

# Some Biological Properties and an *in Vivo* Evaluation of Tyrosine Phenol-Lyase on Growth of B-16 Melanoma<sup>1</sup>

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## SUMMARY

Tyrosine phenol-lyase from *Erwinia herbicola* was purified with the goal of assessing its effect on growth of malignant melanoma. Ammonium sulfate-sodium citrate fractionation and diethylaminoethyl cellulose-hydroxylapatite chromatography were used. The purified enzyme was shown to reduce plasma tyrosine levels when administered to normal C57BL × DBA/2 F<sub>1</sub> mice. The plasma half-life value of the enzyme was found to be 6 to 7 hr. Unlike results reported with glutaminase and asparaginase preparations, the lactate dehydrogenase-elevating virus had no significant influence on plasma clearance of tyrosine phenol-lyase. The enzyme significantly inhibited growth of established B-16 melanoma tumors.

## INTRODUCTION

An exploitable difference between normal cells and some neoplastic cells is the inability of the latter to synthesize certain nonessential amino acids. Investigations along these lines have identified enzymes such as asparaginase (9, 16-18) and glutaminase-asparaginase GA:1.2 (24-26) as potent chemotherapeutic agents. More recently, investigators have shown an enhanced requirement by certain neoplasms for the essential amino acids, phenylalanine, and tyrosine. Because mammalian cells require an exogenous supply of tyrosine and phenylalanine, it is possible to lower plasma levels of these 2 amino acids by dietary restriction. Lorincz *et al.* (13) have reported some success with dietary treatment of several solid murine tumors. Others have demonstrated beneficial effects in both human and murine malignant melanomas by feeding low-phenylalanine and low-tyrosine diets (5, 6, 10, 13). In all of these studies prolonged dietary treatment was necessary to lower plasma phenylalanine and tyrosine levels and to produce objective remissions. Also, the unpalatable nature of the low phenylalanine and tyrosine diets made human compliance difficult (6). The administration of enzymes that degrade phenylalanine and tyrosine *in vivo* would offer the distinct advantage of rapidly depleting amino acid levels and would facilitate continu-

ously maintaining a predetermined level that is required for an optimum therapeutic response.

Abell *et al.* (2) have reported that administration of phenylalanine ammonia-lyase from *Rhodotorula glutinis* inhibits both murine and human leukemic lymphocytes *in vitro* and growth of murine L-5178Y lymphoblastic leukemia *in vivo* (1). Tyrosine phenol-lyase catalyzes the conversion of L-tyrosine to phenol, pyruvate, and ammonia and requires pyridoxal phosphate as a coenzyme. It has been described from *E. herbicola* (11) and *Escherichia intermedia* by Kumagai *et al.* (12). The enzyme from *Erwinia* has a  $K_m$  of 0.28 mM for tyrosine (11). A preliminary study (7) shows that both phenylalanine ammonia-lyase and tyrosine phenol-lyase have antineoplastic activity against B-16 melanoma *in vivo*. We report a purification scheme, some biological properties, and the antimelanoma activity of tyrosine phenol-lyase. Since the ubiquitous LDH-virus<sup>3</sup> has been shown to increase the plasma half-life values of asparaginase (20) and glutaminase-asparaginase (24), the influence of this virus on tyrosine-phenol lyase is also considered.

## MATERIALS AND METHODS

**Enzyme Isolation and Purification.** *E. herbicola* (ATCC 21434) was grown for 24 hr in submerged culture at 28° on a rotary shaker operated at 200 rpm. The growth medium was the same as that described by Enei *et al.* (8) with the exception that yeast extract (20 g/liter) was substituted for hydrolyzed soybean protein.

Enzyme isolation and purification was conducted at 0-7°. All buffers, unless noted, contained 0.5 mM mercaptoethanol and 0.1 mM EDTA. Packed cells (approximately 340 g) were suspended in 2 to 3 volumes of 0.01 M potassium phosphate buffer, pH 6, and were sonically disrupted with a Branson LS75 sonifier for 2 min at 30-sec intervals at 20 kc/sec. The sonic extract was centrifuged at 30,000 × *g* for 45 min. The precipitate was resuspended in 2 volumes of buffer and centrifuged again, and the supernatant and wash were combined. Solid ammonium sulfate was added to 20% (w/v) of saturation and the enzyme-inactive material was removed by centrifugation. Addition of ammonium sulfate to 50% (w/v) of saturation precipitated all of the enzyme activity. The precipitate was collected by centrifugation and dissolved in buffer. Sodium citrate was added to a concentration of 190 mg/ml, and the insoluble portion was removed by centrifugation. An additional amount was added to give a final concentration of 320 mg/ml. The resulting suspension

