T.R.N.C.
NEAR EAST UNIVERSITY
INSTITUTE OF HEAL TH SCIENCES

THE STUDY OF THE KINETIC BEHAVIOR OF HORSE SERUM BUTYRYLCHOLINESTERASE WITH FLUOXETINE

Osman YETKİN

MEDICAL BIOCHEMISTRY PROGRAMME
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SUPERVISOR
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The Directorate of Health Sciences Institute,

This study has been accepted by the Thesis Committee in Medical Biochemistry Programme as Master Thesis

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According to the relevant articles of the Near East University Postgraduate Study - Education and Examination Regulations, this thesis has been approved by the above mentioned members of the thesis committee and the decision of the Board of Directors of the Institute.

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Director of the Institute of Health Sciences

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ABSTRACT

Yetkin O. The Study of the Kinetic Behaviour of Horse Serum Butyrylcholinesterase with Fluoxetine. Near East University, Institute of Health Sciences, Medical Biochemistry Programme, M.Sc. Thesis, Nicosia, 2015.

Butyrylcholinesterase (3.1.1.8; BChE) is a serine esterase which is found in vertebrates. BChE is synthesized in liver and secreted into the plasma, where it makes up approximately 0.1 % of the total serum proteins in humans. BChE plays a role in the detoxification of natural as well as synthetic ester bond-containing compounds. It is also responsible for the elimination of acetylcholine when acetylcholinesterase is inhibited. Alterations in BChE activity is associated with the diseases. Particulary, cholinergic system abnormalities are correlated with the formation of senile plaques in Alzheimer's disease (AD). Current therapeutic approaches use cholinesterase inhibitors in the treatment of AD. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and easily passes through the blood-brain barrier. In this study, it was aimed to study the interaction of hor~ serum BChE with fluoxetine. The molecular weight of the tetrameric, dimeric and monomeric forms of BChE was calculated as 380 kDa, 190 kDa and 95 kDa, respectively. Optimum pH of the enzyme was 8.1. Optimum temperature, energy of activation (Ea) and temperature coefficient (Q_{10}) were calculated as 36.4°C, 1526 cal/mol and 1.19, respectively. In kinetic studies, V, and Km were found to be 20.59 ± 0.36 U/mg protein and 194 ± 14 µM, respectively. Fluoxetine inhibited BChE competitively. Half maximal inhibitory concentration, IC_{50}, and Ki were found to be 104 µM and 363 ± 4.7 µM, respectively. Low Ki value suggests that fluoxetine is a potent inhibitor of BChE even at therapeutic doses but the molecular mechanisms explaining the benefit of BChE inhibition in diseases remain to be elucidated.

Key words: Butyrylcholinesterase, fluoxetine, competitive inhibition

ÖZET

Yetkin O. At Serumu Bütirilkolinesteraz Enziminin Fluoksetin ile Kinetik Davranışının İncelenmesi. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2015.

Bütirilkolinesteraz (3.1.1.8.; BChE) omurgalı canlılarda bulunan bir serin esteraz enzimidir. BChE karaciğerde sentezlendikten sonra plazmaya salınmakta ve insan serum proteinlerinin yaklaşık olarak %0.1'ini teşkil etmektedir. BChE doğal ve ester bağı içeren sentetik bileşiklerin detoksifikasyonunda görev yapmaktadır. Ayrıca asetilkolinesteraz enzimi inhibe edildiğinde asetilkolinin ortamdan uzaklaştırılmasında rol oynamaktadır. BChE aktivitesindeki değişimler hastalıklar ile bağlantılıdır. Özellikle kolinerjik sistem anormallikleri ile Alzherimer hastalığındaki (AD) senil plak oluşumu arasında bir ilişki bulunmaktadır. Günümüzdeki tedavi yaklaşımları AD'nin tedavisinde kolinesteraz inhibitörlerini kullanmaktadır. Fluoksetin kan-beyin bariyerini kolaylıkla geçebilen seçici serotonin gerialım inhibitörüdür (SSRI). Çalışmamızda at serumu BChE enziminin fluoksetin ile etkileşiminin incelenmesi hedeflenmiştir. BC~E'nin tetramerik, dimerik ve monomerik formlarının molekül ağırlıkları sırasıyla 380 kDa, 190 kDa ve 95 kDa olarak hesaplanmıştır. Enzimin optimum pH'sı 8.1 olarak bulunmuştur. Optimum sıcaklık, aktivasyon enerjisi (Ea) ve sıcaklık katsayısı (Q_{10}) ise sırasıyla 36.4°C, 1526 cal/mol ve 1.19 olarak saptanmıştır. Kinetik çalışmalarda, V_m 20.59 ± 0.36 U/mg protein ve K_m ise 194 ± 14 µM olarak bulunmuştur. Fluoxetine BChE enzimini kompetitif (yarışmalı) olarak inhibe etmektedir. Yarım maksimal inhibisyon konsantrasyonu (IC_{50}) ve K_i sırasıyla 104 µM ve 36.3 ± 4.7 µM olarak hesaplanmıştır. Düşük K_i değeri fluoksetinin tedavi dozunda bile potansiyel BChE inhibitörü olarak kullanılabileceğini göstermektedir. Ancak hastalıklarda BChE inhibisyonun sağlayacağı yararın moleküler mekanizması da aydınlatılmalıdır.

Anahtar Kelimeler: Bütirilkolinesteraz, fluoksetin, kompetitif (yarışmalı) inhibisyon

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SYMBOLS AND ABBREVIATIONS

ACh	: Acetylcholine
AChE	: Acetylcholinesterase
AD	: Alzheimer's disease
APS	: Ammonium persulfate
BChE	: Butyrylcholinesterase
BChE-T	: Butyrylcholinesterase-tetramer
BChE-D	: Butyrylcholinesterase-dimer
BChE-M	: Butyrylcholinesterase-monomer
BMI	:Body mass index
BSA	:Bovine serum albumin
BTC	: Butyrylthiocholine iodide
BW284C51	: 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one dibromide
CAD	: Coronary artery disease
CAT	: Cholineacetyltransferase
CBB	: Coomassie Brillant Blue
ChE	: Cholinesterase
CYP	: Cytochrome P-450
dH_2O	: Distilled water
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
DTNB	: 5,5'-Dithiobis(2-nitrobenzoic acid)
E_a	: Energy of activation
HOL	: High-density lipoprotein
IC_{50}	: Half maximal inhibitory concentration
iso-OMPA	:Tetraiso-propylpyrophosphoramide
K_i	: Inhibitor constant
k_{cat}	: Turnover number

