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A SIMPLIFIED AND IMPROVED METHOD OF PREPARATION OF ACETYLCHOLINESTERASE OF THE EEL'S ELECTRIC ORGAN

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SUMMARY

Acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) of the electric organ of the eel was purified according to the following procedure: precipitation of the enzyme at pH 5.1, adsorption by tricalcium phosphate gel, elution by 25% saturated magnesium sulphate, precipitation with 65% saturated magnesium sulphate and chromatography and rechromatography on a DEAE-cellulose column.

The product is almost homogeneous in ultracentrifugation, (sedimentation constant 4 S) and in paper electrophoresis.

INTRODUCTION

Three methods have been used for the extraction of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) from the electric organ of *Electrophorus electricus* (L) (refs. 1–3). They have been reviewed recently[4]. The present paper reports a more extensive study of the second method, proposed by HARGREAVES and collaborators[2,4].

Although this method does not yield a product as pure as that of the first method[1], it is simple and yields reproducible results without long and time-consuming manipulations.

Chromatography on DEAE-cellulose gave a preparation showing a single, though not quite symmetrical, peak in the ultracentrifuge.

MATERIAL AND METHODS

The enzyme source was the electric organ of the eel obtained after sacrifice of the animal and careful dissection. The organ was minced and kept in a refrigerator at 5°, under a thin layer of toluene, until required. Under these conditions the enzyme is stable for 2 or 3 weeks.

The enzyme activity, protein concentration and specific activity were measured by the method described by HARGREAVES *et al.* in previous reports[3,4].

Tricalcium phosphate gel[4,5]: 1.5 l of calcium chloride solution (84 g $CaCl_2 \cdot 2\ H_2O$ per l) are diluted with 1.6 l distilled water and 1.5 l trisodium phosphate solution

Abbreviation: AChE, acetylcholinesterase.

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(152 g $Na_3PO_4 \cdot 12\ H_2O$ per l). The volume is made up to 4 l with distilled water. After the precipitate has settled the supernatant is removed by decantation and the precipitate washed with distilled water. This process is repeated twice. The sedimented gel is centrifuged and washed with distilled water five times and then suspended in 3 l of distilled water.

EXTRACTION AND PURIFICATION

Extraction

After removal of exuded blood, 1 kg of tissue is homogenized for 2 min in a Waring blendor with 2 l of cold distilled water; the homogenate is then centrifuged at 5° and 1200 × *g*. The supernatant is separated and the residue homogenized again with 1 l of water and then centrifuged. The total volume of the combined supernatant is about 3.3 l.

The purification procedure is carried out at 5°.

Adsorption on tricalcium phosphate gel at slightly acid pH

To the combined supernatants 15 ml of 10% acetic acid is added, with continuous mechanical stirring, to bring the pH to 5.2, then 300 ml of the phosphate gel is added. The mixture is kept overnight with continuous stirring. It is centrifuged for 30 min at 3000 × *g* and the supernatant discarded.

Elution by magnesium sulphate solution

The collected gel is suspended in 300 ml of 25% saturated magnesium sulphate solution. The suspension is mechanically stirred until the gel is homogenized; 20% NaOH, about 3 ml, is added to adjust the pH to 7.6–7.8. The suspension is stirred for a few hours (overnight, if convenient) then centrifuged at 3000 × *g* for 20 min.

Dialysis and precipitation of the inactive fraction

The eluate (about 300 ml) is dialysed overnight in cellophane bags against 4 l of distilled water.

The water is replaced by another 4 l of distilled water. At this point an abundant precipitate of inactive material containing some protein and a great deal of lipids is formed. After 8 h the water is replaced once more and the dialysis continued for another night in the cold room.

The residual solution is centrifuged at 3000 × *g* and the clear supernatant liquid is collected.

Precipitation of the active fraction at the isoelectric point

Acetic acid (1%) is added with continuous stirring to pH 5.1. A flocculent precipitate is formed which contains most of the enzyme activity.

The vessel is kept for at least 4 h or overnight and then centrifuged at 3800 × *g* for 20 min. The precipitate is suspended in 50 ml 0.1 M NaCl. NaOH (20%) is added to pH 7.6–7.8. The suspension is stirred until the protein is completely dissolved.

