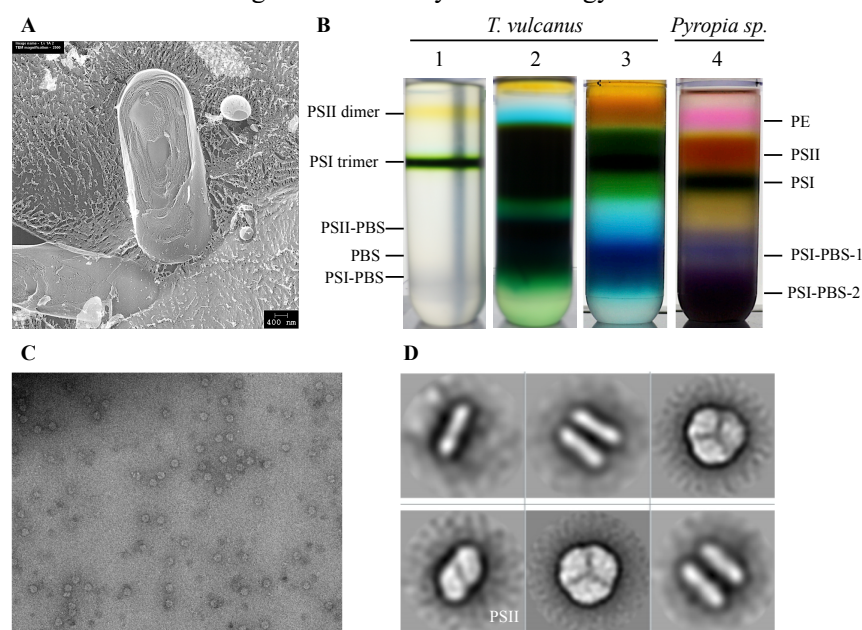


Figure 1. (A) Observation of a cell from *Thermosynechococcus vulcanus* (*T. vulcanus*) by a freeze-fracture technique. (B) Purification of the photosynthetic supercomplexes from *T. vulcanus* (lane 1–3) and *Pyropia sp.* (lane 4) by a trehalose density gradient. PSI, Photosystem I; PSII, Photosystem II; PBS, Phycobilisome; PE, Phycoerythrin. Lane 1, Purified PSI trimer by an anion exchange column chromatography; lane 2, the photosynthetic supercomplexes; lane 3, the purified photosynthetic supercomplexes obtained by a treatment different from that of lane 2; lane 4, the purified photosynthetic supercomplexes from *Pyropia sp.*. (C) An electron micrograph of PSI trimer from *T. vulcanus*. (D) Single particle analysis of the purified PSI trimer from *T. vulcanus*. A small amount of PSII dimers were detected by this analysis.

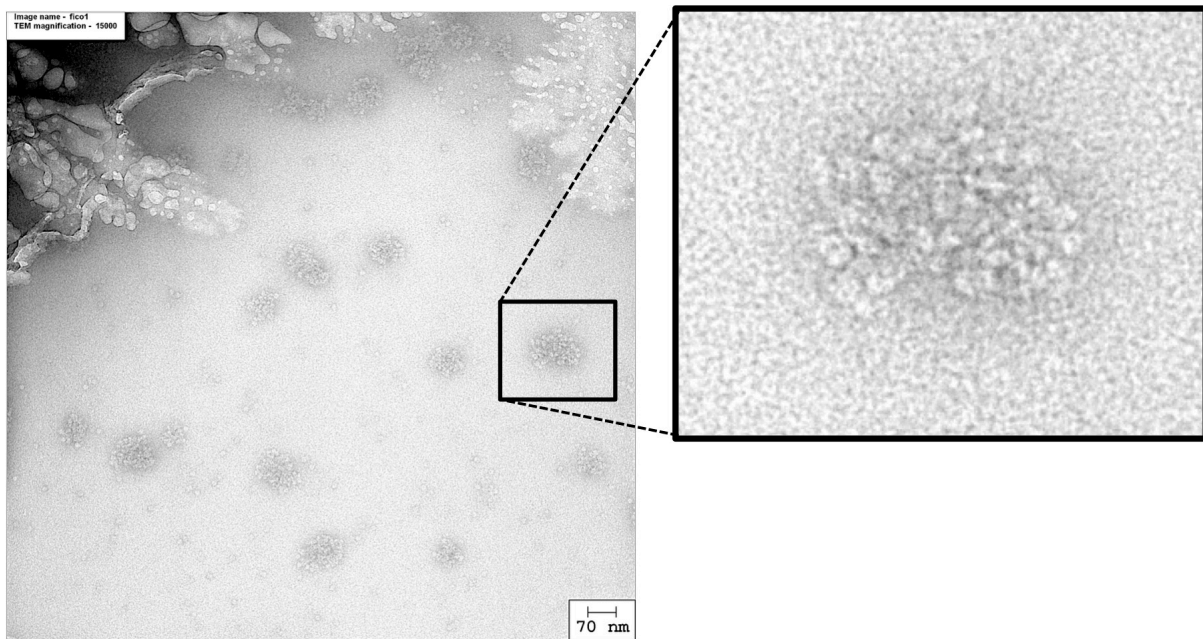


Figure 2. An electron micrograph of PBS from *T. vulcanus*. In the previous method, the intact PBS can not be obtained without using a buffer containing a high concentration phosphoric acid. However, the interactions of photosynthetic proteins with PBS are dissociated when the phosphate buffer is used. By a newly devised method, the photosynthetic supercomplexes can be obtained without using the phosphate buffer.

Enhanced Thermal Z-Isomerization of (all-*E*)-Lycopene by Some Foodstuffs

Masaki Honda^{1*}, Ryota Takemura², Tetsuya Fukaya²

¹ Faculty of Science & Technology, Meijo University, Shiogamaguchi, Tempaku-ku, Nagoya 468-8502, Japan. ² Innovation Division, Kagome Company, Limited, Nishitomiya, Nasushiobara 329-2762, Japan

Abstract: The effect of foodstuffs on thermal Z-isomerization of (all-*E*)-lycopene contained in tomato puree was investigated. When tomato puree was heated with olive oil and some foodstuffs such as *Allium* sp. and *Brassica* sp., the thermal Z-isomerization of (all-*E*)-lycopene was significantly promoted. Since Z-isomers of lycopene are more bioavailable and show a higher antioxidant capacity than the (all-*E*)-isomer, these findings will contribute to effective intake of lycopene.

1. Introduction

Lycopene is an acyclic carotenoid (C₄₀H₅₆) principally responsible for the bright-red color found abundantly in tomatoes. Recently, lycopene has been of interest due to its strong antioxidant properties as well as its potential anticancer and antiarteriosclerosis actions. While lycopene in fresh tomato is accumulated predominantly as the all-*E*-isomer, Z-isomers of lycopene are primarily found in the human body [1]. Several studies have reported that Z-isomers of lycopene are more bioavailable and show a higher antioxidant capacity than the (all-*E*)-isomer [2,3]. Thus, the intake of lycopene Z-isomers, rather than all-*E*-isomer, is preferable for human health, and appropriate methods are required to enable the efficient Z-isomerization of (all-*E*)-lycopene. Very recently, we revealed that a few foodstuffs such as garlic and sesame promoted Z-isomerization of (all-*E*)-lycopene [4,5]. In this study, we investigated foodstuffs, which were able to promote Z-isomerization of (all-*E*)-lycopene contained in tomato puree, other than garlic and sesame.

2. Experimental

2.1 Thermal isomerization of (all-*E*)-lycopene

Olive oil and each foodstuff were added to tomato puree, and the mixture was homogenized by a food processor. Then, the mixture was heated at 80 °C for 1 h. The Z-isomer ratio of lycopene after the heat

treatment was determined by normal-phase HPLC as previously described [7]

2.2 Clarification of components promoting thermal Z-isomerization of lycopene

The effect of characteristic compounds contained in foodstuffs that promoted Z-isomerization of (all-*E*)-lycopene on the thermal Z-isomerization was investigated.

