Biochimica et Biophysica Acta, 327 (1973) 146–156

BBA 67062

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

PER ERIK JONER[a], TORE KRISTIANSEN AND MONICA EINARSSON[b]

[a]*Department of Biochemistry, University of Bergen, Bergen (Norway) and* [b]*Institute of Biochemistry, University of Uppsala, Uppsala (Sweden)*

(Received August 10th, 1973)

SUMMARY

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) A from *Acinetobacter calcoaceticus* has been purified to apparent homogeneity by precipitation with streptomycin, chromatography on DEAE-cellulose, gel filtration on Sephadex G-200 and affinity chromatography. The purified enzyme was compared to a commercial preparation of highly purified asparaginase from *E. coli*. It was found to be of the same purity as the enzyme from *E. coli*. The molecular weight was 105 000 and the enzyme consists of four subunits of molecular weight 25 000. The pH optimum was found to be at approximately pH 8.6, the isoelectric point at pH 5.2 and the Michaelis constant $2 \cdot 10^{-3}$ M. The enzyme also catalyzed the deamination of L-glutamine and D-asparagine but at a rate of approximately 10 and 5 percent, respectively, of that of L-asparagine. Hg^{2+}, Cu^{2+} and *p*-chloromercuribenzoate strongly inhibit the enzyme while Mg^{2+} stimulated the activity slightly. The temperature stability of the enzyme was also studied. The enzyme was devoid of tumor inhibitory power.

INTRODUCTION

During the last years a number of reports have appeared describing the biological effects of L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1). Perhaps the most thoroughly studied of these effects is the tumor inhibitory power of the enzyme. Growth inhibition of several tumors has been reported for L-asparaginase isolated from guinea pig serum[1]. *Escherichia coli*[2–4], *Serratia marcescens*[5], *Erwinia aroideae*[6], *Proteus vulgaris* and *Bacterium cadaveris*[7] and a species of *Achromobacteraceae*[8]. L-Asparaginase from yeast[9], *Fusarium trinctum*[10] and *Bacillus coagulans*[11] on the other hand does not seem to have any tumor inhibitory power. There is as yet no agreement as to the cause of these differences in tumor inhibitory power for enzymes isolated from different sources. Other biological effects that have been described for L-asparaginase includes immunosuppression[12] and growth inhibition of certain viruses in tissue culture[13].

Some years ago it was noted that *Acinetobacter calcoaceticus* was a relatively rich source of L-asparaginase[14]. We have observed that this organism contains two isoenzymes of L-asparaginase, and will hereafter refer to them as L-asparaginase A and L-asparaginase B. In the present paper we describe a purification procedure for L-asparaginase A and report on some of its physicochemical properties as well as its tumor inhibitory capacity.

EXPERIMENTAL PROCEDURE

Materials

Chemicals. All amino acids were chromatographically pure and obtained from Schwartz Bioresearch. DEAE-cellulose, DE 23 was from Whatman. The carrier ampholytes used in isoelectric focusing were purchased from LKB Products. Streptomycin was from Glaxo Laboratories, sodium dodecylsulfate, deoxyribonuclease IIB and ribonuclease type IA were from Sigma. Cytochrome *c*, bovine serum albumin and hemoglobin were from Boehringer Mannheim, trypsin from Worthington, Crasnitin (*E. coli*–asparaginase) from Bayerwerke and γ-globulin from Kabi.

Organism. A. calcoaceticus (Bacterium anitratum) 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 per l distilled water. L-Asparagine was included as nitrogen source, carbon source and inducer while xylose was additional carbon source.

Animals and tumor cells. C3H mice were received from Statens Institutet for Folkehelse, Oslo, Norway. Professor Georg Klein, Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden kindly donated C3H mice bearing 6C3HED lymphoma[15].

Methods

Growth and harvesting of bacteria. The cells were grown in 10–15-liter portions under strong aeration. They were collected in the stationary phase by centrifugation and the cell paste was then frozen and kept at −20 °C.

