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L-Asparaginase Production by *Erwinia aroideae*[1]

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Maximum yields of 1,250 IU (international unit)/g (dry weight of cells) of L-asparaginase were obtained in 8 hr from *Erwinia aroideae* NRRL B-138. Partial purification and concentration of the extracted L-asparaginase yielded a preparation with an activity of 275 IU/ml. Only one L-asparaginase was present as determined by electrophoresis, and the enzyme exhibited a *p*H optimum of 7.5 and a K_m of 3×10^{-3} M.

L-Asparaginase is an effective antileukemia agent in mice and rats (2, 4, 7). Current clinical studies indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man (9). At present, the principal source of L-asparaginase for clinical trials is the bacterium *Escherichia coli* (1). Although production and purification techniques have been developed, they generally provide a quantity of enzyme sufficient for only limited trials. Possibly, alternative sources of L-asparaginase could overcome the problem of antigenic reactions found in some patients (9).

A screen of microorganisms in the Agricultural Research Service Culture Collection at the Northern Laboratory has shown that some bacteria produce larger quantities of L-asparaginase than *E. coli* (10). Of these, *Erwinia aroideae* (Townsend) Holland NRRL B-138 was selected for further study. In addition, extraction procedures were investigated to determine which would liberate the maximum amount of L-asparaginase from the cells. Partial purification techniques were utilized to furnish a product suitable for preliminary antitumor trials in mice.

MATERIALS AND METHODS

Enzyme production. *E. aroideae* NRRL B-138 was used throughout this investigation. The culture was maintained on TGY (glucose, 1.0 g; K_2HPO_4, 1.0 g; yeast extract, 5.0 g; tryptone, 5.0 g; agar, 20.0 g; tap water to 1.0 liter; *p*H adjusted to 7.0) slants, and the same medium (without agar) was utilized for growth and L-asparaginase production studies. Inocula were prepared in 300-ml Erlenmeyer flasks, each containing 50 ml of TGY. After 24 hr of incubation on a Gump rotary shaker (200 rev/min) at 28 C, the contents of the flasks were transferred aseptically to 2.8-liter Fernbach flasks containing 500 ml of TGY broth. For larger scale production runs, 24 hr of growth from the Fernbach flasks was transferred to 20-liter fermentors containing 10 liters of sterile TGY.

Growth studies. Fernbach flasks, prepared as above, were sampled (5-ml samples) at 1-hr intervals. Determinations were made for *p*H, protein concentration, cell count, and L-asparaginase activity.

Production studies. In semipilot-plant trials, 20-liter stainless-steel fermentors were utilized (3). Temperature was held at 28 C, and aeration rates were varied to determine the effect on cell growth and L-asparaginase yield. Cells were harvested after 24 hr by centrifugation and washed with phosphate buffer (Na_2HPO_4, 4.76 g; KH_2PO_4, 4.54 g; Triton X-100, 0.125 ml; distilled water to 1 liter; *p*H adjusted to 7.0).

Extraction procedures. Lysozyme spheroplast formation with subsequent release of L-asparaginase was carried out by the method of Malamy and Horecker (6). Washed cells (300 g) were suspended in 2 liters of 20% sucrose-0.033 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0. Successive treatments of the cells with 0.01 volume of 0.1 M ethylenediaminetetraacetic acid, *p*H 8.0, and 10 μliters of lysozyme (5 mg/ml) per ml of suspension were performed at 5 C. The suspension was stirred gently, and osmotic fragility was checked at 15-min intervals by determining the optical density (OD) at 490 nm. After the decrease in OD had ceased, the suspension was centrifuged at 13,000 × *g* for 2 hr. To analyze for efficiency of extraction, the spheroplasts were resuspended in 2 liters of the original sucrose-Tris solution. Analyses for enzyme content were made on the resuspended spheroplasts and on the supernatant fluid from the final centrifugation.

Purification procedures. All purification steps were carried out at 4 C. Supernatant fluid from the extraction procedure was treated with 0.05-volumes of 1.0 M $MnCl_2$ to partially remove nucleic acid. After the $MnCl_2$ was added, the suspension was stirred for 1 hr, allowed to stand for 15 min, and then centrifuged to remove precipitate. The clear supernatant fluid was treated with 0.06-volumes of 1.0 M $BaCl_2$ and stirred for 20 min. Precipitated polysaccharides were re-

[1] Cited, in part, in Abstracts of Papers, G5-2, 3rd International Fermentation Symposium, New Brunswick, N.J., September 1968.

moved by centrifugation, and the supernatant fluid was retained for further purification.

