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THE CONVERSION OF 3-DEOXYARABINOHEPTULOSONATE 7-PHOSPHATE TO 3-DEHYDROQUINATE BY SORGHUM SEEDLING PREPARATIONS*

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Key Word Index—*Sorghum bicolr*; Gramineae; 3-dehydroquinate synthetase; shikimic acid pathway.

Abstract—Partially purified preparations from etiolated sorghum seedlings catalyzed the conversion of DAHP to DHQ. The reaction catalyzed by DHQ synthetase was stimulated by 0.1 μM to 0.1 mM NAD in the presence O-0.5 mM Co^{2+}. NADH at 1 μM stimulated the reaction as much as 50% but became inhibitory at 100 μM. Co^{2+} at 0.5 mM stimulated enzyme activity 3-fold; Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} were not stimulatory. EDTA at 5 mM inhibited the reaction 95% but its effects were reversed by equal concentrations of Co^{2+}. Phe, Tyr, Trp, *t*-cinnamate, several hydroxylated cinnamates, DHS, quinate, and shikimate at 0.3 mM failed to affect enzyme activity but slight inhibition occurred with DHQ and protocatechuic acid at 0.3 mM, inhibition being 14% and 22%, respectively. DHQ synthetase activity also was detected in spinach leaves and potato tuber tissue. Synthetase activity appeared to increase in response to injury of potato tuber and sweet potato root tissues.

INTRODUCTION

Many studies have been done to demonstrate that plants possess the shikimic acid pathway for the synthesis of the aromatic amino acids first demonstrated in microorganisms [1–3]. Several enzymes of the bacterial pathway have been detected in plant tissues and regulatory enzymes such as chorismate mutase [4] and anthranilate synthetase [5] first studied in microorganisms later were reported to occur in several plants [6–9]. Despite such observations, there is evidence that certain steps in the pathway in plants differ from the corresponding reactions of the pathway in microorganisms. For example, none of the regulatory properties described for the microbial DAHP synthetase has been demonstrated for the enzyme from green algae and higher plants [10–12]. Radioisotope experiments comparing efficiency of quinate and shikimate as precursors of the aromatic amino acids have either yielded ambiguous results or provided data showing quinate to be more effective than shikimate as a precursor of the aromatic amino acids [13, 14]. Such reports emphasize the need for more information on the occurrence and properties of other enzymes in plants involved in the synthesis and further metabolism of shikimic acid. Recently we reported on the properties of shikimate kinase purified from sorghum seedlings [15]. Here we report on the conversion of DAHP to DHQ by a partially purified preparation from etiolated sorghum seedlings and describe certain properties of the enzyme catalyzing that reaction.

RESULTS

Partial purification of DHQ synthetase

Preparations enriched 8-fold with DHQ synthetase activity were obtained by the procedures summarized in Table 1. DHQ synthetase activity in the preparation from DEAE-cellulose chromatography was stable during storage of the preparation at −25° for at least 4 weeks. However, attempts to further purify the enzyme by other procedures including gel filtration and blue

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‡Abbreviations used: DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; DRHP, 3-deoxyriboheptulosonic acid 7-phosphate; DAPH, 3-deoxyarabinoheptulosonic acid 7-phosphate; QA, quinic acid; SA, shikimic acid; PEP, phosphoenol pyruvate; E-4-P, erythrose 4-phosphate.

Table 1. Purification of DHQ synthetase from sorghum seedlings

Step	Volume (ml)	Protein (mg)	Total activity (nano units*)	specific activity (10^{-9} unit/mg protein)	Purification	Recovery
Crude extract†	690	2763	191.2	0.0692	1.00	100
30–60% $(NH_4)_2SO_4$ fraction	63	1179	99.5	0.0844	1.22	52
DEAE-cellulose	106	180	98.3	0.5461	7.89	51

*Units are expressed as moles DAHP utilized per sec at 30°. †Ice bath temperatures were maintained for all steps.

dextran–sepharose chromatography were unsuccessful owing to excessive losses in activity. The preparation from the DEAE cellulose procedure showed no activity for DRHP but contained both DHQ synthetase and dehydroquinase activities.

Conversion of DAHP [1-^{14}C] to DHQ and DHS.

Reaction mixtures with DAHP-[1-^{14}C] contained two radioactive products. R_f values for the two compounds (A and B) were, respectively, solvent I, 0.30, 0.48, solvent II, 0.20, 0.49. When compared with authentic samples of DHQ and DHS, reactions to the periodate–aniline spray reagent were identical as were chromatographic migrations in solvent systems I and II. Thus, the two compounds were: Compound A, DHQ, and compound B, DHS. The sequence of reactions DAHP → DHQ → DHS clearly occurred in the reaction mixture. In all subsequent experiments, enzyme concentration was adjusted to provide a linear rate of DAHP utilization over the first 20 min.

pH optimum for DAHP utilization.

