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CAROTENOID SCIENCE



*Proceedings of the 31st
Annual Meeting on*

Carotenoid Research

*Held in Kyoto, Japan
16-17 September 2017*



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EDITOR

H. HASHIMOTO

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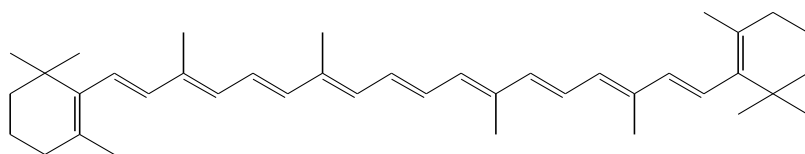
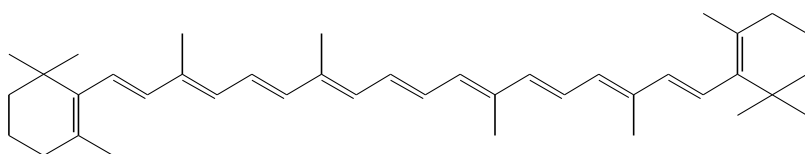
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Japanese Society for Carotenoid Research

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Japanese Society for Carotenoid Research

Preface for the 31st Annual Meeting

The 31st Annual Meeting of Japanese Carotenoid Society was held at Aigaku Hall in Kyoto Pharmaceutical University located at the east end of Kyoto City from 16th to 17th of September, 2017. The scientific program of this meeting consisted of five invited lectures by Prof. Masato Umeda (Kyoto University), Prof. Yukihiro Sugimoto (Kumamoto University), Dr. Daisuke Shibata (Kazusa DNA Research Institute), Prof. Akio Kawata (Kyoto University), and Dr. Ritsuko Fujii (Osaka City University) and 25 oral presentations.

In this meeting, 81 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 (Associate Prof. Kimura, Assistant Prof. Arimitsu, Assistant Prof. Naito, Dr. Yagi, scientific secretary Mrs. Hosokawa, and nine undergraduate students) 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 32nd Annual Meeting of Japanese Carotenoid Society, which will be held in Kumamoto University from 15th to 16th of September, 2018.

Hiroyuki Yasui

Professor of Kyoto Pharmaceutical University

The Meeting Chair of the 31st Annual Meeting of Japanese Carotenoid Society

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Transport and Metabolism of Carotenoids in *Drosophila melanogaster*

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Abstract: Carotenoids and their metabolites are involved in multiple biological processes in various organisms. In animals, carotenoids play a role in one of the most important biological processes—the visual system. Like other animals, the fruit fly (*Drosophila melanogaster*) utilizes dietary carotenoids to produce visual pigments. Because carotenoids and their metabolites are not essential for the growth or development of *Drosophila*, carotenoid metabolism and its role in the visual system can be identified using *Drosophila* as a model system. In fact, many functions of the visual system and the genes associated with carotenoid metabolism in the visual system of animals have been identified using *Drosophila*. Although increasing evidence of the molecular mechanisms underlying carotenoid metabolism in the visual system has been reported, several critical metabolic steps remain to be elucidated. In this article, we review the genes involved in the transport and metabolism of carotenoids in *Drosophila melanogaster*.

1. Introduction

Drosophila melanogaster has been recognized as a useful model system for the analysis of cell biology, physiology, immunology, social biology, and population genetics. Although evolutionarily distant from humans, the orthologues to ~70% of all human disease-associated genes are conserved in *Drosophila* [1]. Although carotenoids and their metabolites are critical for the visual system in *Drosophila*, they are not essential for development and growth; however, in vertebrates, the metabolites of carotenoid (e.g., retinoic acid) also function in the regulation of gene expression via binding to ligand-activated transcription factors. Therefore, *Drosophila* is a suitable model for the analysis of the molecular mechanisms underlying carotenoid metabolism and their function in the visual system [2–4]. The visual system of *Drosophila* was the first sensory system in animals to be genetically analyzed [5, 6], and various mutant lines of flies with affected phototransduction have been isolated. The mutants designated *nina* (neither inactivation nor afterpotential) show reduced rhodopsin levels in photoreceptor cells and altered electroretinograms [7]. Among the six genes that are defective in *nina* mutant strains, the *ninaA*, *ninaC*, and *ninaE* genes were shown to be involved in opsin production. On the other hand, the *ninaB*, *ninaD*, and *ninaG* genes are implicated in the biosynthesis of retinoids (e.g., all-*trans*-retinol) from carotenoids (e.g., zeaxanthin). Like other animals, *Drosophila* must acquire carotenoids from their diet. Dietary carotenoids are absorbed from the gut and transported to neuron and glial cells where they are processed into

the retinoid, 11-*cis*-3-hydroxyretinal, through various enzyme reactions. Finally, 11-*cis*-3-hydroxyretinal is transported to pigment cells to be processed by enzymes and then incorporated into visual pigments in photoreceptor cells. This article introduces the molecules responsible for the transport and metabolism of carotenoids in *Drosophila*.

2. Transport of Dietary Carotenoids

Dietary carotenoids are absorbed from the gut and transported to neuron and glial cells, a process in which two cell surface receptors, *ninaD* and *santa-maria*, have been shown to play pivotal roles. Kiefer *et al.* (2002) found that the visual pigment-deficient phenotype in the *ninaD* mutant was rescued by supplementation with very high amounts of β -carotene, zeaxanthin, or retinal [8], indicating that the *ninaD* mutation abrogates the absorption and transport of carotenoids. The polypeptide encoded by the *ninaD* gene shares significant sequence similarity with the mammalian class B scavenger receptors, SR-BI and CD36. The *ninaD* gene produces two splice variants with different C-terminal membrane-bound domains. Expression of each of these variants in *Drosophila* S2 cells revealed that the longer variant (NINAD-I) was localized at the plasma membrane, whereas the shorter variant (NINAD-II) was localized at the intracellular membrane. It was also reported that the expression of NINAD-I, but not NINAD-II, in S2 cells facilitated the uptake of carotenoids from culture medium. Moreover, ectopic expression of NINAD-I in *ninaD* mutant flies rescued impaired visual pigment synthesis, demonstrating that NINAD-I plays a crucial role in the absorption of carotenoids in *Drosophila* [9] (**Figure 1-i**).

Another scavenger receptor, *santa-maria* (scavenger receptor acting in neural tissue and

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majority of rhodopsin is absent), also plays an important role in the absorption of carotenoids in *Drosophila* [10]. Although NINAD and SANTA-MARIA show high amino acid sequence similarity, ectopic expression of *santa-maria* in *ninaD*-expressing cells of the *ninaD* mutant fly, or vice versa, cannot restore visual pigment synthesis. Moreover, *ninaD* and *santa-maria* are expressed in different tissues of *Drosophila*; *ninaD* is expressed in the gut, and *santa-maria* is expressed in neuron and glial cells, indicating that *ninaD* and *santa-maria* have independent functions in carotenoid absorption. In silkworms and locusts, carotenoids are transported between tissues by lipophorin [11-13], a lipoprotein in insects. Because *Drosophila* also uses lipophorin for inter-organ transport of neutral lipids and carbohydrates [14], lipophorin is expected to play an important role in carotenoid transport from the gut to neuron and glial cells in *Drosophila*. Therefore, it is likely that carotenoids absorbed from the diet by NINAD are transported to neuron and glial cells in which SANTA-MARIA receives the carotenoids from lipophorin (Figure 1-i, -ii). However, further studies are required to fully understand the molecular mechanisms of the inter-tissue transport of carotenoids in *Drosophila*.

3. Visual Pigment Synthesis in *Drosophila*

In neuron and glial cells, carotenoids (zeaxanthin and β -carotene) are metabolized to retinoid for the production of rhodopsin in photoreceptor cells. The *ninaB* gene encodes β -carotene-15,15'-monooxygenase, which catalyzes the symmetric oxidative cleavage of zeaxanthin and β -carotene (Figure 1-iii, -iv) [15-17]. Von Lintig *et al.* (2000) demonstrated the function of NINAB using an *Escherichia coli* strain genetically engineered to produce β -carotene [15]. In the *E. coli* strain expressing the wild-type form of NINAB, a significant amount of retinal and retinol produced via the cleavage of β -carotene was detected. However, no retinoids were detected in an *E. coli* strain expressing a mutant form of NINAB that harbored mutations identified in the *ninaB* mutant fly [15, 16]. The *ninaB* is expressed in neuron and glial cells in which the scavenger receptor SANTA-MARIA absorbs carotenoids from circulation [10], indicating that NINAB and SANTA-MARIA are functionally coupled for retinoid production.

The retinoid produced from carotenoids by NINAB is used for visual pigment synthesis in pigment cells. Both *Drosophila* and vertebrates use the photoreceptor rhodopsin, which comprises opsin protein and a chromophore; this chromophore in *Drosophila* is 11-*cis*-3-hydroxyretinal [18], whereas vertebrates use 11-*cis*-retinal. 3-hydroxyretinal is thought to be generated via two pathways in *Drosophila*. One is the hydroxylation of retinal by a cytochrome P450 [19] (Figure 1-v), and the other is the cleavage of zeaxanthin (Figure 1-iii). Although

(3*S*)-3-hydroxyretinal is used as a chromophore in *Drosophila*, hydroxylation by P450 and cleavage of zeaxanthin give only (3*R*)-3-hydroxyretinoid, which would need to be converted to (3*S*)-3-hydroxyretinoid for production of the photoreceptor. NINAG was reported to play a role in the isomerization of (3*R*)-3-hydroxyretinoid to (3*S*)-3-hydroxyretinoid [20]; however, the detailed mechanism has yet to be elucidated.

Cleavage of zeaxanthin by NINAB yields two molecules with different configurations: 11-*cis*-3-hydroxyretinal and all-*trans*-3-hydroxyretinal (Figure 1-iii). NINAB also produces two retinoid molecules with different configurations from β -carotene, 11-*cis*-retinal and all-*trans*-retinal (Figure 1-iv). Retinoid molecules with the 11-*cis* configuration can be directly used as a chromophore in photoreceptor cells (Figure 1-vi). However, retinoid molecules with the all-*trans* configuration need to be converted to the 11-*cis* configuration in pigment cells before incorporation into photoreceptors. Conversion of all-*trans*-3-hydroxyretinal to 11-*cis*-3-hydroxyretinal starts with the reduction of the carbonyl moiety of retinal by an oxidoreductase, PDH (Photoreceptor dehydrogenase) (Figure 1-vii). The resultant all-*trans*-3-hydroxyretinol is then converted to 11-*cis*-3-hydroxyretinol by light-dependent isomerization via an undetermined pathway (Figure 1-viii) [21]. 11-*cis*-3-Hydroxyretinol is finally oxidized to 11-*cis*-3-hydroxyretinal by an oxidase, RDHB (retinol dehydrogenase B) [22] (Figure 1-ix) and transported to photoreceptor cells (Figure 1-x) where it is incorporated into chromophores (Figure 1-xi). The *pinta* (prolonged depolarization afterpotential is not apparent) gene was shown to be associated with the transport of 11-*cis*-3-hydroxyretinal from pigment cells to photoreceptor cells [17] (Figure 1-vi). The PINTA protein is a *Drosophila* homolog of CRALBP that has high affinity for retinoid [23]. Decreased expression levels of rhodopsin and accumulation of the chromophore 11-*cis*-3-hydroxyretinal have been observed in the neuron and glial cells of a *pinta* mutant, suggesting that chromophore transport is impaired in the *pinta* mutant fly [17].

In photoreceptor cells, 11-*cis*-3-hydroxyretinal is covalently bound to opsin protein to form rhodopsin. Blue light signals evoke the isomerization of 11-*cis*-3-hydroxyretinal to all-*trans*-3-hydroxyretinal (Figure 1-xii), and all-*trans*-3-hydroxyretinal becomes dissociated from opsin (Figure 1-xiii). Dissociated all-*trans*-3-hydroxyretinal reenters the pigment cells to be converted to 11-*cis*-3-hydroxyretinal by PDH (Figure 1-xiv), an unknown isomerase (Figure 1-viii), and RDHB (Figure 1-ix). Regenerated 11-*cis*-3-hydroxyretinal is then transferred to the photoreceptor cells to be used for chromophore synthesis. In vertebrates, the regeneration of retinal takes place within the photoreceptors, whereas

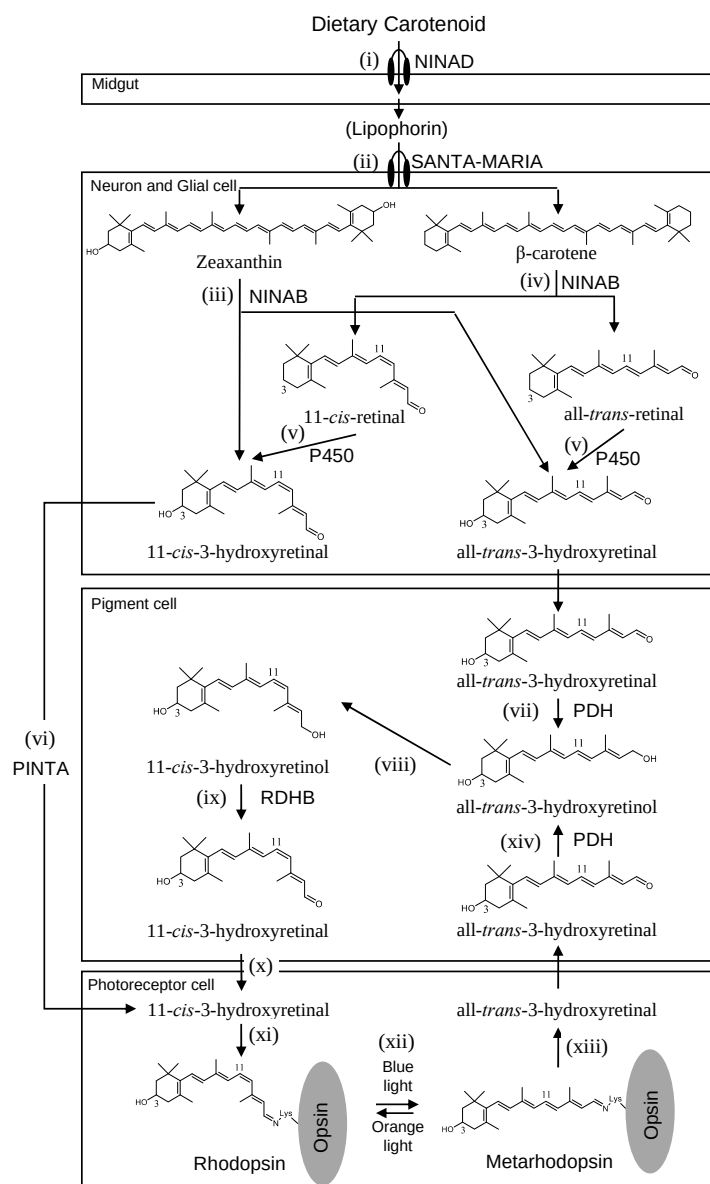


Figure 1. Putative pathway for transport and metabolism of carotenoids in *Drosophila*.

Dietary carotenoids (zeaxanthin and β -carotene) are transported into the gut (i) and then into neuron or glial cells (ii) by the scavenger receptors NINAD and SANTA-MARIA, respectively. Oxygenase NINAB cleaves the carotenoids and produces 11-cis-retinal and all-trans-retinal (iii-iv). P450 is a putative oxidase that catalyzes the hydroxylation of retinal to 3-hydroxyretinal (v). In pigment cells, all-trans-3-hydroxyretinal from neuron and glial cells or from photoreceptor cells is reduced to all-trans-3-hydroxyretinol by PDH (vii, xiv) and isomerization of all-trans-3-hydroxyretinol to 11-cis-3-hydroxyretinol occurs via an unknown mechanism (viii). Finally, 11-cis-3-hydroxyretinol is oxidized to 11-cis-3-hydroxyretinal by RDHB (ix). 11-cis-3-hydroxyretinal is transported into photoreceptor cells by the retinoid-binding protein pinta (vi, x) where it covalently binds to the lysine residue of opsin protein to form rhodopsin, a photoreceptor protein (xi). Blue light signals evoke transformation of 11-cis-3-hydroxyretinal to all-trans-3-hydroxyretinal (xii), and all-trans-3-hydroxyretinal is liberated from opsin (xiii). Liberated all-trans-3-hydroxyretinal is reused to produce 11-cis-3-hydroxyretinal in pigment cells.

Drosophila displays the unique use of two types of cells (pigment cells and photoreceptor cells) to regenerate retinal.

4. Summary and Perspectives

Research on *Drosophila* has revealed the various pathway components of the visual system and carotenoid metabolism in animals. For example, functions of the scavenger receptor (*ninaD*) in carotenoid absorption, and oxygenase (*ninaB*) in the cleavage of carotenoids, were first identified in *Drosophila*. However, several mechanisms such as the inter-organ transport of carotenoids and the regeneration of retinal in the pigment cells remain to be fully revealed. Although carotenoids are not necessary for growth and development in *Drosophila*, several studies have shown that carotenoids are also associated with various nonvisual biological functions. Shen *et al.* (2011) [24] demonstrated the function of the photoreceptor rhodopsin in temperature

discrimination. Impairment of temperature preference was observed in *ninaE* (major opsin in *Drosophila*) and *santa-maria* mutants or wild-type flies reared in vitamin A-depleted medium, suggesting that carotenoids are required for thermosensation. Future studies in *Drosophila* may yield novel carotenoid functions and contribute to a better understanding of carotenoid metabolism in animals.

Acknowledgements

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History of Carotenoid Research in Kyoto Pharmaceutical University

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Abstract: From September 15 to 16, 2017, the 31th Annual Meeting of Japanese Society for Carotenoid Research was held in Kyoto Pharmaceutical University by an organizer, Prof. Hiroyuki Yasui at Department of Analytical and Bioinorganic Chemistry. Originally, Department of Natural Product Chemistry in Kyoto Pharmaceutical University was one of the pioneer research center of carotenoid research in Japan. This group organized the First Meeting of the Japanese Society for Carotenoid Research in Kyoto in 1987. Several researchers in Kyoto Pharmaceutical University contributed progress of carotenoid chemistry and biochemistry. History of carotenoid research in Kyoto Pharmaceutical University was described in this review.

Key Words: history, carotenoid research, Kyoto Pharmaceutical University

In 1949, Dr. Takao Matsuno arrived at Kyoto College of Pharmacy (Kyoto Pharmaceutical University) from Kyoto University. At the early time, he studied citrus essential oils and flavonoids. The discovery of a new antibiotics cephalosporin from *Cephalosporium acremonium* in 1955 prompted him to study marine natural product chemistry. Then, he studied toxic saponin in sea cucumbers [1]. During the research, astaxanthin and related carotenoids were isolated as gonadal pigments of sea cucumbers [2, 3]. Then, Prof Matsuno group (Department of Natural Product Chemistry) comprehensively studied carotenoids in marine and aquatic animals. Several interesting structural carotenoids, such as 4-ketozeaxanthin from spiny lobster [4], parasiloxanthin and dihydroparasiloxanthin from the cat fish [5], salmoxanthin from salmon [6], were determined. These structures were determined by UV-VIS, EI-MS, IR and 80-100 ¹H-MHz NMR spectral data and chemical dramatizations such as acetylation, trimethyl silylation and reaction with NaBH₄ or LiAlH₄. They also studied various fish carotenoids from the comparative biochemical point of view [7]. In 1980s, pulsed Fourier-transform (FT) NMR spectrometry was routinely available for structural elucidation of natural carotenoids. Structure of new carotenoids in tunicates and shellfish such as halocynthiaxanthin, mytiloxanthinone, amoarouciaxanthin A, amarouciaxanthin B, pectenol, pirardixanthins etc were determined by Prof Matsuno, Dr. Masahiro Ookubo, Dr. Takashi Maoka, Dr.

Kiyoshi Katagiri [8]. Through these studies, Prof. Matsuno group revealed metabolism of fucoxanthin in marine invertebrates [8, 9]. Stereochemical studies of natural carotenoids were performed using CD spectrometry, chiral HPLC, and chemical methods [9]. These studies propelled the metabolic study of carotenoids in marine animals. Prof. Matsuno and Dr. Masaaki Katsuyama founded reductive metabolic pathway of astaxanthin to tunaxanthin in fish through research of the carotenoids in fish egg [9, 10]. Furthermore, his group also found that xanthophylls such as astaxanthin were precursor of retinols in fish by feeding experiments [11]. Dr. Eiji Yamashita found several apocarotenoids in marine and aquatic animals [9, 10].

In order to hold International Symposium of Carotenoid in Japan at 1990, Profs Kiyoshi Tsukida (Kobe Pharmaceutical University) and Matsuno and Drs Masayoshi Ito (Kobe Pharmaceutical University) and Wataru Miki (Suntory Institute for Bioorganic Research) founded Japanese Society for Carotenoid Research. The First Meeting on Carotenoid Research in Japan was held in Kyoto at 28-29, November 1987 by Profs Matsuno and Tsukida and Dr. Miki and research group of Kyoto Pharmaceutical University. About 40 carotenoid researchers belonging to the fields of pharmaceutical, chemical, biochemical, physical, agricultural, medical, and fisheries science met together in this meeting (**Figure 1**). Then, 9th International Symposium on Carotenoids was held in Kyoto, 20-25 May 1990 by the chairman of the

organizing committee, Profs Tsukida and Matsuno and secretaries, Drs. Ito and Miki. This symposium attracted approximately 200 participants from more than 20 countries and presented 64 oral presentations and 103 posters, which covered various fields of carotenoid research such as natural product chemistry, analytical chemistry, synthesis, biochemistry, photosynthesis, life science, medical science, fisheries science etc.

After retirement of Prof Matsuno, Prof Mutsuo Kozuka arrived Department of Natural Product Chemistry in 1990. Dr. Miyuki Tsushima and Prof. Kozuka studied anti-tumor promoting effect of natural carotenoids with Prof. Hoyoku Nishino (Kyoto Prefecture University of Medicine) [12]. Dr. Tsushima also studied roles of carotenoid for development of marine invertebrates.

Dr. Maoka (Research Institute of Production Development) collaborated with Prof Keiji Hashimoto, Dr. Yasuhiro Fujiwara, and Dr. Naoshige Akimoto (Graduate School of Pharmaceutical Science, Kyoto University) and studied structural elucidation of complex structural carotenoids such as crassostreaxanthins, pittedosporumxanthins, tobiraxanthins etc, using 2D NMR and MS/MS techniques [13]. Dr. Shinzo Hosoi studied stereostructure of (4R)- and (4S)- β - isocryptoxanthin by analysis of CD spectral data with calculated

chemistry [14].

Prof. Hiroyuki Yasui (Department of Analytical and Bioinorganic Chemistry) have studied the chemical structure-dependent anti-oxidative activity of natural carotenoids using chemiluminescence method and ESR spectrometry [15] and revealed chemical scavenging mechanism of carotenoids against each reactive oxygen species such as singlet oxygen ($^1\text{O}_2$), hydroxyl radical ($\cdot\text{OH}$), and superoxide anion radical ($\cdot\text{O}_2^-$) by using both the LC/MS and ESR spin-trapping methods [16]. Prof. Kentaro Kogure (Department of Physical Chemistry) found scavenging effect of astaxanthin encapsulated in liposomes on hydroxyl radical in aqueous solution [17] and synergistic antioxidative effect of astaxanthin and tocopherol by liposome system [18].

In this year, from September 15 to 16, 2017, Prof. Hiroyuki Yasui organized the 31th Annual Meeting of Japanese Society for Carotenoid Research in Kyoto Pharmaceutical University since 30 years after the first meeting in 1987, where almost 100 persons attended, and 5 invited lectures and 25 oral presentations were talked and discussed with keen interests.

Many researchers in Kyoto Pharmaceutical University contributed and are still contributing progress of carotenoid chemistry and biochemistry.



Figure 1. The First Meeting on Carotenoid Research in Japan held in Kyoto at 28-29, Nov. 1987.

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Cross-talk between prostaglandin and retinoic acid signaling

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Abstract: Prostaglandins (PGs) and retinoic acid (RA) are lipophilic biological mediators, which play essential roles in a broad range of physiological and pathological contexts. These mediators exert their functions by regulating various signaling pathways in several biological situations. In this article, we describe the roles of PGs and RA in colon cancer development and in dendritic cell differentiation and discuss the biological significance of the cross-talk between the signals controlled by these two lipid mediators.

1. Introduction

Prostaglandins (PGs) and retinoic acid (RA) share common features, such as being lipophilic biologically active molecules that are synthesized by oxidative reactions. However, their mechanisms of action are different, as they act on different receptor types. PGs act on G protein-coupled receptors (GPCRs) located on the cell membrane and exert relatively early responses by inducing intracellular second messengers. In contrast, RA binds to nuclear receptors and modulates the transcription of target genes. Both PGs and RA regulate a wide variety of biological processes, including inflammation, immune responses, and cell differentiation, and recent reports demonstrated that cross-talk regulation occurs between their signaling in some physiological or pathological conditions. In this review, we focus on the biological regulation performed by these two mediators.

2. PG synthesis and its receptor signaling

Prostanoids are a group of bioactive lipid mediators synthesized from arachidonic acid (AA) by cyclooxygenase (COX) as the rate-limiting enzyme, and consist of four types of PGs and a thromboxane (TX), as follows: PGE₂, PGD₂, PGF₂α, PGI₂, and TXA₂. PG biosynthesis is triggered by the release of AA from membrane phospholipids by phospholipase A₂ (PLA₂). The COX isozymes, namely, constitutively expressed COX-1 or inducible COX-2, then converts AA into PGH₂, and finally PGH₂ is metabolized to each PG by their respective synthases [1]. The synthesized PGs are then released from the cells and exert their various functions, including the induction of fever, pain, and inflammation by acting on GPCRs expressed on the plasma membrane of neighboring cells [2]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin elicit their effects by inhibiting the enzymatic activity of

COXs [3]. PGE₂, one of the most typical PGs, has four types of receptor subtypes, EP1–EP4, and exerts multiple functions through these receptors by activating different intracellular signaling pathways [4]. EP1 is coupled to intracellular Ca²⁺ mobilization via G_{q/11}. EP2 and EP4 are coupled to the stimulation of adenylyl cyclase via G_s, and EP3 is mainly coupled to the inhibition of adenylyl cyclase via G_i. EP2 and EP4 elicit the activation of phosphoinositide-3 kinase via the β-arrestin pathway [5, 6]. PGs act as local mediators and their action range is limited to their production sites because synthesized PG is immediately inactivated by 15-hydroxyprostaglandin dehydrogenase [7] (**Figure 1**). Recently, PGE₂ has been shown to regulate not only acute physical responses but also chronic diseases, including immune responses and cancer progression, by interacting with various intracellular signaling pathways.

3. Vitamin A metabolism and RA signaling

Vitamin A is an important micronutrient obtained from the diet as retinol, β-carotene, and retinyl esters [8]. β-carotene is cleaved into two molecules of retinal and converted into retinyl esters. Retinyl esters are taken up by target tissue and then converted into retinol [9]. Among the vitamin A metabolites, *All-trans* retinoic acid (atRA) is involved in the most crucial biological effects, such as the regulation of cell growth, differentiation, and immune response [10, 11]. atRA is synthesized from retinol through two oxidative steps. Retinol is oxidized to retinal by cytosolic alcohol dehydrogenases or microsomal retinol dehydrogenases. Then, retinal dehydrogenases (RALDHs) catalyze the irreversible conversion of retinal to atRA [12]. atRA undergoes isomerization into 9-*cis* RA, which serves as a ligand for two families of nuclear receptors, namely, the RA receptor and retinoid X receptor [13]. These receptors form a heterodimer that binds to DNA regulatory sequences and regulates the expression of multiple

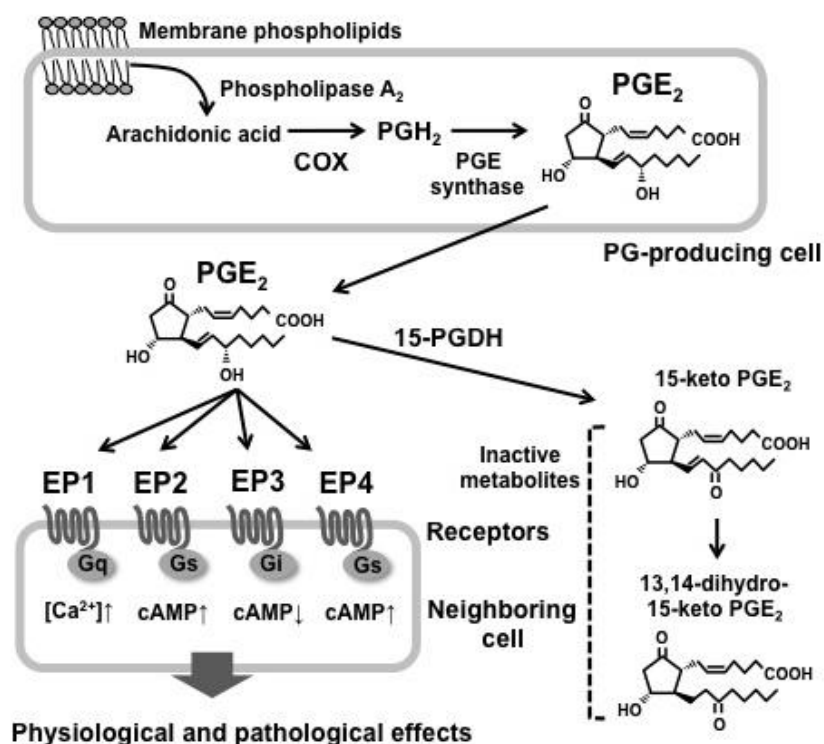


Figure 1. PGE₂ synthesis and its receptors. 15-PGDH: 15-hydroxyprostaglandin dehydrogenase.

target genes in response to ligand binding. RA levels are controlled by specific RA-degrading cytochrome P450s (CYPs), namely, CYP26A1, CYP26B1, and CYP26C1 [14] (**Figure 2**).

4. RA is involved in the regulation of PGE₂ production by adenomatous polyposis coli

Colorectal cancer is one of the most common cancers in the world with high incidence and mortality rates in both men and women [15]. The sequential accumulation of mutations or deletions in tumor suppressor genes and oncogenes is closely associated with the development of this type of cancer [16]. Patients with familial adenomatous polyposis (FAP), characterized by numerous colorectal polyps, harbor mutations in the adenomatous polyposis coli (APC) tumor suppressor gene [17]. APC mutant (*Apc^{min}*) mice mimic the symptoms of human FAP and develop colon cancer upon acquiring further genetic mutations [18]. It has been shown that COX-2 is overexpressed in human colon cancer tissue [19], and treatment of FAP patients and *Apc^{min}* mice with NSAIDs or COX-2 inhibitors reduces the number and size of intestinal polyps, implicating that COX-2 and one of its products, PGE₂ contribute to colon cancer development [20-22]. APC is a negative regulator of the Wnt/β-catenin pathway, and contributes to cytoplasmic degradation of β-catenin by forming a complex with axin and glycogen synthase kinase-3β (GSK3β) [23]. Loss of APC results in β-catenin stabilization and its nuclear translocation. Nuclear

β-catenin binds to T-cell factor/lymphoid enhancer factor transcription factors and activates their target gene promoters, resulting in colon cancer progression [24]. A recent report showed that PGE₂ induces the release of GSK3β from the β-catenin degradation complex and promotes colon cancer growth by enhancing β-catenin signaling [25]. However, what triggers PGE₂ production in these situations remained unclear.

Jones and his colleagues investigated how APC modulates COX-2 expression, using APC mutant zebrafish. They found that APC enhances RA synthesis and promotes colonocyte differentiation [26, 27]. Interestingly, APC mutant zebrafish demonstrated similar phenotypes to RA-deficient zebrafish, including aberrant intestinal differentiation, and this phenotype was rescued by RA treatment [28]. Moreover, both APC mutant and RA-deficient zebrafish showed notably higher COX-2 expression levels than wild type (WT) zebrafish, which were also reduced by RA treatment [29]. CCAAT/enhancer-binding protein elements (C/EBP)-β expression was also enhanced in APC mutant zebrafish, and C/EBP-β knockdown reduced COX-2 expression in this mutant. These results show that APC enhances RA production, and RA reduces COX-2 expression by suppressing C/EBP-β expression. β-catenin protein levels were increased in APC mutant and PGE₂-treated WT zebrafish, similarly to in colon cancer tissue, and both RA and COX-2 inhibitors decreased β-catenin protein levels

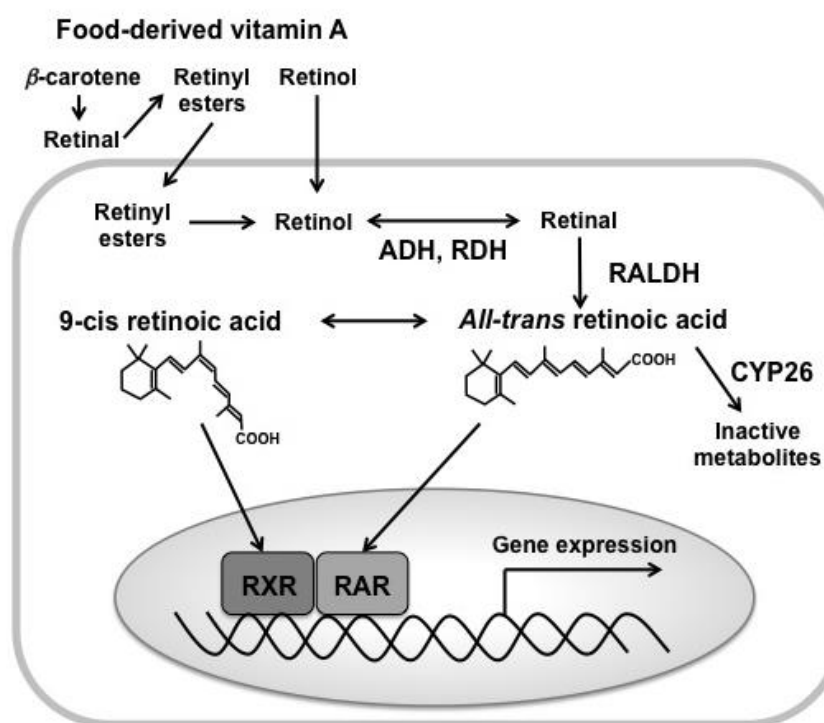


Figure 2. Vitamin A metabolism and RA receptors. ADH: Alcohol dehydrogenase; RDH: Retinol dehydrogenase; RAR: RA receptor; RXR: Retinoid X receptor.

of APC mutants [30]. These reports suggest that RA induced by APC may suppress colon cancer progression by inhibiting COX-2/PGE₂/EP2 receptor-mediated activation of the β -catenin pathway (Figure 3).

