TUMOR INHIBITORY EFFECT OF L-ASPARAGINASE*

Louise T. Mashburn and John C. Wriston, Jr., Department of Chemistry, University of Delaware, Newark, Delaware.

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Guinea pig serum (GPS) has been shown (1-3) to inhibit the growth of the Gardner lymphosarcoma 6C3HED in C3H mice, as well as certain other tumors (1,2). Broome (4) has presented evidence recently that the L-asparaginase activity of GPS is responsible for the anti-lymphoma effect. The experiments described here on the effectiveness of partially purified L-asparaginase of GPS in inhibiting tumor growth support Broome's proposal.

METHODS

L-asparaginase assays were carried out on aliquots (0.3 ml or less) of sample in a total volume of 2.0 ml containing 20 μmoles of L-asparagine. Tris buffer (0.05M, pH 8.6), which was 10^{-5}M with respect to glutathione was used. Incubations were carried out at 37° in a Dubnoff shaker. After 30 minutes the reaction was stopped by addition of 0.1 ml of 1.5M trichloroacetic acid. The precipitated protein was removed by centrifugation and the liberated ammonia determined by nesslerization. A unit of L-asparaginase is that amount of enzyme which will liberate one μmole of ammonia in 30 minutes.

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Frozen GPS (Cappel Laboratories, West Chester, Pa.) was thawed and treated with 30% sodium sulfate according to the method of Meister (5). The protein which precipitated was recovered by centrifugation, redissolved in distilled water and dialyzed against two changes of distilled water followed by two changes of phosphate buffer[1]. A precipitate which formed during dialysis was removed by centrifugation and discarded. The supernatant was applied to a diethylaminoethyl cellulose (DEAE) column (2.5 x 10 cm) which had been washed overnight with phosphate buffer[1]. The column was then washed with two column volumes (76 ml) of buffer[1], and protein eluted by means of a sodium chloride gradient. The result of a typical fractionation is shown in Figure 1,a.

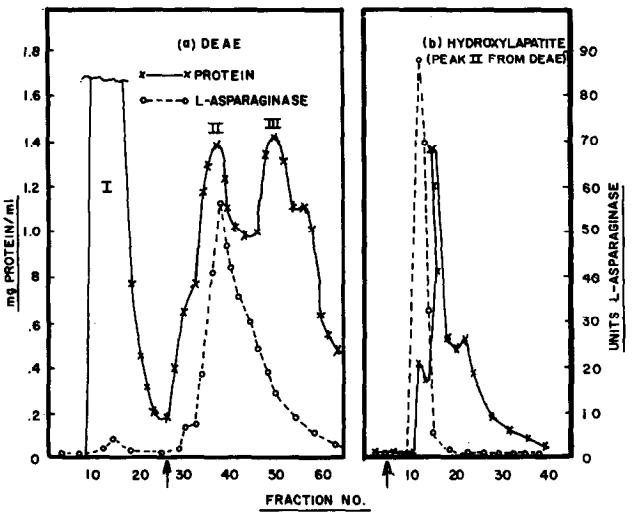

Fig. 1. Column chromatography of GPS proteins precipitated by 30% Na_2SO_4. Arrow indicates point where gradient elution was started. Gradient for DEAE column: 0.01M sodium phosphate buffer, pH 6.6, 10^{-5}M with respect to GSH, to 0.3M NaCl in 0.01M sodium phosphate buffer, pH 6.6. Flow rate, 100 ml/hr. Gradient for hydroxylapatite column: 0.005M Na_2HPO_4 to 0.2M Na_2HPO_4. Flow rate, 16 ml/hr. Reservoir volumes at start of gradient were 220 ml for both columns, fraction volumes 4.2 ml.

[1] Sodium phosphate buffer, 0.01M, pH 6.6, 10^{-5}M with respect to GSH.

Pooled fractions containing the L-asparaginase activity after elution of the DEAE column were applied to a hydroxylapatite (6) column (2.5 x 5 cm) which had been washed overnight with 0.001M sodium phosphate buffer, pH 7.6, 10^{-5}M with respect to GSH. The column was then washed with 20 ml of 0.005M Na_2HPO_4, followed by a disodium hydrogen phosphate gradient for elution. The resulting elution pattern is shown in Figure 1,b.

Protein was measured by the method of Lowry et al. (7). The lymphosarcomas were transplanted by trocar into the right flank of C3H-HeJ mice (Jackson Memorial Laboratories, Bar Harbor, Maine) four days before testing began. The tumor-bearing mice were injected two times daily (intraperitoneally, no less than 6 hours apart) with the test material. The average diameter of the tumors was determined by measuring with calipers in three directions.

