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AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE

THE USE OF AMBERLITE CG-120 FOR DISSOCIATING THE ENZYME–INHIBITOR COMPLEX

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SUMMARY

Acetylcholinesterase from rat brain was solubilized with 1% (w/v) Triton X-100 and purified by affinity chromatography. Two different ligands were investigated. The most efficient purification was obtained when the enzyme was eluted from a column containing the acetylcholinesterase inhibitor N-methyl-3-aminopyridinium iodide covalently linked to Sepharose 2B. An initial recovery of 6% of the applied enzyme increased to 70% after treatment with Amberlite CG-120. The partially purified enzyme had a specific activity of 205 μmoles min^{-1} mg^{-1} and a purification of 162-fold with respect to the brain homogenate and 44-fold with respect to the Triton solubilized enzyme.

The effect of metal cations on the stability of the partially purified enzyme during storage at −20°C was also investigated. The addition of $MgCl_2$ to the purified enzyme prevented the rapid loss of enzyme activity.

INTRODUCTION

Acetylcholinesterase (AChE: acetylcholine hydrolase, E.C. 3.1.1.7.) plays a key rôle in the transmission of the nerve impulse at cholinergic neurones and is found in the central nervous system of all vertebrates[1,2]. During the last decade, the enzyme from a number of animal brains has been purified by affinity chromatography using a variety of ligands[3–8]. Once the enzyme has been bound to the affinity material, the AChE has usually been released by running a solution of an irreversible inhibitor through the column. However, the recoveries of the enzyme activity obtained by this method although adequate have not been very high even after extensive dialysis[5–7]. Another problem has been that the partially purified enzyme loses most of its activity after freeze drying[4] or during storage in the deep freeze at −20°C[5].

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In this paper we have examined two affinity columns for purifying AChE from rat brain and the use of ion exchange materials for removing decamethonium from the eluted enzyme–inhibitor complex. The effect of a number of compounds, including several cations, on the stability of this partially purified enzyme has also been investigated.

MATERIALS AND METHODS

Materials

General chemicals were analytical reagent grade from BDH (Poole, Great Britain) or Fisons (Loughborough, Great Britain).

Materials for the two affinity columns were obtained as follows: Sepharose 2B and 4B from Pharmacia (London, Great Britain); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride from Aldrich (Wembley, Great Britain); anhydrous HBr in glacial acetic acid, isobutyl chloroformate, iodomethane, 9-chloroacridine and 3-aminopyridine from Eastman-Kodak (Rochester, NY, U.S.A.).

The ion-exchange materials used were Amberlite CG-120 (200–400 mesh, Na^+) from BDH, CM-Sephadex C-50 from Pharmacia and Dowex 50W-X4 (50–100 mesh, H^+) from Fluka (Buchs, Switzerland).

Decamethonium bromide, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), acetylthiocholine iodide (ATChI), thyroglobulin, apoferritin, beef liver catalase and lactate dehydrogenase were obtained from Sigma (London, Great Britain). Triton X-100, Folin reagent and bromophenol blue were purchased from BDH, bovine serum albumin (Fraction V) from International Enzymes Ltd. (Windsor, Great Britain). Prepared Gradipore polyacrylamide concave gradient gels (4–26%, w/v), in the form of slabs (70 × 70 × 3 mm) were obtained from Universal Scientific Ltd. (London, Great Britain).

Preparation of the enzyme

Wistar rats of 150 and 250 g body weight were beheaded and the brains removed and stored at −20°C in the deep freeze until required. A 10% (w/v) homogenate of the brains was obtained by grinding the tissue with washed sand in sodium phosphate buffer (30 m*M*, pH 7). The sand was allowed to settle and the supernatant removed by decantation then centrifuged at 100,000 *g* for 1 at 4°C on an MSE SS50 ultracentrifuge. The supernatant from this centrifugation is known as the "naturally soluble" enzyme. The pellet was then resuspended in the same volume of buffer containing Triton X-100 (1% w/v), homogenized and recentrifuged at 100,000 *g* for 1 h. The supernatant is referred to as the "Triton solubilized" enzyme.

