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The entire metabolite spectrum of the green alga *Scenedesmus obliquus* in isotope-labelled form

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Abstract

Modern biological structural and biosynthetic investigations often require the use of suitably isotope-labelled pigments or putative metabolic precursors. We present here, a culture and extraction protocol for *Scenedesmus obliquus*, which allows the simultaneous isolation of the-entire low M+ primary and secondary metabolite spectrum of this green alga. Fractions of amino acids, carbohydrates, lipids, carotenoids and chlorophylls are obtained, labelled in any combination of ^{2}H, ^{13}C and ^{15}N.

Keywords: *Scenedesmus obliquus*; Chlorophyta; Green algae; Isotope labelling; Metabolites; Carotenoids; Chlorophylls; Amino acids; Carbohydrates

1. Introduction

The ability to produce isotope-labelled proteins and nucleic acids, followed by the application of advanced heteronuclear NMR techniques, has dramatically extended the current repertoire of macromolecular structure and function elucidation (Oschkinat, Müller, & Dieckmann, 1994). However, due to the use of prokaryotic expression systems, these investigations are often limited to enzymes and proteins which do not contain cofactors from eukaryotic origin. Photosynthetically relevant pigments, for instance, have so far, apart from very few exceptions (Patzelt et al., 1997; Egorova-Zachernyuk et al., 1997), resisted to any detailed structural studies in their natural protein environment.

Recent advances have also been made in the understanding of the biosynthesis and catabolism of plant metabolites, e.g. the chlorophylls (Rodoni et al., 1997). Such investigations, however, as well as quantitative distribution analyses of plant secondary metabolites, are still hampered by the lack of suitably labelled advanced biosynthetic precursors for NMR investigations or reference compounds for quantitative mass spectrometric analyses.

The green alga, *Scenedesmus obliquus*, has been successfully used for the production of isotope-labelled amino acid mixtures, on which bacteria, such as *Escherichia coli*, could be grown with various labelling patterns (Crespi & Katz, 1972; Markley, 1972; Brodin, Drakenberg, Thulin, Forsén, & Grundström, 1989; Sørensen & Poulsen, 1992). We describe herein, an extension of the existing procedures which allows the production of amino acid/sugar hydrolysates, rigorously purified from toxic algal metabolites and thus suitable for the cultivation of more demanding organisms (Patzelt et al., 1997). Furthermore, a fractionating extraction procedure was devised to simultaneously isolate the low M+ lipophilic metabolites of the alga, including carotenoids and chlorophylls, labelled in any combination of ^{2}H, ^{13}C and/or ^{15}N.

2. Results and discussion

Cultures of *S. obliquus* were grown photoautotrophically in two-tier flasks in a slightly modified inorganic medium, as described before (Sørensen & Poulsen,

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1992; Murashige & Skoog, 1962; Hüsemann & Barz, 1977). A stepwise replacement of H_2O by D_2O led to deuterated cultures, a replacement of CO_2, the sole carbon source, by $^{13}CO_2$ and/or the nitrogen-containing salts by their ^{15}N-isotopomers produced ^{13}C- and/or ^{15}N-labelled cultures. These were used as inocula for larger fermentations in Fernbach flasks or stirred fermenters.

In contrast to previous procedures, the cells were harvested by filtration and the recovered medium was again inoculated. When the growth rate began to decrease, phosphate was supplemented. In this way, one medium preparation could be used for up to five fermentations, thus considerably reducing isotope costs and recycling time. Algal cells with any combination of ^{2}H, ^{13}C and/or ^{15}N could be produced, including random fractional label distributions.

The obtained cell mass was lyophilised and submitted to consecutive dark extractions with hexane, Me_2CO and 20% MeOH in $CHCl_3$, either in the cold or by using a Soxhlet apparatus. The extracts were further purified by TLC or HPLC (e.g. Märki-Fischer, Marti, Buchecker, and Eugster (1983); Schmidt, Connor, and Britton (1994)). The expected (Powls & Britton, 1977) α- and β-carotene and the larger part of the xanthophylls, like lutein, violaxanthin and loroxanthin, were found in the hexane fraction. The rest of the xanthophylls, together with chlorophylls a and b (in a ratio of ca. 10:1), appeared in the Me_2CO extract. $CHCl_3$/MeOH eluted mainly methyl chlorophyllide and phaeophorbide, together with other chlorophyll degradation products, higher M+ products and last traces of the xanthophylls and chlorophylls.