3. Results and Discussions

When tomato puree was heated with olive oil and some foodstuffs such as *Allium* sp., *Brassica* sp., and *Diplotaxis* sp., the thermal Z-isomerization of (all-*E*)-lycopene was promoted. It revealed that some sulfur compounds such as diallyl disulfide promoted thermal Z-isomerization of lycopene.

4. Conclusion

Some foodstuffs such as garlic and onion promoted Z-isomerization of lycopene with cooking.

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* Corresponding author
e-mail: honda@meijo-u.ac.jp

Analysis of the excited energy-states of *cis*- β -Apo-8'-carotenal using time-resolved absorption spectroscopy

Kota Horiuchi¹, Chiasa Uragami¹, Taiki Inoue¹, Nao Yukihiro¹
and Hideki Hashimoto¹

¹ Department of Applied Chemistry for Environment, School of Science and Technology,
Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan.

1. Introduction

β -Apo-8'-carotenal is one of the carotenoids that has a carbonyl group coupled to its polyene backbone and has nine conjugated C=C double bonds. The light energy that is captured by carotenoids is transferred to chlorophylls in order to initiate photosynthetic light reaction. When carotenoids absorb photons, they are excited up to the second excited singlet state (S_2 state) from the singlet ground state (S_0 state). However it is recognized that carotenoids with carbonyl group generate the ICT (Intramolecular Charge Transfer) excited state.¹⁾ The ICT excited state is an excited energy-state that is very important to improve the efficiency of excitation energy-transfer from carotenoid to chlorophyll. It is confirmed that all-*trans*- β -Apo-8'-carotenal (see **Figure 1** for its chemical structure) generates the ICT excited state following photoexcitation when it is dissolved in polar organic solvents.¹⁾ In this study, we focused on the ICT excited state of *cis*- β -Apo-8'-carotenal. We isolated *cis*-isomers of β -Apo-8'-carotenal that were produced by photoisomerization and applied them femtosecond time-resolved absorption spectroscopic studies.

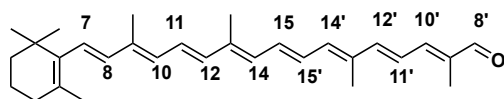


Figure 1. Chemical structure of all-*trans*- β -Apo-8'-carotenal

2. Experimental

To obtain *cis*-isomers of β -Apo-8'-carotenal, we applied photoisomerization of the all-*trans* isomer using iodine as a catalyst. 13'- and 9-*cis* isomers were isolated and purified by means of HPLC.²⁾ We dissolved 13'-*cis*- β -Apo-8'-carotenal in polar and nonpolar solvents (acetone, methanol, and *n*-hexane) and 9-*cis*- β -Apo-8'-carotenal in *n*-hexane. We recorded

femtosecond transient absorption spectra of these *cis*-isomers.

3. Results and Discussion

We found that the 13'-*cis* isomer in polar solvent generates the ICT excited state as has similarly been exemplified by the all-*trans* isomer. In the case of 9-*cis*-isomer, the steady-state absorption exists at the shorter wavelength region than that of the all-*trans* isomer. However, as shown in **Figure 2**, the transient absorption band that represents $S_1 \rightarrow S_N$ transition of 9-*cis* isomer in *n*-hexane is redshifted compared with that of the all-*trans* isomer. From this observation, it is suggested that the energy gap between S_2 and S_0 in the 9-*cis* isomer is larger than that of all-*trans*, but the gap between S_1 and S_N in 9-*cis* is smaller than all-*trans*.

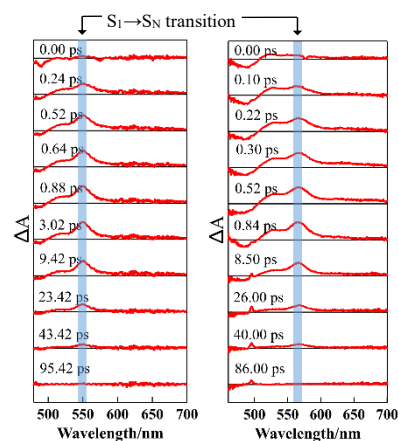


Figure 2. Femtosecond time-resolved absorption spectra of all-*trans* and 9-*cis*- β -Apo-8'-carotenal in *n*-hexane at room temperature

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High-yield production of carotenoid in *Escherichia coli* via re-engineered non-mevalonate pathway

Katsuya Minakata, Tashiro Miki, Shigeko Kawai-Noma and Daisuke Umeno*

*Department of Applied Chemistry and Biotechnology, Graduate school of Technology,
Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan.*

Years of research efforts have markedly improved the amount and types of carotenoids that can be produced by recombinant *Escherichia coli*. Full installation of exogenous Mevalonate pathway has been the gold standard for successfully improving various terpenoids, while the fortification of endogenous non-Mevalonate pathway has been largely unexplored to date. Simple overexpression of the enzymes therein (dxs, dxr, ispDEFGH) does not elevate production level of terpenoids as much. Motivated by high theoretical yield of carotenoid precursors IPP/DMAPP (per glucose), we decided to reconstruct endogenous non-mevalonate pathways on single plasmid, by step-by-step manner. In this presentation, we report the current status of this reconstruction project. Included are: (1) Directed evolution of 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (IspH) using newly developed screening method: from the library of IspH prepared by error-prone pCR, we isolated number of IspH mutants that alone can significantly improve the lycopene production level by three folds. (2) Additional overexpression of 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (IspG) and 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), the enzyme that has been regarded as the rate-limiting step of the non-Mevalonate pathway. (3) Additional expression of the NADH-utilizing mutant of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr). We observed step-by-step increase in the level of lycoepe production up to 3mg-g dry cell weight/24h in flask culture.

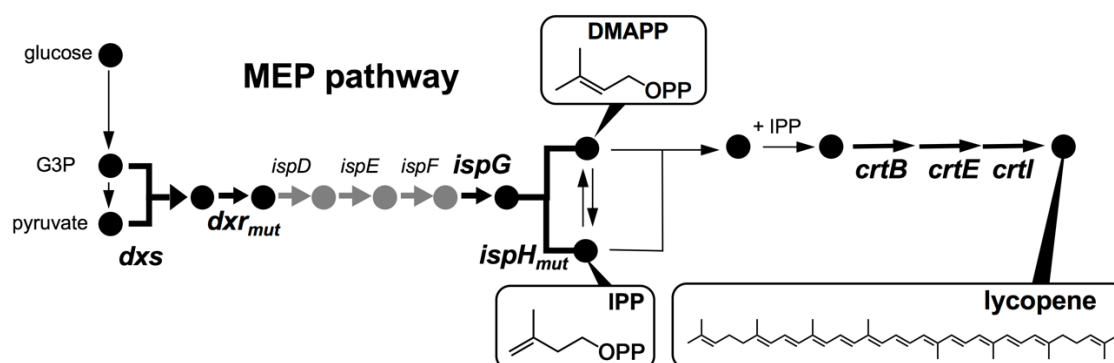


Figure. 1. Re-engineering mevalonate pathway of *Escherichia coli* for lycopene production

* Corresponding author
e-mail: umeno@faculty.chiba-u.jp

Comparison of early stages of carotenogenesis among phototrophs

Shinichi Takaichi¹, Jiro Harada², Kenjiro Sugiyama³

¹Department of Molecular Microbiology, Faculty of Life Science, Tokyo University of Agriculture, Sakuragaoka, Setagaya, Tokyo, 156-8502, Japan

²Department of Biochemistry, Kurume University School of Medicine, Fukuoka 830-0011, Japan

³Department of Applied Chemistry, Kogakuin University, Hachioji, Tokyo, 192-0015, Japan

