BIOPRODUCTION OF USNIC ACID FROM ACETATE BY KAOLINITE IMMOBILIZED CELLS OF *CLADONIA SUBSTELLATA* VAIN.

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ABSTRACT

Cells of the lichen *Cladonia substellata*, immobilized in kaolinite and supplied with acetate, produce at room temperature large amounts of usnic acid which can be recovered from the washing solution.

KEY WORDS: *Cladonia substellata*, immobilization, kaolinite, usnic acid.

INTRODUCTION

Many lichen species produce phenolics which accumulate either on the cortex or on the cell walls of medullary hyphae (Rogers, 1989). These phenolics are mainly orsellinate derivatives, such as depsides, depsidones, and dibenzofurans (Vicente, 1991). Traditionally, these lichen metabolites have been used as powerful antibiotic substances (Vartia, 1973) and cytostatic products against higher plant cells (Oswiecimska et al., 1979) or some human tumor cell lines, such as Hela and KB (Cain, 1961; Oswiecimska et al., 1983; Takai et al., 1979). Usnic acid, one of the best known lichen phenolics, is also used as antitranspirant plant agent (Lasceve and Gaugai, 1990) and as an uncoupler of photosynthesis (Inoue et al., 1987; Monsó et al., 1993; Vavasseur et al., 1991). Moreover, lichens have a very low growth rate (Hill, 1984) and, then, the continuous and indiscriminate use of these plants to obtain active metabolites must be carefully limited. Thus, immobilized cells or enzymes are used in order to produce some active compounds (Mosbach and Mosbach, 1966; Mosbach, 1983; Garcia-Junceda et al., 1991; Vicente et al., 1992) avoiding practically irreversible destruction of large biomass amounts.

The main purpose of this paper is the study of the production of usnic acid by immobilized cells of *Cladonia substellata* discontinuously supplied with acetate as a precursor of lichen phenolics.

MATERIAL AND METHODS

Plant material

Cladonia substellata Vain., collected from sandy soils of Mamanguape (Pernambuco, Brazil), was used throughout this work. Thalli were dried in air flow and stored at 7°C in the dark, no longer than 4 weeks. This lichen species produces some phenolic compounds, such as usnic, stictic, cryptostictic and constictic acids (Ahti et al., 1993), although usnic acid appears as the main secondary metabolite.

Cell immobilization

Thallus samples (5.0 g in dry weight) were gently macerated in a mortar with 20 ml double-distilled water, filtered through a four-layered cheese-cloth and the filtrate centrifuged for 30 min at 3,200 x g. The pellet (about 0.5 g in dry weight) was mixed with fully hydrated kaolinite (20 g in dry weight) and loaded onto a glass column (15 cm x 3 cm i. d.). Solutions (50 ml) of three different concentrations of sodium acetate, 0.1 mM, 1.0 mM and 10 mM, as a precursor of usnic acid, were supplied to three identical columns and maintained at room temperature (about 25°C) for 12 days in white light (125 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Each 48 h, 50 ml of fresh acetate solutions were added to the columns and collected the old washing solutions. These eluates centrifuged at 10,000 x g for 30 min and the pellets were dried and weighed. Supernatants were treated with 50 ml of diethyl ether: ethyl acetate (65:35

v/v), strongly stirred for 1 h, and collected the upper organic phase. The lower aqueous phase was then treated with 50 ml chloroform: acetonitrile (60:40 v/v) and processed as above. Both organic extracts were mixed, dried in air flow and used to analyze usnic acid.

Analysis and quantization of usnic acid

The occurrence of usnic acid in these extracts was firstly detected by thin layer chromatography on Silica Gel F₂₅₄+366 Merck plates, using toluene: dioxane: acetic acid (180:45:5 v/v) as mobile phase (Culbertson, 1972). Spots were revealed under UV light at 254 nm and 366 nm. D-Usnic acid from Sigma Chemical Co. was used as a standard. Quantization of usnic acid was achieved by HPLC as described by Legaz and Vicente (1983), using a Varian 5060 liquid chromatograph equipped with a Vista CDS 401 computer. Solid residues were redissolved in methanol (1.0 mg ml⁻¹) and then chromatographed by layering 10 µl of each solution onto the chromatographic column. D-Usnic acid (1.0 mg ml⁻¹) were used as external standards. Chromatographic conditions were as follows: reverse phase column, (300 mm x 4 mm i.d.), packed with MicroPak MCH-10; mobile phase, methanol: water: acetic acid (80:19.5:0.5 v/v); pressure, 87 atm; flow rate, 1.0 ml min⁻¹; temperature, 22°C; detector, UV set at 254 nm; absorbance units at full scale, 0.005.

