Mycoscience (2011) 52:65–68
DOI 10.1007/s10267-010-0070-7

SHORT COMMUNICATION

Studies on cytochromes of lichenized fungi under optimized culture conditions

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Received: 9 March 2010 / Accepted: 23 August 2010 / Published online: 11 September 2010

Abstract Cytochromes are membrane-bound hemoproteins responsible for the generation of ATP via the electron transport system to fuel the metabolic processes of the organism for their growth. This study reports the properties of cytochromes present in the isolated lichenized fungi of the cultured lichen *Usnea ghattensis* under optimized conditions. The fungal partner of the cultured lichen *Usnea ghattensis* contains *a*, *b* and *c* types of cytochromes. The concentrations of *a*, *b* and *c* type cytochromes were found to be significantly high (0.0967, 0.0900, and 0.1030 mM/mg protein, respectively) in the isolated fungal symbiont of cultured lichen grown in malt-yeast extract medium supplemented with 0.01 mol/l sucrose and 0.01 mol/l polyethylglycol. The results suggest that supplementation of additional carbon sources may play a role in optimizing the growth via activating the cytochrome respiratory system in lichenized fungi.

Keywords Fungal respiratory system · Hemoproteins · Lichen culture · *Usnea ghattensis*

Introduction

Lichen is a symbiotic organism composed of a fungus and a photosynthetic partner (algae). Since the fungal partner is unique in this symbiosis and usually dominates the association, these organisms traditionally were considered as a type of fungi (Muggia et al. 2009). The adaptability of these organisms to extreme environmental conditions, particularly to temperature-induced ones, is of much interest in relation to novel and effective therapeutic compounds, and is reported for many biological activities (Müller 2001; Oksanen 2006; Verma et al. 2008). However, although having enormous biological potential, lichens have been scarcely studied in high-throughput screening programs because of their slow-growing nature, non-availability of biomass in bulk quantity and difficulties in culturing them in vitro.

In the recent past, Fujiwara et al. (1995) studied the cytochrome profile of isolated cultured fungi of the lichen *Cladonia vulcani* in enriched culture medium and reported that the level of cytochromes was lower than in the fast-growing organism *Candida rugosa* yeast. Their results indicate that the respiratory system in isolated cultured fungi of lichen *Cladonia vulcani* is much weaker than that of other fast-growing organisms. Like other lichens, *Usnea ghattensis* is not an exception. This species also grows very slowly in nature, but in our optimized culture conditions (reported elsewhere), its symbionts as cell aggregates grew and produced lichen substances much faster (Behera et al. 2009). Fungi produce a great variety of primary and secondary metabolites and perform many different complex chemical conversions, such as the hydroxylation of polyaromatic hydrocarbons and other compounds, which are mediated by cytochromes. Cytochromes are membrane-bound hemoproteins and present in the cells of all organisms as a fundamental component. Thus, it seems very advantageous to select cytochromes as materials for comparative biochemical studies (Yamanaka and Okunuki 1964).

With this background, an attempt has been made to understand the properties of cytochromes present in the isolated lichenized fungi of the cultured lichen *Usnea ghattensis* under optimized conditions.

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Materials and methods

Collection of natural lichen thalli

The natural thallus of the lichen *Usnea ghattensis* producing usnic acid and norstictic acid in a natural environment was collected from Silver Oak trees in Mahabaleswar, Satara District, Maharashtra State, India. Part of the material was preserved in Ajrekar Mycological Herbarium at Agharkar Research Institute, Pune, India (accession no: 03.391).

In vitro culture

Cell aggregates of symbionts of the lichen *Usnea ghattensis* were obtained using the methodology described by Yamamoto et al. (1985). The following culture medium was used: malt-yeast extract (MYE) medium containing 20 g/l malt extract and 2 g/l yeast extract, solidified with sabouraud dextrose agar (20 g/l) and supplemented with 0.01 mol/l sucrose, 0.01 mol/l polyethylglycol (PEG) and 0.005 mol/l glycine separately and in combination. The inoculum was grown at 18°C, with alternating photoperiods of 8 h light (400 lux)/16 h dark and 50–80% relative humidity in a plant growth chamber for a period of 3 months. The details of the culture optimization procedure have already been presented in our previous paper (Behera et al. 2009).