Solid ammonium sulfate was added to 15% saturation over a period of 15 min with constant stirring. After 30 min for equilibration, the precipitate was removed by centrifugation. A second ammonium sulfate precipitation with a 75% salt saturation yielded a fraction high in L-asparaginase activity. This precipitate was removed by centrifugation and redissolved in 20 ml of 0.01 M K_2HPO_4 buffer at *p*H 8.5. The enzyme was then concentrated to near dryness in a vacuum dialysis apparatus (Carl Schleicher & Schuell Co., Keene, N.H.). Additional 5-ml portions of the same buffer were added to the enzyme in the apparatus, and dialysis was continued until the enzyme was free from any remaining ammonium sulfate. The final product was dissolved in 10 ml of the potassium phosphate buffer and refrigerated at 4 C until further analysis.

L-Asparaginase assay. A 0.1-ml sample of cell suspension or enzyme solution, 0.9 ml of 0.1 M sodium borate buffer (*p*H 8.5), and 1 ml of 0.04 M L-asparagine solution were combined and incubated for 10 min at 37 C. The reaction was stopped by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid. After centrifugation, a 0.1-ml portion of the supernatant fluid was diluted to 8 ml with distilled water and treated with 1.0 ml of Nessler's reagent and 1.0 ml of 2.0 M NaOH. The color reaction was allowed to proceed for 15 min before the OD at 500 nm was determined. The OD was then compared to a standard curve prepared from solutions of ammonium sulfate as the ammonia source. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 μmole of ammonia in 1 min at 37 C.

Protein assay. Determinations were made on whole cell suspensions or crude enzyme preparations by the method of Lowry et al. (5).

RESULTS AND DISCUSSION

Growth and production of L-asparaginase. Cell growth was maximal in 7 to 8 hr (Fig. 1) in 2.8-liter Fernbach flasks containing TGY. Concurrently, the L-asparaginase content also reached a maximum at this time. Yields in Fernbach flasks after an 8-hr period of incubation were 1,250 IU/g (dry weight) of cells. On longer incubation, the wet-cell weight per milliliter and the L-asparaginase concentration appeared to remain constant from 11 to 24 hr. The *p*H dropped to a minimum of 6.8 at 3 hr and then increased in most experiments to a maximum of 8.5. Enzyme production began just before the upward trend of the *p*H curve.

Aeration was varied in 24-hr production runs in 20-liter fermentors (Table 1). Higher aeration rates produced greater cell production; however, total L-asparaginase production was lowered 70%.

Harvest and extraction. Asparaginase activity was completely intracellular during the growth

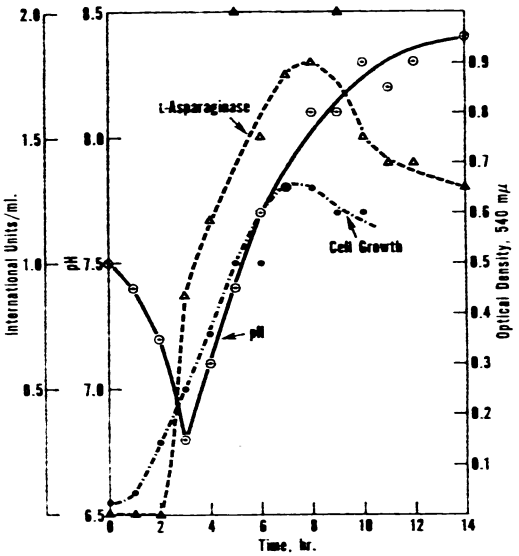

FIG. 1. *Growth, pH, and L-asparaginase production of Erwinia aroideae NRRL B-138 in 2.8-liter Fernbach flasks incubated at 28 C on a Gump rotary shaker (200 rev/min).*

TABLE 1. *Production of L-asparaginase by Erwinia aroideae in 20-liter fermentors with various aeration rates at 28 C*

Aeration (vol/min)	Baffles	Agitation rate (rev/min)	Dry wt of cells from 10 liters (g)	L-Asparaginase activity (IU/g)
0.5	No	300	14	960
1.0	Yes	300	16	580
1.5	Yes	300	18	340
2.0	Yes	350	18	230

period; no activity was detected in the supernatant fluid. After cell harvest, several extraction methods were tried for liberating the maximum amount of L-asparaginase from the cells. Sonic treatment (11) and osmotic shock (8) procedures yielded only 6 to 14% of the enzyme available from 24-hr cells. Lysozyme extraction provided a 40 to 65% release of L-asparaginase from 24-hr cells and a 95% release from 8-hr cells.

