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PURIFICATION OF TWO FORMS OF THE ASSOCIATED 3-DEHYDROQUINATE HYDRO-LYASE AND SHIKIMATE:NADP$^+$ OXIDOREDUCTASE IN *PHASEOLUS MUNGO* SEEDLINGS

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Summary

Two associated enzymes, 3-dehydroquinate hydro-lyase (EC 4.2.1.10) and shikimate:NADP$^+$ oxidoreductase (EC 1.1.1.25), have been purified from *Phaseolus mungo* seedlings. These enzymes were purified 6900- and 9700-fold, respectively, but they were not separable. Moreover, two activity bands of the shikimate:NADP$^+$ oxidoreductase were detected after polyacrylamide gel electrophoresis and the two peaks also have 3-dehydroquinate hydro-lyase activity. The two forms of the associated enzymes showed only small differences in molecular weight, K_m value, pH optimum and the responses to some inhibitors.

Introduction

In *Neurospora crassa* it has been shown that five enzymes (3-dehydroquinate synthase, 3-dehydroquinate hydro-lyase, shikimate:NADP$^+$ oxidoreductase, shikimate kinase and 3-phospho-5-*enol*pyruvylshikimate synthase), which catalyze five sequential steps in the common shikimate pathway, are physically associated in an enzyme aggregate with an approximate molecular weight of 230 000 [1,2]. The aggregate of 3-dehydroquinate hydro-lyase and shikimate:NADP$^+$ oxidoreductase activities could not be dissociated further. Recently, it has been reported that these two enzymes are also associated in several higher plants such as *Quercus pedunculata* [3], *Ginkgo biloba*, *Pisum sativum* [4] and *Phaseolus mungo* [5]. Since these studies with higher plants were carried out using unpurified enzyme preparations, it is not clear yet whether the purified enzymes are still associated. On the other hand, two shikimate:NADP$^+$ oxidoreductase isoenzymes were observed in developing pea seedlings [6]. We also found similar enzymes having two activities in crude enzyme extract from *P. mungo* seedlings.

In the present study, 3-dehydroquinate hydro-lyase and shikimate:NADP$^+$

oxidoreductase were partially purified, and their properties were investigated to know the detailed state of the association of the two enzymes.

Materials and Methods

Materials

Seeds of *P. mungo* were soaked in conc. H_2SO_4 for 10 min and then washed thoroughly with water until the washings became neutral. The seeds were then soaked in water for 24 h in the dark at 25°C, washed with water and used for the enzyme extraction.

Shikimate was prepared from Chinese star-anise (*Illicium anisatum*) by the method of Hattori et al. [7]. 3-Dehydroquinate and 3-dehydroshikimate were synthesized according to the method of Haslam et al. [8]. NADP (Boehringer Mannheim GmbH or Oriental Yeast Co., Ltd.), MTT tetrazolium (3(4,5-dimethyl thiazolyl 2)-2,5-diphenyl tetrazolium bromide) (Wako Pure Chemical Industries, Ltd.), phenazine methosulfate (Tokyo Kasei Kogyo Co., Ltd.) were purchased from the sources mentioned.

Purification procedure

All procedures were followed at 0—5°C, and all buffers used contained 10 mM (Steps 1—6) or 8 mM (following steps) 2-mercaptoethanol unless otherwise stated.

P. mungo seedlings were blended in liquid nitrogen and the frozen powder stored at −20°C, if necessary. The powder (9.6 kg) was suspended in 10 l of 0.05 M Tris · HCl buffer (pH 7.4) containing 960 g of Polyclar AT. The mixture was stirred until the temperature increased to 4°C and then it was centrifuged at 7700 × *g* for 30 min.

Solid $(NH_4)_2SO_4$ was added (30% saturation) and the solution was stirred for 40 min. The resulting precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant (to 50% saturation) and this precipitate was also collected by centrifugation, suspended in the extraction buffer and dialyzed against the same buffer. The dialyzed solution was diluted 30 mg/ml protein and then $(NH_4)_2SO_4$ fractionation (35—45% saturation) was carried out.

The dialyzed solution (228 ml) was applied to DEAE-cellulose column (Whatman DE-23, 5.7 × 32 cm) equilibrated with 50 mM Tris · HCl buffer (pH 7.4). After a 600 ml wash with the same buffer, the enzyme was eluted with a 3.2-l linear KCl gradient from 0—0.3 M at 160 ml/h (40-ml fractions). The active fractions (Nos. 69—82) were combined and concentrated by 60% saturation with $(NH_4)_2SO_4$.

