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Isolation of acetylcholinesterase G4 and G1 molecular isoforms from rat cortex

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KEY WORDS acetylcholinesterase; chromatography; ultracentrifugation; rats

ABSTRACT

AIM: To isolate the G4 and G1 molecular forms of acetylcholinesterase in rat cortex for interpreting the therapeutical effect of the AChE inhibitor drugs. **METHODS**: Size exclusion chromatography and ultracentrifugation were used to isolate and ascertain the G4 and G1 molecular forms of AChE. **RESULTS**: After size exclusion chromatography, two AChE active peaks were gotten. The putative molecular weights of the two highest AChE active fractions were around M_r 239 000 and M_r 68 000 respectively, which represented G4 and G1 AChE isoforms. The sedimentation coefficients of the M_r 239 000 and M_r 68 000 fractions were 10 S and 4 S, which also assigned to G4 and G1 AChE isoforms. **CONCLUSION**: The G4 and G1 isoforms of AChE could be gotten by size exclusion chromatography method.

INTRODUCTION

A decrease of acetylcholine in the brain of patients with Alzheimer disease (AD) has led to extensive research in possible therapeutic approaches with anticholinesterase drugs towards improving the effects of the dwindling amount of acetylcholine[1-2]. Currently, acetylcholinesterase (AChE) inhibitors are the most promising class of drugs for the treatment of AD. AChE, however, can be separated into multiple molecular forms by means of their sedimentation coefficients[3-4]. In mammalian brain, the bulk of AChE is the G4 (10 S) and G1 (4 S) form[5-6]. The presence of multiple molecular forms of AChE makes it difficult to interpret the therapeutical effect of the AChE inhibitor drugs on the brain.

It was reported that AChE inhibitors had differential selectivity on AChE molecular isoforms[7-9]. For interpreting the effect of AChE inhibitor drugs on the brain, exploring the method of isolating G4 and G1 molecular forms of AChE was the first important and indispensable step. In the previous study, G4 and G1 molecular forms of AChE were isolated by 5 % - 25 % (w/v) sucrose gradient ultracentrifugation according to the different sedimentation coefficients of G4 and G1 isoforms, and the relative inhibitory potency of several clinically significant AChE inhibitors on the G4 and G1 isoforms was investigated[7,10].

Several procedures are currently available for isolation of G4 and G1 isoforms from a variety of sources. In the present study, we initiated to isolate the G4 and G1 isoforms of AChE by size exclusion chromatography according to their different molecular weights, which so far had not been extensively established by other researchers.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (n = 10, weighting 200 g ± 35 g, Grade Ⅱ, Certificate № 003) were supplied by Shanghai Experimental Animals Center, Chinese Academy of Sciences.

Tissue sample preparation Rats were decapitated and cortex was removed on the ice. The cortex was homogenized in five volumes of phosphate saline buffer 75 mmol/L, pH 7.4. The homogenate was centrifuged at 13 000 × g for 30 min (4 ℃), and its supernatant was collected and stored at -20 ℃.

AChE activity assay The AChE activity was measured by the principle of Ellman method[11]. AChE incubated with acetylthiocholine iodide 0.3 mmol/L (Sigma Chemical Co) and tetraisopropyl pyrophosphoramide 50 μmol/L (Sigma Chemical Co), a selective inhibitor of butyrylcholinesterase in phosphate

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buffer 10 μmol/L, pH 7.5 for 150 s at 37 ℃. The 5,5′-dithio-*bis* (2-nitrobenzoic acid) was added to reaction mixture to produce the yellow anion of 5-thio-2-nitro-benzoic acid. The rate of color production was measured with SPECTRAmax 96-well plate reader from Molecular Devices (Sunnyvale, CA, USA) at 412 nm. One unit of AChE activity was defined as the number of micromoles of acetylthiocholine hydrolyzed per second and per milligram protein at 37 ℃.

Protein assay Protein concentration was measured with the Coomassie brilliant blue protein-binding method[12] using bovine serum albumin as standard.

