ELŻBIETA PAJDAK and ZDZISŁAW SZAFRAN

PURIFICATION AND PROPERTIES OF L-ASPARAGINASE EC-2 FROM
ESCHERICHIA COLI 055:B5

Department of Clinical Biochemistry and Department of Microbiology,
Institute of Paediatrics, Medical School,
ul. Wielicka 265: 30-663 Kraków, Poland

1. L-Asparaginase has been isolated from aerobically grown Escherichia coli
055:B5 and purified about 140-fold in a three-step procedure involving acidification
to pH 4.5, ammonium sulphate fractionation and column chromatography on
DEAE-Sephadex A-50. The activity of the preparation is 140 U/mg protein.
2. The enzyme acts within a broad pH range (pH 5-9) and is affected neither
by PCMB, N-ethylmaleimide nor metal ions.
3. Molecular weight of the isolated asparaginase is 130 000.

L-Asparaginase (EC 3.5.1.1) is of special interest because of its oncolytic action,
first reported by Kidd (1953) and Broome (1961, 1963a,b). Asparaginase is widely
distributed in microorganisms but only some of the microbial asparaginases show
antilymphoma activity (Roberts et al., 1968; Heineman & Howard, 1969; Boyd &
strain has been previously selected out of 38 strains of Gram-negative bacteria
as the best source of L-asparaginase (Pajdak, 1971) The present paper deals with
purification of the enzyme from E. coli 055:B5 and the culture conditions providing
a high yield of asparaginase.

MATERIAL AND METHODS

Organism. Escherichia coli 055:B5 was obtained from the State Institute of
Hygiene, Warsaw.

Growth and harvesting of bacteria. Cells were grown at 37°C for 14 - 16 h in
the medium containing 4 g of yeast extract and 1 g of L-asparagine per 100 ml.
The cells were harvested by centrifugation at 5°C and kept frozen (—28°C) until
further processing.

[53]
**Estimation of molecular weight.** Sephadex G-200 column (1 x 65 cm) was equilibrated with 0.01 M-Tris-HCl buffer, pH 7.6, containing 0.1 M-KCl. The column was standardized with haemoglobin (Hyland, mol.wt. 66 000), ovalbumin (Serva, 45 000), and aldolase (Serva, 147 000). Blue dextran (Pharmacia, mol., wt. 2 000 000) was used for determination of void volume.

**Polyacrylamide-gel electrophoresis.** Preparation of gel and electrophoretic separation were performed essentially according to Clark (1964). Detection of L-asparaginase activity in polyacrylamide gel has been described elsewhere (Pajdak & Pajdak, 1972).

**Protein determination.** Protein was determined by the method of Lowry et al. (1951). Bovine serum albumin (Cohn fraction V) was used as a standard.

**L-Asparaginase assay.** L-Asparaginase activity was determined by the modified method of Meister (1955). According to this procedure, 0.1 ml of the enzyme solution was added to 2.0 ml of 0.1 M-Tris-phosphate buffer, pH 8.0, containing 20 μmoles of L-asparagine. The samples were incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5 M-trichloroacetic acid and the protein precipitate was removed by centrifugation. The ammonia liberated was determined colorimetrically with the Nessler reagent. The enzyme activity was expressed in standard units (U).

**Reagents.** L-Asparagine was obtained from Reanal (Hungary), acrylamide, N,N'-methylenbis-acrylamide and Amido Black 10B from Serva (G.F.R.), sodium tetraphenylboron from the Institute of Organic Chemistry (Warszawa), Crasnitin (asparaginase from E. coli ATCC 11303) from Bayer (G.F.R.), Cetavlon, DEAE-Sephadex A-50 and Sephadex G-200 from Pharmacia (Sweden), p-chloromercuribenzoate (PCMB) and N-ethylmaleimide from Koch-Light (England).

**RESULTS**

**Effect of culture medium on L-asparaginase production.** As shown in Fig. 1, the maximum induction of L-asparaginase of E.coli 055:B5 was observed at L-asparagine concentration of 1%. Further increase in concentration was ineffective. The additional supply of yeast extract (4%) resulted in an about 40% enhancement of the enzyme yield.

**Purification of L-asparaginase.** The initial steps of purification of the enzyme, i.e. extraction and ammonium sulphate fractionation, were based essentially on the method of Ho et al. (1970). The results obtained are summarized in Table 1.

The frozen cell cake (10g) was suspended in the medium containing 15 ml of 0.05 M-Tris-HCl buffer, pH 6.5, 1.5 ml of 0.2 M-EDTA, pH 6.5, 0.4 ml of 5% (w/v) ethyltrime:thlammonium bromide, and 1.65 g of sodium chloride. The cells were then disrupted by repeated freezing and thawing. The suspension was acidified to pH 4.5 with acetic acid and the supernatant was collected by centrifugation for 10 min at 10 000 rev./min.

The supernatant was then adjusted to pH 8.0 with 0.5 M-NaOH and brought to 0.5 saturation with ammonium sulphate. The pellet precipitated at 0.5 - 0.9 am-
monium sulphate saturation was collected by centrifugation, dissolved in 1 ml of 0.01 M-Tris-HCl buffer, pH 7.6, and dialysed against the same buffer containing 0.01 M-KCl at 8°C.

