Chapter 1

Characterisation of Carotenoids Involved in the Xanthophyll Cycle

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http://dx.doi.org/10.5772/67786

Abstract

Carotenoids are known for versatile roles they play in living organisms; however, their most pivotal function is involvement in scavenging reactive oxygen species (ROS) and photoprotection. In plant kingdom, an important photoprotective mechanism, referred to as the xanthophyll cycle, has been developed by photosynthetic organism to avoid excess light that might lead to photoinhibition and inactivation of photosystems and induce the formation of reactive oxygen species (ROS), resulting in photodamage and long-term changes in the cells caused by oxidative stress. Apart from high-light driven enzymatic conversion of violaxanthin (Viola) to zeaxanthin (Zea) that occurs mostly in higher plants, mosses and lichens, other less known types of the xanthophyll cycle have been hitherto described. The work is aimed at summarising the current knowledge on the pigments engaged in the xanthophyll cycles operating in various organisms.

Keywords: carotenoids, chromatography, diadinoxanthin, diatoms, diatoxanthin, *Phaeodactylum tricornutum*, xanthophyll cycle

1. Introduction

Carotenoids constitute a large group of pigments with over 700 compounds [1]. They comprise of carotenes and their oxygenated derivatives, xanthophylls. Carotenes are polyunsaturated hydrocarbons with 40 carbon atoms, while xanthophylls contain oxygen atoms, most frequently as hydroxyl and epoxide groups, which increase their polarity. Both groups of carotenoids act as accessory light-harvesting pigments or as quenchers of singlet oxygen and chlorophyll triplet states to provide protection against photooxidative damage [2]. The main photoprotective mechanism occurring in photosynthetic organisms is the xanthophyll

cycle—a process of enzymatic reactions of epoxidation and de-epoxidation of xanthophylls [3]. These cyclic conversions can proceed between several pigments including violaxanthin (Viola), antheraxanthin (Anth), zeaxanthin (Zea), diadinoxanthin (Diadino), diatoxanthin (Diato), lutein (Lut), lutein-epoxide (LutE) and oxidised but not epoxidised siphonaxanthin (Siph). Therefore, five types of xanthophyll cycles and additional non-specific cycle have been described [4] (**Figure 1**). The common factor in all of them is conversion of epoxidised xanthophylls to their de-epoxidised forms under strong light to dissipate of excess energy and epoxidation of de-epoxidised xanthophylls in low light or dark [5].

