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Biomolecular Engineering 20 (2003) 249–253

Biomolecular Engineering

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Carotenoids as protective response against oxidative damage in *Dunaliella bardawil*

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Abstract

In the present work the relation between carotenoids production and cell response mechanisms to oxidative damage was studied. High light intensity and nitrogen starvation, both conditions, which may increase the oxidative damage in microalgae, significantly increased total carotenoids content in *Dunaliella bardawil*, the effect of N-starvation being more noticeable when acting synergetically with light on carotenoid production. S-starvation stimulated carotenoids production as much as N-starvation. The use of norflurazon, inhibitor of phytoene desaturase that blocks formation of ε-carotene from phytoene, caused a decrease of carotenoid content down to 5% that of the control cells incubated without the inhibitor. The decrease in the oxygen consumption rate of *D. bardawil* cells exposed to norflurazon suggests a connection between carotenoids desaturation and chloroplastic oxygen species dissipation processes reported in the literature for other algae. It is an indication of the carotenoids involvement in chloroplastic response mechanisms to oxidative damage.

Keywords: *Dunaliella*; Carotenoids; Oxidative stress; Chlororespiration

1. Introduction

Increasing interest on carotenoids production by microalgae is due to the important commercial applications of these natural compounds and to the market demand of carotenoids, specially for pharmaceutical and nutritional applications. Carotenoids have traditionally been commercialized as food additives including colorants, antioxidants and vitamins [1,2]. Their protective ability against oxygen free radicals seems to be responsible for the therapeutic applications of carotenoids as degenerative diseases preventives, anti-cancer agents and immune-system stimulators, claimed by several studies [2–6].

Chemically carotenoids are a wide family of isoprenoids with 40 carbon atoms than comprise the carotenes and their oxygenated derivates, the xantophylls. Physiologically they act as secondary photosynthetic pigments, provitamin factors and as protectors against photooxidative damage of the photosynthetic apparatus. Its ability of protection against photooxidative damage has been associated to the capacity of carotenoids to dissipate the excess of light acting as filter [7] and to their antioxidant properties. Carotenoids are effective singlet oxygen quenchers able to eliminate activated oxygen radical forms. Moreover, it has recently been proposed a role for carotenoids in the chloroplastic dark reduction of oxygen, an oxygen dissipation pathway so-called chlororespiration [8,9].

Despite all the interesting studies on the production of carotenoids in large amounts with the final aim of its industrial exploitaition [7,8] the physiological role of β-carotene in *Dunaliella bardawil* and the signal triggering carotenogenesis are not well established yet.

The present paper focuses on the role of carotenoids against photooxidative damage by studying the effect of different stress conditions and chemicals able to generate reactive oxygen species on carotenoids production and oxygen evolution of *D. bardawil*.

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1389-0344/03/$ - see front matter
doi:10.1016/S1389-0344(03)00065-0

2. Materials and methods

2.1. Microorganism and culture conditions

D. bardawil (UTEX 2538) was kindly provided by the Plant Biochemistry and Photosynthesis Institute (CSIC, Seville). Standard cultures were grown in mineral liquid medium at 25 °C, bubbled with air containing 5% (v/v) CO_2 and continuously illuminated with cool white and daylight from fluorescent lamps (100 μE m^{-2} s^{-1}, at the surface of the flasks). The composition of the culture medium is described in Cao et al. [11].

2.2. Oxygen evolution

The biological activity used to test cell viability was light dependent photosynthetic activity. For photosynthetic and respiratory activities determinations, 1 ml cell culture of the corresponding microalgae was placed in a Clark-type electrode to measure O_2-evolution. Measurements were made at 25 °C under either saturating white light (1500 μE m^{-2} s^{-1}) or darkness (endogenous respiration).

2.3. Analytical determinations

Chlorophyll was determined by heating and extracting with acetone [12], using an absorbance coefficient at 652 nm of 34.5 mg^{-1} ml cm^{-1}. Total carotenoids were determined spectrophotometrically by the following equation:

$$\text{Total carotenoids (mg ml}^{-1}) = 0.0045[(3000\text{Abs}_{470\text{nm}} - 1.63\text{Chl a}]$$

where $Abs_{470\ nm}$ is absorbance 470 nm; Chl a is chlorophyll a (μg ml^{-1}).

Protein content was determined following the method described by Bradford [13].

