The Properties and Large-scale Production of L-Asparaginase from *Citrobacter*

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(Received 4 December 1974; revised 3 April 1975)

SUMMARY

An intracellular L-asparaginase with antitumour activity was purified from a strain of *Citrobacter*. The optimum conditions for enzyme production by fermentation on scales up to 2700 l were investigated. Highest enzyme yield was obtained in corn-steep liquor medium (9.2 %, w/v) at 37 °C. Oxygen limitation was not necessary for high enzyme yield. A total recovery of 4.3 % from nucleic-acid-free extract and a 180-fold increase in specific activity were obtained after purification. The specific activity of the purified preparation was 45 i.u./mg protein. The enzyme hydrolysed D-asparagine and L-glutamine at 7 and 5 %, respectively, of its activity toward L-asparagine, but L-glutaminase activity could be demonstrated only at substrate concentrations above 5 mM. The K_m values for L-asparagine and D-asparagine were 2.6×10^{-5} and 1.4×10^{-4} respectively. The anti-lymphoma activity of the enzyme was demonstrated with Gardner lymphosarcoma and was found only slightly less potent than Crasnitin, the most active asparaginase so far tested in this system.

INTRODUCTION

The effect of L-asparaginase (L-asparagine amidohydrolase, EC. 3.5.1.1) in treatment of experimental and human tumours has been studied extensively and reviewed recently (Capizzi, Bertino & Handschumacher, 1970). The need for large quantities of enzyme for therapeutic work has promoted a search for L-asparaginase in micro-organisms, as they lend themselves to large-scale production. Occurrence of L-asparaginase in various micro-organisms was reported by many workers and recently by Wade, Robinson & Phillips (1971), Arima *et al.* (1972) and Imada *et al.* (1973). L-Asparaginase activity was found in varying quantities in different organisms, and appears to be more common among Gram-negative bacteria. Anti-tumour activity was shown by the enzymes from *Escherichia coli* (Mashburn & Wriston, 1964), *Serratia marcescens* (Rowley & Wriston, 1967), *Erwinia carotovora* (Wade *et al.* 1968), *Er. aroideae* (Peterson & Cigler, 1969), *Mycobacterium tuberculosis* (Reddy *et al.* 1969), *Proteus vulgaris* (Tosa *et al.* 1971), and by a glutaminase/asparaginase enzyme from *Achromobacter* (Roberts, Holcenberg & Dolowy, 1970) but not by L-asparaginase from *Bacillus coagulans* (Mashburn & Wriston, 1964). The enzyme

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from *E. coli* is produced commercially and most of the clinical tests have been done with different commercial preparations of this enzyme (Clarkson *et al.* 1970; Tallal *et al.* 1970). Repeated administration of L-asparaginase to the blood stream caused hypersensitivity, ranging from mild allergic symptoms to anaphylactic shock, in 5 to 20 % of patients (Cooney & Handschumacher, 1970). To overcome this allergic response, L-asparaginases with similar anti-tumour activity but of different antigenic structure are needed to allow continued treatment of a responding but antigenically sensitive patient. So far the *Er. carotovora* enzyme has been shown in clinical trials to be a useful alternative to that of *E. coli* (Crowther, 1971) and we have continued the search for other enzymes. Although a number of papers describe the extraction of L-asparaginase (Wriston & Yellin, 1973), only a few describe large-scale extraction procedures (Arens *et al.* 1970; Ho *et al.* 1970). This paper describes the large-scale production, purification and some of the properties of L-asparaginase from a strain of *Citrobacter*. Particular emphasis is placed on the problems involved in its production on a semi-industrial scale (2700 l) and its purification.

METHODS

Media. Two types of media were used: (i) Media containing different concentrations of corn-steep liquor (CSL) (Garton & Son Ltd, London) were prepared according to Roberts, Burson & Hill (1968). The pH was adjusted to 7.0 unless otherwise stated. (ii) Yeast autolysate media to which various organic acids were added. Yeatex granules were obtained from Bovril Ltd, Burton-on-Trent, Staffordshire. The acids used were standard laboratory reagent grade from BDH or Hopkin and Williams Ltd. Media were prepared in tap water and the pH adjusted to 7.0. Details of media are given in Table 4.

Organism. *Citrobacter*, Imperial College No. c6 from the Salmonella Reference Laboratory, Colindale (No. E101/67), showing high enzyme activity, was chosen for large-scale production.

Culture maintenance. Master cultures of *Citrobacter* c6 were preserved by storage in Pyrex ampoules under liquid nitrogen at -196°C . Cultures for preservation were grown for 24 h at 30°C using 6.14 % (w/v) CSL medium in shaken flasks; sterile glycerol (10 %, v/v) was added at the end of the incubation period and the mixture dispensed in 1 ml quantities into sterile ampoules. The ampoules were cooled slowly to -60°C overnight, before immersing them quickly in liquid nitrogen.

