Cholinesterase in Plasma: Purification and Some Biochemical Properties

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Summary: Cholinesterase from plasma of sheep was partially purified by ion exchange Chromatography on Phospho-Cellulose column, gel filtration on Sepharose-6B and finally by affinity chromatography on Sepharose Con-A. The enzyme was purified 100 fold with specific activity of 440 mU/mg of protein with recovery of about 20%. The enzyme was found to be glycoprotein with Km value of 2.7×10^{-5} M. Molecular weight of the enzyme was estimated to be approximately 400,000 daltons by gel filtration.

Introduction

Cholinesterases are defined as enzymes that catalyze the hydrolysis of choline esters. There are at least two distinct types of such enzymes in human blood [1], one in the red cells and the other in serum [2]. The red cell type, called acetylcholinesterase [E.C.3.1.1.7], hydrolyses acetylcholine more rapidly than butyrylcholine (type I) where as the serum type, called butyrylcholinesterase or non-specific cholinesterase [E.C.3.1.1.8], hydrolyses butyrylcholine more rapidly than acetylcholine (type II) [3]. The two types of enzymes can be classified not only on the basis of tissue localization and substrate specificity; but are also susceptible to differential inhibition [4].

Cholinesterases are found in mammalian liver, pancreas, intestinal mucosa, excitable tissues such as nerve muscles, at the surface of the mammalian erythrocytes and in Cobra venom [5]. The electric organs of Electrophorous electricus and Torpedo marmorata are rich sources of acetylcholinesterase. Cholinesterases are also found in senile plaques even at the initial stages of their formation [6]. Large quantities of acetylcholinesterase were purified and crystallized from the electric organs of Torpedo and eel. The enzymes from bovine erythrocytes membrane [7] and bovine caudate nuclei [8] have been purified. Vertebrate blood sera generally contain multiple forms of cholinesterase and that the electrophoretic heterogeneity of the enzyme in human serum has been reported [9]. These isoenzymes have been the subject of intensive studies from a pharmacological point of view [10]. Cholinesterases from yolk, liver and blood plasma of chicken have also been purified and characterized [11]. Present study deals with the purification of cholinesterase from plasma of sheep and its biochemical properties.

Results and Discussion

Purification of Enzyme

Citrated blood (1volume of a solution containing 0.35% NaCl and 3.5% Na. citrate was added to 4 volume of blood) of sheep, was