2.4. HPLC analysis of carotenoids

Separation and analysis of carotenoids was performed in a Merck Hitashi HPLC, column RP-18. Mobile phase: solvent A, ethyl acetate; solvent B acetonitrile/water (9:1, v/v). Flow rate 1 ml min^{-1}. Gradient: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100% A, as described by Young et al. [14].

2.5. Statistics

Unless otherwise indicated, figures show means of three independent experiments. Each data point represents the mean, and error bars represent standard error of the mean.

3. Results

3.1. Production of carotenoids under nutrient limitation and light excess

The halotolerant chlorophyta *D. bardawil* is well known due to its ability to produce large quantities of β-carotene under different conditions of stress [7]. In fact many conditions that affect microalgae growth negatively seem to induce carotenoid biosynthesis. In Table 1 growth and pigment content of nutrient-deprived cultures of *D. bardawil* are compared. All stress conditions tested including nitrogen, phosphorus and sulfur starvation seemed to activate carotenoids production being nitrogen and sulfur starvation the conditions that drove major carotenoids accumulation. From the results in Table 1 can be inferred that both nitrogen and sulfur starvation led to a major accumulation of carotenoids. Quantitatively, the obtained concentrations of carotenoids are comparable to other data published by other authors [7,10] Thus, both conditions cause strong inhibition in growth rate and light-dependent oxygen production.

Nitrogen starvation resulted in the most effective condition to enhance carotenoids accumulation. In parallel, growth rate and light-dependent oxygen production, sensors of cell viability, decreased significantly in N-starved cells. The synergetic effect of nitrogen starvation and light excess on carotenoids accumulation in *D. bardawil* is shown in Fig. 1. When control *Dunaliella* cells, cultured at 200 μE m^{-2} s^{-1} were illuminated with white light of 1500 μE m^{-2} s^{-1} the carotenoid/chlorophyll ratio increased 2-fold in 14 h. Such effect was enhanced up to 4-fold by limitations in nitrogen supply. In *Dunaliella* cells up to 95% was β-carotene as obtained from the peaks profile by HPLC analysis (data not shown). Apparently, nutrient imbalance produced by N-starvation is responsible for both growth rate and photosynthetic activity reduction, which in parallel is accompanied by a significant increase of the intracellular accumulation of carotenoids. Such sequence of events was not observed under phosphorus starvation, where the significant reduction of light-dependent oxygen production occurred with no increase of the total carotenoids content.

3.2. Protective effect of carotenoids against photoinhibition

For in vivo testing the protective ability of carotenoids against photo-oxidative damage *Dunaliella salina* cell cultures were incubated in the presence of the pyridazone herbicide norflurazon which has been described as inhibitor of the enzyme phytoene desaturase in plants [15]. Phytoene desaturase transforms the first incolor 40-carbon carotenoid, phytoene, into ε-carotene

Table 1
Effect of nutrient starvation on growth and carotenoids accumulation of *D. bardawil*

Nutrients	Growth rate (s^{-1})	Carotenoids (crt) ($\mu g\ ml^{-1}$)	Crt (pg) per cell	Chlorophyll (chl) ($\mu g\ ml^{-1}$)	Crt/Chl	LDOP ($\mu mol\ mg^{-1}$ Chl h^{-1})
Control	0.0601	9.0	0.15	28.1	0.32	427
−Phosphorus	0.0067	7.9	0.15	24.8	0.32	275
−Nitrogen	0.0041	13.1	0.32	19.9	0.66	179
−Sulphur	0.0044	12.7	0.37	16.1	0.79	137

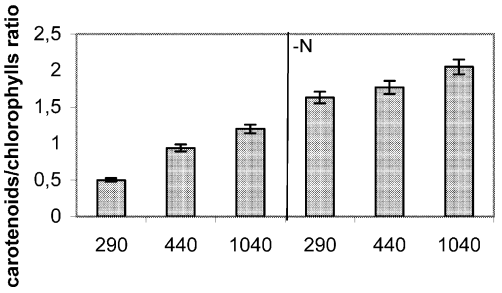

Fig. 1. Synergetic effect of nitrogen starvation and light intensity on carotenoids/chlorophyll ratio (Car/Chl) of *D. bardawil.* Car/Chl ratio was calculated after 14 h incubation of *D. bardawil* cell cultures illuminated with C (100 $\mu E\ m^{-2}\ s^{-1}$), ML (700 $\mu E\ m^{-2}\ s^{-1}$) or HL (1500 $\mu E\ m^{-2}\ s^{-1}$).

