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Isolation and characterization of compactin resistant mutants of an astaxanthin synthesizing green alga Haematococcus pluvialis

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Haematococcus pluvialis mutants resistant to compactin, an inhibitor for 3-hydroxy-3-methylglutaryl coenzyme A reductase that is a key regulatory enzyme in the isoprenoid biosynthesis, were screened for higher astaxanthin producers. At 2 mM compactin, the wild type failed to grow, while two isolated mutants showed fairly good growth. In compactin-free medium, these resistant mutants have accumulated 1.4–2.0 fold higher astaxanthin than the wild type did. Based upon the specific enzyme activity assay, the mutant enzyme was found slightly less sensitive to compactin.

Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione), a highly oxygenated carotenoid, is synthesized by the unicellular green alga, *H. pluvialis* (Kobayashi *et al.*, 1991; recently reviewed by Johnson and Schroeder, 1995). Antioxidative activity of astaxanthin was shown superior to most of the hydrophobic antioxidants such as β-carotene and vitamin E (Miki, 1991), so that the ketocarotenoid has interest for medical purposes (Palozza and Krinsky, 1992) as well as for pigmentation for aquaculture feeds (Choubert and Heinrich, 1993).

Carotenoid biosynthesis is part of the diverse isoprenoid biosynthetic pathway directed for a variety of bioactive compounds in plants (Bach, 1995). It has been reported that the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate catalyzed by HMG-CoA reductase (HMGR) [EC. 1.1.1.34] is a rate-limiting step at the early stage of cholesterol biosynthesis in mammalian cells (Goldstein and Brown, 1990), and compactin, a competitive inhibitor for HMGR, strongly blocked cholesterol formation (Endo, 1985). Moreover, it was found that when higher plants were wounded or attacked by pathogens, HMGR was significantly stimulated at the mRNA synthesis leading to antimicrobial phytoalexins and toxic alkaloids accumulation (Yang *et al.*, 1991; Stermer *et al.*, 1994). Thus, HMGR appears to be one of the key regulatory enzymes in the isoprenoid biosynthesis in a wide range of organisms. However, there has been little information on whether the astaxanthin biosynthesis in the alga is regulated at the HMGR stage.

In order to construct higher astaxanthin producers of the green alga, we have attempted to confer compactin resistance, which would lead to a specific mutation of the HMGR gene, resulting in possibly enhanced carotenoid biosynthesis. The compactin-resistant mutant isolated in this study, showed not only higher astaxanthin levels, but also contained a mutated HMGR enzyme less sensitive to compactin than that of the wild strain.

Materials and methods

Organisms and culture conditions

H. pluvialis NIES-144 was mixotrophically cultivated with acetate as C source in the basal medium for the facilitated growth of the motile vegetative cell, or in the modified medium for astaxanthin production by the immotile resting cysts cells (Kobayashi *et al.*, 1993).

Mutagenesis and screening of compactin-resistant mutants

The algal cell (5×10^4 cell/ml) at the log phase between 1–2 d culture was treated with ethyl methanesulfonate (EMS) as described by Tjahjono *et al.* (1994). Alternatively, the algal cells in a petri dish were illuminated at 35 cm below a UV lamp (15W) for 7 min, and kept for 1 d in the dark. EMS- or UV-treated cells with *ca.* 5–10% viable population were then spread on

2 mM compactin-containing basal medium, and incubated under high light intensity at 140 μμole/m^2·s and 20°C for 10–14 d.

Preparation of membrane-bound enzymes

To prepare algal HMGR, cyst cells were induced by acetate addition at 45 mM to the vegetatively-growing cells at 4 d culture in the basal medium. The induced cyst cells from 1.8 *l* of the medium cultivated for 24 h after the acetate addition were harvested and suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 0.35 M sucrose, 30 mM EDTA, and 10 mM dithiothreitol (DTT), and homogenized with glass beads by BeadBeater (Bio-Spec Product, Okla) for 30 sec, 10 cycles with sufficient cooling intervals. The cell debris was removed at 16,000 *g* for 30 min, and then the supernatant was ultracentrifuged at 105,000 *g* for 1 h at 4°C. The pellet of membrane-bound proteins was suspended in 0.2 M K-phosphate buffer (pH 6.9) containing 25 mM DTT.

