Detection and subcellular localization of a higher plant chorismate synthase

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Chorismate synthase (EC 4.6.1.4) was detected in tissue extracts and chloroplast preparations of pea (*Pisum sativum* L.) in the presence of reduced flavin generated by dithionite. Chorismate synthase and anthranilate synthase were present in intact chloroplasts isolated by density gradient centrifugation.

Chorismate synthase *Anthranilate synthase* *Shikimate pathway* *Amino acid synthesis* *Chloroplast*
(Pisum sativum)

1. INTRODUCTION

Chorismate synthase (5-enolpyruvylshikimate 3-phosphate phospho-lyase; EC 4.6.1.4) catalyzes the final step in the common shikimate (prechorismate) pathway of aromatic biosynthesis (fig.1). The enzyme has been characterized from three microbial sources. In *Neurospora crassa* chorismate synthase is present in a bifunctional enzyme together with a diaphorase activity that reduces FMN in the presence of NADPH [1,2]. In *Bacillus subtilis* chorismate synthase is associated with a similar but separable NADPH-dependent flavin reductase and with a third polypeptide which, when complexed with chorismate synthase, catalyzes the 3-dehydroquinate (DHQ) synthase (EC 4.6.1.3) reaction [3–5]. In both these species the chorismate synthase is readily assayed under aerobic conditions with FMN and NADPH as cofactors. The chorismate synthase partially purified from *Escherichia coli* could, however, only be assayed under strictly anaerobic conditions in the presence of chemically or enzymatically reduced flavin or if the enzyme were treated with dithionite or H_2/platinum [6]. The chorismate synthase of *Klebsiella pneumoniae* was briefly described as being active in the presence of added NADH [7].

No report has appeared of chorismate synthase in any plant species [8]. Its participation in plant aromatic biosynthesis is presumed because of the presence of chorismate-utilizing enzymes such as chorismate mutase and anthranilate synthase [8]. We describe here the detection of an 'anaerobic' chorismate synthase in pea seedlings which is at least partially chloroplastic.

Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHQ, 3-dehydroquinate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane

2. MATERIALS AND METHODS

2.1. *Plant material*

Seeds of *Pisum sativum* L. cv 'Onward' were germinated and grown as described [9]. Shoot tissue was used from seedlings 9–12 days after germination.

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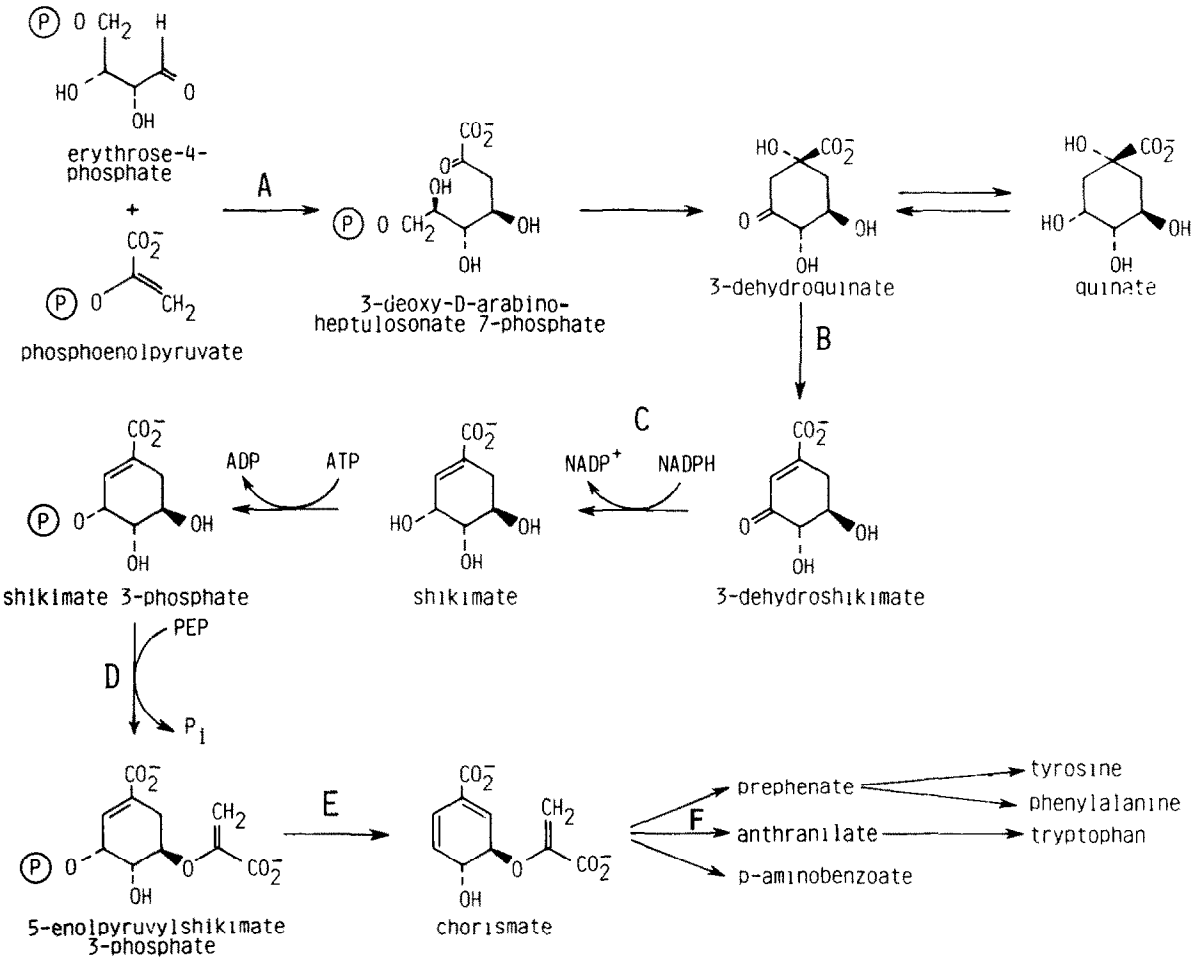

