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SHORT COMMUNICATION

PURIFICATION OF ARGININOSUCCINATE SYNTHETASE FROM COTYLEDONS OF GERMINATING PEAS

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Abstract—Previous attempts to separate argininosuccinate synthetase (L-citrulline: L-aspartate ligase (AMP), E.C. 6.3.4.5), and argininosuccinate lyase (L-argininosuccinate arginine lyase, E.C. 4.3.2.1) from each other in extracts of higher plant tissues have been unsuccessful. Data previously published on plant argininosuccinate synthetase has involved assays utilizing the presence of argininosuccinate lyase. In the present study, conditions were established to improve the stability of the combined enzyme system to storage at 2°, and subsequently the synthetase enzyme was purified to yield a preparation that was free of lyase activity.

INTRODUCTION

PREVIOUS communications[1,2] have dealt with the partial purification and properties of argininosuccinate lyase and argininosuccinate synthetase from extracts of the cotyledons of germinating peas. Neither the procedure used for the partial purification of lyase enzyme,[1] nor that used for the synthetase enzyme[2] was found to be capable of separating the two enzyme activities, which together catalyse the following overall reaction: ^{14}C-(carbamyl) L-citrulline + L-aspartate + ATP $\xrightarrow{Mg^{+2}}$ ^{14}C-arginine + fumarate + AMP + PPi.[2]

When further experiments were initiated on preparations containing both the lyase and synthetase enzymes, it was found that the overall activity was unstable on storage at 2°, in the Tris acetate or potassium phosphate buffers (both 0·1 M, pH 7·9), previously used for the preparation of enzyme extracts (Fig. 1). Thus experiments were carried out firstly to improve this stability, and secondly to obtain a preparation of the synthetase enzyme free of lyase activity.

RESULTS

Four of the buffers used by Good *et al.*[3] were investigated to ascertain their effect on enzyme stability. The four chosen all had pKA values close to that of the pH optimum of the enzyme system (pH 7·9). The buffers were Hepes (pKA at 20°=7·55), Tricine (pKA at 20° = 8·15), Bicine (pKA at 20° = 8·35), and Taps (pKA at 20° = 8·4) all were used at a concentration of 0·1 M, and pH 7·9. Hepes and Tricine were found to have the most marked effect on the preservation of enzyme activity at 2° (Fig. 1).

It was found that the substrate components of the enzyme assay system used for the synthetase enzyme[2] also improved the stability of this system at 2° (Fig. 2). When 3 μmoles of ATP plus 5 μmoles of Mg^{+2} were present per ml of enzyme preparation in tricine buffer (0·1 M, pH 7·9), 52% of the activity remained after 4 days at 2° (Fig. 2). When L-aspartate was used in the amount of 2 μmoles per ml of enzyme preparation, then 57% of the activity

[1] P. D. SHARGOOL and E. A. COSSINS, *Can. J. Biochem.* **46**, 393 (1968).
[2] P. D. SHARGOOL and E. A. COSSINS, *Can. J. Biochem.* **47**, 467 (1969).
[3] N. E. GOOD *et al.*, *Biochemistry* **5**, 467 (1966).

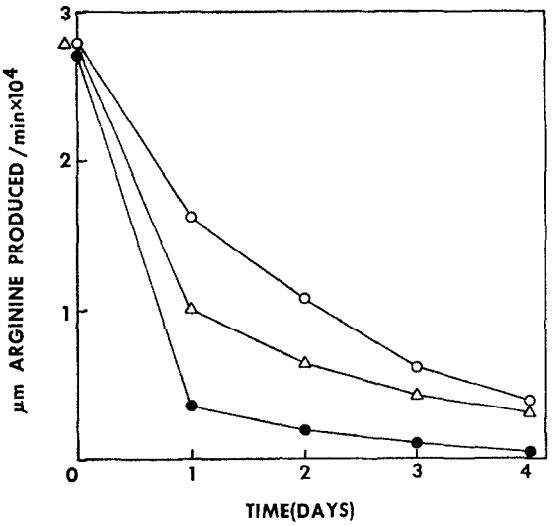

FIG. 1. THE EFFECT ON OVERALL ENZYME ACTIVITY (IN μmoles ARGININE/min × 10^4) OF STORAGE IN VARIOUS BUFFER SYSTEMS (0·1 M, AND pH 7·9). TRIS (OR PHOSPHATE) (—●—●—); TRICINE (—○—○—); HEPES (—△—△—).

remained after 4 days (Fig. 2). These results are in marked contrast to those obtained with Tris or phosphate buffer, when no activity remained after 4 days (Fig. 1). In subsequent experiments L-aspartate (2 μmoles per ml) was used in conjunction with Tricine or Hepes buffers (0·1 M, pH 7·9) to stabilize enzyme preparations to storage at 2°.

