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High-performance liquid chromatographic assay of anthranilate synthase from plant cell cultures

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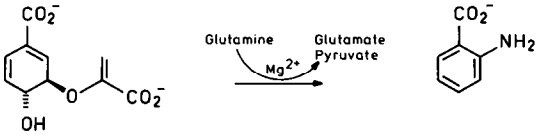
ABSTRACT

An assay is described for the enzyme anthranilate synthase (E.C. 4.1.3.27) from plant cell cultures, based on the fluorimetric detection of anthranilate after high-performance liquid chromatography on a LiChrosorb RP-8 Select B column. Depletion of the substrate chorismate and the presence of interfering enzymes can be followed by UV measurement. The rate of anthranilate formation was linear for at least 3 h at 30°C. The calibration graph was linear for at least 20 n*M* to 95 μM. Anthranilate synthase was measured in *Catharanthus roseus, Tabernaemontana divaricata, Cinchona robusta, Rubia tinctorum* and *Euonymus europaeus*. The highest specific activity was found in *C. roseus* after induction for indole alkaloid production.

INTRODUCTION

Anthranilate synthase (E.C. 4.1.3.27) catalyses the conversion of chorismate to anthranilate utilizing either glutamine or ammonium as amide donor and Mg^{2+} as cofactor (see Fig. 1). This is the first step in the branching of the shikimate pathway to tryptophan biosynthesis. Tryptophan is a key precursor for the indole alkaloids in plants, among which there are several pharmacologically important compounds [1]. Several studies have indicated that loss of control at the anthranilate synthase step



Chorismate Anthranilate

Fig. 1. Reaction scheme of anthranilate synthase.

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can lead to largely unregulated accumulation of tryptophan [2–4]. As part of a programme to study the regulation of the biosynthesis of indole alkaloids, we were interested in a rapid and specific assay for anthranilate synthase. Existing methods measure anthranilate synthase activity fluorimetrically either by following the anthranilate formation directly [5] or after extraction of anthranilate into ethyl acetate from an acidified incubation mixture [5]. These two assays were developed for the bacterial enzyme, but have also been applied to plant enzymes [6–8]. With these assays possible chorismate (substrate) depletion, due to other chorismate–utilizing enzymes, cannot be detected. In crude plant extracts several chorismate–utilizing enzymes are present. We also found that even desalted cell-free plant extracts can cause a considerable background fluorescence in these assays. We therefore developed a high-performance liquid chromatographic (HPLC) assay for anthranilate synthase in which the anthranilate formed is detected by fluorescence and the chorismate decrease can be followed by UV measurement. The product is separated from other fluorescent compounds in the crude extract by reversed-phase chromatography on a column especially selected for basic compounds [9].

EXPERIMENTAL

Chemicals

The inorganic chemicals used were of analytical-reagent grade. Anthranilate and L-glutamine were obtained from Merck (Darmstadt, Germany), leupeptin, phenylmethyl sulphonyl fluoride (PMSF) and pepstatin from Boehringer (Mannheim, Germany) and chorismate (80% pure, barium salt, C1259) and polyvinylpyrrolidone (insoluble form) from Sigma (St. Louis, MO, USA). PD-10 columns were purchased from Pharmacia (Uppsala, Sweden).

High-performance liquid chromatography

The HPLC system consisted of a Model 2248 HPLC pump from LKB (Bromma, Sweden), a Rheodyne Model 7125 injector with a 20-μl loop, a Model 2158 Uvicord SD detector from LKB equipped with an 8-μl flow cell and operating at 280 nm and a Shimadzu (Kyoto, Japan) RF 530 fluorescence detector with a 12-μl flow cell. The excitation wavelength was 340 nm and the emission wavelength 400 nm. All analyses were carried out at room temperature on a 250 mm × 4.0 mm I.D. LiChrosorb RP-8 Select B column (Merck) with a particle size of 7 μm at a flow-rate of 1 ml/min. A guard column (Merck) was always used in combination with the analytical column. The eluent consisted of 50 m*M* H_3PO_4 (final concentration) in water–methanol (65:35, v/v); the pH of the buffer was adjusted to 2.5 with 6 *M* NaOH before addition of methanol. The eluent was filtered through a 0.45-μm nylon (RC 55) filter (Schleicher & Schüll, Dassel, Germany) and finally degassed under vacuum.