Phototrophs including anoxygenic and oxygenic phototrophs contain many kinds of carotenoids. Usually, most carotenoids are derived from lycopene [1,2]. Exceptionally, heliobacteria have acyclic C₃₀-carotene, and some genera of purple bacteria have derivatives of neurosporene. At first, two pathways are known from phytoene to lycopene. (1) Phytoene is desaturated by one enzyme of phytoene desaturase (CrtI) in purple bacteria, filamentous anoxygenic phototrophic bacteria (Harada et al., unpublished) and *Gemmatimonas* (genome), which have PSII-type photosystem. (2) On the other hand, green sulfur bacteria and *Chloracidobacterium* (genome), which have PSI-type photosystem, need three enzymes, phytoene desaturase (CrtP), ζ -carotene desaturase (CrtQ) and *cis*-carotene isomerase (CrtH), but ζ -carotene isomerase gene (*Z-iso*) is absent in the genome. Further, cyanobacteria and plants, which have PSII- and PSI-type photosystems, need four enzymes: CrtP/PDS, CrtQ/ZDS, Z-ISO, and CrtH/CrtISO (Sugiyama et al. unpublished). Most phototrophs produce β - and/or γ -carotene, and their derivatives. *Okenone* producing purple bacteria has *crtY*-type lycopene cyclase from the genome, *Chloroflexus* has functional CrtY (Harada et al. unpublished), and plants have homologous CrtY/CrtL/LCY. *Chlorobaculum* has functional CruA [3], and *Chloracidobacterium* has *crtYc+crtYd* and *cruA* homologous genes. On the other hand, two cyanobacteria (*Synechococcus* PCC 7942 and *Prochlorococcus marinus* MED4) have functional CrtL, and three (*Synechococcus* PCC 7002, *Arthrospira platensis* and *Synechocystis* PCC 6803) have functional CruA and CruP. Consequently, photosynthetic bacteria with PSII-type photosystem have CrtI-type phytoene desaturase and CrtY-type lycopene cyclase, whereas those with PSI-type photosystem have three enzymes for lycopene synthesis and CruA-type lycopene cyclase. On the other hand, cyanobacteria have four enzymes for lycopene synthesis, and lycopene cyclases are not sure. We will discuss about comparison of carotenogenesis pathways among phototrophs.

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This is an abstract of the 32th Annual Meeting on Carotenoid Research, on September 15-16, 2018 at Kumamoto, Japan.

A part of contents has been presented on International Conference on Microbial Photosynthesis, on August 9-12, 2018 at Vancouver, Canada.

This will be published as a research article.

Light Harvesting Function of LH1 Complex Reconstituted with Fucoxanthin

Masayuki Yoshizawa^{1*}, Ren Sato¹, Martin R. Steinell¹, Nao Yukihiro², Chiasa Urugami²
and Hideki Hashimoto²

¹ Department of Physics, Graduate School of Science, Tohoku University,
6-3 Aramaki-aza-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan.

² Department of Applied Chemistry for Environment, School of Science and Technology,
Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1338, Japan.

Abstract: We have applied femtosecond spectroscopy to light-harvesting complex reconstituted with fucoxanthin. Fucoxanthin has intramolecular charge transfer state (ICT) additional to the S_2 and S_1 excited states. Dynamics of excitation energy transfer and a role of ICT have been investigated.

Carotenoids play important roles in photosynthesis. In the antenna complexes, they act as light-harvesting and photo-protecting pigments. Light in blue-green region is absorbed by carotenoids and the energy is transferred to bacteriochlorophyll (BChl) with high efficiency. We have reported dynamics of excitation energy transfer (EET) in LH1 reconstituted with different carotenoids [1]. EET occurs both from the S_2 and S_1 excited states. The EET rate depends on conjugation length of the carotenoids.

In this study, we have investigated EET dynamics in LH1 reconstituted with fucoxanthin, LH1(Fx), using tunable pump pulses. EET in LH1(Fx) has been already reported [2], but the detailed dynamics has not been clear. Figure 1 (a) shows transient absorbance change following the 490 nm pump which excites S_2 in fucoxanthin. Signal of S_2 appears around 900 nm just after excitation. Signal of S_1 /ICT is observed in visible region. Additionally, bleach of Q_y in BChl appears at 890 nm. It means that EET from fucoxanthin to BChl occurs effectively in LH1(Fx). On the other hand, Figure 1(b) shows that signal of fucoxanthin does not appear after excitation of Q_x in BChl by the 590 nm pump. The reverse EET from BChl does not occur in LH1(Fx).

The EET dynamics in LH1(Fx) and the dependence on pump wavelength are going to be discussed more in detail.

* Corresponding author

e-mail: m-yoshizawa@m.tohoku.ac.jp

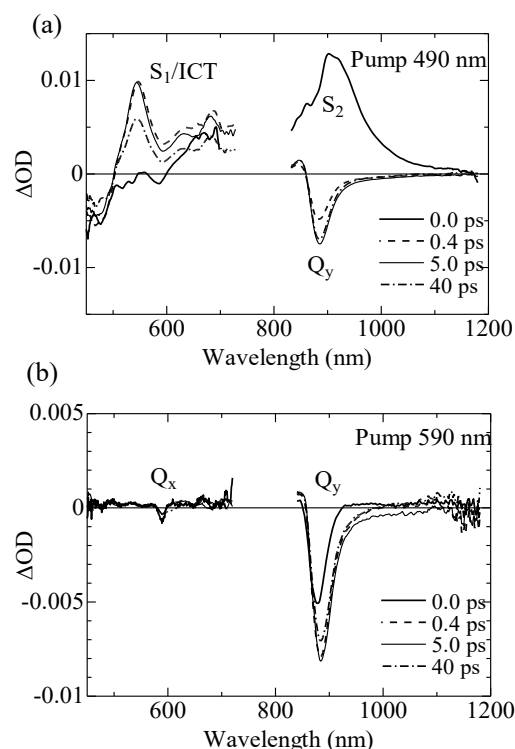


Fig.1 Transient absorbance change of LH1(Fx).
(a) Car S_2 pump, (b) BChl Q_x pump.

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Relationship between *Oryzias* fish body coloration and food resources

K Matsui¹, K Mochida², T Sugiuchi¹, A Fukuda¹, S Fujimoto³, J Kitano⁴, A Ishikawa⁴, D. F. Mokodongan⁵, I. F. Mandagi⁶, A. K. W. Masengi⁶, R. K. Hadiaty⁷ and T Yamahira³

Azabu Univ.¹, Keio Univ.², Ryukyu Univ.³, NIG⁴, Sam Ratulangi Univ.⁵, LIPI⁶

Sulawesi island of Indonesia have an endemic group of *Oryzias* genus (family) fish, its external form and diversity of body color are reported. Once we reported the difference of carotenoids consistent for the body coloration among four populations of *O. woworae* species complex. Unfortunately, the identification of the correct components has not been reached yet. *O. woworae* has a red fins in blueish body and the red color of its fins. It is associated with carotenoids, mostly are carotenoid esters. The carotenoids of wild *O. woworae* composed of so-called "red" carotenoids and carotenoid esters, at 470 nm with peaks of its absorption spectra. Its composition was estimated to be astaxanthin, adonixanthin, 3-OH echinenone and those esters from the results of HPLC and ESI-MS / MS. "Yellow" carotenoid presumed to be a lutein diester was detected from 70 minutes to 90 minutes of analysis but it was shown that the "red" carotenoid occupies a large proportion of its existence ratio.

On the other hand, *O. profundicola* and *O. marmoratus*, which are closely related species living in Sulawesi island, also have a yellow body coloration. The HPLC chromatogram of carotenoid extracted from *O. profundicola* contained a small amount of astaxanthin, lutein and β -carotene, but most of them were lutein esters.

Also, free astaxanthin, lutein, canthaxanthin, and echinenone were detected in HPLC from *O. woworae* reared from eggs with brine shrimps as food. However, the carotenoid composition of *O. marmoratus*, which was reared with brine shrimps, did not form a clear peak in HPLC, detected a small amount of carotenoid free form at MS level, this was clearly difference to those results of *O. woworae*.

Based on the above, the genus *Oryzias* inhabiting Sulawesi island is adapted for the body coloration which develops color by different carotenoid composition, and the difference is due to the difference in the carotenoid resource composition in the food resources, the inheritance of their carotenoid absorption / metabolism. It was shown that the difference may also be affecting. In this presentation, we will consider differences in food resources caused by differences in their habitat in addition to these results.