Isolation of fungi from culture cell aggregate

Fungal cells were isolated by separating the algal cells from culture cell aggregates of the cultured lichen *Usnea ghattensis* by following the methodology of Ascaso (1980) with minor modifications. Briefly, cell aggregates were homogenized in double-distilled water with an electric homogenizer. The homogenate was filtered through a three-layered muslin cloth. The fungal hyphae collected from muslin cloth were suspended in 0.25 M sucrose and vortexed to remove attached algal cells. The resulting suspension was centrifuged at 10,000 rpm for 10 min. The supernatant containing fungal hyphae was filtered through a muslin cloth and resuspended in 1 M potassium iodide to remove the remaining algal cells. The resulting suspension was again centrifuged at 10,000 rpm for 10 min. The supernatant containing fungal hyphae was collected by filtering the supernatant through muslin cloth and storing at −20°C for further use.

Isolation of cytochromes from fungal cells

Cytochromes from fungal cells were isolated by the method described by Fujiwara et al. (1995) with some minor modifications. The fungal cells (4 g) were crushed with a mortar and pestle with liquid nitrogen. The crushed cell powder was suspended in suspension buffer [0.3 M KCl, 100 μM ethylenediaminetetraacetate (EDTA), 10 μM phenylmethane sulphonyl fluoride (PMSF) in 10 ml of 0.1 M Tris-HCL buffer, pH 8.0]. The cell wall degrading enzyme lyticase (β-1,3-glucose) at a concentration of 1 mg/10 ml was added to the suspension. The resulting suspension was gently stirred at 4°C for 24 h. The suspension was centrifuged at 12,000 rpm for 30 min to remove cell debris. The resultant cell-free extract containing all types of cytochromes was dialyzed against a dialysis buffer (100 μM EDTA, 10 μM PMSF in 100 mM phosphate buffer, pH 6.0) in dialysis tubing purchased from Sigma Chemicals, USA. The resulting concentrated fraction was passed through a glass column packed with carboxymethyl cellulose of medium viscosity (Sigma Chemicals, USA) equilibrated with the 100 mM phosphate buffer, pH 6.0. The first fraction containing cytochrome *a* and *b* was collected. The light yellowish/pinkish material adsorbed as a layer on the top of the column was eluted by the elution buffer (100 μM EDTA, 10 μM PMSF, 0.5 M NaCl in 10 mM phosphate buffer, pH 6.0) and used as a partially purified fraction of cytochrome *c*. Both the fractions were again dialyzed to obtain a concentrated fraction of cytochromes for further studies.

Estimation of cytochromes

For the estimation of cytochrome *a*, *b* and *c* in fractions, spectral studies were carried out according to the method of Berry and Trumpower (1987). Pyridine hemichrome and hemochrome spectra were recorded at 588, 558 and 549 nm for cytochrome *a*, *b* and *c* in a UV-Visible spectrophotometer (Shimadzu 1601) by reducing and oxidizing the cytochromes in the presence of sodium dithionite and potassium ferricyanide, respectively. The concentrations of cytochromes were calculated using extinction coefficients 27.20 mM^{-1} for cytochrome *a*, 33.51 mM^{-1} for cytochrome *b* and 29.71 mM^{-1} for cytochrome *c*.

Estimation of proteins

The total proteins in both fractions were estimated by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard for quantification of proteins.

Determination of molecular weight

For determination of molecular weight of cytochromes, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of 15% concentration was performed according to the method of Laemmli (1970). Standard

protein molecular weight markers (Bangalore Genei, India) were used for determination of molecular weight.

Results and discussion

The properties of cytochromes present in the isolated lichenized fungi of cultured lichen *Usnea ghattensis* were studied. Pyridine hemichrome and hemochrome spectra showed the presence of *a*, *b* and *c* type cytochromes in the fractions. The reduction absorption maximum (λ max) 0.050 reordered at 588 nm in the presence of sodium dithionite and 0.044 on oxidation with potassium ferricyanide confirmed the presence of *a* type cytochrome in fraction 1. A *b* type cytochrome was also detected in fraction 1 with λ max 0.047 on reduction and 0.043 on oxidation at 558 nm. Fraction 2 showed λ max 0.054 on reduction and 0.048 on oxidation at 549 nm, confirming the presence of *c* type cytochrome in the fraction (Fig. 1).