Enzyme assay. The activity of the L-asparaginase was usually measured as follows: An appropriate amount of enzyme was diluted with 0.05 M borate buffer pH 8.6 and the reaction started by addition of 50 μl 0.2 M L-asparagine. The total reaction volume was 1.0 ml and the reaction temperature was 37 °C. The reaction was stopped after 10 min by the addition of 50 μl 25% trichloroacetic acid. After centrifugation of the sample 0.5 ml was diluted ten-fold with H_2O, 1.0 ml Nessler's reagent was added and the absorbance read at 420 nm.

The reliability of the method was occasionally checked by determining the amount of L-aspartic acid formed from L-asparagine by thin-layer chromatography. A small amount of reaction mixture was spotted on cellulose thin layer and developed in the solvent, methanol–water (7:3, v/v) and the amino acids determined by the ninhydrin reaction. One international unit of L-asparaginase was taken as the amount of enzyme liberating 1 μmole NH_3 per min using the conditions described above.

Determination of proteins. Protein was estimated employing the method of Lowry *et al.*[16].

Affinity chromatography. The column material used for affinity chromatography was the same as originally used for the purification of L-asparaginase from *E. coli*[17]. The material consists of a matrix of Sepharose 6B to which D-asparagine is covalently attached by means of a spacer — hexamethylenediamine. L-Asparaginase binds to the D-asparagine residues on the column while other proteins pass through. The enzyme can be eluted by using a buffer containing D-asparagine of a sufficient concentration to compete with the matrix-bound D-asparagine for the active site on the enzyme and thus release the enzyme.

Polyacrylamide gel electrophoresis. (a) Without sodium dodecylsulfate: 20–30 μg protein to which glycerol had been added to a final concentration of 25% as well as a small amount of bromophenol blue was applied to 5% acrylamide gel in 0.375 M Tris buffer pH 8.5. The electrophoresis was run at 10 V/cm. The gels were stained by coomassie blue in 9.2% acetic acid with 0.6% methanol for 1 h and destained with 7% acetic acid. (b) With sodium dodecylsulfate: To the protein sample, 20–30 μg sodium dodecylsulfate, dithiothreitol and glycerol were added to final concentrations 0.1–1.0 and 25%, respectively. A small amount of bromophenol blue was added before application to the 5% acrylamide gel and the electrophoresis run at 10 V/cm. After fixing in 20% sulfosalicylic acid overnight the gels were stained by coomassie blue for 3 h and destained as above.

RESULTS

Purification of the enzyme

All purification steps, if not otherwise stated, were carried out in the cold room at 4 °C and all buffers contained 0.006% NaN_3.

Preparation of crude extract. Frozen cells were thawed and homogenized with an equal volume of 0.05 M borate buffer pH 8.6 in a Potter–Elvehjem homogenizer. The cell suspension was treated with 1 mg egg white lysozyme per 100 g cell paste at 37 °C for 15 min, cooled in ice and sonicated in a Bronson sonicator for 4 × 5 minutes with intermediate cooling, not allowing the temperature to rise above 25 °C. Sonicated cells were centrifuged for 30 min at 30 000 × *g*, cell debris resuspended in an equal volume of borate buffer, stirred for some minutes at 0 °C and centrifuged again for 30 min at 30 000 × *g*. To the combined supernatants deoxyribonuclease and ribonuclease (2–5 μg/ml) were added and the solution held at room temperature while stirring for 30 min and then centrifuged for 90 min at 105 000 × *g*.

Streptomycin precipitation. Streptomycin was added to the cell-free crude extract to a final concentration of 1% w/v. After 30 min at 0 °C the precipitate was removed by centrifugation for 30 min at 30 000 × *g* and discarded.

