Biochimica et Biophysica Acta, 438 (1976) 287—295

BBA 67823

PURIFICATION AND PROPERTIES OF L-ASPARAGINASE B FROM *ACINETOBACTER CALCOACETICUS*

PER ERIK JONER

Department of Biochemistry, University of Bergen, Bergen (Norway)

(Received December 22nd, 1975)

Summary

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) B from *Acinetobacter calcoaceticus* has been purified by precipitation with streptomycin, chromatography on DEAE-cellulose and CM-cellulose, gel filtration on Agarose and chromatography on phosphocellulose. The molecular weight of the enzyme was found to be 130 000. The enzyme was rather insensitive to pH changes between 7 and 9. The Michaelis constant was $3 \cdot 10^{-3}$ M. Hg^{2+}, Cu^{2+} and Ni^{2+} as well as high ionic strength inhibited the activity of the enzyme, whereas citrate seemed to stimulate the activity. The enzyme catalyzed the deamination of L-glutamine to about the same extent as L-asparagine. The temperature stability of the enzyme is also reported. The enzyme had a weak tumor inhibitory power.

Introduction

In 1953, J.G. Kidd found that guinea pig serum inhibited the growth of several mouse tumors [1,2]. 7 years later Broome [3] identified L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) as the serum component responsible for this effect. When Mashburn and Wiston [4] in 1964 reported the discovery of a tumor inhibitory L-asparaginase in *Echerichia coli*, an intense activity started in this field and large scale purification of the *E. coli* enzyme and some other bacterial L-asparaginases was developed [5—8].

Clinical application of the enzyme in the treatment of different cancer types in man followed. The enzyme turned out to be most effective against acute lymphatic leukemia, but unfortunately the effect was not permanent. Relapses developed caused by the outgrowth of asparaginase-resistant cell lines. These cells had the ability to synthesize their own L-asparagine and were thus insensitive to the breakdown of L-asparagine in the circulating body fluids caused by injected L-asparaginase. The hope that the enzyme might be the great remedy

in the battle against cancer has therefore been abandoned but there are still reports coming with promising results on the use of L-asparaginase in combination therepy with other drugs (such as the metabolite analogue cytosine arabinoside and others).

Acinetobacter calcoaceticus has been shown to contain two isoenzymes of L-asparaginase called L-asparaginase A and B and a report on the purification and some properties of L-asparaginase A has been published [9]. The two isoenzymes can be separated by chromatography on DEAE-cellulose. L-Asparaginase A is retained by the DEAE-cellulose at pH 8.6 whereas L-asparaginase B passes through.

This report describes a purification procedure for L-asparaginase B and gives some data on the purified enzyme.

Materials

Chemicals. The amino acids used were chromatographically pure and obtained from Schwarz Bioresearch. DEAE-cellulose, DE 23, CM-cellulose, CM 23 and phosphocelloluse, P 11 were from Whatman. Agarose 1.5 m, 200—400 mesh, was from Biorad, streptomycin was a product of Glaxo Laboratories; sodium dodecyl sulfate, deoxyribonuclease IIB, ribonuclease IA and egg-white lysozyme were obtained from Sigma and hemoglobin and ferritin from Boehringer Mannheim. Crasnitin (*Escherichia coli* asparaginase) was purchased from Bayerwerke and γ-globulin from Kabi.

Organism. A. calcoaceticus, National Collection of Type Cultures, NCTC 7363 (England) was obtained from Department of Microbiology, The Gade Institute, School of Medicine, University of Bergen, Norway.

Media. The bacteria were cultivated in a medium containing 3 g KH_2PO_4, 9 g Na_2HPO_4, 0.985 g $MgSO_4$, 0.06 g $CaCl_2$, 2.3 g NaCl, 0.011 g $FeCl_3 \cdot 6H_2O$, 3 g L-asparagine and 3 g D-(+)-xylose or ethanol per l distilled water.

Animals and tumor cells. C3H mice were obtained from Statens Institutt for Folkehelse, Oslo (Norway). Professor Georg Klein, Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden kindly donated C3H mice carrying 6C3HED lymphoma [10].

