Journal of Chromatography, 92 (1974) 349–354

CHROM. 7371

AFFINITY CHROMATOGRAPHY OF TRITON-SOLUBILIZED BRAIN ACETYLCHOLINESTERASE

R. M. DAWSON and H. D. CRONE

Australian Defence Scientific Service, Defence Standards Laboratories, P.O. Box 50, Ascot Vale, Victoria 3032 (Australia)

(First received August 17th, 1973; revised manuscript received January 21st, 1974)

SUMMARY

Ox and rat brain acetylcholinesterase, solubilized with Triton X-100, have been purified by affinity chromatography. Gels substituted with two isomeric inhibitors were used. Protein impurities were eluted with NaCl solutions of low ionic strength, and acetylcholinesterase was recovered by increasing the ionic strength or by eluting with the enzyme inhibitor edrophonium chloride. Acetylcholinesterase of low purity was obtained when Triton was not present in the crude preparation and when it was omitted from eluting solvents. A high degree of purification was observed when the surfactant was present throughout the chromatography.

INTRODUCTION

Purification of acetylcholinesterase (AChE; E.C. 3.1.1.7) by affinity chromatography has been reported by a number of workers in the last few years. Most studies have used a soluble preparation from the electric eel[1–7] or bovine erythrocytes[1,8]. Chan *et al.*[9] used a soluble preparation of AChE from bovine brain tissue[10]. Recently, Yamamura *et al.*[11] reported the first case of affinity chromatography of AChE solubilized by Triton X-100, in this case from guinea-pig brain. The results of affinity chromatography of rat and ox brain Triton-solubilized AChE are reported below; these results supplement those of Yamamura *et al.*

EXPERIMENTAL

AChE was prepared from the caudate nuclei of a single ox brain essentially as described by Crone for rat brain[12]. The particulate fraction prepared in 0.32 *M* sucrose was solubilized in a sufficient volume of 2% Triton X-100 to give a ratio of 8 parts by weight of surfactant to 10 parts of protein. The solubilized preparation was used without further modification.

The preparation of rat brain AChE, solubilized by Triton X-100, has been described[12]. In one instance, the crude preparation was partially purified by gel filtration chromatography[12]. The partially purified enzyme solution was concentrated

to approx. 10 ml using a Centriflo cone and dialysed against 5 m*M* Tris–HCl, pH 8.2 (400 ml/17 h; 500 ml/6 h; 500 ml/17 h).

For another experiment the crude preparation of rat brain AChE was freed of Triton by diafiltration. Diafiltration apparatus was produced by Amicon (Lexington, Mass., U.S.A.) and was used with a Diaflo PM10 ultrafilter membrane. A nitrogen pressure of 0.17 MPa (25 p.s.i.) was maintained in the cell, and 5 m*M* Tris–HCl pH 8.2 was used to keep the volume of solution in the cell constant at 10–15 ml until approximately 500 ml had passed through the membrane.

AChE activity was measured by the pH stat method[13] using 25 ml 150 m*M* NaCl–0.5 m*M* acetylcholine at pH 7.40 and 37°. 1 m*M* NaOH was used as titrant. One milliunit of AChE activity is defined as the hydrolysis of 10^{-9} moles acetylcholine per minute under the above conditions. In some cases a radiometric method[12] was used to locate active fractions.

Protein was determined by the method of Lowry *et al.*[14] using bovine serum albumin as standard.

The affinity gels were prepared from agarose–NH–CH_2–CH_2–NH–CO–CH_2–CH_2–COOH (AF 201; Affitron Corporation, Costa Mesa, Calif., U.S.A.). The ligand, at a concentration of 8 μmoles/ml, was firstly extended with 3,3'-diaminodipropylamine and then with succinic anhydride (100-fold excess of each reagent)[1,15]. [^{14}C]Trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride, prepared with $^{14}CH_3I$, or trimethyl-(*m*-aminophenyl)-ammonium chloride hydrochloride was coupled to the substituted gel[1] (85 μmoles inhibitor per ml gel). Scintillation counting showed that the concentration of bound, labelled inhibitor on the gel was 1.6 μmoles/ml. The scintillation solvent consisted of 2 parts toluene–0.4% PPO–5% dioxan to 1 part Triton X-100 (ref. 16).

