Short Communication

Derepression and Repression of Lysine-sensitive Aspartokinase during *in Vitro* Culture of Carrot Root Tissue[1]

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KATSUHIRO SAKANO
Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

ABSTRACT

The increment of lysine-sensitive aspartokinase (EC 2.7.2.4) activity during *in vitro* culture of carrot (*Daucus carota*, cv. Oogata sanzun) root tissue was explained in terms of derepression caused by an earlier decrease in the endogenous level of lysine, a possible end product repressor. Tissue content of free lysine decreased to about one-third of the initial level after 1 day of culture and no lysine was detected in the 2nd day. Inclusion of lysine (0.1 to 1.0 millimolar) in the culture medium resulted in a specific suppression of increase in lysine-sensitive aspartokinase activity without affecting the increase in threonine-sensitive aspartokinase activity.

Regulation of metabolic processes by feedback inhibition and end product repression of specific enzymes is a common mechanism in bacterial systems. In higher plants, although control by feedback inhibition has been demonstrated for many enzymes, only few instances are known of end product repression (17).

As a regulatory enzyme in the biosynthetic pathway of aspartate family amino acids, aspartokinase, ATP:L-aspartate-4-phosphotransferase (EC 2.7.2.4), has recently been characterized in many higher plants (1–6, 12, 13, 18). Two isozymic forms of the enzyme have been established only in carrot (12).

In vitro culture of carrot root slices leads to a change in the proportions of the two isozymes, as the lysine-sensitive enzyme is preferentially increased (12).

This paper shows that this increase is due to a derepression triggered by the lowered level of endogenous lysine which takes place immediately after slicing and culture of the root tissue.

MATERIALS AND METHODS

Plant Materials. Carrot roots (*Daucus carota*, cv. Oogata sanzun) were purchased from a local market and stored at 2 C under high humidity. Discs (0.6 × 20 mm) were prepared and cultured aseptically in Murashige and Skoog basal medium (11) plus 4.5 μM 2, 4-D and 3% (w/v) sucrose as described previously (12).

Chemicals. L-[2,3-^{3}H]Aspartic acid was purchased from New England Nuclear. All of the amino acids used were reagent grade and L form.

Preparation of Enzyme. Partially purified enzyme solutions were prepared from fresh and cultured carrot root tissue as described previously (12).

Aspartokinase Assay. Aspartokinase activity was assayed with radioactive aspartate as described previously (12). One unit of aspartokinase activity is defined as the amount of enzyme producing 1 nmol of β-aspartylhydroxamate/min at 25 C under the standard assay conditions.

Protein Determination. Protein was determined by the method of Lowry *et al.* (10).

Extraction and Analysis of Free Amino Acids. Free amino acids were extracted by the method described by Heimer and Filner (8). Minced tissue was boiled for 5 min in 70% (v/v) ethanol (5 ml/g fresh weight) and vigorously homogenized for 2 min in a blender. The homogenate was centrifuged at 15,000*g* for 30 min, and the pellet rinsed twice with 70% (v/v) ethanol. The ethanol fractions were pooled and dried *in vacuo*. After extraction twice with petroleum ether to remove carotenoids, the residue was dissolved in 0.2 M Na-citrate (pH 2.2). The solution was clarified by centrifugation and diluted to an appropriate concentration with the same buffer. A portion of the extract was applied to a JEOL-AH-6 automatic amino acid analyzer.

RESULTS

Changes in Endogenous Level of Lysine during *in Vitro* Culture. Table I shows the representative results of one of the several experiments in which the endogenous levels of some amino acids in fresh tissue and tissues cultured for 1 to 4 days were determined. In all of the amino acid listed, the levels dropped significantly on the 1st day. On the 2nd day, the amounts of most of the amino acids were partially restored. The levels continued to decrease on the 3rd and 4th days, except threonine and lysine.

