University of South Bohemia

Faculty of Science

Characterization of Photosystem I in the Red Alga *Porphyridium purpureum*

Master Thesis

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Annotation

Photosynthesis, a fundamental biochemical process on planet Earth, has passed through several steps during the course of evolution since beginning of time. Red algae represent a primitive stage of evolution of photosynthesis in eukaryotes, hence they are important for our understanding of functioning and evolution of photosynthetic energy conversion in general. In the current work, isolation and characterization of the supramolecular complex consisting of photosystem I with three antenna complexes from the red alga *Porphyridium purpureum* is presented. The purification consisted of sucrose gradient centrifugation followed by anion exchange and size-exclusion chromatography. The composition of the PSI-antenna supercomplex was confirmed using electrophoresis and reverse-phase chromatography. Function of the isolated supercomplex was further investigated using time-resolved absorption and fluorescence spectroscopy.

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DECLARATION

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Panagiotis Kosmas

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1 INTRODUCTION

1.1 Nature of Light

Solar radiation is the primary source of energy for life evolution since the beginning of time. The so-called electromagnetic energy is an entity of continuous stream of photons (Kirk, J., 2011). Photons have dual nature, they act as a wave but are also quantized existing in discrete units, i.e. particles (Fig. 1). The electromagnetic spectrum, shown in Fig. 2, is parted into different areas, depending on the wavelength λ (nm) (Metzler, D., 1977). The electromagnetic radiation is characterized by velocity c of 3×10^8 m/s (in vacuum), wavelength λ and frequency ν. These quantities are related as (Kirk, J., 2011):

$$c = \lambda \cdot \nu \tag{1.1}$$

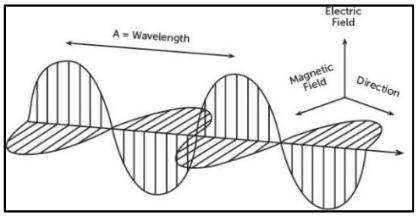


Figure 1: Propagation of electromagnetic radiation

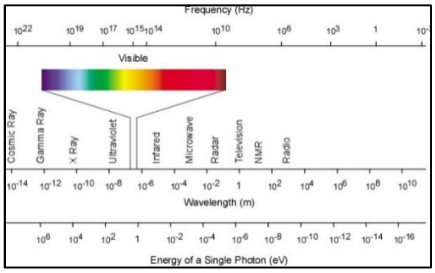


Figure 2: The divisions of electromagnetic spectrum

When the solar radiation has reached Earth's atmosphere, its intensity is reduced because of scattering by dust particles and air molecules and because of absorption by ozone layer, oxygen, water vapor and carbon dioxide that are composing the atmosphere. Finally, some of the scattered solar radiation is reflected and lost in space and the rest will find its way to Earth's surface and is belonging to near IR, visible and near UV part of spectrum (Kirk, J., 2011).

1.2 Photochemistry in Nature

Light affects different aspect of life in different ways. The different processes are mainly based on photochemical reactions that take place when the energy of light is promoting molecules from their ground state (M) to an excited state (M^*). Globally, the most important photochemical process is photosynthesis which converts the solar energy to chemical energy and makes it thus available for all the life on Earth.

The concept of photochemical reactions is similar with that of 'dark' chemical reactions, with the difference that in first one there is a ground state and an excited state of initial molecule (reactant). It is incorrect to consider the light as catalyst. Light always act as a reactant, it is absorbed by the molecules and its energy is promoting their electrons from ground state to an excited state. The energy difference between the two energy states corresponds to the frequency of absorbed photons.

$$E_2 - E_1 = \mathrm{h} \cdot \nu, \tag{1.2}$$

where h is the Planck constant equal to 6.626×10^{-34} J.s. Light is not lowering the activation barrier E_a as only catalysts are doing. Furthermore, it cannot be recovered after the photochemical reaction, or reused.

1.3 Evolutionary Aspects of Photosynthesis

The most important photochemical reaction for life evolution is photosynthesis. The recent studies of gene sequences have given the chance for more precise examination of life evolution. The very first organisms were established on Earth about 4 billion years ago, in simple form of short replicating RNA strands adsorbed on pyrites. It is widely known that bacteria are simpler than cells with organized nuclei and that the photosynthesis of green plants and algae using two distinct photosystems is more advanced than that of anoxygenic bacteria. The chemoautotrophic ancestor of these organisms probably inhabited in the waters of sunless locations where highly reducing substances such as FeS, H_2S and H_2 were the educts for CO_2 fixation by exergonic reaction.

$$4FeS + 4H^+ + CO_2 \rightarrow 4Fe^{3+} + 4S^{2-} + [CH_2O] + H_2O \qquad (1.3)$$

These organisms had no need of sunlight to maintain their vital functions. The hypothesis for the evolvement of photosynthesis was that it might worked as protection mechanism. The Earth contained no oxygen in early ages and there was no ozone layer to block the damaging UV radiation. An organism with pigments was able to survive in such conditions in comparison to an organism without pigments (Archer, M., Barber, J., 2004).

Thoughts about evolution were presenting bacteria containing BChl *a* as the earliest photosynthetic organisms. This statement was coming in contrast with current knowledge about biosynthesis of chlorophylls, which places BChl *a* several steps beyond Chl *a* in the biosynthetic sequence. J. M. Olson and then D. C. Mauzerall suggested that the ancestor of modern photosynthetic organisms was a bacterium about 3,5 billion years ago that contained Chl *a* and either Type I or Type II reaction centers (RCs), performing anoxygenic photosynthesis (Archer, M., Barber, J., 2004).

About 2,8 billion years ago, cyanobacteria appeared as the first organisms that were able to perform oxygenic photosynthesis. They exist as single cell or multicellular filaments. Their name comes from the bluish hue of light-harvesting pigments phycobilins (i.e. phycocyanins, phycoerythrins and allophycocyanin), arranged in phycobilisomes, a water-soluble antenna complexes (Archer, M., Barber, J., 2004). Cyanobacteria are the only phototrophic bacteria that contain two type of photosystems (PSI & PSII), being able to overcome the gap in redox energies from H_2O to the

reductants (Clayton, R., 1980 & Archer, M., Barber, J., 2004). This excessive production of O_2 evolved the Earth's atmosphere into the current day's composition.

At a parallel evolutionary line there was another type of organism, precursor of present-day photosynthetic bacteria. This species contained BChl and was unable to produce O_2. The difference between BChl and Chl *a* is that the first can absorb light at wavelengths greater than 720 nm while the latter can absorb near 430 and 670 nm. This evolutionary line had no future in production of O_2 (oxygenic photosynthesis) because BChl was not sufficiently strong to oxidize H_2O molecules. (Clayton, R., 1980).

Endosymbiosis, a crucial evolutionary step, took part around 1,6 billion years ago. Photosynthetic prokaryotes (cyanobacteria) were engulfed by precursors of non-eukaryotic cells and were evolved into organelles, called chloroplasts, having their own circular DNA and replicating by simple division. Red and green algae are the direct descendants of the primary endosymbiotic event (Papachristodoulou, D. et al., 2014).

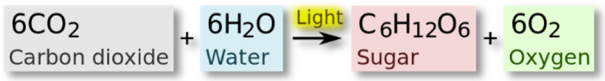
Red algae are economically the most exploited, as they biosynthesize sulphated polysaccharides, carrageenans and agarans and are cultivated for human consumption because of nutritional substances. They can be found in aquatic environment (even below the photic zone), moist soil, tree trunks and attached to rocks on shallow sea beds and basalt ledges, or floating, due to its fragile constitution (Pereira, D. et al., 2018).

The red algae can be seen as an intermediate step between cyanobacteria and the eukaryotic algal or plant species: phycobilisomes continue being the dominant antennae complexes bound to photosynthetic membrane in red algae as they are in cyanobacteria. In addition to that, chlorophyll-binding light-harvesting complexes of the LHC family, integrated in the thylakoid membrane are present in red algae (Neilson, J., Durnford, D., 2010).

During the last 350 million years, the higher land plants (e.g. mosses and liverworts) and vascular plants (e.g. trees and grass) appeared as descendants of green algae (Archer, M., Barber, J., 2004).