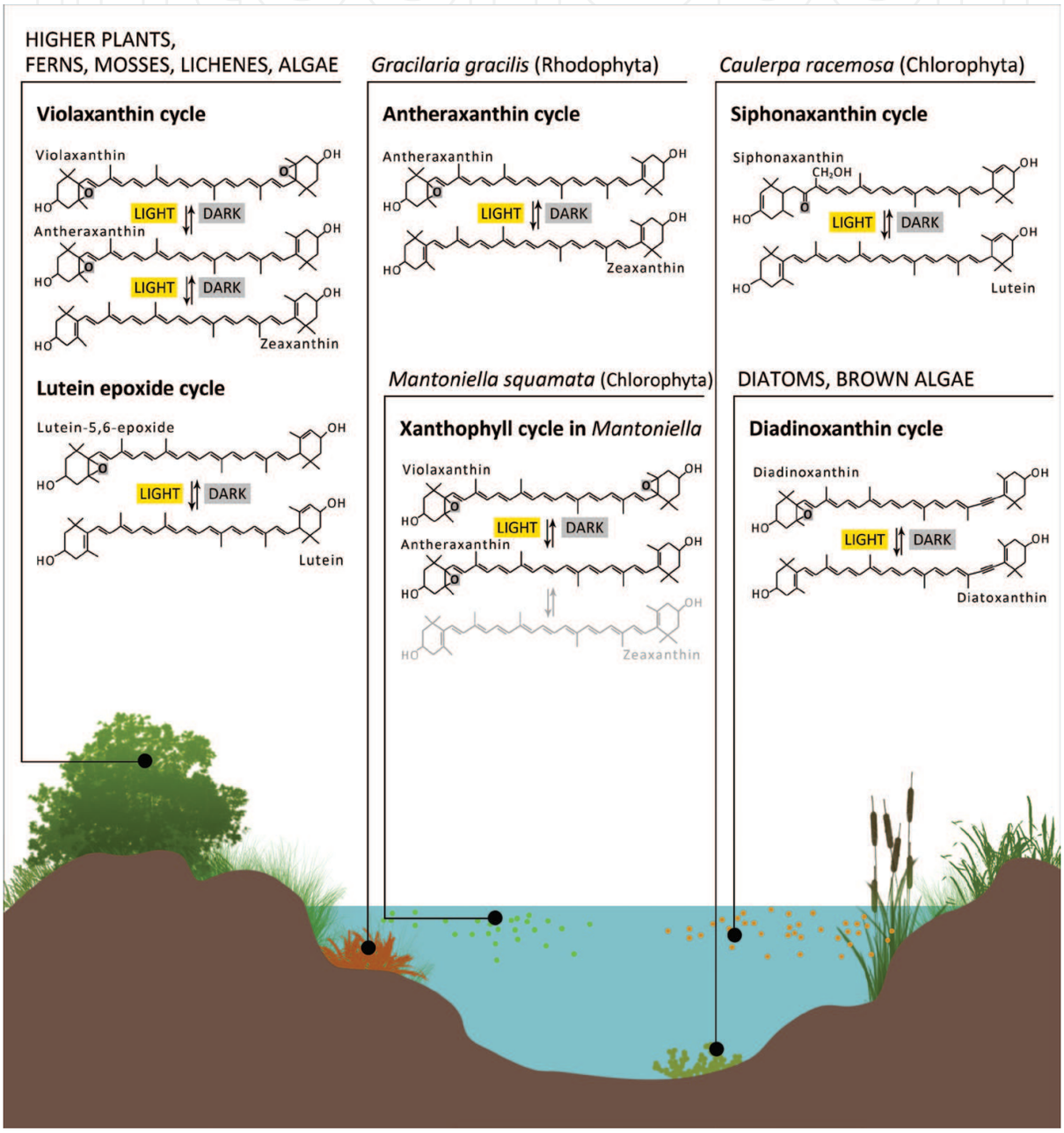

Figure 1. Xanthophyll cycles in photosynthetic organisms.

http://dx.doi.org/10.5772/67786

2. Carotenoids in the xanthophyll cycle

2.1. Violaxanthin, antheraxanthin and zeaxanthin

Viola, Anth and Zea are engaged in the most common xanthophyll cycle, referred to as the violaxanthin cycle (VAZ cycle), see **Figure 1**. Di-epoxy Viola is de-epoxidised to epoxy-free Zea in two-step reaction catalysed by Viola de-epoxidase (VDE) with mono-epoxy Anth as an intermediate product. In absence of photosynthesis, VDE is localised in the thylakoid lumen as inactive monomer; however, it undergoes dimerization and binds to the membrane in an acidic pH caused by the light-driven transmembrane proton gradient [6–8]. Additionally, ascorbate as a donor of protons and monogalactosyldiacylglycerol (MGDG) as lipid-forming inverted hexagonal structures are essential for Viola de-epoxidation [9, 10]. In reverse reactions of the VAZ cycle, product of de-epoxidation—Zea—is epoxidised by Zea epoxidase (ZEP) to Viola, also via Anth. The reaction is observed in low light and in darkness due to lack of VDE activity in such conditions, but in higher plants, it can also proceed in high light [11, 12]. ZEP, localized in chloroplast stroma, is active in neutral pH and requires nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and molecular oxygen as co-substrates to epoxidise rings in Viola and then in Anth [11, 13].

