Biochimica et Biophysica Acta, 480 (1977) 282—294

BBA 67998

L-ASPARAGINASES FROM *CITROBACTER FREUNDII*

LOIS DAVIDSON, MAURICE BURKOM *, SAM AHN **, LIE-CHING CHANG *** and BARRIE KITTO §

Clayton Foundation Biochemical Institute, Department of Chemistry, University of Texas, Austin, Texas 78712 (U.S.A.)

(Received July 1st, 1976)

Summary

Three enzymes which catalyze the hydrolysis of L-asparagine have been identified in extracts of *Citrobacter freundii*. One of these (asparaginase-glutaminase (EC 3.5.1.1) also shows substantial glutaminase activity. This enzyme is extremely labile, is sensitive to inactivation by *p*-chloromercuribenzoate, and is not protected by dithiothreitol. A second enzyme (asparaginase B) is also sensitive to mercurials but is protected from inactivation by dithiothreitol. This enzyme has a relatively low affinity for L-asparagine (K_m = 1.7 · 10^{-3} M). The third enzyme (asparaginase A) is insensitive to inactivation by mercurials, is stable upon long term storage and has a relatively high affinity for L-asparagine (K_m = 2.9 · 10^{-5} M). This enzyme has been purified to homogeneity and has a molecular weight of approx. 140 000; the subunit weight being approx. 33 000. The *C. freundii* asparaginase A produced significant increases in the survival time of C3H/HE mice carrying the 6C3HED lymphoma tumor.

Introduction

Use of the enzyme L-asparaginase (EC 3.5.1.1) for the treatment of acute lymphocytic leukemia was initiated in 1967 [1] and has been found to produce remissions in 60—80% of patients treated regardless of whether they are sensitive or resistant to conventional chemotherapeutic agents [2]. Current programs almost exclusively use an L-asparaginase from the bacterium *Escherichia coli*. Despite the promise of L-asparaginase therapy for human leukemias, there

* Present address: Department of Biochemistry, Colorado State University, Fort Collins, Colo. 80523, U.S.A.

** Present address: University of Texas Southwestern Medical School, Dallas, Texas 75235, U.S.A.

*** Present address: Department of Home Economics, The University of Texas, Austin, Texas 78712, U.S.A.

§ To whom correspondence should be addressed.

are a number of problems associated with such treatment. Most of these problems are fortunately minor, such as mild nausea, some vomiting and anorexia. A major difficulty, though, is that the *E. coli* protein is a foreign protein for humans, and hypersensitivity reactions ranging from mild allergy to anaphylactic shock have been reported in 5—20% of patients treated [2]. Extremely rapid clearance of plasma asparaginase activity accompanies or precedes overt allergic reactions and has been reported in at least two patients who exhibited no other signs of allergy [3]. Extended use of *E. coli* asparaginase therefore poses the problems of loss of effectiveness through rapid clearance of the drug and the potential for production of troublesome anaphylactic reactions. These problems could be overcome by switching to sequential therapy with serologically unrelated L-asparaginases and has prompted us to search for such enzymes. In this paper we report the occurrence of three asparaginases in extracts of *Citrobacter freundii*. One of these enzymes has been purified to homogeneity and, on the basis of its anti-tumor activity in mice, shows potential for the therapy of human leukemias.

Materials and Methods

Nessler's reagent (Banco standardized) was obtained from Capitol Scientific Co., Austin, Texas, 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-hydroxamate, sodium tetraphenylboron, dithiothreitol, *N*-acetyl-L-asparagine, DL-valine, EDTA and bovine serum albumin (fraction IV) were purchased from Sigma Chemical Co. L-Isoasparagine was from Cyclo Chemical Co. Bacto-tryptone (Difco), Bacto-yeast extract (Difco) and Special Agar Noble (Difco) were from Curtin-Matheson. L-[^{14}C] Asparagine was obtained from New England Nuclear. Ampholine carrier ampholytes were purchased from LKB. Sephadex G-200 and Sepharose 4B were from Pharmacia and DEAE-cellulose-52 (Whatman) was purchased from Reeve Angel. Ammonium sulfate (ultra pure) was obtained from Schwartz Mann. Dowex 1-X2 (Cl^-) was purchased from BioRad. All other chemicals were reagent grade.

Bacterial culture. The bacterial organism was isolated from enrichment cultures of mud samples taken from the floor of a tropical rain forest in Puerto Rico by Dr. Dwayne Savage.

