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ANTHRANILATE SYNTHASE FROM *AILANTHUS ALTISSIMA* CELL SUSPENSION CULTURES

ROSAURA M. ROMERO* and MARGARET F. ROBERTS†‡

*CIPRONA, Escuela de Quimica Universidad de Costa Rica/CONICIT San Jose, Costa Rica; †Department of Pharmacognosy, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, U.K.

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Key word index—*Ailanthus altissima*; Simaroubaceae cell suspension culture; anthranilate synthase.

Abstract—Anthranilate synthase (AS) and chorismate mutase (CM) were monitored throughout the growth cycle of cells from *Ailanthus altissima*. Two isoenzymes were found, both of which were inhibited by 20 mM tryptophan: AS-a (by 66%) and AS-b (by 97%). Because AS-a was not always present, the purification and properties relate to AS-b. For this isoenzyme the K_m and V_{max} for chorismate were 73 μM and 93 pkat for L-glutamine were 50 μM and 35 pkat and for $MgCl_2$ were 180 μM and 45 pkat AS-b occurred as a dimer with M_r for the monomers, by SDS–PAGE, of $60(\pm 1.3) \times 10^3$ and $37(\pm 1) \times 10^3$.

INTRODUCTION

Ailanthus altissima is classified in the subfamily Simarouboideae, which contains many pharmacologically active constituents such as β-carbolines, canthin-6-one alkaloids and quassinoids [1, 2]. The wood, root and stem barks of the plant have all been reported to contain these compounds, the quassinoids being the major constituents in the whole plant [3]. Cell cultures of *A. altissima* have been obtained which produce, as major constituents, canthin-6-one alkaloids (100-fold greater than the whole plant) and, in some instances, low levels of quassinoids [4, 5]. Cell cultures of *A. altissima* have been used to investigate the biosynthesis of canthin-6-one alkaloids which are derived from tryptophan via β-carboline-6-propionic acid and 4,5-dihydrocanthin-6-one. Canthin-6-one may be hydroxylated at any one carbon followed by methylation. In *A. altissima* the principal alkaloids are canthin-6-one (C-6-O), 1-hydroxy-C-6-O and 1-methoxy-C-6-O [6–8]. The enzyme *S*-adenosyl- L-methionine methyltransferase, responsible for the formation of 1-methoxy-C-6-O from 1-hydroxy-C-6-O, has been isolated and purified to a single protein [9].

Tryptophan is utilized in microorganisms, as in plants, as a substrate for protein biosynthesis; however, in some plants, most notably those containing indole alkaloids, it also has an important role as precursor of secondary metabolites. The first committed step to tryptophan biosynthesis is the conversion of chorismate to anthranilate, a reaction that is catalysed by anthranilate synthase (AS). AS has been intensively studied and isolated from microorganisms, and in many cases the enzyme complex catalyses other reactions of tryptophan biosynthesis. For example, it can be associated, in microbial species, with anthranilate phospho-ribosyltransferase (PRT), indole-3-glycerolphosphate synthase (InGPS) or phospho-ribosyl anthranilate isomerase. This association of enzymes does not appear to occur in higher plants [10].

AS is an oligomer of nonidentical subunits designated α-subunit and β-subunit where the subunits are associated to give an α,β dimer or an $\alpha\alpha,\beta\beta$-tetramer [11]. The α-subunit catalyses an NH_3-dependent synthesis of anthranilate and the β-subunit binds glutamine, facilitating the transfer of the amide function of glutamine to the α-subunit. The utilization of glutamine by anthranilate synthase therefore requires both subunits, whereas use of ammonia requires only the α-subunit [12]. AS from plants has been investigated, but mainly in crude extracts or after a one-column chromatographic step. The AS from cell cultures of *Ruta graveolens* was partially purified and two isoenzymes were detected [13]. The enzyme was purified to a single protein from cell cultures of *Catharanthus roseus*, and it was found to be a tetramer consisting of two large and two small subunits with M_r of 67,000 and 25,500 respectively [10] but only one AS was detected. The genes for AS from *Arabidopsis thaliana* have been obtained, two genes encoding the α-subunit and three encoding for the β-subunit [14, 15].

