BBA 66578

THE SEPARATION OF THREE ALLOSTERICALLY INHIBITABLE 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASES FROM EXTRACTS OF *NEUROSPORA CRASSA* AND THE PURIFICATION OF THE TYROSINE INHIBITABLE ISOENZYME

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(Received November 22nd, 1971)

SUMMARY

1. A method is described for the complete separation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) isoenzymes inhibited by tryptophan (DAHP synthase (Trp)), phenylalanine (DAHP synthase (Phe)) and tyrosine (DAHP synthase (Tyr)).

2. DAHP synthase (Tyr) is purified to homogeneity as judged by electrophoresis on polyacrylamide gels. The presence of the substrate phosphoenolpyruvate is necessary for stability during purification and subsequent storage.

3. The purification procedure makes use of allosteric ligand dependent reversible changes of molecular weight. Purification by ligand-dependent changes of molecular weight may have wider applications.

INTRODUCTION

The isoenzymic regulatory function 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptulosonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase), the first enzyme specific to the shikimate pathway of aromatic biosynthesis, has been the subject of physiological, biochemical and genetical analysis in this laboratory for some years[1-9]. Although isoenzymes have been detected[5,7,10,11] further understanding of this multicomponent regulatory system has necessitated separation and purification of the various protein species involved. This was made difficult by the extreme instability of the isoenzymes inhibitable by phenylalanine and tyrosine.

Abbreviations: DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; PEP, phosphoenolpyruvate.

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Thus, some reports of the separation of isoenzymes by $(NH_4)_2SO_4$ fractionation and subsequent chromatography[10,11] are difficult to interpret and nearly all remaining activity is inhibitable by tryptophan, therefore representing only 5–15% of the total activity of crude extracts[12].

The present paper describes a procedure for the complete separation of forms of enzyme inhibitable by tyrosine (DAHP synthase (Tyr)), phenylalanine (DAHP synthase (Phe)) and tryptophan (DAHP synthase (Trp)) and the purification to homogeneity of a form of DAHP synthase (Tyr). Use is made of the ability of the substrate phosphoenolpyruvate (PEP) to protect DAHP synthases (Phe) and (Tyr) [3,6,7,12] and of the ability of phenylalanine and tyrosine to dissociate these isoenzymes[6,7,12]. Preliminary reports have appeared elsewhere[13,14].

MATERIALS AND METHODS

The following are general procedures; other methods are described in Results.

Organism and culture

Organism. Wild-type *Neurospora crassa* 74A.

Culture. Maintenance of stocks and small scale culture of mycelium were described previously[3].

Conidia for inoculating liquid cultures were harvested in distilled water, from 2-ml slopes of Vogel's N medium[15] containing sucrose (2%, w/v) and Oxoid Ion agar (1.5%, w/v), following incubation at 34 °C for 24 h in the dark and then 2–3 days at room temperature in daylight.

For large-scale culture, 10-l bottles containing 8 l of Vogel's liquid N minimal medium and sucrose (2%) were inoculated with the conidia from one slope and incubated at 25% for 2.5–3 days with forced aeration. The mycelium was harvested in an M.S.E. basket centrifuge, lyophilised, reduced to a powder in a coffee mill and stored dry at −15 °C until required.

Extracts

The crude extracts used in small scale experiments were prepared from frozen non-lyophilised mycelium with 50 mM KH_2PO_4–NaOH buffer (pH 7.4) as described previously[3].

Assay of the DAHP synthases

Enzymic activity was determined by estimating the amount of DAHP formed in 10 min at 37 °C. The standard reaction mixture contained 5 μmoles Tris–maleate–NaOH buffer (pH 6.4), 125 nmoles PEP, 125 nmoles erythrose 4-phosphate with, when required, 12.5 nmoles L-phenylalanine, L-tyrosine or L-tryptophan, and enzyme in a final volume of 0.20 ml. Reaction was initiated by the addition of enzyme and terminated by the addition of 0.1 ml 10% trichloroacetic acid.