After dialysis against 50 mM Tris · HCl buffer (pH 7.4), the solution (80 ml) was passed through a Sephadex G-100 column (7 × 84 cm) at 60 ml/h (20-ml fractions). The enzyme activities were eluted between fractions Nos. 86 and 96.

These fractions were then applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm). After a 230-ml wash with the same buffer, the adsorbed enzymes were eluted with an 800 ml linear KCl gradient (0—0.25 M) of the buffer at 15 ml/h (10-ml fractions). Both enzyme activities were eluted together and the active fractions (Nos. 88—97) were combined.

This enzyme solution was adjusted to pH 6.7 with HCl and then applied to

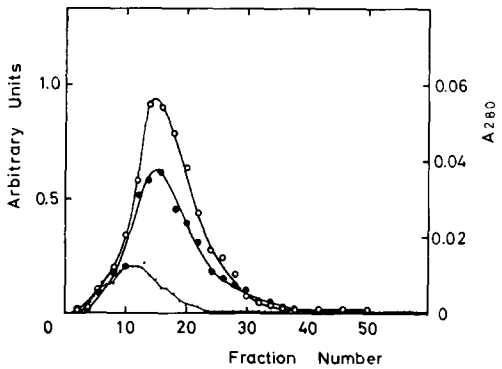

Fig. 1. Chromatography of 3-dehydroquinate hydro-lyase and shikimate:NADP$^+$ oxidoreductase on a CM-Sephadex C-50 column. Details of the chromatographic procedure are described in the text. 3-Dehydroquinate hydro-lyase activity (●), shikimate:NADP$^+$ oxidoreductase activity (○). Protein was monitored at 280 nm (·).

a hydroxyapatite column (2 × 20 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.7). After a 70 ml wash, the enzymes were eluted with an 800 ml linear gradient of phosphate buffer (5—150 mM, pH 6.7) at 25 ml/h (10-ml fractions). The active fractions (Nos. 53—56) were combined and the buffer of enzyme solution was exchanged for 40 mM potassium phosphate buffer (pH 5.8) containing 10% sucrose on Sephadex G-25 column (1.5 × 20 cm).

The sample was then applied to a CM-Sephadex C-50 column (1.5 × 18 cm), continuously eluted with the same buffer. Elution pattern is shown in Fig. 1. The fractions (Nos. 14—32) were collected and concentrated using a hydroxyapatite column. The buffer of the solution was exchanged for 50 mM potassium phosphate buffer (pH 6.8) containing 15% sucrose, and was stored at 4° C.

Enzyme assays

3-Dehydroquinate hydro-lyase activity was assayed by following the formation of 3-dehydroshikimate. The reaction mixture (2 ml) contained 0.2 mmol of Tris · HCl buffer (pH 7.4), 0.75 μmol of 3-dehydroquinate and the enzyme. The increase in absorbance at 234 nm was recorded at 25° C.

Shikimate:NADP$^+$ oxidoreductase activity was assayed by reduction rate of NADP$^+$ in the presence of shikimate at 25° C. The assay mixture (2 ml) contained 0.4 mmol of Tris · HCl buffer (pH 8.6), 10 μmol of potassium shikimate, 1.0 μmol of NADP$^+$ and the enzyme. The reaction was followed photometrically at 340 nm.

Protein in the crude extract and the ammonium sulfate fractions was determined by the method of Lowry et al. [9] and in other cases by the method of Warburg and Christian [10].

Analytical polyacrylamide gel electrophoresis

Electrophoresis was performed using 6% polyacrylamide gels in pH 9.4 systems. Gel length was 7 cm. Activity band of shikimate:NADP$^+$ oxidoreductase on the gel was located as described previously [5].

