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Tumor Inhibitory and Non-Tumor Inhibitory L-Asparaginases from *Pseudomonas geniculata*

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Two enzymes that catalyze the hydrolysis of L-asparagine have been isolated from extracts of *Pseudomonas geniculata*. After initial salt fractionation, the enzymes were separated by chromatography on diethylaminoethyl-Sephadex and purified to homogeneity by gel filtration, ion-exchange chromatography, and preparative polyacrylamide electrophoresis. The enzymes differ markedly in physicochemical properties. One enzyme, termed asparaginase A, has a molecular weight of approximately 96,000 whereas the other, termed asparaginase AG, has a molecular weight of approximately 135,000. Both enzymes are tetrameric. The asparaginase A shows activity only with L-asparagine as substrate, whereas the asparaginase AG hydrolyzes L-asparagine and L-glutamine at approximately equal rates and it is also active with D-asparagine and D-glutamine as substrates. The asparaginase A was found to be devoid of antitumor activity in mice, whereas the asparaginase AG was effective in increasing the mean survival times of both C3H mice carrying the asparagine-requiring Gardner 6C3HED tumor line and Swiss mice bearing the glutamine-requiring Ehrlich ascites tumor line. These differences in antitumor activity were related to differences in the K_m values for L-asparagine for the two enzymes. The asparaginase A has a K_m value of 1×10^{-3} M for this substrate whereas the corresponding value for the AG enzyme is 1.5×10^{-5} M. Thus the concentration of asparagine necessary for maximal activity of the asparaginase A is very high compared with that of the normal plasma level of asparagine, which is approximately 50 μM.

The enzyme L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) has attracted much attention in the past few years because of its antineoplastic activity (for reviews see references 5, 16, 18, and 35). The enzyme has a particularly high therapeutic index when used in the treatment of acute lymphocytic leukemia (32) with more than two-thirds of human patients showing complete remissions. Most of these remissions are, however, short-lived, and there have been a number of difficulties associated with the use of *E. coli* L-asparaginase, which has been the agent of choice in most clinical studies. Prominent among these difficulties have been immune reactions to the foreign protein. Hypersensitivity reactions and an accelerated clearance time of the enzyme from plasma have been noted with continued therapy (2, 20). These immunological problems can be circumvented by sequential therapy with serologically unrelated asparaginases (9, 14), and this has prompted a search for a variety of bacterial asparaginases with antineoplastic properties.

Some bacterial glutaminases and asparaginases with glutaminase activity have also been found to have antineoplastic activity in mice, with these enzymes being effective against tumor lines which are resistant to asparaginase therapy (1, 13, 25, 26, 29, 35).

Factors to consider in examining an asparaginase for potential therapeutic use are that the enzyme should be amenable to rapid isolation in high yield, active under physiological conditions, and cleared relatively slowly from the bloodstream and should show substantial activity at the low substrate levels found in blood. The asparaginase should also not cross-react immunologically with *Escherichia coli* asparaginase. We chose to examine the asparaginases of *Pseudomonas geniculata* for the following reasons. First, this bacterium had been reported by Peterson and Ciegler (19) to produce relatively high levels of asparaginase when grown on a simple medium. Second, it seemed unlikely that *Pseudomonas* asparaginases would cross-react with antibodies to *E. coli* asparaginase. Third, preliminary experiments showed that two enzymes with asparaginase activity were produced

by *P. geniculata*. One of these enzymes showed only L-asparaginase activity, whereas the other possessed both asparagine and glutamine hydrolyzing activity. This offered the opportunity of comparing, from a single source, the antineoplastic activity of these two types of enzyme. The present report details the isolation and properties of the two *P. geniculata* asparaginases. The asparaginase-glutaminase was shown to have antineoplastic activity against both the asparagine-requiring 6C3HED Gardner lymphoma in C3H/HE mice and the glutamine-requiring Ehrlich tumor in Swiss mice.

MATERIALS AND METHODS

The chemicals and reagents used in these studies are the same as those described in our previous publications (6, 7).

C3H/HE and Swiss Webster mice were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine, or from Health Research Laboratories, West Seneca, N. Y. All animals were maintained on the appropriate Purina Laboratory Chow and water.

C3H/HE mice carrying the ascites strain of the Gardner lymphosarcoma 6C3HED-OG were generously supplied by Morton Prager, The University of Texas Southwestern Medical School, Dallas, Tex. Swiss mice carrying the ascites strain of the Ehrlich lymphosarcoma were generously donated by J. Roberts and J. S. Holcenberg of the University of Washington, School of Medicine, Seattle, Wash.

P. geniculata (B.3178) was a gift from R. E. Peterson of the United States Department of Agriculture, Agricultural Research Service, Peoria, Ill.

The organism was maintained by biweekly transfers on agar slants of 5 g of tryptone, 1 g of glutamic acid, 5 g of yeast extract, 2.5 g of dibasic potassium phosphate (TGY) and 20 g of agar per liter of water.

Cells for enzyme purification were grown in 88 liters of dibasic potassium phosphate broth in a 100-liter fermentor (Fermentation Design) at 30°C using 60 ft^3 (ca. 1.68 m^3) of air per min at 200 rpm agitation and a 10% inoculum. Stationary phase was reached in about 5 h. Cells were harvested with a Sharples centrifuge at the beginning of the stationary phase. Yields were approximately 700 g (wet weight) of cells per batch.