Km	: Michaelis constant
Kmapp	: Apparent Michaelis constant
MOPS	: 3-(N-Morpholino)propanesulfonic acid sodium salt
Native-PAGE	: Native-polyacrylamids,gel electrophoresis
OP	: Organophosphates
PAS	: Reripheral ionic site
R	: Gas constant
SOS	:Sodium dodecyl sulfate
SOS-PAGE	: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSRI	: Selective serotonin reuptake inhibitor
SuCh	: Succinylcholine
Tris	: Tris(hydroxymethyl)aminomethane
TCA	: Tricyclic antidepressants
TNB	: 5-thio-2-nitrobenzoate
TEME0	: N, N, N', N'-Tetramethylethylenediamine
010	: Temperature coefficient
Vmax	: Maximum velocity

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1. INTRODUCTION

Vertebrates have two different enzymes that hydrolyse acetylcholine (ACh). Acetylcholinesterase (EC 3.1.1.7; AChE) has a well-defined function in terminating the action of neurotransmitter ACh at the postsynaptic membrane in the neuromuscular junction. The other enzyme also hydrolyses ACh and many other esters, but has no known physiological function. It is called butyrylcholinesterase, pseudocholinesterase, non-specific cholinesterase, acylcholine acylhydrolase or plasma cholinesterase (EC 3.1.1.8; BChE) (Whittaker, 2010).

BChE belongs to the family of serine hydrolases. Active site of the enzyme contains serine amino acid and catalysis by BChE requires catalytic triad: Ser198, Glu325 and His438. BChE shows 50-55% sequence similarity with AChE. BChE's active site (S198, H438 and E325) and oxyanion hole (G116, G117 and A199) are identical to those of AChE (Nicolet et al., 2003). BChE is synthesized in liver and secreted into the plasma (Chatonnet and Lockridge, 1989), where it makes up approximately 0.1 % of the total serum proteins in humans (Ryhanen, 1983). BChE is encoded by one gene (64.57 kb) located on the long arm of chromosome 3 in humans. The mRNA is encoded by 4 exons. There are no alternatively spliced forms for BChE. Same BChE mRNA encodes the soluble, globular, tetrameric BChE in plasma as well as the membrane-bound forms in muscle and brain (Massoulie, 2002). More than 70 natural mutations have been reported in human BChE gene. Although the majority of these mutations are rare, the atypical and the K-variants are relatively common in Caucasian population. Individuals with atypical BChE (070G) show prolonged apnea after succinylcholine administration (Lockrigde and Masson, 2000).

In mammals, AChE is predominantly found in muscle and nervous tissue. In less significant amounts it is also localized in non-neuronal compartments including liver, placenta, lymphocytes where it probably involves in anti-inflammatory response (Wessler and Kirkpatrick, 2008). On the other hand BChE is expressed in many tissues, including lungs, intestinal mucosa, heart, liver and as well as brain (Massoulie, 2002). It is also present

in plasma (Brimijoin and Hammond, 1988). In the human brain, BChE is expressed in significant amounts in glial cells, while AChE predominates in neurons. BChE is also found in amygdala, hippocampus and thalamus (Darvesh and Hopkins, 2003).

Unlike AChE, BChE has no unique physiological substrate. Examination of BChE(+/+) and BChE(-/-) mice did not reveal any physiological alteration in body functions (Li et al., 2008). BChE does not appear to have a significant role in ACh hydrolysis under normal conditions. However, butyrylcholinesterase does have a role in neurotransmission in acetylcholinesterase deficient mice. The AChE(-/-) mice have normal levels of butyrylcholinesterase activity. Treatment of AChE(-/-) mice with OP results in inhibition of butyrylcholinesterase activity and lethality at concentrations well below those that cause lethality in wild-type mice (Chatonnet et al., 2003; Duysen et al., 2001). This finding suggests that butyrylcholinesterase performs the function of the missing acetylcholinesterase in these mice by hydrolyzing acetylcholine.

Although the function of BChE is yet unclear, it was demonstrated that BChE plays a role in the detoxification of natural as well as synthetic ester bond-containing compounds. Therefore, it is also called a bioscavenger enzyme (Ashani et al., 1991; Broomfield et al., 1991). Naturally occurring compounds include physostigmine (also called eserine) in the calabar bean, cocaine from the Erythroxylum coca plant, solanidine in green potatoes, huperzine A from the club moss *Huperzia serrata,* and anatoxin-a(S) an organophosphate in blue-green algae (Mahmood and Carmichael, 1987). The synthetic compounds include organophosphate nerve agents, organophosphate pesticides, carbamate pesticides and Alzheimer drugs donepezil and rivastigmine (Casida and Quistad, 2004; Duysen et al, 2007). BChE inactivates these compounds by reversible or irreversible binding or by hydrolysis.

Growing evidence in basic research and clinically-related studies brings forward that parasympathetic insufficiency and elevated inflammation as underlying mechanism in most of the peripheral and neurological

diseases. Serum cholinesterase activities were found to vary from control values in a number of diseases. Therefore, cholinergic parameters become more important as disease biomarkers. Elevated BChE activity has been shown in stroke (Ben Assayag et al., 2010; Shenhar-Tsarfaty et al., 2010), Alzheimer's disease (Podoly et al., 2009), Parkinson's disease (Sirviö et al., 1987) and rnetabolle syndrome (Shenhar-Tsarfaty et al., 2011) whereas in myocardial infarction (Goliasch et al., 2012), inflammatory bowel disease (Maharshak et al., 2013) and diabetes (Shenhar-Tsarfaty et al., 2011) decreased BChE activity was reported.

Mutant and wild type enzymes have shown that BChE can be used against organophosphate poisoning as a prophylactic agent (Lockridge et al., 1997; Raveh et al., 1997). Currently, the drugs used in Alzheimer's disease are cholinesterase inhibitors (Giacobini, 1997). The studies on effects of different drug groups such as anticonvulsants (Shih et al., 1991), 13-adrenergic agonists (Sitar, 1996) and blockers (Krnic and Bradavante, 1997), anti-depressants (Çokuğraş and Tezcan, 1997), opioids (Galli et al., 1996) and neoplastic agents (Gresl et al., 1996) have increased with the attempt to define a role for BChE in several metabolic pathways.