The organism was maintained by weekly transfer on slants of TGY agar (5.0 g tryptone, 5.0 g yeast extract, 1.0 g glucose, 1.0 g dibasic potassium phosphate, and 20 g Agar per l) [4]. Cells for purification were grown in 88 l of TGY broth in a 100 l (Fermentation Design) fermentor at 34° C under 60 l/min air and 200 rev./min agitation using a 10% inoculum. Stationary phase was reached in 4—5 h. Cells were harvested using a Sharples centrifuge at the beginning of stationary phase.

Assays. Asparaginase and glutaminase activities were routinely determined by direct nesslerization as described by Mashburn and Wriston [5]. However, the substrates were prepared in a 0.1 M Tris · HCl 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 asparaginases was determined by substituting

the various compounds for asparagine in this assay mixture.

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

The thermal stability of the asparaginases was determined by incubating aliquots of the enzymes in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA and 0.0001 M dithiothreitol, at various temperatures for 10 min. The residual activity was then determined by the standard nesslerization assay. Standard nesslerization assays were also carried out at various temperatures to determine the degree of protection of the enzyme activity by substrate.

^{14}C-Labeled substrates were used for kinetic studies at low substrate concentrations. The substrate and product were separated on Dowex 1-X2 (Cl^-) columns and the level of radioactivity determined by liquid scintillation in a Nuclear Chicago Unilux II counter, using the procedure described by Prusiner and Milner [6]. 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 concentration level.

A micro-nesslerization procedure was developed for kinetic studies at 10^{-3} and 10^{-4} M substrate concentrations. 1 ml of substrate and 0.1 ml enzyme dilution were incubated at 37° C for 10 min. 0.1 ml of 1.5 M trichloroacetic acid was added to stop the reaction. To the reaction mixture 1 ml of water and 1 ml of Nessler's reagent were added. This was mixed well and allowed to stand for 10 min, then read on a spectrophotometer at 420 nm. The enzyme was diluted to give less than 20% conversion of the substrate.

The enzymatic decomposition of aspartic acid-β-hydroxamate and glutamic acid-γ-hydroxamate was determined by the method described by Allison [7].

Protein concentration was determined by the method of Lowry et al. [8], using bovine serum albumin for a standard curve.

Electrophoresis in sodium dodecyl sulfate-polyacrylamide gels was performed according to Weber and Osborn [9] except that proteins were denatured in a boiling water bath for 2 min. Ribonuclease A, myoglobin, chymotrypsinogen and ovalbumin were used as standards for subunit molecular weight determination.

Gel filtration for the approximate determination of molecular weight was carried out on Sephadex G-100 in a 2 × 90 cm column, using reverse flow. The buffer was 0.05 M borate, 0.1 M NaCl and 0.001 M EDTA, pH 7.0.

Isoelectric focusing was carried out in polyacrylamide gels (0.5 × 15 cm) using pH 3—10 ampholytes at 4° C as described by Wrigley [10]. 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 activity.

Acrylamide gels were stained for L-asparaginase and L-glutaminase activity using the method described by Pajdak and Pajdak [11] or the method of Cooney et al. [12].

For amino acid analysis purified asparaginase A was lyophilized. Duplicate

samples containing approx. 0.4 mg protein were hydrolyzed in 6 M HCl at 110° C in sealed evacuated tubes for 24 h. Samples were hydrolyzed under similar conditions in *p*-toluene sulfonic acid for tryptophan determinations [13]. Analyses were carried out on a Beckman Model 121 amino acid analyzer.

Rabbit antiserum to *E. coli* asparaginase was prepared by giving an initial intramuscular injection of 0.1 ml of an asparaginase solution (1.0 mg/ml) emulsified with an equal volume of Freund's complete adjuvant to a New Zealand white doe. 2 weeks later the rabbit received the first of 13 weekly subcutaneous injections of 0.1 ml of asparaginase solution. The animal was bled after the thirteenth dose, and the serum was collected and stored frozen until use. Double diffusion was performed by the method of Ouchterlony [14]. Inhibition of enzyme activity by antiserum was determined by incubation of 0.1-ml aliquots of the asparaginases with 0.1 ml of undiluted antiserum for 1 h at 25° C after which residual enzyme activity was measured by the standard nesslerization assay. Normal rabbit serum was used as a control.