In addition to AS, there are other enzymes that utilize chorismate and therefore compete with AS for this substrate, they are chorismate mutase (CM) and isochorismate synthase (ICS). As so much tryptophan in *A. altissima* cell cultures is channelled into alkaloid production, the present work sets out to make a study of AS and the activities of the other two chorismate utilizing enzymes, namely chorismate mutase (CM) and isochorismate synthase (ICS).

‡Author to whom correspondence should be addressed.

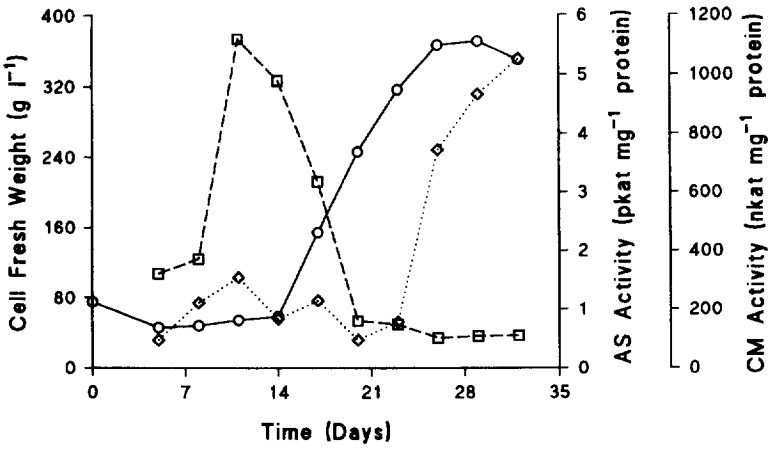

Fig. 1. Time course study of chorismate mutase (CM) and anthranilate synthase (AS) in *Ailanthus altissima* cell suspension culture. Cells were harvested at 3-day intervals throughout the growth cycle of the cells. O—O, fresh wt.; □—□, AS and ◇–◇, CM activities. Assays for protein, AS and CM were as given in Experimental.

RESULTS

Time course study

A detailed time-course study was carried out to investigate *A. altissima* cell suspension cultures for AS activity. In addition to AS, the activity of CM and ICS was monitored, as these two enzymes also utilize chorismate as substrate. During the time-course study of the cell growth cycle, the cells multiplied rapidly after day 14 and underwent an almost five-fold increase in fresh weight. In the cell line used in these experiments, alkaloid content, with C-6-O as the major alkaloid, was maximal at day 22–24 [16]. The activity of AS peaked between days 8 and 17, while the activity of CM increased after day 20. The activity of CM was 150 times the activity of AS; however, no ICS activity was detected during the growth cycle with this cell line (Fig. 1).

Purification of anthranilate synthase

A series of procedures were explored before reaching the final purification scheme and a comparison of separations utilizing precipitations with ammonium sulphate and polyethylene glycol, and chromatography on Piksi agarose A6XL, Q-Sepharose, Orange A, Mono-Q and Superose 12 HR led to the procedure given in the Experimental and Table 1. The suspension cultures used for purification were always harvested between days 10 and 15 of the cell growth cycle, and callus cultures were harvested between days 10 and 23. After harvesting, the cells were immediately frozen in liquid nitrogen and ground. When the cells were not used for extraction on the day they were harvested, after being frozen they were stored at − 80° and maintained AS activity for 2 months.

Cells from callus or suspension cultures frozen in liquid nitrogen were processed as given in the Experimental. Polyethylene glycol (PEG) precipitation gave a good and reproducible fractionation with a higher enzyme activity than ammonium sulphate precipitation. Preliminary experiments indicated that enzyme precipitation commenced at 10% PEG and at 18% PEG enzyme precipitation was optimal for anthranilate synthase. The PEG enzyme fraction was redissolved in buffer, applied to a Q-Sepharose column and the column was eluted with buffer using a discontinuous salt gradient (see Experimental and Fig. 2). Chorismate mutase activity was detected in the fractions obtained from the Q-Sepharose column (Fig. 2). Two peaks with CM activity were present, one of which overlapped the peak for AS activity. The fractions with AS activity from the Q-Sepharose column were pooled and applied to a Mono-Q column. The best running conditions for this column also used a discontinuous salt gradient (see Experimental and Fig. 3).