Cell cultures

Cell suspension cultures were routinely grown at 25°C on a type G10 gyrotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 120 rpm. Subculturing of *Catharanthus roseus* cells was done every 7 days and of the other cell cultures every 14 days using a five-fold dilution of cells. *C. roseus* cells were grown in LS medium [10] containing 3% sucrose, 2 mg/l 1-napthaleneacetic acid and 0.2 mg/l kinetine. The

induction medium was according to Berlin *et al.* [11]. *Cinchona robusta* cells were grown in B5 medium [12] with 2% sucrose, 2 mg/l (2,4-dichlorophenoxy)acetic acid and 0.2 mg/l kinetine. *Rubia tinctorum* cells were grown in B5 medium [12] with 2% sucrose, 2 mg/ml (2,4-dichlorophenoxy)acetic acid, 0.5 mg/l l-naphthaleneacetic acid, 0.5 mg/l indoleacetic acid and 0.2 mg/l kinetine. *Tabernaemontana divaricata* cells were grown in MS medium [13] with 3% sucrose, 1 mg/l (2,4-dichlorophenoxy)acetic acid and 1 mg/l kinetine. *Euonymus europaeus* cells were grown in MS medium [13] with 3% sucrose, 1 mg/l (2,4-dichlorophenoxy)acetic acid and 0.2 mg/l kinetine.

Enzyme extraction

Cells were harvested by suction, washed once with water and immediately frozen in liquid nitrogen. Cells were stored at −80°C. Liquid nitrogen-frozen cells were homogenized for 1 min at maximum speed in a Waring blender equipped with a stainless-steel bucket. All procedures were carried out at 4°C. To the cell powder, 0.05 g of polyvinylpyrrolidone and 1 ml of extraction buffer [0.1 *M* Tris–HCl (pH 7.5)–10% glycerol–1 m*M* EDTA–1 m*M* dithiothreitol–10 μM leupeptin] were added per gram fresh weight of tissue. After thawing, the homogenate was centrifuged at 10 000 *g* for 30 min. The supernatant was always desalted on Sephadex G-25 (Pharmacia PD-10 columns) equilibrated with 0.1 *M* Tris–HCl (pH 7.5). The non-desalted homogenate could be stored at −80°C without loss of enzyme activity for at least 3 months. Protein was determined according to Peterson [14].

Assay of anthranilate synthase

The incubation mixture (total volume 0.5 ml) contained 0.1 *M* Tris–HCl (pH 7.5), 1 m*M* barium chorismate, 20 m*M* L-glutamine, 10 m*M* $MgCl_2$ and 250 μl of desalted enzyme preparation. The incubation was started by addition of chorismate. After incubation for 1 h at 30°C, the reaction was stopped by the addition of 125 μl of 1 *M* H_3PO_4. Blanks were made by adding H_3PO_4 before the incubation. After centrifugation, the samples were analysed by HPLC as described above. The injection volume was 20 μl. The detector attenuation on the fluorescence detector was set according to the amount of enzyme activity in the incubation mixture.

RESULTS AND DISCUSSION

A representative chromatogram obtained with enzyme from *C. roseus* is shown in Fig. 2. The minimum detectable amount of anthranilate, defined as three times the value of the baseline noise, was 0.05 ng. The separation shown in Fig. 2 was achieved on a 3-year-old column, which had been used for multiple purposes, but the requirements on the column are very small with this assay. We always use a guard column in combination with the analytical column, and this guard column has to be replaced regularly when the HPLC system is used to determine anthranilate synthase in crude plants extracts. In the system described here, chorismate and isochorismate are well separated with retention times of 4.8 and 3.5 min, respectively (UV detection, chromatogram not shown). We have therefore also applied this HPLC system to determinations of isochorismate synthase (E.C. 5.4.99.6) [15]. A methanol concentration of 35% is the optimum, owing to strongly adsorbed compounds which elute at higher methanol concentrations.

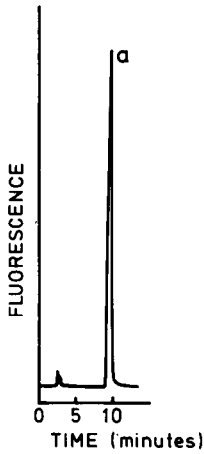

Fig. 2. Determination of anthranilate synthase activity by HPLC. Anthranilate (a) was separated on a LiChrosorb RP-8 Select B column, as described under Experimental. The fluorescence detector was set at high sensitivity, the attenuation was 32 and the injection volume was 20 μl.

The retention of anthranilate on a LiChrosorb RP-Select B column was found to be independent of salt concentration. However, the pH of the eluent has a very strong effect on the retention time of both anthranilate and chorismate. The retention time of anthranilate increased with increasing pH, whereas the retention time of chorismate decreased. For the assay pH 2.5 was found to be optimum. Blanks contain small amounts of anthranilate, which originates solely from the substrate barium chorismate. It is possible to eliminate this blank by using the purer, free acid of chorismate (Sigma, C1761). It is no problem, however, to correct for the blank.