Plasma clearance of asparaginase. Plasma clearance rates of *C. freundii* asparaginase A were determined by injecting 0.2 ml of enzyme solution in 0.05 M borate buffer, 0.1 M NaCl, 0.001 M EDTA, pH 7.0, containing 5 I.U. of asparaginase A activity intraperitoneally into normal and tumor-bearing C3H mice. The mice were bled from the tail vein at various time intervals. The asparaginase activity was determined by micro-nesslerization.

Tumor inhibition assays. Tumor inhibition assays were performed using a modification of the methods of Roberts et al. [15]. The anti-tumor effect of the asparaginase A was evaluated by the effect of the enzyme on the survival time of mice carrying the ascitic form of the Gardner lymphoma 6C3HED. Cells were transplanted by intraperitoneal injection of 10^6 viable ascites cells from a C3H/HE mouse with an 8-day-old ascites tumor. The number of viable cells was estimated by trypan blue exclusion in a hemocytometer. Following tumor transplantation the mice were treated with five units of asparagine A (0.05—0.2 ml) by intraperitoneal injections. The number of surviving mice was recorded daily. Long-term survivors of the treated mice were rechallenged with 10^6 ascites cells 45 days following initial tumor transplantation. Surviving mice were recorded daily and were considered cured and immune if they survived 30 days beyond the rechallenge.

Results

Identification of the organism

The bacterium was identified as *C. freundii* using standard morphological and biochemical tests. It is a gram negative, facultatively anaerobic, monotrichous rod. Its morphology and colony formation closely resemble that of *E. coli*, but growth occurs on some media which inhibit *E. coli*. The TSI test showed fermentation with the production of both acid and gas. The oxidase test was negative. Trimethylene is produced from glycerol. H_2S is produced; indole is not produced; lysine is not decarboxylated and malonate is not utilized. KCN does not inhibit growth.

Enzyme purification

Approx. 700 g of cells were resuspended in 2 l of 0.1 M potassium phosphate

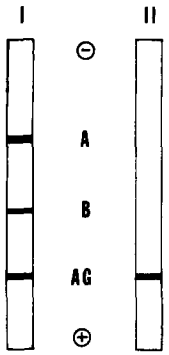

Fig. 1. A diagrammatic representation of polyacrylamide gel electrophoresis of a crude extract of *C. freundii* showing the relative mobility of the asparaginases. Gel I was stained for asparaginase activity and gel II for glutaminase activity. A, B, glutaminase A and B, respectively; AG, asparaginase-glutaminase.

buffer, 0.001 M EDTA, 0.0001 M dithiothreitol, pH 7.5. The cells were ruptured by two passes through a Gaulin continuous flow homogenizer at a pressure of 5500 lb/inch^2. The homogenized cells were centrifuged at 14 000 rev./min in a type 15 rotor in a Beckman L3-40 ultracentrifuge for 2.5 h to remove cell debris. The supernatant was decanted and 1.0 M $MnCl_2$ was added dropwise, with continuous stirring, to a final concentration of 0.05 M. The solution was stirred for an additional 30 min, then allowed to stand overnight at 4° C. The precipitated proteins were removed by centrifugation at 14 000 rev./min for 1 h in a type 15 rotor. Disc gel electrophoresis on this supernatant (the $MnCl_2$ fraction) showed two separate asparaginase enzymes and one asparaginase-glutaminase enzyme (Fig. 1). These enzymes were designated asparaginase A, asparaginase B and asparaginase-glutaminase.

Finely powdered ammonium sulfate was added to the stirred supernatant to give 50% saturation, while the pH was maintained at pH 6.8 with 10% NH_4OH. After standing at 4° C for 30 min, the precipitate was removed by centrifugation. The precipitate was resuspended in a minimum volume of 0.1 M potassium phosphate buffer, 0.001 M EDTA, 0.0001 M dithiothreitol, pH 7.8, and dialyzed overnight against the same buffer. This ammonium sulfate precipitate contained both the asparaginase B and the asparaginase-glutaminase enzymes. The supernatant was treated with finely powdered ammonium sulfate to an 80% saturation, then allowed to stand at 4° C for 30 min. After centrifugation, the supernatant was discarded and the precipitate resuspended in a small volume of 0.01 M potassium phosphate buffer, 0.001 M EDTA, pH 8.0, and dialyzed overnight against this buffer. This fraction contained the asparaginase A enzyme.

