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# AGOUTI SERUM L-ASPARAGINASE

## II — Chromatographic fractionation and characterization of the enzymatically active component

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**SUMMARY**

DEAE — cellulose chromatography of the Meister L-asparaginase prepared from agouti serum yielded a fraction with increased enzymatic activity. The eluted material was collected in aliquotes, and each of these analyzed separately for enzymatic activity, using L-asparagine as substrate and measuring the $NH_3$ produced, by the Berthelot's reaction. The first peak of protein elution contained most of the IgG while the L-asparaginase activity was recovered in subsequent eluates displaying very low protein content. These active fractions were pooled and concentrated, disclosing an immunoelectrophoretic pattern of a mixture of IgG and a beta-$_1$ component. Using macroimmunoelectrophoresis and subsequent enzymatic reaction in order to identify the active component, L-asparaginase behaved as an alpha-$_2$ in the Meister preparation and as a beta-$_1$ component in the chromatographically eluted material. On Sephadex G-200 gel filtration, a single peak was eluted, revealing two components at immunoelectrophoresis, one corresponding to L-asparaginase and the other to IgG, which suggests a similar molecular weight for both.

## INTRODUCTION

Great interest in the study of L-asparaginase was raised by the discovery by KIDD [20, 21] that guinea-pig serum was effective in promoting regression of certain transplanted tumors, and from BROOME's [1] demonstration that this activity was related to the level of L-asparaginase.

*Escherichia coli* L-asparaginase was the most studied as far as antitumor activity [24, 31], physicochemical and pharmacological properties [19, 22, 29, 32] are concerned.

L-asparaginases are widelly distributed in living bodies [4, 33] but the first to be identified was that from guinea-pig serum, its action being demonstrated not only in experimental tumors [2, 3] but also in human leukemia [9]. Its use is thus far restrict, due to difficulties in purification. SULD & HERBUT [34, 35] exhaustivelly studied serum and liver guinea-pig L-asparaginases and proposed specific precipitation by immune-sera to obtain the highly purified enzymes [36].

Although agouti serum (*Dasyprocta sp*) shows high level of this enzyme [15, 27], and proved antitumoral effect [15, 18, 27], its properties are as yet not very well established.

In a previous paper [6] we have analyzed agouti serum L-asparaginase purified by the Meister method [25]. By immunochemical

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procedures this preparation revealed the presence of gammaglobulin and a fraction with alpha-$_2$ mobility besides albumin traces. The present paper deals with column chromatography of the Meister preparation and immunoelectrozymophoretic identification of the enzymatically active component present in specific precipitates.

## MATERIAL AND METHODS

1) *Agouti serum L-asparaginase* — A lyophilyzed enzymatic preparation was used, partially purified by Meister method[25], referred as L-Ase-Meister. This material was kindly supplied us by Dr. Oswaldo Gonçalves de Lima from the "Instituto de Antibióticos do Recife".

2) *Rabbit anti-L-Ase-Meister immune serum* — Prepared as described in a previous paper[6], purified by 50% saturation with ammonium sulfate.

3) *Enzymatic reaction* — A L-Ase-Meister solution (5.74 mg protein/ml) in a volume of 0.10 ml was incubated for 1 hour at 37°C with 0.30 ml 0.10 M L-asparagine (Merck P.A.) and 0.60 ml 0.01 M sodium borate buffer pH 8.5, the reaction being stopped by cooling in an ice-bath. The $NH_3$ catalitically formed was measured colorimetrically, by Berthelot's reaction.

Enzymatic reaction with the eluates from the DEAE-cellulose column was carried out as follows: 0.10 ml of each eluate was mixed with 0.15 ml 0.1 M L-asparagine as substrate and 0.75 ml borate buffer.

4) *$NH_3$ determination by Berthelot's reaction* — L-asparaginase acts on the substrate, L-aspartic acid and $NH_3$ being formed. CHANEY & MARBACH's[7] modification of the Berthelot's reaction was used for $NH_3$ quantitation.

5) *Total protein quantitation* — Whole protein was measured following LOWRY et al.[23].

6) *DEAE-cellulose chromatography* — The ion exchange resin (Nutritional Biochemical Corporation) was prepared according to FAHEY & TERRY[11] and equilibrated with a 0.0175 M phosphate buffer pH 6.3.