1.4 Approaches for Understanding the Mechanism

The general idea of photosynthesis is depicted in equation (1.4), carbon dioxide and water are reacting with light to produce carbohydrates and oxygen:



(1.4)

The first approach for understanding was made in 1650 when Van Helmont grew a willow tree in sandy soil. Five years later the weight of the tree was 110 times more than in the beginning and the soil had the same weight. He suggested that most of weight of the tree has come from the water. A century later Bonnet observed that leaves submerged in water are producing gas bubbles when exposed on the sunlight. In 1771 Joseph Priestley showed that a mouse could not live in a container inside where the air was burned out by a candle flame. But when a spray of mint was applied, the air was restored and after few days the candle could burn again, or the mouse could live for a time. That way he suggested that a plant could produce oxygen. In 1779 Ingenhousz proved that the plants with their green parts and light can freshen the air and at night with respiration as reverse reaction of photosynthesis spoil the air. In 1782 Senebier stated that plants need carbon dioxide to oxygenate the air. Ingenhousz suggested in 1796 that this carbon dioxide had to be the source for all the organic matter in a plant.

Finally, in 1804 de Saussure, being aware of the law of conservation of matter, confirmed van Helmont's proposal about that most of the weight of a plant comes from water. After all ingredients of equation (1.4) were incorporated in life of plants, it remained for Robert Mayer in 1845 to show that the energy from sunlight was stored as chemical energy in organic molecules, the sugars. In the early 20th century the organic chemists R. Willstatter and A. Stoll characterized the green pigment chlorophyll and tried to explain how chlorophyll could participate in the photosynthetic process. No more experiments were suggested until C. B. van Niel in 1920's began to formulate a new view of photochemical process of photosynthesis (Clayton, R., 1980).

1.5 Chloroplasts ─ Photosynthetic Machineries

All kinds of cells in eukaryotic organisms contain specialized organelles for producing the necessary amount of energy that can maintain their function. Eukaryotic cells contain plastids, organelles with two surrounding membranes. Most common type of plastids, the chloroplasts, are absorbing light and together with H_2O oxidation are converting it into biochemical energy (i.e. ATP) and reducing power (i.e. NADPH). Afterwards, these molecules are combined with atmospheric CO_2 to form carbohydrates. Chloroplasts contain a mixture of different pigments with chlorophyll being in excess and resulting to the green color of plant cells. They contain their own DNA, RNA and ribosomes (Papachristodoulou, D. et al., 2014).

In terrestrial plants chloroplasts are mainly placed in the palisade and mesophyll cells of the leaves. On upper epidermis there are molecular diodes, called stomata, that allow the exchange of gases (e.g. CO_2 and O_2) with the environment. In submerged plants the chloroplasts are developed in epidermal cells and in such species, stomata do not exist. Every mature leaf cell can have up to 400 chloroplasts from 4 to 8 μm diameter and about 2 μm thickness in the centre. Some algal species contain up to 100 chloroplasts per cell of about 100 μm diameter (Kirk, J., 2011).

In morphology, chloroplasts (Fig. 3) are surrounded by a continuous outer membrane while an inner membrane encloses the internal compartment. Inside the inner membrane space there is a fluid called stroma. The inner membrane forms sheets, thylakoids which in plants are arranged in stacks called grana. In other organisms, the thylakoid membrane forms typically parallel stacks and typical grana are not formed. In thylakoid membranes are present all the photosynthetic pigments and all the required enzymes for the light-dependent reactions which take place in the intermembrane space. Subsequently, in stroma takes place the CO_2 fixation and the production of carbohydrates with all required enzymes for the so-called Calvin cycle (Lehninger, A., 1984).

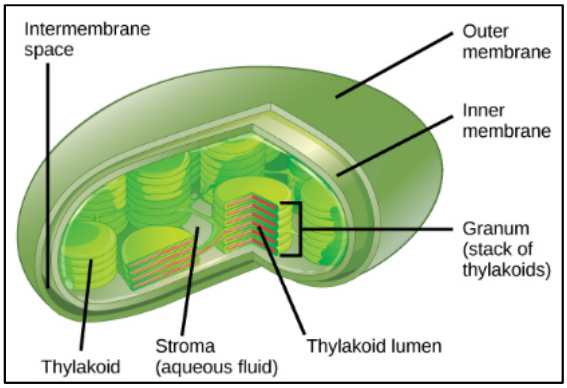


Figure 3: Morphology of a chloroplast

The distribution of chloroplasts within the cell varies with the light intensity, ensuring that majority of light incident on a cell passes through multiple chloroplasts. In low intensity light the chloroplasts tend to move to a position in the cell where the absorption of light is maximized. They distribute themselves adjoining and parallel to those cell walls that face the incident light. The opposite is happening in high intensity light when the chloroplasts move in a way to the most shaded cell walls, parallel to the direction of the light and being shaded by each other (Kirk, J., 2011).

Movement of chloroplasts is protecting them against photoinhibition, an impairment of photosynthesis. Usually, there is a continuous degradation and resynthesis of D1 protein in PSII, but excessive light conditions can lead to imbalance in repair cycle and permanent stall of photosynthesis (Schulze, E., 1995).

1.6 Energetics of Photosynthetic Reactions

Photosynthesis is performed by diverse organisms and can be divided into oxygenic and anoxygenic photochemical reactions. In oxygenic photosynthesis of cyanobacteria, algae and leaves of higher plants, H_2O is used as H_2 donor and O_2 is released. Anoxygenic photosynthesis is performed by anaerobic bacteria that use inorganic compounds (e.g. H_2S) as H_2 donors, producing elemental sulfur. Other bacteria can use organic molecules (e.g. lactate) as hydrogen donors. Van Niel stated that both processes performed by plants or bacteria are similar despite the different H_2 donors and this can become more obvious by a more general form of photosynthetic equation:

$$2H_2D + CO_2 \xrightarrow{\text{Light}} CH_2O + H_2O + 2D \qquad (1.5)$$

H_2D is a random hydrogen donor and D its oxidized form (Lehninger, A., 1984).

Anoxygenic photosynthesis involves a simpler mechanism than the oxygenic photosynthesis of Cyanobacteria. These are not identical for all anaerobic bacteria. Mainly the electron transport chain refers to a cyclic pathway consisting of a reaction center (RC) with its associated antenna proteins and a bc_1 complex. Light absorbed by the antenna excites the reaction center, a special pair of BChl, to P^* and the e^- passing through multiple co-factors is transferred to quinone (Q_B). Ultimately, Q_B is doubly reduced which is accompanied with transfer of two protons from cytoplasmic side. QH_2 is oxidized passing two e^- to bc_1 complex and releasing two protons into the periplasmic side. The bc_1 complex drives electrons to cytochrome c_2 and same time to oxidized quinone. The reduced cytochrome c_2 can transfer an e^- to oxidized P^+ and complete the cycle of electron flow, while the recently reduced quinone is pumping 2 protons from cytoplasmic side during the so called 'Q-cycle'. Some bacteria contain an Fe-S centre instead of quinone as terminal electron acceptor with a subsequently $NADP^+$ reduction via a ferredoxin $NADP^+$ reductase (Archer, M., Barber, J., 2004).

The light-dependent reactions in oxygenic photosynthesis involve e^- transfer between three protein complexes (i.e. photosystem II, cytochrome bf complex and photosystem I) across the thylakoid membrane of chloroplasts. The protons flow from stroma to lumen establishes a pH gradient across the thylakoid membrane and the evolved proton motive force is activating the production of ATP. Z-scheme (Fig. 8) shows how the light absorbed by the two photosystems is providing enough energy for

an electron to flow from H_2O with very positive potential (+0,82 V) until reduction of $NADP^+$ to NADPH which has a very negative potential (-0,32 V) (Lehninger, A., 1984).

