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GUINEA PIG LIVER L-ASPARAGINASE

SEPARATION, PURIFICATION, AND INTRACELLULAR LOCALISATION OF TWO DISTINCT ENZYMATIC ACTIVITIES

J.C. ROGEZ[a], R. PLAQUET[a] and G. BISERTE[b]

[a] *Laboratoire de Biochimie. Groupe de Recherche Enzymologie, Faculté de Médecine 80036 Amiens Cedex and* [b] *Institut de Recherches sur le Cancer et Unité INSERM n° 124 (BP 3567) 59020 - Lille Cedex (France)*

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Summary

Two distinct L-asparaginase (EC 3.5.1.1) activities were detected in guinea pig liver: Asparaginase 1 and Asparaginase 2.

Asparaginase 1 has been purified 272 fold from the crude homogenate; its molecular weight was evaluated by gel filtration to be about 150 000. The purified preparation was shown to be homogeneous by cellulose acetate strip and polyacrylamide disc-gel electrophoresis.

Asparaginase 2 has been purified 63.5 fold from the crude homogenate. Its molecular weight was evaluated by gel filtration to be about 21 500. Cellulose acetate strip electrophoresis demonstrated two bands, one of which corresponded to Asparaginase 1 and the other to Asparaginase 2.

Cellular fractionation in the ultracentrifuge, showed Asparaginase 1 to be present only in the cytosol fraction. Asparaginase 2 which was unstable at 105 000 × *g* seemed mostly localized in the mitochondria and secondarily in the cytoplasmic fraction.

Introduction

Since the studies of Broome [1,2,3] demonstrating that the antilymphomatic activity of guinea pig serum was due to L-asparaginase (EC 3.5.1.1) much research has been devoted to the preparation and study of the biological activity of this enzyme in animals, microorganisms and more recently, in man.

In the vegetable kingdom, L-asparaginase has been demonstrated in microorganisms [4]. Among those it can also be found in numerous families of bacteria, particularly in certain strains of *Escherichia coli* [5—8], where it has been possible to obtain two asparaginases, EC_1 and EC_2. These asparaginases

differ in biological activity, EC_1 being inactive and EC_2 active on the lymphoma of mouse; they differ as well in physical and chemical properties [9,10].

In the animal kingdom, asparaginase activity is equally widely spread (horse, guinea pig, chicken, rabbit, rat, mouse, birds and fish). The serum activity is rare, and is demonstrated only in the guinea pig and some rodents [11]. On the other hand, L-asparaginase can be detected in these animals cited above, as well as in numerous tissues, mostly liver, kidney, brain, spleen, pancreas, and testis.

From the supernatant resulting from ultracentrifugation at 105 000 × *g* of a guinea pig liver homogenate, Suld and Herbut [12,13] have been able to demonstrate only one asparaginasic activity.

The results we place in evidence here, show that there are at least two asparaginase activities in guinea pig liver. The intracellular localization and a partial purification of the two enzymes are presented.

Materials and Methods

Preparation of the guinea pigs

Guinea pigs of the tricolored race, each weighing between 300 and 400 g, were anaesthetized with 1 ml/100 g body weight 10% ethyl carbamate solution, intraperitoneally injected.

To avoid any contamination by serum L-asparaginase, the guinea pigs were then exsanguinated under anaesthesia and perfused with an isotonic saline solution.

Preparation of the homogenate

All operations relating to the preparation of the crude homogenate were performed at +4°C in a cold room.

Each liver, weighing about 20 g was homogenized for 2 min in 70 ml of isotonic saline solution in an Ultraturrax homogenizer* then centrifuged at 10 000 rev./min for 10 min at +4°C in a Beckman J 21 Centrifuge with a JA 20 fixed-angle rotor.

Partial purification of the crude homogenate

To the supernatant resulting from the procedure described above was added drop by drop with constant magnetic stirring, first a solution of 1 M HCl, then a solution of 0.1 M HCl until pH 5.6 was reached.

The results obtained permit us to deduce that both the resultant enzymatic activity and the protein precipitation were equally optimal at this pH value.

