Plant Physiol. (1978) 61, 115-118

Change in the Proportion of Two Aspartokinases in Carrot Root Tissue in Response to *in Vitro* Culture[1]

Received for publication May 25, 1977 and in revised form September 9, 1977

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ABSTRACT

Two isofunctional aspartokinases (EC 2.7.2.4) exist in fresh root tissue of carrot (*Daucus carota*, cv. Oogata sanzun). The threonine-sensitive portion constitutes about 70% of the activity; the lysine-sensitive, less than 20%. Culture of slices of carrot tissue for 3 days reversed the ratio as the lysine-sensitive activity preferentially increased. Inhibition by threonine and lysine was additive in both enzyme preparations from fresh and cultured tissues. The activities were resolved into two distinct fractions of different sensitivity to threonine and lysine by DEAE-Sephadex A-50 column chromatography.

In the biosynthetic pathway of aspartate family amino acids, *i.e.* threonine, lysine, methionine, and isoleucine, aspartokinase, ATP: L-aspartate-4 phosphotransferase (EC 2.7.2.4), catalyzes the first reaction by which aspartate is converted to β-aspartylphosphate in the presence of ATP and Mg^{2+}. This enzyme has been known as a regulatory enzyme under sophisticated control in bacteria (15). Two distinct aspartokinases, each specifically inhibited by either threonine or lysine, have been highly purified and characterized in *Escherichia coli* (14). In certain other bacteria, aspartokinase are, in contrast, cooperatively inhibited by threonine and lysine (15).

In some higher plants, an enzyme exists which is cooperatively inhibited by threonine and lysine (1, 16, 17) or lysine and methionine (13), whereas other plants have been reported to have only one aspartokinase which is inhibited by either threonine (2) or lysine (3, 4). The latter cases pose a serious problem with respect to metabolic control over balanced production of amino acids of the aspartate family, since the presence of excess threonine or lysine could shut off the whole pathway (4).

The present paper shows that there are at least two different aspartokinases in carrot root tissue which are inhibited by either threonine or lysine, and the proportion of the two enzymes changes in response to incubation of slices in liquid culture medium.

MATERIALS AND METHODS

Plant Materials. Carrot roots (*Daucus carota*, cv. Oogata sanzun) were purchased from a local market and stored in a cold room (4 C) under high humidity until used. Discs (0.6 × 18 mm) of phloem parenchymatous tissue of carrot roots were prepared by means of cork borer and sliding microtome under aseptic condition (6), and cultured in 500-ml Erlenmeyer flask (about 50 discs/flask) containing 160 ml of Murashige and Skoog basal medium (10) plus 4.5×10^{-6} M 2,4-D and 3% (w/v) sucrose with shaking in the dark at 27 C. Under these conditions, first cell divisions were observed after about 5 days of culture.

Chemicals. L-[2,3-^{3}H]Aspartic acid was purchased from New England Nuclear and L-β-aspartylhydroxamate was a product of Sigma. All chemicals were reagent grade and amino acids were L form.

Extraction of Enzyme. Fresh or cultured carrot root tissue was homogenized in a blender at maximum speed for 2 min with about twice the tissue weight of ice-cold 0.1 M Na-phosphate buffer (pH 7.2) containing 2 mM EDTA, 2 mM L-threonine, 10 mM 2-mercaptoethanol, 0.1 M potassium acetate. Longer homogenization did not improve the final yield of aspartokinase activity. The homogenate was squeezed through four layers of nylon cloth and the filtrate was centrifuged at 10,000*g* for 10 min. The supernatant fluid was centrifuged again at 70,000*g* for 60 min and the yellow supernatant was passed through a Sephadex G-25 column (4.5 × 30 cm) which was previously equilibrated with the homogenizing buffer. The protein fraction eluted in the void volume was collected, and solid ammonium sulfate was added to 62.5% saturation (38 g/100 ml). The precipitate collected by centrifugation was dissolved in a small amount of the homogenizing buffer minus threonine and dialyzed overnight against three changes of about 200 volumes of the same buffer. The dialyzate was clarified by centrifugation and used as partially purified enzyme preparation. The dialysis could be replaced by gel filtration by a Sephadex G-25 column without changing results. All procedures were carried out at 0 to 4 C. The enzyme preparation remained active without changing regulatory properties for at least 1 month when kept frozen at −20 C.