Fluoxetine (also known by the tradename Prozac) is a widely ysed selective serotonin re-uptake inhibitor for patients with major depression and it has little effect on other neurotransmitters (Guze and Gitlin, 1994). MOiieret al. reported that fluoxetine inhibited the hydrolytic activities of AChE and BChE when acetylthiocholine was used as a substrate for both enzymes (Müller et al., 2002). On the other hand, kinetic behaviour of the BChE by using butyrylthiocholine in the presence of fluoxetine is unknown. It has been known that affinity of BChE to acetylthiocholine is much lower than that of butyrylthiocholine (Pezzementi et al., 2011). In our study it was aim to study the inhibitory kinetic behavior of fluoxetine on equine serum butyrylcholinesterase enzyme.

2. GENERAL INFORMATION

2.1. Cholinesterases

The cholinergic system plays an important role in neurotransmission in peripheral and central nervous systems. The cholinergic neurotransmitter acetylcholine (ACh) is synthesized by cholineacetyltransferase (CAT) (Figure 2.1) and hydrolysed by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Reid et al., 2013) enzymes for terminating its neurotransmitter function (Mesulam et al., 2002a).

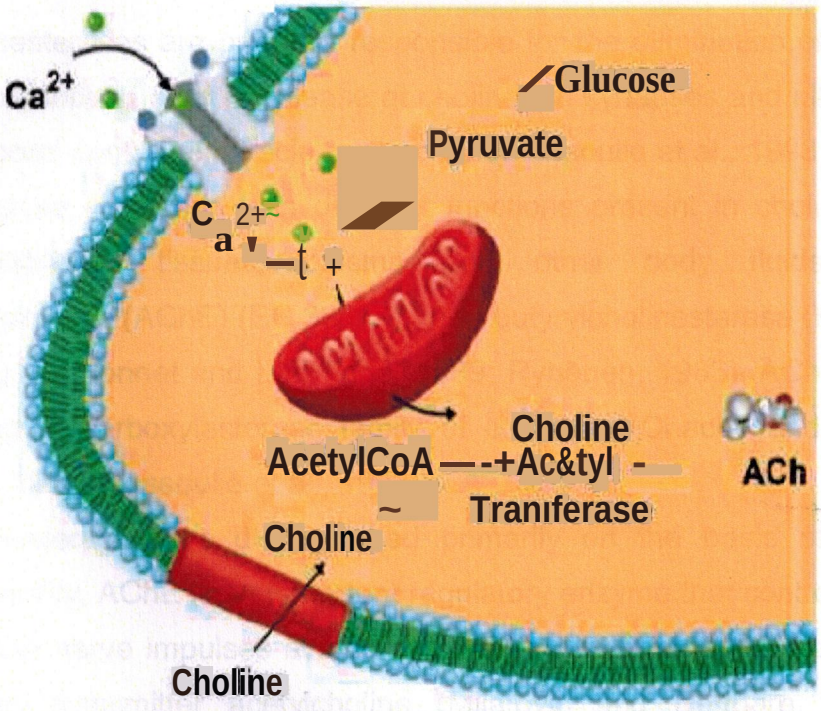
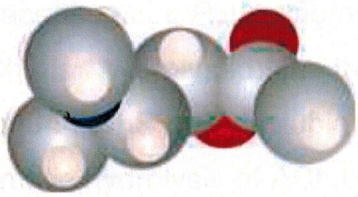


Figure 2.1 Synthesis of acetylcholine (Waymire, 2000)

Acetylcholine (Figure 2.2) plays an important role in the nervous system. It is a neurotransmitter which enables chemical communication between a nerve cell and a target cell. Target cell may be another nerve cell, muscle fiber or gland. The nerve cell releases acetylcholine into the synapse between the two cells. Released acetylcholine binds to specific receptors on

a target cell and passes the signal on to target nerve cell, muscle or gland (Taylor and Brown, 1999).



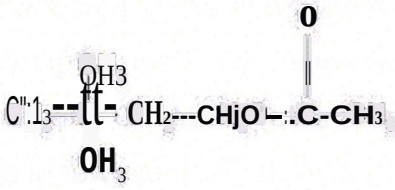


Figure 2.2. Structure of acetylcholine (Waymire, 2000)

Cholinesterases are primarily responsible for the elimination of ACh within one millisecond after its release at cholinergic synapses and allowing precise temporal control of muscle contraction (Massoulié et al., 1993). Two structurally close esterases with different functions present in cholinergic and noncholinergic tissues, plasma and other body fluids are acetylcholinesterase (AChE) (EC 3.1.1.7) and butyrylcholinesterase (BChE) (EC 3.1.1.8.) (Chatonnet and Lockridge, 1989; Ryhänen, 1983). AChE and BChE belong to carboxylesterase family of enzymes (Chacho and Cerf, 1960; Koelle,1984; Massoulié et al., 1993).

Cholinesterases are distinguished primarily on the basis of their substrate specifity. AChE is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter acetylcholine (Milatovic and Dettbarn, 1996; Schetinger et al., 2000). AChE hydrolyzes acetylcholine faster than other choline esters and it is less active on butyrylcholine. BChE is highly efficient at hydrolyzing both butyrylcholine and acetylcholine (Habig and Di Giulio, 1991). BChE is more active on the synthetic substrates, propionylcholine or butyrylcholine, than acetylcholine (Toutant, 1986). Butyrylcholine is not a physiological substrate in human brain and is used to differentiate between the two types of cholinesterases (Giacobini, 2001).

The two enzymes is also distinguished by their affinity or reactivity with various selective inhibitors, such as 1,5-bis(4-allyldimethylammonium-

propylpyrophosphoramide (iso-OMPA) for BChE (Brimijon and Rakonczay, 1986; Radie et al., 1993). The first known anti-cholinesterase agent was physostigmine, also known as eserine, a major alkaloid found in the seeds of the fabaceous plant *Physostigma venenosum* (Zhao et al., 2004).

ACh is hydrolysed in the synaptic cleft by membrane-bound tetrameric G4 AChE or by soluble monomeric G1 AChE (Lane et al., 2006). The enzymatic hydrolysis of ACh occurs in few steps. First, ACh is bound by its quarternary nitrogen atom to anionic site and by carboxyl group to esteratic site of AChE and enzyme-substrate complex is formed. Then acetyl-enzyme intermediate is formed by separating of choline. This acylated enzyme reacts with water to make acid-enzyme complex, which then spontaneously breaks down into acetic acid and AChE. (Stepankova and Komers, 2008).

