Biochemical Pharmacology, Vol. 26, pp. 1441–1443. Pergamon Press, 1977. Printed in Great Britain.

SHORT COMMUNICATIONS

Partial purification and separation of retinal acetylcholinesterase from butyrylcholinesterase by affinity chromatography

(*Received* 10 *September* 1976; *accepted* 10 *December* 1976)

The blood and tissues from several animals have been shown to possess both acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8) activity [1, 2]. The ratio of the two enzyme activities depends upon the tissue and species under study. For instance, the enzyme activity in brain tissue is largely AChE, while cerebrospinal fluid and plasma may contain both enzymes [1–3].

Affinity chromatography has been used in the partial purification of solubilized AChE from the electric tissue of *Electrophorus electricus* and from guinea pig brain [4–6]. In tissues where both enzymes are present, it would be desirable to separate AChE from BuChE during the process of enzyme purification. In this communication, we report on the partial purification and separation of AChE from BuChE in solubilized retinal tissue using affinity chromatography.

Most of the AChE in nervous tissue is associated with membranes [7, 8]. Thus, solubilization of the tissue is a requirement before the enzyme can be applied to the affinity column. Since Triton X-100 had previously been used to solubilize the enzyme from nervous tissue [7], we also used this detergent to solubilize retinal cholinesterase. Guinea pigs (Harthley strain, 300–400 g) were sacrificed by decapitation; the retina was removed from both eyes as described earlier [9]. Retinas from each animal were sonicated in 2 ml of 10 mM phosphate buffer with 50 mM NaCl, pH 7.4 (salt–phosphate buffer). After an aliquot was removed from each sonicate for enzyme activity, the remainder was divided into two equal volumes; one portion was incubated with 0.5% Triton for 30 min at 37°, while the second portion was incubated with buffer only. After incubation all samples were centrifuged at 105,000 *g* for 90 min at 5° in a Beckman ultracentrifuge, model L. The supernatants were separated from the pellets, and the pellets were resuspended by sonicating in salt–phosphate buffer. The cholinesterase activity was determined in all samples (with and without 5×10^{-4} mM BW284C51, a specific inhibitor of AChE activity) according to the method described by Siakotos *et al.* [1]. As a prelude to the purification studies, it was necessary to determine whether Triton affects enzyme activity. The cholinesterase activity [μmoles acetylcholine (ACh) hydrolyzed/hr/retina] before and after Triton treatment was found to be 10.1 (9.5 to 10.7) and 9.5 (9.2 to 9.8), respectively; each retina was found to contain approximately 2.5 mg protein. The effects of treatment with detergent on solubilization and on BuChE and AChE activity of retinal tissue are shown in Table 1. The results indicate that BuChE and AChE are largely membrane bound enzymes, because in the absence of detergent, the enzymes were present in the 105,000 *g* pellet. The higher specific activity of AChE in this pellet is due to the removal of extraneous proteins in the supernatant. Solubilization of the enzymes is indicated by the presence of both enzymes in the 105,000 *g* supernatant after treatment of the crude sonicate with Triton (Triton treatment solubilized 44 per cent of the pellet proteins). The inhibition by BW284C51 indicates that about 6 per cent of the total hydrolysis of ACh is catalyzed by BuChE.

Efforts were made to obtain [^{14}C]L-leucine-labeled retinal cholinesterase for solubilization and affinity

Table 1. Contribution of butyrylcholinesterase to total enzyme activity in retinal tissue