5. PGE₂ suppresses the differentiation of RA-producing dendritic cells in non-intestinal tissue

Vitamin A is one of the most important dietary nutrients for modulating the immune system. Vitamin A deficiency is closely associated with increased susceptibility to infectious diseases, poor response to vaccination and increased mortality in children [31, 32]. Among the vitamin A metabolites, RA has long been recognized as an important modulator of the intestinal immune system [33]. In intestinal tissue, dendritic cells (DCs) are located in gut-associated lymphoid tissue (GALT) and the lamina propria, and DCs play crucial roles in mucosal immune initiation and regulation [34, 35]. DCs derived from the mesenteric lymph node (mLN), but not the spleen or skin-draining peripheral LN (pLN), enhance the expression of gut-specific homing receptors, such as $\alpha 4\beta 7$ and C-C chemokine receptor (CCR) 9 upon T-cell and B-cell priming [36, 37]. mLN-derived DCs also induce transforming growth factor β (TGF β)-dependent Foxp3⁺ regulatory T cell differentiation and immunoglobulin A class switching

of B cells [38–40]. These activities are restricted specifically to CD103⁺ DCs, and RA released from this DC subset appears to contribute to these functions [39–42]. Indeed, RALDH and RA production are observed in CD103⁺ DCs, but not in other immune cell types in the intestine (40). Thus, these RA-producing DCs are very important not only for intestinal immune responses to pathogens but also for the tolerance to food antigens and commensal bacteria. Then, how do gut-associated DCs gain such RA-producing properties? Multiple factors, including RA, the toll-like receptor 2 ligand zymosan, interleukin (IL)-4, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF) have been reported to induce RALDH expression in DCs in *in vitro* and/or *in vivo* studies [43, 44], suggesting that the intestinal environment can locally educate DCs to develop RA-producing activity. However, gut-specific functional properties of DCs cannot be fully explained solely by these RALDH-promoting factors, because GM-CSF also regulates the development of DCs associated with the skin [45].

Stock *et al.* identified the unexpected role of PGE₂ as a negative regulator of RALDH during non-intestinal DC development [46]. They first isolated RALDH⁺, CD11c⁺ DCs from the mLN, skin-draining pLN, and spleen, and confirmed that a large proportion of these sorted DCs upregulated

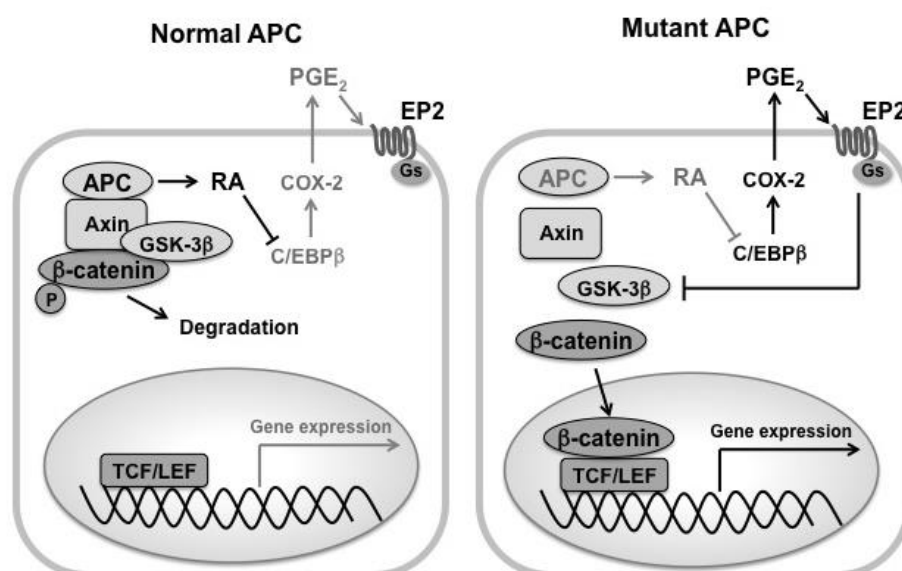


Figure 3. Schematic illustration of β -catenin signaling pathway regulation by RA and PGE_2 in normal and mutant APC situations.

RALDH activity in response to GM-CSF *in vitro*, suggesting that RALDH⁺ DCs have the potential to express RALDH by positive signals. Given that RALDH⁺ DCs are restricted to a minor subset of DCs from mLNs *in vivo*, they hypothesized that RALDH expression is actively suppressed by some negative regulators. In fact, when mouse bone marrow-derived DCs (BM-DCs) were differentiated with GM-CSF, the treatment with conditioned medium from skin stromal cells or 3T3 fibroblasts inhibited RALDH expression. The conditioned medium from indomethacin-treated skin stroma had only a weak ability to suppress RALDH⁺ DCs, indicating that stroma-derived PGs inhibit the differentiation of RALDH-expressing DCs. They also showed that PGE_2 and an EP4 receptor agonist reversed the effects of indomethacin, and that RALDH expression was increased in EP4-deficient BM-DCs, suggesting that PGE_2 /EP4 receptor signaling involves RALDH regulation in mouse DCs. Moreover, systemic treatment of indomethacin to mice reduced splenic PGE_2 levels and increased the number of RALDH⁺ splenic DCs, which can induce CCR9 expression during T-cell activation. This means that the inhibition of PGE_2 synthesis can induce RALDH⁺ DCs in non-intestinal sites and can prime gut-homing T cells at these sites. The inhibitory effects of PGE_2 were also observed in human monocyte-derived DCs. Unlike in mouse BM-DCs, PGE_2 appears to act predominantly on EP2 receptors to inhibit RALDH expression in human DCs. Given that both EP2 and EP4 are G_s -coupled receptors that increase intracellular cAMP levels and that the RALDH promoter contains multiple cAMP response element (CRE)-binding sites [47], Stock *et al.* focused on inducible cAMP early repressor (ICER), which represses the transcription of CRE-responsive genes

[48]. Indeed, PGE_2 induced ICER expression in both mouse and human DCs. Moreover, there appears to be a negative correlation between the expression levels of RALDH and ICER in DCs both *in vitro* and *in vivo*. Thus, based on these results, PGE_2 likely works as a negative factor for RALDH expression in DCs of non-intestinal tissue by a cAMP/ICER-dependent mechanism, and contributes to the tissue specificity of the gut-tropic immune response.

6. Concluding remarks

Recent studies support the idea that PGs and RA exert their functions by regulating various signaling pathways in some physiological and pathological contexts. For example, RA suppresses colon cancer by suppressing PGE_2 synthesis, whereas PGE_2 inhibits RA production from DCs. PGs usually act as autacoids due to their rapid inactivation, and exert various actions by modulating other signaling pathways on target cells. In contrast, RA plays crucial roles in cell differentiation, proliferation, and apoptosis by affecting global gene expression. It is interesting that in some cases these different types of lipid mediators cooperatively regulate common biological processes by utilizing each other's advantages.

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Tools and Databases for an Integrated Metabolite Annotation Environment for Liquid Chromatography–Mass Spectrometry-based Untargeted Metabolomics

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Abstract: An elucidation of the precise chemical flows that occur between organisms and within an organism is required for a proper understanding of biological systems. Untargeted metabolomics is one of the promising methods for such elucidation. An integrated metabolite annotation environment for liquid chromatography–mass spectrometry-based untargeted metabolome analysis was developed by the present authors. The environment includes various computational tools and databases. This paper describes two of the tools and one database, namely, “PowerGetBatch,” which is a data analysis tool; “MassChroViewer,” which is for checking of data quality and annotation; and “Food Metabolome Repository,” which is a database for supporting annotations based on the sample specificities of unknown peaks. The tools and database are available on the authors’ KOMICS website at <http://www.kazusa.or.jp/komics>.

1. Introduction

Living organisms biosynthesize and utilize a huge number of chemical compounds for their sustenance. It is estimated that more than one million metabolites are biosynthesized within all plants [1]. In addition, many unknown metabolites that exist within the human body are considered to derive from food and drug intakes, exposure to diverse elements, and the bio-transformation of known metabolites by intestinal bacteria and the liver [2, 3]. The understanding of biological systems and the development of marker chemicals for discriminating between diseased and healthy conditions thus require the elucidation of the detailed chemical flows that occur between organisms and within a particular organism, as well as an appreciation of the potential metabolic pathways defined by the genomes. Metabolomics is one of the promising technologies for such elucidation.

Metabolomics is a technology that enables the comprehensive detection of the small molecules present in living organisms [4]. However, due to the limited availability of the standard chemicals required for compound identification, only a limited number of metabolites can be identified. Incidentally, when several hundreds of metabolites are identified, it becomes difficult to establish a full picture of the metabolic flows. Hence, researchers have developed an interest in untargeted methods, which do not focus on specific metabolites but attempt to detect all the compound signals including those originating from unknown compounds and the annotation of the unknown compounds. Untargeted metabolome

analyses often utilize liquid chromatography (LC)–mass spectrometry (MS) owing to its high sensitivity, high throughput, applicability to metabolites over a wide polarity range, and the availability of MS/MS or MSⁿ spectra, which provide key information for the elucidation of the chemical structures.

The authors have developed various computational tools and databases for metabolomics, as published on their KOMICS website (<http://www.kazusa.or.jp/komics>) [5]. In this paper, two of the computational tools and one database that were recently developed for LC–MS-based untargeted metabolome analyses are reported, namely, PowerGetBatch, which is a peak detection and alignment software; MassChroViewer, which is a data viewer and annotation tool; and Food Metabolome Repository, which is LC–MS-based untargeted metabolome database containing data obtained from foods for improving annotations. These resources are available on the KOMICS website.

2. Materials and Methods

PowerGetBatch and MassChroViewer were developed using Java 7 (Oracle Corporation). Food Metabolome Repository was developed using SpringBoot (Povital Software, Inc.) and MariaDB 5.5, and run on a Linux server (RedHat EL7.1). The details of the materials and methods for the sample preparation, LC–high-resolution MS analyses, and data processing for Food Metabolome Repository are available on the website (<http://metabolites.in/foods>).

3. Results and Discussion

3.1 PowerGetBatch

PowerGetBatch (<http://www.kazusa.or.jp/komics/software/PowerGetBatch>) is an improved version of a

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previous software developed by the authors, referred to as PowerGet, which is used for peak detection, alignment, and peak characterization in LC–high-resolution MS. The latter software affords a higher processing rate, more comprehensive peak detection, and more accurate peak characterization. These are mainly achieved by three modifications, as described below.

3.1.1 Batch processing

The calculation processes of PowerGetBatch can be executed in batches using a command line (character-based user interface) without the need for graphical user interface (GUI) operations. This enables the distributed processing of a large number of LC–MS data on servers, as well as automation and acceleration of the data processing.

3.1.2 Improved adduct ion estimations

PowerGetBatch enables the precise assignment of an adduct ion to a detected compound peak, and accurate estimation of the weight of the molecule without the adduct. The estimation is critical to the annotation of the compound by querying the compound databases for the molecular mass. The software also significantly increases the detectable variation of the adduct ions by calculating all the possible combinations of the charged units such as the protons and sodium ions with the neutral units such as water and acetonitrile. A similar strategy is also adopted in the CAMERA module of the XCMS software [6].

3.1.3 Improved detection limit of low intensity peaks

PowerGetBatch utilizes enhanced algorithms for peak detection and noise rejection, and is therefore able to appropriately detect lower intensity peaks. One of the key enabling modifications is the introduction of a new parameter, namely, the averaged mass difference between the ions in neighboring scans. This parameter facilitates the efficient discrimination and removal of the noise peaks. The improved peak detection limit significantly enhances the accuracy of the adduct ion estimation by increasing the number of co-eluting peak pairs available for adduct ion calculation.

3.2 MassChroViewer

MassChroViewer is used for the visualization of mass chromatogram data obtained by LC–MS in two-dimension, namely, retention time (x -axis) versus m/z (y -axis). It can also be used to visualize the ion intensities based on the color strength of the dots (Figure 1(a)). The two-dimensional (2D) representation of MassChroViewer enables optimization of the LC–MS process conditions and the data processing parameters, as well as detailed compound annotation as described below.

3.2.1 Data quality check

Subtle differences between the peak signals of samples can be easily detected through the parallel view of 2D mass chromatograms in MassChroViewer. This enables the user to precisely check the qualities of the data and the validity of the performed experiments, such as for the absence of carryover peaks (success of wash runs), reproducibility of the replications, and appropriateness of the blank and mock samples. Most of the vendor software installed in the LC–MS instruments afford data visualization in terms of the total ion current (TIC), extracted ion current (EIC), and mass spectra. However, such data snapshots are not suitable for identifying differences of the lower intensity and unknown peaks between the samples. Two-dimensional representation of mass chromatograms is suitable for data overview [7], and 2D visualization functions are available in some vendor software and free tools such as MZmine2 [8], TOPPView [9], Mass++ [10], and BatMass [7]. However, they provide only limited functions for the parallel viewing of chromatograms, changing of the visualization region by Google map-like intuitive mouse operations, and changing the color strength to focus on the weakly or strongly detected peaks, all of which are required for detailed determination of the differences between mass chromatograms. MassChroViewer provides these functions and is therefore suitable for checking the quality of mass chromatogram data.

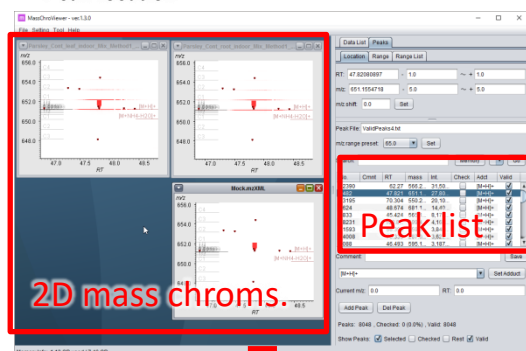
It should, however, be noted that MassChroViewer is not intended as a quality control tool for guaranteeing the quantity, quality, and reproducibility of a huge amount of data obtained by specific experimental methods, neither for the automation of such quality controls.

3.2.2 Optimization of experimental conditions and data processing

A user may determine the appropriateness of the experimental conditions, peak detection, and alignment conditions by visualizing the positions of the precursor ions in an MS/MS analysis (Figure 1(c)) and the detected peaks (Figure 1(a)) in the 2D mass chromatogram. Especially in the case of data-dependent acquisition, visualization of the precursor ions in an MS/MS analysis enables ascertainment of whether the appropriate peaks are being analyzed, as well as optimization of the parameters for data-dependent exclusion (dynamic exclusion). Peak lists such as those generated by peak detection and alignment tools such as PowerGetBatch can be imported by MassChroViewer, and the positions of the peaks can be visualized (Figure 1(a)). This enables the user to check whether the peaks have been appropriately detected, the alignment of the sample peaks, and the proper exclusion of peaks in the control samples. Such checks facilitate optimization of

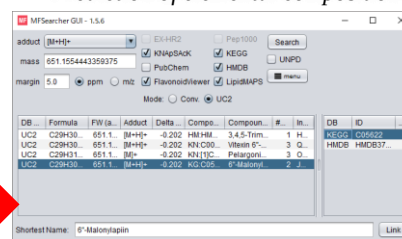
(a) Main window

- 2D mass chromatogram
- Peak location



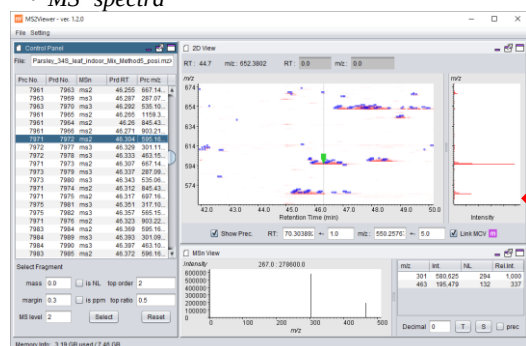
(b) MFSearcher tool

- Compound database search
- Prediction of elemental composition



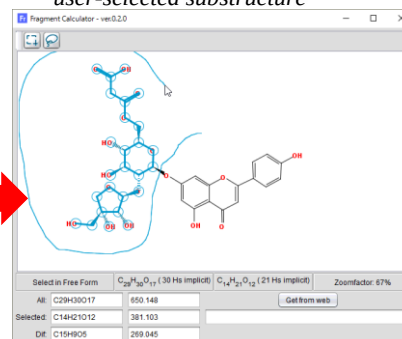
(c) MS2Viewer tool

- Location of precursor ions
- MSⁿ spectra



(d) Fragment Calculator tool

- Chemical structure
- Formula and molecular weight of user-selected substructure



(e) FlavonoidSearch GUI tool

- Prediction of flavonoid aglycone
- Prediction of O-type substituents

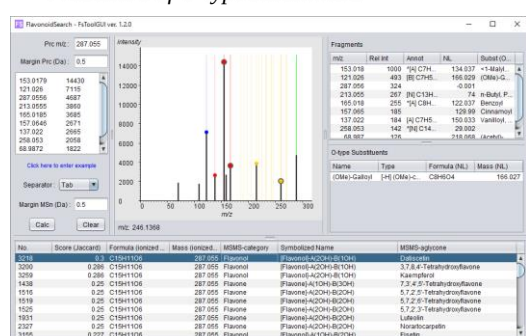


Figure 1. MassChroViewer and integrated tools for compound annotation. (a) The main window of MassChroViewer. Multiple mass chromatograms are displayed in parallel in 2D. A list of the detected peaks can be loaded. The small brown markers in the 2D windows indicate the locations of the detected peaks. (b) MFSearcher tool for compound database search and elemental composition prediction. A mass value selected in the main window can be seamlessly searched for. (c) MS2Viewer tool for browsing the MSⁿ analysis results. The blue dots indicate the positions of the precursor ions. (d) Fragment Calculator tool. The structure of the candidate compounds found by the MFSearcher tool is displayed, as well as the formula and molecular weight of the user-selected substructure. (e) FlavonoidSearch GUI tool for predicting the flavonoid aglycones. O-type substituent candidates can be searched for based on the neutral loss mass value.

the parameters for peak detection and the conditions for peak alignment and selection of valid peaks.

3.2.3 Compound annotations

This section describes several tools that are integrated in MassChroViewer and used for metabolite annotation.

3.2.3.1 MFSearcher for compound database search and prediction of elemental composition

The first step in compound annotation is querying the estimated mass values from the compound databases and predicting the elemental compositions using the exact mass values. The MFSearcher tool [11, 12] used for this purpose is integrated in MassChroViewer, and the mass values selected from the 2D chromatogram and peak list can be seamlessly queried (**Figure 1(b)**). Concise and understandable compound candidates are obtained when using the UC2 database in MFSearcher, whereby the constitutional isomers redundantly recorded in several databases are compiled in a single search result with consideration of the dissociation of the hydrogens and metals [11].

3.2.3.2 Fragment Calculator for visualization of chemical structure

The chemical structures of the candidate compounds found during the database search using MFSearcher are immediately visualized in the Fragment Calculator tool, which can be used to calculate the mass and neutral loss of the user-selected molecule substructure (**Figure 1(d)**). This calculation function enables the user to narrow down the candidate compounds by assigning appropriate and understandable substructures to the MSⁿ fragments available in the MS2Viewer tool (**Figure 1(c)**).

3.2.3.3 FlavonoidSearch for flavonoid annotation

The FlavonoidSearch system [13], which can be used to predict the flavonoid aglycones based on the MSⁿ spectra, is also integrated in MassChroViewer. Flavonoids are secondary metabolites derived from plants. More than 7000 flavonoids have been identified, with many exhibiting bio-activities such as antioxidant, anti-inflammatory, and estrogen-like activities. An MSⁿ spectrum browsing using MS2Viewer can be immediately evaluated by the FlavonoidSearch GUI tool (**Figure 1(e)**). The candidate *O*-type substituents (glycosides and acyls) can also be searched for based on the mass values of the neutral losses.

3.3 Food Metabolome Repository

The distribution of an unknown compound among samples (sample specificity) sometimes constitutes valuable information for annotation of the unknown compound. If sample specificity information

such as the peak is detected in a specific plant family, or the peak is commonly detected in the fermented foods, the candidate compounds can be further narrowed down by referencing literature on the origin of the candidate compounds. However, there have been no reports on databases that can be searched for peaks experimentally detected in a wide range of samples based on the detected mass values.

The present authors developed the Food Metabolome Repository database (<http://metabolites.in/foods>), which contains LC–MS-based metabolome data obtained from various foods. The database can be searched for peaks, including unknown ones, based on the mass values (**Figure 2**). Because foods contain a wide variety of organisms that have been subjected to various cooking and other processes, their constituent chemical compounds are also very diverse. The approximately 230 items of representative Japanese foods listed in the Standard Tables of Food Composition in Japan - 2015 (Seventh Revised Version), published by the country's Ministry of Education, Culture, Sports, Science and Technology, were analyzed by LC–high-resolution MS. The compound peaks were detected using PowerGetBatch, and the candidate compounds and flavonoids were searched for using MFSearcher and FlavonoidSearch, respectively. The obtained information were attached to the peaks (**Figure 2(b)** and **(c)**). The metabolome data acquired from 136 foods are presently available to the public (**Figure 2(a)**).

The LC–MS elution profiles of different foods can be compared using 2D mass chromatogram images in Microsoft PowerPoint (MassChroBook), which can be accessed through the top page of the Food Metabolome Repository. The following features of foods are intuitively recognizable in MassChroBook: 1) a broad diversity of peaks occur in plants (vegetables and fruits), implying, the biosynthesis of various secondary metabolites; 2) medium-to-high-polar compounds are rare in fishes, but few variations of low-polar compounds are observed; 3) many medium-polar compounds occur in fermented foods such as “Natto” (fermented soybeans) and foods known to undergo rapid auto-proteolysis, such as “Prawn” and “Squid,” with many of the peaks supposed to be peptides owing to their detection as multivalent ions.

The search function for detecting the peaks in foods by the mass values is useful for representing the sample specificity of the unknown peaks, enabling the annotation of the unknown compounds (**Figure 2(d)**). A user may further annotate the unknown peaks in publicized foods data using MassChroViewer. The mass chromatogram data in mzXML format and the peak list data, which are readable by MassChroViewer, are available at the peak list page for each food.

(a) Food list

Food Metabolome Repository

Home Food List Search Help

Food List

Show All entries

Item No.	Food Group	Food and Description	#Peak Pos	#Peak Neg	Status
01001	Cereals	Common wheat, bread, white	6,905	4,235	A. Published
01002	Cereals	Common wheat, "udon" (thick wheat noodles), uncooked	5,425	2,909	A. Published
01003	Cereals	Common wheat, "Chikwatu" (tube-shaped steamed wheat dough)	3,479	1,817	A. Published
01004	Potatoes and starches	Konjac, block, made from fine powder	1,337	1,130	A. Published
01005	Potatoes and starches	Potatoes, tuber, raw	2,458	2,119	A. Published
01006	Potatoes and starches	Yam, Chinese yam, "ichamo", tuberous root, raw	2,254	2,585	A. Published
01007	Sugars and sweeteners	Sugars, brown sugar lump	5,440	11,012	A. Published

(c) Peak information

- Database search results
- MSⁿ spectra
- FlavonoidSearch results

Peak Information

04046 Soybeans, natto, "Itohiki-natto" (fermented whole soybean) pos

Peak #20205 MN MID: SE112_5040461_M90

mz: 520.33876 RT: 85.60, Int: 30211600, log(int): 3.36, Adduct: [M+H]⁺

DB search result: (3 names) PC(18:2(6E,4E)(0:0), 2'-inosoyl-sn-glycerol-3-phosphocholine; PC(18:2(6E,4E)(0:0))

Number of candidates found in each DB: ALL: 6253 MS: 1627

FlavonoidSearch top score MS2: 0.000, MS3: 0.000

DB Search Results

Formula	MW	delta ppm	Candidate name (shortest name is shown as representative)	Compound IDs
C26H50O7NP	519.33249	-1.874	PC(18:2(6E,4E)(0:0))	LipidMAPS: LMGPD10000034
C26H50O7NP	519.33249	-1.874	2-inosoyl-sn-glycerol-3-phosphocholine	HMDB: HMDB0562711
C26H50O7NP	519.33249	-1.874	LysPC(18:2(6E,4E)(0:0))	HMDB: HMDB010006 KEGG: R12041000 LipidMAPS: LMGPD10000035

MSn Spectra

Level: 2, Precursor m/z: 520.33893

Splash: splash10-0udi-010000000-07beef9f6833db772

FlavonoidSearch Results

No hits

(b) Peak list

Food Metabolome Repository

[Home](#)
[Food List](#)
[Search](#)
[Help](#)

04046 Soybeans, natto, "Itohiki-natto" (fermented whole soybean)

pos

Positive

Negative

Sample Details

Download

Show peak positions

Clear selection

View

Search in all foods

Show

16109

65.66

943.52507

3.87

[M+H]⁺

(23 names) 22-Hydroxyarabonic acid 28-O-alpha-L-rhamnopyranosyl-(1->4)-beta-D-glucopyranosyl-(1->6)-beta-D-glucopyranosyl ester, Buddleiosaponin IV, Phorbolide A, Acaribioside A, (-)-Acaribioside A, Soyasapogenol...

6253

MS2

0.000

12585

42.15

841.48360

5.45

[M+H]⁺

6253

MS2

0.000

20205

85.60

520.33879

3.36

[M+H]⁺

(3 names) PC(18:2(6E,4E)(0:0), 2'-inosoyl-sn-glycerol-3-phosphocholine, PC(18:2(6E,4E)(0:0))

6253

MS2

0.000

19943

68.68

797.46952

5.36

[M+H]⁺

(16 names) Maltogenin 5-

6253

MS2

0.000

necessary in the experiment-based metabolome database (e.g. Food Metabolome Repository) used for compound annotation, as well as in the environments for integrating all the acquired information (e.g., using MassChroViewer). Enrichment of the experiment-based metabolome database is of particular importance to identifying the unknown compound of immediate interest by confirming the true positives and narrowing down the candidate compounds based on the sample specificities. Improvement in the compound coverage of Food Metabolome Repository would also enhance the applicability of the database in various research fields. For example, it would enable the inclusion of low-polar compounds such as carotenoids and volatiles that can be detected by gas chromatography–MS. These compounds cannot be detected by the current applied analytical methods that are based on reversed phase-LC and MS analysis through electron spray ionization. Inclusion of such compounds would make the database appropriate for the analyses of their functionalities such as the antioxidant function and color with regard to carotenoids, and fragrance and biological interactions in the case of volatiles. Provision for manually curated and high-quality peak would also critically increase the value of an experiment-based database. The current Food Metabolome Repository includes many false positive, as well as potentially false negative, peaks because data obtained by automatic software calculation using a specific set of parameters were deposited in it without further curation. The development of a system for efficient collection of manual curation results by administrators and users, and feeding them back to the repository would facilitate more accurate depiction of the sample specificities, clearer planning of next approaches, and rapid annotation and identification of valuable unknown compounds. The use of an artificial intelligence (AI) application promises to improve the processing of untargeted metabolomics data, especially for the detection of the true positive peaks and compound annotation. Further, the digitalization of human intellect and existing knowledge as was demonstrated in the development of the FlavonoidSearch system for elucidating the chemical structure of flavonoids using MS/MS spectra [13], would also be beneficial to improving the computational annotation of the chemical structures of a broad diversity of compounds.

4. Conclusions

This paper introduced innovative data processing tools and a database recently developed by the authors, namely, PowerGetBatch, which is a peak detection, peak alignment, and peak characterization software; MassChroViewer, which is a quality check and annotation tool; and Food Metabolome Repository, which is an experiment-based metabolome database

for the construction of integrated annotation systems. These tools and database, as well as other metabolomics resources, are available on the KOMICS website of the authors at <http://www.kazusa.or.jp/komics>.

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Comparison of carotenoid contents (β -carotene and lutein) in the blood and follicular fluid of Japanese brown cattle during and after the grazing period

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Abstract: Carotenoid contents of Japanese brown cattle during and after the grazing period were investigated. β -Carotene was presented as a major component along with lutein in the blood and follicular fluid at the estrous period. In the blood, the lower β -carotene level after the grazing season was observed as (918.7 ± 290.3 ng/mL) compared with that during the grazing season as (4325.4 ± 1000.9 ng/mL, $P < 0.01$). In addition, a lower lutein level was observed after the grazing season as (15.1 ± 7.6 ng/mL) compared with that during the grazing season as (189.0 ± 41.7 ng/mL, $P < 0.01$). Moreover, a lower lutein level of the follicular fluid was observed after the grazing season as (11.4 ± 5.7 ng/mL) compared with that during the grazing season as (63.9 ± 40.2 ng/mL, $P < 0.05$). The β -carotene concentration included in the silage is one-seventh to one-twelfth that of fresh green grass and the lutein concentration was one-fifth to one-tenth. These results indicate that lower carotenoids level (in the blood and the follicular fluid) after the grazing season is dependent of the change of feed. More carotenoids might be necessary for breeding cattle, which cannot be fed enough fresh green grass because carotenoids might be especially necessary for bovine reproduction.

Key words: β -carotene, carotenoid, follicular fluid, heifers, lutein

1. Introduction

It is well known that carotenoids are important nutrients not only for humans [1, 2], but also for animals [3-5]. Carotenoids enhance immunity and reproduction performance for livestock [6]. Moreover, β -carotene and lipophilic vitamins such as vitamin A and vitamin E, are important nutrients for reproduction and development of cattle [7, 8]. Lipophilic vitamins and β -carotene concentrations in plasma influence bovine ovarian function [9, 10], and development of follicles [11], and embryos [12]. However the function of carotenoids in cattle is not clear enough. In Japan about thirty years ago, carotenoid-rich green grass was mainly used for cattle feed. The major feed of cattle has been changed from fresh green grass with carotenoids to imported hay

over the last three decades.

Therefore, the accumulation of carotenoids in cattle has dramatically decreased [13, 14]. Japanese livestock industry has serious problems which are the decrease of reproduction performance [14], and tolerance for heat stress in the summer period [15, 16]. It was reported that the serum β -carotene concentration in Japanese black breeding cattle was 673 ± 51 ng/mL (106 to 4,252 ng/mL), and were very low during the summer [17]. Moreover the plasma β -carotene concentration in the three weeks prepartum were greater in ovulatory cows ($2,970 \pm 240$ ng/mL) than in anovulatory cows ($1,530 \pm 140$ ng/mL; $P < 0.001$), and indicated that lower β -carotene concentrations in plasma was a feature of anovulatory cows [13]. β -Carotene played a role independently of vitamin A in the reproductive performance of dairy cows and the β -carotene content in corn silage, which

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is a popular main feed in high-producing dairy cows, was very low [14]. In human medicine it was reported that carotenoids in uterus [18] and in follicular fluid [19], may be useful to prevent and decrease women reproductive disease.

In the previous study, we could demonstrate that almost one month old calves absorbed xanthophylls, one of carotenoids administered, and accumulated them in some organs [5]. However, it is unknown what kind of physiological roles carotenoids play and how carotenoids affect the reproductive functions in adult cattle.

In this study, we tried to investigate accumulation of carotenoids in blood and follicular fluid of heifers. Furthermore, carotenoid contents in grass of grazing land and silage etc. were also investigated. Our purpose is to prove the accumulation of carotenoids in bovine follicular fluid and investigate the role of carotenoids for bovine reproductive performance.

2. Materials and methods

2.1 Animals and diets

Three Japanese Brown cattle [20], which were born in on the farm of the Faculty of Agriculture at Tokai University, were used in this study. Nineteen-month-old female heifers which were born in April on 2013 grew well without getting any disease. Heifers were weighed every two weeks during the experiment. Cattle were fed fresh green grass on grasslands in the Aso region during the grazing season (from April to October), and fed silage etc. after the grazing season. The test period was divided into two periods, based on the grazing seasons (During: during the grazing season in October of 2014, $n=5$; After: after the grazing season in November of 2014, $n=6$). Cattle were fed 10 kg of green grass dry matter (DM) per day per head on grasslands during the grazing season, and 8 kg of (grass silage, whole crop silage, total mixed ration (TMR), corn, and soybean) DM per day per head in the barn after the grazing season. Body weights were increasing well from (Mean $\pm$ SD) 351 ± 31 kg to 382 ± 35 kg.

2.2 Treatments and sample collection

Two blood samples were taken from tail vein by jugular venipuncture into 10 mL heparinized vacutainer tubes (Venoject II, Terumo, Tokyo, Japan) at the estrus period. One of samples was used for carotenoid analyses and immediately stored at -30°C until analyses. The other blood sample was centrifuged within 3 h at $250\times g$ for 10 min at room temperature. The plasma was then removed and stored at -30°C until hormonal analyses.

The time of sample collection was two patterns. One of them is voluntary estrus period. Farm staff visually examined the heifers every AM and PM for sign of estrus. When estrus was observed we detected the estrus time based on trans-rectal palpation and ultrasonographic findings (MyLab™One VET,

Esaote) at the same time. The other time is that heifers were treated by intramuscular injection of 25 mg prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$). After two or three days from treatment we detected the estrus by the same way.