Preparative polyacrylamide gel electrophoresis was carried out according to the procedure of Davis (8) by using a Canalco apparatus (Canalco, Rockville, Md.).

Gel filtration for the determination of approximate molecular weights was carried out on Sephadex G-100 in a column (2 by 90 cm), using reverse flow. The buffer was 0.05 M borate, 0.1 M sodium chloride, and 0.001 M EDTA at pH 7.0. Thin-layer gel filtration with Sephadex G-200 Superfine was also used for molecular weight determination using a Pharmacia TLG apparatus according to directions supplied by the manufacturer (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

Ultracentrifugal analysis of the enzyme was carried out with a Beckman Spinco model E ultracentrifuge at a rotor speed for 54,000 rpm at 20°C. The protein concentration was approximately 6 mg/ml in 0.05 M borate–0.1 M sodium chloride–0.001 M EDTA buffer (pH 7.0). The sedimentation constant was corrected to water at 20°C.

For amino acid analysis purified asparaginase AG was lyophilized. Duplicate samples containing approximately 0.4 mg of protein were hydrolyzed in 6 N HCl at 110°C in sealed evacuated tubes for 24, 48, and 72 h. Samples were hydrolyzed for 72 h under similar conditions in *p*-toluene sulfonic acid for tryptophan determination (15). Analyses were carried out on a Beckman model 121 amino acid analyzer. Serine and threonine values were extrapolated to zero time of hydrolysis. Cysteine was determined as cysteic acid in performic acid-treated samples.

Double diffusion in agar was performed by the method of Ouchterlony (31). A high-potency rabbit antiserum directed against *Escherichia coil* L-asparaginase was the gift of James P. Allison.

The rate of clearance of the *P. geniculata* AG enzyme was determined by injecting 0.4 ml of enzyme solution, containing 10 IU of asparaginase activity in 0.05 M borate buffer, 0.1 M sodium chloride, and 0.001 M EDTA (pH 7.0) intraperitoneally into normal and tumor-bearing C3H mice. The mice were bled from the tail vein at various time intervals and the asparaginase activity was determined by the micronesslerization procedure.

Tumor inhibition assays were carried out by a modification of the methods of Roberts et al. (27). Both the A and AG enzymes were tested against the ascitic form of the asparagine-requiring Gardner lymphoma 6C3HED in C3H/HE mice. The AG enzyme was also tested against the glutamine-requiring Ehrlich tumor in Swiss mice. Cells were transplanted by intraperitoneal injection of 10^6 viable ascites cells from 8 and 12-day-old tumors in the case of the Gardner lymphoma and the Ehrlich tumor, respectively. The number of viable cells was estimated by trypan blue exclusion in a hemacytometer. Various dose schedules were used, as noted below. The number of surviving mice was recorded daily.

RESULTS

Preliminary experiments established that crude extracts of *P. geniculata* contained relatively high levels of both asparaginase and glutaminase activities. When the crude extracts were examined by polyacrylamide gel electrophoresis, two enzymes with asparaginase activity were detected (Fig. 1). One enzyme migrated rapidly to the anode at pH 9, running close to the tracking dye. This enzyme showed only asparaginase activity and was termed asparaginase A. The other enzyme migrated more slowly in the gel and showed both asparaginase and glutaminase activities. It was termed asparaginase AG.

Enzyme purification. Approximately 600 g of *P. geniculata* cells was thawed with warm water and suspended in a 2.4-liter solution of 0.1

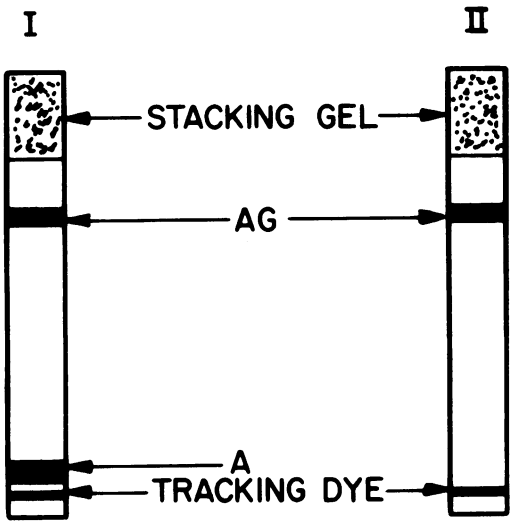

FIG. 1. *Diagrammatic representation of polyacrylamide gel electrophoresis of a crude extract of P. geniculata showing the relative mobility of the asparaginases. Gel I was stained for asparaginase activity and gel II for glutaminase activity. A, Asparaginase; AG, asparaginase-glutaminase.*

M potassium phosphate buffer, 0.001 M EDTA, and 0.1 mM dithiothreitol (pH 7.5), and stirred for about 1 h. The cells were disrupted by two passes through a Gaulin continuous-flow homogenizer at a pressure of 6,000 lb/in^2. Cellular debris was removed by centrifugation in a Beckman model L3-40 preparative ultracentrifuge in a type 15 rotor at 14,000 rpm for 30 min. The supernatant was decanted, and 1.0 M manganese (II) chloride was added dropwise with continuous stirring to the supernatant at 4°C to give a final concentration of 0.05 M manganese chloride. The pH was checked and adjusted with 0.1 M potassium hydroxide to pH 7.8. The solution was stirred for an additional hour, then allowed to stand overnight in a 4°C cold room. The precipitate was removed by centrifugation at 14,000 rpm for 30 min.

