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PURIFICATION OF THE TYROSINE INHIBITABLE 3-DEOXY-D-*ARABINO*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE FROM *SCHIZOSACCHAROMYCES POMBE*

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Summary

A method is described for the purification of the tyrosine inhibitable isoenzyme 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) to homogeneity as judged by polyacrylamide gel electrophoresis.

Introduction

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, DAHP synthase) catalyzes the first reaction of the common aromatic amino acid biosynthesis in microorganisms [1]. In the two yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* this reaction (the formation of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate and P_i from phospho*enol*pyruvate and erythrose 4-phosphate) is catalyzed by two isoenzymes, DAHP synthase(Tyr) and DAHP synthase(Phe). The activity of the former is inhibited by the aromatic end product L-tyrosine, whereas the latter is inhibited by L-phenylalanine [2—5].

In order to study the molecular control mechanism of the two DAHP synthase activities by L-tyrosine and L-phenylalanine respectively, purified preparations of the isoenzymes are needed. This paper describes a procedure for the isolation of DAHP synthase(Tyr) from *S. pombe* that results in a more than 300-fold purification.

Materials and Methods

Organism and growth conditions. Cells of the mutant strain *aro*1-10C/h^+ [4], which is defective in DAHP synthase(Phe), were cultivated aerobically in

yeast extract at 30° C. At the end of log phase cultures were harvested by centrifugation at 0—4° C and washed twice with 0.1 M potassium phosphate buffer (pH 7.4).

Assay of the DAHP synthase. Enzymatic activity was assayed as described previously [4]. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of DAHP in 1 min at 37° C (molar extinction coefficient of the chromogen formed from DAHP at 549 nm = $8.0 \cdot 10^4$ [6]. Specific activity is stated as units per mg protein.

Protein assay. Protein was either determined using Folin's reagent [7] with bovine serum albumin as the standard or spectrophotometrically at 280 nm.

Polyacrylamide gel electrophoresis. The apparatus and methods are described in the brochure "Instructions for the Polyanalyst" provided by Bucheler Instruments, Inc., Fort Lee, N.J., U.S.A. A 4-cm long resolving gel and a 1.5 cm long stacking gel were cast in glass tubes with an inner diameter of 0.5 cm.

Resolving gel: 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide in 0.37 M Tris · HCl buffer (pH 9.1) containing 0.06% (v/v) *N,N,N',N'*-tetramethylenediamine (TEMED) and 0.07% ammonium persulfate.

Stacking gel: 2.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide in 0.045 M Tris/H_3PO_4 buffer (pH 6.7) containing 0.025% (v/v) TEMED, 0.0005% riboflavin and 0.02% ammonium persulfate. The buffer in the upper electrophoresis chamber was 0.045 M Tris/Glycine (pH 8.9), in the lower chamber 0.12 M Tris · HCl (pH 8.1). The samples were layered on the gels in presence of sucrose (20%) and Bromphenol Blue. A current of 1.5 mA per gel tube was applied until the extract had entered the stacking gel, afterwards the current was increased to 3 mA per tube. Protein was located by staining for 1 h with 0.5% Amido Schwarz in 7% acetic acid and power destaining in 7% acetic acid.

Chemicals. Inorganic chemicals were of analytical reagent grade. Organic chemicals were of the highest grade commercially available (supplied by Merck AG, Darmstadt, Germany, and Fluka AG, Buchs, Switzerland). D-erythrose 4-phosphate (barium salt of the diethylacetal) and phospho*enol*pyruvate (monosodium salt) were supplied by Boehringer Mannheim, Germany.

Results

Conditions for optimum yield of the isoenzyme were systematically investigated during the establishment of each purification step (Bracher, M. and Schweingruber, M.E., unpublished). Unless otherwise indicated, all operations were carried out at 0—4° C in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM phospho*enol*pyruvate, and centrifugations were performed at 17 000 × *g* for 30 min.