Follicular fluid was collected at the same time. We detected the uterus and ovaries based on transrectal palpation and ultrasonographic findings. Then 3cc per head of 2% lidocaine for epidural anesthesia was treated immediately. Through rectal, follicular fluid was collected from dominant follicle by ovarian syringe that was improved to absorb the follicular fluid. Moreover the ultrasonographic findings were recorded. The samples were collected 5 times (twice $\times 2$ heads and once $\times 1$ head) during the grazing season and 6 times (twice $\times 3$ heads) in the after the grazing season.

The feed which were cattle fed (green grass in October on 2014 and the other in November on 2014) were also collected for carotenoid analyses and immediately stored at -30°C until carotenoid analyses.

2.3 Sample analyses

2.3.1 HPLC analysis of carotenoids in blood and follicular fluid

Extraction and analysis of carotenoids from blood and follicular fluid were carried out with our routine method [5]. Namely, 2mL of ethanol was added to 1mL of blood or follicular fluid and stirred, followed by extraction of carotenoids with 5 mL of ether: hexane (2:8 v/v) solution from this mixture with stirring. After being let stand for 10 min, the upper layer was filtered through a Millipore membrane filter (Millipore Corporation, Massachusetts, USA) and was evaporated until dry. The residue was dissolved in acetone: hexane (2:8 v/v) and was subjected to HPLC.

HPLC analysis of carotenoids was carried out by our routine methods [5]. HPLC was performed with a Hitachi L-6000 intelligent pump, an L-4250 UV-VIS detector set at 450 nm, and a $5\mu\text{m}$ Cosmosil 5SL-II (250X4.6 mm i.d.) column (Nacalai Tesque, Japan) with acetone: hexane (2:8 v/v) as the mobile phase at a flow rate of 1.0 mL/min.

2.3.2 Hormonal analysis in blood

Plasma estradiol (E2) concentrations were measured by CLIA and plasma progesterone (P2) concentrations were measured by ECLIA (MONOLIS Co, Ltd. (8-31-6 Jindaiji higashimachi Cyofu Tokyo Japan).

2.3.3 Statistical analyses

Diameter (mm) of dominant follicle was frozen at its maximum, and that was calculated by the scanner. As the dominant follicle was assumed to be spherical in shape, the volume of dominant follicle (mm^3) was estimated by $\frac{4}{3}\pi \times (\text{diameter of dominant follicle}/2)^3$. Then follicular accumulated concentration (μg) was estimated by (carotenoids

concentration) $\times$ (volume of dominant follicle). For statistical analysis, Student's t-test were used for comparison of the carotenoid concentrations, diameter of dominant follicle, follicular accumulated concentration, blood E2 level and blood P4 level using a commercial statistical software program for personal computers. $P < 0.05$ was considered statistically significant in this study. This study was approved by the Animal Experimentation of Faculty of Agriculture, Tokai University.

3. Results

3.1 Carotenoids in grass, silage, TMR and corn

Structures of identified carotenoids in the food of cattle and these contents were shown in Figure 1 and Table 1, respectively.

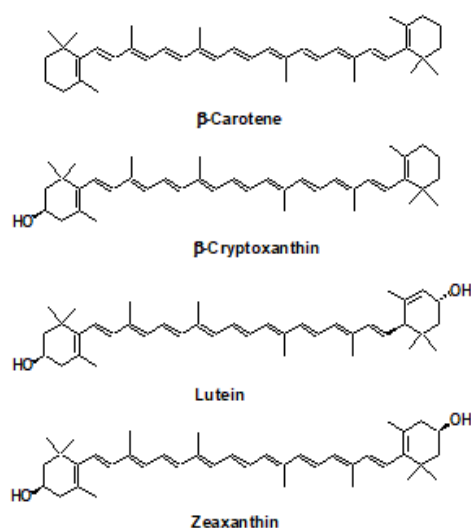


Figure 1. Structures of carotenoids identified in the feed.

β -Carotene was presented as a major component along with lutein as a minor component in grass and silage. Furthermore, zeaxanthin was detected in corn, silage (dry) and TMR. β -Cryptoxanthin was also found in corn. Zeaxanthin was detected in silage, TMR and corn. There is no carotenoid in soybean meal. The lower β -carotene level in silage was observed as (30.3 $\mu\text{g}/\text{mg}$ or 9.7 $\mu\text{g}/\text{m}$) compared with that in grass (195.4 $\mu\text{g}/\text{mg}$). There was a small amount of β -carotene in whole crop silage, TMR and corn. The lower lutein level in silage was observed as (6.2 $\mu\text{g}/\text{mg}$ or 10.9 $\mu\text{g}/\text{mg}$) compared with that in grass (52 $\mu\text{g}/\text{mg}$). Moreover the lower lutein level in whole crop silage and TMR and corn, was observed compared with that in grass. The β -carotene concentration included in the silage is one-seventh to one-twelfth that of fresh green grass and the lutein concentration was one-fifth to one-tenth.

3.2 Carotenoids concentration in blood during and after the grazing season

Among the accumulated carotenoid contents at the estrous period in the blood, β -carotene was

presented as a major component along with lutein as a minor component. In the blood, the lower β -carotene level after the grazing season was observed as (Mean $\pm$ SD; 918.7 ± 290.3 ng/mL) compared with that of during the grazing season as (4325.4 ± 1000.9 ng/mL, $P < 0.01$). In addition the lower lutein level was observed after the grazing season as (15.1 ± 7.6 ng/mL) compared with that of during the grazing season as (189.0 ± 41.7 ng/mL, $P < 0.01$) as shown in Figure 2.

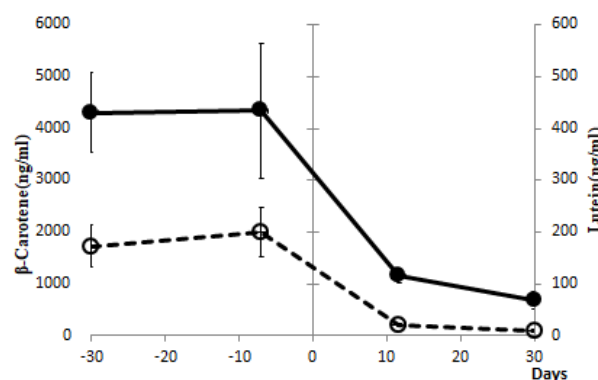


Figure 2. Individual carotenoids contents change between during and after the grazing season. β -Carotene ; —●—, Lutein ; --○--, Date are expressed means $\pm$ SD. Day 0 indicates the end day of grazing.

3.3 Carotenoids concentration and accumulated concentration in follicular fluid

There was no difference in ovarian follicles size by ultrasound measurements in after season as (13.3 ± 1.9 mm) and during season as (12.8 ± 0.8 mm). Although there is no statistical significance, a lower β -carotene level of the follicular fluid was observed in the After period as (968.5 ng/mL $\pm$ 162.8 ng/mL) compared with during the grazing season as (1493.0 ng/mL $\pm$ 681.3 ng/mL, $P = 0.10$). However, a lower lutein level was observed after the grazing season as (11.4 ± 5.7 ng/mL) compared with that of during the grazing season as (63.9 ± 40.2 ng/mL, $P < 0.05$).

Moreover, in estimating follicular accumulated concentration, a lower lutein level after the grazing season was calculated compared with that during the grazing season (16.0 ± 13.2 μg vs. 77.2 ± 62.5 μg , $P < 0.05$) (Table 2, Figure 3).

3.4 Blood hormonal concentration

There was no difference in E2 level after the grazing season as (30.3 ± 5.6 pg/mL) and during the grazing season (27.7 ± 5.7 pg/mL), and P4 level after the grazing season as (0.13 ± 0.03 ng/mL) and during the grazing season (0.14 ± 0.07 ng/mL).

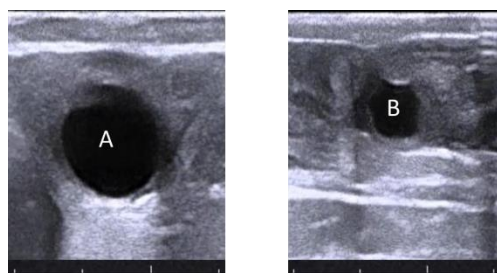


Figure 3. Ultrasound images of the follicle at estrus.

A: A follicle (13.8 mm) before aspirating follicular fluid.

B: A follicle (8.5 mm) after aspirating follicular fluid. Note the scale on this image is 10 mm per line. (10.0 MHz probe)

4. Discussion

The lower β -carotene and lutein level in blood were observed after the grazing season compared with during the grazing period ($P < 0.01$). On the other hand the β -carotene concentration included in the silage is one-seventh to one-twelfth that of fresh green grass and the lutein concentration was one-fifth to one-tenth. These results indicate that lower carotenoids level (in the blood and the follicular fluid) after the grazing season is dependent on the change of feed.

Everybody knows that carotenoid-rich green grass is necessary for cattle. Carotenoids are potential biomarkers for traceability of cows' feeding management [21]. However cattle have not been fed enough fresh green grass in Japan [13, 14]. Oxidative stress during the periparturient and early lactation period may contribute to a number of health disorders in dairy cattle. The antioxidant requirements of cows will likely increase as production demands continue to escalate within the dairy industry [22].

Moreover, the south western Japan, the number of hot days when diurnal temperature exceeds 35 °C was counted more than 20 days in 2004, 23 days in 2006 and 23 days in 2010 (Japan Meteorological Agency), and reproductive performance was deteriorated [15]. Summer heat stress (HS) weakens estrous behavior, suppresses follicular development, and causes early embryo mortality [16, 23]. HS upsets prostaglandin secretions, which would be expected to decrease the motility of the oviductal smooth muscle [24]. The plasma Vitamin E and β -carotene concentrations are positively correlated with the embryo collection results [12]. It was reported that intrafollicular concentration of vitamin A may be used as a physiological indicator of follicular quality and function in cattle. The higher concentrations of vitamin A in healthy follicles might be due to a local conversion of β -carotene into vitamin A in follicular structures [11].

In human, retinol and retinoic acid are essential for a number of reproductive processes. These are investigated in peripheral plasma and follicular fluid

in women undergoing in vitro fertilization. Retinoic acid plays a fundamental role in oocyte development and quality, and may contribute to decreased fecundity of participants with endometriosis [19]. Moreover there are associations between serum antioxidants and endogenous hormones in healthy premenopausal women [25]. Although there was no difference in hormonal blood levels (E2 and P4) between the two periods in our study, it is necessary to further reveal the relationship of hormones and carotenoids in cattle further.

It was found that carotenoid in follicular fluid after the grazing was lower than that during the grazing. However the rate of decrease in follicular fluid was less than those of blood. The reasons may be that carotenoids were accumulated in follicular fluid after the grazing season [11, 19, 26]. The other reason was that the size of dominant follicular may be related to the carotenoids concentration. Further study will be needed to investigate the reasons.

Moreover, carotenoids may be useful for prevention of bovine uterine disease. β -Carotene and other carotenoids protect human skin and tissues from sunlight UV (ultraviolet) [1]. Protection effect from UV light will be useful for bovine embryo transfer and reproductive medicine. Lutein is a xanthophyll type of carotenoid. Since animals cannot synthesize lutein, they must obtain it from diet. It is absorbed from the intestinal epithelium into the blood and circulates systemically to reach the liver and retina and is concentrated in the macula. In human, lutein acts for health of the eye and protects the macula from damage by blue light. Recently lutein has been focused for several biological functions such as an antioxidant and neuroprotective treatments [27-29]. Moreover Xu et al. [30] reported that addition of lutein in bovine diet could improve the production performance and health status of dairy cows. Feeding lutein can enhance the antioxidant capacity of cows.

This study found that follicular fluid concentrations of lutein, a kind of xanthophyll, were significantly higher during grazing period. This suggests the interesting possibility that lutein might be an important component for maintaining reproductive function during breeding season in cattle.

However mechanistic evidence for effects of lutein is not clear in cattle.

These studies indicate that carotenoids concentration in blood and follicular fluid is dependent on the carotenoids concentration in the feed. It was found that in the grazing periods (from April to October) grazing cattle took in carotenoids (β -carotene 2g/day, lutein 0.5g/day). The ideal calving season in Japan for grazing cattle is in April. Cattle which deliver in April can eat a sufficient amount of fresh green grass for six months.

Carotenoids might be especially necessary for bovine calving, milk secretion, conception and maintaining pregnancy. This study indicated that in

the blood β -carotene and lutein level after the grazing season was lower than those during the grazing season. Moreover the lutein level in the follicular fluid was lower than that during the grazing season. Therefore more carotenoids may be necessary for breeding cattle, which cannot be fed enough fresh green grass. The role that carotenoids play in cattle must be studied further.

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Table 1. Carotenoid concentration (ng/g) in grass, silage, TMR and corn

	Grass (dry)	Silage (dry) ^a	Silage (dry) ^b	Whole crop silage (dry)	TMR (dry)	Corn (dry)
β -Carotene	195.4	30.3	9.7	4.6	8.4	0.3
β -Cryptoxanthin	N.D.	N.D.	N.D.	N.D.	N.D.	0.4
Lutein	52	6.2	10.9	7.6	3.3	0.7
Zeaxanthin	N.D.	N.D.	4.0	0.7	0.6	0.7
Others			3.5 ^c	2.6 ^c	1.0 ^c	0.4 ^c
Total	247.4	36.5	24.6	15.4	13.2	2.3

^a The first lot, ^b The second lot, ^c epoxy carotenoids, Soybean meal; not detected, N.D. not detected

Table 2. Carotenoid concentration in follicular fluid and estimated follicular accumulated concentration

	Period	During	After
	The number of days relative the to the end of grazing	-13.8 $\pm$ 12.8	22 $\pm$ 7.8
	n	5	6
Ovarian Follicle	Diameter (mm)	12.8 $\pm$ 0.8	13.3 $\pm$ 1.9
	Volume (mm ³)	1108.8 $\pm$ 220.3	1304.8 $\pm$ 631.9
Follicular fluid concentration (ng/mL)	β -Carotene	1549.6 $\pm$ 773.0	968.5 $\pm$ 162.8
	Lutein	75.7 $\pm$ 35.2 ^{a)}	11.4 $\pm$ 5.7 ^{b)}
Estimated follicular accumulated concentration (μ g) (Concentration $\times$ Volume)	β -Carotene	1743.2 $\pm$ 1189.9	1305.8 $\pm$ 805.8
	Lutein	77.2 $\pm$ 62.5 ^{c)}	16 $\pm$ 13.2 ^{d)}

a, b; c, d ; P < 0.05 Date are expressed means $\pm$ SD

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Carotenoid-Solvent Interaction Studied by the Simulation of Steady-State Optical Absorption Spectra

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Abstract: The chemical structures of the carotenoid molecules are very simple, and one might think that the electric feature of it is easily predicted. However, it still has so much unknown information except the correlation between the electronic energy state and the length of effective conjugation chain of carotenoids. To investigate the electric feature of the carotenoids, the most essential method is measuring the optical absorption spectra, but simulating it from the resonance Raman spectra is also the effective way. From this reason, we studied the optical absorption spectra as well as resonance Raman spectra of 15 different kinds of cyclic carotenoid molecules, recorded in tetrahydrofuran (THF) solutions at room temperature. The whole band shapes of the absorption spectra of all these carotenoid molecules were successfully simulated based on a stochastic model using Brownian oscillators. The parameters obtained from the simulation made it possible to discuss the intermolecular interaction between carotenoids and solvent THF molecules quantitatively.

1. Introduction

Carotenoids are ubiquitous pigments in photosynthesis [1, 2]. They absorb blue-green spectral region of sunlight and transfer the captured energy to chlorophylls that have scant absorption in this spectral range. This singlet-singlet type energy transfer defines the overall efficiency of photosynthetic light reaction. Carotenoids also serve as the scavenger of the excess amount of light exposure. In this case, triplet-triplet type energy transfer from chlorophylls to carotenoids does have an important role. In the light-harvesting pigment-pigment complexes from purple photosynthetic bacteria, carotenoids have an additional role of structural stabilization of the pigment-protein complexes [3]. All of these roles are essential for photosynthetic organisms to survive on the earth.

Carotenoids achieve the above roles of energy transfer through their electronic features, which can be defined by their linear conjugated polyene structure, or more precisely by their electronic structures in low-lying singlet excited states [4-7]. Predicting the electronic structure of the carotenoids looks to be easily done by modern molecule physics, because of their simplicity of chemical structure. However, it is still poorly unknown except the transition from the ground state to the second excited state energy tightly

depends on the length of the conjugation chain and on the polarity of the solvent [8]. The spectral and functional variety of the natural carotenoids is expected to exhibit from the ring group and the several functional groups, but to understand that plenty of information is yet needed. In biological systems the situation is even more complicated because of the presence of pigment-protein interaction. Generally carotenoids exist in the very anisotropic environments, mostly bound to protein and this makes the problem so difficult. To figure out how the carotenoids perform their biological function *in vivo*, it is important to consider the interaction between the carotenoid molecule and the surrounding molecules.

In order to investigate the interaction between pigment and surrounding molecules, measurement of the optical absorption spectra has the essential importance. Since the optical absorption occurs when the irradiated light energy is in resonance with the transition energy of the pigment molecules, the observed absorption spectra sensitively reflects the electronic properties of the pigment molecule perturbed by the surroundings. There are so many studies on optical absorption spectra of carotenoids up to now. Combining the measurement of resonance Raman excitation profiles and the analysis in terms of the Albrecht theory the absorption spectra of β -carotene were simulated [9-11]. The absorption spectra of carotenoids were sometimes correlated with the polarity or polarizability of solvents [8, 12-14].

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The correlation between the frequency of the C=C stretching vibration of carotenoids and the energy of absorption transition has been known for decades, starting just several years after the first laser Raman spectroscopy on carotenoids by Rimai et al. [15]. Similarly, the correlation between the C=C stretching and the conjugation length has been highlighted for decades in numerous reviews [16-18]. Recently, this interesting issue was re-visited by the group of Robert [19]. A good linear relationship between the frequency of C=C stretching mode and the energy of absorption transition of carotenoids with or without end cyclic structures has been interpreted by introducing the idea of effective conjugation length of carotenoids [19,20]. There is a report on even another theoretical approach that correlates solvent polarizability and the transition energies of a carotenoid peridinin in different solvents [21]. In this report, the similar theoretical approach with this presented paper (the cumulant expansion technique) has been employed and the solvent polarizability has been discussed by the Lippert-Mataga equation.

In this study, we propose generally accepted approach to interpret the shape of absorption spectra based on the stochastic theory using the Brownian oscillator model. Sue and Mukamel originally proposed this idea, and used stochastic Gaussian model to interpret solvation dynamics in coherent and spontaneous Raman spectroscopy of β -carotene [22]. More recently, the group of McHale has explored this

approach to β -carotene in non-polar solvent [23]. They used the Brownian oscillator model, and examined temperature effect on the absorption line-shape. The work of Kelly emphasized the importance of Raman excitation profiles in tandem with absorption spectra to quantify displacements, and to distinguish broadening mechanisms [24]. This is actually true, but laborious work is required when we apply this approach to number of carotenoids. Therefore, we have adopted more simple approach, and examined 15 different species of carotenoids. We found new systematic relation about the carotenoid-solvent interaction by simulating the band-shape of absorption spectra using a stochastic model.

2. Materials and Methods

2.1 Isolation and preparation of the studied carotenoids

Figure 1 shows the chemical structure of carotenoids used in this study. Except for β -carotene (9), canthaxanthin (11) and astaxanthin (13), all other carotenoids were isolated and purified from the corresponding plant materials. All-*trans*- β -carotene was purchased from Wako Pure Chemicals Japan and purified by twice recrystallization from benzene solution. All-*trans*-canthaxanthin and all-*trans*-astaxanthin were gift from Kuraray Chemical Company Japan and used as they received.

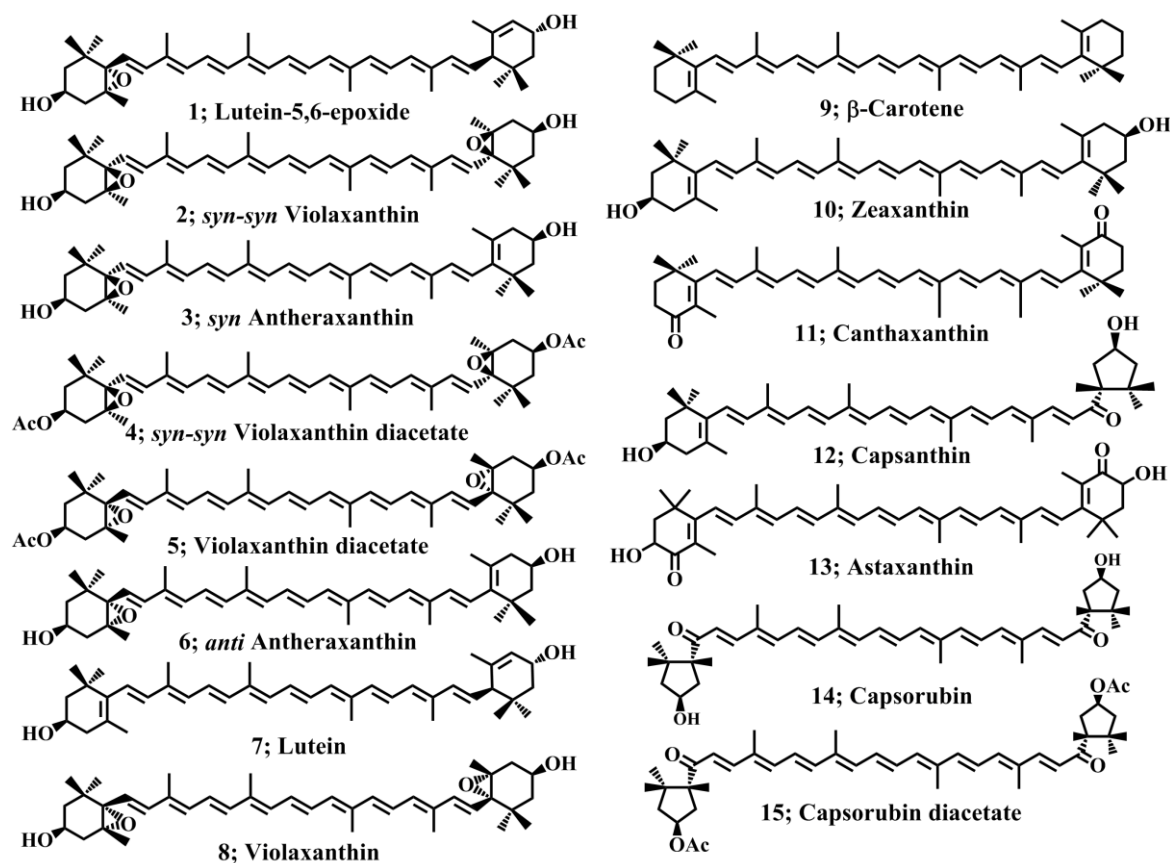


Figure 1. Chemical structure of carotenoids used in this study.

2.2 A general isolation procedure of carotenoids from the corresponding plant materials (fruits, flowers, leaves)

The plant materials were extracted three times with MeOH and once with Et₂O. The methanolic solutions were combined and were transferred into toluene/hexane (~1:1) mixture. This solution was evaporated and the residue was dissolved in Et₂O. The ethereal solutions were combined and this “total extract” was saponified by 30% KOH-MeOH in heterogeneous phase overnight. After washing alkali-free the ethereal solution was evaporated, dried by anhydrous Na₂SO₄ and the residue was partitioned between MeOH/H₂O (9:1) mixture and hexane in a separatory funnel. This procedure resulted in two fractions: fraction of hypophasic carotenoids [(containing more than one OH group and epoxy group(s)) and epiphasic carotenoids (carotenoid hydrocarbons and carotenoids containing only one OH group). After the usual work-up [25] the fraction containing the hypophasic carotenoids was evaporated and crystallized from toluene/hexane (~1:5) mixture. The fraction of epiphasic carotenoids was evaporated and crystallized from toluene/MeOH (~1:5) mixture.

The separation of hypophasic (polar) carotenoids was carried out by column liquid chromatography (CLC) using CaCO₃ (Ph. Hg. VI.; Biogal, Hungary; precipitated pharmaceutical) as adsorbent and toluene/hexane mixtures as eluent. After the identification (UV/VIS spectroscopy) and the usual work-up the separated carotenoids were crystallized from toluene/hexane (~1:5) mixture. The purity control of the crystalline samples was carried out by HPLC. The identification of pure crystalline samples was completed by NMR, IR, CD and MS methods.

2.3 Preparation of the acetylated carotenoids

Capsorubin diacetate (**15**), violaxanthin diacetate (**5**) and *syn-syn* violaxanthin diacetate (**4**) were prepared from the pure crystalline capsorubin [(**14**), isolated from red paprika, *Capsicum annuum*], violaxanthin [(**8**), isolated from the blossoms of yellow pansy, *Viola tricolor*] and *syn-syn* violaxanthin [(**2**), prepared from zeaxanthin diacetate; zeaxanthin (**10**) was isolated from the berries of *Lycium halimifolium* or *Lycium barbarum*] using the well-known acetylation procedure [25].

2.4 Isolation of the other carotenoids from natural sources

Capsorubin (**14**) and capsanthin (**12**) were isolated from red paprika (*Capsicum annuum*) using the general isolation procedure in our laboratory [26-29]. Zeaxanthin (**10**) was isolated from the ripe red berries of *Lycium halimifolium* or *Lycium barbarum* [30]. Violaxanthin (**8**) was isolated from the blossoms of yellow pansy [25, 31]. Lutein (**7**) was isolated from the flowers of *Helianthus annuus*, *Taraxacum*

officinale and from the leaves of *Acer campestre* [25, 32]. Antheraxanthin (**6**) was isolated from the pollen sacs (anthers) of *Lilium candidum*, from the pollen sacs and petals of *Lilium tigrinum* and from the leaves of *Acer campestre* [25, 33-35]. *Syn-antheraxanthin* (**3**) and *anti-antheraxanthin* (**6**) as well as the diastereoisomers of violaxanthin [(*syn-syn*- (**2**) and *syn-anti*-violaxanthin)] were prepared from zeaxanthin-diacetate [(prepared from zeaxanthin (**10**) using the well-known acetylation procedure)] by the well-known method of epoxidation, using monoperphthalic acid in absolute ether solution [29, 30]. Lutein-5,6-epoxide (**1**) was isolated from the flowers of *Helianthus annuus* and from the flowers of *Chelidonium majus* [32, 38].

2.5 Spectroscopic measurements

Optical absorption spectra of carotenoids in tetrahydrofuran (THF) solutions were recorded on JASCO V-670 spectrophotometer at room temperature. These solutions were placed in a quartz cuvette to record the Raman spectra. Resonance Raman spectra were recorded using 514.5 nm excitation light from an Ar⁺ laser (ILT Model 5500A, Japan Laser Inc.,) at room temperature. The 90° scattering from each sample was focused onto a Raman spectrometer (SpectraPro 2300i, Acton Research Corporation, USA) equipped with a liquid nitrogen cooled CCD camera (LN/CCD-576-E/1, Roper Scientific, USA). The spectral resolution of the present set-up for Raman spectroscopy is as large as 5 cm⁻¹, which is good enough to discuss the bandwidths of Raman lines for carotenoids in THF solutions.

3. Theoretical simulation

When we are willing to obtain the information concerning the motional behavior of the solvent molecules surrounding the pigment molecules from the measurements of optical absorption spectra, selection of the appropriate model for theoretical simulation has primal importance. This is because the shapes of the absorption spectra of pigment molecules are determined by the interaction between electrons inherent to the optical transition of the pigment molecules and the surrounding solvent molecules. The motion of solvent molecules could approximately be described as the ensemble of oscillators. Consequently, the effect induced by the electrons of the pigments modulates the frequencies of the oscillators randomly. This modulation could theoretically be written as a stochastic process, based on the interaction between the electron and oscillator [39]. Therefore, for the theoretical simulation of the optical absorption spectra, it is crucial to describe the above effect of the frequency modulation in an appropriate stochastic process.

The cumulant expansion is an appropriate method to describe the frequency modulation of the

oscillator in a stochastic process [39]. This method gives an accurate result even in the case that the oscillators experience random modulation supposing a Gauss process. In this case the expansion would be cut off at secondary term, and the higher cumulant will be eliminated by the central limit theorem. Cumulant expansion is used not only for interpreting the limited case of the local vibration of the system, but also can be widely used for many other cases that express the harmony of the system in which many molecules move coherently, *e.g.* a Brownian oscillator model [40-42]. When one of the oscillators having frequency ω_j receives random modulation, the time dependence of the frequency of this oscillator can be described as,

$$\omega_j(t) = \omega_0 + \omega_1(t) \quad (1),$$

where subscript j means the j -th particle, ω_0 is the standard frequency and $\omega_1(t)$ is the frequency corresponding to the modulation. Now we suppose that $\omega_1(t)$ takes the stochastic process like a Gauss process. In order to simulate the resonance absorption spectra we need to know the exact form of correlation function that describes the motion of the target molecules. However, what we can obtain directly from the Raman measurement is the information concerning the frequency of the oscillators. Therefore, in order to perform the simulation of absorption spectra, we need to know the relationship between the correlation function of the motion and the frequency of the oscillator. Cumulant expansion can solve this issue. The cumulant of the motion correlation function of oscillator and frequency correlation function could be connected to each other. This is the most important point. There are two factors that characterise the random modulation of frequency, *i.e.*, the speed and the magnitude of the change of modulation. These two factors decide the shape of the absorption spectrum.

The electronic transitions of pigment molecules are coupled to the vibrational motions of these molecules. The nuclear motion of a pigment molecule, which is coupled to an electronic transition of the same molecule, is supposed to have two equilibrium positions in the ground and excited electronic states, and can be modelled using independent harmonic modes. In this case the Hamiltonian of the system can be written as below.

$$H_g = \frac{1}{2} \sum_j \hbar \omega_j (p_j^2 + q_j^2) \quad (2),$$

$$H_e = \hbar \omega_{eg}^0 + \frac{1}{2} \sum_j \hbar \omega_j [p_j^2 + (q_j + d_j)^2] \quad (3).$$

Here the subscript g and e means ground and excited electronic states, and $\hbar$ is the Planck's constant. p_j , q_j and d_j are the dimensionless momentum, nuclear coordinate and the deviation of equilibrium position of the j -th mode, respectively. The ω_{eg}^0 is the energy gap between the ground and excited state. In this case, the electronic energy gap ω_{eg} is written as,

$$\omega_{eg} = \omega_{eg}^0 + \frac{1}{2} \sum_j d_j^2 \omega_j \quad (4),$$

and the interaction between the electron and the nuclear motion can be written as

$$U = \hbar \sum_j \omega_j d_j q_j \quad (5).$$

Let us introduce a correlation function $\langle U(\tau_1)U(0) \rangle$, where $U(t) \equiv \exp(iH_g t/\hbar)U \exp(-iH_g t/\hbar)$ and the thermal average $\langle \dots \rangle \equiv \text{Tr}[\dots \rho_g]$ with $\rho_g \equiv \exp(-\beta H_g)/\text{Tr}[\exp(-\beta H_g)]$. Here, $\beta \equiv 1/k_B T$ with the Boltzmann constant k_B and temperature T . The correlation function can be as bellow [39].

$$\begin{aligned} \langle U(\tau_1)U(0) \rangle = \\ \hbar^2 \sum_j \omega_j^2 S_j \{ \exp(i\omega_j \tau_1) \bar{n}_j + \exp(-i\omega_j \tau_1) (\bar{n}_j + 1) \} \end{aligned} \quad (6),$$

where $\bar{n}_j = (\exp(\beta \hbar \omega_j) - 1)^{-1}$ and S_j is the Huang-Rhys (HR) factor, which is described as $S_j = 1/2 d_j^2$. The intensity of absorption spectra is given by the Fourier transformation of a correlation function $J(t_1)$ as [39]

$$\begin{aligned} I(\omega_j) \\ = \frac{1}{2\pi} \int_{-\infty}^{\infty} J(t_1) \exp(i\omega_j t) dt \\ = \frac{1}{\pi} \text{Re} \int_0^{\infty} \exp[-i\omega_{eg} t_1 - \\ g(t_1)] \exp(i\omega_j t) dt \end{aligned} \quad (7),$$

with

$$J(t_1) = \exp[-i\omega_{eg} t_1 - g(t_1)] \quad (8),$$

$$g(t_1) \equiv \int_0^{t_1} d\tau_2 \int_0^{\tau_2} d\tau_1 C(\tau_1) \quad (9),$$

$$C(\tau_1) \equiv \frac{1}{\hbar^2} \langle U(\tau_1) U(0) \rangle \quad (10).$$

This is the equation used in the simulation. There are several vibrational modes and here we describe this by introducing the spectral density $\rho(\omega)$. $\rho(\omega)$ can be defined as,

$$\rho(\omega) \equiv \sum_j S_j \delta(\omega - \omega_j) \quad (11).$$

In terms of the spectral density and the reorganization energy λ , the eq. (9) can be rewritten as,

$$g(t) = -i \frac{\lambda}{\hbar} t + i \int_0^\infty d\omega \rho(\omega) \sin(\omega t) \quad (12),$$

$$+ \int_0^\infty d\omega \rho(\omega) \coth\left(\frac{\beta \hbar \omega_j}{2}\right) \{\cos(\omega t) - 1\}$$

$$\lambda = \int_0^\infty d\omega \rho(\omega) \hbar \omega = \hbar \sum_j S_j \omega_j \quad (13).$$

Usually, the vibrational frequency ω_j is not temporally fixed to a constant value, but modulated as eq. (1) due to interactions. Taking this effect into consideration, we introduce the damping constant γ_j , and rewrite eq. (6) as

$$\langle U(\tau_1) U(0) \rangle =$$

$$\hbar^2 \sum_j \omega_j^2 S_j \{ \exp(i(\omega_j - \gamma_j)\tau_1) \bar{n}_j$$

$$+ \exp(-i(\omega_j + \gamma_j)\tau_1) (\bar{n}_j + 1) \} \quad (14).$$

Note that the γ_j basically corresponds to the peak bandwidth of the Raman lines. In this case, the spectral density $\rho(\omega)$ of eq. (6) should be replaced by

$$\rho(\omega) = \sum_j S_j \frac{\gamma_j}{\pi(\gamma_j^2 + (\omega - \omega_j)^2)} \quad (15).$$

Substituting eq. (14) into eq. (8-10) we can finally obtain the equation to perform the spectral simulation with vibrational modes described by the spectral density $\rho(\omega)$ of eq. (15). The parameters only

needed for spectral simulation are the HR factor of each frequency mode and ω_{eg} . Those parameters can be decided by the fitting of the simulated absorption spectra to the experimentally observed absorption spectra.