Size exclusion chromatography For size exclusion chromatography, a HiPrep™ 16/60 Sepharcryl™ S-200 HR column (Amersham Pharmacia Biotech AB, Sweden) was equilibrated with phosphate saline buffer (pH 7.2, 50 mmol/L) containing edetic acid (5 mmol/L) with FPLC system (Amersham Pharmacia Biotech AB, Sweden). The supernatant extracted from rat cortex was thawed at 4 ℃ and filtered with 0.2 μm syringe filter. Three mL of filtrate was charged on the column. The absorbed protein was eluted with the same buffer as above with the flow rate of 0.5 mL /min. The fractions of 1.5 mL were collected and each fraction was assayed for AChE activity. In order to determine the approximate molecular size of AChE, protein markers such as: myosin, M_r 200 000; calmodulin-binding protein, M_r 130 000; rabbit phosphorylase b, M_r 97 400; bovine serum albumin, 66 200 M_r; rabbit actin, M_r 43 000, and bovine carbonic anhydrase, M_r 31 000 were charged onto the same column and eluted with the same conditions as above.

Sedimentation analyses The fractions eluted from HiPrep™ 16/60 Sepharcryl™ S-200 HR column with AChE activity were layered onto 5 % – 25 % (w/v) sucrose gradients in phosphate saline buffer (50 mmol/L, pH 7.2) containing edetic acid (5 mmol/L). Ultracentrifugation was performed at 160 000 × *g* for 21 h, 4 ℃ in a Beckman SW 41 Ti rotor. About 26 fractions were collected from the flank of each tube and assayed for AChE activity. Catalase from beef liver (11.3 S) and bovine serum albumin (4.3 S) were used as internal sedimentation markers.

RESULTS

Chromatography of AChE Molecular polymorphism of AChE could be elucidated by the procedure of size exclusion chromatography on a HiPrep™ 16/60 Sepharcryl™ S-200 HR column. Protein concentration traces showed three major peaks eluting at fractions 19 – 23, 27 – 35, and 37 – 45 (Fig 1). AChE activity assay of aliquots from all the fractions collected revealed two major active peaks, corresponding to fractions 25 – 27 and 37 – 38 (Fig 1). The fractions containing the highest AChE activity were at 25/26 and 37/37.

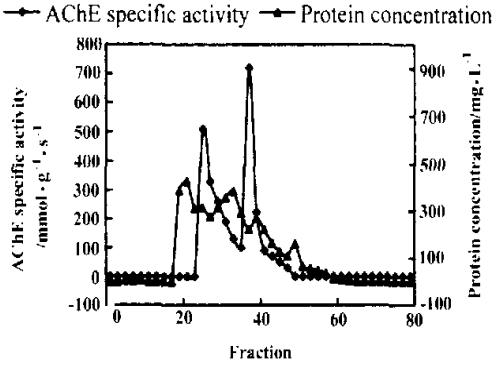

Fig 1. Elution profile of AChE molecular forms in rat cortex. AChE forms were separated by a HiPrep™ 16/60 Sepharcryl™ S-200 HR column chromatography. Protein concentration was measured with the Coomassie brilliant blue protein-binding method.

Chromatography of protein markers With the aim of trying to elucidate the molecular weight of the different AChE peaks, protein markers such as: myosin, M_r 200 000; calmodulin-binding protein, M_r 130 000 rabbit phosphorylase b, M_r 97 400; bovine serum albumin, M_r 66 200; rabbit actin, M_r 43 000, and bovine carbonic anhydrase, M_r 31 000, were loaded on the HiPrep™ 16/60 Sepharcryl™ S-200 HR column and eluted with same conditions as above. A good linear relationship was obtained with the protein markers used. Upon plotting the elution profile of the separated samples against this linear graph, a putative molecular mass of M_r 239 000 and M_r 68 000 was calculated for rat cortex AChE, respectively (Fig 2).

Ultracentrifugation In order to ascertain the sedimentation coefficients of different AChE fractions, the two AChE active fractions separated from HiPrep™ 16/60 Sepharcryl™ S-200 HR column were collected respectively and layered onto 5 % – 25 % (w/v) sucrose gradients in phosphate saline buffer (50 mmol/L, pH

7.2) containing edetic acid (5 mmol/L). Centrifugation was performed at 160 000 × g for 21 h. About 26 fractions were collected from the flank of each centrifugal tube and assayed for AChE activity. Typical profiles of the each peak of AChE in rat cortex were shown in Fig 3. The sedimentation coefficients of two fractions from HiPrep™ 16/60 Sepharcryl™ S-200 HR column chromatography were 10 S and 4 S, which assigned to G4 and G1 AChE forms[5-6].