DEAE chromatography. The supernatant from the streptomycin precipitation was applied on a DEAE-cellulose column equilibrated with 0.05 M borate buffer pH 8.6. The column was washed with two column volumes of the same borate buffer, then followed by a linear salt gradient in the same buffer from 0 to 1.2 M NaCl. The elution pattern given in Fig. 1 indicates that two different L-asparaginases are present in the bacterial extract. One is eluted with the washing buffer while the other is retained by the DEAE-cellulose and is eluted at about 0.7–0.8 M NaCl. The possibility

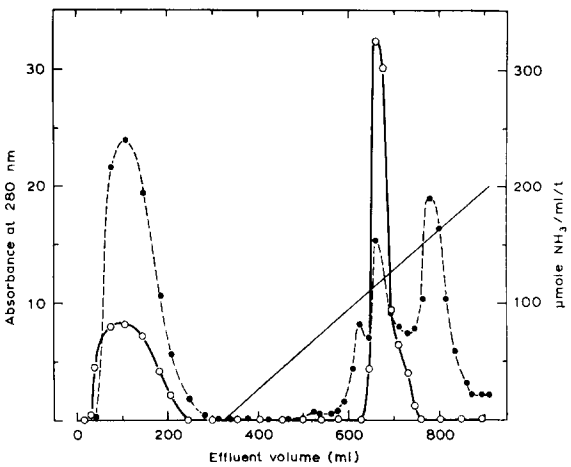

Fig. 1. DEAE-cellulose chromatography of supernatant from Streptomycin precipitation. The column (4 cm × 20 cm) was equilibrated with 0.05 M borate buffer pH 8.6. After washing the protein was eluted with a linear gradient in NaCl from 0 to 1.2 M. Total volume of gradient 600 ml. ●-----●, protein measured by absorption at 280 nm; ○—○, enzyme activity.

that the first peak of activity from the DEAE chromatography was due to overloading the column was ruled out by passing the first peak through another DEAE-cellulose column. No activity was retained by the second column under identical conditions. The enzyme that was retained on the DEAE column had a higher specific activity than the first peak. The present report describes the purification of the species of L-asparaginase retained on the DEAE-cellulose column which we for the sake of simplicity call L-asparaginase A. The purification and properties of the other isoenzyme, L-asparaginase B, will be the subject of another report.

The enzyme eluted from the DEAE-cellulose by the salt gradient was concentrated on a Diaflo ultrafiltration unit and subjected to gel filtration.

Sephadex G-200 gel filtration. The Diaflo concentrate was applied on a Sephadex G-200 column equilibrated with 0.05 M borate buffer pH 8.6. Column dimensions were 2.5 × 100 cm. The peak containing the L-asparaginase activity was pooled, dialyzed against 0.05 M borate buffer containing 0.5 M NaCl and concentrated to a small volume on a Diaflo filter.

Affinity chromatography. This was carried out using a column material described in Methods. The column material was equilibrated with 0.05 M borate buffer pH 8.6 containing 0.5 M NaCl. The enzyme material was applied on the column which was then washed with 3 column volumes of the equilibrating buffer. A substantial amount of protein came off the column by this washing procedure, however, all the enzymatic activity was retained. Desorption of the enzyme was effected with the same buffer containing 0.1 M D-asparagine. A sharp activity peak with low absorption at 280 nm as shown in Fig. 2 was obtained. Thus a large degree of purification was achieved in this step. Table I gives a summary of the purification acquired in the different steps of the purification procedure.

The enzyme eluted from the specific adsorbent was then concentrated by

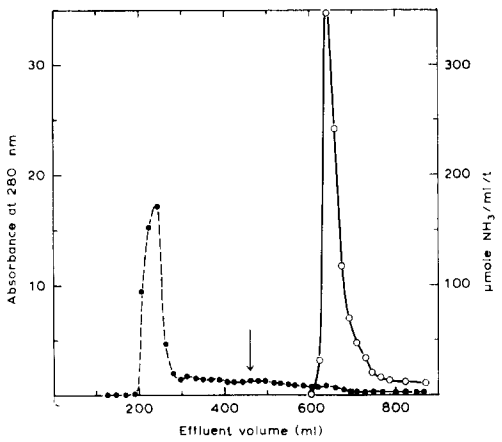

Fig. 2. Affinity chromatography of enzyme from Sephadex gel filtration. The column (2.5 cm × 30.6 cm) was equilibrated with 0.05 M borate buffer pH 8.6 containing 0.5 M NaCl. Desorption, denoted by an arrow, was carried out using the same buffer with 0.1 M D-asparaginase included. ●-----●, protein measured by absorbance at 280 nm; ○—○, enzyme activity.

