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# Carotenogenesis in *Haematococcus lacustris*: Role of Protein Tyrosine Phosphatases

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**In the present study, we examined the inhibitory effects of protein tyrosine phosphatase (PTPase) inhibitors, including sodium orthovanadate (SOV), ammonium molybdate (AM), and iodoacetamide (IA), on cell growth, accumulation of astaxanthin, and PTPase activity in the photosynthetic algae *Haematococcus lacustris*. PTPase activity was assayed spectrophotometrically and was found to be inhibited 60% to 90% after treatment with the inhibitors. SOV markedly abolished PTPase activity, significantly activating the accumulation of astaxanthin. These data suggest that the accumulation of astaxanthin in *H. lacustris* results from the concerted actions of several PTPases.**

**Keywords:** *Haematococcus lacustris,* astaxanthin, protein tyrosine phosphatase (PTPase), PTPase inhibitor, sodium orthovanadate (SOV)

The application of the photosynthetic machinery of algal cells to the production of highly bioactive compounds has gathered tremendous interest [6, 5, 8, 12]. Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione), a highly valued carotenoid pigment found in green algae, is desirable for use as a food supplement and in cosmetics and various pharmaceutical applications owing to its powerful antioxidant properties [9]. *Haematococcus lacustrus* is a green unicellular microalga, capable of expressing at levels of up to 80% of dry cell weight [14]. *H. lacustris* accumulates astaxanthin and its intermediate carotenoids under various stress conditions, such as exposure to high light irradiance and high temperature, nitrate deficiency, and oxidation *via* reactive oxygen species [7]. *H. lacustris* is considered to be one of the best natural sources of astaxanthin, and the production of large amounts of astaxanthin by this organism is under intensive investigation [10, 14, 16].

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The attachment and removal of phosphate groups from proteins represents an important aspect of signaling pathways that regulate cell proliferation and differentiation in plants [2, 1]. This process depends on the antagonistic interplay between protein kinases and serine/threonine/tyrosine phosphatases, in response to a variety of signals, including hormones, mitogens, and cytokines [3, 15, 17]. Several protein kinases in *H. lacustris* have been examined; however, the activities of protein tyrosine phosphatases (PTPases) in this organism have yet to be investigated. The phosphorylation status or activity of proteins might be important in regulating the photosynthetic state and activity of microalgae. Therefore, we examined the influence of sodium orthovanadate (SOV), ammonium molybdate (AM), and iodoacetamide (IA), which are known to be inhibitors of PTPases [4, 13] as well as nonspecific protein tyrosine phosphatases, alkaline phosphatases, and a number of ATPases, on the function of PTPases in cell growth and astaxanthin production in *H. lacustris*.

*H. lacustris* was purchased from the Culture Collection of Algae at the University of Texas at Austin (UTEX), and was cultivated photoautotrophically in modified Bold's basal medium (MBBM). A single colony of cells grown on an agar plate was inoculated into 120 ml of medium adjusted to pH 6.5±0.5, in a 250-ml Erlenmeyer flask. The cells were incubated at 25°C with continuous shaking (175 rpm) and illuminated at 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ with fluorescent lamps (Model FL 18D; OSRAM Korea Co., Ansan, Korea). For high light stress, cells were illuminated at 400 $\mu E \cdot m^{-2} \cdot s^{-1}$. To evaluate the influence of PTPase inhibitors on cell growth and astaxanthin production, cells were cultured in the presence or absence of SOV, AM, and IA at various concentrations ranging from 0 to 5.0 mM. Separate experiments were performed in 0.4-l bubble column photobioreactors (PBR) containing 0.4 l of MBBM medium supplemented with 5% $CO_2$ at an aeration rate of 0.2 $_{VVM}$ and 95% air under continuous illumination at 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the column surface. Compact fluorescent lamps (Model DULUX L; OSRAM Korea, Ansan, Korea) were used for external

illumination of PBRs. The temperature and pH were kept at 25°C and 6.5±0.5, respectively, during cultivation.