Fig.1. The shikimate pathway in plants and micro-organisms. The lettered steps are those catalyzed by enzymes mentioned in the text: (A) DAHP synthase; (B) DHQ dehydratase; (C) shikimate:$NADP^+$ oxidoreductase; (D) EPSP synthase; (E) chorismate synthase; (F) anthranilate synthase.

2.2. *Enzyme extraction and chloroplast preparation*

Seedling tissue extracts were prepared by homogenization in 2 ml/g fresh wt 50 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 1 mM EDTA and 1 mM benzamidine hydrochloride (buffer A) containing 1 mM phenylmethylsulphonyl chloride. The extract was filtered through two layers of cheesecloth and centrifuged at 80000 × *g* for 1 h at 4°C.

Chloroplasts were prepared by the method of Nakatani and Barber [10]. Washed chloroplast were lysed by dilution into 5–10 ml buffer A and centrifuged at 100000 × *g* for 1 h at 4°C. Prior to density gradient analysis washed chloroplasts were resuspended in 5 ml of 0.33 M sorbitol (pH 7.8 with Tris) and applied to a 30 ml continuous gradient of 10–70% (v/v) Percoll (Pharmacia, Milton Keynes, England) in 20 mM Tricine-KOH (pH 7.5), 0.25 M sucrose. The gradient was centrifuged at 7000 × *g* for 15 min at 4°C.

2.3. *Enzyme assays*

Chorismate synthase was assayed fluorimetrically using forward-coupling to anthranilate synthase [1,11]. The assay mixture contained in a final volume of 3 ml: 200 μM EPSP, 10 μM FMN, 10 mM L-glutamine, 50 mM KCl, 5 mM $MgCl_2$,

50 mM Bis-Tris-HCl (pH 7.0) and 60 pkat/ml partially purified *N. crassa* anthranilate synthase with no detectable chorismate synthase activity [12]. Sufficient solid sodium dithionite was carefully added to decolourize the (initially yellow) complete reaction mixture containing the sample (30 μl) to be assayed. The mixture was incubated at 25°C for 30 min and the reaction then terminated by the addition of 50 μl of 6 M HCl. The anthranilate formed was extracted with 3 ml ethyl acetate and the aqueous and organic layers separated by a brief centrifugation. The fluorescence (λ_{ex} 315 nm, λ_{em} 390 nm) of the ethyl acetate layer was measured with a Hitachi-Perkin Elmer model MPF-2A spectrofluorimeter. Assay blanks lacked substrate.

