Purification and General Properties of Argininosuccinate Lyase from Jack Bean, *Canavalia ensiformis* (L.) DC

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1. Argininosuccinate lyase (EC 4.3.2.1) from jack bean [*Canavalia ensiformis* (L.) DC] seeds was purified 532-fold from an acetone–butanol-dried powder. 2. The enzyme functions reversibly and exhibits maximum stability at 16°. 3. At 16° it has a half-life ($t_{1/2}$) of 263 min. 4. The enzyme is both cold-labile ($t_{1/2}$ 131 min. at 0°) and heat-inactivated ($t_{1/2}$ 74 min. at 38°); inactivation appears to be irreversible. 5. Treatment of the acetone–butanol-extracted powder with sodium dodecyl sulphate increased the sensitivity of the enzyme to temperature ($t_{1/2}$ 70 min. at 0°; $t_{1/2}$ 23 min. at 38°). 6. Addition, to the purified enzyme, of a fraction containing lipid from the seed increased the half-life to about 510 min. at either 0° or 38°. 7. Arginine or homoarginine, and to a smaller extent some other amino acids or fumarate, protected the enzyme from cold-inactivation. 8. Reactivation attempts with both the cold- and heat-inactivated enzyme failed. 9. The K_m value for argininosuccinate at pH 7.5 is 1.3×10^{-4} . 10. The enzyme was inactivated completely within 15 min. at 16° by 0.5 mM-*p*-hydroxymercuribenzoate, and subsequent exposure to 5 mM-cysteine had no restorative effect.

Argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) catalyses a reversible non-hydrolytic cleavage of L-argininosuccinate to L-arginine and fumarate (Ratner, Anslow & Petrack, 1953), and is operative in the ornithine–urea cycle. Isolation of the enzyme from plant material was first achieved by Davison & Elliott (1952), by isoelectric precipitation of an aqueous extract of dry pea meal. Walker & Myers (1953), demonstrated the presence of argininosuccinate lyase in commercial jack-bean meal and in *Chlorella pyrenoidosa*. Subsequently, Buraczewski, Kleczkowski & Reifer (1960) reported increased amounts of arginine in wheat and rye-grass seedlings that had been supplied with L-argininosuccinate. Although Ennis & Gorini (1961) demonstrated the occurrence of this enzyme in various strains of *Escherichia coli*, their study did not include a description of purification procedures. While our investigation was in progress, Cohen & Bishop (1966) published a procedure for the isolation of argininosuccinate lyase of high specific activity from *Neurospora crassa*.

Havir, Tamir, Ratner & Warner (1965) developed a procedure for preparing crystalline argininosuccinate lyase from ox liver. Their crystallized enzyme was stable at 38° in both tris and phosphate

buffer systems, but was reversibly cold-labile in tris buffer. Thermal reactivation of the cold-inactivated enzyme occurred after several minutes at 38°. Arginine and argininosuccinate (2 mM) and phosphate (50 mM) prevented cold-inactivation.

Thiol-group titration studies of the fully active crystallized enzyme demonstrated the presence of one freely available thiol group/enzyme molecule. However, the inability of a wide variety of reagents to inhibit the enzyme led Havir *et al.* (1965) to suggest that this thiol group is not essential for enzymic activity.

This paper summarizes results from our studies of the argininosuccinate-cleaving enzyme present in vascular-plant material. Our investigations reveal several differences between the argininosuccinate lyase isolated from jack bean and that from ox liver.

EXPERIMENTAL

Materials

L-Argininosuccinate (barium salt) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Free argininosuccinic acid was prepared from the barium salt by the addition of 1.0 ml. of 0.5 M- K_2SO_4 /50 μ moles of substrate. Sodium dodecyl sulphate was purchased from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A., and free base L-arginine from Sigma Chemical Co., St Louis, Mo., U.S.A. Crystalline fumarase, disodium fumarate, and all amino acids, except L-arginine, were obtained from Cal-

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biochem. Jack-bean (*Canavalia ensiformis*) seeds were bought from Mr Ernest Nelson, Waldron, Ark., U.S.A.

Calcium phosphate gel was prepared by the procedure of Keilin & Hartree (1938) as outlined by Dixon & Webb (1964), and was aged for 6 months in the dark at 2°.

Methods

Arginine assay. Arginine assays were conducted with double-strength reagents in a final volume of 6.0 ml. by the Sakaguchi (1925) method as modified by Van Pilsum, Martin, Kito & Hess (1956). Over the range 0.02–0.2 mM, E_{518} was proportional to arginine concentration.

Definition of activity. One unit of enzyme catalyses the formation of 1 μ mole of arginine/min. under the described conditions. Specific activity is defined as milliunits/mg. of protein. Protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine plasma albumin as a standard.