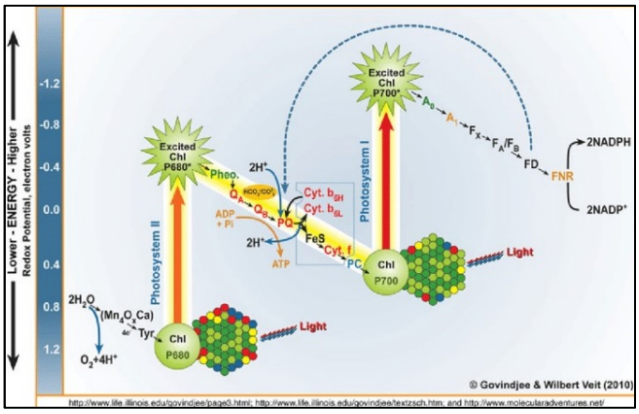


Figure 4: Diagram with redox potentials of electron transfer from H_2O to $NADP^+$

Starting in photosystem II, photons are absorbed by antenna chlorophylls and through resonance energy transfer are led to the reaction center P680 (P stands for pigment and 680 for the wavelength, in nm, of maximal absorbance) which consists of a pair of chlorophyll *a* molecules bound to proteins. The excited reaction center P680* is transferring an e^- to the first member (i.e. pheophytin) of the electron transport chain. The formed $P680^+$ is a strong oxidant. A water-splitting enzyme consisting of a four manganese ions cluster as catalytic center can restore the $P680^+$ to the ground state by extracting 4 e^- from two H_2O molecules and releasing 4 H^+ along with O_2 into the thylakoid lumen. It is necessary to extract all 4 e^- so that no release of hazardous intermediate oxygen free radicals occurs (Papachristodoulou, D. et al., 2014).

Afterwards, e^- goes from the reduced pheophytin to a plastoquinone (Q) reducing it to plastoquinol (QH_2) which is released into the hydrophobic region of the membrane enclosing the energy of two photons. Q comes from quencher because plastoquinone quenches the fluorescence of P680 (Metzler, D., 1977). The next complex is called cytochrome bf. This complex is transferring 2 e^- from plastoquinol (QH_2) to plastocyanin (Pc) and 2 protons (H^+) are released during first half of Q-cycle into lumen while in second half of the cycle a second plastoquinone (Q) is reduced by taking up two protons from stromal side and then this plastoquinol (QH_2) is reoxidized to release these protons into the lumen (Stryer, L., 1988).

Next comes the photosystem I, the second light-converting complex containing 13 polypeptide chains (>800 kDa). Light is absorbed by its accessory antenna of chlorophyll and then it excites the reaction centre primary donor P700 to P700*. The

electron is transferred to an acceptor chlorophyll A_0, forming A_0^-, the most powerful reductant biomolecule known, and to a series of iron-sulfur centers until reduce the next electron carrier ferredoxin (Fd), a 2Fe-2S cluster. The last electron carrier, ferredoxin-NADP reductase, reduces $NADP^+$ to NADPH in stroma. Same as in photosystem II, $P700^+$ is a strong oxidant which will accept an e^- from reduced plastocyanin (Pc) (Stryer, L., 1988 & Papachristodoulou, D. et al., 2014).

When the ratio of NADPH:$NADP^+$ is very high, then the $NADP^+$ is unavailable to accept electrons from reduced ferredoxin. In that case an additional process called cyclic photophosphorylation takes place and the e^- flows back to cytochrome *bf* and from there back to the oxidized form of P700 through plastocyanin. Cytochrome *bf* is pumping protons into lumen, while photosystem II is not involved during this cyclic pathway and no O_2 production occurs (Stryer, L., 1988).

ATP production is catalyzed by ATP synthase, an CF_1 – CF_0 complex. CF_0 subunit is loaded with protons from lumen and is submerged in thylakoid membrane. CF_1 subunit catalyzes the ATP formation from ADP and P_i and stands on the stromal side of the membrane (Stryer, L., 1988).

Cyclic photophosphorylation is more productive in terms of ATP synthesis. In the non-cyclic pathway 4 photons must be absorbed by PSII to generate one O_2 molecule and 4 protons will be released into lumen. The 2 QH_2 will be oxidized by cytochrome *bf* and liberate 2*4 = 8 protons into lumen. In PSI 4 photons will be absorbed and the electrons from reduced plastocyanin will reduce 4 ferredoxin molecules. In total 8 photons pump 12 electrons that flow through 12 CF_0 subunits back to stroma to produce 3 ATP and additionally 2 NADPH from 4 reduced ferredoxins. In cyclic pathway, 4 photons absorbed by PSI will pump 8 protons into lumen through cytochrome *bf* and 2 ATP will be produced without NADPH production (Berg, J., Tymoczko, J., Stryer, L., 2002).

Summarizing, two light quanta must be absorbed one by each photosystem to pass one e^- from H_2O to $NADP^+$. Generation of one O_2 molecule requires transfer of four e^- that makes eight light quanta. Equation 1.4 subscribes six O_2 molecules that means forty-eight light quanta must be absorbed. One mol of photons has energy of 41 kcal at 700 nm. Depending the wavelength of absorbed light, green cells require approximately 48*41 = 1968 kcal to produce 1 mol of glucose in stromal membrane phase (Lehninger, A., 1984).

1.7 Light Harvesting Complexes

All photosynthetic organisms, except Halobacteria, contain photosynthetic complexes with two distinct functions. The antenna systems or light harvesting complexes (LHCs) collect light and guide the excitation energy to reaction centers (RCs) where it is transformed into electrochemical energy. The antennae size and composition vary and they can be either part of the membrane or attached on inner or outer surface. The reaction centers are always integrated into photosynthetic membrane (Fong, F., 1982).

The antennae systems (Fig. 5) consist of pigments bound to proteins. In 1932, Emerson and Arnold found out that a large array of pigments is involved in transmitting light energy to the photochemical reaction and estimations were about 2400 chlorophylls for production of one O_2 molecule. Antennae complexes have more pigments than RCs increasing the effective absorption photons and the diversity of their pigment types allow the absorption of wider range of frequencies from the solar spectrum. They also regulate the energy flow to the RCs. According to Förster's theory, the efficiency of photon's transmittance depends on the angle between emission and absorption dipoles, the fluorescence rate constant and the normalized area of overlap of the donor fluorescence and acceptor absorption spectra divided by the sixth power of distance between the two molecules (Rowan, K., 1989 & Golbeck, J., 2014).

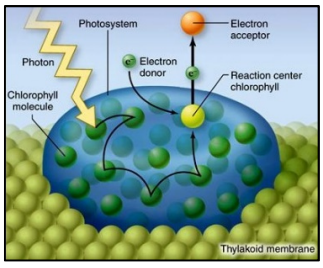


Figure 5: Schematic depiction of energy transfer in a light harvesting complex

The most abundant antenna pigment in all photosynthetic organisms is chlorophyll *a*, bacteriochlorophyll in bacteria. The molecular structure consists of a tetrapyrol ring with a magnesium atom chelated at the centre of the ring structure. Chl *a* has absorption maxima at 430 nm and 663 nm in acetone (80%).

Carotenoids are accessory pigments in harvesting of light that transfer the energy to the chlorophylls. They are distributed into two classes: the carotenes and the xanthophylls. There structure contains two cyclic end-groups linked by a C_{18} chain of conjugative double bonds and additionally at least one atom of oxygen for xanthophylls.

In Rhodophyta (red Algae) exist *a*- and *b*- carotene and the 3-hydroxy derivatives: lutein and zeaxanthin (Rowan, K., 1989).

Carotenoids play triple role in photosynthesis: i) they serve light-harvesting function, complementing the chlorophylls; ii) they protect against the reactive oxygen species. By quenching chlorophyll triplet states, they prevent formation of reactive singlet oxygen and they also quench singlet oxygen directly; iii) the third role of carotenoids is in quenching of excess singlet energy (Allen, J., 2008).