For the calculation, we used the experimentally obtained data from the Raman spectra as summarized in Table 1. To describe the interaction between an electron in the pigment and surrounding solvent molecules, we also introduced the low frequency mode around 250 cm⁻¹ as its frequency and with 400 cm⁻¹ full width at half maximum (FWHM). These values are employed by referring to the work by author's group for the successful simulation of transient grating signals from β -carotene [40].

4. Results and Discussion

Chemical structures of carotenoid molecules used in this study are displayed in **Figure 1**. We have investigated 15 different species of naturally occurring carotenoids with cyclic end-structures. All of these carotenoids have very similar conjugated polyene backbone (isoprenoid skeleton). Except capsanthin (**12**), capsorubin (**14**), and capsorubin diacetate (**15**), each carotenoid has cyclohexane rings at both ends. Of course the way of conjugation of C=C bond within the cyclohexane ring to the polyene backbone is different among each carotenoid. Also, there are varieties of functional groups depending on the species of carotenoids. These characteristic structural features do affect the transition energy of carotenoids as well as the interaction between carotenoids and solvents.

Figure 2 shows the optical absorption spectra of all the carotenoid molecules recorded in this study. These absorptions correspond to the transition from the ground (S_0) state to the optically allowed (S_2) state [43]. Even though the absorption spectra were recorded with the same solvent THF, the spectral features are different depending on the species of carotenoids. The carotenoids that have epoxide group at both ends (**1**, **2**, **4**, **5**, **8**) show well-resolved structure in their absorption spectra. These structures are due to vibronic transitions of these carotenoids. On the other hand, the carotenoids that have carbonyl groups conjugated to the polyene chain (**11**, **12**, **13**, **14**) show structure-less absorption spectra. The other carotenoids (**3**, **6**, **7**, **9**, **10**, **15**) show vibronic structure but its separation is not good enough. The whole spectral band-shape should be discussed introducing the interaction between carotenoids and surrounding solvent molecules.

Figure 3 shows the resonance Raman spectra of all the carotenoid molecules recorded in this study. Even though the chemical structural difference among carotenoids is obvious, overall spectral features of the Raman spectra look similar to each other. This is due to the appearance of the integrity of all-*trans*

conformation of these carotenoids [44]. The all-*trans* carotenoids give rise to three characteristic strong Raman lines around 1520 cm^{-1} , 1155 cm^{-1} , and 1005 cm^{-1} , which are assigned to in-phase C=C stretching (ν_1), C-C stretching (ν_2) and methyl in-plane rocking (ν_3) vibrational modes, respectively. In the case of all-*trans* carotenoids, both ν_1 and ν_2 modes are observed with similarly strong intensity in resonance Raman spectra. When there is a contamination of *cis* isomers, a weak Raman line should appear around 1140 cm^{-1} region [45]. Nevertheless, the overall similarity, the frequency of ν_1 Raman line shows apparent difference among the carotenoids. The optical absorption spectra were simulated based on stochastic theory as explained above. The fixed parameters we used for theoretical simulation are ν_1 and ν_2 Raman frequencies and their bandwidths, which were determined experimentally (see **Table 1**). The other four important parameters, *i.e.* ω_{eg} and three HR factors of the ν_1 , ν_2 and low frequency modes, were determined by the fitting (see **Table 2**. The HR factors of the low frequency mode are not shown). The spectra shown in dotted line in **Figure 2** are the results of spectral simulation. Satisfactory fitting was achieved for 0-0, 0-1, and “0-2” vibronic bands; the “0-2” vibronic bands show slight deviation. There are some discrepancies in the higher vibronic transition regions. This is due to the anharmonicity of the potential energy surfaces and inevitable within the

limit of harmonic approximation [10].

In order to test the validity of thus determined HR factors, we compared the ratio of the HR factors of ν_1 and ν_2 modes and that of the areas under the peaks of experimentally observed ν_1 and ν_2 Raman lines. The result is shown in **Figure 4** (a). It is expected that the ratio of HR factors is proportional to the ratio of the integrated peak intensities of Raman lines within the limit of linear electron-phonon coupling [46]. Except for the case of carotenoids with two carbonyl groups like canthaxanthin (**11**), astaxanthin (**13**), capsorubin (**14**) and capsorubin diacetate (**15**), rest of the carotenoids show good linear relation, a fact which support the validity of determination of the HR factors of these carotenoids. In fact, the ratio of the HR factors of ν_1 and ν_2 modes show good agreement with the previous reports for violaxanthin, lutein, and β -carotene [23, 47-50]. The deviation of the above two carbonyl containing carotenoids from this trend is mainly due to the ambiguity of the spectral fitting, since these carotenoids show broad and structure-less absorption spectra. This has already been exemplified by the work of Kelley [24]. Capsorubin diacetate (**15**) shows somewhat well resolved absorption spectrum. However, this particular carotenoid shows relatively broad band shape for ν_1 and ν_2 Raman lines, and the relative intensity of the ν_2 Raman line against the ν_1 Raman line is obviously small than those of other carotenoids. This might be a cause of the deviation.

Table 1. The ν_1 and ν_2 mode Raman frequencies (in cm^{-1}) and FWHM of these Raman lines (in cm^{-1}) determined experimentally by resonance Raman measurements of 15 different species of carotenoids. All the data were obtained at room temperature in THF solution. Spectral resolution was 5 cm^{-1} .

Carotenoid	ν_1		ν_2	
	Frequency	FWHM	Frequency	FWHM
1; Lutein-5,6-epoxide	1527	18	1155	15
2; syn-syn Violaxanthin	1527	18	1155	12
3; syn Antheraxanthin	1526	18	1156	12
4; syn-syn Violaxanthin diacetate	1526	18	1155	12
5; Violaxanthin diacetate	1525	18	1154	12
6; anti Antheraxanthin	1524	18	1155	15
7; Lutein	1524	18	1156	15
8; Violaxanthin	1524	16	1153	12
9; β -Carotene	1522	18	1156	12
10; Zeaxanthin	1519	20	1152	13
11; Canthaxanthin	1518	24	1156	18
12; Capsanthin	1516	24	1155	15
13; Astaxanthin	1516	24	1154	18
14; Capsorubin	1515	21	1154	15
15; Capsorubin diacetate	1514	20	1155	15

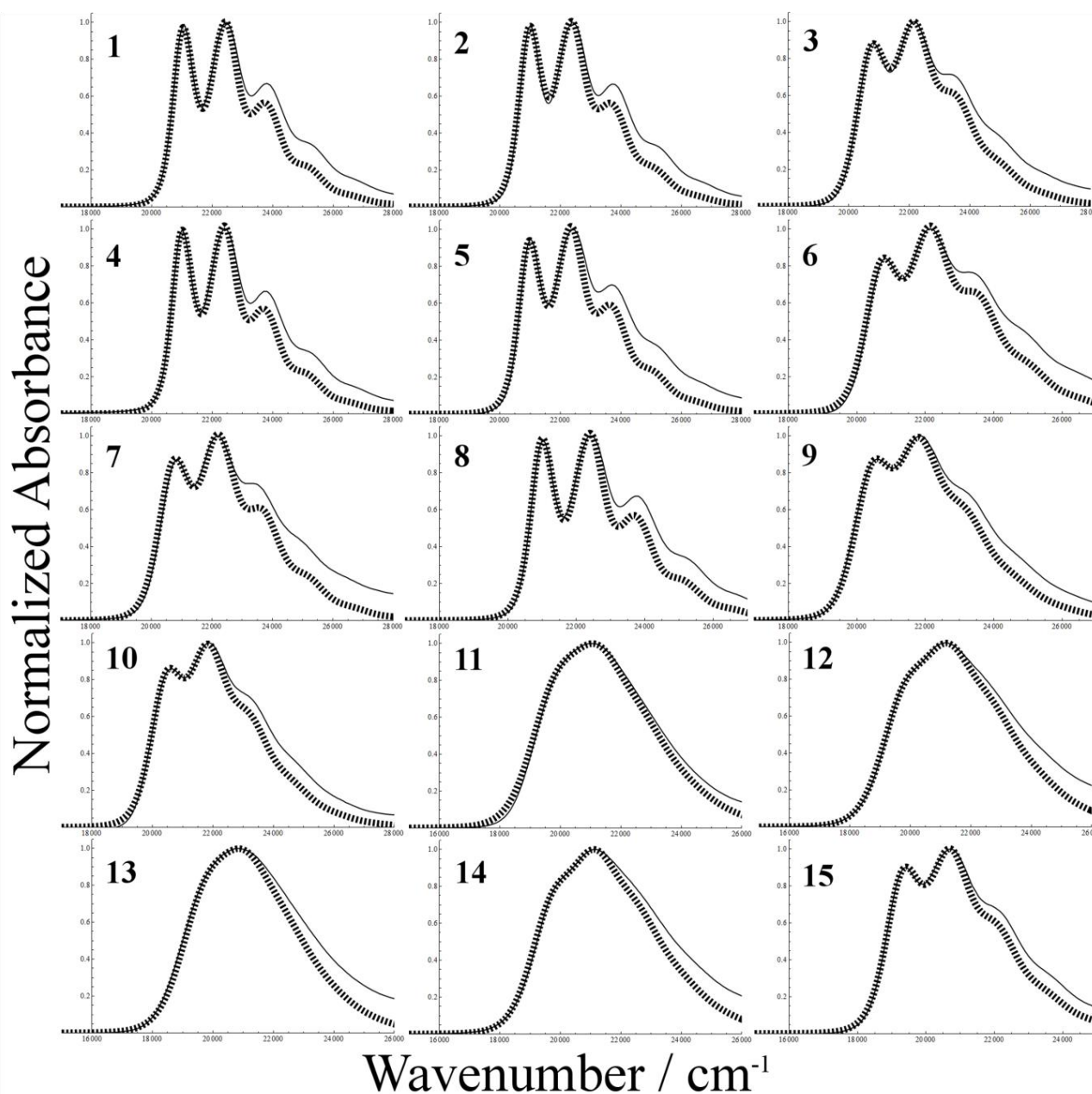


Figure 2. Optical absorption spectra of 15 different species of carotenoids in THF solutions measured at room temperature. Solid lines show the experimentally observed absorption spectra, and dotted lines show the results of spectral simulation. The number displayed at each corner corresponds to the numbers for carotenoids shown in **Figure 1**.

Next we discuss about the reorganization energy λ . This physical parameter can be a good measure of the strength of carotenoid-solvent interaction. In our simulation, however, λ is automatically calculated using spectral density $\rho(\omega)$ and cannot directly be sorted out. Therefore in return, we recalculated the λ value using the second formula of eq. (13). However the reorganization energy calculated from this equation includes both the inner and the outer sphere reorganization energies. To discuss the carotenoid-solvent interaction, we only need the outer sphere reorganization energy λ_{out} , which is defined by the low frequency mode ($j = \text{low}$) component of eq. (15), *i.e.*,

$$\rho_{\text{out}}(\omega) = S_{\text{low}} \frac{\gamma_{\text{low}}}{\pi(\gamma_{\text{low}}^2 + (\omega - \omega_{\text{low}})^2)} \quad (13-1),$$

$$\lambda_{\text{out}} = \int_0^\infty d\omega \rho_{\text{out}}(\omega) \hbar \omega \quad (16).$$

Because the integral of eq. (13-1) does not converge by using eq. (16) as it is, we approximate the frequency ω of under integrand by the peak value ω_{low} of this mode:

$$\lambda_{\text{out}} \cong \int_0^{\infty} d\omega \rho_{\text{out}}(\omega) \hbar \omega_{\text{low}} \quad (17).$$

$$\lambda_{\text{out}} = 0.6778 S_{\text{low}} \hbar \omega_{\text{low}} \quad (17-1).$$

Substituting eq (13-1) and $\omega_{\text{low}} = 250 \text{ cm}^{-1}$, $\gamma_{\text{low}} = 400 \text{ cm}^{-1}$ [40] to equation above, we obtain,

The outer sphere reorganization energies calculated by eq. (17-1) are written in **Table 2**. **Figure 4** (b) shows

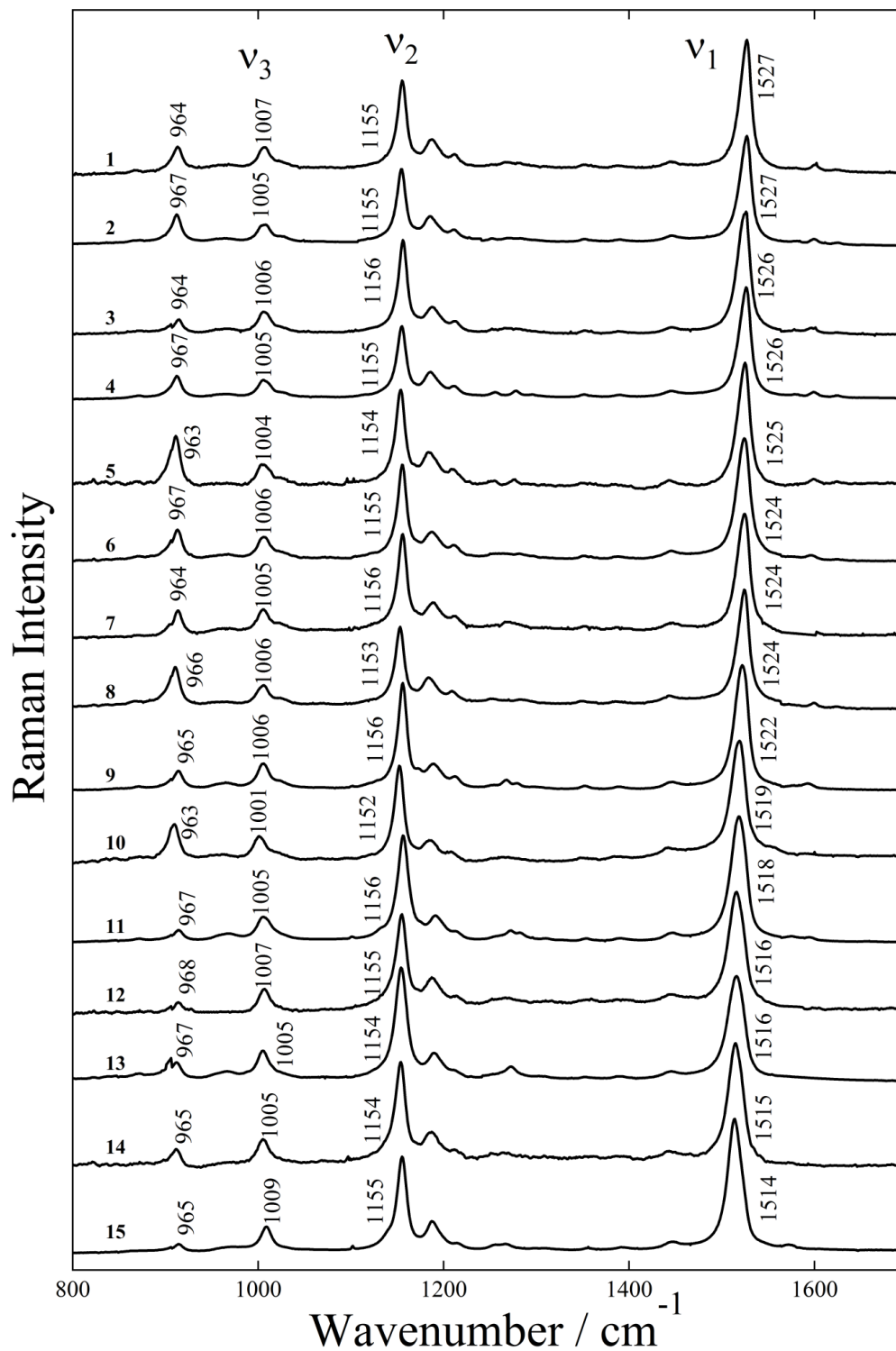


Figure 3. Resonance Raman spectra of 15 different species of carotenoids recorded at room temperature in THF solutions. The number displayed at each left corner of the Raman spectrum corresponds to the numbers for the carotenoids shown in **Figure 1**.

Table 2. The Huang-Rhys (HR) factors of ν_1 and ν_2 modes as well as ω_{eg} (in cm^{-1}) used to simulate the absorption spectra of 15 different species of carotenoids in THF solution. The outer sphere reorganization energies were calculated using eq. (17-1). FWHM of 0-0 bands were determined independently by spectral deconvolution of the absorption spectra using Voigt functions. 0-0 Transition energies (in cm^{-1}) was determined experimentally from the second derivative wave shape of the absorption spectra.

Carotenoid	Huang-Rhys Factors		ω_{eg}	Outer Sphere Reorganization Energy	ν_2 / ν_1 of Raman Line Area Size	FWHM of 0-0 Band	0-0 Transition Energy
	ν_1	ν_2					
1; Lutein-5,6-epoxide	0.68	0.5	22700	381.3	0.875	795	21053
2; <i>syn-syn</i> Violaxanthin	0.645	0.5	22650	423.6	0.903	792	21008
3; <i>syn</i> Antheraxanthin	0.77	0.4	22450	635.4	0.756	1089	20747
4; <i>syn-syn</i> Violaxanthin diacetate	0.67	0.5	22650	381.3	0.918	849	21008
5; Violaxanthin diacetate	0.68	0.5	22650	423.6	0.941	776	21008
6; <i>anti</i> Antheraxanthin	0.85	0.4	22550	635.4	0.848	1153	21008
7; Lutein	1	0.15	22500	677.8	0.673	1235	20747
8; Violaxanthin	0.67	0.5	22650	389.7	0.884	804	21008
9; β -Carotene	0.65	0.5	22150	728.6	0.849	1311	20408
10; Zeaxanthin	0.725	0.45	22150	720.2	0.828	1358	20367
11; Canthaxanthin	1	0.05	21550	—	0.846	1565	19724
12; Capsanthin	1.1	0.08	21650	1322	0.600	1748	19685
13; Astaxanthin	0.7	0.4	21300	—	1.06	1949	19646
14; Capsorubin	1	0.25	21600	—	0.867	1771	19685
15; Capsorubin diacetate	0.65	0.5	20950	—	0.589	1127	19268

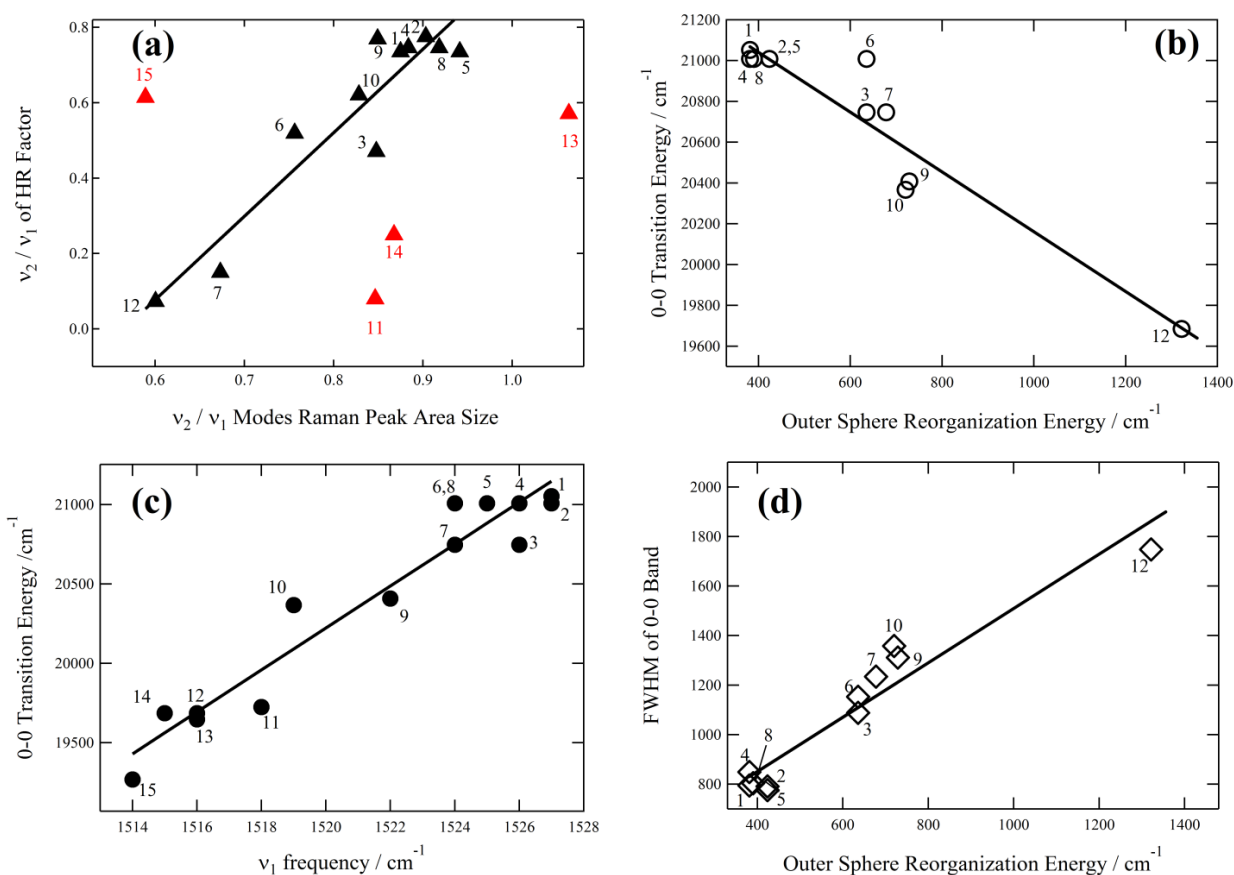


Figure 4. The plot of the ratio of Huang-Rhys (HR) factors of ν_2 mode / ν_1 mode against the ratio of area size of Raman line is shown in (a). Plot of 0-0 transition energies against (b) outer sphere reorganization energies and (c) ν_1 frequencies of 15 different species of carotenoids are shown, respectively. The plot of FWHMs of 0-0 band determined by spectral deconvolution against outer sphere reorganization energy is shown in (d). The numbers displayed in all these panels correspond to those shown in Figure 1.

the plot of 0-0 transition energies against thus calculated outer sphere reorganization energies of carotenoids except canthaxanthin (**11**), astaxanthin (**13**), capsorubin (**14**) and capsorubin diacetate (**15**). These carotenoids were not included since they showed the ambiguity for the determination of HR factors. Here beautiful linear relationship can be seen between the 0-0 transition energies and the outer sphere reorganization energies. The proportional relationship between the 0-0 transition energies and the outer sphere reorganization energies means that the carotenoid whose 0-0 transition energy is large (small), *i.e.* effective conjugation is short (long), has more severe (weak) interaction between the surrounding solvent (THF). This is quite an interesting new finding and should be addressed more precisely in the future work.

Finally we discuss the overall band-shape of the absorption spectra. Depending on the species of carotenoids, some show structured absorption spectra and some other show structure-less absorption spectra. The reason why this kind of difference can be seen is solely due to the difference of the inhomogeneous band-broadening of each of the vibrational band. In order to make clear this point, we have performed a phenomenological evaluation (beside from the Stochastic approach) by the spectral deconvolution of the absorption spectra using several Voigt functions. The examples of the deconvolution are shown in Figure 5. Both the structure-less absorption spectrum of astaxanthin (**13**) (Figure 5 (a)) and structured absorption spectrum of *syn-syn* violaxanthin diacetate (**4**) (Figure 5 (b)) are successfully deconvoluted with 5 series of Voigt profiles. Since the profile appear at 0-0 transition energy is not affected by unwanted effect due to the overlap of several vibrational modes, we here phenomenologically refer the bandwidth of the lowest energy Voigt profile (0-0 band) to as the inhomogeneous bandwidth. The higher energy sub-bands of the Voigt profile correspond to the 0-1, 0-2, transitions and so on. Figure 4 (c) shows the plot of 0-0 transition energies against ν_1 Raman frequencies of all the carotenoids. As has already been exemplified [19], the 0-0 transition energies have a good linear relationship with the ν_1 Raman frequencies. This can be explained by taking the idea of effective conjugation length into account. When the effective length of conjugation is short (long) the 0-0 transition energy becomes large (small) [20]. Therefore, our results successfully support and expand the previous study. In what follows, we will discuss further new findings of carotenoid-solvent interaction. The FWHM of the 0-0 band is plotted against the outer sphere reorganization energy is shown in Figure 4 (d) (for this plot we also exclude the four carotenoids same as Figure 4 (b)). Here again a good linear relationship can be seen. This result demonstrates that the overall spectral feature can be quantitatively discussed based on the magnitude of the

outer sphere reorganization energy. When the outer sphere reorganization energy is large (small), inhomogeneous band broadening is large (small). This correlation defines the overall shape of the absorption spectra of carotenoids. In other words, electron-phonon coupling between carotenoid and surrounding solvent molecules is represented by the outer sphere reorganization energy in our model simulation. The outer sphere reorganization energy is calculated from using the HR factor of low frequency mode, and from the linear correlation shown in Figure 4 (d), this could suggest that the variation of S value of the low frequency mode (data not shown) may become the measure of the inhomogeneous band broadening of the absorption spectra of various carotenoid molecules. Unfortunately, the exact S values used here are not always physically reasonable. However, we think that the present finding may open a door to explore the carotenoid-surrounding interaction based on stochastic model, especially focusing on the low frequency phonon modes. This idea should be correctly addressed by further study using, for example, four-wave mixing spectroscopy [40-42, 51]. The work is now under progress.

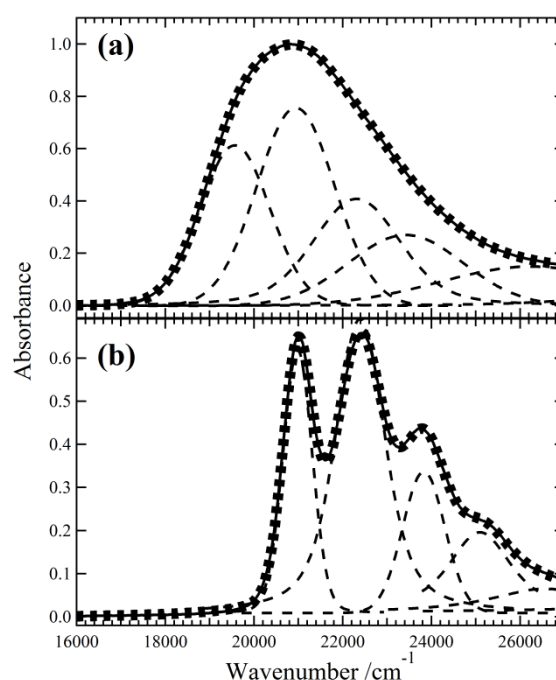


Figure 5. Examples of spectral deconvolution using Voigt functions. (a) shows the result of astaxanthin (**13**), and (b) shows that of *syn-syn* violaxanthin (**4**). Solid lines show the experimentally observed absorption spectra and broken lines show the results of spectral deconvolution. The bold dish lines show the summation of all the Voigt profiles.

5. Conclusions

In this study we have demonstrated the simulation of optical absorption spectra of 15 different species of carotenoids in the same solvent based on the stochastic theory using Brownian oscillator model. We found that the outer sphere reorganization energy can be good measures to quantitatively discuss the carotenoid-solvent interaction.

Acknowledgement

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A New Acetylenic Carotenoid 6'-Epimonadoxanthin from the Rosary Goby *Gymnogobius castaneus*

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Abstract: Carotenoids of the rosary goby *Gymnogobius castaneus* (Juzukakehaze in Japanese) were investigated. A new acetylenic carotenoid (3*R*,3'*R*,6'*S*)-7,8-didehydro- β,ϵ -carotene-3,3'-diol, named 6'-epimonadoxanthin, was isolated along with 18 known carotenoids. The structure of 6'-epimonadoxanthin was determined by UV-Vis, ESI-TOF MS, MS/MS, ¹H-NMR, and CD spectral data.

1. Introduction

Gobies are small fish (less than 10cm in length) belonging to Gobiidae, and inhabit brackish and freshwater environments. Tunaxanthin, lutein, zeaxanthin, alloxanthin, diatoxanthin, and trihydroxy- β -carotene have been reported as characteristic carotenoids in gobies by Matsuno et al [1,2]. Rosary goby *Gymnogobius castaneus* (Juzukakehaze in Japanese) is a small freshwater goby (5~6 cm body length) and inhabits in middle stretches of a river, lake, and lagoon in East Japan. Its tail fin shows bright orange color. This goby is edible and uses for 'tukudani'.

In the present investigation, a new acetylenic carotenoid named 6'-epimonadoxanthin was isolated along with 18 known carotenoids. The present work describes the isolation and structural elucidation of this new carotenoid from the rosary goby *G. castaneus*

2. Experimentals

The rosary goby *G. castaneus*, which fish was identified by Dr. Takahiko Mukai, Graduate School of Regional Studies, Gifu University, collected in Aomori prefecture at October, 2014 were purchased from local fish market.

Extraction, isolation, identification, and structural elucidation of carotenoids were according to our routine methods [3]. One kg of fish was extracted with acetone at room temperature. The acetone extract was partitioned with n-hexane:ether (1:1, v/v) and water. The n-hexane:ether layer was evaporated to dryness. The residue was saponified with 5% KOH/MeOH at room temperature for 1 hr. Then, carotenoid was extracted with n-hexane:ether (1:1, v/v) and washed with water. After evaporating of extract solution, individual carotenoids were separated by according to the methods described previously [3]. Shortly, after saponification, carotenoids were separated by silica gel chromatography, followed by HPLC using silica gel and ODS columns. Identification of individual

carotenoids were accomplished by UV-VIS, ESI TOF MS, and partly by ¹H-NMR and CD spectral data

3. Results and Discussion

The known 18 carotenoids (**Figure 1**) were isolated from the rosary goby *G. castaneus* along with a new acetylenic carotenoid. A new acetylenic carotenoid (**Figure 2**) was obtained as minor component (30 μ g from 1 kg of fish. 4.5 % of the total carotenoid). This carotenoid showed absorption maxima at 423, 445, 474 nm. The molecular formula of this compound was determined to be C₄₀H₅₄O₂ by ESI TOF MS data. The characteristic products ions of carotenoids in MS/MS spectrum, M+Na-92 and M+Na-106 were observed [4]. These data were resembled to those of monadoxanthin [(3*R*,3'*R*,6'*R*)-7,8-didehydro- β,ϵ -carotene-3,3'-diol] [5]. ¹H-NMR data (**Figure 2**), which was assigned by ¹H-¹H COSY experiment, indicated the presence of 7,8-didehydro-3-hydroxy- β -end group and 3,6-*cis*-3-hydroxy- ϵ -end group and all-*trans*-polyene chain with acetylenic group at 7,8-position. Therefore, the structure of this carotenoid was determined to be 7,8-didehydro- β,ϵ -carotene-3,3'-diol with 3,6-*cis*-configuration. This structure was correspond to 3' or 6' epimer of monadoxanthin, (3*R*,3'*R*,6'*R*)-7,8-didehydro- β,ϵ -carotene-3,3'-diol [5]. The absolute configuration of this carotenoid was analyzed by CD spectral data. The CD spectrum of this compound was shown in Figure. 3. The additivity rule of CD is used for estimation for chirality of the carotenoid having different end groups in both side of polyene chain [6]. This CD spectrum of this compound was resembling to the additive CD spectrum of (3*R*,3'*R*)-alloxanthin and (3*R*,6*S*,3'*R*,6'*S*)-tunaxanthin as shown in **Figure 3**. Therefore, the absolute configuration of this compound was assigned to be (3*R*,3'*R*,6'*S*). This carotenoid, (3*R*,3'*R*,6'*S*)-7,8-didehydro- β,ϵ -carotene-3,3'-diol was correspond to 6'-epimer of

monadoxanthin [(3*R*,3'*R*,6'*R*)-7,8-didehydro- β,ϵ -carotene-3,3'-diol]. Therefore, this carotenoid was named for 6'-epimonadoxanthin. This carotenoid was assumed to be metabolite of

diatloxanthin. Possible metabolic pathway from diatloxanthin to 6'-epimonadoxanthin was shown in Figure 4.