Assay of acetylcholinesterase

The enzyme activity was determined by the spectrophotometric method of Ellman *et al.*[9] as described by Reavill and Plummer[8]. One unit of activity represents the hydrolysis of 1 μmole of acetylthiocholine iodide per minute at 30°C.

Estimation of protein

Protein was determined by the method of Lowry *et al.*[10] using crystalline bovine serum albumin as standard. When Triton X-100 was present, a gelatinous

precipitate was formed but this interference was overcome by centrifuging the precipitate (1000 *g* for 5 min) and incorporating Triton X-100 in the reagent blank and standard[11,12].

Affinity column 1 (MAC-agarose)

The ligand, [1-methyl-9-(N$^{\beta}$-aminocaproyl)-β-aminopropylamino]acridinium bromide hydrobromide, was prepared as previously described and coupled to Sepharose 4B activated with cyanogen bromide[8,13].

Affinity column 2 (MAP-agarose)

The affinity material was built up stepwise by a method based on the procedures used by Berman and Young[14] and Goodkin and Howard[15]. A solution of 1,4-diaminobutane was added to the cyanogen bromide activated Sepharose 2B and after washing, succinic anhydride was added. The succinylated resin was treated with the ligand, N-methyl-3-aminopyridinium bromide, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

Full experimental details for the preparation of the ligands and their coupling to the Sepharose have been presented in a previous report[8].

Conditions for use of the affinity columns

All operations were carried out at 4°C.

Binding. The Triton solubilized enzyme (50–100 ml) was passed through each column which had previously been equilibrated with 30 m*M* sodium phosphate buffer (pH 7) containing 1 % (w/v) Triton X-100.

Elution. The standard elution buffer was 30 m*M* sodium phosphate, pH 7.0, containing Triton X-100 (1 % w/v) and all solutions were prepared in this medium. The column was first washed with 10–15 column volumes of buffer until the eluate gave a zero reading of protein. The elution was then continued with a volume of the standard buffer containing the competitive inhibitor decamethonium bromide (at various concentrations) and 2–5 ml fractions were collected. Finally, the column was washed with four column volumes of the elution buffer containing 1 *M* NaCl.

Washing. The column was prepared for further use by washing with 6 *M* guanidine hydrochloride followed by 40–60 column volumes of the standard elution buffer.

Removal of the inhibitor from the decamethonium–enzyme complex

Dialysis. The fractions containing the peak of the AChE activity were combined and dialysed for up to 150 h against five changes of 2 l each of the elution buffer.

Ion exchange resins. The ability of three cation-exchange resins (CM-Sephadex, Dowex 50W-X4 and Amberlite CG-120) to remove the decamethonium from the enzyme–inhibitor complex was studied together with their effect on the stability of the Triton solubilized preparation. The resins were washed twice then suspended in cold buffer (20 %, w/v) and added to the free AChE or to the enzyme inhibited with 50 m*M* decamethonium bromide. Equal volumes of resin suspension and the enzyme preparation were mixed and after stirring gently, the resin was removed by centrifugation at 1000 *g* for 3 min. The enzyme activity in the supernatant was then determined.

RESULTS

MAC-agarose column

Triton solubilized enzyme (100 ml) was passed through the affinity column: fractions were then collected and assayed for acetylcholinesterase. However, only 8% of the enzyme activity and 9% of protein became bound to the resin. The column was prepared twice but identical results were obtained and so this procedure was abandoned.

Binding of the enzyme to the MAP-agarose

The Triton solubilized enzyme preparation (50 ml) was passed through the column (10 × 1.5 cm) which had been equilibrated with the standard buffer. About 90% of the activity and 80% of protein remained bound to the column. When the eluate gave a zero reading for protein, 25 ml of a solution of the competitive inhibitor decamethonium bromide (10 m*M* in buffer) were applied to the column but only 1% of the total activity was collected after this treatment. A further 25 ml of the elution buffer containing 100 m*M* decamethonium bromide were applied and 10% more of the total activity was eluted from the column. The low yield of acetylcholinesterase activity could be due to the presence of the inhibitor so the active fractions were pooled and extensively dialysed for 72 h and five changes of 2 l of standard buffer in an attempt to remove the decamethonium. However, there was no change in the enzyme activity of the dialysed eluate suggesting that the inhibitor remained firmly bound to the enzyme. Attempts were therefore made to remove the inhibitor by other means.

