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Purification and Characterization of Acetylcholinesterase from Sheep Liver and Inhibition by Some Painkillers

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In this study, acetylcholinesterase (AChE; EC 3.1.1.7) was purified from sheep liver by affinity chromatography. The purification rate was found 3541.7 fold. The purification control of enzyme was done with sodium dodesilsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimal pH, ionic strength and temperature of enzyme were determined. The optimal pH and the optimum temperature were 7.5–8.5 and 35°C respectively. The highest activity was seen in concentration of 0.2 M $(NH_4)_2SO_4$ as ionic strength. On the other hand, the inhibition effects of some painkiller, (paracetamol + caffein), (neostigmin methylsulfate) and (paracetamol + propifenazon + caffein) were investigated in this study. According to results, the I_{50} values are 1.27×10^{-3}, 1.02×10^{-4} and 1.236 M respectively. Also, K_i values are determined as 1.246×10^{-3}, 4.326×10^{-5} and 1.646×10^{-3} mol^{-1} min^{-1} respectively.

Key Words: Purification, Characterisation, Inhibition, Acetylcholinesterase.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7) catalyzes the hydrolysis of acetylcholine, a neurotransmitter substance which functions in certain portions of the nervous system. The reaction catalyzed by acetylcholinesterase occurs enzymically in two steps. In the first step the enzyme serves as a remarkable nueleophile,

$$(CH_3)_3N^+C_2H_4O{-}\overset{\overset{O}{\|}}{C}{-}CH_3 + H_2O \leftrightarrow (CH_3)_3N^+C_2H_4OH + CH_3COOH$$

In the second step, the enzyme serves as an excellent leaving group. The nucleophilic group is the hydroxyl group of a specific serine residue[1].

$$(CH_3)_3N^+C_2H_4O{-}\overset{\overset{O}{\|}}{C}{-}CH_3 + E: \leftrightarrow CH_3{-}\overset{\overset{O}{\|}}{C}{-}E + (CH_3)_3N^+C_2H_4OH$$
$$\updownarrow$$
$$CH_3COOH + E$$

Later, such enzymes were found in blood. Both serum and red cells hydrolyse choline esters more rapidly than other esters at low substrate concentrations. The red cell type has been called acetylcholinesterase. Acetylcholinesterase is found in all conducting tissues in all species of animals, which have been investigated.

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Acetylcholinesterase is mainly found in the brain, in nerve cells (especially end plates), in muscle and in erythrocytes. Acetylcholinesterase was first isolated by extraction from the electric organ of *Torpedo marmorata* in 1938, following the discovery of an extraordinary concentration of the enzyme in this tissue[2]. The optimum pH of the AChE of plasma and erythrocytes is around 8.0. Both enzymes have a distinctly different optimum substrate concentration[3].

The purpose of this study is to purify acetycholinesterase enzyme from sheep liver by affinity chromatography, to characterize, to compare purified enzyme from liver with that of the other living species and to investigate whether a painkiller could inhibit.

EXPERIMENTAL

The chemicals used in the present study are: sepharose 4B, 1-ethyl-3-(3-dimethyl-aminopropyl)carbadiimide hydrochloride (EDCl), sephadex G-25, decamethonium bromide, standard bovine serum albumin, coomassie brillant blue G-250, coomassie brillant blue R-250, N,N,N′,N′-tetramethylethylene diamine (TEMED), trichloroacetic acid (TCA), sodium dodesilsulfate (SDS), dialysis bag, NaOH (Sigma), sodium carbonate, sodium bicarbonate, trihydroxymethyl aminomethane (Tris), NaCl, cyanide bromide (CNBr), sodium citratedehydrate, HCl, acetic acid, β-mercaptoethanol, propanol, methanol (Merck). The sheep liver samples were obtained from slaughterhouse at Van, Turkey.

Preparation of the homogenate: The liver samples were dissected and put in petri dishes. After washing the tissues with physiological saline (0.9% NaCl), the liver samples were cut into parts with a blender. The ground liver samples were homogenized for 5 min in a solution containing 0.836 g $Na_2HPO_4 \cdot 2H_2O$ and 8.25 g glucose (1 : 5 w/v) using a glass-porcelain homogenizer (20 kHz frequency ultrasonic, Jencons Scientific Co.) and then centrifuged at 7000 x g for 30 min. After the membrane and intact cells were broken down, the supernatant was removed. All processes were carried out at 4°C. The pH of the homogenate was adjusted at 8.0 by adding sodium phosphate (25 mM) and solid phosphate salts.