The dialyzed 0—50% $(NH_4)_2SO_4$ fraction containing asparaginase B and the asparaginase-glutaminase enzyme was applied to a 5.5 × 50 cm column containing diethylaminoethyl (DEAE)-cellulose equilibrated with dialysis buffer, then eluted with 0.1 M potassium phosphate buffer, 0.001 M EDTA, 0.0001 M dithiothreitol, pH 7.8, until the 280 nm absorbance returned to 0.1. Asparaginase B was eluted from the column with 0.5 M NaCl, 0.001 M EDTA, 0.0001 M dithiothreitol, pH 6.2. The asparaginase-glutaminase enzyme remained on

the column and could not be eluted without drastic losses of activity. Asparaginase B was concentrated and dialyzed against 0.1 M potassium phosphate buffer, 0.001 M EDTA, 0.0001 M dithiothreitol, pH 8.0. Disc gel electrophoresis was performed upon the asparaginase B enzyme to be sure that it had been separated from the asparaginase-glutaminase and asparaginase A enzymes.

The dialyzed 50—80% $(NH_4)_2SO_4$ fraction containing asparaginase A was applied to a 2.5 × 150 cm column of DEAE-cellulose equilibrated with the dialysis buffer. The column was washed with dialysis buffer until the 280 nm absorbance returned to 0.1. The enzyme was eluted with a 1.6 l linear gradient from 0.01 M potassium phosphate buffer, 0.001 M EDTA, pH 8.0, to 0.2 M potassium phosphate buffer, 0.001 M EDTA, pH 7.8. The asparaginase fractions were pooled, concentrated by ultrafiltration to 50 ml and dialyzed against 0.005 M potassium phosphate buffer, 0.001 M EDTA, pH 8.0. The enzyme solution was then applied to a second DEAE-cellulse column, 2.5 × 50 cm, equilibrated with dialysis buffer. A linear gradient was begun after the 280 nm absorbance dropped below 0.1. The gradient was 300 ml of 0.005 M potassium phosphate buffer, 0.001 M EDTA, pH 8.0, to 300 ml of 0.1 M potassium phosphate buffer, 0.001 M EDTA, pH 7.8. The asparaginase A fractions were pooled, concentrated to less than 10 ml and applied to the bottom of a 5 × 80 cm column of Sephadex G-200 in a Pharmacia K-50 column with flow adaptors. The enzyme was eluted by ascending chromatography using a 0.05 M sodium borate buffer, 0.001 M EDTA, pH 7.0, containing 0.1 M NaCl.

Because of the extremely labile nature of the asparaginase-glutaminase enzyme, it is difficult to estimate the relative quantities of each of the enzymes in the crude extract. The asparaginase A was purified 1623-fold to a final specific activity of 133 I.U./mg and was judged homogeneous by polyacrylamide and sodium dodecyl sulfate gel electrophoresis. The purified asparaginase A represented 18% of the total asparaginase activity of the crude extract.

The asparaginase B was purified 19-fold to a specific activity of 1.6 I.U./mg. The enzyme was not pure but was completely separated from the other *C. freundii* asparaginases. It was used in this state for the comparative studies described below.

For comparative purposes the asparaginase-glutaminase enzyme was partially purified by valine-Sepharose chromatography [16]. A sample of the 0—50% $(NH_4)_2SO_4$ fraction containing both asparaginase B and asparaginase-glutaminase enzymes was applied to a valine-Sepharose column in a 0.5 M potassium phosphate buffer containing 10^{-3} M EDTA and 10^{-4} M dithiothreitol. The B enzyme was eluted with 0.4 M buffer and the asparaginase-glutaminase enzyme was eluted with water.

Enzyme stability

The asparaginase-glutaminase enzyme was very unstable with large losses of activity occurring during ammonium sulfate fractionation and ion-exchange chromatography. This enzyme was almost completely inhibited by incubation with 10^{-4} M *p*-chloromercuribenzoate for 30 min. The addition of 10^{-4} M dithiothreitol did not stabilize the enzyme for storage. Asparaginase B was also completely inhibited by *p*-chloromercuribenzoate under the same conditions. The addition of 10^{-4} M dithiothreitol allowed storage for up to 6 months

without noticeable loss of activity. Asparaginase A was not inhibited by *p*-chloromercuribenzoate and did not have any appreciable loss of activity on storage at 4° C for 12 months, even in the absence of dithiothreitol.