After centrifugation, the resultant supernatant was added at laboratory temperature and with magnetic stirring with a 30% solution of sodium sulfate, at a proportion of 12 ml of saline solution for 10 ml of enzyme solution, according to the technique described by Suld and Herbut [12]. Afterwards, the suspension was put aside for 0.5 h at ambient temperature. The precipitate was

* Ultraturrax Janke and Kunkel KG. Staufen.

TABLE I

PARTIAL PURIFICATION PROCEDURES OF GUINEA PIG LIVER CRUDE HOMOGENATE

Purification step	Vol (ml)	Total protein (mg)	Total enzymatic activity (I.U.)	Specific activity (I.U./mg protein)	Yield (%)	Purification factor
Crude extract	60	1200	78	0.065	100	1
pH 5.6 precipitation	55	440	56	0.13	70	2
Na_2SO_4 precipitation	10	70	56	0.80	70	12.5

collected by centrifugation at 14 000 rev./min for a duration of 15 min at 20° C, then dissolved in 10 ml of 0.02 M sodium phosphate buffer, pH 7.35.

Table I summarizes the different stages and characteristics of the products obtained. This partially purified supernatant serves as a working solution for the demonstration of the two L-asparaginasic activities.

Determination of the enzymatic activity

L-Asparaginase or L-asparagine amidohydrolase (EC 3.5.1.1) hydrolyses L-asparagine to give L-aspartic acid and ammonia.

We measured the ammonia generated, after automatic dialysis against a solution of sodium chloride at 9 per 1000, by the method of Berthelot with phenol-hypochlorite. The dosage was performed with a Technicon autoanalyser according to the modified technique of Girard et al. [13].

Determination of protein concentration

The colorimetric assay of proteins was performed according to the method of Lowry and Rosebrough [14], adapted to the Technicon autoanalyser equipped with a 425 nm interference filter.

Gel filtration on Sephadex G 200

Sephadex G 200 (Pharmacia Fine Chemicals) was placed in suspension and equilibrated in a solution of 0.02 M NaCl. The gel was then allowed to expand at 4° C, the saline solution being replaced daily. After about 72 h, the gel was debubbled with a vacuum pump and placed in a glass column (1 cm × 60 cm) previously filled with a solution of 0.02 M NaCl from which all gas had been removed. The gel was sedimented for 48 h first at free flow, then slowly (4 to 5 drops/min). As soon as the column had been prepared 2 ml of partially purified solution were placed at the top of the column. The elution was then performed with a solution of 0.02 M sodium chloride at a speed of 9.2 ml/h. The collected fractions were 2.3 ml in volume.

Chromatography on DEAE-Cellulose

Powdered DEAE-cellulose (DEAE 52 Whatman) in moistened form was placed in suspension with gentle hand agitation in a pH 7.35 0.02 M sodium phosphate buffer. Then, the exchanger was equilibrated with successive rinses and decantations with the same buffer.

DEAE-Cellulose from which gas had been removed by vacuum pump was

introduced into a glass column (1.8 × 52 cm)), then sedimented for 48 h with free flow.

2 ml of partially purified solution were placed at the top of the column. Elution was obtained by augmenting the ionic force of the milieu according to the technique of Suld and Herbut [15].

Preparative liquid film electrophoresis

The apparatus used was an Elphor-Vap; the buffer in which the electrodes were immersed was a buffer of 0.0256 M citric acid and 0.24 M Tris, pH 8.6.

The buffer of the electrophoretic chamber consisted of a borate buffer at pH 8.6 (500 ml of 0.1 M boric acid in 0.1 M KCl; 120 ml of 0.1 M sodium hydroxyde; 380 ml of deionized water).

5 ml of the partially purified solution were injected at a speed of 2 ml/h in the chamber, which is maintained at 5°C during the entire operation. The speed of injection of buffer into the chamber was 100 ml/h; the difference of potential applied to the electrodes was 1900 V with an intensity of 100 mA.

Cellulose acetate block preparative electrophoresis

0.5 ml of the partially purified solution were deposited with the aid of a Pasteur pipette in a depression 2.5 cm from the cathode border of a block of cellogel* (4 × 17 cm).

The electrode buffer was veronal/HCl at a pH of 8.4 (sodium veronal 17 g; 1 M HCl 23.5 ml; deionized water q.s.p. 1000 ml).

