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Purification and Properties of an L-Glutaminase-L-Asparaginase from *Pseudomonas acidovorans*

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An enzyme that catalyzes the hydrolysis of both glutamine and asparagine has been purified to homogeneity from extracts of *Pseudomonas acidovorans*. The enzyme having a ratio of glutaminase to asparaginase of 1.45:1.0 can be purified by a relatively simple procedure and is stable upon storage. The glutaminase-asparaginase has a relatively high affinity for L-asparagine (K_m = 1.5×10^{-5} M) and L-glutamine ($K_m = 2.2 \times 10^{-5}$ M) and has a molecular weight of approximately 156,000, the subunit molecular weight being approximately 39,000. Injections of the enzyme produced only slight increases in the survival time of C3H/HE mice carrying the asparagine-requiring 6C3HED Gardner lymphoma and of white Swiss mice carrying the glutamine-requiring Ehrlich lymphoma.

Since the introduction of L-asparaginase for clinical use in 1967 (7), the enzyme has been used extensively for the production of rapid remissions in patients with acute lymphocytic leukemia (see 1, 3-5, 35 for reviews). The enzyme causes selective death of asparagine-dependent tumor cells by depriving these cells of asparagine. Most of the clinical programs to date have employed an L-asparaginase from *Escherichia coli*. This enzyme has been found to produce rapid remissions in 60 to 80% of patients treated (1), but untoward side effects are not uncommon. In 5 to 20% of the patients, allergic reactions have been noted (1) and, on occasion, the anaphylactic shock has been sufficiently severe for treatment to be discontinued even though clinical improvement of the leukemia was evident. Increasingly rapid clearance of asparaginase from the bloodstream also occurs with extended therapy, and this has been observed even in the absence of overt allergic symptoms (18, 19). These problems can be alleviated by the sequential use of serologically unrelated asparaginases (15), and this has prompted us to examine a variety of bacterial asparaginases that might be used for this purpose.

In this paper we report the purification of a glutaminase-asparaginase from *Pseudomonas acidovorans* and describe some of the properties of this enzyme, including the molecular weight, subunit composition, and amino acid composition. The enzyme produces only marginal increases in the survival time of C3H/HI mice carrying the 6C3HED Gardner tumor and of Swiss mice carrying the ascitic form of the Ehrlich lymphoma.

MATERIALS AND METHODS

Nessler reagent (Banco Standardized) was obtained from Capitol Scientific Co., Austin, Tex. L- and D-asparagine, L- and D-glutamine, L- and D-aspartic acid, L-glutamic acid, L-aspartic acid-β-hydroxamate, L-glutamic acid-γ-monohydroxamate, sodium tetraphenylboron, dithiothreitol, *N*-acetyl-L-asparagine, DL-valine, ethylene diaminetetraacetic acid (EDTA), and bovine serum albumin (fraction IV) were purchased from Sigma Chemical Co., St. Louis, Mo. Tryptone, yeast extract, and Special agar-Noble (all from Difco) were from Curtin-Matheson. L-[U-^{14}C]asparagine and L-[U-^{14}C]glutamine were obtained from New England Nuclear Corp. L-Isoasparagine was obtained from Cyclo Chemical Co. Ampholine carrier ampholytes were purchased from LKB Instruments Inc. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., and carboxymethyl cellulose (CM-52, Whatman) was purchased from Reeve Angel. Ammonium sulfate (ultrapure) was obtained from Schwarz/Mann. Dowex 1-X2 (Cl^-) was purchased from Bio-Rad Laboratories. All other chemicals were reagent grade.

The bacterial organism was isolated from enrichment cultures of mud samples taken from the floor of a tropical rain forest in Puerto Rico by Dwayne Savage. The organism was maintained by weekly transfer on slants of tryptone-fructose-yeast (TFY) agar containing (per liter); 5.0 g of tryptone, 5.0 g of yeast extract, 1.0 g of fructose, 1.0 g of dibasic potassium phosphate, and 2.0 g of agar. Cells for purification were grown in 88 liters of TFY with a 10%

inoculum in a 100-liter (Fermentation Design) fermentor at 30°C under 60 liters of air per min and 200 rpm agitation. Stationary phase was reached in 6 to 7 h. Cells were harvested at the beginning of stationary phase with a Sharples centrifuge.

Asparaginase and glutaminase activities were routinely determined by direct nesslerization as described by Mashburn and Wriston (13). However, the substrates were prepared in a 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0). One unit of activity is that amount of enzyme necessary to catalyze the formation of 1 μmol of ammonia per min under the conditions of the assay. The substrate specificity of the enzyme was determined by substituting the various compounds for asparagine in the assay mixture.