Preparative polyacrylamide gel electrophoresis

Preparative electrophoresis was carried out on a PREP-DISC (CANALCO) with the column PD 2/70 according to the method described in instruction manual. 5 cm of 6% acrylamide gel was used as separating gel and 2 cm of 3% acrylamide gel as stacking gel. The sample (1.5 ml) in 50 mM potassium phosphate buffer (pH 6.8) containing 8 mM 2-mercaptoethanol, 15% sucrose and a small amount of Bromophenol Blue was layered on the stacking gel. Amperage was corrected to 7 mA before the marker dye reached the interface of the two gels, then increased to 15 mA and maintained throughout the run. Samples were collected at 100-s intervals with a 30 ml/h flow rate. To prevent enzyme from denaturating, each fraction was immediately mixed with 0.3 ml of 50 mM phosphate buffer (pH 6.8) containing 16 mM 2-mercaptoethanol and 80% sucrose.

Results

Purification

A summary of the purification is shown in Table I. 3-dehydroquinate hydro-lyase and shikimate:$NADP^+$ oxidoreductase were not separated by these purification procedures. The hydro-lyase and the oxidoreductase were purified approximately 6900- and 9700-fold from the crude extract with 0.8 and 1.1% yields, respectively. When the purified sample was subjected to analytical gel electrophoresis, four bands were stained by Coomassie Brilliant Blue. Two bands of the oxidoreductase activity were observed by specific staining and these bands agreed with the upper two protein bands (Fig. 2). The hydro-lyase activity could not be detected on the gel by the method of staining for its activity.

Separation of two forms of the associated enzymes by preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide gel electrophoresis was performed to locate the both enzymes on the gel. As shown in Fig. 3, two peaks (I and II), each having both enzyme activities, were eluted from the gel, indicating two forms of the associated enzymes. Analytical gel electrophoresis of fractions No. 58, 64 and 67 was performed and the oxidoreductase activity on the gel was staind (Fig. 3, inset). Since activities of peak II were contaminated with those of peak I, fraction Nos. 56—60 (Type I) and Nos. 66—72 (Type II) were combined separately.

Comparison of the two forms of the associated enzymes

Some properties of the hydro-lyase and the oxidoreductase were compared between Type I and Type II. The molecular weights were estimated using polyacrylamide gel electrophoresis by the method of Hedrick and Smith [11]. The slopes of two activity bands of the oxidoreductase were the same, suggesting that they had the same molecular weight (approx. 57 000, Fig. 4) but slightly different net electric charge.

K_m values of the two enzymes were determined according to the Lineweaver-Burk method [12]. K_m values of 3-dehydroquinate hydro-lyase of the two forms for 3-dehydroquinate were similar (Type I; $1.3 \cdot 10^{-4}$ M, Type II; $1.8 \cdot$

TABLE I

PURIFICATION OF ASSOCIATED 3-DEHYDROQUINATE HYDRO-LYASE AND SHIKIMATE:$NADP^+$ OXIDOREDUCTASE FROM *PHASEOLUS MUNGO* SEEDLINGS

Purification steps	Volume (ml)	Protein (mg)	Total activity (units *)		Specific activity (units/mg protein)		Yield (%)	
			DHQase **	SORase ***	DHQase	SORase	DHQase	SORase
Crude extract	9850	250 000	390	4320	0.0016	0.017	100	100
30—55% $(NH_4)_2SO_4$	1010	125 000	297	3680	0.0024	0.030	76.1	85.3
35—45% $(NH_4)_2SO_4$	230	26 400	203	2630	0.0077	0.100	52.1	61.0
DEAE-cellulose	680	5 370	117	1770	0.0217	0.328	30.0	40.9
Sephadex G-100	293	481	86.3	1050	0.180	2.18	22.1	24.4
DEAE-Sephadex A-50	131	82.1	50.3	658	0.613	8.02	12.9	15.3
Hydroxyapatite	42	2.22	9.95	124	4.48	55.7	2.55	2.87
CM-Sephadex C-50	120	0.271	2.92	45.3	10.8	167	0.749	1.05

* One unit of enzyme activity is defined as the activity required to produce 1 μmol of product per min under the assay conditions.