The constant intensity applied for each block was 25 mA for a 250 V potential difference. The migration lasted 4 h.

The block was then cut longitudinally into two sections; the first was colored with amidoschwarz. Decoloration was carried out in successive rinses of washing liquid (100 ml of glacial acetic acid; 900 ml of Technical grade methanol). The second position of the block was cut apart with a razor blade, taking into account the position of the bands appearing on the first section of the block.

The elution of the zones of the latter (fragmental) portion of the block is effected by simple compression of the gel in a syringe.

Cellulose acetate strip analytic electrophoresis

This technique permits the verification of the degree of purification of the different stages of the fractionation. 2—3 ml of the preparations were placed with the aid of an applicator on strips of cellogel (2.5 × 17 cm).

The buffer used for electrophoresis was a pH 9.2 veronal buffer: (8.2 g of sodium veronal; 1000 ml deionized water, q.s.p.).

Electrophoresis was performed for 75 min, with a potential difference of 200 V. The bands were then colored with amidoschwarz and decolored with successive rinses in a solution of acetic methanol.

*Polyacrylamide gel preparative electrophoresis***

Operations were performed with a separation gel (7% cross linked) in a

* Cellogel Chemetron 20129 Milano.
** (Canalco Prep-Disc Equipment)

Tris · HCl, pH 9 buffer; above this gel, a staking gel (2 to 3% cross linked) in a Tris · HCl, pH 8.6 buffer was used.

The sample gel, the cathodic and anodic pH 8.5 buffers, and the Tris · HCl pH 9 elution buffer, were prepared as described in the Canalco manual. A constant current of 10 mA was applied for 15 h. The elution rates were respectively 3 ml/min with the largest column (PD_2 320) and 1.5 ml/min with the middle sized column (PD_2 150).

3-ml fractions were collected as soon as the tracking-dye was eluted at the bottom of the column. 70 fractions were thus collected, and aliquots were assayed for asparaginase activity. The active fractions were then pooled and concentrated by dialysis against powder of polyethylene glycol 20 000 (Schuchardt, Munchen).

Preparative ultracentrifugation in a sucrose gradient

We used a Beckman L_3 50 centrifuge equipment with a SW 41 T rotor with swinging buckets.

The cellulose nitrate tubes were filled with a gradient of 5—20% sucrose. Linearity of the gradient was verified with the aid of an ABBE refractometer, after fractionation of the gradient. 0.2 ml of the purified solution were placed on the surface of the sucrose solution. The temperature of the rotor chamber was fixed at +4° C, and the speed of centrifugation at 41 000 rev./min (286 000 × *g*) and maintained for increasing periods of time from 3 to 7 h. The resulting solution was then fractioned and an aliquot taken from each fraction to permit determination of the enzymatic activity and proteins.

Cellular fractionation of the crude homogenate of guinea pig liver

The liver having been removed according to the technique previously described, was placed in suspension in a solution of physiological saline of 0.25 M sucrose and ground down in the Potter-Elvehjem homogenizer at +4° C.

The homogenate was fractionated according to the method of Appelmans et al. [16]. The purity of each fraction was controlled by electronic microscopy.

Results

Demonstration of two distinct asparaginasic activities in guinea pig liver homogenate

Fig. 1 shows the distribution of enzymatic activities of the fractions collected after gel filtration chromatography on Sephadex G 200 of the partially purified homogenate. Two distinct peaks of activity were shown to be present, and were designated Asparaginase I and Asparaginase II in the order of their elution.

Similarly, ion exchange chromatography on DEAE-Cellulose of the partially purified homogenate (Fig. 2a) demonstrated the existence of two peaks. None of them corresponded to serum asparaginase as established by performing a chromatography on a mixture of liver homogenate and serum (Fig. 2b).

We verified that the order of elution of asparaginases on DEAE-cellulose corresponded with that obtained with Sephadex G 200.

