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A Simple Spectrophotometric Assay for Arogenate Dehydratase

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A simple spectrophotometric assay for arogenate dehydratase, the enzyme that catalyzes the formation of L-phenylalanine from L-arogenate, is presented. The method couples the arogenate dehydratase reaction with that of an aromatic aminotransferase partially purified from *Acinetobacter calcoaceticus*. In the presence of 2-ketoglutarate, phenylpyruvate formation is measured at 320 nm at basic pH. The method was compared with two other methods already in use in our laboratory for arogenate dehydratase. The new method is simple, quick, fairly sensitive, and especially suitable for the screening of a large number of samples.

Arogenate dehydratase catalyzes the formation of L-phenylalanine from L-arogenate (Fig. 1). In some prokaryotic systems this enzyme can also use prephenate as an alternative substrate (cyclohexadienyl dehydratase) and coexists with a specific prephenate dehydratase. In other prokaryotic systems this enzyme activity is the sole dehydratase in operation for L-phenylalanine biosynthesis, is specific for L-arogenate, and is feedback-inhibited by L-phenylalanine (1–3). The arogenate pathway constitutes the only route to phenylalanine biosynthesis in higher plants (4,5). Difficulties in the assay of this enzyme activity are considerable. The available assay methods depend upon measurement of phenylalanine. These include separation of radiolabeled dansylarogenate[1] from dansylphenylalanine by thin-layer chromatography (6), selective oxidation of unutilized arogenate followed by fluorometric estimation of phenylalanine (7), separation of the phenylalanine formed from arogenate on a high-performance liquid chromatography (HPLC) column after derivatization with *o*-phthalaldehyde in order to facilitate fluorometric detection (8), and coupling the formation of phenylalanine with phenylalanine ammonia lyase (PAL) so that absorption of cinnamic acid at 290 nm can be monitored (9). All of the above methods are either arduous and time consuming or not sensitive enough.

This article describes a simple spectrophotometric assay procedure that couples the formation of phenylalanine with aromatic aminotransferase in the presence of 2-ketoglutarate so that phenylpyruvate absorption at 320 nm can be measured at basic pH.

MATERIALS AND METHODS

Biochemicals. L-Phenylalanine, 2-ketoglutarate (2-KG), dithiothreitol (DTT), *o*-phthalaldehyde (OPA), β-mercaptoethanol, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). DE52 was purchased from Whatman, Inc. (Clifton, NJ). Prephenate (PPA) was isolated

[1] Abbreviations used: dansyl-, 5-dimethylaminonaphthalene-1-sulfonyl-; PAL, phenylalanine ammonia lyase; 2-KG, 2-ketoglutarate; DTT, dithiothreitol; OPA, *o*-phthalaldehyde; PPA, prephenate; AGN, arogenate; Buffer A, 50 mM K phosphate buffer (pH 7.0) containing 1 mM DTT.

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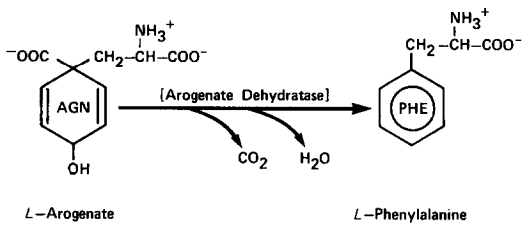

FIG. 1. The arogenate dehydratase reaction of L-phenylalanine biosynthesis.

as the barium salt from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (10) and was converted to the potassium salt before use. Potassium arogenate (AGN) was isolated from a triple auxotroph of *Neurospora crassa* as previously described (11). Prephenate is available from Sigma, but L-arogenate is not yet available from commercial sources. All other chemicals were standard reagent grade.