DEAE-Sephadex A-50 Column Chromatography. The enzyme preparation after ammonium sulfate fractionation was dialyzed against 0.1 M Na-phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.05 M potassium acetate and the dialysate was applied to a DEAE-Sephadex A-50 column (1.5 × 30 cm) which was previously equilibrated with the same buffer. Aspartokinase was eluted with a 150-ml linear potassium acetate gradient of 0.05 to 1 M in the same buffer, and 2.5-ml fractions were collected.

Aspartokinase Assay. Aspartokinase activity was assayed by a method similar to that described by Aarnes and Rognes (2). The standard assay medium contained (in μmol) the following components, in a final volume of 100 μl: TES (pH 7.2), 5; L-[2-3-^{3}H]aspartate, (0.83 μCi/μmol), 1; $MgCl_2$, 1; ATP, 0.8; 2-mercaptoethanol, 1; hydroxylamine hydrochloride, neutralized with KOH, 40; and enzyme. Following incubation at 25 C for 60 to 120 min, the reaction was stopped by adding 50 μl of 20% (w/v) trichloroacetic acid, containing 30 mM carrier β-aspartylhydroxamate. The precipitate was removed by centrifugation and an aliquot (10–20 μl) of the supernatant was applied to a strip of Whatman 3MM paper (2 × 20 cm). After

[1] This work was partly supported by Grants 139011 and 164191 from the Ministry of Education of Japan.

electrophoresis in 0.05 M acetate-pyridine buffer (pH 4) for 60 min at 30 v/cm, the paper was dried and β-asparatylhydroxamate (near the origin) was located by spraying 5% (w/v) $FeCl_3$-N HCl without loss of activity, and unreacted aspartic acid (near the anodic end of the paper) by 0.2% (w/v) ninhydrin in ethanol with about 50% loss of activity. No radioactivity was detected between the two spots.

The hydroxamate spot was cut out and the radioactivity in it was determined by Beckman LS-250 liquid scintillation system after combustion in Packard 305 sample oxidizer. The incorporation of tritiated aspartate into β-aspartylhydroxamate as a function of time was linear for 120 min, and enzyme activity was proportional to the amount of extract added to the reaction mixture over the range of 0 to 6 mg of protein/ml. A control was run without incubation. A unit of aspartokinase activity is defined as the amount of enzyme which produces 1 nmol of β-aspartylhydroxamate (corresponding to about 1,800 dpm)/min under the standard assay condition.

RESULTS

The assay method is not specific for aspartokinase if asparagine synthetase is present (2, 16), since asparagine would also produce β-aspartylhydroxamate. The possible interference by asparagine synthetase was examined by replacing hydroxylamine with NH_4Cl (12), or glutamine (5). However, no incorporation of radioactive aspartate into asparagine was observed, indicating no interference by asparagine synthetase with the aspartokinase assay under the conditions employed.

Increase of Aspartokinase Activity during Culture of Tissue Slices. Figure 1 shows the time course analysis of aspartokinase activity during culture of carrot root tissue slices in liquid medium. The activity was low in the tissue before culture, but gradually increased for the first 2 days after culture and then rapidly to the maximum level on the 3rd day. A rapid decreae followed thereafter. The activity on the 3rd day was about 12 times the initial level in this experiment. The maximum increment reached in 3 days was 10 to 16 times that of the tissue before culture. The initial level varied with storage length; before storage, the initial level was in the range of 0.02 to 0.05 unit/g wet wt, but it was raised to 0.1 to 0.2 after a storage of about 4 months. In spite of the change in initial level, however, the time-dependent change of aspartokinase activity during culture was similar to the one shown in Figure 1.

Inhibition of Aspartokinase Activity by Threonine and Lysine. Figure 2 shows the inhibition of aspartokinase activity in the partially purified preparations from fresh tissue and the tissue slices cultured for 3 days as a function of concentration of threonine and lysine.

The enzyme activity of fresh tissue was highly inhibited by threonine (71%, at 5 mM) and only slightly by lysine (16%, at 5 mM). In contrast, the enzyme preparation from the cultured tissue slices was strongly inhibited by lysine (78%, at 5 mM) but weakly by threonine (8%, at 5 mM). In either case, maximum inhibition was observed at concentrations as low as 2 to 3 mM for both threonine and lysine.

Property of Increased Aspartokinase Activity. Table I shows the analysis of increased aspartokinase activity during culture of tissue slices for 3 days in terms of sensitivity to inhibition by threonine and lysine, each at 5 mM concentration.