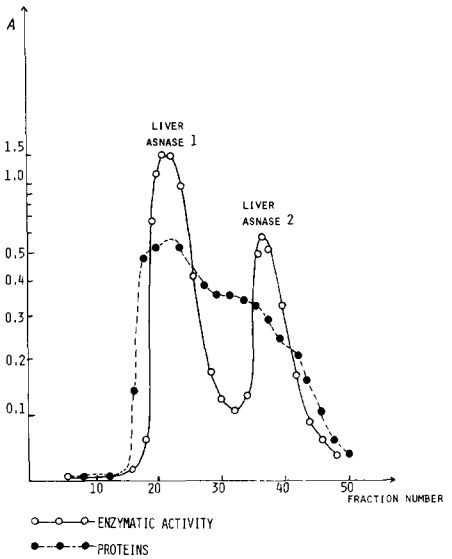

Fig. 1. Gel filtration chromatography of a partially purified homogenate of guinea pig liver on a G 200 Sephadex column (1 cm X 60 cm) equilibrated at +4°C with a 0.02 M NaCl solution, and eluted at 9.2 ml/h. The volume of each collected fraction was 2.3 ml.

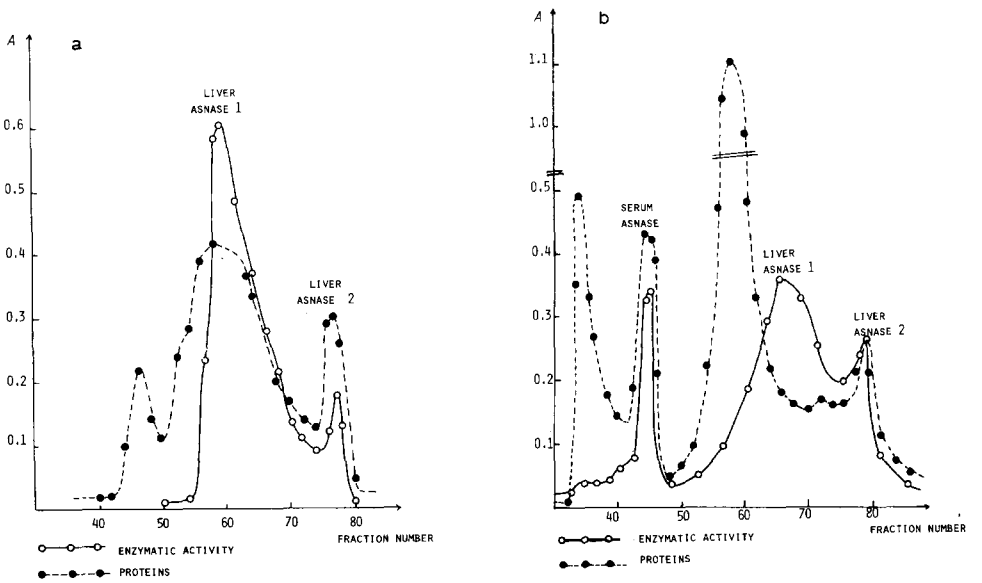

Fig. 2(a). DEAE-Cellulose chromatography of a partially purified homogenate of guinea pig liver (1.8 X 52 cm) column equilibrated at +4°C with a 0.02 M, pH 7.35 sodium phosphate buffer and eluted with a 0.5 M sodium chloride gradient, at 6 ml/h. Fractions of 4.5 ml were collected. (b) DEAE-Cellulose chromatography of a mixture of partially purified guinea pig liver homogenate and guinea pig serum. The technical operations were as in (a).