Organisms and growth conditions. Acinetobacter calcoaceticus ATCC 14987 and *Erwinia herbicola* 33243 were obtained from the American Type Culture Collection (Rockville, MD). *A. calcoaceticus* was grown at 30°C as described by Kaplan and Rosenberg (12) except that the medium was supplemented with 0.5% (w/v) casamino acids (Difco). *E. herbicola* was grown at 28°C in M9 medium as described by Winkler and Stuckman (13). The organisms were grown to the late exponential phase of growth, harvested by centrifugation, washed twice with 50 mM K phosphate buffer (pH 7.0) containing 1 mM DTT (Buffer A), and stored at −80°C until used.

Preparation of cell extracts. Frozen cell pellets of *A. calcoaceticus* and *E. herbicola* were suspended in Buffer A and disrupted by sonication, and the resulting extract was centrifuged at 150,000*g* for 1 h at 4°C. The supernatant is termed as crude extract.

Protein estimations were made by the method of Bradford (14) using bovine serum albumin as a reference standard.

Partial purification of aromatic aminotransferase from A. calcoaceticus. Approximately 100 mg of crude extract protein was loaded onto a DEAE-cellulose (DE52) column (1.5 × 20.0 cm) equilibrated with Buffer A. After the column was washed with 2 bed vol of the equilibration buffer, the bound proteins were eluted with a linear gradient of KCl (0.0–0.3 M) contained in the equilibration buffer in a total volume of 300 ml. Fractions of 2.2 ml were collected and were assayed for A_{280} and the aromatic aminotransferase activity.

Assay of aromatic aminotransferase. The reaction mixture in a final volume of 0.2 ml contained Buffer A, 1 mM L-phenylalanine, 5 mM 2-KG, and a suitable amount of enzyme. After incubation at 37°C for 20 min, the reaction was stopped by adding 0.8 ml of 2.5 N NaOH, and the absorbance was read at 320 nm. The enzyme activity was calculated using an extinction coefficient of 17,500 for phenylpyruvate (15). One unit of enzyme catalyzes the formation of 1 nmol of phenylpyruvate per minute at 37°C.

DE52 column chromatography. E. herbicola possesses a cyclohexadienyl dehydratase in addition to a specific prephenate dehydratase, and these two activities were separated by DE52 column chromatography.

Approximately 100 mg of crude extract protein of *E. herbicola* was fractionated on a DE52 column exactly as described above, and the column fractions were assayed for absorbance at 280 nm and for arogenate dehydratase activity by methods described below.

Coupled assay for arogenate dehydratase. The reaction mixture in a final volume of 0.2 ml contained Buffer A, 1 mM K arogenate, 10 mM 2-KG, 150 units of partially purified aromatic aminotransferase, and a suitable amount of enzyme. After incubation at 37°C for 20 min, 0.8 ml of 2.5 N NaOH was added and the absorbance was read at 320 nm.

OPA:HPLC method. Arogenate dehydratase activity was assayed as described previously (6). The reaction mixture in a final volume of 0.2 ml contained Buffer A, 1 mM K arogenate, and a suitable amount of en-

zyme. After incubation at 37°C for 20 min, 0.05 ml of 0.5 N NaOH was added and the phenylalanine formed was estimated by HPLC as described by Lindroth and Mopper (8).

Arogenate dehydratase assay with prephenate as substrate. Since the cyclohexadienyl dehydratase from *E. herbicola* can also use prephenate as substrate, the enzyme was also assayed as prephenate dehydratase. The reaction mixture in a final volume of 0.2 ml contained Buffer A, 1 mM K prephenate, and a suitable amount of enzyme. After incubation at 37°C for 20 min, 0.8 ml of 2.5 N NaOH was added and the absorbance was read at 320 nm.

RESULTS AND DISCUSSION

The elution profile of aromatic aminotransferase activities from *A. calcoaceticus* is shown in Fig. 2. Two peaks of activity eluted in the gradient fractions, a small leading peak of activity and a larger trailing peak of activity. The major peak of activity was pooled (fractions 153–168), concentrated by ultrafiltration on a PM-10 membrane (Amicon Corp.), and dialyzed against buffer A. This concentrated sample had 4.7 mg of protein and 1500 units of enzyme activity per milliliter. This aminotransferase exhibited a K_m of 1.2 mM for L-phenylalanine (as determined from a double-reciprocal plot) and did not use L-arogenate as amino-group donor. Both L-tyrosine and L-tryptophan were good amino-group donors, though less effective than L-phenylalanine. The aminotransferase uses oxaloacetate better than 2-KG as the keto-group acceptor. However, oxaloacetate absorbs at 320 nm and thus could not be used in the coupling assay. Pyruvate was a poor keto-group acceptor. A 0.1-ml aliquot of the concentrated sample (150 units) was used in the coupled-assay protocol.