Methods

Growth and harvesting of bacteria

The bacteria were grown under strong aeration in 15-l portions and collected in the stationary phase by continuous flow centrifugation. The cell paste was frozen and kept at −20° C.

Enzyme assays

L-asparaginase activity. This was measured in the following way: an appropriate amount of enzyme was diluted with 0.05 M borate buffer, pH 8.6, and the reaction was started by addition of 50 µl L-asparagine, the final volume being 1 ml. The reaction mixture was incubated at 37° C for 10 or 20 min. At the end of the reaction period 50 µl trichloroacetic acid was added to terminate the reaction. After centrifugation at 15 000 × *g* for 5 min 0.5 ml of each sam-

ple was diluted 10-fold with water, 1 ml Nessler's reagent was added and the absorbance was read at 420 nm.

L-glutaminase activity. The activity of the enzyme towards L-glutamine was measured in two different ways. (1) 10—25 μl of a reaction mixture identical to that mentioned above and incubated with enzyme for the same period but with L-glutamine as a substrate instead of L-asparagine was spotted on Whatman No. 1 chromatography paper, developed in methanol/water (7 : 3, v/v) and the amino acids determined by the ninhydrin method. One unit of enzyme activity is defined as the amount of enzyme converting 1 μmol of L-glutamic acid per min under the conditions described. (2) This method of measuring L-glutaminase activity is based on the precipitation with sodium tetraphenylborate in acid solution of the NH_3 liberated in the reaction [11]. To the reaction mixture described above HCl was added to a final concentration of 0.1 M to stop the reaction. After centrifugation at 15 000 × g for 50 min 0.1 ml 0.1 M sodium tetraphenylborate was added to the supernatant fluid and another centrifugation at 15 000 × g for 30 min followed. The precipitate consisting of ammonium tetraphenylborate was dissolved in ethanol and the absorbance read at 250 nm. One unit of L-glutaminse is defined as the amount of enzyme liberating 1 μmol NH_3 per min under the conditions described.

Protein determinations.

All determinations of protein were carried out according to the method of Lowry et al. [12].

Polyacrylamide gel electrophoresis

20—40 μg protein to which had been added glycerol and a little bromphenol blue (final glycerol concentration of 25%) was applied to 7.5% acrylamide gel in Tris buffer, pH 8.5. The electrophoresis was run at 10 V/cm. The gels were stained by 0.2% Coomassie blue in 46% methanol with 9.2% acetic acid for 1 h and destained with 7% acetic acid.

Results

Purification of the enzyme

The first steps of the purification procedure, which were identical to those described for L-asparaginase A, have been given in detail earlier [9] and will therefore only be summarized here: frozen cells were thawed, treated with egg-white lysozyme, sonicated, then treated with DNAase and RNAase and centrifuged. The crude extract thus obtained was precipitated with streptomycin and the supernatant applied on a DEAE-cellulose column at pH 8.6. The two isoenzymes were separated at this step. L-Asparaginase A was retained on the DEAE-cellulose column while L-asparaginase B passed through (Fig. 1 in ref. 9). The further purification of L-asparaginase B is described below.

CM-cellulose chromatography

The enzyme activity which passed through the DEAE-cellulose at pH 8.6 was dialyzed against 0.05 M phosphate buffer, pH 6.0, and applied to a CM-cellulose column with a settled bed volume of 90 ml (2.4 × 20 cm) equilibrated with

the same phosphate buffer. The column was washed through with two bed volumes of equilibrating buffer and then eluted with a gradient in the same buffer with concentrations of NaCl from 0 to 1.0 M (twice 100 ml). Fig. 1 shows the elution pattern of the CM-cellulose column. The enzyme activity corresponding to L-asparaginase B eluted at a concentration of approx. 0.3 M NaCl.

Agarose gel filtration

The pooled enzyme activity from the CM-cellulose column was concentrated by Diaflo ultrafiltration and applied on an Agarose column (2.4 × 80 cm). The enzyme rapidly lost activity if kept in phosphate buffer with NaCl, whereas 0.1 M citrate buffer, pH 6.0, seemed to stabilize the enzyme. The elution therefore was carried out with this buffer.