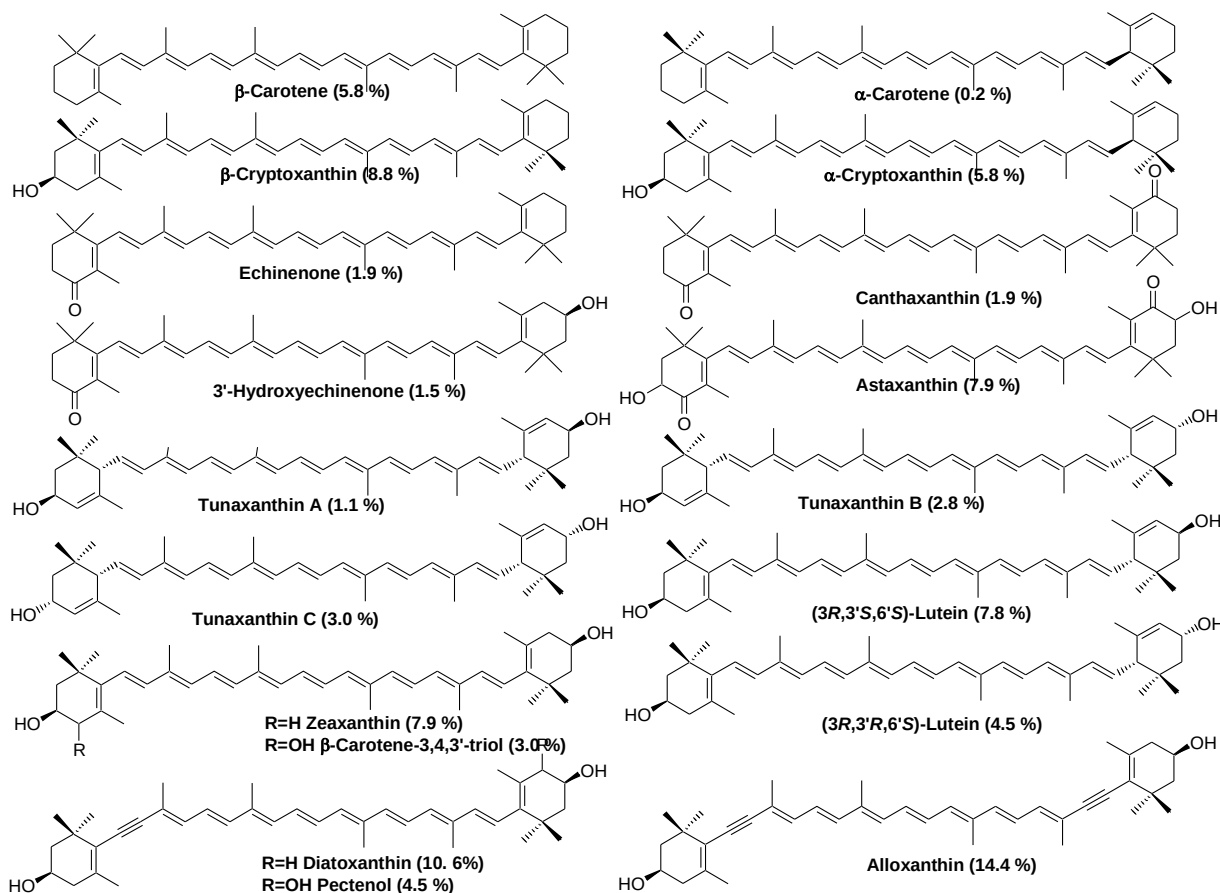


Figure 1. Structures of identified 18 known carotenoids in the rosary goby *Gymnogobius castaneus* (percentage composition of total carotenoid).

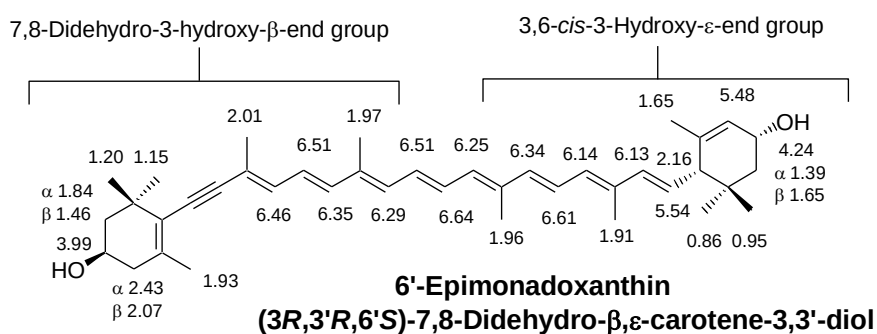


Figure 2. Structure and ^1H -NMR assignment in CDCl_3 of 6'-epimonadoxanthin.

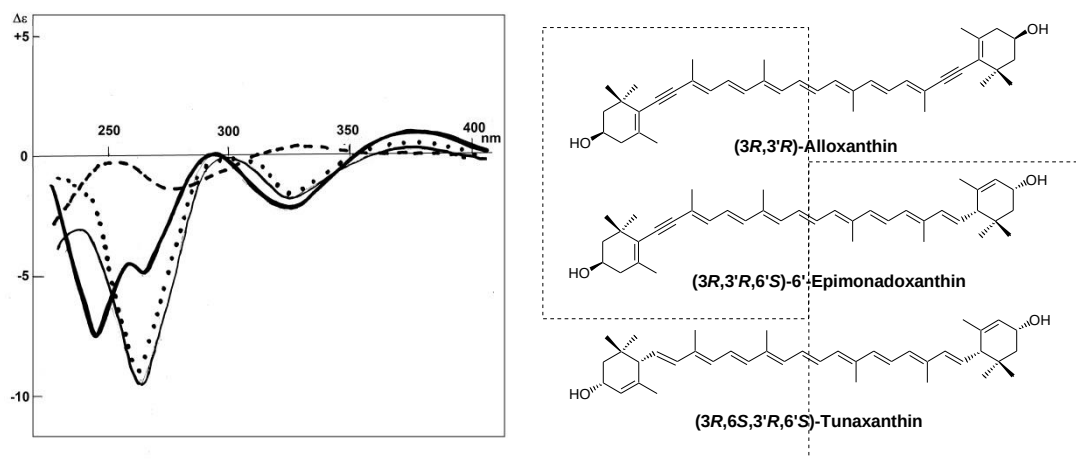


Figure 3. CD spectrum of (3R,3'R,6'S)-6'-epimonadoxanthin (—), (3R,3'R)-alloxanthin (1/2 intensity) (·····), and (3R,6S,3'R,6'S)-tunaxanthin (1/2 intensity) (----) in ether at room temperature and additive spectrum of (3R,3'R)-alloxanthin and (3R,6S,3'R,6'S)-tunaxanthin (1/2 intensity) (—).

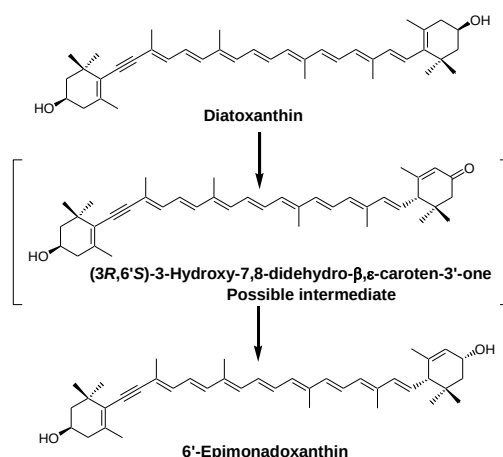


Figure 4. Possible metabolic pathway from diatoxanthin to 6'-epimonadoxanthin.

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Effects of paprika xanthophyll on chronic inflammation in adipocyte

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Abstract: Paprika (*Capsicum annuum* L.) contains various carotenoids such as capsanthin (CA), capsorubin (CS), cucurbitaxanthin A (CU), cryptocapsin, β -cryptoxanthin, capsanthin epoxide, zeaxanthin, and β -carotene. We previously reported that paprika carotenoids show anti-inflammatory effect in adipocyte cells. In this study we examined preventing the effect of paprika carotenoids (CA, CS and CU) on low-grade chronic inflammation was evaluated in 3T3-L1 adipocyte cells and RAW264.7 macrophage cell co-culture system. These carotenoids suppressed inflammatory adipocytokines expression and medium protein level in a co-culture of adipocyte and macrophage cells. Furthermore, CU suppressed cyclooxygenase-2 (COX-2) protein expression. These findings suggest that paprika carotenoids ameliorates low-grade chronic inflammation in adipocyte cells.

Obesity has been regarded as a major risk factor for type-2 diabetes, hypertension, and dyslipidemia. The cluster is called metabolic syndrome, of which the incidence is a worldwide problem.

Adipocytes secrete biologically active mediators called adipocytokines. Monocyte chemoattractant protein-1 (MCP-1), IL-6, and tumor necrosis factor- α (TNF- α) are known to be elevated in obesity conditions. These adipocytokines induce low-grade chronic inflammation, developing into insulin resistance and type-2 diabetes.

Paprika carotenoids ameliorated chronic inflammation in obesity condition adipocyte cells [1]. Paprika carotenoids showed suppressive effects of chronic inflammation in co-culture of adipocyte and macrophage cells. MCP-1, TNF- α , resistin, and IL-6 mRNA expression was suppressed in treatment with paprika carotenoids cells. However, the Paprika carotenoids used in the experiment was mixture of CA (44.3%), CS (12.8%), and other carotenoids including CU. So, the active component was unclear. The aim of this study was to analyze anti-inflammatory effect of paprika major xanthophyll (CA, CS and CU) in 3T3-L1 adipocyte cells and RAW264.7 macrophage cell co-culture system (**Fig. 1**).

CA, CS, and CU were purified from paprika oleoresin (LKT Laboratories Inc.). 3T3-L1 preadipocyte cells and RAW 264.7 macrophage

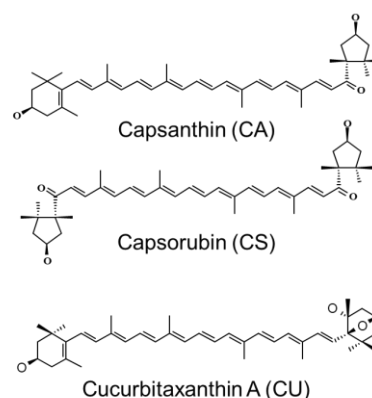


Fig. 1 Carotenoid structure of capsanthin (CA), capsorubin (CS), and cucurbitaxanthin A (CU)

cells were cultured respectively in DMEM with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. After 3T3-L1 cells reached confluence, they were incubated for an additional 24 hr. Then adipocyte differentiation of 3T3-L1 preadipocytes was initiated using differentiation medium I containing 10 μ g/mL insulin, 0.5 mmol/L isobutylmethylxanthine, and 0.1 μ mol/L dexamethazone for 48 hr. The medium was then replaced with DMEM containing 5 μ g/mL insulin (differentiation medium II), and changed to fresh medium every 48 hr for fresh medium II. After 3T3-L1 cells were cultured in differentiation medium II for 144 hr, then RAW 264.7 cells (5×10^4 cells/well) were plated onto 24-well-plate cultured differentiated 3T3-L1 cells. They were

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treated with DMEM medium containing carotenoids (10 μ M) for 24 hr. Troglitazone (TR) was used as a positive control compounds which improve low-grade chronic inflammation in adipocyte cells. Subsequently, cells and culture medium supernatants were collected. After that mRNA expression was analyzed real-time quantitative RT-PCR analysis. Concentrations of inflammatory factors in the culture supernatants were determined using ELISA. COX-2 protein expression were analyzed by western blot analysis.

Paprika carotenoids showed suppressive effects of chronic inflammation in co-culture of adipocyte and macrophage cells. Co-cultured cells showed increased TNF- α , IL-6, and MCP-1 mRNA expression. TNF- α and IL-6 mRNA expression tended to be suppressed in treatment with paprika carotenoids cells (Fig. 2). MCP-1 was significantly ($p < 0.05$) suppressed in treated with CU and CS cells. Moreover, these carotenoids decreased IL-6 protein concentration level in the medium.

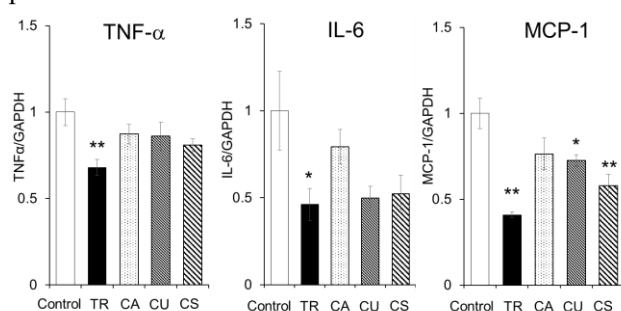


Fig.2 Suppressive effect on inflammatory cytokine mRNA expression in adipocyte and macrophage co-cultured cells. Control: Control, TR: Troglitazone (10 μ M), CA: Capsanthin (10 μ M), CU: Cucurbitaxanthin A (10 μ M), CS: Capsorubin (10 μ M). ** $p < 0.01$ vs. Control * $p < 0.05$ vs. Control

The cyclooxygenase-2 (COX-2) is an inducible enzyme expressed in inflammation-related cells such as macrophages, producing large amounts of prostaglandins. Excess production of prostaglandins causes inflammatory disorders. COX-2 mRNA expressions were markedly down-regulated

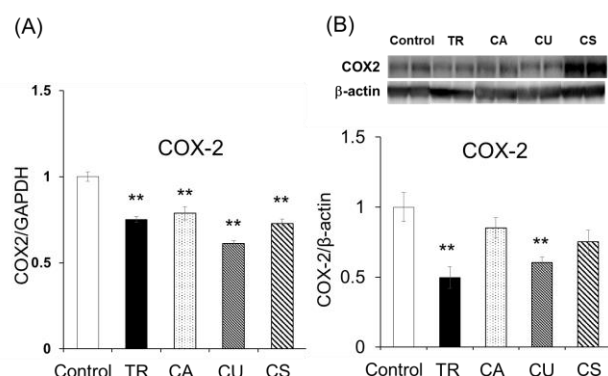


Fig.3 (A) COX-2 mRNA expression in adipocyte and macrophage co-cultured cells. (B) COX-2 protein expression in adipocyte and macrophage co-cultured cells. Control: Control, TR: Troglitazone (10 μ M), CA: Capsanthin (10 μ M), CU: Cucurbitaxanthin A (10 μ M), CS: Capsorubin (10 μ M). ** $p < 0.01$ vs. Control

($p < 0.05$) in treated with paprika carotenoids cells compared to Control cells (Fig. 3 (A)). In addition, COX-2 protein expression was significantly

($p < 0.05$) suppressed in treated with CU cells (Fig. 3(B)). These results suggest that the major xanthophyll of paprika ameliorates inflammatory changes in adipocyte cells induced by macrophage migration. Previous report suggested that the mixture of paprika carotenoids ameliorated chronic inflammation in adipocyte cells. CA contained approximately half of the percentage of paprika carotenoids showed the same effects. Furthermore, CU significantly suppressed COX-2 mRNA and protein expression compared with Control cells. It was reported that anti-oxidation and bioavailability of CU were higher than other paprika xanthophyll [2, 3]. Therefore, CU is considered to be an attractive substance from the viewpoint of the functionality.

Acknowledgments

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Seasonal Variation in the Proximate Composition and Carotenoid Profile of *Halocynthia aurantium* in the Okhotsk Sea

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Abstract: In the Okhotsk Sea, *Halocynthia aurantium* caught along with scallops is discarded, as it is a bycatch. In order to promote the utilization of *H. aurantium* as a process material, the proximate composition and carotenoid profile were analyzed in the muscle of *H. aurantium*. The results revealed the following: (1) from summer to fall, there was a gradual decrease in the moisture and ash contents, however the protein, lipid, and glycogen contents increased, (2) unlike the proximate composition, the carotenoid content did not show a clear trend, (3) the major carotenoids identified included β -carotene, alloxanthin, astaxanthin, mytiloxanthin, pectenolone, halocynthiaxanthin, and their fatty acid esters, and (4) the muscle of *H. aurantium* contains several useful carotenoids, therefore it can be used as a functional food.

1. Introduction

H. aurantium, known as “akaboya” in Japan, belongs to the class Ascidiacea. It inhabits only along the coast of Hokkaido, Japan. In the Okhotsk Sea in Hokkaido, *H. aurantium* is caught along with scallops. *H. aurantium* is a seston feeder (feeding on particulate organisms or suspended matter in the sea). Furthermore, the species competes with the scallops for feed, therefore they are considered an interruption to the development of scallops and discarded as a bycatch. On the other hand, there is a growing concern over the decrease in fishing resource in the Okhotsk Sea. This decrease in the fishing resource has caused chronic shortage of process material for the fish processing industry. In this milieu, *H. aurantium* must be utilized as a food material and not discarded as a fishery waste. However, only a few studies have concentrated on *H. aurantium* as a food material. In the present study, monthly variation (from summer to fall) in the proximate composition of *H. aurantium* was investigated. Further, the gonad tissue was observed under a microscope to elucidate the relationship between changes in proximate composition and gonad maturation. Recently, various health-promoting properties of carotenoids, such as antioxidative, anticarcinogenic, and anti-obese activities, have been reported [1]. Ookubo *et al.* [2]

evaluated the carotenoid profile of ten ascidians and reported the presence of carotenoid in the muscle of ascidians. However, there are no studies on the carotenoid profile of *H. aurantium*. Therefore, in the present study, the carotenoid profile of the muscle of *H. aurantium* was carried out to encourage its use as a functional food.

2. Experimental

2.1 Materials

H. aurantium (total weight 183.2 ± 40.3 g, $n=86$, **Figure 1**) caught along with scallops at

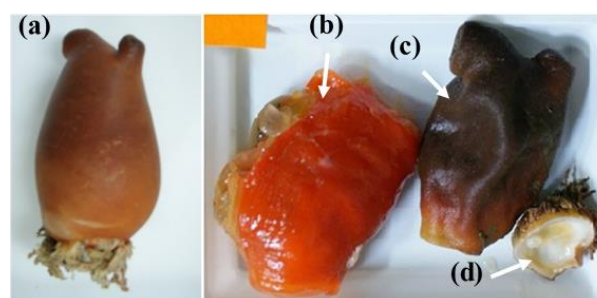


Figure 1. *Halocynthia aurantium*. (a) Whole, (b) Mantle body*, (c) Upper part of tunic, (d) Basal part of tunic with attachment villi. *Mantle body is composed of muscle and viscera. The muscle was analyzed in this experiment.

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Mombetsu Coast were used in the present study. The samples obtained from June to October in 2015 and 2016 were analyzed. Immediately after obtaining the sample, mantle was separated from tunic. The mantle is composed of muscle and viscera. In the present study, only the muscle was analyzed. For a comparative study, *H. roretzi* known “maboya” collected from Funka Bay, Japan in September 2016 was also analyzed.

2.2 Analysis of proximate composition

Moisture content was determined after drying the sample at 105°C for 24 h. Protein content was estimated by Kjeldahl method using a nitrogen to protein conversion factor of 6.25. Lipid content was determined by Soxhlet extraction method. Ash content was determined by combusting the sample at 550°C for 8 h. Glycogen content was determined by anthrone method.

2.3 Microscopic observation of gonad

One gram of gonad was fixed for 24 h in a modified Davidson's solution [3] containing: formalin, 20 parts; 95% ethanol, 30 parts; glacial acetic acid, 10 parts; and distilled water, 30 parts. The fixed gonad was preserved in 70% ethanol, dehydrated progressively in a graded series of ethanol. The dehydrated gonad was embedded in paraffin and sections (8 µm thick) were cut using a microtome. The sections were then stained with hematoxylin and eosin and examined under a light microscope.

2.4 Extraction and determination of total carotenoid

Five grams of muscle sample was taken in a 50 mL glass centrifuge tube and dehydrated by adding 20 mL of 40% acetone. The mixture was homogenized twice at 10,000 rpm for 1 min, and then centrifuged at the 3,000 rpm at 20°C for 20 min. After centrifugation, 99.5% acetone was added to the residue, stirred using a glass bar for 1 min, and centrifuged again at 3,000 rpm at 20°C for 20 min. The extraction with 99.5% acetone was repeated 3 times. The supernatant, containing carotenoids, was evaporated under reduced pressure. The carotenoids obtained was dissolved in 25 mL acetone and absorbance was measured at 450 nm. Total carotenoid content was calculated using the absorption coefficient ($E_{cm}^{1\%}=2,400$) at maximum wavelength.

2.5 Identification of carotenoid

Identification of carotenoid was accomplished by UV-Vis, FAB-MS, 1H-NMR and 13C-NMR [4].

3. Results and discussion

3.1 Proximate composition, glycogen, and total carotenoid

The moisture, protein, lipid, ash, and glycogen contents of *H. aurantium* ranged from 80.4%–86.4%, 7.3%–9.1%, 0.9%–1.9%, 1.9%–2.6%, and 2.2%–4.7%, respectively. The range of total carotenoid content was 8.5–18.0 mg/100 g. From summer to fall, the moisture and ash contents decreased gradually. However, the protein, lipid, and glycogen contents exhibited an increasing trend. The total carotenoid content with a large standard deviation between months within the same year did not exhibit a clear trend (**Figure 2**). Park et al. [5] investigated the proximate composition of the muscle of *H. roretzi* cultured at the coast of Korea. The study reported that from April to October, the moisture content decreased and the protein, glycogen, and fat contents increased gradually. These observations suggested that seasonal variation in proximate composition might be a common occurrence in ascidians. In the present study, in *H. roretzi*, the moisture, protein, lipid, ash, glycogen, and total carotenoid contents were 75.4%, 9.4%, 3.5%, 2.3%, 5.8%, and 1.0 mg/100 g, respectively (**Table 1**). In comparison with this data, the moisture content was higher, while the lipid content was lower in *H. aurantium*. Furthermore, the total carotenoid content, was significantly high in *H. aurantium*.

3.2 Histological observation of gonad

Ascidians are hermaphrodites with both ovary and testis in a single organism. The ovary in recovery stage (**Figure 3a**) was observed in *H. aurantium* from June to September (**Figure 4a**). The number of individuals with a developing ovary increased from 50% in July to 100% in October. On the contrary, the testis in immature stage (**Figure 3c**) was observed in individuals in June and August. However, the number of individuals with immature testis was not more than 10% (**Figure 4b**). The number of individuals with both ovary and testis in developing stages reached 100% in October. Further, the histological observation suggested that the spawning season of *H. aurantium* was after October. Therefore, the variation in proximate composition in *H. aurantium* was considered a seasonal variation, and is related to gonad maturation.

3.3 Carotenoids composition

The major carotenoids identified included β-carotene and alloxanthin ester (9.4%–33.1%), astaxanthin ester (8.4%–23.8%), mytiloxanthin ester and halocynthiaxanthin-3-ester (13.4%–29.5%), astaxanthin (11.6%–18.9%), pectenolone

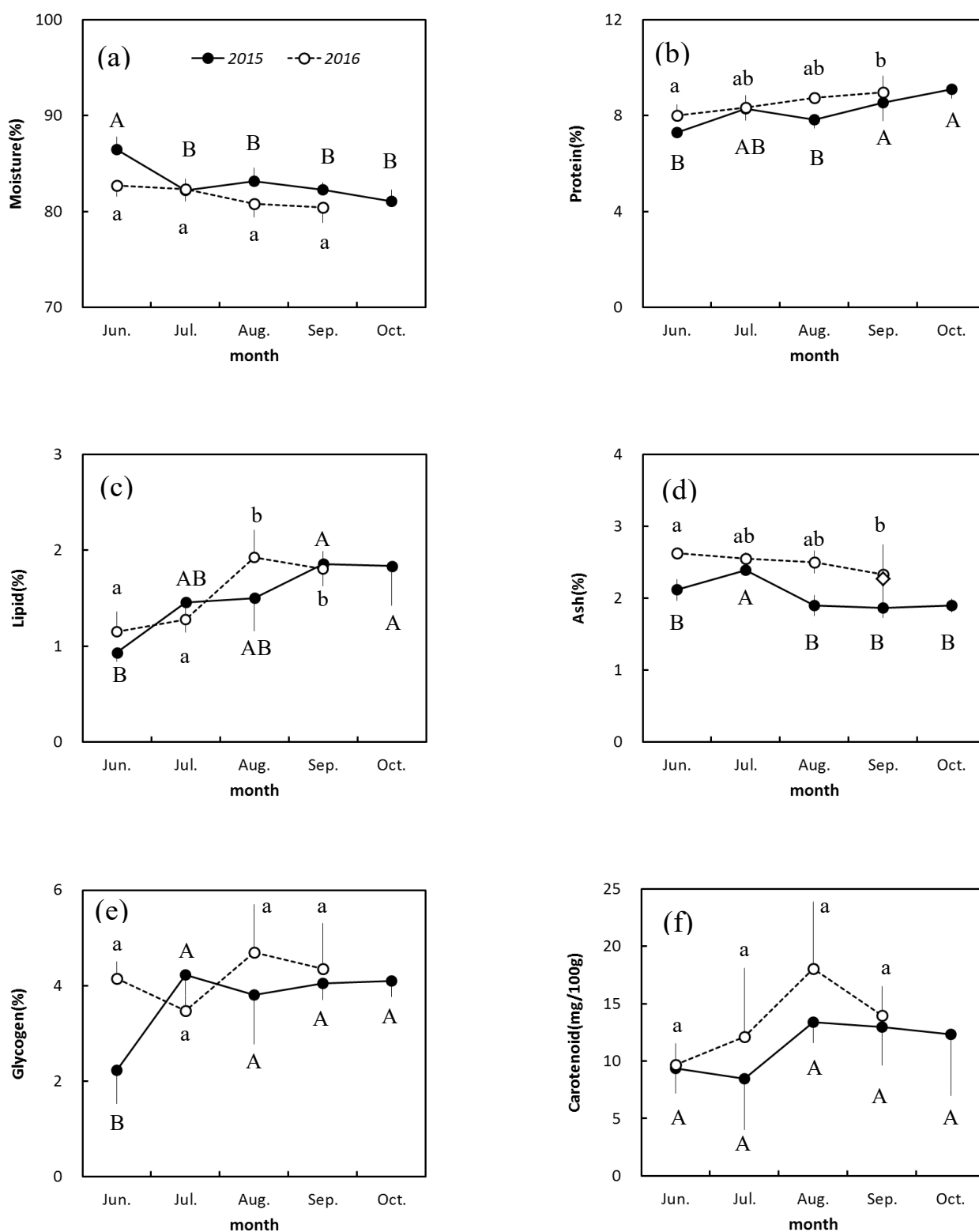


Figure 2. Change of proximate components in muscle of *Halocynthia aurantium* (a) moisture, (b) protein, (c) lipid, (d) ash, (e) glycogen, (f) carotenoid. Error bars indicate standard deviation ($n=4-5$). Different large letters indicate significant among 2015 ($p<0.05$). Different small letters indicate significant among 2016 ($p<0.05$).

Table 1. Proximate composition, glycogen and carotenoid of *H. roretzi*

Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Glycogen (%)	Carotenoid (mg/100g)
75.4±1.0	9.4±0.1	3.5±0.5	2.3±0.1	5.8±0.9	1.0±0.3

H. roretzi were taken in Funka Bay, Hokkaido September 2016.

Average ± standard deviation, $n=5$.

(0.9%–5.8%), alloxanthin and diatoxanthin (1.0%–18.9%), mytiloxanthin (1.0%–3.5%), and halocynthiaxanthin (3.7%–10.1%). To the best of our knowledge, the present study is the first report the presence of halocynthiaxanthin-3-ester in ascidians (**Figure 5**). Further, the major carotenoids identified in *H. aurantium* were similar to that in *H. roretzi*. However, in September 2016, the quantity of astaxanthin and astaxanthin ester in *H. aurantium* was higher than that of *H. roretzi* (**Table 2**). The relatively high percent of astaxanthin and astaxanthin ester can be considered as one of the characteristic features of *H. aurantium*. There was increase in the concentrations of β -carotene, alloxanthin ester, and astaxanthin ester from June to August. Similarly, the concentrations of alloxanthin, mytiloxanthin, and pectenolone increased from June to August. However, astaxanthin, mytiloxanthin ester, and halocynthiaxanthin did not exhibit a clear trend (**Figure 6**). Most microorganisms and algae synthesize β -carotene *de novo* from isoprenyl diphosphate *via* phytoene and lycopene [6]. Therefore, β -carotene and alloxanthin are derived from algae, such as diatoms, on which *H. aurantium* feed. It has been reported that dietary β -carotene is an oxidative metabolite of astaxanthin in animals [7]. Similarly, dietary fucoxanthin from diatoms is a metabolite of mytiloxanthin *via* fucoxanthinol and halocynthiaxanthin in shellfish and tunicate. Pectenolone is an oxidative metabolite of diatoxanthin [7]. The seasonal variation in the carotenoids can be correlated to the increase in algal population, such as diatoms, which accelerate feeding in *H. aurantium* from summer to fall. The carotenoids in the muscle of *H. aurantium* might move into their gonads, therefore the carotenoids in gonad must be examined in future.

3.4 Biological functions of the carotenoids

The major carotenoids in *H. aurantium*, such as halocynthiaxanthin, mytiloxanthin, alloxanthin, astaxanthin, and pectenolone have been reported to have several biological activities. Mytiloxanthin and pectenolone exhibited similar antioxidative activity for quenching singlet oxygen, and also

inhibited lipid peroxidation, similar to that of astaxanthin [8, 9]. Furthermore, mytiloxanthin showed significant hydroxyl radical scavenging activity, which was significantly higher than that of astaxanthin [9]. These carotenoids have also exhibited antitumor activity [10–12]. The results of the present study indicate that the muscle of *H. aurantium* can be used as a functional food, since it is rich in valuable carotenoids.

Acknowledgments

We thank Mombetsu Fishery Cooperative for providing the samples.

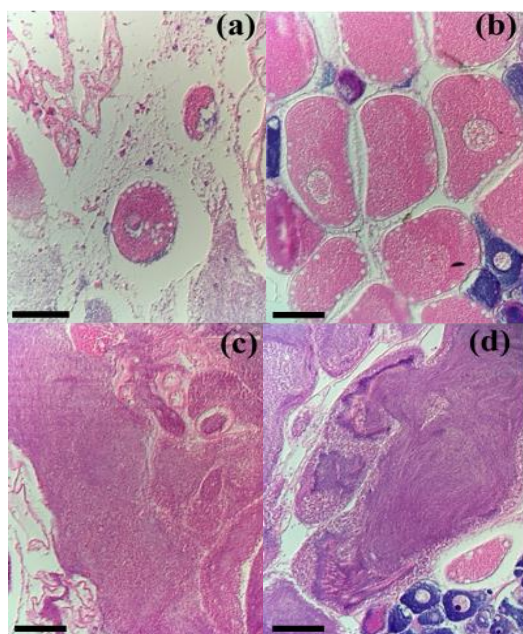


Figure 3. Light micrographs of gonad of *H. aurantium* (a) Ovary in recovery stage, (b) Ovary in developing stage, (c) Testis in immaturity stage, (d) Testis in developing stage. Scale bar: 100µm.

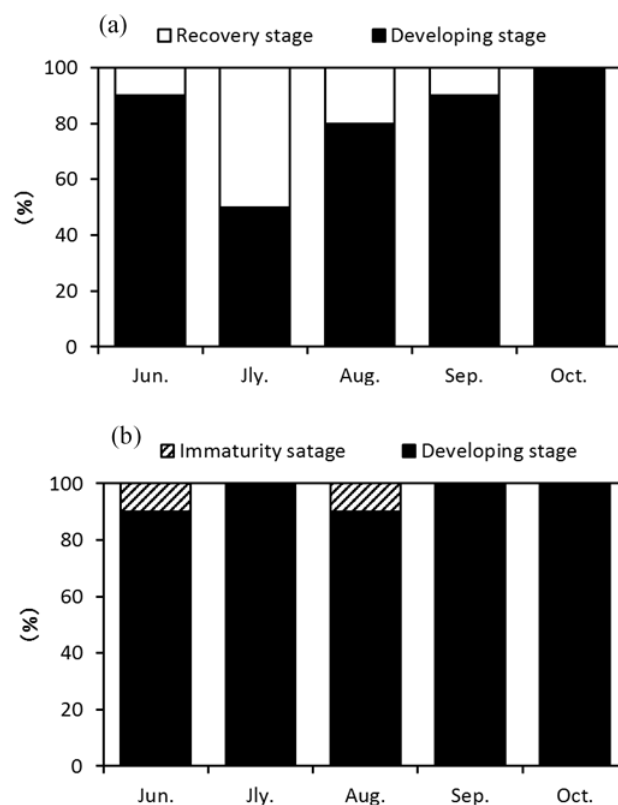


Figure 4. Formative stage of gonad of *Halocynthia aurantium*.

(a) Ovary (n=10). (b) Testis (n=10).

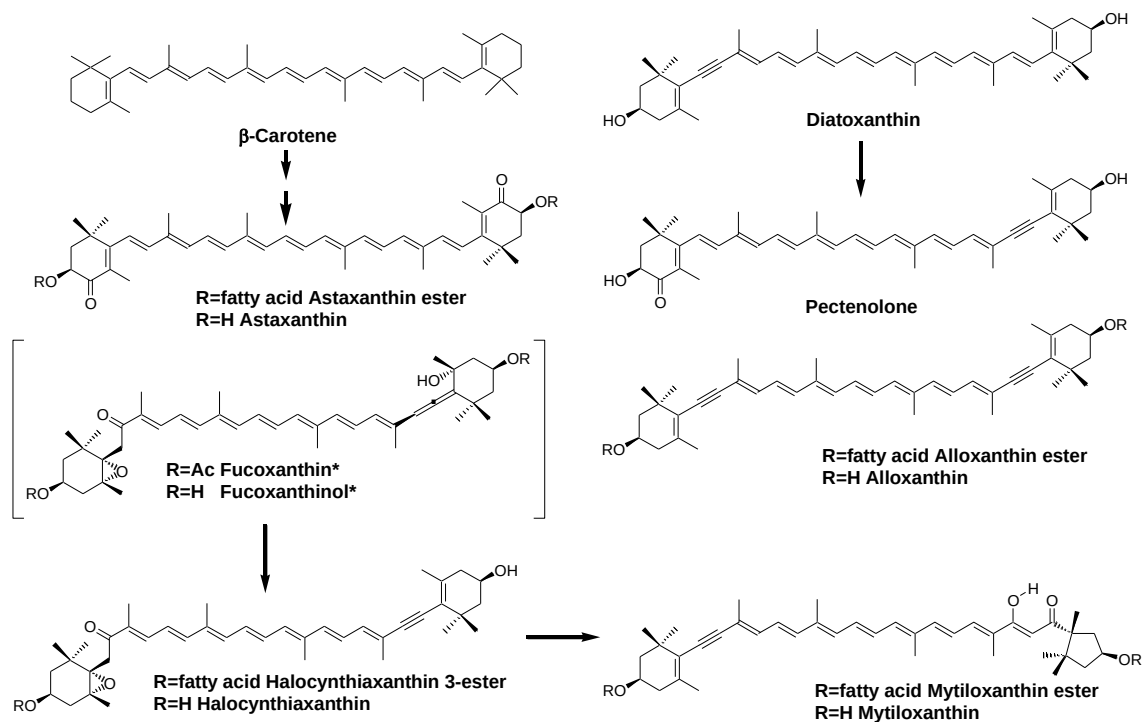


Figure 5. Structures of identification carotenoids in *H. aurantium* and their metabolic pathways.