The crude extract prepared from *E. herbicola* was assayed for arogenate dehydratase activity by the coupled assay and the prephenate dehydratase assay. Specific activities of 39.5 and 86.4 nmol phenylpyruvate formed per minute per milligram protein were obtained, respectively. When DE52 column fractions from *E. herbicola* were assayed for prephenate dehydratase activity, two peaks of activity were detected in the column fractions. A large peak of activity passed through the column without retardation while a second smaller peak of activity was detected in the gradient fractions (Fig. 3a). The latter peak of activity coincided with the elution position of chorismate mu-

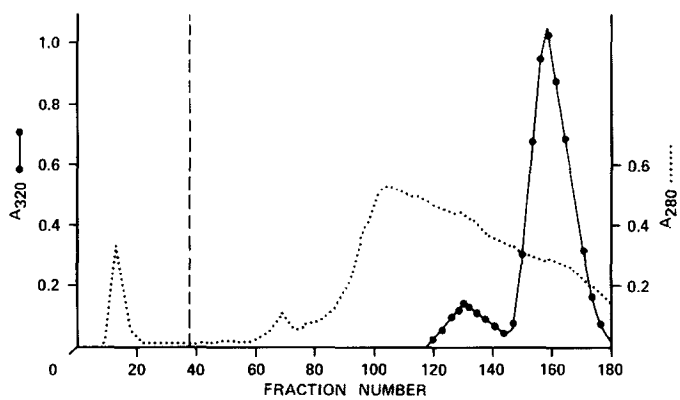

FIG. 2. Elution profiles from a DE52 column of aromatic aminotransferases from *A. calcoaceticus*. DE52 chromatography was performed as described under Materials and Methods. Vertical dashed lines indicate the onset point of gradient elution. Aromatic aminotransferase activity is expressed as A_{320}. The distribution of protein as measured by A_{280} is shown by dotted lines.