The VAZ cycle occurs in higher plants, ferns, mosses, lichens and some groups of algae [3, 4]. However, in few species of algae, two specific xanthophyll cycles with Viola, Anth and Zea have been observed (**Figure 1**). In *Mantoniella squamata* (Chlorophyta), Viola is converted mainly to Anth, which is rapidly epoxidised back to Viola, and Zea occurs in low amount in these cells. It is a result of reduced affinity of VDE to Anth [14]. Second modification in the VAZ cycle has been described in two Rhodophyta species *Gracilaria gracilis* and *Gracilaria multipartita* in which Viola does not occur so de-epoxidation and epoxidation proceed only between Anth and Zea [15].

2.2. Diadinoxanthin and diatoxanthin

In several algal groups including diatoms, phaeophytes, dinophytes and haptophytes, mainly Diadino and Diato are involved in the xanthophyll cycle therefore named diadinoxanthin cycle (DD cycle). In addition, also the VAZ cycle can be observed in these organisms during strong light stress [16, 17], see **Figure 1**. In DD cycle, mono-epoxy Diadino is converted to epoxy-free Diato by a Diadino de-epoxidase (DDE or VDE), and the reverse reaction is catalysed by a Diato epoxidase (DEP or ZEP). Both enzymes have comparable properties and are able to convert Diadino/Diato as well as Viola/Anth/Zea [17]. De-epoxidation occurs in high light, in decreased pH in thylakoid lumen and in the presence of ascorbate, however, at lower concentration than that in plants [18, 19]. Unlike plants, in diatoms, epoxidation does not occur in high light since the proton gradient between thylakoid lumen and chloroplast stroma inhibits this reaction [20, 21]. It was reported that the rates of Diadino de-epoxidation and Diato epoxidation are several times higher than the conversions of Viola and Zea in plants and green algae [20].

2.3. Lutein, lutein epoxide and siphonaxanthin

Lut is an epoxy-free xanthophyll bound to antenna proteins and is essential for their stability in higher plants, while LutE occurs in significant amount in some species only [22], see **Figure 1**. Although the presence of LutE does not mean its involvement in cyclic conversions with Lut, the fully operative or truncated LutE cycle has been reported in several plant species [4]. In the first case, the initial LutE pool is fully recovered in the dark, which is not observed in truncated cycle. Both reactions of the LutE cycle are catalysed by VDE and ZEP, which are also engaged in the VAZ cycle but their rates are 2- or 3-fold lower, which might be a result of decreased affinity of enzymes to Lut and LutE or stronger binding of these pigments to antenna proteins [4].

Another xanthophyll cycle is operating in green algae *Caulerpa racemosa* in which interconversions between Siph and Lut have been reported [23]. During illumination, Siph is converted to Lut, and the reverse reaction proceeds in low light. Despite the mechanism of the Siph cycle is still unknown, this suggests a photoprotective role [22].

3. *In vitro* assays of the xanthophyll cycle

Studies on de-epoxidation and epoxidation of pigments involved in the xanthophyll cycle, the mechanism and conditions of these reactions, enzyme properties and factors regulating their efficiencies are usually performed *in vivo* by treatment of the organisms studied with stress or genetic modifications. Such experiments allow to observe holistic effects in natural or semi-natural conditions but to analyse specific parameters of a single reaction, it is more convenient to perform them in fully controlled system.

Among two reaction types in the xanthophyll cycles, de-epoxidation of Viola has been extensively studied, and *in vitro* assay was developed [24]. The system comprises phosphatidylcholine liposomes with MGDG and Viola suspended in sodium citrate buffer with sodium ascorbate and VDE isolated from wheat. It was concluded that MGDG is an essential component of lipid membrane, which allows to bind VDE to the membrane which is necessary for its activity. Additionally, de-epoxidation of Viola to Anth seemed to be more sensitive to MGDG concentration than the second step of the reaction. Although this assay has been tested with Viola as a substrate, it is highly probable that Diadino can be also used for such assay. Considerable progress in these studies is the use of purified recombinant enzymes preferably from several species which allow for comparative analysis of their properties.