Agar slope cultures for use in the development of laboratory inocula were obtained by removing a master culture from liquid nitrogen and allowing it to attain ambient temperature; the thawed bacterial suspension was used to inoculate nutrient agar slopes, which were incubated at 30°C for 24 h and then stored at 2 to 4°C for a maximum period of 2 months.

Growth studies. Preliminary experiments were conducted in 500 ml conical flasks containing 100 ml medium sterilized by autoclaving at 121°C for 20 min. Each flask was inoculated with 2 ml of an overnight nutrient broth culture. Incubation, unless otherwise stated, was at 37°C for 18 h on a rotary shaker, operated at 200 rev./min, radius of gyration 45 mm. Under these conditions an oxygen transfer rate of 38 ml oxygen (at s.t.p.)/100 ml/h was recorded by means of the sulphite oxidation technique (Cooper, Fernstorm & Miller, 1944).

Pilot-scale fermentations. For the development of a laboratory inoculum, the primary seed stage was first prepared in 500 ml conical flasks containing 100 ml of 6.14 % (w/v) CSL medium. Each flask was inoculated with a loopfull of cells derived from a refrigerated

Table 1. *Fermentation conditions*

Fermenter dimension or characteristic	Fermenter scale (l)			
	5	60	400	3000
Liquid volume (l)	3.4	60	400	2700
Agitator shaft speed (rev./min)	400	183	153	72
Air flow rate (l/min)	2.0	60	400	2700
Air gauge pressure (atm)	~ 0.1	0.7	0.7	0.7
Oxygen transfer rate (ml O ₂ at s.t.p./100 ml/h)	140	26	120	120
Volume of inoculum (l)	0.01	1	1	10

slant culture of the organism, and incubated at 30 °C for 24 h on a rotary shaker. Then to prepare the secondary seed stage, CSL medium (1 l; 6.14 %, w/v) was dispensed into 4 l conical flasks each fitted with a side-arm, and sterilized by autoclaving at 121 °C for 1 h. Each flask was inoculated with 10 ml primary seed-stage culture and incubated at 30 °C for 18 h on a rotary shaker.

Production-stage fermentations. Fermenters of conventional design, ranging from 5 to 2700 l capacity, were employed. The characteristic features of the 5, 60 and 400 l fermenters were described by Banks, Mantle & Szczymbak (1974); the features of the 2700 l fermenters were similar to those described for 400 l fermenters.

Fermenters were batched with CSL medium (6.14 and 9.20 %, w/v, CSL, for seed-stage and production-stage fermentations, respectively), containing 0.01 % (v/v) polypropylene glycol antifoam (polyglycol P.2000; Dow Chemical Co. Ltd). Further polypropylene glycol was added, as necessary, during the fermentation to control foaming. Medium was always sterilized *in situ* with the fermenter. Five-litre vessels were sterilized by autoclaving for 1 h at 121 °C, whilst all other vessels were sterilized by live steam injection for 20 min at 121 °C.

Fermentation conditions are given in Table 1. Secondary seed-stage laboratory inoculum was used to initiate all 5, 60 and 400 litre-scale fermentations, which were subsequently incubated at 37 °C for up to 24 h. Inoculum for 2700 l fermentations was provided in the form of a 60 litre-scale seed-stage fermentation grown for 20 h at 37 °C, under conditions of low aeration. The 2700 litre-scale fermentations were terminated after 8 h incubation (corresponding to the time of maximum enzyme yield, see Fig. 3) by diverting oxygen from the sparger to the fermenter head, thus effectively depriving the micro-organism of oxygen, and by reducing the temperature of the fermenter contents to 5 °C by passing chilled water through the vessel jackets.

Automatic pH control was employed in 5 l fermenters only. Control, within ± 0.1 pH unit, was achieved by the addition of sterile 10 % (v/v) sulphuric acid.

Dissolved oxygen concentration. A Hersch meter connected to a Teflon coil containing oxygen-free argon as the carrier gas (after Roberts & Shepherd, 1968) was used.

Oxygen consumption. Input air and exhaust gas from fermenters were dried by passage through chilled water condensers followed by silica gel. The oxygen content of the dried gasses was determined with a Servomex Paramagnetic oxygen analyser OA 184. The oxygen consumption of the culture was calculated from the difference between input and output measurements.

CO₂ evolution. Exhaust gas was dried by passage through a chilled water condenser followed by anhydrous CaSO₄, and the CO₂ content determined by a Hilger and Watt infrared gas analyser (SC/F). A correction for the CO₂ present in input air was applied.

Fig. 1. Manifold of automated system for kinetic studies. The final concentration of potassium borate buffer was 25 mM. Other reagents employed were 0.3 % sodium potassium tartrate and a 10 % solution of Koch & McMeekin (1924) Nessler reagent. S.M.C., single mixing coil; D.M.C., double mixing coil; ↑, 20 ft delay coil introduced for experiments studying the effect of temperature.

Total nitrogen was determined by the continuous flow method of *Technicon Auto-Analyser* No. N3a.

Cell counts. Total cell counts were determined with a standard haemocytometer counting chamber (0.02 mm depth) with Thoma ruling. Viable cell counts were determined by the Miles & Misra (1938) technique.