The necessity of free sulphydryl groups for enzyme activity was investigated by the addition of *p*-choloromercuribenzoate to assay mixtures. Activity was found to diminish rapidly with concentrations between 10^{-4} and 10^{-3} M and fell almost to zero with 10^{-3} M *p*-chloromercuribenzoate. Addition of 10^{-4} to 10^{-3} M 2-mercaptoethanol to enzyme preparations did not improve their stability in Hepes or Tricine and mercaptoethanol at 10^{-2} M was found to markedly decrease the stability.

After the stability of enzyme preparations to storage at 2° had been improved, a number of methods were tried in order to separate the lyase and synthetase activities in G200 Sephadex-treated fractions.[2] Analytical polyacrylamide gel electrophoresis of these fractions yielded a large number of protein bands but attempts to elute these bands using Hepes or Tricine buffer (0·1 M, pH 7·9) were unsuccessful. Preparative polyacrylamide gel electrophoresis using Tris, Hepes or Tricine buffers at 2° was also unsuccessful. Attempts to adapt the affinity chromatography technique of Cautrecasas *et al.*[4] to the purification of argininosuccinate synthetase, were also unsuccessful. No fractions possessing synthetase activity, or the combined synthetase plus lyose activity were found after using any of the above methods.

However, electrofocussing of G200 Sephadex fractions,[2] using ampholine gradients of pH 3–10, or 4–6, was found to yield a fraction (pH 4·2), that contained synthetase activity only (^{14}C-(carbamyl) L-citrulline + L-aspartate + ATP $\xrightarrow{Mg^{2+}}$ ^{14}C-argininosuccinate + AMP + PPi). After subjecting to analytical polyacrylamide gel electrophoresis, the pH 4·2 fraction appeared to be essentially homogeneous when compared with the material before electrofocussing treatment. When fractions from the pH 4 3 region of the electrofocussing run were mixed with the pH 4·2 fraction, then the overall reaction yielded

[4] P. CUATRECASAS *et al.*, *Proc. Nat. Acad. Sci.* **61**, 636 (1968).

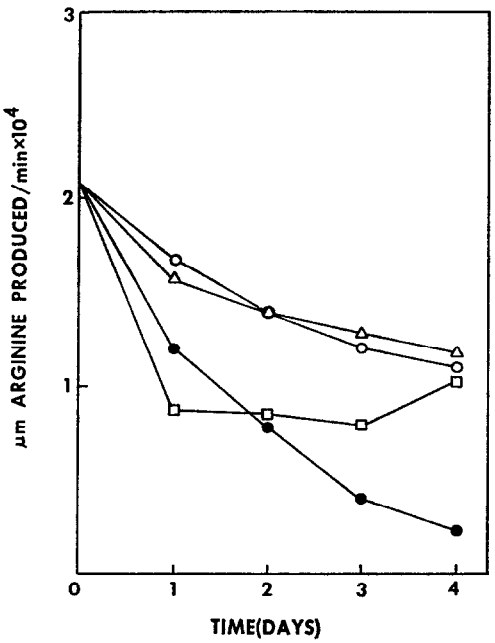

FIG. 2. THE EFFECT ON OVERALL ENZYME ACTIVITY OF STORAGE IN THE PRESENCE OF ATP (3 μmoles/ml) + Mg^{+2} (5 μmoles/ml) —○—○—; ASPARTATE (2 μmoles/ml) —△—△—; CITRULLINE (2 μmoles/ml) + ATP (3 μmoles/ml) —□—□—, ALL DISSOLVED IN 0·1 M, pH 7·9 TRICINE. TRICINE ALONE —●—●—.

^{14}C-labelled arginine was regained. Thus the pH 4·3 fraction must contain argininosuccinate lyose activity. From Table 1 it can be seen that the final preparation of argininosuccinate synthetase obtained, was purified more than 400-fold over the crude ammonium sulphate fraction.[2]

TABLE 1. STAGES IN PURIFICATION OF ARGININOSUCCINATE SYNTHETASE

Stage of purification*	Total enzyme units* (μmoles/min/ml × 10^3)	Specific activity (units/mg protein × 10^3)
1. Crude $(NH_4)_2SO_4$ fraction	115·5	0·03
2. Sephadex G-50 treated enzyme	376·2	0·16
3. Sephadex G-200 treated enzyme†	8·0	0·06
4. Enzyme from electrofocussing†	0·51	12·8

* For details of fractionation and units of enzyme activity see Ref. 2.
† The total enzyme units for stages 3 and 4, are expressed per 2 ml of enzyme.

