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Extraction, Purification and partial Characterization of a Carotenoid Binding Protein (CBP) from the Epidermis of the Monarch Butterfly Larvae (*Danaus plexippus*)

Nan Fang

Florida International University, nfang001@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

EXTRACTION, PURIFICATION AND PARTIAL CHARACTERIZATION OF A
CAROTENOID BINDING PROTEIN (CBP) FROM THE EPIDERMIS OF THE
MONARCH BUTTERFLY LARVAE (*DANAUS PLEXIPPUS*)

A dissertation submitted in partial fulfillment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Nan Fang

2016

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Nan Fang and entitled Extraction, Purification and partial Characterization of a Carotenoid Binding Protein (CBP) from the Epidermis of the Monarch Butterfly Larvae (*Danaus plexippus*), is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Watson Lees

Bruce McCord

Piero Gardinali

Manuel Barbieri

John T. Landrum, Major Professor

Date of Defense: June 17, 2016

The dissertation of Nan Fang is approved.

Dean Michael R. Heithaus
College of Arts, Sciences and Education

Andrés G. Gil
Vice President for Research and Economic Development
And Dean of the University Graduate School

Florida International University, 2016

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DEDICATION

I would like to dedicate this dissertation to my parents: Yulan Wang and Xiaochun Fang for their enduring love and unconditional support throughout this journey. Also to my loved ones, for their love, understanding and encouragement which helped me to complete this work.

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ABSTRACT OF THE DISSERTATION
EXTRACTION, PURIFICATION AND PARTIAL CHARACTERIZATION OF A
CAROTENOID BINDING PROTEIN (CBP) FROM THE EPIDERMIS OF THE
MONARCH BUTTERFLY LARVAE (*DANAUS PLEXIPPUS*)

by

Nan Fang

Florida International University, 2016

Miami, Florida

Professor John T. Landrum, Major Professor

This dissertation describes the purification and partial characterization of CBP from the epidermis of the monarch butterfly larvae (*Danaus plexippus*). A yellow protein-carotenoid complex was extracted from the yellow pigmented epidermal tissue from monarch butterfly larvae by homogenization. Additional steps in the purification process included differential precipitation with ammonium sulfate, cation and anion chromatography, and lastly size exclusion chromatography. Polyacrylamide gel electrophoresis demonstrates that a single protein was isolated (M-LBP) having a ~60 kDa molecular weight, the value has subsequently been confirmed by HR-tandem MS. Lutein is the sole carotenoid bound by M-LBP with a stoichiometry of the binding of 2: 1. Immunohistochemistry results show that M-LBP has no cross-reactivity to antibodies for silk worm CBP (*Bombix mori*) but does have cross-reactivity with antibodies for horn worm epidermal CBP (*Agrius convolvuli*). Binding affinities were determined using surface plasmon resonance for the carotenoids lutein (K_D

= 18.6 ± 0.7), R,R-zeaxanthin ($K_D = 990 \pm 60$), R,S-zeaxanthin ($K_D = 60 \pm 2$). Tryptophan fluorescence lifetimes were determined for the apoprotein and compared to those of the native M-LBP. Tryptophan fluorescence lifetimes were found to be 3.9 ns and 3.0 ns, respectively for these two forms of the protein, indicating that upon dissociation of the carotenoid from the protein the tryptophan fluorophore adopts a position where it has less interaction with the polar surface environment.

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LIST OF ABBREVIATION AND ACRONYMS

FULL NAME	ABBREVIATION
Age related macular degeneration	AMD
Arbuscular mycorrhizal fungi	AM fungi
Ammonium persulfate	APS
Beta Carotene Dioxygenase 1	BCDO1
Beta Carotene Dioxygenase 2	BCDO2
Beta-carotene cleavage oxygenase	BCO
Bovine serum albumin	BSA
C-terminal calmodulin-homology domain	CaM
Carotenoid binding protein	CBP
Carotenoid cleavage dioxygenases	CCD
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS
Dimethyl sulfoxide	DMSO
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride	EDC
Ethylenediaminetetraacetic acid	EDTA
Fluorescence resonance energy transfer	FRET
Glutathione S-transferase	GST

Glutathione S-transferase pi 1	GSTP1
High resolution Mass	HR-MS
Low density lipoprotein	LDL
Light harvesting complex I	LH1
Light harvesting complex II	LH2
Monarch butterfly	MBL
β -Mercaptoethanol	β -ME
2-(N-morpholino)ethanesulfonic acid	MES
Monarch butterfly larvae lutein binding protein	M-LBP
N-hydroxysuccinimide	NHS
Nominal Molecular Weight Limit	NMWL
Orange carotenoid protein	OCP
Phosphate-buffered saline	PBS
Red carotenoid protein	RCP
Reactive oxygen species	ROS
Response unit	RU
Ground state	S ₀
Excited singlet state	S ₁

Surface plasmon resonance	SPR
Scavenger receptor class B type I	SRB-1
Steroid acute regulatory protein	StAR protein
Silkworm carotenoid binding protein	SW-CBP
Tetramethylethylenediamine	TEMED
(hydroxymethyl)aminomethane	Tris
Polysorbate 20	Tween-20
Very low density lipoprotein	VLDL

Chapter 1 Overview

1.0 Introduction

Carotenoids are naturally occurring pigments synthesized in plants and microorganisms (algae, fungi, bacteria) (Britton, 1993). They are richly colored and vary from red and orange to yellow but can, through their interactions with other carotenoids or covalent binding to amines, even produce a blue color in the tissues in which they are found. (Bjerkeng, 2008a). The localization of carotenoids in tissues, cells, and/or organelles and the manner in which it is localized depends upon the functional role that it fulfills (Yamamoto and Bassi, 1996). Some carotenoids are found associated with membranes and membrane rich organelles, for example, the yellow colored carotenoids, lutein and zeaxanthin, found in sunflower petals and the lycopene present in tomatoes (Lee et al., 2000; Ronen et al., 1999). In others they may be bound to proteins (Vishnevetsky et al., 1999). Carotenoids are synthesized in the leaves of photosynthetic plants and function as essential components of the light harvesting system (Hencken, 1992). Although almost universally essential in higher animals, e.g., as pro-vitamin A, the absence of the isoprene pathway makes synthesis of carotenoids impossible for animals. They obtain these pigments from their diet. The absorption of carotenoids by higher animals occurs in the intestine (Harrison, 2012). A variety of different functions are recognized for carotenoids including, coloration, modification of membrane stability, and antioxidant activity (Britton, 2008). When metabolized they form a variety of

cleavage products that may have additional important functions similar to those of vitamin A (retinal) (Britton, 2008). Evidence has been found that carotenoid metabolites function as transcription co-factors in bacteria and it is reasonable to anticipate this function may also be a significant one in higher animals (Britton, 1995). The importance of carotenoids found in humans includes their function in the human retina where they play an important role protecting the human photoreceptors against the photooxidative damage, a role first proposed by Kirschfeld (Kirschfeld, 1982).

1.1 Structure and Classification

There are approximately 700 different carotenoid derived natural pigments that have been isolated from natural sources, and more than 500 unique carotenoid structures have been identified. The most abundant structural motif in carotenoids consists of a skeleton of 40 carbon atoms (tetraterpenes) (2004; Mercadante et al., 2004). The biosynthetic pathway incorporates four 5-carbon isoprenoid units concatenated 'head-to-tail' to form a C₂₀ unit that is subsequently linked together in a 'head-to-head' manner to produce the characteristic centro-symmetric carotenoid skeleton. The principal differences among carotenoids arise from the structure and substitution pattern of the end-groups which may be either cyclic or acyclic containing or lacking additional functional groups, most commonly oxygen containing oxo- or hydroxyl groups. The linear sequence of conjugated double bonds within the central polyene chain produces what is often perceived to be a rigid backbone of the molecule. In

reality, the polyene chain is relatively flexible and it exists as a large array of conformational isomers arising from the rotation around the single bonds of the polyene (Strain, 1948). This conjugated system is also responsible for the strong absorption of visible light by carotenoids. In a typical C40 carotenoid there are most often 9 conjugated bonds resulting in the ability of the carotenoid to absorb light in the visible spectrum. The quantum mechanically allowed $\pi \rightarrow \pi^*$ transition makes carotenoids intense absorbers. The longer the conjugated polyene system the higher the wavelength of the maximum absorption will be for the molecule. In nature, the extent of the conjugation of the central polyene can vary depending on the structure of the end-group with the result that there are a large number of differently colored carotenoid pigments found in nature (Vershinin, 1999a). In most carotenoids the polyene chain has an all-*trans* isomeric structure which is more stable than those isomers in which one or more of the double bonds is found in a '*cis*' geometry. Strictly speaking, the geometry of the individual double bonds in the polyene chain should be described by the E/Z convention however, the use of '*cis*' and '*trans*' is widely accepted in the literature and focuses strictly on the geometrical relationship of the polyene carbon atoms of the double bonds equating the '*cis*' with Z and '*trans*' with E. However, the presence of Z-carotenoid isomers characterized by the presence of '*cis*' double bonds is known to occur in a sizeable number of naturally occurring carotenoids (Britton et al., 2009). Moreover, isomerization of the all-*trans* isomer occurs in carotenoids during the steps in food processing, particularly pasteurization (Cazzonelli, 2011). Carotenoids are broadly classified into two

types (Fig. 1). The hydrocarbon carotenoids are known as carotenes and are represented by examples such as α -carotene, β -carotene and lycopene. The oxygenated derivatives are known as xanthophylls and the most well-known among these lutein, zeaxanthin, astaxanthin, and cryptoxanthin (Britton, 1995). Also, by their derivatives, carotenoids can be categorized into provitamin A carotenoids and non-provitamin A carotenoids (Rao and Rao, 2007).

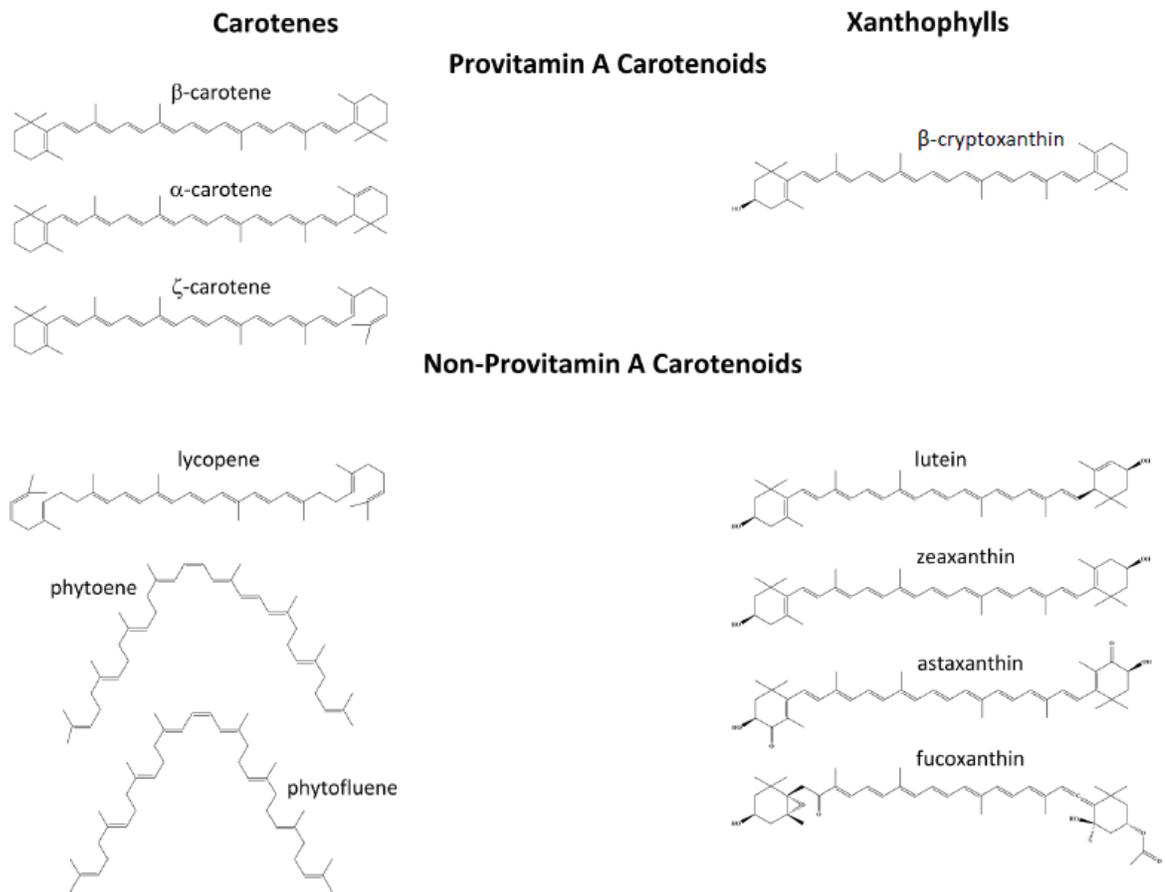


Fig. 1. Chemical structures of carotenoids.

1.2 Functions

As has already been noted, carotenoids are most widely recognized for the role they serve as natural colorants, and in many instances, this is crucial to the survival of a species. Extensive investigations of carotenoid functions have focused their roles in photosynthesis, as anti-oxidants and influence on membrane stabilization, in addition to their ability to act as pigments.

1.2.1 Coloration and Signaling

Coloration not only provides the distinguishing color characteristic of different organisms, it also provides some essential ecological signals. Differences in color have a crucial influence on behavior and actions within and between species (Cazzonelli, 2011).

1.2.1.1 Coloration in plants

Carotenoids are universally present in plants and photosynthetic organisms which produce a wide variety of structural variants. They are responsible for the yellow, orange and red color in many flowers and fruits. They may also contribute to coloration of leaves and stems. Aside from carotenoids plants produce anthocyanins that responsible for red-purple pigmentation, and in some plants tetrapyrrole derived structures produce intense coloration (Britton, 1983). Carotenoids have been found to exist in all of the different anatomical structures of flowers and seeds: sepals, pollen, anthers, and petals, although not necessarily in all of these structures in every flower type (Hirschberg, 2001).

Familiar examples are lutein in marigold flower petals (*Tagetes erecta*) (Rivas, 1991) and zeaxanthin and lutein in the white horse chestnut (*Aesculus hippocastanum*) flower pollen (Schulte et al., 2009). Crocin and crocetin, are the major carotenoids found in flower of *Crocus sativus*, they are frequently used in food coloration as the spice saffron (Abdullaev, 2002). This visible color attracts the attention and attracts the insects essential for pollination. In fruits, carotenoids also provide an essential biological signal to attract animals for seed dispersal. Thus, carotenoids serve vital role in plant reproduction (Cazzonelli, 2011). Carotenoids are found in different parts of fruit, such as skin, flesh, and seeds , For example, multiple carotenoids, violaxanthin, β -carotene, zeaxanthin, lutein and neoxanthin have be found in flesh of mango (*mangifera indica*) (Chen et al., 2004).

By contrast, in green plants, the distinctive yellow carotenoid color is obscured by the more dominant absorption of the green chlorophyll. Spinach is an abundant source of β -carotene, but it appears in green since chlorophyll is the predominant pigment (Lessin et al., 1997). The authentic carotenoid colors are only seen in exceptional cases. For example, high concentrations of keto-carotenoids provide the red color in the young leaves of the dawn redwood, *Metasequoia glyptostroboides* (Czeczuga, 1987). Carotenoids are also prominent in autumn leaves of deciduous trees, with the loss of chlorophyll; masked carotenoids appear are responsible for the natural autumn yellow to orange or colors. One of the examples is the yellow leaf of sugar maple, *Acer saccharum*

(Archetti et al., 2009). Leaf color will vary between species and depends on the identity and quantity of the carotenoids present.

1.2.1.2 Coloration in animals

Coloration ensures animals are recognized or camouflaged depending on need. Color in the animal kingdom serves a host of functions but particularly important is signaling. Coloration has a role in the mate selection in a wide variety of species of birds, fish, and reptiles. In many insects coloration also serves a protective function against predation, referred to as aposematism (Heath et al., 2013). Certain types of Lepidoptera (butterflies), have toxic compounds in the body tissue, the presence of carotenoids are positively related to the presence of these toxic compounds suggesting that carotenoids provide warning coloration to repel predators (Rothschild et al., 1986). Carotenoids are present in the feathers of many bird species. In the house finch, (*Haemorrhous mexicanus*), coloration of feathers ranges from pale yellow and orange to red. A dietary carotenoid source is essential to maintain the color of feathers. The diversity of color among populations is due to the amounts and types of the carotenoids deposits in the feathers (Inouye et al., 2001). Considering research into the identity of carotenoids in feathers has shown that birds are capable of metabolizing carotenoids to produce those responsible for characteristic coloration even though they cannot synthesize the carotenoid carbon skeleton (Stradi et al., 1995). It is hypothesized that a specific protein is responsible for transport of each carotenoid into the developing feathers or integuments in the

case of fishes where it is ultimately deposited. The presence of these carotenoids in numerous organisms enables them to distinguish sexual maturity and social status (Pike et al., 2010). For instance, the color and pattern of fishes can change seasonally. In the breeding season, the epidermal color of male guppies (*Poecilia reticulata*) changes to red or orange as a result of carotenoid accumulation. The females are attracted by the carotenoid-colored males. Thus, the males with well-developed carotenoid coloration are more successful in mating (Hurtado-Gonzales et al., 2014). Carotenoids are found in many predator species, the predators match the body color pattern with their surrounding environment to increase their chances of being successful during hunting (Bjerkeng, 2008b). Astaxanthin is found in the dark blue carapace of lobster (*Homarus gammarus*) (Zagalsky, 2003) and isolated from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*) (Shahidi and Synowiecki, 1991).

Carotenoids are present in the reproductive organs of many organisms (Bjerkeng, 2008b). For instance, astaxanthin and canthaxanthin are found in the fish eggs and fry. Lutein is isolated from the yolk of most eggs (for example barn swallows (*Hirundo rustica*), (Saino et al., 2003). Astaxanthin, all-*trans*-retinol, lutein and canthaxanthin are detected in eggs from adult Chinook salmon (*Oncorhynchus tshawytscha*) (Li et al., 2005).

1.2.1.3 Coloration in microorganisms

Although most of photosynthetic microorganisms are colored green as a result of the presence of chlorophyll, some other non-photosynthetic microorganisms are colored by carotenoids (Britton, 2008). Carotenoid, deoxyoscollol 2-rhamnoside is detected in the orange colored aerobic bacterium *Gemmatimonas aurantiaca* (*Gemmatimonadetes*) (Takaichi et al., 2010). Astaxanthin is responsible for the brown color of mold (*Dictyostelium discoideum*) (Staples and Gregg, 1967). There are 17 triterpenoid carotenoids found in the pathogenic bacterium *Staphylococcus aureus*, the main pigment is staphyloxanthin, the presence of the carotenoids are associated with its virulence (Marshall and Wilmoth, 1981).

1.2.2 Interaction of carotenoids with light

Directly or indirectly sunlight is the main energy source for all life on earth. However, light must be harvested and energy rich products are synthesized and enter geobiochemical circulation (Britton, 2008). Sunlight drives the photosynthetic process in plants and photosynthetic microorganisms to generate sugars that are ultimately critical for other organisms such as animals and humans. On the other hand, excess of light causes problems and is extremely dangerous to cells. The over-exposure to sunlight damages or destroys cells and tissues (Britton, 2008). Thus, protection against excess light is essential to the survival of photosynthetic organisms. Furthermore, light serves as a signal for communication between living organisms (Weissleder and Ntziachristos, 2003). It

requires sensitive photoreceptors to detect and transfer these signals. Surprisingly, carotenoids are involved in all of these varied processes (Bannister, 1985).

1.2.2.1 Light harvesting

Carotenoids play a number of important roles in photosynthesis. Carotenoids can serve as accessory light harvesting pigments. Carotenoids have a strong absorption at around 450 nm in the visible spectrum, a region that is not covered by chlorophyll. Carotenoids are structurally located close to chlorophyll in the light harvesting protein complex. The light energy absorbed by a carotenoid produces an excited singlet state and can transfer energy to chlorophyll by singlet-singlet energy transfer. Thus light absorbed by carotenoids complements that absorbed by the chlorophyll and increases the overall efficiency of the photosynthesis process (Siefermann-Harms, 1987).

1.2.2.2 Photoprotection

Not only do carotenoids serve to harvest the light, they can protect organisms against the damaging effects of light. Carotenoids provide a regulatory mechanism to avoid lethal damage due to the excess light exposure during the photosynthetic process. Carotenoids quench the excess energy through a triplet-triplet energy-quenching mechanism. When chlorophylls absorb excess light at a rate that exceeds the ability of the photosystem to functionally transfer it in a productive redox process the chlorophyll excited state can transfer energy to oxygen producing reactive singlet oxygen (Frank and Cogdell, 1996). Energy

transfer from triplet excited-state chlorophylls to ground state singlet carotenoids generates the carotenoid triplet and quenches the chlorophyll (Frank and Cogdell, 1996). The triplet carotenoid relaxes without activating oxygen safely shunting the excess energy into waste heat. The zeaxanthin cycle in plants and algae depends on this process (Gilmore et al., 1994). In the zeaxanthin cycle a reversible conversion of zeaxanthin into violaxanthin functions as a regulatory mechanism. Zeaxanthin is epoxidized to form violaxanthin at low light levels and de-epoxidized at high levels of ambient light. Zeaxanthin efficiently quenches the chlorophyll excited state preventing the transfer of energy from chlorophyll to oxygen and generation of reactive singlet oxygen (Gilmore et al., 1994). By contrast, violoxanthin is incapable of this process.

1.2.2.2 Carotenoids and photoreceptors

Light is a signal which stimulates tremendous number of physiological actions in many living organisms. Light signals at different wavelength are collected by different photoreceptor types and carotenoids are one of these photoreceptors. Evidence shows that β -carotene serves as an internal screening pigment in corn seedlings in phototropism. The carotenoid enhances the light sensitivity gradually and affects the ability of growing tip of the seedling to navigate toward the source of light (Vierstra and Poff, 1981). Another example is the carotenoid involved in phototactic response in higher plants. Zeaxanthin serves as blue light photoreceptor in guard cell in leaves of cocklebur (*Xanthium strumarium*) to regulate the interaction between light and CO₂ concentration.

Zeaxanthin stimulated stomatal opening when the light has a high blue light percentage which resulting the increase of CO₂ concentration in the guard cell in leaves (Messinger et al., 2006).

1.2.2.3 Antioxidant Activity

Reactive oxygen species (ROS) and free radicals are now recognized to play significant roles in a number of pathophysiologic processes that can cause damage and injury to living organisms (Palozza, 1998). There is strong evidence supporting the hypothesis that carotenoids in the light harvesting protein are functional antioxidants that protect the light harvesting system itself. They protect the chloroplast and photosynthetic apparatus from the action of photogenerated singlet oxygen and other reactive species capable, such as free radical and peroxy compounds, from inducing chemical degradation of the photosynthetic apparatus. (Palozza, 1998) Carotenoids can quench reactive oxygen species to reduce the risk of oxidation of the local environment in the chloroplast (Krinsky, 1989). Carotenoids also prevent damage from free radicals. Free radicals generated by many redox processes in biological systems can interact with the oxygen and produce peroxy radicals which react with unsaturated double bonds in fatty acids (Stahl and Sies, 2003). In their role as antioxidants, carotenoids can react with peroxy radicals in three ways, electron transfer which forms a carotenoid cation and reduces the radical, hydrogen subtraction which forms carotenoid radical and an unreactive hydroxyl group, and radical addition which forms a complex carotenoid peroxy radical. All of three carotenoid products are

very stable (Young and Lowe, 2001). Thus, the carotenoids help to scavenge the peroxy radicals and prevent cellular damage (Young and Lowe, 2001).

1.2.3 Carotenoids influence membrane structures

In living organisms, lipophilic bilayer membranes form important barriers to maintain the integrity of the cell. They isolate and compartmentalize cellular functions. In the bilayer membrane, the hydrophobic acyl chains face inward and the hydrophilic head groups of the phospholipids face the surrounding aqueous environments of the membrane exterior (Britton, 2008). Many molecular species are embedded within membranes. These include large proteins as well as small lipophilic compounds. Their lipophilic nature allows carotenoids to be incorporated within membranes. To minimize the energy of these membrane systems different carotenoids are incorporated within the bilayer in structurally unique geometries (Gruszecki and Strzałka, 2005). In particular, the polar xanthophylls, lutein and zeaxanthin, have preference to span the membrane placing the two hydrophilic hydroxyl bearing end-groups in the polar regions on opposing sides of the membrane (Gruszecki and Strzałka, 2005). The orientation of less polar carotenoids, such as β -carotene and lycopene, are dependent on the van der Waals interactions with the hydrophobic core of bilayer membrane. Thus, β -carotene is buried in the lipophilic core where it adopts a random orientation (Fig. 2) (Gruszecki and Strzałka, 2005). Carotenoids appear to serve multiple biological functions in association with these specific localizations in the membrane. These include maintenance membrane structural integrity and

regulation lipid motion (Gruszecki and Strzałka, 2005). The polar carotenoids, such as lutein and zeaxanthin, increase the stability of the membrane thereby limiting lipid movement. Less polar carotenoid, β -carotene increases the flexibility of lipids in the hydrophilic head area (Wisniewska et al., 2006). In addition, carotenoids such as β -carotene and zeaxanthin appear to enhance the ability of small molecules to insert into the polar region of membrane (Gruszecki and Strzałka, 2005). Moreover, the carotenoid when present in the membrane are able to intercept lipophilic reactive oxygen species and free radicals mitigating the potential for degradation of the double bonds present in the phospholipids present within the membrane and responsible for maintaining the optimal balance in membrane fluidity (Gruszecki and Strzałka, 2005). It has been suggested that the access of polar end groups of the xanthophylls to the aqueous surround of the membrane enables them to assist in maintaining a redox balance between lipophilic membrane antioxidants such vitamin E and polar reducing agents such as glutathione and ascorbate (Gruszecki, 2009).

