Process Biochemistry 46 (2011) 1056–1062

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Bioproduction of resveratrol and viniferins by an elicited grapevine cell culture in a 2 L stirred bioreactor

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ARTICLE INFO

Article history:
Received 19 October 2010
Received in revised form 11 January 2011
Accepted 17 January 2011

Keywords:
Bioproduction
Plant cell cultures
Resveratrol
Stilbenes
Stirred bioreactor
Elicitation

ABSTRACT

A cell suspension culture was developed from calli of grape rootstock 41B in order to study the bioproduction of resveratrol. While 41B grape cultures produced no resveratrol, methyljasmonate (MeJA) elicitor treatment activated its production in a dose dependent manner. The concentration of 0.2 mM MeJA was optimal for efficient production and high accumulation of resveratrol (150 mg/L) in flask experiments. Microscopic analysis of cells monitored for viability showed that MeJA elicitor triggered expression of resveratrol fluorescence within the cells. These results led to scale-up of the culture in a 2 L stirred bioreactor where a resveratrol production of 209 mg/L being secreted into the liquid medium, corresponding to 90% of the total production. Liquid/liquid extraction of the culture medium and a solid/liquid extraction of the cells showed that other stilbenes were also produced. For the first time, *trans*-ε-viniferin, *trans*-δ-viniferin, and a *trans*-3-methylviniferin as well as *trans*-piceatannol were identified in a 2 L bioreactor cell cultures of grapevine. Furthermore, a one step FPLC method was developed for the purification of resveratrol and ε-viniferin.

1. Introduction

Grape stilbenes are a family of plant polyphenols in which *trans*-3,5,4′-tri-hydroxystilbene named resveratrol is best known for its numerous biological activities. Resveratrol is a key precursor of different oligostilbene dimers (ε-viniferin and δ-viniferin) [1,2], trimers (α-viniferin) [3] and tetramers (r- and r-2-viniferins) that have been characterized in grape plants [4], while stilbene dimer glucosides were identified in grape cell cultures [5]. There is considerable interest in resveratrol due to its biological properties (antioxidant and disease control) that may contribute to increase lifespan of diverse organisms [6–8]. The role(s) of resveratrol in human health may include (i) protection of the cardiovascular system [9] by inhibiting platelet aggregation [10], (ii) prevention and treatment of some forms of cancer [11] and (iii) modulation of *Sirtuin-1* gene expression [12] that may have effects against diabetes [13].

Related to the French paradox, nutraceutical use of resveratrol has been possible since the glycosylated derivatives, piceid and polydatin, can be obtained in large and inexpensive quantities from the Japanese knotweed (*Polygonum cuspidatum*, *Fallopia japonica*) for conversion into the aglycone. More recently, resveratrol oligomers that are not present in Japanese knotweed but useful for the cosmetic industry [14,15] have been obtained from grapevine as raw materials. For example, the stilbene ε-viniferin is now being used in cosmetic applications and also has been introduced in nutraceutical formulations. The economics for production of these oligomers from grapevine remain limiting since large amounts of costly organic solvents are required for their extraction and purification [14,16]. This has led to alternative strategies for the bioproduction of resveratrol and/or derivatives using microorganisms and plant cells [17]. The aim is to produce pure resveratrol, and potentially viniferins, by the mean of biotechnological processes without using plants but with sustainable protocols of extraction, as realized for example for taxanes (www.phytonbiotech.com).

While cultivation of grapevine under natural conditions will lead to the production of resveratrol and related derivatives, these secondary metabolites are also viewed as phytoalexins with antibiotic properties that are biosynthesized *de novo* and are secreted