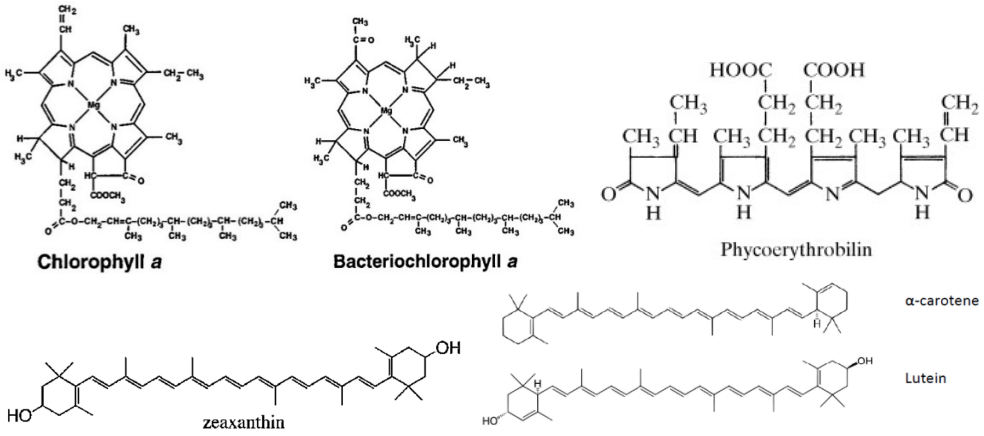


Figure 6: Molecular structures of photosynthetic pigments

Other pigments, called phycobilins (Fig. 6), are found in certain algae (i.e. Rhodophyta, Cryptophyta and Cyanobacteria) divided into four classes: phycoerythrins, phycoerythrocyanins, phycocyanins and allophycocyanins with absorption peaks from blue to red light region of spectrum respectively (Kirk, J., 2011). They are covalently bound to apoproteins constituting the phycobiliproteins that are water-soluble. Their structure is similar to chlorophylls, but the final steps of biosynthesis involve oxidative opening of the porphyrin ring resulting in linear tetrapyrroles (Rowan, K., 1989). The phycobiliproteins are organized in phycobilisomes, prolate hemi ellipsoids with 300 Å height and 450 Å width. The phycoerythrin forms the outer layer and phycocyanin is placed between phycoerythrin and allophycocyanin. The latter is in contact with the photosynthetic membrane (Fong, F., 1982). Phycobiliproteins become highly fluorescent after removal from phycobilisomes due to absence of photosynthetic reaction centers which would receive the energy efficiently (Lakowicz, J., 2006).

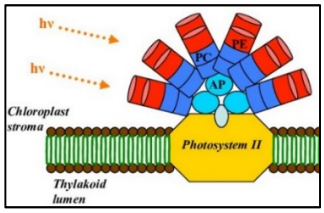


Figure 7: Schematic structure of phycobilisome

1.8 Photosystems

The photosynthetic apparatus based on two photosystems operating in series evolved in Cyanobacteria. It was transmitted to eukaryotes via endosymbiosis by which an engulfed cyanobacterium became the chloroplast. This explains several similarities between red algae and Cyanobacteria. In both cases, the PSII complex contains phycobilisomes as primary photosynthetic antenna instead of chlorophyll a binding proteins that are contained in PSII of higher plants and green algae. On the other hand, similarly to plants, red algae contain normally intrinsic chlorophyll-based light harvesting complexes in PSI.

The reaction center of PSII in red algae is mainly composed by D1 and D2 proteins, associated with cytochrome c_{550} and 12 kDa psbU protein and surrounded by chlorophyll a-binding inner antenna proteins CP47 and CP43. The rest extrinsic proteins of the core complex differ among organisms except 33 kDa psbO that is common in all oxygen-evolving photosynthetic organisms with addition of 16 kDa psbQ and 23 kDa psbP in green algae and higher plants (Bumba, L. et al., 2004).

Among organisms, PSI core shows some differences but in general it has two large membrane intrinsic subunits, PsaA and PsaB, and some small extrinsic or intrinsic proteins located around the periphery of the two large subunits. The eukaryotic peripheral antenna complex, known as light-harvesting complex I (LHCI), includes four distinct chlorophyll a/b binding (Lhca1-4) proteins. Differences in the antenna system resulted in different organization of the complex (Drop, B. et al., 2011 & Germano, M. et al., 2002). PSI core in cyanobacteria exists independently without associated antennae or in some cases with phycobiliproteins as light harvesting antennae. PSI core in cyanobacteria forms a trimeric assembly while in eukaryotic organisms it exists as a monomer. PSI in red algae *C. caldarium* and *C. merolae* include two types of peripheral light-harvesting antenna complexes, comprising Lhcr proteins and the PSI-specific phycobilisome sub-complex that hinders the trimerization of the core complex. This evidence suggested an efficient excitation energy transfer from phycobilisomes to both photosystems (Busch, A. et al., 2010)

The core components of PSI have not changed much during evolution time. Although, the light-harvesting antenna system has transformed quite enough to adapt to diverse environmental habitats of each organism. The LHCI apoproteins have differences in sequences, numbers and associated pigments for each organism. The

study report of red algal *Cyanidioschyzon merolae* has revealed the single-particle cryogenic electron microscopy (cryo-EM) structure of PSI-LHCR. *C.merolae* has three Lhcr genes in comparison to higher plants which have four Lhca subunits. In (Fig. 7a, 7b), two forms of the PSI-LHCR supercomplex were shown. The one had three Lhcr antennae (PSI-3Lhcr) and the other had five Lhcr antennae (PSI-5Lhcr) (Pi, X. et al., 2018).

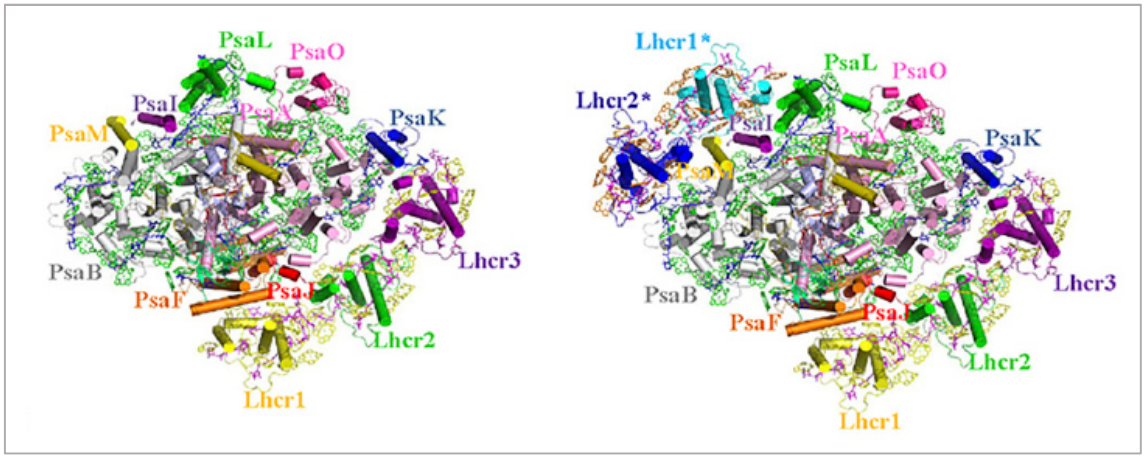


Figure 8: The structures of the PSI-3Lhcr form (left) and PSI-5Lhcr form (right) viewed along the membrane from the stromal side

2 AIMS OF THE THESIS

Red algae (Rhodophyta) are direct descendants of a primary endosymbiotic event by which photosynthesis was adopted by eukaryotic organisms. Through secondary endosymbiosis, a red alga was engulfed by a non-photosynthetic host and the endosymbiont was reduced to an organelle being present in a large and diverse group of algae including major global primary producers (e.g. the diatoms). Phycobilisomes remained dominant antenna system for PSI in red algae, while light harvesting antenna complexes (LHC) were associated with PSI. After secondary and tertiary endosymbiotic events, (LHCs) were adapted to diverse photosynthetic lineages acclimated to the environmental conditions (Neilson, J., Durnford, D., 2010 & Litvin, R. et al., 2016).

Due to their evolutionary importance, the red algae are important model organism for investigation of evolution of structure and function of the integral light-harvesting complexes of the LHC family. In spite of this, photosystem-antenna complexes of red algae have remained little studied. A high-resolution structure of a PSI-antenna complex became available only recently (Pi, X. et al., 2018, Antoshvili, M. et al., 2018). It was for the first time that the structure of the PSI-antenna complexes in red algae was unambiguously demonstrated to be different from the green algae and plants, although indications of this had been presented earlier (Bina, D. et al., 2017). Moreover, almost all the recent research in red algae has been focused on a narrow group of thermophilic extremophiles (i.e. Cyanidiales), most probably because they contain specialized enzymes useful for applications in biotechnology and pharmacy (Thangaraj, B. et al., 2011).

The aims of the thesis are i) to establish a method for purification of a well-defined photosystem I–antenna supercomplex from a mesophilic unicellular red alga *Porphyridium purpureum*; and ii) perform basic spectroscopic characterization of its function.

