PURIFICATION OF L-ASPARAGINASE FROM *E. COLI* BY SPECIFIC ADSORPTION AND DESORPTION

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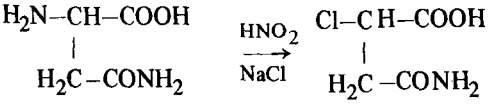
The effect of L-asparaginase in treatment of certain types of leukemia is now well established. The history of L-asparaginase as a therapeutic agent has been reviewed by Broome [1]. Recently, immunosuppressive properties of the enzyme have been reported [2, 3].

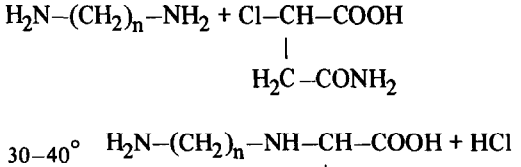
A 900-fold purification of L-asparaginase from guinea pig serum was obtained by Yellin and Wriston [4] after a series of steps involving sodium sulfate fractionation, gel filtration on Sephadex G-200, and chromatography on DEAE-cellulose and hydroxylapatite. The two distinct asparaginases EC-1 and EC-2 of *E. coli* have been purified from cell-free extracts by Campbell et al. [5] who used similar procedures, and by Roberts et al. [6] by means of ethanol fractionation, chromatography on DEAE-cellulose and CM-Sephadex, and polyacrylamide electrophoresis. Whelan and Wriston [7] reported a 2000-fold purification of *E. coli* B asparaginase by heat denaturation, gel filtration, chromatography on DEAE-cellulose and hydroxylapatite, and polyacrylamide electrophoresis. Large-scale purification of EC-2 for clinical use at present requires a costly multi-step process giving a product which is only partially pure.

We believe that techniques developed in our laboratory [8–11] might lead to a faster and simpler purification method based on specific adsorption and desorption on a matrix to which an inhibitor had been covalently bound. Reversible inhibition by D-asparagine or L-asparaginase from *Micobacterium phlei* was reported by Grossowicz and Halpern [12], and rat liver and guinea pig liver asparaginases were found by De-Groot and Lichtenstein [13] to be inhibited by a series of α-*N*-alkyl derivatives of DL-asparagine. Mor and Lichtenstein [14] showed that carbobenzoxy derivatives of L- and DL-phenylalanine, L-tyrosine, and L-cysteine were competitive inhibitors of rat liver asparaginase. Our first attempt to prepare an adsorbent for *E. coli* asparaginase was by direct coupling of D-asparagine by way of the amino group, using the CNBr method. The coupling was successful but the product did not adsorb any enzyme. We have found that direct coupling of small molecules nearly always leads to adsorbents of low efficiency. One of the probable reasons is that the close proximity to the matrix of the active molecule reduces the probability of complementary specific sites being brought into sufficiently close contact. To overcome this problem, we decided to insert a spacer molecule between D-asparagine and the carbohydrate matrix. We settled for a procedure involving the following steps:

Step 1. By a Walden conversion L (+)-β-chlorosuccinamic acid was prepared by treatment of D(−)-asparagine with nitrous acid in a large excess of sodium chloride [15]:



Step 2. As spacers, we used putrescine (n = 4) or hexamethylenediamine (n = 6) in large excess. These were reacted with chlorosuccinamic acid to form a monoamino derivative:



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Step 3. Coupling of the monoamino derivative by way of the free amino group to Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the CNBr method.

The best results so far have been obtained with hexamethylenediamine as a spacer, in which case the adsorbent was prepared as follows: 2.6 g chlorosuccinamic acid obtained in step 1 was added to 10 g melted hexamethylenediamine at 40° and the mixture left for 80 hr with stirring. To improve the yield and reduce elimination of Cl^- from chlorosuccinamic acid by hydrolysis leading to formation of maleamic acid [15], no water was added during step 2. The oily reaction product was concentrated under vacuum from a large excess of water in a Büchi rotating evaporator at 40° three times in succession. To the final volume of 15 ml, 40 ml 0.5 M $NaHCO_3$ was added. Due to the strong basicity of the derivative, the solution had to be adjusted to pH 8.5 with HCl before addition to 100 ml Sepharose 6B, representing 6 g dry gel, which had been activated with 6 g CNBr as described previously [11]. The suspension was gently rocked overnight at room temperature and then washed extensively at pH 8.5 and 3.0 on a Büchner funnel. A column 32 × 45 mm (36 ml) was filled with adsorbent and cycled several times between pH 3.0 and 8.5.