Phosphocellulose chromatography

The fractions containing enzyme activity from the Agarose gel filtration were pooled, concentrated by ultrafiltration on Diaflo, dialyzed against 0.05 M phosphate buffer and applied on an equilibrated phosphocellulose column (1.4 × 24 cm). After application of the enzyme material, the column was washed through with 0.05 M phosphate buffer, pH 6.0, until very little ultraviolet-absorbing material was present in the eluate. Then a gradient from 0 to 0.5 M KCl in the same buffer was applied to the column (twice 100 ml). Enzyme activity appeared in a peak at a concentration of KCl of approx. 0.1 M (Fig. 2). A summary of the purification procedure is given in Table I.

Properties of L-asparaginase B

Molecular weight. The molecular weight of the purified enzyme was estimated by gel filtration on Sephadex G-200 column (1.4 × 96 cm) using hemoglobin, γ-globulin, crasnitin (*E. coli* asparaginase) and ferritin as reference pro-

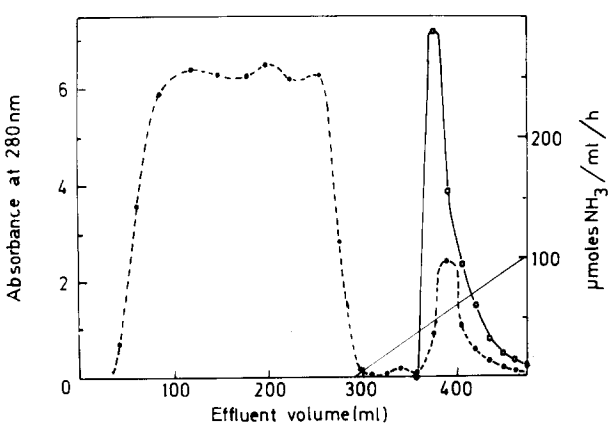
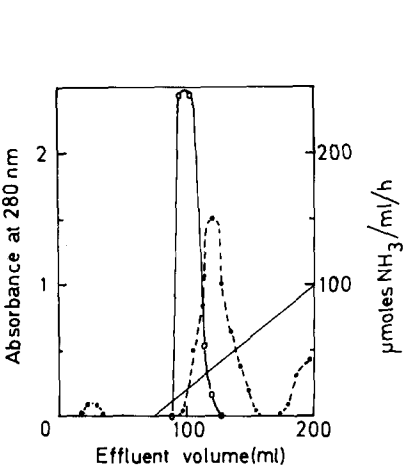


Fig. 1. CM-cellulose chromatography of enzyme material from DEAE-cellulose chromatography, i.e. material that was not retained by the DEAE-cellulose. The column (2.4 × 20 cm) was equilibrated with 0.05 M phosphate buffer, pH 6.0. After washing, the protein was eluted with a linear gradient in NaCl from 0 to 1.0 M. Total volume of gradient, 200 ml. o- - - - -o, protein measured by absorbance at 280 nm; o———o, enzyme activity.



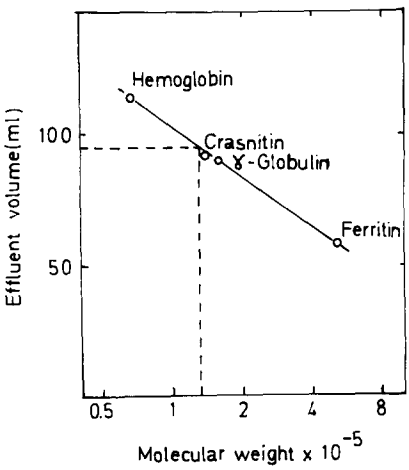


Fig. 2. Phosphocellulose chromatography of enzyme material from CM-cellulose chromatography. The column (1.4 X 24 cm) was equilibrated with 0.05 M phosphate buffer pH 6.0. After washing, the protein was eluted with a linear gradient in KCl from 0 to 0.5 M. Total volume of gradient 150 ml. o- - - - -o, protein measured by absorbance at 280 nm; o———o, enzyme activity.