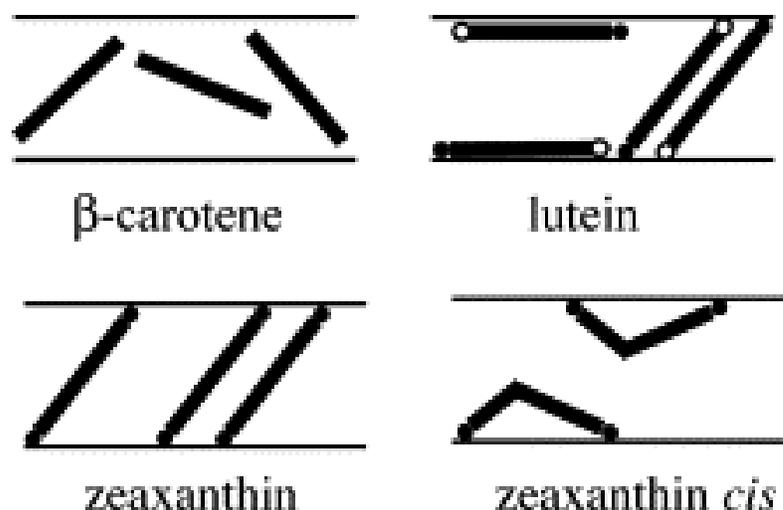


Fig. 2. The localization of different carotenoids in hydrophobic environment of lipid membranes (Gruszecki and Strzałka, 2005).

1.2.4 Carotenoids derivatives

Carotenoids are metabolized to provide abundant essential active derivatives. Pro-vitamin A carotenoids are the precursor molecules of vitamin A. Vitamin A refers to the group of nutritional compounds including retinol, retinal and retinoic acid (Bauernfeind, 1972). Retinal formed by the oxidative cleavage of the pro-vitamin A carotenoids, such as α -carotene and β -carotene, is an essential component of rhodopsin all animals (Arathi et al., 2015). Retinoic acid serves as a hormone that regulates epidermal growth in mammals and also as a transcription regulator for differentiation during embryonic development (Kam et al., 2012). Other carotenoid derivatives serve various functions in plants and microorganisms (Vershinin, 1999b). Abscisic acid is a growth development hormone in plants and is formed from zeaxanthin by a cleavage mechanism (Vershinin, 1999a). Strigolactones are another group of essential carotenoid

derivatives, they are the branching factors for some microscopic symbionts such as arbuscular mycorrhizal fungi (AM fungi) (Akiyama and Hayashi, 2006). Strigolactones are also responsible for regulating the plant architecture. Strigolactones can inhibit the shoot branching of the plant by preventing the outgrowth of the leaf axillary buds (Gomez-Roldan et al., 2008).

1.3 Carotenoids in humans

Although hundreds of carotenoids have been found in nature, there are only about 60 carotenoids that are present in the average human diet. About half of them have been found in human blood and tissue (Gerster, 1997). Convincing evidence shows these micronutrient components are inversely correlated to chronic human diseases. The intake, absorption, distribution, and health benefits of carotenoids in humans have been and continue to be comprehensively investigated.

1.3.1 Intake, Absorption of carotenoids in humans

As exogenous pigments, humans must obtain carotenoids from food sources. The majority of the dietary carotenoids are obtained from green, yellow or orange fruits and vegetables such as corn, pumpkins, tangerines, red bell peppers and spinach, broccoli, kale, carrots (Cazzonelli, 2011). Other common food sources of carotenoids include eggs and salmon. Carotenoids are also found in some spices, saffron and paprika, which have been used since antiquity (Bauernfeind, 1972). The degradation of carotenoids starts during food processing and is generally initiated by the heat of cooking or pasteurization. The

release of the carotenoids trapped in the cellular matrix is accelerated by the mechanical mastication and enzymatic digestion with saliva. After carotenoid rich foods have been consumed, the lipophilic carotenoids that can be absorbed as components of micelles by the cells that line the microvilli of the digestive tract. Carotenoids are solubilized in micelles that are formed by fats and bile salts. Absorption is a non-specific process and lacks a mechanism to select effectively for different carotenoids on any basis other than their intrinsic solubility in micelles (Borel et al., 1996). Carotenoid containing micelles are absorbed intact by intestinal epithelium cells. Within the cells of the digestive tract micelles are broken apart and the components are reassembled into chylomicrons that are excreted from the cell into the hepatic portal circulation. This step involves exclusion of some carotenoids that are selectively transported back across the cell membrane into the digestive tract (Yonekura and Nagao, 2007). This includes most epoxy carotenoids. The mechanism of this selective exclusion is not clearly understood. Arriving in the liver, chylomicrons are again repackaged and carotenoids are ultimately released into circulation in the lipoproteins, HDL, LDL, and VLDL (Yonekura and Nagao, 2007).

1.3.2 Distribution of carotenoids in human tissue

In humans, carotenoids enter target tissues along with the other components bound to the lipoproteins, a process that is typically regulated by such cell receptors as scavenger receptor class B type I (SRB-1). It is believed that similar mechanisms operate in most higher organisms (Bohn et al., 2015).

The majority of hydrocarbon carotenes such as α -carotene, β -carotene and lycopene are transported by low density lipoprotein (LDL) and very low density lipoprotein (VLDL) , while the oxygenated carotenoids lutein, zeaxanthin and cryptoxanthin are predominantly delivered by high density lipoprotein and to a lesser extent by low density lipoprotein (Yeum and Russell, 2002). The detectable amounts of these carotenoids found in different organs varies widely (Parker, 1989). β -carotene, lycopene, lutein and zeaxanthin have been found in human liver. In another study β -carotene was reported to be present in adrenals, liver, testes and adipose tissue. Canthaxanthin, β -carotene, and lycopene accumulate in human skin with carotenoid rich dietary (Furr and Clark, 1997). In many cases carotenoids are not highly concentrated nor is there evidence of a selective accumulation, the relative tissue concentrations of carotenoids in human liver, kidney, and lung are very similar to those in serum (Kaplan et al., 1990). Surprisingly, specific carotenoids are accumulated in high concentrations within the human retina (Bone et al., 1985). The human macular pigment consists of lutein and zeaxanthin to the exclusion of other carotenoids. The highest concentration of carotenoids found anywhere in human body is the macular pigment of the retina which is present at a concentration that is 100 times higher than serum (Landrum and Bone, 2001).

1.3.3 Carotenoids and human health

Carotenoids are reported to reduce the risk of several diseases including cancer and assist in the maintenance of good health. Although the actions by

which carotenoids prevent cancer remains unclear but evidence shows that a diet rich in carotenoids is inversely related with certain types of cancer. It is highly likely that this activity of the carotenoids is related to their antioxidant function of carotenoids (Ziegler, 1989). By contrast investigation of β -carotene uptake and risk of lung cancer shows that β -carotene may increase the risk of lung cancer in smokers (Michaud et al., 2000). In another epidemiological investigation the consumption of total carotenoids was found to be inversely proportional to the risk of lung cancer. In a 14-year study of a large cohort of Finnish male smokers the risk of lung cancer was shown to be reduced by the uptake of total carotenoids, such as lycopene, β -cryptoxanthin, lutein, and zeaxanthin (Holick et al., 2002). In a 6 year study of 58,000 Dutch men dietary intakes of β -cryptoxanthin, lutein, and zeaxanthin were inversely associated with lung cancer risk (Voorrips et al., 2000). Another study indicated that consumption of lutein/zeaxanthin, β -carotene and vitamin A are inversely associated with the risk of premenopausal breast cancer. (Voorrips et al., 2000) Carotenoids can also play a critical role in the prevention of cardiovascular diseases. It is known that the consumption of vegetables and fruits is associated with a reduction in the risk of heart disease (Wang et al., 2016). Given the complexity and diversity of nutrition and human diets, it is very hard to tell which single nutrient in vegetables and fruits contribute most to this cardioprotective activity. The carotenoids are especially likely candidates for this bioactivity (Kohlmeier and Hastings, 1995). One of the most critical signs of cardiovascular disease is the development of atherosclerosis which can be measured by the thickness of the inner layer

(intima thickness) of the carotid arteries. The measurement of thickness of carotid artery inner layer is considered an effective way to monitor the development of heart disease (Bots and Grobbee, 2002). Higher carotenoid concentrations in blood have been associated with a thinner intima layer of the carotid arteries (Rissanen et al., 2003). Thus, a high level of carotenoids in blood is inversely related with risk of cardiovascular disease because of their ability to reduce the rate of atherosclerosis which arises from oxidative inflammatory processes (Krinsky and Johnson, 2005b).

Carotenoids can prevent vitamin A deficiency (de Pee and West, 1996). As an important nutrient, vitamin A can be obtained by food sources directly or produced from pro-vitamin A carotenoids biosynthetically. Vitamin A is essential to the visual function, and responsible for maintaining healthy skin and soft tissues (Blomhoff et al., 1991). Vitamin A deficiency also interferes with normal fetal development (Krinsky and Johnson, 2005b).

Some animal studies have shown carotenoids may strengthen immune responses (Hughes, 1999). Evidence shows carotenoids can enhance cell-mediated immune responses in humans (Hughes et al., 1997). Cell-mediated responses can be triggered by antigen-presenting cells. Blood monocytes, the principle antigen-presenting cell in human blood are used as the indicator to see whether carotenoids could boost the immune response. A double-blind study was conducted in 25 healthy male non-smokers (Hughes et al., 1997). The volunteers were randomly assigned to take β -carotene supplement or placebo for short

period. After the treatment, a significant increase in the percentage of blood monocytes was found in the group taking the β -carotene supplement. This phenomenon supports the hypothesis that β -carotene and possibly other carotenoids could enhance the cell-mediated immune responses in short time periods (Hughes et al., 1997).

Carotenoids also comprise the macular pigment. Two carotenoids, lutein and zeaxanthin, are visible as a yellow spot in the center of the retina (Fig. 3) (Handelman, 2001). They absorb damaging blue light reducing the risk of photo-oxidative damage (Krinsky and Johnson, 2005a). The macular pigment additionally benefits visual function by reducing chromatic aberration and improving visual acuity. Within the human retina, the macular region has a high level aerobic activity and is abundantly illuminated producing an environment favorable for the production of singlet oxygen. Age related macular degeneration is the predominant cause of blindness in older adults. An irreversible process involving nerve loss, consequently lead to age related macular degeneration that cannot be cured (Bone et al., 1997). Efforts aimed to prevent or postpone the progression are deemed the most likely means to reduce the prevalence of this disease. Carotenoids lower the risk of age-related macular degeneration (Bone et al., 1997; Landrum and Bone, 2001). Epidemiological studies show that a diet high in carotenoids, particularly lutein and zeaxanthin, is associated with lower risk of age related macular degeneration (AMD) (Age-Related Eye Disease Study 2 Research, 2013; Landrum and Bone, 2001). Cataract is another common eye disease among elderly. Excess UV light and oxidants lead to the protein

degeneration in the lens, the structural changes accompanying damage to these proteins causes lens opacities, cataracts. Some studies show a lutein and zeaxanthin rich diet may prevent or slow the progress of cataracts (Krinsky et al., 2003).



Fig. 3. The accumulation of carotenoids, lutein and zeaxanthin on the central of macular (Snodderly et al., 1984).

1.4 Carotenoid binding proteins

We have seen that carotenoids play multiple functions in biological systems. Accumulation and localization of high levels of specific carotenoids in living organisms must occur via selective transport processes that are mediated by proteins capable of discriminating between the many carotenoids transported

within the circulation. In many instances carotenoid proteins with highly selective binding sites must be involved (Reboul and Borel, 2011). Due to their hydrophobic character, carotenoids are insoluble in the polar aqueous cytosol. Interaction of carotenoids with proteins allows these lipophilic pigments to be mobilized in the polar aqueous environment within the cell or interstitial media. Numerous carotenoid binding proteins have been found in biological systems of algae, bacteria, plant and mammal (Britton and Helliwell, 2008).

1.4.1 Carotenoid binding proteins in plants and microorganisms

1.4.1.1 Light harvesting complex (photosynthetic proteins)

Found in plants and photosynthetic bacteria, the light harvesting complex is a collection of protein subunits that incorporates the different types of photosynthetic pigments and spatially organizes them to facilitate their absorption of light and transfer of energy (Croce and van Amerongen, 2014). Carotenoids are among of the photosynthetic pigments present in the light harvesting complex. These carotenoids inadvertently provide pigmentation for the organism, but more importantly are directly involved in the steps associated with photochemical functions. These include light harvesting, dissipation of excess energy, photo-protection, and stabilization of the protein quaternary structure (Croce et al., 1999). Two types of light harvesting complexes have been isolated, complex I (LH1) and complex II (LH2). Both types are assembled in a similar way, they consist of similar polypeptides, α and β . Well-known as the core antenna complex, LH1 directly associates with the photosynthetic reaction center to form

a complex, bringing together the pigments involved in the photosynthesis, meanwhile LH2 serves as the peripheral antenna complex to expand the light harvesting capacity, the amount of LH2 is always influenced by the external environment, such as light intensity (Kuhlbrandt, 1995). For instance, the level of LH2 generated under low light condition is higher to compensate the low total light absorption (Scheuring and Sturgis, 2005). One of the extensively investigated examples of the light harvesting complex is the LH2 from purple non-sulfur bacteria, *Rhodospseudomonas acidophila*. The bacteriochlorophyll a (Bchl a) is the principal light and energy absorber, but the carotenoids, rhodopin glucoside and lycopene, serve as secondary antenna in the photosynthetic process increasing the efficiency of conversion of visible light energy in ATP (Georgakopoulou et al., 2004). The singlet-singlet energy transfer between carotenoids and chlorophyll helps to regulate the rate of light (Holt et al., 2005). Bchl a and carotenoids are non-covalently bound to the hydrophobic apoprotein in the light harvesting complex. The crystal structure of LH2 from *Rhodospseudomonas acidophila strain 10050* has been determined from single crystals that were grown from the isolated protein complex *in vitro* (Fig 4). The complex consists of two low molecular weight concentric cylinders of helical protein subunits (α , β) with Bchl a, carotenoid and detergent molecules, β -octylglucoside. The carotenoid to Bchl a ratio is 1:2. Each carotenoid is located in a spatially critical position within the complex. It is not attached to a single protein subunit but inserts through the depth of membrane bound protein and across the hydrophobic core of both α and β subunits (McDermott et al., 1995).

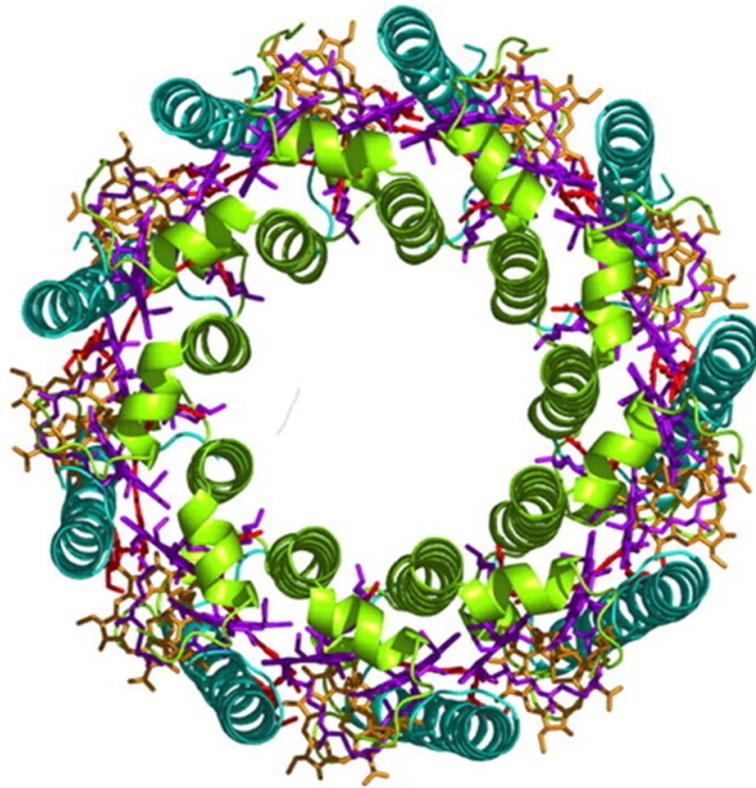
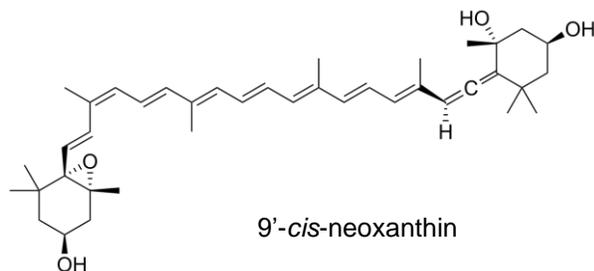


Fig. 4. The crystal structure of the light-harvesting antenna complex (LH2) from *Rhodospseudomonas acidophila* strain 10050 (McDermott et al., 1995).

The light harvesting complex present in higher plants also has carotenoid components that play a crucial role in photosynthesis. The major light harvesting complex found in higher plants is LH2. The structure of LH2 has been determined by electron microscopy (Liu et al., 2004). LH2 exists as a trimer and every monomer consists of three transmembrane α -helices: A, B and C; 8 chlorophyll a, 6 chlorophyll b, and 4 carotenoids. In each monomer, two α -helices are held together by ion pairing of polypeptide residues (Liu et al., 2004). Lutein is the major carotenoid and there are two luteins found per one monomer. The all-*trans* lutein molecules adopt the characteristic carotenoid S-shape in the

hydrophobic pocket of the monomer (Liu et al., 2004). The two luteins sit at the ends of helices and form an internal cross-brace at the supercoil of helices A and B (Fig. 5). The distance between the lutein end-groups and each helix is equal and the lutein molecules are symmetrically hydrogen-bonded to the polypeptide residue. Lutein 1 is attached to Gln 197, Ser 160 and Leu 164. Lutein 2 is bonded to Asp 47, Ala 49, Trp 97 and Ala 100. The interaction of two luteins at the monomer-monomer interface provides a sturdy structural tie between polypeptides and forms a strong link between two helices (Liu et al., 2004). Thus, not lutein only serves as the secondary antenna pigment and protects the plant from photo-induced oxidation, the incorporation of carotenoids is essential for protein stabilization (Kuhlbrandt et al., 1994). The third carotenoid, assigned as 9'-*cis*-neoxanthin, is situated near helix C. As the case with two luteins, it is also hydrogen-bonded to polypeptide residues through the hydroxyl functional groups present on the end-groups.



Amino acid side chains of the protein and chlorin rings from chlorophyll define a hydrophobic '*canyon*' that fits the curved polyene chain of 9'-*cis*-neoxanthin. This binding site has high specificity for 9'-*cis*-neoxanthin (Ruban, 2010). The fourth carotenoid found in LH2 is zeaxanthin/violaxanthin. Zeaxanthin bound is

favorably positioned to quench the excited state of the nearby chlorophyll enabling it to modulate the rate of electron hole pair formation in high light environment (Wentworth et al., 2000). Violaxanthin does not quench the chlorophyll excited state and the violaxanthin deepoxidase of the zeaxanthin cycle is upregulated under high ambient light conditions (Havaux et al., 2000).

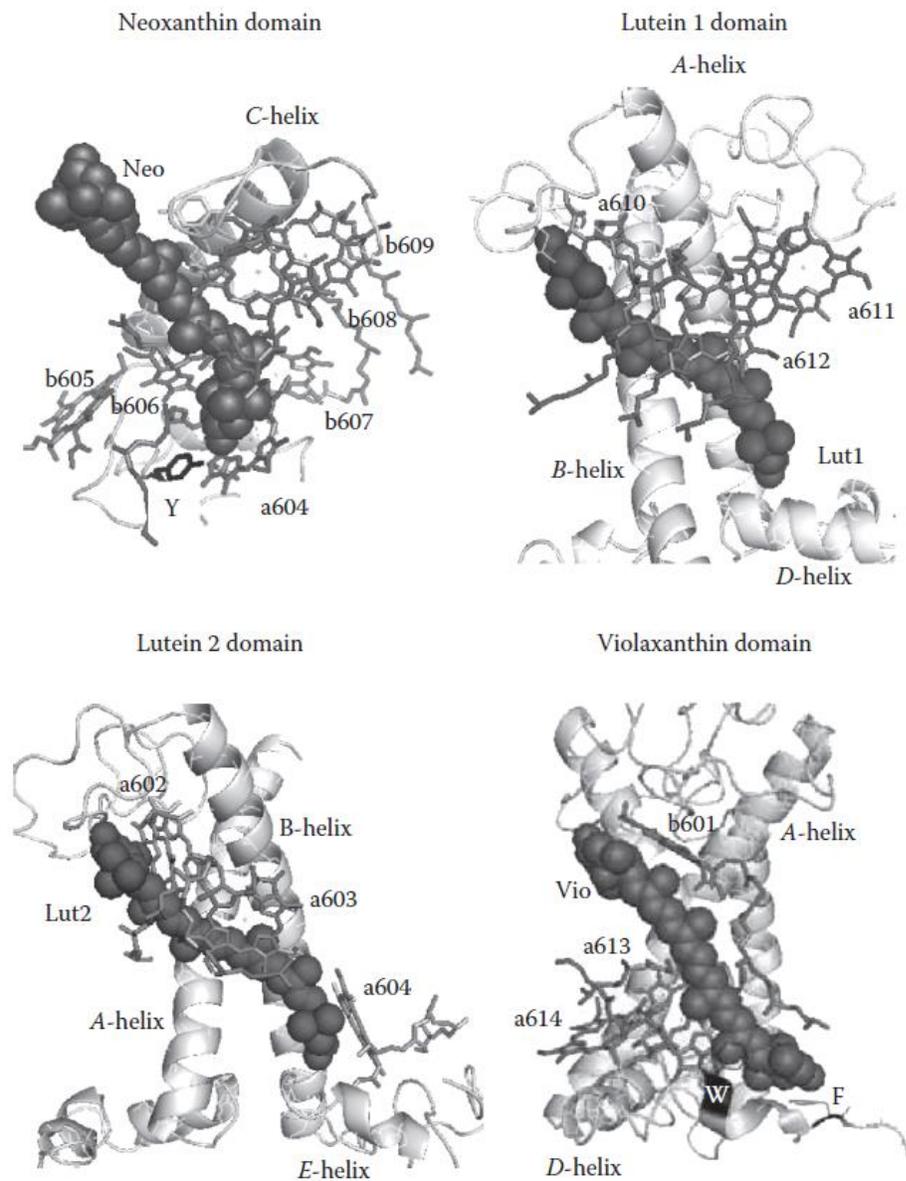


Fig. 5. The localization and arrangement of carotenoids on the domains of LH2 (Ruban, 2010).

1.4.1.2 Orange carotenoid protein (OCP)

In addition to the role in light harvesting, carotenoid binding proteins are involved in the photoprotection in photosynthetic organisms. A well-studied example is the orange carotenoid protein found in cyanobacteria, the presence of orange carotenoid protein helps the cyanobacteria to accommodate a wide range of photic environmental conditions. Orange carotenoid protein was characterized by Holt and Krogmann. It is a 35 kDa protein and belongs to lipocalin protein family (Pilbrow et al., 2012a). Lipocalins are a group of proteins that share some common characteristics. Although the lipocalins vary in size, the protein domains are typically in the range of about 18-20 kDa. Six or eight continuous antiparallel strands comprise each β -sheet in the tertiary structure of lipocalins. With the distinctive hydrophobic inner pocket binding site in the domain, lipocalins possess the potential to bind and transport small, lipophilic molecules. Tuning non-covalent binding site in these proteins controls the strength of the small molecule interaction with the protein and exerts influence on the properties of these ligands. For instance, a spectral shift of carotenoid absorption occurs when it binds to lipocalin (Britton and Helliwell, 2008). With the exception of the interaction with small lipids, lipocalins in higher organisms are involved in the immune modulation, stress factor response, and signal transduction. Not only this orange carotenoid protein, but many other carotenoid binding proteins belong to lipocalin family, these include crustacyanin from crustaceans and glutathione-S-transferase pi isoform 1 (GSTP1) from human retina (Grzyb et al., 2006). A keto carotenoid, 3'-hydroxyechinenone, is the carotenoid ligand present

in the orange carotenoid protein (Kay Holt and Krogmann, 1981). In 2003, the crystal structure study revealed the orange carotenoid protein is a homodimer with one monomer per one carotenoid (Fig. 6). The monomer consists of two major domains: an α/β domain and an all helical domain. 3'-hydroxyechinenone sits very deep in both domain and only 3.4% of this carotenoid is exposed to the aqueous environment. The keto end of 3'-hydroxyechinenone inserts into a hydrophobic protein pocket in α/β domain and hydroxyl end is buried within the helical domain. The oxygen on the keto end is H-bonded to the Trp 290 and Tyr 203 in the C-terminal domain. The binding of 3'-hydroxyechinenone to protein controls the orientation of the carotenoid and produces a stable protein-pigment environment (Kerfeld et al., 2003). Evidence shows that orange carotenoid protein is localized near the exterior of the cell (Kirilovsky and Kerfeld, 2012). The excess light strikes the surface of the cell and induces the formation of a active form of protein carotenoid complex. The active form interacts with the phycobilisome which is the light-harvesting antenna of cyanobacteria. A consequence of these events is that the phycobilisome fluorescence emission and energy transfer to the reaction center is reduced. Hence, the interaction of the orange carotenoid protein with the phycobilisome allows the cyanobacteria to acclimate in different photo environmental conditions (Boulay et al., 2008).