A cell pellet (2.5 g) of *H. lacustris* cultured in the absence of PTPase inhibitors was washed once with 0.5 l of 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 4.0 mM $MgCl_2$, 5.0 mM DTT, and a mixture of protease inhibitors (Sigma, U.S.A.), and the final pellet was resuspended in 25 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 1.0 M NaCl. The cells were then disrupted by three passages through a French-press (TS series; Constant Systems Ltd., Engineering Research Center [ERC], Inha University, Korea), and centrifuged at 10,000 ×*g* for 60 min to remove unlysed cells. All procedures were performed at 4°C, unless otherwise indicated. The supernatant was stored at −80°C until use. PTPase activity was determined spectrophotometrically using *p*-nitrophenyl phosphate (*p*NPP) (Sigma, U.S.A.) as the substrate. Protein samples (0.1−1.0 μg) were added to 0.1 ml of 5.0 mM *p*NPP dissolved in 20 mM Tris-HCl buffer (pH 7.0) to initiate the reaction, and incubated for 0−60 min at various temperatures. The reaction was quenched by addition of 0.9 ml of 1.0 M $Na_2CO_3$. Absorbance was measured at 405 nm against a control sample that consisted of the reaction with enzyme boiled at 100°C for 5 min before assaying. A unit (U) of PTPase activity was defined as the amount of enzyme needed to release 1.0 μmole of *p*-nitrophenol per minute. In addition, peptide substrates (tyrosine phosphatase assay kit; Boehringer Mannheim, Germany) were used according to the manufacturer's instructions. For the determination of free phosphate generated during dephosphorylation of the phosphotyrosine peptide, the reaction was terminated by the addition of the malachite green/ammonium molybdate complex, which binds to the inorganic phosphate formed in the reaction, generating a brilliant green color. The absorbance was measured at 620 nm using a spectrophotometer (HP8453B; Hewlett Packard, Waldbronn, Germany). The total amount of free inorganic phosphate released in the reaction reflects the relative amount of tyrosine phosphatase activity in the sample. All results were analyzed in triplicate, with a value of $P \leq 0.05$ as an indication of statistical significance.

We initially investigated the effect of various putative PTPase inhibitors on growth of the green alga *H. lacustris*. Cell growth was significantly affected by increasing the concentration of IA, SOV, or AM. Among these, SOV had the strongest effect in promoting cell growth and astaxanthin production. Addition of SOV at 2.5 mM (final) to *H. lacustris* ($2\times10^6$ cells/ml) inhibited growth when compared with the control. Untreated cells were green with small red specks visible inside them, presumably due to the production of carotenoids, within one day under normal light conditions, whereas mixed populations of red and enlarged cells were visible after treatment with SOV for 3 days (Fig. 1). The observed morphology of SOV-treated cells is similar to

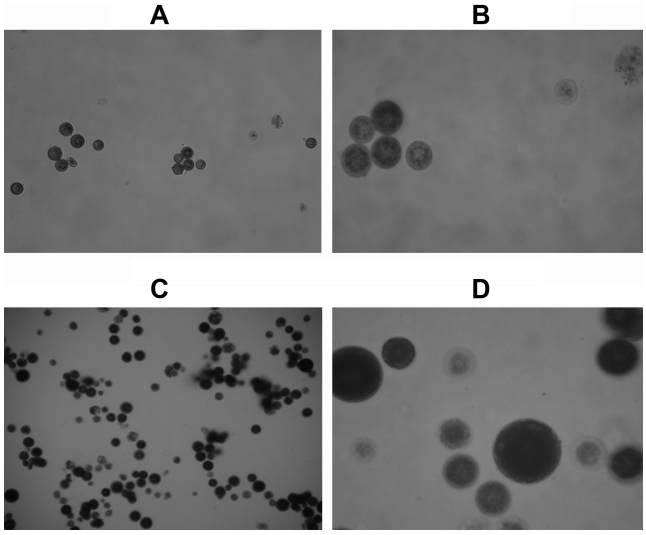


**Fig. 1.** Influence of SOV on the growth of *Haematococcus lacustris* cells.
**A** and **B** show the morphology of wild-type cells in the absence of SOV; **C** and **D** show the morphology of cells cultured in the presence of SOV. Cells were treated with 2.5 mM SOV for 3 days and their morphology was visualized at 100×(**A, C**) and 400×(**B, D**) magnification.

that following the cell maturation process that occurs when the alga accumulates astaxanthin under conditions of nitrate depletion and/or high light stress (data not shown). Whereas high light stress can induce this phenotype after cultivation for several days, approximately 3 to 4 weeks are required to observe similar results under normal light (data not shown). Our data indicate that inactivation of intracellular