Threonine level continued to increase from day 2 to 4. Lysine continued to decrease for the first 2 days, reaching an undetectable level, then began to recover on the 3rd day.

The absolute values of amino acid levels in both fresh and cultured tissues fluctuated as much as 20 to 100% depending on the lot of carrot roots used, but the initial rapid decrease and the following temporal increase in the levels of most of the amino acids were always observed.

The significant decrease in the levels of endogenous amino acids may be a reflection of the activated protein synthesis which occurs soon after slicing and culturing the tissue *in vitro* (9). Such a decrease in amino acid level is universally seen in plant storage tissue when mechanically injured, or cultured after injury (16).

The level of threonine was restored earlier than that of lysine and accumulated in a relatively large amount as compared with lysine.

Repression of Lysine-sensitive Aspartokinase by Lysine. If the increase in lysine-sensitive aspartokinase activity during the first 3 days of culture of carrot tissue slices (12) is a result of derepression caused by the observed fall in lysine level, the increase should be suppressed when the endogenous lysine level is elevated in some way. This was tested by culturing tissue slices in the presence of lysine.

Table II shows the effects of lysine on the increases in fresh

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weight, protein content, and aspartokinase activity during culture of carrot root tissue for 3 days.

Lysine applied in the range 0.1 to 1.0 mM was significantly inhibitory to the increase in fresh weight, but partially to that of protein content except at 0.1 mM where the increase was as much as control. Lysine significantly suppressed the increase in aspartokinase activity. The higher the concentration, the greater the suppression. Lysine suppressed only the increase in lysine-sensitive activity without affecting the increase in threonine-sensitive activity which was approximately doubled during the culture at all of the lysine concentrations examined. About 3.4 μmol of the amino acid accumulated per g of the cultured tissue when 1 mM lysine was added.

DISCUSSION

In a previous paper (12), we reported a change in the proportion of isofunctional aspartokinases during culture of carrot root tissue slices *in vitro*. In the tissue before culture, total aspartokinase activity was low, threonine-sensitive activity represented about 70% of the total, and lysine-sensitive activity about 20%. During *in vitro* culture for 3 days lysine-sensitive activity increased to about 70 times the initial level, whereas threonine-sensitive activity only doubled. Total enzyme activity dropped significantly on the 4th and 5th days.

Table I. Endogenous levels of some amino acids during culture of carrot root tissue

Amino acid	Culture period (day)				
	0	1	2	3	4
	(μmoles/g fresh wt)				
Lys	0.08	0.03	ND[3]	0.01	0.04
His	0.14	0.07	0.09	0.05	0.07
Arg	1.02	0.05	0.08	0.04	0.04
Asp[1]	2.41	0.75	1.45	0.57	0.48
Thr	5.26	0.96	2.47	2.76	3.88
Ser	5.73	1.78	3.50	2.02	1.42
Glu[2]	4.13	1.12	2.59	1.28	1.33
Gly	0.18	0.07	0.16	0.08	0.09
Ala	5.24	1.18	3.07	1.33	2.03
Val	1.16	0.32	0.36	0.14	0.14
Met	0.08	0.04	0.05	0.04	0.03
Ile	0.48	0.09	0.10	0.04	0.04
Leu	0.33	0.04	0.03	0.01	0.01
Tyr	0.16	0.03	0.07	TR[4]	TR[4]
Phe	0.35	0.03	0.04	0.04	TR[4]

1. Aspartic acid plus asparagine.
2. Glutamic acid plus glutamine.
3. Not detected.
4. Trace amount.

Present results indicate that the preferential increase in lysine-sensitive aspartokinase during culture is due to a derepression triggered by the decreased level of endogenous lysine, a possible end product repressor.

