Antioxidant in a model biomembrane – astaxanthin and its esters mixed with DPPC in Langmuir films

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Abstract. Amphiphilic properties of astaxanthin molecule and its esters make it possible to include it in self-organized structures based on lipid membrane components. Astaxanthin and its mono- and diesters were isolated from the extract of the microalga Haematococcus Pluvialis, purified by column chromatography, and identified by thin-layer chromatography. The absence of impurities in astaxanthin and its esters was confirmed by means of high performance liquid chromatography method with detection in the ultraviolet-visible region and mass spectrometric detection. The model systems of the cell membrane lipid bilayer — Langmuir films of mixtures of DPPC with astaxanthin and its mono- and diesters, were formed at the air-water interface and studied by a complex of methods. It was found that an increase in the amount of astaxanthin, as well as the addition of its esters to the Langmuir films of DPPC, leads to an increase of values of area per molecule at the surface pressure isotherm rise and decrease of Langmuir film collapse pressure. The addition of astaxanthin mono- and diesters prevents the phase transition in the DPPC monolayer more strongly than unesterified astaxanthin.

1. Introduction

Astaxanthin and other carotenoids are involved in the course of biochemical processes in living organisms as antioxidants, they protect the membranes of living cells from destruction by free radicals and active oxygen forms that are produced when the skin is exposed to UV radiation from sunlight. The level of antioxidants in the body decreases with increasing oxidative stress (smoking, sunburn, aging, etc.), the source of their intake can be food, as well as dietary and biologically active additives [1], that are promising in the complex treatment of pathologies and diseases caused by aging [2–4]. It is assumed that carotenoids with a maximum absorption of 400–500 nm, including astaxanthin, perform protective functions in the human body, protecting the skin and eyes from excessive illumination of the visible part of the spectrum [4, 5], from photoaging of the skin [6]. Therefore, the development of photoprotective cosmetics and dietary supplements is relevant, astaxanthin carotenoid being one of the promising ingredients [7] that has antioxidant [8, 9], anti-inflammatory [10, 11], and wound healing [12, 13] effects. The potential application of astaxanthin is not limited to dermatology. Prospects for its use as a hepatoprotector [14], in complex therapy for the treatment of neurodegenerative diseases and brain injuries [15–17], consequences of diabetes mellitus [18], and age-related eye pathologies [19, 20] have been noted.

From the microalgae Haematococcus Pluvialis [5], containing up to 5% astaxanthin, it is extracted by organic solvents in the form of mono- and diesters [6]. It is known that the bioavailability of astaxanthin is higher than that for its esters [1, 21]. In living organisms, astaxanthin exhibits
antioxidant activity both in the cytoplasm and in the lipid membrane, since it is able to integrate into it, increase its rigidity and prevent the penetration of active oxygen forms and other radicals through the membrane [2, 3, 22]. Due to the amphiphilic structure of the astaxanthin molecule, the properties of its derivatives [23], as well as mixtures with lipid components, can be studied on model biological membranes – Langmuir monolayers at the air/water interface [24-28]. The results obtained on such simplified systems can be extrapolated to biomembranes [22, 28]. The aim of this work was to study the self-assembly of mixtures of astaxanthin and its esters with DPPC in Langmuir films at the air/water interface.

2. Experimental part
Commercially available DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, Sigma-Aldrich, USA), astaxanthin (Sigma-Aldrich, USA), as well as its mono- and diesters isolated from the microalgae Haematococcus Pluvialis extract (Sigma-Aldrich, USA) and purified by column chromatography on silica gel (mobile phase – mixture of acetone : hexane, gradient elution). Components were identified by thin-layer chromatography. On average, the microalgae Haematococcus Pluvialis extract contains: 75% astaxanthin monoesters, 20% astaxanthin diesters and 5% astaxanthin.

Astaxanthin and its esters were separated by high performance liquid chromatography (HPLC) with detection in UV-visible range and mass detection (Agilent 1200 series with a diode-matrix detector and 6120 Quadrupole LC/MS, Agilent Technologies Inc., USA). Astaxanthin monoesters were separated on a 150×2.1 mm reverse phase column, 5 µm particle size, Orbit-C18. Astaxanthin diesters were separated on a 250×4.6 mm column, 5 µm particle size, Eclipse-C18. The detection was performed on a UV-visible detector at a wavelength of 470 nm, as well as a mass spectrometric detector in the mode of positive ions in the range of 830–870 m/z, 870–900 m/z for astaxanthin monoesters and 1050–1150 m/z for astaxanthin diesters. The mobile phase was 50% (V/V) acetonitrile in water (Millipore, USA) (A) and 100% acetonitrile (B) for astaxanthin monoesters. The flow rate was 700 µl/min, the volume of the injected sample was 20 µl. The elution program was started with 25% A and 75% B and continued in gradient elution mode (0–12 min) to 100% B. From 12 minutes to the end it was isocratic mode of 100% (V/V) of the mobile phase B. The mobile phase was 100% (V/V) acetone : water (Millipore, USA) 90 : 10 (A) and acetone : water (Millipore, USA) 98 : 2 (B) for astaxanthin diesters. The flow rate was 800 µl/min, the volume of the injected sample was 20 µl. The elution program started with 100% A and continued in gradient elution mode (0–50 min) until 100% B.