Whereas the over-all activity increased 14.8-fold, the portion sensitive to threonine increased only slightly (1.7 times) compared to the huge increase in the activity insensitive to threonine (46.9 times). With lysine, almost the reverse of the threonine condition occurred. Activity sensitive to lysine increased 72.1 times whereas the lysine-*in*sensitive activity increased only slightly at the same time.

These results indicate that more than 80% of the increased

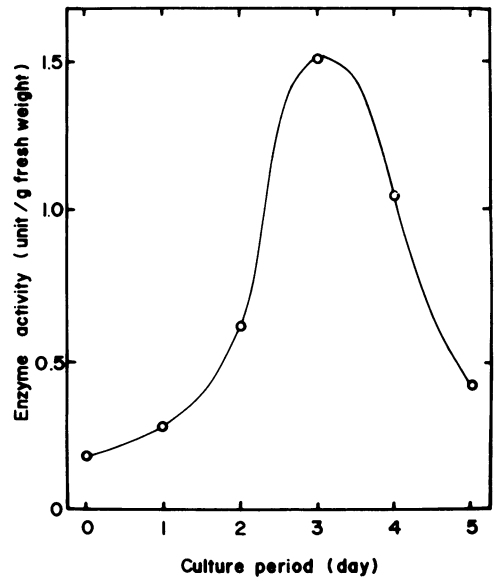

FIG. 1. Changes in aspartokinase activity during culture of carrot root tissue slices in liquid medium. Activities were assayed as described under "Materials and Methods" and expressed as units/g wet tissue wt.

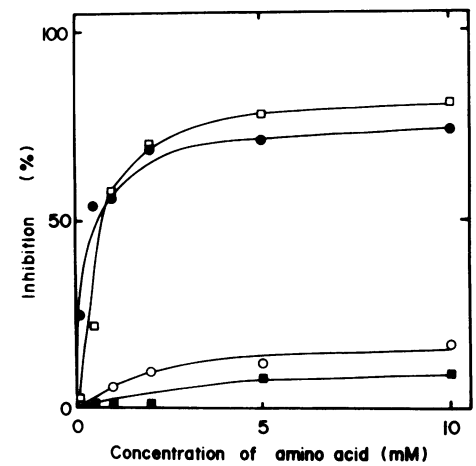

FIG. 2. Inhibition of aspartokinase activity from fresh tissue (○, ●) and tissue slices cultured for 3 days (□, ■) as a function of concentration of threonine (●, ■) or lysine (○, □). Activities for control where threonine or lysine was omitted from reaction mixture were 0.125 and 1.84 unit/g wet wt for preparations from fresh and cultured tissues, respectively.

Table I. Increase in aspartokinase activity during culture of carrot root tissue slices accompanied by a change in ratio of sensitivity to threonine and/or lysine

Aspartokinase activities were assayed in the presence or absence of either L-threonine or L-lysine, each at 5 mM. Data are from the same experiment as for Figure 2.

Fractions	Fresh		Cultured (3 days)		Increment[2]
	activity[1]	% of total	activity[1]	% of total	
Total	124.6	100	1841	100	14.8
Thr (5 mM)					
sensitive[3]	88.5	71	147	8	1.7
insensitive[4]	36.1	29	1694	92	46.9
Lys (5 mM)					
sensitive[3]	19.9	16	1435	78	72.1
insensitive[4]	104.7	84	405	22	3.9

1. Activities were expressed as unit x 10^3 per g wet tissue weight.
2. Ratio of activity of cultured tissue to that of fresh tissue.
3. Fraction of activity inhibited by amino acid.
4. Fraction of activity not inhibited by amino acid.

activity was a lysine-sensitive enzyme wheres the threonine-sensitive enzyme remained at essentially the same level throughout the culture period.

Additive Inhibition by Threonine and Lysine. Table II shows the inhibition of aspartokinase in the preparations from fresh tissue and the tissue cultured for 3 days by threonine, lysine, homoserine, and their combinations at 5 mM concentrations.

In both preparations from fresh and cultured tissues, the per cent inhibition observed in the simultaneous presence of both threonine and lysine was approximately equal to the sum of the per cent inhibitions when threonine and lysine were added separately. Additivity was not observed in the combination of threonine and homoserine, or lysine and homoserine, in which inhibition caused by threonine or by lysine was essentially unaffected, or rather lowered by the simultaneous presence of homoserine, although homoserine itself was slightly inhibitory when added alone.