Comparison of properties of C. freundii asparaginases A and B

The two *C. freundii* asparaginases were found to differ considerably in their pH optima. Asparaginase A has a broad range of activity with an optimum at approx. 7.5. The asparaginase B is active over a much narrower pH range with the optimum being pH 8.5, although the enzyme still displays approx. 90% of optimal activity at physiological pH values. The two enzymes also differed in thermal stability with the asparaginase A being 50% inactivated after 10 min at 67° C while the asparaginase B was 50% inactivated at 51° C under the same conditions. Both enzymes were significantly protected from thermal inactivation by the presence of substrate (0.01 M L-asparagine); the temperatures for 50% inactivation being 78 and 68° C for asparaginases A and B, respectively.

An examination of the substrate specificity (Table I) showed the two enzymes to have generally similar properties. Both show relatively little activity with L-glutamine or with the D-isomers of asparagine or glutamine. Nor do these D-isomers cause marked inhibition of activity with L-asparagine as substrate. Neither of the enzymes showed detectable ammonia production upon prolonged incubation with the asparagine derivatives L-isoasparagine or *N*-acetyl-L-asparagine. However, when the asparaginase B was incubated with L-aspartic acid for

TABLE I

SUBSTRATE SPECIFICITY OF *C. FREUNDII* ASPARAGINASES A AND B

All substrate were used at concentration of 0.01 M in 0.1 M Tris · HCl, pH 8.0. The asparaginase enzymes were incubated with the subatrates for 10 min at 37° C. Ammonia was determined by nesslerization. Substrates not showing activity were reassayed for 100 min. Relative activity was based upon the amount of asparagine converted to ammonia as 100% velocity.

Substrate	Relative activity	
	Asparaginase A	Asparaginase B
L-Asparagine	100	100
D-Asparagine	5.0	1.4
L-Glutamine	3.0	4.6
D-Glutamine	0	3.2
L-Asparagine + D-asparagine	95.0	92.0
L-Asparagine + L-glutamine	90.0	86.0
L-Asparagine + D-glutamine	100	104.0
L-Aspartyl-β-hydroxamic acid *	32.0	66.0
D-Aspartyl-β-hydroxamic acid *	6.0	0
L-Glutamyl-γhydroxamic acid *	1.8	5.6
L-Aspartic acid	0	9.0
D-Aspartic acid	0	0
L-Glutamic acid	0	0
L-Isoasparagine	0	0
N-Acetylasparagine	0	0
Acetamide	0	0
Propionamide	0	0
Butyramide	0	0
Succinamide	0	0

* Incubated with the enzymes for 10 min. Hydrolysis was determined as described in Materials and Methods.

100 min a color reaction was noted in the standard nesslerization assay. This anomaly was not further investigated.

Both enzymes exhibited typical Michaelis-Menten kinetics at low L-asparagine concentrations; the apparent K_m values for this substrate being $2.9 \cdot 10^{-5}$ M for asparaginase A and $1.7 \cdot 10^{-3}$ M for asparaginase B. The isoelectric point of asparaginase A was pH 5.75 and that of asparaginase B, pH 5.19.

Physicochemical properties of asparaginase A

The molecular weight of the *C. freundii* asparaginase A was estimated by gel filtration on Sephadex G-100. This enzyme had the same elution volume as a sample of *E. coli* L-asparaginase and an approximate molecular weight of 140 000 was calculated using Squires' [17] equation. The subunit molecular weight of the *C. freundii* enzyme was determined by electrophoresis in polyacrylamide-sodium dodecyl sulfate gels using appropriate standards. Only a single protein band was noted upon electrophoresis, the mobility of the band corresponding to a molecular weight of approx. 33 000. These results suggest that *C. freundii* asparaginase A is tetrameric. The amino acid composition of the enzyme is shown in Table II, together with the compositions of other bacterial asparaginases for comparison. The various asparaginases show strong similarities in composition. The *C. freundii* asparaginase A was found to con-