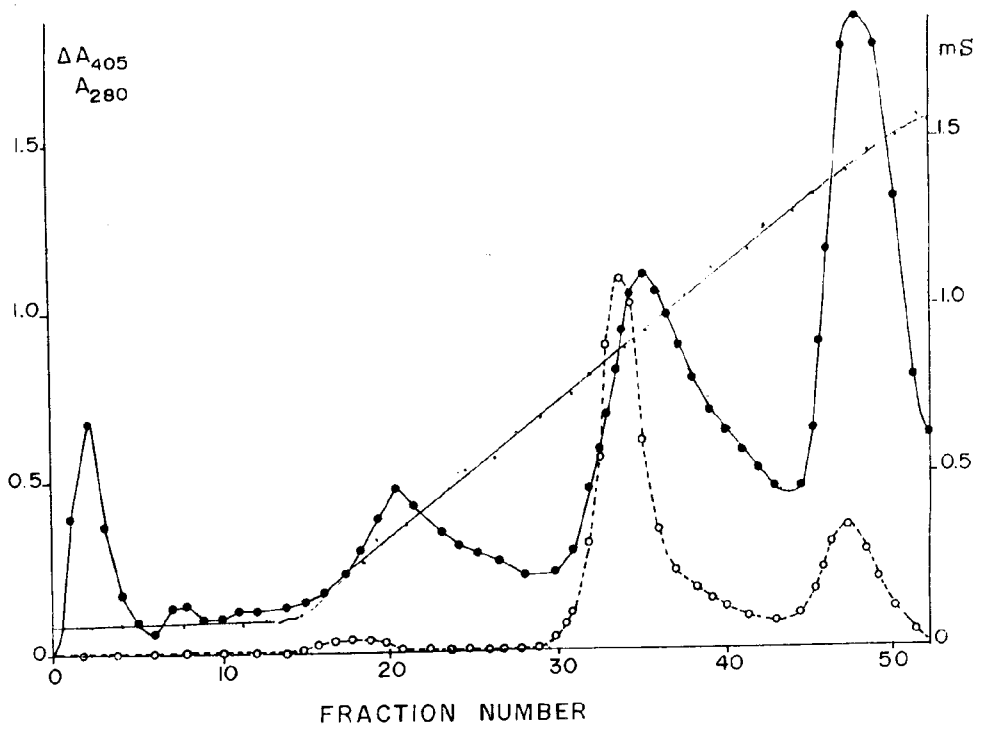

Fig. 1: P-Cellulose chromatography of sheep blood plasma. 40 mL sample was applied to the column (30x2.5 cm), 15 mL fractions were collected at the flow rate of 40 mL/h. Ordinates: Protein at 280 nm (●——●), activity at 405 nm (o----o) and conductivity mS (.——.).

centrifuged at 3000 x g for 10 min. The supernatant was dialyzed against 0.01 M acetate buffer pH 4.5 and the precipitate so formed was centrifuged and discarded.

Phospho-cellulose(P-Cellulose)Chromatography

The supernatant (40 mL) was applied to the P-Cellulose column (30 x 2.5 cm) previously equilibrated and washed with 0.01 M acetate buffer pH 4.5. The activity was completely bound to the column and was not eluted even washed with several volumes of buffer. This was followed by elution with linear salt gradient between 0 and 0.15 M NaCl in the same buffer (total volume 400 mL). The activity peak eluted at 0.08 M NaCl with slight shift from the protein peak leading to purification. The results are shown in figure 1. The most active fractions were pooled together and concentrated by 70% $(NH_4)_2SO_4$ precipitation. The specific activity increased to 15mU/mg of protein with recovery of 65-70% and purification factor of 4 by this step.

Gel Chromatography on Sepharose-6B

The sample after P-Cellulose chromatography (8 mL) was applied on Sepharose- 6 B column (55 x 3.2 cm) which was previously equilibrated with 0.01M Tris-HCl buffer pH 7.0 and eluted with the same buffer at flow rate of 40 mL/h. The elution profile is shown in figure 2. Asymmetry of the activity peak was observed. The most active fractions (A region) were pooled and concentrated 5 times by ultra filtration using YM-5 membrane. A 10 fold further purification was achieved with recovery of 45 % from this column step.

Affinity Chromatography on Concanvalin A-Sepharose-4B

A small column (15 mL) was packed with Sepharose Con A gel. The gel was equilibrated with 0.1 M Tris-HCl pH 7.0 containing 1 mM Mn^{++}, 1 mM Ca^{++} and 1% α-D-methyl glucoside. The enzyme sample after gel filtration was dialyzed against the