Epoxidation of Zea was investigated in semi-defined system [25]. Thylakoids of *npq1* mutant of *Arabidopsis thaliana* were the source of an active ZEP suspended in Hepes buffer with sorbitol, $MgCl_2$ and ethylenediaminetetraacetic acid (EDTA). Zea was mixed with MGDG and incorporated into thylakoids by sonication, and also sodium ascorbate, FAD and NADPH were added. During 2 hours, the amount of Zea was reduced by 38%. However, isolated thylakoids contain not only an active enzyme but also additional compounds that could play an essential role in Zea epoxidation. Therefore, such semi-defined system may not be applicable to study of purified enzyme activity.

In vitro assay of Zea epoxidation has also been reported [26] using recombinant *Capsicum annuum* β-cyclohexenyl epoxidase isolated from *Escherichia coli*. Reaction was carried out in phosphate buffer with FAD, NADPH, Zea, MGDG, digalactosyldiacylglycerol (DGDG), ferredoxin, ferredoxin: $NADP^+$ oxidoreductase and β-cyclohexenyl epoxidase. It has been reported that epoxidase is able to accept NADP only via reduced ferredoxin activity in the presence of NADPH, ferredoxin oxidoreductase and ferredoxin, and in these conditions, a significant conversion of Zea into Viola was observed.

4. Production of the xanthophyll cycle carotenoids

The production of carotenoids with well-known beneficial effects is of great importance for various industries including food, cosmetology, pharmacy and medicine and can be performed both by extraction from plans, algae, fungi, yeast and bacteria or through chemical synthesis [27, 28]. Both methods have some advantages and disadvantages but the choice is usually dependent on availability of extraction or synthesis procedure [29]. Due to many technologies of chemical synthesis have been developed and the cost of this production often is relatively low, the majority of carotenoids is obtained chemically. However, a consequence of that way is usually the production of stereoisomers mixture with reduced biological activity or even having side effects. Such disadvantages are not the case of natural pigments extraction; however, difficulties with yield and separation efficiency are present. Chemical and physical properties of various carotenoids are similar which results in limited separation capacity.

Most of the xanthophyll cycle carotenoids such as Anth, Zea, Viola, Lut and LutE are commercially available (data are given by international carotenoid society), but Diadino and Diato production has been developed only recently [30]. The procedure consists of total pigments extraction from marine diatom *Phaeodactylum tricornutum* followed by saponification and pigments partitioning and finally purification by open-column chromatography (**Figure 2**).

