PARTIAL PURIFICATION AND ANTILYMPHOMA ACTIVITY OF *SERRATIA MARCESCENS* L-ASPARAGINASE *

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In 1961, Broome suggested that the inhibitory effect of guinea pig serum on the Gardner lymphosarcoma in mice is due to L-asparaginase (E. C. 3.5.1.1) (Broome, 1961, 1963). Several other asparaginases have been found since then that possess antitumor activity, including those from the serum of several species of rodents related to the guinea pig (Holmquist, 1963; Old et al., 1963); guinea pig liver (Suld and Herbut, 1965); E. *coli* (Mashburn and Wriston, 1964); and *Serratia marcescens* and several other micobial sources (Boyd, 1965). We wish to report the partial purification and antilymphoma activity of L-asparaginase from *Serratia marcescens*.

Materials and Methods

Serratia marcescens, American Type Culture Collection #60, was obtained from the collection of the Department of Biological Sciences at the University of Delaware. It was carried on agar plates of trypticase soy broth (Baltimore Biological Laboratories) as well as in lyophilized form. Five ml seed cultures in trypticase soy broth were incubated for one day at room temperature, spread over the surface of

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22 by 34 cm Pyrex dishes containing trypticase soy broth plus 1.5% agar (Difco) to a depth of one cm, and incubated at 37-40° for 20 hours. The bacteria were scraped from the surface of the agar with a bent glass rod, washed in .05M Tris-HCl, pH 8.6, suspended in fresh Tris (one ml/gram wet cells) and frozen until ready for use. Bacteria grown commercially in liquid medium, with aeration, were unsatisfactory. Enzyme was liberated by sonication of the Tris suspension for three five-minute intervals, with cooling in ice water, using a Branson sonifier.

Protein concentration was determined according to Waddell (1956); the concentration in column effluents was estimated from the optical density at 280 mu or 215 mu.

Routine asparaginase assays were performed essentially as described previously (Yellin and Wriston, 1966b), except that .05M Tris-HCl, pH 8.6, was used as the buffer instead of borate. A unit of activity is defined as that amount of enzyme which will catalyze the formation of one umole of ammonia per hour (Meister, 1955). Specific activity is expressed as units per milligram of protein.

DEAE-cellulose (Whatman microgranular DE-52, 1.0 meg/gram) was treated as recommended by the manufacturer. The resin was packed under hydrostatic pressure in a Fischer-Porter chromatographic tube 5.8 cm in diameter, with the bed supported by a coarse sintered glass disc 0.54 inches in diameter. In a typical experiment a column with 100 ml bed volume gave a flow rate of 400-700 ml/hour under a hydrostatic head of approximately 125 cm.

BioGel P-300 (BioRad Laboratories, 50-150 wet mesh) was equilibrated with buffer at room temperature for 2-3 days, then degassed, cooled, and packed in a Sephadex K50 Laboratory column (Pharmacia) by pouring in sections. The column was operated using the upward flow technique.

Hydroxylapatite columns were prepared by suspending one part by weight of calcium hydroxylapatite (BioRad Laboratories, in .001 M

phosphate buffer) with three parts of cellulose powder (Whatman) in an excess of buffer and packing under hydrostatic pressure.

Protein solutions were concentrated using a Diaflo 400 ultrafiltration cell (Amicon Corp.) under 40-50 psi of nitrogen. All pH measurements were made at room temperature. Except where otherwise stated, all operations with the enzyme took place at 0-5°.

Female C3H mice were transplanted with the Gardner lymphosarcoma (6C3HED) as previously described (Yellin and Wriston, 1966a). The mice were treated after easily palpable tumors had formed (7-9 days), either with a single intraperitoneal injection of enzyme or with a series of two injections per day for four successive days. The enzyme was also tested on the 6C3HED-RGl tumor, a subline resistant to guinea pig serum asparaginase.

Results

The bacteria were disrupted by sonication, then centrifuged for 45 minutes at 27,000 g, and the precipitate discarded. Manganese chloride (.05 volumes of 1.0 M) was added dropwise to the supernatant, and the mixture stirred for 1-2 hours, then heated in a 55° water bath for 5 minutes after the mixture reached 50°. After cooling, the enzyme preparation was centrifuged for 45 minutes at 27,000 g and the precipitate discarded.