The method used to form the chromogen from DAHP was a modification of previous methods[16,17]. After terminating the enzymic reaction, each assay mixture was incubated with 0.6 ml periodate (0.01 M $NaIO_4$ in 0.03 M H_2SO_4)at 37 °C for 30 min followed by the addition of 0.5 ml of 2% (w/v) $NaAsO_2$ in 0.5 M HCl. After 4 min, 2 ml of 0.3% (w/v) of 2-thiobarbituric acid in 5 mM NaOH was added to each

tube. The tubes were covered with foil, heated in a boiling water bath for 8 min and cooled rapidly by plunging into cold water. The chromogen was immediately extracted into 3 ml of water-saturated cyclohexanone. The emulsion was broken by centrifugation and the absorbance at 549 nm of the cyclohexanone layer was measured.

One unit of enzyme activity is defined as that amount of enzyme required to catalyse the formation of 0.1 μmole of DAHP in 10 min at 37 °C. Isoenzymes inhibitable by the aromatic amino acids[6,7] were determined by subtracting the activity measured in the presence of the inhibitor concerned from activity in the absence of inhibitors. Specific activities are expressed as units per mg protein. The amount of DAHP was calculated by using a value of $8.00 \cdot 10^4$ for the molar extinction coefficient of the chromagen formed from DAHP at 549 nm. This value was determined with an authentic sample of DAHP under the conditions described above.

Protein assays

Protein was determined using Folin's reagent[18] with bovine serum albumin as the standard.

Chemicals

PEP sodium salt was purchased from Calbiochem, Los Angeles, Calif., U.S.A. or the monocyclohexylammonium salt was prepared and converted to the sodium salt according to the method of Clark and Kirby[19]. Erythrose 4-phosphate dimethylacetaldicyclohexylammonium salt was obtained from Calbiochem and Fine Chemicals of Australia, Melbourne, Victoria, and was converted to the free phosphate by the method of Ballou[20]. Protamine sulphate was obtained from Mann Research Laboratories N.Y., U.S.A. DAHP was a gift from Professor N. Giles and was prepared in the Chemistry Department, Yale University. Agarose (Bio-Gel A-0.5 m, 100–200 mesh), DEAE-cellulose (Cellex-D) and hydroxylapatite (Bio-Gel HTP) were purchased from BioRad Laboratories, Richmond, Calif., U.S.A. and Sephadex G-25 (coarse grade) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The cellulose powder used was Whatman column chromedia CF-11.

Reagents for acrylamide gel electrophoresis were from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. All other chemicals used were of analytical reagent grade.

DEAE-cellulose chromatography

Anion-exchange chromatography of desalted $(NH_4)_2SO_4$ fractions was on 1.2 cm × 23.5 cm columns of DEAE-cellulose. The columns were eluted with a non-linear salt gradient[21] using eight of the nine chambers of a Technicon "Autograd" gradient former. All chambers contained 67 ml of 20 mM KH_2PO_4–NaOH buffer (pH 7.4) and chamber numbers 2, 4, 6 and 8 were supplemented with 0.25, 0.27, 0.54 and 0.40 M NaCl, respectively. The flow rate was 45 ml/h and 4.4 ml fractions were collected.

Polyacrylamide gel electrophoresis

The apparatus and methods were essentially the same as described by Davis[22]. A 1-cm long 3% acrylamide, 0.63% (w/v, final concn) *N,N'*-methylene-bis-acrylamide stacking gel, and a 5.5-cm long, 7.5% acrylamide, 0.1% (w/v, final concn)

N,N'-methylene-bis-acrylamide-resolving gel were cast in glass tubes with a uniform bore of 0.57 cm. Gels were photopolymerised with riboflavin (13.3 μM final concn) and contained PEP (0.5 mM).

The Tris–glycine buffer system of Davis[22] has a pH of 9.1–10.0 in the resolving gel and was unsuitable for electrophoresis of DAHP synthase which is stable at pH 7.4–8.5 (ref. 23). The triethanolamine *N*-Tris(hydroxylmethyl)methyl-2-amino-sulphonic acid system of Orr[24] was used since the resolving gel is at pH 6.8–8.0.