*Fucoxanthin and fucoxanthinol were not found in *H. aurantium*. This tunicate accumulates fucoxanthin from dietary algae and metabolized to halocynthiaxanthin and mytiloxanthin.

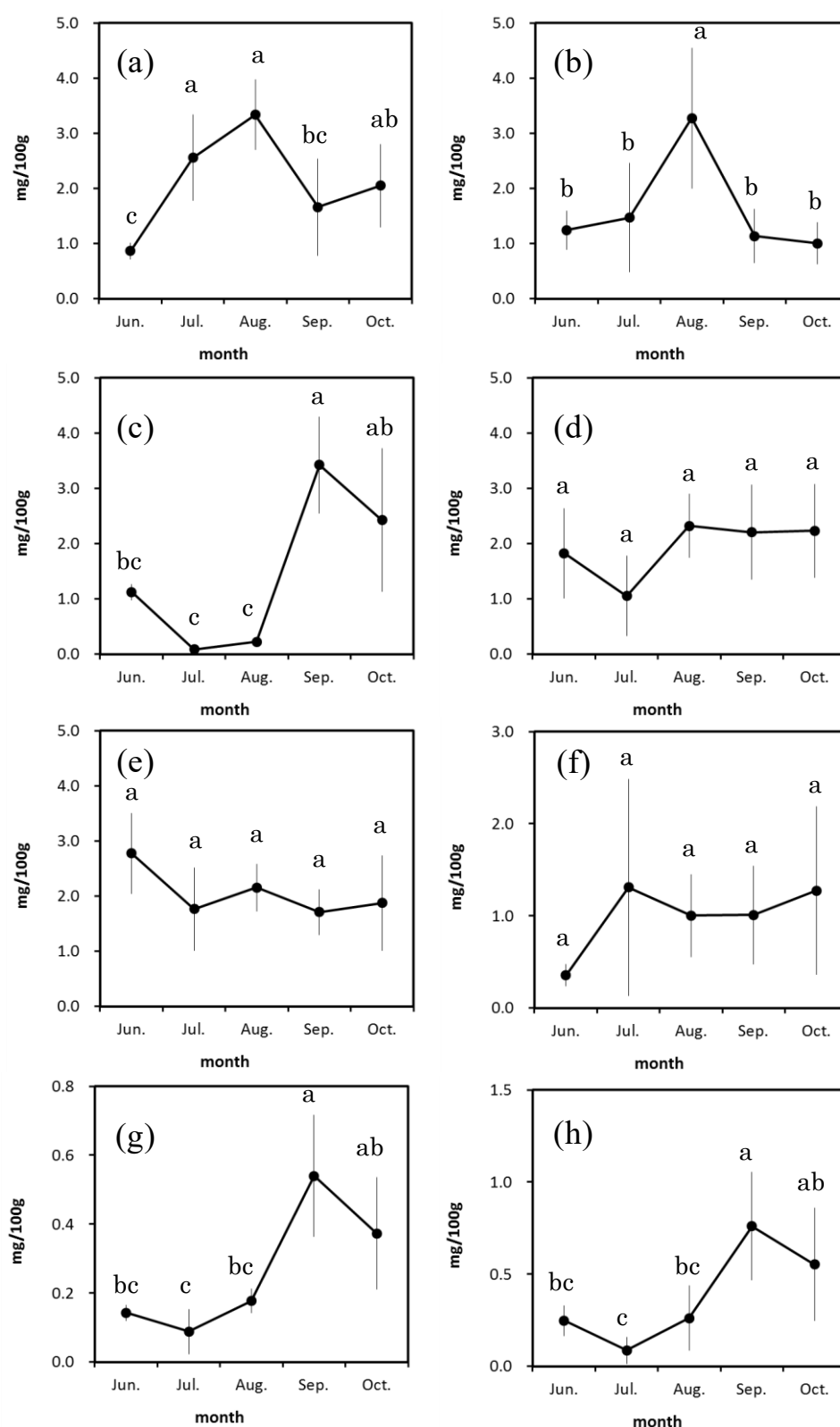


Figure 6. Change of carotenoid components identified in muscle of *Halocynthia aurantium*. (a) β -carotene^{*1}, (b) astaxanthin ester, (c) alloxanthin^{*2}, (d) astaxanthin, (e) mytiloxanthin ester^{*3}, (f) halocynthiaxanthin. (g) mytiloxanthin, (h) pectenolone. Error bars indicate standard deviation ($n=4$). Different letters indicate significant ($p < 0.05$). ^{*1}Contains alloxanthin ester. ^{*2}Contains diatoxanthin. ^{*3}Contains halocynthiaxanthin-3-ester.

Table 2. Carotenoid composition in muscle of *H. aurantium* and *H.*

Year	<i>H. aurantium</i>					<i>H. aurantium</i>	<i>H. roretzi</i>
	(2015)					(2016)	
Month	Jun.	Jly.	Aug.	Sep.	Oct.	Sep.	Sep.
Compound	Composition (%) ^{*4}						
β-Carotene ^{*1}	9.4	33.1	25.4	12.2	17.5	11.5	9.8
Astaxanthin ester	13.3	16.6	23.8	8.5	8.4	10.1	2.6
Mytiloxanthin ester ^{*2}	29.5	22.0	16.3	13.4	15.2	27.5	32.4
Astaxanthin	18.9	11.6	17.2	16.6	18.8	17.2	2.5
Pectenolone	2.7	0.9	1.9	5.8	4.3	6.2	8.7
Alloxanthin ^{*3}	12.3	1.0	1.7	27.4	18.9	11.6	22.9
Mytiloxanthin	1.6	1.0	1.3	4.1	3.0	3.5	3.8
Halocynthiaxanthin	3.7	13.1	7.5	7.6	10.1	7.4	2.5
Others	8.6	0.8	5.0	4.6	3.8	5.0	14.8

^{*1} Contains alloxanthin ester

^{*2} Contains halocynthiaxanthin-3-ester

^{*3} Contains diatoxanthin

^{*4} Composition is average ($n=4-5$).

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Synthesis of Myxol and Deoxymyxol Stereoisomers and their HPLC separation

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Abstract: Total syntheses of myxol stereoisomers (**1a,b**) and deoxymyxol (plectanixanthin) stereoisomers (**2a,b**) were accomplished by Wittig reaction of (*S*)- and (*R*)-C₁₀-phosphonium salts **7a,b** with C₃₀-apocarotenals **8** and **9**. The phosphonium salts **7a,b** were derived from aldehydes **4a,b** possessing a cyclopentylidene ketal moiety, which were prepared via Sharpless asymmetric epoxidation of allylic alcohol **3**. In order to determine the absolute configuration of natural products, a HPLC analytical method for stereoisomers **1a,b** and **2a,b** was established by using a chiral column.

1. Introduction

Carotenoids having a 1,2-dihydroxy- ψ -end group, such as myxol (**1**) and deoxymyxol (alias name: plectanixanthin) (**2**), have been isolated from several microorganisms and most of these carotenoids exist as glycosides and fatty acid esters [1–4]. Their absolute configurations have been postulated by comparing their CD spectra with that of fungal carotenoid plectanixanthin, whose chirality has been determined as 2'*R* by synthetic approach [5]. However, it was demonstrated that the CD spectrum of plectanixanthin was non-conservative with weak Cotton effect and was strongly temperature dependent [5,6].

In order to exactly determine the absolute configuration of naturally occurring myxol and deoxymyxol, we synthesized stereoisomers of these analytical method.

into the aldehyde **4a** via Sharpless asymmetric epoxidation by use of (+)-diisopropyl tartrate (DIPT), Ti(O^{*i*}Pr)₄ and cumene hydroperoxide. This was then condensed with C₅-phosphonate **10** to give the corresponding dienolate, which was subjected to LiAlH₄ reduction followed by MnO₂ oxidation and subsequent ketal-hydrolysis to afford an isomeric mixture of the dihydroxy aldehyde **6a**. After protecting of the secondary hydroxyl group in **6a** with triethylsilyl (TES) group and subsequent reduction with NaBH₄, the resulting allylic alcohol was transformed into the desired (*S*)-tri-*n*-butyl- phosphonium salt **7a** via the corresponding chloride.

The phosphonium salt **7a** was then condensed with apocarotenals **8** and **9** and then desilylated to provide (3*R*,2'*S*)-myxol (**1a**) and (2'*S*)-deoxymyxol (**2a**), whose ¹H-NMR spectral data were identical with those reported [2a,6].

(3*R*,2'*R*)-Myxol (**1b**) and (2'*R*)-deoxymyxol (**2b**) were synthesized in a similar fashion starting from asymmetric epoxidation of allylic alcohol **3** using (–)-DIPT in place of (+)-DIPT.

The CD spectra of myxol stereoisomers (**1a**) and (**1b**) look very similar to one another, whereas those of deoxymyxol stereoisomers (**2a**) and (**2b**) showed antisymmetrical curves having extremely weak Cotton effects each other. These spectra indicate that the stereochemistry at C2'-position has little effect on the shape of their CD spectra.

HPLC conditions for identification of each stereoisomers **1a,b** and **2a,b** were then established by using a column carrying a chiral stationary phase (CHIRALPAK IA; Daicel). This HPLC analytical method was applied to some natural specimens. The details will be described elsewhere.

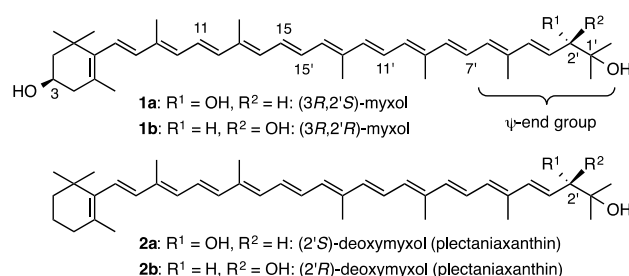
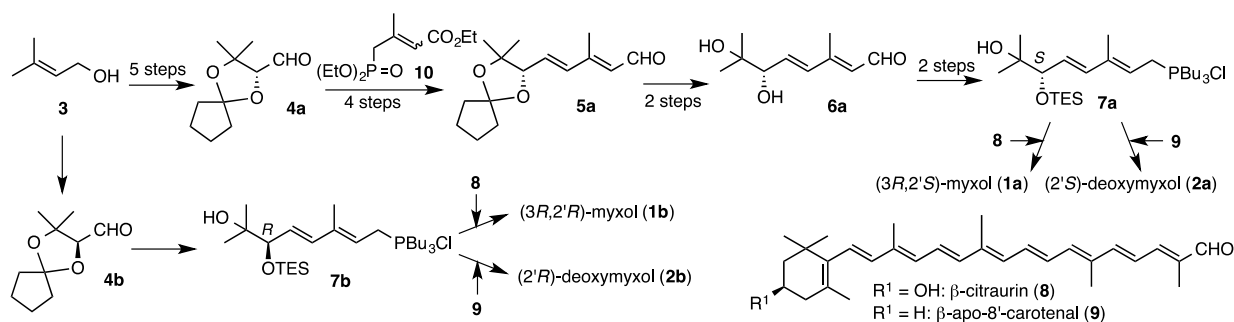


Figure 1. Chemical structures of myxol and deoxymyxol (plectanixanthin).carotenoids **1a,b** and **2a,b** and established their HPLC

2. Results and Discussion

First, we prepared the (*S*)-C₁₀-phosphonium salt **7a** as shown **Scheme 1**. According to the known method [7], 3-methyl-2-butenal (**3**) was transformed



Scheme 1.

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Total synthesis of apocarotenoid, paracentrone, for elucidation of marine photosynthetic energy-transfer mechanism

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Abstract: The stereocontrolled total synthesis of the C31 apocarotenoid, paracentrone, was accomplished by sequential cross-coupling reactions using C5 boron and iodine-substituted dienes, which were building blocks developed for extension of the conjugated polyene systems at both their terminals. The time-resolved absorption spectroscopic measurement of paracentrone confirmed that the ICT (intramolecular charge transfer) state appeared as in the case of fucoxanthin.

1. Introduction

Paracentrone (**1**, **Figure 1**) isolated from the sea urchin *Paracentrotus lividus* in 1969 by Weedon et al. is a C31 apocarotenoid [1]. Paracentrone is presumed to be a metabolite of the C40 carotenoid, fucoxanthin (**2**) [2,3-6]. Fucoxanthin is a typical light harvesting pigment for photosynthesis in the sea and transfers energy with a very high efficiency (>80%) in the fucoxanthin–chlorophyll *a*–protein (FCP complex) [7], whose structure has not yet been confirmed. This super function is thought to be due to the unique chemical structure of **2**. Since paracentrone (**1**) possesses the same allene and carbonyl conjugated polyene framework as that of **2**, this apocarotenoid **1** is regarded as one of the most intriguing fucoxanthin analogs and is expected to provide further important information about the structural characteristics for super energy-transfer. For example, it is very interesting to know whether or not reconstitution of the modified FCP complex using **1** instead of **2** would be possible and also to know the role of the terminal cyclohexane moiety in **2** for its energy-transfer ability. The ability of **1** as a light harvesting pigment in photosynthesis besides its biological activities,

however, has not been revealed to date.

The synthesis of **1** has been independently reported by Haugan's [8] and Katsumura's [9] groups. Since both use Wittig olefination for construction of the polyene framework, the obtained **1** was a mixture of *E/Z* stereoisomers. Meanwhile, a wide variety of the Pd-catalyzed C(sp²)–C(sp²) cross-coupling reactions was reported to give the corresponding conjugated polyene products with retention of the olefin geometry [10]. In order to realize the stereocontrolled synthesis of **1** having an all-*trans* geometry, we designed the new C5 dienes **3** and **4** (**Figure 2**) as bidirectionally extensible building blocks for construction of the conjugated polyene framework of **1** by sequential Pd-catalyzed cross-coupling reactions. To date, several bifunctional diene units, *e.g.*, boron and halogen-substituted [11] and boron and tin-substituted [12] dienes, have been developed for the synthesis of conjugated polyene-containing compounds [13], in particular, the protocol using vinyl *N*-methyliminodiacetic acid (MIDA) boronate derivatives developed by Burke's group is very

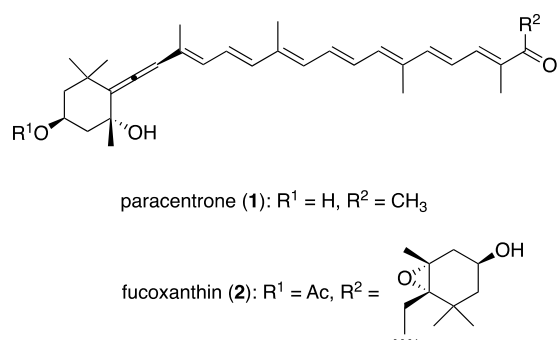


Figure 1. Paracentrone (**1**) and fucoxanthin (**2**)

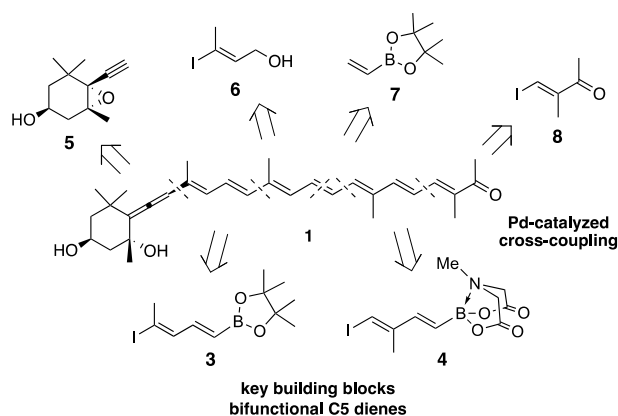


Figure 2. Synthetic plan

attractive [11]. In this paper, we describe the stereocontrolled synthesis of **1** by sequential Pd-catalyzed cross-coupling reactions using **4** and **10** as key building blocks.

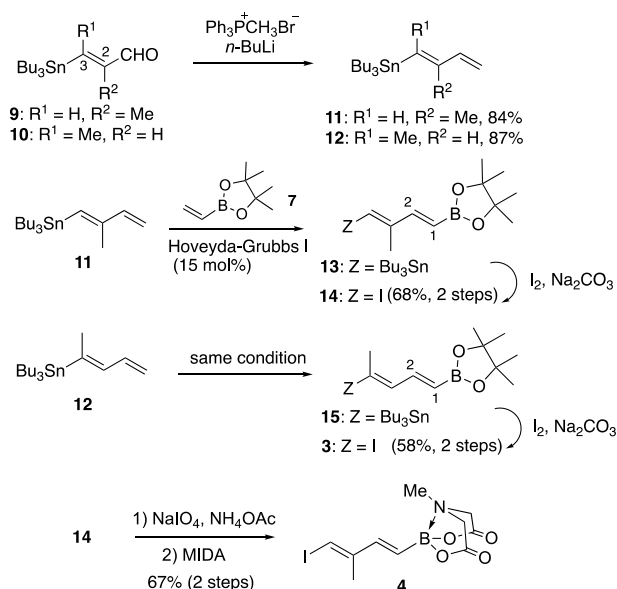
2. Synthesis

2.1 Synthetic plan

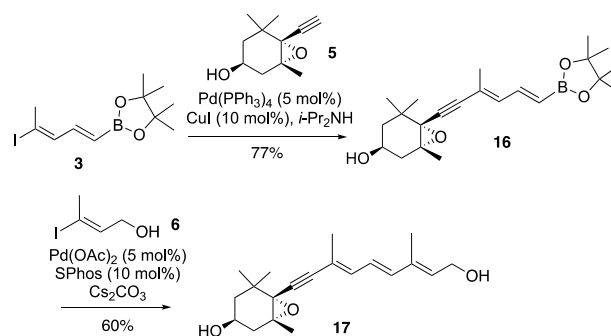
As shown in **Figure 2**, we envisioned that compound **1** would be synthesized by the sequential cross-couplings of six fragments containing bifunctional the C5 diene units **3** and **4** with iodine and boron at both terminals.

2.2 Synthesis of C5 dienes

For the synthesis of the C5 dienes **3** and **4**, we found that the olefin cross-metathesis was suitable for bifunctional dienes **13** and **15** containing alkyltin and boronate substituents (**Scheme 1**). Several catalyst screenings for the cross-metathesis reaction of **11** [14], which was obtained by the Wittig olefination of **9** [15] with $\text{Ph}_3\text{P}^+\text{CH}_3\text{Br}^-$ in 84% yield, with the vinylboronic acid pinacol ester **7** revealed that the Hoveyda–Grubbs I catalyst (15 mol%) effectively produced the desired all-*trans* **13** [16] (CH_2Cl_2 , reflux, 19 h) [17]. The obtained vinylstannane was transformed into the desired 3-methylvinyl iodide **14** in 2 steps upon treatment with iodine as a single stereoisomer in 68% yield. The 4-methyl isomer **3** [18] having an all-*trans* geometry [19] was also stereoselectively synthesized from **10** [20,21] in the same manner as that of **14**. The transformation of a pinacol ester in **14** into the corresponding MIDA ester in 2 steps was successful by the reported protocol [22] to give the desired **4** [23] as a single isomer in 67% yield. The olefin cross-metathesis was thus effective for the synthesis of bifunctional dienes.



Scheme 1. Synthesis of C5 dienes **3** and **4**

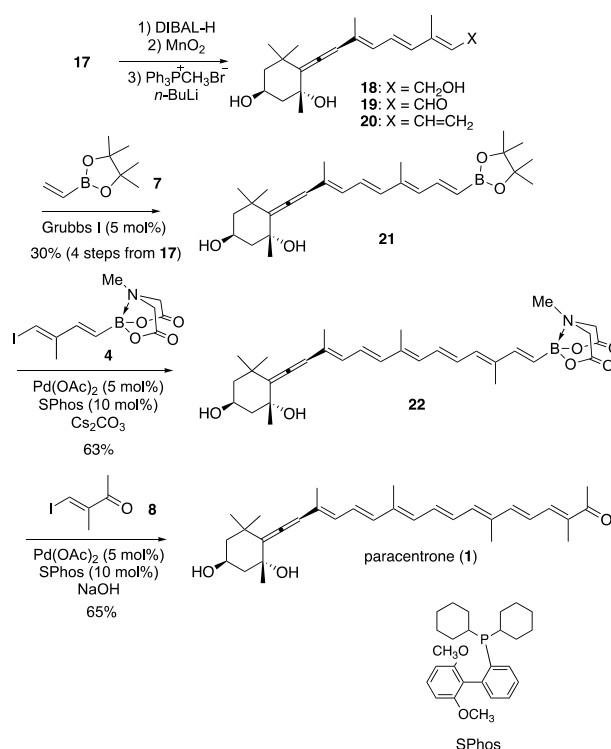


Scheme 2. Sequential cross-coupling of bifunctional diene **3**

2.3 Synthesis of paracentrone

With the key C5 bifunctional diene building blocks **3** and **4** in hand, we investigated extending the polyene chain at both ends of **3**. The diene **3** was subjected to the Sonogashira reaction with epoxy acetylene **5** [24], which was synthesized from (–)-actinol in 8 steps [20], under the standard reaction conditions ($\text{Pd}(\text{PPh}_3)_4$, CuI , $i\text{-Pr}_2\text{NH}$) to afford the vinylboronate **16** in 77% yield (**Scheme 2**). The obtained vinylboronate **16** was connected with the allyl alcohol **6** [25] in the presence of $\text{Pd}(\text{OAc})_2$, SPhos, and Cs_2CO_3 to afford the diol **17** with all-*trans* form in 60% yield. This indicated that diene **3** is a useful building block for the stereoselective elongation of polyene chains.

The formation of the allenic alcohol was attempted by the $\text{S}_{\text{N}}2'$ reduction of the ethynyl epoxide **17** with DIBAL-H (**Scheme 3**) [26]. Such an allene



Scheme 3. Synthesis of paracentrone **1**

formation is generally used for the synthesis of allene-containing carotenoids, such as paracentrone (**1**) [9], fucoxanthin (**2**) [27a] and peridinin [20,24]. The reaction of **17** with DIBAL-H produced **18** as a single isomer. Oxidation of **17** by MnO_2 followed by the Wittig olefination with $\text{Ph}_3\text{P}^+\text{CH}_3\text{Br}^-$ gave the desired tetraene **20** [28]. The resulting **20** was subject to the cross-metathesis reaction with the vinylboronic acid pinacol ester **7** in the presence of the Grubbs I catalyst to afford the desired vinylboronate **21** in 30% yield in 4 steps from **17**. The obtained **21** was subjected to the Suzuki–Miyaura reaction with the C5 vinyl iodide **4** ($\text{Pd}(\text{OAc})_2$, SPhos, Cs_2CO_3 , DMSO, 35 °C, 17 h) to produce the MIDA boronate **22** as the sole product in 63% yield. Finally, the Suzuki–Miyaura reaction of **22** with vinyl iodide **8** ($\text{Pd}(\text{OAc})_2$, SPhos, NaOH, THF– H_2O , rt, 20 min) produced paracentrone (**1**) [29] with an all-*trans* geometry in 65% yield [30,31,32]. The spectral data of the synthesized **1** were in good agreement with the reported data [8,9].

2.4 Excited state property of **1**

During the course of our studies toward understanding the highly efficient energy transfer mechanism of carbonyl carotenoids during photosynthesis, we reported the syntheses of fucoxanthin (**2**) and its polyene chain length modified analogs [27] along with the results of their ultrafast time-resolved optical absorption analysis [33]. The spectroscopic analysis of the obtained **1** revealed that the S_1/ICT lifetime of **1** was shorter in methanol than in benzene (Figure 3). This result is similar to fucoxanthin, indicating that paracentrone possesses the intramolecular charge transfer (ICT) energy level in the excited state, which is postulated to be deeply related to the highly efficient energy-transfer in marine photosynthesis. Based on this fact, it was found that the polyene structure where the carbonyl group and allene were conjugated was important for the appearance of the ICT state, and the cyclohexane moiety on the right side was not involved.

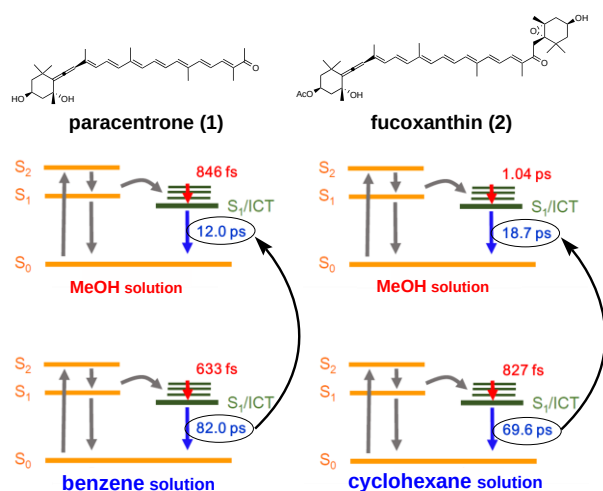


Figure 3. ICT lifetime of **1**

3. Conclusion

The stereocontrolled synthesis of the all-*trans* paracentrone (**1**) was achieved by sequential cross-coupling reactions using the bifunctional C5 dienes **3** and **4** as the key building blocks. Time-resolved absorption spectroscopic measurement of the obtained **1** confirmed that the remarkable ICT state appeared as in the case of fucoxanthin (**2**). Attempts at the reconstitution of the light harvesting complex consisting of the compound **1** – chlorophyll – protein are now in progress.

Acknowledgments

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- [16] The tin and boronate-substituted diene **16** is also a new bidirectionally extensible C5 building block.
- [17] When lower amount of the Hoveyda-Grubbs I catalyst (5 mol %) was employed, the cross-metathesis reaction of **11** with **7** was very slow (CH₂Cl₂, reflux, 19 h, >50% recovery of **11**). The Grubbs I catalyst (15 mol%) was also effective for the cross-metathesis reaction of both **11** and **12** with **7** (CH₂Cl₂, reflux, 41 h) to give **13** and **15**, which upon treatment with iodine, furnished **14** (51%, 2 steps) and **3** (52%, 2 steps), respectively.
- [18] Compound **3**: IR (neat) 2978, 2929, 1614, 1379, 1357, 1328 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.07 (dd, *J* = 10.7, 17.4 Hz, 1H), 6.87 (brd, *J* = 10.7 Hz, 1H), 5.49 (d, *J* = 17.4 Hz, 1H), 2.60 (d, *J* = 1.3 Hz, 3H), 1.27 (s, 12H); ¹³C NMR (75 MHz, CDCl₃) δ 143.1, 142.6, 103.1, 83.4, 28.7, 24.7, [note: the carbon attached to boron was not observed due to quadrupole broadening caused by the ¹¹B nucleus]; HRMS (APCI) *m/z* [M+H]⁺ calcd for [C₁₁H₁₉BIO₂]⁺ 321.0519, found 321.0519.
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- [23] Compound **4**: IR (neat) 3007, 2958, 1755, 1611, 1457, 1336, 1292, 1259, 1237, 1158, 1113, 1087, 1030, 1004, 990, 956 cm⁻¹; ¹H NMR (400 MHz, d₆-acetone) δ 6.71 (d, *J* = 17.8 Hz, 1H), 6.64 (s, 1H), 5.83 (d, *J* = 17.8 Hz, 1H), 4.23 (d, *J* = 17.0 Hz, 2H), 4.05 (d, *J* = 17.0 Hz, 2H), 3.02 (s, 3H), 1.99 (s, 3H); ¹³C NMR (100 MHz, d₆-acetone) δ 168.8, 147.2, 142.7, 85.9, 62.1, 47.2, 19.8, [note: the carbon attached to boron was not observed]; [18] HRMS (ESI) *m/z* [M+H]⁺ calcd for [C₁₀H₁₂BINO₄]⁺ 347.9904, found 347.9908.
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- [29] Paracentrone (**1**): mp 145–149 °C; IR (neat) 3361, 2922, 2853, 1923, 1734, 1653, 1647, 1606, 1368, 1278, 1229, 1154, 956 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.14 (d, *J* = 10.8 Hz, 1H), 6.75–6.55 (m, 5H), 6.39 (d, *J* = 11.2 Hz, 1H), 6.36 (d, *J* = 15.2 Hz, 1H), 6.26 (d, *J* = 10.8 Hz, 1H), 6.12 (d, *J* = 11.6 Hz, 1H), 6.03 (s, 1H), 4.32 (m, 1H), 2.36 (s, 3H), 2.26 (brd, *J* = 11.8 Hz, 1H), 1.992 (s, 3H), 1.985 (s, 3H), 2.00–1.95 (m, 1H), 1.94 (s, 3H), 1.81 (s, 3H), 1.55 (brs, 2H), 1.45–1.30 (m, 2H), 1.35 (s, 3H), 1.34 (s, 3H), 1.07 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 202.4, 199.4, 144.5, 140.0, 137.9, 137.0, 136.2, 135.6, 132.6, 132.2, 132.1, 129.4, 128.4, 125.6, 123.7, 117.7, 103.2, 73.0, 64.3, 49.5, 49.0, 35.8, 32.1, 31.4, 29.3, 25.6, 14.0, 12.9, 12.7, 11.7; HRMS (ESI) *m/z* [M+Na]⁺ calcd for [C₃₁H₄₂NaO₃]⁺ 485.3032, found 485.3039.
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- [31] Other geometric isomers were not detected by ¹H NMR.
- [32] Pure paracentrone (**1**) was obtained by crystallization from Et₂O and *n*-hexane without purification by HPLC.
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Yellow Carotenoids, as Metabolites of Astaxanthin, in the Prawn *Penaeus japonicus*

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Abstract: Yellow carotenoids, metabolites of astaxanthin, in the prawn, *Penaeus japonicus* were investigated. A new carotenoid, peaeusxanthin (**1**) was isolated along with 3,4,3',4'-tetrahydroxypirardixanthin (**2**), and curustaxanthin (**3**).

1. Introduction

The prawn, *Penaeus japonicus* (*Marsupenaeus japonicus*) [Kurumaebi in Japanese] is one of the largest species of prawns and one of the most economically important species of shrimp in Japan. Cultured specimens account for more than 80 % of the prawn market in Japan. This prawn is mainly cultured in Okinawa, Kagoshima, and Kumamoto Prefecture. The market value of the prawn is predominately based on the visual appeal of their body color. Therefore, pigmentation is an important factor for aquaculture of the prawn. It is well known that predominant pigment of the prawn is astaxanthin [1, 2]. Thus astaxanthin was used for pigmentation of the prawn [3, 4]. It was reported that prawn not only accumulated astaxanthin in their body but also metabolized to yellow carotenoids [5-7]. In the present investigation, we described the structures of yellow carotenoids in the prawn, which administrated astaxanthin for pigmentation.

2. Experimentals

2.1 Feeding experiment

The prawns were fed to satiation with experimental food, containing 25, 50, and 100 ppm of astaxanthin (PANAFERD-AX[®]) one time a day for 8 weeks. After the time, carapace, body, and head were removed from the prawn. The carotenoids were extracted from these organs with acetone and were analyzed by our routine methods [8]. PANAFERD-AX[®] is a dried powder of astaxanthin-producing bacterium *Paracoccus carotinifaciens*, which was produced by JXTG Nippon Oil & Energy Corporation. Carotenoid composition of PANAFERD-AX[®] is as follows; astaxanthin (67%), adonirubin (21%), canthaxanthin (3%), adonixanthin (3%), 3'-hydroxy-echinenone etc (3%).

2.2 Characterization of yellow carotenoids

Yellow carotenoids fraction obtained from preparative HPLC was saponified with 5% KOH/MeOH as usual manner. They were re-purified by silica gel HPLC afforded three yellow xanthophylls. They were characterized by UV-VIS, ESI TOF MS, ¹H-NMR, and CD spectral data [8].

Peaeusxanthin (1): UV-VIS: 425, 445, 473 nm. ESI TOF MS: *m/z* 598.4012 (*M*⁺, calcd. for C₄₀H₅₄O₄, 598.4022). ¹H-NMR δ (CDCl₃): 0.97 (3H, s, CH₃-17'), 1.07 (3H, s, CH₃-16), 1.09 (3H, s, CH₃-17), 1.10 (3H, s, CH₃-16'), 1.57 (1H, dd, *J* = 12.5, 7.5 Hz, H-2ax), 1.69 (1H, dd, *J* = 12.5, 12.5 Hz, H-2eq), 1.84 (3H, s, CH₃-18'), 1.89 (3H, s, CH₃-18), 1.92 (3H, s, CH₃-19'), 1.97 (6H, s, CH₃-20, 20'), 1.98 (3H, s, CH₃-19), 2.23 (1H, d, *J* = 17 Hz, H-2'ax), 2.53 (1H, d, *J* = 17 Hz, H-2'eq), 2.64 (1H, d, *J* = 9 Hz, H-6'), 3.87 (1H, m, H-3), 3.96 (1H, d, *J* = 3.5 Hz, H-4), 5.55 (1H, dd, *J* = 15, 9 Hz, H-7'), 6.05 (1H, br.s, OH), 6.07 (1H, d, *J* = 15 Hz, H-7), 6.17 (1H, d, *J* = 11 Hz, H-10'), 6.17 (1H, d, *J* = 15 Hz, H-8), 6.21 (1H, d, *J* = 15 Hz, H-8'), 6.27 (2H, m, H-14, 14'), 6.38 (2H, d, *J* = 15 Hz, H-12, 12'), 6.61 (1H, dd, *J* = 15, 11 Hz, H-11 or 11'), 6.64 (1H, dd, *J* = 15, 11 Hz, H-11 or 11'), 6.65 (2H, m, H-15, H-15'). CD λ (Δε) : 218 (+4.0), 225 (0), 240 (-4.8), 248 (0), 258 (+7.6), 273 (0), 288 (-15.1), 325 (-4.7).