Preparation of samples for assay of enzyme activity and protein content. The cells were harvested by centrifuging 10 ml samples for 10 min at 38000 *g* in an MSE highspeed centrifuge at 4 °C, and resuspended in 0.05 M-potassium phosphate buffer pH 8.0.

Samples from extraction steps (ii), (iii), (iv) and (v) (see below) were dialysed overnight at 5 °C against 0.05 M-potassium borate buffer pH 8.0. All samples were diluted in this buffer.

Assay of L-asparaginase, L-glutaminase and protein. The automated assay system was of the continuous-flow type and consisted of three analytical channels running simultaneously (Bascomb & Grantham, 1975). The modified manifold differed from the above in providing the enzyme samples through the revolving sampler plate, and using only one probe and a continuous supply of substrate. For kinetic studies an automated single-channel system measuring the ammonia released after a 1 min incubation period was used (Fig. 1). The sensitivity range was 0.05 to 0.5 $\mu\text{mol NH}_3/\text{ml}$, which is equivalent in this system to 0.05 to 0.5 i.u. ($\mu\text{mol NH}_3$ released/min).

Large-scale extraction of L-asparaginase. Methods commonly used for laboratory purification of enzymes were adapted to large-scale usage. The control of temperature at all stages was of paramount importance and standard equipment was modified to achieve efficient cooling. Unless otherwise specified, the product was maintained at 4 ± 1 °C. The extraction steps were as follows:

(i) Harvest of cells. At the end of the fermentation the culture broth was chilled and held without agitation for 1 h. The medium solids which settled were discarded with the first 50 l of culture medium. Bacteria were then removed from the bulk of the culture by centrifugation in a Westfalia model SAMR 3036 operating at 420 l/h. The final volume of bac-

terial suspension was brought up to 150 l with 0.02 M-potassium borate buffer pH 8.0. The suspension was thoroughly mixed with a Silverson high-speed emulsifier model DX and kept for up to 66 h.

(ii) Cell disruption. The bacteria were ruptured by passing the chilled suspension through an APV Manton-Gaulin sub-micron dispersor model K3, operating at a gauge pressure of 500 atm and at a rate of 250 l/h. The suspension was mixed and chilled to 8 °C between passes. Maximum temperature attained during this stage was 22 °C. Four passes were necessary to disrupt most of the cells and solubilize about 90 % of the enzyme.

(iii) Removal of nucleic acids. MnCl_2 (1 M) (Rowley & Wriston, 1967) was added with vigorous agitation to the suspension at the rate of 1 to 2 l/min, to give a final concentration of 0.1 M. After 1 h mild agitation, the precipitate was removed by repeated centrifugation in a Sharples No. 6 tubular bowl centrifuge at a feed rate of 100 l/h. The volume of the supernatant was brought to 150 l with cold 0.02 M-potassium borate buffer pH 8.0, and the temperature lowered to 0 °C. A clear supernatant was not obtained.

(iv) Acetone fractionation. A volume of acetone at -10 °C, equal to 0.28 of the volume of the supernatant of step (iii), was added slowly and with continuous stirring, temperature being maintained below 0 °C. The suspension was agitated for 30 min and the temperature lowered to -5 °C. The precipitate (about 17 kg) was removed by centrifugation and discarded. Temperature at this stage never exceeded 2 °C. A volume of acetone at -12 °C, corresponding to 0.62 of the original volume, was added to the completely clear supernatant held at -5 °C. The suspension was gently agitated for 30 min at -10 °C and centrifuged. The temperature during this stage was rigorously controlled at -10 °C. The supernatant was discarded and the precipitate (about 7 kg) resuspended immediately in 40 l (total volume) of cold 0.05 M-potassium borate buffer pH 8.0. The suspension stood overnight at 5 °C, and was then centrifuged. The volume of the supernatant was brought up to 40 l with the same buffer.

(v) Ammonium sulphate fractionation. The fraction of protein precipitating between 0.21 and 0.52 g crystalline ammonium sulphate (general purpose reagent)/ml (about 2.5 kg) was dissolved in 0.05 M-potassium borate buffer pH 8.0 and brought to a total volume of 5 l. Some finely divided material remained in suspension.

(vi) DEAE column fractionation. The solution from step (v) was ultrafiltered by a Sartorius membrane filter No. 12134 (pore size 15 nm) until the ammonium sulphate concentration was below 0.01 M. The dialysed filtrate was brought to a volume of 20 l and allowed to flow at a rate of 4 l/h through an 8 l column (15 × 45 cm) of DEAE cellulose (grade DE-11; Whatman Biochemicals, Maidstone, Kent), and equilibrated on the column by washing with 2 to 3 volumes of 0.05 M-potassium borate buffer pH 8.0. The column was eluted with a 40 l 0 to 0.3 M gradient of KCl in borate buffer. The effluent was collected in 800 ml fractions and the most active fractions combined.

