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A SIMPLE AND COST EFFECTIVE WAY TO PURIFY ACETYLCHOLINESTERASE ENZYME FROM BOVINE (*BOSTAURUS*) BRAIN

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ABSTRACT

AChE is the vital enzyme in the cholinergic nervous system. Hydrolysis of the Acetylcholine (ACh) in the nerve ending is the main function of this enzyme. By this way it maintains homeostasis of neurotransmitter in both central nervous system and peripheral nervous system. In both pharmacological and toxicological way AChE provides a vital role. The aim of the study was to develop a simple protocol for the purification of Acetylcholinesterase enzyme (AChE) and to extent an easy and worthy purification method for further characterization. The purification of AChE from bovine brain (Bostaurus) using gel chromatography on Sephadex G-200 was studied. The affinity matrix was synthesized by coupling an inhibitor, acetate activated Sephadex G-200 with flow rate of 0.5ml/min. In this study the enzymes were purified 5 folds with aspecific activity of 18.95 UI/mg proteins.

KEY WORDS

Acetylcholinesterase, Enzyme purification, Bovine brain, Gel chromatography, Sephadex G-200.

INTRODUCTION

Acetylcholinesterase, also known as acetylhydrolase, is a hydrolase type enzyme that hydrolyzes theneurotransmitter acetylcholine in the nerve endings.It belongs to enzyme familycarboxylesterase.[1,2] Itis the primary target of inhibition by organophosphoruscompounds such as nerve agents and pesticides. AChE is found at mainly neuromuscular junctions and cholinergic brain synapses between brain cells and muscle cells.[3-5]

AChE has a very strong catalytic activity in the nerve endings. A single molecule of AChE is capable to catalyze 25000 molecules within a second.[6-9] During neurotransmission, Acetylcholine is released from the nerve into the synaptic clefts and binds to the ACh receptor on the post synaptic membrane. AChE is also located on the post synaptic membrane and terminates the transmission by hydrolyzing the ACh by Acetyl co-A and Choline. The choline is taken up by the pre-synaptic membrane and ACh is synthesized by combining with acetyl co-A through the action of cholineacetyltransferase.[10-12]

AChE is mainly found in conducting tissues like nerve and muscle cells, central and peripheral tissue, motor and sensory fibers and cholinergic and non-cholinergic fibers. Activity of AChE is higher in motor neurons than in sensory neurons.[13-15]

AChE is encoded by single AChE gene in mammals, but in some intervertibrates multiple AChE gene were found. There are 3 known forms of AChE are $AChE_T$, $AChE_H$ and $AChE_R$. $AChE_T$ is the major form of AChEfound mostly in brain muscle and other tissues. $AChE_H$is primarily found in erythroid tissue and $AChE_R$found in *Torpedo sp.*and mice, which is thought to be involved in the stress response and possibly inflammatory response.[16,17]AChE is a

very important enzyme in biological systems because of its nerve impulse transmission. Besides this it is the target for organophosphate and carbamate pesticides.

AChE was first isolated by the extraction from the electric organ of *Torpedo marmorata* in 1938. Purification of this enzyme from electric tissue of *Electrophorus electricus* by fractional precipitation of the enzyme at carefully controlled ammonium sulphate concentration and controlled pH values routinely give a specific increase in specific activity of the enzyme. But this method provides a lots of background noise in spectrophotometer. Furthermore this process is a laborious process and also not a cost effective way. The result is in the enzyme yield of less than 10%.In the current study a cost effective and less laborious method we develop to purify AChE from a very common and affordable source, bovine brain.

MATERIALS

Bovine brain,Wash buffer,Extraction buffer,Dilution buffer,DTNB (Sigma chemical company, USA),ATCI (Sigma chemical company, USA),Ammonium Sulphate (Sigma chemical company, USA) and Sephadex G-200 gel (Sigma chemical company, USA).

Formulation of reagents:

Wash buffer: 10mM Tris buffer.

Extraction Buffer: 50mM Tris buffer + 10% Triton-X + 50mM $MgCl_2$ + 50mM NaCl.

DTNB: 0.7mM solution.

ATCI: 0.35mM solution.

Procedure:

A. Preparation of Crude enzyme extract:

The bovine brain (10gm) was weighted, cut into small pieces and grinned into a mortar and pestle with 50ml of homogenization buffer, pH 7.4. The temperature was maintained at 4^0C by putting ice in the outer chamber of the homogenizer. The suspension was filtered through double layer of muslin cloth in the cold room. The filtrate was collected and clarified further by centrifugation at 10000 rpm for 25 minutes at 4^0C. This clear supernatant was used as crude enzyme extract.