Step 1: preparation of the crude cell extract. 60 g of freshly harvested cells were suspended in buffer to a final volume of 80 ml and disrupted with 120 ml glass beads (0.25—0.5 mm) in a Dyno-Mill, keeping the temperature below 3° C. The glass beads were washed with 25 ml of buffer and the cell debris were spun at 48 000 × *g*, leaving all the activity in the slightly turbid yellow supernatant.

Step 2: removal of nucleic acids. Protamine sulfate was added as a suspension in buffer to the crude extract to yield a final concentration of 0.1%. The mixture was stirred for 10 min and centrifuged. The precipitate was discarded.

Step 3: ammonium sulfate fractionation. Saturated cold $(NH_4)_2SO_4$ solution was added dropwise to the supernatant of Step 2 to a final concentration of 35% saturation. After stirring for 1 h the resulting precipitate was removed by centrifugation and discarded. Subsequently the $(NH_4)_2SO_4$ saturation of the supernatant was raised to 50%. The precipitate was collected by centrifugation and resuspended in 5 ml buffer. (During the salting out process the pH was kept between 7.0 and 7.5 by addition of some drops of 10 M NaOH).

Step 4: Sephadex G-150 filtration. The 35—50% $(NH_4)_2SO_4$ fraction from Step 3 was applied to a Sephadex G-150 gel column (4 × 80 cm) equilibrated with elution buffer (Fig. 1). The fractions with highest specific activity were pooled and concentrated on a Diaflo ultra filtration membrane (PM 30, provided by Amicon, Lexington, Mass., U.S.A.) to about 40 ml.

Step 5: DEAE-Sephadex A-50 chromatography. The pool from Step 4 was diluted to a buffer molarity of 0.05 M and applied to an anion exchanger column of DEAE-Sephadex A-50 (1 × 35 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, containing 0.1 mM phospho*enol*pyruvate. The column was eluted with a linear NaCl gradient (0.05—0.2 M) in a total volume of 100 ml equilibration buffer (Fig. 2). The optimal fractions were pooled and concentrated by vacuum dialysis against the same buffer to a final volume of 2.5 ml.

Step 6: chromatography on hydroxyapatite. The concentrated pool from Step 5 was applied to a 1 × 10 cm column of Bio-Gel HTP (Bio Rad, Richmond, Calif., U.S.A.) and eluted with a linear gradient of potassium phosphate (0.05—0.2 M) containing 0.1 M phospho*enol*pyruvate (Fig. 3). The pooled fractions were concentrated to 2.7 ml by vacuum dialysis against 0.02 M buffer containing 0.1 mM phospho*enol*pyruvate.

Step 7: DEAE-cellulose chromatography. Pooled and concentrated fractions from Step 6 were finally chromatographed on a 1 × 9 cm DEAE-cellulose column with a linear buffer gradient (0.02—0.2 M) containing 0.1 mM phos-

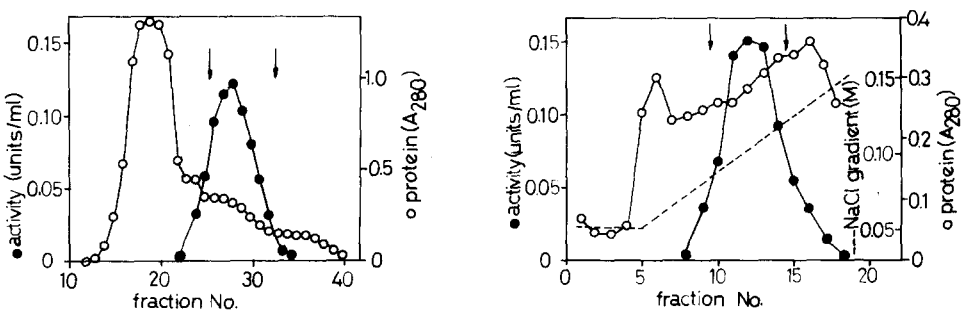

Fig. 1. Gel filtration of DAHP synthase(Tyr) on Sephadex G-150. 119 mg protein (Step 3, Table I) were layered on a 4 × 80 cm column and eluted at 50 ml/h with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM phospho*enol*pyruvate. 16.8-ml fractions were collected. •, DAHP synthase(Tyr) activity (units/ml); ○, protein (A_{280nm}). Fractions between arrows were pooled.