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containing the precipitated enzyme activity was centrifuged. The precipitate was dissolved in and dialyzed against 0.01 M potassium phosphate buffer, pH 6, for 30 hr. Following dialysis, the solution was applied to a DEAE-cellulose column (80 x 2.5 cm) that had been equilibrated with 0.1 M potassium phosphate buffer, pH 6.5. The enzyme was eluted with 700 ml of a linear gradient of 0 to 1 M KCl in the equilibrating buffer at a flow rate of 1.2 ml/min. Ten-ml fractions were collected and assayed for tyrosine phenol-lyase activity. Active fractions were combined and concentrated by ultrafiltration using an Amicon PM-10 membrane. The ultrafiltrate was dialyzed against 0.01 M phosphate buffer, pH 6, for 30 hr with 4 or 5 changes of buffer. Following dialysis, the solution was applied to a hydroxylapatite column (35 x 2.6 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 6. The adsorbed enzyme was eluted with 500 ml of a linear gradient of 0.01 M potassium phosphate buffer, pH 6, to 0.5 M potassium phosphate buffer, pH 7, at a flow rate of 0.7 ml/min. The fractions with enzyme activity were pooled and dialyzed for 36 hr against 0.01 M potassium phosphate buffer, pH 7.4, containing no mercaptoethanol or EDTA. The dialyzed enzyme solution was sterilized by filtration through 0.45- $\mu$ m Millipore filters, lyophilized, and stored at 4° for further use. The lyophilized enzyme was reconstituted when needed, with a sterile solution of pyridoxal phosphate (50  $\mu$ g/ml) in 0.01 M phosphate buffer, pH 7.4, containing no mercaptoethanol or EDTA.

**Enzyme Assay.** Tyrosine phenol-lyase was assayed by measuring the amount of pyruvate formed from L-tyrosine. One IU of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1  $\mu$ mole of pyruvate per min. A continuous spectrophotometric assay system was devised by modifying the method of Morino and Snell (15). The reaction mixture contained 142  $\mu$ moles of  $K_2HPO_4$  buffer, pH 7.8, 94 nmoles of pyridoxal phosphate, 200 nmoles of NADH, 1 IU of lactate dehydrogenase, 2.5  $\mu$ moles of L-tyrosine, and enzyme in a total volume of 3 ml. The reaction was carried out at 28°. The rate of decrease in absorbance of NADH at 340 nm was determined using a Beckman DB spectrophotometer. Protein was determined by the method of Lowry *et al.* (14), and specific activity was expressed as units per mg of protein. Addition of plasma to reaction mixtures did not affect the enzyme assays under the conditions used. The plasma levels of lactate dehydrogenase of normal and LDH-virus-infected mice were determined by the method of Bergmeyer *et al.* (3), and the enzyme activity was reported as Wroblewski units (27).

**Electrophoresis.** Twenty-five  $\mu$ g of tyrosine phenol-lyase were applied to polyacrylamide gels and electrophoresis was carried out using a current of 4.0 ma for 1.5 hr in Tris-glycine buffer, pH 8.3, according to the method of Davis (4). The gel was stained with Coomassie brilliant blue and stored in 7% acetic acid.

**Mice Used.** C57BL  $\times$  DBA/2 F<sub>1</sub> (hereafter called BDF<sub>1</sub>) female mice (17 to 22 g) and BDF<sub>1</sub> male mice (22 to 26 g) were obtained from the Charles River Breeding Laboratory, Wilmington, Mass. The female mice were used in all studies except Tumor Study B (Chart 1) where the male mice were used. All mice were housed in metal cages in groups of 3 to 5 mice to a cage. Purina laboratory chow and water were

