HPLC ASSESSMENT OF VIOLAXANTHIN CYCLE’S FUNCTION UNDER EXCESSIVE LIGHT IN MOUGEOTIA SP. ALGAE

1Muntean E., 2V. Bercea, 3N. Dragoș, 4Nicoleta Muntean

1University of Agricultural Sciences and Veterinary Medicine, Faculty of Agriculture, 3-5 Mănăștur Street, 400372 Cluj Napoca, Romania, e-mail: edimuntean@yahoo.com
2Institute of Biological Researches Cluj Napoca
3Babeș-Bolyai University Cluj Napoca
4Institute of Public Health Cluj Napoca

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Abstract. The protective function of xanthophyll cycle was investigated in the green algae Mougeotia sp. Agardt (AICB 560) originating from the collection belonging to Institute of Biological Research Cluj Napoca, using excessive light. High performance liquid chromatography was used to analyze carotenoids involved in violaxanthin cycle in this matrix. Separations were performed on an Agilent 1100 system, using a Nucleosil 120-5 C18 column and the following mobile phases: A - acetonitrile : water (9 : 1) and B - ethyl acetate. The flow rate of the mobile phase was 1 ml/min. and the solvent gradient was as follows: from 0 to 20 min. - 10% to 70% B, then from 20 to 30 min. - 70% to 10% B. Chromatographic analysis demonstrated a particular behavior of AICB 560 under intense illumination: the major de-epoxidation product is not zeaxanthin, but antheraxanthin. More than that, the illumination conditions affect the whole carotenoid biosynthesis, starting with the violaxanthin cycle’s pigments precursor; β-carotene.

INTRODUCTION

Photosynthetic organisms convert the light and use this energy to perform the metabolic reactions required for life. The chlorophylls are key molecules in these systems but they absorb efficiently only the red and blue portions of the light spectrum; light absorption is improved by carotenoids, which can absorb other portions of the light spectrum. Carotenoids are also essential photoprotective and antioxidant pigments. Thus, carotenoids are involved in the xanthophyll cycle (also called the violaxanthin cycle), which is a mechanism which protects photosynthetic organisms from damage by excessive light (Garcia-Mendoza, 2000).

The xanthophyll cycle is a two-stage reversible transmembrane interconversion of three oxygenated carotenoids: zeaxanthin, antheraxanthin and violaxanthin. It starts when exposure to high light levels occurs, exceeding a maximum that can be used productively by the photosynthetic apparatus, when a reversible violaxanthin de-epoxidation leads to antheraxanthin and finally to zeaxanthin; the resulting zeaxanthin accumulates in the chloroplast thylakoids, the excessive energy being afterwards dissipated as heat (Demming-Adams, 1996). This reaction is a relatively rapid one (it occurs in minutes), being catalyzed by violaxanthin de-epoxidase located in the thylakoid lumen of the chloroplasts (Hager, 1994). In darkness or under low irradiance (lower than that required for saturation of photosynthesis), zeaxanthin is re-epoxidated back to violaxanthin by zeaxanthin epoxidase (Hager, 1980), the monoepoxide antheraxanthin being again an intermediate in this reversible redox process (figure 1).
These light-dependent interconversions of violaxanthin and zeaxanthin in chloroplasts was firstly observed in higher plants (Saphoznikov, 1957; Yamamoto, 1962); since then, it was established that it has a photoprotective role, removing the excess excitation energy from the photosynthetic antennae (Darko, 2000; Demming-Adams, 1996; Gilmore, 1993; Goss, 1998; Masojidekl, 2004).

![Figure 1. The xanthophyll cycle](image)

Xanthophyll cycle is not only a mechanism which protects photosynthetic organisms from damage by excessive light, but it also fulfills a regulatory task in the biosynthetic pathway of carotenoids in several algal classes, as it allows the accumulation of xanthophylls intermediate during high light conditions while neither light-harvesting pigments nor chlorophylls are synthesized (Lohr, 1999).

The aim of this paper is to establish whether the xanthophyll cycle is functional and how this mechanism works under excessive light in the AICB 560 strain of green algae Mougeotia sp. Agardt. For investigation the light-dependent adaptation of the pool size of the violaxanthin cycle pigments, the carotenoid analysis was achieved using one of best techniques available to date - high performance liquid chromatography (Darko, 2000; Muntean, 2005).