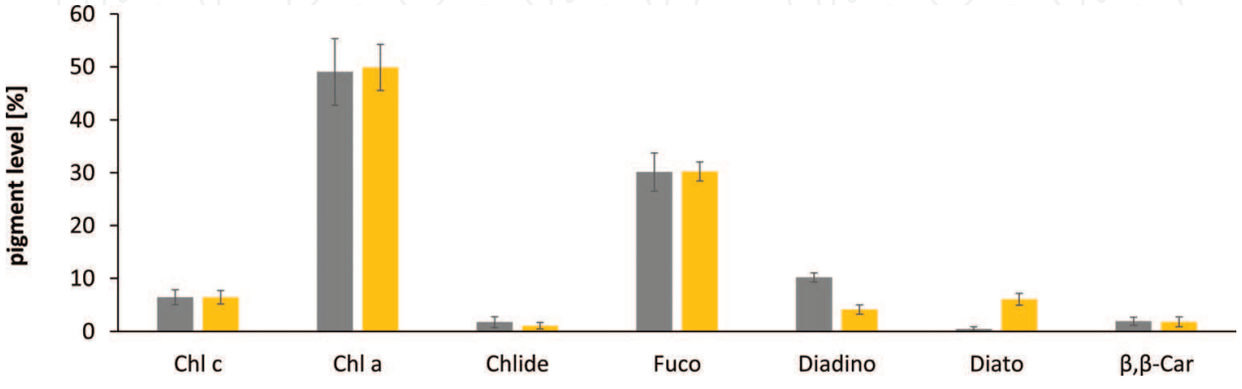

Figure 2. A process of isolation and purification of diadinoxanthin and diatoxanthin illustrating the successive steps comprising: diatoms cultivation under different light conditions, total pigments extraction, carotenoids separation by partitioning and purification of diadinoxanthin and diatoxanthin by open-column chromatography.

4.1. Diatoms as a rich source of natural pigments

Diatoms are microalgae widely distributed in marine and freshwater environment. They contain two types of photosynthetic pigments which are involved in light harvesting (chlorophyll *a*, chlorophyll *c* and fucoxanthin) and photoprotection (β-carotene, diadinoxanthin, diatoxanthin, zeaxanthin, antheraxanthin and violaxanthin). Three of above-mentioned pigments including Fuco, Diadino and Diato occur only in few algal groups; therefore, they might be considered as diatom-specific carotenoids. The quantitative composition of above pigments is dependent on growth conditions.

Pigment content is regulated mostly by light which increases the level of photoprotective carotenoids which are involved in the xanthophyll cycle [31]. Therefore, light stress can be used to produce the highest possible content of Diadino or Diato in the cells.

4.2. Diatoms cultivation

Diatoms *P. tricornutum* Bohlin, strain CCAP 1055/1, were cultivated in f/2-Si medium [32] made with seawater supplemented with inorganic nutrients and vitamin mixture. The temperature of 15°C was proved to be an optimal for increased level of both xanthophylls [30]. Cells were grown under white light of the intensity of 100 μmol photons m^{-2} s^{-1} in a 16/8 hours day/night photoperiod. Cells used to Diadino purification were collected after dark phase, while in the case of Diato, purification cells were illuminated with white light of the intensity of 1250 μmol photons m^{-2} s^{-1} for 2 hours. Several light conditions during diatoms growth have been tested, which resulted in high level of both xanthophylls (even 19% of Diadino or 17% of Diato); however, *cis* isomers of Diadino and Diato and also Zea, Anth and Viola were detected in addition in these conditions [30]. These additional xanthophylls have similar physical and chemical properties, including polarity, which has crucial impact on separation efficiency, and therefore, obtained Diadino and Diato were contaminated by them. Summarising, to obtain pure Diadino and Diato, diatoms should be cultivated under specific light conditions which result in increased biosynthesis of these pigments and simultaneously do not cause induction of the VAZ cycle and *cis* isomers formation. The pigment composition in *P. tricornutum* cultivated in such conditions is given in **Figure 3**, and the levels of Diadino or Diato in these samples were 10 and 6%, respectively.

4.3. Pigment extraction and saponification

An essential aspect of pigment quantification and its collecting for further purification is a step of extraction which requires selection of an appropriate method for a particular purpose. To analyse the amount of pigments in the cells, it is important to extract each of them with the same yield to avoid disproportion between their content. Since pigment composition of each organism varies and includes pigments with different polarity and hence the solubility in organic compounds, the extraction technique should be selected individually. On the other hand, to obtain a particular pigment, special attention should be paid to the extraction yield and to minimising formation of degradation products. In view of these issues, many methods of pigment extraction have been developed. The most applicable methods are solvents that