TABLE II

AMINO ACID COMPOSITION OF BACTERIAL ASPARAGINASES

Amino acid	*Citrobacter freundii* Asparaginase A (M = 32 800)	*Alcaligenes eutrophus* [b] (M = 34 500)	*Escherichia coli* [c] (M = 33 300)	*Acineto-bacter* [d] (M = 33 000)	*Proteus vulgaris* [e] (M = 30 000)	*Erwinia* [f] (M_r = 33 370)
Lysine	21.0	22.4	21.0	24.4	22.8	18.2
Histidine	4.8	4.2	3.1	8.9	3.1	5.4
Arginine	9.8	12.3	7.2	10.0	6.4	16.2
Tryptophan	1.8	2.9	1.0	4.3	1.2	0
Aspartic acid	39.3	33.5	50.7	36.7	40.0	35.1
Threonine	28.0	20.6	32.6	16.3	22.4	26.3
Serine	19.0	18.8	14.6	18.9	13.9	17.5
Glutamic acid	25.4	30.0	18.7	23.5	21.8	20.9
Proline	13.5	14.3	11.0	10.2	11.4	12.5
Glycine	28.7	28.5	28.5	22.2	27.3	33.4
Alanine	31.2	42.6	31.1	36.2	31.2	29.0
Cysteine	2.1	0	1.9	0	2.0	0
Valine	26.5	26.1	35.0	28.5	30.7	29.7
Methionine	5.0	4.9	3.9	7.4	5.2	6.7
Isoleucine	11.7	16.0	12.2	16.4	14.6	16.9
Leucine	23.2	23.3	22.3	24.0	22.6	28.3
Tyrosine	11.0	10.0	11.0	8.0	8.8	12.5
Phenylalanine	8.5	8.3	8.0	9.0	10.3	6.7

[a] Determined by analysis of samples hydrolyzed in *p*-toluene sulfonic acid.
[b] Data from Allison [7].
[c] Data from Ho et al. [29].
[d] Data from Roberts et al. [30].
[e] Data from Tosa et al. [31].

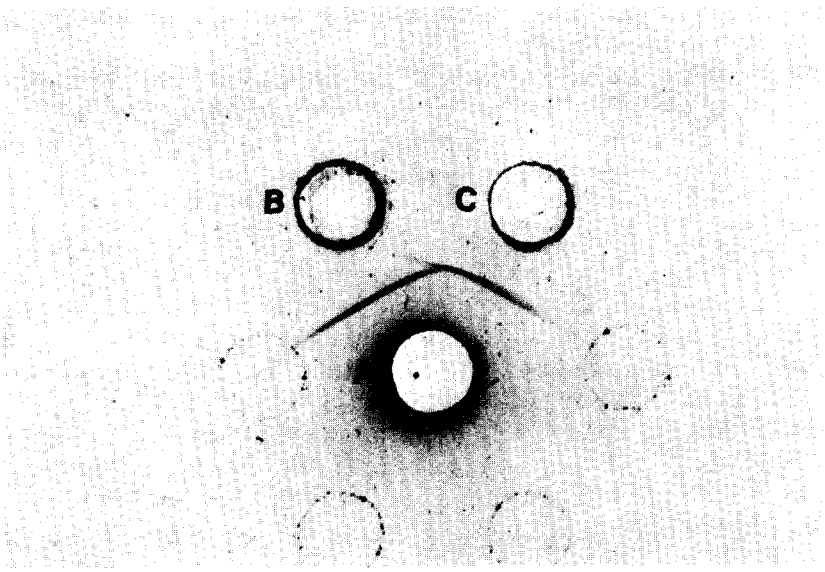

Fig. 2. Double diffusion in agar of an antiserum directed against *E. coli* asparaginase (well A) with *C. freundii* asparaginase A (well B) and *E. coli* asparaginase (well C).

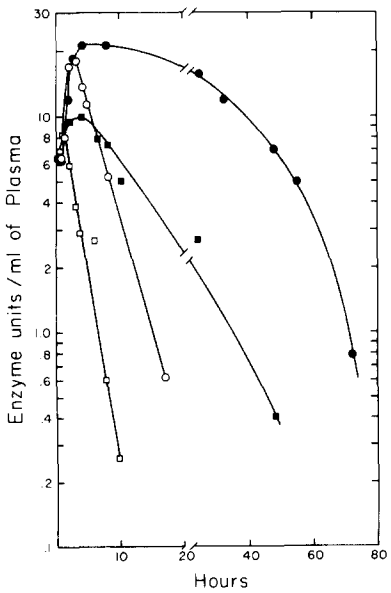

Fig. 3. Plasma clearance of L-asparaginase in C3H mice. At zero time, C3H mice received intraperitoneal injections of five units of either *C. freundii* asparaginase A or 10 units of *E. coli* asparaginase. Tumor-bearing mice had received transplants of 10^6 6C3HED ascites cells 4 days previously. At the specified intervals mice were bled from the tail vein and asparaginase activity determined by the micro-nesslerization assay method. ●, *E. coli*, tumor-bearing mouse; ■, *E. coli*, normal mouse; ○, *C. freundii*, tumor-bearing mouse; □, *C. freundii*, normal mouse.

tain two residues of cysteine per subunit even though this enzyme, unlike the asparaginase-glutaminase and asparaginase B, is insensitive to inhibition by *p*-chlormercuribenzoate.