An advantage of the HPLC assay described here is that it is linear over a much larger product concentration than the spectrofluorimetric assay in which anthranilate is extracted into ethyl acetate. The calibration graph is linear at least from 0.4 pmol to 1.9 nmol of anthranilate per 20-μl injection volume which corresponds to linearity from 20 nM to 95 μM.

In all the plant cell cultures it was impossible to measure anthranilate synthase in a non-desalted cell-free extract. Desalting is not a prerequisite for the HPLC method as such; anthranilate synthase is simply not active in our non-desalted crude extracts. This is probably due to the presence of tryptophan [7], a potent inhibitor of anthranilate synthase.

A major advantage of the present HPLC method is that both the formation of the product anthranilate and the disappearance of the substrate chorismate can be followed. In assays of partially purified anthranilate synthase preparations, the amount of anthranilate formed can be directly correlated with the amount of chorismate reacted. However, in crude enzyme preparations several chorismate-utilizing enzymes are present. We were unable, for instance, to measure anthranilate synthase in *C. robusta* suspension cells elicited for anthraquinone production (elicited with autoclaved and freeze-dried *Phytophthora cinnamomi*, 0.5 mg/ml, the cells being harvested after overnight culture). By injection into the HPLC system from an incuba-

tion mixture it was obvious that the chorismate was used up within the first 5 min; this proved to be caused by a very high chorismate mutase (E.C. 5.4.99.5) activity, making it impossible to measure anthranilate synthase in elicited *C. robusta*. Chorismate mutase has only chorismate as substrate and has no cofactor requirements.

The crude anthranilate synthase enzyme was very unstable; the enzyme lost 25% of its activity in 3.5 h when kept on ice in Tris–HCl buffer without additives. The stability was increased substantially when protease inhibitors and glycerol were added. The rate of anthranilate formation was linear for at least 3 h as measured with the *C. roseus* enzyme, when using enzyme that had been desalted on a PD-10 column equilibrated with extraction buffer plus 0.2 m*M* PMSF and 1 μM pepstatin.

In Table I the specific activity of anthranilate synthase is shown in different cell cultures harvested in the exponential and stationary phase, respectively. It is remarkable that the anthranilate synthase activity in desalted *T. divaricata* preparations was so low, as this is an indole alkaloid-producing cell line. In order to determine if the *T. divaricata* cell-free extracts contained an inhibitor of anthranilate synthase, we tried to mix *C. roseus* and *T. divaricata* cell-free extracts. This experiment showed that the desalted *T. divaricata* cell-free extracts were not inhibitory for the *C. roseus* enzyme. It was possible to increase the specific activity of anthranilate synthase in *T. divaricata* by further adding pepstatin and PMSF as protease inhibitors. Probably the *in vivo* activity of anthranilate synthase in *T. divaricata* is higher. Work to elucidate this is in progress. Interestingly, a slight induction of anthranilate synthase in *C. roseus* was found (Table I) after induction for production of indole alkaloids. It is well known that tryptophan decarboxylase (E. C. 4.1.1.28) is induced upon induction of *C. roseus* for indole alkaloid production [16]. However this is, as far as we know, the first time that plant anthranilate synthase has been reported to be induced after transfer to production medium for indole alkaloids. The enzyme level found in *R. tinctorum* and

TABLE I

SPECIFIC ACTIVITY OF ANTHRANILATE SYNTHASE IN DIFFERENT CELL CULTURES

Cell culture	Age of culture (days)	Anthranilate synthase (pkatal/mg protein)[b]
Catharanthus roseus	2	5.3
	4	5.3
	7	5.0
	3[a]	5.4
	6[a]	7.1
Tabernaemontana divaricata	6	0.1
	14	0.1
Cinchona robusta	5	1.1
	12	1.3
Rubia tinctorum	5	1.7
	12	0.7
Euonymus europaeus	7	1.5
	14	3.0

[a] The cells were induced by subculture to induction media.

[b] Values are averages for duplicate samples; pkatal = pmol anthranilate formed per second.

E. europaeus, two non-indole alkaloid-producing cell lines, probably represents the enzyme level necessary for sustaining the cell culture with tryptophan for protein synthesis.

In conclusion, a reliable HPLC assay has been developed for anthranilate synthase from both crude and purified enzyme preparations. No extraction into organic solvents is needed and the assay is linear over a wide range. A major advantage is that substrate depletion and the presence of interfering enzymes using chorismate as substrate can easily be detected when both fluorescence and UV detection are used.

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