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Short communication

In vivo O-dealkylation of resorufin and coumarin ethers by the green alga *Chlorella fusca* analysed by a rapid and sensitive high-performance liquid chromatographic assay

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Abstract

The O-dealkylation of resorufin or coumarin ethers is known to be a cytochrome P450-mediated reaction. It is used as a reference to characterize the corresponding enzyme activity and its state of induceability. In adopting this approach, an in vivo O-dealkylase assay was developed (a) using the unicellular green alga *Chlorella* as the biotransforming system and (b) applying a rapid and sensitive high-performance liquid chromatography (HPLC) assay which makes use of the wide pH stability of modified aluminium oxide packings. Separation was achieved isocratically at pH 9.8 using an Aluspher RP select B analytical column. The eluate was monitored by fluorescence detection with a detection limit of ≤3.5 pg for both resorufin and umbelliferone. The time needed for HPLC analysis is about 2 min, indicating that the method is suitable for automation.

1. Introduction

Cytochrome (Cyt) P450, the terminal oxygenase of the mixed function oxygenase system (mfo), is documented to be widely distributed among mammals, avians, fishes, insects, plants and unicellular organisms [1]. Although there are other oxygenase systems such as the flavin containing monooxygenase, Cyt P450 plays a major role in the metabolism of a broad range of xenobiotics. The first step (phase 1) in the biotransformation of drugs or xenobiotics is usually a Cyt P450-mediated reaction [2]. In plants, the involvement of Cyt P450 isoenzymes in the biotransformation of xenobiotics is described particularly for herbicides [3,4]. In the case of green algae, representing the main part of primary producers in aquatic systems, references to Cyt P450 implications are rare [5] and systematic research is lacking. A common method for determining Cyt P450 activities or state of induction is to measure the O-dealkylation of alkoxycoumarins or alkoxyresorufins (ACOD, AROD) by a direct fluorimetric assay [6,7]. This approach allows one to distinguish different P450 isoforms [8,9]. In pharmacology and ecotoxicology it is used, e.g., for pharmacokinetic studies [10,11] and effect monitoring [12,13]. In contrast to a direct fluorimetric assay, HPLC analysis prevents interferences and allows the determination of both the substrate and metabolite. However, HPLC assays already described

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[14,15] showed limitations due to insensitivity or a long duration of analysis. This paper presents a simple, very sensitive and rapid HPLC method that was used to determine the O-dealkylase activities of the green alga *Chlorella fusca*.

2. Experimental

2.1. Chemicals

Methoxy-, ethoxy- and pentoxyresorufin (7-MR, 7-ER, 7-PR) were synthesized from resorufin (Res) by the method of Prough et al. [16], including the modifications of Klotz et al. [17]. The sodium salt of resorufin was purchased from Sigma (Deisenhofen, Germany) and iodomethane, 1-iodoethane and 1-iodopentane from Merck (Darmstadt, Germany). All preparative separations were performed on a 310-25 LiChroprep Si 60 column (Merck). The resorufin ethers were judged greater than 98% pure by HPLC with a combination of diode-array and fluorescence detection. Analytical standards were obtained from Boehringer (Mannheim, Germany) and Lambda (Graz, Austria). Acetone stock solutions were stored at −30°C. 7-Ethoxycoumarin (7-EC) and umbelliferone (7-HC) were obtained from Sigma, 7-methoxycoumarin (7-MC) from Lambda and glycine and organic solvents (analytical-reagent or HPLC grade) from Riedel-de Haën (Seelze/Hannover, Germany). Ingredients required for alga nutrient media were purchased from Merck.

2.2. Equipment

The HPLC equipment consisted of an L-6200A HPLC pump, an AS-4000 autosampler including a cooling sample rack, an F-1000 fluorescence detector, an L-4500 diode-array detector (not necessary for O-dealkylase assay) and D-6000 HPLC software (Merck). Quantification was effected by the external standard method.

Compounds were separated on an Aluspher 100 RP select B column (125 mm × 4 mm I.D.) with an appropriate guard column (4 mm × 4 mm I.D.) (Merck).

2.3. Mobile phase

Separations of resorufin and alkoxyresorufin and of unbelliferone and alkoxycoumarin were performed isocratically with acetonitrile (solvent A) and 10 m*M* glycine in water adjusted to pH 9.8 with NaOH (solvent B). Solvent A to B ratios were as follows: MROD assay, 40:60; EROD assay, 50:50; PROD assay, 55:45; MCOD assay 35:65; and ECOD assay, 42:58. Total flow-rate was 1.5 ml/min in all instances. The excitation and emission wavelengths were tuned to 370 and 455 nm respectively, for umbelliferone–alkoxycoumarin analysis and to 540 and 600 nm, respectively, for resorufin–alkoxyresorufin analysis.