Investigation of Intramolecular Charge-Transfer Character of β -Apo-8'-carotenal using Stark Absorption Spectroscopy

Taiki Inoue¹, Shota Seto², Tomoko Horibe², Kota Horiuchi¹, Chiasa Uragami¹, Hideki Hashimoto¹

¹Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1338, Japan.

²Fuji Chemical Co. Ltd., 55 Yokohoonji, Kamiichi, Nakaniikawa, Toyama 930-0397, Japan

Carotenoids are natural pigments that serve important roles in the process of natural photosynthesis. They contribute to an efficient energy conversion by absorbing blue to green region of sunlight in which chlorophylls can hardly absorb. An energy state called intramolecular charge transfer (ICT) excited state is required in realizing a highly efficient energy transfer from carotenoids to chlorophylls in the primary process of photosynthesis. Carotenoids that show strong ICT character upon photoexcitation are expected to have high energy-transfer efficiency in photosynthetic light-harvesting. The strength of ICT character upon photoexcitation can be estimated by measuring the static dipole-moment change upon photoexcitation ($|\Delta\mu|$). For example, a carotenoid peridinin was found to have a large (20-30 Debye) ¹⁾ $|\Delta\mu|$ and the energy-transfer efficiency from peridinin to chlorophyll *a* in the natural light-harvesting system from dinoflagellates is reported to be higher than 90%.²⁾ In this study, we focused on one of the carotenoids, β -Apo-8'-carotenal. The chemical structure of β -Apo-8'-carotenal contains an aldehyde group attached to the conjugated polyene system, therefore, it is expected to have an ICT character in the excited state.³⁾ In this study, we conducted measurements with all-*trans* and 13-*cis* isomers of β -Apo-8'-carotenal to obtain a better understanding between the ICT character of a carotenoid and its geometric structure.

13-*cis* isomer was produced by photo-isomerization of all-*trans* isomer. From the steady-state and Stark absorption spectra, the presence of an ICT excited state of β -Apo-8'-carotenal was confirmed. $|\Delta\mu|$ and the angle ($\theta_{\Delta\mu}$) between $\Delta\mu$ and transition dipole-moment of all-*trans* and 13-*cis* isomer were successfully determined (**Figure 1**). All-*trans* isomer has a larger $|\Delta\mu|$ and a smaller $\theta_{\Delta\mu}$

compared with those of 13-*cis* isomer. From these results, we conclude that there is a possibility that $|\Delta\mu|$ becomes larger as $\theta_{\Delta\mu}$ approaches 0°.

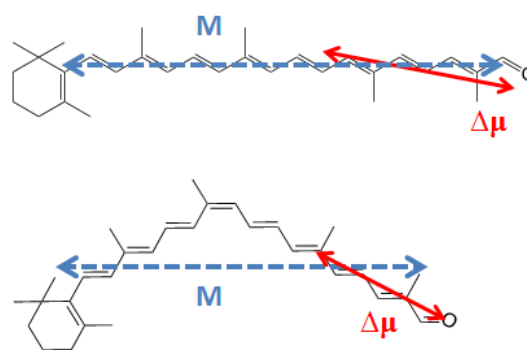


Figure 1. Static dipole-moment change upon photoexcitation ($\Delta\mu$) and its direction against transition dipole-moment (M) of all-*trans* (left) and 13-*cis* (right) isomers of β -Apo-8'-carotenal

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Defect of photosynthetic activity in the *cruC* gene mutant of the green sulfur bacterium *Chlorobaculum tepidum*, which lost the ability to synthesize carotenoid glucoside ester

Jiro Harada¹, Chihiro Azai², Taku Inoue², Shogo Fujimoto³, Shinji Masuda⁴
and Daisuke Kosumi^{3,5}

¹*Department of Medical Biochemistry, Kurume University School of Medicine, Kurume, Fukuoka 830-001, Japan.*

²*College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 522-8577, Japan.*

³*Graduate School of Science and Technology, Department of Physics, Kumamoto University, Chuo-ku, Kumamoto 860-8555, Japan.*

⁴*Center for Biological Resources and Informatics, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan.*

⁵*Institute of Pulsed Power Science, Kumamoto University, Chuo-ku, Kumamoto 860-8555, Japan.*

Carotenoid is a photosynthetic pigment used for harvesting or dissipation of light energy in pigment-protein complexes in all the photosynthetic organisms. There is an enormous range of carotenoid species; most of them are well characterized chemically and spectroscopically by photochemical property and photosynthetic function. Some of carotenoid are glycosylated at one or two edges of the polyene backbone mostly with glucoside or its fatty acid ester. However, the carotenoid glycosides have remained virtually unknown about their function, especially effect of the glycosylation on photosynthesis. OH- γ -carotene glucoside ester and OH-chlorobactene glucoside ester are carotenoid glycosides bound specifically to the photosynthetic reaction center complex of the green sulfur bacterium *Chlorobaculum tepidum* [1]. Other carotenoids of *C. tepidum* are not glycosylated and not bound to the RC complex the glycosylations of OH- γ -carotene and OH-chlorobactene would thus be required specifically for some RC functions. In this study, we constructed a *C. tepidum* mutant strain, Δ *cruC*, in which the *cruC* gene encoding glycosyl transferase of carotenoid [2] is insertionally inactivated. Photosynthetic function of the glycosylation was explored by fluorescence lifetime analysis in the Δ *cruC* mutant using ultrafast spectroscopy. When the most upper antenna system, chlorosome, in the *Chlorobaculum tepidum* cell was excited dominantly, the mutational effect was observed on fluorescence decays only at longer wavelength regions attributable to energy transfers to

bacteriochlorophylls *a* in the reaction center complex. The glycosylated carotenoids would have a crucial role for effective quench of excess excitation energy in the *Chlorobaculum tepidum* reaction center complex.

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Suppression process of the generation of triplet bacteriochlorophyll *a* by carotenoid in the LH1 antenna pigment-protein complexes from a purple photosynthetic bacterium *Rhodospirillum rubrum*

Hiroki Sato^{1*}, Nao Yukihiro¹, Chiasa Uragami¹, Masazumi Fujiwara², Alastair T. Gardiner³
Richard J. Cogdell³ and Hideki Hashimoto¹

¹ Department of Applied Chemistry for Environment, School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan.

² Department of Molecular Materials Science, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan.

³ Institute of Molecular, Cell and Systems Biology, College of Medical Veterinary and life Sciences, University of Glasgow, Avenue G12 8QQ, Glasgow, UK.

1. Introduction and Experimental

In photosynthesis of purple photosynthetic bacteria, carotenoid plays an important role in light-harvesting, which can efficiently absorb light around 500 nm spectral region where the solar radiation density is the most abundant. On the other hand, when the excess amount of light is irradiated to the photosynthetic system, triplet excited state of Bacteriochlorophyll (Bchl) *a* is spontaneously generated. The triplet Bchl *a* causes the generation of harmful singlet oxygen under aerobic and semi-aerobic conditions, which leads to the destruction of biological systems. To prevent this carotenoid inactivates triplet Bchl *a* and singlet oxygen by triplet-triplet excitation energy transfer. The triplet carotenoid thus generated safely dissipates its energy to the surroundings as heat [1]. This process is called as the photoprotective function of carotenoid. This presented work is mainly concerned with this process.

In the recent work we have reported the photoprotective mechanisms of a carotenoid spheroidene in the peripheral LH2 system from *Rba. sphaeroides* strain 2.4.1. using time-resolved absorption spectroscopy in the wide temporal range from 100 femtoseconds to sub-millisecond with high enough time resolution [2]. In this study we have extended this work and investigated the similar but distinct processes in the LH systems from *Rsp. rubrum* strain S1. *Rsp. rubrum* is a curious organism that has only LH1 antenna system, in which carotenoid spirilloxanthin is bound to, although *Rba. sphaeroides* has both LH2 and LH1. The experiments have also

been performed on the carotenoidless strain G9+ of *Rsp. rubrum* in order to make a good comparison for the LH systems with or without carotenoid.

2. Results

We confirmed that in the case of *Rsp. rubrum* strain S1 triplet Bchl *a* is successfully inactivated with the aid of carotenoid, while that in the case of strain G9+ triplet Bchl *a* was generated by excess light exposure, which decays extremely slowly. In addition, as a result of comparison with the resent work, it was suggested that LH1 shows energy dissipating ability superior to LH2.