The effect of pH upon the reaction velocity was determined with 0.01 M asparagine and 0.01 M glutamine dissolved in a buffer containing 0.02 M each of sodium citrate, sodium barbital, monobasic potassium phosphate, and boric acid. Either 1 N HCl or 1 N NaOH was used to adjust the buffer to the desired pH. Assays were performed by the standard nesslerization procedure.

The thermal stability of the asparaginases was determined by incubating portions of the enzyme in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.001 M EDTA at various temperatures for 10 min. The residual activity was then determined by the standard nesslerization assay. Standard nesslerization assays were also done at various temperatures to determine the degree of protection of the enzyme activity by the substrates.

^{14}C-labeled substrates were used for kinetic studies at low substrate concentrations. The substrate and product were separated by Dowex 1-X2 (Cl^-) columns, and the level of radioactivity was determined by liquid scintillation in a Unilux II counter (Nuclear-Chicago Corp.) by using the procedure described by Prusiner and Milner (20). The amount of enzyme used for K_m determinations was adjusted to give less than 20% conversion of substrate to product at the lowest substrate level.

Enzymatic decomposition of aspartic acid-β-hydroxamate and glutamic acid-γ-monohydraxamate was determined by the method of Frohwein (6).

Protein concentration was determined by the method of Lowry et al. (12). Bovine serum albumin was used as a standard. Electrophoresis was performed according to the procedure of Ornstein and Davis (16) except that the polarity was reversed and basic fuchshin was used as a tracking dye.

Electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels was performed according to Weber and Osborn (31) except that proteins were denatured in a boiling-water bath for 2 min. Ribonuclease A, myoglobin, chymotrypsinogen A, and ovalbumin were used as standards for subunit molecular weight determination. Gel filtration to determine approximate molecular weights was performed on a Sephadex G-200 column (2 by 90 cm) using reverse flow. Isoelectric focusing was performed in polyacrylamide gels (0.5 by 15 cm) using pH 3 to 10 ampholytes at 4°C as described by Wrigley (34). Gels were then removed, sliced into 2-mm pieces, placed in 1 ml of water, and allowed to stand for 2 h. The pH of each fraction was determined and the fractions were then assayed for asparaginase and glutaminase activity. Acrylamide gels were stained for L-asparaginase and/or L-glutaminase activity using the method described by Pajdak and Pajdak (17).

A Beckman-Spinco model E ultracentrifuge was used for ultracentrifugal analysis of the enzyme. The sedimentation constant was determined at a rotor speed of 52,000 rpm and at a temperature of 19°C. The value was corrected to water at 20°C. The protein concentration was approximately 4 mg/ml in 0.05 M borate-0.1 M sodium chloride-0.001 M EDTA buffer (pH 7.0).

For amino acid analysis, the purified glutaminase-asparaginase enzyme was lyophilized. Duplicate samples containing approximately 0.4 mg of protein were hydrolyzed in 6 N HCl at 110°C for 24 h in sealed evacuated tubes. Samples were hydrolyzed under similar conditions in *p*-toluene sulfonic acid for tryptophan determinations (11). Analyses were done on a Beckman 121 amino acid analyzer.

Plasma clearance rates of the *P. acidovorans* glutaminase-asparaginase were determined by injecting intraperitoneally (i.p.) into normal and tumor-bearing C3H mice: 0.2 ml of enzyme solution in 0.05 M borate buffer-0.1 M sodium chloride-0.001 M EDTA (pH 7.0) containing 10 IU of asparaginase activity. The mice were bled from the tail vein at various time intervals and the asparaginase activity was determined by the micronesslerization assay.

The method of Ouchterlony (28) was used for immunodiffusion tests. The rabbit antiserum to *E. coli* asparaginase (1.0 mg/ml) was prepared by giving an initial intramuscular dose of 0.1 ml of the asparaginase emulsified with an equal volume of Freund complete adjuvant to a New Zealand white rabbit. After 2 weeks, the rabbit received a subcutaneous injection of 0.1 ml of the asparaginase solution. The injections were continued weekly for 13 weeks. After the thirteenth dose, serum was collected and stored frozen until use.

Tumor inhibition assays were performed by a modification of the methods of Roberts et al. (25). The antitumor effect of the glutaminase-asparaginase enzyme was evaluated by the effect of the enzyme on the survival time of mice carrying the ascites form of the Gardner lymphoma 6C3HED or the Ehrlich lymphoma. Cells were transplanted by i.p. injection of 10^6 viable ascites cells from a C3H/HE mouse with an 8-day-old 6C3HED tumor or a white Swiss mouse with a 12-day-old Ehrlich tumor. The number of viable cells was estimated by trypan blue exclusion in a hemocytometer. After tumor transplantation, the mice were treated with 5 U of enzyme (0.5 to 0.1 ml) by i.p. injection. The number of surviving mice was recorded daily.