Acid hydrolysis of the extracted algae finally gave a mixture of amino acids and sugars, which could further be split up by ion-exchange chromatography (Crespi & Katz, 1972); the typical amino acid composition is given in Patzelt et al. (1997). The carbohydrates consist mainly of glucose, with minor amounts of mannose and galactose (Takeda, 1996).

In order to confirm the identity of the isolated compounds and to estimate the degree of isotope labelling, all extracts were analysed by mass spectrometry. Fig. 1 shows the $[M]^+$ peaks of β-carotene from unlabelled, ^{2}H-, ^{13}C- and ^{13}C, 60% ^{2}H-labelled cultures. Typically, isotope enrichments of larger than 97% were obtained.

The described fermentation and extraction protocol for *S. obliquus* is easy to set up using common laboratory equipment and allows the production of isotope-labelled algal metabolites under very economic conditions, both with respect to expenses and labour time. Any biologically relevant combination of ^{2}H, ^{13}C and ^{15}N can be achieved and the entire metabolite spectrum can be isolated. In particular, the availability of labelled photopigments should greatly facilitate further physiological and structural investigations, so far limited by a shortage of these compounds.

3. Experimental

3.1. Strain collection of S. obliquus in different isotope compositions and adaptation to novel isotope patterns

The upper reservoir of two-tier culture flasks (2×250 ml) was filled with 50 ml of a medium con-

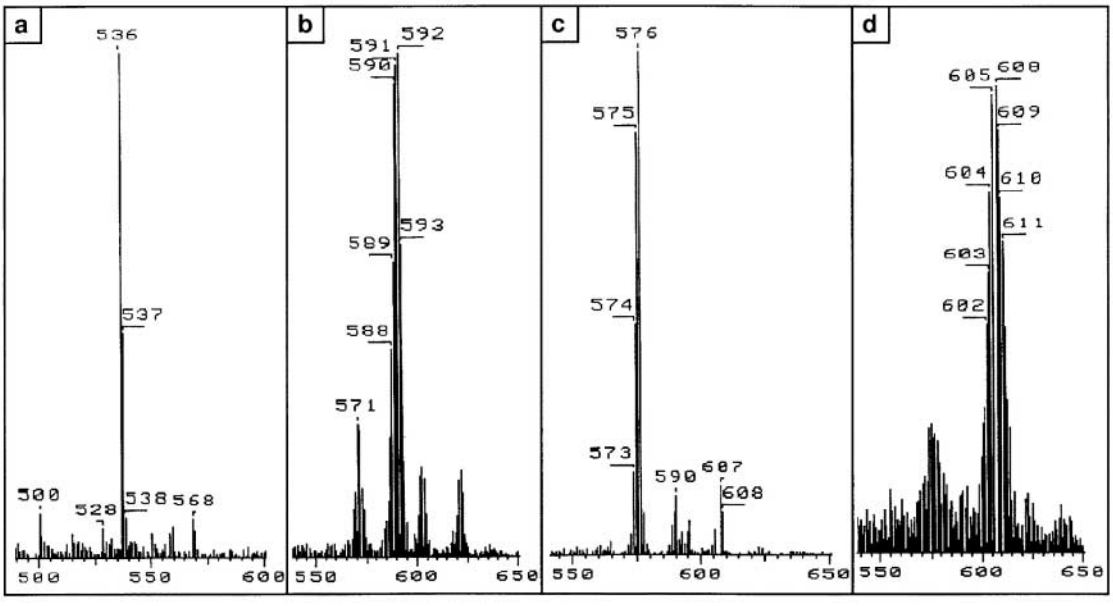

Fig. 1. Molecular ion peaks (EI-MS) of β-carotene isolated from cultures of the following isotope compositions: (a) unlabelled, (b) ^{2}H-labelled (calculated for $C_{40}{}^{2}H_{56}$: *m*/*z* 592), (c) ^{13}C-labelled ($^{13}C_{40}H_{56}$: *m*/*z* 576) and (d) 60% ^{2}H- and 100% ^{13}C-labelled ($^{13}C_{40}H_{23}{}^{2}H_{33}$: *m*/*z* 609).