(vii) Hydroxylapatite-brushite column fractionation. Ammonium chloride was added to the combined fractions from step (vi) to give a final concentration of 0.1 M. A 2 l column (7.5 × 40 cm) containing 500 ml of a packed calcium hydroxylapatite suspension mixed with 1500 ml of a packed brushite suspension (Skarstedt & Bascomb, 1974) was equilibrated with 3 column volumes of 0.05 M-potassium borate buffer containing 0.1 M-ammonium chloride. The combined fractions were loaded at a rate of 250 ml/h, the column washed with a further column volume of 0.1 M-ammonium chloride in potassium borate buffer pH 8.0, and eluted with a 6 l 0 to 0.3 M-potassium phosphate gradient in the borate buffer containing 0.1 M-ammonium chloride (pH 8.0). The effluent was collected in 120 ml fractions and the most active combined.

(viii) Sephadex G-200 column fractionation. The combined fractions from step (vii) were placed in an Amicon ultrafiltration cell, 400 ml capacity, fitted with a PM-10 membrane, and the volume reduced to 50 ml at an applied gauge pressure of nitrogen of 1.7 atm. The sample was then applied to three 67 × 77 cm columns arranged in series, each containing 1400 ml Sephadex G-200 (Pharmacia) supported by glass beads (Sachs & Painter, 1972) and equilibrated with 0.05 M-potassium borate buffer pH 8.0. The column was eluted with the same buffer at a rate of 100 ml/h. The first eluate (1800 ml) was discarded. The further eluate (2400 ml) was collected in 60 ml fractions, and the most active fractions combined.

(ix) Sterilization and lyophilization. The combined fractions from step (viii) were ultrafiltered as above, and the volume reduced to 20 ml. This solution was applied to an 1100 ml column of Sephadex G-25, washed and eluted with distilled water adjusted to pH 8.0 at a flow rate of 225 ml/h. The first eluate (500 ml) was discarded. The next eluate (110 ml) was collected and sterilized by passage through a Millipore filter (pore size 0.22 μ m). The resulting solution was shell-frozen and lyophilized under a pressure of less than 0.005 mmHg. The dry material was removed aseptically and stored in a sterile container at -15 °C until used in the tests for anti-tumour activity.

Molecular weight determination. A column of Sephadex G-200 measuring 1.8 × 62 cm (156 ml) was prepared and calibrated using the following proteins: yeast β -D-fructosidase (270000 daltons), yeast alcohol dehydrogenase (145000 daltons), *Clostridium histolyticum* collagenase (107000 daltons), horseradish peroxidase (40000 daltons) and denatured lysozyme (14400 daltons). For each determination, the fraction number was normalized with blue dextran, which was always considered to elute at fraction 25.2. The sample consisted of 0.5 ml enzyme, and fractions of 36 drops were collected under conditions of gravity-dependent flow at a rate of 15 ml/h.

Assay of biological activity. The Gardner lymphosarcoma (Connors & Jones, 1970) was obtained from Dr J. D. Broome in 1968 and was routinely passaged in ascitic form by the intraperitoneal injection of about 5×10^6 cells in 0.1 ml of 0.90 % (w/v) NaCl, and in solid form by the subcutaneous implantation of tumour fragments. Solid tumours were also obtained from the ascites cells by subcutaneous injection. From time to time the ascites line was discontinued and new ascitic tumour obtained by intraperitoneal injection of a suspension of the solid tumour. The tumour was passaged in CBA/LAC mice, not in the C₃H strain in which it was originally induced.

For routine screening of asparaginase preparations, groups of mice received an intraperitoneal injection of approximately 5×10^6 ascites cells on day zero and then on day 3 were treated with the agent under test, administered intraperitoneally in 0.1 ml saline. Drug treatment was repeated daily for a total of 5 days. Several dose levels of the test agent were used and the survival times of treated groups compared with the survival times of controls. Since there is an inverse relationship between survival time and number of viable cells injected, the percentage increase in life span (% i.l.s.) of treated groups, compared with controls, is a direct measure of tumour-cell kill (Connors & Jones, 1970).

RESULTS AND DISCUSSION

Choice of strain

Over a thousand strains were screened for L-asparaginase activity by the method of Bettelheim, Kissin & Thomas (1970). These included: recognized serotypes of *E. coli* and *Salmonella*, identified strains of species of enterobacteria and other rod-shaped Gram-negative bacteria, and micrococci, all from the Reference Laboratories in the Central Public Health Laboratory, Colindale; *E. coli* strains from the Pathology Department, University College Hospital, London; *Salmonella* strains from Nationale *Salmonella* Zentrale, Hygiene-Institute, Hamburg; a collection of Gram-negative and Gram-positive strains isolated from soil and a few strains recently isolated from various environmental sources. An atypical *Citrobacter*, Imperial College strain c6, was chosen for large-scale production of asparaginase.