RESULTS

Identification of the organism. The bacterium was identified as *P. acidovorans* using standard morphological and biochemical tests. It is a gram-negative, aerobic, amphitrichous rod. It does not grow autotrophically with hy-

drogen and will not utilize arginine, betaine, and testosterone. Denitrification does not occur. It is lipolytic. Organic growth factors are not required; fructose is the only sugar that can be used as a sole carbon source. Other utilizable compounds are all saturated dicarboxylic acids from malonate to sebacate, ethanol, glycollate, norleucine, L-tryptophan, and acetamide.

Enzyme purification. Examination of crude extracts of *P. acidovorans* by polyacrylamide gel electrophoresis showed the presence of only a single band staining for asparaginase activity. This band also showed glutaminase activity. Cells of approximately 600 g were suspended in 2 liters of 0.1 M potassium phosphate buffer–0.001 M EDTA, pH 7.5. The cells were ruptured by two passes through a Gaulin continuous flow homogenizer at 5,500 lb/in^2. The homogenized cells were centrifuged at 14,000 rpm in a type 15 rotor in a Beckman L3-40 preparative ultracentrifuge for 2.5 h to remove the cell debris. The supernatant was decanted and 1.0 M manganese chloride was added dropwise, with continuous stirring, to a final concentration of 0.05 M. The solution was stirred for an additional 30 min and then allowed to stand overnight at 4°C. The precipitated proteins were removed by centrifugation at 14,000 rpm for 1 h in a type 15 rotor. Finely powdered ammonium sulfate was added to the stirred supernatant to 85% saturation while the pH was maintained at pH 6.8 with 10% ammonium hydroxide. After standing at 4°C for 30 min, the precipitate was removed by centrifugation. The precipitate was then resuspended in a minimum volume of 0.02 M potassium phosphate buffer–0.001 M EDTA (pH 6.2) and dialyzed overnight against the same buffer. The dialyzed solution was applied to the top of a column (5.5 by 50 cm) containing CM-52 cellulose equilibrated with dialysis buffer and then eluted with dialysis buffer until the absorbancy at 280 nm returned to 0.3. The glutaminase-asparaginase enzyme was eluted from the column using a gradient of 1 liter of 0.02 M potassium phosphate buffer–0.001 M EDTA (pH 6.2) and 1 liter of 0.2 M potassium phosphate buffer–0.001 M EDTA (pH 7.8). The glutaminase-asparaginase fractions were pooled, concentrated by ultrafiltration to 50 ml, and dialyzed against 0.02 M potassium phosphate buffer–0.001 M EDTA (pH 6.2). The dialyzed enzyme solution was then applied to a second CM-52 cellulose column (2.5 by 50 cm) equilibrated with dialysis buffer. A linear gradient was begun after the absorbancy at 280 nm dropped below 0.1. The gradient was 500 ml of dialysis buffer (0.02 M potassium phosphate buffer–0.001 M EDTA, pH 6.2) and 500 ml of 0.1 M potassium phosphate buffer–0.001 M EDTA, pH 7.5. The glutaminase-asparaginase fractions were pooled, concentrated by ultrafiltration to less than 10 ml, and applied to the bottom of a Sephadex G-200 column (5 by 80 cm). The enzyme was eluted by ascending chromatography with a 0.05 M sodium borate buffer (pH 7.0) containing 0.1 M sodium chloride and 0.001 M EDTA. A summary of the purification procedure is given in Table 1.

The purified glutaminase-asparaginase enzyme represented 19% of the activity in the crude extract. The enzyme was purified 2,205-fold for the asparaginase activity and 2,059-fold for the glutaminase activity. The final specific activities were 86 IU/mg for the asparaginase and 104 IU/mg for the glutaminase. The glutaminase-asparaginase was judged homogeneous by polyacrylamide and SDS-gel electrophoresis. The enzyme preparation was judged to be homogeneous under nondenaturing and denaturing conditions by polyacrylamide electrophoresis. Only a single protein band was observed in both instances. Sedimentation velocity runs in the ultracentrifuge showed only a single sedimenting species.

