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Higher Plants Contain L-Asparate Oxidase, the First Enzyme of the Escherichia coli Quinolinate Synthetase System*+

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Summary: Cotton callus cells contain an L-aspartate oxidase which does not appear to be active with D-aspartate, L-glutamate or D- or L-alanine. The enzyme requires for activity a dialyzable cofactor with an apparent molecular weight of 1,050. Since L-aspartate oxidase is the first enzyme of the pathway for de novo synthesis of the pyridine ring in Escherichia coli, this finding suggests that higher plants may use the L-aspartate-dihydroxyacetone phosphate pathway for de novo pyridine nucleotide biosynthesis.

Quinolinic acid (QA) is a precursor of the pyridine nucleotides in a wide variety of organisms. In Escherichia coli QA is synthesized from L-aspartate and dihydroxyacetone phosphate by two enzymes which we have designated A and B (1-3). Recent studies in our laboratory have shown that in E. coli, B protein, which catalyzes the first step in QA synthesis, is an FAD-requiring L-aspartate oxidase (4). The details of QA synthesis in higher plants have not been definitely elucidated, but the majority of isotopic studies indicate that QA is probably formed from aspartate and a 3-carbon glycolytic intermediate (5,6). These findings suggested that higher plants may contain the same QA synthetic pathway as E. coli. Therefore, we examined plant tissues for the presence of L-aspartate oxidase and A protein activities. Cultured cotton callus tissue proved to be a good source of L-aspartate oxidase, but all activity was lost when purification was attempted. This activity loss was found to be due to loss of a dialyzable cofactor during purification. Herein we describe some properties of cotton tissue L-aspartate oxidase and its as yet unidentified cofactor.

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Materials and Methods: Cotton callus tissue (Acala 44) was grown as previously described (7) except that niacin was omitted from the growth medium. Callus tissue was used for L-aspartate oxidase or Small Molecule Cofactor (SMC) preparation 2-4 weeks after transfer to fresh medium. L-aspartate oxidase was partially purified from callus tissue as follows: Step 1 - 100 gm of callus and 100 gm of polyvinylpyrrolidone were ground in 100 ml of 50 mM KPi, pH 8.0, (buffer 1) in an ice cold mortar for 10 min. This mixture was filtered through 4 layers of cheesecloth, the filtrate was centrifuged at 30,000 x g for 30 min and the pellet was discarded. Step 2 - the crude extract from Step 1 was dialyzed against 2 liters of 10 mM KPi, pH 8.0, (buffer 2) for 6 h. Fresh buffer was then substituted, dialysis was continued for another 6 h, and the extract was applied to a 2.3 x 20 cm column of DEAE cellulose equilibrated with buffer 2. The column was washed with 2.0 liters of buffer 2 and the enzyme was eluted with a 0 to 0.4 M KCl gradient in 400 ml of the same buffer. Step 3 - the active fractions from the DEAE cellulose column were combined and concentrated to 2.0 ml with an Amicon PM-30 membrane filter and applied to a 1.6 x 100 cm column of Sephadex G-200 equilibrated and developed with buffer 2. L-Aspartate oxidase activity was assayed by the catalytic decarboxylation of [4-^{14}C]oxaloacetate formed from L-[4-^{14}C]aspartate as previously described (8). The standard assay contained in a total volume of 0.5 ml: L-[4-^{14}C]aspartate, 0.5 mM; FAD, 20 μM; bovine serum albumin, 500 μg; Bicine buffer, pH 8.0, 0.1 M; enzyme; and, in partially purified preparations, SMC. The reaction mixture was incubated at 25°C for 20 min and the product [4-^{14}C]oxaloacetate was assayed (8). Protein was determined by the method of Lowry et al. (9). A unit of enzyme activity is defined as that amount which will decarboxylate 1 μmole of oxaloacetate per minute at 25°C.

Results and Discussion: Our first attempts at purification of cotton callus tissue L-aspartate oxidase were unsuccessful until we discovered that this enzyme requires a readily dissociable dialyzable cofactor, other than FAD, for activity. As shown by the data in Table I, dialysis of a crude homogenate of cotton callus tissue results in almost total loss of L-aspartate oxidase activity. The enzyme activity is restored by addition of

Table I

Requirement of Cotton L-Aspartate Oxidase for a Dialyzable Cofactor other than FAD

Enzyme Activity		
Before Dialysis	After Dialysis	
	- boiled supernatant	+ boiled supernatant
units x 10^3	units x 10^3	
0.16	0.007	0.20

Crude extract (Step 1) was dialyzed against 10 mM KPi, pH 8.0, overnight. Assays were carried out under standard assay conditions with or without addition of 0.1 ml of boiled supernatant which was prepared by heating a 1/0.5 water homogenate of callus tissue in a boiling water bath for 5 min followed by centrifugation at 30,000 x g for 30 min.