Finely powdered ammonium sulfate was added to the supernatant to bring the concentration to 70% saturation. The solution was stirred in an ice bath and the pH was maintained at pH 7.0 with 10% ammonium hydroxide. The sample was centrifuged, after standing in centrifuge bottles for 30 min, at 14,000 rpm in a type 15 rotor for 30 min. The supernatant was discarded and the pellet was dissolved in a minimal volume of 0.1 M potassium phosphate buffer, 0.001 M EDTA, and 0.1 mM dithiothreitol (pH 7.5). This solution was dialyzed against a solution of 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 0.01 M NaCl, 0.001 M EDTA, and 0.1 mM dithiothreitol (pH 8.3). The dialysis buffer was changed twice. The last dialysis buffer was used to equilibrate a diethylaminoethyl (DEAE)-Sephadex column (7.5 by 50 cm). The enzyme sample containing both the asparaginase-glutaminase (AG) and the asparaginase (A) enzymes was applied to the equilibrated column. The column was eluted with dialysis buffer until the absorbance of the elutant at 280 nm was below 0.2. A 6-liter linear gradient from 0.01 to 0.5 M NaCl in the same buffer was then applied to the column. The AG enzyme was eluted in the breakthrough peak. The fractions containing this activity were pooled and concentrated by ultrafiltration. Asparaginase A was bound to the column and was eluted at a salt concentration of approximately 0.4 M. The tubes containing the asparaginase A activity were pooled and concentrated by ultrafiltration.

The asparaginase A enzyme was dialyzed against a solution of 0.02 M Tris-hydrochloride buffer, 0.1 M NaCl, 0.001 M EDTA, and 0.1 mM dithiothreitol (pH 8.3). The dialyzed sample was applied to a second DEAE-Sephadex column (3 by 40 cm) under the same conditions as those of the first column. Asparaginase A was eluted from the column at 0.4 M NaCl. The fractions containing activity were combined and concentrated by ultrafiltration to 10 ml and dialyzed against a solution of 0.02 M Tris-hydrochloride buffer, 0.001 M EDTA, and 0.1 mM dithiothreitol (pH 8.0). Preparative polyacrylamide gel electrophoresis was used for further purification of asparaginase A (see above). Asparaginase A was the first protein component eluted from the gel after the tracking dye. The fractions containing asparaginase activity were pooled and concentrated by ultrafiltration.

The asparaginase-glutaminase (AG) which was found in the breakthrough peak of the first DEAE-Sephadex column was concentrated by ultrafiltration and dialyzed against a solution of 0.05 M potassium phosphate buffer and 0.001 M EDTA (pH 4.5). The dialyzed AG enzyme was applied to a CM-Sephadex column (7.5 by 50 cm) equilibrated with dialysis buffer. The column was eluted with dialysis buffer until the absorbance at 280 nm was below 0.2. A linear gradient was then started with 2,000 ml of 0.05 M potassium phosphate buffer–0.001 M EDTA (pH 4.5) to 2,000 ml of 0.1 M potassium phosphate buffer–0.001 M EDTA (pH 7.0). The fractions with AG activity were pooled and concentrated by ultrafiltration to 10 ml. The concentrated AG sample was then applied to the bottom of a column of Sephadex G-200 (5 by 80 cm). The enzyme was eluted by ascending chromatography by using 0.05 M borate buffer–0.001

M EDTA (pH 7.0) containing 0.1 M sodium chloride. The active fractions were concentrated and dialyzed against 1.2 M potassium phosphate buffer–0.001 M EDTA (pH 7.5). Hydrophobic chromatography, using a valine-Sepharose column (24) equilibrated with the same buffer, was used to further purify the enzyme. A gradient of 50 ml of 1.2 M to 50 ml of 0.5 M potassium phosphate buffer (pH 7.5) containing 0.001 M EDTA was used to elute the enzyme. Fractions containing enzyme activity were pooled and concentrated. A summary of the purification procedures is given in Table 1.

The asparaginases A and AG comprised 40 and 60%, respectively, of the asparaginase activity found in the crude extract. The asparaginase A was purified 1,116-fold to a final specific activity of 96 IU/mg, whereas the asparaginase-glutaminase AG was purified 1,375-fold to a final specific activity of 151 IU/mg. Both purified enzymes were judged homogeneous by polyacrylamide and SDS gel electrophoresis. The purified asparaginase A represented 9.5% and the asparaginase AG represented 10% of the total activity of the crude extract. Both enzymes showed no appreciable loss of activity with storage at 4°C for 6 months in the presence of 0.1 mM dithiothreitol.

Comparison of the properties of asparaginases A and AG. The molecular weight of both enzymes was estimated by gel filtration on Sephadex G-100. Approximate molecular weights of 135,000 for the asparaginase-glutaminase AG and 94,000 for the asparaginase A were calculated by Squire's equation (30). Thin-layer gel filtration on Sephadex Superfine G-200 gave approximate molecular weights of 147,000 and 96,500 for the AG and A enzymes, respectively. A single symmetrical peak was observed in schlieren photographs of a sedimentation velocity run in the ultracentrifuge of the AG enzyme. The $s_{20,w}$ was 6.93S. The subunit molecular weight of the two enzymes was determined by SDS-polyacrylamide gel electrophoresis. Only a single protein band was noted in both cases. The mobility of the band corresponded to a molecular weight of 34,500 for the asparaginase AG and 24,000 for the asparaginase A. The amino acid composition of the AG enzyme is given in Table 2 together with the composition of other bacterial asparaginases for comparison.