Identity of strain

Imperial College strain c6, a Gram-negative rod-shaped bacterium, gave positive results in the following standard tests: decarboxylation of arginine and ornithine; growth on MacConkey agar and on Simmons' citrate agar (Oxoid); motility at 37 °C; presence of catalase, nitrate reductase and urease; production of acid from adonitol, arabinose and lactose, of acid and gas from glucose and of indole and H₂S (as tested by lead acetate paper); utilization of malonate. It was fermentative in the Hugh & Leifson oxidation-fermentation test. It gave negative results in decarboxylation of lysine, production of acid from starch and sucrose, oxidation of gluconate, liquefaction of gelatin in 14 days, presence of oxidase, PPA and Voges Proskauer tests. Variable results were obtained with the TSI test. These results are very similar to those described for *Citrobacter* matrix biotype II (Bascomb *et al.* 1973).

Growth and enzyme production

Effects of medium. The effect of nitrogen source, at 0.2 % N, on growth and L-asparaginase production was studied in 500 ml flasks using six commercial products available for large-scale fermentations (Table 2). Growth as measured by viable count was best on yeast autolysate but more enzyme was produced with CSL (5.7 %, w/v). The optimum concentration of CSL for growth and enzyme production was tested in 5 l fermenters. Highest yield of bacterial protein (Table 3) was obtained at the highest CSL concentration, while maximum enzyme production was at 8.6 to 9.8% (w/v).

CSL medium formed a precipitate during heat sterilization, and was therefore not desirable for growing bacteria intended for extraction of intracellular enzymes. Also, variability of enzyme yields between different large-scale fermentations was too high. The effect on growth and enzyme production of media containing Yeatex plus acetic, citric or lactic acids at concentrations of C and N equal to those in 9.2 % (w/v) CSL, was tested (Table 4). The medium containing Yeatex plus lactic acid yielded enzyme activities greater than Yeatex plus acetic or citric acid, but much smaller than CSL medium. Experiments with various concentrations of Yeatex plus lactic acid showed that 3 % (w/v) Yeatex plus 0.9 % (v/v) lactic acid may produce enzyme in yields similar to those achieved in CSL, but at ten times the cost.

Effects of temperature. Experiments with 60 l fermenters containing 9.2 % (w/v) CSL medium, grown for 24 h at 24, 27 and 37 °C, showed (Fig. 2) that the cell yield was similar at the three temperatures but that the highest enzyme activity was obtained at 37 °C. At 37 °C, increase in enzyme activity paralleled the increase in protein during the

Table 2. *Effect of different nitrogen sources on the growth and L-asparaginase production of Citrobacter c6*

The dry commercial preparations were dissolved in distilled water to a concentration of 0.2 % N and the pH adjusted to 7.0. Portions (75 ml) of medium in 500 ml conical flasks were autoclaved at 121 °C for 20 min. Each flask was inoculated with a loopful of cells derived from an overnight nutrient-broth culture and incubated at 37 °C on a rotary shaker for 24 h.

Medium	$10^{-9} \times$ Viable cells/ml	L-asparaginase (i.u./ml)
Soybean meal	0.96	0
Ground-nut meal	1.00	0
Pharmamedia	1.54	0
Scotaferm (Borthwick, Glasgow)	2.20	0.07
Yeast autolysate (Yeatex)	11.00	0.47
Corn steep liquor	7.00	0.88

Table 3. *Effect of CSL concentration on the growth and L-asparaginase production of Citrobacter C6*

Bacteria were grown in 5 l fermenters at 37 °C. Samples (10 ml) were taken at 3 h intervals during a 24 h fermentation and the maximum values obtained.

CSL concentration (%, w/v)	Maximum bacterial protein (mg/ml)	Maximum L-asparaginase (i.u./ml)
6.14	1.81	1.24
7.37	2.50	1.27
8.60	2.91	1.33
9.82	2.60	1.33
11.05	3.31	1.26
12.28	3.34	0.61

Table 4. *Effect of organic acids on the growth and L-asparaginase production of Citrobacter C6*

Bacteria were grown in 5 l fermenters at 37 °C. Media were prepared in tap water, with the pH adjusted to 7.0. Samples (10 ml) were taken at 3 h intervals during a 24 h fermentation and the maximum values obtained.

Medium	Maximum bacterial protein (mg/ml)	Maximum L-asparaginase (i.u./ml)
CSL (9.2 %, w/v)	1.93	1.03
Yeatex (3.09 %, w/v)		
+ Acetic acid (0.96 %, v/v)	1.18	0.37
+ Citric acid (1.12 %, w/v)	1.21	0.41
+ Lactic acid (1.12 %, w/v)	1.72	0.59

exponential phase but during the stationary phase enzyme activity decreased. In production, bacteria were therefore harvested at the beginning of the stationary phase.

Effects of pH. Application of pH control during the fermentation at values between 6.0 and 8.0 showed that bacterium and enzyme yields were small at pH 6.0. Above this value growth was similar to that of the control fermentation; however, L-asparaginase production at pH values below 7.5 was markedly less than in fermentations without pH control in which the pH rose from 7.0 to 8.5.