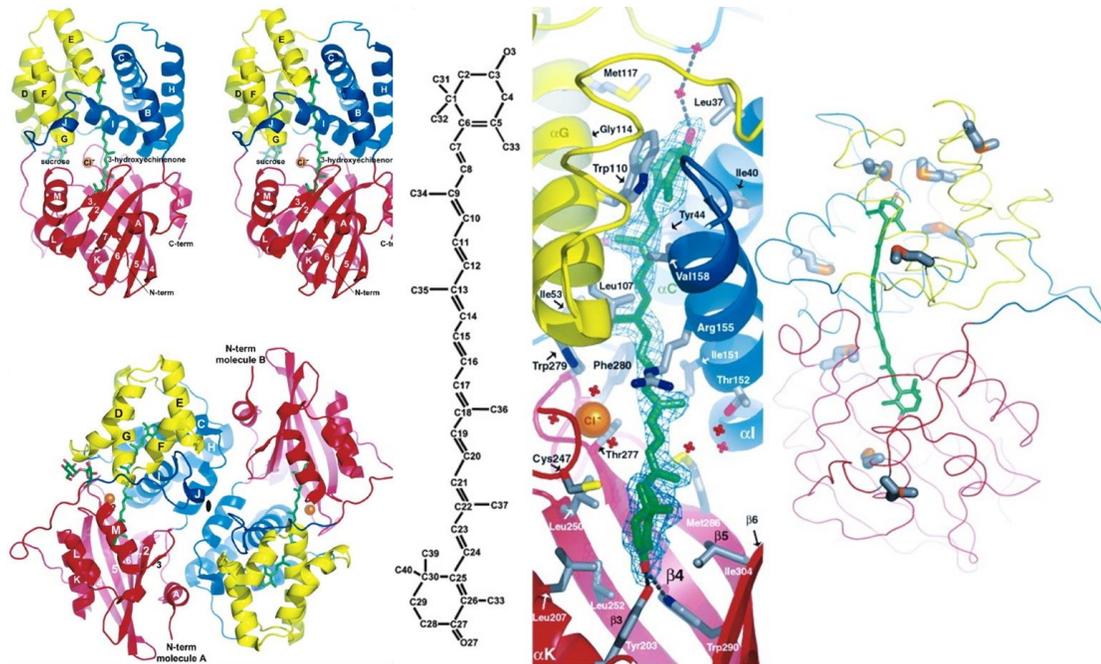


Fig. 6. Left: crystal structure of OCP extracted from cyanobacterium (*Arthrospira maxima*). Right: Carotenoid 3-Hydroxyechinenone binding site on RCP (Kerfeld et al., 2003).

1.4.1.3 Red carotenoid protein (RCP)

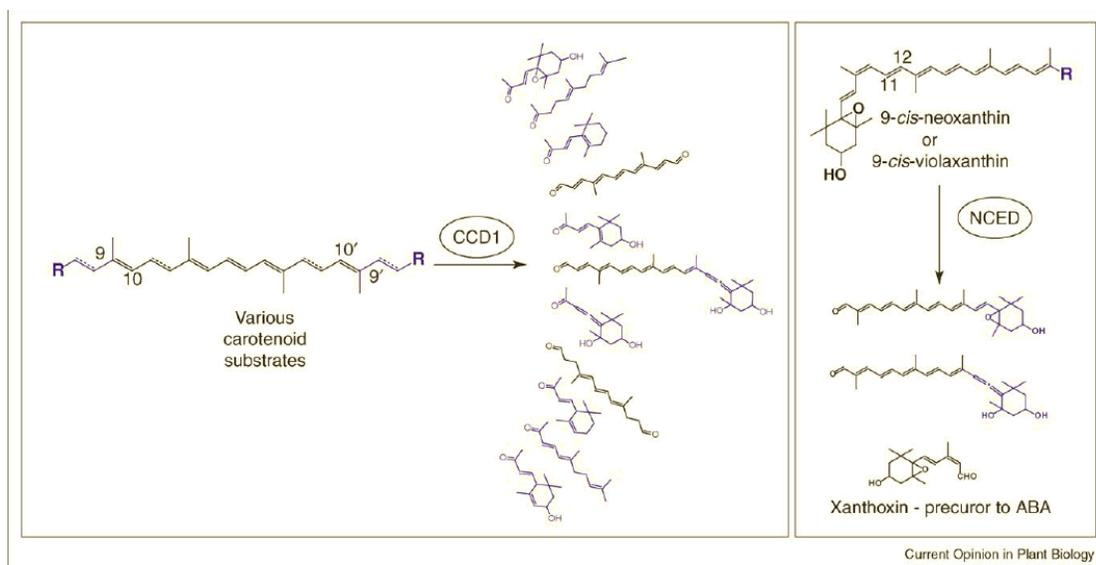
Red carotenoid protein, a 16 kDa protein, appears to be very closely related to the orange carotenoid protein. Like OCP it binds the keto carotenoid, 3'-hydroxyechinenone, and can be extracted from cyanobacteria. The main difference between these proteins is that the red carotenoid protein does not have the C-terminal domain found in the orange carotenoid protein. This structural feature is responsible for a conformational change and causes the excited state life-time of 3'-hydroxyechinenone in, red carotenoid protein to be different from that of OCP. RCP has a lifetime of 5.5 ps compared 3.3 ps in orange carotenoid protein (OCP). Thus the absence of C-terminal domain alters

the properties of 3'-hydroxyechinenone. In RCP, the exposed hydrophobic end of 3'-hydroxyechinenone penetrates further into the lipid membrane. This feature enhances the interaction between the exposed RCP 3'-hydroxyechinenone and lipid membrane and it is proposed that this offers additional protection for the photosynthetic organisms (Chábera et al., 2011).

1.4.1.4 Carotenoid cleavage enzymes

Carotenoid cleavage enzymes are found in both plants and animals (Cunningham and Gantt, 1998). Plants possess a capability to metabolize carotenoids and several carotenoid cleavage enzymes have been reported (Cunningham and Gantt, 1998). Enzymatic metabolism leads to the cleavage of carotenoids producing apocarotenoids. The family of carotenoid cleavage dioxygenases (CCD) is a good example of these enzymes. CCDs can cleave the double bond of polyene chain at different positions. These enzymes produce the wide variety of apocarotenoids observed in plant kingdom (Fig. 7). CCD1 and CCD7 cleave the double bond of zeaxanthin or lutein at 9-10 to form β -ionone. β -ionone is a volatile compound and is responsible for the pleasant flavor characteristic of many fruits and vegetables. It also serves as an attractant pollinator and increases the likelihood that pollinators will ensure viable seed production and dispersion. 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) cleave neoxanthin at the 11, 12 position to form abscisic acid a plant hormone responsible for dormancy and drought tolerance. In addition, CCD7 and CCD8 produce the novel hormone strigolactone to regulate the axillary branch growth.

There are some similarities among the features found in the various CCDs. First of all, a ferrous ion is required for enzyme catalysis. Second, a conserved peptide sequence at the carboxyl end is shared in all CCDs. Third, the active site complex consists of four histidine which bind the ferrous ion and is present in all CCDs (Auldridge et al., 2006). CCDs are found in animals also. In animals, these enzymes show preference for particular carotenoids. Beta-carotene cleavage oxygenase (BCO)-BCO1 cleaves only pro-vitamin A carotenoids and only at 15, 15' position. BCO2 cleaves a wider range of carotenoids and cleaves 8' and 10' position. Lutein, zeaxanthin and lycopene are cleaved by BCO2 (Amengual et al., 2013). These enzymes have active sites that fit the carotenoid structure with more or less specificity (Kloer and Schulz, 2006). β -carotene-15,15' dioxygenase (Beta Carotene Dioxygenase 1 (BCDO1)) and β -carotene-9,10 dioxygenase (Beta Carotene Dioxygenase 2 (BCDO2)) are the CCDs involved in the retinoid biosynthesis in most higher animals (von Lintig and Vogt, 2000).



CCDs that cleave 9,10 (CCD1) and 11,12 (NCED) double bonds have been identified in numerous species. CCD1 is a promiscuous enzyme that is able to utilize phytoene, ζ -carotene, lycopene, β -carotene, δ -carotene, zeaxanthin, violaxanthin, and neoxanthin as substrates *in vitro* to

Fig. 7. CCDs cleave and derivative apocarotenoids (Auldrige et al., 2006).

Table 1. Major carotenoid binding proteins in plants and microorganisms

Protein	Major Carotenoid	Molecular weight	Source	Major function	Reference
LH2	Lutein	129	Purple non-Sulfur bacteria	Light harvesting	(Li et al., 2000)
LH2	Lutein	129	Spinach leaf	Light harvesting	(Ruban and Horton, 1999)
OCP	3'-hydroxyechinenone	35	Cyanobacteria	Photoprotection	(Kerfeld et al., 2003)
RCP	3'-hydroxyechinenone	23	Cyanobacteria	Photoprotection	(Chábera et al., 2011)
CCD1	N/A	14	Tomato (higher plants)	Carotenoid cleavage	(Simkin et al., 2004)

1.4.2 Animal Carotenoid Binding Proteins

1.4.2.1 Crustacyanins

Carotenoid binding proteins are not only found in bacteria and plants, they are widely found within the animal kingdom. Crustacyanin, another carotenoid binding protein belonging to lipocalin family, is a remarkable blue-colored protein that is widespread in crustaceans. The unique color of crustacyanin is caused by the presence of the carotenoid, astaxanthin. The dark blue carotenoid binding protein provides camouflage that protects crustaceans from predators. Interestingly, the native red astaxanthin is released from the protein complex during cooking processes (Pilbrow et al., 2012b). Crustacyanin is a 320 kDa, water-soluble protein isolated from carapace of lobster (*Homarus gammarus*) named α -crustacyanin (Wald et al., 1948). This native macromolecule is comprised of 8 heterodimeric protein subunits and binds sixteen astaxanthin molecules. Two astaxanthin molecules are embedded in each heterodimer. The α -crustacyanin complex can irreversibly dissociate into eight 40 kDa β -crustacyanin subunits which consist of one heterodimer each with two astaxanthins (Wade et al., 2009). The heterodimer in β -crustacyanin is composed of two different separable apoprotein subunits, one of 21 kDa while another is 19 kDa. Five apoprotein subunits are classified by electrophoresis on the basis of molecular weight into two types. Type 1 is the group of 21 kDa subunit (A_1 , C_1 and C_2), Type 2 includes the 19 kDa subunits (A_2 , A_3). Only one gene per each type has been identified which suggests that the apoprotein

subunits in the same type arise from the same gene (Quarmby et al., 1977). The UV studies demonstrate that the binding of astaxanthin to crustacyanin induces a large bathochromic shift in its natural absorption of light. In α -crustacyanin, the maximum absorption is 632 nm and is red shifted by 160 nm compared to the maximum absorption 472 nm of the unbound astaxanthin. In β -crustacyanin, the maximum absorption is found to be 580 nm is red shifted by ~100 nm compared the unbound astaxanthin. (Cianci et al., 2002). The interaction between this astaxanthin and the protein has been intensively investigated. Details on the tertiary structure of β -crustacyanin help to explain more about this protein. The crystal structure of the A_1/A_3 dimer of β -crustacyanin has been determined (Fig. 8). Similar to the typical structure of lipocalins, each subunit contains two β -sheets consisting of antiparallel β -strands. The two monomers, A_1 and A_3 , interface with each other. The two astaxanthins are located at the center of A_1/A_3 loop region, both subunits share the astaxanthins equally (Cianci et al., 2002). The structural nature of astaxanthin binding is highly regulated (Fig. 9). Astaxanthins bind with the monomer non-covalently, both C1-C6 end-rings of the astaxanthins are nestled into hydrophobic pockets in A_1 and A_3 . Two sets of Pro, Phe and Ile residues from each monomer are attached to the astaxanthin (Fig. 9). The specific alignment of the astaxanthin polyene chains forms in a coplanar fashion enhances the pi-pi interaction between the astaxanthins. The astaxanthin keto group at O4' and hydroxyl group O3' are hydrogen bonded with the peptide residues such as His. The hydrogen bonding shortens the distance between two astaxanthin producing a stronger interaction between the pair and an electronic

polarization further that leads to excitation interactions. All of these features alter the conformational structure and spectral properties and cause the bathochromic shift of astaxanthin bound to crustacyanin. Hence, the color change is induced by a carotenoid-carotenoid interaction (Chayen et al., 2003).

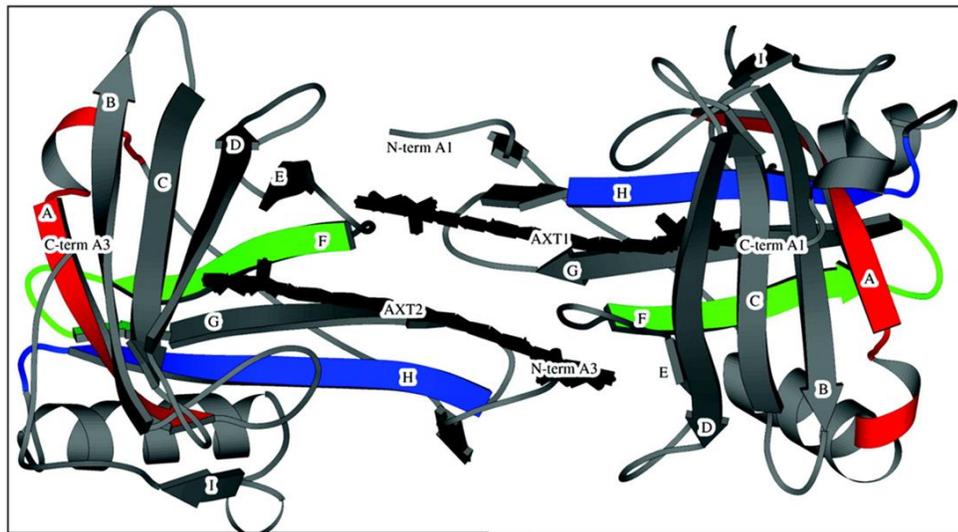


Fig. 8. Crystal structure of β -crustacyanin isolated from carapace of lobster (*Homarus gammarus*) (Chayen et al., 2003).

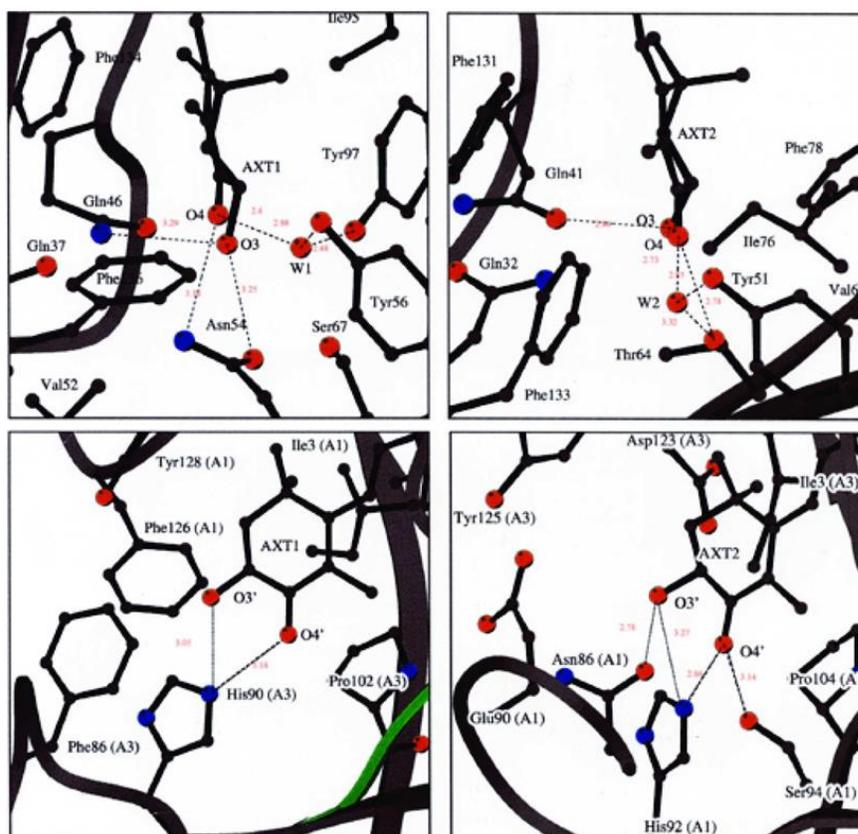


Fig. 9. Astaxanthin binding site of β -crustacyanin (Chayen et al., 2003).

1.4.2.2 α -Actinin

In addition to lobsters, carotenoid binding proteins are also found in other marine organisms. Many fish also concentrate carotenoids in tissues. A particularly well-known example is the accumulation of astaxanthin within the muscle of salmon. The unique and bright orange color characteristic of the flesh in salmon is due to the accumulation of carotenoid in the muscle. The carotenoids are acquired from algae and crustaceans in the food chain of wild salmon. Farmed salmon are fed astaxanthin and/or canthaxanthin as supplements (Storebakken et al., 1987). One astaxanthin binding protein, α -actinin, has been identified from Atlantic salmon (Matthews et al., 2006). This

protein has been isolated and identified by SDS-PAGE. The 105 kDa protein has been sequenced and the result matches the α -actinin gene. Astaxanthin was shown to bind to the myofibrillar protein, α -actinin, in 1:1 ratio. A minor fraction of the carotenoid is identified in lipid bilayers (Matthews et al., 2006). α -actinin is a group of actin-crosslinking proteins which are classified into four subtypes. α -Actinins bind to a variety of different molecules such as stress fiber, focal adhesion, lymphocyte targeting integrin, and regulatory enzymes. α -actinin possesses multiple functions in the cell including the regulation of various receptors and connection of the cytoskeleton to different transmembrane proteins (Otey and Carpen, 2004). In salmon, astaxanthin is transported through the blood. α -Actinin serves as cell surface receptor facilitating uptake of astaxanthin into the muscle cell (Saha et al., 2006). Its unique structure is the key to α -actinin's ability to bind astaxanthin. α -Actinin is a homodimer containing antiparallel β -pleated sheet motif, each monomer consists of 2 N-terminal calponin homology domains (CH1, CH2), 1 C-terminal calmodulin-homology domain (CaM) and a rigid central rod domain formed by 4 spectrin repeats (R1-R4) (Fig. 10) (Ylänne et al., 2001). CH1, CH2 and CaM consist the actin binding head of α -actinin. Electron microscopic study shows that the protein is twisted from left end to right end (Fig. 11). R1 and R2 are parallel to the x-axis while R3 and R4 are twisted to form a 12 degree angle between R2 and R3. The twist of R3 and R4 leads to the curvature of the dimer interface and this curve provides a high affinity binding site for astaxanthin. The hydrophobic core of the coiled

repeats has a high probability of being the binding site of astaxanthin α -actinin.
(Otey and Carpen, 2004).

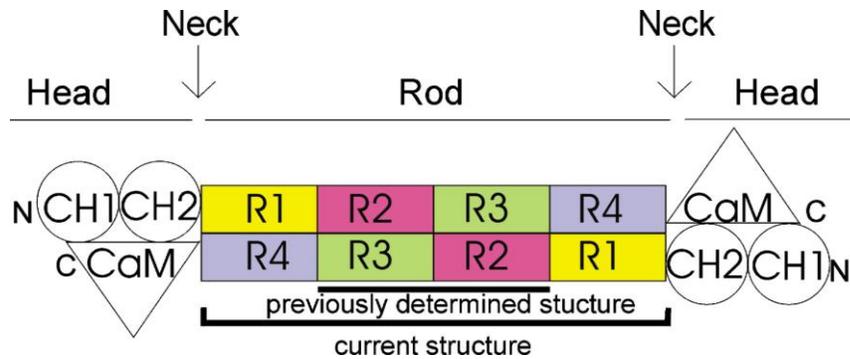


Fig. 10. Structural domain of α -actinin (CH1, CH2: two N-terminal calponin-homology domains, CaM: C-terminal calmodulin-homology domain, R1-R4: four spectrin repeats which form the dimeric central rod of the domain) (Yläänne et al., 2001).

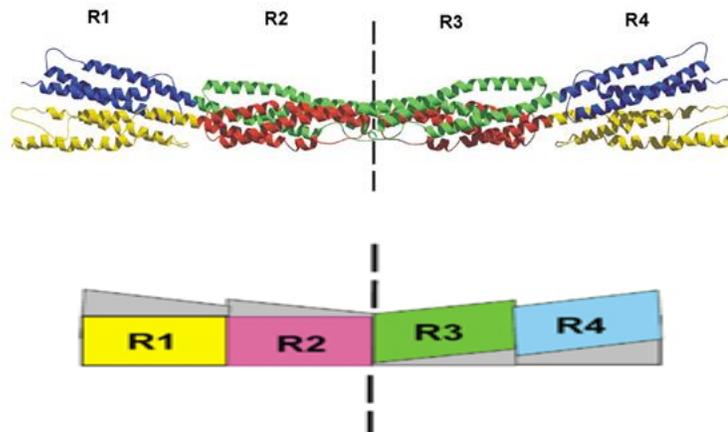


Fig. 11. Up: Overall structure of α -actinin rod domain Down: Localization of R1-R4 repeats (CH1, CH2: two N-terminal calponin-homology domains, CaM: C-terminal calmodulin-homology domain, R1-R4: four spectrin repeats which form the dimeric central rod of the domain) (Yläänne et al., 2001).

1.4.2.3 Carotenoid binding protein from ferret liver

Well-known for their capability to absorb β -carotene in a similar manner to humans, ferrets have been found to be a good model to study carotenoid metabolism and bioavailability. As the predominant storehouse of carotenoids, liver tissue is likely to possess its own carotenoid binding proteins (Rao et al., 1997). Some carotenoid binding proteins have also been found in liver tissue of rodents (Blomhoff et al., 1985). A single 67 kDa carotenoid binding protein was isolated from ferret liver. It shows high binding affinity to β -carotene and does not bind retinol, zeaxanthin, lycopene, or astaxanthin. Hence, the β -carotene is bound to this carotenoid binding protein with high specificity. This β -carotene carotenoid binding protein appears to play an important role in transport and storage of β -carotene in biological systems (Rao et al., 1997).

1.4.2.4 Silkworm carotenoid binding protein (SW-CBP)

Carotenoids are well known to be found in high concentrations in insects (Kayser, 1982). A carotenoid binding protein that is responsible for the yellow or orange coloration in the cocoons of the silk worm has been identified. Approximately 90% of the carotenoid found bound to this protein is lutein. Although it binds lutein with a high affinity, the carotenoids, α -carotene and β -carotene, also bind to SW-CBP. Specificity of the protein for a particular carotenoid is clearly believed to be important to its ability to function appropriately. It is unclear what advantage may exist in its lack of fidelity and ability to bind the α - and β -carotenes in addition to lutein is unknown. There is a specific transport

pathway that ensures delivery of lutein to the silk gland. Lutein is carried in the hemolymph from the gut on a lipoprotein, called lipophorin. Lipophorin is a general, non-specific all-purpose lipoprotein that is responsible for transporting a wide range of lipophilic compounds. Lipophorin transports lutein to the cells of the silk glands where it binds to the SW-CBP and is concentrated within the silk of the cocoon (Jouni and Wells, 1996). Thus, the lutein binding protein SW-CBP is responsible for the yellow-orange color of the cocoon (Tabunoki et al., 2004). Furthermore, SW-CBP has been sequenced and found to belong to the steroid acute regulatory (StAR) protein family (Tabunoki et al., 2002). In humans, there are 15 different protein variants that belong to the StAR family (Alpy and Tomasetto, 2005). The widely observed StAR proteins are important for the transport and regulation of hydrophobic molecules, including sterols. A transport protein, the StAR protein functions to regulate cholesterol transfer within mitochondria. This transport process is the rate-limiting step controlling the production of steroid hormones. StAR proteins also have various other functions associated with lipid transfer between intracellular organelles, lipid trafficking, lipid metabolism and modulation of signaling events (Stocco, 1999). StAR proteins are most commonly present in steroid-producing cells, including theca cells and luteal cells in the ovary, leydig cells in the testis and additional cell types in the adrenal cortex (Manna et al., 2009). To help us understand the mechanism of lipid transfer, the StAR-related lipid transfer (StART) domains have been well studied. Metastatic lymph node 64 protein (MLN 64) is the closest homology to StAR proteins. The crystal structure of human MLN 64 START

domain has been determined (Fig. 12) (Tsuji-shita and Hurley, 2000). MLN 64 StART is comprised of four α -helices and a β -sheet consisting of nine antiparallel strands. The β -sheets are twisted to form a U-shaped unclosed β -barrel. A hydrophobic tunnel is enveloped inside this domain, the tunnel has two openings at each end and a wide central chamber which is large enough for transport of small lipophilic molecules. StAR shuttles lipid molecules via this interior hydrophobic tunnel. MLN 64 and StAR START domains both bind cholesterol *in vitro*, both domains have the same cholesterol binding stoichiometry of 1:1 (Tsuji-shita and Hurley, 2000).