Determination of HMG-CoA reductase activity

The standard HMGR assay method for pea seedlings (Russell, 1985) was modified due to the negligibly low activity by the original method. Thus, as a result of varying the amounts of HMG-CoA, the reaction (100 μl in total) was performed by adding 30 μl of 420 nmol [3-^{14}C]-HMG-CoA (0.74 GBq/ml, Amersham Corp.), which was six-fold higher substrate concentrations than the original method. The labeled reaction product developed on silica gel 60 plate (0.25 mm, Merck) was exposed to Fuji Imaging Plates, and the sizes of the emerged bands were calibrated by a Bio-Imaging Analyzer BAS 1000 system (Fuji Photo Film, Tokyo).

Analysis

The cell number was counted with a haemacytometer, and astaxanthin concentration was determined at 480 nm (Kobayashi *et al.*, 1993). Astaxanthin was found as the major carotenoid over 85–95% throughout the experiments. Protein was determined by the method of Bradford (1976). All the analyses were carried out for duplicated or triplicated cultures, and their samples.

Chemicals

Compactin (2-methylbutanoic acid 1,2,3,7,8,8a-hexahydro-7-methyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl)ethyl]-1-naphthalenyl ester, also termed as ML-236B) was provided from Sankyo Co. Ltd. (Tokyo) as the lactone form. For saponification, compactin was suspended in water at 250 mM, mixed with equimolar NaOH solution, and incubated at 90–95°C for 30 min.

Results and discussion

Growth inhibition by compactin

To examine the effect of compactin on the algal growth, the wild type was cultivated with varying compactin concentration up to 2.0 mM in basal medium (Fig. 1). At 1.7 mM of compactin addition, the cell growth was strongly inhibited. The growth inhibition seems likely to be due to the impaired sterol biosynthesis by the addition of compactin or mevinolin, 7-methylated at the napthalenyl ring of compactin (Bach and Lichtenthaler, 1983), since sterols are generally indispensable components for cell membrane fluidity. The sterols in the vegetative algal cells consist of ergosterol and some minor unidentified mixtures (Williams *et al.*, 1966).

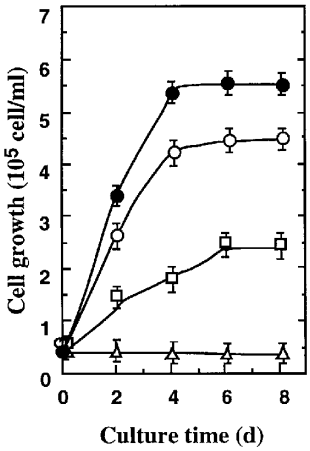

Figure 1 Effect of compactin on cell growth of the wild type. No addition (●), 1.3 mM (○), 1.7 mM (□), 2.0 mM (△) of compactin.

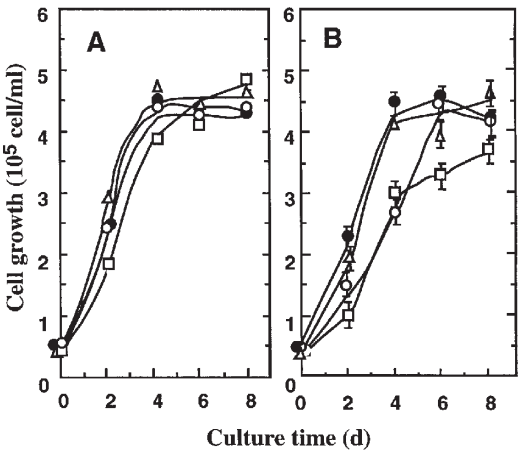

Figure 2 Effect of compactin on cell growth of the compacting resistant mutants Mb (A) and M15 (B). The concentrations of compactin in the basal medium: no addition (■), 1.3 mM (○), 1.7 mM (□), 2.0 mM (△).

Isolation of compactin-resistant mutants

With extensive screening for compactin-resistant mutants after mutagenesis, several positively-growing colonies were recovered from compactin-containing plate after 2 week cultivation.

Among the apparent mutants, it was demonstrated that two resistant mutants named Mb derived from UV method, and M15 from EMS treatment were capable of forming dark red colonies due to the massive astaxanthin accumulation at 2 mM compactin plates whereas the wild type completely failed to grow, which was also shown in the liquid medium. In compactin-free plate, the strains Mb, M15 and the wild type generated all dark red colonies, where color differences of their colonies were hardly detected. Upon colony purification, and the successive transfer to compactin-containing basal media every 4 d over a half year, it has been verified that the compactin resistance was stably maintained in both strains Mb and M15. However, some other mutants failed to propagate under the selective pressure (data not shown).