The glutaminase-asparaginase enzyme was stable upon storage at 4°C for 3 to 4 months in buffer without appreciable loss of activity. The enzyme was, however, sensitive to inhibition by *p*-chloromercuribenzoate. Upon incubation with 10^{-4} M *p*-chloromercuribenzoate for 30 min, the enzyme lost 26% of its activity. It was

TABLE 1. *Purification of P. acidovorans glutaminase-asparaginase*

Fraction	Enzyme activity (IU)		Total protein (mg)	Sp act (IU/mg)		Yield (%)
	Glutaminase	Asparaginase		Glutaminase	Asparaginase	
Crude extract	14,292	11,196	289,884	0.051	0.039	100
$MnCl_2$ fraction	10,420	9,552	170,006	0.061	0.056	70 to 80
Ammonium sulfate fraction	7,280	6,448	65.472	0.111	0.098	49 to 58
First CM-52 chromatography	5,139	4,094	1,568	3.6	2.6	35
Second CM-52 chromatography	4,033	3,315	463	8.7	7.2	28
Sephadex G-200 chromatography	2,886	2,356	27.3	104	86	19

not inhibited by incubation with 10^{-5} M *p*-chloromercuribenzoate for this time.

Biochemical properties of *P. acidovorans* glutaminase-asparaginase. Both the glutaminase and asparaginase activities were exhibited over a broad range of pH. Although the pH optimum for both activities is 9.5, the enzymes had 70% of the optimum activity at pH 7.4. The thermal stability for both the glutaminase and asparaginase activity was identical, with both being 50% inactivated after 10 min at 50°C. The enzyme was significantly protected from thermal inactivation by the presence of substrate (0.01 M L-asparagine or 0.01 M L-glutamine); the temperature for 50% inactivation was 79°C for both glutaminase and asparaginase activities.

An examination of the substrate specificity, based on the activity with L-asparagine as 100%, shows almost 150% activity with L-glutamine, 50% activity with D-asparagine, and 58% activity with D-glutamine (Table 2). With the mixed substrates, L-asparagine plus D-glutamine, L-asparagine plus L-glutamine, and L-asparagine plus D-glutamine, the enzyme gave 99%, 110%, and 74% activity, respectively. The prolonged incubation with the asparagine derivative L-isoasparagine did not produce any ammonia; however, with *N*-acetyl-L-asparagine, the enzyme did produce ammonia amounting to 5% of the total asparaginase activity.

The enzyme showed typical Michaelis-Menten kinetics at low substrate concentrations. The apparent K_m value for glutamine was 2.2×10^{-5} M and for asparagine was 1.5×10^{-5} M. The isoelectric point was pH 7.2 for both the glutaminase and asparaginase activities.

The immunological cross-reactivity of the *P. acidovorans* glutaminase-asparaginase with *E. coli* asparaginase was tested by double diffusion in agar using a rabbit antiserum directed against the *E. coli* enzyme. No cross-reaction was observed.

Physicochemical properties. The molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200. An approximate molecular weight of 156,000 was calculated by the equation of Squire (27). A single peak was observed in Schlieren photographs from a sedimentation velocity run in the ultracentrifuge using the purified enzyme. The sedimentation coefficient corrected to water at 20°C was 7.76×10^{-13} *s*. The subunit molecular weight was determined by electrophoresis in polyacrylamide-SDS gels using appropriate standards. Only a single protein band was noted upon electrophoresis, with the mobility of the band corresponding to a molecular weight of approximately 39,000. These results suggest that the *P. acidovorans* glutaminase-asparaginase enzyme is tetrameric. For comparison, the amino acid composition of the enzyme with the compositions of other bacterial asparaginases is shown in Table 3. The *P. acidovorans* glutaminase-asparaginase has an amino acid composition generally similar to that of other bacterial asparaginases with the exception of the arginine content which is markedly higher than that in other asparaginases. The proline, alanine, and leucine contents of the *P. acidovorans* enzyme are slightly higher than those of other asparaginases.

Antitumor activity. The results of several experiments to test the antineoplastic activity of the *P. acidovorans* glutaminase-asparaginase are given in Table 4. I.p. injections of the *P. acidovorans* enzyme produced only slight

TABLE 2. *Substrate specificity of P. acidovorans glutaminase-asparaginase*

Substrate[a]	% Relative activity
L-Asparagine	100
D-Asparagine	50
L-Glutamine	147
D-Glutamine	58
L-Asparagine + D-asparagine	99
L-Asparagine + L-glutamine	110
L-Asparagine + D-glutamine	74
L-Aspartic acid-β-hydroxamate[b]	91
D-Aspartic acid-β-hydroxamate[b]	29
L-Glutamic acid-γ-monohydroxamate[b]	98
L-Aspartic acid	0
D-Aspartic acid	0
L-Glutamic acid	0
L-Isoasparagine	0
N-Acetyl-L-asparagine	5.2
N-CB2-L-asparagine	0
N-CB2-DL-asparagine	0
N-CB2-L-asparagine-*p*-nitrophenyl	0
N-t-BOC-L-asparagine	0
N-t-BOC-L-asparagine-*p*-nitrophenyl	0
Acetamide	0
Propionamide	0
Butyramide	0
Succinamide	0

