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Production of carboxyatractyloside and atractyloside by cell suspension cultures of *Atractylis gummifera*

T. Kephalas*, F. Alikaridis, K. Pantelia, D. Papadakis

Department of Biological Chemistry, School of Medicine, University of Athens, 11527 Athens, Greece

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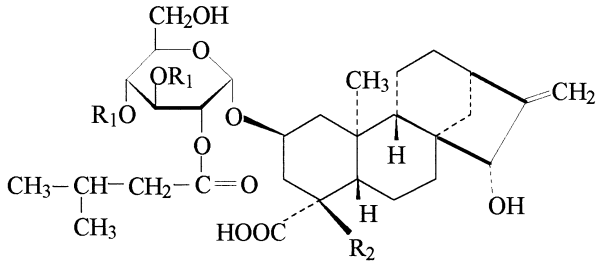
Abstract

Cell suspension cultures were grown from explants of *Atractylis gummifera* in several culture media. The accumulated biomass differed in the various culture media. The presence of carboxyatractyloside and atractyloside was examined in relation to the different culture media, by means of HPLC. Carboxyatractyloside was identified in the biomass produced in only one of the culture media employed, while atractyloside was not present in detectable amounts in any of the biomasses.

Keywords: Atractylis gummifera; Compositae; Cell suspension; Cultures; Norkaurane; Atractyloside; Carboxyatractyloside; HPLC

1. Introduction

Atractylis gummifera (Compositae) grows on the Mediterranean shores. Its rhizomes contain atractyloside (**1**)



1. R_1= SO_3H , R_2= H : Atractyloside
2. R_1= SO_3H , R_2= COOH: Carboxyatractyloside

(Piozzi et al., 1966; Piozzi, Quilico, Fuganti, Ajello, & Sprio, 1967), a toxic glycoside which strongly inhibits translocation of adenine nucleotides across the mitochondrial membrane (Pfaff, Klingenberg, & Heldt, 1965) and carboxyatractyloside (**2**) (Danieli, Bombardelli, Bonati, & Gabetta, 1972), which shows an in vivo toxicity 10 times greater than that of atractyloside (Luciani, Martini, & Santi, 1971). Carboxyatractyloside has also been tested in the wheat coleoptile bioassay and on intact plants and was found to significantly inhibit the growth of the tested plants (Culter & Cole, 1983).

In the present work, cell suspension cultures in four different culture media were employed and the presence of atractyloside and carboxyatractyloside was examined, by means of HPLC.

2. Results and discussion

Sterilized seeds of *Atractylis gummifera* were germinated in Nitsch and Nitsch solid medium (Nitsch & Nitsch, 1969) and the explants were used to grow cell suspension cultures, in four different culture media described as A, B, C and D in the experimental section. The biomass produced after 8 months of culture was collected, dried and weighed. The mass production in medium D was approximately three times higher than that in any other culture media. The dried materials were extracted by an aqueous solution of Me_2CO and the condensates were tested for the presence of atractyloside and carboxyatractyloside, by means of HPLC. A linear gradient system of phosphate buffer with MeOH on a reversed phase nova-pak C-18 column was developed for the separation of the compounds of interest. Carboxyatractyloside and atractyloside exhibited good resolution in this system (R_t 13 min and 26 min, respectively). Neither of the compounds of interest was identified in detectable amounts in the samples derived from the culture media A, B and C.

Atractyloside was found in trace amounts in the sample derived from the medium D cell culture. On the other

* Corresponding author.

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hand, carboxyatractyloside was present in the same biomass at a concentration of 0.4% of dry wt. The corresponding amount of carboxyatractyloside in fresh rhizomes of the plant has been reported to be 0.6% (fresh weight) (Danieli et al., 1972). It thus appears that under our culture conditions the production of the above glycosides was greater than that from the intact plant rhizomes.

3. Experimental

3.1. Culture methods

Atractylis gummifera seeds were surface sterilized (20 min) with sodium hypochloride soln (2% w/v) and germinated on nutrient solid medium Nitsch and Nitsch (1969). The one-week old explants were cut in small pieces and transferred to several solid culture media for calli creation. The media used were:

(A) Murashige and Skoog (1962) salt base supplemented with sucrose (50 $g \cdot l^{-1}$), casein hydrolysate (500 $mg \cdot l^{-1}$), myoinositol (100 $mg \cdot l^{-1}$), thiamin (10 $mg \cdot l^{-1}$), pyridoxine (1 $mg \cdot l^{-1}$), nicotinic acid (1 $mg \cdot l^{-1}$), kinetin (0.2 $mg \cdot l^{-1}$) and 2,4-dichlorophenoxy acetic acid (2,4-D) (0.2 $mg \cdot l^{-1}$).

(B) Linsmaier and Skoog (1965) supplemented with 2,4-D (0.2 $mg \cdot l^{-1}$) and naphtyl acetic acid (NAA) (0.2 $mg \cdot l^{-1}$).

(C) Gamborg, Miller and Ojima (1968) and

(D) Gamborg et al. (1968) supplemented with 2,4-D (2 $mg \cdot l^{-1}$), NAA (0.5 $mg \cdot l^{-1}$), IAA (0.5 $mg \cdot l^{-1}$) and kinetin (0.2 $mg \cdot l^{-1}$).

Cells were grown on solid culture in the above media under continuous light conditions at 25°C and subcultured every week in fresh media.

Suspension cultures were initiated by transferring 1 g of fresh callus tissue into 100 ml of liquid culture media (A–D), in a 250-ml flask and placed on an orbital shaker (100 rpm) under the above light and temperature conditions. Suspension cell cultures were subcultured at intervals of 20 days for 8 months. The resulting biomass from all the different media was filtered through a Büchner funnel, freeze dried and weighed. The total amount collected from media A–D was 1.6, 2.8, 2.2 and 6.4 g (dry wt), respectively.

3.2. Extraction and isolation

The method described by Danieli et al. (1972) was used. The dried material was extracted at 40°C with Me_2CO (10 $ml \cdot g^{-1}$). The extraction was repeated three times using a mixture of Me_2CO–H_2O (9:1) (5 $ml \cdot g^{-1}$) at the same temperature. The combined extracts were evaporated at reduced pressure, at a temperature below 40°C and condensed to 2% of the initial volume. The aq. condensate was extracted three times with $CHCl_3$, using each time twice the volume of the condensate, centrifuged and the aq. phase was evaporated at 40°C under vacuum to dryness. The dried material dissolved in 2 ml of MeOH was used for HPLC analysis.

3.3. HPLC analysis

HPLC was carried out with two HPLC pumps connected with an HPLC controller, monitored at 220 nm by a UV detector and quantified by an integrator. A nova-pak C-18 (150×3.9 mm ID) column was used and the samples were eluted by a linear gradient for 35 min from 25 to 60% MeOH in a phosphate buffer 0.18 M, pH 3.9, containing EDTA (440 $mg \cdot l^{-1}$), octyl sulfate sodium salt (120 $mg \cdot l^{-1}$) and triethylamine (165 $\mu l \cdot l^{-1}$), at a flow rate of 1 ml/min. In this system the R_t of the compounds of interest were identical with the R_t of authentic samples, commercially available (R_t carboxyatractyloside 13 min, R_t atractyloside 26 min).

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