2.2. Butyrylcholinesterase

Human butyrylcholinesterase (EC 3.1.1.8; BChE) is a serine esterase which is present in vertebrates (Lockridge, 1990). Butyrylcholinesterase is also known as acylcholine acylhydrolase, pseudocholinesterase, non-specific cholinesterase. Natural substrate of BChE is not knGw~ and the enzyme is named after an artificial substrate, butyrylcholine (Reubsaet and Ringvold, 2005). Reaction mechanism is shown in Figure 2.3.

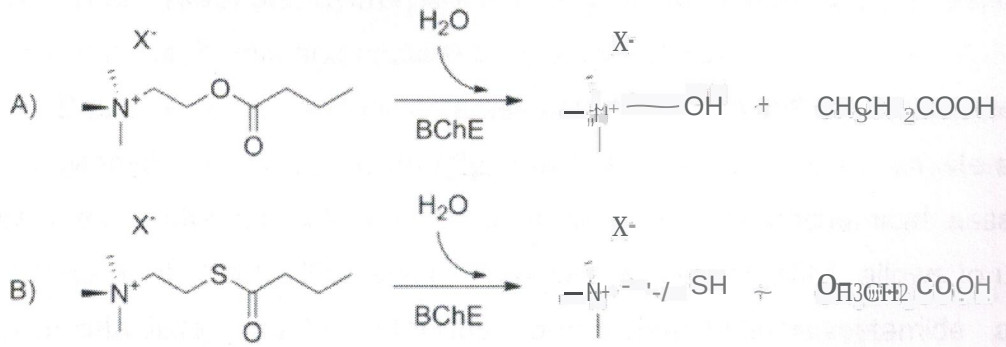


Figure 2.3. Esterase and thioesterase activity of BChE on conversion of butyrylcholine (A) into choline and butyrylthiocholine (B) into thiocholine with simultanous releasing of butyric acid. (Pohanka, 2013).

Human serum cholinesterase, is a globular, tetrameric molecule with a molecular mass of approximately 340 kDa (Haupt et al., 1966). BChE is mainly localized in neuroglial cells and can also be found in cholinergic synapses, neurons and endothelial cells (Darvesh et al., 1998; Darvesh and Hopkins, 2003).

The importance of BChE in cholinergic neurotransmission is further supported by the observation that AChE-knockout mice survive to adulthood. This study shows that BChE is able to compensate for the lack of AChE, allowing the continued regulation of cholinergic neurotransmission (Li et al. 2000; Xie et al., 2000).

AChE is inhibited, while BChE is activated by an increase in substrate (Ach) concentration. In humans, AChE is more abundant in the central nervous system, end plate of skeletal muscle and erythrocyte membranes while BChE is more abundant in serum (Massoulié et al., 1993).

The physiological role of BChE remains unclair (Chatonnet and Lockridge, 1989; Mack and Robitzki, 2000). Because BChE is relatively abundant in plasma (about 3 mg/liter), and can degrade a large number of ester containing compounds, it plays important pharmacological and toxicological roles (Lockridge and Masson, 2000). For instance, BChE is a potential detoxifying enzyme to be used as a prophylactic scavenger against neurotoxic organophosphates such as nerve gas soman (Allon et al., 1998; Raveh et al., 1993) and hydrolyses a variety of xenobiotics such as aspirin, succinylcholine, heroin and cocaine (Lockridge. 1988).

Beside esterase and thioesterase activity, both AChE and BChE exert aryl acylamidase activity. Currently, there are number of cholinesterase substrates useful for colorimetric, fluorimetric or electrochemical assays (Pohanka at al., 2009). The aryl acylamidase activity of BChE allows to use o-nitroacetanilide, m-nitroacetanilide, o-nitrophenyltrifluoroacetamide and 3(acetamido) N,N,N-trimethylanilinium as chromogenic substrates (Masson et al. 2007). The o-nitroacetanilide is probably the most frequently used chromogenic substrate of acylamides. The main disadvantage of aryl

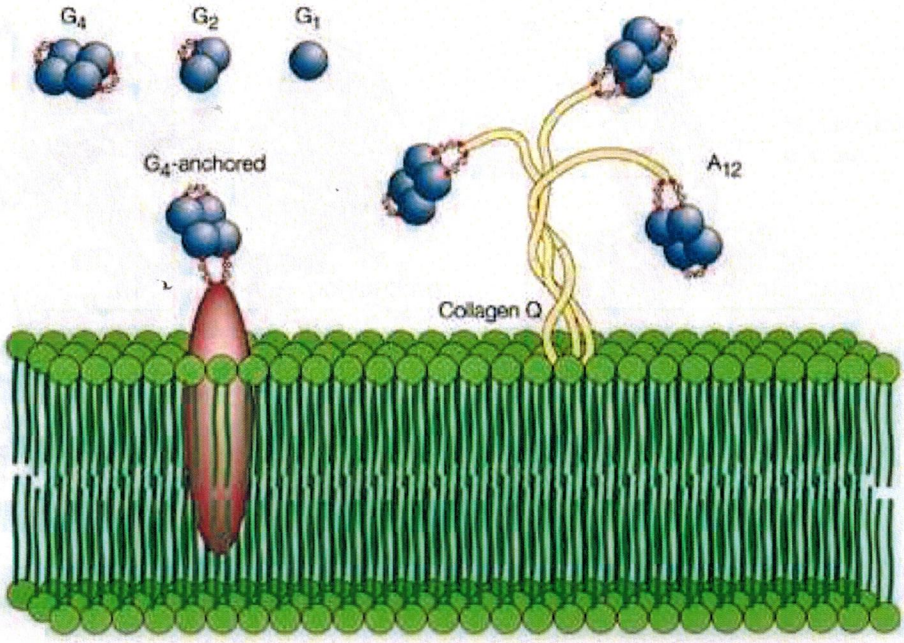


Figure 2.4. Models of BChE structure (Darvesh et al., 2003)

Structure and action mechanism of esteratic active center

The active site within the structure of human BChE gap is schematized and the peripheral ionic site (PAS) is found at the mouth of the gorge. Asp70 and Tyr332 residues of PAS are initial binding sites of the charged substrates and have a bond that controls the operate design of the BChE situation gorge (Tougu, 2001).

