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Note

High-performance liquid chromatography of anthranilate synthase using gel filtration and a post-column reactor

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Anthranilate synthase is feedback inhibited by tryptophan (Trp) and controls the synthesis of Trp used for protein synthesis and synthesis of important secondary products in many organisms[1]. Plant cell cultures selected using 5-methyltryptophan (5MT) usually overproduce Trp and have an altered anthranilate synthase that is less sensitive to feedback inhibition[2–4]. When plants are regenerated from 5MT-selected *Nicotiana tabacum* cells, the plants do not overproduce Trp and do not contain an altered anthranilate synthase[5,6]. However, new cell cultures initiated from these plants again overproduce Trp and have an altered enzyme.

Extracts of 5MT-selected *N. tabacum* cells were analyzed using Sephacryl S-200 gel filtration chromatography and two forms of anthranilate synthase were detected with molecular weights estimated to be 200 000 and 150 000 (ref. 6). The larger form of the enzyme was less sensitive to inhibition by Trp. Wild-type cell extracts contained a barely detectable amount of this enzyme form. Only the Trp-sensitive form was observed in extracts of whole plants. It has been proposed that 5MT selects for cells that have an alteration in the control for the expression of this less sensitive enzyme form, resulting in overexpression of the enzyme which cause an overproduction of Trp[6].

A high-performance liquid chromatographic (HPLC) method has been developed for the analysis of plant cell extracts to aid in the rapid analysis of the multiple forms of anthranilate synthase in plant cells and tissues. Analysis of anthranilate synthase using Sephracryl S-200 is tedious and time consuming, and lack of an adequate direct detection procedure limits use of disc gel electrophoresis[7].

EXPERIMENTAL

Materials

The 5MT-resistant cell lines used were: *N. tabacum* L. cv. Xanthi, TX2-4, a clone from 5MT-resistant cells[5]; and *Daucus carota* L. var. sativa, C123 (ref. 8). Chorismate was prepared as described by Gibson[9]. The water used in the preparation of the HPLC mobile phase was glass distilled. All other chemicals were reagent grade and obtained from commercial sources.

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Preparation of anthranilate synthase samples

Cell extracts were prepared using a nitrogen pressure cell as described in Brotherton *et al.*[6]. After centrifugation at 27 000 *g* for 10 min to remove cell debris, 10 ml of *N. tabacum* extract was mixed with 5 ml 10 m*M* Trp. After a brief incubation period, 5 ml of DEAE-cellulose suspension (0.25 g dry DEAE-cellulose in 200 m*M* Tris–HCL, pH 7.5) was added with mixing, incubated 5 min and centrifuged 5 min at 27 000 *g*. The supernatant was decanted and the pellet washed two times with 20 ml HPLC mobile phase buffer using centrifugation to collect the pellet between each wash. Anthranilate synthase was dissociated from the DEAE-cellulose by adding 10 ml HPLC mobile phase buffer and 1 ml room temperature saturated ammonium sulfate, followed by centrifugation as above. The supernatant was combined with two volumes of room temperature ammonium sulfate in water (final concentration 66%), centrifuged and the pellet resuspended in the original extraction buffer. The final sample was centrifuged in a Beckman Microfuge Model B for 2 min. Samples contained between 5 and 20 units/ml of enzyme activity. One unit equals 1 nmole of anthranilate produced per minute at 30°C as measured in the assay described by Brotherton *et al.*[6]. The same procedure was used for *D. carota* extracts except no Trp solution was used.

HPLC separation of anthranilate synthase forms

The separation was performed using a Waters Assoc. (Milford, MA, U.S.A.) Model M6000 pump, Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector, Kratos (Ramsey, NJ, U.S.A.) Model FS970 Spectrofluoromonitor (excitation 320 nm, emission 370 nm filter). A 600 × 7.5 mm TSK-Gel G4000SW column equipped with a 100 × 7.5 mm TSK-Gel SW precolumn (Toyo Soda, Tokyo, Japan) was used.

The post-column reactor consisted of a Sage (Orion Research, Cambridge, MA, U.S.A.) Model 341 syringe pump, and the mixing tee and 2.5-ml reaction coil from a Kratos Model URS 050 post-column reactor connected in the eluent path with a minimum length of tubing. A Waters Assoc. Model 441 absorbance detector (280 nm) was connected upstream from the post-column reactor to measure protein absorbance. Data were collected using strip chart recorders.