State of retinal tissue	Treatment	Cholinesterase activity [^{14}C]ACh (μmoles hydrolyzed/mg protein/hr)	Cholinesterase activity [^{14}C]BuCh (μmoles hydrolyzed/mg protein/hr)
Crude sonicate	None	4.00 (3.70–4.30)*	0.80 (0.74–0.86)*
Crude sonicate	BW284C51	0.24 (0.18–0.30)	0.73 (0.67–0.79)
Crude sonicate	Triton X-100†	4.00 (3.80–4.20)	0.82 (0.63–1.01)
Crude sonicate	Triton X-100 + BW284C51‡	0.26 (0.22–0.30)	0.77 (0.60–0.94)
Supernatant	None	1.00 (0.90–1.10)	0.23 (0.13–0.33)
Supernatant	BW284C51	0.07 (0.06–0.08)	0.21 (0.11–0.31)
Supernatant	Triton X-100	4.80 (4.40–5.20)	1.07 (0.86–1.28)
Supernatant	Triton X-100 + BW284C51	0.37 (0.27–0.47)	0.97 (0.78–1.16)
Pellet	None	7.20 (6.70–7.70)	0.68 (0.59–0.77)
Pellet	BW284C51	0.46 (0.40–0.52)	0.63 (0.50–0.76)
Pellet	Triton X-100	0.30 (0.00–0.60)	0.02 (0.01–0.03)
Pellet	Triton X-100 + BW284C51	0.06 (0.04–0.08)	0.04 (0.01–0.07)

* Each study was conducted on six animals; data are presented as the mean with 95 per cent confidence limits.

† Treatment with Triton X-100 was as follows: crude sonicate was incubated with 0.5% Triton for 30 min at 37°, then centrifuged at 105,000 *g* to yield supernatant and pellet fractions.

‡ When BW284C51 was used, it was added to the enzyme preparation and incubated for 10 min at 37° prior to assaying for enzyme activity.

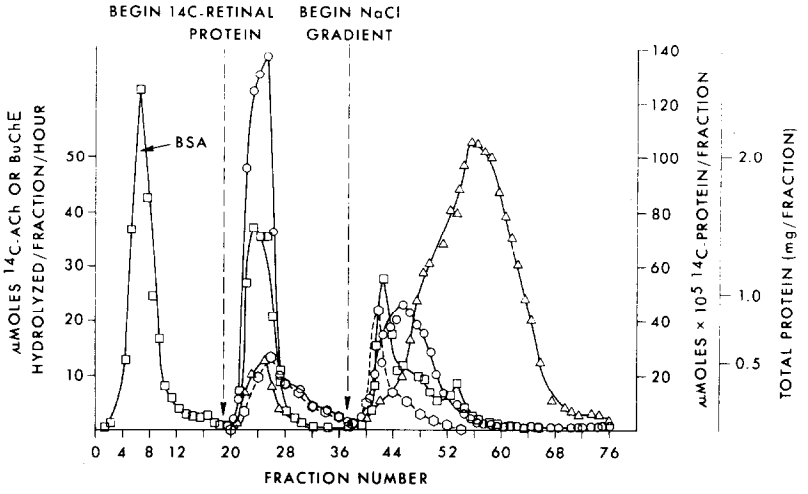

Fig. 1. Purification of cholinesterase from solubilized ^{14}C-labeled retinal tissue by affinity chromatography. AChE activity (△——△); BuChE activity (◯——◯); total protein (□——□); and ^{14}C-protein (○——○).