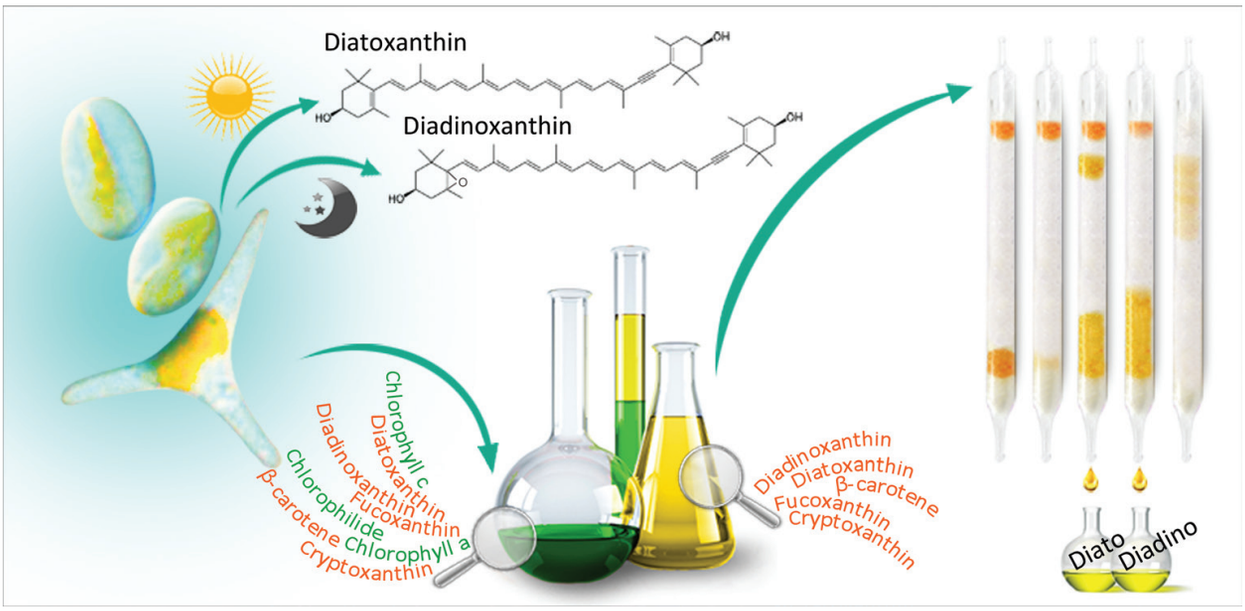

Figure 3. Pigment composition in *Phaeodactylum tricornutum* cultivated at 15°C under white light of the intensity of 100 μmol photons m^{-2} s^{-1} in a 16/8 hours day/night photoperiod. One day old cells were collected after dark phase (dark) or after illumination with white light of the intensity of 1250 μmol photons m^{-2} s^{-1} for 2 hours (light) [30].

comprise acetone, methanol and water in varying ratios, while the most common homogenisation techniques include grinding, sonication, heating and shaking [33].

The aim of pigment extraction from *P. tricornutum* in described protocol was to recover the highest amount of Diadino and Diato neglecting the efficiency of other pigments extraction. The composition of solvent mixture and the proportion between the solvent volume and the number of cells were found to be essential for the yield of this step. Pigments were extracted from frozen cells, which were earlier harvested by centrifugation of liquid diatoms culture, using a medium composed of methanol, 0.2-M ammonium acetate and ethyl acetate (81:9:10, v/v/v) in a ratio of 10 mL per 2×10^9 cells [30].

Saponification and liquid-liquid partitioning are widely practised techniques of carotenoids purification, which allows to remove chlorophylls and lipids—compounds abundantly present in pigment extracts. In general, saponification is performed by adding methanol or ethanol and aqueous solution of KOH in concentration of approx. 5–10% into pigment extract and then incubation of the mixture in darkness for several hours. Numerous modifications of this method have been described [34]. Before performing this step, it should be considered whether the carotenoid is stable in alkali because some of them are not, that is, astaxanthin, fucoxanthin and peridinin [35, 36].

After saponification, chlorophylls and carotenoids are separated through the partitioning with mixture of solvents of varied polarity. Lipophilic carotenoids dissolve in the upper phase, which usually contains petroleum, while chlorophylls dissolve in methanol or ethanol in the lower phase. However, the presence of epoxy, hydroxy and other groups in pigments cause decrease in polarity of chlorophylls, while the polarity of some carotenoids is enhanced [34]. Therefore, the composition of mixture for partitioning should be more complex and specific for the extract.