Table II
Purification of the L-Aspartate Oxidase (B Protein of the QA Synthetase System) from Cotton Callus Tissue

Step	Total Activity	Total Protein	Specific Activity	Purification	Yield
	(Units x 10^3)	(mg)	(Units x 10^3/mg)	(Fold)	(%)
1. Crude extract	447	37.5	11.9	1.0	100
2. DEAE-cellulose chromatography	247	5.5	44.9	3.8	55
3. Sephadex G-200 chromatography	199	0.8	147.1	20.9	43

Standard assay conditions were used.

"boiled juice" prepared by heating a water extract of callus tissue for 10 min in a boiling water bath and removing the precipitate by centrifugation at 15,000 x g for 30 min. This small molecule cofactor (SMC) was further purified by passage through a 1 x 18 cm Dowex-50 H^+column from which it was eluted with water. The active fractions were pooled, reduced in volume to 1 ml and applied to a Bio-gel P-2 column (1.1 x 200 cm) which was eluted with distilled water. This preparation of SMC, which was free of FAD, was used in all subsequent experiments.

The data in Table II show a partial purification of L-aspartate oxidase from cotton callus tissue. Figure 1 shows the elution pattern of cotton L-aspartate oxidase from a Sephadex-G-200 column and the absolute dependence of the partially-purified enzyme on added SMC for activity. The apparent molecular weight of cotton L-aspartate oxidase, determined on an analytical Sephadex G-200 column (data not shown), is 83,000 daltons which is very similar to the value of 85,000 daltons previously reported for E. coli B protein (L-aspartate oxidase) (1). Cotton L-aspartate oxidase is also similar to the E. coli B protein (L-aspartate oxidase) in several other respects (1). Its K_m for L-aspartate of 0.66 mM (data not shown) is very similar to the low-concentration K_m of 0.63 found for the E. coli enzyme (4). Cotton L-aspartate oxidase does not appear to act on L-glutamate, D-aspartate or D- or L-alanine. Like the E. coli enzyme, cotton L-aspartate oxidase is

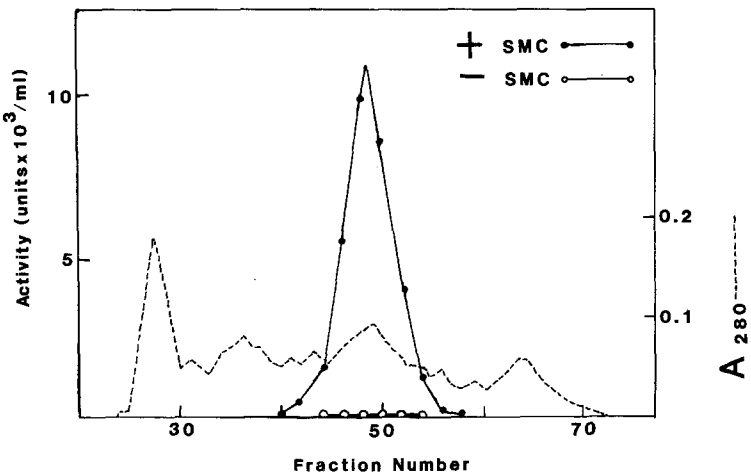
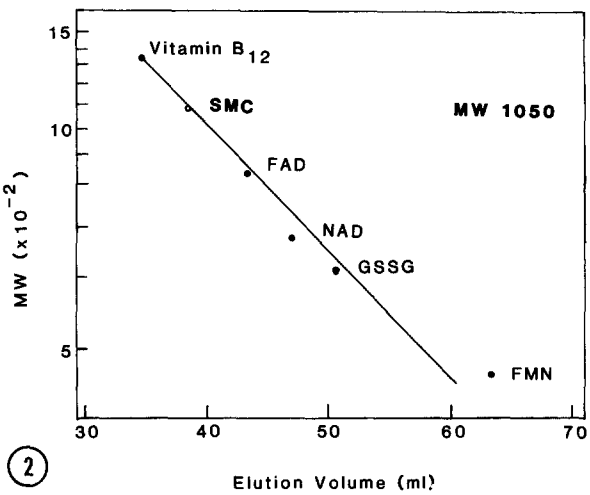


Fig. 1. Elution profile of L-aspartate oxidase activity from Sephadex G-200 column. Enzyme activity was measured under standard assay conditions with and without addition of partially purified SMC.

inhibited by NAD (about 50% at 1.0 mM) but is less sensitive to mesotartrate inhibition than the E. coli enzyme (70% inhibition versus 95% inhibition at 100 mM). As far as we can ascertain, the present work is the first report of a specific L-aspartate oxidase from plant tissue. Tsukamoto (10) previously reported a flavin-linked L-glutamate oxidase from spinach leaves which has a low rate of L-aspartate oxidase activity; our cotton enzyme is inactive with L-glutamate.