Both the *P. geniculata* enzymes have a relatively high pH optimum, with that of the A enzyme being pH 9.0 and that of the AG enzyme, pH 9.5. The pH activity curves are, however, broad, with both enzymes showing at least 50% of their optimal activity at pH 7.4. The isoelectric points of the enzymes differ greatly, that of the asparaginase A being 3.80 and that of the asparaginase AG being 6.97. There was no separation of the asparaginase and glutaminase activities of the AG enzyme by isoelectric focusing. Marked differences in thermal stability between the two enzymes was also observed. After incu-

TABLE 1. *Purification of P. geniculata asparaginase*

Fraction	Enzyme activity (IU)		Total protein (mg)	Sp act (IU/mg)	
	Asparaginase	Glutaminase		Asparaginase	Glutaminase
Crude extract	46,592	29,120	266,560	0.17	0.11
$MnCl_2$	39,000	22,750	150,000	0.26	0.15
Ammonium sulfate	32,500	19,500	70,000	0.46	0.28
Asparaginase AG					
First DEAE-Sephadex chromatography	14,550	13,380	12,500	1.16	1.07
CM-Sephadex chromatography	13,100	12,500	450	28.9	27.8
Sephadex G-200 chromatography	5,590	5,240	81.7	68	64
Valine-Sepharose chromatography	4,620	4,340	30.6	151	142
Asparaginase A					
First DEAE-Sephadex chromatography	12,090		7,400	2.0	
Second DEAE-Sephadex chromatography	5,970		624	9.6	
Preparative polyacrylamide electrophoresis	440[a]		4.7	96	
	4,400[b]		47	96	

[a] Preparative polyacrylamide gel electrophoresis was run on 1/10 of the material from the second DEAE-Sephadex chromatography.

[b] Theoretical values for total sample.

TABLE 2. *Amino acid composition of bacterial asparaginase*

Amino acid	*P. geniculata* AG (mol/ 34,500 g)	*C. freundii*[a] A (mol/ 32,800 g)	*A. eutrophus*[b] (mol/ 34,500 g)	*E. coli*[c] (mol/ 33,300 g)	*Acinetobacter*[d] (mol/ 33,000 g)	*P. vulgaris*[e] (mol/ 30,000 g)	*E. carotovora*[f] (mol/ 33,370 g)	*P. acidovorans*[g] (mol/ 39,000 g)
Lysine	15.9	21.0	22.4	21.0	24.4	22.8	18.2	19.9
Histidine	4.1	4.8	4.2	3.1	8.9	3.1	5.4	5.8
Arginine	10.9	9.8	12.3	7.2	10.0	6.4	16.2	32.7
Tryptophan	9.5[h]	1.8	2.9	1.0	4.3	1.2	0	4.2
Aspartic acid	31.5	39.3	33.5	50.7	36.7	40.0	35.1	36.8
Threonine	21.1	28.0	20.6	32.6	16.3	22.4	26.3	23.9
Serine	16.9	19.0	18.8	14.6	18.9	13.9	17.5	18.3
Glutamic acid	33.4	25.4	30.0	18.7	23.5	21.8	20.9	30.4
Proline	7.9	13.5	14.3	11.0	10.2	11.4	12.5	17.8
Glycine	32.8	28.7	28.5	28.5	22.2	27.3	33.4	30.5
Alanine	42.8	31.2	42.6	31.1	36.2	31.2	29.0	47.7
Cysteine	4.5	2.1	0	1.9	0	2.0	0	1.0
Valine	32.1	26.5	26.1	35.0	28.5	30.7	29.7	30.4
Methionine	8.3	5.0	4.9	3.9	7.4	5.2	6.7	4.6
Isoleucine	17.6	11.7	16.0	12.2	16.4	14.6	16.9	13.3
Leucine	24.2	23.2	23.3	22.3	24.0	22.6	28.3	28.6
Tryosine	7.4	11.0	10.0	10.0	8.0	8.8	12.5	8.8
Phenylalanine	5.3	8.5	8.3	8.3	9.0	10.3	6.7	9.6

[a] Data from Davidson et al. (7).
[b] Data from J. P. Allison (Ph.D. thesis).
[c] Data from Ho et al. (10).
[d] Data from Roberts et al. (26).
[e] Data from Tosa et al. (33).
[f] Data from Cammack et al. (3).
[g] Data from Davidson et al. (6).
[h] Determined by analysis of samples hydrolyzed in *p*-toluene sulfonic acid.

bation at 60°C for 10 min, the A enzyme retained 30% of the initial activity, whereas the AG enzyme was completely inactivated. At 50°C for 10 min the AG retained only 19% of the original activity and both asparaginase and glutaminase activities were reduced at the same rate. The presence of the substrate L-asparagine provided considerable protection against thermal inactivation. Fifty percent inactivation occurred after 10 min of incubation at 66°C for the A enzyme and at 71.5°C for the AG enzyme under these conditions.

