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PURIFICATION AND CHARACTERIZATION OF BIFUNCTIONAL DEHYDROQUINASE-SHIKIMATE: NADP OXIDOREDUCTASE FROM PEA SEEDLINGS

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Key Word Index—*Pisum sativum*: Leguminosae; pea; shikimate dehydrogenase; dehydroquinase; shikimate pathway.

Abstract—The bifunctional enzyme dehydroquinase (DHQase, EC 4.2.1.10)-shikimate: NADP oxidoreductase (SHORase, EC 1.1.1.25) has been purified 6500-fold to homogeneity from *Pisum sativum* shoot tissue. A rapid purification procedure using high performance liquid chromatography was used to isolate the enzyme from chloroplast preparations. The purified enzyme is monomeric with M_r 59 000. Chromatofocusing separates three isoenzymes, two of which are chloroplastic. DHQase and SHORase (forward reaction) show pH optima at pH 7 and apparent K_m values of 2.7×10^{-5} M (dehydroquinate), 2.1×10^{-4} M (dehydroshikimate) and 1.5×10^{-5} M (NADPH). Chloride is a competitive inhibitor of DHQase. The SHORase reaction has an ordered (sequential) kinetic mechanism and is unaffected by the presence of DHQ.

INTRODUCTION

The central steps of the common shikimate (prechorismate) pathway of aromatic biosynthesis in plants interconnect with the pathways of quinate metabolism and the formation of gallic and protocatechuic acids [1]. This area of alicyclic and aromatic metabolism is of considerable phytochemical interest since large endogenous pools of quinate and its conjugates and derivatives of gallic acid occur widely among higher plant species [1]. Shikimate itself is accumulated comparatively rarely [2–4] and the biochemical regulation of these competing processes of accumulation and primary and secondary biosynthesis is largely unexplored. Bifunctional enzymes containing the sequential shikimate pathway activities DHQase and SHORase have been isolated from the moss *Physcomitrella patens* [5] and chloroplasts of spinach (*Spinacia oleracea*) [6] and there is evidence that this association occurs generally in plants [7–9]. In *Zea mays* a similar bifunctional enzyme combining DHQase and quinate: NAD oxidoreductase has been reported [10].

SHORase has been partially purified from a variety of plant species for kinetic investigations [9, 11–20]. Both SHORase and DHQase are associated with chloroplasts [6, 21], although an extraplastidic shikimate pathway using isoenzymes has been suggested [22, 23]. We have therefore purified the DHQase–SHORase from pea seedling shoot tissue and chloroplasts to study its kinetic and molecular properties and its occurrence as isoenzymes.

RESULTS

Enzyme purification

Extracts of pea seedling shoot tissue after filtration and centrifugation contained SHORase (routinely assayed in the reverse direction as shikimate dehydrogenase) at 0.7–1.8 nkat/mg protein (6.6–12.2 nkat/g FW) and DHQase at 0.1–0.2 nkat/mg protein (0.7–1.1 nkat/g FW). The mean ratio SHORase:DHQase was 10.6 (s.d. 1.4, $n = 8$). Both activities purified at a constant ratio; a representative purification is given in Table 1. The individual steps were: ammonium sulphate precipitation, ion exchange chromatography on DEAE-Sephacel, affinity chromatography on ADP-Sepharose, hydroxylapatite chromatography, and high performance anion-exchange and gel permeation chromatography. After the final step the SHORase and DHQase activities had been purified by more than 6500-fold.

Molecular properties

That the purified SHORase and DHQase activities were contained in a single bifunctional enzyme was shown by the presence of only one polypeptide visualized by silver staining of polyacrylamide gels in the presence of SDS (Fig. 1) and the presence of one protein peak during high performance gel filtration (Fig. 1). The subunit M_r (electrophoresis) was $59\,000 \pm 1000$ and the corresponding native M_r (gel filtration) was 50 000; the enzyme is therefore monomeric. Gel filtration estimates of the M_r of SHORase from seven higher plant species were in the range 52 000–73 000 [6–9, 15, 20].