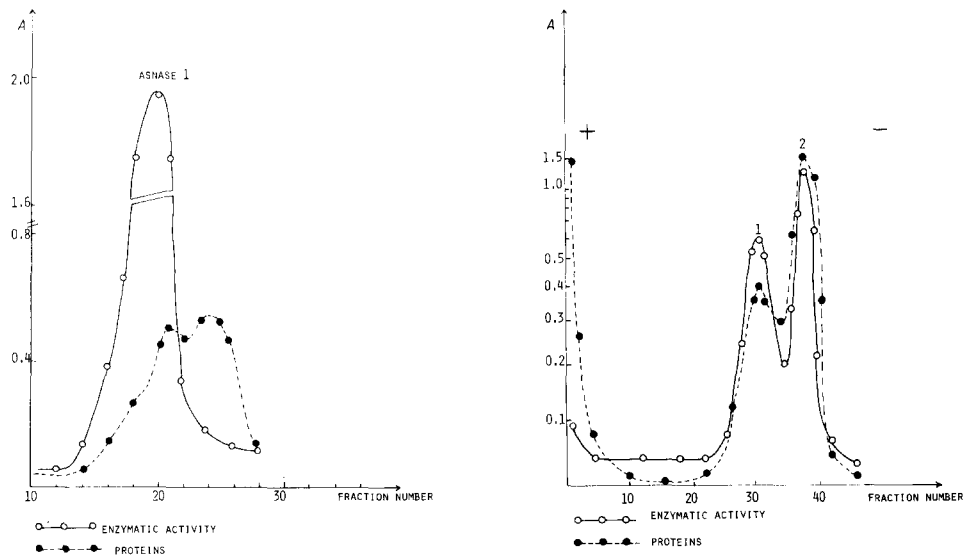

Fig. 3. Preparative liquid film electrophoresis of a partially purified guinea pig liver homogenate (see text for experimental conditions).

Fig. 4. Preparative ultracentrifugation in a 5—20 per cent sucrose gradient of a partially purified guinea pig liver homogenate, at +4° C, 41 000 rev./min (286 000 × *g*) for 7 h.

Preparative liquid film electrophoresis of the partially purified liver homogenate indicated the existence of two peaks of asparaginase activity (Fig. 3).

On studying separately by chromatography on Sephadex G 200, each of the two peaks thus obtained by preparative film liquid electrophoresis, we demonstrated the anodic peak to contain principally Asparaginase I, and the cathodic peak Asparaginase II. This result was confirmed by electrophoresis on Cellogel block and on Cellogel strip where Asparaginase I migrates farther toward the anode side than enzyme II.

A partially purified Asparaginase I obtained from a gel filtration was subjected to a preparative density 5—20% sucrose gradient ultracentrifugation for 7 h at 286 000 × *g* and showed only one peak which was identifiable as Asparaginase I in a second gel filtration chromatography.

On the other hand, we established that in submitting enzyme II, separated by gel filtration on Sephadex G 200, to an acceleration of 286 000 × *g* for 7 h, its activity on L-asparagine disappeared nearly completely.

In conclusion, by virtue of the reproducibility of the results obtained and the variety of physico-chemical methods used, it is possible to demonstrate in the guinea pig liver homogenate, the activities of two distinct asparaginases which may be distinguished by their different isoelectric points and molecular weights.

Determination of the molecular weights of Asparaginases I and II

Molecular weights were determined by gel filtration on Sephadex G 200. We used the following markers: fibrinogen (M_r = 340 000); immunoglobulin G (IgG) (M_r = 150 000); bovine serum albumin (M_r = 67 000); crystallized tryp-