The active site of human BChE contains a catalytic triad residues, Ser198, Glu325 and His438, determining the esteratic activity of the enzyme (Suarez et al., 2006). The long and narrow active site gorge is about 20 A deep and includes two sites of ligand interaction: an acylation site at the base of the gorge with the catalytic triad and a peripheral site at its mouth. In BChE and AChE, the hydrolysis is carried out by a "catalytic triad" of Ser, His and Glu in the active center (Harel et al., 1993).

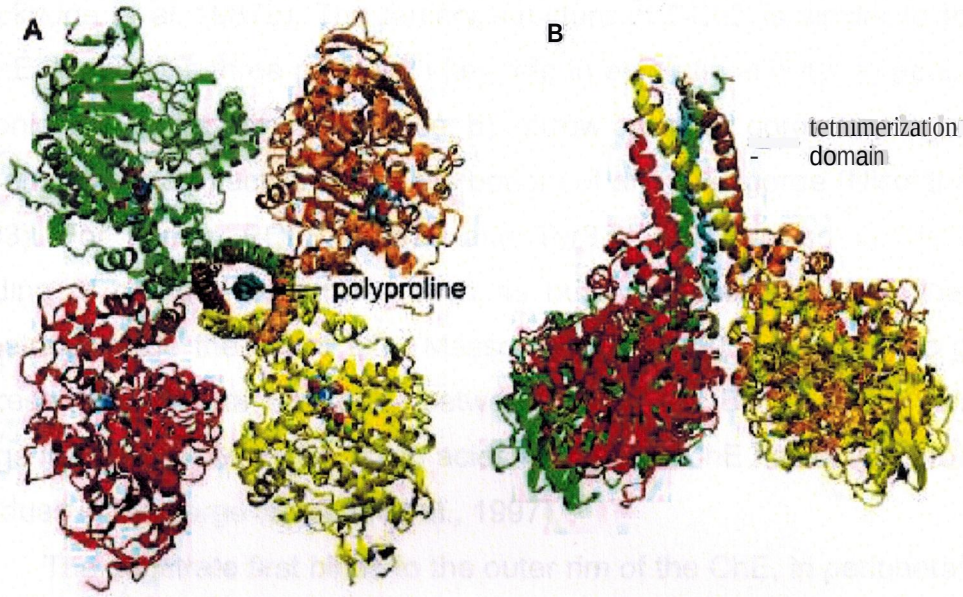


Figure 2.5. Structure of the BChE tetramer. A. Top view of the BChE tetrameric protein shows four identical subunits (each 85 kDa) interacting with each other via a four-helix bundle at the C-termini. A polyproline-rich peptide lies in the center of the four-helix bundle which is a tetramerization domain. B. Side view of the modeled BChE tetramer shows the four helix bundle projecting out of the globule. The polyproline-rich peptide in the center of the four-helix bundle is hydrogen bonded to Trp543, Trp550, Trp557 and three other hydrophobic residues, all on the same side of the amphiphilic helix. The polyproline-rich peptide is not released from the tetramer when BChE is diluted. The dissociation constant for polyproline/BChE is estimated to be in the nanomolar range ($K_d = 10^{-9}$ M) or even lower (Pan et al., 2009).

The most immanent form of BChE is a tetramer (Figure 2.5). Each subunit of the tetramer has 574 amino acids and nine carbohydrates linked to nine asparagine residues. Forty amino acids at the C-terminal are responsible for tetramerization of subunits. The total weight of one subunit is approximately 85 kDa. Similarity between AChE and BChE resulted in finding that there is a 53.8% identity between the enzymes in their sequence

(Lockridge et al., 1987b). The tertiary structure of BChE is similar to that of AChE. It contains three parts with key role in esteratic activity: a) peripheral anionic site on the enzyme surface, b) narrow aromatic gorge leading inside the enzyme and c) active site at the bottom of aromatic gorge (Nicolet et al., 2003). For human BChE, Asp70 and Tyr332 are involved in the initial binding of charged substrates such as butyrylcholine and allow them to penetrate inside the active site (Masson et al., 1999). The aromatic gorge represents the major difference between AChE and BChE. AChE has the gorge lined by 14 aromatic amino acids residues, BChE has only 8 aromatic residues in the gorge (Saxena et al., 1997).

The substrate first binds to the outer rim of the ChE, in peripheral site, but the hydrolysis occurs inside the enzyme, in the bottom of a gorge, which is divided into four main subsites: esteratic site, oxyanion hole, anionic subsite and acyl pocket. The esteratic site contains the catalytic structure of the enzyme and includes a serine, histidine and a glutamate residue (Figure 2.6). (i) the serine residue induces a nucleophilic attack to the carbon of the carbonyl group of the ester substrate; (ii) histidine stabilizes the serine intermediate by strong hydrogen bonds; (iii) the negative charge of glutamate stabilizes the histidinium cation (Houghton et al., 2006).

The oxyanion hole contains hydrogen donors which stabilize the tetrahedral intermediate of the substrate that is formed during the catalytic process.

The anionic subsite (choline-binding subsite or hydrophobic subsite) contains several aromatic residues, which are important for the binding of quaternary ammonium ligands by TT-cation interactions. The number of aromatic amino acids differs according to the enzyme. Some aromatic amino acid residues present in the acyl pocket and in the peripheral site of AChE are replaced by aliphatic amino acids in BChE. As aliphatic amino acids are smaller than aromatic amino acids, these differences allow larger substrates to enter the active site of BChE. The active site of BChE can hydrolize larger acyl groups, such as those with four carbons (e.g. butyrylcholine) or aromatic rings (e.g. benzoylcholine) (Houghton et al., 2006).