Enzyme assay. The standard assay mixture contained 4.0 μ moles of freshly prepared argininosuccinate, 100 μ moles of potassium phosphate buffer, pH 7.5, and excess of fumarase. After 2 min. equilibration at 30°, the total volume was adjusted to 2.0 ml. by the addition of not more than 35 milliunits of enzyme. Similarly incubated reaction mixtures containing all of the components except substrate were used as controls. Argininosuccinate itself does not produce a significant colour in the modified Sakaguchi reaction.

After incubation for 20 min. at 30°, 3.0 ml. of 10% (w/v) trichloroacetic acid was added. After centrifuging at 20 000g for 10 min., duplicate samples (1.0 ml.) of the supernatant solution were assayed for arginine. Care was taken to prevent contamination of the arginine-assay tubes with precipitated protein.

Argininosuccinate lyase isolated directly from hydrated seeds showed far less activity than an equivalent sample of acetone–butanol-dried powder, and it was suspected that the very high concentration of lipid present interfered with enzymic activity and its measurement. Experiments supported this conclusion. Further, lipids interfered with all procedures used to purify this enzyme directly from the hydrated seed. An acetone–butanol-dried powder therefore served as the starting material for the enzyme used in most of the studies reported here.

RESULTS

Purification of the enzyme. Jack-bean seeds, submerged in water for 24 hr., were peeled and then stored overnight at –20°. Freezing of the seeds, before the preparation of the acetone–butanol-dried powder, increased the yield of enzyme. The frozen seeds were blended with 2 vol. of freshly distilled acetone at –20°. The resulting mash was stirred with an additional 3 vol. of cold acetone, and the suspension filtered by suction.

Freshly distilled butan-1-ol (3 vol.) at –20° was mechanically stirred with the acetone-extracted cake for 4 min. in a sodium chloride–ice bath. The suspension was filtered (20–30 min.) at 2°. Butanol treatment removes acetone-insoluble phospholipids (Morton, 1955).

The acetone–butanol-dried cake was spread out at room temperature until the residual butanol evaporated. After drying, the meal was ground into a fine powder in a mortar and stored at –20°. The weight of the final powder was 40–45% of that of the hydrated seeds.

Step I. Preparation of the homogenate. Unless otherwise indicated, the buffer system was 50 mM-potassium phosphate buffer, pH 7.5, and centrifuging was conducted at 12 000g for 13 min. Acetone–butanol-dried powder (45 g.) was extracted with 250 ml. of buffer for 60 min. at 2°. After filtration through four layers of cheesecloth and centrifuging, the supernatant solution was diluted with buffer to 215 ml.

To minimize inhibition by arginine and canavanine, the activity was determined after dialysing 10 ml. samples of the supernatant solution for 8 hr. at 16°. The main solution was dialysed against approx. 600 ml. of buffer containing 0.1 mM-EDTA.

Step II. Ammonium sulphate fractionation. The homogenate was adjusted to 25% saturation by adding saturated ammonium sulphate at pH 6.6. After standing at 2° for 10 hr., the turbid solution was centrifuged for 20 min. (After 10 hr. a gradual decrease in the enzyme activity of the solution occurred.) The precipitate was discarded. The supernatant solution, adjusted to 37% saturation with ammonium sulphate, was kept for 90 min. and centrifuged.

At this stage of purification the enzyme became cold-labile, and, unless otherwise indicated, all subsequent purification steps were conducted at room temperature. The precipitate was suspended in 20 mM-phosphate buffer, pH 6.8, homogenized and diluted to 100 ml.

Step III. Negative adsorption with calcium phosphate gel. Calcium phosphate gel suspension (10 ml., 32.1 mg. dry wt./ml.) and 440 μ moles of L-arginine were added to the suspended residue of step II. The resulting mixture was stirred mechanically for 15 min. and centrifuged at 31 000g for 12 min.

Step IV. Second ammonium sulphate fractionation. Saturated ammonium sulphate, pH 7.75, was added to the supernatant solution to make it 35% saturated. After 45 min., the precipitated protein was removed by centrifugation at 31 000g for 15 min. and dissolved in 15–16 ml. of buffer. Sometimes the precipitate was stored overnight at –20° in ammonium sulphate solution.

Step V. Sephadex gel filtration. The dissolved protein from step IV was passed through a column (2.5 cm. $\times$ 45 cm.) of Sephadex G-200. The enzyme was eluted with buffer at 16° containing 4 mM-L-arginine hydrochloride, at a flow rate of 36 ml./hr. Fractions (3 ml.) were collected (Fig. 1) and the contents of the three tubes containing the most

enzyme activity were pooled for further purification. As shown in Fig. 1, excellent separation of the enzyme from other proteins was achieved with Sephadex.