The time courses of changes in lysine level (Table I) and aspartokinase activity (12) are clearly in the opposite direction; the enzyme activity was low while lysine level was high in the fresh tissue before culture, but after 2 days of culture when enzyme activity was increasing rapidly, no free lysine was detected in the tissue. On the 3rd day of culture when enzyme activity was maximum, free lysine began to accumulate. With the accumulation of lysine, enzyme activity dropped significantly on the 4th and 5th days. The opposite relationship between aspartokinase activity and free lysine level during culture strongly suggests that lysine is a repressor of lysine-sensitive aspartokinase as established in *Escherichia coli* (15).

Exogenous lysine suppressed the increase in lysine-sensitive aspartokinase activity during culture without affecting the concomitant increase in threonine-sensitive activity, although growth inhibition was observed to some extent.

These results indicate that lysine is an end product repressor of lysine-sensitive aspartokinase in carrot root tissue.

The possibility that the increase in the enzyme activity may be due to the change in the level of inhibitor or activator can be excluded by the following observations: (*a*) the enzyme preparations used in the present experiments were free from low mol wt substances such as threonine and lysine. In fact, full activity could be recovered after the final step of enzyme preparation (dialysis), even if the extraction buffer contained either threonine or lysine at 5 mM, indicating that these inhibitors are removable simply by dialysis; (*b*) about 80 to 90% of the activity in the partially purified enzyme preparation could be recovered after chromatography on DEAE-Sephadex A-50 (12), or Sephadex G-200 column (unpublished data), suggesting that the enzyme preparation contained no inhibitor or activator of high mol wt.

These observations suggest that the increase in the enzyme activity during culture is due to *de novo* synthesis, although there is no direct evidence so far.

In summary, in the tissue before culture, lysine-sensitive aspartokinase would be in a repressed state. Activation of protein synthesis after slicing and culturing the tissue would result in a consumption of endogenous amino acids including lysine. As

Table II. Repression of lysine-sensitive aspartokinase by lysine. Values are from one representative experiment of three independent experiments.

	Initial tissue	Tissues cultured for 3 days with lysine (mM)			
		0	0.1	0.5	1.0
Fresh weight (mg/disc)	200	291	230	233	218
Protein (mg/disc)	0.153	0.524	0.561	0.373	0.373
Total aspartokinase (act[1]/disc)	13.7	251	183	85.5	55.2
(act[1]/mg protein)	89.7	482	327	230	148
Thr-sensitive act[2] (act[1]/disc)	11.7	25.5	25.8	21.4	25.1
Lys-sensitive act[3] (act[1]/disc)	0.9	173	124	56.4	22.2

1. Activity expressed as units x 10^3.
2. Activity inhibited by 5 mM threonine.
3. Activity inhibited by 5 mM lysine.

lysine level lowered, derepression of lysine-sensitive enzyme would occur. When the enzyme accumulated in sufficient amount to allow lysine synthesis to exceed consumption, lysine would begin to accumulate, which, in turn, would cause another repression of enzyme synthesis.

The threonine-sensitive aspartokinase activity increased only 2-fold as compared with the huge increase in lysine-sensitive enzyme during the same culture period. The corresponding threonine-sensitive enzyme in *E. coli* is repressible by threonine and isoleucine (7). During culture of carrot tissue slices, endogenous levels of threonine and isoleucine were also subject to a significant decrease for the 1st day. However, the restoration of levels was observed as early as the 2nd day, and on the 3rd day, threonine level recovered even to about half of the initial value. If the control of the synthesis of threonine-sensitive enzyme is similar to that of the corresponding enzyme in *E. coli*, the observed smaller increase in the enzyme level may be attributable to the relatively earlier restoration of threonine and isoleucine levels which follow after their initial decrease. The threonine level is restored earlier than the lysine level, although both amino acids derive from a common precursor, aspartic acid. This may indicate that the levels of biosynthetic enzymes which specifically lead to lysine synthesis are rather low as compared with those which specifically lead to threonine synthesis; the flux of biosynthetic intermediates is preferentially directed to threonine synthesis as reported in *Brevibacterium flavum* (14).

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