The HPLC mobile phase buffer was 50 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, 0.05 m*M* EDTA, 2.0 m*M* magnesium chloride, 10 m*M* glutamine, 2.0 m*M* dithiothreitol, 5% glycerol. The reactant solution added in the post-column reactor was: blank = mobile phase buffer; control = 1 mg/ml chorismate in mobile phase buffer; Trp = 0.5 m*M* Trp and 1 mg/ml chorismate in mobile phase buffer. The mobile phase buffer and the reactant solution were filtered through 0.45-μm Millipore (Bedford, MA, U.S.A.) filters prior to use.

The mobile phase flow-rate was 0.1 ml/min. The post-column reactant solution flow-rate was 0.01 ml/min. The separation was performed at room temperature and the post-column reactor coil at 30°C. Samples applied to the system were 50 or 100 μl.

RESULTS AND DISCUSSION

HPLC analysis of a 66% ammonium sulfate fraction of a cell extract produced numerous background fluorescent peaks that obscured the anthranilate fluorescence.

DEAE-cellulose was used to purify partially and concentrate the anthranilate synthase prior to HPLC analysis. Prior addition of Trp was necessary for recovery of *N. tabacum* enzyme activity from DEAE-cellulose under the conditions used here. *D. carota* enzyme activity was recovered without use of Trp.

Trp and the post-column reactant solution also contributed background fluorescence. Chorismate routinely contains fluorescent impurities which can be minimized by careful chorismate preparation as outlined by Gibson[9]. The detection conditions selected represent a compromise where fluorescence due to Trp, chorismate, chorismate impurities and protein is decreased relative to that of the reaction product anthranilate. The low mobile phase buffer flow-rate allows for 25 min residence in the reaction coil. This allows a substantial production of anthranilate from chorismate although the total conversion is less than 1%. The lowest enzyme activity conveniently measured is 5 nmol/min · ml in the sample prior to application. With *N. tabacum*, an estimated 5% of the 1 unit of enzyme activity applied to HPLC was detected in the post-column reactor.

The results obtained with extracts of two 5MT-resistant cell lines are shown in Fig. 1. In Fig. 1A, two forms of anthranilate synthase were separated and detected in an extract of *N. tabacum*. Addition of 50-μM Trp reduced the amplitude of the second peak but not the first peak. In Fig. 1B, one form of anthranilate synthase is observed in the extract from *D. carota*. The same peak, reduced in amplitude, was observed when Trp was added to the post-column reactant solution.

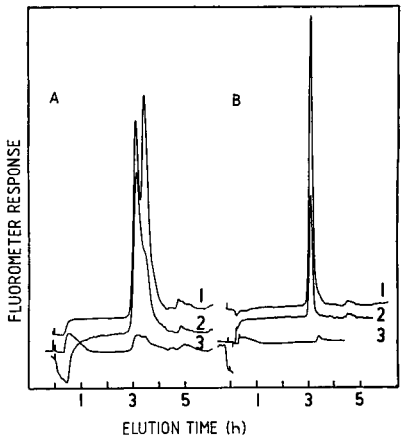

Fig. 1. Analysis of anthranilate synthase from extracts of 5MT-resistant *N. tabacum* (A) and *D. carota* (B). Analyses: 1 = control, 1 mg/ml chorismate in post-column reactant solution; 2 = Trp, 50 μM Trp and 1 mg/ml chorismate in post-column reactant solution; 3 = blank, HPLC mobile phase buffer without substrate added as post-column reactant solution. Samples of 50 μl were injected for each analysis.

The positive association of the observed fluorescent peaks with anthranilate synthase activity is suggested by (a) the absence of the peak when anthranilate synthase substrates are not present, (b) the inhibition of the anthranilate production when Trp is present, and (c) the similarity of the enzyme profiles with profiles obtained using other chromatographic methods. Using Sephacryl S-200, two forms of

anthranilate synthase are observed in *N. tabacum* extracts, the first form eluted being resistant to Trp inhibition[6].

The HPLC method has been used to extend our investigations to other species (*Nicotiana otophora, Solanum tuberosum, Datura innoxia*). Further preliminary sample preparation procedures are being developed for plant extracts where the DEAE-cellulose procedure has so far proven insufficient for removing interfering cellular components.

The HPLC technique described here is an improvement over former chromatographic[6] and electrophoretic[7] methods since the use of the post-column reactor makes the enzyme measurement rapid and sensitive. Thus information like molecular size, number of forms and feedback inhibition characteristics can be determined relatively efficiently.

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