Kinetic properties

Apparent K_m values deduced from steady-state kinetic analyses with the same preparation of the purified DHQase–SHORase are given in Table 2. The values for SHORase at pH 7 are very similar to the limiting K_m values obtained with partially purified pea enzyme [17]. Dowsett *et al.* [18] found a much higher K_m for NADPH (2.8×10^{-4} M at pH 8.0), which may however have reflected the presence of NADPH-utilizing activities in the partially purified SHORase from pea epicotyl. The K_m for

Table 1. Purification of DHQase-SHORase from pea shoot extract

Step	Volume (ml)	Protein (mg)	SHORase (nkat)	SHORase (nkat/mg)	DHQase (nkat)	DHQase (nkat/mg)	SHORase* / DHQase	Recovery (%)	Purification (fold)
1. 80000 *g* centrifugation	425	1530	1471	0.96	144.5	0.094	10.2* (9.8–11.4)	100†	1†
2. $(NH_4)_2SO_4$	55	506	1331	2.63	134.8	0.266	9.9 (8.2–12.3)	90.5	2.7
3. DEAE-Sephacel	110	66	757	11.5	73.7	1.12	10.3 (10.0–10.9)	51.5	12
4. ADP-Sepharose	22.5	3.8	621	163	54.0	14.2	11.5 (10.8–12.8)	42.2	170
5. Hydroxyl-lapatite	15	1.7	389	229	39.8	23.4	9.8 (9.2–10.6)	26.4	238
6. Mono-Q	1.0	0.08	192	2400	22.6	283	8.5 (8.1–9.4)	13.1	2500
7. TSK G2000 SW	2.0	0.014	90.2	6443	9.4	686	9.4 (8.2–10.7)	6.1	6711

* Mean ratio derived from triplicate determinations (values in parentheses represent the maximum and minimum derivable values).
† For SHORase.

dehydroshikimate (DHS) was considerably higher than that for DHQ (Table 2); the presence of 2.0×10^{-4} M DHQ did not affect the K_m for DHS. Shikimate exhibited a similarly high K_m (Table 2); analyses of SHORase from plant sources at pH 7.4–9.9 have given a wide range of K_m values but with a mean of over 3.5×10^{-4} M [9, 11–20, 24, 25].

Kinetic analyses of the forward SHORase reaction at varying concentrations of substrate and coenzyme indicated an ordered (sequential) mechanism. This is consistent with the proposed mechanism from kinetic and isotopic exchange studies in which the coenzyme binds to the enzyme in advance of the substrate [17, 18]. Both the forward SHORase reaction and DHQase showed pH optima at pH 7.0 (phosphate buffer) whereas the shikimate dehydrogenase reaction was optimally active at pH 10.0 (carbonate/bicarbonate buffer). Chloride and several organic acid anions inhibit the monofunctional DHQase of *Escherichia coli* and the DHQase activity of the *arom* pentafunctional enzyme from *Neurospora crassa* with K_i values $1.3–10.2 \times 10^{-2}$ M [26]. Chloride was a competitive inhibitor of the DHQase activity from pea tissue with a K_i of *ca* 5×10^{-2} M.

Chloroplastic and extrachloroplastic isoenzymes

Washed chloroplasts suspended in 0.33 M sorbitol exhibited highly latent SHORase and DHQase and other shikimate pathway activities. The ratio of enzyme activities in the absence to those in the presence of sorbitol was 21:1 (SORase), 53:1 (DHQase), 50:1 (EPSP synthase) and 30:1 (DAHP synthase), the lower values representing the limits of detection for the assays. This is the first report of latency in shikimate pathway enzymes and confirms their intraplastidic location as deduced from density gradient sedimentation studies [21].