Immunological properties

The immunological cross-reactivity of the *C. freundii* asparaginase A and *E. coli* asparaginase was examined by double diffusion in agar, using a rabbit antiserum directed against the *E. coli* enzyme. As shown in Fig. 2, a reaction of partial identity was observed, with spurring of the precipitin bands. Inhibition of the enzymatic activity of both enzymes was observed on incubation with the *E. coli* asparaginase antiserum. After a 1 h incubation period the *E. coli* enzyme showed only 27% of the original activity while the *C. freundii* asparaginase A retained 72% of the initial activity.

Plasma clearance of asparaginase A

The rate of clearance of *C. freundii* asparaginase A and *E. coli* asparaginase from the blood plasma of C3H/HE mice was determined by taking bleedings from the tail vein at various intervals after intraperitoneal injections of the enzymes. These experiments were carried out on both tumor-bearing and non-tumor-bearing mice and representative results are shown in Fig. 3. In normal mice *C. freundii* asparaginase A was cleared from the plasma with a half-life of about 0.8 h, compared with a half-life of 4 h for the *E. coli* enzyme. In tumor-bearing animals the clearance of the enzymes from the plasma was markedly slower, with half-lives of about 7 and 20 h being recorded for *C. freundii* asparaginase A and *E. coli* enzymes, respectively.

Anti-tumor activity of C. freundii asparaginase A

Intraperitoneal injections of *C. freundii* asparaginase A produced significant increases in the survival times of C3H/HE mice infected with the 6C3HED

TABLE III

ANTI-TUMOR ACTIVITY OF *C. FREUNDII* ASPARAGINASE A

Mice were rechallenged with 10^6 ascites cells 45 days after tumor implantation. This was more than 30 days after the mean survival time of the untreated controls.

Treatment	No. of mice	Mean survival time (days)	Range	Increase in mean survival time (days)	No. of mice rechallenged	No. of immune mice
None	5	9.6	9—10	—	—	—
25 I.U. *C. freundii*, day 1	5	17.2	16—18	7.6	—	—
25 I.U. *C. freundii*, day 7	5	22.2	18—37	12.6	—	—
5 I.U. *C. freundii*, days 2, 4, 6, 8, 10 [a]	5	23.3 [b]	22—26	13.7; 2 cured,	2	2
5 I.U. *C. freundii*, days 2, 4, 6, 8, 10 [a]	5	29.3 [c]	24—42	17.1; 1 cured,	1	1
5 I.U. *C. freundii*, days 2, 4, 6, 8, 10 [a]	5	30 [b]	26—36	17.8; 2 cured,	2	1
Heat inactivated *C. freundii*, days 2, 4, 6, 8, 10 [a]	3	12.7	12—13	1.0	—	—
25 I.U. *E. coli*, day 7	5	—	—	— 5 cured	5	3

[a] These results were obtained using three different enzyme preparations.
[b] Mean survival for 3—5 mice.
[c] Mean survival for 4—5 mice.

Gardner lymphoma. The results of representative experiments are given in Table III. The most successful regime tested was the injection of a total of 25 units of asparaginase A given in five unit doses on days 2, 4, 6, 8 and 10 after tumor implantation. This procedure produced from a minimum of a 9.9 day increase in survival time, compared with untreated animals, to a complete cure in 30% of the animals. A complete cure was defined as a survival of 45 days after tumor implantation, with no visible signs of tumor at this time. The cured mice were resistant to a rechallenge with 10^6 6C3HED ascites cells. By comparison 25 units of *E. coli* asparaginase given on day 7 after tumor implantation (determined from previous studies to be the optimal dose) produced cures in all the test animals and 60% of these mice were resistant to subsequent rechallenge with 10^6 ascites cells. Heat-inactivated *C. freundii* L-asparaginase A was without beneficial effect on tumor growth.