TABLE I

PURIFICATION PROCEDURE FOR L-ASPARAGINASE A

Purification step	*Total volume (ml)*	*Total protein (mg)*	*Total units*	*Specific activity*	*Enrichment (fold)*	*Recovery (%)*
Crude extract	150	3600	1350	0.4	—	100*
Streptomycin supernatant	150	3300	1320	0.4	—	100
DEAE chromatography	15	522	680	1.3	3	50
Sephadex G-200 filtration	10	160	400	2.5	6	31
Affinity chromatography	8	6.4	205	32.0	80	15

* This represents the total activity of both L-asparaginase A and B while the remaining steps only represent L-asparaginase A. This means that the resulting enrichment of this species of enzyme actually is twice as high as is shown in the table since both isoenzymes are present in approximately the same amount in the crude extract.

ultrafiltration and kept at −20°C. Little loss of activity was observed after prolonged storage at this temperature.

Purity of the enzyme. The purified enzyme was analyzed by polyacrylamide gel electrophoresis with and without sodium dodecylsulfate present together with a commercially available *E. coli* L-asparaginase of high purity (Crasnitin, Bayerwerke). The gel pattern shown in Fig. 3 indicates that L-asparaginase A from *A. calcoaceticus* is relatively pure. Both enzymes gave a single protein band in the presence of sodium dodecylsulfate. The purified enzyme was also analyzed by analytical ultracentrifugation and as shown in Fig. 4 a single symmetrical peak was obtained suggesting a high degree of purity.

Properties of L-*asparaginase A*

Molecular weight, subunit structure and isoelectric point. The molecular weight

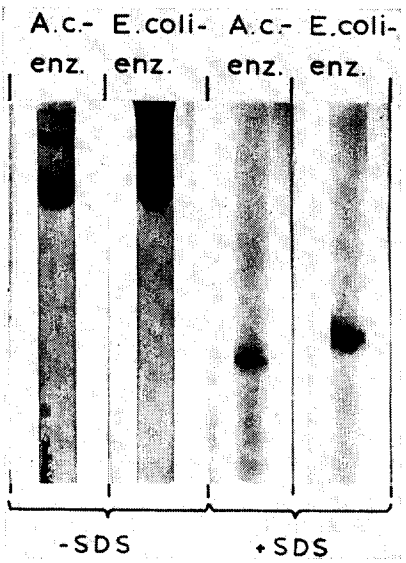

Fig. 3. Polyacrylamide gel electrophoresis pattern of purified L-asparaginase from *A. calcoaceticus* (A.c.) and L-asparaginase from *E. coli*. 20–30 μg protein was used for each run and both enzymes were analyzed in the presence and absence of sodium dodecylsulfate (SDS). Time of run was 15 min without sodium dodecylsulfate and 5 h when in the presence of sodium dodecylsulfate. Further experimental details are given in Methods.

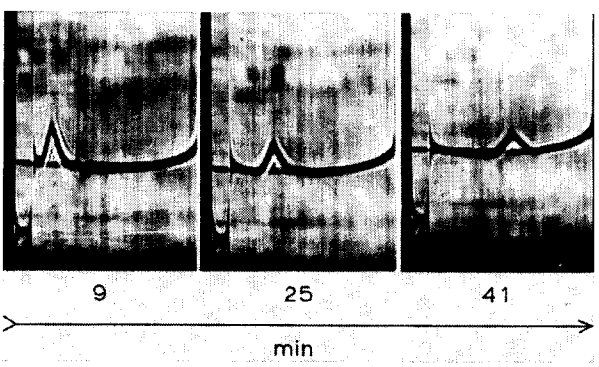

Fig. 4. Analytical ultracentrifugation of purified L-asparaginase A. The protein concentration was 3 mg/ml and the buffer used was 0.05 M borate pH 8.6. The pictures were taken at the above indicated time intervals after full speed of 59 780 rev./min had been attained.

of the enzyme was estimated by gel filtration and analytical ultracentrifugation. From the gel filtration data on Sephadex G-100 shown in Fig. 5 the molecular weight of the enzyme was found to be approx. 105 000. The sedimentation coefficient obtained by analytical ultracentrifugation was 6.2 S suggesting a similar weight to that obtained by gel filtration.