DHQase–SHORase could be rapidly purified from the lysed chloroplasts by two consecutive HPLC steps. Active fractions from anion-exchange HPLC on Mono-Q material were diluted and chromatofocused on a Mono-P column (Fig. 2). The chromatofocusing step gave two peaks of DHQase–SHORase which when analysed by SDS-PAGE revealed a single polypeptide of M_r 59000 (Fig. 2). The entire purification procedure from the beginning of chloroplast preparation from shoot tissue to the completion of the chromatofocusing step could be completed within 6 hr.

Chromatofocusing of partially purified shoot extracts revealed three separable forms of DHQase–SHORase (Fig. 3). The two major isoenzymes were routinely separated (unresolved) from the minor isoenzyme by Mono-Q chromatography (step 6 in Table 1); chromatofocusing of the relevant fractions at this stage showed that the bulk of the SORase–DHQase is associated with the two higher-pI isoenzymes as shown in Fig. 2. The third minor isoenzyme with the lowest pI could not be purified to homogeneity; preparations with the highest specific activities contained a polypeptide with M_r 59000. A similar pattern of isoenzymes (three in leaf extracts, two of which were present in chloroplast preparations) was observed with spinach, whereas Fiedler and Schultz reported four forms resolved by gel electrophoresis, all of which were chloroplastic [6]. Since the compartmentation of SHORase is highly age-dependent and is also influenced by light regime and nutrient status [22], the isoenzyme content is probably determined by the precise developmental state of the plant tissue used as the enzyme source.

DISCUSSION

The M_r of the DHQase–SHORase purified from pea shoot tissue and chloroplasts is close to the sum of the

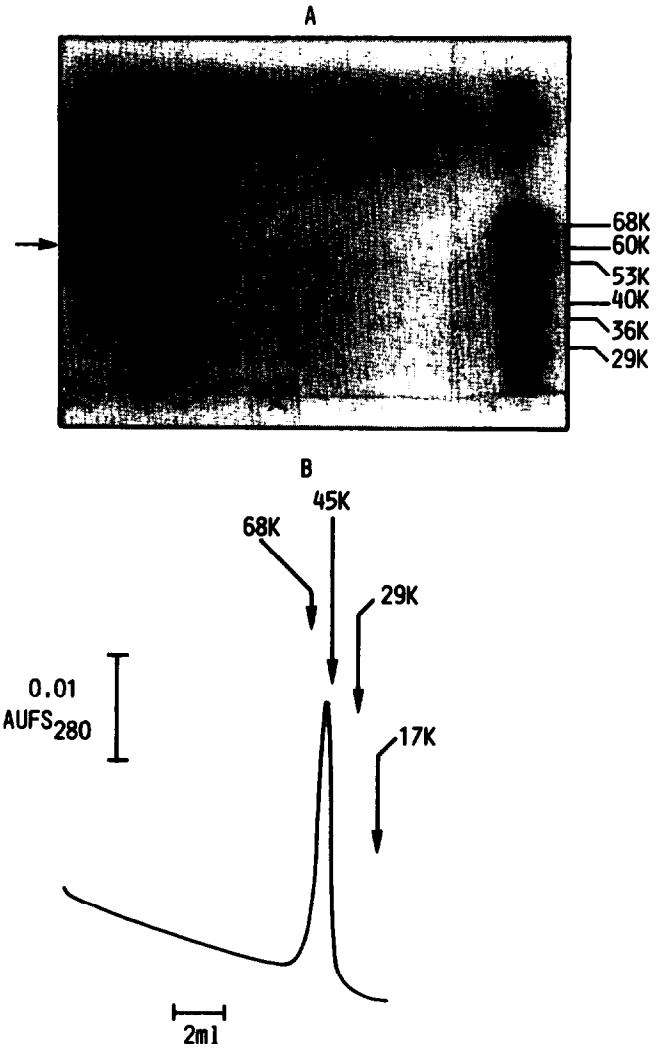

Fig. 1. (A): SDS-PAGE of purified DHQase–SHORase from pea shoot extract. 8 % acrylamide gel. Proteins stained with silver nitrate. The DHQase–SHORase band is marked with an arrow; diffuse bands at M_r 65 000 and 72 000 are artefacts which are also observed in the absence of protein samples. (B): High performance gel filtration of purified DHQase–SHOrase. Column: Superose 12.