3 EXPERIMENTAL METHODS

3.1 Thylakoid Membranes Purification

The initial steps of the isolation procedures followed the established procedures of our laboratory. Cells were harvested by centrifugation at 5000 g, discarding the supernatant. The cells were stored at -80 ºC. Sample of cells was suspended in 50 mM HEPES pH 7,5 with protease inhibitor and transferred into glass homogenizer and after the cells were broken in EmulsiFlex-C5 high pressure cell disruptor (Avestin Inc., Canada) at 10,000 psi. Unbroken cells were removed by centrifugation for 5 min at 1.000 g and the solution were centrifuged for 40 min at 60.000 g. Supernatant containing highly fluorescent phycobiliproteins was discarded. The thylakoid membranes in pellet were collected, mixed with 1 ml HEPES buffer and transferred into small glass homogenizer. Following, small fraction from pellet (10 µl) was mixed with 1 ml acetone 80% and the concentration of chlorophyll a was determined by measurement of absorption. The rest amount of pellet was solubilized with 10% dodecyl-β-maltoside (β-DM) to final concentration 4% (w/v) for 45 min. Afterwards, the homogenate was centrifuged for 30 min at 30.000 g and the insolubilized material collected in the pellet was discarded.

Meanwhile, mixture of continuous sucrose density gradient (0.1-1.1 M in 50 mM HEPES pH 7,5, 0,02% β-DM) was prepared. The volume was split into 6 centrifuge tubes and they were stored in the freezer. Progressive thawing of the sucrose gradients was required. The supernatant composed of solubilized thylakoid membranes was placed onto the sucrose gradient and they were fractionated by ultracentrifugation overnight. The different bands were collected with a syringe withdrawing the material from the bottom of each band, starting with the highest. Their volume was concentrated by centrifugation with membrane filters and the precipitate was transferred into cryo-tubes, plunged in liquid N_2 and stored at -80°C (Gardian, Z. et al., 2007).

3.2 Methods used for PSI purification and characterization

Following the sucrose gradient, the PSI complex was further purified by combination of chromatographic steps. These are briefly described below.

3.2.1 Anion-Exchange Chromatography

Ion exchange chromatography is a useful method for separation of both organic and inorganic anions and cations. The principle is based on an ion-exchange process between the charged groups of stationary phase and the solute ion of the mobile phase. The stationary phase of the column consists of different type of resins depending on the analyte. Ion-exchange groups with a fixed charge are attached on the resin and near these functional groups are located the counter ions, rendering the whole entity electrically neutral. In our case, the employed anion exchanger was DEAE, diethylamino ethanol, a tertiary amine that carries a positive charge, hence binds negatively charged molecules. This means that the process works when the pH is above the protein's isoelectric point.

In the present case, open-column anion-exchange chromatography was performed using DEAE (Diethyl Aminoethanol) attached to Sepharose, a polymer resin (DEAE-Sepharose CL6B, Sigma) as a stationary phase. The polymer Sepharose consists of agarose gel about 0,5% concentration and is extremely porous with exclusion limits of up to 10^7 Daltons.

The sample was manually loaded on top of the column bed using a Pasteur pipette. The loaded sample was washed thoroughly with salt-free buffer and then eluted by increasing concentration of NaCl in the 5 mM-300 mM range. The working buffer was typically 50 mM HEPES, 0,02% β-DM, pH 7,5. Peristaltic pump operating at the flow rate of 1 mL/minute was used. The analysis was performed in the dark at 4°C.

3.2.2 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is differing from other separation methods (e.g. ion-exchange chromatography, reverse-phase liquid chromatography) which are based on interactions of the solutes with the mobile phase and stationary phase. SEC separation principle arise from the differences in molecular size and the penetration ability of different molecules into the stationary phase to different extents. SEC is used in preparative separations of biological origin macromolecules. The selection of the

stationary phase is important. The pore size of the material must match with the size of the desired molecules for separation. Large molecules will be excluded from all the pores of the stationary phase and will elute in the interstitial volume of the column, V_o. Smaller molecules will manage to penetrate all the pores of the stationary phase and will elute at the total elution volume of the column, V_t. Molecules of intermediate size will elute between V_o and V_t. Other crucial point for the selection of the stationary phase is compatibility with the mobile phase and the analytes. In SEC, any interaction with the stationary phase will lead to retention and consequently increase in elution volume (Lough, W., Wainer, I., 1995 & Katz, E. et al., 1998). In our setup we used Superdex 200 Increase 10/300 GL column (GE-Healthcare).

3.3 Pigment Composition Analyses

Reversed-phase chromatography was used to analyze pigment composition of our samples. In this method, the column consists of a hydrophobic silica based stationary phase modified with C_8 or C_{18} covalently bonded and a polar water-organic mobile phase is used. Sample molecules separation is determined by their hydrophobicity. More polar (hydrophilic) compounds are eluted first because they are held less strongly by the hydrophobic stationary phase and appear in chromatogram in the very beginning. In contrast more hydrophobic (non-polar) compounds are held more strongly and elute last. In order to modify the retention, parameters such as temperature and polarity of the column or the mobile phase can be adjusted. Specifically, decrease of retention can be achieved by usage of more polar column (cyano, C_4), less polar mobile phase and higher temperature (Snyder, L. et al., 1997).

In the present case, the analysis was done using following system: Pump Controller Delta 600, a manual injection system and a PDA 2996 detector (Waters, USA). Pigments analyzed on a reverse-phase Zorbax SB-C18 column (4,6 * 150 mm, 5 µl, silica-based, non-end capped; Agilent, USA). A linear gradient was used in the form: solvent A (80:20 methanol: 0,5 M ammonium acetate (aq., pH 7,2 v/v)), solvent B (90:10 acetonitrile: water), solvent C (100% ethyl acetate). After chromatographic separation, the pigment molar ratios were estimated from the areas under the chromatograph peaks displayed at different wavelengths corresponding to the respective extinction coefficients. The following molar extinction coefficients (in $dm^3\ mmol^{-1}\ cm^{-1}$) were used: Chl *a*: 78 at 662 nm, zeaxanthin: 134 at 453 nm, β-carotene: 134 at 453 nm (Litvın, R. et al., 2016).

3.4 Protein Composition Analyses

Protein complexes from different fractions of thylakoid membranes were analyzed into zones by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). SDS is a surface-active agent that is forming negatively charged protein-detergent complexes, under conditions which allow denaturation of proteins, with identical mobilities in solution despite their original charge. Additionally, a molecular weight size marker with known sizes of proteins is used as a distinct band onto the gel for estimation of the sizes of the proteins in sample. After the current is applied, these complexes on all track slots are migrating in parallel bands across the gel towards the positively charged anode depending on their size. Proteins with higher molecular weight are moving slower through the gel's pores compared to smaller molecular weight proteins that can penetrate the gel easier and move faster through it (Deyl, Z., 1979).

Electrophoresis buffer was composed of 470 ml distilled water, 25 ml running buffer and 5 ml SDS (10%). 15 µl of each sample was mixed with 1 µl reducing agent and 2,5 µl LDS sample buffer and pipetted into its own well in the gel which was previously immersed in electrophoresis buffer in electrophoresis apparatus. Voltage was applied for 25 min. After finishing with migration of proteins, the gel was kept in solution of acetic acid and Coomassie Brilliant Blue dye under shaking for 2 hours. In the end, the gel was washed one time with ethanol and was left for distaining in ethanol solution overnight (Litvın, R. et al., 2016).

3.5 Optical Spectroscopy

Analysis of interaction of light with matter forms a basis of a set of powerful methods that are widely used in chemistry to obtain information about the composition of the studied samples as well as progress of chemical reactions occurring in the sample. In the course of the present work methods based on analysis of both the absorption and emission of light were utilized, a brief overview of those follows.

3.5.1 Absorption Spectroscopy

Absorption spectroscopy is based on measurement of attenuation of radiation by a sample as a function of wavelength of incident radiation. The attenuation is typically described by a quantity called absorbance, which according to Lambert-Beer law is proportional to concentration (c) of the absorbing species:

$$A = c \cdot \varepsilon \cdot d,$$

where d is the length of path the light travels through the sample and ε is a constant called the extinction coefficient, specific for a given chemical species. The absorbance is related to intensity of light:

$$A = -\log (I_S/I_0),$$

where I_S is the intensity measured after the passage of the light beam through the sample and I_0 is the incident intensity. The plot of absorbance as a function of incident wavelength is the sample's absorption spectrum.