The subunit structure of the enzyme was studied by gel electrophoresis in the presence of sodium dodecylsulfate. The results are given in Fig. 6. In all experiments only one protein band was observed and the molecular weight of this subunit was estimated to be 25 000. This indicated that the native enzyme contains four subunits

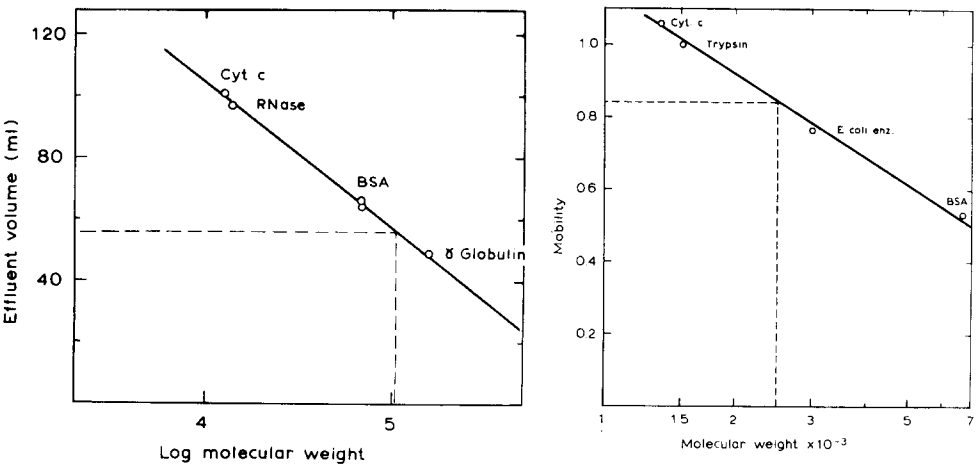

Fig. 5. Elution volume of L-asparaginase A on a Sephadex G-100 column (1.5 cm × 95 cm) compared to some standard proteins of known molecular weight[18]. The column was equilibrated with 0.02 M Tris buffer containing 0.01 M β-mercaptoethanol. Standard proteins were cytochrome *c* (cyt *c*), ribonuclease (RNase) ,bovine serum albumin (BSA) and γ-globulin.

Fig. 6. Mobility of L-asparaginase A in sodium dodecylsulfate polyacrylamide gel electrophoresis compared to some standard proteins. The mobility is defined as $\frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before stain}}{\text{distance of dye migration}}$. The standard proteins used were cytochrome *c* (cyt. *c*), trypsin, *E. coli* L-asparaginase (*E. coli* enz.) and bovine serum albumin. For further experimental details see Methods.

of the same molecular weight. Several experiments showed that it was possible to dissociate the enzyme into subunits by other reagents and conditions than those described above. The monomeric unit also possessed enzymatic activity. This aspect is under further investigation and will be reported elsewhere. The isoelectric point of the enzyme was determined by isoelectric focusing in a pH gradient[20] and found to be at pH 5.2.

Effect of pH and substrate concentration. Fig. 7 shows the activity of the enzyme at various pH values using different buffer systems. The activity of the enzyme varied to some extent with the nature of the buffer, however, in all cases the pH optimum appeared to be between pH 8.4 and 8.8.

The effect of various concentrations of L-asparagine was also investigated. At a high concentration of L-asparagine substrate inhibition was observed, Fig. 8. The apparent Michaelis constant was estimated to be $2 \cdot 10^{-3}$ M, using the Lineweaver–Burk method.