Anthranilate synthase (EC 4.1.3.27) was assayed in a continuous fluorimetric assay [12]. The assay contained in final volume of 3 ml: 100 μM chorismate (to initiate assay), 10 mM L-glutamine, 50 mM KCl, 5 mM $MgCl_2$ and 50 mM Bis-Tris-HCl (pH 7.0). EPSP synthase (EC 2.5.1.19), DHQ dehydratase (EC 4.2.1.10), shikimate:$NADP^+$ oxidoreductase (EC 1.1.1.25), cytochrome-*c* oxidase (EC 1.9.3.1) and catalase (EC 1.11.1.6) were assayed as described in [9,13].

2.4. *Protein and chlorophyll determinations*

Protein was determined using the Coomassie dye binding assay [14] with bovine serum albumin (BSA) as protein standard. Total chlorophyll was measured spectrophotometrically [15].

3. RESULTS

No chorismate synthase could be detected in pea shoot extracts or chloroplast lysates using aerobic assay conditions suitable for the assay of *N. crassa* chorismate synthase [1,11,12]:

(i) by monitoring chorismate formation spectrophotometrically at 275 nm from 20–200 μM EPSP in the presence of 10 μM FMN or FAD and 20 μM NAD(P)H;
(ii) by monitoring anthranilate formation fluorimetrically in the presence of EPSP, FMN or FAD, NAD(P)H, glutamine and an excess of *N. crassa* anthranilate synthase.

The inclusion of up to 1 mg/ml BSA in the assay mixture (as required for the chorismate synthase from *B. subtilis* [3]) and 0.1 mM dithiothreitol (required for maximal activity with *E. coli* chorismate synthase [6]) failed to stimulate activity.

In vitro chorismate synthase activity was observed when reduced flavin was generated by the addition of dithionite (table 1). The EPSP-dependent anthranilate formation in the coupled assay system was dependent on the presence of FMN, glutamine and exogenous anthranilate synthase. The levels of chorismate synthase measured were comparable to those found for EPSP synthase, the previous enzyme in the shikimate pathway (table 1 and [9,13]).

Chorismate synthase was present together with other shikimate pathway enzyme activities at a higher specific activity in chloroplast lysate preparations, suggesting an association with plastids (table 1). By density gradient analysis chorismate synthase was found in fractions containing intact chloroplasts that were contaminated with only trace amounts of mitochondrial and peroxisomal enzyme markers (fig.2).

Anthranilate synthase, the initial enzyme in the pathway from chorismate to tryptophan, was also present in chloroplast lysates and intact chloroplasts (table 1 and fig.1). This enzyme has been found associated with etioplasts from pea seedlings [16] and with leucoplasts from *Juglans regia* seeds [17] but has not previously been described as chloroplastic. The levels of anthranilate synthase in tissue extracts and chloroplast lysates were approx. 10% of the levels of chorismate synthase (table 1). Similar low levels of anthranilate synthase were quantified in extracts

Table 1

Shikimate pathway enzyme levels in extracts and chloroplast lysates from pea seedling shoot tissue

Enzyme	Specific activity (nkat/mg protein)	
	Shoot extract	Chloroplast lysate
Chorismate synthase	0.011	0.060
Anthranilate synthase	0.001	0.007
EPSP synthase	0.043	0.087
DHQ dehydratase	0.094	0.188
Shikimate:$NADP^+$ oxidoreductase	0.984	1.85