Purification and properties. Purification, as previously described, gave a final product with an activity of 275 IU/ml and a 56% recovery of total enzyme from the cells. This partially purified enzyme was characterized by determination of *p*H optimum of the enzyme (Fig. 2), electrophoretic studies (Fig. 3), and Lineweaver-Burk data for K_m calculation (Fig. 4). Maximum activity occurred between *p*H 7 and 8; enzyme activity

was essentially absent below *p*H 4.5. Only one asparaginase was present as determined by electrophoresis (and subsequent incubation of the cellulose strip with L-asparagine). Varying substrate concentrations gave a K_m of 3.0×10^{-3}, indicating good affinity of the enzyme for substrate. Serological tests with antibodies sensitive to the L-asparaginases from *E. coli* and *Serratia marcescens* proved that the L-asparaginase from *E. aroideae* NRRL B-138 is immunologically distinct. Antitumor properties were investigated by injecting tumor-bearing mice with a dose of 3.6 IU of this L-asparaginase. Within 4 days, tumor regression averaged 6 mm. A single dose of 5 IU injected intraperitoneally into each of three mice was sufficient to cause complete remission of the implanted tumors. In later tests, an enzyme solution exhibited some toxicity. This toxicity, which appeared in only some of the test animals, was probably caused by proteinaceous material other than the L-asparaginase present in the partially purified extracts. Indications are that increased purification would give a relatively nontoxic product suitable for intensive animal testing.

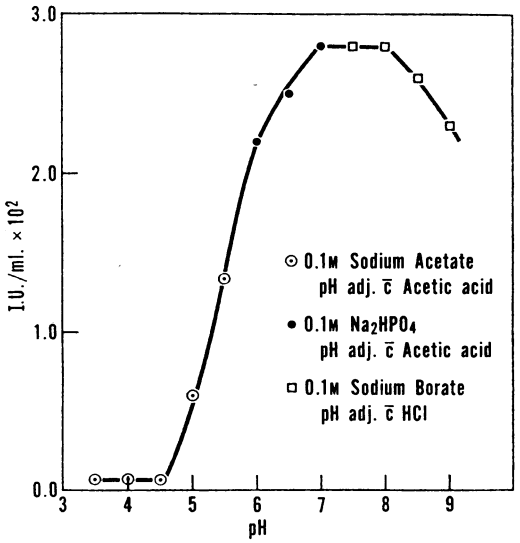

FIG. 2. *Effect of pH on activity of* L-*asparaginase from partially purified extracts of Erwinia aroideae.*

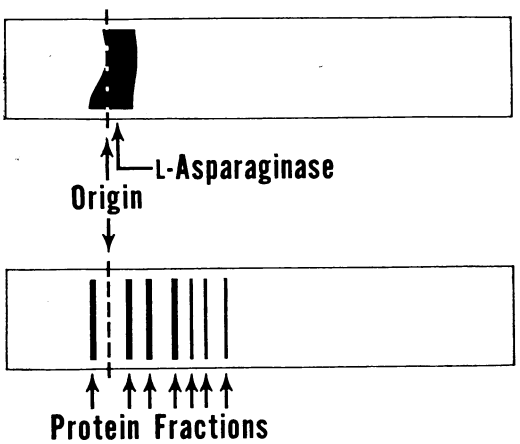

FIG. 3. *Electrophoresis of partially purified extract of Erwinia aroideae showing seven protein fractions and one* L-*asparaginase fraction.*

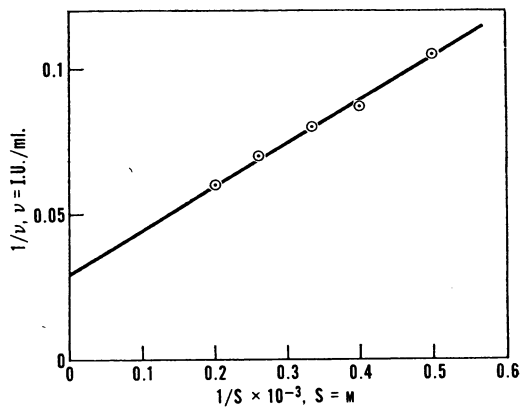

FIG. 4. *Lineweaver-Burk plot of* L-*asparaginase activity from Erwinia aroideae as a function of substrate concentration.*

Alternative sources of enzyme to that now in therapeutic use are desirable in treatment of human neoplasia to overcome the problem of antigenic reactions. *E. aroideae* lends itself to large-scale production of L-asparaginase for human antitumor therapy. High yields in short growth periods should make available greater quantities of this enzyme.

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