**MATERIAL AND METHODS**

**Reagents and materials.** The carotenoid references were provided by F. Hoffmann-La Roche, Basel, Switzerland. All solvents for chromatography were HPLC grade purity (ROMIL Chemicals) and they were filtered through 0.45µm microfibre filters (Whatman), then degassed in an ultrasonic bath, under vacuum, before use. Solvents for extraction were of p.a. quality, freshly distilled.

**Special precautions.** For avoiding artifacts, all operations were carried out in reduced light, avoiding samples heating at more than 40°C. Prior to injection in HPLC systems, carotenoid solutions were filtered through 0.45 µm Whatman filters.

**Matrix.** Experiments were carried out using the green algae Mougeotia sp. Agardt (strain AICB 560) originated from the collection of the Institute of Biological Researches Cluj Napoca. The AICB 560 strain was grown in a Bold nutritive solution that was mixed by
introducing air containing 5% CO₂, under continuous illumination (300 μmol.m⁻².s⁻¹, measured with a Hansatech Quantum Sensor QSPAR), at an average temperature of 20°C, for 15 days.

**Experimental conditions.** The AICB 560 strain was subjected to two high light irradiations (4500 and 1500 μmol.m⁻².s⁻¹) and after each one a recovery stage followed (in the dark); algal suspension samples were collected during each illumination and recovery stage.

**Extraction.** The algal sample suspensions were saponified overnight (10 hours), using 10 ml solution 30% KOH in methanol, at room temperature. Carotenoids were then extracted using diethyl ether; the etheric layer was separated and washed repeatedly with brine, then with distilled water until free of alkali. The aqueous layers were re-extracted with small volumes of diethyl ether until colorless, then the organic layers were combined, washed several times with distilled water and evaporated to dryness under reduced pressure. The saponified extracts were dissolved in 10 ml ethyl acetate, being then subjected to HPLC analysis.

**High performance liquid chromatography** analysis was performed using an Agilent 1100 HPLC System consisting in a solvent degasser, a quaternary Agilent G1311A pumping system, a Rheodyne 7125 injection valve with a 20 μl loop, an Agilent G1314A UV/ VIS detector, a Agilent G1316A column thermostat and a computer running Chemstation software (Agilent Technologies) for data analysis. On both systems separations were performed using the same Nucleosil 120 - 5 C₁₈ column (250 mm length and 4.6 mm i.d., 5 μm particle size) and the following mobile phases: A - acetonitrile : water (9 : 1) and B - ethyl acetate. The flow rate was 1 ml/ min. and the solvent gradient was as follows: from 0 to 20 min. - 10 to 70% B, then from 20 to 30 min. - 70 to 10% B (Muntean, 2005). Separations were performed at 35°C, being monitored at 450 nm; quantitative HPLC analysis was based on external standard method. Carotenoids identification was completed based on HPLC co-chromatography with authentic carotenoid standards (Britton, 1996).

**RESULTS AND DISCUSSIONS**

The HPLC chromatogram from figure 2 displays the carotenoids separated from the saponified extract of *Mougeotia sp.* control sample. The target carotenoids identified are the xanthophylls violaxanthin, lutein, zeaxanthin (missing here, due to illuminating conditions utilized for growing) and 5,6-epoxy-β-carotene; besides, carotenes α-carotene, β-carotene, 9Z-β-carotene and 15Z-β-carotene were also identified. The chromatographic profile is a simple one, dominated by only two major carotenoids: lutein and β-carotene.

![Figure 2: HPLC chromatogram of carotenoids from the Mougeotia sp. control sample Peak identities are: 1. violaxanthin, 2. antheraxanthin, 3. lutein, 5. 5,6-epoxy-β-carotene, 6. α - carotene, 7. β - carotene, 8. 9Z - β - carotene, 9. 15Z - β - carotene.](image-url)
The HPLC chromatogram from the figure 3 shows the algal response to a high light irradiation (4500 \( \mu \text{mol.m}^{-2}.\text{s}^{-1} \)). Here, the chromatographic pattern shows two major carotenoids: lutein and antheraxanthin, while among minor carotenoids 5,6-epoxy-\( \beta \)-carotene disappeared. The recovery after irradiation with 4500 \( \mu \text{mol.m}^{-2}.\text{s}^{-1} \) is revealed by the chromatogram from figure 3b. The new chromatographic pattern is dominated by three major carotenoids: lutein, \( \beta \)-carotene and violaxanthin. Quantitative data are presented in table 1.