RESULTS AND DISCUSSION

Kaolinite-immobilized cells were maintained for 12 days at room temperature. During this time period, no bacterial contamination was observed, probably as a result of the antibiotic activity of lichen phenolics (Vartia, 1973). However, purple bacteria developed from this time, although they could not survive in liquid media containing 10 µM usnic acid. When 7,000 units ml⁻¹ penicillin was included in acetate-containing washing solutions, no bacterial development was observed without significant changes of the pattern of usnic acid production.

The occurrence of usnic acid in the washing solutions was confirmed by TLC for the complete time of experimentation. A spot showing quenching fluorescence properties was detected

Fig. 1. Structural formulae of usnic acid (a) and reduced usnic acid (b).

with a R_f value of 0.97, identified as usnic acid (Culbertson, 1972). A spot with a R_f value of 0.83 only appeared after 8 days of cells incubation in 1.0 mM or 10 mM acetate, corresponding to a substance tentatively identified as oxidized usnic acid (Avalos and Vicente, 1987). Analysis by HPLC of the residue obtained from the washing solution containing 10 mM acetate, after 10 days contacting lichen cells, showed that a large amount of usnic acid (retention time, 11.28 min; formula a in Fig. 1) was produced whereas a substance with a retention time of 8.92 min (oxidized usnic acid; formula b in Fig. 1) appeared at very low amounts (Fig. 2A). This compound was not detected by analyzing samples obtained from washing liquid containing 0.1 mM acetate (Fig. 2B). Stictic, constictic and cryptostictic acids were never detected. This indicated that the synthesis of usnic acid might be a favored pathway over that of depsidone (stictic acid series) production, which only was started after long times of incubation with higher concentrations of acetate. Standard D-usnic acid showed a linear response in the detector for a range of mass injected varying from 2.0 µg to 20 µg (Fig. 2C). The time-course of usnic acid production is shown in Fig. 3. Large amounts of this phenolic were recovered after the second day of immobilization, probably as a result of the washing of usnic acid crystallized on the cell wall of lichen cells at the moment of immobilization (Ahmadjian and Jacobs, 1985). Latter, production of usnic acid initially decreased to continuously increase from the fourth day of incubation. This increase was dependent on the concentration of acetate supplied to the

Fig. 2. Chromatographic traces of 10 mM acetate-containing medium (A) or 0.1 mM acetate-containing medium (B) obtained after 10 days of contact with the immobilized cells of *C. sub-stellata*. (C) Direct calibration of response in the detector of standard D-usnic acid.

Fig. 3. Time-course of usnic acid production by lichen cells immobilized in kaolinite supplied with (●) 0.1 mM, (▲) 1.0 mM or (■) 10 mM acetate. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

cells. Total recovery of usnic acid from the overall process was 1.52 g from 0.1 mM acetate, 2.80 mg from 1.0 mM acetate and 3.69 g from 10 mM acetate. Since the respiratory pathway of acetate in lichens excludes the use of this metabolite via acetyl-CoA carboxylase (Vicente, 1991), an excess of acetate would increase cell respiration and phenolics would be only produced when the amount of intracellular acetate did not enhance fungal respiration. Losses of lichen cells lower than 10% in dry weight were observed in the washing liquid after elution from the kaolinite column during the complete time of storage.

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BIOPRODUKCJA KWASU USNINOWEGO Z OCTANU
PRZEZ KOMÓRKI *CLADONIA SUBSTELLATA* VAIN IMMOBILIZOWANE W KAOLINIE

STRESZCZENIE

Komórki porostu *Cladonia substellata* immobilizowane w kaolinie i zaopatrywane octanem, produkują w temperaturze pokojowej duże ilości kwasu usninowego, który można odzyskać z roztworu wymywającego.

SŁOWA KLUCZOWE: *Cladonia substellata* L., immobilizacja, kaolin, kwas usninowy.