chromatography by injecting the amino acid directly into the vitreous chamber of the eye. After the guinea pigs had been anesthetized with ether, one μCi [^{14}C]L-leucine, sp. act. 27.7 mCi/m-mole, was injected into the eye in a volume of 0.02 ml. Two hr after [^{14}C]L-leucine administration, twelve retinas were removed, rinsed with copious amounts of 0.9% NaCl, pooled and sonicated in salt–phosphate buffer. The sonicated tissue was centrifuged at 105,000 *g* for 90 min at 5°. After removing the supernatant, the pellet was dispersed and sonicated in 10 ml salt–phosphate buffer containing 0.5% Triton and incubated for 30 min at 37°. The preparation was then diluted with additional buffer to give a final concentration of 0.1% detergent. The Sepharose complex used for affinity chromatography was prepared as described by Broomfield *et al.* [5]. It was aminobutyl-Sepharose 4-B, to which a specific competitive inhibitor of cholinesterase (*p*-carboxyphenyl trimethyl ammonium iodide, *p*-quat) was covalently attached. An 0.4 × 18.7 cm column of the material was prepared and treated with bovine serum albumin (BSA) to cover non-specific binding sites. After applying 10 ml BSA (1.3 mg/ml) in salt–phosphate buffer containing 0.1% Triton (column buffer) to the column, unbound BSA was removed by washing the column with column buffer until only traces of protein were present in the effluent (Fig. 1). The ^{14}C-solubilized retinal tissue was then applied to the column at a rate of 0.16 ml/min; 1.6-ml fractions were collected. After washing the column virtually free from unbound ^{14}C-proteins, the column was eluted with a linear gradient of NaCl (0–1 M). Excess salt was removed from the enzyme rich fractions by dialyzing overnight in large volumes of column buffer. All fractions were assayed for ^{14}C-protein, BuChE and AChE activity and total protein. Unbound proteins and 5 per cent of the total cholinesterase activity applied to the column were removed by washing (fractions 10 through 38). Fifty-six per cent of the total BuChE activity was present in these fractions. The remaining BuChE activity was fairly well separated from the AChE activity with a peak of activity in fraction 42. Only AChE activity was present in fractions 52–76 with the peak activity in fractions 54–56. In order to further purify the AChE, fractions 52 through 68 were pooled and divided into two portions. One portion was applied to a virgin column not pretreated with BSA, while the second portion was placed on a column pretreated with BSA as before. The columns were washed to remove unbound ^{14}C-proteins and eluted with a linear gradient of choline chloride (0–1 M). Results obtained upon elution from the virgin column are illustrated in Fig. 2. Elution using choline chloride produced an enzyme peak (tube 22) with an activity of 357 μmoles ACh hydrolyzed/hr/mg of protein. As the concentration of choline increased up to fraction 36, increasing amounts of ^{14}C-protein were also eluted. Elution of proteins from the BSA-pretreated column (not shown) with choline chloride resulted in an elution pattern for AChE activity and ^{14}C-proteins similar to that

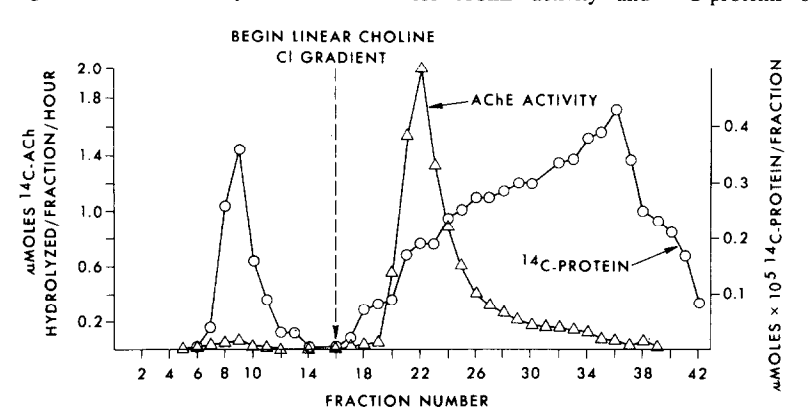

Fig. 2. Rechromatography of partially purified retinal AChE on a virgin affinity column and elution by choline chloride (0–1 M).

Table 2. Cholinesterase activities and protein at various stages of enzyme purification

Treatment*	Specific enzyme activity (μmoles substrate hydrolyzed/hr/mg)		Total enzyme activity (μmoles substrate hydrolyzed/hr)		Total protein (mg)
	BuChE	AChE	BuChE	AChE	
Before chromatography					
Crude sonicate	0.45	4.0	17.5	155.0	38.4
Triton-treated pellet†	0.98	8.6	14.7	130.0	15.1
Initial chromatography					
Fractions 19–36	0.15	0.9	1.1	6.7	7.3
Fractions 37–51	1.85	3.8	9.8	20.3	5.3
Fractions 52–68	0.17	46.0	0.2	55.5	1.2
Rechromatography					
Fraction 22		357.0		2.0	0.0056
Fractions 18–39				9.5	

* For chromatography details, see Figs. 1 and 2.
† Enzyme activities applied to the affinity column.

observed in Fig. 2, but the enzyme activity of the peak fractions was only 15 μmoles ACh hydrolyzed/hr/mg of protein, suggesting that BSA was released along with the enzyme by choline chloride.