[a] All substrates were used at 10 mM concentrations in 0.1 M Tris-hydrochloride, pH 8.0. Assays were performed by standard nesslerization. Those substrates not showing activity were reassayed for 100 min. Relative activity was based upon the amount of asparagine converted to ammonia as 100% activity.

[b] Incubated with the enzyme for 10 min. Hydrolysis was determined as described in Materials and Methods.

TABLE 3. *Amino acid composition of bacterial asparaginases*

Amino acid	mol/39,000 g of *P. acidovorans* GA[a]	mol/34,500 g of *P. geniculata*[a,b] AG	mol/32,800 g of *Citrobacter freundii*[c] asparaginase A	mol/34,500 g of *A. eutrophus*[d]	mol/33,300 g of *E. coli*[e]	mol/33,000 g of *Acinetobacter*[f]	mol/30,000 g of *P. vulgaris*[g]	mol/33,370 g of *Erwinia*[h]
Lysine	19.9	15.9	21.0	22.4	21.0	24.4	22.8	18.2
Histidine	5.8	4.1	4.8	4.2	3.1	8.9	3.1	5.4
Arginine	32.7	10.9	9.8	12.3	7.2	10.0	6.4	16.2
Tryptophan	4.2[i]	9.5	1.8	2.9	1.0	4.3	1.2	0
Aspartic acid	36.8	31.5	39.3	33.5	50.7	36.7	40.0	35.1
Threonine	23.9	21.1	28.0	20.6	32.6	16.3	22.4	26.3
Serine	18.3	16.9	19.0	18.8	14.6	18.9	13.9	17.5
Glutamic acid	30.4	33.4	25.4	30.0	18.7	23.4	21.8	20.9
Proline	17.8	7.9	13.5	14.3	11.0	10.2	11.4	12.5
Glycine	30.5	32.8	28.1	28.5	28.5	22.2	27.3	33.4
Alanine	47.7	42.8	31.2	42.6	31.1	36.2	31.2	29.0
Cysteine	1.0	4.5	2.1	0	1.9	0	2.0	0
Valine	30.4	32.1	26.5	26.0	35.0	28.5	30.7	29.7
Methionine	4.6	8.3	5.0	4.9	3.9	7.4	5.2	6.7
Isoleucine	13.3	17.6	11.7	16.0	12.2	16.4	14.6	16.9
Leucine	28.6	24.2	23.2	23.0	22.3	24.0	22.6	28.3
Tyrosine	8.8	7.4	11.0	10.0	11.0	8.0	8.8	12.5
Phenylalanine	9.6	5.3	8.5	8.3	8.0	9.0	10.3	6.7

[a] GA, glutaminase-asparaginase; AG, asparaginase-glutaminase.
[b] Data from Smith (unpublished data).
[c] Data from Davidson et al. (Biochim. Biophys. Acta, in press).
[d] Data from J. P. Allison (Ph.D. dissertation, University of Texas, Austin, 1973).
[e] Calculated from Ho et al. (8).
[f] Calculated from Roberts et al. (24).
[g] Calculated from Tosa et al. (30).
[h] Calculated from Cammack et al. (2).
[i] Determined by analysis of samples hydrolyzed in *p*-toluene sulfonic acid.

TABLE 4. *Antitumor activity of P. acidovorans glutaminase-asparaginase enzyme*

Tumor	Treatment	No. of mice	Mean survival time (days)	Range	Increase in mean survival time
6C3HED	None	53	11.67	11 to 12	
	5 IU of *P. acidovorans* GA[a] on days 2, 4, 6, 8	5	18.20	14 to 22	6.53
	25 IU of *P. acidovorans* GA on day 7	5	14.20	13 to 15	2.53
	Heat-inactivated *P. acidovorans* GA on days 2, 4, 6, 8, 10	5	12.0	11 to 13	0.33
Ehrlich	None	3	16.0	14 to 18	
	5 IU of *P. acidovorans* on days 2, 4, 6, 8, 10	5	18.4	14 to 26	2.4

[a] GA, glutaminase-asparaginase.

increases in the survival time of C3H/HI mice infected with the 6C3HED Gardner lymphoma. Under the same conditions, *E. coli* asparaginase produced complete cures in essentially 100% of the test animals (Allison, Davidson, and Kitto, unpublished data). The enzyme also produced slight increases in the survival time of the white Swiss mice infected with the Ehrlich lymphoma. Mice infected with the Ehrlich lymphoma did not have any visible signs of tumor while treated with the enzyme. However, when the treatment was terminated, the tumor grew rapidly.