(3R,4S,5R,6R,3'R,4'S,5'R,6'R)-Tetrahydroxypirardixanthin (2): UV-VIS: 419, 439, 468 nm. ESI TOF MS: *m/z* 604.4518 (*M*⁺, calcd. for C₄₀H₆₀O₄, 604.4492), ¹H-NMR δ (CDCl₃): 0.80 (6H, s, CH₃-17, 17'), 0.93 (6H, d, *J* = 6.5 Hz, CH₃-18, 18'), 1.06 (6H, s, CH₃-16, 16'), 1.43 (2H, dd, *J* = 15, 3 Hz, H-2, 2' ax), 1.53 (2H, dd, *J* = 9.5, 9.5 Hz, H-6, 6'), 1.84 (4H, overlapped, H-2, 2' eq and H-5, 5'), 1.93 (6H, s, H-19, 19'), 1.96 (6H, s, H-20, 20'), 3.17 (2H, dd, *J* = 10, 3.5 Hz, H-4, 4'), 4.01 (2H, m, H-3, 3'), 5.45 (2H, dd, *J* = 15.5, 9.5, H-7, 7'), 6.05 (2H, d, *J* = 15.5 Hz, H-8, 8'), 6.11 (2H, d, *J* = 11 Hz, H-10, 10'), 6.24 (2H, m, H-14, 14'), 6.35 (2H, d, *J* = 15 Hz, H-12, 12'), 6.62 (2H, dd, *J* = 15, 11 Hz, H-11,

11'), 6.63 (2H, m, H-15, 15'). CD λ ($\Delta\epsilon$) : 238 (0), 263 (-4.0), 320 (0).

Crustaxanthin (3): UV-VIS: (425), 449, 475 nm. ESI TOF MS: m/z 600.4210 (M^+ , calcd. for $C_{40}H_{56}O_4$ 600.4179).

3. Results and Discussion

The total carotenoid amount in the carapace of control group, astaxanthin 25 ppm administrated group, astaxanthin 50 ppm administrated group, and astaxanthin 100 ppm administrated group were 41.8, 82.6, 116.3, and 128.7, respectively. It was found that the total carotenoids in the prawn were increased with administration dose dependency of supplementation of astaxanthin. Carotenoid compositions were almost similar to both each parts of the prawn and experimental groups. Namely, astaxanthin diester consisted as major components (30~40 % of the total carotenoids), followed by astaxanthin monoester (25~25%), astaxanthin (12~20 %), yellow xanthophyll ester (20~25 %) and other minor carotenoids (1~2%). This indicated that absorbed astaxanthin was mainly accumulated as fatty acid esterified from in the prawn. Furthermore, contents of yellow xanthophyll were also increased by supplementation of astaxanthin. This suggested that these yellow xanthophylls were metabolized from astaxanthin in the prawn.

Yellow carotenoid fraction obtained by silica gel HPLC was saponified with 5%KOH/MeOH and submitted to preparative HPLC with reversed phase system. Three yellow carotenoids, including a new compound, were obtained.

The yellow new carotenoid, named peaeusxanthin (1), showed UV-VIS absorption maxima at 425, 445, 473 nm indicating the presence of β,ϵ -carotene type conjugated double bond system [9]. The molecular formula of this compound was determined to be $C_{40}H_{54}O_4$ by HR ESI TOF MS. 1H -NMR of this compound showed a presence of a 3,4-*cis*-3,4-dihydroxy- β -end group, a 3-keto-4-hydroxy- ϵ -end group and an all-*trans*-polyene chain [10]. This was also confirmed by COSY and NOESY experiments. Relative stereochemistry of this compound was confirmed by

NOESY correlation data as shown in **Figure 1**. From these spectral data, the structures of this carotenoid was determined to be 3,4,4'-trihydroxy- β,ϵ -caroten-3'-one. The second yellow carotenoid (2) having a molecular formula of $C_{40}H_{60}O_4$ showed absorption maxima at 419, 439, 468 nm. 1H -NMR data were indicated in experimental section. These spectral data were identical with (3*S*,4*R*,5*S*,6*S*,3'*S*,4'*R*,5'*S*,6'*S*)-tetrahydroxypirardixanthin isolated from the spindle shell *Fushinus perplexus* [11]. On the other hand, this compound showed mirror image CD spectrum that of tetrahydroxypirardixanthin isolated from the spindle shell *F. perplexus* [11]. Therefore, (3*R*,4*S*,5*R*,6*R*,3'*R*,4'*S*,5'*R*,6'*R*) stereochemistry was postulated for this compound. The other yellow carotenoid having a molecular formula of $C_{40}H_{56}O_4$ showed UV-VIS absorption maxima at (425), 449, 475 nm was identified to be crustaxanthin (3).

The ratio of crustaxanthin, isoastaxanthin, and tetrahydroxypirardixanthin in the carapace of the prawn is 2:5:3.

In 1987, Katagiri et al first isolated tetrahydroxypirardixanthin as reductive metabolite of astaxanthin from the prawn [5]. Subsequently, Schiedt et al., isolated isoastaxanthin, tetrahydroxypirardixanthin, and crustaxanthin from the prawns *P. japonicus* and *P. vannamei* by astaxanthin supplemented experiment [6,7].

In the present investigation, it was found that the amount of crustaxanthin (3), tetrahydroxypirardixanthin (2), and penaeusxanthin (1) were increased by administration of astaxanthin with dose dependency. These yellow carotenoids are not found in PANAFERD-AX[®]. From the results of investigations, reductive metabolic pathways of astaxanthin in the prawn was proposed as shown in **Figure 2**. It was also assumed the presence of 4,4'-dihydroxy- ϵ,ϵ -carotene-3,3'-dione, symmetrical carotenoid having 3-keto-4-hydroxy- ϵ -end group at the both side of polyene chain, in the prawn. However, we could not found 4,4'-dihydroxy- ϵ,ϵ -carotene-4,4'-dione in this prawn in the present investigation.

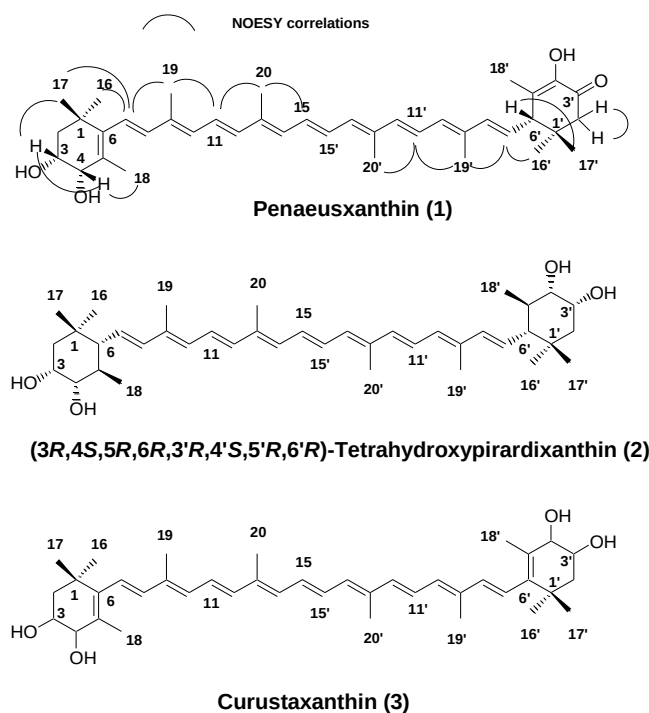


Figure 1. Structures of yellow carotenoids in the prawn.

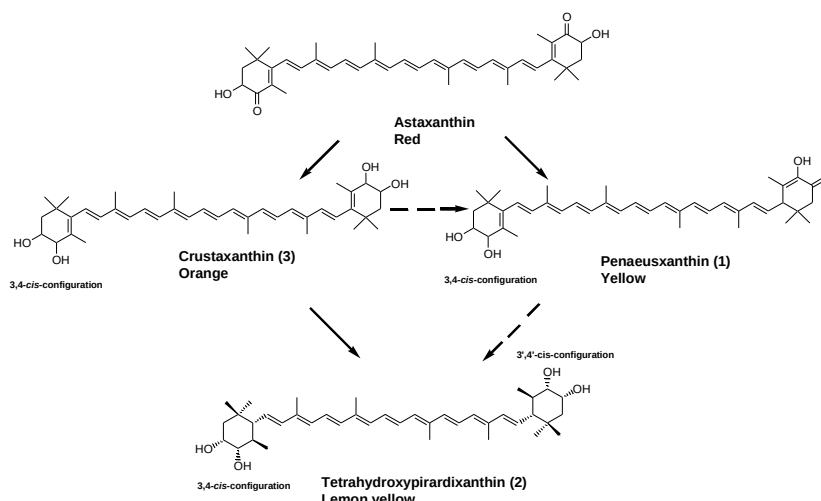


Figure 2. Reductive metabolic pathways of astaxanthin in the prawn.

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Stabilization of reconstituted LH1 complex from a purple photosynthetic bacterium *Rhodospirillum (Rsp.) rubrum* G9+ with fucoxanthin

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Abstract: Fucoxanthin (Fx) is a carotenoid that is mainly bound to the light-harvesting (LH) complexes from brown algae and diatoms [1-3]. It produces intra-molecular charge transfer (ICT) excited-state in polar environments following photoexcitation up to the excited state owing to the presence of a carbonyl group attached to the polyene chain. This ICT state plays a key role in the highly efficient (~95%) excitation energy-transfer from fucoxanthin to chlorophyll *a* in the LH complex from brown algae. The efficiency of excitation energy-transfer (EET) from carotenoids to bacteriochlorophylls depends on the extent of conjugation of the carotenoids in the bacterial light-harvesting systems. We were successful, for the first time, to incorporate fucoxanthin into the LH complexes from a purple photosynthetic bacterium, *Rhodospirillum rubrum* G9+ (a carotenoidless strain) [4]. Femtosecond pump-probe spectra of reconstituted LH1 complex were recorded in order to determine the efficiency of EET from fucoxanthin to bacteriochlorophyll (Bchl) *a* when they are bound to the LH1 apo-proteins. This previous study shows the necessity to stabilize the reconstituted LH1 complex in order to further investigate the detailed mechanisms of fucoxanthin to Bchl *a* EET. In this study, the trials to improve the stability of the reconstituted LH1 complexes were performed by consolidating the reconstituted complexes with a polymer. If stabilization of the reconstituted LH1 complex can be realized, it is expected that various trials will be available to explore ways to enhance light harvesting efficiency in artificial photosynthesis

1. Introduction

The light-harvesting systems of purple photosynthetic bacteria are ring-shaped antenna pigment-protein complexes called LH1 and LH2. They collect solar energy and efficiently transfer the captured energy to the reaction center (RC) [5]. Carotenoids are bound to both peripheral LH2 and core LH1 pigment-protein complexes in purple photosynthetic bacteria. The LH1 complex is composed of 15-16 heterodimer, consists of two bacteriochlorophyll (Bchl) *a*, one carotenoid and a pair of LH1 membrane proteins and is formed in a ring shape so as to surround the RC

Carotenoid in the LH1 complex has a role of light absorption in the wavelength region where Bchl *a* has scant absorption. Carotenoids capture solar energy and then the captured energy is transferred to Bchl *a*. The origin of the main absorption band of carotenoids is

assigned to the $S_0 \rightarrow S_2$ transition. Carotenoids have another singlet-excited state below the S_2 state in energy. This state is called as S_1 and cannot be accessed through a one photon transition from the S_0 state [6-8]. The wavelength of photons that carotenoids can absorb is in a region where the radiation intensity of sunlight shows its maximum. Therefore, the EET efficiency from carotenoid to Bchl *a* is a key factor that determines the total EET efficiency of photosynthesis. The efficiency of EET from carotenoid to Bchl *a* in the LH complexes depends on the number of conjugated C=C double bonds (*n*) of carotenoid [9]. In the natural LH1 complex, the EET efficiency from carotenoid to Bchl *a* is known to be 36-78% [10].

Rhodospirillum (Rsp.) rubrum G9+, a blue-colored photosynthetic bacterium used in this study, is a carotenoidless mutant. This bacterium has only LH1 complex as the antenna pigment-protein complex. The unique characteristics of the LH1 from *Rsp. rubrum* G9+ is that B880 ring-shape LH1

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complex convert to B820 heterodimer and then to B780 monomer subunit by increasing the detergent (β -OG) concentration (*vice versa*), as illustrated in **Figure 1**. The shape of the LH1 complex can easily be determined by the measurement of the absorption spectrum.

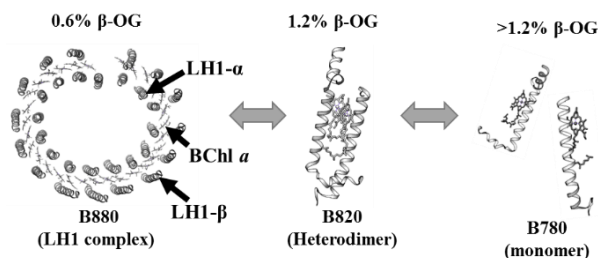


Figure 1. Schematic illustration of dissociation/reassembly of the LH1 complexes from a purple photosynthetic bacterium *Rsp. rubrum* G9+

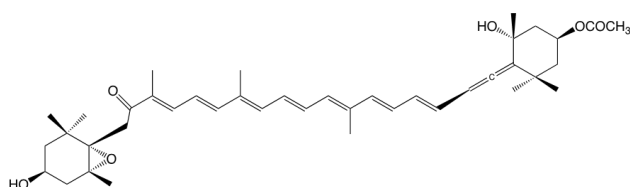


Figure 2. Chemical structure of fucoxanthin, a carotenoid from a brown alga, to be incorporated into the reconstituted LH1 complex.

Fucoxanthin used for reconstitution is a type of closed end-ring carotenoid that can be isolated from brown algae and diatoms (See **Figure 2** for its chemical structure). Highly efficient EET from carotenoid to chlorophyll *a* has been reported for a marine brown alga *Cladosiphon okamuranus* TOKIDA (Okinawa Mozuku) [2]. In this alga, fucoxanthin is bound to the light-harvesting fucoxanthin-chlorophyll *a/c* protein (FCP). Fucoxanthin in FCP shows nearly 100% EET efficiency to chlorophyll *a* [11]. The origin of this highly efficient EET is due to the involvement of an intra-molecular charge-transfer (ICT) excited state that is coupled to the S_1 state of fucoxanthin. However, the detailed generation mechanisms of the ICT excited state are not clarified yet. Therefore, this study takes aim to investigate the generation mechanisms of the ICT excited state that enables highly efficient EET.

In the previous study, using the controllability of the shape of the LH1 complexes that is characteristic of *Rsp. rubrum* G9+, we were successful to reconstitute fucoxanthin into the LH1 complex, which was considered to be impossible so far [4]. Femtosecond time-resolved absorption measurements were performed in order to investigate the EET efficiency from fucoxanthin to Bchl *a*. The results revealed the presence of two types of fucoxanthin that were present in the reconstituted LH1 complex. One

can transfer excitation energy from fucoxanthin to Bchl *a* and the other did not transfer excitation energy at all. It was suggested that the reconstituted LH1 complex has two pools of fucoxanthin, one is successfully bound close proximity to Bchl *a* and the other that is not correctly bound close enough to Bchl *a* (see **Figure 3**). However, in order to proceed further experiments, improvement of the stability of the reconstituted LH1 complexes was required.

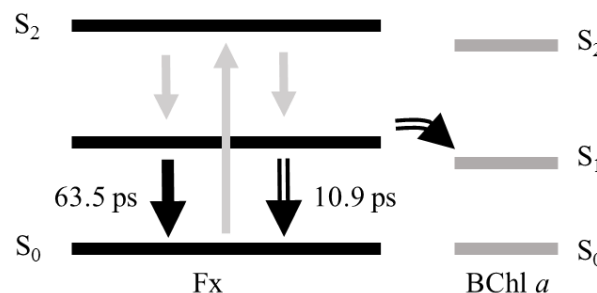


Figure 3. The model to analyze data sets of femtosecond transient absorption spectra of the reconstituted LH1 complexes from a purple photosynthetic bacterium *Rsp. rubrum* G9+ with fucoxanthin

This study aims to improve the stability of the reconstituted LH1 complexes by consolidating it with a polymer for the purpose to investigate the generation mechanisms of the ICT excited state of fucoxanthin that enables highly efficient EET.

2. Experimental

2.1 Isolation and purification of B820 heterodimers from the native LH1 complex of *Rsp. rubrum* G9+

Cells of *Rsp. rubrum* G9+ were photosynthetically grown under anaerobic conditions with C-succinate medium modified from Cohen-Bazire *et al.* at 27 °C [12]. The cells were harvested by centrifugation (18,800 g × 20 min at 4 °C), resuspended in 20 mM MES buffer (pH 7.0), and stored in a freezer (−30 °C) until use. Chromatophores of *Rsp. rubrum* G9+ were prepared as described previously [13].

Native LH1 complex was isolated from the freshly prepared chromatophores of *Rsp. rubrum* G9+ according to the reported protocol [14, 15]. The B820 heterodimers were isolated from the native LH1 complex as described previously [16–18] following solubilization with 1.2% octyl- β -glucopyranoside (β -OG) in 50 mM phosphate buffer (pH 7.0) with the presence of 10 mM sodium ascorbate. The B820 heterodimers were further purified by means of sucrose density gradient (0.6 – 0.8 M) ultracentrifugation (165,000 g × 16 hrs. at 4°C) and stored in a freezer (−30 °C) until required.

2.2 Isolation and purification of fucoxanthin

Fucoxanthin was extracted and purified from a brown alga *Cladosiphon okamuranus* TOKIDA (Okinawa-Mozuku) as reported previously [19]. The purity of the fucoxanthin was confirmed to be >95% by $^1\text{H-NMR}$ spectroscopy. For the spectroscopic experiments a solution of fucoxanthin in acetone ($\text{OD}_{443} = 1 - 2$) was dropped, under a vigorous stream of N_2 gas, onto the 50 mM phosphate buffer (pH 7.0) solution containing 1.2% β -OG. Fucoxanthin was thus solubilized in the buffer solution in detergent micelles.

2.3 Reconstitution of fucoxanthin into the LH1 complex

The reconstitution of carotenoid into the LH1 complex was performed using the reported protocol [16] with a slight modification. The acetone solution of fucoxanthin ($\text{OD}_{443} = 2$) was very slowly dropped, under vigorous flow of N_2 gas, into a 50 mM phosphate buffer (pH 7.0) solution of B820 ($\text{OD}_{820} = 0.4 - 0.6$) containing 1.2% β -OG at 0 °C. Fucoxanthin was added until its absorption band became three times as large as the Q_x absorption band of Bchl *a*. The solution was then diluted with the same volume of 50 mM phosphate buffer (pH 7.0) with 10 mM sodium ascorbate so that the final concentration of β -OG becomes 0.6%. This last procedure (dilution of the detergent concentration) induces the reassembly of the B820 heterodimers to form the LH1 ring structure [15]. During this process fucoxanthin is incorporated into the LH1 complex [4].

2.4 Consolidation of the reconstituted LH1 complex with a polymer

The reconstituted LH1 complex that was prepared does not have sufficient concentration for time-resolved absorption spectroscopic measurements. Therefore, the solution was concentrated using an ultrafiltration membrane. The absorbance of the LH1 complexes at the maximum of their Q_y transition of Bchl *a* was adjusted to $\text{OD}_{880} = 1.1-1.2$. Thus concentrated LH1 complexes were added to the solution of a polymer PVA-217. The reconstituted LH1 complexes were solidified on a glass slide using a spin coating method and stabilized to a ring-shaped LH1 composite. Steady-state and time-resolved absorption spectroscopic measurements were applied to thus fabricated samples.

2.5 Steady-state spectroscopic measurements

The absorption spectra of the sample before and after the femtosecond time-resolved absorption measurements were recorded on a JASCO V-670 UV-vis spectrophotometer at room temperature (24 °C).

2.6 Femtosecond time-resolved absorption measurements

Details of femtosecond pump-probe spectroscopic measurements are described elsewhere [19, 20]. The excitation pulse at 500 nm used to excite the $\text{S}_0 - \text{S}_2$ absorption band of fucoxanthin was generated by an optical parametric amplifier (Spectra Physics, OPA-800CF). The excitation intensity was set to 70- 120 nJ/pulse and the beam was focused on to the sample with a diameter of 200 μm . The instrumental response function of the system, determined by cross-correlation between excitation and probe pulses, was about 130 fs. The cross-correlation function was used to determine the precise zero time delay at each probe wavelength. The uncertainty in the zero time delay was less than 20 fs after chirp compensation.

The liquid sample was placed in a static quartz cuvette that has an optical path-length of 2 mm. The optical density of the sample was set to be 0.5 at the excitation wavelength of 500 nm. The sample solution was continuously stirred with a small magnetic stirring bar during the measurements. The integrity of the sample was checked by comparison of the steady-state absorption spectra of the sample before and after the measurements. The hardened polymer film sample that was placed on a glass slide was subjected to the time-resolved measurement as it is. All the time-resolved measurements were performed at room temperature.

3. Results and Discussions

3.1 Steady-state absorption spectra before and after the concentration protocol

The results of steady-state absorption measurements before and after the concentration of the reconstituted LH1 complex solution are shown in **Figure 4**. Since both spectra show exactly the same spectral pattern, it is concluded that the concentration protocol does not give any damage and the reconstituted LH1 complexes are intact.

3.2 Steady-state absorption spectra before and after the consolidation with a polymer

Figure 5 shows the comparison of the steady-state absorption spectra of the reconstituted LH1 complexes before and after hardening complexes with a polymer. These two spectra show marked difference. When we look at the solid-line spectrum around 500 nm, the absorbance shows the increase in its intensity along the decrease of the wavelength. This is due to the increase of the background of the

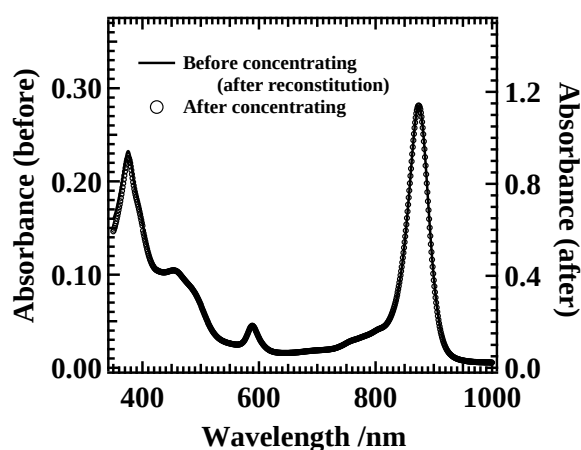


Figure 4. Steady-state absorption spectra before (solid line) and after (dotted line) the concentration of the reconstituted LH1 complex solution.

spectrum caused by the scattering from the sample film that is not favorable for time-resolved absorption spectroscopic measurements. In addition, apparent increase of the absorbance around 780 nm is seen. This signal is due to the presence of the monomeric Bchl *a* that is released from the pigment-protein complexes. This means that the reconstituted LH1 complex might be damaged during the protocol to solidify the sample with a polymer. However, we force to apply the time-resolved spectroscopic measurements to this sample, since this was the best sample so far that we could obtain at this stage.

3.2 Femtosecond time-resolved absorption measurements

The results of the time-resolved absorption spectroscopic measurements are shown in **Figure 6**. In this figure, difference absorption spectra recorded at 1 ps after excitation are compared. The results of 3 independent measurements are shown for the samples consolidated with a polymer (lower panel in **Figure 6**). Those results suggest that it was not easy to obtain consistent results for the samples of the reconstituted LH1 complexes solidified in the polymer. We further need to explore more suitable polymers in order to stabilize the samples [21, 22].

4. Conclusion

As a summary, our first trial to stabilize the reconstituted LH1 complex using a polymer was in vain. We aimed for the stabilization by solidification with a polymer, but the shape of the LH1 complex was changed by consolidation. Further investigation is necessary with varying conditions such as type and concentration of polymers. If we were successful for

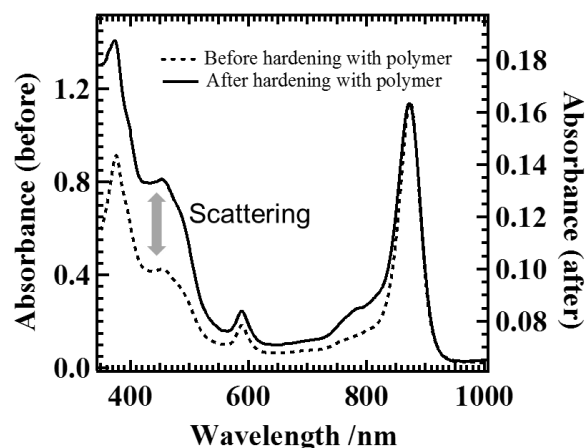


Figure 5. Steady-state absorption spectra of the reconstituted LH1 complexes before (dotted line) and after (solid line) hardening the complexes with a polymer.

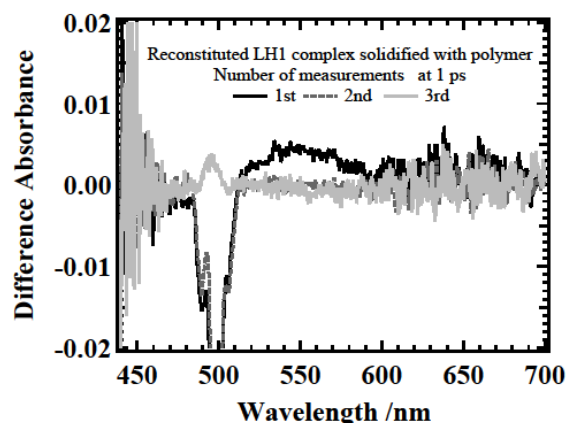
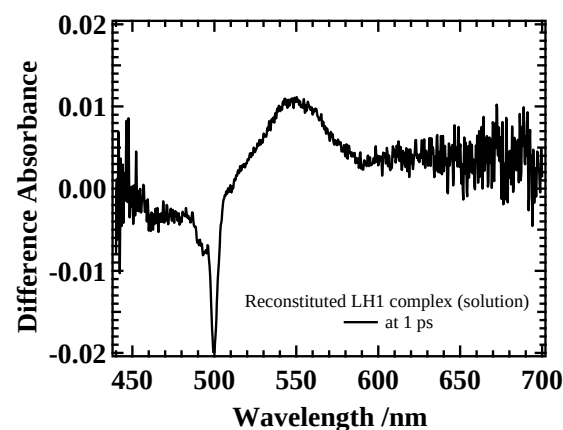


Figure 6. Femtosecond time-resolved absorption spectra of the reconstituted LH1 complex in solution (upper panel) and (b) that fixed with a polymer film (lower panel) at room temperature following excitation up to the S_2 state of fucoxanthin with 500 nm laser pulse.

this, time-resolved absorption spectroscopic measurements should be accurately applied in order to

obtain new knowledge on the generation mechanisms of the ICT excited state of fucoxanthin that enables highly efficient energy transfer.

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Photoprotective Function in the LH1 Antenna Pigment-Protein Complex from A Purple Photosynthetic Bacterium *Rhodospirillum rubrum*

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Abstract: Carotenoids (Cars) in the light-harvesting (LH) antenna systems from phototrophs not only efficiently absorb the energy of sunlight, but also safely dissipate the captured energy to the surroundings when they are exposed excess amount of solar light. We tried to elucidate this photoprotective function of Cars using strain S1 with Cars and carotenoidless strain G9+ of a purple photosynthetic bacterium *Rhodospirillum* (*Rsp.*) *rubrum* that has only core LH1 antenna pigment-protein complex as its light-harvesting apparatus. Based on the measurements of sub-nanosecond pump-probe transient absorption spectra, we suggest that triplet-triplet excitation energy transfer from bacteriochlorophyll (Bchl) *a* T₁ to carotenoid (Car) is promoted by excess light exposure in strain S1, while stable radicals of Bchl *a* with long lifetime were generated in strain G9+. The measurement of the ambient light-harvesting antenna complex LH2 used in the previous study resulted in the lifetime of Car T₁ as 5.5 μ s, while that of LH 1 showed substantially shorter lifetime than LH 2 of 3.6 μ s. From this result, we suggest that the Cars bound to LH1 should have excellent energy dissipation capability than those of LH2.

1. Introduction

In photosynthesis of purple photosynthetic bacteria, light-harvesting (LH) antenna pigment-protein complex has not only the LH function that absorbs solar light, but also it has the function to effectively release the energy as heat against excess light exposure. This is called as photoprotective function. Carotenoid (Car) plays key roles to perform both these functions [1]. Purple photosynthetic bacteria mainly have two different kinds of LH complexes, called LH1 and LH2. The LH1 complex surrounds the reaction center (RC) where charge separation takes place, while LH2 complexes exist around the LH1-RC complex and they are called peripheral antenna. The LH1 complex forms a ring shape structure with 15 or 16 subunits, each of which is composed of two bacteriochlorophyll (Bchl) *a* and one Car and a pair of polypeptides (see **Figure 1a**). Elucidation of the photoprotective function of the ambient LH2 antenna complex from *Rhodobacter* (*Rba.*) *sphaeroides* stain 2.4.1 has already been reported and 100% triplet-triplet excitation energy

transfer from Bchl *a* to Car was observed [2]. In addition, ultrafast spectroscopic data suggests that a molecular assembly of Bchl *a* in LH2 efficiently suppresses a generation of triplet Bchl *a* by singlet-singlet annihilation [2]. *Rsp. rubrum* used in this experiment is a curious organism that has only LH1 complexes (see **Figure. 1b**) as antenna. Since LH1 surrounds RC and a large ring is formed with more subunits than LH2, elucidating its photoprotective function may give us a good hint in order to construct robust artificial photosynthetic antenna systems [3].

In the photosynthesis of purple photosynthetic bacteria solar light energy around 500 nm region of the solar spectrum, where the radiation density is the most abundant, is captured by Car and is transferred to Bchl *a* that is bound to the LH pigment-protein complexes [4, 5]. This process is categorized as the singlet-singlet excitation energy-transfer. The energy is eventually trapped by RC, where charge separation takes place so as to initiate the photosynthetic light reactions [6].

When the excess amount of light is irradiated to the photosynthetic systems, triplet excited state of Bchl *a* is spontaneously generated. Triplet Bchl *a* induces the production of harmful singlet oxygen

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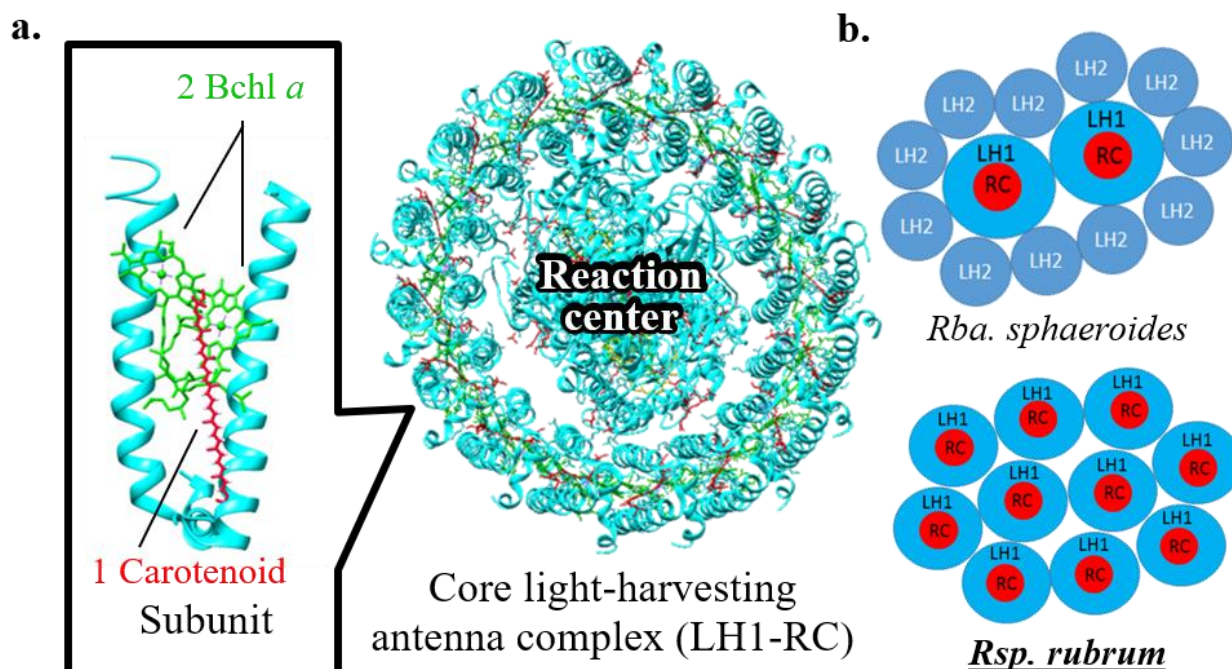


Figure 1 a. Three-dimensional structure of a LH1-RC complex (PDB: 3WMM) with its sub-unit (inset in the left-hand side) **b.** Schematic illustration of the molecular assembly of the LH systems in the photosynthetic membranes of *Rba. sphaeroides* and *Rsp. rubrum*.

under aerobic condition. To prevent this, Car can scavenge the triplet Bchl *a* or singlet oxygen by triplet-triplet excitation energy-transfer. Thus, produced triplet Car safely dissipate its energy as heat to surrounding apo-proteins. This is called as a photoprotection function of Car [1]. Since excitation energy transfer in the primary process of photosynthesis occurs in the range of femtosecond to sub-millisecond time regime, time-resolved absorption spectroscopy has been applied to clarify the detailed mechanisms of this process.