Fig. 3. Elution volume of L-asparaginase B on a Sephadex G-200 column (1.4 X 94 cm) compared to that of some standard proteins of known molecular weight. The enzyme eluted at a volume of 95 ml corresponding to a molecular weight of 130 000. The column was equilibrated with 0.05 M phosphate buffer, pH 6.0, containing 0.01 M β-mercaptoethanol. Standard proteins were hemoglobin, crasnitin (*E. coli* asparaginase), γ-globulin and ferritin.

teins. The enzyme eluted at a volume corresponding to a molecular weight of about 130 000 (Fig. 3).

Effect of pH on the enzymatic activity. The activity of the enzyme was registered in several buffers as a function of pH (Fig. 4). The enzyme seemed to be rather insensitive to pH changes in the medium at least in the region between 7 and 9 in all buffers tested. Below 7 and above 9 the activity declines.

Effect of substrate concentration. The effect of substrate concentration on enzyme activity was studied and these investigations showed that high concentrations of L-asparagine inhibited enzyme activity. The apparent Michaelis constant of the enzyme was estimated by the Lineweaver-Burk method to be $3 \cdot 10^{-3}$ M.

TABLE I

PURIFICATION PROCEDURE FOR L-ASPARAGINASE B

Purification step	Total volume (ml)	Total protein (mg)	Total units	Specific activity	Enrichment (fold)	Recovery (%)
Crude extract	150	3600	1350	0.4	—	—
DEAE cellulose chromatography	200	1200	600	0.5		100
CM cellulose chromatography	10	180	350	1.96	4	58
Agarose filtration	10	30	210	7.0	14	35
Phospho-cellulose chromatography *	2	1.0	70	70.0	140	11.6

* The purified enzyme from this step was rather unstable so the material from the previous step was used in studying the properties of the enzyme.

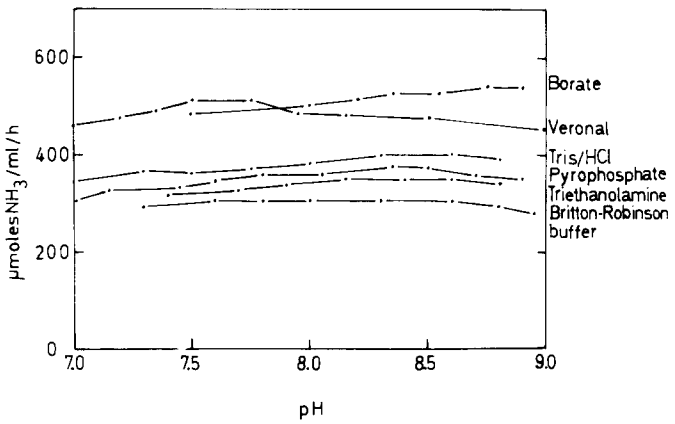


Fig. 4. The activity of L-asparaginase B as a function of the pH of the medium in different buffer systems. The reaction temperature was 37° C.

Inhibitors and activators. Table II shows the effect of several metal ions and some other compounds on the activity of the enzyme. Hg^{2+}, Cu^{2+} and Ni^{2+} strongly inhibited the activity of the enzyme whereas *p* chloromercuribenzoate ($pClHgBzO^-$), Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} did not. β-Mercaptoethanol and azide did not seem to affect the activity while citrate stimulated the activity. High concentrations of aspartate, one of the products of the reaction catalysed, inhibited the activity and so did high salt concentrations.

Specificity. The enzyme deaminated L-glutamine to the same extent as L-asparagine while D-asparagine was deaminated to 30% of activity. Neither formamide nor acetamide was deaminated by the enzyme (Table III).

TABLE II

INHIBITORS AND STIMULATORS OF L-ASPARAGINASE B

Compound added	Concentration (M)	Activity (%)
O	—	100
Hg^{2+}	10^{-3}	12
Cu^{2+}	10^{-3}	22
Co^{2+}	10^{-3}	99
Ni^{2+}	10^{-3}	60
Ba^{2+}	10^{-3}	102
Ca^{2+}	10^{-3}	99
Zn^{2+}	10^{-3}	105
Mg^{2+}	10^{-3}	101
Cd^{2+}	10^{-3}	103
$pClHgB_2O^-$	10^{-5}	100
Citrate	10^{-2}	130
β-Mercaptoethanol	10^{-3}	102
Potassium aspartate	10^{-2}	85
Potassium aspartate	$5 \cdot 10^{-2}$	65
KCl	$5 \cdot 10^{-1}$	34
NaCl	$5 \cdot 10^{-1}$	40
Na_2SO_4	$2 \cdot 10^{-1}$	36
NaN_3	10^{-3}	110