B. Precipitation with Ammonium Sulphate:

The crude extract was precipitated with super saturated ammonium sulphate salt. Because of low density, as compare to ammonium sulphate solution, the precipitate rose to the surface on standing. Centrifuge this mixture at 3000 rpm for 25 min. The bottom layer was withdrawn. Finally the precipitate dissolved in homogenization buffer and used as a one-step purified enzyme sour

C. Gel Filtration:

a. **Activation of gel powder:**
Sephadex G-200 powder was suspended in 10% acetic acid in a beaker and left it to swell for over-night. It was stirred by glass rod after short intervels to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality.

b. **Packing of the column:**
This is very critical step in all types of column chromatographic experiment. The gel suspension was taken in a filtering flask and desecrated by vacuum pump. A column of desired length was packed uniformly with gel suspension.

c. **Equilibrium of the column:**
After completion of column packing it was equilibrated with the eluate buffer (0.1M sodium acetate buffer, pH 5.2). The buffer was contained to run through the column until the pH of elute become same as the pH of elute buffer.

d. **Application of the sample:**
Before loading the sample, the outlet tube of the column was opened and the eluent buffer from the top of the gelbed was

allowed to diffuse into the gel. The crude extract was then loaded on the top of the bed. After diffusion of the sample, about 1ml of the eluent buffer was poured on the top of the gel bed and was allowed to diffuse. Then the additional amount of the buffer was added, so that the space about 3-4cm above the gel bed was filled with eluent. The buffer was allowed to flow continuously at a flow rate of 0.5ml/min. Column fractions are collected as a pure source of enzyme.

RESULTS AND DISCUSSION

Bovine brains were used as a source of acetylcholinesterase enzyme. Crude enzyme extract was prepared by homogenization of bovine brain with Tris-HCl buffer containing Triton X-100 and centrifugation at 10,000 rpm for 20 min. The enzyme solution was the subjected to two steps for purification.

Step I: Ammonium sulphate precipitation: Crude enzyme extract was 70% saturated with ammonium sulphate, left for few hours at 4^0C to complete precipitation of proteins and centrifuged at 12,000 rpm for 30 minutes. The resulting precipitates were solubilized with the extraction buffer that yielded more than two fold purification of acetylcholinesterase enzyme as shown in the Table-1.

Step II: Size exclusion chromatography: Enzyme solution, after purification with ammonium sulphate precipitation method, was applied to the size exclusion gel chromatography using Sephadex G-200 as the stationery phase previously equilibrated with elution buffer for further purification. More than 120 fractions of 5 ml elute were collected and their protein concentration as well as acetylcholinesterase activity was measured. The elution profile of enzyme solution on Sephadex G-200 has been shown in Figure 1.

There was no activity found in the first 53 fractions. Acetylcholinesterase activity began to appear afterward, increased gradually and reached at the peak level at around 70^{th} fraction. A second peak appeared at fraction 98^{th} and reached at the highest level at fraction 99, representing the lower activity of enzyme. This result indicated that the acetylcholinesterase, eluted in two different peaks, were of two different types having two different molecular weights. The existence of acetylcholinesterase in different molecular forms is well established.

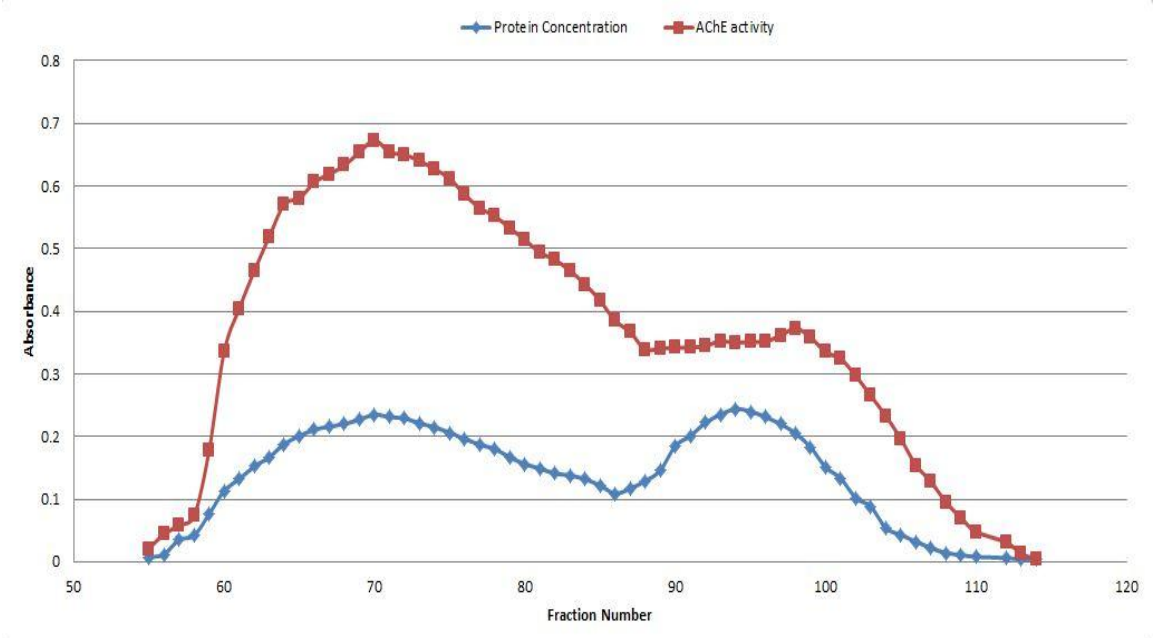
Table 1: Acetylcholinesterase Activity of the different fractions obtained from Sephadex G-200 column chromatography of enzyme solution