Enzyme solutions to be electrophoresed were dialysed 12 h against 500 vol. of 2 mM phosphate buffer (pH 7.4) containing PEP (0.1 mM), then concentrated by ultrafiltration in dialysis tubing under vacuum. For each gel a sample was prepared by mixing 3 μl of tracking dye (50 mg/100 ml bromophenol blue in water), 1 drop of glycerol and 100 μl (approx. 100 μg) of protein. The mixture was applied to the top of the stacking gel and electrophoresis was at 4 °C and 2.5 mA per tube. Protein bands were detected with amido black[22].

Preparation of chromatographic materials

DEAE-cellulose. The anion exchanger was regenerated in 1 M NaOH and washed with 20 mM phosphate buffer (pH 7.4) prior to use.

Hydroxylapatite. Hydroxylapatite powder was suspended in 50 mM phosphate buffer (pH 7.4) and allowed to settle. To improve flow rates, half this volume of settled cellulose powder, equilibrated in the same buffer, was added and the well-mixed slurry used to pour columns.

RESULTS

Production of DAHP synthase in wild-type mycelium

The relative amounts of DAHP synthase isoenzymes present in wild-type mycelium as reported by Doy[3] were for a fully grown culture. In the present study, mycelium was grown under different conditions (see Materials and Methods) and harvested when the mycelium was still growing. The production of DAHP synthase during growth is summarised in Fig. 1. The yield of activity was greatest after 3 days growth and cultures were harvested at this time. The dry wt of mycelium after lyophilisation was about 21% of the wet wt and there was a yield of 25–30 g dry wt per 8 l of culture medium.

The separation of three DAHP synthase isoenzymes

After removal of nucleic acid with protamine sulphate the bulk of DAHP synthase activity precipitated over a large concentration range of $(NH_4)_2SO_4$ (Table I). A 45–70% satn $(NH_4)_2SO_4$ fraction containing all three isoenzymes was chromatographed on agarose gel to remove $(NH_4)_2SO_4$ and unwanted protein (Fig. 2). Isoenzymes inhibitable at least 90% by tryptophan (DAHP synthase (Trp)), phenylalanine (DAHP synthase (Phe)) and tyrosine (DAHP synthase (Tyr)) (see Materials and Methods) were then separated on DEAE-cellulose by means of a non-linear salt gradient (Fig. 3). Instability of activity, presumably because of the removal of PEP previously present in the buffers, was minimised by the inclusion of PEP (final concn 0.1 mM) in the fraction tubes.

This procedure resulted in the complete separation of three differently inhi-

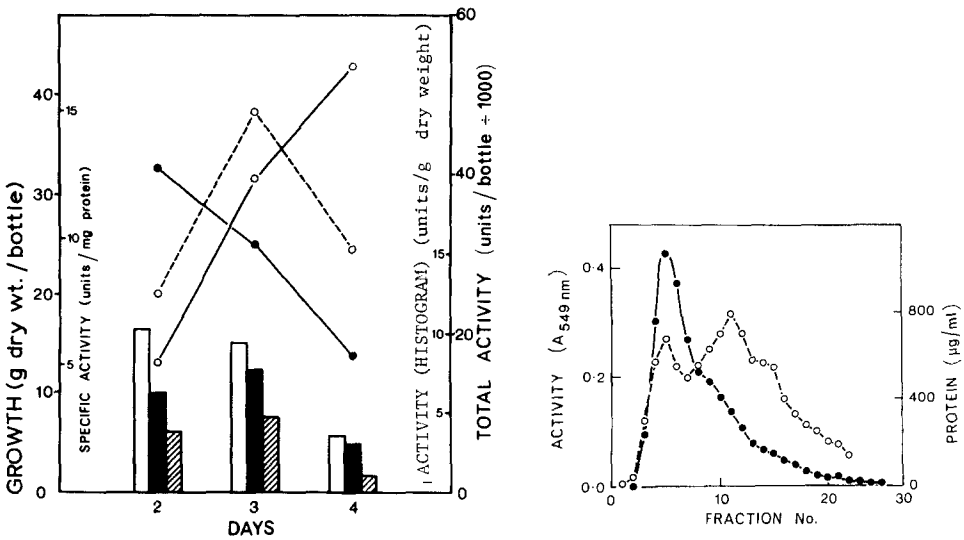

Fig. 1. Production of DAHP synthase activity during large scale culture of wild-type mycelium. ●—●, specific activity; ○---○, total activity; ○—○, growth. The histogram shows the amount of each isoenzyme present: □, Phe-inhibitable, ■, Tyr-inhibitable, ▨, Trp-inhibitable. For experimental details see Materials and Methods.