Fig. 2. Effect of temperature on the growth and L-asparaginase production of *Citrobacter c6*. Bacteria were grown in 60 l fermenters. Samples (10 ml) were taken at 3 h intervals and assayed by an automated analytical system as described. (a) Protein content of bacteria, with fermentation at (■) 37 °C, (●) 27 °C, and (▲) 24 °C. (b) L-Asparaginase activity, with fermentation at (□) 37 °C, (○) 27 °C, and (△) 24 °C.

Fig. 3. The course of a typical 3000 litre-scale production-stage fermentation. (a) ■, Bacterial protein; □, total asparaginase activity; △, total glutaminase activity; ▲, total nitrogen content of medium; ●, pH. (b) ●, Dissolved-oxygen concentration; ▲, total oxygen uptake; ○, total carbon dioxide evolution; ■, log cell count.

Large-scale fermentation

The course of a typical 2700 litre-scale production-stage fermentation is illustrated graphically in Fig. 3. There was a progressive rise in pH throughout the fermentation, from 7.0 to 8.5. The amount of nitrogen utilized (0.5 mg/ml) corresponded well with the final cell dry weight recorded (5.1 mg/ml). Exponential growth (as determined by bacterial protein or bacterial dry weight measurements) occurred during the first 5 h of the fermentation, after which the dissolved oxygen concentration fell below 5% of the saturation

Table 5. *Effect of aeration on the growth and L-asparaginase production of Citrobacter C6*

Bacteria were grown in 5 l fermenters at 37 °C. Samples (10 ml) were taken at 3 h intervals during a 30 h fermentation and maximum values obtained.

Agitator shaft speed (rev./min)	Impeller diameter (cm)	Aeration efficiency* (h ⁻¹)	Maximum asparaginase yield†	
			i.u./ml	i.u./mg protein
350	5.0	18.0	0.77 (27)‡	0.90 (27)
400	5.0	26.6	1.06 (30)‡	0.88 (7)
350	6.4	60.8	1.06 (21)	0.91 (6)
400	6.4	93.0	1.04 (12)	0.84 (6)
350	7.8	163	1.03 (12)	0.84 (5)
400	7.8	251	0.99 (9)	0.52 (6)

* Measured by the gassing-out technique (Wise, 1951).

† The times (h) at which the yields were maximum are given in parentheses.

‡ Fermentations not complete in the allotted time.

value, suggesting that the oxygen supply became limiting at this stage; thereafter, a 2 h period of approximately linear growth was observed, total oxygen uptake remaining constant throughout. Total L-asparaginase activity increased rapidly after 3 h to a final value of 0.73 i.u./ml culture fluid after 7 h. Glutaminase activity also increased throughout the fermentation, though the increase was negligible during the period of presumed oxygen limitation. During the same period L-asparaginase activity more than doubled, with the result that the specific activity of L-asparaginase and the L-asparaginase/L-glutaminase ratio increased as the fermentation progressed. After 7 h, there was a sudden drop in oxygen consumption and a rise in dissolved oxygen concentration, whilst both cell multiplication and asparaginase production ceased.

Since, in a normal production-stage fermentation, the oxygen supply appeared to become limiting, causing a change from exponential to linear growth, and a considerable amount of L-asparaginase synthesis occurred during the period of presumed oxygen limitation, it was considered desirable to investigate the effect of aeration on enzyme production. Initial experiments were conducted on the 5 l fermenter scale with 6.14 % (w/v) CSL medium at 37 °C. Culture aeration was varied by altering the agitator shaft speed and impeller diameter, both of which factors are known to have a profound effect upon oxygen transfer rates under oxygen-limiting conditions. Culture aeration had little effect upon the ultimate yields of L-asparaginase achieved (Table 5); however, aeration had a profound effect upon the rate of fermentation. Specific enzyme activity was depressed at the highest aeration efficiency value, 251 h⁻¹. A single fermentation was conducted on this scale under anaerobic conditions (by gassing with nitrogen); no significant growth or L-asparaginase production occurred under these conditions.

Two further experiments were carried out, each using 2 × 400 l fermenters (Table 6). One fermentation was operated under standard conditions, whilst conditions of high aeration were employed for the other fermentation to avoid oxygen limitation. In the first experiment oxygen supply did become limiting for a short period (about 30 min) in the fermenter employing high aeration. In the second experiment, oxygen-limitation conditions were completely avoided. The results indicated that conditions of oxygen limitation are not necessary for good enzyme yield; however, the specific enzyme activity was greater under conditions of lower oxygen supply.

Schwartz, Reeves & Broome (1966) and Cedar & Schwartz (1968) claimed that production

Table 6. *Effect of aeration on the growth and L-asparaginase production of Citrobacter C6*

Bacteria were grown in 400 l fermenters. Samples (10 ml) were taken at 3 h intervals during a 24 h fermentation and the maximum values obtained.

Agitator shaft speed (rev./min)	Impeller diameter (cm)	Maximum oxygen uptake (ml O ₂ at s.t.p./100 ml/h)	Oxygen limitation	Maximum asparaginase yield*	
				i.u./ml	i.u./mg protein
Expt 1					
153	28.5	.	+	0.87 (9)	0.31 (9)
306	28.5	156	±	0.60 (7)	0.21 (6)
Expt 2					
153	28.5	101	+	0.83 (9)	0.28 (9)
306	32.5	153	—	0.67 (7)	0.19 (7)

* The times (h) at which the yields were maximum are given in parentheses.