Substrate specificity. The purified enzyme also catalyzed the deamination of L-glutamine and D-asparagine. However, the rate as compared to the natural substrate L-asparagine was only 10% and 5%, respectively.

Inhibitors and stimulators. The effect of a number of metal ions and some other compounds on the enzymatic activity were investigated and the results are shown in Table II. Hg^{2+}, Cu^{2+} and *p*-chloromercuribenzoate strongly inhibited the activity of the enzyme whereas Zn^{2+}, Ni^{2+} and Co^{2+} gave less inhibition. NH_3 inhibited the enzyme

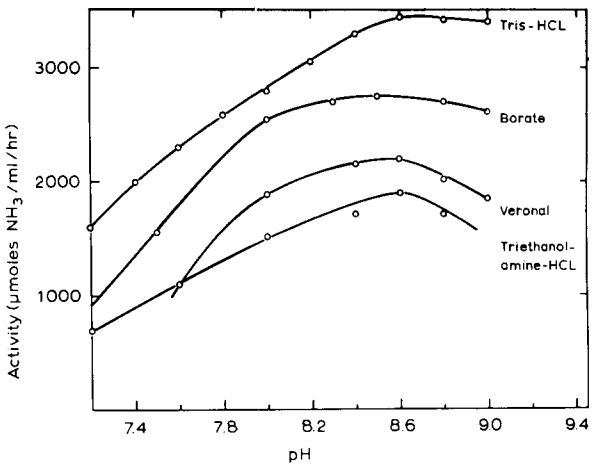

Fig. 7. Activity of L-asparaginase A as a function of pH in different buffer systems. Reaction temperature 37 °C.

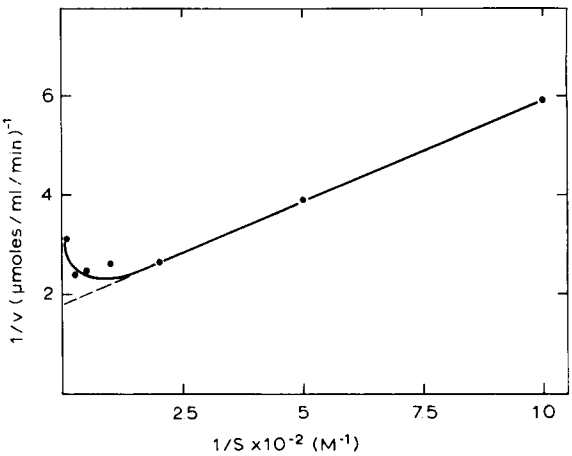

Fig. 8. Effect of concentration of L-asparagine plotted in Lineweaver–Burk plot.

slightly at the concentrations used. Aspartic acid, Ca^{2+} and Ba^{2+} on the other hand did not affect the enzymatic activity.

The inhibitory effect of *p*-chloromercuribenzoate could be reversed by β-mercaptoethanol. This reagent did not, however, stimulate the activity of the enzyme. On the contrary, prolonged incubation of the enzyme in the presence of β-mercaptoethanol *i.e.* three days at 4 °C completely inactivated the enzyme. A control without β-mercaptoethanol retained full activity under identical conditions. These results indicate that SH groups and probably also disulfide bonds are of importance for the maintenance of catalytic activity. Mg^{2+} was found to stimulate the activity of the enzyme a small amount.

Temperature stability. The stability of the enzyme at various temperatures is shown in Fig. 9. No loss of activity was observed when the enzyme was incubated at

TABLE II

INHIBITORS AND STIMULATORS OF L-ASPARAGINASE A

The compounds were added to the reaction mixture in the final concentration indicated below and the activity measured as described in Methods.