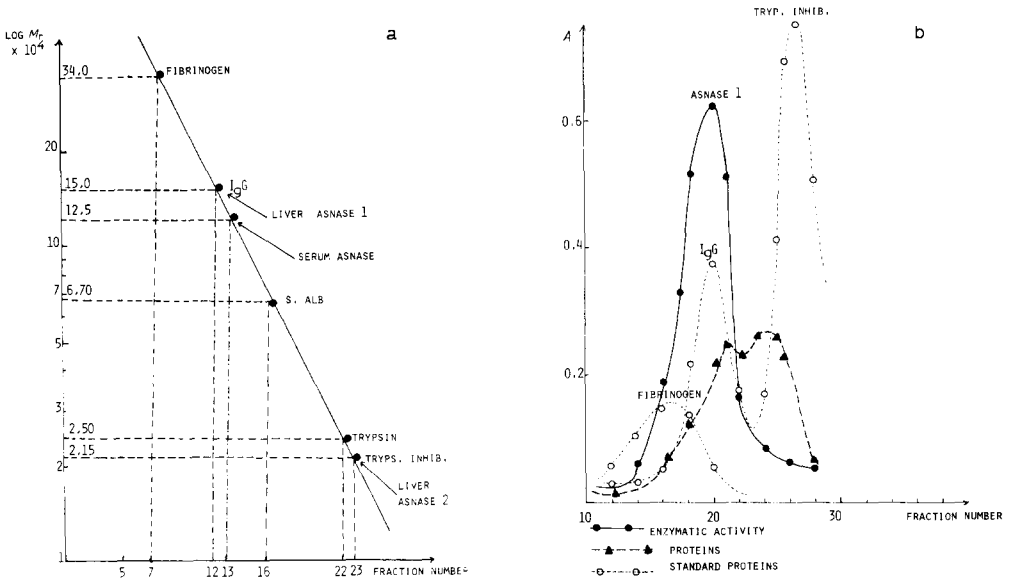

Fig. 5(a). Molecular weight estimation by gel filtration on the G 200 Sephadex column of Asparaginase I and II of guinea pig liver. Ordinate: $\log_{10}$ molecular weight X 10^4; abcissa : fraction number of the highest concentration of each reference protein. (b) Molecular weight verification by preparative ultracentrifugation of a mixture of fibrinogen (M_r = 340 000) immunoglobulin G (M_r = 150 000) and soya bean trypsin inhibitor (M_r = 21 500). All technical operations were effectuated as in Fig. 4.

sin (M_r = 25 000), and trypsin inhibitor of soya (M_r = 21 500).

The molecular weights so determined were 150 000 (±25 000) for Asparaginase I and 21 500 (±2000) for Asparaginase II (Fig. 5a).

The molecular weight of enzyme I was verified with ultracentrifugation at 286 000 X g using as markers, fibrinogen, immunoglobulin G, and trypsin inhibitor (Fig. 5b).

We verified similarly the molecular weight of the enzyme present in serum, obtaining a value of 125 000 (±20 000), corresponding to the molecular weight indicated in the literature (Yellin [17]).

Localisation of Asparaginases I and II within the hepatic cell

The different cellular fractions were obtained according to the method of fractionation of Appelmans et al. [16], mentioned above. The distribution of asparaginasic activity measured in the various fractions (mitochondrial, lysozomal, microsomal and cytoplasmic) is depicted in Fig. 6. Apart from the residual activity normally existant in non-homogenized cells, two fractions contain an important asparaginasic activity: the mitochondrial and the cytoplasmic.

The mitochondrial fraction was resuspended in the 0.02 M sodium phosphate buffer at pH 7.35 and then homogenized in the Ultraturrax, and centrifuged at 14 000 rev./min at +4°C. The supernatant was placed at the top of a column of G 200 Sephadex. Gel filtration showed this mitochondrial fraction to contain virtually exclusively Asparaginase II with traces of Asparaginase I.

In the cytoplasmic fraction it was possible to localise only Asparaginase I with Sephadex G 200 gel filtration, which produced a single peak of molecular weight 150 000.

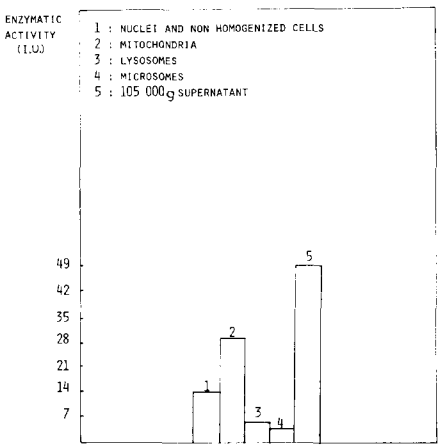

Fig. 6. Cellular fractionation of guinea pig liver. The surface of each rectangle is proportional to the whole asparaginasic activity of each separated fraction obtained from one crude homogenate.

This does not necessarily mean that Asparaginase II is absent from the cytosol of the living cell. A centrifugation at 105 000 × *g* is necessary for the preparation of the cytosol fraction; this acceleration has been shown by us to deactivate irreversibly Asparaginase II.

It is necessary to mention as well that the sum of Asparaginase II activity in the various fractions is less than the activity of the total cellular homogenate; this would suggest the presence of Asparaginase II in the cytosol and its destruction at 105 000 ×*g*.

Purification of Asparaginase I

The different stages of purification are presented in Table II. From a crude homogenate of guinea pig liver containing both enzymatic activities, we isolated the Asparaginase I activity by centrifugation at 105 000 × *g* for 1 h. This homogenate underwent a partial purification analogous to the one described in Table I.