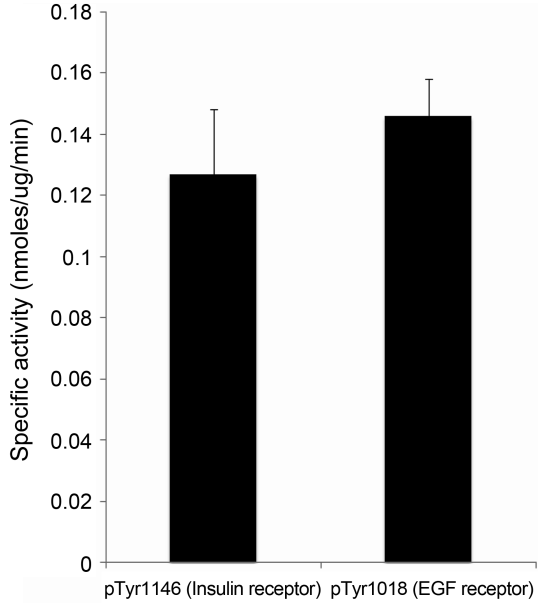


**Fig. 2.** Determination of intracellular PTPase activity.
Intracellular PTPase(s) activity was determined using phosphotyrosine peptides $pTyr^{1146}$ (insulin receptor) and $pTyr^{1018}$ (EGF receptor) as specific substrates.

proteins by SOV results in activation of the astaxanthin biosynthetic pathway in the cells.

Next, we examined *H. lacustris* PTPase activity by measuring the concentration of inorganic phosphate generated from the model phosphotyrosine peptide substrates $pTyr^{1146}$ (insulin receptor) and $pTyr^{1018}$ (epidermal growth factor [EGF] receptor). As shown in Fig. 2, intracellular PTPase(s) derived from crude cell extracts displayed similar activity against $pTyr^{1018}$ and $pTyr^{1146}$. In addition, the activity of PTPase(s) derived from crude extracts of cells illuminated at 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ was determined in the presence of various inhibitors. As shown in Fig. 3, SOV treatment resulted in the strongest inhibition of PTPase activity, ranging from 75% to 95%. Similar results were observed using *p*NPP as the enzyme substrate for PTPase(s) in crude extracts of cells cultivated with or without high light stress. Furthermore, the inhibition observed with SOV was similar to that obtained with AM. These two compounds resulted in greater than 90% inhibition when using *p*NPP as the enzyme substrate and about 70% when using pTyr1018. In contrast, IA displayed a lower level of inhibition of about 30% to 60% using the same substrates (Fig. 3). Our data suggest that the intracellular PTPase(s) may be highly sensitive to AM and/ or SOV, whereas the inhibitory action of IA may indicate an important role for thiol groups (SH) in the catalytic site of the PTPase(s). Further research is needed to identify individual PTPase proteins and their substrate and inhibitor specificities, particularly those that may be involved in the stress response. Additional experiments that seek to evaluate the relationship between maturation and the activity of PTPase(s) and or kinase(s) in *H. lacustris* are in progress.

Finally, we investigated carotenoid formation during cultivation of *H. lacustris* in the presence of 0 to 5.0 mM SOV, to define the concentration that promotes the maximum production of astaxanthin. Cells were grown in MBBM supplemented with $CO_2$ at an aeration rate of $0.2_{VVM}$ at 24°C under continuous illumination at 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ using white fluorescent light, reaching a maximum density of $2.4 \times 10^5$/ml. Following acetone extraction, the concentration