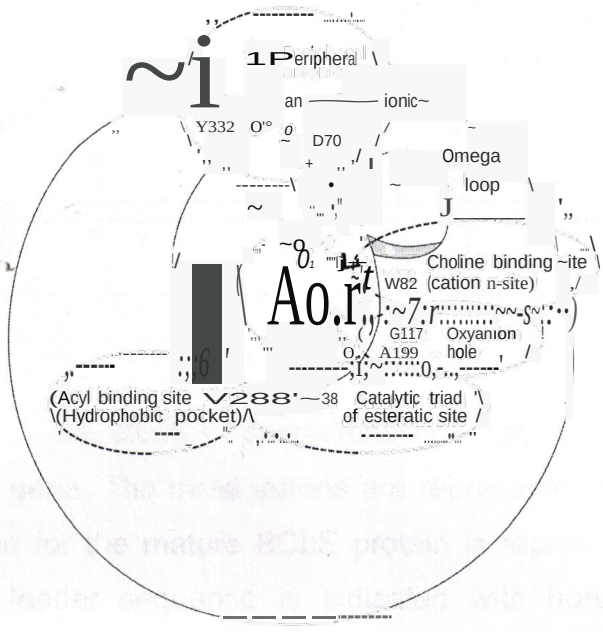


Figure 2.6. Substrate binding sites of BChE (Çokuğraş, 2003)

2.2.2. Genetic Variants of BChE

AChE and BChE are exactly distinct enzymes encoded by two different but related genes (Arpagaus et al., 1990). AChE and BChE contribute 65% amino acid sequence similarity (Nachmansohn and Wilson, 1951) and there is one gene for BChE, located on chromosome 3q26, with multiple nucleotide variations identified at this locus (Figure 2.7) (Lockridge and Masson, 2000).

In human, point mutations and frameshifts in BChE gene localyzed on chromosome 3q26 cause the different BChE genotypes that have different levels of enzyme activity. Atypical BChE (Asp70Gly mutant or dibucain resistant mutant) is the best known variant and has reduced activity, because Asp70 plays an important role for initial binding of positively charged substrates to active site gorge. K variant (Ala539Thr mutant), J variant \Glu497Val mutant) and fluoride resistant variants (Thr247Met or Gly390Val mutants) also show reduced BChE activities.

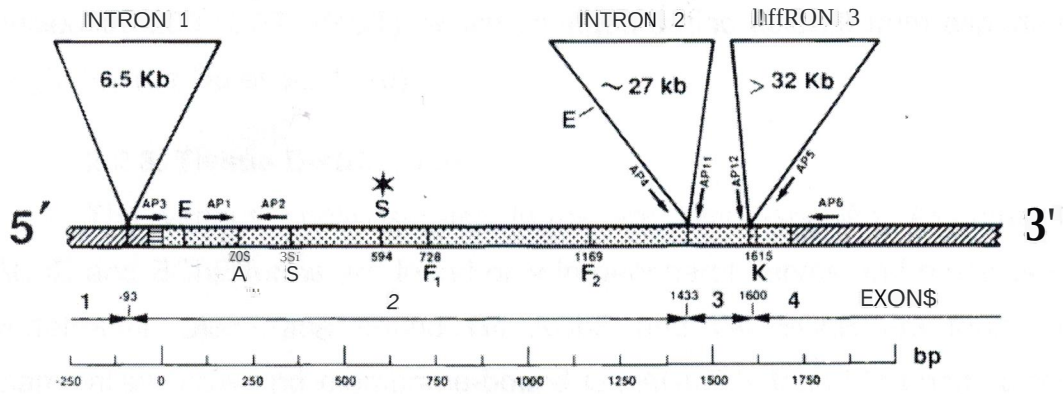


Figure 2.7. Structural characteristics of the human butyrylcholinesterase gene. The three intrans are represented by triangles, and the coding region for the mature BChE protein is represented by the dotted portion. The leader sequence is indicated with horizontal bars. Amplification primers for exon 2 (AP3 and AP4), exon 3 (APII and AP12), and exon 3 (AP5 and AP6) are indicated by arrows. (La Du et al, 1990)

Furthermore, approximately 20 different silent genotypes have been recognized with 0-2% of normal activity. On the otherhand, C5+ variant (combination of BChE with an unidentified protein), Cynthiana variant (increased amount of BChE than normal level) and Johannesburg variant (increased BChE activity with normal enzyme level) have increased activity than usual BChE (La Du et al., 1990; Lockridge, 1990).

Direct sequencing of the atypical genomic DNA revealed a change in only one of the 1722 bases coding for 574 amino acids of the atypical BChE subunit (La Du et al., 1990). The polymorphic forms of the K-variant enzyme (Ala/Thr) at position 539 depend on the base present at position 1615 (GCA/ACA, respectively) (Bartels et al., 1989). A frame-shift mutation at nucleotide position 351, which changes codon 117 from GGT to GAG (McGuire et al., 1989; Nogueira et al., 1990), explains one type of silent phenotype (La Du et al., 1990). One structural mutation at nucleotide 728 (ACG to ATG; Thr to Met at codon 243) was found in two members of one family with the fluoride-resistant trait. Atypical mutation was defined at

nucleotide 209 (GAT→GGT), which changed amino acid 70 from aspartate to glycine (La Du et al., 1990).

2.2.3. Tissue Distribution

The various cholinesterase forms are tissue-specific. Asymmetric AChE and BChE forms are found only in peripheral nerves and muscles of vertebrates. Membrane bound G4 AChE and G4 BChE are found in mammalian brain and membrane-bound G2 AChE is found in erythrocytes (Massoulie and Bon, 1982).

BChE corresponds to only 10% of total ChE in the normal brain, being more abundantly expressed in liver, lung and heart tissues and is predominantly present in plasma (Jbilo et al., 1994). In the normal brain, BChE activity has been located in all regions that receive cholinergic innervation. It is mainly found in glial cells and in endothelial cells, whereas AChE is located in neurons and axons (Mesulam et al., 2002b).

AChE is predominant in muscles and nervous system, where it is usually accompanied by a lower level of BChE, especially at early developmental stages. BChE is also expressed in other tissues and most notably is synthesized in the liver and secreted into the plasma. BChE, but not AChE, is expressed in the chorionic villi of human embryos. Whereas the role of AChE in cholinergic transmission is unambiguous, the function of BChE remains unsolved (Zakut et al., 1991).

AChE is found exuberant in brain, muscle and blood corpuscle membrane, whereas BChE has higher activity in liver, intestine, heart, excretory and respiratory organs (Nachmansohn and Wilson, 1951).

2.2.4 Functions of BChE

BChE which is synthesized by the liver, is the most abundant cholinesterase in human serum (Prody et al., 1987) and the assay of BChE activity is also considered as a liver function test (Boopathy et al., 2007). Although the exact physiological function of BChE is still unclear, the assay of serum BChE activity is especially used in the diagnosis of pesticide poisoning and in the assessment of patients with prolonged apnea after

administration of succinylcholine during anesthesia (Nelson and Burritt, 1986). BChE is involved in the degradation of succinylcholine, used as a myorelaxant in surgical operations. It also hydrolyzes drugs such as heroin and physostigmine and activates the antiasthmatic prodrug bambuterol (Tunek and Svensson,1988).