For affinity chromatography, performed in a cold room at 5°, the enzyme solution was applied to the column in 5 m*M* Tris–HCl, pH 8.2, and eluted with 30–40 ml of this solvent. Up to 4% of the AChE was eluted at this stage. All subsequent solvents used (most of which contained 0.05% Triton X-100; see Results) were buffered to pH 8.2 with 5 m*M* Tris–HCl. Columns were of 1-cm diameter and consisted of 5.0–5.6 ml gel. Fraction volumes were generally between 5 and 10 ml. After completion of the experiment, the column was washed with 1 *M* NaCl–5 m*M* Tris–HCl, pH 8.2 (150–450 ml) and 5 m*M* Tris–HCl, pH 8.2 (150 ml) before re-use.

RESULTS AND DISCUSSION

A preliminary experiment showed that ox brain AChE had no affinity for a substituted gel without the AChE inhibitor attached to it, in 5 m*M* Tris–HCl pH 8.2, in contrast to the results of Schwyzer and Frank[6], who reported that a gel with a similar substitution pattern bound 15% of eel AChE applied to it at ionic strength 0.17.

Several chromatographic experiments were performed in which Triton X-100 (0.05%) was included in all solvents. The columns were eluted with sodium chloride solutions of increasing molarity, and in some cases this was followed by elution with an inhibitor of AChE, edrophonium chloride —ethyl-(3-hydroxyphenyl)-dimethylammonium chloride— in 1 *M* NaCl. The actual elution conditions, the yield of AChE at each stage, and the extent of purification achieved, are shown for each experiment

in Table I. The specific activity of the purified AChE was too high to be measured accurately, due to the limits of sensitivity of the protein assay (20 μg/ml) at the low concentrations of AChE in the solutions isolated. The extents of purification shown in Table I are, therefore, expressed as minimum values.

In Table I and the following discussion, a column of substituted gel coupled

TABLE I

ELUTION OF AChE FROM AFFINITY COLUMNS USING SOLVENTS CONTAINING 0.05% TRITON X-100

AChE source	*Activity (milliunits)*	*Column type*	*Recovery of AChE (%)*			*Purification*
			NaCl (<500 mM)	*1 M NaCl (30–45 ml)*	*Edrophonium* (50 ml)*	
Rat	2,500	*para*	>32**	14		>36
Ox	5,300	*para*	37***	52		>13
Ox	5,300	*meta*	0§	4	61	
Ox	16,900	*meta*	2§	5	55	>35
Rat	1,370	*meta*	12§	12	61	>17

* 1 *M* NaCl–10 m*M* edrophonium chloride.

** Gradient elution, 0–400 m*M* NaCl (250 ml); not all fractions assayed for enzyme activity; see Fig. 1.

*** 100 m*M* NaCl (56 ml).

§ 100 m*M* and 500 m*M* NaCl, total volume 75–90 ml.

with trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride or trimethyl-(*m*-aminophenyl)-ammonium chloride hydrochloride is described as a *para* column or *meta* column, respectively.

Effects of varying elution conditions

For the experiment in which a gradient elution (0–400 m*M* NaCl) was applied to a *para* column in the chromatography of rat brain AChE (Table I), the overlap of major protein and enzyme bands (Fig. 1) caused the yield of AChE with maximum specific activity (eluted with 160–400 m*M* NaCl) to be only 24%. On the other hand, highly purified ox brain AChE was recovered in greater than 50% yield by elution of a *para* column with 1 *M* NaCl (see Table I) after a preliminary elution with 0.1 *M* NaCl. Moreover, a considerable saving in time was achieved by this approach, due to the lower volumes of solvent required.

Structural specificity of the ligand

The superiority of *meta* columns in general (disregarding the nature of the spacer "arm") over corresponding *para* columns for purification of brain AChE has been reported by Chan *et al.*[9] and Yamamura *et al.*[11]. Berman and Young[1] made a similar observation with respect to bovine erythrocyte AChE. The experiments summarised in Table I confirm that AChE is bound more firmly to the *meta* column than to the *para* column. Theoretically a higher yield of pure AChE might be expected from the *meta* column, due to easier separation of enzyme from protein impurities.