Enzyme preparations at this stage of purification are not stable to prolonged dialysis or refrigeration, as they contain a non-dialysable component which inactivates the asparaginase, especially near pH 7.5. Dialysis was avoided by adjusting the pH to 8.6 with 3M KOH, centrifuging for 45 minutes at 27,000 g and discarding the precipitate. The enzyme was then diluted with four volumes of water to lower the ionic strength to a level below that of the first buffer used in DEAE chromatography. A column containing 100 ml of DEAE equilibrated with .01 M Tris-HCl, .05M in KCl, pH 8.6, was loaded with an enzyme preparation

containing two grams of total protein, and the column rinsed with this buffer until the optical density at 215 mu of the effluent fell below 0.2; the enzyme was then eluted with .01M Tris-HCl, .10M in KCl, pH 8.6. Ten ml fractions were collected and the most active fractions pooled. Pools from several such columns were combined and ultrafiltered prior to gel filtration.

Gel filtration was performed on BioGel P-300 columns equilibrated with .01 M potassium phosphate buffer, pH 6.9, with a diameter of 5.0 cm, and a height of from 65 to 80 cm. Elution was continued with this buffer; and a typical elution profile is shown in Figure 1.

The enzyme pool from the BioGel column was applied directly to a column of hydroxylapatite and cellulose, containing about one ml settled bed volume per mg. of protein, and rinsed with .01 M potassium phosphate, pH 6.9. The enzyme was eluted with .08M potassium phosphate, pH 6.9. The enzyme so obtained is stable to brief dialysis and refrigeration and stable

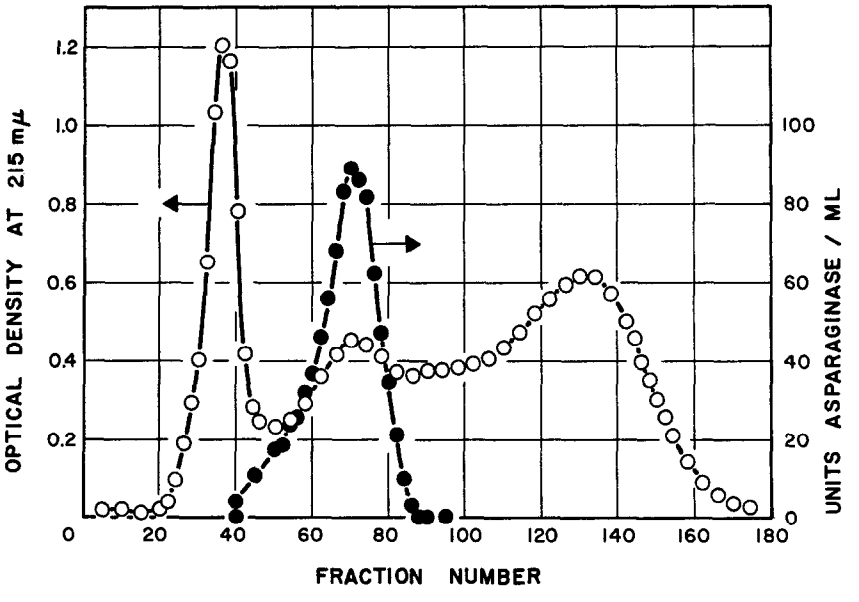

Figure 1. Gel Filtration of Serratia marcescens Asparaginase on BioGel P-300. Fraction volume, 9.5 ml.

to freezing and thawing and to storage in the frozen state. Table 1 shows a typical fractionation of Serratia marcescens asparaginase. It may be seen that a 240-fold purification has been achieved with an overall recovery of L-asparaginase of approximately 10%.

TABLE 1

Purification of Serratia marcescens Asparaginase

Step	Total Units	Total Protein (mgs)	Specific Activity	Enrichment (fold)
Crude extract	102,600	6430	16	-
$MnCl_2$ and heat	54,800	1520	36	2.3
DEAE	29,600	55.5	532	33.5
Gel Filtration	13,800	6.6	2080	130
Hydroxylapatite	9,700	2.5	3860	240

TABLE 2

Antilymphoma Activity

of Serratia marcescens Asparaginase

Number of Animals	Treatment	Average Survival (days)
4	Buffer	21.5
3	25 units	22.7
4	50 units	Complete regression
4	100 units	Complete regression
3	200 units	Complete regression

The animals were injected subcutaneously with 0.2 ml of a tumor suspension containing 5.6×10^5 cells on day zero. On day eight a single i.p. injection/mouse was administered as indicated above; the asparaginase had a specific activity of 4050 units/mg.

The antilymphoma activity is demonstrated by the data in Table 2. A single injection of 50 units was sufficient to cause complete regression in each of the four mice in the group. In another experiment, two injections per day of 110 units each of highly purified Serratia marcescens asparaginase were given on days 7-10 to mice bearing the GPS-resistant tumor 6C3HED-RG1. No increase in survival time was noted compared to controls treated with buffer, while mice bearing the GPS-sensitive tumor, treated in identical fashion, showed a complete regression. This indicates that the antilymphoma activity is due to the asparaginase and not to trace amounts of some other component.

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