The substrate specificity of the A and AG enzymes also offers an interesting comparison (Table 3). The asparaginase A has a very restricted specificity, showing no detectable activity with the D-isomers of asparagine and glutamine or with L-glutamine. A number of asparagine analogs also gave no detectable ammonia production, with the exception of the L-asparagine-*t*-butyl ester which showed slight but detectable ammonia production upon prolonged incubation with enzyme A. By contrast, the AG enzyme showed essentially equivalent activities with both L-asparagine and L-glutamine and relatively high activity with both D-isomers. A number of asparagine derivatives and analogs also gave slight ammonia production on prolonged incubation with this enzyme. Marked

TABLE 3. *Substrate specificity of P. geniculata asparaginases A and AG*

Substrate[a]	Relative activity	
	Asparaginase A	Asparaginase AG
L-Asparagine	100	100
D-Asparagine	0	44
L-Glutamine	0	94
D-Glutamine	0	24
L-Aspartic acid	0	0
L-Asparagine-butyl ester	0.9	1.4
L-Isoasparagine	0	2.3
L-Carbamyl-L-cysteine	0	6.0
L-Aspartyl-β-hydroxamic acid[b]	94	256
L-Glutamyl-γ-hydroxamic acid[b]	0	282
N-acetyl-L-asparagine	0	4.1
Carbobenzyloxy-L-asparagine	0	0
Succinamide	0	0

[a] All substrates were used at concentrations of 7.5 mM in 0.1 mM in 0.1 M Tris-hydrochloride buffer at pH 7.5. After a 10-min incubation, ammonia released was determined by nesslerization. Those substrates not showing activity were reassayed for 100 min. Relative activity was based upon the amount of L-asparagine converted to ammonia as 100% activity.
[b] Incubated with the enzymes for 10 min. Hydrolysis was determined as described in the text.

differences were also seen between the ability of the A and AG enzymes to hydrolyze the hydroxamic acid analogs of glutamine and asparagine. Although the A enzyme hydrolyzes both L-asparagine and L-asparatic acid-β-hydroxamate at approximately equal rates, it will not hydrolyze L-glutamic acid-γ-hydroxamate. On the other hand, the AG enzyme hydrolyzes both these analogs at rates considerably greater than with L-asparagine as substrate. The enzymes exhibited typical Michaelis-Menten kinetics at low substrate concentrations, the apparent K_m values for asparagine being 1×10^{-3} M for the asparaginase A and 1.5×10^{-5} M for the asparaginase AG. The K_m value for glutamine is 3.1×10^{-5} for the asparaginase AG.

Immunological properties. The cross-reactivity of the two *P. geniculata* enzymes with antibodies to *E. coli* asparaginase was tested with a high-potency rabbit antiserum by double diffusion in agar. As shown in Fig. 2, neither of the *P. geniculata* enzymes showed cross-reactivity by this technique.

Antitumor activity. Intraperitoneal injections of the *P. geniculata* asparaginase AG produced significant increases in the mean survival time (MST) of C3H/HE mice inoculated with the asparagine-requiring Gardner 6C3HED lymphoma. Results of representative experiments are given in Table 4. The most successful treatment schedule tested was a single 30-IU injection of the AG enzyme given 7 days after tumor implantation. This gave an average increase of 5 days in mean survival time. One of the *P. geniculata* asparaginase AG-treated mice which survived for 42 days after tumor inoculation (30 days longer than the mean survival time of untreated controls) was rechallenged with 10^6 ascites cells to determine whether immunity to the tumor had been established. The mouse rapidly succumbed to the tumor. This may be contrasted with five mice treated with *E. coli* asparaginase, three of which, when rechallenged

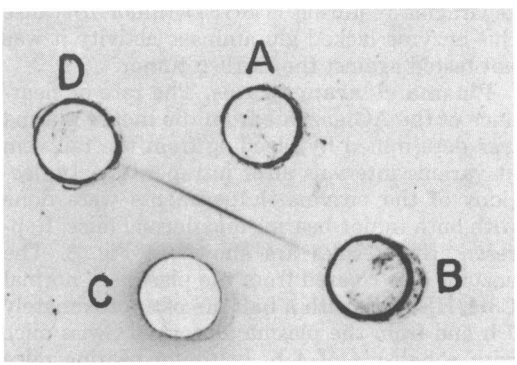

FIG. 2. *Double diffusion in agar of an antiserum directed against E. coli asparaginase (A) with P. geniculata asparaginase A (B), P. geniculata asparaginase AG (D), and E. coli asparaginase (C).*

TABLE 4. *Antitumor activity of P. geniculata asparaginases A and AG*

Tumor	Treatment	No. of mice	Mean survival time (days)	Range	Increase in mean survival time (days)
6C3HED	None	5	13	13	
	15 IU *P. geniculata* A, day 7[a]	5	13	13	0
	6.7 IU *P. geniculata* A, days 2,4,8	5	13	13	0
6C3HED	None	4	14.5	14–15	
	15 IU *P. geniculata* A, day 7[a]	5	15.6	12–20	1.1
	6.7 IU *P. geniculata* A, days 2,4,8	5	13.6	12–14	
	15 IU *P. geniculata* AG, day 7[b]	5	15.8	15–17	1.3
6C3HED	None	5	12.2	10–13	0
	10 IU *P. geniculata* AG, days 2,4,8[b]	5	12.4	7–20	0.2
	5 IU *P. geniculata* AG, days 0,1,2,3,4	5	12.8	7–28	0.4
	30 IU *P. geniculata* AG, day 7	5	18	14–28	7.8
	25 IU *P. geniculata* AG, day 7[b]	5	16.2	13–18	4.0
	Heat-inactivated *P. geniculata* AG, day 7	5	11.2	9–14	0
Ehrlich	None	5	17.8	17–18	
	5 IU *P. geniculata* AG, days 1,3,5,7,9[b]	5	31.2[c]	20–49	13.5
	5 IU *P. geniculata* AG, days 0,1,2,3,4,5	5	18.2	1–53	0.4