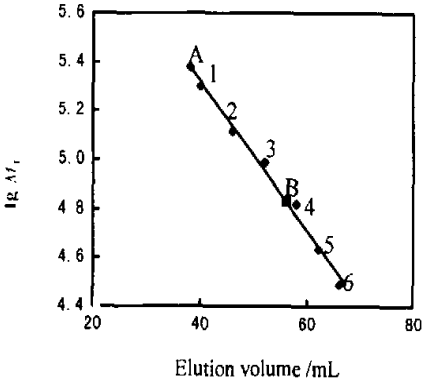

Fig 2. Size exclusion chromatography of rat cortex AChE molecular isoforms. Open diamonds indicated point of elution of the following protein markers with their respective molecular weights: (1) myosin, M_r 200 000; (2) calmodulin-binding protein, M_r 130 000; (3) rabbit phosphorylase b, M_r 97 400; (4) bovine serum albumin, M_r 66 200; (5) rabbit actin, M_r 43 000, (6) bovine carbonic anhydrase, M_r 31 000. The filled diamond (A) indicated the point of elution of the first AChE highest active fraction from the HiPrep™ 16/60 Sepharcryl™ S-200 HR column, whilst the filled square (B) indicated the second highest AChE active fraction.

DISCUSSION

It is always the case that whenever one works on the material with biological significance, the first important and indispensable step in this task is to explore methods for its optimal separation. To gain insights into the AChE isoforms selective inhibition of inhibitors, isolating the G4 and G1 molecular forms of AChE is essential.

Size exclusion chromatography separates proteins on the basis of their molecular size. HiPrep™ 16/60 Sepharcryl™ S-200 HR column provides good preparative purifications by its excellent separation properties with narrow particle size distribution and steep selective curves. In this study, size exclusion chromatography was used to separate AChE to its isoforms for which have different molecular weights. It was shown two AChE active peaks after chromatography. The putative molecular weights of the two highest AChE active fractions were around M_r 239 000 and M_r 68 000 compared to the protein markers eluted with the same conditions. The M_r 239 000 and M_r 68 000 fractions were represented G4 and G1 forms respectively, which was more or less coincidental with the previous documents[4,13].

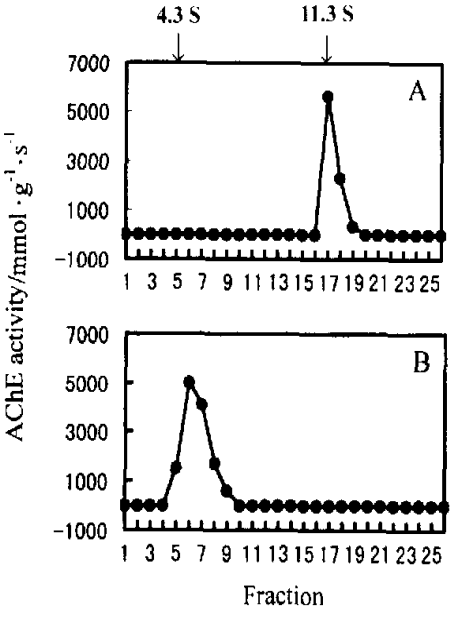

Fig 3. (A) Sedimentation profile of the first AChE active fractions from HiPrep™ 16/60 Sepharcryl™ S-200 HR column; (B) Sedimentation profile of the second AChE active fractions from HiPrep™ 16/60 Sepharcryl™ S-200 HR column. Sedimentation coefficients were assayed by ultracentrifugation in a Beckman SW 41 Ti rotor with beef liver catalase (11.3 S) and bovine serum albumin (4.3 S) as internal.

On the other hand, AChE was separated into multiple molecular forms by means of their sedimentation coefficients. The sedimentation coefficients of G4 and G1 were 10 S and 4 S[3]. To ascertain the sedimentation coefficients of AChE with different molecular weights, ultracentrifugation was performed. As expected, the two AChE active peaks were 10 S and 4 S respectively, which assigned to G4 and G1 AChE forms. As a result, the G4 and G1 isoforms of AChE could be gotten with size exclusion chromatography method.