DISCUSSION

Both Shargool and Cossins[2] and Grabarek-Bralczyk *et al.*[5] have previously experienced difficulty in trying to separate the argininosuccinate lyase and synthetase enzymes extracted from higher plant tissues. Both groups of workers were unable to obtain a separation of the two enzymes using ammonium sulphate fractionation, a technique successfully employed by Ratner and Petrack[6] with mammalian enzymes. The reasons for the discrepancy are not known.[5]

[5] J. GRABAREK-BRALCZYK *et al.*, *Acta. Biochim. Polon.* **16**, 151 (1969).
[6] S. RATNER and B. PETRACK, *J. Biol. Chem.* **191**, 693 (1951).

The results presented here show that the argininosuccinate synthetase enzyme isolated from pea cotyledons is markedly unstable to periods of storage in 0·1 M, pH 7·9 Tris acetate or potassium phosphate buffers at 2°, but that stability can be improved by the use of buffers containing Hepes or Tricine, and substrates of the synthetase enzyme. The response to phosphate buffer is in marked contrast to the lyase enzyme isolated from pea cotyledons which showed no diminution in activity after storage at 2° in 0·1 M potassium phosphate buffer (pH 7·9) for 18 hr.[1]

By subjecting the purest enzyme preparations previously obtained (G200 Sephadex fractions) to electrofocussing, the synthetase enzyme was finally freed of lyase activity, as judged by the inability of the preparation to convert ^{14}C-labelled argininosuccinate to ^{14}C-arginine. This preparation of argininosuccinate synthetase appeared to be essentially homogeneous as judged by polyacrylamide gel chromatography.

Rosenthal and Naylor[7] have recently purified argininosuccinate lyase from Jack beans, but as far as the author is aware the present paper is the first report of the isolation of argininosuccinate synthetase from a plant source.

EXPERIMENTAL

Tricine, Hepes, Taps and Bicine,[3] were all obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. L-Citrulline (carbamyl)-^{14}C, was obtained from Amersham Searle, Don Mills, Ontario. Sephadex gels came from Pharmacia (Canada) Ltd , Montreal, P.Q. Cotyledons were obtained from seeds of *Pisum sativum* L. (var. Homesteader), germinated for 24 hr.

The partial purification of argininosuccinate synthetase, and its assay using ^{14}C-labelled citrulline, were carried out as previously described [2] An additional colorimetric assay procedure used to monitor synthetase activity in fractions from columns, was that of Gerhard and Pardee [8] In order to ascertain the effect of various substances on the stability of the lyase-synthetase mixture, the enzymes were equilibrated with the desired solution at the Sephadex G50 stage of purification.[2]

Analytical chromatography was performed using a 5% polyacrylamide gel and the Tris buffer system of Davies.[9] Gels were stained using Coomassie blue dye, and the method of Chrambach *et al.*[10] The preparative polyacrylamide gel electrophoresis apparatus was purchased from Quickfit Inc., Fairfield, New Jersey.

For affinity chromatography, the technique of Cuatrecasas *et al.*[4] was modified as follows. Activated Sepharose, with either L-asparate or L-citrulline, was packed into a 1 × 25 cm column. Enzyme from the G50 stage of purification[2] was run through the column and eluted with the remaining components of the synthetase assay mixture Thus, when aspartate was coupled to the column, citrulline, ATP, and Mg^{+2} were used in the elution buffer, at concentrations equivalent to those used in the isotope assay mixture.[2] When citrulline was coupled to the column, aspartate was substituted for citrulline in the eluting buffer. Subsequent attempts to obtain an active enzyme preparation utilized elution with 0·1 M acetic acid.[4]

Electrofocussing was carried out using an 8101 electrofocussing column (L K B. Produktor Co., Stockholm, Sweden). The operating instructions were modified slightly, in order to keep the protein fractions separated in solution, and prevent precipitation and subsequent mixing. Thus 3 g of Ampholine solution were used instead of 1 g. Also only one 2 ml G200 Sephadex fraction (usually the peak one), equivalent to approximately 9 mg of protein was used per run. Separation was effected in about 35 hr, at 2°, using 300V

Samples from all columns were monitored for protein at 280 nm. Protein was also estimated by the method of Lowry *et al.*[11] with bovine serum albumin as standard. Buffer interference[12] was eliminated either by dialysing the samples against phosphate buffer or by preparing the standards in the appropriate Good buffer.

Acknowledgements—The author wishes to thank the National Research Council of Canada for a grant in aid of research.

[7] G. A. ROSENTHAL and A. W. NAYLOR, *Biochem. J.* **112**, 415 (1969).
[8] J. C. GERHART and A. B. PARDEE, *J. Biol. Chem.* **237**, 891 (1962).
[9] B. J. DAVIS, *Ann. N.Y Acad. Sci.* **121**, 404 (1964).
[10] A. CHRAMBACH *et al.*, *Anal Biochem.* **20**, 150 (1967).
[11] O. H. LOWRY *et al.*, *J. Biol. Chem.* **193**, 265 (1951).
[12] J. D. GREGORY and S. W. SAJDFRA, *Science* **169**, 97 (1970).