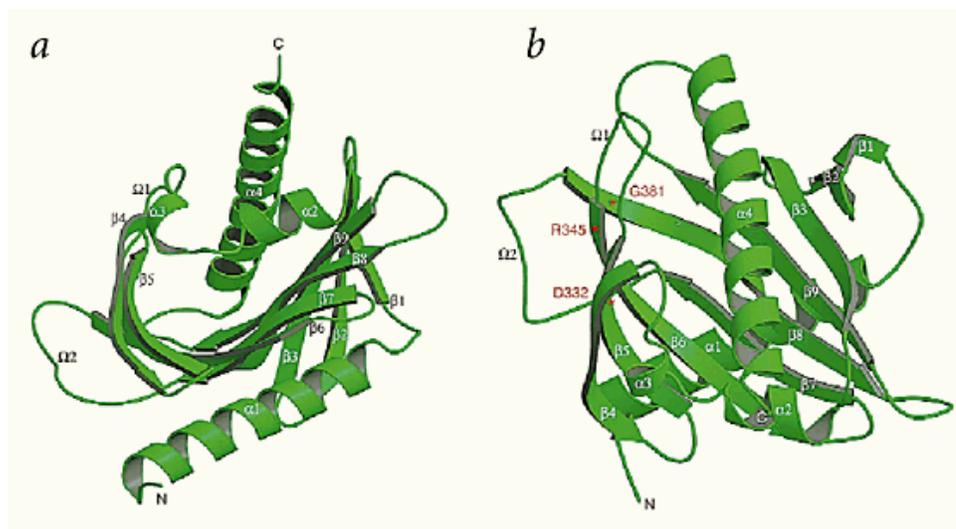


Fig. 12. Ribbon domain of MLN64-START, a/b are different view of MLN 64-START (b is rotated 90° by x-axis) (Li et al., 2011).

1.4.2.5 Carotenoid binding proteins found in the human retina

Carotenoid binding proteins in human retina are important to protect the retina from photooxidative damage but their properties and actions are not fully

understood. Two carotenoid proteins have been isolated from the human retina, a zeaxanthin binding protein (GSTP1, Glutathione S-transferase pi 1) and a lutein binding protein (identified as StAR D3, a steroidogenic acute regulatory protein) . (Li et al., 2010)

1.4.2.5.1 Glutathione S-transferase pi 1 (GSTP1)

The zeaxanthin binding protein, GSTP1, is a 23 kDa membrane associated protein. The sequence of the zeaxanthin binding protein was obtained by use of the Mascot search engine using high resolution Mass (HR-MS) to identify fragments produced by lysis from the protein extract. Identification of the protein as the pi isoform of GSTP1 was based on the match of sequence in fragments combined with corresponding match of molecular weight based on SDS PAGE analysis (Bhosale et al., 2004). GSTP1 is a well-known detoxification enzyme that belongs to the glutathione S-transferase (GST) family. Glutathione-S-transferase is responsible for the conjugation of glutathione to a variety of substrates typically toxic xenobiotic or metabolites, thus targeting the molecule for excretion. Therefore this enzyme is best known for its role in the elimination toxic chemicals including endogenous species, examples of which include adenine, acrolein, benzyl isothiocyanate, 4-vinylpyridine and propenal (Hayes and Strange, 2000). In addition to its detoxification functions, GST serves other biological functions. GST interacts with some of the protein kinases involved signal transduction. GSTs (GSTM1, GSTP1 and GSTT1) are also considered to be the risk factor for acute leukemia (Ye and Song, 2005).

The binding affinity of different carotenoids to GSTP1 has been determined by surface plasmon resonance. Dissociation constant (K_D) is determined to compare the binding affinity. The larger the K_D value, the smaller the binding affinity. Zeaxanthin and *meso*-zeaxanthin show the highest binding affinity and lutein shows little of no affinity for this protein (Table 2). Hence, the interaction of GSTP1 with zeaxanthin is strong and specific and further supports the hypothesis that zeaxanthin may have unique and essential functions within the retina (Vachali et al., 2012).

Table 2. K_D value of GSTP1 with carotenoids

Carotenoids	Astaxanthin	β -Carotene	Lutein	<i>Meso</i> - Zeaxanthin	Zeaxanthin
K_D GSTP1	1.16 \pm 0.02	1.09 \pm 0.01	1.30 \pm 0.01	0.18 \pm 0.02	0.14 \pm 0.02

1.4.2.5.2 StARD3

After the identification of zeaxanthin binding protein GSTP 1, a lutein binding protein was also found in the human retina (Li et al., 2011). The discovery of lutein binding protein was made by looking for cross-reactivity of antibodies prepared from SW-CBP to proteins extracted from human retina. Comparison of the homology between SW-CBP and all 15 human StAR proteins revealed that human StARD3 has the highest homology with SW-CBP. Western blot provides solid evidence that StARD3 is the lutein binding protein in human retina (Bhosale et al., 2009). The binding affinities of a number of carotenoids

with recombinant StARD3 have been measured by surface plasmon resonance. Of the carotenoids investigated, lutein binds to StARD3 with the highest affinity having a K_D value of 0.59 μ M. By contrast, a K_D value of 1.6 μ M for zeaxanthin illustrates that the binding affinity is considerably lower but StARD3 appears to be less selective in its binding of carotenoids in contrast to GSTP1 (Table 3) (Li et al., 2011). This is consistent with the report that SW-CBP is able to bind as much as 10% β -Carotene (Tabunoki et al., 2002).

Table 3. K_D values of StARD3 with carotenoids

Carotenoids	Astaxanthin	β -Carotene	Lutein	<i>Meso</i> - Zeaxanthin	Zeaxanthin
K_D StARD3	2.09 \pm 0.09	2.30 \pm 0.06	0.59 \pm 0.03	1.63 \pm 0.07	1.60 \pm 0.01

Table 4. Major carotenoid binding proteins in animals

Protein	Major carotenoid	Molecular weight (kDa)	Source	Major function	Reference
Crustacyanin	Astaxanthin	320	Lobster carapace	Colorant	(Wade et al., 2009)
α -actinin	Astaxanthin	105	Salmon flesh	Anti-oxidant	(Ylänne et al., 2001)
CBP	β -carotene	67	Ferret liver	Carotenoid shuttle	(Rao et al., 1997)
GSTP1	Zeaxanthin	23	Human retina	Anti-oxidant	(Bhosale et al., 2004)
StARD3	Lutein	29	Human retina	Anti-oxidant	(Li et al., 2011)
SW-CBP (StAR)	Lutein	33	Silkworm silk gland	Cocoon colorant	(Tabunoki et al., 2004)

1.4.3 Summary of Carotenoid binding proteins

In conclusion, numerous carotenoid binding proteins have been identified and characterized from various living organisms and they have been found to have a variety of functions. Carotenoids bound to the proteins of the photosynthetic system are essential to both plants and microorganisms. In animals, non-specific carotenoid binding proteins, such as HDL and LDL can serve to transport carotenoids as well as other small lipid molecules. Specific carotenoid binding proteins possessing well defined binding sites that interact exclusively with one or a limited number of carotenoids also have been identified. The interactions between these proteins and their partner carotenoids are very

important for their functions. In efforts to understand the interaction of carotenoids with protein through the amino acid side chains, x-ray structures of several carotenoid binding proteins have successfully been investigated. Evidence shows that carotenoids possess their own unique binding site within each protein (Britton and Helliwell, 2008). Carotenoids are bound non-covalently to the hydrophobic core of their partner proteins. In reviewing carotenoid binding proteins it is evident that their binding sites share similar or common features. The x-ray structures of carotenoid binding proteins reveal that the presence of helix domains and β -sheets are the dominating motifs that are common present at the carotenoid binding site (Zagalsky, 1976). The helix is a secondary protein structure that amino acids unwind, twist and coil; the helical domain in the protein can form hydrophobic supercoil region which fits carotenoid. Except the helix, β -sheet is another important feature for carotenoid binding, the β -structured amino acids facilitate the formation of antiparallel β -pleated sheet (Zagalsky, 1976). The β -sheet of these carotenoid binding proteins are twisted forming a U-shaped unclosed β -barrel, this hydrophobic tunnel enables the localization of small lipid molecules such as the carotenoid (Britton and Helliwell, 2008). To summarize the related literature, there are three different types of carotenoid binding protein based on carotenoid binding motif. In the first, of which LH2 is the prime example, the binding site is formed exclusively by α -helices of the protein with carotenoids located at ends of two helices where they form an internal cross and bridge between these structures, (Liu et al., 2004). The second type is typified by orange carotenoid protein and the carotenoid binding protein consists of α/β

domain and helical domain. One end of carotenoid inserts into the hydrophobic protein pocket in a β -sheet and another end is associated with the α -helical coil (Kerfeld et al., 2003). The third and final structural type of carotenoid binding proteins has a pocket comprised only of β -sheets. A notable instance is β -sheet dimer β -crustacyanin isolated from lobster (Zagalsky, 2003). In β -crustacyanin, the carotenoid end-ring is hydrogen bonded to the peripheral amino acid side chains of β -pleated sheets. Each carotenoid inserts into the hydrophobic core of two subunits. These subunits sit on both sides of the carotenoid to form a loop region that connects two dimer (Zagalsky, 2003). Although the x-ray structure of carotenoid binding proteins from some marine life, plant and microorganism have been studied, our knowledge of animal carotenoid binding proteins especially mammals is still missing. The utility of identifying more model systems and the ability to express recombinant proteins should be helpful to investigate the carotenoid-protein interaction.

1.5 Research goals for Monarch butterfly larval CBP

The recruitment of xanthophylls to function as antioxidants and to protect the human macula from light induced damage is of acute interest because of its implications for human health. The uptake and accumulation of the carotenoids lutein and zeaxanthin by the macula is a process shepherded by the selective carotenoid binding proteins, GSTP1 and StARD3 as described above. Comparison of similar carotenoid binding proteins found in unique biochemical systems of different species can be an insightful approach and can help us

develop a more complete understanding of both the function and general binding mechanisms exploited to manipulate carotenoids in nature. Significantly, despite our successes in the x-ray crystallographic studies of a number of the carotenoid binding proteins, factors controlling the specificity and the selective binding of carotenoids in various proteins remain incompletely understood. Further, even such basic details as the specific cells where carotenoid binding proteins are produced and concentrated remain obscure. Study of these carotenoid binding proteins also is hampered by their lack of availability and a methodology for preparing samples in suitable quantities for intensive study.

Thus, the discovery and study of model systems that produce lutein or zeaxanthin binding proteins will enable detailed investigation of the properties of these carotenoid binding proteins generally and will enable experiments that can provide insight into their function in humans. Insects which comprise the most diverse class of higher animals on earth are known for their extensive coloration (Landrum et al., 2009). Although there are many pigments and physical structures that contribute to insect coloration and display, frequently coloration arises the result of the concentration of carotenoids (Britton and Goodwin, 2013). Evidence shows carotenoid composition in insects corresponds principally to the dietary supply during the larvae stage (Feltwell and Valadon, 1974). For instance, β,β -carotene and astaxanthin are found in locusts species such as *Locusta migratoria migratorioides* and *Schistocerca Gregaria* (Goodwin and Srisukh, 1948). β,β -carotene and lutein are found in many butterflies. The order, Lepidoptera, is a large and genetically diverse but common group of insects and

as such is a potentially practical animal model for the study of carotenoid binding proteins. The carotenoid accumulation in specific tissues and anatomical structures by insect reflects ability to absorb, metabolize and to actively transport dietary carotenoids (Britton and Goodwin, 2013). For this reason, the diversity of insect species may be anticipated to be an abundant source of novel carotenoid binding proteins. Moreover, insect carotenoid binding proteins essential for utilization and mobilization of carotenoids may be anticipated to provide a rich natural showcase where the evolutionary diversity as well as convergence among these proteins can be provide many unique opportunities to employ comparative studies to unravel the intricacies of carotenoid-protein binding interactions (Landrum et al., 2009). Surprisingly, there have been relatively few reports in the literature that carefully and completely describe the identities of the carotenoids present in insects and even fewer that have identified the proteins essential to the processes of chaperoning their accumulation and localization. An exception is the silk worm (*Bombix mori*) in which a protein belonging to the StAR family has been identified and characterized. It functions in the mobilization and accumulation of lutein in the silk gland (Tabunoki et al., 2004). The concentration of lutein within the silk gland of the silk worm determines the extent to which colored silk is produced. This carotenoid transport system in the silk worm is genetically regulated (Tabunoki et al., 2004). Significantly, it was this insect protein which enabled identification of the corresponding human lutein binding protein responsible for lutein accumulation in the human retina (Li et al., 2011).

Monarch butterfly larvae (*Danaus plexippus*) have been chosen for its intensive yellow coloration resulting from carotenoid. The monarch butterfly is a milkweed butterfly and is one of the most abundant the best known of all North American butterflies (Jesse and Obrycki, 2003). Previous work in our lab has shown that the larval monarch butterflies specifically accumulate the carotenoid lutein only the yellow colored regions of the epidermis. HPLC analysis demonstrated that of all the carotenoids present in the diet only lutein is found in the yellow pigmented regions (Fig. 13) (Landrum et al., 2009). Although the functional role of lutein in the monarch butterfly larvae (MBL) epidermis is distinctly different from that of lutein in the retina, the highly specific accumulation of a single xanthophyll exclusively within a small region is a notable convergence in the transport systems of these two widely separated species, humans and monarch butterflies. The exclusivity of lutein accumulation within yellow regions and its absence in black or white colored regions, strongly implicates a finely regulated mechanism controls the transport, localization and binding of the carotenoids in these organisms (Landrum et al., 2009) and this is due to the presence of a specific carotenoid binding protein (Fig. 14).

The goal of this project is to isolate and purify the carotenoid binding protein responsible for the specific accumulation of lutein in the epidermis of monarch larvae. This system is anticipated to be an excellent comparative model for the study of the carotenoid binding. We expect that the monarch carotenoid binding protein will be a productive model for understanding carotenoid binding systems. Understanding the carotenoid binding proteins, the mechanisms by which they

facilitate accumulation and transport of xanthophylls in larval butterflies will produce new perspectives on the role, function, and action of the analogous proteins present in the human macula, particularly their role in maintaining optimal ocular health.

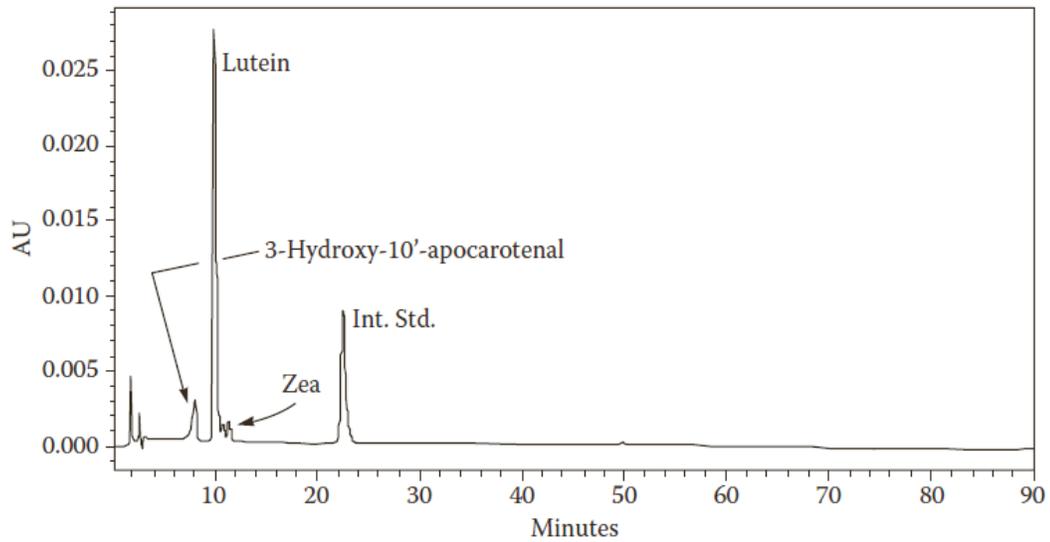


Fig. 13. HPLC chromatogram of the extract obtained from a yellow-pigmented sample of Monarch epidermis (Landrum et al., 2009).

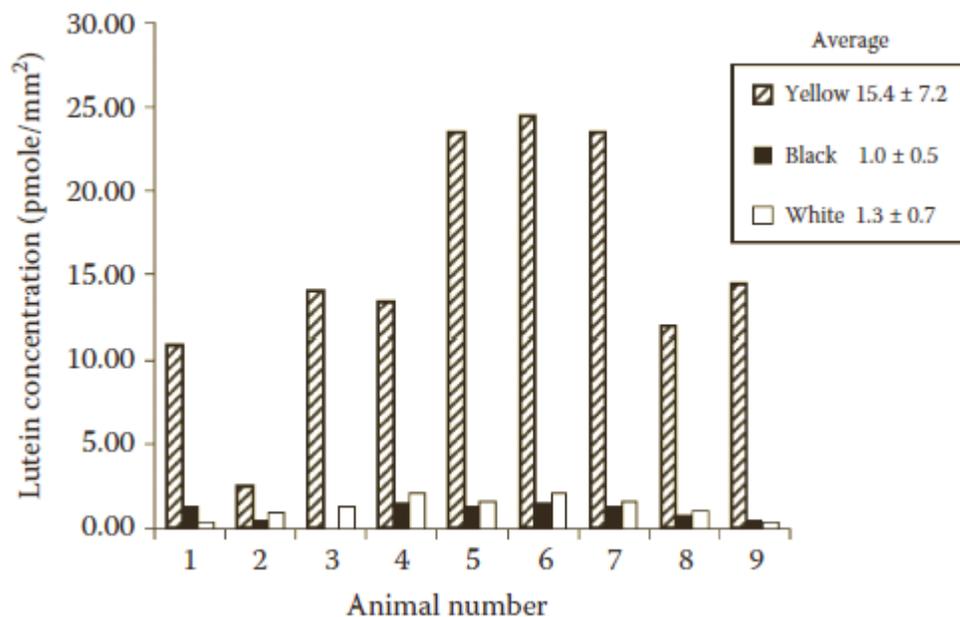


Fig. 14. Comparison of lutein concentration (pmole/mm²) presents in yellow, black, and white colored region from nine individual monarch butterfly larvae (Landrum et al., 2009).

Chapter 2: Purification and identification of carotenoid binding protein from monarch butterfly larvae (*Danaus plexippus*)

2.0 Introduction

In this chapter a description of the purification of the carotenoid binding protein (CBP) found in the epidermis of monarch butterfly larvae (*Danaus plexippus*) is described. The protein which is the focus of this work is restricted to the larval stage of monarch butterfly (MBL) and is responsible for the distinctive yellow coloration in the epidermis of the larvae.

The purification steps of M-LBP follow the methodologies described by others for human retina CBP and silkworm CBP (Bhosale et al., 2009; Tabunoki et al., 2002). In our experimental purification method is a combination of those used by Bernstein et al. and Tsuchida et al. High speed centrifugation, ammonium sulfate precipitation and a series of protein chromatography steps were combined.

2.1 Experimental procedures

2.1.1 Animals

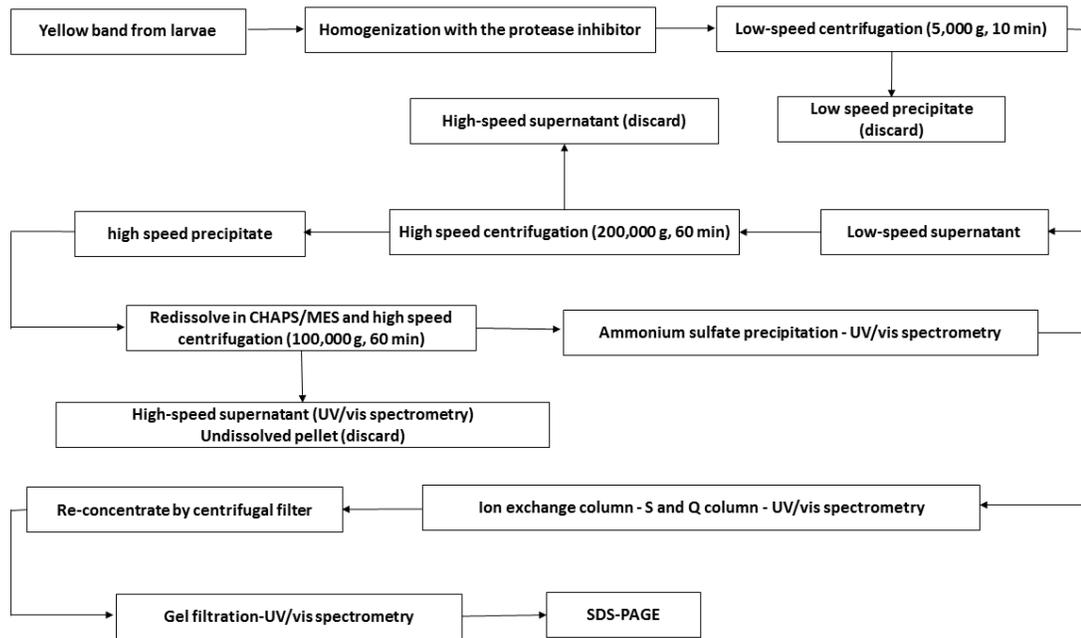
The monarch butterfly larvae (*Danaus plexippus*) used in the experiment were grown and collected after the 5th instar in suburban South Florida 7-10 days after hatching. Animals were frozen and stored at -20 °C for brief periods prior to dissection and extraction of the protein from the epidermis.

2.1.2 Isolation of the Monarch lutein binding protein (M-LBP)

2.1.2.1 Sample dissection and high-speed centrifugation

Extraction of proteins from the MBL epidermis follows Scheme 1. Typically, 200 MBL were carefully dissected and the gut was removed to minimize contamination of tissue with the intestinal contents which is rich in carotenoids and chlorophyll. Following dissection the internal surface of each tissue sample was carefully rinsed with phosphate buffer (PBS: 10 mM phosphate, 150 mM NaCl, pH 6.5) and any fat bodies adhering to the inner surface were removed by gentle scraping. The white and black pigmented epithelium bands were separated by cutting the epidermis into sections leaving only the yellow M-LBP containing sections to be included in subsequent extraction steps. The yellow epidermal tissue was homogenized in 25 mL of cell lysing solution containing protease inhibitors (Thermo Scientific Pierce #PI88665) in Tris (hydroxymethyl aminomethane) buffer (20mM Tris, 1 mM CaCl₂, 2 mM MgCl₂, pH7.4) using a ground glass tissue homogenizer. The resulting homogenate was centrifuged at a low speed (5,000 g) in an Eppendorf centrifuge 5804 for 10 min, to separate suspended membranous debris and cellular particulates during the homogenization step. After separation of the debris using low-speed centrifugation, the proteins present in the supernatant were pelleted by high speed centrifugation (200,000 g) for 60 min. The resulting protein pellet was solubilized by sonication on ice into 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)/ 2-(N-morpholino) ethanesulfonic acid (MES) buffer

(25mM CHAPS, 20 mM MES, 1 mM CaCl₂, 2mM MgCl₂, pH 5.5). Subsequently, the homogenized sample was pelleted by a further centrifugation at 100,000 g for 60 min, the undissolved precipitation was discarded.



Scheme. 1. Monarch butterfly larvae purification steps

2.1.2.2 Differential Ammonium Sulfate Precipitation

The resolubilized proteins were differentially precipitated by treatment with ammonium sulfate. The protein solution was chilled in an ice/water bath to maintain a temperature of 0°C during this procedure. The yellow M-LBP rich fraction was separated from other soluble proteins by progressive, step-wise precipitation, using increasingly higher concentrations (15-25%, 25-35%, 35-45%, 45-55%, 55-65%, 65-75%, and 75-85%) of ammonium sulfate. Precipitates were collected by centrifugation at each progressive ammonium sulfate concentration. Each fraction was subsequently resolubilized in 2 mL CHAPS/MES buffer and the carotenoid and protein levels were compared by determining the A_{450}/A_{280} ratio in Cary 17 UV/Visible Spectrometer. The carotenoid rich protein fraction was subsequently purified using several chromatography steps.