2.4. In vivo O-dealkylase assay

Organism and culture conditions

The unicellular green alga *Chlorella fusca* var. *vacuolata* Shihira et Krauss, strain 211-15, culture collection Pringsheim (Göttingen, Germany), was grown photoautotrophically at 28 ± 0.5°C in an appropriate sterilized medium [18] adjusted to pH 7.6. Cultures were aerated with sterilized, water-saturated air, enriched with CO_2 (1.5–2.0%, v/v) and illuminated by a combination of two types of fluorescent tubes (L36W/41 Interna, L36W/11 daylight; Osram, Berlin, Germany) with an intensity of 13–18 W/m^2 (22–33 klux). Cells were synchronized by light–dark changes of 14–10 h and a periodic dilution to a standard cell density of 1×10^6/ml. The cell number and cell volume distribution were analysed using a Coulter Counter Model ZB Industrial and a Coulter Channelizer C-256 (Coulter Electronic, Luton, UK). For statistical treatment (mean cell volume of population, calculated as median or average of cell volume distribution), data were transferred directly to a microcomputer (Victor VPC; Victor Technologies, Scotts Valley, CA, USA) [19,20].

Assay procedure

An adequate volume of a stock solution of alkoxyresorufin/-coumarin was transferred into an erlenmeyer flask, acetone was evaporated and the flasks were filled completely with the nutrient medium, closed and incubated for about 1–2 h at 48°C in the dark with continuous stirring until dissolution was complete. The high hydrophobicity of 7-pentoxyresorufin requires acetone as a mediator. Therefore, 7-PR stock solution was applied directly to nutrient media, resulting in a final acetone concentration of 0.25%. At t_2–t_3 of the cell cycle, algae were harvested (3250 *g* 5 min), washed twice, resuspended and adjusted to a biovolume of 0.8 ± 0.02 μl/ml (average cell volume × cell number). Aliquots of 2.5 ml of algae suspension were filled in 10-ml centrifuge tubes, each equipped with a 15-mm stirrer bar. Test-tubes were placed in a water-bath at 28°C with a multi-point magnetic stirrer (Variomag; H+P, Munich, Germany) adjusted to the maximum rpm and illuminated as described above. The layout made it possible to test up to 48 samples simultaneously. The assay was started by adding 2.5 ml of alkoxyresorufin/-coumarin solution to each test-tube, resulting in a final biovolume of 0.4 ± 0.01 μl/ml. After an adequate time of incubation the tubes were immediately put in a precooled (0°C) centrifuge and algae were separated at 3250 *g* for 5 min. Supernatants were analysed by HPLC as outlined above. The injection volume was 20 μl.

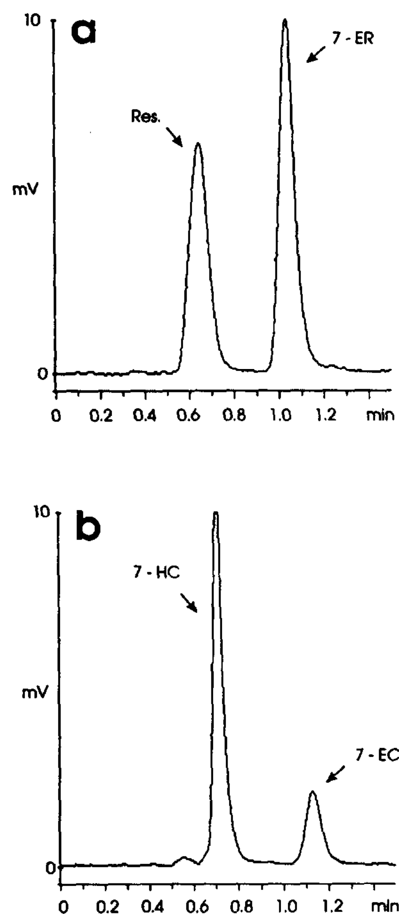

Fig. 1. Standard chromatograms of (a) resorufin–7-ethoxyresorufin and (b) 7-hydroxycoumarin–7-ethoxycoumarin (7-HC, Res 1.0×10^{-8} *M*; 7-EC, 2.0×10^{-5} *M*; 7-ER, 5.0×10^{-6} *M*). Substances were dissolved in alga nutrient media. Injection volume, 20 μl. Conditions as stated in the text.