Precipitation with 65% saturated magnesium solution

The volume of the preparation is adjusted to 60 ml with the saline solution and 40 ml saturated $MgSO_4$ (at room temperature) is added. $MgSO_4$ at 40% saturation produces a slight precipitate with little enzyme activity.

The vessel is left 3–4 h at 5° and then centrifuged at 3800 × *g* for 20 min. The supernatant is collected, its pH adjusted to 6.2–6.4, and 71.5 ml of saturated $MgSO_4$ is added. At this point an abundant precipitate is formed, containing most of the enzyme activity. After a few hours or next morning the precipitate is centrifuged at 3800 × g, brought to pH 7.6 by adding some drops of concentrated NaOH and stirred until complete dissolution is achieved.

The solution is dialysed for one night against 4 l of distilled water. The water is replaced next morning and the dialysis continued for another 3 h.

DEAE-cellulose column chromatography

The cellulose is first washed with 0.01 M Tris–acetate buffer (pH 7.2). The column of 1.2 cm diameter, 12 cm length, contains 1.5 g of the cellulose in the same buffer.

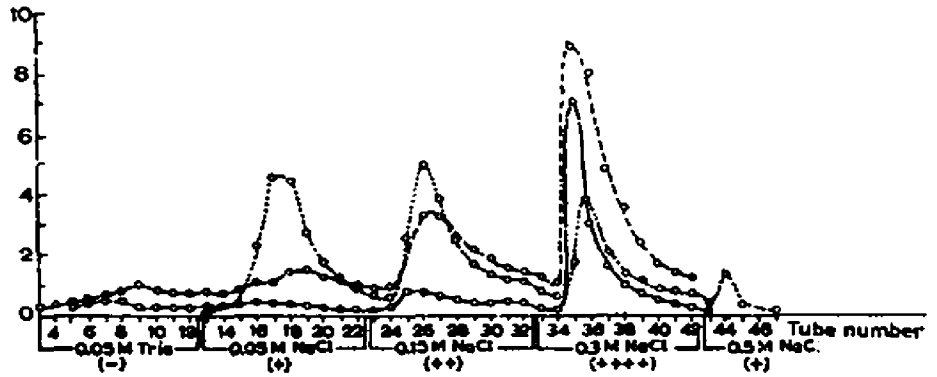

Fig. 1. DEAE-cellulose chromatography. O.....O, solution eluted from the phosphate gel; O- - - -O, dialysed magnesium sulphate (65% satd.) precipitate; O—O, rechromatography of 0.3 M NaCl eluate. Ordinate: protein concentration expressed in mg. The relative AChE activities are indicated by *minus* and *plus* signs.

The total dialysed solution is passed through the column and the column is eluted with 100 ml each of 0.05 M Tris buffer (pH 7.2) and 0.05 M Tris buffer containing 0.05 M, 0.15 M, 0.30 M, 0.50 M NaCl, respectively.

The effluent is collected in 10-ml fractions. Absorbancy is measured at 280 mμ, and in the fractions containing the bulk of the protein the enzyme activity was measured.

As indicated in Fig. 1, most of the AChE is eluted at a salt concentration of 0.3 M.

Rechromatography on DEAE-cellulose column

The combined fractions 34–37 of the first DEAE column (Fig. 1), obtained by processing 2 kg of tissue separately, are dialysed twice against 50 times their volume

TABLE I

SUMMARY OF THE PURIFICATION PROCESS

	Volume (ml)	*Total protein (mg)*	*Total units*	*Specific activity*	*Purification*	*Recovery*
(1) Aqueous extract	3200–3400	20 000–40 000	800 000–1 600 000	20–80	—	—
(2) After elution from Ca phosphate	280–320	1000–1600	600 000–1 200 000	600–800	20 ×	75%
(3) Dissolved precipitate at pH 5.2	50–65	400–800	400 000–850 000	1000–1400	50 ×	50%
(4) Dissolved precipitate with 65% satd. $MgSO_4$	50–65	200–300	300 000–650 000	1500–2400	75 ×	25%
(5) Eluted with 0.3 M NaCl from DEAE column	40	20–30	60 000–180 000	3000–6000	200 ×	10%
(6) Rechroatographym 5)	20	6–12		9500–12 000	—	—

of distilled water, each time for 8 h. The dialysed preparation is rechromatographed as before. Fractions 35 and 36 containing enzyme of the highest specific activity are combined (Table I, Stage 6).