The formation and investigation of Langmuir films properties was performed on the Langmuir Minitrough Extended (KSV, Finland) with a maximum interfacial surface area of 558 cm². Purified and demineralized water (Millipore, USA) with a specific resistance of 18.2 MΩ·cm (at 25 °C) was used as a subphase. The subphase was thermostated at 20 °C. The solutions of DPPC and its mixtures with 1 wt. % of astaxanthin, 1 wt. % of astaxanthin monoesters and 1 wt. % of astaxanthin diesters in chloroform with a concentration of 1 mg/ml were prepared. Langmuir films were formed after the spreading of solutions and evaporation of the solvent and were studied in the compression mode with a constant rate of 15 cm²/min. Surface pressure was measured by the Wilhelmy method using a rough platinum plate with an accuracy of 0.1 mN/m. Surface potential was measured by the vibrating electrode method using a SPOT sensor (KSV, Finland) with an accuracy of 1 mV. The morphology of Langmuir films directly on the water surface was visualized using a BAM-300 Brewster microscope (KSV, Finland). The obtained micrographs corresponding to the interfacial surface of 100×100 µm² were geometrically corrected taking into account the observation at the Brewster angle (53.1°).

3. Results and discussion
Model systems of the cell membrane lipid bilayer – the Langmuir films of mixtures of DPPC with astaxanthin and its mono- and diesters were formed at the air-water interface and were studied by a complex of methods. The surface pressure vs. mean molecular area (π-A) compression isotherms and
the surface potential vs. mean molecular area ($\Delta U$-$A$) compression isotherms are shown in figure 1, surface morphology of Langmuir films visualized by Brewster angle microscopy is shown in figure 2.

**Figure 1.** The $\pi$-$A$ compression isotherms (a) and the $\Delta U$-$A$ compression isotherms (b) of the Langmuir films of DPPC (1), DPPC mixed with 1 wt. % of astaxanthin (2), DPPC mixed with 1 wt. % of astaxanthin monoester (3), DPPC mixed with 1 wt. % of astaxanthin diester (4) at the air/water interface at 20 °C. The empty circles in plot (a) correspond to Brewster angle micrographs in figure 2.

**Figure 2.** Brewster angle micrographs for the surface of compressed Langmuir films: DPPC to a surface pressure of 5.8 mN/m (a), DPPC mixed with 1 wt. % of astaxanthin compressed to a surface pressure of 8.1 mN/m (b) at a subphase temperature of 20 °C. Scale bar 50 um.

The addition of astaxanthin, as well as its mono-and diesters in the amount of 1 wt. % of in DPPC leads to a change in the shape and parameters of the $\pi$-$A$ and the $\Delta U$-$A$ compression isotherms of Langmuir films. The shape of the $\Delta U$-$A$ isotherms for mixed Langmuir films is characterized by the disappearance of the “plateau” section. The magnitude of the jump in the surface potential decreases to $295\pm5$ mV both in the case of astaxanthin and its esters, and the maximum value at the point of collapse of the monolayer is reduced to $595\pm2$ mV in the case of astaxanthin and to $555\pm2$ mV in the
case of astaxanthin esters. It was found that the mean molecular area at the beginning of growth in surface pressure increased from 90±1 to 93±1 Å² per molecule with the addition of astaxanthin, as well as to 122±1 and 145±1 Å² per molecule with the addition of astaxanthin mono- and diesters, respectively. A change in the shape of the π-A isotherms in the region of the DPPC phase transition in mixed Langmuir films was revealed. The bend at the plateau exit point was still noticeable for DPPC mixed with astaxanthin, but was no longer evident for DPPC mixed with astaxanthin esters. A significant reduction in the size of condensed phase aggregates is noticeable in the Brewster angle micrograph (figure 2b) with the addition of 1 wt. % of astaxanthin to DPPC Langmuir film compared to the pure DPPC Langmuir film (figure 2a). The bending on at the π-A isotherm associated with the end of the phase transition of the Langmuir film from the liquid expanded to the liquid condensed state is observed with increasing values of the mean molecular area (in Å² per molecule) during the transition from pure DPPC (57±1) to its mixtures with astaxanthin (62±1), with astaxanthin monoester (76±1), with astaxanthin diester (90±1). The surface pressure values (in mN/m) at that point also increase with the transition from pure DPPC (7.0±0.1) to its mixtures with astaxanthin (8.0±1), its mono (12.3±1) and diesters (12.5±1). Thus, the addition of mono- and diesters of astaxanthin increases the surface pressure and the mean molecular area more strongly, and therefore loosens the DPPC lipid monolayer more strongly than non-esterified astaxanthin. The stability of the Langmuir films of DPPC mixed with astaxanthin and its esters, determined by the value of the surface pressure at the collapse point, is lower compared to the Langmuir film of pure DPPC. The addition of unesterified astaxanthin reduces the stability of the Langmuir film from 55±1 mN/m at 38±0.5 Å² per molecule to 48.5±1 mN/m at 40±0.5 Å² per molecule, and to 45.0±1 mN/m at 45±1 and 55±1 Å² per molecule for astaxanthin mono- or diesters, respectively.

4. Conclusion
The study of astaxanthin and its esters in the model systems of the cell membrane lipid layer – Langmuir films – showed that the structure formation of DPPC from a liquid-expanded to a liquid-condensed layer is hindered both in the presence of astaxanthin and astaxanthin mono - and diesters. The shift of the surface pressure and surface potential isotherms in the direction of increasing area per molecule, when adding astaxanthin and its esters to the Langmuir films of DPPC, was detected. The addition of astaxanthin mono-and diesters more strongly prevents the phase transition in the DPPC monolayer than non-esterified astaxanthin. As a result, the stability of Langmuir films of DPPC mixed with astaxanthin and its esters is lower than that of the Langmuir film of pure DPPC. The results obtained on model systems will be taken into account in the development of stable lipidized forms of astaxanthin and its esters with antioxidant properties able to protect the skin from active oxygen forms.

5. References
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