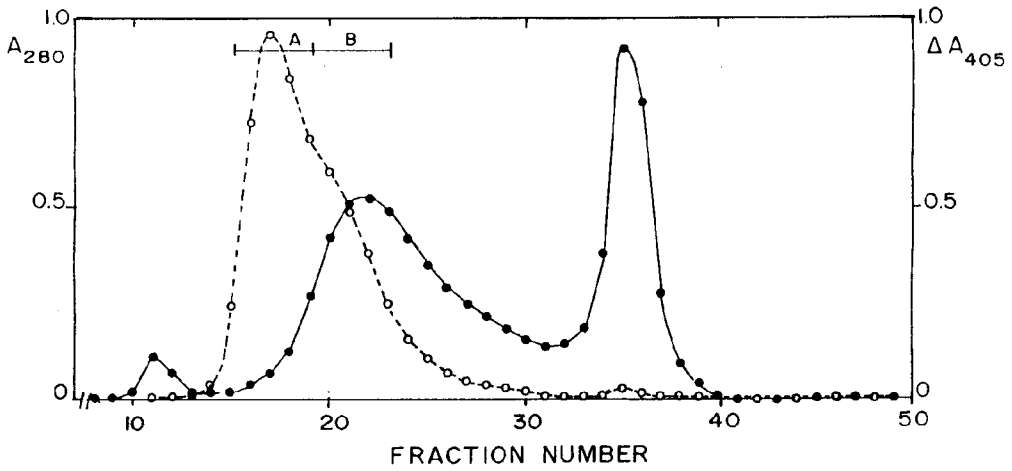

Fig. 2: Gel filtration on Sepharose 6 B. 10 mL fractions were collected at the flow rate of 40 mL/h. Ordinates: Protein at 280 nm (●——●), activity at 405 nm (o----o).

same buffer and applied to the affinity column and eluted with the same buffer. Most of the proteins ran un-retarded through the gel, while cholinesterase activity was completely adsorbed. Subsequent elution with 0.1 M Tris-HCl pH 7.0 containing 1 mM Mn^{++}, 1 mM Ca^{++} and 3% α-D-methyl mannoside gave an enzyme that was purified 2.5 times as compared to the material applied to the column (Figure 3). The recovery from the column was 80%. The summary of the purification of cholinesterase from the plasma of sheep is presented in table 1. Concanvalin A-Sepharose had already yielded satisfactory results in the purification of the acetylcholinesterases of Torpedo californica [12], human erythrocytes, the electric organ of Electricus and plaice body muscle [13]; showing that the enzymes are glycoprotein in nature, which are similar to our findings.

The enzyme preparation appeared to be not homogeneous as many bands, though faints, were observed on 7.5 % acrylamide gel electrophoresis in a Tris-glycine buffer pH 8.5 [14]. The presence of these few contaminating enzymes was further investigated in the purified sample. Only the trace amounts of acid phosphatase (at pH 5.0), alkaline phosphatase (pH 8.0) acid diesterase (pH 4.6), alkaline diesterase (pH 8.0) and ribonuclease (pH 7.0) were detected after incubating the enzymes for 24 h with their specific substrates.

Sepharose-6B which was previously calibrated with standard proteins. Figure 4 shows that apparent molecular weight was approximately 400,000 ±

Biochemical Properties

The molecular weight of cholinesterase was determined by gel filtration chromatography on a 10,000. Similar results have also been obtained from cholinesterases of chicken's liver and yolk [11].

The function of Butyrylcholinesterase remains a puzzle, it has no known specific natural substrate, although it is capable of hydrolyzing acetylcholine.

Substrate specificity study was carried out on various cholinesters and the results are shown in table 2. Acetylcholine chloride, butyrylcholine chloride, propionylcholine chloride and acetycholine bromide were found to be good substrates. Succinylcholine chloride did not act as a substrate for our enzyme.

Table 1: Purification of Sheep Plasma Cholinesterase.

Steps	Vol.(mL)	Act.(mU/mL)	T.Ac.(mU)	Prot.(mg/mL)	S.A.(mU/mg)	P.F	Rec.%
Plasma	40	122	2240	30	4.06	1	100
P-Cellulose	8	186	1488	11.27	16.50	4.06	66.4
Sepharose -6B	44	14.7	646.8	0.07	210.0	51.65	28.8
Ultrafiltration	8	69	552	0.38	181.5	44.63	24.6
Sepharose Con A	20	22	440	0.05	440.0	108.21	19.6

Vol. Volume; Act. Activity; T.Act. Total activity; Prot. Protein; S.A. Specific activity; P.F. Purification factor; Rec. Recovery.