Plasma clearance. The rate of clearance of the *P. acidovorans* glutaminase-asparaginase from the blood plasma of C3H/HE mice was compared with that of the *E. coli* asparaginase in both normal and 6C3HED tumor-bearing mice. As shown in Fig. 1, the clearance of the *P. acidovorans* enzyme was much more rapid, in both instances, than that of the *E. coli* asparaginase. The half-lives of the *P. acidovorans* and

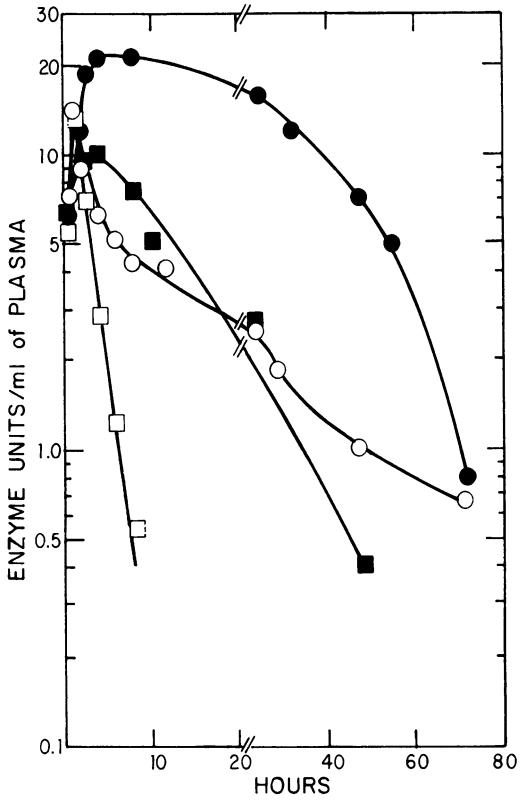

FIG. 1. *Plasma clearance of* L-*asparaginase in C3H mice. At zero time, C3H mice received i.p. injections of 10 U of P. acidovorans asparaginase (15 U of glutaminase) or 10 U of E. coli asparaginase. Tumor-bearing mice received transplants of* 10^6 *6C3HED ascites cells 4 days previously. At the specified intervals, mice were bled from the tail vein and the asparaginase activity was determined by the micronesslerization assay method. Symbols:* (●) *E. coli, tumor-bearing mouse;* (■) *E. coli, normal mouse,* (○) *P. acidovorans, tumor-bearing mouse; and* (□) *P. acidovorans, normal mouse.*

E. coli enzymes were 1.5 and 3.0 h, respectively, in normal mice. The comparable values in tumor-bearing mice were 4 h for the *P. acidovorans* enzyme and 20 h for the *E. coli* asparaginase. The slower clearance in tumor-bearing mice has been ascribed to the presence of lactic dehydrogenase virus which almost invariably accompanies the 6C3HED tumor (22, 23).

DISCUSSION

For an asparaginase to be ideally suited for use in antineoplastic therapy, it should satisfy a variety of criteria. The organism that is selected should produce the asparaginase in high yield, and it should be capable of being grown in large quantities on a simple and inexpensive medium. The procedures developed for purification of the enzyme should be as rapid and simplified as possible, providing pure enzyme in high yield. The purified enzyme should have long term stability on storage, maximal activity at a physiological pH, and a K_m for substrate below the concentration of the substrate in the blood. Most importantly, the enzyme should be capable of producing rapid remissions of the neoplastic state, the potential for which is well indicated by experiments with mouse tumor lines.

The glutaminase-asparaginase we isolated from *P. acidovorans* meets many, but not all, of these criteria. The organism grows well on the simple medium described, with the stationary phase being reached in 6 to 7 h. The enzyme can be purified to homogeneity rapidly and with an overall yield of 20% by a simple procedure involving only salt fractionation, two ion-exchange chromatography steps, and gel filtration. The pure enzyme lost no appreciable activity upon storage at 4°C for 4 months.