2.1.2.3 Ion-exchange chromatography and gel filtration

The carotenoid rich precipitate obtained following ammonium sulfate precipitation was sequentially separated from other proteins in the mixture first by anion and then cation exchange chromatography. The A_{450}/A_{280} ratio was recorded for the resulting extract produced after each step. An Econo Gradient Pump system (Bio-rad #7319001, Hercules, CA) was used for ion exchange chromatography. Prior to running the protein sample, the anion exchange column anionic Bio-Scale Mini Macro-Prep High Q Cartridge (Bio-rad #7324120, Hercules, CA) was prepared according to the following procedure. First, the column is washed with 25 mL of deionized water. After washing, the column was

regenerated by treatment with 10 mL of low salt buffer (50 mM L-histidine, 0.5 M NaCl, 8 mM CHAPS, pH 5.6) followed by 20 mL of high salt buffer (50 mM L-histidine, 2 M NaCl, 8 mM CHAPS, pH 5.6). In the final step the column was equilibrated with 15 mL low salt buffer and 15 mL of running buffer (50 mM L-histidine, 8 mM CHAPS, pH 5.6). The yellow M-LPB rich precipitate produced from ammonium sulfate fractionation step was resolubilized in CHAPS/MES buffer (pH 5.5). A 2 ml aliquot of 2 mg/mL protein sample was pumped into the column at a flow rate of 1 mL/min. Typically, 3 mL of the protein eluent containing the highest A_{450}/A_{280} ratio were combined and collected. The eluent collected from anion exchange chromatography was re-concentrated using a spin column, Amicon Ultra-15 Centrifugal filter unit-30,000 Nominal Molecular Weight Limit (NMWL) (EMD Millipore #UFC910008, Darmstadt, Germany) centrifuging at 7,500 g for 15 min. The re-concentrated M-LBP rich fraction was resolubilized in cation exchange buffer (100 mM sodium phosphate, 8 mM CHAPS, pH 7.5) to reach a final volume of 0.8 mL, and subsequently injected on a cationic Bio-Scale Mini Macro-Prep High S Cartridge (Bio-rad #7324130, Hercules, CA). The cation exchange column was regenerated and equilibrated as described above following the anion chromatography step with cation exchange buffer. After collection the eluent was re-concentrated using a spin column. Typically, a 0.3 mL sample was obtained.

Following the ion exchange steps, a final gel filtration step was used to further purify the protein extract. The sample was loaded on a Bio-Scale™ Mini Bio-Gel P-6 Desalting Cartridges (Bio-rad #7324502, Hercules, CA) and eluted

with running buffer (30 mM sodium phosphate, 8 mM CHAPS, pH = 6). Later on, to achieve a better resolution, gel filtration step was performed using a Perkin Elmer Flexar Autosampler LC system with a Yarra™ 3 μm SEC-2000 (Phenomenex #00H-4512-K0, Torrance, CA) size exclusion column. The column was equilibrated with running buffer (100 μl 100 mM phosphate, 8 mM CHAPS, pH 7.0) at a flow rate of 0.5 mL/min for 60 min. 100 μl 2 mg/mL aliquots of the concentrated M-LBP were injected at a 0.5 ml/min flow rate with running buffer. The eluent peak was monitored by a Flexar UV detector at 280 nm and a single peak was collected eluting at 13 minutes (Fig 15).

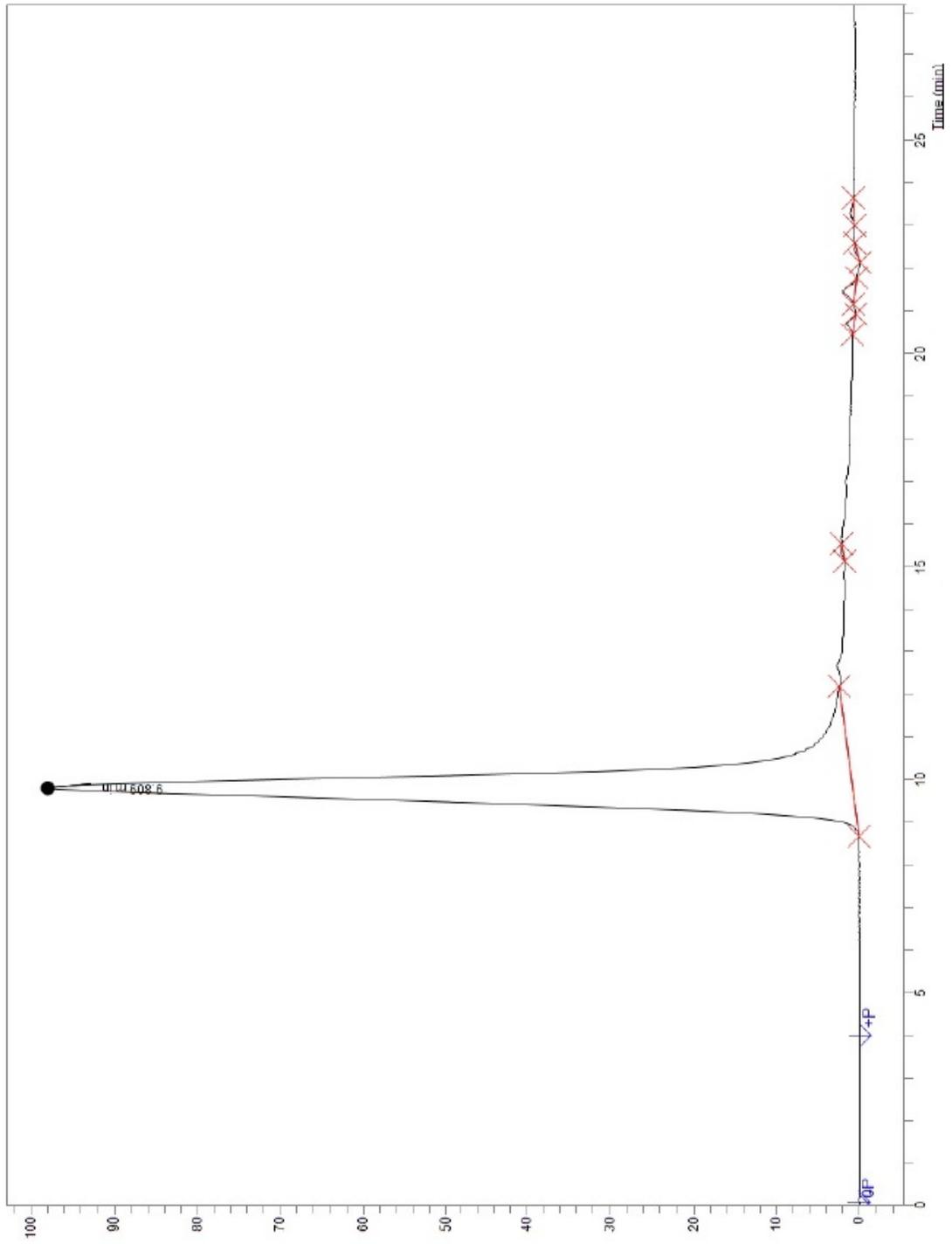


Fig. 15. Yarra™ 3 μm SEC-2000 size exclusion column of purified M-LBP

2.1.3 Identification of the Monarch lutein binding protein, M-LBP

After each step in the purification process, the purity of protein sample was tested by SDS polyacrylamide gel electrophoresis. A commercial ladder containing protein standards was run in an adjacent lane so that protein molecular weight could be estimated. In addition to SDS PAGE a native gel electrophoresis was run on the purified protein fraction after final purification step.

Mass spectrometry was carried out to confirm the protein molecular mass via direct injection of intact protein.

Antibodies from silkworm and sweet potato hornworm carotenoid binding proteins for immunological tests were provided by Drs. Tsuchida (National Institute of Infectious Diseases) and Dr. Shirai (Shinshu University) from Japan. Cross reaction of the M-LBP with antibodies was performed to test homology of M-LBP with previously described carotenoid binding proteins.

2.1.3.1 Electrophoresis

Electrophoresis of the native protein conducted in the absence of sodium dodecyl sulfate (SDS) and was run using a 8% Tris-HCl polyacrylamide gel was prepared according to the following procedure. To make the resolving gel, 2.5 mL resolving buffer (1.5M Tris HCl, pH 8.8), 4.8 ml deionized water and 2.7 mL 30% acrylamide/bis solution, 37.5:1 (Bio-rad #1610158, Hercules, CA) were mixed well. This was followed by 0.1 mL fresh ammonium persulfate (APS) solution (10% w/v) and 10 μ l of tetramethylethylenediamine (TEMED) (Bio-rad

#1610800, Hercules, CA) that was added into the solution and thoroughly mixed. Bio rad Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-rad #1658004, Hercules, CA) was used to conduct the gel electrophoresis. The resolving gel was poured into the gel cassette to a level 5mm below the top of the well in the comb. 2 ml ethanol was added on the top of resolving gel to make a flat surface and exclude O₂. After pouring, the gel was allowed to sit for 10 min to ensure complete polymerization. The stacking gel which consists of 2.5 ml stacking buffer (0.6M Tris HCl, pH 6.8), 6.5 ml deionized water and 1.25 ml 30% Acrylamide/Bis Solution, 37.5:1 (Bio-rad #1610158, Hercules, CA) was prepared in the same manner as resolving gel. After the resolving gel was polymerized, the ethanol on the surface was removed by pipette, the stacking gel was then poured to fill the top of the gel cassette and the comb was inserted to form the sample wells. Subsequently, 10 µl of the 1 mg/mL native M-LBP solution was mixed with 10 µl sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol) and then loaded into the sample well. The protein sample was run in the tris/glycine running buffer (25 mM Tris, 192 mM glycine, pH 8.3). Figure 16 shows the native protein runs as a single band on the gel.

Protein extracts produced at each stage of the purification process were monitored by SDS PAGE providing a qualitative estimation of homogeneity of these samples. In the sample preparation step, 15 µl protein sample was mixed with 5 µl 4xLaemmli Sample Buffer (Bio-rad #1610747, Hercules, CA). The reducing reagent, β-mercaptoethanol (β-ME) was included in the loading buffer

to ensure that all protein disulfide bonds were reduced and the protein tertiary structure fully disrupted. The sample mixture was heated at 95 °C in a water bath for 10 min to make sure the protein was fully denatured. For SDS PAGE the resolving gel, stacking gel and running buffer were made as described above with exception that the 1g SDS was added into 1L of running buffer, and 10% (w/v) SDS was added to gel. In addition to the sample, a 'ladder' containing reference proteins was run in one lane of the gel. The protein 'ladder' used was Precision Plus Protein™ all blue pre-stained protein standards (Bio-rad #1610373, Hercules, CA). After the running, the protein bands in the purified extracts were visualized by staining with PageBlue™ protein staining solution (ThermoFisher Scientific, # 24620, USA). The molecular weight of the M-LBP was estimated from the SDS-PAGE by comparison with the pre-stained proteins.

2.1.3.2 Determination of protein concentration by Bradford assay

Bradford assay was used to determine the M-LBP concentration (Kruger, 1994). A calibration curve was established using Bovine serum albumin (BSA). Different amount of BSA; 0 mg, 0.1 mg, 0.2 mg, 0.5 mg, 1 mg, 2 mg, 5mg, were used as the standard to determine the concentration M-LBP. 20 µl of each protein sample was combined with 200 µl protein assay dye reagent, coomassie brilliant blue G-250 (bio-rad, #5000006, Hercules, CA), in a 96 well micro-plate. The UV absorptions were measured by BioTek Synergy HTX Multi-Mode Reader.

2.1.3.3 Immunohistochemical tests

Cross-reaction of the M-LBP with rabbit antibodies prepared from silk worm carotenoid binding protein, SW-CBP (a gift from Dr. Kozo Tsuchida, National Institute of Infectious Disease, Japan) and epidermal carotenoid binding protein, EH-CBP, from sweet potato hornworm, (*Agrius convolvuli*), (a gift from Dr. Koji Shirai, Shinshu University, Japan) were assessed to determine the possibility that the M-LBP represents an homologous protein to either of these previously described protein. Both, western blot and dot blot assays were conducted to test the protein homologies between this three insect species.

For western blots, 10 μ l of each 1 mg/mL protein sample, M-LBP, EH-CBP and SW-CBP was run by SDS-PAGE on an 8 % Tris-HLC polyacrylamide gel. During the staining process, the PVDF membrane was cut into appropriate size, and soaked in methanol for 2 min. The PVDF membrane was subsequently incubated in cold (0°C) transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3) for 5 minutes. Meanwhile, the gel, filter papers and sponges were also soaked in the ice cold transfer buffer and equilibrated for 3-5 minutes. The protein was transferred from the gel to the methanol activated PVDF membrane as described below. First, the sandwich clip was unfolded and placed in a tray filled with transfer buffer (positive charged side down, negative charged side up), the buffer soaked sponge was placed on the positive charged side. The filter paper, PVDF membrane, gel, filter paper, sponge were then placed on the first sponge in sequence. Bubbles caught between PVDF membrane and gel

were carefully removed. Finally the negative charged side of sandwich clip was folded back to the positive charged side and clipped tightly. The transfer sandwich with gel and membrane was placed into the transfer chamber and transfer performed by running at 25 V overnight at 4 °C in a Mini Trans-Blot® Electrophoretic Transfer Cell (bio-rad, # 1703930, Hercules, CA). On the second day, the protein bands were transferred from gel to PVDF membrane and the membrane has been placed in a clean, small tray for conducting the western blot. The blocking of PVDF membrane was accomplished by soaking membrane in 10 ml of blotting buffer which is 1% bovine serum albumin (BSA) in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Polysorbate 20 (Tween-20)) (for 1 hour with shaking at room temperature. 10 µl 1 mg/mL primary antibody (1st Ab) was diluted in the blotting buffer in a 1:3,000 ratio and incubated for 1 hour with shaking at room temperature. The membrane was then washed 3 times with 10 ml of TBST buffer for 5 min with shaking at room temperature. 2 µl 1 mg/mL secondary antibody (2nd Ab) anti-rabbit IgG with Horse Radish Peroxidase (HRP) (abcam #ab7621, Cambridge, MA) was diluted in 10 ml TBST buffer in a 1:5,000 ratio for 1 hour with shaking at room temperature. Subsequently, the membrane was washed 3 times with 10 ml TBST buffer for 5 min with shaking at room temperature. For signal development, chemiluminescence detection reagent SuperSignal™ ELISA Pico Chemiluminescent Substrate 1 and 2 (Thermo Fisher, #37070, USA) were mixed in a 1:1 ratio and added to the membrane for 5 min period at room temperature. A LI-COR C-DiGit Blot Scanner was used to visualize the chemiluminescence signal.

In a second immunoassay was performed on the protein extract as a dot-blot. The dot-blot is a simplification of western blot intended to detect presence of protein in an impure protein extract or in cases where transfer of the protein is incomplete limiting the reliability of the western blot assay. Instead of running an SDS PAGE, the sample is directly deposited on the membrane. The dot-blot prevents the loss or smearing of the protein sample by skipping the SDS-PAGE step, and thus provides a more distinct result. In this experiment a grid was drawn on the PVDF membrane by pencil and 4 μl of each 1 mg/mL protein sample was applied to one of the grid sections. The membrane was left to dry for 10 min. The membrane was subsequently treated as described above following the western blot to assess cross-reaction.

2.1.3.4 Mass spectrometry

Mass spectrometric analysis was performed with the intact protein to confirm the molecular mass estimated by SDS-PAGE.

Direct injection of the intact protein using Electrospray tandem MS (Agilent 6530 Accurate-Mass Quadrupole-Time of Flight LC/MS) was conducted to determine molecular mass. This experiment was performed with the help from Vanesa Thompson from Dr. Anthony DeCaprio's lab. Prior to running the protein sample the instrument was cleaned with methanol and a blank run was performed to establish background levels. The syringe was cleaned three times with 50:50 methanol: water. The mixture consisted of a spray mix (0.1% Formic acid in 50:50 acetonitrile: water) injected by the syringe and run for a 10 minute

period. After allowing the baseline to stabilize for about two minutes, deionized water was injected and served as the blank baseline. The baseline spectrum was recorded for later reference. After running the blank the protein sample, 100 μ l 1mg/mL M-LBP was washed four times with deionized water in a spin column, Amicon Ultra-2 Centrifugal filter units-10,000 NMWL (EMD Millipore # UFC201024, Darmstadt, Germany) to remove all the reagents in the solution. Subsequently, the M-LBP was redissolved in deionized water and 10 μ l 0.2 mg/mL protein sample was injected in the MS inlet. Data were collected over a mass range of the system set to cover 100 to 4000 m/z. After ensuring sufficient time to allow the electrospray to stabilize producing a response of about 150 to 200 ions per scan data were collected. It was necessary to combine scans to improve the signal to noise response and produce a meaningful mass spectrum. Background subtraction produced a clean and smooth spectrum. The highest molecular mass of the protein was extracted for M-LBP by deconvolution which was performed by qualitative analysis software BioConfirm from Agilent.

Electrospray analysis of M-LBP protein following lysis with trypsin was conducted at the University of Utah in collaboration with the Paul Bernstein. This analysis provided mass matches of the resulting polypeptides of the purified M-LBP with the NCB library using a Mascot database search.

2.1.3.5 Preparation of the apo-protein

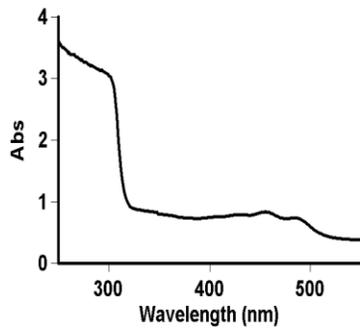
Extraction of the carotenoid from M-LBP to produce an apoprotein was conducted in a manner similar to that described by Rao et al (Rao et al., 1997).

500 μ l 2 mg/mL aliquots of the M-LBP concentrate in CHAPS/MES buffer (25mM CHAPS, 20 mM MES, 1 mM CaCl₂, 2mM MgCl₂, pH 5.5) was extracted with 500 μ l of a hexane solution containing 5% v/v 1-butanol. After shaking gently the sample was placed in a refrigerator at 4 °C for 8 hours. Low speed centrifugation (3000 g) at 4 °C for 10 min ensured separation of the organic and aqueous phases. The organic supernatant was removed and dried under nitrogen gas for subsequent HPLC quantification of the extracted carotenoid. This process was repeated three times to ensure quantitative removal of the carotenoid from the protein. The UV-visible spectrum was used to monitor the extent to which the carotenoid was removed. The extracted carotenoid was characterized by HPLC. The dried carotenoid was redissolved in ethanol and injected into a reversed-phase HPLC column (Phenomenox ODS Ultracarb 3 μ m, 250 mm \times 4.6 mm column), the mix solvent acetonitrile/methanol/TEA (85%/15%/0.1% v/v) was used as the mobile phase. An authentic sample of lutein extracted from marigold flowers was used as an authentic comparative standard.

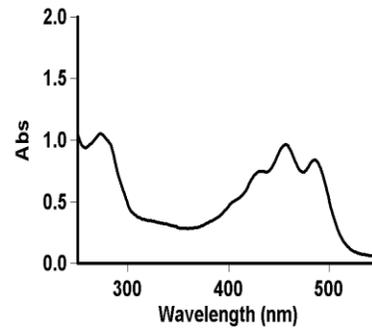
2.2 Results

Purification of the M-LBP followed, with minor modifications, the procedure used successfully by Tsuchida et al, Rao et al. and Bernstein et al (Bhosale et al., 2009; Rao et al., 1997; Tabunoki et al., 2002). For the characterization of SW-CBP, the ferret liver β -carotene binding protein and the carotenoid binding proteins found in the human macula (Bhosale et al., 2009; Tabunoki et al., 2004; Tabunoki et al., 2002). By comparing the 280 nm absorption of the protein

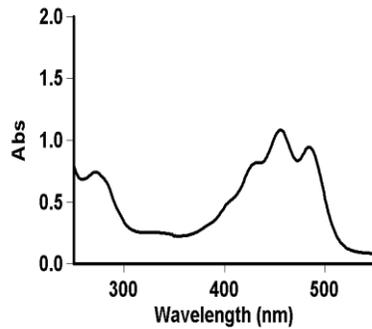
aromatic residues to the of 450 nm characteristic carotenoid the UV-Visible the extent of the purification was readily assessed. The A_{450}/A_{280} of initial protein extract could not be obtained because the solution remained turbid after low speed centrifugation step. After the first high-speed centrifugation step the protein sample was found to have an A_{450}/A_{280} ratio of 0.1: 1. Following ammonium sulfate differential precipitation the protein fraction precipitated in the 35-45% fraction had the highest A_{450}/A_{280} ratio (1: 1). SDS-PAGE separation of protein components undertaken at this step revealed that the extract still contained several proteins and subsequent purification steps were undertaken, including both anion and cation ion-exchange chromatography, and gel exclusion chromatography. Each step yielded an improvement in the A_{450}/A_{280} ratio. (Fig.16) During the purification process, the absorption corresponding to the carotenoid chromophore at 450 nm increased consistently relative to the 280 nm amino acid aromatic residue absorption of the protein. The A_{450}/A_{280} ratio reached a maximal value of ~2.9: 1 in the final chromatography step and corresponds to an enrichment by a factor of 25x compared to the extract obtained after the first high speed centrifugation step (Table 5).



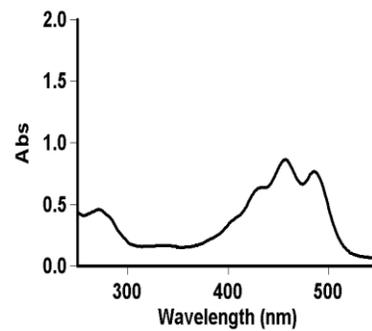
a. UV/Vis spectrum of the protein extract after the initial ultra-centrifugation step.



b. UV/Vis spectrum of the fraction of the protein extract collected by precipitation in 35-45% prepared from after the



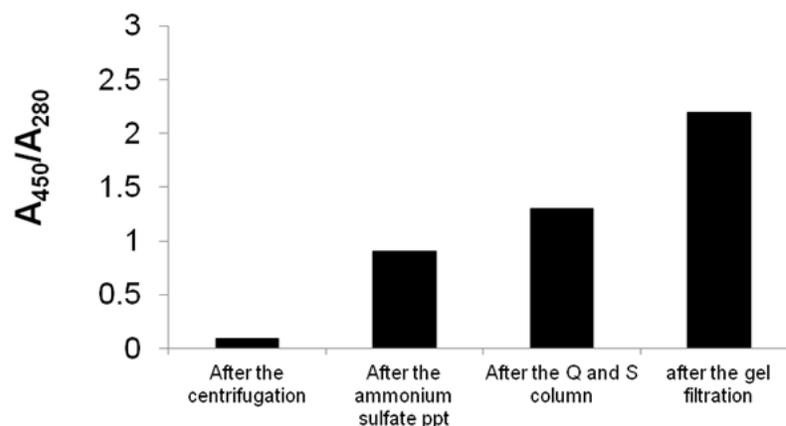
c. UV/Vis spectrum of the purified protein after ion exchange column (Q and S) chromatography.



d. UV/Vis spectrum of purified protein after gel filtration.

Fig. 16. UV/vis spectrum during the protein purification steps.

Table 5. The A_{450}/A_{280} ratio increase of M-LBP during the purification steps.



Gel electrophoresis carried out on the native protein after purification (Fig. 17) clearly demonstrates that the carotenoid is tightly bound to protein and no apparent dissociation was observed during electrophoresis. A single, (although broad) bright, yellow band was observed for the native protein after the electrophoresis. A subsequent SDS-PAGE also produced a single solid band aligning closely to a position corresponding to molecular weight of ~60kDa as determined by comparison with known standards present in the ladder in the adjacent lane (Fig. 18).

Measurement of the protein molecular mass was subsequently confirmed by electrospray tandem mass spectrometry through direct injection of the purified protein yielding a mass of 60,645 Da, consistent with the SDS-PAGE result (Fig. 18).

In order to determine the M-LBP amino acid sequence, M-LBP was digested by trypsin and fragments masses were determined using high resolution-tandem mass spectrometry. A Mascot search was employed to masses of individual

fragments to those previously reported. A search matching masses of M-LBP peptide fragments against the NCBI database reveals there are 62 proteins with matches. Among this total number of 62, 27 hits are from *Danaus plexippus*. Among the 27 hits from *Danaus plexippus* are listed with their scores and molecular mass in Table. 6.



Fig. 17. Native gel electrophoresis of purified M-LBP shows a single band of protein.

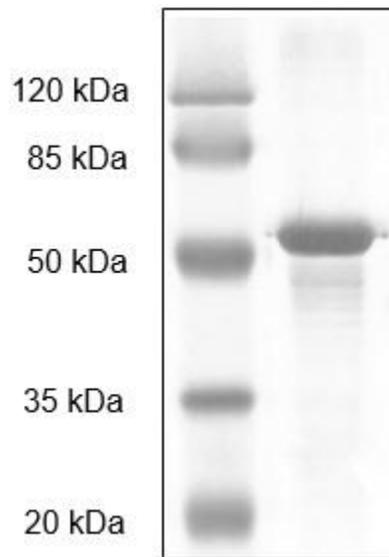


Fig. 18. SDS-PAGE of purified M-LBP (right)* with protein ladder (left).