Number of Fractions	Protein concentration (Abs taken at 280nm)	Acetylcholinesterase activity (Abs taken at 412nm)		Difference	Enzyme activity (UI/L)
		Positive Value	Background		
1-53	0	0	0	0	0
54	0.003	0.008	0.002	0.006	140.76
55	0.007	0.032	0.011	0.021	492.66
56	0.012	0.064	0.020	0.044	1032.24
57	0.035	0.089	0.031	0.058	1360.68
58	0.042	0.112	0.039	0.073	1712.58
59	0.076	0.218	0.041	0.177	4152.42
60	0.113	0.380	0.045	0.335	7859.10

61	0.132	0.456	0.052	0.404	9477.84
62	0.152	0.521	0.057	0.464	10885.44
63	0.167	0.584	0.065	0.519	12175.74
64	0.188	0.639	0.068	0.571	13395.66
65	0.201	0.651	0.071	0.580	13606.80
66	0.211	0.678	0.072	0.606	14216.76
67	0.216	0.695	0.076	0.619	14521.74
68	0.220	0.712	0.079	0.633	14850.18
69	0.227	0.735	0.081	0.654	15342.84
70	0.235	0.758	0.085	0.673	15788.58
71	0.231	0.741	0.087	0.654	15342.84
72	0.229	0.733	0.083	0.650	15249.00
73	0.221	0.720	0.080	0.640	15014.40
74	0.215	0.705	0.078	0.627	14709.42
75	0.206	0.689	0.077	0.612	14357.52
76	0.196	0.658	0.072	0.586	13747.56
77	0.187	0.631	0.066	0.565	13254.90
78	0.180	0.615	0.063	0.552	12949.92
79	0.167	0.593	0.060	0.533	12504.18
80	0.156	0.572	0.058	0.514	12058.44
81	0.149	0.547	0.053	0.494	11589.24
82	0.141	0.531	0.049	0.482	11307.72
83	0.138	0.512	0.047	0.465	10908.90
84	0.132	0.487	0.044	0.443	10392.78
85	0.122	0.458	0.041	0.417	9782.82
86	0.109	0.422	0.037	0.385	9032.10
87	0.117	0.409	0.042	0.367	8609.82
88	0.129	0.384	0.045	0.339	7952.94
89	0.146	0.392	0.051	0.341	7999.86
90	0.185	0.399	0.057	0.342	8023.32
91	0.201	0.405	0.063	0.342	8023.32
92	0.222	0.411	0.066	0.345	8093.70
93	0.235	0.423	0.071	0.352	8257.92
94	0.244	0.427	0.077	0.350	8211.00
95	0.240	0.434	0.083	0.351	8234.46
96	0.231	0.441	0.089	0.352	8257.92

97	0.220	0.456	0.095	0.361	8669.06
98	0.205	0.472	0.099	0.373	8750.58
99	0.183	0.462	0.104	0.358	8398.68
100	0.151	0.432	0.097	0.335	7859.10
101	0.133	0.411	0.087	0.324	7601.04
102	0.102	0.378	0.081	0.297	6967.62
103	0.088	0.341	0.076	0.265	6216.90
104	0.054	0.305	0.072	0.233	5466.18
105	0.043	0.263	0.067	0.196	4598.16
106	0.032	0.217	0.063	0.154	3612.84
107	0.022	0.184	0.058	0.128	3002.88
108	0.014	0.146	0.051	0.095	2228.70
109	0.011	0.117	0.047	0.070	1642.20
110	0.008	0.088	0.040	0.048	1126.08
112	0.006	0.067	0.036	0.031	727.26
113	0.004	0.041	0.028	0.013	304.98
114	0.003	0.023	0.019	0.004	93.84

Figure 1: AChE activities of Sephadex G-200 column fractions.



200 µl samples are taken from these three steps to compare purity, enzymatic activity, specific activity.

Enzymatic activity (UI/L) = 23460 × (DO_{412nm} t_{0s} – DO_{412nm} t_{70s}).

Where, 23460 is the activity coefficient.