The measurement of absorbance as a function of time can be used to study the time course of chemical reactions. In pump-probe spectroscopy, the absorbance of a sample is first recorded in the dark to capture the initial state using a weak measuring light ("probe"). The sample is then exposed to an intense pulse of light ("pump") and further absorbance recordings (using the "probe" beam) are taken at defined time intervals after the pulse. Using multichannel detectors, it is possible to record absorbance at different wavelengths simultaneously, hereby acquiring a set of time-dependent absorbance spectra that characterize the dynamics of the light-induced reactions. Typically, the initial dark spectrum is subtracted from the spectra acquired after the pump pulse. In the resulting difference spectra, the positive features correspond to the absorbance of the products formed by the pump pulse; negative features indicate depletion of the reactants.

3.5.2 Fluorescence Spectroscopy

Following absorption of a photon, the molecule resides in an excited state, lifetime of which is finite. During its return to the ground state, light can be emitted. If the process does not involve a change of electron spin, the process is called fluorescence. The spectral dependence of fluorescence emitted from a sample carries information about the nature of the emitting species, time-resolved measurement of fluorescence yields information about the dynamics of the excited state of the probed molecules.

Following a short pulse of light that promotes the molecules in the sample to the excited state, the fluorescence signal typically decays exponentially, starting from an initial value F_0:

$$F = F_0 \cdot \exp(-t/\tau),$$

where t is time and τ corresponds to the lifetime of the excited state. This is related to the rates of all the possible routes of deexcitation as:

$$\tau = 1 / (k_f + k_{IC} + k_{ISC} + \ldots),$$

where k's correspond to the rate constants of respective de-excitation processes, such as fluorescence (f), internal conversion (IC, release of heat), intersystem crossing (ISC, formation of triplet state), etc.

Using the knowledge of τ and the rate constants allows one to compute the yield of the particular de-excitation pathway; thus

$$\Phi_f = k_f / (k_f + k_{IC} + k_{ISC} + \ldots) = k_f \cdot \tau,$$

corresponds to the yield of fluorescence, the ratio of photons emitted to photons absorbed by the sample. Yields for other processes can be expressed in analogous manner.

Similar to absorbance, fluorescence measurements can be performed in the steady-state and time-resolved manner. Steady-state fluorescent measurement is performed with constant illumination of sample with a continuous beam of light and the emission spectrum is recorded.

In the present work, time-resolved fluorescence was measured using the time-correlated single photon counting approach (TCSPC). Here, the sample is subjected to a series of short (~10 ps) pulses delivered at high frequency (~MHz) by a laser diode and the statistics of arrival times of photons to the detector following each pulse is acquired. The resulting histogram corresponds to the kinetics of the fluorescence decay.

3.5.3 Instrumentation

The steady-state absorbance of all fractions was measured by uv/vis spectrophotometer Shimadzu UV-2600 (Shimadzu, Japan). For spectroscopic determination of chlorophyll concentration, a spectrophotometer UV300 (Spectronic Unicam, UK) was used. Pigment were measured in 80 % (v/v) acetone solution according to Lichtenthaler (Litvın, R. et al., 2016). Time-resolved absorption spectroscopy was performed using home-built multichannel spectrometer with microsecond resolution utilizing pulsed Xe-lamp light sources and photodiode array detection (Bina, D. et al., 2006). Time-resolved spectra were acquired and analyzed by locally written scripts (provided by the supervisor).

Fluorescence steady-state emission spectra were recorded by using a Jobin-Yvon Spex Fluorolog II spectrometer. For fluorescence emission, the excitation wavelength was 435 nm and the slit width 4 nm. The emission spectra were recorded in 1 nm steps from 640 to 780 nm, with 4 nm slit width.

Time-resolved fluorescence was measured using the FluoTime 300 (PicoQuant, Germany). Analysis was performed using the FluoFit software (PicoQuant). Fluorescence was excited using a blue laser diode LDH-P-C-485. Instrument response function was acquired by measuring the temporal profile of actinic pulse scattered using colloidal silica suspension.

4 RESULTS

4.1 Algal Culture

The cultures of *P. purpureum* were grown in artificial seawater f/2-Si medium (Guillard, R., Ryther, J., 1962) in 1000 or 3000 mL Erlenmeyer flask placed on the lab bench. The cultivation was carried out under ambient illumination (typically 20-50 μmol(photons)·m^{-2}·s^{-1}, and ambient laboratory temperature. The cultures were constantly stirred. The cells were harvested after 2-4 weeks of cultivation. A typical absorption spectrum of our *P. purpureum* culture upon time of the harvest is shown in (Fig. 9). Due to lack of environmental control, the cultures showed some variation in density as well as the pigment content, and Chl a / phycobilisome ratios, as shown in appendix A. Nevertheless, the results described in the following sections, pertaining to the isolation and purification of the PSI-Lhcr complex were stable.

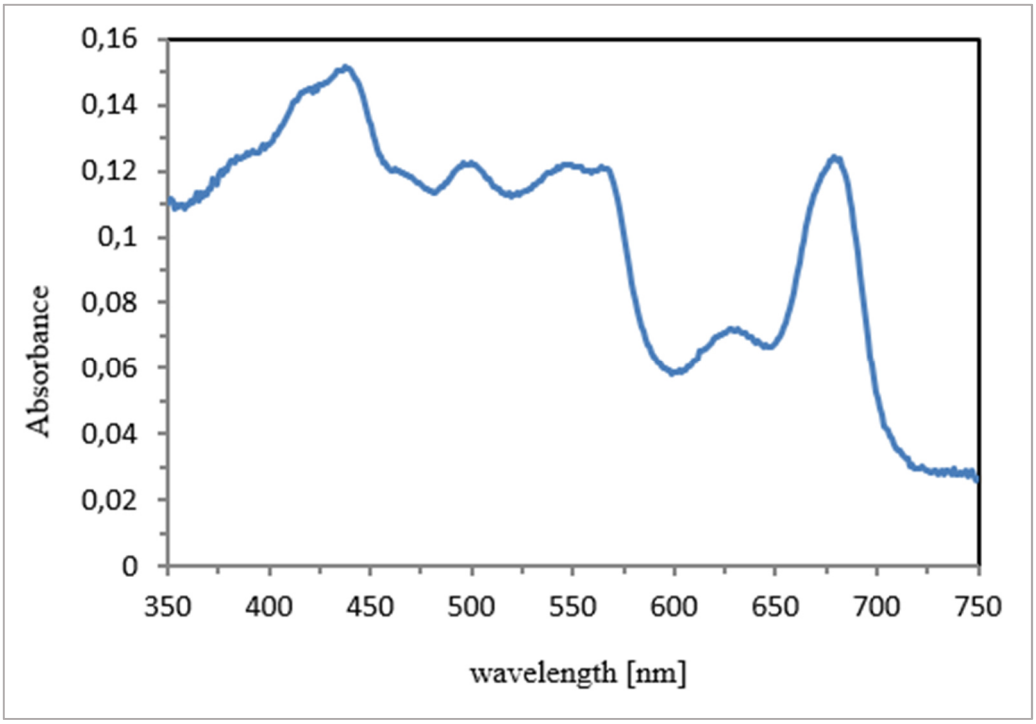


Figure 9: Example of absorption spectrum of P. purpureum culture, measured using integration sphere equipped spectrometer (Shimadzu UV2600)

4.2 Fractionation of Thylakoid Membranes from P. Purpureum

Ultracentrifugation of red algal *Porphyridium Purpureum* cells culture resulted in five sucrose gradient bands, zones, shown in (Fig. 10). A straightforward initial assignment of the major components of the zones can be made based on color of the zones and using comparison to literature, such as Litvin, R. et al. (2016), Gardian, Z. et al. (2007) and Bumba, L. et al. (2004).

Band 1 contained free pigments, band 2 contained phycobilisomes. It was noted that phycobilisome zones exhibited strong visible fluorescence when observed in ambient light. The faint green band at the top of zone 2 was likely to contain free Lhc or fragments of Chl a-binding proteins. Zone 3 consisted of phycobilisomes and presumably PSII particles. The green bands of zones 4 and 5 contained the larger Chl a-binding complexes, most likely supercomplex PSI, based on comparison to other algae.