![Graph](image)

Fig. 1. Effect of L-asparagine and yeast extract on L-asparaginase production by *E. coli* 055:B5. Yeast extract was added to the medium at concentration of: 1 g (○), 2.5 g (●) or 4 g (△) per 100 ml, in the presence of asparagine at the concentration indicated.

The dialysed solution was applied on DEAE-Sephadex A-50 column (1 × 45 cm) equilibrated with 0.01 M-Tris-HCl buffer, pH 7.6, containing 0.01 M-KCl. The flow rate was 0.2 ml/min. The enzyme solution was eluted with KCl concentration gradient 0.1 - 0.4 M in the same buffer. The effluent fractions were assayed for asparaginase activity and protein content. The elution diagram is presented in Fig. 2.

### Table 1

**Purification of L-asparaginase from *Escherichia coli* 055:B5**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Activity</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total</td>
<td>specific (U/mg protein)</td>
<td></td>
</tr>
<tr>
<td>Cell extract</td>
<td>470</td>
<td>460</td>
<td>0.99</td>
<td>100</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>45</td>
<td>184</td>
<td>4.0</td>
<td>40</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 0.1-0.9 saturation</td>
<td>8</td>
<td>129</td>
<td>16.0</td>
<td>16</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>0.4</td>
<td>36</td>
<td>149.0</td>
<td>141</td>
</tr>
</tbody>
</table>

By the described simple three-step procedure the enzyme was purified 140-fold; its specific activity was about 140 U/mg protein.

**Properties of L-asparaginase.** The enzyme shows a very broad pH optimum ranging from pH 5 to 9 (Fig. 3). The electrophoretic mobility of the L-asparaginase from *E. coli* 055:B5 in polyacrylamide gel was exactly the same as that of L-asparaginase from *E. coli* ATCC 11303 (Crasnitin). On gel filtration on Sephadex G-200 (Fig. 4) the molecular weight of the enzyme was found to be 130 000, similarly as for the Crasnitin preparation. Neither metal ions (1 mM) nor PCMB or N-ethylmaleimide at concentrations of 0.1 and 1.0 mM affected the activity of the L-asparaginase isolated from *E. coli* 055:B5.
Fig. 2. DEAE-Sephadex A-50 chromatography of L-asparaginase from *E. coli* 055:B5. The fraction separated at 0.5-0.9 ammonium sulphate saturation was used, as described in text. ○, Protein concentration; ●, L-asparaginase concentration; ---, KCl concentration.

Fig. 3. pH optimum of L-asparaginase from *E. coli* 055:B5; △, glycine-HCl buffer; □, sodium phosphate buffer; ○, Tris-phosphate buffer.

Fig. 4. Molecular weight determination of L-asparaginase from *E. coli* 055:B5 by Sephadex G-200 gel filtration. △, Ovalbumin; ○, haemoglobin; □, aldolase; ●, L-asparaginase (Crasniti); ▲, purified L-asparaginase from *E. coli* 055:B5.

**DISCUSSION**

The yield of L-asparaginase from *E. coli* 055:B5 under the described conditions was relatively high (1.2 U/mg dry cells) as compared with the results obtained by other authors. Roberts *et al.* (1968) reported that *E. coli* HAP, which is considered...
a high asparaginase producer, synthesizes 0.95 U/mg of dry cells; Bilimoria (1969) obtained only 0.36 U/mg of dry cells from E. coli U.M.

It is well documented that in Escherichia coli two l-asparaginases are present: EC-1 and EC-2; only one of them (EC-2) is effective in treatment of leukaemia. These two forms differ in solubility in ammonium sulphate solution, chromatographic properties and susceptibility to hydrogen ion concentration (Campbell et al., 1967). The precise quantitative determination of both forms is not possible, however the activity of the preparation at pH 5.0 could be ascribed to the EC-2 form (Bilimoria, 1969; Kaczurka, 1971). Basing on this assumption it seems that our preparation consists mainly of the EC-2 form (97%) and is but slightly contaminated with EC-1 (3%). Chromatography on DEAE-Sephadex A-50 resulted in separation of l-asparaginase into two peaks. Molecular sieving on Sephadex G-200 showed that both these forms of asparaginase had the same molecular weight. The nature of the chromatographic heterogeneity of the preparation has not been elucidated.

The pH dependence observed with our enzyme preparation was similar to that reported by Boyd & Phillips (1971). According to Evseev et al. (1967) l-asparaginase of E. coli B does not contain free SH groups, similarly as our preparation which was found to be insensitive to p-chloromercuribenzoate and N-ethylmaleimide.

Molecular weight, pH dependence, electrophoretic mobility and immunological properties (Pajdak & Pajdak, 1974) of our preparation resemble closely those of the Casmirn-Bayer asparaginase. Examination of its anti-tumour activity would be of considerable interest.

REFERENCES

Broome, J. D. (1963a) *J. Exp. Med.*, 118, 99 - 120.
Oczyszczanie i Własności L-Asparaginazy EC-2
Ze Szczepu Escherichia Coli 055:B5

Streszczenie


2. Enzym wykazuje aktywność w szerokim zakresie pH (pH 5 - 9) i nie jest wrażliwy na działanie PCMB, N-etylomaleimidu i jonów metali.

3. Ciężar cząsteczkowy uzyskanego preparatu L-asparaginazy wynosił 150 000.

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