Two anthranilate synthase enzymes were detected in callus and cell suspension cultures and designated AS-a and AS-b (Fig. 3). Both activities were not always present in all the enzyme preparations. On the occasions where the two AS isoenzymes were detected, each was reinjected onto the Mono-Q column, and then only the injected peak was detected. AS-b was finally loaded onto a Superose 12 HR column and was eluted as a single peak of activity. A summary of the purification is provided in Table 1. The activity of AS-b isolated from callus cells was, at the Q-Sepharose step of purification, of the order of 11.5 nkat (specific activity: 5630 pkat mg^{-1} protein compared with 2.2 nkat (specific activity 572 pkat mg^{-1}protein for the suspension cultures. These values varied with the sample being processed, but were always higher for the callus cultures.

Properties of anthranilate synthase-b

The M_r of AS-b was shown to be $77(\pm 3) \times 10^3$ on a precalibrated FPLC Superose 12 HR column. The

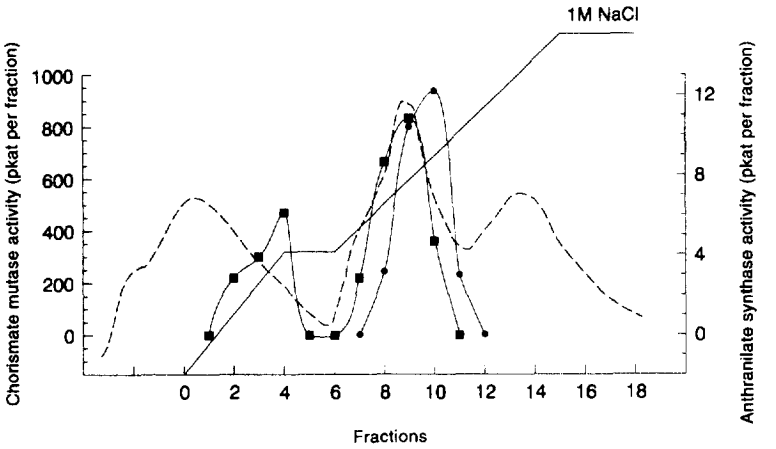

Fig. 2. Elution profile for *Ailanthus altissima* anthranilate synthase (AS) and chorismate mutase (CM) from the Q-Sepharose column. Extracts were prepared from cells harvested at day 14 of culture and protein precipitated with polyethylene glycol. This method and the assays for AS, CM and protein are given in Experimental. - - -, UV absorbance (protein);—●—AS activity;—■— CM activity.