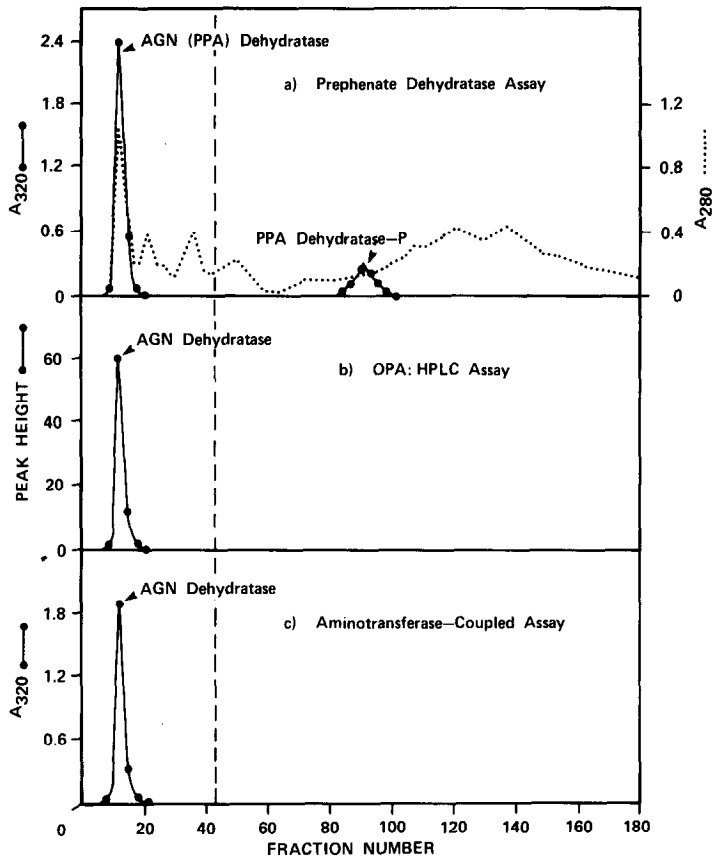

FIG. 3. Elution profiles from a DE52 column of arogenate dehydratase from *E. herbicola* (a) by an indirect assay using prephenate as substrate, (b) by the OPA:HPLC assay method, and (c) by the aminotransferase-coupled assay described in this paper. DE52 chromatography was performed as described under Materials and Methods. Vertical dashed lines indicate the onset point of gradient elution. Arogenate dehydratase activity is expressed as A_{320} for (a) and (c) and as peak height for (b). The distribution of proteins as measured by A_{280} is shown by the dotted line (a).

tase activity and is referred to as prephenate dehydratase-P, a catalytic component of the bifunctional P-protein of phenylalanine biosynthesis (16). Arogenate dehydratase from purple sulfur bacteria (Superfamily-B prokaryotes) can use prephenate as an alternative substrate (1). *E. herbicola* is an enteric bacterium possessing cyclohexadienyl dehydratase in addition to the bifunctional P-protein as key enzymes making up the dual pathways of phenylalanine biosynthesis (see Ref. (1) for details). When the column fractions were assayed with L-arogenate as substrate by the OPA:HPLC method, a single peak of activity (Fig. 3b) that coincided with the first peak of activity shown in Fig. 3a was obtained. When the column fractions were assayed by using the aminotransferase-coupled assay described in this paper, the same peak of activity was again obtained (Fig. 3c).

To compare the three methods, the specific activity of arogenate dehydratase in the peak fraction (fraction 12) was estimated by the OPA:HPLC assay, by the aminotransferase-coupled assay, and by the prephenate dehydratase assay. The results are presented in Table 1. The specific activity for arogenate dehydratase by the aminotransferase-cou-

pled assay was about 35% lower than the value obtained by the OPA:HPLC assay method. The results show that the arogenate dehydratase of *E. herbicola* exhibits a preference for prephenate over arogenate as substrate. The aminotransferase-coupled assay is linear with increasing protein concentration up to an absorption value of 2.0 at 320 nm and the phenylpyruvate formation is linear with respect to reaction time after the first 2 min (data not shown). Unlike the PAL-coupled assay, the aminotransferase-coupled assay could be used for arogenate dehydratase assay in crude cell extracts, as the interference due to protein absorption at 320 nm is very little. The present method may be useful in screening large number of fractions such as in a column profile for the arogenate dehydratase activity from plants (4,5) and microorganisms (2,3) that use only arogenate as substrate. The present assay may be particularly useful in systems where arogenate dehydratase is very sensitive to end product inhibition (4,5) because L-phenylalanine formed is removed by the coupling enzyme.

Although we have not checked the ability of commercially available aminotransferases to function in the coupled assay, they would be suitable for use in the coupled assay if they qualify on three criteria: (i) a low K_m for L-phenylalanine exists, (ii) L-arogenate will not function as an amino-group donor, and (iii) arogenate dehydratase activity does not contaminate the aminotransferase preparation (*A. calcoaceticus* does not possess arogenate dehydratase).

TABLE 1

COMPARISON OF SPECIFIC ACTIVITIES OBTAINED WITH AROGENATE DEHYDRATASE IN THE PEAK FRACTION (FIG. 3) BY VARIOUS ASSAY METHODS

Method	Specific activity[a]
OPA:HPLC assay with arogenate as substrate	1.56
Aminotransferase-coupled assay with arogenate as substrate	0.97
Assay with prephenate as substrate	1.97

[a] One unit of enzyme catalyzes the formation of 1 μmol of product per minute under the assay conditions described under Materials and Methods. Specific activities are expressed as units per milligram protein.

ACKNOWLEDGMENTS

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