by two consecutive desaturations. Phytoene desaturase inhibition partially blocks carotenoids synthesis and, therefore, reduces cell response against oxidant species including free radicals. It was first checked the inhibitory effect of norflurazon on *D. bardawil* cells by incubating the cells in the presence of the inhibitor and following the production of carotenoids under high irradiance conditions (1000 $\mu E\ m^{-2}\ s^{-1}$). Results are shown in Fig. 2. The incubation with norflurazon did not affect the chlorophyll concentration, which accounted for 80% of that in *D. bardawil* cells under same conditions without norflurazon. Carotenoids content of *D. bardawil* cells grown under high irradiance (HL) without norflurazon accounted for 2.7-fold that of control cells grown under low irradiance (100 $\mu E\ m^{-2}\ s^{-1}$). Nevertheless, cells grown at 1000 $\mu E\ m^{-2}\ s^{-1}$ in the presence of norflurazon (HL/NF) contained 80% less carotenoids that non-treated cells (Fig. 2). As it can be observed in Fig. 2 light-dependent oxygen production decreases 50% with respect to control cells as consequence of the low level of protective carotenoids.

The protective effect of carotenoids against oxygen species has also been associated to an oxygen consumption chloroplastic process called chlororespiration. In conditions of low PSI activity or PSII/PSI imbalance, phytoene desaturase activity would supply electrons from carotenoids oxidation to reduce oxygen by plastoquinone pool reduction via a terminal oxidase according Fig. 3.

To study the role of carotenoids on dissipation of oxygen species excess the theoretical electron flow from phytoene to the plastoquinone pool was blocked by norflurazon, specific inhibitor of phytoene desaturase. Oxygen consumption in dark due to endogenous respiration was measured and compared with control cells. As shown in Fig. 4, the interruption of electron flow from phytoene to plastoquinone pool results in a slight decrease (less than 10%) of respiratory activity with respect to control cells. The use of DCMU, inhibitor of PSII, completely inhibits light-dependent oxygen production and the respiratory activity.

4. Discussion

The production of carotenoids occurs when microalgae cells grown in full nutrient culture media are incubated under specific conditions that limit cell

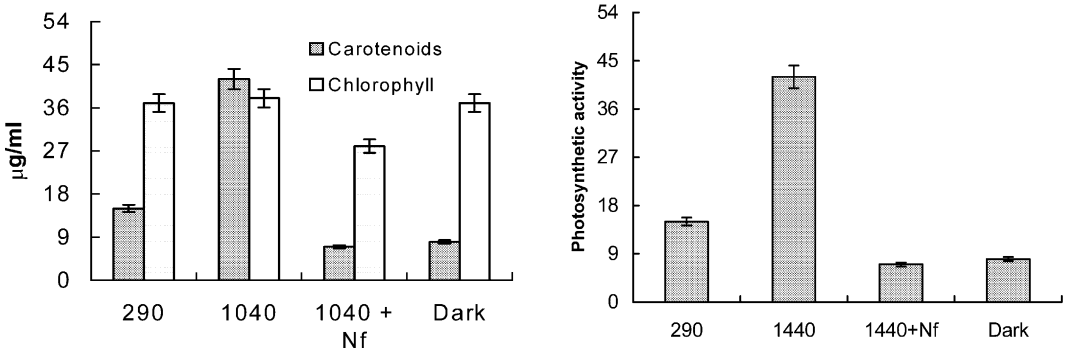

Fig. 2. Effect of norflurazon on high light stressed *Dunaliella* cells. Chlorophyll, total carotenoids and photosynthetic activity were measured in non-treated (HL) and treated (HL/NF) cells.

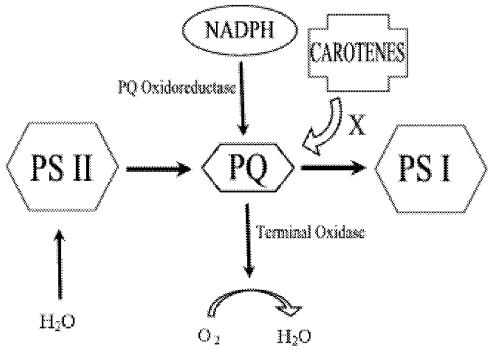

Fig. 3. Oxygen consumption chloroplastic process so-called chlororespiration. PSI, photosystem I; PSII, photosystem II; PQ, plastoquinone pool.

growth including nutrient imbalance, illumination of high intensity and high concentration of salt [7,16]. Our results show that the obtained concentrations of carotenoids produced by *Dunaliella* cells in nitrogen-deficient culture medium, are according with data reported in literature for *Dunaliella* producing carotenoids under same conditions [7,10]. Thus, when incubated in sulfur-deficient culture medium the ratio carotenoids/chlorophyll in *Dunaliella* increases (20%) with respect to that in nitrogen-deficient culture media, which could be of interest for practical purposes.