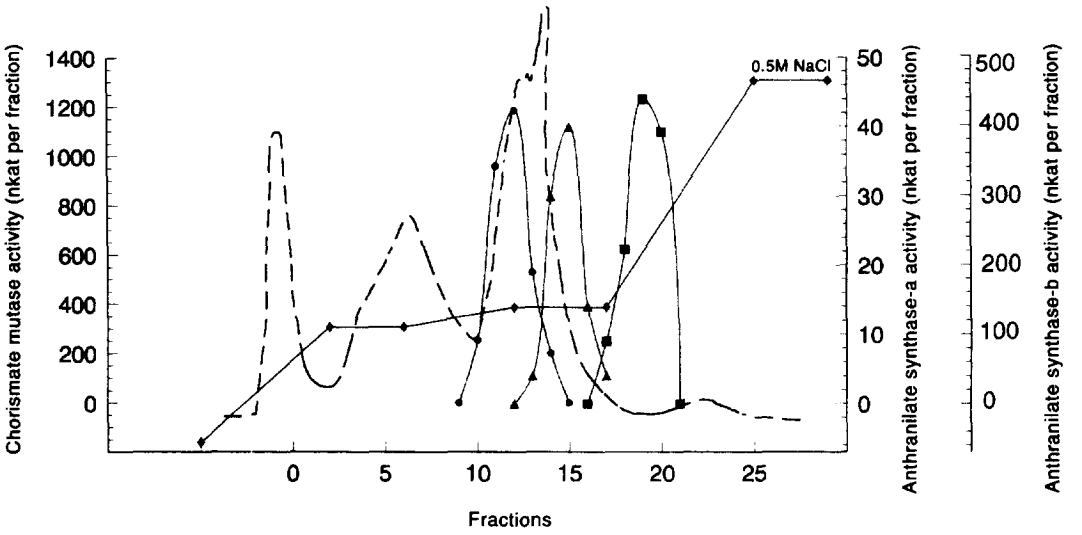

Fig. 3. Separation of anthranilate synthase (AS) a and b and chorismate mutase (CM) using chromatography on a Pharmacia Mono-Q column. The extract eluted on this column contained the fractions (7–12) with AS and CM activity from the Q-Sepharose column. Enzyme activities were measured using the methods given in the Experimental:—●— CM activity; —▲— AS-a and —■— AS-b activities; - - UV absorbance; ◆–◆ NaCl gradient.

M_r was also determined from samples obtained from the Mono-Q column, using vertical SDS-PAGE. Two bands were obtained, one with M_r corresponding to $60(\pm 1.3) \times 10^3$ and another with $37(\pm 1) \times 10^3$.

The pH optimum of the enzyme from the FPLC Mono-Q state, using citrate, phosphate and Tris buffers, fell between 7.3 and 8 (Fig. 4). The temperature at which maximum activity occurred was 35° and the optimum incubation time was 60 min. The activation energy, calculated using the Arrhenius equation, was 66 kJ mol^{-1}.

Kinetic properties

The Michaelis–Menten constants (K_m) and the maximum velocity for the enzyme substrates, chorismate and L-glutamine, and for the cofactor Mg^{2+}, were determined with the enzyme obtained from the FPLC Mono-Q column. From the Lineweaver–Burk equation, the K_m and V_{max}, respectively, were determined to be 73 μM and 93 pkat for chorismate, 50 μM and 1 pkat for L-glutamine and 180 μM and 45 pkat for $MgCl_2$.

Table 1. A typical purification of anthranilate synthase from *Ailanthus altissima* cell suspension cultures

Purification step	Total Protein (mg)	Total Activity (pkat)	Specific Activity (Pkat (mg protein)$^{-1}$)	Yield (%)	Purification (Fold)
Crude extract	36.5	2540	69.6	100	–
PEG	7.08	119	16.8	4.7	0.24
Q-Sepharose	3.90	2230	572	87.9	8.22
Mono-Q					
AS-a	0.56	1100	1955	43.1	28.1
AS-b	0.31	955	3080		

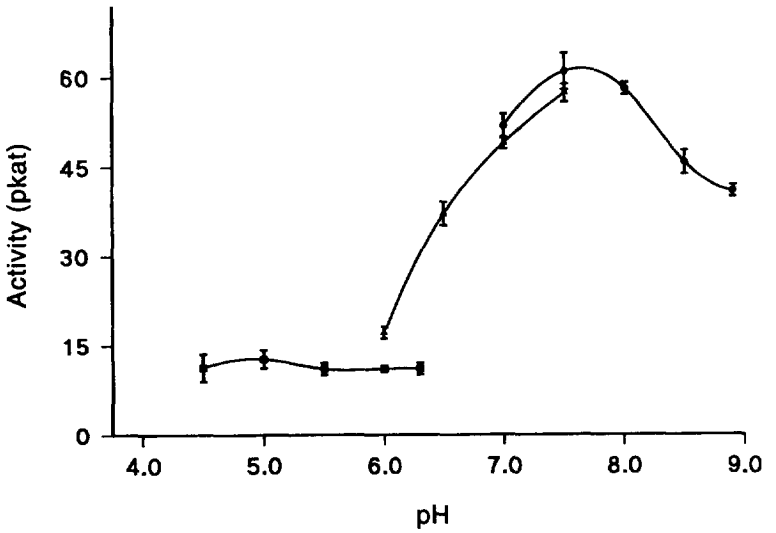

Fig. 4. Anthranilate synthase-b activity in response to changes in pH. The assay method for anthranilate synthase is as given in Experimental. bBuffers used were citrate, —■—; phosphate, —▲—; TRIS, —●—.