Discussion

The antineoplastic effects of asparaginase stem from the fact that certain tumor lines require exogenous asparagine for growth whereas normal cells have much less stringent requirements for this amino acid. McCoy and his colleagues [18—20], working with tissue culture lines, noted this nutritional requirement of certain tumor lines well before the antineoplastic effects of L-asparaginase had been demonstrated.

The use of *E. coli* asparaginase for producing tumor remission has been limited by problems of hypersensitivity and the rapid clearance of the enzyme from the bloodstream associated with long-term therapy. This has prompted a search for other sources of asparaginases which possess satisfactory antineoplastic activity. For example, while asparaginases from *E. coli*, *Serratia marcescens* and *Erwinia caratovora* are effective in mice for this purpose, the asparaginases of *Saccharomyces cerevisae* and *Bacillus coagulans* are not [5,21—23]. The most obvious requirements for an asparaginase to be of therapeutic use are that it be relatively easy to isolate in quantity; that it be active at physiological pH and temperature; that the enzyme should not be very rapidly cleared from the bloodstream and that the enzyme show appreciable activity at the substrate levels found in blood (approx. 0.03—0.05 mM asparagine). With these requirements in mind we can ask how well they are fulfilled by the *C. freundii* asparaginases. It should first be pointed out that the occurrence of multiple asparaginases, with differing properties, in a single species of bacterium is by no means unusual. Similar findings have been reported in *E. coli* [14], *Mycobacterium tuberculosis* [24] and a *Pseudomonas* species [25]. Of the three asparaginases found in *C. freundii* the asparaginase-glutaminase enzyme is manifestly unsuitable for therapeutic use because of its extreme lability both during purification and as the partially purified enzyme. The *C. freundii* asparaginase B, though it is relatively labile and sensitive to inhibition by *p*-chloromercuribenzoate, could be protected from inactivations during storage by inclusion of a reducing agent. This enzyme shows satisfactory activity at a physiological pH and is only moderately sensitive to thermal inactivation. A major factor which suggests that this enzyme would be of little therapeutic use is the low affinity for substrate. With a K_m of $1.7 \cdot 10^{-3}$ M the asparaginase B

would be of very limited effectiveness in reducing blood asparagine levels well below the normal values of 0.03—0.05 mM.

By contrast, the *C. freundii* asparaginase A is very stable, can be readily isolated by simple purification procedures and can be stored without loss of activity for long periods of time. The enzyme shows optimal activity in the physiological pH range; the K_m ($2.9 \cdot 10^{-5}$ M) is similar to that of the antineoplastic *E. coli* asparaginase. The *C. freundii* asparaginase A is also similar to this *E. coli* enzyme in molecular weight, subunit composition and isoelectric point. The *C. freundii* enzyme does, however, show limited cross-reactivity with *E. coli* asparaginase, as shown by the immunological studies. The degree to which this would reduce the clinical effectiveness of the *C. freundii* asparaginase A, following *E. coli* asparaginase therapy, can only be assessed by further animal testing.

The clearance of the *C. freundii* asparaginase A from blood plasma was found to be considerably more rapid than the *E. coli* enzyme, both in the presence and absence of tumor growth. The slower clearance in the presence of tumor is probably due to the presence of lactate dehydrogenase-elevating virus [26]. That the clearance rate determined for the *C. freundii* enzyme may not be a major limitation in its use for therapeutic purposes is suggested by the fact that although an asparaginase-glutaminase from *Alcaligenes faecalis* is cleared at a rate similar to that of the *C. freundii* asparaginase, the *A. faecalis* enzyme is nevertheless an effective anti-tumor agent [27,28]. The limited experiments carried out on the effect of the *C. freundii* asparaginase A against the 6C3HED lymphoma of mice indicate that the enzyme possesses significant antineoplastic activity in this system, although with the doses tested it is somewhat less effective than the *E. coli* asparaginase. The results to date suggest that the *C. freundii* asparaginase A may have potential for the therapy of human leukemia.

Acknowledgements

We are indebted to Dr. Dwayne Savage for collection of the organism and to him, Christine Haegerty and Paul Szaniszlo for its identification. We thank James Lemburg and John Omnaas for assistance in carrying out the amino acid analyses and James Allison for helpful discussions. This work was supported in part by grants IC-93A and T-555 from The American Cancer Society.

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