Fig. 2. Agarose sieving of DAHP synthase from a 45–70% $(NH_4)_2SO_4$ fraction in the presence of PEP. ●—●, total activity; ○---○, protein. Chromatography (sample of 30 mg protein) was on agarose (2.5 cm × 39 cm column) equilibrated and eluted with 50 mM phosphate buffer (pH 7.4) containing 0.1 mM PEP. The flow rate was 30 ml/h and 3-ml fractions were collected. Fractions 4–9 were pooled.

bitable Neurospora DAHP synthase isoenzymes. Previous reports show considerable overlap of these isoenzyme activities[7,10,11] and recovery of activity is poor unless PEP is present to stabilize DAHP synthases (Phe) and (Tyr)[7]. Thus, in preliminary experiments of this laboratory (unpublished) and in reports from other workers, where PEP was omitted, it was difficult to identify DAHP synthases (Phe) and (Tyr) by inhibition characteristics[10,11].

Because it was undesirable to take broad (45–70% satn) $(NH_4)_2SO_4$ fractions

TABLE I

FRACTIONATION OF DAHP SYNTHASE ACTIVITY WITH $(NH_4)_2SO_4$

A crude extract was prepared from 10 g wet wt of wild-type mycelium (see Materials and Methods) and after removal of nucleic acids, solid $(NH_4)_2SO_4$ was slowly added to give the desired saturation at 4 °C. The mixture was then stirred for 45 min and the precipitate collected by centrifugation. Redissolved precipitates were dialysed for 4.5 h against 3 times 1 l of 50 mM phosphate buffer (pH 7.4) before assay.

$(NH_4)_2SO_4$ satn (%)	*Activity (units)*	*Spec. act. (units per mg protein)*
30–40	33	2.56
40–50	100	3.03
50–60	168	5.63
60–70	356	12.4
70–80	28	1.23

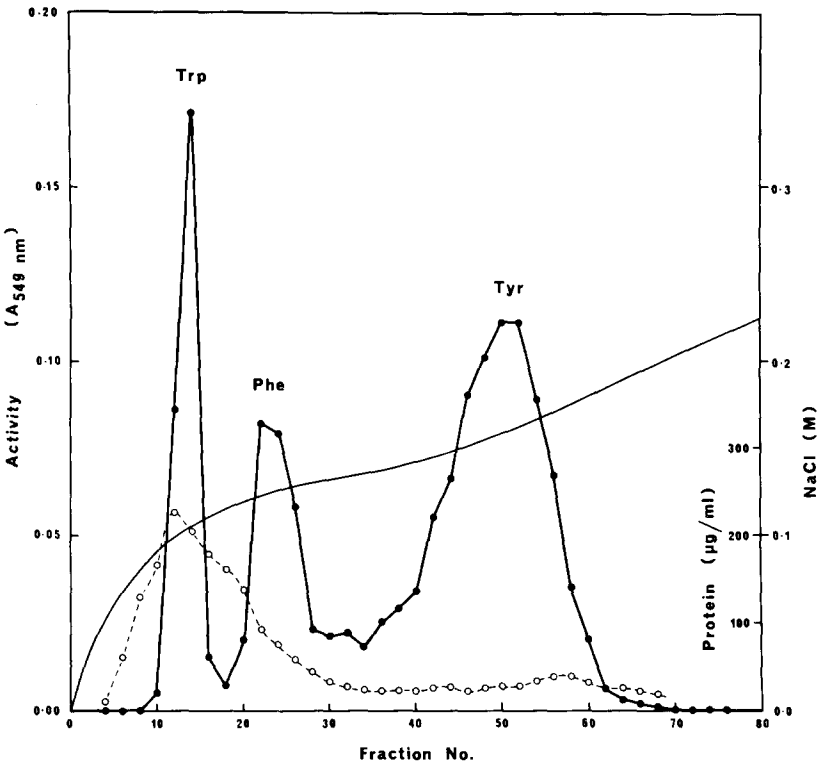

Fig. 3. Separation of DAHP synthase isoenzymes from a desalted 45–70% $(NH_4)_2SO_4$ fraction by chromatography on DEAE-cellulose. ●—●, DAHP synthase activity, inhibition as indicated; ○---○, protein; ———, NaCl gradient. A sample of 25 mg of protein was applied to the column. For experimental details see Materials and Methods.