Esterases in human plasma have an important role in the disposition of drugs. They participate in activation of ester prodrugs, for example, the prodrug bambuterol is converted to the anti-asthma drug terbutaline and isosorbide-based prodrugs release aspirin. A second role is to inactivation of drugs. For example, esterases in plasma inactivate the local anesthetics procaine and tetracaine, the muscle relaxants, succinylcholine and mivacurium, and the analgesics, aspirin, and cocaine (Li et al., 2005).

Detoxification

Acetylcholinesterase-inhibiting chemical exposures interact with a variety of circulating enzymes in humans, including BChE and AChE. BChE is present more than 10 times higher than the level of AChE in whole blood. It is known to provide protection from adverse effects of carbamates, organophosphates and other chemicals by acting as a scavenger, binding them molecule-for-molecule, thereby sparing circulating levels of AChE (Lockridge and Masson, 2000).

Although the real substrate(s) is still unknown, BChE can hydrolyze hydrophobic and hydrophilic carboxylic or phosphoric acid ester containing compounds. Its toxicological and pharmacological importance becomes clear when an individual exposures to poisonous compounds targeting to acetylcholine binding sites. Loss of AChE function leads to muscle paralysis, seizure and may cause death by asphyxiation. BChE can be considered as an endogenous scavenger of anticholinesterase compounds. BChE detoxifies them before they reach to AChE at physiologically important target sites (Çokuğraş, 20Ö3).

Succinylcholine (SuCh)

The importance of BChE in the hydrolysis of several drugs, particularly in the hydrolysis of succinylcholine (SuCh), is well established. The classical pharmacogenetic studies by Kalow et al., during the 1950's, made it clear that people with a hereditary deficiency in their serum BChE show an exaggerated response to succinylcholine, if given the standard amount of this muscle relaxant (Kalow and Genest, 1957).

SuCh is a neuromuscular blocking drug used for endotracheal intubation during operation, endoscopies and electroconvulsive therapy. It is hydrolyzed by BChE to succinylmonocholine and choline. Whereas the diester is a powerful muscle relaxant, monoester is not. When SuCh is injected intravenously, about 90% of its dose is hydrolyzed by BChE within 1 min and rest amount reaches the nerve-muscle junctions and binds to a receptor. In result, the nerve-end plate is depolarized and losses sensitivity to acetylcholine. SuCh administration to individuals carrying no or reduced BChE activity variants results in prolonged apnea, since a large overdose reaches to the nerve-muscle junctions. In order to avoid from this result, the assay of serum BChE activity is used in the assessment of patients with prolonged apnea after administration of SuCh during anesthesia.~.~ prolonged apnea occurs, well-timed intravenous administration of highly purified human serum BChE decreases the duration of the induced apnea (Viby-Mogensen, 1981).

Organophosphates (OPs) and Carbamates

Organophosphorus pesticides inhibit esterase enzymes, especially acetylcholinesterase in synapses and on red-cell membranes, and butyrylcholinesterase in plasma (Lotti, 2001). Exposure to organophosphate pesticides disrupts neurotransmission by inhibiting AChE resulting in acetylcholine accumulation within the junction and neural overstimulation results in death due to cardiovascular and respiratory collapse (Tama, 2007).

Diagnosis of organophosphorus poisoning should be confirmed with an assay to measure butyrylcholinesterase activity in plasma (or acetylcholinesterase in whole blood) (Lotti, 2001). AChE activity has been proposed as a biomarker of exposure to neurotoxic compounds in aquatic organisms. AChE is mainly inhibited by organophosphorus compounds and carbamates, which are pesticides that are widely used in agriculture (Cajaraville et al., 2000).

Cocaine

BChE plays an important role in cocain metabolism. It is the major detoxification enzyme of both natural (-) cocain and unnatural (+) cocain in plasma. The inactive metabolites produced by BChE is ecgonine methyl ester and benzoic acid that are rapidly excreted from circulation by kidney (Hoffman et al., 1996; Matter et al., 1996). Cocain abuse is a medical problem in all around of the world. Symptoms of cocaine toxicity include grand-mal seizure, cardiac arrest, stroke, elevated body temperature. Animal studies showed that administration of purified human serum BChE protected mice and rats from the lethal effects of cocaine as well as from hypertention and arrythmia (Mattes et al., 1997; Sun et al., 2001). Although BChE protects against cocain toxicity, it acts slowly. Turnover number (k_{cat}) of natural (-) cocaine is found to be as 3.9 min^{-1}. To increase the catalytic efficiency of BChE towards cocaine by increasing its binding affinity and hydrolysis rate, different mutants of the enzyme have been tested. It is found that Ala328Tyr mutant has an improved cocaine hydrolase activity (Xie et al., 1999).

Aspirin

Aspirin is one of the examples of negatively charged substrates of BChE. BChE is the major plasma esterase involved in hydrolysis of aspirin to salicylate. Usual and atypical BChEs can hydrolyze aspirin with the same kinetic manner (Masson et al., 1998).

Antidepresants

Amitriptyline, fluoxetine, sertraline as clinical antidepresssants are used worldwide. Besides of their confirmed efficiency, especially amitriptyline is characterized by anticholinergic side effects including memory impairment, delirium, behavioural toxicity and cardiovascular dysfunctions (Morttgomery and Kasper, 1995). Reason of these side effects is the inhibition of AChE and BChE activities. It is reported that AChE from cerebral cortex (Barcellos et al., 1998) and erythrocyte membrane (Müller et al., 2002) is inhibited by imipramine, desipramine and amitriptyline at high concentrations. Amitriptyline is a partial competitive inhibitor of human serum BChE (Çokuğraş and Tezcan, 1997). Long-term treatment with amitriptyline causes acquired BChE and AChE deficiency at relatively close to the clinical levels. If these patients have to be operated on because of emergency, the possibility of succinylcholine apnea must be considered (Çokuğraş, 2003).

Heroin

Heroin is hydrolyzed by BChE to 6-acetylmorphine which penetrates the blood-brain barrier and is hydrolyzed to morphine by the enzymgs.in the brain. BChE is the only enzyme in human serum that hydrolyzes heroin. Persons having silent BChE variants are not able to hydrolyze heroin (Lockridge et al., 1980).