In reactions with KPi buffer the utilization of DAHP was optimum between pH 7 and 7.5, with half maximal velocity occurring at pH 6.5 and 8.

Cofactor requirements.

In the presence of 10 μM NAD DAPH utilization was stimulated 3.5-fold by 0.5 mM Co^{2+}. Other metal salts tested at 0.5 mM including $MnCl_2$, $MgCl_2$, $FeSO_4$, $CaCl_2$, $ZnCl_2$, and $CuSO_4$ had no effect on DAHP utilization. EDTA 5 mM inhibited the reaction 95% but the inhibition was reversed by 5 mM Co^{2+} In the presence of 0.5 mM Co^{2+}, NAD at 0.1 μM stimulated the reaction 15% and from 1 μM to 100 μM stimulated the reaction 40%; NADH at 1 μM stimulated the reaction 50% but became slightly inhibitory (10%) at 100 μM. In addition, NADP at 10 and 100 μM had no effect on DAHP utilization.

Effect of intermediates and products of the shikimic acid pathway.

DHQ and protocatechuic acid at 0.3 mM inhibited DAHP utilization 14% and 22%, respectively. Other compounds tested at 0.3 mM and without effect were DHS, quinic acid, shikimic acid, *t*-cinnamic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, phenylalanine, tyrosine, and tryptophan.

Distribution of DAHP utilization in plants.

A limited survey of plants revealed that apparent DHQ synthetase activity based upon DAHP utilization occurred in preparations from several plants (Table 2). However, amounts detected in specialized tissues such as leaves in heads of cabbage and lettuce, cauliflower florets, and carrot roots were less than 0.05 p unit/g fr. wt. DHQ synthetase activity could not be detected in freshly sliced sweet potato root but was detected in the tissue 24 hr after slicing. Similar increase in DHQ synthetase activity was noted in potato tuber tissue after slicing.

Table 2. DHQ synthetase activity from various sources

Plant	Activity (pico unit/g fr. wt)	Activity (pico unit/mg protein)
Etiolated sorghum seedling (3 days old)	1.49	0.28
Sorghum stem (3 months old)	0.25	0.18
Spinach leaves	1.43	0.20
Sweet potato roots	*	†
Sweet potato roots (24 hr after slicing)	0.20	0.03
White potato tuber	0.40	0.05
White potato tuber (24 hr after slicing)	0.94	0.17

*Less than 0.05; †less than 0.01.
A unit of activity is expressed as moles substrate utilized per second.

DISCUSSION

The results of these studies on sorghum, demonstrating the conversion of DAHP to DHQ, lend further support for the occurrence in higher plants of the same shikimic acid pathway that has been demonstrated in microorganisms and confirms the reports of the occurrence of the enzyme in mung bean [16] and in tobacco callus tissue [17]. The sorghum DHQ synthetase resembles the mung bean [16] and the bacterial enzyme [17, 18] being stimulated by Co^{2+} and NAD. However, Co^{2+} -specific enzymes in plants are rare and the general nutritional requirement for the element in plants has not been conclusively demonstrated. In this connection, it is interesting to note that none of the common divalent cations could replace Co^{2+} in activating the sorghum and the mung bean DHQ synthetases [16]. The identity of the metal ion associated with the enzyme *in vivo* remains to be determined

Presumably, the effect of NAD on the reaction is due to its participation in the oxidation of DAHP to a diketone intermediate. It is proposed that the latter compound undergoes P_i elimination yielding a product that undergoes a NADH-dependent reduction to yield a dideoxy diketone which cyclizes to form 5-DHQ [17,18].

The stimulatory effect of NADH in these studies undoubtedly is due to its conversion to NAD by contaminating NADH oxidizing enzymes in the DHQ synthetase preparation. Higher concentrations of NADH were inhibitory here as was the case with the bacterial DHQ synthetase [17], the site of inhibition presumably occurring at the NAD-dependent oxidation of DAHP to a diketone intermediate [17, 18]. Although DHQ synthetase from sorghum seedlings was slightly inhibited by DHQ and protocatechuic acid, the low level of inhibition and the high concentration required to inhibit does not suggest regulatory function of these compounds.

The increase in DHQ synthetase activity in response to slicing of sweet potato roots and potato tubers is reminiscent of the injury stimulated increase in those tissues of phenylalanine ammonia lyase and other enzymes of phenolic biosynthesis [19]. The observation of the parallel increase in activity of that enzyme with increases in products of the shikimic acid pathway lends further support for the role of the enzyme in the synthesis of the aromatic amino acids. It also seems significant that DHQ synthetase activity was not detected in those tissues (e.g. cabbage heads) expected to have low requirements for the aromatic amino acids.