Table 2. Apparent K_m values for DHQase-SHORase

Substrate	Co-substrate (mM)	pH	K_m (mM)
Shikimate	NADP (2.0)	10.6	1.5
NADP	Shikimate (4.0)	10.6	0.032
Shikimate	NADP (2.0)	7.0	0.69
NADP	Shikimate (4.0)	7.0	0.013
Dehydroshikimate	NADPH (0.35)	7.0	0.21
NADPH	Dehydroshikimate (0.9)	7.0	0.011
Dehydroquinate	—	7.0	0.027

subunit M_r values of the separable enzymes from *E. coli*: DHQase, dimeric with subunit M_r 29 000 [26, 27]; SHORase, monomeric, M_r 32 000 [28]. The pentafunctional *arom* polypeptide has been proteolytically cleaved to yield a fragment of M_r 68 000 containing the DHQase and SHORase activities [29] and this has been further reduced by protease action to a bifunctional fragment of M_r 63 000 [30]. These data are consistent with the hypothesis that the plant bifunctional enzyme and the pentafunctional *arom* enzyme evolved by the fusion of genes similar to those in *E. coli* [30]. The amino acid sequence of the pentafunctional *arom* enzymes from *Saccharomyces cerevisiae* and *Aspergillus nidulans* also show extensive homologies with those of the five corresponding *E. coli* enzymes [31–33].

The functional significance of the DHQase–SHORase association in plants is however not readily apparent. The individual activities are catalytically independent in that the pea DHQase has a similar K_m (2.7×10^{-5} M) to that