3. Results

Fig. 1 shows typical chromatograms for ECOD and EROD assays. The retention times for resorufin and umbelliferone are 0.65 and 0.71 min, respectively. The corresponding *k'* values (the hold-up time was 0.56 min determined with acetone and diode-array detection) are 0.15 and 0.27, which indicates only weak retention. For all other assays they were the same; the *k'* values for resorufin and coumarin ethers are 0.98 (7-MR), 0.84 (7-ER), 1.86 (7-PR), 1.07 (7-MC) and 1.02 (7-EC). Therefore, the analysis takes no longer than 1.5–2.0 min. There was no or only minor interference from ingredients of the medium which eluted together with resorufin or umbelliferone. The detection limits, defined by a signal-to-noise ratio of 3:1, were 2.6 pg for umbelliferone and 3.4 pg for resorufin, equivalent to a molarity of the medium supernatants of 0.8 nmol/l. The column packing was stable for over 300 days at pH 9.8.

Fig. 2 depicts the rate of umbelliferone/resorufin production versus substrate concentration. Because this curve resulted from an in vivo assay, it is the summation of all kinetic processes

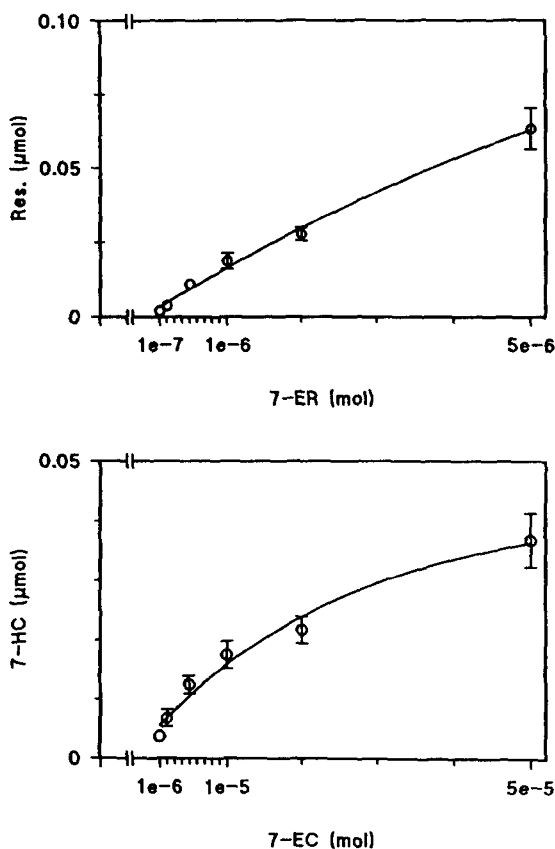

Fig. 2. Kinetic plots of umbelliferone (7-HC)/resorufin (Res) formation by *Chlorella fusca* (0.4 μl/ml) at $t_{60\,min}$ versus 7-EC/7-ER concentration. Values are means ±S.D.

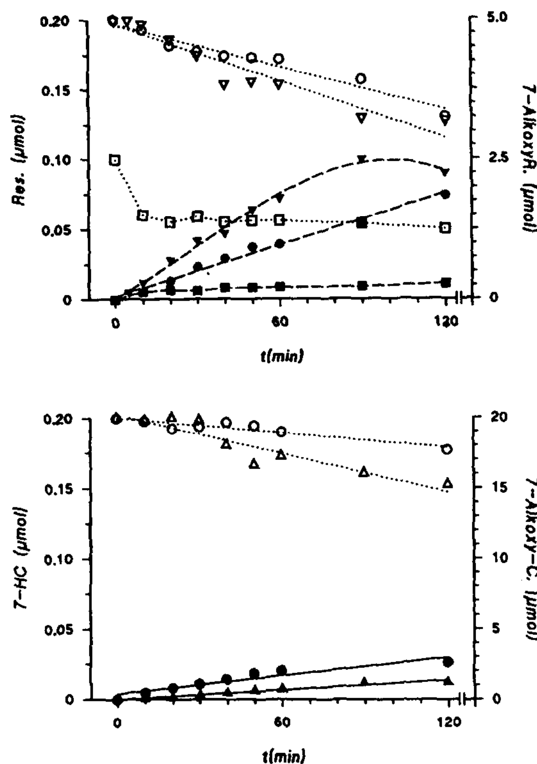

Fig. 3. Kinetic plots of umbelliferone (7-HC)/resorufin (Res) formation (solid symbols) and decrease of O-dealkylase substrates (open symbols) by *Chlorella fusca* (0.4 μl/ml) versus time. Substrate concentrations at t_0: 7-MC, 7-EC, 2.0×10^{-5} *M*; 7-MR, 7-ER, 5.0×10^{-6} *M*; 7-PR, 2.5×10^{-6} *M*. △, ▲ = MCOD; ○, ● = ECOD; ▽, ▼ = MROD; ○, ● = EROD; □, ■ = PROD.

