Chromatography of Human Serum Cholinesterase

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Human serum cholinesterase has been purified by chromatography on calcium phosphate and on the anion exchange resin Dowex 2. One milligram of the most highly active material, obtained by calcium-phosphate chromatography alone, hydrolyzes 13 000 micromoles of acetylcholine in 1 h at 37.5°. The chromatographic experiments show that this is not a homogeneous protein but that further purification can be achieved by rechromatographing on the same adsorbents.

Several methods for the partial purification of serum cholinesterase (ChE) have been developed¹. The most active preparation from human serum has been obtained by low-temperature alcohol fractionation according to Surgenor and Ellis². In the present communication, experiments will be described showing that an additional purification of the enzyme material can be achieved by chromatography on calcium phosphate^{3,4} and on an anion exchange resin (Dowex 2)⁵.

MATERIALS AND METHODS

Enzyme. A partially purified preparation of human serum ChE, fraction IV-6-3 of Surgenor and Ellis² (prepared by AB. Kabi, Stockholm), was used as the starting material for the chromatographic experiments.

Chromatographic Techniques. The preparation of calcium phosphate (hydroxylapatite) and details of the chromatographic procedure have been described^{3,4}. Sodium phosphate buffers, pH 6.83, were used in all experiments. The enzyme was adsorbed at low ionic strength and eluted with increasing buffer concentrations.

An extensive description of protein chromatography on the anion exchange resin Dowex 2 has been published recently⁵. With this adsorbent, tris(hydroxymethyl) amino-methane (THAM)-HCl buffers, pH 7.3, were used. As in the case of calcium phosphate, adsorption of the protein was effected at low ionic strength and elution by increasing buffer concentrations.

All chromatographic runs have been performed at +4°.

The protein concentration of the eluate fractions was determined by ultraviolet absorption measurements at 280 mμ in the Beckman DU spectrophotometer using 1 cm cells. The protein components of the separate fractions were identified by paper electrophoresis; the solutions were concentrated by pressure dialysis before application to the paper.

Measurement of Enzyme Activity. For determining the ChE activity of the fractions, an indicator method was used. The substrate mixture employed had the following composition: (a) 0.01 M Na barbital; (b) 0.02 M KH_2PO_4 ; (c) 0.3 M KCl; (d) 0.0014 M HCl; (e) 0.05 M butyrylcholine iodide; and (f) 0.005 % bromothymol blue. To 3 ml of this solution, 0.1 ml of the sample was added, and the optical density at 610 $\text{m}\mu$ was immediately determined in a Beckman B spectrophotometer using 1 cm cells. The solution was then allowed to stand at 22° for 30 min. after which time the optical density was read again. The measured change in optical density allowed the estimation of the activity from a standard curve constructed by carrying serial dilutions of a solution of fraction IV-6-3 through the entire procedure. The activity unit was defined as the activity obtained when a solution containing 1 mg of IV-6-3/ml was used as sample.

The simplicity of the method described made it suitable for following the activity in the eluate fractions, but it could not be employed for accurate determinations of the specific activity of the starting material and purified fractions. For this purpose, the method of Hestrin⁶ was used, and the activity was expressed as the number of micro-moles of acetylcholine (AcCh) hydrolyzed in 1 h at 37.5° per mg of protein.

RESULTS

Fig. 1 gives part of the elution curve from an experiment with 360 mg of IV-6-3 on a column (15 × 4 cm) with 190 ml of calcium phosphate in equilibrium with 0.01 M phosphate buffer. The sample was dissolved in 35 ml of the same buffer and applied to the column. Elution was first carried out with 0.05 M buffer and fractions of about 5 ml volume were collected. The two zones that are obtained in this elution range are the ones shown in Fig. 1. They account for about 40 % of the added activity but contain only 10 % of the protein material applied. The remainder of the adsorbed proteins could be eluted with 0.3 M buffer but the specific activity in the zones obtained in this elution range was never higher than that of the starting material.

Fractions 40—44 from the experiment illustrated in Fig. 1 were combined (fraction A) and used for rechromatographing both on calcium phosphate and

Fig. 1. The first part of a chromatogram obtained with 360 mg of IV-6-3 on a column with 190 ml of calcium phosphate. Elution was here carried out with 0.05 M phosphate buffer, pH 6.83. Open circles denote extinction at 280 $\text{m}\mu$, and the blocks show the ChE activity.

Fig. 2. Rechromatographing of fractions 40—44 from the experiment shown in Fig. 1 on a column with 7.5 ml of calcium phosphate. The first zone (tubes 18—22) was eluted with a gradient and the second one (tubes 29—32) with 0.1 M phosphate buffer, pH 6.83. Open circles denote extinction at 280 mμ, and the blocks show the ChE activity, zero activity being marked with a line.

on Dowex 2; the specific activity in the combined sample was about 10 times that of the starting material. The distribution of ultraviolet-absorbing material and activity seen in Fig. 1 indicate that a separation of the enzyme from contaminating proteins by step-wise elution would offer difficulties. Thus, gradient elution⁷ was tried, but it was found that the enzyme zone was too wide to allow accurate measurements of concentration and activity. Instead the

Fig. 3. Chromatography of 40 mg of IV-6-3 on a column with 180 ml of Dowex 2. The arrows indicate the start at the top of the column of the different elution agents. Open circles denote extinction at 280 mμ, and the blocks show the ChE activity, zero activity being marked with a line.