for large scale purification, the distribution of the isoenzymes in narrover $(NH_4)_2SO_4$ fractions was examined. The response of DAHP synthase activity in these fractions to tryptophan, phenylalanine and tyrosine was complex, including inhibitory and stimulatory effects as well as the sum of the inhibitions being less than 50% of the total activity[23]. In our hands, these results gave no reliable indication of the amount of each isoenzyme present, in contrast to the inhibition analysis of $(NH_4)_2SO_4$ fractions from extracts of *Escherichia coli*[17] where clear and reproducible results were obtained.

Three $(NH_4)_2SO_4$ fractions were then analysed by chromatography on DEAE-cellulose by the technique described in Fig. 3. Each peak of DAHP synthase activity eluted (Fig. 4) was identified by inhibition analysis (see Materials and Methods) and by comparison of its elution position with the data of Fig. 3.

It was now clear that DAHP synthase (Trp) could be separated from DAHP synthases (Phe) and (Tyr) by $(NH_4)_2SO_4$ fractionation. These latter isoenzymes could then be separated by DEAE-cellulose chromatography.

Large scale purification of DAHP synthase (Tyr)

All procedures were carried out at 4 °C and centrifugation was at 16 770 × *g*

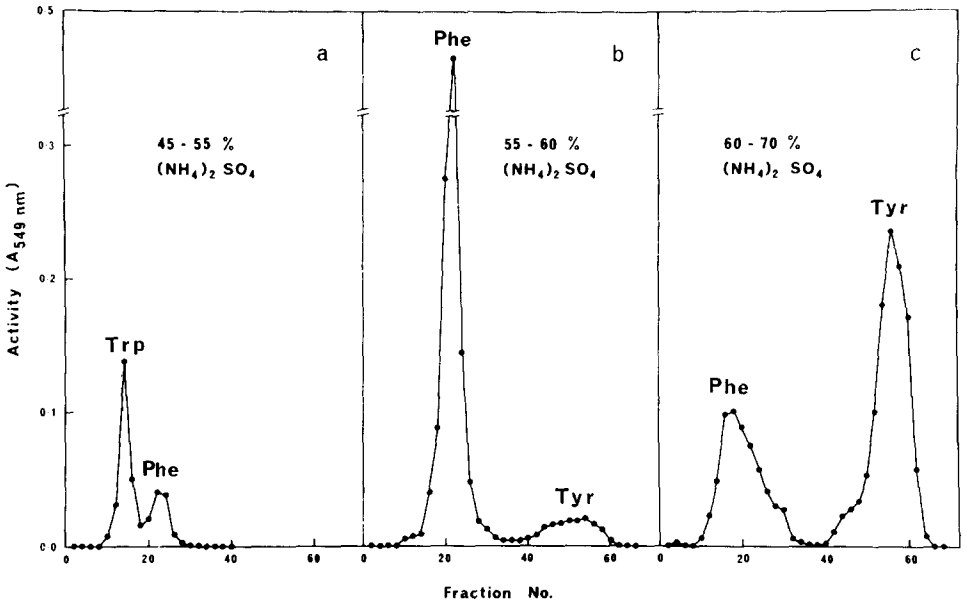

Fig. 4. Distribution of DAHP synthase isoenzymes in $(NH_4)_2SO_4$ fractions. Desalted 45–55 (a), 55–60 (b) and 60–70% (c) fractions (each 150 mg protein) were chromatographed on DEAE-cellulose columns as described for Fig. 3. ●—●, total DAHP synthase activity; activity in each peak was inhibited at least 90% by the amino acid indicated (see Materials and Methods.)

for 20 min. All buffers used were KH_2PO_4–NaOH (pH 7.4, molarity as indicated) containing PEP (0.1 mM) except during DEAE-cellulose chromatography when PEP was added to each fraction tube to give a final concentration of 0.1 mM in each collected fraction. Constant flow rates during chromatography were maintained with a peristaltic pump.