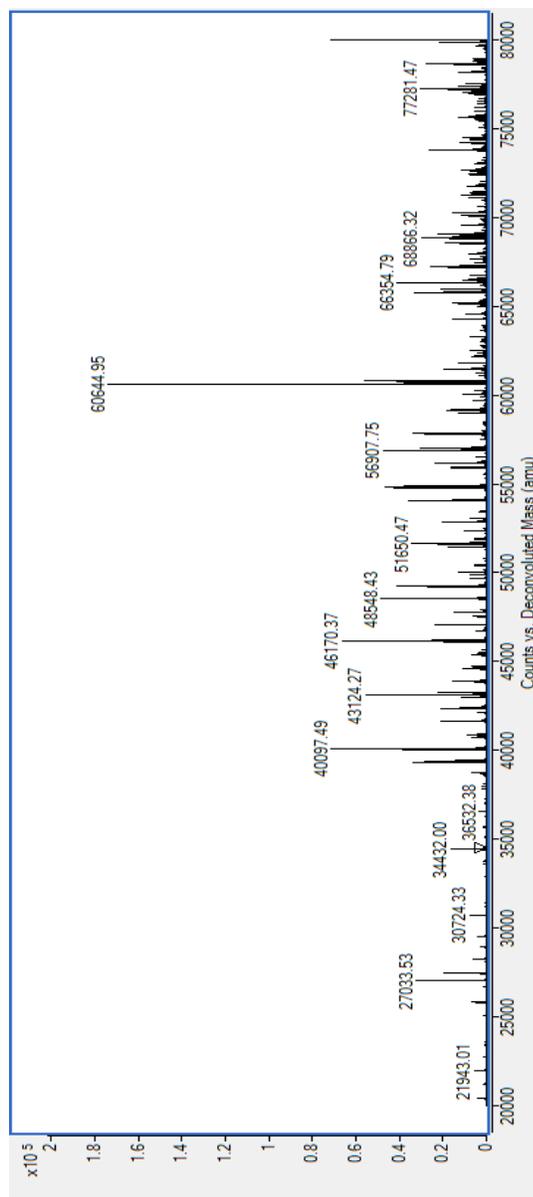


Fig. 19. HR-Mass Spectrum of purified M-LBP showing the 60,644 Da peak.

Table 6. Proteins with Matches in a Mascot Search of polypeptides produced by trypsin cleavage of M-LBP

Protein	Score	Mass
sarco/endoplasmic reticulum calcium ATPase	670	112684
Apolipoprotein-3	361	20542
putative serine protease-like protein 2	341	39265
putative plasma membrane associated protein, S3-12-like protein	330	68400 *
hypothetical protein KGM_13587	311	48845 *
cuticular protein CPR2	217	14255
hypothetical protein KGM_08664	215	11547
cuticle protein 1	183	11964
putative voltage-dependent anion-selective channel isoform 1	155	30116
hypothetical protein KGM_08146	143	36566
arylphorin-type storage protein	142	164790
cytochrome P450 4G49	125	42133 *
heat shock protein 60	125	56882 *
hemocyte aggregation inhibitor protein precursor	113	48073 *
glutathione S-transferase	104	23615
cyclophilin A	98	17830
reticulon/nogo	87	28035
mitochondrial aldehyde dehydrogenase	83	56289 *
progesterone receptor membrane component 2	80	13857
cuticular protein CPR3	77	14177
small GTP-binding protein	74	22385
cuticular protein RR-1 motif 46	69	N/A
catalase	67	56770 *
transmembrane trafficking protein	67	21294
putative stretch regulated skeletal muscle protein	66	5787
cytochrome P450 CYP4G48	64	23198
aquaporin AQP-Gra2	54	27102

Repetitive extraction of the buffer solution containing the M-LBP with equal volumes of a hexane solution with 5% v/v 1-butanol successfully removed the carotenoid from the protein. A UV-Visible spectrum obtained of the resulting apoprotein confirmed the absence of the 450 nm carotenoid absorbance (Fig. 20). Collection of the resulting hexane fraction containing the extracted carotenoid (Fig. 21), comparison to an authentic lutein standard and UV-vis spectrum of extracted carotenoid demonstrated that the bound carotenoid is exclusively lutein (Fig. 22). No other carotenoids were detected in this extract.

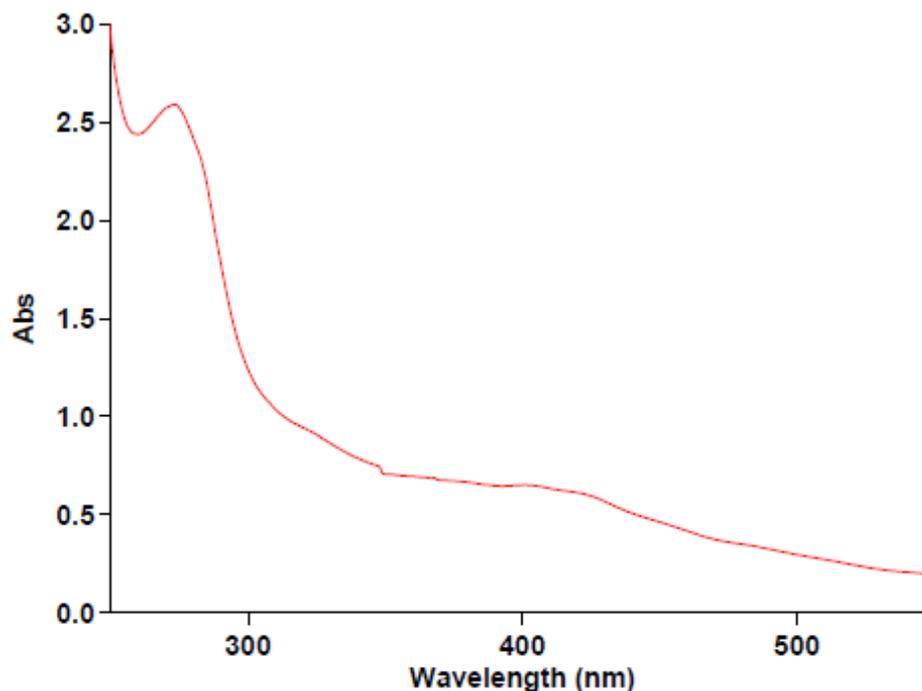


Fig. 20. UV-Visible spectrum of apoprotein showing the the absence of a significant lutein contribution at 450 nm.

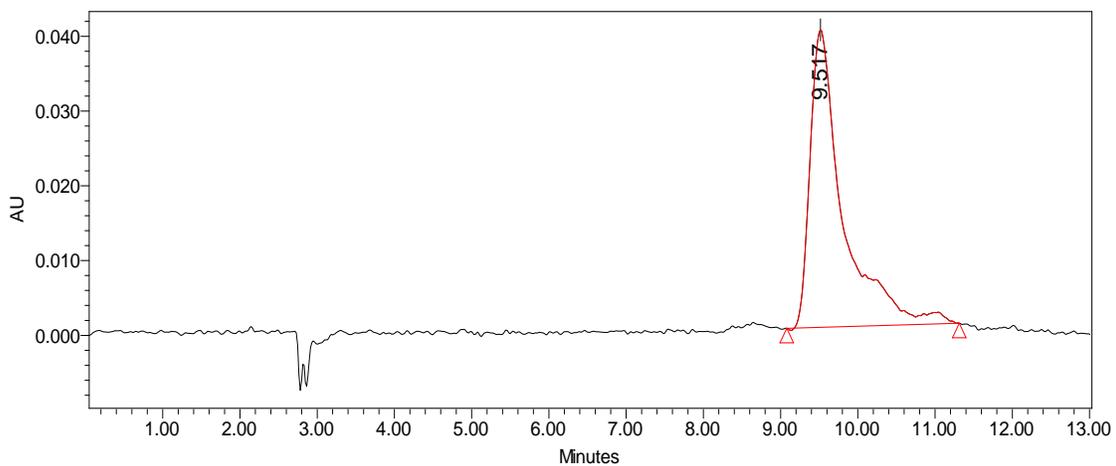


Fig. 21. HPLC chromatogram of extracted lutein from the apoprotein preparation, a small shoulder is consistent with the presence of a small quantity of a *cis*-isomer.

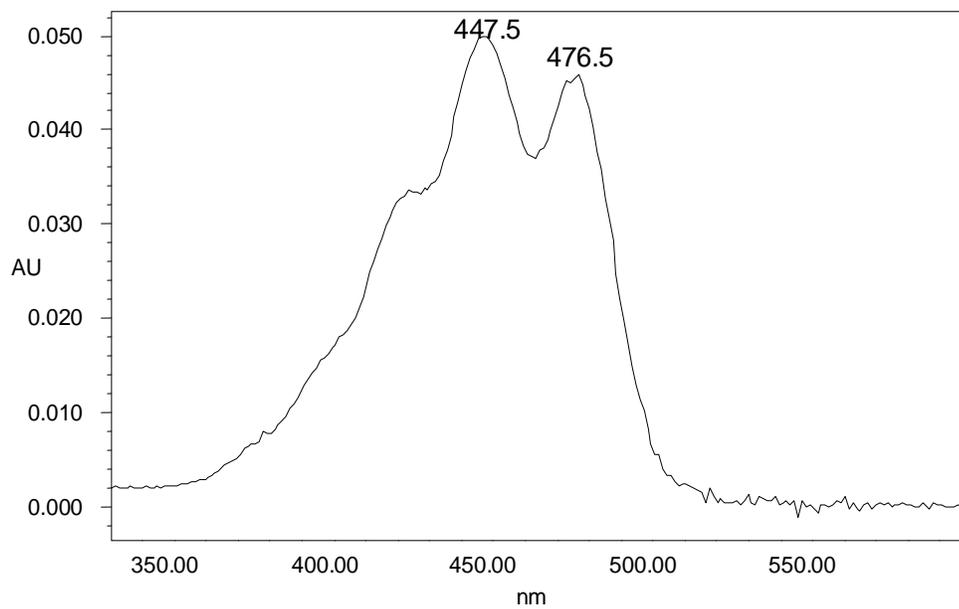


Fig. 22. UV/Vis spectrum of the extracted lutein.

To determine the protein to carotenoid ratio, the purified M-LBP was precipitated and weighed, the mass of lutein was calculated by UV absorption at A_{450} using the lutein mole extinction coefficient: 145,000 L/mol cm. We found, the mass ratio of protein to carotenoid is 58.7:1. Using the estimated protein molecular weight of 60 kDa a mole ratio of protein to carotenoids of 1.8:1 was determined. In another method, the protein concentration is determined by Bradford assay, we found the protein to carotenoid ratio is 1.7:1 which is consistent with the first estimation.

In experiments designed to assess homology of the M-LBP to other xanthophyll binding proteins that have been previously studied, a Western blot experiment was employed. Two lutein binding proteins, EH-CBP and SW-CBP, and their rabbit antibodies, from related species, the silk worm and the horn worm were tested. The results are shown in Fig. 23 & 24, M-LBP failed to bind rabbit anti-SW-CBP antibody. By the contrast, M-LBP shows a strong binding affinity with rabbit anti-EH-CBP antibody. In a dot-blot analysis, consistent results were obtained showing binding affinity of M-LBP with rabbit EH-CBP antibody but not with SW-CBP antibody (Fig. 25 & 26).

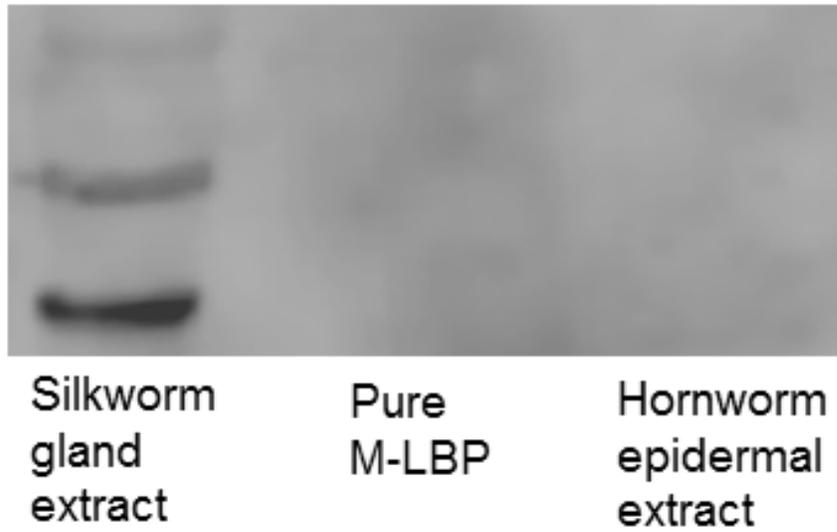


Fig. 23. Western blots of SW-CBP, M-LBP and EH-CBP after reaction with anti-SW antibody. Only SW-CBP shows a cross reactivity with anti-SW antibody.

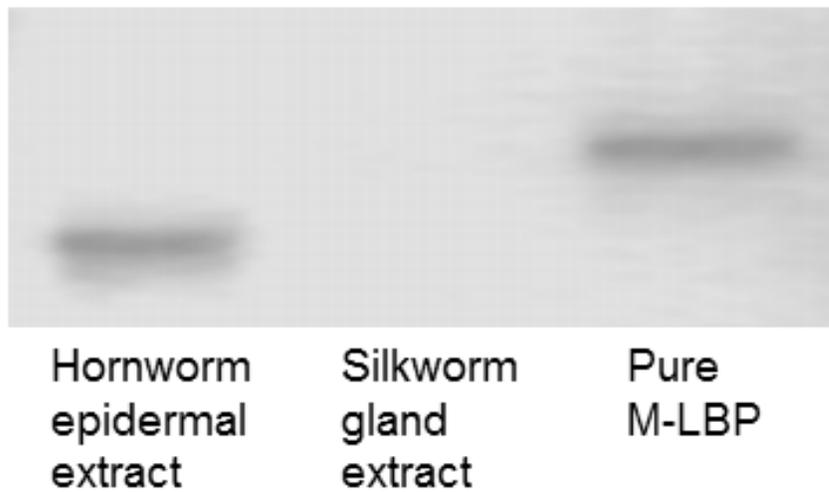


Fig. 24. Western blots of SW-CBP, M-LBP and EH-CBP with anti-HR antibody showing that both M-LBP and EH-CBP have cross react with anti-EH antibody. SW-CBP has no cross reactivity with this antibody.

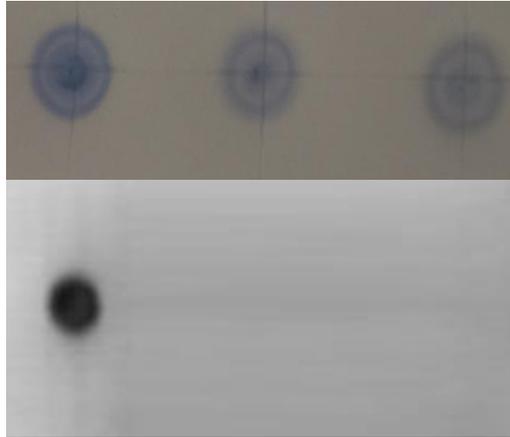


Fig. 25. Dot blots with SW-CBP, M-LBP and EH-CBP showing interaction with anti-SW antibody. Upper row: SW-CBP, M-LBP and EH-CBP were applied to the PVDF membrane and stained with coomassie blue. Lower row: dot blot shows only SW-CBP has cross reactivity with anti-SW antibody.

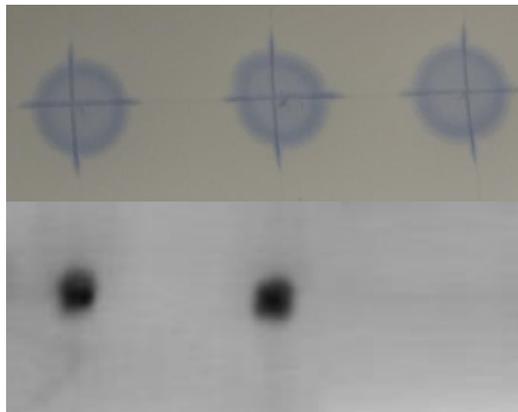


Fig. 26. Dot blots with SW-CBP, M-LBP and EH-CBP showing interaction with anti-EH antibody. Uper row: EH-CBP, M-LBP and SW-CBP were applied to the PVDF membrane and stained with coomassie blue. Lower row: dot blot shows both M-LBP and EH-CBP show reactivity with anti-EH antibody, only SW-CBP shows no cross reactivity).

2.3 Conclusion and discussion

The characteristic yellow-striped pattern of epidermal pigmentation in monarch butterfly larvae (*Danaus plexippus*) is the result of high concentrations of lutein. Purification of the protein homogenate obtained from the epidermis in a buffer system incorporating the CHAPS surfactant yields a carotenoid/protein complex that migrates as a single band during native protein electrophoresis. HPLC analysis of the carotenoid extracted from this protein extract demonstrates that lutein is the sole carotenoid present. Although zeaxanthin and β -carotene were detectable in the foliage of the larval diet foliage neither of these was detectable in the epidermis or the purified protein. Measured peak positions and relative intensities in the UV-visible spectrum of the carotenoid in ethanol after solvent extraction from protein match those of authentic lutein samples and the literature, $\lambda_{\max} = 447.5$ and 476.5 nm (lit. values 445 & 474) for the two major peaks and the QI/QII ratio is 60% (lit. value 60%) (Britton et al., 2004; Mercadante et al., 2004). This result contrasts with the result reported for the SW-CBP isolated from the silk gland. Although SW-CBP predominantly binds lutein it shows some promiscuity and an ability to bind the isomeric xanthophyll, zeaxanthin and even small amount of β -carotene a much less polar carotene lacking the hydroxyl functionality. SW-CBP appears to have a lower fidelity to its primary ligand than M-LBP. (Tabunoki, 2002) The carotenoid binding affinity of SW-CBP has been determined and the comparison of binding affinity between SW-CBP and M-LBP is discussed as a topic in Chapter 3. The fully purified

protein obtained after size exclusion chromatography (see purification, Scheme I) shows an enrichment of the carotenoid content by a factor of 25 compared to the extract obtained after the first high speed centrifugation step and exhibits an A_{450}/A_{280} ratio of 2.5/1 (Fig. 15).

As determined by SDS PAG electrophoresis and confirmed by HR-tandem MS, M-LBP yields a molecular mass of 60 kDa. The masses of polypeptides produced by trypsin cleavage of M-LBP were analyzed by HR-tandem mass spectrometry. Mascot and the matches are shown in Table 6. 62 candidates were produced by the Mascot search containing 27 hits that belong to *Danaus plexippus*. After filtering these results for the protein molecular weight of ~60 kDa, 7 candidates remained (labeled with * in table 6). These data do not provide sufficient information to unambiguously assign the identity of M-LBP. The search result including multiple proteins is due to the high sensitivity of the method and the likely presence of trace quantities of other proteins within the sample used for this analysis. Protein matches to species other than *Danaus plexippus* are ascribed to cross-contamination in the handling of the sample or background contamination in the mass spectrometric system. Unambiguous determination of the identity of the protein will require further HD-MS analysis.

An estimate of the molar mass of the protein per carotenoid based on a nominal protein aromatic amino acid composition was calculated by using the following equation (Layne, 1957).

$$\text{mg of protein per ml} = 1.45 \times (A_{280} - 1.15 \times A_{450}) - 0.74 \times (A_{260} - 2.27 \times A_{450})$$

By using the A_{260} , A_{280} , & A_{450} values, (figure 16d). Based on the 60 kDa protein molecular weight, an estimate of the stoichiometry of lutein binding is estimated to be 1.8 carotenoid molecules per protein. This ratio has also been confirmed by protein concentration determined by Bradford assay (Kruger, 1994). We tentatively conclude the nominal lutein/protein stoichiometry is 2: 1. This stoichiometry distinguishes the M-LBP from previously reported proteins which all appear to bind only a single carotenoid. Comparing the carotenoid binding proteins from most close species, M-LBP has a molecular weight of 60 kDa, approximately double those of EH-CBP and SW-CBP SW-CBP has a reported molecular weight of 33 kDa and EH-CBP is reported to be 28 kDa. Both of SW-CBP and EH-CBP possess a 1:1 ratio of protein to carotenoid while the protein to carotenoid ratio is 1:2. These data suggest it is a possibility that M-LBP maybe a dimer.

A Western Blot of M-LBP using SW-CBP antibody reveals no cross-reactivity exists between these two proteins and they are likely completely different proteins. EH-CBP antibody does cross react but the 60kDa molecular weight of M-LBP is twice that of, 27kDa. The possibility must be considered that the M-LBP is a dimer of the previously described EH-CBP from the horn worm epidermis. Moreover, M-LBP binds two lutein molecules also suggesting the possibility of a dimer. Moreover, both of M-LBP and EH-CBP are expressed in the epidermis.

The identity of the EH-CBP has been suggested to be the juvenile protein of the hornworm (Shirai, 2006). The complete genome has been published for both species, horn worm and monarch. Results from a mass spectrometric analysis of peptides produced from a sample M-LBP revealed peptide matches from 27 monarch proteins. A cross search of the monarch genome for a match to the hornworm juvenile protein (EH-CBP) was also undertaken. The proposed sequence of the EH- CBP does not match any of the proteins that were found in the MASCOT search based on lysis of our M-LBP. Given the cross-reaction to the EH-CBP antibody we are led to consider the possibility that the assignment of EH-CBP to the hornworm juvenile protein may be in doubt.

Chapter 3: Surface plasmon resonance (SPR) studies on the interaction of M-LBP with carotenoids

3.0 Introduction

Evidence shows only the single carotenoid, lutein, is present in the yellow epidermal stripes of monarch butterfly larvae. It is bound there to a protein that is highly specific M-LBP that is presumed to mediate the transportation, deposition and stabilization of carotenoid into the target tissue. To better understand the binding interaction between M-LBP and carotenoids, the protein binding affinities were determined by surface plasmon resonance. The binding affinities of M-LBP to five carotenoids, lutein, zeaxanthin, R,S-*meso*-zeaxanthin, astaxanthin, and β -carotene were determined. SPR is a convenient, sensitive, real-time optical method for measurement of the on/off rates of interaction between the ligands and the protein. It is especially attractive because it can be carried out without the use of labeled molecules (Karlsson, 2004). Due to the high sensitivity and reproducibility, SPR provides very reliable analytical consistency (Homola et al., 1999). SPR instruments are composed of a sensor chip possessing a coating by ultra-thin metal film on the functional side, a flow channel, a probe laser source and a diode array detector (Fig. 26) (Cooper, 2002). To determine the molecular interaction, one of the participant species must be immobilized on the inner metalized surface of the sensor chip. The corresponding binding component is introduced through the flow channel at controlled flowrate and concentration. The interaction of the analyte and its

binding partner on the surface of the chip results in a minor structural change and shifts the polarity at the thin surface which in-turn alters the refractive index and induces a shift in the angle of reflection of the probe beam angle. (See Figure 27) The shift in angle of the probe beam is monitored and recorded. The result change in the refractive index is plotted as a response versus time (Cooper, 2002).

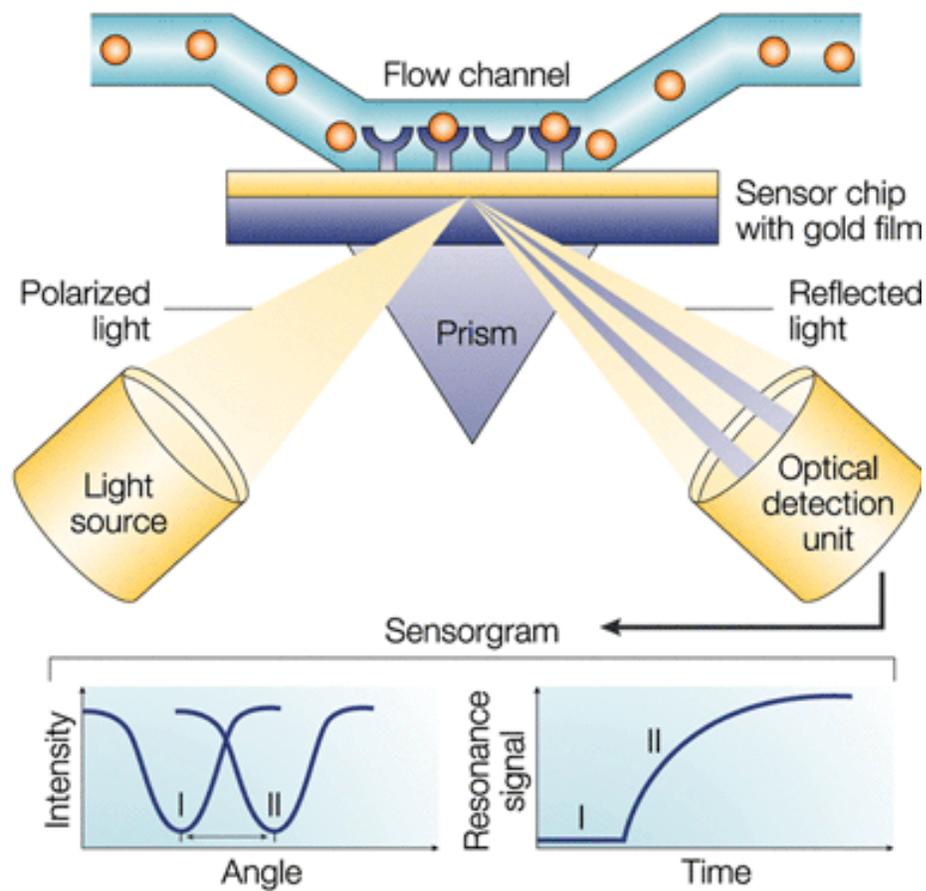


Fig. 27. Schematic diagram of SPR apparatus, the effect of changes at the sensor chip surface on the signal and the resultant sensorgram. (Cooper, 2002).