RESULTS AND DISCUSSION

Figure 2 shows that there is a direct relationship between L-asparaginase activity and tumor inhibitory activity, regardless of the specific activity of the L-asparaginase used for testing.

The specific activity at various stages of purification and the recovery of L-asparaginase activity are shown in Table I. There is no readily apparent explanation for the low recovery of activity in our purified preparations as compared to those of Tower et al. (8) who recently reported 70% recovery of 100-fold purified L-asparaginase from GPS. Mardashev and Shao-Khua (9) and Broome[2], however, have also reported low

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[2] J. D. Broome, in press.

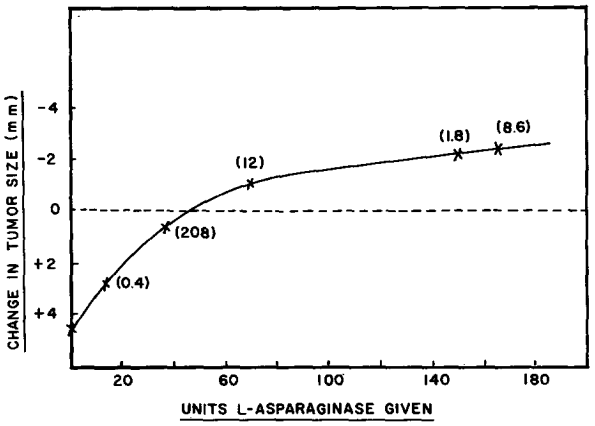

Fig. 2 Effect of L-asparaginase on Tumor Growth. Numbers in parentheses represent specific activity (units L-asparaginase/mg protein) of samples tested. All points are the average of four animals. Testing was carried out as described under Methods, the test period was three days.

TABLE I

PURIFICATION OF GUINEA PIG SERUM L-ASPARAGINASE

	PREP'N. 1 *				PREP'N. 2 **			
	FRACTION NO.	SP. AC. UNITS/mg PROT.	PURIF X	RECOV. %	FRACTION NO.	SP. AC. UNITS/mg PROT.	PURIF X	RECOV. %
GUINEA PIG SERUM	–	1.8	–	–	–	1.5	–	–
Na_2SO_4 PPT.	–	8.6	5.3	58	–	5.6	3.8	71
DEAE PEAK I	(10-18)	0.4	–	–	–	–	–	–
PEAK II	(36-44)	34	19	25	(43-49)	13	8	7
PEAK III	(45-52)	12	6.6	–	(53-59)	42	29	14
HYDROXYLAPATITE	(12-13)	208	116	10	(28-35)	308	205	3
HYDROXYLAPATITE	–	–	–	–	(30)	600	400	0.1

*DEAE column eluted with rapid NaCl gradient, as described in legend for Fig. 1, a; fractions tested for anti-lymphoma activity.

**DEAE column eluted with slow NaCl gradient, as described in text; first L-asparaginase peak from DEAE column (Peak II, tubes 43-49) applied to hydroxylapatite column. Arrows indicate point at which gradient elution was started.

recoveries of purified L-asparaginase from DEAE columns. The most highly purified preparation which we have tested in mice was purified 116-fold, but the 400-fold purified preparation (Table I) could not be tested since all activity was lost upon standing 24 hours in the cold.

It is worth noting that when a slower rate of gradient change is used on the DEAE column, (0.01M sodium phosphate buffer pH 6.6, 10^{-5}M with respect to GSH, to 0.15M NaCl in the same buffer) the L-asparaginase is divided into two peaks. When the second of these asparaginase peaks is applied to the hydroxylapatite column, no L-asparaginase activity was recovered, although 98% of the protein was eluted, while 50% of the L-asparaginase activity of the first peak was recovered (Table I, Prep'n. 2). If the two peaks are due to a dissociation of the L-asparaginase into subunits, or to minor modifications of the molecule as suggested by Mardashev and Shao-Khua (9), this might account for the low recovery and instability of the highly purified enzyme reported here.

Several L-asparaginase samples of intermediate specific activity (approximately 50 units/mg of protein) were tested for anti-lymphoma activity. These preparations showed an initial inhibitory effect on tumor growth which was not sustained throughout the 4-day injection period. When these samples were again assayed for L-asparaginase activity, it was noted that there had been a complete loss of L-asparaginase activity. This observation also supports the view that L-asparaginase of GPS is responsible for the anti-lymphoma activity.

We are continuing efforts to improve the recovery of purified GPS asparaginase, and in addition are studying the mechanism of action of the anti-lymphoma activity.

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