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Evaluating the carotenoid-solvent interaction by theoretical simulation of steady state absorption spectra

Chiasa Uragami¹, Keisuke Saito², Masayuki Yoshizawa³, Péter Molnár⁴, Hideki Hashimoto¹

¹*School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hogo, 669-1337 Japan*

²*School of Engineering, The University of Tokyo, 4-6-1, Komaba, Meguro-ku, Tokyo, 153-8904, Japan*

³*Graduate School of Science, Tohoku University, 6-3 Aramaki-aza-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan*

⁴*Department of Pharmacognosy, University of Pécs, Medical School, Szigeti str 12, H-7624 Pécs, Rokos u. 2, Hungary*

The chemical structure of carotenoid consists of three components which is, isoprenoid chain, end-ring group and functional group. From this simple conformation, one could think that the electronic feature of carotenoid is easily estimated. If we focus on only to the isoprenoid chain of carotenoids, it is known that there is a correlation between the effective conjugation length and the electronic energy state. This correlation shows that the electronic feature of carotenoid develops a tendency to follow the estimate of the modern molecular physics [1, 2]. However, as it is shown in **Figure 1**, even carotenoids which has same effective conjugation length, the vibrational structure of absorption spectra is completely different. This shows the difficulty of estimating the absorption spectra of carotenoids. To estimate and understand the electronic feature of carotenoids, there is still lack of information.

One of the easiest ways to investigate the electronic character of carotenoid is to measure the absorption spectra. Even though, simulating the absorption spectra from resonance Raman spectra is also an effective way. From this reason, we simulated the optical absorption spectra of 15 different kinds of carotenoids using the values which is obtained from the resonance Raman spectra. Every Raman spectra was measured at room temperature with tetrahydrofuran (THF) solution. Whole shape of absorption spectra was successfully simulated for all carotenoids using stochastic model based on the Brownian oscillator theory.

In this presentation, we will show the quantification of interaction between carotenoid molecules and the THF molecules using the parameters obtained from the simulation.

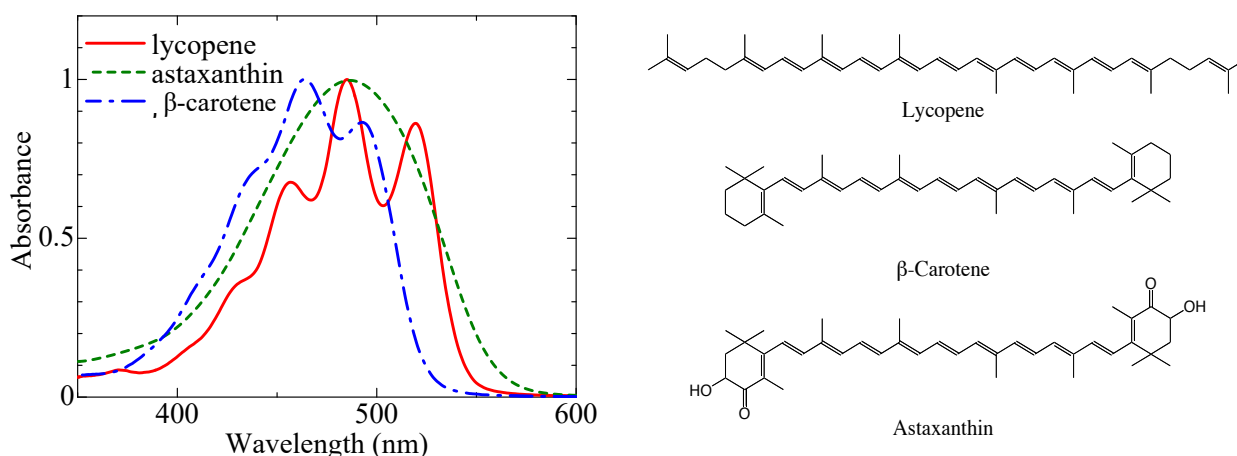


Figure 1. The absorption spectra (left) and the chemical structure of carotenoids (right).

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Isolation and functional analysis of carotenoid biosynthesis genes from human gut and probiotic bacteria

Wataru Matsumoto¹, Haruka Nanaura¹, Miho Takemura¹, Takashi Maoka² and Norihiko Misawa^{1*}

¹Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University,

1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

²Research Institute for Production Development,

15 Shimogamomorimotocho, Sakyo, Kyoto 606-0805, Japan

Abstract: Some human gut and probiotic bacteria can produce C30 carotenoids, while a little is known about their carotenoid biosynthesis. In this study, we isolated carotenoid biosynthesis genes from several human gut and probiotic bacteria, and analyzed them using an *Escherichia coli* functional expression system. It was consequently revealed that proteins encoded by the *phytoene desaturase-like* (*pdl*) and *4,4'-diapophytoene synthase-like* (*dsl*) genes derived from *Leuconostoc mesenteroides* possess CrtN (4,4'-diapophytoene desaturase) and CrtM (4,4'-diapophytoene synthase) activities, respectively.

1. Introduction

More than 750 carotenoids have been isolated from the nature so far, and many of them possess C40 basic backbone. On the other hand, some human gut and probiotic bacteria can produce carotenoids of C30 basic backbone (C30 carotenoids), e.g., *Lactobacillus plantarum* was reported to produce C30 carotenoids such as 4,4'-diaponeurosporene [1]. Whereas, a little is known about carotenoid biosynthesis in the intestinal bacterial flora, since they are often difficult to cultivate, and their carotenoid levels always remain a limited amount. We have analyzed the functions of carotenoid biosynthesis genes using carotenoid substrates-accumulating *Escherichia coli*, one of the most suitable hosts for carotenoid biosynthesis research. In this study, we tried to analyze the functions of carotenoid biosynthesis genes isolated from human gut and probiotic bacteria.

2. Experimental

2.1 Search for carotenoid biosynthesis genes from genomic DNA of human gut and probiotic bacteria

We searched for carotenoid biosynthesis genes in human gut and probiotic bacteria using BLAST with genome DNA database in NCBI (<https://www.ncbi.nlm.nih.gov/>).

2.2 Isolation of carotenoid biosynthesis genes by PCR

Genome DNA was extracted from human gut and probiotic bacteria, which were thought to possess carotenoid biosynthesis genes. We designed primers to isolate phytoene desaturase-like (*pdl*), 4,4'-diapophytoene synthase-like (*dsl*), and geranylgeranyl diphosphate synthase-like (*gsl*) genes. Using these primers, we performed PCR cloning.

2.3 Expression of carotenoid biosynthesis genes in recombinant *E. coli*

The *pdl* and *dsl* genes were inserted into pUC18, and introduced into WT, phytoene-producing, or 4,4'-diapophytoene-producing *E. coli* (JM101 (DE3)).

2.4 Growth condition of recombinant *E. coli* for carotenoid analysis

Each recombinant *E. coli* grew in 2YT medium at 37 °C overnight. Then, we inoculated it into new 2YT medium and cultured at 20 °C for 48 h.

2.5 Carotenoid extraction from *E. coli*, and HPLC-PDA analysis

Carotenoids were extracted from recombinant *E. coli* with chloroform/methanol (1/1), dried by centrifugal evaporation, and then suspended with ethyl acetate.

HPLC-PDA analysis was performed using Waters Alliance 2695-2996 system with a photodiode array detector [2].

* Corresponding author: Norihiko Misawa
e-mail: n-misawa@ishikawa-pu.ac.jp

3. Results and Discussions

3.1 Discovery of phytoene desaturase-like gene from several gut and probiotic bacteria

We found the *crtM* and *crtN* genes in *Lactobacillus plantarum*, and *pdl* in *Bacteroides ovatus*, *Clostridium perfringens*, *Leuconostoc mesenteroides*, and *Parabacteroides distasonis*. We also found *dsl* in *L. mesenteroides*, and *gsl* in *L. plantarum* and *L. mesenteroides*.