[a] These results were obtained from two different enzyme preparations.
[b] These results were obtained from three different enzyme preparations.
[c] Mean survival time for 4 of 5 mice. One mouse survived for 42 days and was then rechallenged (see text).

in the same manner, showed no signs of tumor growth. The AG enzyme was also tested against the glutamine-requiring Ehrlich lymphoma in Swiss mice. Here the most effective schedule tested was the injection of 5 IU of the AG enzyme every other day from day 1 to day 9 after tumor implantation. This schedule produced an increased mean survival time of 11 days. Heat-inactivated *P. geniculata* asparaginase AG was devoid of antitumor activity. Intraperitoneal injections of the asparaginase A did not produce any significant increase in mean survival time of C3H/HE mice injected with the asparagine-requiring 6C3HED tumor. Because this enzyme lacked glutaminase activity it was not tested against the Ehrlich tumor.

Plasma clearance rates. The rate of clearance of the AG enzyme from the mouse plasma was determined by bleeding from the tail vein at various intervals after intraperitoneal injections of the enzyme. Experiments were done with both tumor-bearing and normal mice. Representative results are shown in Fig. 3. The enzyme was cleared from the plasma of normal C3H/HE mice with a half-life of approximately 7 h and from the plasma of normal Swiss mice with a half-life of 4 h. In tumor-bearing mice clearance of the AG enzyme was slowed, but to different extents with the two tumor lines. In C3H/HE mice carrying the 6C3HED Gardner tumor the half-life of the AG enzyme was approximately 27 h, whereas in Swiss mice carrying the Ehrlich lymphoma it was 6.5 h. The markedly slower clearance rate from the tumor-bearing C3H mice can most likely be attributed to the presence of lactic dehydrogenase elevating virus in such animals (22, 23).

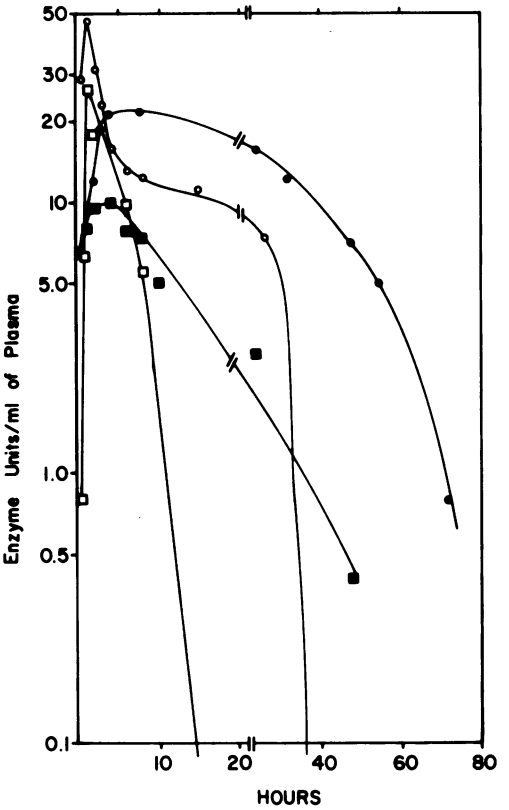

FIG. 3. *Plasma clearance of L-asparaginase in C3H mice. At zero time, C3H mice received intraperitoneal injections of either 10 IU of P. geniculata asparaginase AG or 10 IU of E. coli asparaginase. Tumor-bearing mice had received transplants of 10^6 6C3HED ascites cells 4 days previously. At the specified intervals mice were bled from the tail vein, and asparaginase activity was determined by the micronesslerization assay method.* (●) *E. coli, tumor-bearing mouse;* (■) *E. coli, normal mouse;* (○) *P. geniculata, tumor-bearing mouse;* (□) *P. geniculata, normal mouse.*

DISCUSSION

In this paper we describe the isolation of two asparaginases from *P. geniculata* using a relatively straightforward purification procedure. The two enzymes were found to differ markedly both in their physicochemical properties and in their ability to affect the growth of murine neoplasms. The asparaginase A is similar in catalytic properties to an asparaginase isolated from *Mycobacterium tuberculosis* (12). The substrate specificity of the *P. geniculata* asparaginase AG is similar to the asparaginase found in *Alcaligenes eutrophus* (J.P. Allison, Ph.D. thesis, University of Texas, Austin, 1973). The apparent K_m values for the two *P. geniculata* enzymes also differ greatly.

Both the asparaginase and glutaminase activities of the *P. geniculata* asparaginase AG reside in a single enzyme and share a common active site. Similar findings have been reported for asparaginases from *A. eutrophus* (Allison, Ph.D. thesis), an *Achromobacter* species (26) and from two different pseudomonads (17, 21).