Removal of the inhibitor with ion exchange materials

The effect of three cation exchange resins on the enzyme was examined to see if they could dissociate the decamethonium–enzyme complex.

The activity of the detergent extract was measured and taken as the 100%

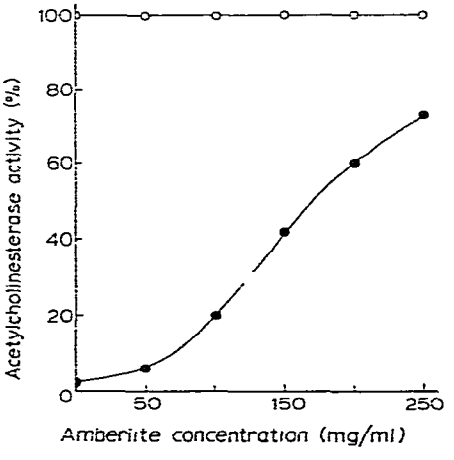

Fig. 1. The effect of Amberlite CG-120 on the activity of acetylcholinesterase in the presence (●) and absence (○) of 50 m*M* decamethonium bromide.

value. A 1-ml volume of this solution was mixed with 1 ml of a solution of 100 m*M* decamethonium bromide and the activity measured again. The activity was reduced to 2.2% of the original activity by this treatment and could not be restored by the addition of Dowex 50W-X4 or CM-Sephadex. In contrast to this, the Amberlite was very effective at removing the inhibitor and more than 70% of the enzyme activity could be recovered after treatment with this resin. The restoration of the activity depended on the concentration of Amberlite present and 250 mg/ml was the highest concentration that could be used in practice (Fig. 1). Amberlite itself had no effect on the uninhibited enzyme. If the enzyme was inhibited by 10 m*M* decamethonium bromide, then addition of Amberlite to 50 mg/ml restored the activity to 98% of its original value but unfortunately a 10 m*M* solution of the inhibitor was unable to remove the enzyme from the column.

The Triton solubilized enzyme preparation was passed through four columns as before and each column was eluted with buffer containing a different concentration of decamethonium bromide (10, 25, 50 and 100 m*M*). The corresponding enzyme recoveries after treatment with Amberlite were 0, 35%, 70% and 75% respectively.

Preparation of the partially purified enzyme

In the light of the above results, 50 m*M* decamethonium bromide was used to remove the enzyme from the MAP-agarose and the eluate treated with washed Amberlite to remove the inhibitor. The fractions were then analysed for protein and enzyme activity and those that contained AChE were pooled and used for the experiments on the storage of the enzyme. A summary of the purification using the above method is shown in Table I. Further elution of the column with 1 *M* NaCl removed a further 9% of the enzyme activity but this fraction was contaminated with other proteins (Fig. 2) and was not used for any other experiments.

TABLE I

PURIFICATION OF ACETYLCHOLINESTERASE BY AFFINITY CHROMATOGRAPHY ON MAP-AGAROSE

	Protein (mg)	*Enzyme activity (μmoles min^{-1})*	*Specific activity (μmoles min^{-1} mg^{-1})*	*Yield (%)*	*Purification (times)*
Brain homogenate	—	—	0.078	100	1
Triton extract					
Total applied	90	25.8	0.287	85	3.7
Affinity column					
Recovered in peak	1.4	17.64	12.6	60	162

Storage of the partially purified enzyme

The preparation of acetylcholinesterase from the affinity column was stored in the deep freeze at −20°C but after 1 week the enzyme had lost virtually all of its activity. Mixing some of the crude Triton extract with the partially purified enzyme increased the stability slightly but most of the activity was gone after 7 days storage (Fig. 3). In contrast to this, the initial extract with detergent retained all its activity on storage.