The effect of inhibitors on enzyme activity

The activity of AS-b was measured at various concentrations of chorismate and L-tryptophan. The presence of L-tryptophan caused a change in the curves (Fig. 5) and when the reciprocal activity was plotted against tryptophan concentration, a concave upward curvature was observed. These results were used to obtain the inhibition constant, K_i which, for AS-b, was 1.5 μM. AS-b was also inhibited by D-tryptophan and tryptamine. A concentration of 100 μM D-tryptophan inhibited the enzyme by 38%, whereas 100 μM tryptamine inhibited the AS-b activity by 27%. When L-tryptophan was used to inhibit AS-b, 20 μM resulted in 97% inhibition whereas AS-a, when present, was inhibited by only 66%.

DISCUSSION

Ailanthus altissima cell suspension cultures utilize large quantities of L-tryptophan for the production of canthin-6-one alkaloids. The formation of these alkaloids constitutes most of the biosynthetic activity at the secondary metabolism level. The activities of the enzymes (AS, CM or ICS) which compete for chorismate, the precursor of tryptophan, were examined during the cell growth cycle of suspension cultures of *A. altissima*. No isochorismate synthase activity was detected in the cultures used in these experiments. Because no secondary metabolites requiring isochorismate such as anthraquinones and phylloquinone have been detected either in cell cultures or in *A. altissima* plants, and as it is not known whether phylloquinone occurs as a vitamin in cell suspension cultures [17], the lack of ICS activity in the cultures can be rationalized. Another possibility is that ICS activity was below the detection limit of the assay, which is given as 5 pmol [18]. Anthranilate synthase activity was detected throughout the cell growth cycle, with a maximum between days 8 and 17. This increased activity correlates with the increase in alkaloid level in cell suspension cultures after day 20 [16]. However, CM activity, which

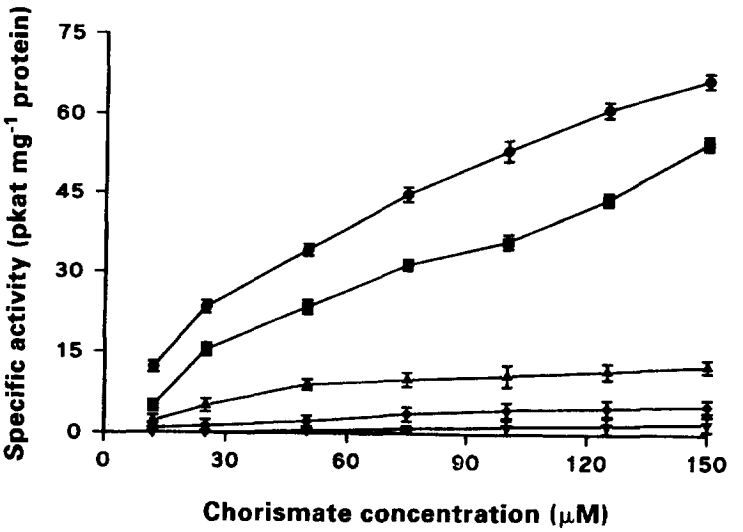

Fig. 5. Tryptophan inhibition: anthranilate synthase-b at different chorismate and tryptophan concentrations. Experiments used protein fractions isolated from the Mono-Q column using the assay method for anthranilate synthase given in the Experimental. ●0 μM; ■, 2.5 μM;▲, 5 μM;◆, 10 μM;▼, 20 μM.

was also active throughout the cell growth cycle, increased after day 20, the activity of this enzyme being 150 times the activity of the AS enzyme. In previous work production of the major coumarin, scopoletin, reached a maximum level at day 12 and isofraxidine production reached a maximum at day 28 [16].