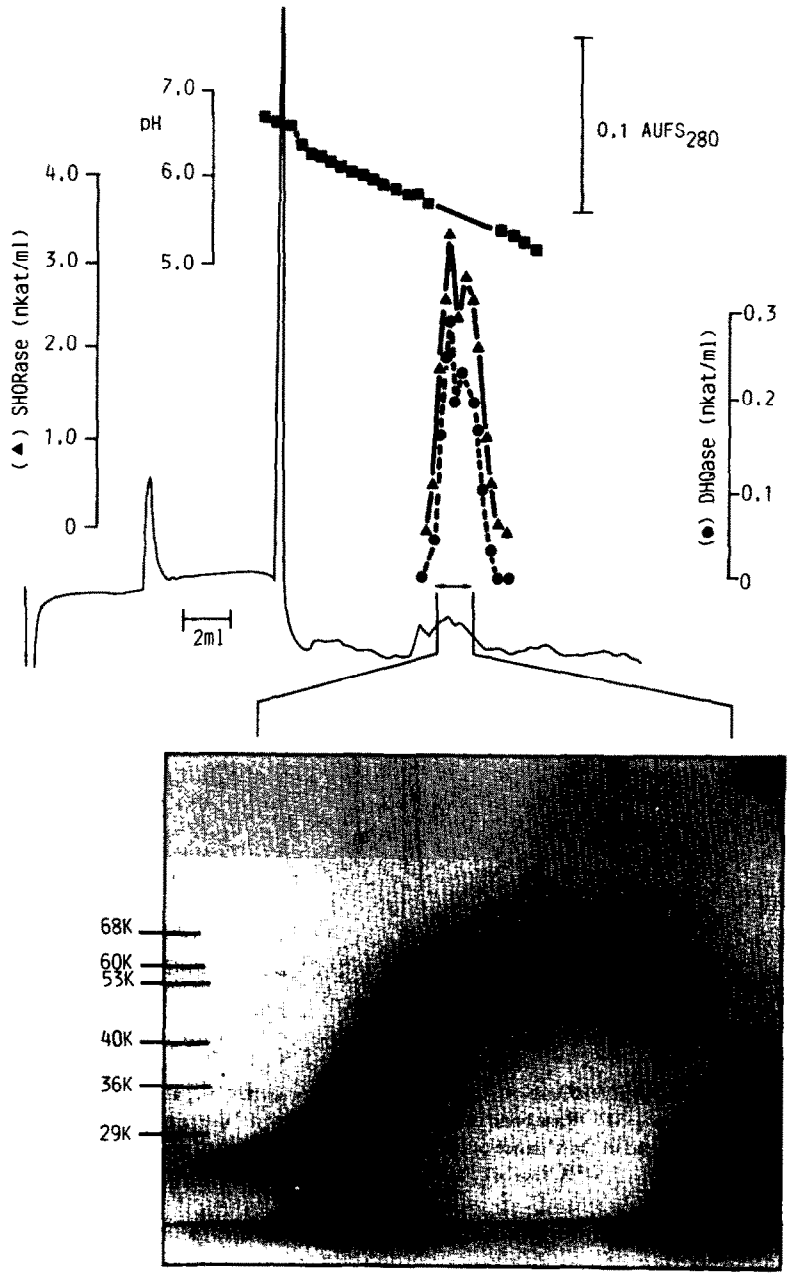

Fig. 2. (A): Chromatofocusing of partially-purified DHQase–SHORase from pea chloroplasts. Column: Mono-P. (B): SDS-PAGE of DHQase–SHORase fractions after chromatofocusing. 10% acrylamide gel. Proteins stained with silver nitrate

found for the *E. coli* monofunctional enzyme (1.8×10^{-5} M; [26]) and retains the sensitivity to inhibition by chloride; the forward reaction of pea SHORase is, furthermore, not affected by the presence of DHQ. In the absence of any regulatory effects idiosyncratic to the plant bifunctional enzyme, a consequence of a close proximity of the active sites could be that of protecting the common intermediate (DHS) from the action of competing enzymes; this phenomenon has been described as substrate channelling [34]. DHS can be converted by plant tissues and extracts to protocatechuic and gallic acids by direct aromatization via dehydration or dehydrogenation [35–39]. These reactions appear to be constitutive in higher plant species whereas in *N. crassa* quinate induces the formation of a catabolic pathway involving a monofunctional DHQase and DHS dehydratase [40]. In the bifunctional plant DHQase–SHORase the DHS may remain bound to the enzyme, equilibrating poorly with any pool(s) of free DHS, unless the metabolic flux through DHQase is high. A channelling effect has been demonstrated with two membrane-bound plant enzyme systems, phenylalanine ammonia lyase–cinnamate 4-hydroxylase [41–43] and the four-step conversion of tyrosine to 4-hydroxybenzaldehyde [44], and with the

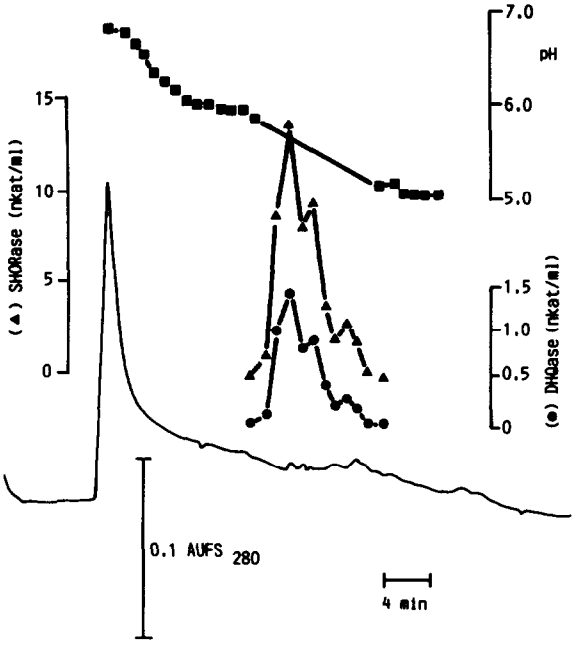

Fig. 3. Chromatofocusing of partially-purified DHQase–SHORase from pea shoot extract. *Ca* 250 μg of protein applied to Mono-P column after ADP-Sepharose step in purification procedure (*ca* 300-fold purified). Conditions as for Fig. 2.