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doi:10.1016/j.procbio.2011.01.019

following pathogen perception [18]. A relationship between the capacity of grapes to produce stilbenes and their resistance to disease was clearly established for powdery mildew [19]. In addition biosynthesis of these phytoalexins may be artificially induced by treating cells with elicitors that mimic a pathogen attack and trigger their defense mechanisms [20]. This property was shown in grapevine leaves where β-aminobutyric acid could elicit expression of stilbene synthesis and accumulation when cells were inoculated with *Plasmopara viticola* [21]. Stilbenes were also produced in grapevine cell suspension cultures when they were treated with various elicitors. For example the use of chitosan induced resveratrol and mono-glucosylated stilbene biosynthesis in *Vitis vinifera* cells [22,23]. Methyljasmonate (MeJA) appears to be a better inducer [24–27], since treated cells produced 840 mg stilbenes/L over 8 days, including mainly piceids whereas only 0.72% of the total was resveratrol [28]. The most powerful elicitor to trigger resveratrol production seems to be cyclodextrin, stimulating cultures that accumulate greater than 5000 mg resveratrol/L in the culture medium [27,29]. In particular, resveratrol was shown to be mainly released in the culture medium whereas piceids were accumulated within the cells [17]. Despite the interest in using *trans*-resveratrol and its derivatives for health purposes, production by cell suspension cultures has been limited to small volumes of culture. There are currently few available examples for production of these metabolites using a larger bioreactor. A scale-up to a 2 L bioreactor of grapevine cell suspension cultures from *V. vinifera* cv. Gamay Fréaux var. Teinturier was optimized to produce stilbenes reaching 360 mg/L but these cultures only accumulated *trans*-piceid [30]. More recently, a 1 L fed-batch bioreactor was used for the production of resveratrol derivatives [23]. The present study describes a grape cell suspension culture line derived from grape rootstock 41B that accumulates resveratrol and different stilbene oligomers following elicitation with MeJA. The scale up in a 2 L bioreactor was performed and extracts were analyzed for stilbene production by HPLC, FPLC and UPLC–MS that led to the identification of resveratrol and viniferins for the first time in such a volume of culture.

2. Materials and methods

2.1. Cell culture flask experiments

Stock cell suspension cultures derived from rootstock 41B (*V. vinifera* cv. Chasselas × *Vitis berlandieri*) were maintained in Erlenmeyer flask of 300 mL containing 100 mL of a modified Murashige and Skoog (MS) liquid culture medium [31] supplemented with 0.5 mg/L of benzylaminopurine (BAP), 0.2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose. Cell suspensions were cultured in darkness at 23 °C on a rotary shaker at 110 rpm and subcultured every week. Cell growth and production time courses were initiated and conducted in triplicate by introducing 10 g of fresh filtered cells in 300 mL flasks containing 90 mL of MS medium.

2.2. Bioreactor experiment

Two hundred milliliters of the stock cell suspension in exponential phase was transferred to a 1 L Erlenmeyer flask and the volume was adjusted to 400 mL with the MS medium. The flask was maintained in darkness at 23 °C on a rotary shaker at 80 rpm. After one week of culture, the 400 mL of suspension was transferred using a peristaltic pump into the 2 L tank of a stirred glass bioreactor equipped with a marine turbine (New Brunswick Scientific, Bioflo 3000). The volume was then adjusted to 2 L with MS medium by pumping. A control culture without elicitation (150 g FW of inoculum) was performed during 22 days at 23 °C in darkness. The speed of the turbine was set to 50 rpm and the aeration rate was 0.025 vvm. The same protocol was used for the MeJA elicited culture with an inoculum of 100 g FW cells that were cultivated for 18 days. Aliquots of 20 mL of suspension were removed with the sample tool of the bioreactor to follow the growth. At the end of the culture, cells and medium were separated by filtration under reduced pressure and then frozen at −80 °C until utilization.

2.3. Elicitor treatment

After 5 days of culture, corresponding to cells in the exponential phase, 500, 1000 and 2500 μL of a solution of methyljasmonate (MeJA) (Sigma) mixed in 50% EtOH (40 mM) were introduced into the flasks containing 100 mL of cell suspension to obtain, respectively, the final concentrations of 0.2, 0.4 and 1 mM MeJA. To control for the effect of EtOH, replicate experiments were performed by the addition of the same volume of ethanol without MeJA. Cell growth and resveratrol production were monitored for 10 days. All experiments were performed in triplicate. Cell integrity was monitored by microscopic observations (visible, epifluorescence without or with fluoresceine diacetate (FDA)). Using bioreactor, after 14 days of culture, while the cells are in exponential phase, 10 mL of 0.4 mM MeJA was added to obtain a final concentration of 0.2 mM. Cells were harvested 4 days later, as the maximal production of resveratrol at 96 h after elicitation appeared as a characteristic of the cell suspension 41B.