Gel filtration experiments give an approximate molecular weight of 96,000 for *P. geniculata* asparaginase A and 135,000 for asparaginase AG. The subunit molecular weights are 24,000 for asparaginase A and 34,500 for asparaginase AG. These values indicate a tetrameric structure for both enzymes. The asparaginase A is smaller than most tumor inhibitory asparaginases. However, the *P. geniculata* asparaginase AG is comparable in size to the antineoplastic asparaginases of *Erwinia carotovora*, *Proteus vulgaris*, *E. coli*, *A. eutrophus*, and *Citrobacter freundii*, as well as the glutaminase-asparaginase of *Aci-*

netobacter glutaminasificans with molecular weights of 135,000, 120,000, 139,000, 138,500, 141,000 and 138,000, respectively (3, 7, 11, 26, 28, 33, 34; and Allison, Ph.D. thesis). The antineoplastic glutaminase A from *P. aeruginosa* has a molecular weight of 137,000 (29). A few tumor inhibitory enzymes have lower molecular weights, such as a glutaminase-asparaginase from one pseudomonad (1), which is reported to have a molecular weight of 59,000, and glutaminase-asparaginase from another pseudomonad with a molecular weight of 25,000 (21).

The amino acid compositions of bacterial asparaginases (Table 1), although showing some broad similarities, differ quite substantially for some residues. The most noteworthy values in the case of the *P. geniculata* asparaginase AG are the relatively high contents of tryptophan, methionine, and cysteine.

The two *P. geniculata* asparaginases differ widely in isoelectric point but this is true of many asparaginases, and there appears to be no discernible relationship between the isoelectric point of such enzymes and their antineoplastic potency (7). No cross-reactivity was observed between an antibody directed against *E. coli* asparaginase and either of the two *P. geniculata* asparaginases.

Of most immediate practical interest is the difference between the *P. geniculata* enzymes in their effect on murine neoplasms (Table 3). The asparaginase A was ineffective in increasing the mean survival time of C3H mice carrying the 6C3HED lymphosarcoma. This can most reasonably be related to the fact that blood asparagine levels must be reduced below 10^{-5} M before protein synthesis is severely limited and the asparaginase A has an apparent K_m value of 10^{-3} M. On the other hand, the *P. geniculata* asparaginase AG was found to be quite effective in prolonging the survival times of both C3H mice carrying the asparagine-requiring 6C3HED tumor and Swiss mice bearing the glutamine-requiring Ehrlich tumor. This enzyme has a relatively high affinity for both asparagine and glutamine (as measured by the apparent K_m), is not inactivated by prolonged incubation in mouse serum at 37°C and is not inhibited by high levels of aspartate or glutamate. Another factor favoring the antineoplastic activity of the *P. geniculata* asparaginase AG is the relatively slow clearance of this enzyme from the plasma of tumor-bearing animals. The half-times of the AG enzyme for plasma clearance in C3H mice with the 6C3HED tumor are comparable to those obtained with *E. coli* asparaginase (6). A problem noted with the *P. geniculata* asparaginase AG is that multiple daily intraperitoneal injections of the enzyme in both C3H and Swiss mice lead to considerable weight loss in the animals. This effect is much reduced by longer spacing between injections. Given this caveat, the *P. geniculata* asparaginase AG appears a promising candidate for further investigation of its potential for the treatment of human leukemia.

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LITERATURE CITED

1. **Broome, J. D., and I. Schenkein.** 1970. Further studies on the tumor inhibitory activity of a bacterial glutaminase-asparaginase, p. 95-105. *In* M. Boiron (ed.), La L-asparaginase. Centre National de la Recherche Scientifique, Paris.
2. **Burchenal, J. H., and D. A. Karnufsky.** 1970. Clinical evaluation of L-asparaginase. Cancer **25**:241-243.
3. **Cammack, K. A., D. I. Marborough, and D. S. Miller.** 1972. Physical properties and subunit structure of L-asparaginase isolated from *Erwinia caratovora*. Biochem. J. **126**:361-379.
4. **Cooney, D. A., A. L. Moore, and R. Zhagats.** 1972. A colorimetric technique for the demonstration of L-asparaginase in electropherograms. Clin. Chim. Acta **40**: 249-257.
5. **Crowther, D.** 1971. L-asparaginase and human malignant disease. Nature (London) **229**:168-171.
6. **Davidson, L., D. R. Brear, P. Wingard, J. Hawkins, and G. B. Kitto.** 1977. Purification and properties of an L-glutaminase-L-asparaginase from *Pseudomonas acidovorans*. J. Bacteriol. **129**:1379-1386.
7. **Davidson, L., M. Burkom, S. Ahn, L. C. Chang, and B. Kitto.** 1977 L-asparaginase from *Citrobacter freundii*. Biochim. Biophys. Acta **480**:282-294.
8. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. N. Y. Acad. Annals **121**:404-427.
9. **Goldberg, A. I., D. A. Cooney, J. P. Glynn, E. R. Homan, M. R. Gaston, and H. A. Milman.** 1973. The effects of immunization to L-asparaginase on antitumor and enzymatic activity. Cancer Res. **33**:256-261.
10. **Ho, P. P. K., E. Milikin, J. Bobbitt, E. Grinnan, P. Burck, B. Frank, L. Boeck, and R. Squires.** 1970. Crystalline L-asparaginase from *Escherichia coli* B. V. Purification and chemical characterization. J. Biol. Chem. **245**:3708-3715.
11. **Holcenberg, J. S., D. C. Teller, J. Roberts, and W. C. Dolowy.** 1972. Physical properties of *Acinetobacter* glutaminase-asparaginase with anti-tumor activity. J. Biol. Chem. **247**:7750-7758.
12. **Jayaram, H. N., T. Ramakrishnan, and C. S. Vaidynathan.** 1968. L-asparaginases from *Mycobacterium tuberculosis* strains $H_{37}R_v$ and $H_{37}R_a$. Arch. Biochem. Biophys. **126**:165-174.
13. **Katsumata, H., R. Katsumata, T. Abe, O. Takenaka, and Y. Unada.** 1972. Purification and physiochemical properties of L-glutaminase from *Pseudomonas*. Biochim. Biophys. Acta **289**:405-409.
14. **King, O. Y., J. R. Wilbur, D. M. Munaford, and W. W. Sutow.** 1974. Therapy with *Erwinia* L-asparaginase in children with acute leukemia after anaphylaxis to *E. coli* L-asparaginase. Cancer **33**:611-614.
15. **Liu, T. U., and Y. H. Chang.** 1971. Hydrolysis of proteins