Lyophilized *E. coli* cells (Strain B, Worthington ECB 6HA) were disrupted in a 10 kc Raytheon ultrasonic disintegrator and an aliquot of the supernatant extract applied to the adsorbent column at a rate of 16 ml/hr. The elution curve is shown in fig. 1. Asparaginase activity was assayed by the phenate-hypochlorite method [16] (Berthelot reaction) which proved much easier to handle and gave more consistent results than the Nessler procedure. We used a ready-made reagent kit (Harnstoff-Farb-Test TC-UR-II) purchased from C.F.Boehringer and Söhne, Mannheim, W.Germany. When putrescine was used as a spacer, the corresponding adsorbent retarded most of the asparaginase activity so strongly that a fair separation between active and inactive material was obtained. In 0.05 M borate buffer pH 8.6, a decrease in salt concentration led to an increase in affinity both for active and inactive material, whereas a higher salt concentration caused insufficient separation of asparaginase activity from the bulk of extracted material. Evidence for specific interaction between enzyme and adsorbents based on both types of spacers is provided by the fact that no selective retardation of

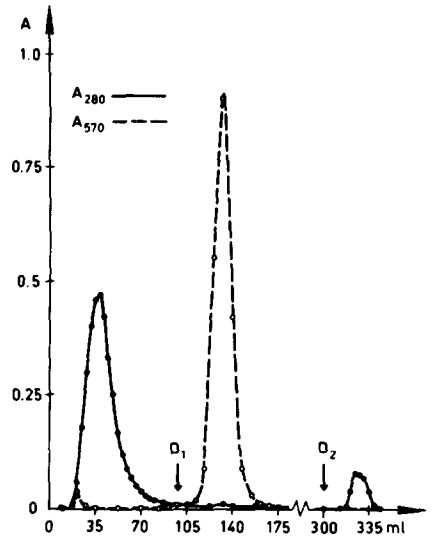

Fig. 1. Elution of L-asparaginase from specific adsorbent. Buffer during adsorption phase: 0.05 M borate, containing 0.3 M NaCl and 0.006% NaN_3, pH 8.6. Desorption with 10^{-3} M D-asparagine in buffer started at D_1, desorption of residual non-specifically bound material with 2 M NaCl in buffer started at D_2. Broken line indicates asparaginase activity.

asparaginase activity was obtained in the presence of 10^{-3} M D-asparagine in buffer and sample during the run, whereas 10^{-3} M L-glutamine barely affected retardation. Due to the small amount of enzyme so far isolated, the specific activity of the purified asparaginase has not been established with certainty. It is at least 10^4 units/mg but may be much higher. This value compares favourably with non-specifically purified enzyme till now reported in the literature.

A crucial question concerning the feasibility of large-scale production of L-asparaginase according to the method described is the rate of hydrolysis of the amide group on the adsorbent. Campbell and Mashburn [17] found that D-asparagine in free solution was hydrolysed by EC-2 asparaginase at 6% the rate of L-asparagine at the optimum pH 7.4, decreasing to 5.5% at pH 8.6. The present adsorbent column has been put through a series of more than ten adsorption-desorption cycles with no obvious reduction in capacity. A decrease in retardation of the enzyme can be counteracted by lowering the ionic strength at the cost of increased non-specific adsorption. However, desorption with D-asparagine strongly favours

the release of enzyme, and residual non-specifically adsorbed material can be desorbed with 2 M NaCl prior to the next cycle. Experiments aimed at optimizing conditions are now under way with a 3 l column; a detailed report of this will appear elsewhere.

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