A step-wise process of elution was used with the buffer sequence described by FERRI & PRIGENZI[13], in a 17.5 × 2.4 cm column with flow rate of 22 drops/minute. Two ml aliquotes were collected in tubes.

Before chromatography, the L-Ase-Meister (200 mg dissolved in 6.0 ml of 0.0175 M phosphate buffer pH 6.3) was dyalized overnight against the same buffer.

The equipment utilized was the LKB 7000 Ultro Rac Fraction Collector coupled with a LKB 8300 Uvicord II, LKB 8300 Control Unit and a LKB 6250-3 Chopper Bar Recorder (*).

7) *Identification of L-asparaginase in specific immuneprecipitates (Immunoelectrozymophoresis)* — GRABAR & WILLIAMS'[16,17] immunoelectrophoresis by FERRI & COSSERMELLI's macromethod[12] was used to analyse the eluates, and final identification of the enzymatically active arc was performed according to URIEL[37], with some minor modifications.

7.1 — Upon immunoelectrophoresis of L-Ase Meister or its fractions (eluted from the column), the gel was freed of soluble proteins by washing with 0.15 M saline in order to be submitted to further enzymatic reaction.

7.2 — Substrate slide. A 34 × 200 mm glass slide layered with 12 ml of 1% buffered agar containing 0.0625 M L-asparagine was used as substrate.

7.3 — Identification of the L-asparaginase-containing arc. The substrate slide was closely applied upside down to the immunoelectrophoresis slide, avoiding bubbles between the agar surfaces. After 1 hour incubation (37°C) both slides were traced with filter paper (Whatman Nr 1) strips. After a few minutes contact, the strips were dried at 95°C and then sprayed with a 0.2% ninhydrin (P.A. Merck) acetone

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solution and developed in an incubator at 95°C for 5 minutes.

## RESULTS

### 1) *Enzymatic activity of L-asparaginase (Meister preparation)*

In a series of six experiments it was found that 574.0 μg of L-Ase-Meister protein liberates 17.23 μmoles of $NH_3$ from L-asparagine under our assay conditions.

In our preparation, 2.0 mg protein corresponds to 1.0 IU (*) with a 0.5 SA. Using the Meister definition, 1.0 U.M. corresponds to 33.3 μg of protein with a S.A.M. of 189 U.M./mg N protein.

### 2) *DEAE-cellulose chromatography of L-Ase Meister*

Figure 1 shows the chromatographic pattern. In tubes 20 through 40 (first peak), a protein component was eluted which showed immunoelectrophoretically, IgG as the main component, along with other minor ones.

Each eluate was individually analyzed for enzymatic activity. As shown in Fig. 1, all activity was concentrated in the region between tubes 110 through 150. Tubes 120 through 140 were pooled and analysed for protein content, immunoelectrophoretic composition (Fig. 2) and enzymatic activity.

According to Meister's definitions this material exhibited 1.0 Unit Activity in 5.83 μg of protein and a S.A.M. of 1.075 U.M./mg N protein. Therefore, according to the international criterium, the material contained 1 IU/0.35 mg protein and a SA of 2.86.

Compared to the original activity of L-Ase-Meister, these results show that the chromatographic procedure has increased 5.7 times the specific activity.