A summary of the the purification process is presented in Table I. The yield of final product is low.

Ultracentrifugation

Ultracentrifugation was carried out in a Spinco Analytical ultracentrifuge model E, at 24°. The protein concentration was 0.5% in 0.1 M NaCl.

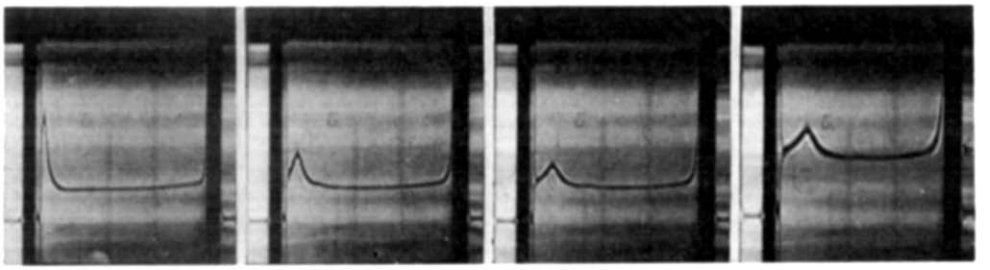

Fig. 2. Ultracentrifugation patterns at 205 000 × *g* of acetylcholinesterase of the highest purification grade.

Fig. 2 shows the pattern obtained with the purest preparation. It consists virtually of one peak, corresponding to a sedimentation constant of $s^{\circ}_{20,w} = 4 \cdot 10^{-13}$.

Electrophoretic mobility

The purified preparation was compared with human serum in a Spinco paper-

electrophoresis apparatus, in 0.07 M barbiturate buffer (pH 8.6) 0.3 mA during 14 h at 26°. The protein was stained with bromphenol blue.

The results are presented in Fig. 3. The purified protein appears to be almost homogeneous and to migrate approximately at the rate of α_2-globulin. This is at variance with the data of previous reports[2,3] in which the preparations were not

Fig. 3. Paper electrophoresis of purified AChE (A) and of a normal human serum (B).

submitted to exhaustive dialysis and isoelectric precipitation. Obviously, the removal of lipids and other impurities results in a greater mobility of the protein.

Stability

It was claimed by BJORSK[7] that Tris buffer is harmful to snake-venom AChE, isolated by cellulose chromatography. Experiments carried out in our laboratory showed that the purified enzyme of the electric organ is stable for 1 week at 5° in distilled water, in 0.1 M NaCl, in 0.1 M sodium acetate, or in 0.05 M Tris solution (pH 6.8). After 10 days losses of activity can be observed, 50% of the initial value is found on the 14th day. Thus eel AChE is not destroyed in Tris solutions during the purification process.

DISCUSSION

The method here proposed yields an almost pure preparation. It gives more reproducible results than earlier methods proposed from this laboratory[2,6].

The previous communication[4] reported two main components on ultracentrifugation, a larger one with a sedimentation constant of 4 S and a smaller one with a sedimentation value of 14 S. In a short communication[8] a comparison between one of our preparations and the preparation obtained by the ammonium sulphate-precipitation technique[1] showed that the latter also yielded more than one component, in fact there were 3 peaks corresponding to components of sedimentation constants 3.9, 6.05 and 11.9 S. The present method gives a preparation showing only a single peak of sedimentation constant 4 S.

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THE THERMAL INACTIVATION OF ACETYLCHOLINESTERASE

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SUMMARY

Thermal inactivation of acetylcholinesterase (Acetylcholine acetyl-hydrolase, EC. 3.1.1.7) from mammalian erythrocytes is accompanied by a decrease in the Michaelis constant K_s, while the substrate inhibition constant K_{ss} remains unchanged. The time course involves two first-order processes, suggesting (a) the presence of two enzymes, or (b) the formation of a second less active enzyme from the native enzyme both species inactivating independently. The second suggestion would explain the K_s and K_{ss} data, if the difference in the two enzymes resides in the spacing of the anionic and esteratic sites. A non-specific salt effect was found, salt stabilising the enzyme solution. The pH range for optimum thermostability is 6.5–7.5, and energies and entropies of inactivation were determined from temperature coefficients of the rate constants.

Abbreviation: AChE, acetylcholinesterase.

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