The activities of AS and CM have been examined in several plant cell cultures. In all cases the activity of CM was measured in nkat mg^{-1} protein whereas AS activity was measured in pkat mg^{-1} protein. In a comparison of the specific activities of these two enzymes from cell suspension cultures of *Catharanthus roseus*, *Tabernaemontana divaricata*, *T. pandacaqui*, *Cinchona robusta*, *Rubia tinctorum* and *Euonymus europaeus*, the specific activity of CM was up to 1000-fold higher than the specific AS activity [16]. The enzymes of the phenylalanine pathway are very active, for example, in *Cinchona robusta*. However in *Catharanthus roseus* cultures, where CM activity was by comparison very low, other enzymes of the phenylalanine pathway also have reduced activity. The activity of AS in plants is low compared with that of microorganisms. Neglecting the differences in assay conditions, the specific activities of AS from plants have been reported to range between 0.1 pkat mg^{-1} protein in cell suspension cultures of *Tabernaemontana divaricata* [19] and 33–150 pkat mg^{-1} protein in callus tissue of *Nicotiana tabacum* [20]. In microorganisms the reported AS activities range from 12 pkat mg^{-1} protein in *Euglena gracilis* [21] to 1830 pkat mg^{-1} protein in *Serratia marcescens* [22]. The values for the specific activities of 270 pkat mg^{-1} protein for crude extract of callus tissue and 70 pkat mg^{-1} protein for crude extracts of cell suspension cultures of *A. altissima* are high in comparison with other plants. Two peaks of activity for anthranilate synthase activity were detected on the Mono-Q column. Isoenzyme AS-b was always present; however, the occurrence of AS-a was variable. The fact that AS-a was not always present, might be due to instability and decomposition during the isolation process.

AS-b was found to operate optimally at a pH between 7.3 and 8, and at a temperature of 35°, although 30° was routinely used in assays to assist comparison with other investigations [17]. The optimal pH for AS activity (glutamine dependent) in *Catharanthus roseus* was between 7.5 and 8.3 [10]. For *R. graveolens* AS, an optimum reaction temperature between 37° and 40° was reported although the value for the optimum pH was not given [23].

Comparison of the *A. altissima* AS K_m values of 72 μM, 50 μM and 180 μM, for chorismate, L-glutamine and Mg^{2+} respectively with AS from other plant sources shows some interesting differences in the affinity of AS for these substrates. For example for *Catharanthus roseus* AS, the K_m values are 67 μM, 370 μM, and 260 μM for chorismate, L-glutamine and the cofactor Mg^{2+}, respectively. For AS from two cell lines of *Ruta graveolens* K_m values are 220 μM and 280 μM for chorismate, 250 μM and 180 μM for glutamine, and 600 μM and 550 μM for Mg^{2+} [23]. For *Daucus carota* normal cells and cells resistant to growth inhibition by D, L-5-methyltryptophan K_m values are 46 μM and 26 μM for chorismate, 249 μM and 237 μM for L-glutamine and 101 μM and 26 μM for Mg^{2+} [24].

SDS-PAGE electrophoresis of *A. altissima* protein fractions from the Mono-Q column showed two bands corresponding to a M_r of 60 000 k and 37 000. Based on these results an estimated M_r value for the dimer AS-b is 97 000 which is much larger than the M_r value of 77 000 obtained from gel filtration. In *Catharanthus roseus* the M_r is 143 000 k measured by gel filtration, but by SDS–PAGE it was 184 000. In this case the explanation

given was that the native AS would be a very compact molecule giving a smaller Stokes radius than that which would be expected from the sum of the subunits [10]. In other plants M_r values for AS vary from 95 300 k in *Pisum sativum* to 220 000 for *R. graveolens* [23]. In *Arabidopsis thaliana*, genes were isolated encoding an α-subunit of 66 000 and a β-subunit of 30 000 [14, 15].