The partially purified homogenate was submitted to preparative electrophoresis on a cellulose acetate block.

TABLE II

PURIFICATION STEPS OF GUINEA PIG LIVER L-ASPARAGINASE I

Purification step	Vol (ml)	Total protein (mg)	Total activity (I.U.)	Specificity activity (I.U./mg protein)	Yield (%)	Purification factor
Crude extract	75	1687.5	207.5	$12 \cdot 10^{-2}$	100	1
105000 × *g* supernatant	63	787.5	194	$24 \cdot 10^{-2}$	93	2
pH 5.6 precipitation	60	510	138	$27 \cdot 10^{-2}$	66	2.3
Na_2SO_4 precipitation	5	62.5	114	1.82	55	15
Preparative electrophoresis on Cellogel block	5	1.68	55	32.7	26	272

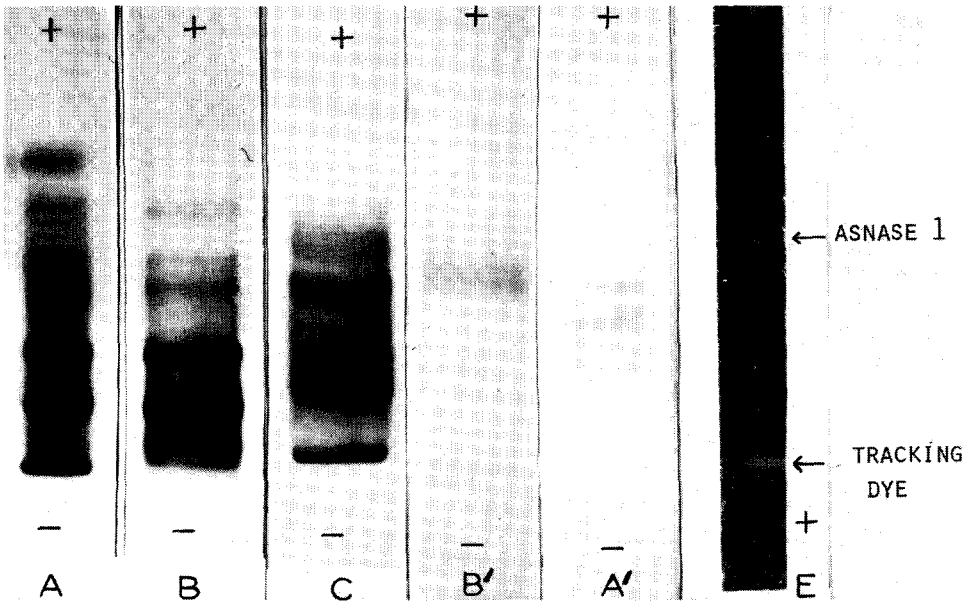

Fig. 7. Different stages of purification according to table II of guinea pig liver asparaginases I and II. Cellulose acetate strip analytical electrophoresis of A: crude guinea pig liver homogenate; B: 105 000 X *g* supernatant; C: after pH 5.6 precipitation and Na_2SO_4 salting-out. B′: after cellogel block preparative electrophoresis. E: analytical polyacrylamide gel electrophoresis control of the B′ fraction. A′: cellulose acetate strip analytical electrophoresis of the pooled active fractions obtained after two successive preparative polyacrylamide electrophoresis (see text).

After each stage of the fractionation an aliquot of the preparation was set aside for analytic electrophoresis on cellogel strips (Fig. 7A, 7B, 7C and 7B′).

In these conditions, it may be ascertained that the purified preparation of Asparaginase I is present as one homogenous band (Fig. 7B′). The specific activity of this enzyme is 272 times higher than in the crude homogenate.

We similarly verified that this purified preparation was homogeneous in analytical electrophoresis on polyacrylamide gel (Fig. 7E). (Band 1 corresponds to enzymatic activity; band 2 to the tracking-dye).

Purification of Asparaginase II

The different stages of purification are presented in Table III. The crude homogenate underwent a partial purification analogous to that described in Table I.

The partially purified homogenate was submitted to gel filtration chromatography on Sephadex G 200. The Asparaginase II thus isolated underwent preparative electrophoresis on cellogel block.