Table II. Additive inhibition of aspartokinase from fresh and cultured tissues of carrot root by threonine and lysine

Partially purified preparations of aspartokinase from fresh tissue and the tissue cultured for 3 days in liquid medium were assayed in the presence of L-threonine, L-lysine, and L-homoserine (HS), either alone or in combination, each at 5 mM.

Addition	Fresh		Cultured (3 days)	
	activity[1]	inhibition (%)	activity[1]	inhibition (%)
None	108.4	-	1694	-
Thr	31.4	71	1559	8
Lys	90.6	16	369	78
HS	93.8	13	1628	4
Thr + Lys	21.5	80	284	83
Lys + HS	89.5	17	520	69
HS + Thr	37.8	65	1569	7

1. Activities were expressed as units x 10^3 per g wet tissue weight.

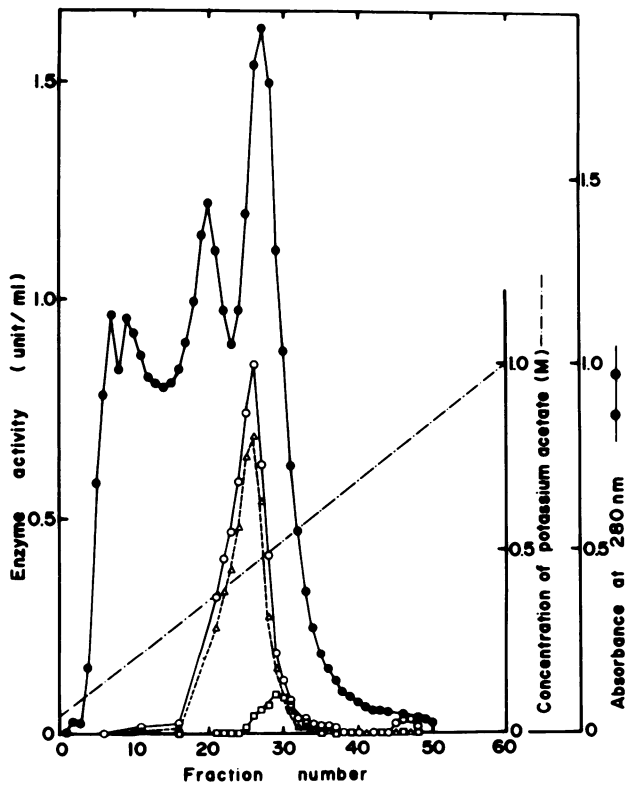

FIG. 3. DEAE-Sephadex A-50 column chromatography of aspartokinase preparation from 150 g of fresh tissue. Elution of aspartokinase was performed by increasing the concentration of potassium acetate (—·—). Aspartokinase activities were assayed as described under "Materials and Methods," without inhibitors (○), plus 10 mM L-lysine (△), and plus 10 mM L-threonine (□), expressed as units/ml of fraction. Solid circles (●) indicate absorbance at 280 nm.

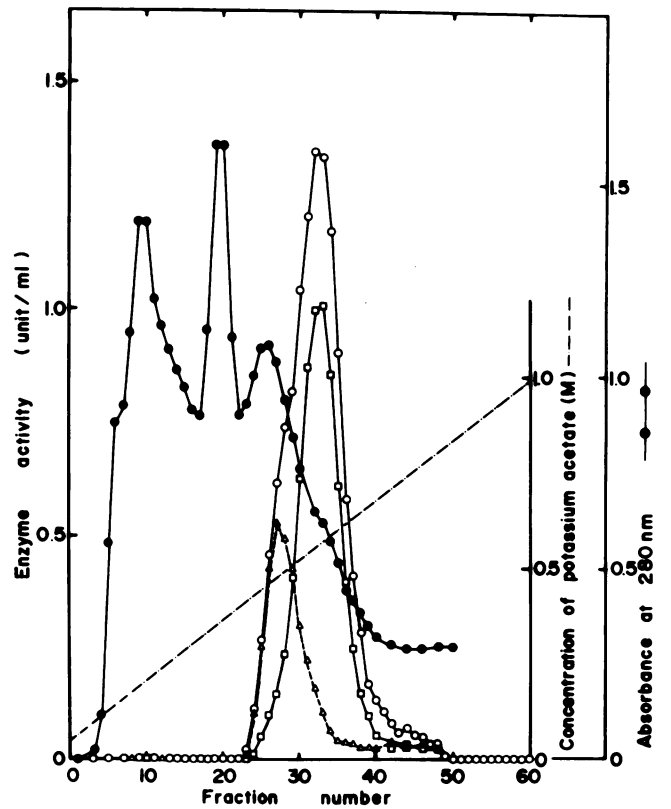

FIG. 4. DEAE-Sephadex A-50 column chromatography of aspartokinase preparation from 80 g of the tissue cultured for 3 days. Methods and symbols are as for Figure 3.