3.2 PDL of *L. mesenteroides* has CrtN activity

CrtM and CrtN of *L. plantarum* were found to have 4,4'-diapophytoene synthase and 4,4'-diapophytoene desaturase activities, respectively, as reported [1]. Among the *pdl* gene products (PDLs), the *L. mesenteroides* PDL was observed to convert 4,4'-diapophytoene into 4,4'-diaponeurosporene, indicating that it has CrtN activity (Fig 1). On the other hand, the PDLs of *B. ovatus*, *C. perfringens*, and *P. distasonis* didn't show any CrtN activity.

3.3 DSL of *L. mesenteroides* has CrtM activity

The *dsl* gene product (DSL) of *L. mesenteroides* converted farnesyl diphosphate (FPP) into 4,4'-diapophytoene. This indicates that it has CrtM activity.

4. Conclusion

PDL and DSL of *L. mesenteroides* were found to have CrtN and CrtM activities, respectively. This suggests that *L. mesenteroides* can biosynthesize C30 carotenoids.

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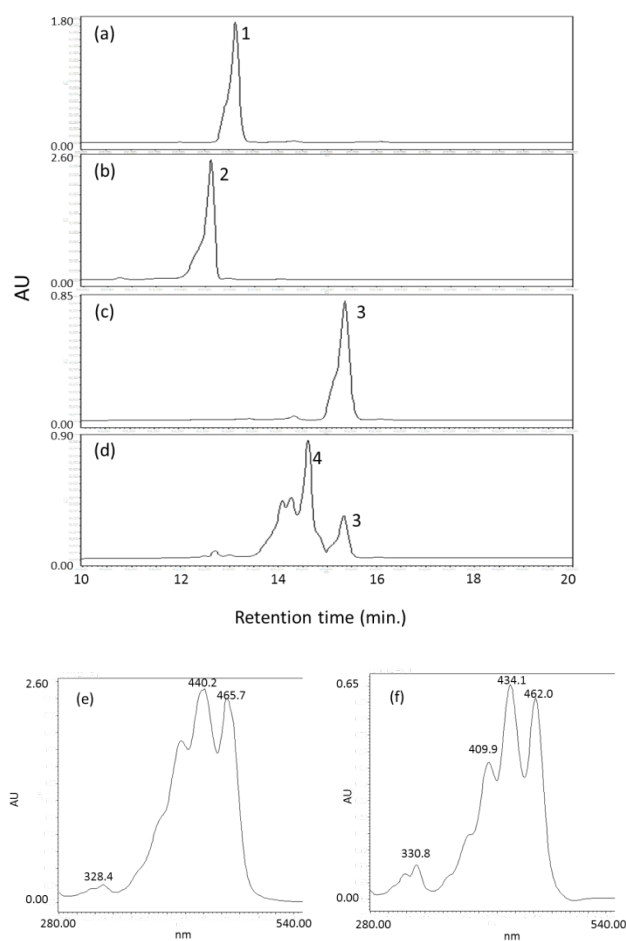


Fig. 1 HPLC analysis of carotenoids produced by the recombinant *E. coli*. HPLC chromatograms of the extracts from *E. coli* that carried (a) *crtM* of *L. plantarum*; (b) *crtM* of *L. plantarum* plus *pdl* of *L. mesenteroides*; (c) *crtE* and *crtB* of *Pantoea ananatis*; (d) *crtE* and *crtB* of *P. ananatis* plus *pdl* of *L. mesenteroides*. 1, 4,4'-diapophytoene; 2, 4,4'-diaponeurosporene; 3, phytoene; 4, neurosporene. UV-visible absorbance spectra of carotenoids produced by the recombinant *E. coli*. (e) peak2; (f) peak4.

Analysis of Anti-Inflammatory Profile of Siphonaxanthin

Yuki Manabe, Nami Tomonaga and Tatsuya Sugawara

*Graduate School of Agriculture, Kyoto University
Kitashirakawa Oiwake-cho, Sakyo, Kyoto, 606-8502, Japan.*

Anti-inflammatory activities of siphonaxanthin were evaluated using monocyte/macrophage models. Siphonaxanthin significantly suppressed both NF- κ B and IRFs activation induced by lipopolysaccharide, triacyl lipopeptide, TNF- α , IL-1 β , and advanced glycation end products. It also suppressed double strand RNA- and IFN- γ -induced IRFs activation. We also evaluated and compared the anti-inflammatory activities of the other carotenoids. As a result, we found that anti-inflammatory profiles of siphonaxanthin and fucoxanthin resemble with each other. Since a carbonyl group at C-8 position is a unique common chemical structure of them, it may be an important for their characteristic profiles. The anti-inflammatory profile of tauroursodeoxycholate, a well-known inhibitor of ER stress, also resemble with that of siphonaxanthin, and moreover, siphonaxanthin significantly inhibited lipopolysaccharide-induced mRNA expression of ER stress marker genes. Taken together, a relief of ER stress could be one of the mechanisms by which siphonaxanthin shows the anti-inflammatory activity.

ζ-Carotene Isomerase (Z-ISO) in Oxygenic Phototrophs

Keisuke Nakazawa¹, Shinichi Takaichi², Masaharu Yamada¹, Kenjiro Sugiyama¹

¹Department of Applied Chemistry, Kogakuin University, Hachioji, Tokyo, 192-0015, Japan

²Department of Molecular Microbiology, Faculty of Life Science, Tokyo University of Agriculture, Sakuragaoka, Setagaya, Tokyo, 156-8502, Japan

Two pathways from phytoene to lycopene are known. Most bacteria require only phytoene desaturase (CrtI). Oxygenic phototrophs require four enzymes: phytoene desaturase (PDS, CrtP), ζ-carotene desaturase (ZDS, CrtQ), ζ-carotene isomerase (Z-ISO) and *cis*-carotene isomerase (CrtISO, CrtH). The gene encoding Z-ISO has been functionally identified in only two higher plants, *Arabidopsis thaliana* and *Zea mays*, but little study has been reported in other organisms. In this study, we found that Z-ISO homologs were widely distributed in the genome of higher plants, green algae, and cyanobacteria, which have PSII- and PSI-type photosystems; however there was no evidence for Z-ISO homologs in the genome of green sulfur bacteria and Chloracidobacterium, which have PSI-type photosystem. Furthermore, we isolated and functionally analyzed the Z-ISO genes from the cyanobacteria *Arthrospira platensis*, the algae *Euglena gracilis*, and *Arabidopsis*. The deduced amino acid sequences of the *Arthrospira* Z-ISO and *Euglena* Z-ISO have 56% and 63% similarities with functional Z-ISO from *Arabidopsis*, respectively. To identify the catalytic activity, the Z-ISO genes of *Arthrospira*, *Euglena*, and *Arabidopsis* were transformed into *Escherichia coli* strains that could mainly produce 9,15,9'-tri-*cis*-ζ-carotene in dark condition, respectively. In the resulting *E. coli* transformants cultured under dark condition, 9,9'-di-*cis*-ζ-carotene was accumulated predominantly. These results indicated that the Z-ISO genes were involved in the isomerization of 9,15,9'-tri-*cis*-ζ-carotene to 9,9'-di-*cis*-ζ-carotene in dark condition. This is the first functional analysis of Z-ISO as a ζ-carotene isomerase in cyanobacteria and alga species.

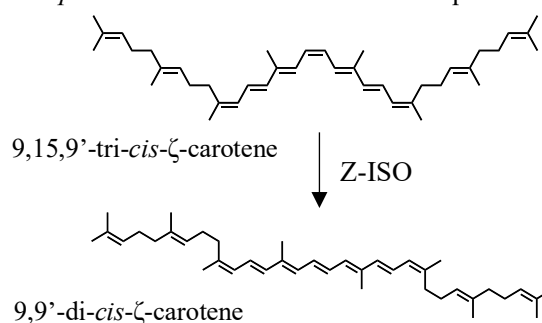


Figure 1 ζ-carotene isomerization by Z-ISO

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This will be published as a research article.

Molecular Distortion of Carotenoids and Optical Properties of Their Nanocrystals

Ryuju Suzuki^{1*}, Kazuma Yasuhara², Satoshi Okada¹ and Shigeru Deguchi¹

¹ Japan Agency for Marine-Earth Science and Technology (JAMSTEC),
2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan.