Preparation of affinity gel: 20 mL of sepharose 4B and 20 mL of water were combined and 4 g of CNBr was added to this suspension. The mixture was titrated to pH 11 in an ice bath by stirring with a magnet and maintained at that pH for 8–10 min. The reaction was stopped by filtering the gel on a Buchner funnel and washed with cold 0.1 M $NaHCO_3$ buffer (pH 10). 1-Ethyl-3-(3-dimethyl-aminopropyl)carbadiimide hydrochloride, using saturated EDCl solution in the same buffer, was coupled to sepharose 4B-EDCl activated with CNBr. The reaction was completed by stirring with a magnet for 90 min. In order to remove excess of 1-ethyl-3-(3-dimethyl-aminopropyl) carbadiimide hydrochloride from the sepharose 4B-EDCl gel, the mixture was washed with abundant water. The affinity gel was suspended in buffer A (50 mM sodium phosphate, pH = 8), then packed in a column (1.3 × 60 cm) and equilibrated with the same buffer. The flow rates for washing and equilibration were adjusted by peristaltic pump 20 mL/h.

Purification of AChE by affinity chromatography: The homogenate was applied to the affinity column. Homogenate sample obtained previously was loaded on sepharose 4B-EDCl affinity coulmn and the flow rate was adjusted to 20 mL/h.

The column was washed with buffer B (0.4 NaCl, 50 mM sodium phosphate, pH 8) until the A_{280} nm of effluent was ≤ 0.01. The AChE was eluted with buffer C (0.1 M NaCl, 50 mM sodium phosphate, pH 8, containing 10 mM decamethonium). The enzyme activity was measured in final fractions and the activity containing tubes were collected together. Then the enzyme solution was dialyzed in buffer A for 3–4 h with two changes of buffer[4–8].

Activity determination: For the colorimetric assay, according to Ellman's method, reaction mixtures were made up in 50 mM tris, pH 7.4 containing 1 mM DTNB and acetylthiocholine iodide at a final concentration of 0.8 mM. The reaction was performed at 25°C and monitored at 412 nm[9].

Protein determination: Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method by using bovine serum albumin as a standard[10].

Optimal pH determination: For the optimal pH determination the enzyme activity was measured in 1 M tris-HCl and phosphate buffers within the pH of 6.0 to 11.0. In brief, a 50 µL enzyme sample (specific activity 11.2 EU/mg protein, concentration 0.034 mg protein/mL) was added to incubation mixture containing 1 mM DTNB and acetylthiocholine iodide at a final concentration of 0.8 mM. The reaction was performed at 25°C.

The effect of temperature on AChE activity: Enzyme activity was measured between 10 and 60°C at optimal pH for this purpose. The enzyme activity was measured as follows: a 50 µL enzyme sample (specific activity 11.2 EU/mg protein, concentration 0.034 mg protein/mL) was added to the incubation mixture containing 1 mM DTNB and acetylthiocholine iodide at a final concentration of 0.8 mM.

Kinetic Studies: In order to determine I_{50} values of the enzymes, regression analysis graphs were drawn by using % inhibition values by a statistical packing program on a computer at five different concentrations. The inhibitor concentrations causing up to 50% inhibition were determined from the graphs. Also, The kind of inhibition was determined by means of a Lineweaver-Burk plot[11]. We have found the K_i values by writing the values of slopes found in the equation $K_i = 2.3003 \times \text{slope}/I$. All kinetic studies were performed at 25°C at optimal pH (50 mM tris-HCl, pH 7.2).

SDS polyacrylamide gel electrophoresis (SDS-PAGE): Laemmli's procedure was carried out in 4 and 10% acrylamide concentrations for running and stacking gel, respectively, to control of enzyme purity SDS added into the gel solution at 10%. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. The staining was made for about 2 h in the solution of 0.1% Coomassie Brillant Blue R-250 + 50% methanol + 10% acetic acid. Finally the washing was carried out in the solution of 50% methanol + 10% acetic acid + 40% distilled water until protein bands were cleared[12].