The data in Table 2 provide additional information on the recovery of esterase activities and protein during the various stages of enzyme purification. The AChE and BuChE activities recovered from the initial chromatography were 64 per cent and 75 per cent respectively. The recovery of total and ^{14}C-protein was 91 and 79 per cent, respectively. The higher recovery of total protein probably represents further removal of BSA from the column. Additional esterase activity and protein could be eluted with 3 M NaCl. Due to the very low concentrations of protein in the fractions collected during choline elution, total protein was only measured in the tube having the peak enzyme activity; 76 per cent of the enzyme activity applied was recovered in choline eluates.

Although the enzyme derived from normal retinal tissue was highly purified by affinity chromatography, the amount of ^{14}C-protein in the purified AChE fraction (Fig. 2, tube 22) was very low (0.2×10^{-5} μmoles). This low level and the fact that there was no close correlation between AChE activity and ^{14}C-protein (Fig. 2) indicate that very little if any AChE was synthesized in the normal animals during the 2 hr period. In contrast, the "rapid" return of AChE activity in animals poisoned with pinacolyl methylphosphonofluoridate (Soman) [10] has provided presumptive evidence of *de novo* synthesis during a 2 hr period. This synthesis could be due to induction of synthesis either by Soman or by abnormally low levels of functional enzyme [2, 11]. Preliminary attempts to purify retinal cholinesterase from Soman-treated animals resulted in abnormally low specific activities when compared to the purification realized with control tissue (90-fold purification). This finding suggested that Soman-inactivated enzymes and/or proteins were absorbed to and eluted from the affinity chromatography column right along with the active enzyme. As a result, retinal tissue from unpoisoned animals was used in this investigation.

Both AChE activity and BuChE activity are found in guinea pig retinal tissue and are largely membrane bound; BuChE activity contributes to only about 6 per cent of the total hydrolysis of ACh.

The application of affinity chromatography in purification of retinal AChE enabled us to separate the majority of extraneous proteins from the peak enzyme fractions. About 99.5 per cent of the proteins were eliminated, with the peak AChE fraction showing a 90-fold increase in specific activity. This technique of protein purification also resulted in the separation of most of the AChE from BuChE.

Biochemistry Group, Biomedical Laboratory, Edgewood Arsenal, Aberdeen Proving Ground, MD 21010 — LARREL W. HARRIS
Department of Biology, Towson State University, Towson, MD 21204 — FREDERICKA KUNDIG
Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724, U.S.A. — HENRY I. YAMAMURA

REFERENCES

1. A. N. Siakotos, M. G. Filbert and R. Hester, *Biochem. Med.* **3**, 1 (1969).
2. T. L. Yaksh, M. G. Filbert, L. W. Harris and H. I. Yamamura, *J. Neurochem.* **25**, 853 (1975).
3. S. Johnson and F. F. Domino, *Clinica chim. Acta* **35**, 421 (1971).
4. N. Kalderon, I. Silman, S. Blumberg and Y. Dudai, *Biochim. biophys. Acta* **207**, 560 (1970).
5. C. A. Broomfield, J. D. Morrisett, D. W. Reichard and H. I. Yamamura, *Trans. Am. Soc. Neurochem.* **3**, 59 (1972).
6. H. I. Yamamura, D. W. Reichard, T. L. Gardner, J. D. Morrisett and C. A. Broomfield, *Biochim. biophys. Acta* **302**, 305 (1973).
7. H. D. Crone, *J. Neurochem.* **18**, 489 (1971).
8. L. W. Harris, *M.S. Thesis*, Towson State University, Towson, Md. (1975).
9. L. W. Harris, J. H. Fleisher and H. I. Yamamura, *Eur. J. Pharmac.* **14**, 38 (1971).
10. L. W. Harris, H. I. Yamamura and J. H. Fleisher, *Biochem. Pharmac.* **20**, 2927 (1971).
11. W. Domschke, G. F. Domack, S. Domschke and W. D. Erdmann, *Arch. Tox.* **26**, 142 (1970).