Step VI. Third ammonium sulphate fractionation. The pooled eluate was adjusted to 25% saturation with ammonium sulphate solution (pH 7.75). After 30 min. the precipitated protein was collected by centrifugation at 31000g for 13 min. and the supernatant solution was adjusted to 33% saturation with ammonium sulphate. The protein precipitate was allowed to form for 45 min., collected by centrifuging for 13 min. at 31000g and dissolved in 2–3 ml. of buffer. A summary of the purification procedure for the isolation of the enzyme is given in Table 1.

In certain experiments enzyme treated with sodium dodecyl sulphate was used. Such treated enzyme was prepared by the addition of 3.5 g. of sodium dodecyl sulphate to the acetone–butanol-dried powder before extraction with the buffer (see Step 1). Argininosuccinate lyase prepared in

this manner is referred to below as 'treated' enzyme.

Characterization of the reaction. Identification of fumarate as one of the reaction products was established by incubating in a spectrophotometer cuvette 3 μ moles of L-argininosuccinate, 25 milliunits of enzyme and 100 μ moles of buffer, pH 7.5, (final vol. 3.0 ml.). A linear increase in E_{240} occurred, due to fumarate formation (Racker, 1950). The addition of fumarase resulted in a rapid decrease in E_{240} .

To determine whether the enzyme catalyses arginine-dependent fumarate utilization, 3.0 ml. of a mixture containing 1.2 μ moles of fumarate, 100 μ moles of phosphate buffer, pH 7.5, 25 milliunits of enzyme and either water or 5.0 μ moles of arginine was monitored at 240 nm. A linear decrease in extinction occurred only when arginine was present, indicating that the argininosuccinate-cleaving reaction is reversible.

Effect of substrate concentration. The K_m value for argininosuccinate at pH 7.5 was about

Fig. 1. Elution of protein from step IV from Sephadex G-200 (see the text). Argininosuccinate lyase activity was assayed in the standard system, with 0.2 ml. of each fraction. $E_{280}^{1\text{cm}}$ was measured on fractions diluted 16-fold. ●, Arginine formed; ○, E_{280} .

Table 1. *Purification of argininosuccinate lyase from acetone–butanol dried Canavalia ensiformis seeds*

Step	For details see the text.				
	Volume (ml.)	Total protein (mg.)	Total enzyme (milliunits)	Specific activity (units/mg. of protein)	Yield (%)
I. Extract	215	9250	10295	1.1	
II. First $(\text{NH}_4)_2\text{SO}_4$ fraction	100	490	9935	20.3	97
III. Calcium phosphate gel supernatant	110	240	8965*	37.4	87
IV. Second $(\text{NH}_4)_2\text{SO}_4$ fraction	16	89	7440	83.6	72
V. Sephadex G-200 eluate	9	5.5	1695*	308.2	16
VI. Third $(\text{NH}_4)_2\text{SO}_4$ fraction	2	1.4	820	585.7	8

* Corrected for arginine inhibition.

Table 2. *Effect of various temperatures on the activity of 'treated' (see the text) argininosuccinate lyase after standing for 90 min. in 50 mM-potassium phosphate buffer, pH 7.5*

The reaction mixture contained 100 μ moles of potassium phosphate buffer, pH 7.5, 4.0 μ moles of argininosuccinate, excess of fumarase and 347 μ g. of enzyme solution (specific activity 78 milliunits/mg. of protein). The final volume was 2.0 ml. Incubation was conducted for 20 min. at 30°.

Temp.	L-Arginine formed in 20 min. (μ mole)
Initial activity	0.50
0°	0.22
11	0.41
16	0.42
22	0.34
30	0.17
38	0.02

Table 3. *Inactivation rate constants and half-life values for argininosuccinate lyase*

See the text for details of treatment.

Treatment	Temp.	Rate constant (min. ⁻¹)	Half-life (min.)
None	0°	5.30×10^{-3}	131
	16	2.64×10^{-3}	263
	38	9.39×10^{-3}	74
Sodium dodecyl sulphate	0	9.96×10^{-3}	70
	16	3.02×10^{-3}	229
	38	2.95×10^{-2}	23

1.3×10^{-4} M. The substrate concentration used in the standard assay mixture was virtually saturating.

Cold-lability and heat-inactivation. The effect of standing at various temperatures on the activity of 'treated' argininosuccinate lyase is shown in Table 2. Stability of the 'treated' and untreated argininosuccinate-cleaving enzyme is compared at 0°, 16° and 38° in Table 3.

The data of Table 3 reveal a decrease in the half-life ($t_{1/2}$) for the 'treated' enzyme compared with the untreated enzyme. At 16° the half-life difference was small, but at 0° and 38° the difference was about twofold and threefold respectively. In contrast, argininosuccinate lyase prepared directly from the hydrated seed rather than from an acetone-butanol-dried powder had a half-life of about 510 min. at either 0° or 38°.