RESULTS AND DISCUSSION

In this study acetylcholinesterase was first purified from sheep liver. The purification steps included preparation of homogenate and sepharose 4B-EDCl affinity chromatography. Sepharose 4B was chosen as a matrix because of better flow properties than other matrices. At first, free hydroxyl groups of the matrix were activated with cyanogen bromide[13]. Table-1 shows a purification of homogenate

characterized with a specific activity of 1671.71 EU/mg proteins, a yield of 49% and a purification coefficient of 3541.71.

TABLE-1
PURIFICATION OF LIVER HOMOGENATE FROM SHEEP

Purification steps	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	300	392.7	185.39	0.472	100	0
Affinity column	50	0.0541	90.44	1671.7	49	3541.7

The AChE from liver homogenate was purified by using the affinity gel with elution buffer of (0.1 M NaCl, 10 mM sodium phosphate pH 8.0 containing 10 mM decamethonium). The eluates were plotted by arraying out protein determination at 280 nm and AChE activity for homogenate (Fig. 1). Specific activities for plasma and erythrocytes were calculated by using homogenate and purified enzyme solution.

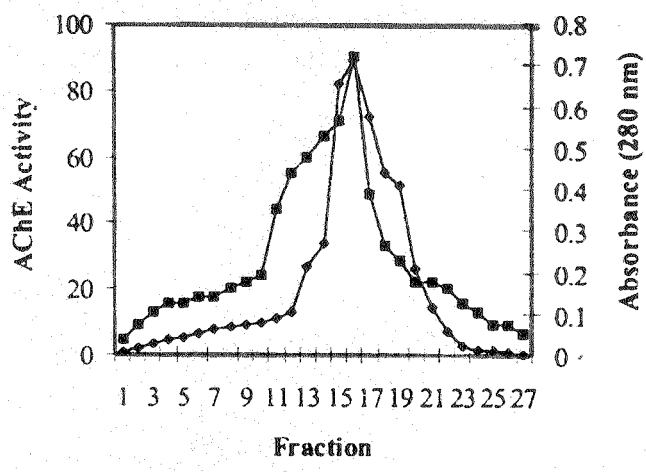

Fig. 1. Purification of homogenate AChE by affinity chromatography, the columns (1.3 × 60 cm) eluted by buffer C at pH 8. Buffer at 20 mL/h flow rate for fraction volumes of 6 mL

Optimal pH of AChE for liver has been determined as 7.5–8.5 using 1 M tris-HCl and shown in Fig. 2. The plot temperature for enzyme activity was

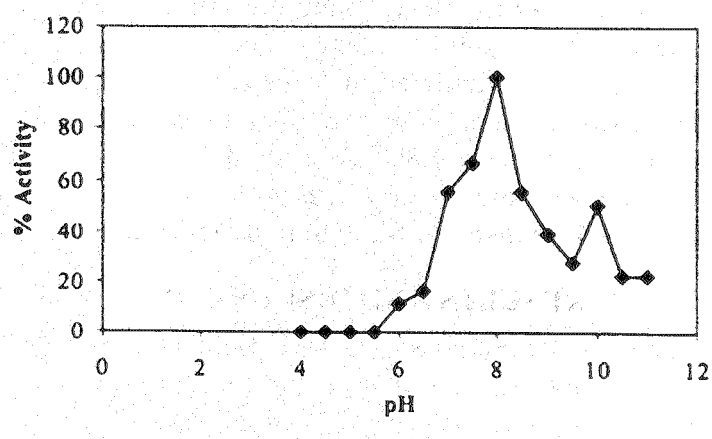

Fig. 2. Activity-pH plot of liver AChE

measured at 35°C and shown in Fig. 3. The highest activity was seen in concentration of 0.2 M $(NH_4)_2SO_4$ as ionic strength and shown in Fig. 4.