For experiments with rat and ox brain AChE chromatographed on a *meta*

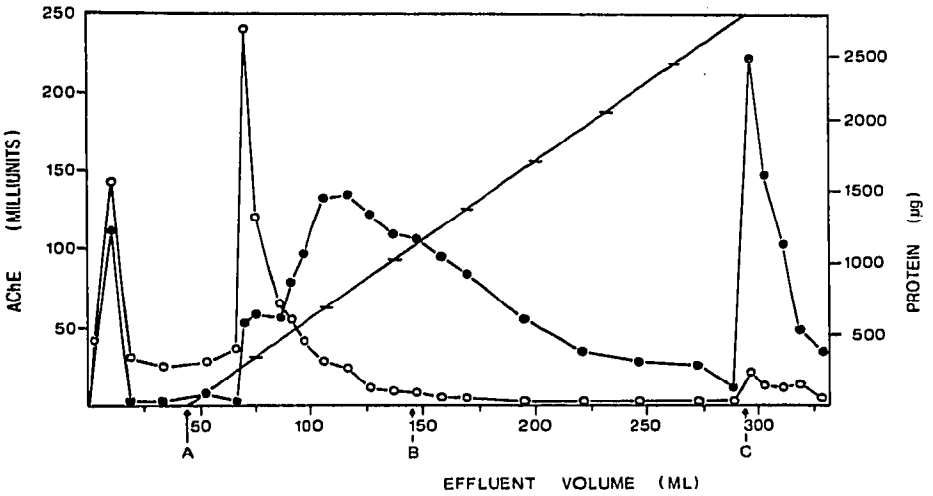

Fig. 1. Elution profile for chromatography of rat brain AChE on a *para* column. ●—●, AChE activity; ○—○, protein content. Each point represents a fraction from the column. +—+, Molarity of NaCl; increments of 50 m*M* are shown. A = Commencement of gradient elution, 0–400 m*M* NaCl. B = Point at which subsequent fractions during gradient elution have specific activity greater than 36 times higher than that of crude AChE. C = Elution with 1 *M* NaCl.

column, an inhibitor of AChE (edrophonium chloride) was required to elute the enzyme (Table I). Edrophonium chloride was then removed from the purified enzyme solution by diafiltration (see Experimental). The solution remaining in the diafiltration cell was removed immediately when the nitrogen pressure was released, but was found to possess low enzymatic activity (10% of AChE applied to the column). No increase in recovery was observed if the cell volume (5–12 ml) was kept constant with 160 ml of a solution of 150 m*M* NaCl–5 m*M* Tris–HCl–0.005% Triton, pH 8.2. Apparently AChE had adsorbed onto the membrane, even in the presence of Triton, for on repeated extraction of the membrane with stirred solutions of 1 *M* NaCl–5 m*M* Tris–HCl–0.05% Triton, pH 8.2, at atmospheric pressure for 1–3 h, a substantial amount of AChE could be recovered (Table I; figures indicate total recovery of AChE).

Chromatography in the absence of Triton X-100

Crone has shown that Triton-solubilized rat brain AChE adsorbs onto Sepharose on removal of Triton from the system[12], and can be recovered in 47% yield when Triton is added to the eluting solvent. An experiment in which extensively dialysed Triton-solubilized rat brain AChE was applied to a *para* column and elution performed with Triton-free solvents is summarised in Table II. A high ionic strength (1.0) was required to elute significant amounts (25%) of AChE, and a second peak of enzyme activity appeared when Triton was added to the solvent, consistent with the results of Crone. Addition of EDTA (1 m*M*) to the solvent (1 *M* NaCl), prior to introduction of Triton, had little effect on the elution of AChE, even though EDTA has been found to aid considerably the solubilization of brain AChE[10,17]. The material eluted with 1 *M* NaCl was re-chromatographed, after removal of NaCl by diafil-

TABLE II

AFFINITY CHROMATOGRAPHY IN THE ABSENCE OF TRITON X-100

Rat brain AChE (4,700 milliunits) was dialysed and applied to a *para* column.