A purple photosynthetic bacterium, *Rsp. rubrum* strain S1, mainly possesses carotenoid spirilloxanthin that has a long 13 conjugated double bonds (see **Figure 2** for its chemical structure). This strain carries out singlet-singlet excitation energy transfer (EET) from Car to Bchl *a* with only 27% efficiency. On the contrary, because of its long conjugated double bonds, it is suggested that this strain carries out efficient triplet-triplet EET from Bchl *a* to Car [7]. Therefore, in this study, we performed time-resolved absorption spectroscopic measurements in order to further investigate its photoprotective mechanisms. We performed the time-resolved experiments on both strains of *Rsp. rubrum* with (strain S1) or without (strain G9+) Car and compared the results.

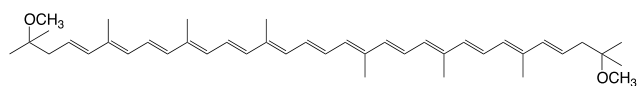


Figure 2. Chemical structure of spirilloxanthin.

2. Experimental section

2.1 Cell growth and preparation of chromatophore membranes

Cells of *Rsp. rubrum* strain S1 and G9+ were photosynthetically grown under anaerobic conditions with C-succinate medium modified from Cohen-Bazire et al. at 27 °C [8, 9]. Both of the grown bacteria were harvested by centrifugation (18,800 g × 10 min at 4 °C), dissolved in 20mM MES buffer (pH 7.0) and stored in a freezer at -30°C until use [9].

The cells of *Rsp. rubrum* were subjected to French pressure cell in order to disrupt the cell walls for the purpose to collect chromatophores. Chromatophores were prepared by successive centrifugation (27,000 g × 30 min at 4 °C) and ultracentrifugation (258,000 g × 90 min at 4 °C) [10,11].

2.2 Preparation of LH1 complex from *Rsp. rubrum* strain G9+

The chromatophores obtained from *Rsp. rubrum* strain G9+ were solubilized using 1.2% octyl-β-glucopyranoside (β-OG) in 50 mM phosphate buffer (pH 7.0) to obtain B820 heterodimer complex. This complex was further purified with a sucrose density gradient (from 0.6 M to 0.8 M linear gradient) ultracentrifugation method (165,000 g × 16 hrs. at 4 °C) and stored in a freezer at -30 °C until use [12-14]. The solution of B820 heterodimer complex was dialyzed overnight against Tris-HCl (pH8.0) solution in order to decrease the concentration of

β -OG. The LH1 complex from *Rsp. rubrum* strain G9+ was reassembled with this procedure [15].

2.3 Preparation of LH1 complex from *Rsp. rubrum* strain S1

Chromatophores of *Rsp. rubrum* strain S1 was homogenized with 50 mM phosphate buffer (pH 7.0) and set to $OD_{883} = 39$. LDAO was added to the solution in which the final detergent concentration to be 0.5% and stirred in a refrigerator (60min). Ultracentrifugation ($105,000\text{ g} \times 90\text{ min}$ at 4°C) was performed and the supernatant solution was obtained. Then the solution was adjusted to $OD_{883} = 28$ with 50 mM phosphate buffer (pH 7.0). Again, LDAO was added to the solution in which final detergent concentration to be 0.4% and then, stirred in a refrigerator (60min), then second ultracentrifugation ($105,000\text{ g} \times 90\text{ min}$ at 4°C) was carried out. The supernatant solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) for 3 days at 4°C [9]. Further purification was done using an anion exchange column chromatography (Whatman, DE-52). First, 50 mM Tris-HCl buffer (pH 8.0) containing 0.03% LDAO was added and the column condition was equilibrated. Crude LH1 solution was subjected to the column, and then 50 mM Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100 and 25 mM NaCl was added, then free Bchl *a* and Car were eluted. Subsequently, the column was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.03% LDAO and 25 mM NaCl, and then LH1 complex solution was eluted by 50 mM Tris-HCl buffer (pH 8.0) containing 0.03% LDAO and 200 mM NaCl. In order to remove NaCl, the LH1 solution was passed through a gel filtration column (Healthcare bioscience, PD-10). Finally, the purified LH1 solution was diluted with 50 mM Tris-HCl buffer (pH 8.0) in order to decrease LDAO concentration [16].

For time-resolved absorption spectroscopic studies, sodium dithionite is added to the sample solutions for the purpose to deactivate the singlet oxygen species. To this end, we found that LDAO is not a suitable detergent. Therefore, we swapped the detergent from LDAO to β -OG.

2.4 Steady-state absorption measurements

Steady-state absorption spectral measurements were carried out using JASCO V-670 UV-vis spectrophotometer [17]. The intensity of the absorption maxima of Bchl *a* Q_y transition of each sample was adjusted to $OD = 0.3$ in a quartz cuvette with 2 mm optical pathlength for the usage of time-resolved absorption studies.

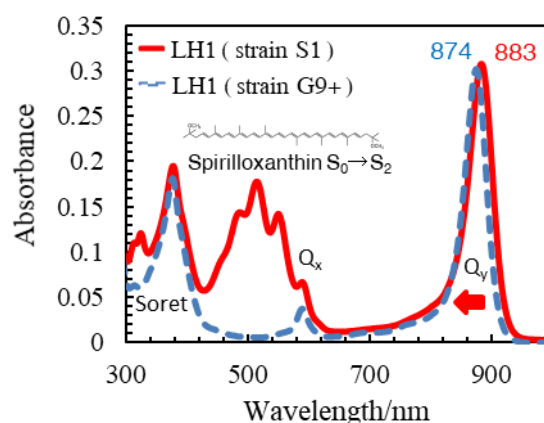


Figure 3. Steady-state absorption spectra of *Rsp. rubrum* S1 and *Rsp. rubrum* G9+.

2.5 Sub-nanoseconds time-resolved absorption spectroscopic studies

Details of the sub-nanosecond pump-probe spectroscopy is described elsewhere [18, 19]. Excitation pulses at 880 nm was generated by OPA (Spectra Physics, OPA-800 CF). These were used to excite the absorption band of Bchl *a* Q_y of each sample. The condition of excitation pulse is as follows: intensity 50 nJ/pulse, pulse width 100 fs, and the beam diameter 200 μm . For the detection, a sub-nanosecond transient absorption spectrometer (EOS Vis-Nir, Ultrafast Systems) was used. The probe pulse was 400 to 900 nm supercontinuum, and the pulse width was 500 ps. The sample was placed in a quartz cuvette with 2.0 mm optical path length, and a small amount of sodium dithionite was added immediately before the measurement. During the measurement, the solution was stirred with a micro magnetic bar, and the cuvette was filled with nitrogen. All optical measurements were performed at room temperature.

3. Results & Discussion

3.1 Steady-state absorption spectra

Figure 3 shows steady-state absorption spectra of the LH1 complexes from *Rsp. rubrum* strain S1 and G9+. Both strains show the absorption bands assignable to the Soret and Q_x band of Bchl *a*, respectively, at 400 nm and at around 600 nm. In addition, for strain S1, absorption derived from Q_y appears around 880 nm and for strain G9+ it appears around 870 nm. The difference of this absorption peak shift would be caused by the presence of Car (spirilloxanthin) in strain S1, which shows three absorption peaks at 420-580 nm. The presence of Car should induce the additional electrostatic interaction to Bchl *a*, which results in the red-shift of Q_y absorption band of Bchl *a* of strain S1 [7].

3.2 Subnanosecond time-resolved absorption spectral measurements

Figure 4 shows the set of transient absorption spectra recorded at selected delay time after photo-excitation of LH1 from *Rsp. rubrum* strain S1. In the near infrared region, at 0.2 ns delay time, bleaching signal around 890 nm and transient absorption band near 860 nm are observed. These signals originate from the Q_y band of Bchl *a* and decays within 2.0 ns. When the signal of Bchl *a* disappears, the signal around 500 nm gradually increases and then slowly decays. It could be considered that the two signals around 570 nm are transient absorption band of triplet state of Car, and the negative signal around 500 nm is the ground state bleaching of Car. From these results, triplet-triplet EET from Bchl *a* to Car in LH1 of *Rsp. rubrum* strain S1 is confirmed.

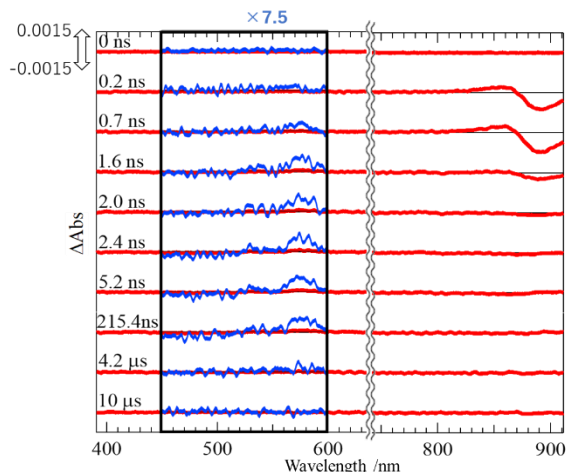


Figure 4. The result of transient absorption spectra with selected delay time after photo-excitation of LH1 from *Rsp. rubrum* strain S1. The intensity of surrounded part (450~600 nm region) is expanded with a factor of 7.5.

Figure 5 shows the result of transient absorption measurements of LH1 from *Rsp. rubrum* strain G9+. In the near infrared region, similar results with the strain S1 are obtained. However, the signal decays extremely slower than the case of strain S1. This is because strain G9+ does not have Cars and the triplet-triplet EET process cannot take place. This means that Bchl *a* of strain G9+ cannot deactivate the excess amount of light energy through triplet state of Car. Consequently, Car could be the essential factor for photoprotective function.

Figure 6 shows the result of global and target analysis of the set of time-resolved absorption spectra of LH1 from strain S1 (upper panel) and G9+ (lower panel) using the Glotalan program [20, 21]. The species associated difference absorption spectra (SAS) of Bchl *a* Q_y band (solid line) of strain S1 decays faster than that of strain G9+. This is probably due to

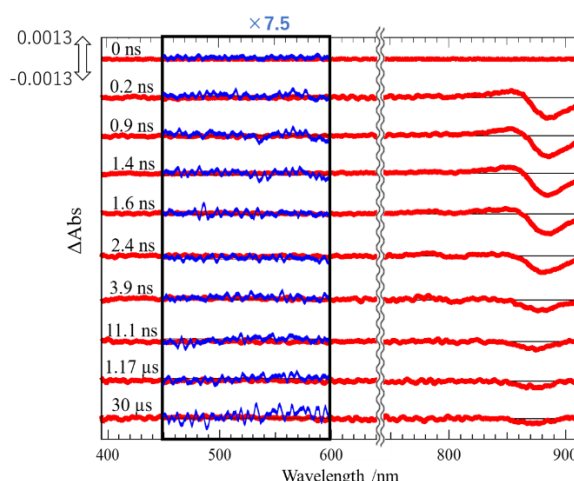
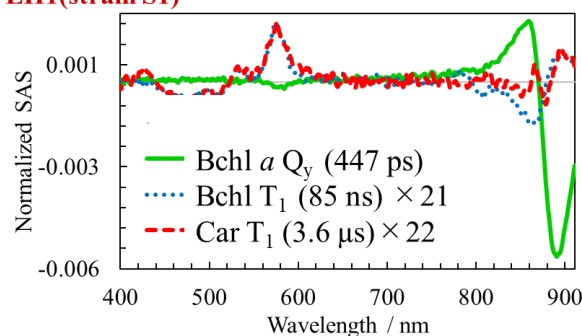


Figure 5. The result of transient absorption spectra with selected delay time after photo-excitation of *Rsp. rubrum* strain G9+. The intensity of surrounded part (450~600 nm region) is expanded with a factor of 7.5.

LH1(strain S1)



LH1(strain G9+)

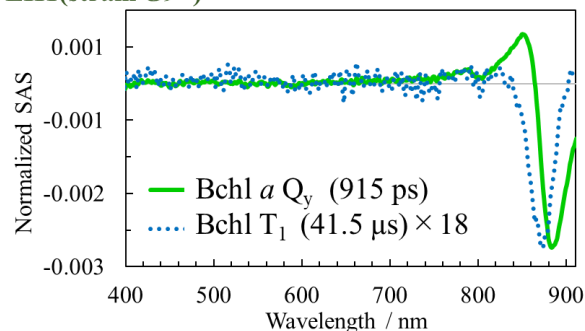


Figure 6. The results of global and target analyses against transient absorption spectra of the LH1 from *Rsp. rubrum* S1 and G9+.

the fact that the presence of Car enhances energy dissipation in stain S1. The SAS of Bchl *a* T_1 band (dotted line) in strain S1 decays with short life time, but strain G9+ decays with long life time. This means that in the case of strain S1, photoprotective function of Car is dominantly performed and the excitation energy deactivates smoothly. On the other hand, in the strain G9+, Bchl *a* T_1 has long lifetime that is different from strain S1. This means that the generation of

stable radical species that have strong oxidation power might be enhanced by excess light exposure. For the SAS of Car T_1 band (dashed line), strain S1 decays with a time constant of 3.6 μ s, but it is considerably faster than the corresponding component in LH2 used in the previous study [2]. This might be caused by the fact that the ratio of Car/Bchl a of LH1 is larger than LH2. Also LH1 has larger number of subunits than LH2. Therefore, LH1 might show the much more excellent energy dissipation performance.

4. Conclusion

Figure 7 shows the summary of energy transfer processes in LH1 from *Rsp. rubrum* with and without spirilloxanthin. In the case of the strain S1, the time constant of excitation energy transfer from the triplet excited state of Bchl a to the triplet excited state of Car generated by excess light exposure was determined to be 85 ns. However, in the case of the strain G9+, the triplet state of Bchl a decayed with a lifetime of 41.5 μ s. Therefore, Car suppresses the generation of triplet Bchl a , and is considered to have an important role to protect photosystem from excess light exposure. In addition, the lifetime of the triplet state of Car was shorter in LH1 than in LH2 that is used in the previous study. Consequently, LH1 shows excellent energy dissipation ability superior to LH2.

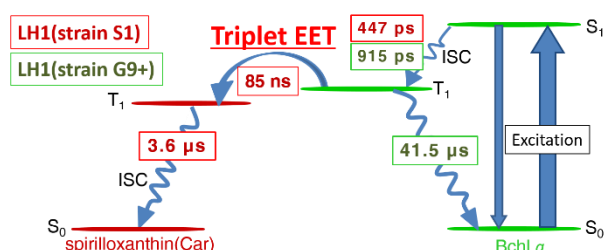


Figure 7. Scheme of energy dissipation pathway of LH1 from *Rsp. rubrum*.

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Isolation and Purification of Capsanthin from Red Paprika (*Capsicum annuum* L.) and its Application to the Stark Spectroscopic Measurements

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Abstract: Capsanthin is one of the main carotenoid components of red paprika. It has a unique chemical structure in which both the keto-carbonyl group and a five-numbered ring are coupled to either end of the conjugated polyene backbone. It is also an interesting carotenoid that is expected to produce the ICT (Intramolecular Charge Transfer) excited state. In this study, we isolated and purified capsanthin and measured mass and NMR spectra. The NMR spectra were recorded using three different organic solvents; benzene-d₆, chloroform-d₁ and methanol-d₄. All the ¹H and ¹³C signals were determined. We also investigated the electronic state of capsanthin by measuring Stark absorption (electroabsorption) spectra for the purpose to identify the generation of the ICT state.

1. Introduction

Capsanthin is a red pigment that has been used as a natural coloring agent for a long time [1]. It also has a strong antioxidant activity and can be used to prevent lifestyle-related diseases [2]. Capsanthin has a keto-carbonyl group that is coupled to the polyene chain with 10 conjugated C=C bonds (see **Figure 1** for its chemical structure). Carotenoids that possess this structure have been reported to generate the intramolecular charge-transfer (ICT) excited state [3].

Peridinin and fucoxanthin are carotenoids that have a keto-carbonyl group coupled to conjugated polyene chains. The lowest excited singlet state (S₁ state) of carotenoids is optically forbidden, and the second excited singlet state (S₂ state) can only be accessed by one photon transition from the ground state. The ICT excited state is coupled to the S₁ state, and it relates to the highly efficient excitation energy transfer from carotenoid to chlorophyll in algae and diatoms [4, 5]. As has already been mentioned, one of the molecular factors that these carotenoids produce the ICT excited state is the presence of the keto-carbonyl group coupled with the conjugated double bonds. However, only few carotenoids are so far found to show the ICT state, and hence the information is not enough to characterize this unique excited state. In this study, we try to extend the previous study and try to

find the new carotenoid that expresses the ICT state. To this end, we have applied the Stark absorption spectroscopy to capsanthin.

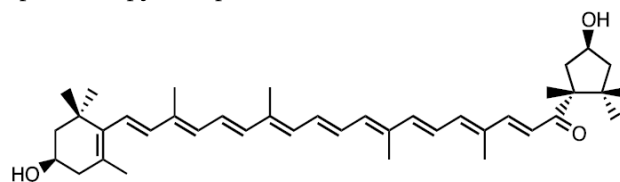


Figure 1. Chemical structure of capsanthin.

2. Experimental

2.1 Sample preparation

All the pigments were extracted with methanol from the freeze-dried red paprika (*Capsicum annuum* L.). The methanol solution was then saponified with KOH to hydrolyze the carotenoid esters. The saponified carotenoids were then extracted with benzene using the ordinary protocol. Capsanthin was isolated from this crude carotenoid mixture solution by silica-gel column chromatography (Eluent: 0~30% THF/benzene (v/v)). Capsanthin was further purified using both normal and reversed phase TLC. For identification of the purified capsanthin, the 1D and 2D ¹H- and ¹³C-NMR spectroscopies in benzene-d₆, chloroform-d₁, and methanol-d₄ as well as mass spectroscopy were applied (The NMR and mass spectral data are shown in **Appendices**). From these

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

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

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

Here, $\mathbf{M}$ is the transition dipole moment and $\mathbf{m}$ is the unit vector of $\mathbf{M}$. $\mathbf{X}$ and $\mathbf{Y}$ is the polarizability and hyper polarizability tensor of transition dipole moment. $\Delta \boldsymbol{\alpha}$ and $\Delta \boldsymbol{\mu}$ is the changes of polarizability and static dipole moment on photoexcitation. This Lyptay's equation is based on the experimental results, and the change $\Delta A(\nu)$ in the absorption spectra can be represented by a linear combination of zero, first and second order derivatives of the absorption spectra $A(\nu)$ [8].

3. Results and Discussion

The solid line of **Figure 3** shows the steady-state absorption spectra of capsanthin measured at room temperature, and the dashed lines show the results of spectral deconvolution using 6 series of Gauss profiles. To implement the optimized fitting, the small band that exists in the lowest energy (Band 1) is necessary. This energy band can be assigned to the transition that generates the ICT state of carotenoids [9]. In **Figure 4** (a), again the absorption spectrum of capsanthin (zero order derivative, solid line) and the series of Gaussian profiles are shown (dashed lines). **Figure 4** (b) and (c) shows the first and the second order derivative of each energy sub-band. The solid lines of **Figure 5** are the Stark absorption spectra of capsanthin measured at room temperature at the polarization angle χ set to 0° , 30° , 54.7° , 70° , and 90° . The dashed lines shown in **Figure 5** are the results of spectral fitting using the derivative waveforms of the

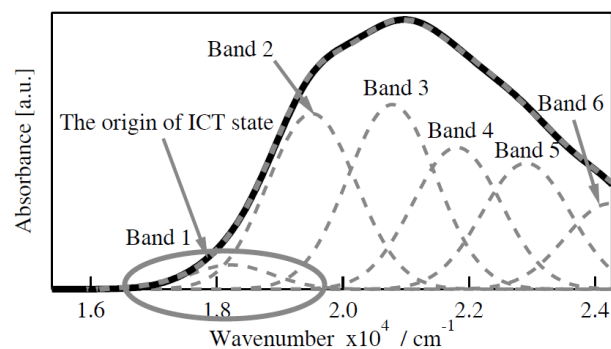


Figure 3. Steady-state absorption spectrum (solid line) and the results of waveform deconvolution (dashed lines) of capsanthin in PMMA at room temperature.

energy sub-bands shown in **Figure 4** (b), (c). Since the value of the coefficient of zero order derivative of the equation (a), A_χ was very small in the fitting, the angle dependency was evaluated only with the coefficients B_χ and C_χ . Band 1 has the largest contribution of the ICT state, so we further investigated the angular dependence of the Stark signals originated from this energy sub-band. The results of the analyses on the angular dependence of B_χ and C_χ using equation (c) and (d) are shown in **Figure 6**. **Figure 6** clearly shows

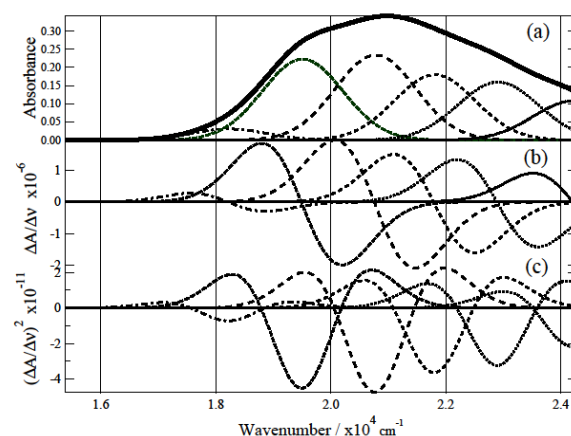


Figure 4. (a) Absorption spectra of capsanthin in PMMA film at room temperature. The first (b) and second (c) order derivative of each Gaussian sub-band used for the fitting of Stark absorption spectra.

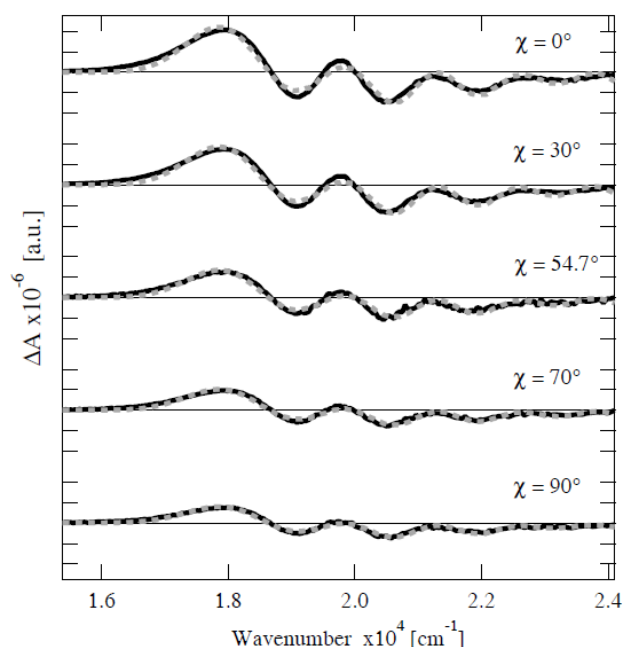


Figure 5. Stark absorption spectra (solid line) and the results of spectral fitting (dashed line) of capsanthin in PMMA at room temperature.

that the accuracy of the spectral fittings is good enough for the determination of both B_χ and C_χ . Using the value of C_χ at the magic angle 54.7° , the magnitude of the change in static dipole moment ($\Delta\mu$) upon photoexcitation was calculated to be 7.57 Debye (here, local field correction factor of PMMA, $f = 1.1$ was applied). In the previous studies, $|\Delta\mu|$ values of fucoxanthin and peridinin were determined, and they were 17.0 and 20.8 Debye, respectively [10, 11]. Since the number of conjugated double bonds of capsanthin is 9 and those of fucoxanthin and peridinin are 7, it was confirmed that the magnitude of $|\Delta\mu|$ decreases when the number of conjugated double bonds increases.

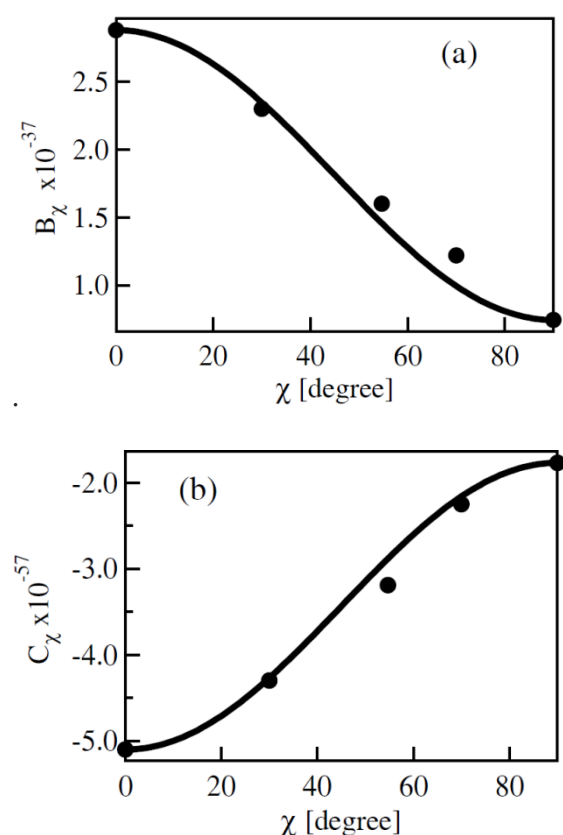


Figure 6. The analysis of the polarization angular dependence of (a) B_χ and (b) C_χ of Band 1 in **Figure 3**.

4. Conclusion

The steady state absorption spectrum of capsanthin was able to deconvolute with 6 Gaussian profiles. It was found that an energy band derived from the ICT state exists in the lowest energy skirt of the absorption band. Stark absorption spectra showed clear polarization angular dependence. The magnitude of $|\Delta\mu|$ upon photoexcitation of the ICT band (Band 1) of capsanthin was determined to be 7.57 Debye. Comparison of this result with the previous ones on other carotenoids suggests that when the number of conjugated double bonds increases, the magnitude of $|\Delta\mu|$ decreases. This finding clearly demonstrates how

difference in chemical structure can affect the strength of ICT character of carotenoids.

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Appendices

¹H-NMR spectra of capsanthin were measured in benzene-d₆, methanol-d₄ and chloroform-d₁ and ¹³C-NMR spectra of capsanthin was measured with benzene-d₆ and methanol-d₄. All the ¹H- and ¹³C- signals were assigned based on 2D spectral measurements and by referring to the assignment of capsanthin in chloroform-d₁ [12]. The data are shown as Chemical shifts in the following **Table A, B** and **C**. Coupling pattern and coupling constants are shown in round brackets.

Table A. ¹H-NMR Chemical Shifts (ppm) of capsanthin (6.00-8.00 ppm)

	7H	8H	10H	11H
Benzene	6.34 (d, 15.8)	6.44 (d, 16.3)	6.43 (d, 11.2)	6.90 (dd, 14.9)
Methanol	6.04 (s)	6.04 (s)	6.07 (d, 10.6)	6.57-6.70 (m)
Chloroform	6.13 (s)	6.13 (s)	6.16 (d, 11.2)	6.59-6.72 (m)
	7'H	8'H	10'H	11'H
Benzene	6.62 (d, 15.2)	7.85 (d, 15.2)	6.50 (d, 13.9)	6.69 (dd, 14.9)
Methanol	6.45 (d, 15.0)	7.19 (d, 15.1)	6.521 (d, 10.8)	6.57-6.70 (m)
Chloroform	6.44 (d, 15.2)	7.33 (d, 15.0)	6.55 (d, 11.5)	6.59-6.72 (m)
	12H	14H	15H	
Benzene	6.58 (d, 13.9)	6.41 (d, 11.8)	6.82(dd, 13.0)	
Methanol	6.30 (d, 14.6)	6.21 (d, 11.0)	6.57-6.70 (m)	
Chloroform	6.36 (d, 14.8)	6.26 (d, 11.7)	6.59-6.72 (m)	
	12'H	14'H	15'H	
Benzene	6.50 (d, 13.9)	6.42 (d, 13.7)	6.73 (dd, 12.5)	
Methanol	6.518 (d, 14.8)	6.32 (d, 10.5)	6.57-6.70 (m)	
Chloroform	6.52 (d, 14.6)	6.35 (d, 11.2)	6.59-6.72 (m)	

Table B. ¹H-NMR Chemical Shifts (ppm) of capsanthin (1.00-2.50 ppm)

	2Ha	2Hb	3H	4Ha	4Hb
Benzene	1.58 (d, 6.6)	1.76 (d, 4.6)	3.94 (br, m)	2.37-2.38 (m)	2.07-2.14 (m)
Methanol	1.33 (d, 4.6)	1.66 (d, 10.26)	3.82 (br, m)	2.24 (dd, 17.7)	1.91-1.94 (m)
Chloroform	1.46-1.50 (m)	1.76-1.78 (m)	4.00 (br, m)	2.39 (dd, 16.3)	2.00-2.07 (m)
	2'Ha	2'Hb	3'H	4'Ha	4'Hb
Benzene	1.78 (d, 3.9)	2.02-2.04 (m)	4.41 (br, m)	3.19 (dd, 14.3)	1.63 (dd, 14.3)
Methanol	1.61 (d, 5.3)	1.825-1.835 (d, 5.5)	4.29 (br, m)	2.79 (dd, 14.2)	1.38 (d, 3.5)
Chloroform	2.00-2.07 (m)	1.71 (dd, 13.6)	4.51 (br, m)	2.96 (dd, 14.4)	1.46-1.50 (m)
	16Me	17Me	18Me	19Me	20Me
Benzene	1.21 (s)	1.22 (s)	1.86 (s)	2.03 (s)	2.01 (s)
Methanol	0.98 (s)	0.97 (s)	1.64 (s)	1.87 (s)	1.89 (s)
Chloroform	1.07 (s)	1.07 (s)	1.74 (s)	1.97 (s)	1.97 (s)
	16'Me	17'Me	18'Me	19'Me	20'Me
Benzene	1.01 (s)	1.30 (s)	1.43 (s)	1.89 (s)	1.90 (s)
Methanol	0.73 (s)	1.11 (s)	1.26 (s)	1.88 (s)	1.89 (s)
Chloroform	0.84 (s)	1.21 (s)	1.37 (s)	1.96 (s)	1.99 (s)

Table C. ^{13}C -NMR Chemical Shifts (ppm) of capsanthin

	Benzene	Methaol		Benzene	Methanol
1C	37.16	38.29	1'C	44.02	45.13
2C	48.87	49.87	2'C	51.25	51.81
3C	64.61	65.71	3'C	70.15	70.77
4C	42.94	43.47	4'C	45.92	46.10
5C	126.98	127.77	5'C	59.10	60.25
6C	137.92	139.67	6'C	201.63	205.57
7C	126.38	127.14	7'C	121.64	122.43
8C	138.96	140.00	8'C	146.67	148.60
9C	※0	※0	9'C	133.98	135.17
10C	131.95	132.91	10'C	140.53	142.70
11C	125.93	※2	11'C	124.50	125.52
12C	137.98	139.05	12'C	141.96	143.93
13C	137.67	139.33	13'C	136.19	137.50
14C	133.07	134.03	14'C	135.50	137.00
15C	132.03	133.32	15'C	130.28	※2
16C	28.87	29.33	16'C	26.13	26.61
17C	30.44	31.07	17'C	25.28	25.69
18C	21.79	22.15	18'C	21.29	22.32
19C	※1	※3	19'C	※1	※3
20C	※1	※3	20'C	※1	※3

※0 ; n. d.

※1 ; 12.67 or 12.71 or 12.80 or 12.89

※2 ; 126.86 or 131.37

※3 ; 13.00 or 13.05 or 13.14

Furthermore, the mass spectrum was measured for capsanthin, by the electrospray ionization method. The results are shown in **Figure A**.

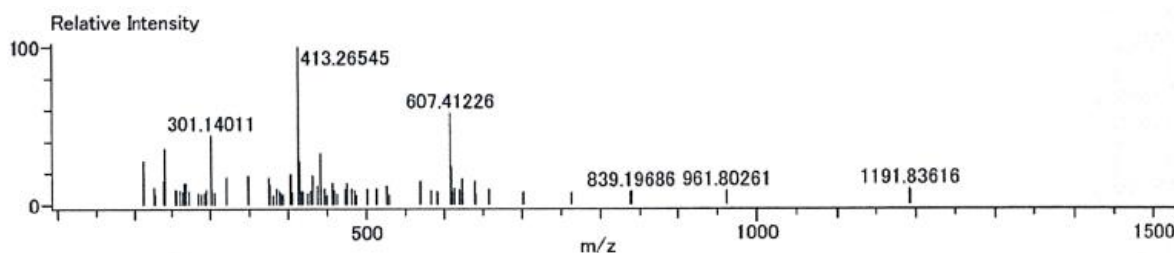


Figure A. Mass spectrum of capsanthin

In **Figure. A**, the peak at $m/z = 607$ is the parent ion ($C_{40}H_{56}O_3$) that the sodium ion as the positive ion is adducted to the capsanthin molecule ($M = 584$). According to the list of compounds in **Table D**, we determined that the finally obtained substance was pure capsanthin.

Table D. Compound candidates at $m/z = 607$

Mass	Intensity	Calc. Mass	Mass Difference [mDa]	Possible Formula	Unsaturation Number
607.41226	2337.31	607.41271	-0.45	$^{12}C_{40}^{1}H_{56}^{23}Na_1^{16}O_3$	12.5
		607.41031	1.95	$^{12}C_{38}^{1}H_{57}^{23}Na_2^{16}O_3$	9.5
		607.41512	-2.86	$^{12}C_{42}^{1}H_{55}^{16}O_3$	15.5

Reference

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