Compound added	*Concentration M*	*% Activity*
0	—	100
Hg^{2+}	10^{-3}	0
Hg^{2+}	10^{-4}	0
Cu^{2+}	10^{-3}	0
Cu^{2+}	10^{-4}	2.7
Co^{2+}	10^{-3}	65
Ni^{2+}	10^{-3}	50
Ba^{2+}	10^{-3}	100
Ca^{2+}	10^{-3}	100
Zn^{2+}	10^{-3}	40
p-Chloromercuribenzoate	10^{-5}	0
NH_3	10^{-3}	80
Asp	10^{-2}	100
Mg^{2+}	$5 \cdot 10^{-4}$	110

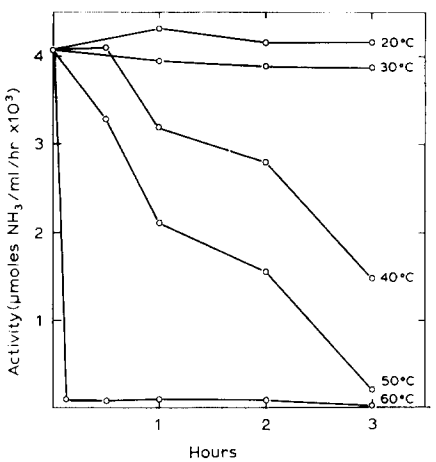

Fig. 9. Stability of L-asparaginase A at different temperatures. Activities were measured after 0, 1/2, 1, 2 and 3 h incubation of the enzyme at the specified temperatures.

20 °C and 30 °C for 3 h. Higher temperatures led to an increasing degree of inactivation. Thus incubation at 60 °C led to complete inactivation in approx. 5 min.

Tumor inhibitory power. The ability of L-asparaginase A to prevent tumor growth was tested in the system 6C3HED lymphoma/C3H mice in the following way: Four groups of 8 mice in each were transplanted intraperitoneally with the same amount of tumor cells. On the following day one group received an injection with buffer only, another group with *E. coli* L-asparaginase, one group with the same amount of L-asparaginase A from *A. calcoaceticus*. The fourth group did not receive any injection. The results of this study are given in Table III. The only mice who survived, and still were alive 6 months after the test, were those who had been treated with L-

TABLE III

TUMOR INHIBITORY POWER OF L-ASPARAGINASE A

The animals were injected intraperitoneally with 0.2 ml of a tumor cell suspension containing 10^7 cells each. On the following day single intraperitoneal injections were given as indicated below. 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*	*Number of survivors*
A	Buffer	0
B	27 I.U of L-asparaginase from *E. coli*	8
C	27 I.U of L-asparaginase from *A. calcoaceticus*	0
D	0	0

asparaginase from *E. coli*. Thus L-asparaginase A from *A. calcoaceticus* was completely devoid of tumor inhibitory power under the conditions of the experiment.

DISCUSSION

The present work deals with L-asparaginases from *A. calcoaceticus*. Two different L-asparaginases denoted A and B were detected and this paper describes the purification and properties of L-asparaginase A. Studies on L-asparaginase B will be the subject of a forth-coming communication.

L-Asparaginase A was purified partly by conventional protein purification procedures and partly by affinity chromatography on a specific adsorbent earlier used for purification of one of the isoenzymes of L-asparaginase from *E. coli*[16]. The adsorbent has been successfully applied to the enzyme from *A. calcoaceticus*. It appears, however, that L-asparaginase A binds more strongly to the specific adsorbent as evidenced by the much higher concentration of D-asparagine required for release of the enzyme, *i.e.* 0.1 M compared to 0.001 M for the corresponding enzyme from *E. coli*.

In many ways L-asparinase A from *A. calcoaceticus* resembles the tumor inhibitory enzyme from *E. coli*. It has approximately the same molecular weight and subunit composition (105 000 compared to 133 000 for the *E. coli* enzyme[19]), the same pH optimum, approximately the same isoelectric point and specificity. However, in sharp contrast to the enzyme from *E. coli* L-asparaginase A is devoid of tumor inhibitory power. Another point of difference between these two enzymes is their K_m values. The K_m for the enzyme from *E. coli* according to P. Ho *et al.*[19] and Broome *et al.*[21] is $1.2 \cdot 10^{-5}$ M whereas the K_m for L-asparaginase A from *A. calcoaceticus* is approx. 180-fold larger, namely $2 \cdot 10^{-3}$ M. This might be a possible explanation for the marked difference with regard to the tumor inhibitory power. If the tumor inhibition results from a draining out of the L-asparagine pool of the tumor cells, it might be that L-asparaginase A is not able to remove enough L-asparagine from the circulation for effective tumor inhibition.