In the previous study, the separation of G1 and G1

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isoforms of AChE was achieved with ultracentrifugation method depends on their sedimentation coefficients[8,9]. In this study, we isolated the G4 and G1 isoforms of AChE with size exclusion chromatography according to their different molecular weights. Sepharcryl size exclusion chromatography is a faster method than ultracentrifugation by which getting AChE isoforms was only 4 h, whereas ultracentrifugation was 18 - 21 h[7-9]. Moreover Sepharcryl column could give high-resolution with biochemical stability. Separation of G4 and G1 isoforms of AChE by size exclusion chromatography appears to be appropriate method. These size-exclusion-chromatographied samples could be used for detecting the AChE isoforms selectivity inhibition of inhibitors. It will be of benefit to interpret the therapeutical effect of the AChE inhibitor drugs for AD.

REFERENCES

1 Candy JW, Perry RH, Perry EK, Irving D, Blessed G, Fairbrain AF, *et al*. Pathological changes in the nucleus basalis of Meynert in Alzheimer's and Parkinson's disease. J Neurol Sci 1983; 59: 277 - 89.
2 Hammod P, Brimijoin S. Acetylcholinesterase in Huntington's disease and Alzheimer's disease-simultaneous enzyme assay and immunoassay of multiple brain regions. J Neurochem 1988; 50: 1111 - 6.
3 Brimijoin S. Molecular forms of acetylcholinesterase in brain, nerve and muscle: nature, localization and dynamics. Prog Neurobiol 1983; 21: 291 - 322.
4 Massoulie J, Bon S. The molecular forms of cholinesterase and acetylcholinesterase in vertebrate. Annu Rev Neurosci 1982; 5: 57 - 106.
5 Bon S, Vigny M, Massoulie J. Asymmetric and globular forms of AChE in mammals and birds. Proc Natl Acad Sci USA 1979; 76: 2540 - 50.
6 Grassi J, Vigny M, Massoulie J. Molecular forms of acetylcholinesterase in bovine caudate nucleus and superior cervical ganglion: solubility properties and hydrophobic character. J Neurochem 1982; 387: 457 - 69.
7 Bajgar J, Bisso GM, Michalek H. Differential inhibition of rat brain acetylcholinesterase molecular forms by 7-methoxytacrine *in vitro*. Toxicol Lett 1995; 80: 109 - 14.
8 Ogane N, Giacobini E, Messamore E. Preferential inhibition of acetylcholinesterase molecular forms in rat brain. Neurochem Res 1992; 17: 489 - 495.
9 Taylor P, Sckhumacher M, Maulet Y, Newton M. A molecular perspective on the polymorphism of acetylcholinesterase. Trends Pharmacol Sci 1986; 8: 321 - 3.
10 Polinsky RJ. Clinical pharmacology of rivastigmine: a new generation acetylcholinesterase inhibitors for the treatment of Alzheimer's disease. Clin Ther 1998; 20: 634 - 47.
11 Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7: 88 - 95.
12 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 - 54.
13 Zhu MC, Xin YB, Sun MJ, Fang YZ. Purification and properties of acetylcholinesterase from human brain. Sci in China (Series B) 1993; 36: 1207 - 15.

大鼠皮层乙酰胆碱酯酶 G4 和 G1 分子亚型的分离

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关键词 乙酰胆碱酯酶；色谱法；超速离心法；大鼠

目的：分离大鼠皮层乙酰胆碱酯酶 G4 和 G1 分子亚型以阐明乙酰胆碱酯酶抑制剂药物的作用. **方法**：使用分子排阻层析和超速离心方法分离和确证乙酰胆碱酯酶的 G4 和 G1 分子亚型. **结果**：经过分子排阻层析得到两个乙酰胆碱酯酶的活性峰，与标准蛋白质的洗脱峰相比，推测其分子量为 M_r 239 000 和 M_r 68 000. 通过超速离心法确证其沉降系数为 10 S 和 4 S，分别为乙酰胆碱酯酶 G4 和 G1 分子亚型. **结论**：乙酰胆碱酯酶的 G4 和 G1 分子亚型可以使用分子排阻层析的方法分离得到.

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