involved, including substrate absorption, enzymatic transformations and release of products. Higher substrate concentrations are not soluble without adding organic solvents (see below). Therefore, V_{max} could not be achieved. However, it is clear that alkoxyresorufin is a more suitable substrate. This becomes clearer by comparing the metabolite formation versus the time of incubation, which is shown in Fig. 3.

In addition to the methyl and ethyl ethers, the pentyl ether of resorufin was tested. In the first 120 min there is a linear increase in umbelliferone/resorufin formation accompanied by a decrease of substrate in the medium. Only with 7-MR as O-dealkylase substrate after 90 min did the resorufin formation begin to stagnate, followed by a decrease in resorufin in the medium. For the alkoxyresorufins the order of turnover is 7-MR > 7-ER > 7-PR, indicating that the preference for one or another substrate is not only a function of its chain length. For the alkoxycoumarins it is 7-EC > 7-MC. In the dark, the dealkylase activities decreased immediately (EROD −88%, ECOD −92%), which can be correlated with the much lower reduction power available.

As shown in Table 1, the inter-assay variances of the different O-dealkylases determined over 3 months are ca. 20%. This relatively high variance seems to depend on differences in the degree of dissolution of the substrates. The intra-assay variances are typically ≤10%. Therefore, to make use of the O-dealkylase activities of *Chlorella fusca* for the purposes of determining

Table 1
Resorufin and umbelliferone formation by *Chlorella fusca*

Substrate	μmol resorufin (umbelliferone) × h^{-1}	*n*
7-Methoxyresorufin	0.0843 ± 0.0175	10
7-Ethoxyresorufin	0.0579 ± 0.0128	14
7-Pentoxyresorufin	0.0083 ± 0.0024	8
7-Methoxycoumarin	0.0108 ± 0.0059	9
7-Ethoxycoumarin	0.0200 ± 0.0051	16

O-Dealkylase inter-assay variation over a period of 3 months. Biovolume of *Chlorella fusca* was adjusted to 0.4 ± 0.01 μl/ml. Values are means ± S.D., *n* = number of assays. 7-MC, 7-EC, 2.0 × 10^{-5} *M*; 7-MR, 7-ER, 5.0 × 10^{-6} *M*; 7-PR, 2.5 × 10^{-6} *M*. Conditions are described in the text.

potential inducers and inhibitors, great efforts have to be made to dissolve the coumarin and resorufin ethers.

Whereas it is common to use organic solvents as mediators for better dissolution, in this instance 0.1% acetone led to more than a 60% decrease in umbelliferone formation and 20% less EROD activity. MROD was inhibited significantly by acetone concentrations ≥0.2%. On the other hand, resorufin formation from 7-PR increased slightly with a higher concentration (0.25–3.0%) of acetone.

4. Discussion

The excitation and emission spectra and fluorescence intensity of umbelliferone and resorufin are affected by the degree of dissociation of the acid hydroxy group; the pK_a values are 7.75 and 6.1, respectively [21,22]. Therefore, full dissociation and maximum fluorescence occur at pH 9.8. In contrast to common RP-modified silica-based column packings, which hydrolyse under alkaline conditions, aluminium oxide-based packings are stable over a wide range of pH (2–12) [23]. This makes the appropriate "RP" phases (Aluspher RP select B is a polybutadiene-coated aluminium oxide [24]) suitable especially for separations of basic compounds, because protonation is suppressed and the chromatographic conditions are well defined. In the application presented this approach is inverted and ionization of the metabolites resorufin and umbelliferone is induced, leading to an improved sensitivity and minimum retention, whereas the retention of the hydrophobic alkoxyresorufins and -coumarins depends only on the concentration of acetonitrile. The detection limit of the applied HPLC method allows the supernatant to be analysed without further substance enrichment. Unlike other O-dealkylase assay procedures, using microsomes as the biotransforming system, it has been shown that it is possible to determine O-dealkylase activities of a unicellular green alga in vivo. Further investigations will demonstrate the usability of such a biotransforming system of Cyt P450, e.g., to establish the ecotoxicological relevance of environmental pollutants.

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