Characterization of compactin-resistant mutants

The two isolated mutant strains Mb and M15 were first cultivated to examine whether the two mutants could exhibit resistance against the varied concentrations of compactin (Fig. 2). The growth curves of strain Mb were nearly identical irrespective to compactin concentration. In the case of strain M15, cell growth (as cell number) was found slightly lower than that of the wild type because of the weak cell aggregation during the cultivation.

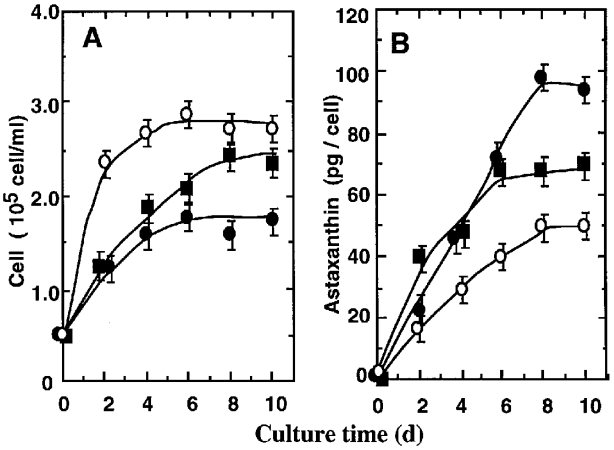

Figure 3 Cell growth (A) and astaxanthin production (B) of the compactin resistant mutants, Mb (■), M15 (●), and the wild type (○). The cultivation was carried out in modified medium under high light intensity to examine the carotenoid production by the cyst cells.

Subsequently, the two compactin-resistant mutants were examined for astaxanthin production by cultivating in the Fe^{2+}-rich modified medium under high light intensity (Kobayashi *et al.*, 1991; Kobayashi *et al.*, 1993). Under these culture conditions, the alga mostly propagated as immotile cysts, accompanied with massive astaxanthin accumulation. As shown in Fig. 3, the compactin-resistant mutants Mb and M15 had an astaxanthin content of 70 pg/cell, and 98 pg/cell, respectively; this level is 1.4–2.0 fold higher than that of wild type (50 pg/cell) in a 10 d culture.

HMGR activity assay

To examine how the mutants acquired the compactin resistance, we have analyzed the HMGR enzyme activities of the mutants and the wild strain grown on the compactin-free medium. As shown in Table 1, specific enzyme activity of HMGR for the resistant strain Mb were found almost comparable to that of the wild type in the absence of compactin. In contrast, at 1.0 mM compactin addition, a specific activity of the mutant HMGR remained at 86% of the control for strain Mb, whereas the wild type HMGR activity was reduced to 63% of the control value. At 2.0 mM compactin addition, no HMGR activities could be detected in both strains. Therefore, it was suggested that the mutant HMGR seemed slightly less sensitive to compactin.

In view of the known mechanisms of acquired drug resistance as reviewed by Stalker (1989), three possible strategies could be proposed: 1) increase in quantity for the drug-targeted enzyme (or protein), which would reduce the inhibitory effect of the drug, 2) conformational change of the targeted protein, causing decreased affinity to the drug, 3) lowered intracellular drug concentration by enzymatic detoxification, or by transport-deficient mutation. Several lines of mutants resistant towards compactin, or mevinolin have been isolated as HMGR-overproducing mutants in chinese hamster

Table 1 Specific HMGR activities from the wild type and a compactin-resistant mutant Mb grown in the absence of compactin.

Compactin (mM)	Specific activity of HMGR (pmol/mg-protein·h)	
	Wild type	Resistant mutant
No addition	1.36 ± 0.08 (100)a	1.43 ± 0.11 (100)[a]
1.0	0.86 ± 0.06 (**63.2**)	1.23 ± 0.09 (**86.0**)
2.0	N.D.[b]	N.D.

[a]Relative residual activities are shown in parentheses taking each no addition as the control (100%).
[b]N.D., Not detectable.

cells (Skalnik *et al.*, 1985), and in a halophilic archaebacterium (Lam and Doolittle, 1992). From the HMGR inhibition assay in this study, however, it seems possible that the mutant HMGR enzyme would rather possess an altered structure in a presumed compactin-binding site.

As recently reviewed on plant HMGR by Bach (1995), there are a small gene family for HMGR isozymes, which play distinct roles in response to the various environmental or developmental signals for the regulation of the biosynthesis of a variety of isoprenoid compounds. Thus, it remains to be elucidated whether an analogous HMGR gene family exists in the green alga, and if so it would be of interest to know which HMGR isozyme(s) is involved in the increased astaxanthin formation.

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