In the case of Diadino and Diato purification, a saponification was performed through addition of ethanol and 60% aqueous solution of KOH (10:1, v/v) and stirring the mixture in darkness overnight in a cold room. To separate phases following solvents were added consecutively: hexane and diethyl ether (1:1, v/v), extraction petroleum and water (4:1:2, v/v/v). Then, to remove alkali, collected carotenoid fraction was washed three times with one volume of distilled water. The efficiency of a single partitioning was more than 90% so repetitions which resulted in increasing of Fuco level were not advisable. After chlorophylls and carotenoids separation, the level of Diadino or Diato in extract increased to approx. 40 or 20%, respectively, and mixtures contained also β,β-Car, cryptoxanthin and fucoxanthin derivatives. This preliminary purification step was essential for further purification by open-column chromatography.

4.4. Diadinoxanthin and diatoxanthin purification

The final step of Diadino and Diato purification was separation of carotenoids present in the mixture after saponification and partitioning by open-column chromatography. This technique is commonly used in preparative scale and therefore is applicable for industrial use. Development of appropriate chromatographic conditions consists mainly in selection of the solid phase and composition of eluents, but also in the amount of pigment mixture applied onto the column, the volume of eluents, pressure applied to the column.

Since xanthophylls are susceptible to isomerization under acidic conditions, a modified silica gel, which is chemically converted into a basic form, was used. To separate and elute pigments, the mixtures of hexane and acetone were used. Hexane:acetone (90:10, v/v) mixture allows for β,β-Car elution. Second mixture (80:20, v/v) removes cryptoxanthin epoxide from the column. Continued use of this mixture allows to separate and collect Diato. Then, third mixture (70:30, v/v) allows to collect Diadino. To remove Fuco derivatives from the column and to reuse the silica gel, pure acetone was used.

Described procedure allowed to obtain all-*trans* Diadino and all-*trans* Diato of a purity of 99.9%, and the final efficiency was estimated to be 63 and 73% for Diadino and Diato, respectively. Due to the use of popular reagents, simplicity of the procedure, possibility of diatoms cultivation in bioreactors and estimated low costs, the method is widely accessible and might be performed both in analytical and preparative scale.

5. Interaction of the xanthophyll cycle carotenoids with membranes

It has been in 1974 when the hypothesis was formed postulating that carotenoids present in some prokaryotic membranes play the similar role as cholesterol in animal membranes [37]. Since then, numerous studies have been carried out to get a deep insight into the molecular mechanism of carotenoid-membrane interactions. A large body of research has been performed using liposomes as model systems of biological membranes, mostly due to their simplicity, stability, and well-characterised properties.

5.1. Localisation within model membranes

The issue of localisation and orientation of carotenoids in the membrane has been addressed mainly by analysing the position of the maxima in the UV-VIS spectral region of these pigments upon their incorporation into lipids matrix as well as by linear dichroism and X-ray diffraction [38]. Generally, xanthophylls are oriented perpendicularly to the membrane surface with their hydrophilic groups anchored in the two opposite polar regions of the lipid bilayer. Accordingly, to minimalise the energy of the system, zeaxanthin molecule with its two hydroxyl groups located at 3 and 3′ position is thought to span the membrane. Alternatively, all polar groups of a xanthophyll molecule can remain with contact with the same polar headgroup region of the membrane. Such orientation has been proposed for xanthophylls in a conformation *cis* [39]. Interestingly, lutein characterised by the rotational freedom around the 6′–7′ single bond is believed to adopt 2 orthogonal orientations: one roughly vertical and the other horizontal to the lipid bilayer [40, 41].