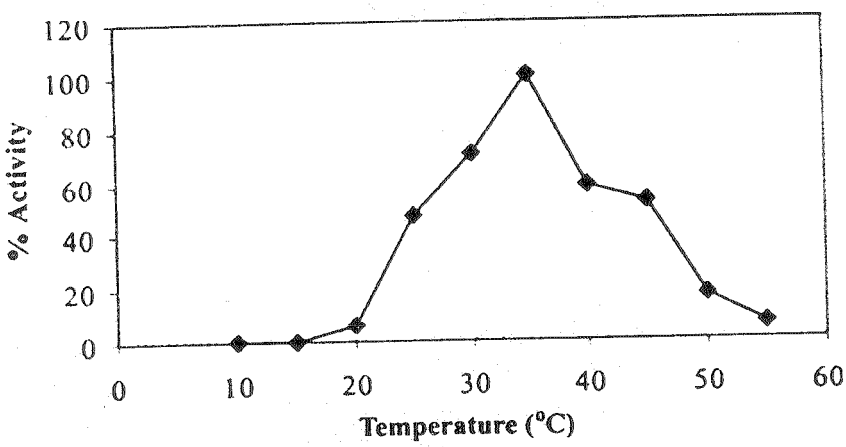

Fig. 3. Activity-temperature plot of liver AChE

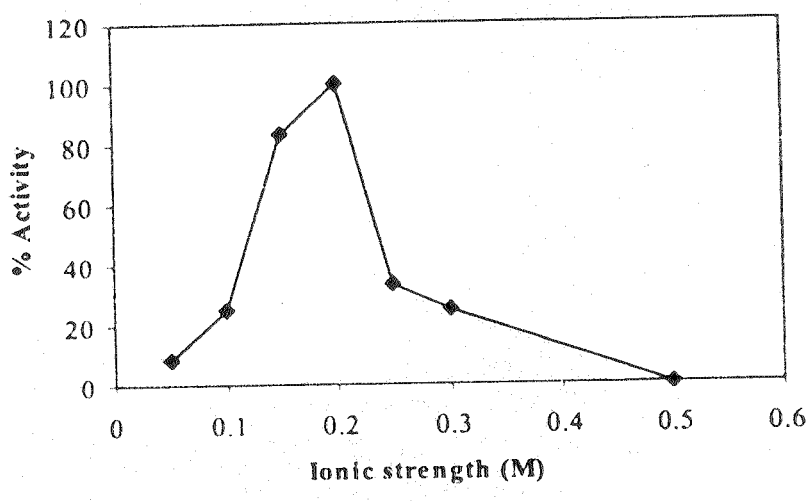

Fig. 4. Activity- ionic strength plot of liver AChE

There was a 3541.7-fold purification of liver AChE. The purification of AChE was controlled with SDS-polyacrylamide gel electrophoresis.

On the other hand, the inhibition effects of some painkiller, (paracetamol + caffeine), (neostigmin methylsulfate) and (paracetamol + propifenazon + caffeine) were investigated in this study. According to results, all painkillers inhibit AChE in a competitive manner (Figs. 5–7). The I_{50} values are 1.27×10^{-3} M, 1.02×10^{-4} M and 1.236 M respectively. Also, K_i values were determined as 1.246×10^{-3}, 4.326×10^{-5} and 1.646×10^{-3} mol^{-1} min^{-1} respectively.

So far, no report is available to study the effect of those chemicals *in vitro* on the liver of this species. Therefore, we could not have a chance to compare our results with the previous results. But, our results dealing with characterization of enzymes were in accordance with previous studies on different species[14–16]. In addition, it is difficult to compare data from different laboratories regarding the inhibition effect, because of high variability in analyzing enzymes *in vitro* and *in vivo* due to inconsistent factors like treatment time and manner, purity and species tissue differences, etc.