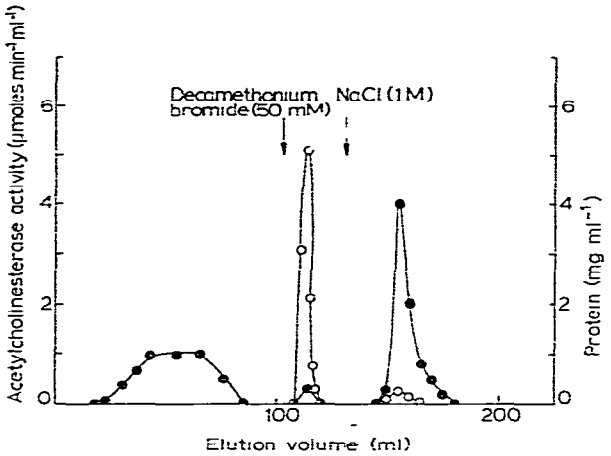

Fig. 2. Purification of acetylcholinesterase by affinity chromatography on column of MAP-agarose. ○, Acetylcholinesterase activity; ●, protein.

The effect of a number of compounds on the stability of acetylcholinesterase was therefore investigated by using the Triton solubilized enzyme that had been inactivated with 50 m*M* decamethonium then reactivated with 250 mg/ml of Amberlite. Storage of this preparation at −20°C resulted in a rapid fall in activity similar to that of the partially purified enzyme (Fig. 4). Storage in the presence of lecithin (5 mg/ml), acetylcholine chloride (150 μM) or sodium phosphate buffer (0.5 *M*, pH 7) had no effect on this rapid decline in activity. However, a number of cations did improve the stability of this preparation and of these, Mg^{2+} was by far the most effective with 80% of the activity retained in the presence of 0.2 *M* $MgCl_2$ (Fig. 4). The protective effect of Mg^{2+} was even more pronounced in the case of the partially

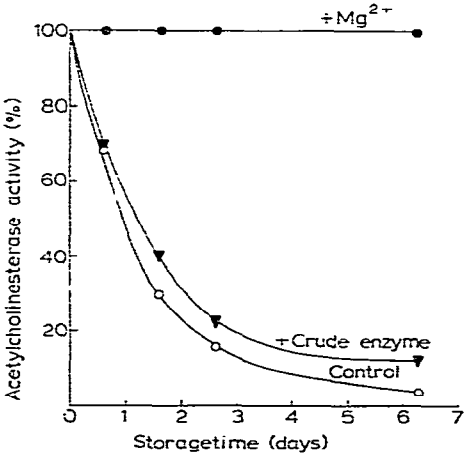

Fig. 3. The stability of partially purified acetylcholinesterase during storage at −20°C. The enzyme was purified by affinity chromatography as described in the text then stored at −20°C by itself (○) and in the presence of the crude Triton extract (▼) or 0.2 *M* $MgCl_2$ (●).

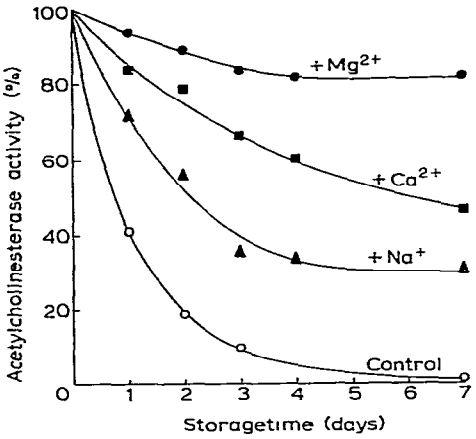

Fig. 4. The stability of Triton solubilized acetylcholinesterase during storage at −20°C. All preparations were inhibited with 50 m*M* decamethonium bromide then reactivated with 250 mg/ml of Amberlite CG-120 as described in the text. Samples were then stored in the presence of 0.2 *M* salt. ○, Control; ▲, NaCl and KCl; ■, $CaCl_2$; ●, $MgCl_2$.

purified enzyme when 0.2 *M* $MgCl_2$ effectively protected the enzyme against loss of activity in the deep freeze (Fig. 3).