3.1 Experimental

3.1.1. Instrumentation

SPR measurements were conducted on an automated SensiQ Pioneer optical biosensor (SensiQ Technologies, Inc., Oklahoma City, OK) equipped with a HC1000 sensor chip (XanTec bioanalytics GmbH, Germany) at 25 °C. The sensing surface is a planar glass slide coated with a ~50 nm gold film. SensiQ is a miniature of dual-channel, semi-automated SPR system. Since the Kretschmann configuration is applied to SensiQ system the metal film is attached onto the glass chip directly. The polarized monochromatic light strikes the glass and penetrates to the metal film and is reflected to the photodiode array. The angle shift of the reflected beam is measured by determine the position on the photodiode array.

3.1.2. Carotenoid preparation

Five carotenoids lutein, zeaxanthin, R,S-*meso*-zeaxanthin, astaxanthin, and β -carotene were dissolved in the sucrose monolaurate (2 mM) (Mitsubishi Chemicals, Tokyo, Japan) (Fig. 28).

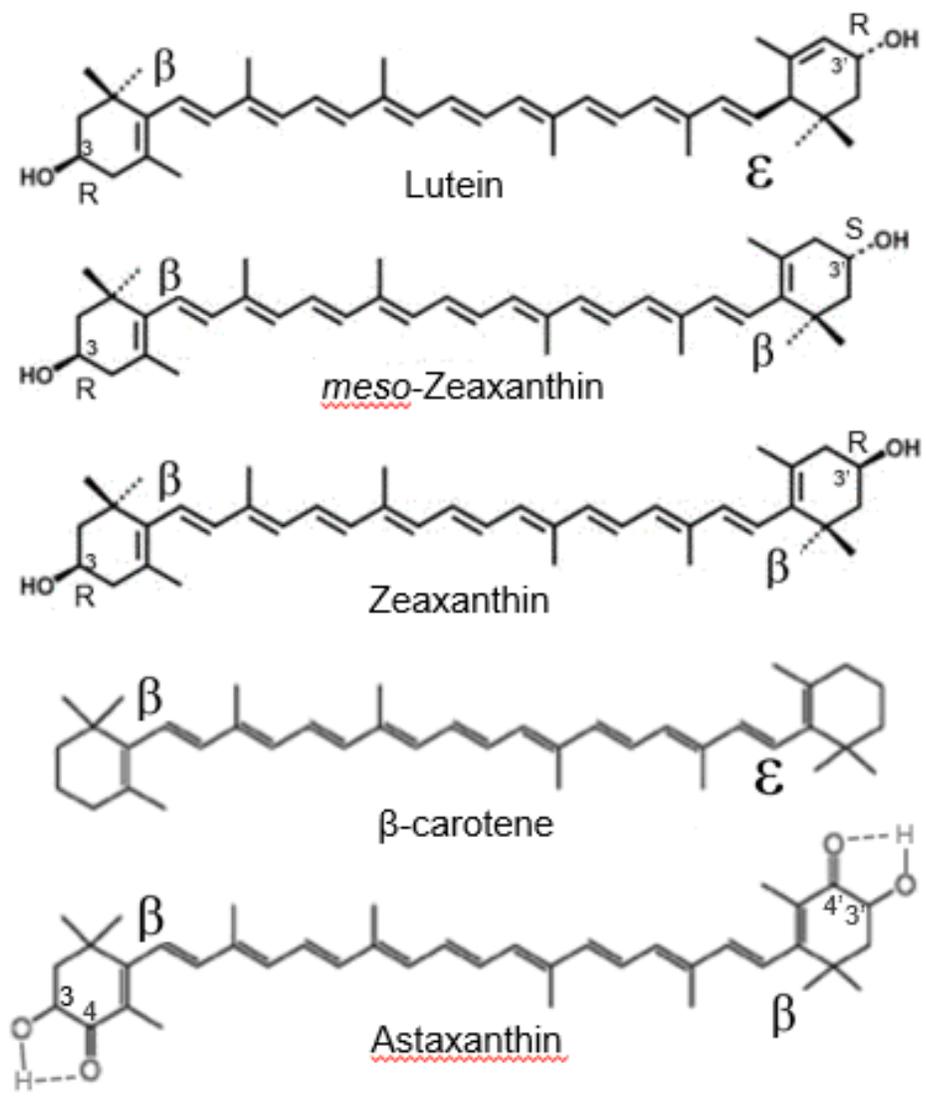


Fig. 28. The structures of carotenoids tested in SPR

3.1.3. Protein immobilization

The SPR signal is referred to a sensorgram as response unit (RU). RUs are directly proportional to the measured change in the angle of reflection of the probe laser beam from the glass sensor inner surface. 1 RU is equal to 10^{-6} refractive index units, which represents approximately 1 pg of protein/mm² (Roper, 2007). Binding of the carotenoid to the bound protein produces a change in the refractive index that is evidence by a change in the angle of the probe beam reflected and a change in the RU in the sensorgram. The M-LBP apoprotein was extracted from purified M-LBP by hexene/1-butanol mixture. The concentration of apoprotein was measured using a Bradford assay to produce a final concentration of 1 µg/µl. The M-LBP apoprotein was immobilized on the sensor chip surface following standard amine coupling methods (Vachali et al., 2013). The flow channel was washed with degassed water followed by a degassed running buffer (10 mM PBS, 0.01% (v/v) Tween-20, 1 mM EDTA, 5%(v/v) DMSO, pH 7.4) to prime the instrument. Four aliquots of ~ 1ml each at 200 µl/min flow rate. To ensure an absence of all the interfering components and any bound ligands are dissociated the functional polymer, surface is moisturized and conditioned with a 1 minute pulse of degassed SPR running buffer containing 50 mM HCl added at 10 minute intervals. After the clean-up step, the degassed SPR running buffer was left running at a low flowrate 10 µl/min prior to further preparation. To immobilize the M-LBP apoprotein, the amine coupling reagent mixture containing N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-

dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (GE Healthcare, Wauwatosa, WI) in 1:1 ratio was injected to the system to activate the surface modifying the polymer headgroup to produce an N-hydroxysuccinimide ester. This step was followed by treatment with M-LBP apoprotein diluted in 10 mM sodium acetate (pH 5.0) at concentration of 10 $\mu\text{g}/\text{mL}$ and injected at a flowrate of 10 $\mu\text{l}/\text{min}$. The injected M-LBP apoprotein forms a covalently bound complex with N-hydroxysuccinimide ester on the sensor chip through amines from the protein amino acid residues. A reference surface was prepared in an identical manner using human serum albumin (HAS). Subsequently, sodium ethanolamine hydrochloride (1M, pH 8.5) was injected to blocking and deactivates the remaining non-bonded N-hydroxysuccinimide ester groups. Immediately after the deactivation of N-hydroxysuccinimide ester group, the SPR system was switched to SPR running buffer to wash all of the coupling reagents from the system prior to measurements (see Fig. 29).

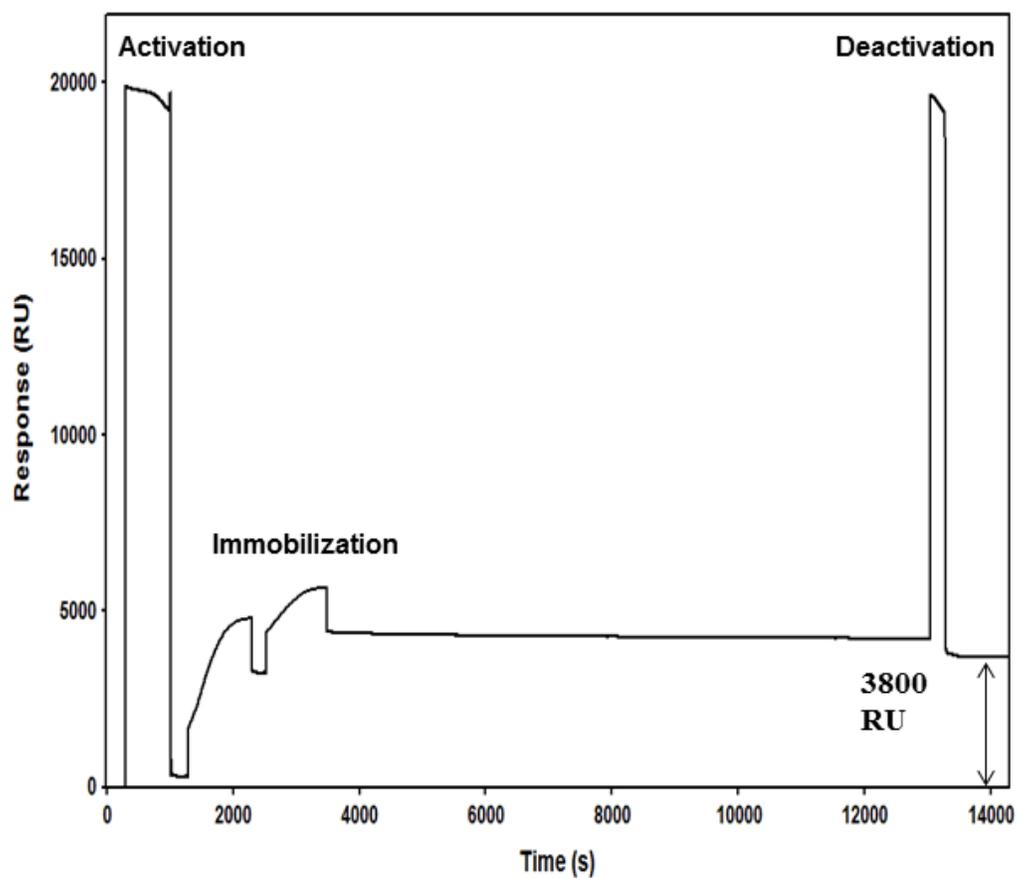


Fig. 29. Sensorgram of M-LBP immobilization process (RU versus Time)

3.1.4. Protein binding affinity measurements and data analysis

Before each run, SPR response data (sensorgrams) were zeroed on both the response and time axes. First, bulk refractive index was tared by comparison to the response of an unmodified reference surface compared to the response obtained by the surface to which the protein is coupled. Second, any systematic interferences with the flow cells was removed by subtraction of the average buffer injection response from the carotenoid binding response. For each measurement a single carotenoid was injected. Kinetic binding rate, k_b , was determined by measuring the response of the surface bound apo-protein to the stepped gradient of the carotenoid solution. The SPR studies were carried out using the SPR one-stepTM injection method (SensiQ Technologies, Inc., Oklahoma City, OK). The carotenoid concentration was stepped to a concentration of 1 μ M, with the exception of β -carotene for which a limit of 200 nM was used. In a similar analysis the kinetic dissociation rate, k_d , was determined by monitoring the response of the SPR when the carotenoid concentration is stepped to zero. K_D values are determined from the ratio of k_b to the k_d values, k_d/k_b . SPR sensorgrams were re-zeroed for each single run. Kinetic rate constants were extracted by QdatTM analysis software (Biologic Software, Australia). The equilibrium dissociation constants (K_D) were determined using QdatTM analysis software (Table. 7).

3.2 Results

This investigation of the binding specificity and affinity was undertaken using the apo-protein coupled to the active surface of the SPR sensor. Due to their hydrophobic nature, carotenoids are insoluble in aqueous solution and prone to stick to the sensor surface. Use of sucrose monolaurate enables the carotenoids to be nano-dispersed in the aqueous solution. The sensorgrams consist of the Response Units (RU) versus Time (s). Measurements were obtained for the carotenoids lutein, zeaxanthin, R,S-*meso*-zeaxanthin, astaxanthin, and β -carotene and were recorded (Fig. 30 to Fig. 32). The equilibrium dissociation constants (K_D) were converted by QdatTM analysis software (Tab. 7).

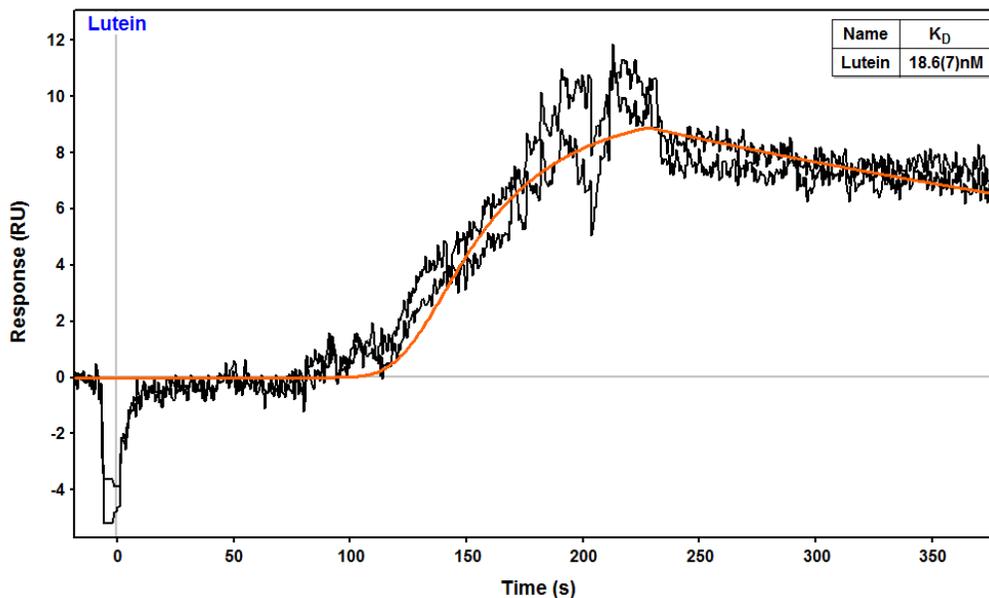


Fig. 30. SPR response of lutein bound to M-LBP via one-stepTM injection method.

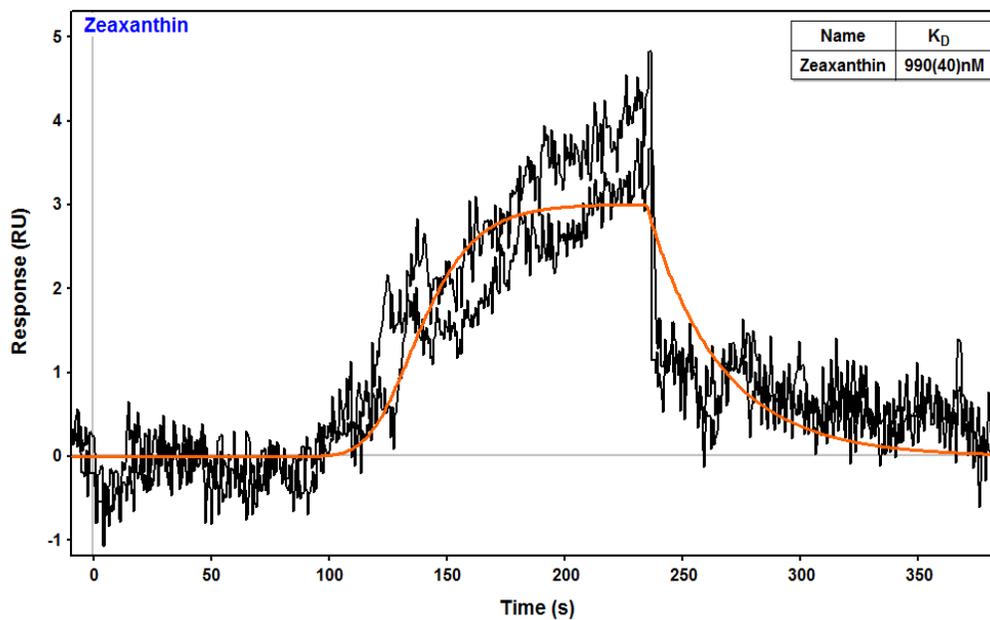


Fig. 31. SPR response of zeaxanthin bound to M-LBP via one-stepTM injection method.

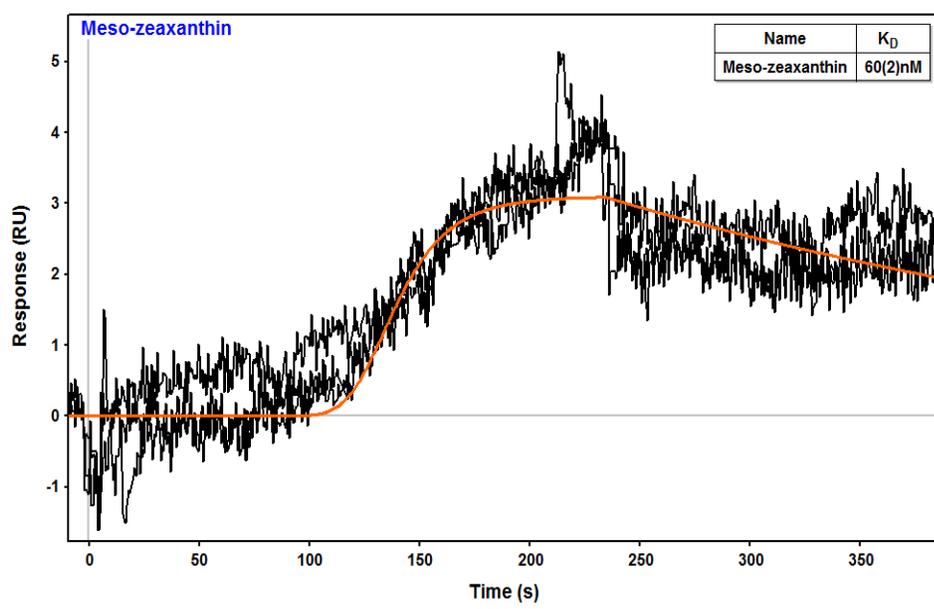


Fig. 32. SPR response of *meso*-zeaxanthin bound to M-LBP via one-stepTM injection method.

Table 7. Equilibrium dissociation constants (K_D) of M-LBP apoprotein with five carotenoids

Carotenoids	K_D (nM)
Lutein	18.6 ± 0.7
Zeaxanthin	990 ± 60
<i>meso</i> -Zeaxanthin	60 ± 2
Astaxanthin	N/A
β -Carotene	N/A

3.3 Conclusion and discussion

Table 7 summarizes the results obtained from SPR measurements of M-LBP apoprotein with five structurally unique carotenoids. In this study, lutein was found to have the highest affinity for binding to M-LBP with a K_D value 18.6 ± 0.7 nM. The K_D value of *R,S-meso-zeaxanthin* is 60 nm, the carotenoid with lowest binding affinity to M-LBP apoprotein is zeaxanthin ($K_D = 990 \pm 60$ nM). Astaxanthin and β -carotene did not bind appreciably under these conditions. Although the binding constant for *R,S-meso-zeaxanthin* ($K_D = 60 \pm 2$ nM) was much stronger than that of *R,R-zeaxanthin* and approached a value comparable to that of lutein. *R,S-meso-zeaxanthin* is not a naturally occurring carotenoid formed by higher plants. As such it is not a dietary component available to the monarch larvae in nature. The comparison of the K_D values of these carotenoids demonstrates that binding of lutein ($K_D = 18.6 \pm 0.7$ nM) is favored over zeaxanthin ($K_D = 990 \pm 60$ nM) by a factor of 50x. These measurements

demonstrate that the binding of lutein to the native protein is a high fidelity interaction specific for this carotenoid. The broadly similar structures and sizes of these carotenoids make it evident that the selectivity of binding must arise from congruence between the topology of carotenoid end-group, as determined by the conformation and the stereochemistry of the hydroxyl substituent of the ionone ring (Fig. 32). The shape of the pocket interacting with the carotenoid end-group including positions of potential H-bonding side-chains are anticipated to be crucial determinants of these interaction.

Comparison of structural differences between lutein which has the highest binding affinity and that β -carotene and astaxanthin which did not measurably bind to M-LBP shows that the 3, 3'-hydroxyl group on the end-ring of lutein is required for carotenoid binding. Failure to observe measureable binding of β -carotene to the protein demonstrates the essential nature of the presence of the 3'-hydroxyl substituent for the docking of the carotenoid within the protein pocket. Comparing lutein and astaxanthin, it is seen that a strong intra-molecular hydrogen bond exists between the 3'-hydroxyl and the adjacent 4'-carbonyl oxygen in astaxanthin that appears to interfere the docking of the hydroxyl group to the protein.

Comparing the carotenoid binding affinities we observe that the differences in the structures of the carotenoids is restricted exclusively to a single end-group on the carotenoid. The right hand ring (Fig. 33) of these top three carotenoids has a strong influence on the binding affinity. A 3.2 times greater relative binding

strength for lutein relative to R, S-*meso*-zeaxanthin. This difference in binding strength suggests that the binding pocket has a preference to interact with carotenoids possessing an ϵ -ring over those with a β -ring. We have previously reported that the ϵ -ring prefers to adopt a lowest energy conformational geometry placing the mean plane of the ring at a 70° angle to the polyene chain, a shape we liken to the relationship of the blade of a hoe and its handle. This contrasts with the optimum conformational geometry adopted by the β -ring with an angle of 170° , essentially co-linear, to the polyene chain and comparable to the blade of a spade and its handle. Lutein and *meso*-zeaxanthin share an identical stereochemical configuration for the 3'-hydroxyl group (on the ϵ -ring of lutein and the β -ring of *meso*-zeaxanthin). A comparison of the relative binding affinity of *meso*-zeaxanthin to that of zeaxanthin indicates that the hydroxyl group stereochemistry has a strong influence on K_D . The stronger binding constant for *meso*-zeaxanthin $K_D = 60$ as compared to $K_D = 990$ for zeaxanthin suggests that inversion of the absolute configuration has a larger influence on K_D than ring type. The binding strength of *meso*-zeaxanthin is 17 times that of zeaxanthin. *Meso*-zeaxanthin and lutein share an identical S stereochemical configuration of the 3'-hydroxyl ring directing the hydroxyl group toward the same side of the ring in each carotenoid. Presumably there is a critical H-bonding interaction that prefers the hydroxyl group to be directed spatially to one side of the carotenoid; this matches that in lutein and *meso*-zeaxanthin but not zeaxanthin. It is possible to speculate this requires the end-ring in zeaxanthin to rotate 180° about the C6-

C7 bond to place the hydroxyl group in the correct orientation at a significant cost in energy. When both the end-ring and the hydroxyl group are different from that of lutein, as seen in zeaxanthin, the combined effect on the binding strength is a reduction by a factor of 1/54.

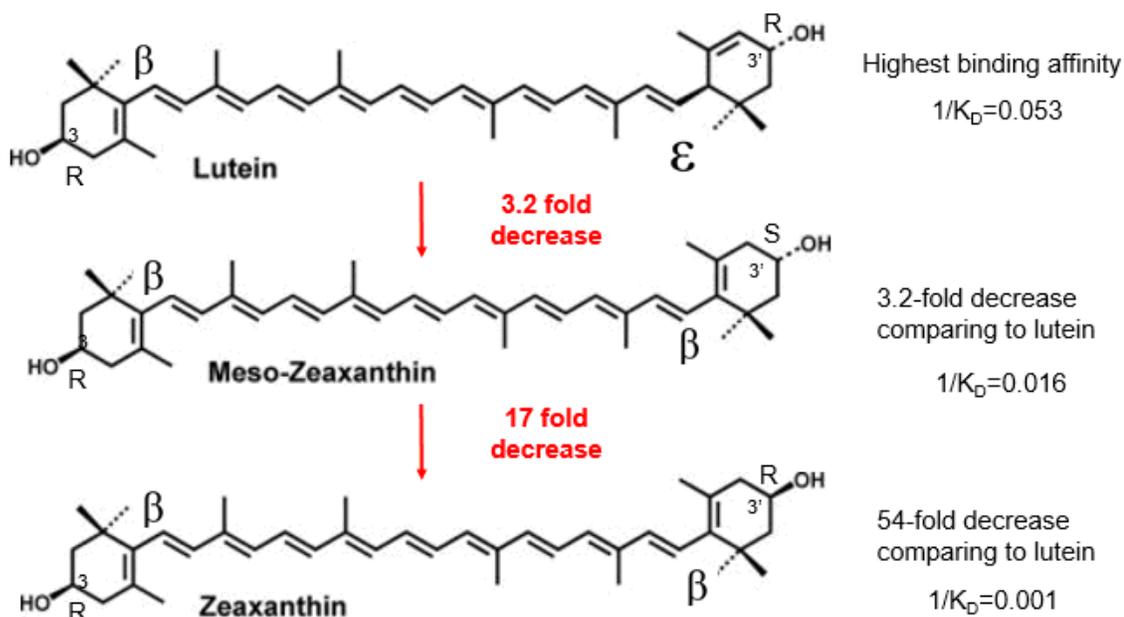


Fig. 33. Comparison of K_D values of three carotenoids

The protein binding affinities of carotenoids with other lutein binding protein have been assessed (Table 8). Human retina lutein binding protein (StARD3) and the carotenoid binding protein found in the silk gland of the silk worm (SW-LBP) are also known to specifically bind lutein. The Human retina zeaxanthin binding protein (GSTP1) also binds lutein, although the binding affinity for lutein is not as strong as zeaxanthin. As mentioned previously, both StARD3 and SW-LBP belong to the StARD protein family (Li et al., 2011). The versatility of StAR proteins in their ability to transport a variety of lipophilic components, specifically

cholesterol, phosphatidyl choline, and ceramides, is widely recognized but it has only recently become clear that they are the critical proteins responsible for specific mobilization of lutein in different tissues for a variety of species (Alpy and Tomasetto, 2005). Comparison of the K_D values measured for these proteins with that of the M-LBP show that these lutein binding protein possess strong binding affinity and a relatively high selectivity towards lutein over other carotenoids. SW-LBP and StARD3 although produced by remarkably dissimilar species are homologous proteins and as such are anticipated to share many characteristic features. StARD3 shows considerable homology as indicated by Western blot and cross-reactivity of this protein to the rabbit antibody for SW-LBP. Comparison of lutein binding constants measured for SW-LBP and StARD3 shows further similarity at the functional level. Both of them show their highest binding specificity with lutein. Moreover for StARD3 zeaxanthin ($K_D=1.60 \mu\text{M}$) and *meso*-zeaxanthin ($K_D=1.63 \mu\text{M}$) have values that are essentially identical. For SW-LBP, the K_D value of zeaxanthin ($1.24 \mu\text{M}$) is also very close to K_D value of *meso*-zeaxanthin ($1.14 \mu\text{M}$). SW-LBP has a three-fold lower (stronger binding) K_D value (0.18nM) for lutein as compared to StARD3. SW-LBP also has a stronger binding affinity zeaxanthin and *meso*-zeaxanthin compared to StARD3. For SW-LBP and StARD3, binding selectivity of lutein favored over zeaxanthin is less than 10 times while lutein selectivity of M-LBP over zeaxanthin is more than a factor of 50. The immunohistochemistry demonstrates StARD3 has positive homology and cross-reactivity against SW-LBP derived rabbit antibody (Li et al., 2011). In contrast, immunohistochemistry demonstrates that M-LBP has no

homology and cross-reactivity against SW-LBP derived rabbit antibody. The specific structural factors responsible for the selectivity of these carotenoid binding proteins toward lutein as a ligand, (as opposed to other structurally similar carotenoids), remain unresolved. Crystal structures have been solved for 3 related StAR proteins, metastatic lymph node 64 (MLN64) (Tsujiata and Herley, 2000), Human phosphatidylcholine transfer protein (Roderick et al 2002), and Mus cholesterol-regulated START protein (START D4) (Romanowski, 2002). Although it is anticipated that the site of cholesterol binding likely overlaps that for lutein it appears that a structure containing carotenoid ligand will be needed to establish the detailed binding geometry in sufficient detail to enable meaningful conclusions. GSTP1 shows relatively high binding affinities for zeaxanthin ($K_D=0.14 \mu\text{M}$) and *meso*-zeaxanthin ($K_D=0.19 \mu\text{M}$). This result is consistent with the early finding that GSTP1 is the protein specific for zeaxanthin binding in human retina (Bhosale et al., 2004). The binding selectivity of zeaxanthin and *meso* zeaxanthin is favored over other carotenoids by almost a factor of 10 times. The binding affinities of GSTP1 with lutein ($K_D=1.30 \mu\text{M}$), astaxanthin ($K_D=1.26 \mu\text{M}$) and β -carotene ($K_D=1.19 \mu\text{M}$) are relatively low. Compared with M-LBP, which shows the highest binding affinity towards lutein, the binding pattern of GSTP1 is strikingly different. This evidence suggests the carotenoid binding site of M-LBP is topologically different from that found in these other well-known carotenoid binding proteins (SW-LBP, StARD3 and GSTP1).