Alanine, isoleucine, and valine have been reported as activators of aspartokinases from seedlings of pea (2) and corn (3). In carrot aspartokinase, however, these amino acids were without effect, or, slightly inhibitory.

Resolution of Threonine- and Lysine-sensitive Aspartokinases by DEAE-Sephadex A-50 Column Chromatography. An aspartokinase preparation concentrated from 150 g of carrot tissue before culture was applied to and eluted from a DEAE-Sephadex A-50 column (Fig. 3).

The majority of aspartokinase activity which was inhibited by threonine was eluted at a concentration of potassium acetate of about 0.4 M, whereas the lysine-sensitive minor fraction was eluted at about 0.5 M.

In the case of a preparation from cultured tissue, the corresponding two activities were eluted at almost the same salt concentrations as for those from the tissue before culture, respectively. However, the threonine-insensitive (lysine-sensitive) activity was the major enzyme component (Fig. 4). There was an additional small fraction of activity which was less sensitive to either threonine or lysine (fraction 40–50 in Fig. 4).

DISCUSSION

The change in the ratio of isofunctional aspartokinases during culture of carrot root tissue slices is probably one of the earliest manifestations of the change in metabolism necessary to produce new callus cells. Activation of protein and nucleic acid syntheses are essential processes which must produce other metabolic changes such as activation of glycolytic, pentose phosphate, and respiratory enzymes. In this connection, Komamine and Shimizu (7) found that phosphoribosyl-pyrophosphate synthetase, a key

enzyme of purine nucleotide synthetic pathway, increased at a very early stage of callus formation during which RNA synthesis was activated remarkably in a carrot root explant culture. The results in this paper indicate that the activity of aspartokinase, a key enzyme required for synthesis of the amino acid building blocks which permit macromolecular syntheses, increased to an equally remarkable extent at an even earlier stage leading to callus cell formation.

Although aspartokinase has been extracted and characterized in many higher plants no one has previously shown more than one enzyme in one tissue or in one plant, except that Davies and Miflin (9) found (unpublished data) two aspartokinases in carrot tissue culture which are similar to the ones presented in this paper. Aarnes and Rognes (2) obtained an aspartokinase preparation from pea seedlings which was inhibited by threonine but not by lysine. Aarnes (1) suggested the presence of two aspartokinases, one threonine- and the other lysine-sensitive, in several species of plants. Bryan *et al.* (3) and Cheshire and Miflin (4) reported a lysine-sensitive aspartokinase from etiolated shoots of maize which showed little sensitivity to threonine.

In light of the present results on carrot tissue, it is possible that other plants could also have at least two isofunctional aspartokinases which vary in amount at different stages of growth or physiological states of the plant.

Under such circumstances, therefore, the possible minor component might be overlooked unless plant material from more than one physiological state was chosen, and unless the methods for enzyme preparation and assay of enzyme were sensitive enough to detect it.

In *E. coli* (15), the synthesis of aspartokinases is under the control of end product repression: threonine-sensitive enzyme by threonine and isoleucine, and lysine-sensitive one by lysine. A third enzyme found in *E. coli* K_{12} is reported to be repressed by methionine (11).

In this respect, the increase and decrease of aspartokinase in carrot root tissue during culture (Fig. 1) could be a reflection of change in endogenous amino acid level, *i.e.* a rapid initiation of protein synthesis which occurs immediately after slicing and culture (7) could result in a lowered amino acid level, leading to a derepression of the enzyme. Repression might take place when the enzyme is synthesized in a sufficient amount to allow amino acid production to surpass consumption. Such an oscillatory repression has been suggested by Masters and Pardee (8).

Work supporting this hypothesis is in progress.

Acknowledgments—The authors wish to express their gratitude to M. Shimokoriyama for his valuable discussions. They are also grateful to S. G. Wildman of the University of California, Los Angeles, for his help in preparing this manuscript.

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