A point of objection against this explanation is the K_m of $5.2 \cdot 10^{-5}$ M obtained

for L-asparaginase from *Fusarium trinctum*[10] which also is unable to inhibit tumor growth, as well as the K_m value of $3 \cdot 10^{-3}$ M reported for the tumor inhibitory enzyme from *Erwinia aroideae*[6].

Another possible explanation for the differences in tumor inhibitory power could be that the rate of clearence of the different L-asparaginases from the serum of the test animals varies. In the case of L-asparaginase A this has not yet been determined. The report of Boyd *et al.*[22] emphasises the importance of this aspect with regard to tumor inhibition.

ACKNOWLEDGEMENT

We are very grateful to Dr Andreas Myking, Department of Experimental Pathology, University of Bergen, Haukeland Hospital, Bergen, Norway for expert advice and help with the animal experiments and tumor transplantations. The excellent technical assistance of Sissel Brynjulfsen, Kari Skjelfjord Pettersen and Terje Björndal is gratefully acknowledged. This work was in part financed by the Norwegian Cancer Society.

REFERENCES

1 Broome, J. D. (1963) *J. Exp. Med.* 118, 99–120
2 Mashburn, L. T. and Wriston, J. C. (1964) *Arch. Biochem. Biophys.* 105, 450–452
3 Roberts, J., Prager, M. D. and Bachynsky, N. (1966) *Cancer Res.* 26, 2213–2217
4 Campbell, H. A., Mashburn, L. T., Boyse, E. A. and Old, L. J. (1967) *Biochemistry* 6, 721–730
5 Rowley, B. and Wriston, J. C. (1967) *Biochem. Biophys. Res. Commun.* 28, 160–165
6 Peterson, R. E. and Ciegler, A. (1969) *Appl. Microbiol.* 18, 64–67
7 Tosa, T., Sano, R., Yamato, K., Nakamura, M., Ando, K. and Chitaba, I. (1971) *Appl. Microbiol.* 22, 387–392
8 Roberts, J., Holcenberg, J. S. and Dolowy, W. C. (1972) *J. Biol. Chem.* 247, 84–90
9 Broome, J. D. (1965) *J. Natl. Cancer Inst.* 35, 967–974
10 Scheetz, R. W., Whelan, H. A. and Wriston, J. C. (1971) *Arch. Biochem. Biophys.* 142, 184–189
11 Law, A. S. and Wriston, J. C. (1971) *Arch. Biochem. Biophys.* 147, 744–752
12 Schwartz, R. S. (1969) *Nature* 224, 275–276
13 Maral, R. and Werner, G. H. (1971) *Nat. New Biol.* 232, 187–188
14 Jyssum, K. and Joner, P. E. (1966) *Acta Path. Microbiol. Scand.* 67, 139–148
15 Boyd, J. W. and Phillips, A. W. (1971) *J. Natl. Cancer Inst.* 46, 1271–1276
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
17 Kristiansen, T., Einarsson, M., Sundberg, L. and Porath, J. (1970) *FEBS Lett.* 7, 294–296
18 Andrews, P. (1964) *Biochem. J.* 91, 222–233
19 Ho, P. P. K., Milliken, E. B., Bobbitt, J. L., Grinnan, E. L., Burch, P. J., Frank, B. H., Beock, L. V. D. and Squires, L. W. (1970) *J. Biol. Chem.* 245, 3708–3715–3724
20 Vesterberg, O. and Svensson, H. (1966) *Acta Chem. Scand.* 20, 820–834
21 Broome, J. D. (1968) *Trans. N.Y. Acad. Sci.* 30, 690–704
22 Boyd, J. W. and Phillips, A. W. (1971) *J. Bacteriol.* 106, 578–587