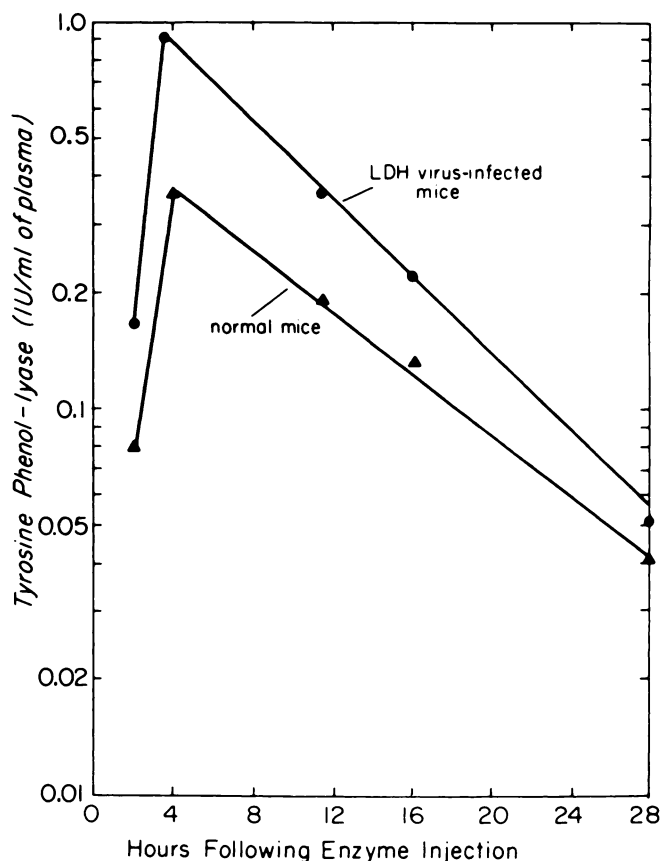


Chart 1. Influence of the LDH-virus on plasma clearance of tyrosine phenol-lyase. A group of mice were given injections of the LDH-virus 4 days prior to injection of enzyme. Mice not given injections of the virus were kept isolated from the infected group. At time 0, partially purified tyrosine phenol-lyase, 240 IU/kg (specific activity = 0.63), was injected i.p. into all mice. At the indicated times 3 mice from each group were bled and the samples were pooled. Plasma enzyme levels were determined as described in "Materials and Methods."

given *ad libitum* to all mice except where indicated. All mice were acclimatized for at least 7 days prior to use. Certain mice were infected with the LDH-virus by injecting 0.1 ml i.p. of a 1:10 dilution of serum from LDH-virus-infected mice.

**Effect of Tyrosine Phenol-Lyase on Tyrosine and Phenylalanine Plasma Levels.** Mice were infected with the LDH-virus 7 days prior to enzyme administration. Blood samples were collected from the tail vein prior to and at 7 and 26 hr after injection of enzyme. About 0.3 ml of blood was removed from each mouse into heparinized capillary tubes. The tubes were quickly stoppered and centrifuged at 4° for 10 min. The samples were prepared for amino acid analysis by the following procedure developed by Spackman (D. Spackman, personal communication). The plasma from groups of 3 mice was pooled and transferred into tared vials containing 15% sulfosalicylic acid, and the weight of plasma was determined. The samples were centrifuged, and the resulting pellet was washed 3 times with cold 5% sulfosalicylic acid. The supernatant and washings were pooled, adjusted to pH 2.6 with NaOH, and diluted to a final volume of 2.5 ml with 0.2 N sodium citrate buffer, pH 2.6. Tyrosine and phenyl-

alanine levels were determined on a JEOL model JLC-5AH amino acid analyzer.

**Half-life Determination.** Blood samples were removed by orbital bleeding (21) into prechilled heparinized tubes. Each sample was centrifuged at 4° and the plasma was assayed for tyrosine phenol-lyase activity as described.

**Tumor Implantation and Measurements.** B-16 melanoma was obtained through the courtesy of Dr. Vernon Riley. Transplantation and tumor volume measurements were performed according to the techniques described by Riley *et al.* (24). Tumor diameters were measured with calipers, the measurements were averaged, and the mean tumor volumes were calculated.