Table 7. *Purification of L-asparaginase from Citrobacter C6*

Step	Volume (l)	Protein (g)	10 ⁻³ × enzyme activity (i.u.)	Specific activity (i.u./mg protein)	Recovery† (%)	Purification†
Cell suspension (two enzymes present)	150	6104	1735	0.24	—	—
Ruptured cells (two enzymes present)*	150	6150	1410	0.24	—	—
MnCl ₂ supernatant*	150	5270	790	0.25	100	—
Acetone precipitation*	40	534	529	0.99	67	× 4
Ammonium sulphate precipitation and dialysis*	10	160	273	1.88	35	× 7.5
DEAE-11 column fractionation	5.6	54	197	3.65	25	× 15.0
HAP‡ column fractionation in 0.1 M-NH ₄ Cl	0.58	6.0	132	21.90	17	× 88.0
Sephadex G-200 column fractionation and lyophilization	(750 mg)	0.75	34	45.28	4.3	× 181.0

* Dialysed sample.

† Calculation based on assay of dialysed sample of nucleic acid-free extract.

‡ Hydroxyapatite.

of L-asparaginase II was stimulated more than 100-fold when *E. coli* K12 was grown anaerobically under conditions where amino acids were utilized as carbon source. In their experiments, bacteria were allowed to multiply under aerobic conditions then transferred to anaerobic conditions. Increase in enzyme activity was detected 20 min after the transfer.

A set of experiments in 5 l fermenters with 9.2 % (w/v) CSL medium was conducted to investigate this effect on *Citrobacter* C6. Anaerobic conditions were introduced after 4 and 8 h with and without the addition of 0.5 % glucose; no growth or enzyme synthesis occurred during anaerobiosis. Addition of glucose at 4 or 8 h did not change the effect of anaerobiosis.

Unlike the situation with *E. coli* K12, the production of L-asparaginase by *Citrobacter* C6 grown in CSL medium was not stimulated by oxygen deprivation.

Large-scale extraction of L-asparaginase

A summary of the results obtained during purification of the enzyme from a typical 2700 l fermentation is given in Table 7.

The presence of MnCl₂ or acetone or more than 2 mM-ammonia in assay samples inter-

ferred with the Nessler reaction, used for assay of enzyme activity; therefore, samples from certain extraction stages had to be dialysed before assay. However, Rowley & Wriston (1967) reported loss of enzyme activity during dialysis at these stages of the purification and we noted similar effects. Moreover, the crude extract of *Citrobacter* C6 contained two L-asparaginases separable by DEAE-11 column fractionation, one of which was sensitive to MnCl_2 treatment; the one we were purifying was resistant. Calculations of the recovery of enzyme at later stages were therefore based on the activity surviving MnCl_2 and dialysis treatments.

The use of acetone for extraction on such a large scale was made possible by controlling the temperature rigorously at all stages of the fractionation, achieved by supplying ethylene glycol to the jackets of all vessels and pipes and to the cooling coil of the centrifuge, and by insulating all equipment extensively. The enzyme recovery under these conditions was 67 to 70 % of the nucleic acid-free material. Much of the enzyme activity which was not recovered in the second acetone precipitate was detected in either the first acetone precipitate or the second acetone supernatant. The enzyme activity discarded amounted to 16 % of the original, bringing the accountable enzyme recovery from the acetone step to 83 %. This procedure could be generally applicable to large-scale purification of other enzymes.

The six-fold increase in specific activity obtained by hydroxylapatite fractionation was due to the presence of 0.1 M- NH_4Cl in all solutions, causing a shift of the elution position of L-asparaginase away from the co-fractionating impurities (Skarstedt & Bascomb, 1974). This improvement is also generally applicable, since all enzymes have one or more small molecules to which they are specifically sensitive, whether the small molecule is a substrate, an activator or an inhibitor. In our experiments, the small ion used, NH_4^+ , is a mild reversible inhibitor.

An overall purification of 180-fold, with a yield of 4.3 %, was obtained in the experiment detailed in Table 7. The specific activity of the final product in the most successful experiment was 79 i.u./mg protein. Arens *et al.* (1970), working with equivalent volumes of culture broth, obtained 4.5 % recovery but only a 100-fold purification.

Properties of the purified enzyme

Temperature dependence. The effect of temperature on enzyme activity was studied on the modified analytical system where enzyme reaction time was 1 min (Fig. 4). Maximum enzyme activity occurred at 52 °C. Enzyme activity at 37 °C, the physiological temperature at which the enzyme is expected to function during therapy, is 79 % of the maximum. Similar behaviour was reported for L-asparaginase from other bacterial sources (Jayaram, Ramakrishnan & Vaidyanatham, 1968; Cooney & Davis, 1970; Law & Wriston, 1971; Tosa *et al.* 1972).

pH dependence. The effect of pH on enzyme activity is shown in Fig. 5. Maximum activity occurred over the whole range from pH 8 to 11. A similar pattern is shown for L-asparaginase activity of glutaminase/asparaginase enzyme from *Achromobacter* (Roberts, Holcenberg & Dolowy, 1972). Both enzymes show a broader maximum than is reported for L-asparaginases from other sources (Wriston, 1971).