² Nara Institute of Science and Technology (NAIST),
Takayama-cho, Ikoma, Nara 630-0192, Japan.

Abstract: To understand and to control the optical properties of carotenoids nanocrystals are required us to practical use in the foodstuffs and pharmaceutical industry. In this study, we tried to understand the peculiar absorption spectrum of carotenoids nanocrystals by structural analysis using electron microscopes and resonance Raman spectroscopy. In particular, we focused on the molecular distortion of carotenoids as a novel viewpoint, and investigated the influence of changing effective π -conjugation length due to molecular distortion on the optical properties of nanocrystals.

1. Introduction

Carotenoids have been practical used as natural pigment for foodstuffs and cosmetics, and recognized one of familiar pigment on daily basis. In general, carotenoids are nanocrystallized to use in industry, but carotenoids nanocrystals show peculiar absorbance spectrum. As optical properties of organic crystals depend on the size and/or morphology of crystal, it is important to reveal the correlation between the structure and optical properties of crystal. The absorbance spectra of carotenoids in solution is strongly related to the length of π -conjugated system in polyene chain, and that π -conjugated system becomes shortened by the molecular distortion. On the other hand, optical properties of nanocrystals have been discussed from the viewpoint of molecular arrangement and intermolecular interaction, assuming that polyene chains have ideally elongated π -conjugated system¹⁾. While carotenoids in bulk crystals are known to have less distorted molecules, crystals have possible to accumulate the molecular distortion depending on the crystal growth process.

In this research, we attempted to understand the peculiar absorption spectrum of carotenoids nanocrystals by analyzing the influence of shortening effective π -conjugation length due to molecular distortion on the optical properties of carotenoids crystal.

2. Experimental

2.1 Materials

Carotenoids (β -carotene, lycopene, astaxanthin and lutein: **Figure 1**) were provided by San-Ei Gen F.F.I inc.. Bulk crystals of carotenoids were obtained by recrystallization.

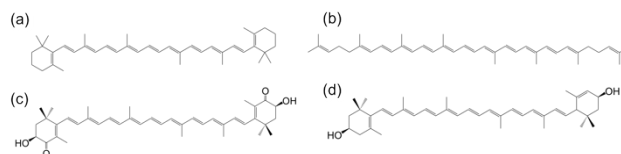


Figure 1 Chemical structures of carotenoids. (a) β -carotene, (b) lycopene, (c) astaxanthin and (d) lutein.

2.2 Fabrication of carotenoids nanocrystals

Carotenoids nanocrystals were fabricated by reprecipitation method²⁾. Respective carotenoids (5 mM) dissolved in tetrahydrofuran (THF) solution (0.2 mL) was injected into vigorously stirred water or any amount THF mixed aqueous solution (9.8 mL), carotenoids nanocrystals were immediately formed in an aqueous dispersion liquid.

3. Results and Discussions

The absorbance peaks of each carotenoid nanocrystals dispersion were blue shifted compared to those of solution and bulk crystal similar to previous report¹⁾. Each carotenoid was nanocrystallized under 100 nm in size (**Figure 2(a)**). From powder XRD patterns, both peaks and halo were observed in each carotenoid nanocrystals. This result suggested that the

* Corresponding author
e-mail: ryujus@jamstec.go.jp

crystal area and amorphous area would exist in carotenoids nanocrystals. In addition, from cryo-TEM observation, a high contrast area was observed inside the carotenoids nanocrystals as shown in **Figure 2(b)**, and electron beam diffraction patterns revealed that carotenoids had high crystallinity in the high contrast area.

Next, we measured resonance Raman spectra to analyze the molecular distortion of carotenoids in crystal. In carotenoids, a characteristic C=C stretching mode derived from the π - π^* transition of the polyene chain observes near 1520 cm^{-1} , and a correlation appears between the peak position and the effective π -conjugation length in polyene chains³⁾. Comparing the C=C stretching mode of the solution state and bulk crystal, the peak of the solution state shifted to higher wavenumber than that of bulk crystal, because carotenoids molecules in solution state have molecular distortion (**Figure 3**). In addition, the C=C stretching mode of fabricated carotenoids nanocrystals shifted to the high wavenumber with greater broadening compared to that of bulk crystal. This result suggests that polyene chains in nanocrystals have shorter effective π -conjugation length than in the bulk crystal.

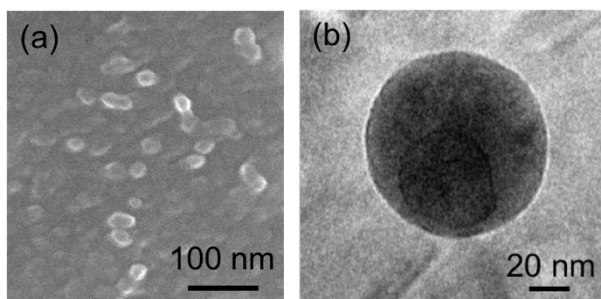


Figure 2 (a) SEM image and (b) cryo-TEM image of β -carotene nanocrystals

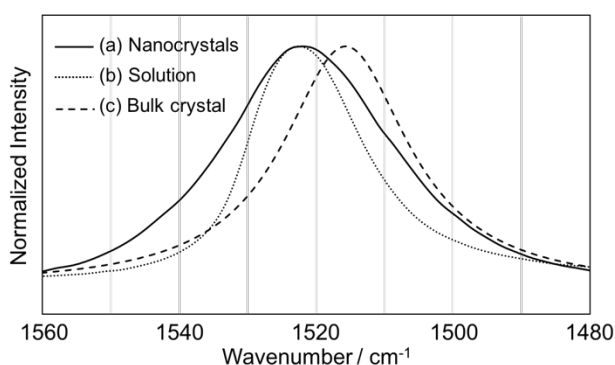


Figure 3 Resonance Raman spectra of β -carotene. (a) nanocrystals, (b) solution, and (c) bulk crystal

We considered that shortening of the effective π -conjugation length due to the molecular distortion appeared peculiarly in nanocrystals. In addition, we supposed that molecular distortion is accumulated in the amorphous area in nanocrystals, and then tried peak fitting in C=C stretching mode. Assuming two kinds of molecules of crystal origin and amorphous origin, we performed peak fitting using these two components. Amorphous origin component was gotten by adopting the peak position and the FWHM of bulk crystal as crystal origin component. As a result, the amorphous origin component shifted to the high wavenumber compared to the C=C stretching mode of the solution. It is suggested that nanocrystals have shorter effective π -conjugation length than solution state. This finding supports that the absorbance peak of nanocrystals was blue-shifted compared to that of solution state. In addition, it was possible to quantify the component ratio of crystal origin and amorphous origin. For example, the amorphous component of β -carotene nanocrystals was estimated at about 60%. These results suggest that shortening of the effective π -conjugation length due to molecular distortion would be one of the factors that the absorption peak was blue-shifted in carotenoids nanocrystals.

4. Conclusion

We analyzed the peculiar absorption spectrum of carotenoids nanocrystals from the viewpoint of molecular distortion present in the crystal. Our analysis using the resonance Raman spectrum would become novel proposal to understand the correlation between the structure and the optical properties of carotenoids nanocrystals from “shortening degree of the effective π -conjugation length” and “abundance ratio” of the distorted molecules present in the crystal.