partially purified enzyme catalysing the two-stage formation of (−)kaurene from *trans*-geranylgeranyl pyrophosphate [45]. In the pentafunctional *arom* enzyme from *N. crassa* free shikimate does not enhance the rate of the overall reaction from non-saturating levels of an earlier substrate, suggesting that a channelling of intermediates on the *arom* enzyme surface occurs [46].

EXPERIMENTAL

Plant material. Seeds of *Pisum sativum* L. cv Onward were germinated and grown as described previously [47]. Shoot tissue was taken from 9 to 12-day old plants.

Enzyme purification. All steps (unless otherwise noted) were performed at 4°. 250 g.fr.wt of shoot tissue was homogenized in 500 ml buffer A (50 mM Tris–HCl, pH 7.5, 0.4 mM DTT, 1 mM EDTA, 1 mM benzamide hydrochloride [BE] and 1 mM phenylmethylsulphonyl fluoride [PMSF]). The brei was filtered through muslin and centrifuged at 80 000 *g* for 1 hr. The supernatant was brought to 35% saturation with solid $(NH_4)_2SO_4$ and centrifuged (12 000 *g*, 20 min); the supernatant was then adjusted to 55% saturation and the pptd protein collected by centrifugation. The ppt. was dissolved in 50–75 ml buffer B (20 mM Tris–HCl, pH 7.5, 0.4 mM DTT, 1 mM BE) containing 1 mM PMSF, dialysed for 16 hr against 2 l. buffer B and applied to a 2.5 × 12 cm column of DEAE-Sephacel (Pharmacia Ltd., Milton Keynes, U.K.) equilibrated with buffer B. The column was washed with buffer B until the *A* of the eluate at 280 nm was less than 0.1 and eluted with a 200 ml linear gradient of 0–100 mM KCl in buffer B. Active fractions were combined, dialysed as before and applied to a 1.5 × 11.5 cm column of ADP-Sepharose (Pharmacia) equilibrated with buffer B. The column was washed with buffer B until the *A* was below 0.1 and then eluted with 0.2 mM NADPH in buffer B. Active fractions were combined, dialysed against 2 l buffer C (10 mM K-Pi, pH 7.0, 0.4 mM DTT, 1 mM BE) and applied to a 1 × 6.5 cm column of hydroxylapatite (Bio-Rad) equilibrated in buffer C. The column was washed with buffer C until the *A* was below 0.05 and then eluted with 0.1 M K-Pi, pH 7.0, 0.4 mM DTT, 1 mM BE. Active fractions were dialysed against buffer B and applied at room temp. to a HR5/5 Mono-Q column (Pharmacia). The column was eluted with a 20 ml linear gradient of 0–200 mM NaCl in buffer B using a Fast Protein Liquid Chromatography apparatus (Pharmacia); 0.33 ml fractions were collected. Chromatography on Mono-Q resolved major (90–95% of the activity) and minor peaks of DHQase–SHORase [21]. Active fractions corresponding to the major peak were combined and applied in 0.5 ml aliquots to a 0.75 × 60 cm column of TSK G2000 SW (LKB) at room temp. the mobile phase was 50 mM Tris–HCl, pH 6.9, 0.4 mM DTT, 1 mM EDTA, 1 mM BE at a flow rate of 0.2 ml/min provided by a model 303 pump (Gilson, France); 0.2 ml fractions were collected. After analysis by SDS-PAGE, active fractions were combined and concd by dialysis against 50% (v/v) glycerol in buffer A.