**Materials.** All chemicals used were reagent grade. Anhydrous sodium citrate was obtained from Merck and Co., Inc., Rahway, N. J. Pyridoxine monohydrochloride; pyridoxal phosphate, A grade; NADH, A grade; and lactate dehydrogenase (beef heart), A grade; were obtained from Calbiochem, Los Angeles, Calif. ICN Pharmaceuticals, Inc., Cleveland, Ohio, supplied the enzyme-grade ammonium sulfate; L-tyrosine was obtained from Aldrich Chemical Co., Milwaukee, Wis. Whatman DEAE-cellulose (DE-32) was obtained from H. Reeve Angel and Co., Clifton, N. J., and hydroxylapatite was from Clarkson Chemical Co., Williamsport, Pa.

## RESULTS AND DISCUSSION

**Purification and Some Properties of the Enzyme.** The overall purification of tyrosine phenol-lyase is summarized

Table 1

*Purification of tyrosine phenol-lyase*

Approximately 340 g of cells (wet weight) obtained from 40 liters of medium were used as a source of enzyme.

Step	Total units	Total protein (mg)	Specific activity
1. Cell homogenate	5,160	43,000	0.12
2. Ammonium sulfate fractionation	4,810	24,050	0.20
3. Citrate fractionation	2,353	5,115	0.46
4. DEAE-cellulose chromatography	1,550	2,183	0.71
5. Hydroxylapatite chromatography	1,128	946	1.15

in Table 1. The yield of enzyme was 22% with a purification of 10-fold. The enzyme showed a single band on acrylamide gel electrophoresis.

The lyophilized enzyme was stable for at least 4 months. Reconstituted enzyme solutions were less stable and retained 75, 64, and 36% of their original activity at 22 hr when standing at 0°, 27°, and 37°, respectively.

The influence of purified tyrosine phenol-lyase on plasma tyrosine and phenylalanine levels is shown in Table 2. A single dose of 160 IU/kg significantly reduced the plasma tyrosine levels at 7 hr after injection. At 26 hr after injection levels the reduction was less pronounced. Phenylalanine levels were not significantly altered.

**Influence of the LDH-Virus on the Plasma Half-life Value.** The presence of the LDH-virus was ascertained by comparing plasma LDH levels from randomly selected normal and infected mice. Enzyme levels are markedly elevated in infected mice (19). Plasma from normal mice gave LDH values of 508 units with a standard deviation of 101 units, whereas plasma from mice previously given injections of the virus gave values of 4208 units with a standard deviation of 1310 units. The plasma half-life of tyrosine phenol-lyase calculated from the data in Chart 1 is approximately 6 to 7 hr. The data indicate that infection with the LDH-virus has no significant influence on clearance of this enzyme. These results are in marked contrast to those reported for asparaginase EC-2 (20) and glutaminase-asparaginase GA:1.2 (24), where in the presence of virus the *in vivo* half-life of these enzymes is considerably increased.

**Antimelanoma Activity.** Since the LDH-virus has been reported to cause immunological and other alterations in the host (22, 23) and since consistently higher enzyme plasma levels were obtained in the LDH-virus infected mice (Chart 1 and unpublished data), all of the tumor-bearing mice in this study were given injections of the virus 2 to 4 days prior to treatment.

The results of 4 experiments using enzyme therapy are shown in Chart 2. Significant inhibition of tumor growth is evident. Minimum mean tumor volume of treated tumors: mean tumor volume of control tumors values during the enzyme treatment period were as follows: Experiment A, 0.03 at Day 4; Experiment B, 0.78 at Day 2; Experiment C, 0.32 at Day 5; and Experiment D, 0.14 at Day 3. In Experiment B, tumor inhibition was less pronounced and this weak response may be a result of the relatively large tumor

Table 2

*Plasma tyrosine and phenylalanine levels after administration of tyrosine phenol-lyase*

A single injection of tyrosine phenol-lyase (160 IU/kg) was given to 2 groups of 3 mice. Two groups of 3 additional mice receiving 0.9% NaCl solution served as controls. All mice were bled at the indicated times.