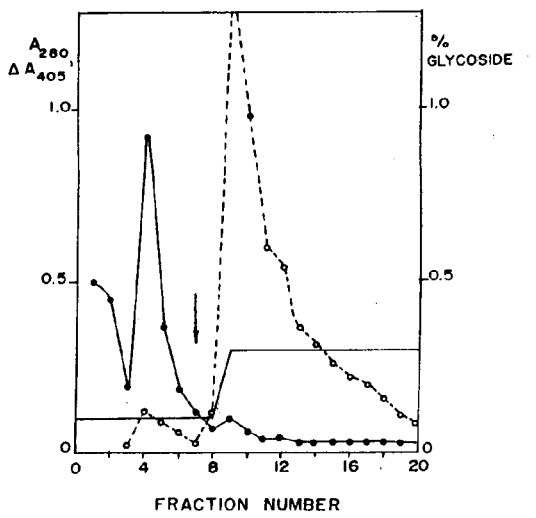

Fig. 3: Affinity chromatography on Con A-Sepharose. 8 mL sample was applied to the column. The arrow indicates the start of elution with 3% α D-methylmannoside. 5 mL fractions were collected at the flow rate of 20 mL/h. Ordinates: Protein at 280 nm (●——●), activity at 405 nm (o——o), % glycoside (———).

Table 2: Substrate Specificity for Cholinesterase from Sheep Plasma.

Substrate	% Activity
Butyrylcholine chloride	108
Propionylcholine chloride	104
Acetylcholine chloride	100
Acetylcholine bromide	101
Acetyl β-methylcholine chloride	12
Benzylcholine chloride	12
Succinyldicholine chloride	0

Activity against butyryl choline choloride was taken as 100%.

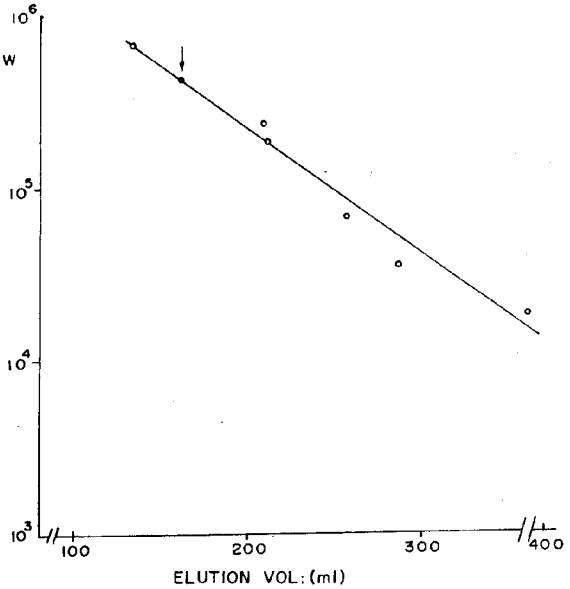

Fig. 4: Estimation of molecular weight of the cholinesterase by gel filtration on Sepharose 6 B. 5 mL enzyme preparation after Phospho-Cellulose chromatography were applied to the column (55 x 3.2 cm) and equilibrated with 0.01 M Tris-HCl buffer pH 7. 10 mL fractions were collected at the flow rate of 40 mL/h. Proteins used were myoglobin (M.W. 18,000), pepsin (35, 000), bovine serum albumin (67,000), bovine-γ-globulin (180,000), catalase (230,000), thyroglobulin (660, 000). Elution volumes of calibration proteins (o) and elution volume of chrolinesterase (●). The arrow (A) points the position of cholinesterase peak.

Acetyl β- methylcholine chloride is as good substrate as acetylcholine chloride for acetylcholinesterase from Tarpedo marmorata, mammalian brain [15] and rat diaphragm [16], but acetyl β- methylcholine chloride was found to be a poor substrate for cholinesterase from sheep plasma (Table 2) which forms the basis to distinguish between acetylcholinesterase and cholinesterase, since the substrate specificity and kinetic consideration can classify the two enzymes [3].

The most useful tool for distinguishing between acetylcholinesterases or cholinesterases and less specific esterases is probably the inhibition pattern by eserine (physostigmine), since the esterases which are not inhibited by 10^{-5}M eserine can not be referred to as cholinesterases [17]. The inhibition pattern in the presence of eserine for sheep plasma cholinesterase is shown in table 3. This proves that this enzyme is of type II cholinesterase. Loss of acetylcholinesterase in Alzheimer disease has been reported [18], leading to the formulation of a "Cholinergic hypothesis", and the work has mainly centered on the inhibitors of the enzymes. Four of the cholinesterase inhibitors viz, tacrine, donepezil, rivastigmine, and galantamine are currently used for treatment of Alzheimer disease [19].