The procedure uses $(NH_4)_2SO_4$ fractionation to separate DAHP synthase (Trp) from the other isoenzymes (Step 3); DAHP synthases (Phe) and (Tyr) are then partly purified together by making use of the reversible effect of the allosteric ligands on molecular weight[6,7], then separated on DEAE-cellulose (Step 6).

Step 1: Preparation of the crude cell extract. Lyophilized, powdered, wild-type mycelium (200 g) was homogenised in buffer (2 l, 50 mM) and after 1 h cell debris was removed by centrifugation.

Step 2: Removal of nucleic acids. Protamine sulphate (4.8 g) as a suspension in buffer (100–200 ml, 50 mM) was added to the crude extract. The mixture was stirred 5–7 min and immediately centrifuged. The precipitate was discarded.

Step 3: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the supernatant to 55% satn at 4 °C (32 g $(NH_4)_2SO_4$/100 ml), the mixture stirred for 45 min and the precipitate collected by centrifugation. Protein precipitating between 55 and 70% satn (obtained by adding a further 10 g $(NH_4)_2SO_4$ per 100 ml supernatant) was collected by centrifugation and dissolved in a minimum volume of buffer (20 mM) containing phenylalanine and tyrosine (each 0.1 mM).

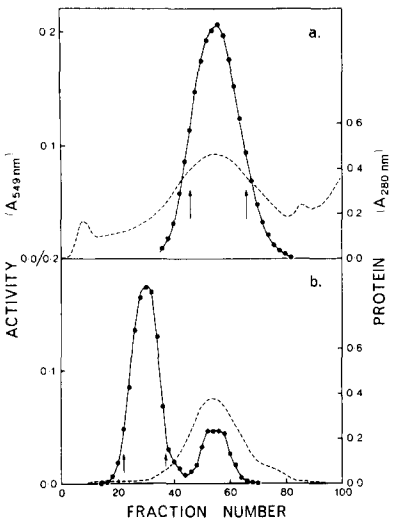

Fig. 5. Purification on agarose of DAHP synthases (Phe) and (Tyr) by allosteric ligand-dependent changes of molecular weight. ●—●, DAHP synthases (Phe) *plus* (Tyr) activity; — — —, protein. (a) Purification Step 4, chromatography in the presence of phenylalanine *plus* tyrosine (each 0.1 mM). (b) Purification Step 5, chromatography of pooled fractions from Step 4 without phenylalanine or tyrosine. Columns were eluted at 60 ml/h and 10-ml fractions were collected. Fractions (between arrows) were pooled and subjected to the next purification step. For other experimental procedures see text.

Step 4: First agarose sieving. The dissolved $(NH_4)_2SO_4$ precipitate was applied to a column of agarose (5 cm × 100 cm) previously equilibrated with the buffer mixture used in Step 3. The inclusion of the amino acid inhibitors in the equilibrating buffer maintains the isoenzymes in the 'low' molecular form (Fig. 5a).

Step 5: Second agarose sieving. Pooled fractions (arrows, Fig. 5a) from Step 4 were concentrated to 50–60 ml by ultrafiltration (Diaflo, Amicon Corp. Massachusetts, U.S.A.: PM 10 membrane). After passage through a 3 cm × 60 cm column of Sepha-

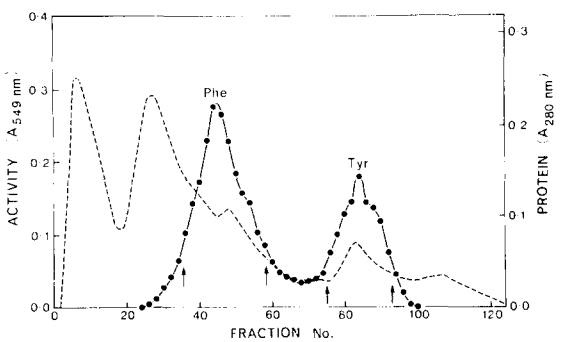

Fig. 6. Separation of DAHP synthases (Phe) and (Tyr) by chromatography on DEAE-cellulose. ●—●, DAHP synthase activity; — — —, protein. The column was eluted at 45 ml/h and 5-ml fractions were collected. Fractions containing only DAHP synthase (Tyr) activity (between latter two arrows) were pooled and subjected to the next purification step. Other experimental details are described in the text.

dex G-25 (coarse grade) to remove the phenylalanine and tyrosine, the sample was chromatographed on the column used in Step 4 but now equilibrated in buffer (20 mM) without inhibitors (Fig. 5b).