The tryptophan biosynthetic pathway was first elucidated with microorganisms and the available information indicates that tryptophan is produced from chorismate in plants in the same way. In most of the plants and microorganisms studied, the enzyme was found to be feedback-inhibited by L-tryptophan. There are, however, some exceptions. In addition to L-tryptophan, the enzyme can be inhibited by other tryptophan analogues such as 5-methyltryptophan [25] and 5-hydroxytryptamine [26]. The two AS isoenzymes detected in *A. altissima* were inhibited by 20 mM tryptophan, but at different levels: 66% inhibition was obtained for AS-a and 97% inhibition for AS-b. AS-b was also inhibited by tryptamine and D-tryptophan and was not affected by L-phenylalanine, L-tyrosine and indole acetic acid. Other enzymes from plants have shown similar inhibition effects with these compounds; for example AS from *Catharanthus roseus* [10].

Two peaks of CM were detected when the enzyme extract was eluted from a Q-Sepharose column. Chorismate mutase was not, however, characterized in this study.

The two AS enzymes described here showed different levels of regulation by L-tryptophan. The presence of two isoenzymes has been detected in other plants. Two AS isoforms, one feedback-sensitive and the other feedback-resistant to tryptophan were separated from *Solanum tuberosum* cell suspension cultures which were resistant to growth inhibition by D, L-5-methyltryptophan [28]. Also in *N. tabacum*, a tryptophan-sensitive AS isoenzyme was found in the organelle fraction, while a tryptophan-resistant AS isoenzyme occurs in the cytosol [29, 30]. The presence of a complete pathway in plastids has been well investigated using isolated chloroplasts [31–33]; however, it was found that the isolated chloroplasts could not account for the total production of aromatic amino acids, suggesting that the balance of production comes from the cytoplasm [34]. Further evidence for the cytosolic compartmentation comes with the finding of two isoforms of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and CM in several plants species [26, 35, 36]. It has been proposed that each pathway, the plastidic and the cytosolic, has a specialized role [37, 38]. The rationale for the hypothesis that the chloroplastic pathway is for amino acid synthesis, and the cytosolic is for support of the chloroplastic pathway and for synthesis of secondary products, is that the chloroplastic isoenzymes of DAHP and CM are regulated by the aromatic amino acids, while the cytosolic forms are either unregulated or are inhibited by caffeic acid [39, 40]. The presence of two isoenzymes for both AS and CM in *A. altissima*, may support the hypothesis of the two aromatic amino acid pathways, once the location of the enzymes is determined. The fact that the two AS showed different regulation by tryptophan gives evidence of this possibility. In some instances, for example in *Arabidopsis*, the AS α-subunit to both isoenzymes have putative chloroplast transit peptides at their amino-terminal end and conserved amino acids involved in feedback inhibition by tryptophan [14, 15]. In *Arabidopsis* no differences were found between the components of isoenzymic systems, and the existence of the two amino acid pathways must ultimately be based on the location of the enzymes and their genes.

EXPERIMENTAL

Plant material. Cell suspension cultures of *A. altissima* (Mill.) Swingle were maintained in M&S medium (Imperial Labs, U.K.) containing 1 mg l^{-1} of 2,4-D (Sigma), 0.1 mg l^{-1} of kinetin (Flow Laboratories) and 5% sucrose under continuous illumination at 25°. These were the standard medium and growing conditions for all cell culture experiments described below.

Enzyme assays. The assays for AS and ICS were taken from refs [17, 19], but with a modification which used an incubation mixture of 250 μl instead of 500 μl. CM was assayed according to [41] and [16], except that a saturated soln of EtONa was substituted for the NaOH soln because a ppt formed with NaOH.

Protein. Measured according to [42] using BSA and the microwell technique recommended by Sigma.