5.2. The influence on the physical-chemical properties of membranes

The orientation of carotenoids within lipid bilayer enables them to interact with alkyl chains of lipids via van der Waals interactions, which results in modifications of structural and dynamical properties of membranes. Among experimental methods employed to investigate the effect of carotenoids on the properties of biomembranes are electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) as well as fluorescence and permeability measurements. An excellent overview of this research is presented in [42]. Considering xanthophylls, Viola, Zea and Lut were reported as modulators of membrane fluidity, increasing it in the ordered phase of the phospholipid bilayer and acting conversely in the liquid crystalline phase [43, 44]. Moreover, in the presence of xanthophylls, an increase of the penetration barrier to molecular oxygen into the hydrophobic region of membrane was observed [45]. In general, polar carotenoids were capable of influencing the thermotropic phase behaviour of phospholipid membranes. As a result, main phase transition ($P_{\beta'} \rightarrow L_{\alpha}$) was shifted to lower temperature in a concentration dependent manner accompanying by a decreased cooperativity and the molar heat capacity of the $P_{\beta'} \rightarrow L_{\alpha}$ transition [46]. Lutein and zeaxanthin affected alkyl chains of lipid bilayers by restriction of molecular motions of both CH_2 and the terminal CH_3 groups [47, 48]. Furthermore, xanthophylls, especially Lut, were responsible for an increase in the thickness of lipid membranes composed of lecithins with myristoyl and palmitoyl moieties [41, 49].

Recently, experiments in atomic force microscope (AFM) showed that the presence of Viola or Zea embedded in the liposomes at a concentration up to 1 mol% does not significantly affect the morphology of the vesicles. However, adhesive forces were 10 times higher for dipalmitoylphosphatidylcholine (DPPC) membranes enriched in Zea, than those observed for an untreated system [50].

Finally, it is worth mentioning that unlike the xanthophylls of the VAZ cycle, diatom-specific carotenoids including Diadino and Diato have hitherto been much less studied in terms of the effect they can exert on the structural and dynamic properties of biomembranes. Interestingly, based on measurements carried out most recently in our laboratory by use of DSC technique and fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in phospholipid liposomes (manuscript in preparation), it seems that Diadino and Diato affect the lipid bilayer much stronger than Viola and Zea.

6. Conclusion

Along with the characterisation of physiological roles, various carotenoids play being involved in the xanthophyll cycle, *in vitro* assays of both epoxidation and de-epoxidation reactions enable to study the molecular mechanism of the cycle. Moreover, it is interesting to address the question of effect carotenoids have on biological membranes, as it helps to put some light on their antioxidant activity. This in turn seems to play a key role in terms of nutrition and human health. In view of this, the issue of isolation and purification of diatom-specific xanthophylls, yet less described in literature seems to be of great importance. Recently, developed method of all-*trans* diadinoxanthin (Diadino) and all-*trans* diatoxanthin (Diato) purification from *P. tricornutum* comprises four-step procedure and is dedicated to both analytical and preparative scale.

Particular attention is paid to natural carotenoids that apart from the photoprotective function in photosynthetic organism have been recognised as exhibiting beneficial activities for humans and animals and used for commercial and industrial applications. Diatoms seem to be a promising source of unique bioactive compounds, with diadinoxanthin and diatoxanthin as representatives of the xanthophyll cycle pigments. Given that until now neither Diadino nor Diato were commercially available in amounts greater than those used as standards in high-performance liquid chromatography (HPLC), the efficiency of the described purification procedure reaching up to 73% makes the method economically feasible.

Acknowledgements

The Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education. HPLC analysis was carried out with the equipment purchased, thanks to the financial support of the European Regional Development Fund in the framework of the Polish Innovation Economy Operational Program POIG.02.01.00-12-167/08. A method of diadinoxanthin and diatoxanthin purification has been reported as patent applications P.412178 and P.412177 (Kuczynska & Jemiola-Rzeminska, 2015) and PCT/PL2016/000047 (Kuczynska & Jemiola-Rzeminska, 2016).

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