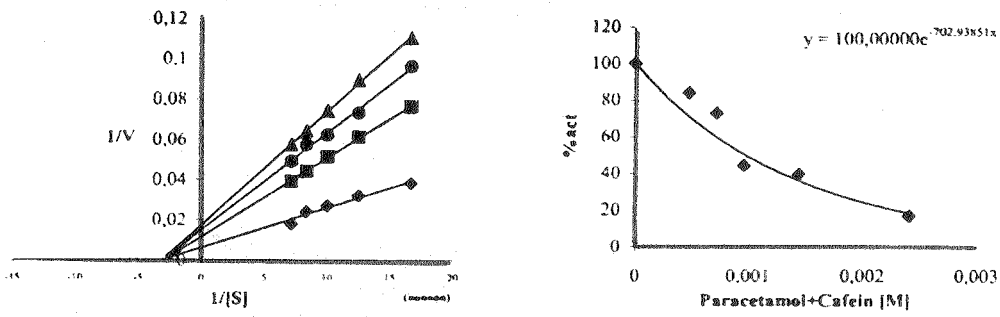

Fig. 5. Lineweaver-Burk plot in 5 different substrates (DTNB and acetylthiocholine iodide) concentrations and in 3 different paracetamol + caffeine concentrations for determination of inhibition kind, and the inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from 5 different concentration activity-[I] regression analysis graphs for paracetamol + caffein

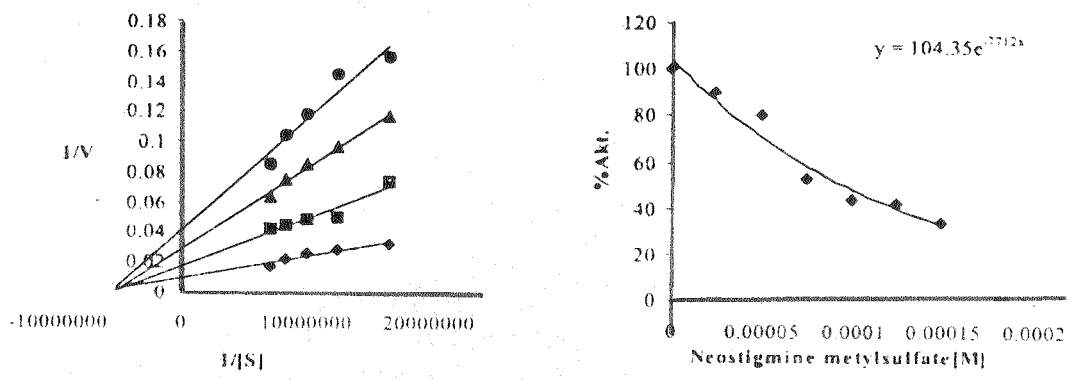

Fig. 6. Lineweaver-Burk plot in 5 different substrate (DTNB and acetylthiocholine iodide) concentrations and in 3 different neostigmin methylsulfate concentrations for determination of inhibition kind and the inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from 5 different concentration activity-[I] regression analysis graphs for neostigmin methylsulfate

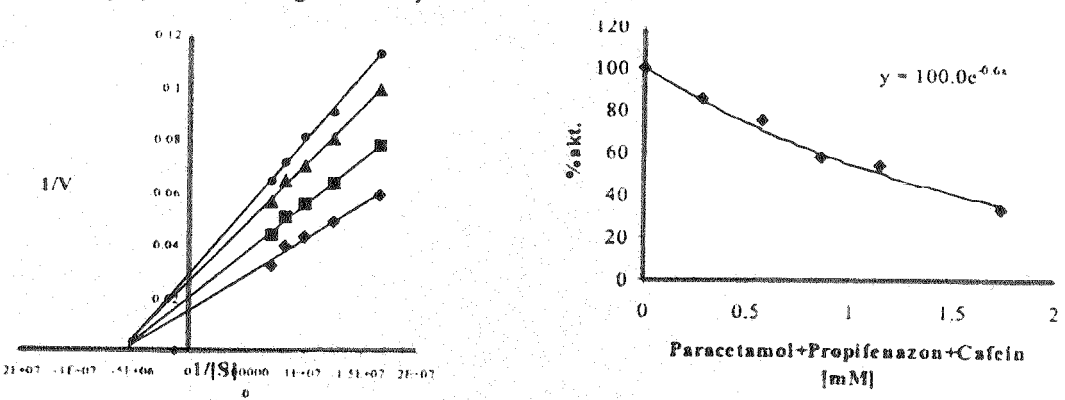

Fig. 7. Lineweaver-Burk plot in 5 different substrate (DTNB and acetylthiocholine iodide) concentrations and in 3 different paracetamol + propifenazon + caffein concentrations for determination of inhibition kind and the inhibitor concentrations causing up to 50% inhibition (I_{50}) were determined from 5 different concentration activity-[I] regression analysis graphs for paracetamol + propifenazon + caffein

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