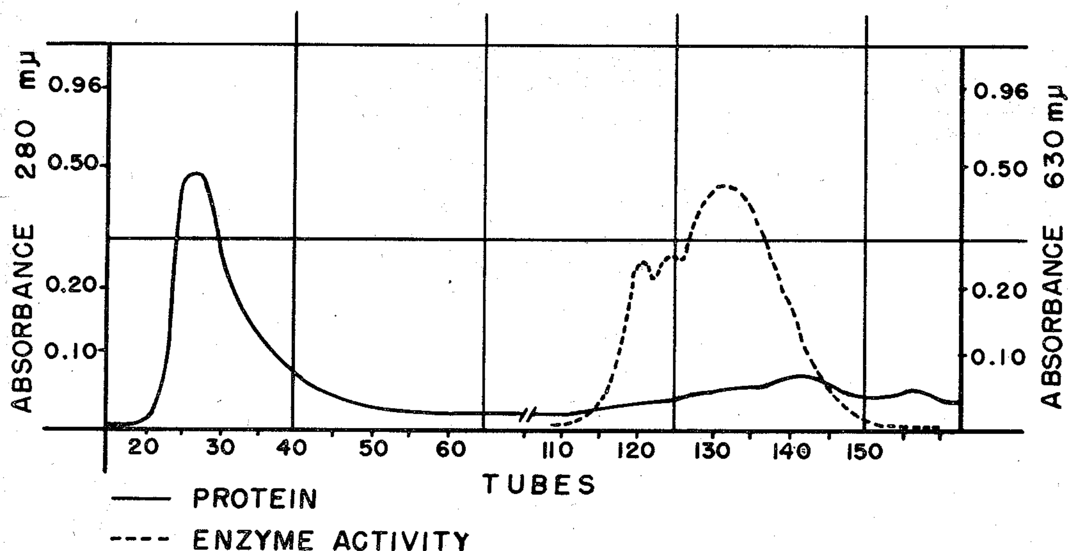


Fig. 1 — Step-wise elution chromatography pattern of partially purified agouti serum L-asparaginase on a DEAE-cellulose column. For chromatography procedure and enzymatic reactions, see text. Ammonia measured by Berthelot's reaction.

(*) International Unit (IU) was defined as the smallest amount of L-asparaginase that catalytically liberates 1 μmol of $NH_3$ from L-asparagine per minute, at 37°C, and Specific Activity (SA) as the number of IU per mg of protein. Meister used as Unit (U.M.) the quantity of enzyme which catalyzes the formation of 1 μmol of $NH_3$ per hour under the assay conditions, and he considers Specific Activity (S.A.M.) as the number of Units (U.M.) per mg N protein

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The pool of enzymatic eluates (concentrated 5×) revealed by immunoelectrophoresis a component in the beta-$_1$ region and another one with the IgG mobility (see Fig. 2).

This active material (pool) was tentatively rechromatographed in TEAE-cellulose and Sephadex G-200 columns. No further purification was achieved, since both components were eluted together.

3) *Identification of enzymatic component*

L-Asparaginase activity in the precipitin arcs after immunoelectrophoresis was detected by a technique similar to the used by URIEL [37].

As shown in Fig. 3, the arc in the beta-$_1$ region (arrow in L-120) is superposable to the L-aspartic acid spot revealed by ninhydrin (arrow in L-121). Therefore, in the same filter paper tracing (L-121) the L-aspartic acid spot produced by L-Ase-Meister appears in the alpha-$_2$ region.

## DISCUSSION

Before assembling the system to measure the $NH_3$ liberated from L-asparagine by L-asparaginase, the influence in the Berthelot's reaction (CALICH [5]) of every component present in the enzymatic reaction was studied. It was verified that L-asparagine at the beginning of the catalysis contributes with 42% of the absorbance, and with only 1.8% after 1 hour incubation. Since in the reaction L-asparagine is converted into L-aspartic acid and $NH_3$, the influence of color development by L-aspartic acid was also controlled; only 3% of the absorbance was found to be to this component. The protein compo-

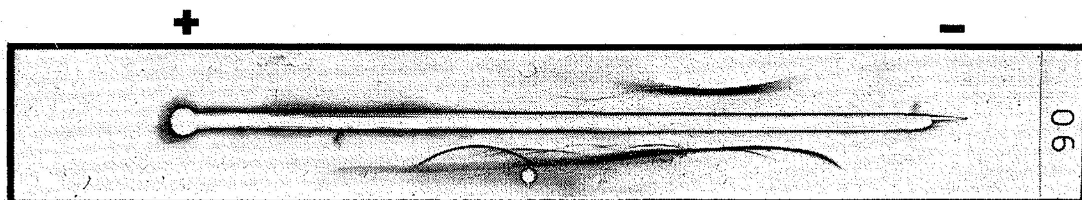


Fig. 2 — Immunoelectrophoresis: upper well — concentrated pool of active eluates obtained from DEAE-cellulose column; trough — rabbit anti-L-Ase-Meister immune serum; lower well — L-asparaginase — partially purified preparation (Meister method).