Fig. 4. Rechromatographing of fractions 40–44 from the experiment shown in Fig. 1 on a column with 34 ml of Dowex 2. The second zone (tubes 13–14), containing most of the activity, was eluted with 0.37 M THAM-HCl, pH 7.3. Open circles denote extinction at 280 mμ, and the blocks show the ChE activity, zero activity being marked with a line.

following procedure was adopted: After dialysis against 0.01 M phosphate buffer, 4.5 ml of the combined sample was applied to a column (9.5×1.0 cm) with 7.5 ml of calcium phosphate. Elution was first carried out with a linear gradient (discussed by Drake⁷ as "case 4") between 0.03 and 0.08 M phosphate buffer, pH 6.83. The fraction volumes were 3.8 ml. When the first zone (tubes 18–22 in Fig. 2) had emerged (at about 0.04 M phosphate), the gradient was interrupted and elution with 0.1 M buffer was started. With this concentration a second zone (tubes 29–31 in Fig. 2) was eluted containing about 70 % of the activity applied. The most highly purified fraction (tube 29) was about 70 times more active than fraction IV-6-3.

Fig. 3 illustrates an experiment with 40 mg of IV-6-3 on a column with 180 ml of Dowex 2 in equilibrium with 0.04 M THAM-HCl. In this figure, as well as in Fig. 4, the arrows indicate the start at the top of the column of the different eluting agents. As can be seen, most of the material added is adsorbed at 0.04 M buffer concentration while three separate zones are obtained for the other three steps. Both the first and the second zone contain activity but most of it is present in the second one, the recovery being about 50 %.

Fig. 4 gives the results of an experiment in which ChE chromatographed once on calcium phosphate is run on Dowex 2. Five milliliters of the same solution used in the experiment in Fig. 2 were applied directly to a column with 34 ml of the resin. The first and last zone together account for about 80 % of the ultraviolet absorbing material while approximately 50 % of the activity applied was found in the second zone. The specific activity in tube 13 is approximately 40 times higher than that of IV-6-3.

The identification of the protein components is illustrated in Fig. 5, which shows a paper electrophoresis run on the starting material (denoted "0") and on material from the first two zones of an experiment similar to that in Fig. 3. The starting material is seen to be made up mainly of α_2 -globulin with smaller

Fig. 5. Paper electrophoresis of IV-6-3 ("0") together with the first ("5") and second zone ("14") from a chromatographic experiment on Dowex 2, comparable to the one shown in Fig. 3.

amounts of albumin and β -globulin. The latter two components, with small amounts of α_2 -globulin, are obviously eluted in the first zone ("5"), while most of the α_2 -globulin, together with the enzyme, appears in the second zone ("14"). In the most highly purified fractions, the only protein component that could be identified had a mobility close to that of α_1 -globulin. However, zone electrophoresis on cellulose columns (unpublished experiments) showed that the enzyme activity migrates somewhat slower than α_2 -globulin, so that this component cannot be ChE.

Table 1 summarizes the maximum specific activities obtained in the experiments described herein.

The enzyme can also be purified directly from human serum using step-wise elution on Dowex 2 as described for the experiment illustrated in Fig. 3.

Table 1. Specific activity of ChE fractions.

Fraction	Maximum specific activity (μ M AcCh hydrolyzed per mg of protein in 1 h at 37.5°)
Starting material (Cohn fraction IV-6-3)	175
One chromatographic run on calcium phosphate (tube 42, Fig. 1)	1 940
One chromatographic run on Dowex 2 (tube 20, Fig. 3)	423
Rechromatographing of "fraction A" on Dowex 2 (tube 13, Fig. 4)	7 700
Rechromatographing of "fraction A" on calcium phosphate (tube 29, Fig. 2)	13 000

The specific activity of the enzyme obtained after one chromatographic run is about 50 times that of human serum. The purification directly from serum can also be carried out by a displacement technique as described by Boman and Westlund ⁵.

DISCUSSION

Mutual displacement phenomena play an important role in chromatography ^{4,5} both on calcium phosphate and on Dowex 2. This is shown in Figs. 2 and 3, where it can be seen that the zones of the major part of the ultraviolet-absorbing material and of the enzymic activity do not coincide. This also demonstrates that even the most highly purified material is not homogeneous. The small amounts obtained prohibited the use of other purity criteria, such as electrophoresis and ultracentrifugation.

It should be stressed that the exact elution concentrations given here for the calcium phosphate experiments apply only to hydroxylapatite. Thus, the detailed directions for the preparation of the adsorbent ⁴ must be strictly adhered to. It is recommended that, before a new batch of calcium phosphate is used in a preparative chromatographic run, the elution concentrations are determined in an orientating experiment with a smaller column and smaller amounts of protein.

While the uneven distribution of protein and activity demonstrates inhomogeneity, it also indicates that further purification can be obtained by additional rechromatographing on these same adsorbents. On Dowex 2, ChE and α_2 -globulin are eluted in the same range of THAM—HCl concentration, while these substances can be separated on calcium phosphate columns. Thus, it may be possible to use calcium phosphate alone for the purification of the enzyme, and, in any case, the procedure must start with chromatography on this adsorbent. It is, however, impossible as yet to decide which adsorbent is capable of giving the highest degree of purification on rechromatographing. A possible advantage of combining the two methods is the fact that the eluate from the calcium phosphate column can be applied directly to Dowex 2, thus obviating the need for dialysis. The main difficulty in carrying the purification further appears to be the fact that the concentration of ChE in serum is very low compared that of contaminating proteins. Thus, considerably larger amounts of starting material than those employed here would be necessary.

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