It is only in recent years that attention has specifically been paid to the antioxidant properties of carotenoids and their derived physiological roles and applications. The carotenoids can exist in a ground state or in one of two excited states after the absorption of light energy. In terms of its antioxidant properties carotenoids can protect the photosystems in one of four ways: by reacting with lipid peroxidation products to terminate chain reactions; by scavenging singlet oxygen and dissipating the energy as heat; by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen; or by the dissipation of excess excitation energy through the xanthophylls cycle [17]. Recently there is evidence for a chloroplastic oxygen removing

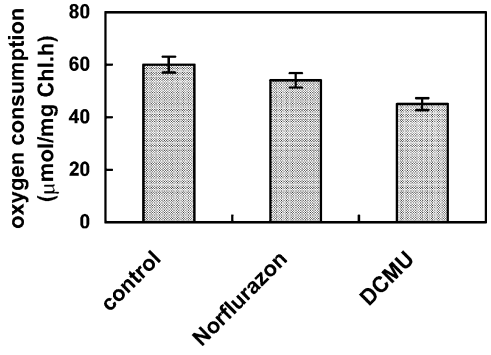

Fig. 4. Endogenous oxygen consumption of *D. bardawil* in the presence of inhibitors of photosynthesis. *D. salina* cell cultures were added either norflurazon or DCMU 0.25 mM. Oxygen consumption was measured after 24 h incubation.

pathway so-called chlororespiration. There is strong evidence that a chlororespiratory pathway operates in the chloroplasts of higher plants [18]. The evidence suggests that this pathway involves the dark reduction of plastoquinone in the thylakoid membrane by NADPH or NADH. The subsequent oxidation of plastoquinol ultimately terminates with the reduction of molecular oxygen. The existence of a chlororespiratory plastoquinol oxidase suggests the existence of yet another pathway through which electrons from PSII can be diverted to oxygen. Bennoun [8] recently suggested that electrons from phytoene desaturation can also be diverted to oxygen via chlororespiration.

Our results are in agreement with the concept that suggests a possible role for carotenoids desaturation pathway in the above mentioned chloroplastic oxygen removing process. Results in Table 1 show a decrease of light-dependent oxygen production and growth rate values of *D. salina* grown in both N- and S-starved culture media. Such loss of photosynthetic activity runs in parallel to an increase in carotenoids cell content. PSII can be selectively inactivated by nitrogen starvation [19]. Several studies documented losses of PSII reaction centre proteins under continuous nitrogen limitation in chemostats [20] whereas there is no agreement on the apparent effect of nitrogen starvation on PSI. When PSII is partially inhibited by N-starvation the electron flow from PSII and from NADPH (PSI) to oxygen decreases. The increase observed in carotenoid content of *D. salina* grown in N-starved culture medium is in agreement with major electron demand to the terminal oxidase responsible for further oxygen reduction to water. Moreover, the increase of carotenoid content of cells grown in N-starved culture medium compared with that of cells grown in full nutrient culture medium is more significant than that observed due to illumination with light of high intensity (Fig. 1). The damage on PSII under N-starvation could be followed by a higher electron-flow derived from carotenoids desaturation to oxygen dissipation in chloroplast. Such electron flow would proceed from phytoene desaturation [8] as suggested from both carotenoids and oxygen consumption decrease in the presence of norflurazon (Figs. 2 and 3). Significative less loss of photosynthetic activity in parallel to a minor increase of carotenoid content was observed in P-starved cell cultures.

5. Conclusion

Light of high intensity and nitrogen starvation significantly increased total carotenoids content in *D. bardawil*, but such accumulation of carotenoids can be also induced by S-starvation and, to less extend (50%), by P-starvation. Under culture conditions driving carotenoids accumulation in *D. bardawil* the use of

norflurazon, inhibitor of carotenoid biosynthesis, lets suggest a connection between carotenoids desaturation and chloroplastic oxygen species dissipation processes reported in the literature for other algae. It is an indication of the carotenoids involvement in chloroplastic response mechanisms to oxidative damage.

Acknowledgements

This work has been supported by the Dirección General de Investigación from the Ministerio de Ciencia y Tecnología and Plan Andaluz de Investigación.

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