Purification of DHQase–SHORase from chloroplasts. Washed chloroplasts were prepared by the method of ref. [48] and lysed in 10–20 ml buffer B; the stromal fraction was separated by centrifugation at 100 000 *g* for 1 hr at 4°. The supernatant was filtered through a 0.2 μm filter and applied to a Mono-Q column; DHQase–SHORase was eluted as described above. Active fractions were combined, diluted ten-fold with 25 mM BisTris–HCl, pH 6.7 or 7.0, containing 0.4 mM DTT and 1 mM BE, and applied to a HR5/20 Mono-P column (Pharmacia). A pH gradient was generated using 10% (v/v) Polybuffer 74 (Pharmacia), pH 5, containing 0.4 mM DTT and 1 mM BE; 0.5 ml fractions were collected at a flow rate of 1 ml/min using an FPLC apparatus. Shoot extracts were chromatofocused similarly after overnight dialysis against the initial (high pH) buffer.

Enzyme assays. DHQase, SHORase (shikimate dehydrogenase), EPSP synthase and 3-deoxy-*arbino*-heptulosonate 7-phosphate [DAHP] synthase were assayed as described previously [21, 49] except that in the colorimetric DAHP synthase assay the final chromophore was extracted into cyclohexanone before spectrophotometric determination [50]. SHORase (dehydroshikimate reductase) was assayed by monitoring the decline in *A* at 340 nm in 100 mM K-Pi, pH 7.0. DHS, prepared enzymatically from DHQ [47], was standardized with *E. coli* SHORase [47]. DHQ sols were standardized with *E. coli* DHQase [46] and a molar extinction coefficient at 234 nm of 1.2 × 10^4 cm^2/mmol for DHS.

Latency assays. Washed chloroplasts were resuspended in 1 ml 0.33 M sorbitol (pH 7.8 with Tris). DHQase and SHORase assays were performed in 50 mM KPi, pH 7.0, in the presence of absence of 0.33 M sorbitol and using 2 μl of chloroplast suspension. For EPSP synthase 25 μl of chloroplast suspension was added to the assay mixture without substrate (± sorbitol), incubated at 25° for 5 min and centrifuged for 1 min at 8500 *g*; the EPSP was added to the supernatant to initiate the reaction. For DAHP synthase 25 μl of chloroplast suspension was incubated with the substrates and 1 mM $CoCl_2$ in 50 mM BisTris–propane–HCl, pH 7.4 (± sorbitol).

Electrophoresis. SDA-PAGE was by the method of ref. [51] using 8 or 10% (w/v) acrylamide gels. Protein staining was by the ammoniacal $AgNO_3$ method [52]. M_r calibration proteins were: bovine serum albumin (BSA), 68 000; bovine liver catalase, 60 000; bovine liver glutamate dehydrogenase, 53 000; rabbit muscle aldolase, 40 000; rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 36 000; bovine erythrocyte carbonic anhydrase, 29 000.

M_r *determination.* M_r of the purified DHQase–SHORase was determined by high performance gel filtration with Superose 12

(Pharmacia) in a FPLC apparatus. The mobile phase was 50 mM K-Pi, pH 7, containing 0.15 M NaCl, at a flow rate of 0.5 ml/min. The eluate was monitored at 214 nm. M_r calibration proteins were: BSA, 68 000; chicken ovalbumin, 45 000; carbonic anhydrase, 29 000; sperm whale myoglobin, 17 000.

Protein and chlorophyll determination. Protein was determined by Coomassie dye binding [53] using BSA as standard. For protein concns less than 0.1 mg/ml, this method was modified to give improved precision; the dye soln was diluted ten-fold with H_2O and 0.95 ml added to 50 μl sample (this method gives a linear calibration in the range 0.1–1.5 μg protein per assay). Total chlorophyll was determined spectrophotometrically in EtOH [54].

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