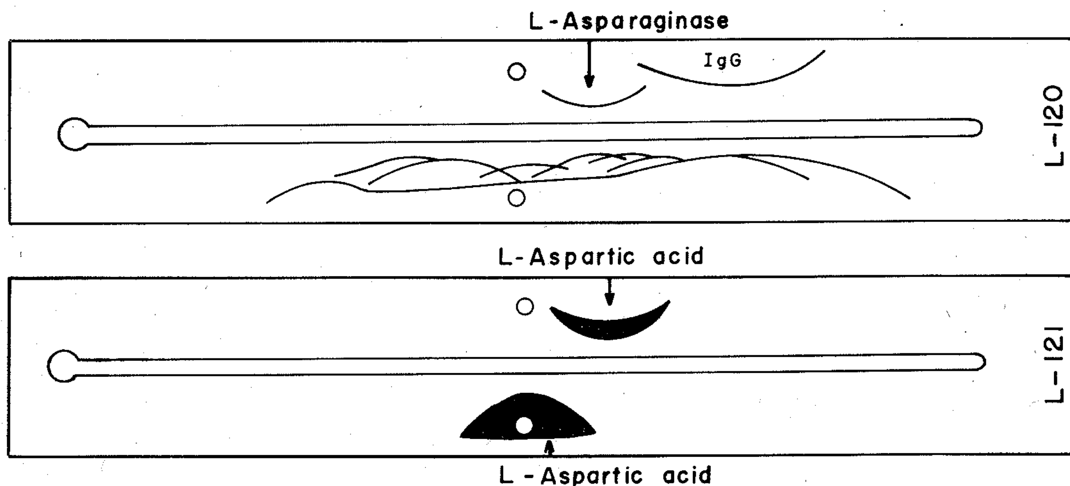


Fig. 3 — Identification of L-asparaginase precipitin arc (schematic). L-120: upper well — concentrated pool of eluates from DEAE-cellulose column; trough — rabbit anti-L-asparaginase (Anti-L-Ase-Meister); lower well — Meister preparation. L-121: filter paper developed with ninhydrin after tracing on agar gel plate with L-asparagine (substrate).

nent of the system (L-Ase-Meister) was found ineffective in color development. Therefore, it was established that the minimal time required to minimize the influence of each component was one hour, using the amounts mentioned in Material and Methods, for L-asparagine and L-asparaginase. Several other investigators [8, 10, 30, 32] have also used Berthelot's reaction to verify the L-asparaginase activity.

By comparing enzymatic activity of agouti serum L-asparaginase (Meister preparation) with that extracted from guinea-pig, we concluded that the former has a specific activity of 189 Units (Meister)/mg protein N, while SULD & HERBUT [34] found 66.1 Units (Meister)/mg protein N for the guinea-pig L-asparaginase purified by the same method. This fact demonstrates that the agouti serum is actually more active than the guinea-pig's.

SULD & HERBUT [34] found 453 U.M./mg protein N after DEAE-cellulose chromatography of the guinea-pig serum Meister preparation. In our eluted fraction the potency of 1,075 U.M./mg protein N was achieved. Furthermore, the purification index was 6.8 for guinea-pig preparation and 5.7 for our agouti serum respectively.

Besides the increased activity after DEAE-cellulose chromatography, the eluted material still had two components recognized as IgG and L-asparaginase. Sephadex G-200 gel filtration did not improve the purification, due to the similarity of MW between L-asparaginase and IgG. *E. coli* L-asparaginase has a MW ranging 126,000-141,000 Daltons [14, 19, 22, 26] and guinea-pig liver L-asparaginase was found to have a MW of 150,000 [28], which leads to the assumption that the MW of agouti serum L-asparaginase is rather close to 150,000.

Immunoelectrozymophoresis has shown that after DEAE-cellulose chromatography the active component changed its electrophoretic mobility from alpha-$_2$ to beta-$_1$.

## RESUMO

*L-Asparaginase de soro de cutia. II — Fracionamento cromatográfico e caracterização do componente enzimaticamente ativo*

O fracionamento cromatográfico em coluna de DEAE-celulose aumenta a atividade específica de L-asparaginase de soro de cutia purificada pelo método de Meister. A análise imunoeletroforética do material obtido após cromatografia em coluna, revela a presença de um componente com localização beta-$_1$ e outro correspondente à IgG. A imunoeletroforese e a atividade enzimática em lâmina demonstra que o componente beta-$_1$ do material obtido da cromatografia e um componente alfa-$_2$ da preparação do soro de cutia purificado segundo Meister são enzimaticamente ativos, demonstrando haver mudança na mobilidade eletroforética da enzima após cromatografia. A L-asparaginase de soro de cutia é eluída junto com a IgG por recromatografia (gel filtração) em coluna de Sephadex G-200, deixando antever que a enzima possui PM próximo ao da IgG (150.000).

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