The effects of culture conditions on the cytochrome content of isolated fungi of the lichen *Usnea ghattensis* grown in MYE media with different carbon and nitrogen sources were studied. The contents of cytochrome *a*, *b* and *c* in fungi isolated from symbionts grown in MYE medium supplemented with 0.01 mol/l sucrose were found to be 0.0219, 0.0238 and 0.024 mM/mg protein, respectively, whereas a slightly high content of cytochromes was found: 0.0579 mM cytochrome *a*, 0.0732 mM cytochrome *b* and 0.0504 mM/mg protein cytochrome *c* in the MYE medium supplemented with 0.01 mol/l sucrose, 0.01 mol/l PEG and 0.005 mol/l glycine. In comparison to these media, the contents of cytochromes were found to be significantly high in the isolated fungi of symbionts grown in the MYE medium supplemented with 0.01 mol/l sucrose and 0.01 mol/l PEG. The content of cytochrome *a* was found to be 0.0967 mM, cytochrome *b* 0.0900 mM and cytochrome *c* 0.1030 mM/mg protein, respectively (Fig. 2). The comparison of these results with the available reports on cytochromes from isolated fungi of lichens (Fujiwara et al. 1995) showed that the concentration of cytochromes present in the isolated fungi of the lichen *Usnea ghattensis* was much higher. On determination of molecular weights of cytochromes by SDS PAGE, the molecular weight of cytochrome *a* was estimated as 32 kD, cytochrome *b* 19 kD and cytochrome *c* 11.5 kD (Fig. 3).

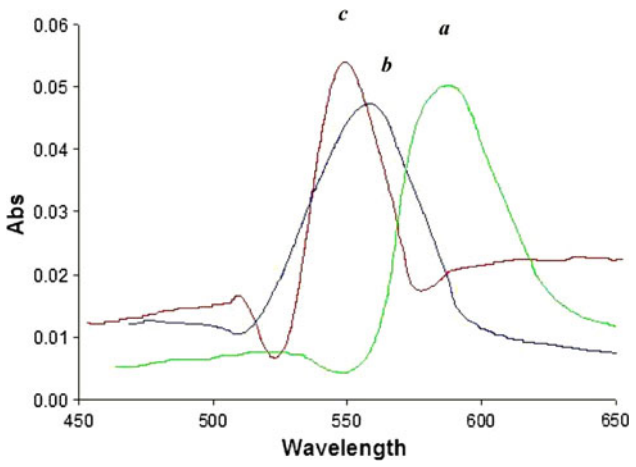

Fig. 1 The reduction absorption spectrum of cytochrome *a*, *b* and *c* isolated from fungal cells of the lichen *Usnea ghattensis*. The cytochromes were reduced by the sodium dithionite

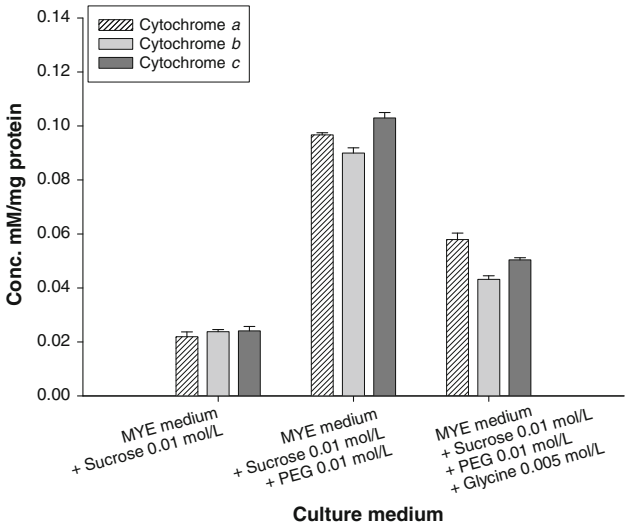

Fig. 2 Effect of culture conditions on the cytochrome content in isolated fungi of the lichen *Usnea ghattensis*