EXPERIMENTAL

Plant material. Sorghum seed were germinated in the dark at 30° and etiolated seedlings harvested after 3 days. The stems of the seedlings were removed, frozen in liquid N_2, and pulverized to a fine powder in a Waring Blendor. The powder was stored at −15° until used for enzyme extraction. Spinach plants

(*Spinacia oleracea*), cabbage heads (*Brassica oleracea* var. *capitata*), lettuce heads, (*Lactuca sativa*), cauliflower heads (*B. oleracea* var. *botrytis*); carrot roots (*Daucus carota* var. *sativa*), sweet potato roots (*Ipomoea batatas*), and potato tubers (*Solanum tuberosum*) were purchased at a local market.

The procedure of enzyme preparation. Pulverized frozen material was combined with the same weight of 100 mM KPi buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and α-toluene sulfonyl flouride (160 mg/kg fr. wt of tissue) which was first dissolved in 2-propanol (40 mg/ml). The mixture was allowed to thaw and then squeezed through 4 layers of cheesecloth. The resulting homogenate was centrifuged for 20 min at 20000 *g*. The supernatant liquid obtained was the crude extract shown in Table 1. To the crude extract, solid $(NH_4)_2SO_4$ was added to 30% satn. The pH of the soln was adjusted to 7 by addition of M KOH. The ppt. was removed by centrifugation at 20000 *g* for 20 min. To the supernatant liquid was added solid $(NH_4)_2SO_4$ to 60% satn. The resulting ppt. was collected by centrifugation at 20000 *g*, and the ppt. suspended in one-tenth the amount of 10 mM KPi buffer containing 10 mM 2-mercaptoethanol. The sample was dialyzed against 10 mM KPi buffer, pH 7, containing 10 mM 2-mercaptoethanol at 4° until the conductivity of the dialysate equalled that of the dialyzing buffer. The dialyzed preparation was applied to a column (2.6 × 40 cm) of DEAE-cellulose (DE-52) which had been equilibrated with 10 mM KPi buffer, pH 7. The column was washed with 100 ml of the equilibrating buffer, then the enzyme eluted, 60 ml/cm^2/hr with a linear gradient formed by 250 ml of 10 mM pH 7 KPi buffer and 250 ml of 300 mM pH 7 KPi buffer containing 0.1 mM $CoCl_2$. Fractions (5 ml) were collected and assayed for enzyme activity.

Preparation of DAHP-[1-^{14}C]. E-4-P, PEP-1-^{14}C, and a DAHP synthetase preparation from cauliflower florets (Step IV, purification procedure, ref. [12]) were used in this preparation. The reaction mixture, total vol. 1.5 ml, which consisted of 150 μmol PIPES buffer (pH 6.6), 7.5 μmol DTT, 3 μmol $MnCl_2$, 3 μmol PEP-[1-^{14}C] cyclohexyl ammonium salt (sp. act. 10 mCi/mmol), 3 μmol E-4-P Na salt, and 150 μl enzyme soln containing 48 nanounits of DAHP synthetase activity, was incubated for 30 min at 30°. DAHP in the reaction mixture was isolated by Dowex 1-X8 (HCO_2H form), and purified as Ba salt by the procedure of ref. [20].

Chemicals. E-4-P was prepared from G-6-P di Na salt according to the method of ref. [21]. DHQ and DHS were prepared from quinic acid and shikimic acid, respectively, by the methods of ref. [22]. All other chemicals were obtained from commercial sources.

Identification of the enzymatic reaction products. DAHP-[1-^{14}C], 8 × 10^4 dpm, 1.4 nmol, was incubated under the same reaction conditions of the enzyme assay with an enzyme preparation containing 50 nanounits of DHQ synthetase activity. After 20 min the reaction was stopped by the addition of one vol. of 8% TCA. The mixture was lyophilized, and the resulting residue suspended in 1 ml H_2O. An aliquot 0.25 ml of the sample was chromatographed on Whatman No. 1 with QA, SA, DHQ and DHS as markers in solvent system I: *n*-BuOH–HOAc–H_2O (4:1:5): or solvent system II: MeCoEt–Me_2CO–H_2O–HCO_2H (80:4:12:2). Radioactive areas on the chromatograms were detected by a chromatographic strip counter and the spots of QA, SA, DHQ, DHS were detected by the spray reagents of ref. [23].

Enzyme assay. The activity of DHQ synthetase was detemined by the disappearance of DAHP. The incubation mixture contained 25 μmol of KPi buffer (pH 7.5), 0.15 μmol unlabeled DAHP, 0.25 μmol $CoCl_2$, 0.005 μmol NAD and 100 μl enzyme soln in a total vol. of 500 μl at 30°. The reaction was started by the addition of DAHP; for the control, an aliquot, 200 μl, of the reaction mixture was immediately removed and mixed with 200 μl of 8% TCA. Another 200 μl of the reaction mixture was removed after 30 min and similarly treated with 8% TCA. DAHP in the TCA-treated sample was estimated by the procedure of ref. [12].

Protein determination. Protein in the enzyme was determined by the biuret method [24]. BSA was used as the protein standard.

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