Acknowledgement

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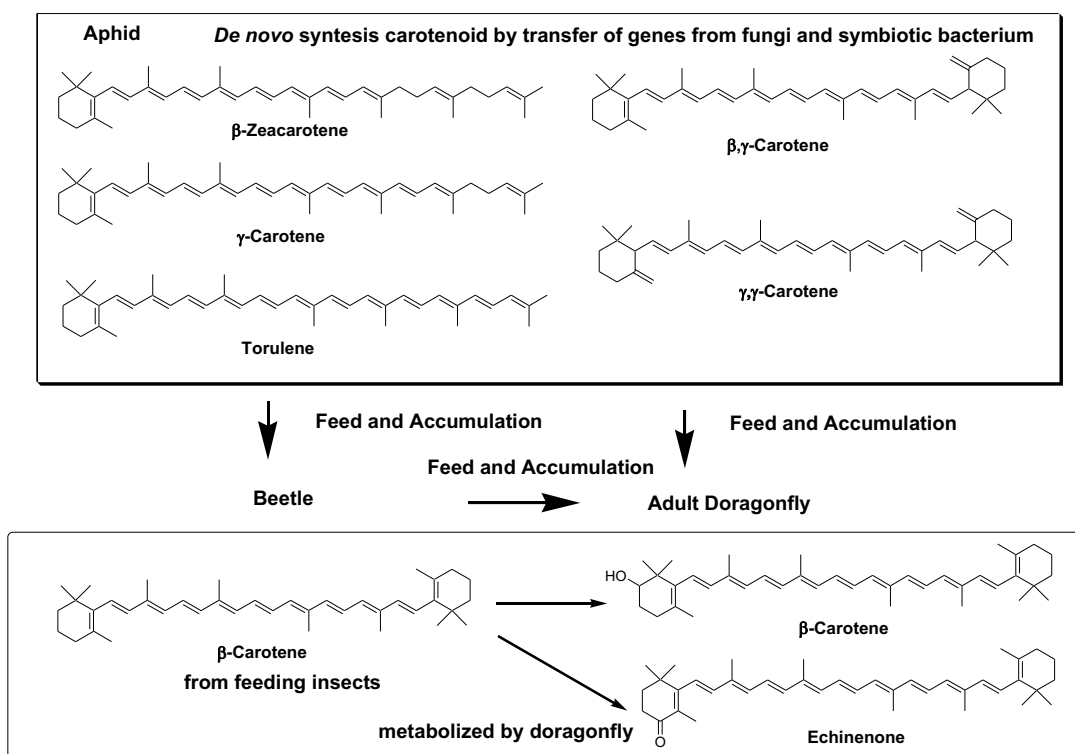
Origin of Carotenoids in Dragonfly

Takashi Maoka^{1*} and Naoki Kawase²

¹Research Institute for Production Development, 15 Shimogamo-morimoto-cho, Sakyo-ku, Kyoto 606-0805, JAPAN

²Minakuchi Kodomono Mori Museum, Minakuchi, Koka City, JAPAN

Origin of carotenoids in dragonfly was investigated. Larvae of dragonfly inhabits aquatic environment and feeds aquatic insect, crustacean, tadpole, small fish etc. On the other hand, adult of dragonfly feeds not only flying insect, such as fly, mosquito, butterfly, moth, aphid, planthopper and beetle, but also spider. β -Carotene, β -cryptoxanthin, lutein, and fucoxanthin, which were ingested from dietary aquatic animals, were found to be major carotenoids in larvae of dragonfly. On the other hand, carotenoid pattern of adult dragonfly is unique. β -Zeacarotene, β , ψ -carotene (γ -carotene), and β,γ -carotene were presented as major components along with torulene and γ,γ -carotene in adult dragonfly. β -Zeacarotene, and β , ψ -carotene (γ -carotene) are known to be intermediates of torulene biosynthetic pathway and are presented in fungi, aphid, and beetle. β , γ -Carotene and γ,γ -carotene, which have a unique γ -end group, are also found in fungi and aphid. Therefore, adult dragonfly accumulates these carotenenes from aphid and beetle through food chain. β -Caroten-2-ol and echinenone were found both adult and larvae of dragonfly. They assumed to be oxidative metabolites of β -carotene. Similar results were obtained damselfly. The result of the present study was indicated as following Figure. Major carotenoids.



Structures of Yellow xanthophylls and Mechanism of Chiral Conversion of Hydroxy group in Astaxanthin in the Prawn

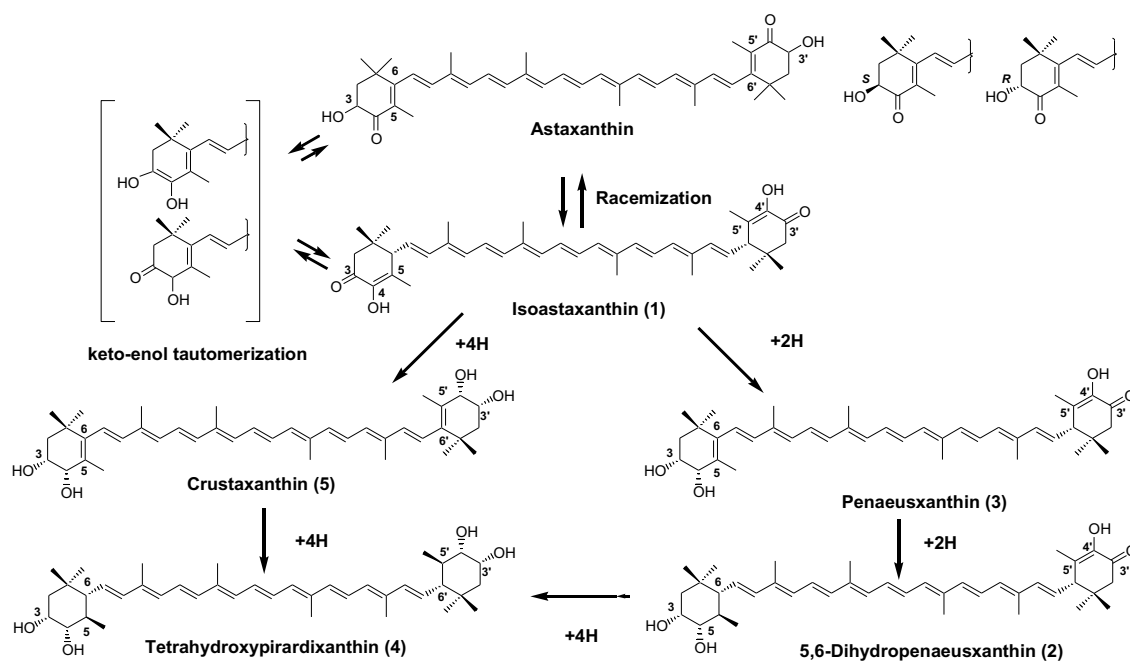
Takashi Maoka^{1*}, Yuki Kawashima² and Mikihiro Takagi²

¹Research Institute for Production Development (15 Shimogamo-morimoto-cho, Sakyo-ku, Kyoto 606-0805, JAPAN)

²JXTG Nippon Oil & Energy Corporation, Specialty Chemicals & Materials Company (W Building, 1-8-15 Konan, Minato-ku, Tokyo 108-8005, JAPAN)

Yellow xanthophylls, isoastaxanthin (**1**) and 5,6-dihydropenaesuxanthin (**2**) were isolated from the prawn in addition to penaeusxanthin (**3**), crustaxanthin (**5**), and tetrahydroxypirardixanthin (**4**), which were reported in previous symposium.

The structures of isoastaxanthin (**1**) and 5,6-dihydropenaesuxanthin (**2**) were deterred by UV-VIS, ESI TOF MS, ¹H-NMR and CD spectral data. It was reported that chiral conversion was occurred in the prawn¹⁾. Chiral conversion at the 3 (3')-hydroxy group in astaxanthin could be explained by the presence of isoastaxanthin (**1**), having a 3-keto-4-hydroxy-ε-end group, as an intermediate. Namely, the 3 (3')-hydroxy group in astaxanthin was oxidized once to a carbonyl group with double-bond translation from C5-C6 (C5'-C6') to C4-C5 (C4'-C5') to form isoastaxanthin (**1**). Then, the 3-keto-4-hydroxy-ε-end group in isoastaxanthin (**1**) was reversibly converted to the 3-hydroxy-4-keto-β-end group to form astaxanthin. Another possible mechanism of astaxanthin racemization is keto-enol tautomerization of the 3 (3')-hydroxy group, as shown in the following Figure.



1) Schiedt, K.; Bischof, S.; Glinz, E. *Pure Appl Chem.* **63**, 89-100 (1991); Schiedt, K.; Bischof, S.; Glinz, E. *Methods in Enzymology.* **214**, 148-168 (1993).