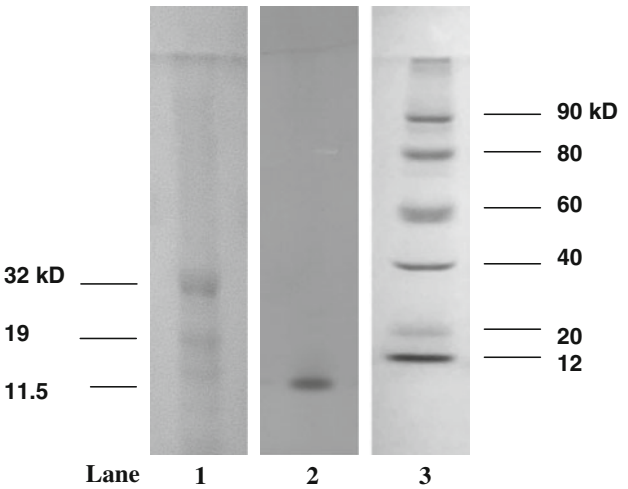

Fig. 3 Gel photograph of SDS-PAGE of cytochromes from isolated fungal cells of the cultured lichen *Usnea ghattensis*. *Lane 1* cytochrome *a* and *b* isolated from the first fraction; *lane 2* cytochrome *c* isolated from second fraction; *lane 3* standard protein molecular weight markers

Cytochromes are membrane-bound hemoproteins that contain heme groups and carry out the electron transport system. Fungal cytochromes constitute two electron transport systems—one in the mitochondria and one in the endoplasmic reticulum. The mitochondrial cytochrome system is called the respiratory chain and is responsible for cellular respiration and generation of ATP for energy production (Yoshida 1988).

In our present study, the addition of sucrose and PEG to the MYE medium in combination triggered the mitochondrial cytochrome system of the fungal symbiont and resulted in a high concentration of cytochromes and increment in the growth of culture biomass. Maximum culture biomass and metabolite production was observed in MYE medium supplemented with sucrose and PEG compared to the medium supplemented with other carbon and nitrogen sources reported earlier by us (Behera et al. 2009). However, the exact reason behind this high concentration of cytochromes obtained in isolated fungi of the cultured lichen *Usnea ghattensis* in relation to different carbon and nitrogen sources is still unknown. But it seems that supplementation of additional carbon sources may play a role in growth optimization of lichenized fungi via activating the cytochrome respiratory system.

Since we could not determine the subtypes of cytochromes in isolated lichenized fungi of *Usnea ghattensis*, further detailed molecular studies are needed to understand their role and mode of action during the growth of culture biomass under optimized conditions.

Acknowledgments We are very grateful to the Director of Agharkar Research Institute, Pune, India, for providing research facilities and financial support by the Department of Biotechnology (DBT) (grant no. BT/PR 3133/BCE/08/237/2002), Council of Scientific and Industrial Research (CSIR) India (grant no. 9/670(034)/2006-EMR-I).

References

Ascaso C (1980) A rapid method for the quantitative isolation of green algae from lichens. Ann Bot 45:483–484

Behera BC, Verma N, Sonone A, Makhija U (2009) Optimization of culture conditions for lichen *Usnea ghattensis* G Awasthi to increase biomass and antioxidant metabolite production. Food Tech Biotech 47:7–12

Berry AE, Trumpower LB (1987) Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. Anal Biochem 161:1–15

Fujiwara T, Ohashi T, Hamade R, Kinoshita Y, Yamamoto Y, Fukumori Y, Yoshimura I, Yamanaka T (1995) Cytochromes in the cultured mycobiont of a lichen, *Cladonia vulcani sav*. Plant Cell Physiol 36:183–186

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 27:680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin–phenol reagent. J Biol Chem 193:265–275

Muggia L, Schmitt I, Grube M (2009) Lichens as treasure chests of natural products. SIM News 59(3):85–97

Müller K (2001) Pharmaceutically relevant metabolites from lichens. Appl Microbiol Biotechnol 56:9–16

Oksanen I (2006) Ecological and biotechnological aspects of lichens. Appl Microbiol Biotechnol 73:723–734

Verma N, Behera BC, Makhija U (2008) Antioxidant and hepatoprotective activity of a lichen *Usnea ghattensis* in vitro. Appl Biochem Biotechnol 151:167–181

Yamamoto Y, Mizuguchi R, Yamada Y (1985) Tissue culture of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agric Biol Chem 49:3347–3348

Yamanaka T, Okunuki K (1964) Comparative biochemistry of cytochrome *c*. J Biol Chem 239:1813–1817

Yoshida Y (1988) Cytochrome P450 of fungi: primary target for azole antifungal agents. Curr Top Med Mycol 2:388–418