Molecular weight. This was determined by the elution position on a Sephadex G-200 column and corresponded to a molecular weight of 166000 daltons, which is higher than those reported for L-asparaginase from *E. coli* B (133000 ± 5000 daltons; Ho *et al.* 1970), *Er. carotovora* (135000 ± 10000 daltons; Cammack, Marlborough & Miller, 1972), and from *B. coagulans* (86000 daltons; Law & Wriston, 1971). A molecular weight of 171000

Fig. 4

Fig. 5

Fig. 4. Effect of temperature on the activity of L-asparaginase from *Citrobacter* c6. The purified lyophilized preparation was assayed by the automated kinetic analytical system, reaction time 1 min. Concentrations in the reaction mixture were 13 mM L-asparagine, and 25 mM-potassium phosphate buffer pH 8.0.

Fig. 5. Effect of pH on the activity of L-asparaginase from *Citrobacter* c6. The purified lyophilized preparation was assayed by the automated kinetic analytical system, reaction time 1 min. Concentrations in the reaction mixture were 13 mM L-asparagine, and 25 mM-citrate-phosphate-borate buffer.

daltons was reported for L-asparaginase from *Fusarium tricinctum* (R. W. Sheetz, as quoted by Wriston, 1971).

Enzyme specificity. The substrate specificity of the purified freeze-dried enzyme was determined with the analytical system where enzyme reaction time was 15 min. The enzyme catalyses the hydrolysis of L-asparagine, D-asparagine and L-glutamine. The activities towards D-asparagine and L-glutamine were 7.4 and 5 %, respectively, of the activity towards L-asparagine. However, L-glutaminase activity could be demonstrated only at substrate concentrations above 5 mM. No ammonia was released from L-alanine, adenosine, L-aspartic acid, cytidine, L-glutamic acid, L-leucine, L-methionine, L-β-phenylalanine, succinamic acid, L-serine, L-threonine, L-tryptophane or urea.

Kinetic studies. A K_m of 2.6×10^{-5} M was found for the hydrolysis of L-asparagine by purified enzyme by the modified analytical system (reaction time 1 min) and the Hofstee (1959) plot. This is comparable with the K_m found for enzymes from *E. coli* B (1.25×10^{-5} ; Ho *et al.*, 1970), *Er. carotovora* (1.0×10^{-5} ; Howard & Carpenter, 1972) and *P. vulgaris* (2.6×10^{-5} ; Tosa *et al.*, 1972), and lower than *S. marcescens* (1×10^{-4} ; Wriston, 1971). The hydrolysis of D-asparagine was determined by the normal analytical system (reaction time 15 min). A K_m value of 1.4×10^{-4} M was found, showing a much lower affinity of the enzyme for this compound.

Anti-lymphoma activities. Table 8 is the result of a comparative test between the effects of a Bayer L-asparaginase (Crasnitin) on the growth of the Gardner lymphosarcoma, and L-asparaginase preparation from *Citrobacter* c6. The latter is curative at a dose level of 80 i.u./kg and only slightly less potent than Crasnitin, which is the most active asparaginase so far tested in this system. It also has no obvious toxicity at the highest dose so far used (7500 i.u./kg). Factors that might affect the anti-lymphoma activity of L-asparaginase include pH optimum, K_m value and the half-life of the enzyme in the host (Wriston, 1971; Wriston & Yellin, 1973). The pH optimum for *Citrobacter* L-asparaginase is slightly higher than those of proven anti-lymphoma activity from *E. coli* and *Er. carotovora*, but

Table 8. *Effect of Crasnitin and Citrobacter asparaginase on the growth of Gardner lymphosarcoma*

Dose (i.u./kg)	Crasnitin		Citrobacter L-asparaginase	
	Average survival time (days)	Increase in life span (%)	Average survival time (days)	Increase in life span (%)
0	13·6	—	13·6	—
5	20·3	49	15·3	12·5
20	> 59·0	'Cures'	22·0	62
80	> 59·0	'Cures'	> 59·0	'Cures'

the activities of the Citrobacter enzyme at pH 7·3 (the pH of the mouse serum) and at pH 6·8 (the pH of the mouse ascitic fluid) are 80 and 73 % respectively of the maximum activity. This, and the low K_m value, might explain the relative potency of the Citrobacter enzyme.

We thank Professor Sir Ernst Chain for interest and support during this work. We also thank the Pilot Plant staff for their assistance with large-scale fermentation and enzyme extraction, and Miss Angela Barker and Mr C. A. Grantham for skilled technical assistance. The work was supported by the Cancer Research Campaign.

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