The preparation thus enriched in Asparaginase II presents a specific activity of 8.25 and a coefficient of purification of 63.5. Unfortunately, the poor yield of the purification, particularly at the stage of gel filtration, and the instability of the purified enzyme did not permit us the final electrophoretic assay of the Asparaginase II preparation. Nevertheless, the use of preparative column electrophoresis on polyacrylamide gel permitted us from a crude homo-

TABLE III

PURIFICATION STEPS OF GUINEA PIG LIVER L-ASPARAGINASE II

Purification step	Vol (ml)	Total protein (mg)	Total activity (I.U.)	Specific activity (I.U./mg protein)	Yield (%)	Purification factor
Crude extract	90	4636	633.6	$13 \cdot 10^{-2}$	100	1
pH 5.6 purification	80	1523	448	$29 \cdot 10^{-2}$	70	2.2
Na_2SO_4 precipitation	15	152	348	2.3	54	17.5
Sephadex G 200 (gel filtration)	310	43.4	68.2	1.5	10.7	12.5
Preparative electrophoresis on cellogel block	5	1	8.25	8.25	1.3	63.5

genate of guinea pig liver to obtain an enzymatic solution containing both asparaginasic activities and presenting in analytic electrophoresis on cellogel strip only two bands of proteins both active on L-asparagine (Fig. 7A'). In comparison with the mobility of Asparaginase I (Fig. 7B') it is possible to deduce that the more mobile of the proteins corresponds to enzyme I and the less mobile one most likely to enzyme II.

Discussion

Greenstein [19] described (rat liver) two asparaginase activities, (Asparaginases I and II) the so called Asparaginase II being keto-acid activated. Later, Meister [20] demonstrated that it was due to a coupling of an asparagine transaminase and of an α-keto ω-amidase.

As in the present work, we detected in the guinea pig liver two distinct L-asparaginase activities, we verified that both of them were unable to split either L-glutamine or several amides (formamide, acetamide, propionamide, butyramide, valeramide, benzamide, oxalamide, succinamide, L-leucinamide) and that their activities were not enhanced by α-keto acids as well as pyruvic, oxaloacetic and ketoglutaric.

Suld and Herbut [15] demonstrated one asparaginasic acitivity in a guinea pig liver homogenate. Our research demonstrates the existence of two asparaginases, which may be separated by means of electrophoresis and which are of different molecular weights (150 000 and 21 500). One of them (Asparaginase I) is conceivably identical to the one isolated by Suld and Herbut. As these authors worked on a homogenate centrifuged at 105 000 $\times$ g, it is not possible for the enzyme with which they were concerned to have been Asparaginase II, which becomes inactive when submitted to this acceleration.

In the course of several purifications, we have never observed either the transformation of Asparaginase I into Asparaginase II or the inverse transformation.

Asparaginase II of the lesser molecular weight (21 500) has a high enzymatic activity and therefore is not comparable to the subunits of *E. coli* asparaginase, which are enzymatically inactive (Whelan and Wriston [18]).

We have shown that enzyme I is localized in the cytoplasm and enzyme II in the mitochondria.

However, it is impossible for us to affirm whether or not Asparaginase II exists in the cytosol. In effect, the preparation of cytosol necessitates an acceleration of 150 000 × *g* which destroys Asparaginase II virtually completely. We have remarked nevertheless that the asparaginase II activity located in the mitochondria is less than the whole asparaginase II activity of the crude uncentrifuged homogenate.

The purification of Asparaginase I permitted us to obtain a product which was homogeneous in both cellogel band and polyacrylamide gel electrophoresis.

The purification of Asparaginase II was more difficult, since it often migrates simultaneously with an important protein fraction, and the enzymatic activity is significantly less stable, mostly in ultracentrifugation. We would also mention that when the fractions separated by ultracentrifugation were pooled, Asparaginase II activity did not reappear. It seems actually difficult to discuss the reason for this instability under high acceleration but we observed also that ultrafiltrations under high pressure of nitrogen (about 7 kg/cm^2) destroyed both asparaginase activities.

As frequently reported in enzyme research, the more purified guinea pig liver asparaginase II is, the more unstable it becomes. The product ultimately obtained consists of a mixture of the two asparaginases.

Acknowledgements

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