with *p*-toluene sulfonic acid. J. Biol. Chem. **246**: 2842–2848.
16. **McCreadie, K. B., D. H. W. Ho, and E. J. Freireich.** 1973. L-asparaginase for the treatment of cancer. Cancer J. Clin. **23**:220–227.
17. **Nikolaev, A. Ya., L. P. Evseeve, E. S. Tyul'panova, and A. Kh. Abdumalikov.** 1969. Isoenzymes of asparaginase in *Pseudomonas* sp. Biokhimiya **34**:352–355.
18. **Oettgen, H. F., P. A. Stephensen, M. K. Schwartz, R. D. Leeper, L. Tallal, C. C. Tan, B. D. Clarkson, R. B. Golbey, I. H. Krakoff, D. A. Karnofsky, M. L. Murphy, and J. H. Burchenal.** 1970. Toxicity of *E. coli* L-asparaginase in man. Cancer **25**:253–278.
19. **Peterson, R. E., and A. Ciegler.** 1969. L-asparaginase production by various bacteria. Appl. Microbiol. **17**: 929–930.
20. **Peterson, R. G., M. S. Mitchell, R. L. Capizzi, and R. E. Handschumacher.** 1969. Immunological modification of L-asparaginase activity. Pharmacologist **11**: 234–235.
21. **Ramadan, M. E. A., F. El-Asmar, and D. M. Greenberg.** 1964. Purification and properties of glutaminase and asparaginase from a *Pseudomonad*. I. Purification and physical chemical properties. Arch. Biochem. Biophys. **108**:143–149.
22. **Riley, V., H. A. Campbell, and C. C. Stock.** 1970. Asparaginase clearance: influence of the LDH-elevating virus. Proc. Soc. Exp. Biol. Med. **133**:38–42.
23. **Riley, V., D. Spackman, M. A. Fitzmaurice, J. Roberts, J. S. Holcenberg, and W. C. Dolowy.** 1974. Therapeutic properties of a new glutaminase-asparaginase preparation and the influence of the lactate dehydrogenase-elevating virus. Cancer Res. **34**:429–438.
24. **Rimmerman, R. A., and G. W. Hatfield.** 1973. Phosphate-induced protein chromatography. Science **182**: 1268–1270.
25. **Roberts, J., J. S. Holcenberg, and W. C. Dolowy.** 1970. Antineoplastic activity of highly purified bacterial glutaminases. Nature (London) **227**:1136–1137.
26. **Roberts, J., J. S. Holcenberg, and W. C. Dolowy.** 1972. Isolation, crystallization and properties of *Achrombacteraceae* glutaminase-asparaginase with antitumor activity. J. Biol. Chem. **247**:84–90.
27. **Roberts, J., M. D. Prager, and W. Bachynsky.** 1966. The anti-tumor activity of *Escherichia coli* L-asparaginase. Cancer Res. **26**:2213–2217.
28. **Shefrin, S., B. G. Solis, and I. M. Chaikin.** 1973. L-asparaginase from *Erwinia carotovora*. Physiochemical properties of the native and succinylated enzyme. J. Biol. Chem. **248**:3464–3469.
29. **Soda, K., M. Oshima, and T. Yamamoto.** 1972. Purification and properties of isozymes of glutaminase from *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **46**:1278–1284.
30. **Squire, P. G.** 1964. A relationship between the molecular weights of macromolecules and their elution volumes based upon a model for Sephadex gel filtration. Arch. Biochem. Biophys. **107**:471–478.
31. **Stollar, D., and L. Levine.** 1963. Two-dimensional immunodiffusion. Methods Enzymol. **6**:848–854.
32. **Tallal, L., C. Tan, H. Oettgen, N. Wallner, M. McCarthy, L. Helson, J. Burchenal, D. Karnofsky, and L. E. Murphy.** 1970. *E. coli* L-asparaginase in the treatment of leukemia and solid tumors in 131 children. Cancer **25**:306–320.
33. **Tosa, T., R. Sano, K. Yamamoto, M. Nakamura, and I. Chibata.** 1972. L-asparaginase from *Proteus vulgaris*. Purification, crystallization and enzymatic properties. Biochemistry **11**:217–222.
34. **Whelan, H. A., and J. C. Wriston, Jr.** 1969. Purification and properties of asparaginase from *Escherichia coli* B. Biochemistry **8**:2386–2393.
35. **Wriston, J. C., and T. O. Yellin.** 1973. L-asparaginase: a review, p. 185–248. *In* A. Meister (ed.), Advances in enzymology, vol. 39. John Wiley and Sons, Inc., New York.