TABLE III

SPECIFICITY OF L-ASPARAGINASE B

The assay was done as described in Methods, i.e. enzyme was added to a mixture of 10 μmol of the substrates indicated in a volume of 1 ml and the reaction was stopped by addition of 50 μl 25% trichloroacetic acid, and the amount of NH_3 formed in 20 min at 37° C was estimated.

Substrate	Activity
L-Asparagine	100
D-Asparagine	30
L-Glutamine	100

Temperature stability. The enzyme was stable at moderately elevated temperatures for several hours as seen from Fig. 5. Even at 50° C, little activity was lost in 3 h. Freezing of the enzyme at −20° C on the other hand completely and irreversibly destroyed the activity.

Storage. The enzyme material eluted from the Agarose column was stable for several weeks if kept in 0.1 M citrate buffer, pH 6.0, at 4° C. Till now we have failed in our efforts to stabilize the enzyme material eluted from the phosphocellulose column with the usual stabilizing agents such as protein, substrate, glycerol etc.

Purity. Polyacrylamide-gel electrophoresis of the enzyme material eluted from the phosphocellulose column revealed two bands and thus indicated that the enzyme was probably not purified to homogeneity.

Tumor inhibitory power

The possible tumor inhibitory power of the enzyme was tested in the system 6C3HED lymphoma/C3H mice. After several passages of lymphoma cells in the form of ascites cells transferred by intraperitoneal injections, the test was performed in the following way: 24 C3H mice were all given injections with 10^7

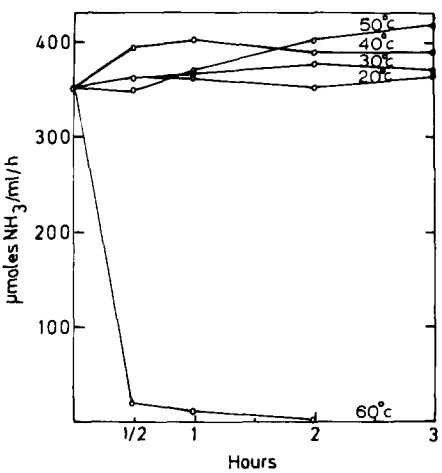


Fig. 5. Stability of L-asparaginase B at different temperatures. Activities were measured after 0, 0.5, 1, 2 and 3 h.

TABLE IV
TUMOR INHIBITORY POWER OF L-ASPARAGINASE B

The animals were injected intraperitoneally with 0.1 ml of a tumor suspension containing 10^7 cells each. On the following day single intraperisoneal injections were given as indicated above. The buffer used was 0.05 M Tris, pH 7.4 (at 37° C) containing 0.1 M NaCl. The enzymes were dissolved in the same buffer.

Test group	Treatment after injection of tumor cells	Number of survivors on day				
		10	11	12	13	14
A	Buffer	0	0	0	0	0
B	20 I.U. of L-asparaginase from *E. coli*	8	8	8	8	8
C	20 I.U. of L-asparaginase from *A. calcoaceticus*	7	7	5	5	0

lymphoma cells in each. On the following day the animals were divided in three groups. Group A received 0.6 ml 0.05 M Tris · HCl buffer, pH 7.4, (at 37° C) containing 0.1 M NaCl. Group B received 0.6 ml of a solution of 20 I.U. *E. coli* asparaginase dissolved in the same buffer and group C received 0.6 ml of a solution of 20 I.U. L-asparaginase B (specific activity 7 I.U./mg protein) in the same Tris buffer. The result is shown in Table IV. The experiment showed that L-asparaginase B possessed a weak tumor-inhibitory power as compared to the controls. Though the enzyme did not prevent the death of the mice, it did keep them alive for some more days than those that did not receive any enzyme treatment.