DISCUSSION

The first affinity column prepared was MAC-agarose as this had been used by Leavill and Plummer[8] to purify AChE from pig cerebral cortex. Results showed that the column was not effective at binding AChE. In view of this another column was prepared of MAP-agarose which had previously been used by Goodkin and Howard[6] to purify AChE from rat brain synaptosomal membranes. The N-methyl-3-aminopyridinium–agarose was quite efficient in binding rat brain AChE since 92% of the enzyme remained bound to the column. The AChE could be eluted from the column with 50 m*M* decamethonium bromide but the enzyme–inhibitor complex was non-dialysable and so several cation exchange resins were tried with a view to binding the strong positive charge of the inhibitor and thus removing it from the enzyme. Of the resins tested, Amberlite CG-120 proved to be extremely efficient at removing the inhibitor without inactivation of the enzyme (Fig. 1). The effectiveness of this resin is probably due to two main factors, one could be the high binding affinity for the inhibitor and the other the size of the resin particle which allows access of the enzyme–inhibitor complex. The procedure is very rapid and passing the eluate from the affinity column through a second column of Amberlite leads to a partially purified enzyme free of inhibitor which can be used for further studies (Fig. 2). A yield of 70% of the applied enzyme activity was obtained with a specific activity of 12.6 μmoles min^{-1} (mg protein)$^{-1}$ a purification of 162-fold with respect to the crude enzyme. These results are similar to those of Adamson[4] who obtained a preparation from mouse brain with a specific activity of 87 μmoles min^{-1} mg $protein^{-1}$; Adamson used a choline chloride gradient to elute the enzyme and obtained a single peak at a concentration of 40 m*M*. A good recovery was only obtained for rat brain if the concentra-

tion of inhibitor was in the range 40–50 m*M* and so it seems reasonable to postulate that electrostatic and hydrophobic forces are involved in the binding of the enzyme to the affinity column. It was pointed out by Dawson and Crone[5] that the more efficient the purification, the more difficult is the problem of removing the inhibitor from the purified product.

A common characteristic of purified mammalian brain AChE prepared by any procedure is its low stability after storage in the deep freeze[4,5]. In contrast to this, the AChE in the crude Triton extract of rat brain was very stable when stored at −20°C and this stability was unchanged by the addition of Amberlite. However, when the Triton solubilized enzyme was inactivated with decamethonium bromide and reactivated with Amberlite, a dramatic change in the stability of the enzyme took place. Storage of this preparation at −20°C resulted in a rapid loss of enzyme activity (Fig. 4). Decamethonium with its two positive centres probably binds to the active site and also a peripheral anionic site[16,17]. This could result in a distortion of the enzyme into a less stable form which remains even when the inhibitor is removed. The rapid fall in the activity of the AChE was very similar to that of the partially purified enzyme so this inhibited and reactivated preparation was used in screening a number of compounds for their potential stabilizing effect on the enzyme. Another very practical advantage of using this preparation was the much shorter time taken to obtain this preparation compared with the enzyme from the affinity column.

The presence of a substrate often improves the stability of an enzyme but acetylcholine chloride (150 μM) had no effect on the rapid loss of activity experienced at −20°C. Sometimes high ionic strength or a phospholipid detergent stabilises an enzyme but 0.5 *M* sodium phosphate (pH 7) or lysolecithin (5 mg/ml) failed to halt the rapid decline in the activity of the enzyme stored at −20°C. The addition of some of the crude Triton extract also had little effect on the stability of the enzyme (Fig. 3).

Protein–lipid complexes can be stabilized by mono and divalent metal cations[18] and so the effect of several metal ions on the stability of the AChE was examined. In the presence of 0.2 *M* concentration of salt, the stability of the preparation increased as follows $Mg^{2+} > Ca^{2+} > K^{+} > Na^{+}$ > enzyme alone (Fig. 4). The partially purified enzyme was therefore suspended in 0.2 *M* $MgCl_2$ then frozen at −20°C. This treatment protected the AChE during storage at −20°C with very little loss of activity.

MAP-agarose with decamethonium bromide as the eluting inhibitor was very effective at purifying AChE from rat brain. The inhibited enzyme could be reactivated with Amberlite and the purified enzyme stored at −20°C in 0.2 *M* $MgCl_2$ without loss of activity.

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