The structure of the cofactor required for cotton L-aspartate oxidase activity remains to be elucidated. It is an anion at neutral pH and is easily separable from FAD by ion exchange or gel filtration chromatography. The apparent molecular weight of SMC is 1,050 as determined by gel filtration on Bio-Gel P-2 (Figure 2). From our present experiments it is not clear whether SMC is required in addition to a tightly bound flavin nucleotide or whether SMC plays the role of electron carrier between substrate and O_2 which flavin nucleotides fulfill in other amino acid oxidases. Based on its apparent molecular weight it might be postulated that SMC is a larger derivative of FAD or FMN. However, our most purified and active fractions of SMC display neither a visible yellow color nor the characteristic UV spectrum of flavins (Figure 3).



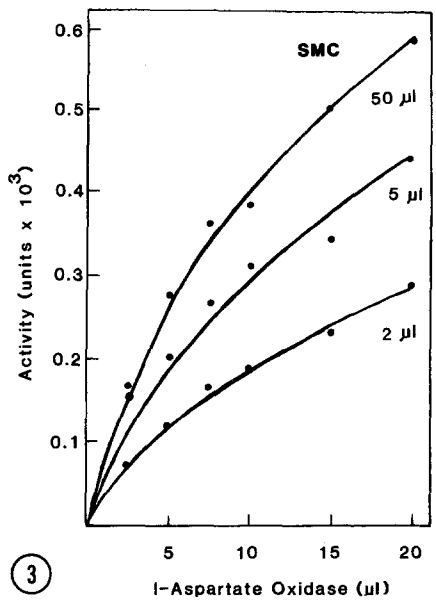


Fig. 2. Molecular weight of SMC from cotton callus tissue as determined by gel filtration on Bio-Gel P-2.

Fig. 3. Cotton callus tissue L-aspartate oxidase activity as a function of SMC and enzyme concentrations. Assays were carried out using Step 3 enzyme at the concentrations of SMC and enzyme indicated.

The finding in plant tissue of enzyme activity which catalyzes the first step in the synthesis of QA from L-aspartate in E. coli suggests, but certainly does not prove, that higher plants have the L-aspartate-dihydroxyacetone phosphate pathway for pyridine nucleotide synthesis. On the other hand, a recent paper (10) reports that "niacin" is synthesized from the benzene ring of DL-[benzene ring-U-^{14}C]tryptophan and L-[5-^{3}H]tryptophan but not from L-[U-^{14}C]aspartate or [U-^{14}C]glycerol in sections of corn seedlings incubated with these precursors for 24 and 48 hours. This report contradicts the conclusions of earlier isotopic work which found no incorporation of DL-[7a-^{14}C]tryptophan into the pyridine ring of niacin in corn and tobacco plants (11). Extensive work in several laboratories [see reviews (5) and (6)] also indicates that the pyridine ring of the alkaloids nicotine and ricinine, which are formed from niacin, is synthesized from aspartate and a three-carbon glycolytic intermediate. A final resolution of these apparent discrepancies must await isolation and

purification of the enzymes of pyridine nucleotide biosynthesis of higher plants.

References

1. Suzuki, N., Carlson, J., Griffith, G., and Gholson, R. K. (1973) Biochim. Biophys. Acta 304, 309-315.
2. Griffith, G. R., Chandler, J. L. R., and Gholson, R. K. (1975) Eur. J. Biochem. 54, 239-245.
3. Wicks, F. D., Sakakibara, S., and Gholson, R. K. (1978) J. Bacteriol. 136, 136-141.
4. Nasu, S., Wicks, F. D., and Gholson, R. K. (1982) J. Biol. Chem. 257, 626-632.
5. Leete, E. (1965) Science 147, 1000-1006.
6. Dawson, R. F., Christman, D. R., and Byerrum, R. U. (1971) Methods Enzymol. 18B, 90-113.
7. Ruyack, J., Downing, M. R., Chang, J. S., and Mitchell, E. D., Jr. (1979) In Vitro 15, 368-373.
8. Nasu, S., and Gholson, R. K. (1982) Biochim. Biophys. Acta 740, 240-252.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. N. (1951) J. Biol. Chem. 193, 265-275.
10. Tsukamoto, A. (1962) Plant and Cell Physiol. 3, 293-307.
11. Tarr, J. B., and Arditti, J. (1982) Plant Physiol. 69, 553-556.
12. Henderson, L. M., Someroski, H. F., Rao, D. R., Wu, P. H. L., Griffith, T., and Byerrum, R. U. (1959) J. Biol. Chem. 234, 93-95.