Protection against cold-lability and heat-inactivation. The instability of the 'treated' enzyme at 0° and 38° could be overcome to different degrees by the addition of the reagents listed in Table 4. A high degree of protection against cold-lability was conferred by addition of substrates fostering the reversal of argininosuccinate cleavage. Complete protection against cold-lability was provided by arginine or by arginine plus fumarate.

Table 4. *Capacity of various reagents to protect argininosuccinate lyase from cold-lability and heat-inactivation*

Sodium dodecyl sulphate-treated enzyme (0.95 ml.) was mixed with 4 μ moles of the indicated reagents in a final volume of 1.0 ml. After standing at the given temperature for 90 min., 0.1 ml. samples were removed for assay. The reaction mixture contained 100 μ moles of potassium phosphate buffer, pH 7.5, 4.0 μ moles of argininosuccinate and excess of fumarase in a final volume of 2.0 ml. Arginine was assayed, as described in the text, after 20 min. incubation at 30°.

Additions	% of initial activity remaining after 90 min. at 0°	% of initial activity remaining after 90 min. at 38°
Water	35	8
Arginine	96	—
Canavanine	67	30
Fumarate	62	26
Arginine + fumarate*	94	—
Water	39	6
Citrulline	52	17
Ornithine	48	—
Glycine	40	—
Aspartate†	39	6
Homoarginine	90	—
Water	36	9
Cysteine	48	17
Methionine	57	20
Reduced glutathione	42	12
Water	38	—
Isoleucine	46	—
Phenylalanine	43	—
Valine	71	—

* Two μ moles of each.

† Aspartic acid (0.10 ml.) treated with 0.90 ml. of enzyme solution.

Excellent protection was provided by homo-arginine and very good protection by valine, canavanine, methionine and fumarate.

Reversal of cold-lability. 'Treated' and untreated enzyme was exposed to 0° for 90 and 110 min. respectively in the presence or absence of 4 mM-L-canavanine, and then reactivation at 38° for 8 min. was attempted. No reactivation occurred, in contrast with the results of Havir *et al.* (1965), who found that cold-inactivated argininosuccinate lyase from ox liver could be fully reactivated by incubation for 6 min. at 38°. Although canavanine is capable of decreasing cold-inactivation of the enzyme, this protecting agent does not enhance thermal reactivation of either the treated or the untreated enzyme.

Lipid recombination study. A single experiment was performed to ascertain if highly purified argininosuccinate lyase could be protected against heat-inactivation by the addition of lipid material

from hydrated seeds. The lipid material was extracted as follows.

Approx. 50g. of hydrated seeds were blended with 125ml. of 50mM-potassium phosphate buffer, pH7.5. After centrifugation, the supernatant solution was adjusted to 40% saturation with saturated ammonium sulphate, pH6.6. After 90min. the suspension was centrifuged. Most of the lipid floated as a cake; this material was dialysed exhaustively against water at 0°. A suspension of the enzymically inactive 'lipid fraction' (containing some protein) in 50mM-potassium phosphate was added to an equal volume of highly purified enzyme, and the mixture was exposed to 38° for 60min. The enzyme, thus protected, synthesized nearly five times as much arginine as did enzyme similarly warmed in the absence of the 'lipid fraction'.

Disc electrophoresis (Davis, 1964) of the third ammonium sulphate fraction at pH7.2 and pH8.6 revealed a single, minor, contaminant band migrating above the argininosuccinate lyase.

Effect of thiol reagents. Incubation of a control sample of enzyme for 30min. at 16° caused a decrease in activity of 7%; with 5mM-iodoacetate and 5mM-N-ethylmaleimide the decrease was 23% and 57% respectively. *p*-Hydroxymercuribenzoate was much more effective, causing complete loss of activity within 15min. at a concentration of 0.05mM; this was not reversed by 5mM-cysteine. Both untreated and 'treated' enzymes were inhibited similarly.

Enzyme storage. Normally enzyme preparations were stored as a pellet in ammonium sulphate solution, after the final centrifugation at step IV or VI, at -20° for 2-4 days with little loss in activity. Dissolved enzyme, however, was stored in the presence of 4mM-L-canavanine at -20°. In both cases the enzyme was rapidly frozen in an acetone-solid carbon dioxide bath before storage. The enzyme can also be stored as a freeze-dried preparation.

DISCUSSION

This is the first report of highly purified argininosuccinate lyase from seed plants. The 523-fold purified enzyme appeared to contain only minor contamination as judged by disc electrophoresis at pH7.2 and pH8.6. With increasing purity a decrease in stability was noted, and a lipid-containing fraction derived from the seed restored stability. Jack-bean argininosuccinate lyase purified from an acetone-butanol-dried powder is a cold-labile and heat-sensitive enzyme (Table 2). Loss of activity is apparently irreversible. It is suggested that this enzyme is, *in situ*, associated with and stabilized by lipid material.

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