** 3-Dehydroquinate hydro-lyase

*** Shikimate:$NADP^+$ oxidoreductase

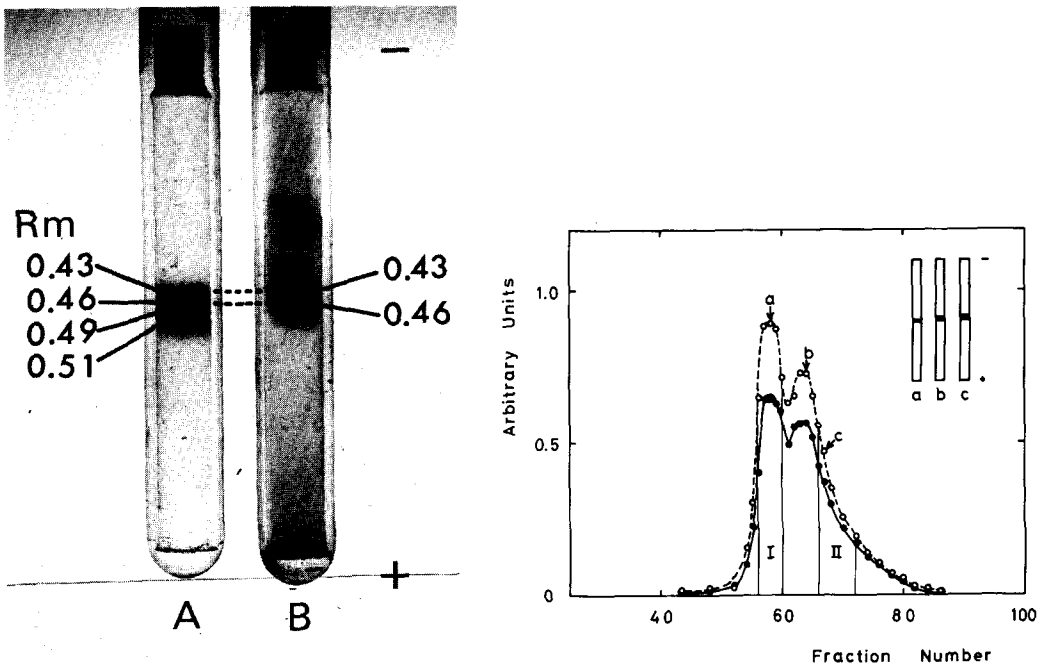

Fig. 2. Photographs of polyacrylamide gel electrophoresis of purified enzymes. (A) Protein stained by Coomassie Brilliant Blue. (B) Shikimate:$NADP^+$ oxidoreductase activity, stained as described in the text.

Fig. 3. Elution pattern of 3-dehydroquinate hydro-lyase and shikimate:$NADP^+$ oxidoreductase from preparative polyacrylamide gel electrophoresis. Details of the experimental procedure are given in the text. 3-Dehydroquinate hydro-lyase activity (●), shikimate:$NADP^+$ oxidoreductase activity (○). Shikimate:$NADP^+$ oxidoreductase activity on the polyacrylamide gel after electrophoresis was stained as described in the text. (in inset, a; fraction No. 58, b; No. 64, c; No. 67).

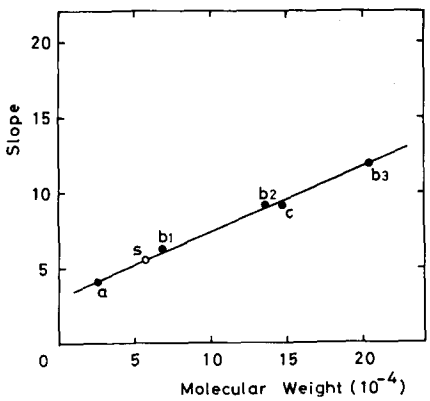

Fig. 4. Determination of molecular weight was made by the method of Hedrick and Smith [11]. a: chymotrypsinogen; b_1, b_2, b_3: bovine serum albumin (monomer, dimer, trimer); c: aldolase; s: two shikimate:$NADP^+$ oxidoreductases. Experimental conditions are given in the text.

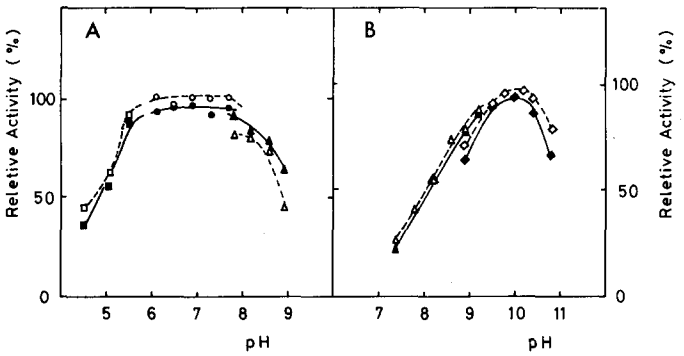

Fig. 5. Effect of pH on the activities of two forms of 3-dehydroquinate hydro-lyase (A) and shikimate: $NADP^+$ oxidoreductase (B). Enzyme activities were assayed as described in the text except that the buffer used in the reaction mixture was varied as shown, □■; 0.1 M succinate · NaOH buffer, ○●; 0.1 M potassium phosphate buffer, △▲; 0.1 M Tris · HCl buffer, ◇◆; 0.1 M glycine · NaOH buffer. And ———; activity of Type I, — — —; activity of Type II. The activity at each pH was preseuted as % of the maximum activity.