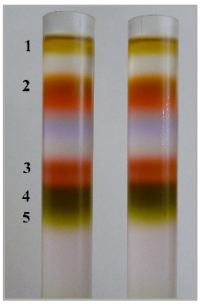


Figure 10: The results from sucrose gradient ultracentrifugation run. The green bands correspond to zones containing a large amount of Chl a

The five bands were collected for concentrating their volume and their absorption spectra are shown in Fig. 11. Band 4 had the highest ratio of chlorophyll, absorbing maximally at 680 nm, while Band 2 had maximum at 557 nm and 621 nm, in the phycobilisomes absorbance range (470-650 nm). Presence of these absorption features in all the bands indicated that traces of phycobiliproteins were present in all the gradient zones and further purification was necessary.

Comparison of the present results to previous recent data from red algae, such as Gardian, Z. et al. (2007) and Bumba, L. et al. (2004) show that in our case the separation of PSII and PSI in the sucrose gradient step was more successful, as indicated by the red-shift of the Chl a absorption maximum at ~680 nm between zones 3 and 4/5. The assignment of the different complexes was further supported by measurement of the fluorescence emission spectra at the liquid nitrogen temperature, 77 K. Since the main interest of this work lies in the investigation of the PSI supercomplex,

excitation into Chl a absorption band at 435 nm (so called Soret band) was used. Zone Z2 showed a rather broad emission band peaking at ~678 nm. Zone Z3 exhibited a narrow peak with a maximum at about 690 nm, flanked on both sides by broad shoulders at about 670 and 715 nm. Emission spectrum of this zone corresponds very well to the crude PSII zone observed by Bumba, L. et al. (2004). The spectra of the bottom zones 4 and 5 showed pronounced maxima at ~675 nm and 715 nm. Such spectrum is typical of PSI-Lhc super-complexes (Bumba, L. et al., 2004, Gardian, Z. et al., 2007, Tichy, J. et al., 2013). The zone 5 was usually very weak and difficult to resolve. Thus, the intense zone 4 was used for further analyses.

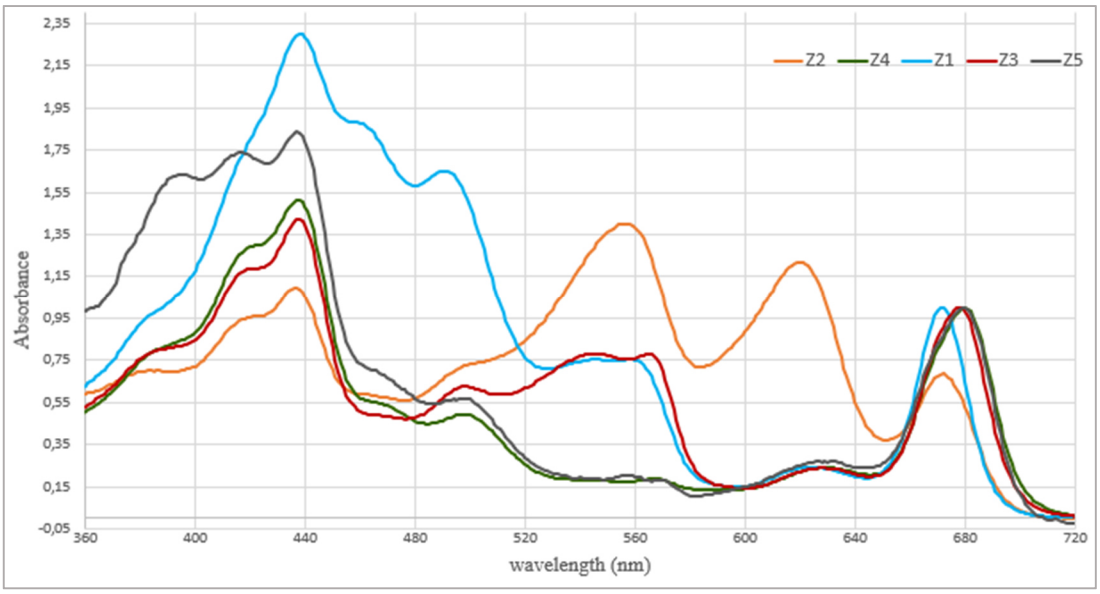


Figure 11: Normalized absorption spectra of sucrose gradient bands

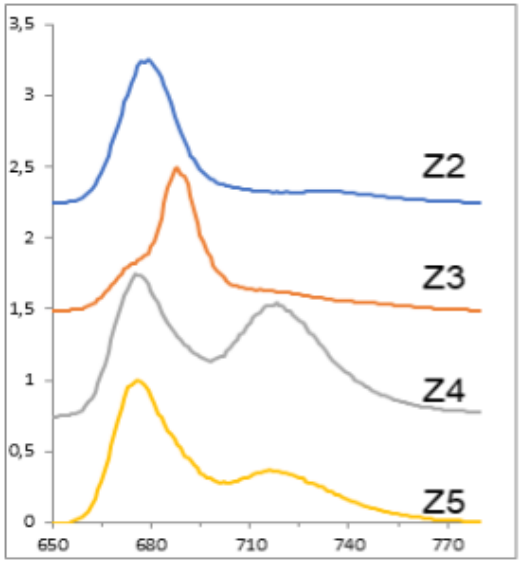


Figure 12: Emission spectra at 77 K of sucrose gradient bands shown in Fig 10. Excitation at 435 nm

4.3 Purification of PSI by Anion-Exchange Chromatography

Zone 4 identified by spectroscopy as containing the majority of PSI was loaded onto a DEAE Sepharose column equilibrated with HEPES buffer with 0 mM NaCl. The anionic groups of the sample were supposed to attach on the functional groups of the column and separation process would carry through by several washing steps of different salt concentrations until whole of the sample was eluted.

At first the column was washed with 60 ml of salt-free buffer. This elution was collected in 10 ml fractions. Then the salt concentration was increased to 5 ml. After 35 ml washing by 5 mM NaCl, the rest of bound sample was eluted by NaCl gradient from 5 to 300 mM and collected by 2 ml fractions. Example chromatograms from this analysis are shown in Fig. 13. Contrary to our expectation a large portion of the Chl a-containing material did not bind to the column at all. Remaining small amount of the Chl a-containing complexes eluted at salt concentration below 100 mM. As shown below, protein composition of the two Chl a-containing fractions from anion-exchange chromatography were the same. Note that the initial non-binding fractions eluting at salt-free buffer, were collected in 10 mL batches, as opposed to the 2mL fractions collected during NaCl gradient. Hence, the total amount of PSI material that left the column at 0 mM NaCl was much larger that what eluted later.

Phycobilisomes eluted at the end of the gradient, above 200 mM NaCl. To see whether the binding of the sample to DEAE column was adversely affected by the buffering agent (HEPES) we performed the same analysis in the Tris buffer, one of the buffers recommended for anion-exchange chromatography (https://www.bio-rad.com/). However, the same result was obtained, excluding the buffer effect.

We also performed the same experiment with the PSI complex containing zone from alga *Nannochloropsis oceanica*, under identical conditions. This is also shown in (Fig. 13). As seen, the *N. oceanica* PSI bound to the DEAE column well with no material eluting at 0 mM NaCl.

In conclusion, the DEAE chromatography was shown to be useful for separation of PSI from contaminating phycobilisome fraction. However, since majority of the PSI did not bind to the DEAE column and was eluted immediately, this method is not suitable for separation of protein-complexes from free pigment and thus further purification step using the size-exclusion chromatography was applied.