Fig. 2. Anion exchange chromatography of DAHP synthase(Tyr) on DEAE-Sephadex A-50. 25.1 mg protein (Step 4, Table I) were adsorbed to a 1 × 35 cm column and eluted at 12 ml/h with a linear salt gradient of 0.05—0.2 M NaCl in a total volume of 100 ml 0.05 M buffer pH 7.4. 6-ml fractions were collected. - - - - - -, NaCl gradient (M). Other symbols are as in Fig. 1.

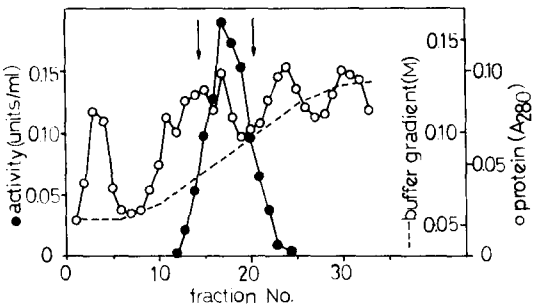

Fig. 3. Chromatography of DAHP synthase(Tyr) on hydroxapatite (Bio-Gel HTP, Bio Rad). 3.28 mg protein (Step 5, Table I) were adsorbed to a 1 × 10 cm column and eluted at 9 ml/h with a linear gradient of potassium phosphate buffer pH 7.4 containing 0.1 mM phospho*enol*pyruvate from 0.05 to 0.2 M (total volume 100 ml). 3-ml fractions were collected. ------, buffer gradient (M). Other symbols are as in Fig. 1.

pho*enol*pyruvate (Fig. 4). Optimal fractions were pooled again and concentrated to 2 ml by vacuum dialysis against 0.1 M buffer containing 1 mM phospho*enol*pyruvate and 0.02% NaN_3.

The final yield was 0.074 mg DAHP synthase(Tyr) as measured by the method of Lowry et al. [7]. The purification procedure is summarized in Table I. Proteins in the various stages of purification were visualized by electrophoresis on polyacrylamide gels (Fig. 5). It is obvious that the final enzyme preparation does not contain any significant amounts of protein other than DAHP synthase(Tyr). Treatment of the purified isoenzyme with 0.1% sodium dodecyl sulfate (100°C, 10 min) and following analysis on a dodecyl sulfate polyacrylamide gel also showed a single band (results not shown here).

This is the first time that a DAHP synthase isoenzyme of a yeast species has been purified to apparent homogeneity. Attempts to purify the enzyme by means of affinity chromatography on an agarose-L-tyrosine column (Miles-Yeda Ltd., Elkhart, Ind., U.S.A.) were unsuccessful. Retardation of the corresponding isoenzyme from *S. cerevisiae* has been reported [8].

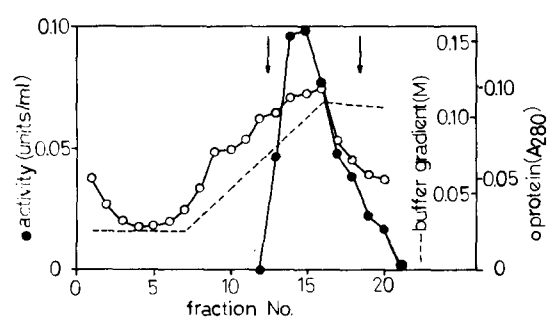

Fig. 4. Anion exchange chromatography of DAHP synthase(Tyr) on DEAE-cellulose. 0.425 mg protein (Step 6, Table I) were adsorbed to a 1 × 9 cm column and eluted at 9 ml/h with a linear gradient of potassium phosphate buffer pH 7.4 from 0.02 to 0.2 M (total volume 20 ml). 1.5-ml fractions were collected. Symbols are as in Fig. 3.