Table. 8. Equilibrium dissociation constants (K_D) of lutein binding protein with carotenoids

Carotenoids	Proteins			
	M-LBP(nm)	SW-LBP(μ m)	StARD3(μ m)	GSTP1(μ m)
Lutein	18.6 \pm 0.7	0.18 \pm 0.01	0.59 \pm 0.03	1.30 \pm 0.02
Zeaxanthin	990 \pm 60	1.24 \pm 0.03	1.60 \pm 0.01	0.14 \pm 0.02
<i>meso</i> - Zeaxanthin	60 \pm 2	1.14 \pm 0.05	1.63 \pm 0.07	0.19 \pm 0.03
Astaxanthin	N/A	0.62 \pm 0.02	2.09 \pm 0.09	1.26 \pm 0.02
β -Carotene	N/A	0.89 \pm 0.02	2.30 \pm 0.06	1.19 \pm 0.02

Chapter 4: Spectroscopic properties of protein M-LBP

4.0 Introduction

Both the protein and the carotenoid possess their own unique spectroscopic properties. These include fluorescence lifetimes, emission & excitation spectra, and absorption spectra. For native carotenoids, the spectroscopic properties are determined by unique structure of particular carotenoid (Eftink, 2006). The conjugation length (number of conjugated double bonds) is the key factor that determines spectroscopic properties of carotenoid (Gillbro and Cogdell, 1989). On another hand, the local environment associated with carotenoid is also contributing factor and influences stability of both the ground state and excited state energies. In our study, we are interested to learn how the protein carotenoid interaction will contribute to spectroscopic properties for both protein and carotenoid.

Protein fluorescence spectroscopy is widely used to study protein conformation. Fluorescence is observed when a high energy singlet excited state molecule relaxes to an electronic ground state via the emission of a photon (Eftink, 2006). Energy from an external light source is absorbed by the molecule and generates a short-lived excited singlet state (S_1) from ground state (S_0). The S_1 can exist for very finite time, typical singlet state lifetimes range from a fraction to a few nanoseconds (ns) lifetime (Lakowicz, 2013). During this short period, the fluorophore interacts with the molecular environment transferring small amounts of energy through collisions and conformational changes. As consequence, a

portion of the total energy absorbed by fluorophore is lost and the molecule relaxes to a thermally equilibrated singlet excited state (S_2) having a marginally lower energy level. S_2 is the origin of the fluorescence emission. Energy from fluorophore is emitted as a photon and the fluorophore is returned to the ground state (Lakowicz, 2013). Since S_2 has a lower energy than S_1 the energy of emitted photon is lower than that originally absorbed and thus the fluorescence emission is observed at a longer wavelength than the excitation. The wavelength shift from excitation to emission is called Stokes shift and it is this shift that allows the emission photons to be easily detected in fluorescence emission techniques (Lakowicz, 2013). Other processes including fluorescence resonance energy transfer (FRET), intersystem crossing, and quenching may occur and contribute to returning the excited molecule back to S_0 without emission of light (Lakowicz, 2013). In contrast to DNA which has a very weak intrinsic fluorescence, proteins have unique and intense intrinsic fluorescence. There are three common amino acids present in protein that possess a fluorophores, phenylalanine, tyrosine, and tryptophan. Only the aromatic side chains of these three amino acids produce a strong fluorescence. Tryptophan is excited around 280 nm and has a typical the emission wavelength around 348 nm. Tyrosine is excited around 274 nm and its emission wavelength is near 303 nm. Phenylalanine possess the shortest absorption around 257 nm and emission wavelengths around 278 nm (Bender, 2012). Tyrosine and tryptophan are used experimentally because the quantum yield (emitted photons versus absorbed photons) of phenylalanine is too low to obtain a good fluorescence result. Tryptophan is the dominant intrinsic

fluorophore, one protein may own just one or a few tryptophan residues, and the present of small numbers of tryptophan residues in the one protein facilitates the interpretation of spectroscopic results (Bender, 2012). Measurement of fluorescence emission is widely used in protein study to characterize the nature of the environment of the fluorescence residue. Several photophysical parameters of the fluorescence emission have been used to understand protein interactions *in vitro* and *in vivo*: emission wavelength, emission lifetime, wavelength of the peak absorption, polarization, and quantum yield (Yan and Marriott, 2003). The tryptophan steady state and lifetime measurement are used to probe protein lutein interaction in our study. The steady state measurement of fluorescence emission is the average fluorescence of a fluorophore determined by intensity versus wavelength. Minor changes in protein conformation or environment can lead to a shift of in the maximum emission wavelength. (Weljie and Vogel, 2002). The fluorescence lifetime is the determination from the emission constant rate (Γ) (sec^{-1}) at which the excited state returns to the ground state and is equal to $1/\Gamma$. The lifetime can be measured by a sum of constant rate (Γ) (Suhling et al., 2005). In this particular case, tryptophan fluorescence lifetime value was measured for both M-LBP and its apoprotein. Because the tryptophan lifetime is very sensitive to the local environment, the lifetime difference between M-LBP and apoprotein provides information of how lutein influences the solvent exposure and interactions of tryptophan residue.

4.1 Experimental

4.1.1 Protein preparation

M-LBP and apoprotein are obtained as described previously in Chapter 2. Absorption spectra were recorded using a single beam UV-vis spectrometer (Cary 50, Varian) to produce a protein absorption of 1 Au.

4.1.2. Tryptophan fluorescence Spectrum

Measurement of emission spectra was carried using a PC1 fluorimeter (ISS, Champaign, IL) to compare the emission of tryptophan residues within the M-LBP and its apo-protein. Fluorescent measurements of M-LBP and its apoprotein were carried out in CHAPS/MES buffer (25mM CHAPS, 20 mM MES, 1 mM CaCl₂, 2mM MgCl₂, pH 5.5) were placed in two 0.2 x 1.0 cm quartz cell. The apo-protein and M-LBP were excited at 275 nm, the emission spectras were recorded.

4.1.3. Time-resolved fluorescence measurement-lifetime

The fluorescence lifetime measurements were conducted on a ChronoFD fluorometer (ISS, IL, Champaign). The external 280 nm light emitting diode was frequency modulated in the range between 5 and 250 MHz. The emission beam was filtered through a 320 nm long pass filter (Andover Inc., Salisbury, MA) and a photomultiplier tube (PMT) (R928, Hamamatsu) served as a detector. Time-resolved fluorescence decay for M-LBP and apo-protein were recorded and decay data were analyzed by da Vinci software (Max-Plank-Institute, Germany)

and fit with three discrete exponential decays. The lifetimes of the tryptophan excited states were determined in both samples.

4.2. Results

The tryptophan fluorescence emission arising from aromatic residues in M-LBP and its apo-protein was undertaken to assess the influence that binding of the carotenoid has on the local environment of these amino acid side chains. Emission from the M-LBP and apo-protein excited at 275 nm is principally attributed to tryptophan and in M-LBP emission ($\lambda_{\text{max}}= 318$) is red-shifted by 18 nm relative to the apo-protein ($\lambda_{\text{max}}= 300$) (Fig.34 & 35). The presence of lutein in the protein produces a significant shift in the emission spectra.

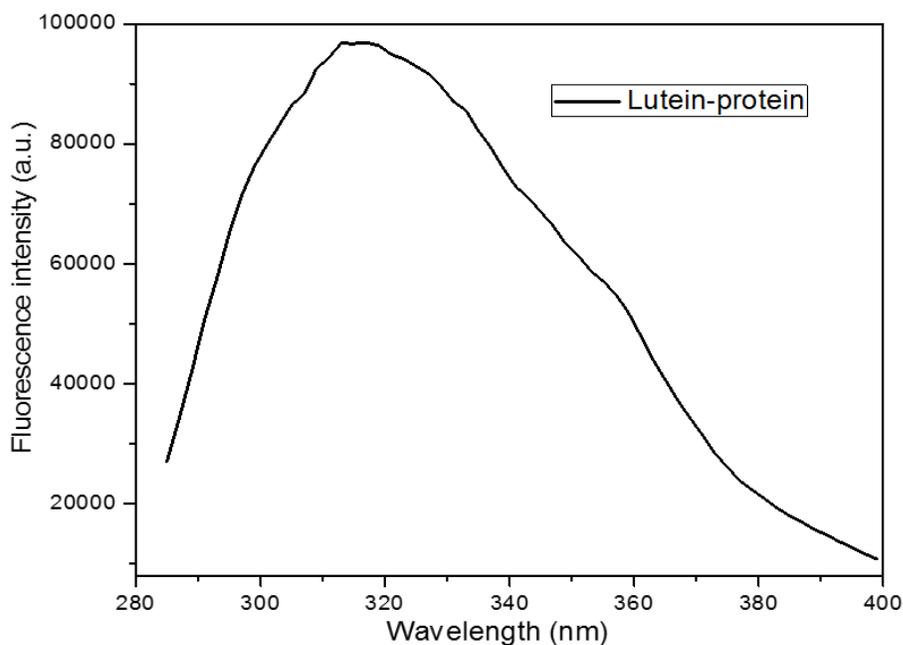


Fig. 34. Tryptophan emission spectrum of M-LBP (Excitation wavelength at 275 nm, emission wavelength at 318 nm)

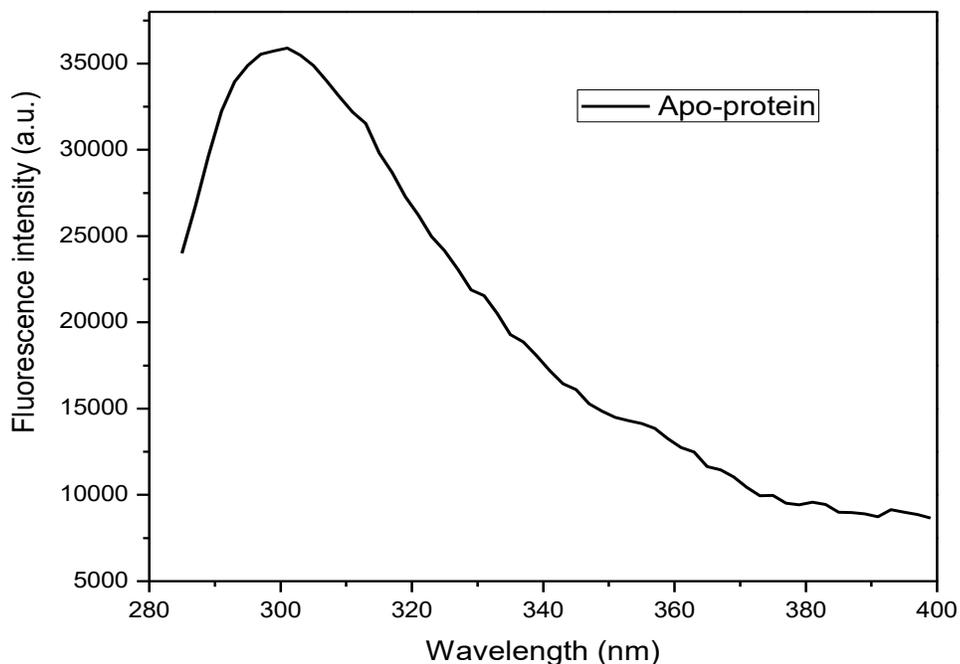


Figure 35. Tryptophan emission spectrum of apo-protein (Excitation wavelength at 275 nm, emission maximum at 300 nm).

Since the tryptophan fluorescence lifetime is very sensitive to small changes in local environment, the measurement of lifetime of the tryptophan emission provides evidence of structural changes in the protein induced by loss of the lutein ligand. The tryptophan lifetime was determined using phase modulation fluorescence spectroscopy via frequency-domain method (Lakowicz and Balter, 1982). In this method, the sample is excited by a light source which is modulated or pulsed in a certain waveform. A consequence of the modulation is that the fluorescence emission from the sample will have a similar waveform that is modulated but has a phase-shift from that of the excitation curve. By determination of modulation ratio (M) and phase-shift (ϕ), the emission lifetime of fluorophore can be deduced (Lakowicz and Gryczynski, 2002). The phase decay shift and modulation ratio of M-LBP and its apoprotein were monitored (Fig. 36).

Lifetime data were recovered using da Vinci software (Max-Planck-Institute, Germany) and was fit to three discrete exponential decay components (Table 9). The lifetime data were analyzed using a sum of three discrete triple exponentials. In contrast with mono-exponential decay, the multi-exponential decay provides a better fit to fluorescence decay data. (Siegel et al., 2001). Measurement of the tryptophan excited-state lifetimes by time-resolved fluorescence decay shows that the binding of lutein by M-LBP produces a significant measurable difference in τ (average) for tryptophan emission within the protein. The apo-protein lifetimes vary from 0.2-6.9ns compared to those for the lutein bound form of the protein which range from 0.5-7.4ns.

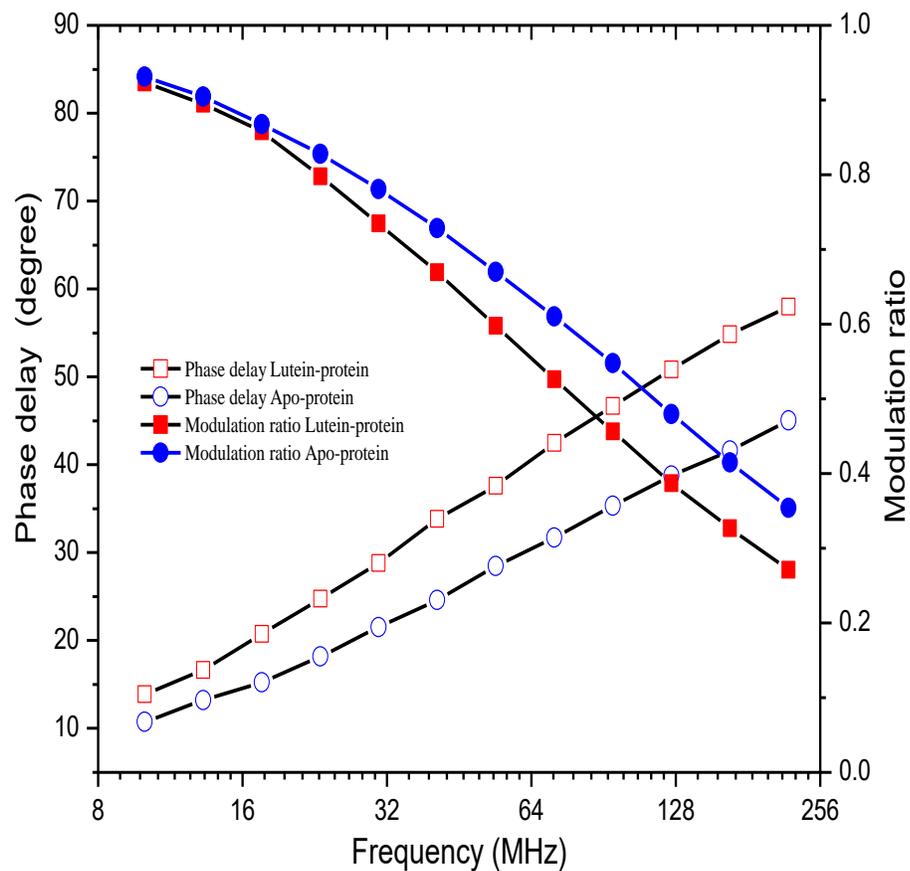


Fig. 36. Tryptophan Phase decay shift (φ) and modulation ratio (M) of M-LBP and its apoprotein

	$\tau_1(\text{ns})^a$	$\alpha_1(\%)^a$	$\tau_2(\text{ns})^a$	$\alpha_2(\%)^a$	$\tau_3(\text{ns})^a$	$\alpha_3(\%)^a$	$\tau_{(\text{average})}(\text{ns})$	χ^2
Apoprotein	0.2	0.91	1.6 ± 0.1	0.28	6.9 ± 0.3	0.05	3.0	4.4
M-LBP	0.5 ± 0.1	0.29	2.3 ± 0.1	0.19	7.4 ± 0.4	0.05	3.9	0.97
Data recovered using Vinci software with three discrete exponential decays.								

Table 9. Tryptophan emission lifetime parameters of M-LBP and its apoprotein (τ : lifetime, α : normalized pre-exponential decay factor, χ^2 : Chi-square)

4.3. Conclusion and discussion

Extraction of the carotenoid from the protein has a modest but significant influence on the tryptophan fluorescence within M-LBP. This modest effect argues against a close contact between the lutein and tryptophan side-chains. Close association between lutein and tryptophan the carotenoid would be expected to quench the tryptophan excited-state in the native protein. Both the wavelength maximum and the average tryptophan lifetime are shifted in the apoprotein as compared to the native form. The 318 nm to 300 nm blue-shift in the tryptophan fluorescence is accompanied by a change in the average lifetime from 3.9 to 3.0 ns, for the native and apoprotein, respectively. Both measurements are consistent with the local tryptophan environment becoming less polar upon extraction of the lutein molecules from the protein. It appears that upon extraction of the carotenoid from the protein structural reorganization enables the tryptophan side-chains to be tucked deeper within protein and thereby reducing contact with the more polar surface.

4.4. Ongoing collaboration

The interaction of the carotenoids with its immediate local environment in solution or within a protein has a large influence on its spectroscopic properties. In an ongoing collaboration with Dr. Frank (University of Connecticut, Storrs, CN) and Dr. Tomas Polivka (University of South Bohemia, Ceské Budejovice, Czech Republic). The objective of this work is to further characterize the carotenoid excited-states in M-LBP and related proteins through the use of femtosecond

transient absorption of carotenoid excited state. As a consequence of the existence of multiple singlet excited states an accurate, quantitative description of the carotenoid excited state properties is complicated. The excitation from ground state to excited state occurs when carotenoids absorb the external light with wavelength from 450 nm to 550 nm (Pan et al., 2011). The initially formed excited state will release energy and relax to a thermally equilibrated excited state having lower energy level by a process referred to as internal conversion over an extremely brief time period, typically a few hundred femtoseconds. This excited state has a fluorescent emission lifetime that is on the order of picoseconds (Pan et al., 2011). The explanation of the nature of the process by which the initial carotenoid excited state undergoes internal conversion between high and low excited states in carotenoid binding protein remains a controversial topic.

Initial results of this study have demonstrated that the lutein molecule in M-LBP has a distinct transient absorption spectrum of the initially formed S1 excited state contrasted to that of lutein in methanol solution and that of lutein bound to the human protein, StARD3. These results show the presence of a feature on the transient absorption spectrum of the 'hot' S1 excited state carotenoid associated with conformational disorder suggests that M-LBP has less conformational disorder than lutein in methanol solution but somewhat more disorder than that of StARD3. This result is consistent with the observed order of K_D values for lutein in these two proteins, $K_D = 18.6 \text{ nM (M-LBP)} > K_D = 0.59 \text{ nM}$

(StARD3) where the lower K_D is indicative of a stronger binding interaction (Fuciman et al., 2015).

Chapter 5 Conclusion and Future work

5.1 Conclusion

The work described in this dissertation has focused on purification and characterization of M-LBP from monarch butterfly larvae (*Danaus plexippus*). This carotenoid binding protein is a novel protein and appears to be distinctly unique from other carotenoid binding proteins that have been described for other species. Purification of the protein homogenate from the larval epidermis requires a buffer system incorporating the CHAPS surfactant and yields a carotenoid/protein complex that migrates as a single band during native protein electrophoresis. HPLC analysis of the carotenoid extracted from this protein extract demonstrates that lutein is the sole carotenoid present although multiple carotenoids are found in larval diet. Using polyacrylamide gel electrophoresis determination it was demonstrated that M-LBP has a molecular mass of ~60 kDa. This value is consistent with the result obtained by HR-tandem MS (60,645 kDa). The stoichiometry of the M-LBP is two lutein molecules per protein, distinguishing it further from SW-CBP which binds a single carotenoid. The dissociation constants demonstrate that the 3'-hydroxyl group is essential to carotenoid binding and that a 3'-S absolute configuration of that hydroxyl group is the optimal spatial orientation for binding. Immunohistochemistry results show that M-LBP has no cross-reactivity to silk worm SW-CBP (*Bombix mori*) but has cross-reactivity with horn worm epidermal HE-CBP (*Agrius convolvuli*). Binding affinities were determined using surface plasmon resonance. The values of the

dissociation constants for several carotenoids were determined; lutein ($K_D = 18.6$), R,R-zeaxanthin ($K_D = 990$), R,S-*meso*-zeaxanthin ($K_D = 60$), β -carotene (NA) and astaxanthin (NA). Lutein, R,R-zeaxanthin, R,S-zeaxanthin. Tryptophan fluorescence lifetimes determined for the native and apoprotein were compared. Tryptophan fluorescence lifetimes were found to be 3.9 ns and 3.0 ns, respectively, for the native and apoprotein, indicating that upon dissociation of the carotenoid the tryptophan fluorophore adopts a position where it has less interaction with the polar surface environment.

5.2 Future Work

There remain many characteristics of this protein that would further assist in our understanding of the interaction of carotenoids with proteins and their functional properties.

5.2.1. Protein crystallization study

The molecular structure of M-LBP and its carotenoid binding motif would provide fine detail of the nature of the protein carotenoid interaction (Rosenbaum and Zukoski, 1996). In addition to the nature of the binding pocket and closest contacts the 3D structure of M-LBP the conformation of the carotenoid within the site would provide further clarity of the nature of the influence that the protein has on the energy of the electronic states of the molecule (Drenth, 2007).

5.2.2. Antioxidant function study

Carotenoids are well known to function as antioxidant and can scavenges reactive oxygen species and free radicals (Young and Lowe, 2001). It is an interesting question that how the carotenoid fulfills its antioxidant function when it associated with protein. An investigation of antioxidant behavior of the protein-carotenoid could provide further understanding of the advantages conferred on antioxidant system when the carotenoid is bound as compared to its activity and properties in homogenous conditions.

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VITA

NAN FANG

Born, Maanshan, Anhui, China

2005-2010

Bachelor of Medicine, Clinical pharmacology
Anhui Medical University, Clinic College
Hefei, Anhui, China

2011-2016

Doctoral Candidate
Florida International University
Miami, Florida

Teaching Assistant
Florida International University
Miami, Florida

PUBLICATIONS AND PRESENTATIONS

Fang, N. (July, 2014). Extraction, purification and partial characterization of a carotenoid binding protein (CBP) from the epidermis of the monarch butterfly larvae (*Danaus plexippus*). Presented at the 17th International Symposium on Carotenoids, Salt Lake City, Utah.