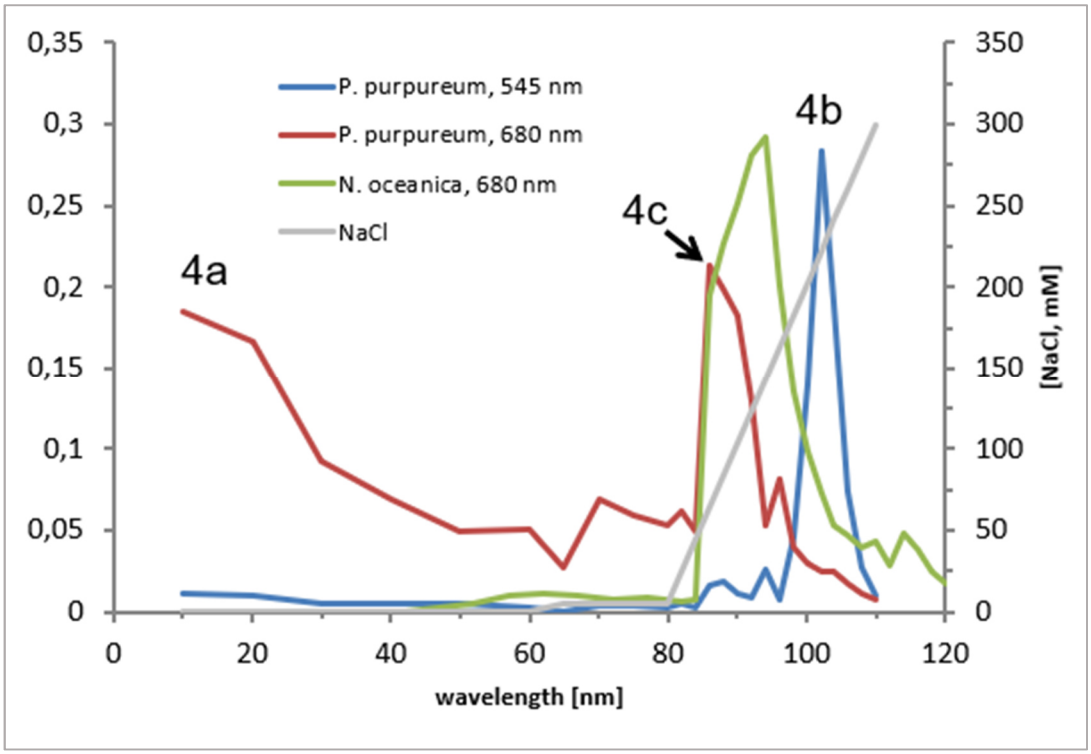


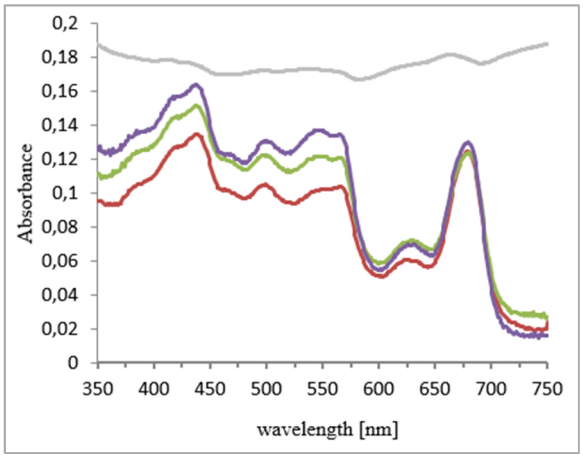
Figure 13: Chromatograms from the DEAE anion exchange chromatography of the P. purpureum PSI-containing sucrose gradient. The labels 4a-c indicate fraction collected for protein analysis, as described in the section 4.4

5 Conclusions

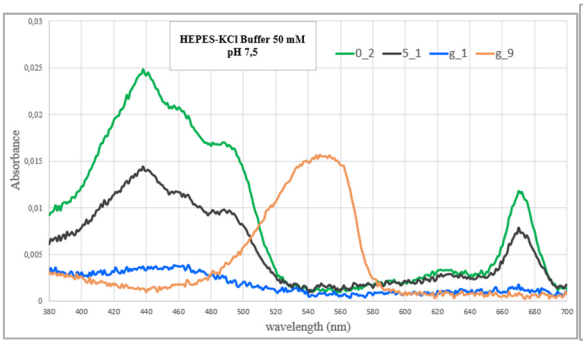
- Structure of the basic PSI-Lhcr antenna supercomplex in mesophilic red algae consists of PSI core associated with three Lhcr.
- This complex can be isolated in functionally intact form useful for functional studies.
- Photoprotective function of carotenoid against Chl a triplets in intact PSI-antenna complexes was demonstrated.

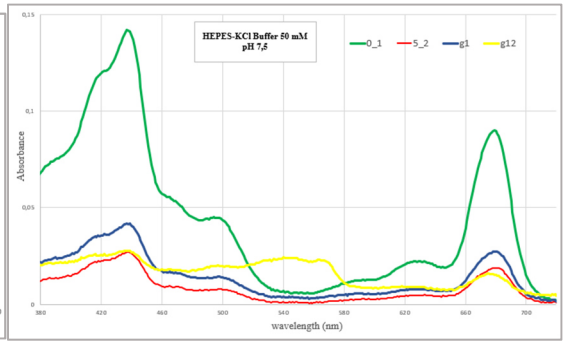
APPENDIX

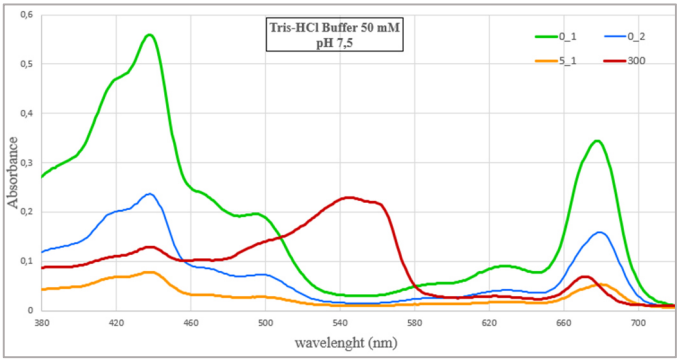
Appx A: Absorption spectra of three different *P. purpureum* cultures used in the present work. Note that the spectra were acquire using spectrometer (Shimadzu UV2600) equipped with an integration sphere. An example of cell culture spectrum measured in regular mode without the integration sphere is shown in grey line.



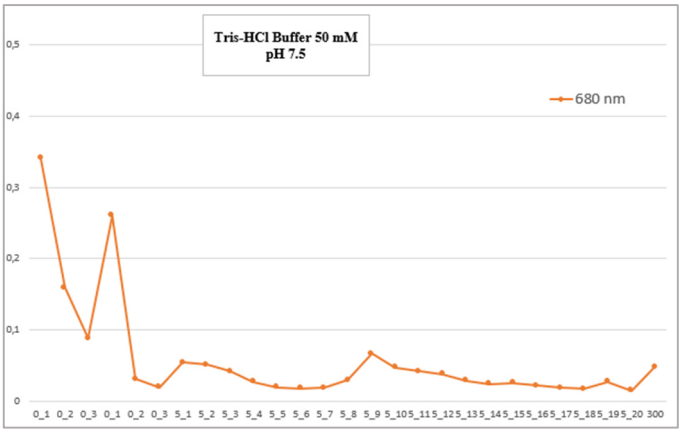
Appx B: Repetitions of absorbance spectra from anion exchange chromatography eluates with different buffers



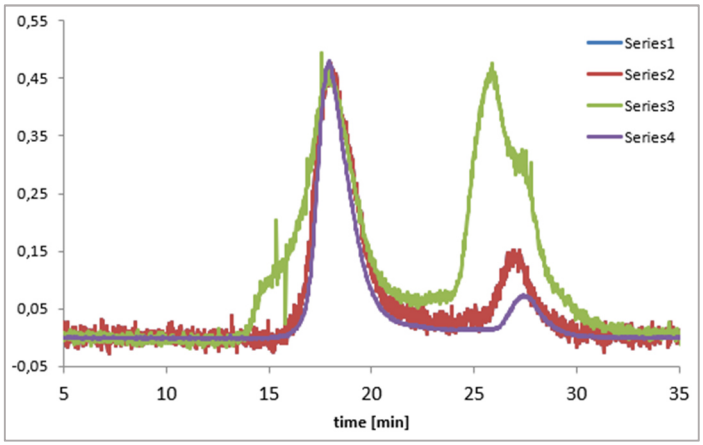




Appx C: Repetitions of spectra variations from anion exchange chromatography eluates with different buffers



Appx D: Size-exclusion chromatogram of *Nannochloropsis oceanica* purified PSI samples



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