The enzyme isolated from *P. acidovorans* catalyzes the hydrolysis of both glutamine and asparagine, the ratio of L-glutaminase activity to L-asparaginase activity being 1.45:1.0. The following evidence suggests that both activities are catalyzed by the same enzyme and by the same catalytic site. (i) The two activities could not be separated by ion-exchange chromatography or isoelectric focusing, and the ratio of glutaminase to asparaginase activities remained constant throughout the purification of the enzyme. (ii) An equimolar mixture of L-asparagine and L-glutamine was hydrolyzed at a lower rate than L-glutamine alone. (iii) Both activities were inactivated by heat at the same rate, and addition of either L-asparagine or L-glutamine increased the thermal stability of the enzyme. (iv) Both activities showed a very similar response to variation in pH.

While many bacterial asparaginases, such as those from *E. coli*, *Serratia marcescens*, and *Erwinia caratovora*, show little or no glutaminase activity, the dual specificity of the *P. acidovorans* enzyme for both glutamine and asparagine is shared by enzymes from several other *Pseudomonas* species, from *Acinetobacter glutaminasificans*, and from *Alcaligenes eutrophus* (8-10, 14, 21, 33; J. P. Allison, Ph.D. dissertation, University of Texas, Austin, 1973). The pH optima of 9.5 for both the glutaminase and asparaginase activities of the *P. acidovorans* enzyme are considerably above the pH of blood, but due to the broad nature of the pH-activity curve, approximately 70% of optimum activity is retained at pH 7.4.

Asparagine does not become severely limiting for protein synthesis until the concentra-

tion is reduced to 10^{-5} M or below (35) and, consequently, for an asparaginase to be an effective antitumor agent, the enzyme must possess a high substrate affinity. The substrate affinity of the *P. acidovorans* glutaminase-asparaginase, as measured by K_m values of 2.2×10^{-5} and 1.5×10^{-5} for glutamine and asparagine, respectively, compares favorably with the values reported for antineoplastic asparaginases from other sources (8, 10, 24 and 29; see 35 for review).

Gel filtration gave an approximate molecular weight of 156,000 for the *P. acidovorans* glutaminase-asparaginase while SDS electrophoresis indicated a subunit molecular weight of 39,000. These results are consistent with a tetrameric structure for the enzyme. The *P. acidovorans* enzyme is somewhat larger than the tumor-inhibitory L-asparaginases from *E. caratovora*, *Proteus vulgaris*, and *E. coli* and the glutaminase-asparaginase of *A. glutaminasificans*, with molecular weights of 135,000, 120,000, 139,000 and 138,000, respectively (2, 8, 24, 30). The antineoplastic glutaminase A from *P. aeruginosa* has been reported to have a molecular weight of 137,000 (26). On the other hand, Ramadan et al. (21) reported a molecular weight of only 25,000 for a tumor-inhibitory asparaginase-glutaminase from a *Pseudomonad* species.

The isoelectric points (pI) of asparaginases and asparaginase-glutaminases vary considerably. The *P. acidovorans* enzyme with a pI of 7.2 falls into a group with relatively basic pI. These include the asparaginase-glutaminases of *A. glutaminasificans* (pI, 8.43; 24), *A. eutrophus* (pI, 9.04; J. P. Allison, Ph.D. dissertation, University of Texas, Austin, 1973), and *P. geniculata* (pI, 6.97; Smith, unpublished data), and the asparaginase of *E. carotovora* (pI, 8.6; 2). By contrast the *E. coli* asparaginase has a pI of 4.8 to 5.2 (2, 8, 32). There is no apparent correlation between the pI of these enzymes and their antitumor activity.

While the biochemical properties of the *P. acidovorans* glutaminase-asparaginase are generally comparable with those of antineoplastic asparaginases and glutaminases, the antitumor activity of the *P. acidovorans* enzyme was disappointingly low in our tests with mice. The enzyme prolonged the survival time by only 6.53 days of C3H mice with 6C3HED lymphoma, and its effect on the Ehrlich lymphoma was even less. The *P. acidovorans* enzyme also caused the C3H mice with the 6C3HED tumor to lose a large amount of weight after the third injection of 5 IU on day 8. A single injection of 25 IU on day 7 also caused large weight losses. No weight loss was observed in mice injected with heat-inactivated enzyme. Growth of the glutamine-requiring Ehrlich tumor was inhibited by injections of the *P. acidovorans* enzyme only as long as treatment was continued. When treatment was discontinued, the tumor grew rapidly and killed the white Swiss mice. The very limited effectiveness of the *P. acidovorans* enzyme may be due, in part, to the relatively rapid clearance of the glutaminase-asparaginase from plasma.

In summary, although the in vivo testing of the *P. acidovorans* glutaminase-asparaginase has been limited, and different dose and schedule regimens may be more satisfactory, the present results suggest that this enzyme would not be an effective replacement for *E. coli* asparaginase in clinical use.