Table 3: Effect of Eserine Upon cholinesterase activity.

Eserine Concentration (M)	%Act.
0	100
1.43×10^{-8}	93.8
2.86×10^{-8}	87.2
1.43×10^{-7}	83.0
2.86×10^{-7}	78.7
1.43×10^{-6}	24.5
2.86×10^{-6}	10.6
1.43×10^{-5}	1.0
2.86×10^{-5}	0

Table 4: Effect of Acetylthiocholine Iodide as Substrate on the Cholinesterase Activity.

Substrate Concentration (M)	Actvity (ΔA_{405})
1×10^{-3}	0.135
5×10^{-4}	0.12
2×10^{-4}	0.115
1×10^{-4}	0.105
5×10^{-5}	0.09
2.5×10^{-5}	0.065
2×10^{-5}	0.058

Michaelis-Menten constant of the cholinesterase for acetylthiocholine iodide was estimated from Lineweaver-Burk plot [20]. The enzyme activities were assayed in the presence of substrate concentrations between 1×10^{-5} and 1×10^{-3} M. The results are shown in table 4. By the method of least square, the value was calculated, which was found to be 2.7×10^{-5} M. The pH optimum of the enzyme was 8.2 - 8.5 and temperature optimum was 45°C. However, Mesulam and coworkers [21] have reported that acetylcholinesterase in plaques and tangles, shows a lower pH optimum and a reduced sensitivity to physostigmine.

Experimental

Materials

Acetylthiocholine iodide and butyrylthiocholine iodide were purchased from Sigma Chemical Co; 5′, 5′-dithiobis (2-nitro) benzoic acid (DTNB) from Merck, Sepharose-6B and Concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals AB. All other chemicals used, were of analytical grade (Aldrich, Merck, and Fluka).

Methods

Enzyme assay

Cholinesterase activity was determined according to Ellman [22] method with little modification as previously described by Saeed et al. [11]. The incubation mixture consisted of 3 mL of 0.25 mM coloring reagent (DTNB) in 0.05 M phosphate buffer pH 7.2, 800 µL of water and 100 µL of enzyme. The reaction was started by adding 100 µL of 0.031 M acetylthiocholine iodide as substrate. The yellow color produced was measured spectrophotometrically at 405 nm after 30 minutes. Blank was also run, where water was used instead of enzyme.

One unit of enzyme is defined as the amount of the enzyme that catalyses the hydrolysis of 1 µmole of acetylthiocholine iodide per minute under above mentioned conditions, and the specific activity as units per mg of protein.

In substrate specificity study or cholinesterase activity determination, the rate of hydrolysis of various cholinesters was determined by Hestrin method [23] based on un-reacted acetylcholine chloride substrate which was converted into acetylhydroxamic acid by alkaline hydroxylamine. The reaction mixture consisting of 2.5 mL of 0.001M substrate in 0.2 M phosphate buffer pH 7.2 and 0.2 mL enzyme solution was incubated for 30 min at 37°C. After incubation, 0.5 mL of freshly prepared alkaline hydroxylamine (1 volume of 2.5 N NaOH and 1 volume of 1 M NH_2OH), 1.0 mL of 1 M citrate buffer pH 1.4 and 1.0 mL of 0.7 M $FeCl_3$ were added. The contents were mixed vigorously and after 20 minutes the absorption at 490 nm was measured. Two suitable blanks were run simultaneously with enzyme assay, which were prepared as, one devoid of enzyme and another devoid of substrate.

Acid phosphatase, alkaline phosphatase, phosphodiesterases and ribonuclease were determined by the method of Willems et al. [24]

Protein assay

Protein concentrations were determined by Lowry method [25], using bovine serum albumin as standard protein. While protein concentrations of the column effluent fractions were estimated by measuring their absorptions at 280 nm.

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