taining (in mg l^{-1} for unlabelled cultures): KH_2PO_4, 340; $CaCl_2{\cdot}2H_2O$, 440; $MgSO_4{\cdot}7H_2O$, 370; $MnSO_4{\cdot}4H_2O$, 22.3; $ZnSO_4{\cdot}4H_2O$, 8.6; H_3BO_3, 6.2; KI, 0.83; $Na_2MoO_4{\cdot}2H_2O$, 0.25; $CuSO_4{\cdot}5H_2O$, 0.025; $CoCl_2{\cdot}6H_2O$, 0.025; Na_2EDTA, 37.3; $FeSO_4{\cdot}7H_2O$, 27.8; KNO_3, 1900 and NH_4NO_3, 1650. The pH was adjusted to 6 prior to sterilisation by filtration (0.45 μm). The lower reservoir contained a 2 M K_2CO_3/$KHCO_3$ buffer (K^+ conc.), pH 7.5 (100 ml). After inoculation (4 ml), the flasks were sealed and shaken at 60 rpm at 30°C under constant illumination for 14 d. The cells remained vital for a further 4 weeks, if stored at 4°C in the absence of light.

For the cultivation of deuterated algae, the salts were dissolved in D_2O and lyophilised ×3, and the medium was prepared in D_2O or in the required mixture of H_2O and D_2O. For ^{15}N-cultures, KNO_3 and NH_4NO_3 were replaced by the corresponding ^{15}N-labelled salts; for ^{13}C-cultures, the carbonate buffer was prepared using $K_2{}^{13}CO_3$. The adaptation of cultures from H_2O to D_2O was performed in three steps (50, 90 and 100%). However, ^{13}C- and/or ^{15}N-cultures were grown without prior adaptation; the first harvest was not used for the inoculation of preparative fermentations.

3.2. Preparative fermentations

For preparative fermentations, the media described above were inoculated (1:10) in Fernbach flasks (1 l in a 2.5 l flask) or in a 12 l stirred fermenter, equipped with a ring of 12 fluorescent tubes (standard plant light). The algae were grown for 14–20 d, depending on the isotope composition, at 30°C under constant illumination. For cultures without labelled carbon, a steady current of 5% CO_2 in compressed air was blown over (Fernbach flasks) or through the fermentation broth. For ^{13}C-labelled cultures, the atmosphere was sealed and the gas cycled through a ^{13}C-carbonate buffer of the composition described above. After the desired cell density was attained ($OD_{800} \approx 6$), 90% of the fermentation vol. was harvested over a sterile filter (GF10 (glass fibre)). The filtrate was re-introduced into the fermenter for a further growth cycle and the retained algal cells were frozen and lyophilised. Typically, between 1.8 and 3 g dry biomass were obtained per l fermentation broth per harvest. Each medium preparation could be used for 4–5 growth cycles.

3.3. Fractionation of metabolites

The dry algal cell mass (e.g. 100 g) was submitted to fractionating extractions with hexane, Me_2CO and $CHCl_3$–MeOH (4:1), either in a Soxhlet apparatus or at cold temps. All operations were performed in the dark. The hexane fr. (4.2 g) contained mainly non-polar lipids and carotenoids. Me_2CO eluted the rest of the xanthophylls and chlorophylls a and b (3.5 g). The $CHCl_3$ fr. (6.3 g) contained some more chlorophyll but mainly chlorophyll degradation products and macromolecular cell constituents.

The residues of extraction were hydrolysed in 1 M HCl (1 M DCl for ^2H-labelled material, 48 h, reflux) and the resulting neutralised slurry was filtered over a pad of activated charcoal. Evapn of solvent and lyophilisation yielded a white solid amino acid/sugar mixt. (80% w/w from the dry cells), with a typical amino acid content of 45–55%.

3.4. Analyses of extracts

The chemical identity of the organic extracts was always monitored by TLC (e.g. silica gel, hexane–EtOAc, 5:1) or HPLC [C_{18}, grad. Me_2CO–H_2O (1:4)- to Me_2CO]. Isotope compositions were determined by EI-MS (70 eV). Labels of the amino acid mixt. were analysed by GC-MS [DB1 (30 m), 70 eV] of the pentafluoropropionamide isopropyl ester derivatives.

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