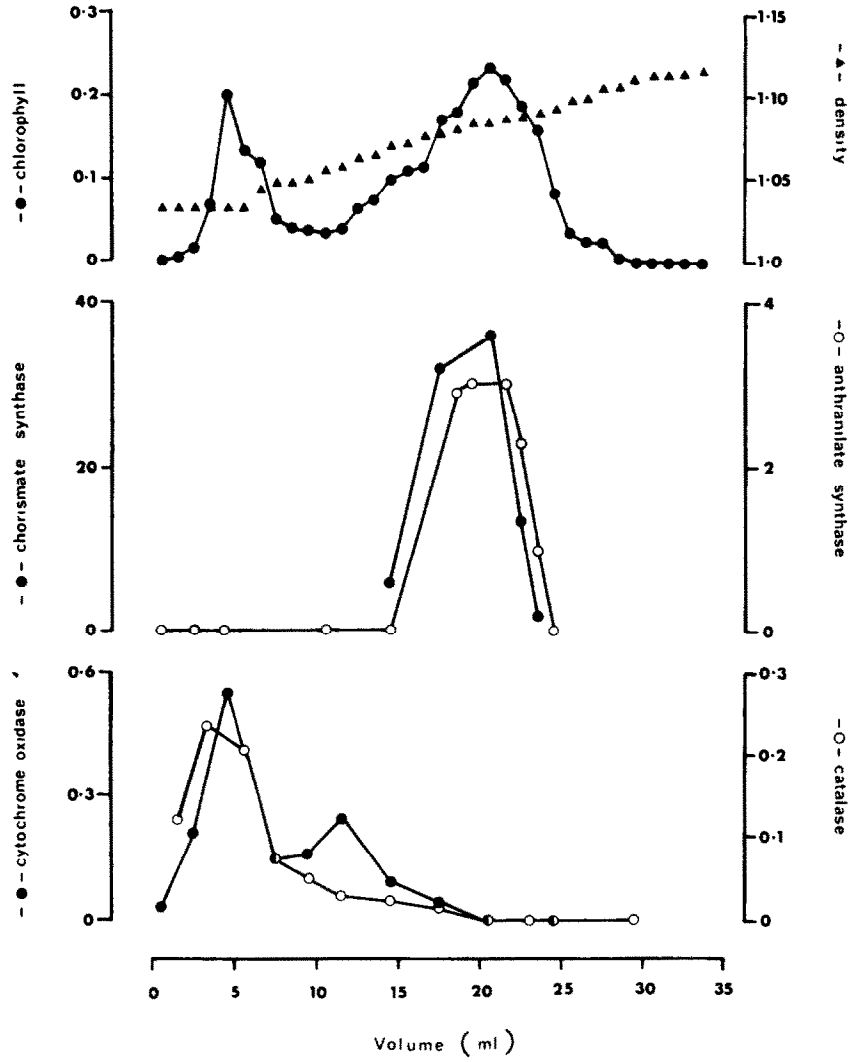

Fig.2. Percoll density gradient analysis of washed chloroplast fraction from pea shoot tissue. Units: chlorophyll (mg/ml), density (g/ml), chorismate synthase and anthranilate synthase (pkat/ml), cytochrome oxidase (nkat/ml), catalase (μkat/ml).

of pea embryos (6.3 pkat/mg protein [18]) and higher plant cell cultures (0.1–14.8 pkat/mg [19–22]).

4. DISCUSSION

The conditions required for the assay of chorismate synthase from the four microbial sources [1–3,6,7] and pea seedlings indicate the reduction of a redox site in the enzyme (which has been suggested to be either Fe(III) [6] or a disulphide bond [1]) as a prerequisite for enzyme activity. Most of the available evidence supports a model involving the participation of reduced flavin and a diaphorase-like second enzymic activity [1–3,6,7]. The lack of chorismate synthase activity in *E. coli* and pea tissue extracts under aerobic conditions implies either that the putative diaphorases are easily inactivated or that the correct redox cofactors have not been defined. The chloroplastic localization of plant chorismate synthases may for example implicate a photosynthetic electron

transport chain component as an alternative electron donor in the general sequence: NAD(P)H–flavin–enzyme. The activation of the *N. crassa* and *E. coli* chorismate synthases by dithionite alone in the absence of exogenous flavin [1,6] is as yet unexplained but does not preclude the presence of enzyme-bound flavin [6]. Long lag times (25–30 min) were observed for dithionite-activation [1,6] and this aspect has not been investigated with the pea seedling enzyme.

The association of chorismate synthase and anthranilate synthase (fig.2), DAHP synthase, DHQ dehydratase, shikimate:$NADP^+$ oxidoreductase and EPSP synthase [13] with intact chloroplasts and the incorporation of $^{14}CO_2$ into aromatic amino acids by isolated chloroplasts [23] provide strong evidence for the presence of a complete plastidic shikimate pathway. An apparent paradox is that there is no obvious source for the phospho*enol*pyruvate required in the DAHP synthase and EPSP synthase steps since pea chloroplasts do not contain a functional glycolytic pathway because of the absence of phosphoglyceromutase [24] and phospho*enol*pyruvate shows only minimal uptake by chloroplasts [25]. Chloroplasts do however contain phospho*enol*-pyruvate hydratase and pyruvate kinase [24] which suggests that plastidic phospho*enol*pyruvate can be derived from pyruvate and/or 2-phosphoglycerate transported from the cytosol [25]. These transport processes represent potential means of regulation of metabolic flux through the shikimate pathway. A more direct control, feedback inhibition by tryptophan, has been observed at an undefined site between shikimate and chorismate [23]. We are currently attempting to purify pea seedling chorismate synthase in order to characterise its cofactor requirements and regulatory properties.

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