Discussion

The present work describes the purification and some of the properties of L-asparaginase B, one of the two isoenzymes of L-asparaginase that has been found in *Acinetobacter calcoaceticus*. The other isoenzyme, L-asparaginase A, has been the subject of an earlier report [9]. The two isoenzymes have some properties in common, but differ in several respects. The molecular weights of the two enzymes are not very different, 105 000 for L-asparaginase A and 130 000 for L-asparaginase B. Their apparent Michaelis constants are also of the same magnitude and they are inhibited by some heavy metals to about the same extent. But there are more differences than there are similarities as to the properties we have studied in this laboratory. Thus the A enzyme has pH optimum around 8.6 while the B enzyme has a rather constant activity between pH 7 and 9. The A enzyme deaminates L-glutamine and D-asparagine only about 10 and 5%, respectively, of the activity towards L-asparagine whereas the B enzyme has activity of 100 and 30% towards those substrates and should thus properly be called a L-asparaginase-glutaminase. The A enzyme is further inhibited 100% by *p*-chloromercuribenzoate at a concentration of 10^{-5} M while the B enzyme is unaffected by this compound. Another striking dissimilarity between the two enzymes is that the A enzyme binds strongly to a biospecific ad-

sorbent, a matrix of Sepharose 6B to which D-asparagine is covalently attached by means of hexamethylenediamine as a spacer. The B enzyme, however, does not seem to bind to this adsorbent. The B enzyme also is more temperature-stable than the A enzyme. It can withstand temperatures as high as 50° C for 3 h without loss of activity while the A enzyme is increasingly inactivated above 30° C under the same experimental conditions. Another difference is the reaction of the two isoenzymes to β-mercaptoethanol. The A enzyme is completely and irreversibly inactivated in the presence of 10^{-2} M mercaptoethanol in 3 days at 4° C while B enzyme is not affected by this treatment. As to the tumor inhibitory power of the two isoenzymes, the A enzyme was completely devoid of this property while the B enzyme seems to possess a weak tumor inhibitory power under the condition of the experiment. Roberts et al. [13] have published reports on an asparaginase-glutaminase isolated from an Achromobacteraceae soil organism later designated as the type strain of *Acinetobacter glutaminasificans* (ATCC 27197). They do not report the finding of more than one asparaginase in their strain. This could be explained by their treatment of the crude bacterial extract by dropwise addition of H_3PO_4 to pH 6.5 followed by removal of the precipitate formed. This may have removed the other isoenzyme. Their enzyme showed a high degree of tumor inhibitory power and had a K_m of $4.8 \cdot 10^{-6}$ M. In other respects their enzyme seems to resemble ours. The pH profile between pH 7 and 9, the molecular weight, the lack of inhibition by $pClHgBzO^-$ and the specificity towards glutamine/asparagine are all properties similar to our enzyme's.

Acknowledgements

The exellent technical assistance of Karin Wiig Johannessen is gratefully acknowledged. The work was in part financed by The Norwegian Cancer Society.

References

1 Kidd, J.G. (1953) J. Exp. Med. 98, 565—582
2 Kidd, J.G. (1953) J. Exp. Med. 98, 583—606
3 Broome, J.D. (1961) Nature 191, 1114—1115
4 Mashburn, L.T. and Wiston, Jr., J.C. (1964) Arch. Biochem. Biophys. 105, 450—452
5 Roberts, J., Prager, M.D. and Bachynsky, N. (1966) Cancer Res. 26, 2213—2217
6 Campbell, H.A., Mashburn, L.T., Boyse, E.A. and Old, L.J. (1967) Biochemistry 6, 721—730
7 Rowley, B. and Wriston, J.C. (1967) Biochem. Biophys. Res. Commun. 28, 160—165
8 Peterson, R.E. and Ciegler, A. (1969) Appl. Microbiol. 18, 64—67
9 Joner, P.E., Kristiansen, T. and Einarsson, M. (1973) Biochim. Biophys. Acta 327, 146—156
10 Boyd, J.W. and Philips, A.W. (1971) J. Natl. Cancer Inst. 46, 1271—1276
11 Kohler, M. (1953) Z. Anal. Chem. 138, 9—18
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Roberts, J., Holcenberg, J.S. and Dolowy, W.C. (1972) J. Biol. Chem. 247, 84—90