10^{-4} M). K_m values of the oxidoreductase of Type I and Type II for $NADP^+$ were the same ($0.25 \cdot 10^{-4}$ M). Two K_m values of the oxidoreductase Type I for shikimate were found (K_{m_1}; $2.2 \cdot 10^{-4}$ M, K_{m_2}; $9.1 \cdot 10^{-4}$ M). The same was true for Type II.

The optimal activities of the hydro-lyase appeared at pH 6.0—8.0, and there was no significant difference between Type I and Type II. The oxidoreductase of Type I and Type II also showed the same optimal activities at pH 10 (Fig. 5).

Effect of some inhibitors on the activity of the oxidoreductase was compared between Type I and Type II. The results are shown in Table II. *p*-Chloromercuribenzoate was a potent inhibitor of both forms of the enzyme and this inhibition was reversed by cysteine. Protocatechuate, 3,5-dihydroxybenzoate and *p*-hydroxybenzoate moderately inhibited the activities. There was no significant difference in the inhibitory effects between Type I and Type II of the oxidoreductase.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON SHIKIMATE:$NADP^+$ OXIDOREDUCTASE OF TYPE I AND TYPE II

Inhibitor	Final concentration	Inhibition (%)	
	(mM)	Form I	Form II
p-Chloromercuribenzoate	1.0	82	74
p-Chloromercuribenzoate + cysteine	1.0 10	14	14
Protocatechuate	1.0	21	14
3,5,-Dihydroxybenzoate	3.0	21	13
p-Hydroxybenzoate	3.0	29	14

Discussion

In several higher plants, 3-dehydroquinate hydro-lyase and shikimate:$NADP^+$ oxidoreductase were shown to be associated in crude enzyme preparations [3—5]. Balinsky and Davies have purified the hydro-lyase about 7.6-fold from cauliflower [13] and the oxidoreductase about 78- and 209-fold from *P. sativum* [14,15]. They mentioned that the 78-fold purified oxidoreductase was free of the hydro-lyase activity. In the present study, the hydro-lyase and the oxidoreductase were purified from *P. mungo* about 6900- and 9700-fold, respectively. However the two enzymes were not separated by the purification procedures. These results suggest that, in higher plants, 3-dehydroquinate hydro-lyase and shikimate:$NADP^+$ oxidoreductase exist as a single protein or an aggregate of two enzyme proteins. Similar results with *N. crassa* were reported by Giles et al. [1,2] who found that the hydro-lyase and the oxidoreductase were present in small component (20 000 daltons) which could not be dissociated further. These enzymes were encoded in a gene cluster together with three other enzymes in the shikimate pathway [1]. In some higher plants, these two enzymes were detected similarly in various organs or in subcellular fractions [16] and their activities also change in parallel in developing mung bean seedlings [17].

In the present study two distinct types of associated enzyme activities were shown to have no significant difference in their properties. In *P. sativum*, two shikimate:$NADP^+$ oxidoreductase isoenzymes which have similar properties were reported by Rothe [6], but it was not examined whether these isoenzymes associated with the hydro-lyase. On the other hand Boudet et al. [18] found two 3-dehydroquinate hydro-lyase in *Zea mays*, one type associated and the other type not associated with the oxidoreductase. The latter type of activity was not found in the present study.

It has been reported that quinate is metabolized through shikimate pathway [19]. Recently, quinate:NAD^+ oxidoreductase [20] and 3-dehydroquinate synthase [21] were found in *P. mungo* seedlings, indicating that 3-dehydroquinate was formed independently from 3-deoxy-D-arabino-heptulosonic acid 7-phosphate and from quinate. It is of interest to know whether the two forms of the associated enzymes in the present study, function separately corresponding to the two pathway of 3-dehydroquinate formation.

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