Solvent	*Volume (ml)*	*Recovery of AChE (%)*
5 m*M* Tris–HCl	33	0
0–250 m*M* NaCl; stepwise increase in molarity	380	7
1 *M* NaCl	62	25
1 *M* NaCl–1 m*M* EDTA	40	4
1 *M* NaCl–0.02% Triton X-100	85	15
Total	600	51

tration. A linear gradient of NaCl (0–800 m*M*, 250 ml) was passed through the column, and yielded 5% of the applied activity. On elution with 1 *M* NaCl–0.05% Triton (27 ml) a further 10% only of enzyme was recovered. Apparently, absorption of AChE onto affinity gels in the absence of Triton is less reversible than in the case of gel permeation chromatography.

Purification was poor for affinity chromatography in the absence of Triton, and the fraction of highest specific activity, eluted with 1 *M* NaCl, was only a little over threefold purer than the crude enzyme, relative to protein content.

Yamamura *et al.* achieved a high purification of Triton-solubilized guinea-pig brain AChE using Triton-free solvents for elution[11]. However, the enzyme was applied to the column in a relatively high concentration of Triton (0.7%) and under their conditions a sufficiently high Triton concentration was probably present in the column at all stages of the chromatography.

Stability of purified AChE

The stability of purified ox brain AChE is uncertain. One sample appeared to be quite stable when kept at 5° for several weeks, but another sample lost 27% of its activity over 7 weeks in a deep freeze. The concentration of enzyme may be critical. Nevertheless, because of the possibility of deterioration of AChE with time, the yields in Tables I and II should be considered as minimum quantities, since most chromatographic experiments, including assays, took 6–8 days to complete.

Homogeneity of AChE

In some experiments, AChE was eluted from the columns as two separate fractions by different solvent systems, but there is no evidence to indicate that this is due to the presence of isoenzymes, a point that is being further examined.

To summarise, affinity chromatography is a simple, efficient means of purifying Triton-solubilized brain AChE, provided Triton is present at all stages of the chromatography. Further, the choice of eluting solvents is flexible, and depends on the relative importance attached to speed of operation (which is restricted by the slow flow-rates of affinity gel columns), purity of the enzyme, and yield of purified product. The quickest purification may not give the highest yield, and the most efficient purification (potentially one on a *meta* column) involved the problem of removing an enzyme inhibitor from the purified product.

ACKNOWLEDGEMENT

The authors are grateful to Mr. M. Poretski for technical assistance.

REFERENCES

1 J. D. Berman and M. Young, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 395.
2 Y. Dudai, I. Silman, N. Kalderon and S. Blumberg, *Biochim. Biophys. Acta*, 263 (1972) 138.
3 Y. Dudai, I. Silman, M. Shinitzky and S. Blumberg, *Proc. Nat. Acad. Sci. U.S.*, 69 (1972) 2400.
4 Y. Ashani and I. B. Wilson, *Biochim. Biophys. Acta*, 276 (1972) 317.
5 T. L. Rosenberry, H. W. Chang and Y. T. Chan, *J. Biol. Chem.*, 247 (1972) 1555.
6 R. Schwyzer and J. Frank, *Helv. Chim. Acta*, 55 (1972) 2678.
7 D. W. Reichard, B. T. Currie and C. A. Broomfield, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 32, No. 3, Part I (1973) Abstr. No. 1894.
8 M. J. Jung and B. Belleau, *Mol. Pharmacol.*, 8 (1972) 589.
9 S. L. Chan, D. Y. Shirachi, H. N. Bhargava, E. Gardner and A. J. Trevor, *J. Neurochem.*, 19 (1972) 2747.
10 S. L. Chan, D. Y. Shirachi and A. J. Trevor, *J. Neurochem.*, 19 (1972) 437.
11 H. I. Yamamura, D. W. Reichard, T. L. Gardner, J. D. Morrisett and C. A. Broomfield, *Biochim. Biophys. Acta*, 302 (1973) 305.
12 H. D. Crone, *J. Neurochem.*, 18 (1971) 489.
13 R. M. Dawson and H. D. Crone, *J. Neurochem.*, 21 (1973) 247.
14 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 P. Cuatrecasas, *J. Biol. Chem.*, 245 (1970) 3059.
16 M. S. Patterson and R. C. Greene, *Anal. Chem.*, 37 (1965) 854.
17 E. C. Hollunger and B. H. Niklasson, *J. Neurochem.*, 20 (1973) 821.