The elution profiles of Step 4 and Step 5 sievings (Fig. 5) show that the isoenzymes are separated effectively from the bulk of the protein by this technique. That portion of the activity between the arrows (Fig. 5b). was subjected to the next step in purification.

Step 6: DEAE-cellulose chromatography. Pooled fractions from the second agarose sieving were applied to a column of DEAE-cellulose (2.5 cm × 27 cm) equilibrated in buffer (20 mM). The column was developed with a linear NaCl gradient (50–350 mM) in buffer (20 mM, 500 ml per chamber). DAHP synthase (Phe) was eluted before DAHP synthase (Tyr) (Fig. 6).

Step 7: Chromatography on hydroxylapatite. Pooled fractions containing DAHP synthase (Tyr) from Step 6 were diluted 1:2 (v/v) with buffer (20 mM) and applied to a hydroxylapatite column (2.5 cm × 20 cm). In some cases the eluate was first concentrated before dilution to decrease the time required in applying the sample to the column. The column was eluted with a linear phosphate gradient (50–250 mM, 500 ml per chamber) at a flowrate of 45 ml/h and 5-ml fractions were collected. DAHP synthase (Tyr) activity which eluted as a single symmetrical peak, was obtained in Fractions 110–140.

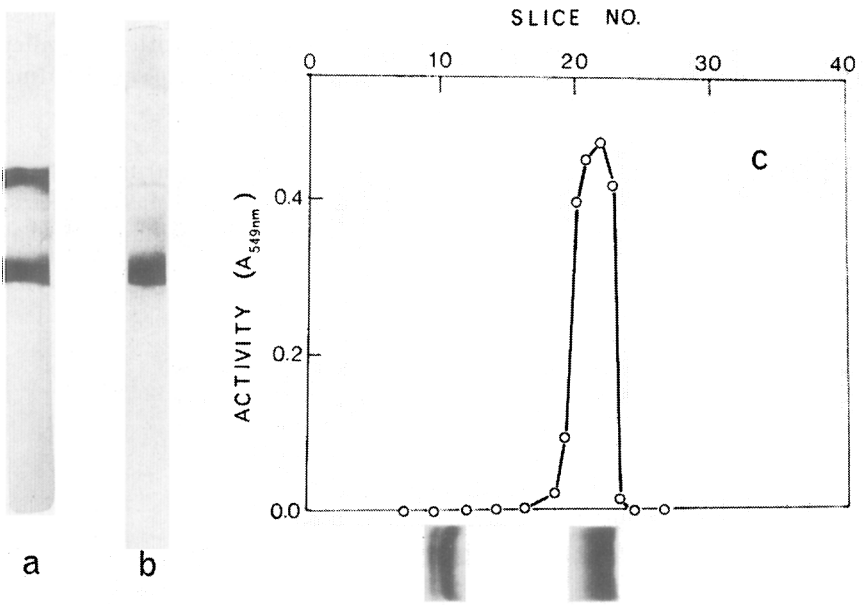

Fig. 7. Polyacrylamide-gel electrophoresis of purified DAHP synthase (Tyr). (a) Freshly prepared; (b) after storage for three weeks; (c) distribution of DAHP synthase activity (○—○) relative to protein. An identical gel to (a) was sliced into 1-mm dics and each disc eluted with 2 times 0.1 ml of 50 mM phosphate buffer (pH 7.4)containing PEP. Each eluate was assayed for DAHP synthase activity. For other experimental details see Materials and Methods.