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LITERATURE CITED

1. **Burchenal, J. H., and D. A. Karnufsky.** 1970. Clinical evaluation of L-asparaginase. Cancer 25:241-243.
2. **Cammack, K. A., D. I. Marlborough, and D. S. Miller.** 1972. Physical properties and subunit structure of L-asparaginase isolated from *Erwinia carotovora*. Biochem. J. 126:361-379.
3. **Capizzi, R. L., R. Peterson, D. A. Cooney, W. A. Creasy, and R. B. Handschumacher.** 1969. L-asparaginase therapy of acute leukemia: biochemical and clinical observations. Proc. Am. Assoc. Cancer Res. 10:12.
4. **Cooney, D. A., and R. E. Handschumacher.** 1970. L-asparaginase and L-asparagine metabolism. Annu. Rev. Pharmacol. 10:421-440.
5. **Crowther, D.** 1971. L-asparaginase and human malignant disease. Nature (London) 229:168-171.
6. **Frohwein, Y. Z., M. Friedman, J. Reiger, and N. Grossowicz.** 1971. Sensitive and rapid assay for L-asparaginase. Nature (London) New Biol. 230:158-159.
7. **Hill, J. M., J. Roberts, E. Loeb, A. Khan, A. MacLellan, and R. W. Hill.** 1967. L-asparaginase therapy for leukemia and other malignant neoplasms. Remission in human leukemia. J. Am. Med. Assoc. 202:882-888.
8. **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.
9. **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.
10. **Howard, J. B., and F. H. Carpenter.** 1972. L-asparaginase from *Erwinia carotovora*. Substrate specificity and enzymatic properties. J. Biol. Chem. 247:1020-1030.
11. **Liu, T. U., and Y. H. Chang.** 1971. Hydrolysis of proteins with *p*-toluene sulfonic acid. J. Biol. Chem. 246:2842-2848.
12. **Lowry, O., N. J. Rosebrough, A. L. Farr, and R. J.**

Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265–275.

13. **Mashburn, L. T., and J. C. Wriston, Jr.** 1964. Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. Arch. Biochem. Biophys. 105:450–452.
14. **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.
15. **Ohnuma, T., J. F. Holland, and P. Meyer.** 1972. *Erwinia carotovora* asparaginase in patients with prior anaphylaxis to asparaginase from *E. coli*. Cancer 30:376–381.
16. **Ornstein, L., and B. J. Davis.** 1964. Anodic systems. I. The standard gel system of Ornstein and Davis, p. 24–25. *In* Disc electrophoresis. Canalco, Rockville, Md.
17. **Pajdak, E., and W. Pajdak.** 1972. A simple and sensitive method for detection of L-asparaginase by polyacrylamide gel electrophoresis. Anal. Biochem. 50: 317–320.
18. **Peterson, R. G., R. E. Handschumacher, and M. S. Mitchell.** 1971. Immunological responses to L-asparaginase. J. Clin. Invest. 50:1080–1090.
19. **Peterson, R. G., M. S. Mitchell, R. L. Capizzi, and R. E. Handschumacher.** 1969. Immunological modification of L-asparaginase activity. Pharmacologist 11:234–235.
20. **Prusiner, S., and L. Milner.** 1970. A rapid radioactive assay for glutamine synthetase, glutaminase, asparaginase synthetase and asparaginase. Anal. Biochem. 37:429–438.
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. **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.
25. **Roberts, J., M. D. Prager, and W. Bachynsky.** 1966. The anti-tumor activity of *Escherichia coli* L-asparaginase. Cancer Res. 26:2213–2217.
26. **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.
27. **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.
28. **Stollar, D., and L. Levine.** 1963. Two-dimensional immunodiffusion, p. 848–854 *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 6. Academic Press Inc., New York.
29. **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.
30. **Tosa, T., R. Sano, K. Yamamoto, M. Nakamura, and I. Chibata.** 1973. L-asparaginase from *Proteus vulgaris*. Subunit and amino acid composition. Biochemistry 12:1075–1079.
31. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.
32. **Whelan, H. A., and J. C. Wriston, Jr.** 1969. Purification and properties of asparaginase from *Escherichia coli* B. Biochemistry 8:2386–2393.
33. **Whelan, H. A., and J. C. Wriston, Jr.** 1974. Purification and properties of L-asparaginase from *Serratia marcescens*. Biochim. Biophys. Acta 365:212–222.
34. **Wrigley, C. W.** 1968. Analytical fractionation of plant and animal proteins by gel electrofocusing. J. Chromatogr. 36:362–365.
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.