2.4. Stilbene extraction

Cells and medium were filtered under reduced pressure and the biomass weighted to obtain the fresh weight (FW). Cells and medium were then stored at −80 °C until extraction. Medium was extracted with the same volume of AcOEt and the organic phase was evaporated to dryness using a rotavapor (Laborata 4000 Efficient, Heidolph). The concentrate was resuspended in 1 mL of MeOH for the flasks experiments and in 15 mL of MeOH for the bioreactor medium extract. Cells were freeze-dried to get the dry weight (DW) and then refluxed for 30 min in MeOH. The solution obtained was filtered, concentrated using the rotavapor, and resuspended in 1 mL MeOH (flask extract) and 50 mL MeOH (bioreactor extract).

2.5. Microscopic observations of the cells

The cells were monitored by fluorescence microscopy using an Olympus BH2 microscope equipped with a UV light source (BH2-RFL-T3; Olympus) and a fluorescent filter (BP495; Olympus). To determine their viability, cells (1 mL) were incubated for 5 min in a 1% FDA solution diluted with acetone in darkness before microscopic observation. Viability was then estimated using Malassez cell counting.

2.6. HPLC analysis

Ten microliters of the methanolic extracts were injected onto a Waters Spherisorb C18 S5ODS2 4 HPLC column (6 mm × 250 mm). The HPLC system used was a Waters apparatus equipped with a 510 pump, a 600 gradient controller, a 2487 UV–vis detector and a 717 automatic sampler maintained at 4 °C. Elution solvents used were A: CH_3CN (Acros Organics) and B: H_2O (Direct Q5 Millipore). The gradient performed was the same as described in [32]. UV detection was monitored at 285 and 307 nm, respectively.

2.7. FPLC separation

The FPLC protocol for separation of stilbenes was adapted from Gu et al. [33]. Five hundred microliters of cell methanolic extract were applied with a 500 μL injection loop to a Superdex 75 (GE Healthcare) column (280 mm × 5 mm) using an Äkta Prime chromatography system (GE Healthcare). The elution gradient was produced by the mixing different proportions of solvent A (30% EtOH/30% CH_3COOH) and solvent B (milliQ water). The flow applied for the elution was 1 mL/min and the column was pre-equilibrated with 30% of solvent A and 70% of solvent B. After injection, the gradient was the following: 75 min 30% A/70% B; 75 min 60% A/40% B; 100 min 100% A/0% B; 100 min 30% A/70% B. The FPLC analysis was realized in triplicate.

2.8. UPLC–MS analysis

A Waters ACQUITY™ ultra-performance liquid chromatography consisting of binary solvent manager, sample manager, photodiode array detector and MasLynx 4.1 software was equipped with mass spectrometry as an electrospray ionization source (UPLC–ESI/MS). The UPLC profiling was performed on a 50 mm × 2.1 mm BEH C18 column packed with 1.7 μm particles (Waters) utilizing a gradient elution profile. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 100% MeCN (solvent B). The gradient for 8 min was as follows: 0 min, 100% A; 0.6 min, 92% A; 6.0 min, 70% A; 6.50 min, 50% A; 7.0 min, 70% A; 7.50 min, 92% A; 8.0 min, 100% A. The column temperature was maintained at 40 °C with a constant flow rate of 0.3 mL/min. Chromatograms were analyzed at 305 nm and 320 nm for stilbenes. The injection volume was 3 μL and all samples were passed through 0.2 μm syringe filter before analyses. MS was performed in the negative ion mode, at a capillary voltage 3.45 kV, cone voltage 30 V and source temperature at 150 °C with a gas flow rate of 450 L/h.

3. Results and discussion

3.1. Cell growth and resveratrol production in Erlenmeyer flasks

The growth curves of *Vitis* 41B cell suspension showed a similar profile as determined by their fresh (FW) or dry (DW) weight. Cells grew exponentially for 14 days followed by a 5 days stationary