Homogeneity of the purified isoenzyme

Freshly prepared DAHP synthase (Tyr) electrophoresed on polyacrylamide gel showed the presence of two protein bands in about equal amount (Fig. 7a). The pattern was reproducible with enzyme obtained from different preparations. The more mobile component has an $R_F = 0.40$, and the slow component an $R_F = 0.18$ with respect to the bromophenol blue marker. DAHP synthase activity was associated with the more mobile band (Fig. 7c). When the same sample of enzyme was stored for 2–3 weeks, the activity increased 2-fold (Table II) and on electrophoresis (Fig. 7b) only one band, corresponding in mobility to the active component from the previous gel (Fig. 7a), was detected. This demonstrates that an inactive protein was converted to an active enzyme during storage and suggests that the preparation is pure in the sense that it does not contain significant amounts of proteins which do not have DAHP synthase (Tyr) potential.

The yield of the enzyme is 1–3 mg and the preparation contains no dehydroshikimate reductase, chorismate mutase or prephenate dehydrogenase activity. A summary of the purification is presented in Table II.

TABLE II

PURIFICATION OF DAHP SYNTHASE (Tyr)

Purification step	*Vol. (ml)*	*Total protein (mg)*	*Total tyrosine-inhibitable activity (units)*	*Spec. act. (units per mg protein)*	*Yield (%)*	*Inhibition* of total DAHP synthase activity (%)*		
						Phe	*Tyr*	*Trp*
(1) Crude cell extract	1845	32 518**	25 020	0.77	100	47	32	45
(2) Protamine sulphate-treated extract	1905	18 812**	18 436	0.98	74	44	24	40
(3) $(NH_4)_2SO_4$ fractionation (55–70% satn)	60	4 138**	14 243	3.44	57	74	46	3
(4) First agarose sieving	330	2 163**	10 682	4.94	43	85	44	29
(5) Second agarose sieving	213	335	4 062	12.1	16	64	42	1
(6) DEAE-cellulose chromatography	107	15.2	3 240	213	13	5	96	−4
(7) Hydroxylapatite	145	1.88	2 592	1378	10	2	98	−5
Storage (3 weeks)	16.8	1.88	5 019	2670	20	−14	99	−6

* *Minus* inhibition represents stimulation of activity by the ligand.
** Estimated after dialysis against 0.05 M phosphate buffer.

Storage of purified enzyme

The purified enzyme was concentrated to approx. 10 ml and was stable when stored in the presence of PEP (1 mM) and NaN_3 (0.02% antimicrobial agent) at 4 °C for 2–3 months. Loss of activity after this time is presumably due to the hydrolysis of protecting PEP, since activity is restored to the original level by the addition of a further portion of the substrate to the preparation. The enzyme has now been stored without loss of activity, under these conditions for 1 year.

DISCUSSION

The procedures outlined describe for the first time from any source, the complete separation of DAHP synthases (Trp), (Phe) and (Tyr) and the purification of a substrate (PEP) stabilised form of DAHP synthase (Tyr).

Attempts to use affinity chromatography[25] as a purification technique for Neurospora DAHP synthase (Tyr) were unsuccesful, although retardation of the corresponding isoenzyme from yeast has been reported[26].

Methods are being developed for the purification to homogeneity of DAHP synthases (Phe) and (Trp) from the appropriate stages of the present procedure. DAHP synthase (Phe) from Step 6 may be treated in a manner similar to Step 7 to give several products of high purity but which are still unstable[23]. DAHP synthase (Trp) from the 0–55% $(NH_4)_2SO_4$ fraction of Step 3 may be kept at −10 °C for at least 2 years and has been accumulated for large scale purification by methods different from the above (C. H. Doy and P. J. Hoffmann, unpublished results).

The subunit structure, kinetic and other properties of the purified DAHP synthase (Tyr) are under investigation. If stable forms of the other DAHP synthases can be purified to homogeneity, comparative biochemical–genetical studies of evolutionary and mechanistic significance will become possible[12]. A notable feature, already recognised in this system, being the interaction between different isoenzymic species[8] would be open to closer investigation.

The successful utilisation of controlled molecular weight changes, in response to the specific ligand tyrosine, as a means of purifying DAHP synthase (Tyr), illustrates a method of high specificity for the purification of enzymes. Thism ethod should be applicable to other systems, providing a method for the purification of enzymes subject to gross molecular weight changes in the presence of specific ligands.

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