When the Mougeotia culture was exposed to a high light irradiation (4500 \( \mu \text{mol.m}^{-2}.\text{s}^{-1} \)), the content of antheraxanthin increased strongly as a result of de-epoxidation (figure 3, table 1); the concentration of zeaxanthin is quite small, proving that de-epoxidation is not complete and stops at antheraxanthin stage. Comparing the chromatograms from figure 2 and 3a, it is obvious that the whole carotenoid pattern was affected by the light stress, not only the xanthophylls involved in the xanthophyll cycle. Carotenoids were converted to xanthophylls and this can explain the high proportion of antheraxanthin; the high concentration of antheraxanthin can be explained only admitting a \textit{de novo} synthesis of this by \( \beta \)-carotene (a metabolic precursor of antheraxanthin). This chromatogram emphasize another important aspect in the studied matrix: the xanthophyll cycle converts violaxanthin mainly in antheraxanthin, not in zeaxanthin; this finding agrees with results reported for \textit{Mantoniella squamata} (Goss, 1998), where they were attributed as consequences for the mechanism of
enhanced non-photochemical energy dissipation. The recovery after such a high light stress lead to a reversible epoxidation, the final higher violaxanthin level being correlated with a strong decrease in antheraxanthin concentration while the decrease in zeaxanthin concentration is weaker (figure 3b, table 1).

Table nr.1: The concentrations of target carotenoids [µg/ml algal suspension]

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Control sample</th>
<th>Irradiation with 4500 µmol.m².s⁻¹</th>
<th>Recovery after irradiation with 4500 µmol.m².s⁻¹</th>
<th>Irradiation with 1500 µmol.m².s⁻¹</th>
<th>Recovery after irradiation with 1500 µmol.m².s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violaxanthin</td>
<td>0.02</td>
<td>0.01</td>
<td>0.10</td>
<td>0.04</td>
<td>0.94</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>0.10</td>
<td>0.60</td>
<td>0.05</td>
<td>1.60</td>
<td>0.18</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.00</td>
<td>0.05</td>
<td>0.02</td>
<td>0.11</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The algal response to a light irradiation with 1500 µmol.m².s⁻¹ can be seen in the HPLC chromatogram from figure 4, being similar with that recorded for 4500 µmol.m².s⁻¹

![HPLC chromatogram of carotenoids from the Mougeotia sp. samples irradiated with 1500 µmol.m².s⁻¹: a.) illumination; b.) recovery. Peak identities are same as in figure 3.](image)

After irradiation with 1500 µmol.m².s⁻¹, the violaxanthin level decreased again and the antheraxanthin concentration is close to that of lutein, while the increase in zeaxanthin
concentration is quite small (table 1, figure 4a). The carotenes’ concentrations were higher than in the previous case – that of illumination with 4500 µmol.m⁻².s⁻¹, indicating a different response in carotenoid biosynthesis. The recovery after irradiation with 1500 µmol.m⁻².s⁻¹ is different than the previous one; here, the concentration of violaxanthin is much higher, while 5,6-epoxy-β-carotene is missing (table 1, figure 4b). The high violaxanthin level is again correlated with a strong decrease in antheraxanthin concentration and a weaker decrease in zeaxanthin concentration.

CONCLUSIONS

The presented research demonstrates that the xanthophyll cycle’s regulatory mechanism is functional in the studied algal strain, but its contribution to non-photochemical quenching is not as significant as in higher plants (Bercea, 2005). The small amounts of zeaxanthin recorded during experiments suggests that this strain poses another dissipation mechanism(s) which operates together with xanthophyll cycle.

The whole analytical procedure is adapted to study the carotenoids involved in the xanthophyll cycle in algae, being very simple, fast and relatively cheap. By using a C₁₈ column and a simple gradient, we have successfully separated the polar xanthophylls and the nonpolar carotenes from one another from algal matrix; these separations include the differentiation of lutein and zeaxanthin and the carotenes α - carotene, β - carotene and β - carotene isomers 9Z and 15Z using an uncomplicated method.

BIBLIOGRAPHY

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