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE OF DAHP SYNTHASE(Tyr)

The detailed purification procedure is given in the text. Enzymatic unit and specific activity are defined in Materials and Methods. The protein was determined by the method of Lowry [7].

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units per mg protein)	Purifcation (-fold)
(1) Crude extract	58	725	24.1 *	100	0.033	1.0
(2) Protamine sulfate	58	558	25.6 *	106	0.046	1.39
(3) Ammonium sulfate fractionation (35—50% satn.)	7	119	18.3 *	76	0.154	4.67
(4) Sephadex G-150	41.7	25.1	13.25	55	0.528	16.0
(5) DEAE-Sephadex A-50	2.5	3.28	4.1	17	1.25	37.85
(6) Hydroxyapatite	2.7	0.425	1.52	6.3	3.57	108.2
(7) DEAE-Cellulose	2.0	0.074	0.795	3.3	10.75	326.0

* Determined after dialysis for 16 h against 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM phospho*enol*pyruvate.

In 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM phospho*enol*pyruvate and 0.02% NaN_3 the purified isoenzyme was stable for 2—3 months. It showed a broad pH optimum (pH 6.2—7.8) and was inhibited in this pH range by 1 mM L-tyrosine as it has been described for the crude cell-free extract [5]. The activity-stimulating effect of $CoCl_2$ described for the crude extract [4] was lost during the purification procedure. This phenomenon is not yet understood but has also been observed in *Salmonella typhimurium* [9] and *Escherichia coli* [10].

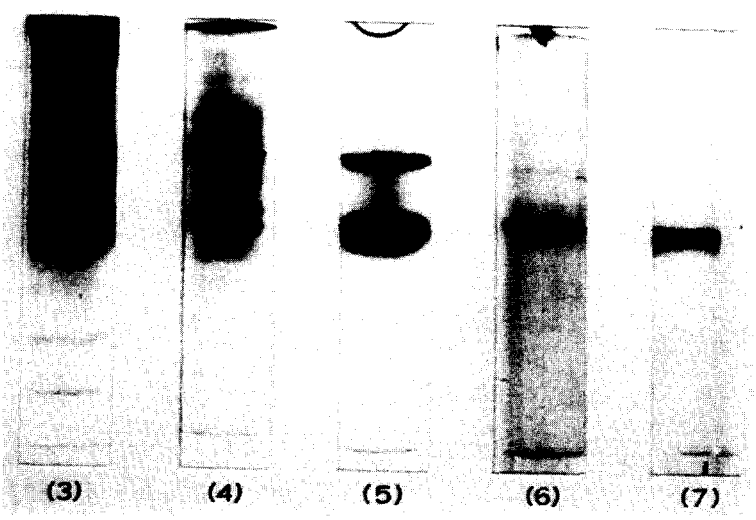

Fig. 5. Electrophoresis of various extracts on polyacrylamide gel (7.5% acrylamide, pH 9.3, 4 cm long gels). Numbers of the gels correspond to the numbers of the purification steps in Table I. Amounts of protein electrophoresed: (3) 80 μg (4) 16 μg, (5) 28 μg, (6) 1.2 μg, (7) 5 μg.

A molecular weight of 75 000 has been reported for the enzyme [5]. Treatment of the purified isoenzyme by sodium dodecyl sulfate and subsequent analysis on dodecyl sulfate gel indicates that the reported form is a dimer (Bracher, M., unpublished). The nature of the two subunits and their role in the feedback control of the activity by L-tyrosine are the subject of further studies.

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