Treatment	Time (hr after treatment)	Tyrosine (nmoles/ml)		Phenylalanine (nmoles/ml)	
		Group 1	Group 2	Group 1	Group 2
0.9% NaCl solution	0	64	92	60	74
	7	85	60	81	73
Tyrosine phenol-lyase	0	87	129	81	113
	7	34	32	73	84
	26	67	75	101	91

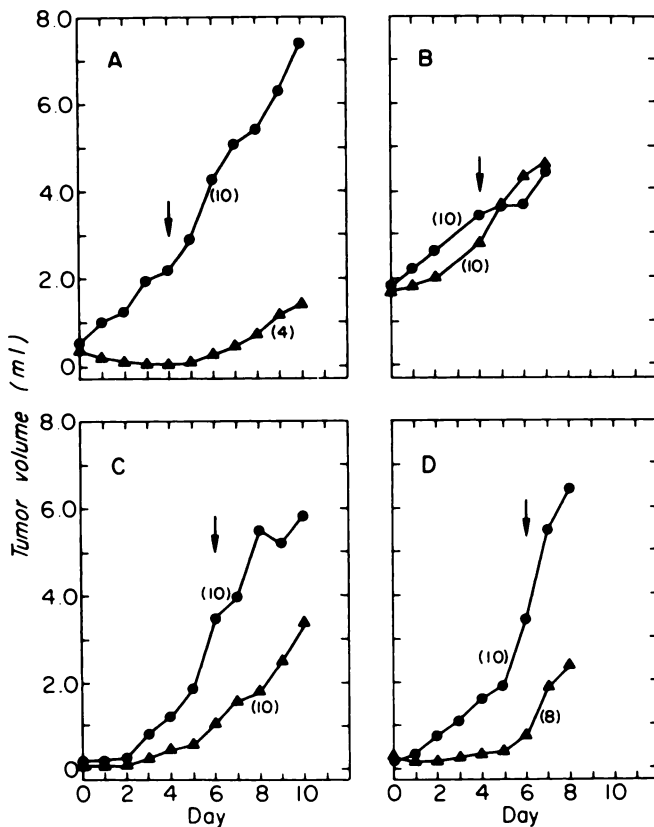


Chart 2. Influence of tyrosine phenol-lyase on the growth of B-16 melanoma. All mice were given injections of the LDH-virus 2 to 4 days prior to enzyme administration. Mice were treated starting at Day 0, 7 to 14 days after tumor implantation, with tyrosine phenol-lyase, 100 IU/kg, every 12 hr. Control mice were tumor-bearing mice that were similarly treated with a 0.9% NaCl solution or a pyridoxal phosphate and buffer solution as indicated. Value in parentheses, the number of mice/group. Arrow, last day of treatment. ▲, treated; ●, control. Experiments A, B, and C used the same protocol, whereas Experiment D differed in that all mice were fed a vitamin-enriched diet that contained 1 mg of pyridoxine hydrochloride and 1 mg of pyridoxal phosphate per g of powdered laboratory chow. All mice in this experiment were given i.p. injections of 1 mg of pyridoxal phosphate at the time of enzyme treatment. Mean tumor volume data for treated and controls were significantly different from Days 2 to 10 ( $p < 0.005$ ) for Experiment A, from Days 2 to 4 ( $p < 0.025$ ) for Experiment B, from Days 5 to 10 ( $p < 0.005$ ) for Experiment C, and from Days 2 to 10 ( $p < 0.005$ ) for Experiment D.

volume at the initiation of the treatment. In Experiment D an attempt was made to increase the level of circulating cofactor in order to improve enzyme efficiency. The chemotherapeutic response obtained, however, was not substantially different from the other experiments.

Dietary restriction of tyrosine and phenylalanine has been repeatedly shown to have a beneficial effect in both animal and human melanomas and in other tumors (5, 6, 13). In these studies 4 to 6 weeks of dietary treatment was necessary to appreciably lower plasma levels of the 2 amino acids. The data in Table 2 indicate that administration of tyrosine phenol-lyase rapidly reduces plasma tyrosine when injected (160 IU/kg) into normal mice. Considering the half-life value of 6 to 7 hr, the dosage schedule of 100 IU/kg every 12 hr presumably kept tyrosine levels in the experimental mice reduced throughout the treatment period. Pyruvate, ammonia, and phenol, the products of the enzymatic reac-

tion, were not tested for *in vivo* antimelanoma activity; however, phenol (42  $\mu\text{g/ml}$ ) had no effect on growth of B-16 melanoma *in vitro* whereas the enzyme was cytotoxic (C. Linden and G. W. Elmer, unpublished results). No attempt was made to restrict dietary intake of tyrosine and phenylalanine in these experiments, and a more thorough evaluation of the therapeutic potential of this new agent would involve concurrent restriction of these amino acids as well as investigation of antineoplastic activity in other tumor systems.

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