Time-course study. Cell suspension cultures of *A. altissima* were filtered aseptically and 3 g fr. wt inoculated into each of 30 flasks containing 40 ml M&S medium with hormones and sucrose. Triplicate samples were harvested by filtration under vacuum every third day throughout the growth cycle and the final fr. wt of each was determined. The harvested cells were immediately frozen in liquid N_2. Extraction buffer: 0.1 M Tris–HCl at pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM leupeptin, 0.2 mM phenylmethylsulphonylfluoride, 10 mM L-glutamine. This buffer was used for all enzyme isolations. When a sample was to be assayed, for each g fresh wt, 0.1 g of polyvinylpyrrolidone (PVP) and 1 ml of extraction buffer were added to the frozen cells. After thawing, the mixture was centrifuged at 12 000 *g* for 25 min. The supernatant was desalted on Sephadex G-25 (Pharmacia PD-10 columns) previously equilibrated with the extraction buffer. The activity of the enzymes AS, CM and ICS was measured in the desalted supernatant.

Purification of anthranilate synthase. A. altissima cell suspension cultures were harvested 10 days after subculturing and isolated by filtration under vacuum. The cells were immediately frozen in liquid N_2 and homogenized in a coffee grinder (Moulinex) for 30 sec. After this all operations were conducted at 4°. Enzyme was extracted as described in the time-course study. After centrifugation at 12 000 *g*, finely ground PEG 6000 was added slowly to a final concn at 10% (w/v). while stirring. After the addition of PEG, the soln was stirred for an additional 25 min, and the soln then centrifuged, 12 000 *g*

30 min. To the supernatant additional PEG was added to give a final concn of 18% and after stirring for 25 min, the pptd protein was obtained by centrifugation under the same conditions as before. The pellet was dissolved in extraction buffer and centrifuged for 25 min at 12000 *g* and the supernatant was applied to a column that had been packed with Q-Sepharose (2.7 × 9.9 cm) and equilibrated with extraction buffer. The column, linked to a Pharmacia FPLC system, was eluted at 1 ml min^{-1} with 120 ml of buffer followed by a 50-ml linear NaCl gradient, 0–0.2 M in the same buffer. The column was then eluted with 25 ml 0.2 M NaCl and finally the linear salt gradient was continued with 50 ml to 1 M NaCl; 3-ml frs were collected with an automated fr. collector. and the proteins eluted from the column were detected with a detector at 280 nm linked to a chart recorder. The AS active fractions were desalted on Sephadex PD-10 columns equilibrated with the extraction buffer. The desalted fractions were loaded onto a Mono Q HR 5/5 column (Pharmacia), previously equilibrated with buffer containing: 0.1 M Tris–HCl, pH 7.5, 10% glycerol, 10 mM L-glutamine 10 mM leupeptin and 1 mM DTT. The column was eluted at 0.5 ml min^{-1} with 5 ml of the same buffer, followed by an 11 ml linear NaCl gradient in the same buffer from 0 to 0.15 M. Further elution of 5 ml at 0.15 M NaCl was followed by a continuation of 5 ml of the linear salt gradient to 0.2 M NaCl. A further 5 ml elution at 0.2 M NaCl was followed by a continuation of 9 ml of the linear salt gradient to 1 M NaCl. Fractions of 0.5 ml were collected. The proteins eluted from the column were monitored using the same detection system described for the Q-Sepharose column.

M_r determination. Gel filtration method. The Pharmacia Superose 12 HR 10/30 column was calibrated with the following proteins; carbonic anhydrase (29000), bovine albumin (66000), alcohol dehydrogenase (150000), β-amylase (200000) and apoferritin (443000). For M_r determination, a 200 μl sample of protein from the previous separation on the Mono-Q column was loaded and eluted with a buffer containing 0.1 M Tris-HCl, pH 7.5, 10% glycerol, 10 mM L-glutamine and 1 mM DTT.

SDS–PAGE gel electrophoresis method. Electrophoresis was performed by the method of [43] using polyacrylamide minigels (7.5%, 1 mm thick). The gel was run on a vertical electrophoresis unit with a constant current of 40 mA at 21°. Gel running buffer 25 mM Tris–HCl, 192 mM glycine and 0.1% SDS at pH 8.3. The gel was developed in about 1 hr and was stained with Coomassie brilliant blue. The M_r markers α_2-macroglobulin (190000), β-galactosidase (125000), fructose-6-phosphate kinase (88000), pyruvate kinase (65000), fumarase (56000), lactic dehydrogenase (38000) and triosephosphate isomarase (33500).

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