Table 2: Purity of AChE enzyme at different stage

Enzyme purity level	Protein conc. (mg/ml)	Enzymatic Activity (UI/ml)	Specific Activity (mU/mg)	Purity increases
Crude Enzyme	9.64	11.213	1.163	1
Step 1	3.83	14.005	3.656	3.14
Step 2	1.02	19.237	18.859	16.21

Figure 2: Specific activity of AChE at different stages of purification.

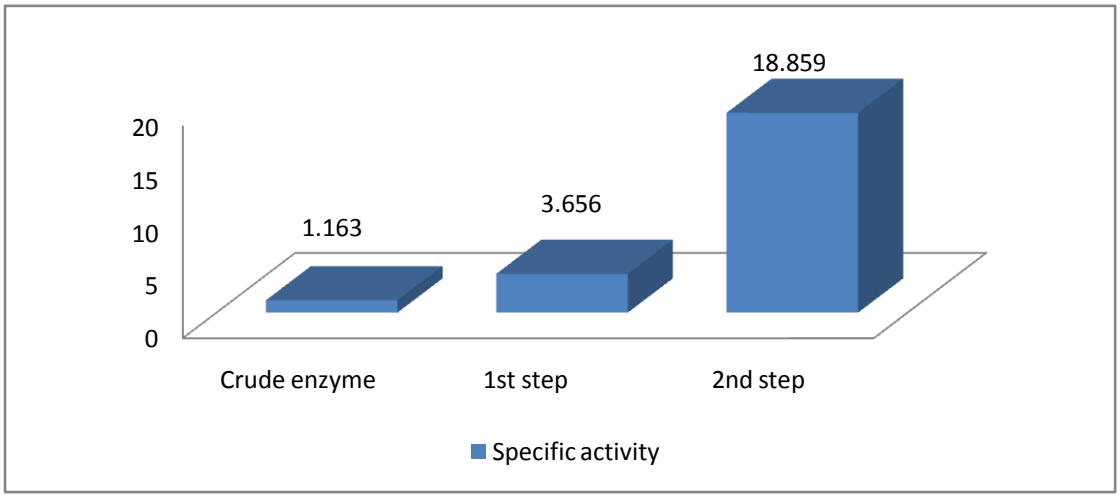
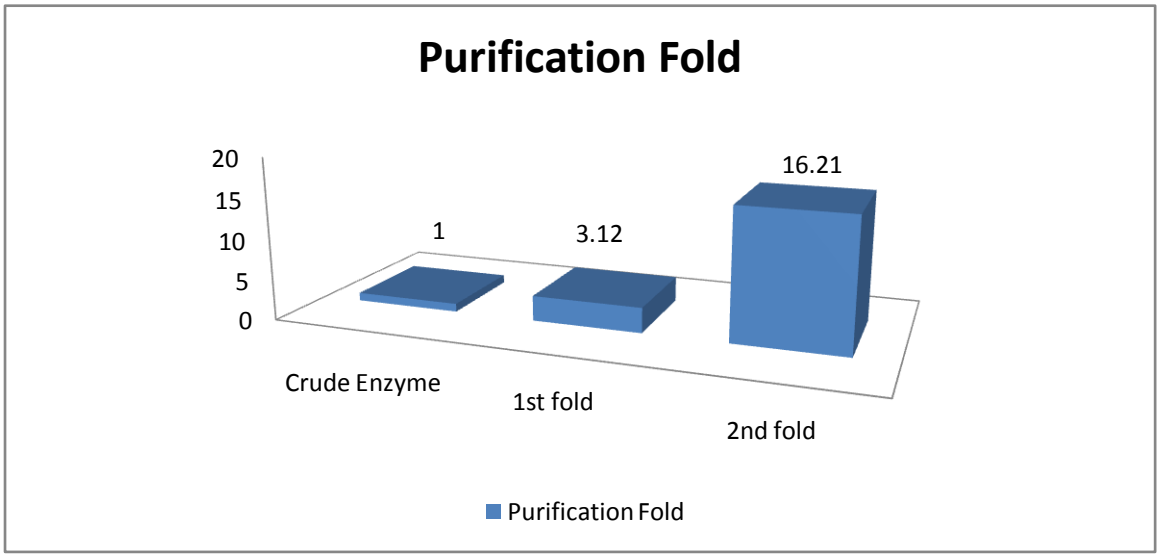


Figure 3: Purification of AChE increases at different stages



In our study we found that after precipitation with super saturated ammonium sulphate solution both specific activity and purity of the crude enzyme increases. But after gel filtration the specific activity of the enzyme and purity increases in many folds.

Lastly from our study we can conclude that, bovine brain is a potent source of neurotransmitters which can be used as a source

of AChE. By precipitation with super saturated ammonium sulphate solution the purity and specific activity of the enzyme increases. But after gel filtration of AChE extracts the specific activity and purity profile of that enzyme increases in many folds.

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