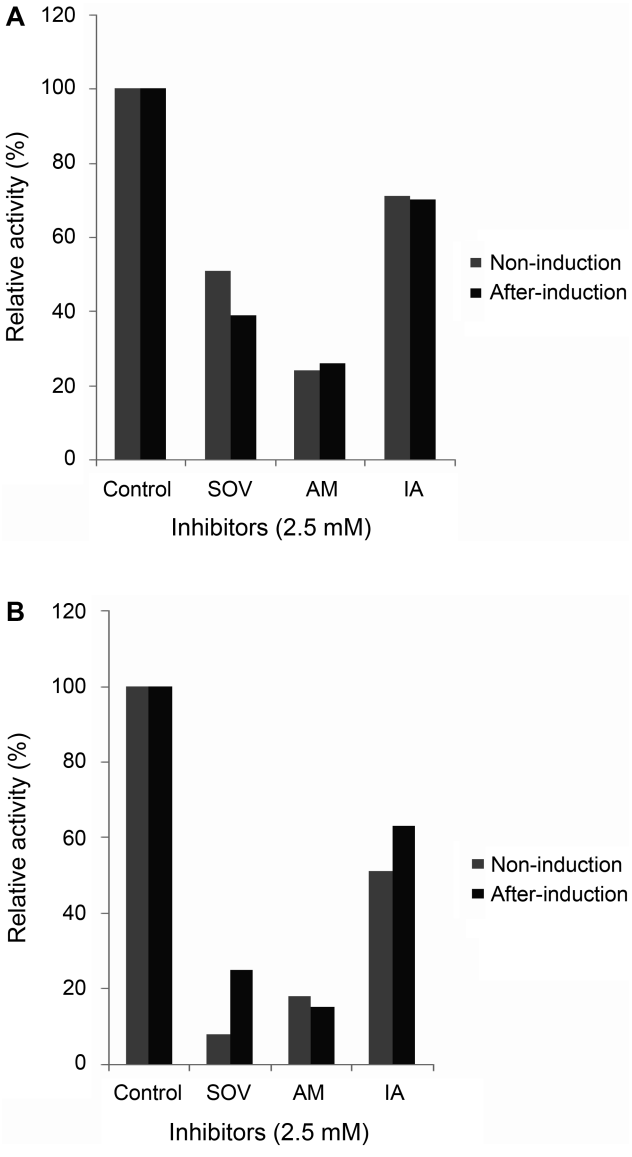


**Fig. 3.** Influence of inhibitors on PTPases activity.
Intracellular PTPase(s) activity of *H. lacustris* was estimated *in vitro* in the presence or absence of several inhibitors in a reaction mixture containing 1.0 mM $pTyr^{1018}$ (**A**) and 5.0 mM *p*NPP (**B**) as substrates. SOV, sodium orthovanadate; AM, ammonium molybdate; IA, iodoacetamide.

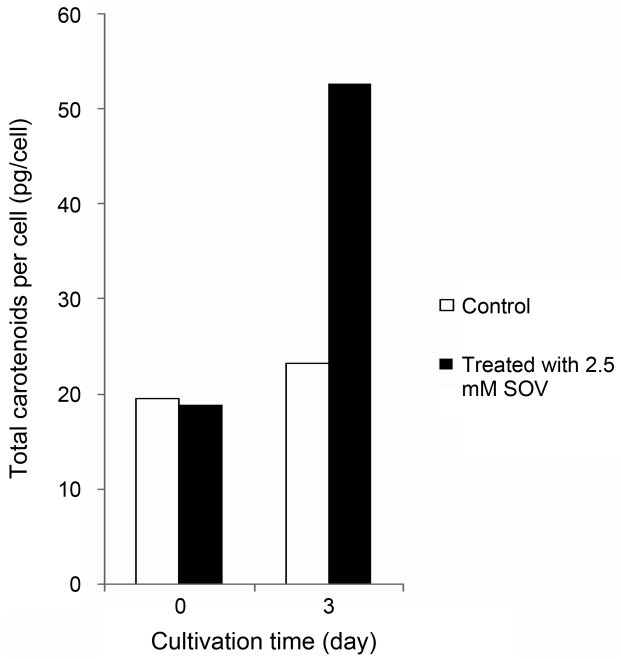


**Fig. 4.** Total carotenoid accumulation per cell (pg/cell) in a culture of *H. lacustris* after 3 days exposure to 2.5 mM SOV.
Control cells (□) and cells treated with 2.5 mM SOV (■). SOV, sodium orthovanadate.

of astaxanthin was calculated from a calibration curve using synthetic astaxanthin (A9335; Sigma Chemical Co., St Louis, MO, U.S.A.) as the standard. For astaxanthin concentrations of less than 10 mg/ml, the following calculation was used: astaxanthin concentration (mg/l)=$0.0045 \times A_{475}$ [11]. When comparing total carotenoid content, expressed as the concentration of the predominant carotenoid astaxanthin per biomass unit, it was found that cells exposed to the optimized concentration of SOV (2.5 mM) for 3 days accumulated a significantly larger amount of carotenoids than control cells, amounting to a 2-to 2.5-fold increase in astaxanthin production (Fig. 4). Our results strongly suggest that, in addition to well-known environmental stress factors, inhibition of PTPase(s) activity by SOV or AM can effectively stimulate astaxanthin production in *H. lacustris*. Taken together, these results strongly suggest that PTPase(s) may play a critical role in cell growth and astaxanthin accumulation in this alga. However, the physiological functions of PTPase(s) have yet to be clearly elucidated; therefore, further studies are in progress to determine the physiological/biochemical role of PTPase(s) in carotenogenesis in *H. lacustris*.

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