Fat metabolism

Butyrylcholinesterase (BChE) is a serine hydrolase which is related to lipid metabolism and has been associated to metabolic syndrome risk variables, such as body mass index (BMI), waist-hip ratio, waist circumference, weight, cholesterol and triglyceride levels (Alcantara et al., 2005; Benyamin et al., 2011; Furtado-Alie et al., 2008; Iwasaki et al., 2007; Randell et al., 2005; Souza et al., 2005). Although the role of BChE in metabolic pathways is not fully defined, it has been proposed that it could be responsible for the hydrolysis of choline esters, which are products of the

free fatty acid metabolism and liver lipogenesis. In obese individuals, there is a tendency of the BChE activity to be higher than in non-obese, which may be related to the increased availability of free fatty acids characteristic of obesity (Alcantara et al., 2005; Furtado-Alie et al., 2008; Randell et al., 2005).

AChE and BChE are often found in blood and are related to options of the metabolic syndrome. The metabolic syndrome is characterized by abdominal obesity, low levels of high-density lipoprotein **(HOL),** sterol, elevated fasting aldohexose levels and hypertriglyceridaemia with cardiovascular disease during involvement of AChE and BChE in lipid metabolism (Rao et al., 2012).

2.3. Relationship between Butyrylcholinesterase activity and diseases

Biomarkers are measurable biochemical, physiological, and behavioral alterations in an organism and can be recognized as associated with an ôccured or possible health impairment or disease (Manno et al., 2010).

The parasympathetic neurotransmitter ACh is extremely labile and difficult to use in diagnosis (Soreq and Seidman, 2001), which is why the use of its hydrolyzing enzymes are utilized as an indirect measurement for parasympathetic dysfunction.

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive decline, impaired abilities to perform activities of daily living and behavioral problems (Darreh-Shori and Soininen, 2010). Cognitive symptoms of AD are related with cholinergic system. ACh synthesizing choline acetyltransferase deficiency, reduced choline uptake and ACh release observed in AD patients cause presynaptic cholinergic deficiency (Berson et al., 2012). AChE and BChE involve in regulation of cholinergic transmission and the proteinase activity causes the development of Alzheimer's disease as a result of the production of 13-amyloid plaques (McClintock, 1989). BChE was found to be co-localised with senile plaques in the central nervous system, and plays a role in the progressive 13-amyloid aggregation and senile plaques maturation (Gomez-Ramos and Moran,

1997). BChE cleaves the amyloid precursor protein, which is found in abundance in normal brain, to 13-amyloid protein in AD. Then 13-amyloid proteins deposit and constitute j3-amyloid plaques (Guillozet et al., 1997).

The therapy of AD at early and moderate levels is based on acetylcholinesterase inhibitors such as synthetic donepezil and galantamine. These inhibitors cause peripheral and central side effects including gastrointestinal disturbances, insomnia, fatigue or depression (Lane et al., 2006). The serious side effects caused by licensed drugs used to treat AD have forced researchers to investigate safer AChE or BChE inhibitors from natural sources (Wszelaki et al., 2010).

Parkinson's disease is classically characterized as a motor neurodegenerative disorder. The motor symptoms in Parkinson's disease are degenerated dopamine-ACh balance due to reduced striatal dopaminergic tone and following cholinergic overactivity (Calabresi et al., 2006). Anticholinergic drugs were given to improve motor aspects of the disease in past years. Benmoyal-Segal et al. declared that serum AChE activity was reduced in Israeli Parkinson's disease patients, as compared with controls, but the AChE homologous enzyme, BChE, was not (Benmoyal-Segal et al., 2005).

BChE levels are strongly influenced by inflammation, sensitively decreasing in the acute inflammatory phase and promptly increasing when inflammation improves (Hubbard et al., 2008).

In patients on chronic hemodialysis, BChE was used as a prognostic marker, beside to the other traditional parameters (anthropometric indices, serum protein content, immune response indexes) (Guarnieri et al., 1980; Kaizu et al., 1998).

Together with serum albumin concentration, BChE levels were described as direct markers of malnutrition and indirect index of inflammatory activity in Crohn's disease (Khalil et al., 1980).

Anorexia nervosa patients developed severe and acute liver failure with increase of serum transaminases and reduction of BChE levels. (De Caprio et al., 2006).

Serum BChE has been associated with the development of coronary artery disease (CAD) (Alcantara et al., 2002; Calderon-Margalit et al., 2006). Calderon-Margalit et al. demonstrated that individuals in the lowest quintile of BChE activity had significantly higher rates of all-cause and cardiovascular mortality (Calderon-Margalit et al., 2006). Goliasch et al. demonstrated a strong association between decreased serum butyrylcholinesterase and long-term adverse outcome in patients with known CAD, which was stronger in stable CAD patients than in those with acute coronary syndrome (Goliasch et al., 2012).

2.4. Fluoxetine

Fluoxetine is a racemic mixture of two enantiomers, which are S-enantiomer and R-enantiomer. S-enantiomer is ~1.5 times more effective in the inhibition of serotonin reuptake than the R-enantiomer (Gram, 1994). The pharmacological distinction between enantiomers is the active metabolite norfluoxetine, with the S-enantiomer having ~20 times higher reuptake blocking potency than the R-enantiomer (Fuller et al., 1992). The concentration of racemic fluoxetine is normally less than the concentration of racemic norfluoxetine. In blood, the concentrations of the N-de~~ylated metabolite are higher for S-norfluoxetine than for R-norfluoxetine (Baumann and Rochat, 1995).

After oral intake, fluoxetine is almost completely absorbed. Through hepatic first-pass metabolism, the oral bioavailability is below 90% (Catterson and Preskorn, 1996). The deposition is highest in lungs, an organ enriched with lysosomes. High volume of distribuiton (Vd) of fluoxetine is considered to be associated with lysosomal accumulation (Daniel and W6jcikowski 1997a; 1997b). In spite of the high V_d, similar with TCAs, accumulation in the brain is lower than for other SSRIs shown *in vitro* in brain slices (Daniel and W6jcikowski, 1997b).

Fluoxetine has a long half-life ($t_{1/2}$), changing between 1-4 days. For norfluoxetine, $t_{1/2}$ varies between 7 and 15 days (Gram, 1994; Benfield et al., 1986). Fluoxetine exhibits nonlinear kinetics, indicated by a

disproportionate increase in its blood concentrations after dose escalation (Caccia et al., 1990).

Fluoxetine exposes extensive metabolic conversion, leading to the active metabolite norfluoxetine and multiple other metabolites (Figure 2.8).

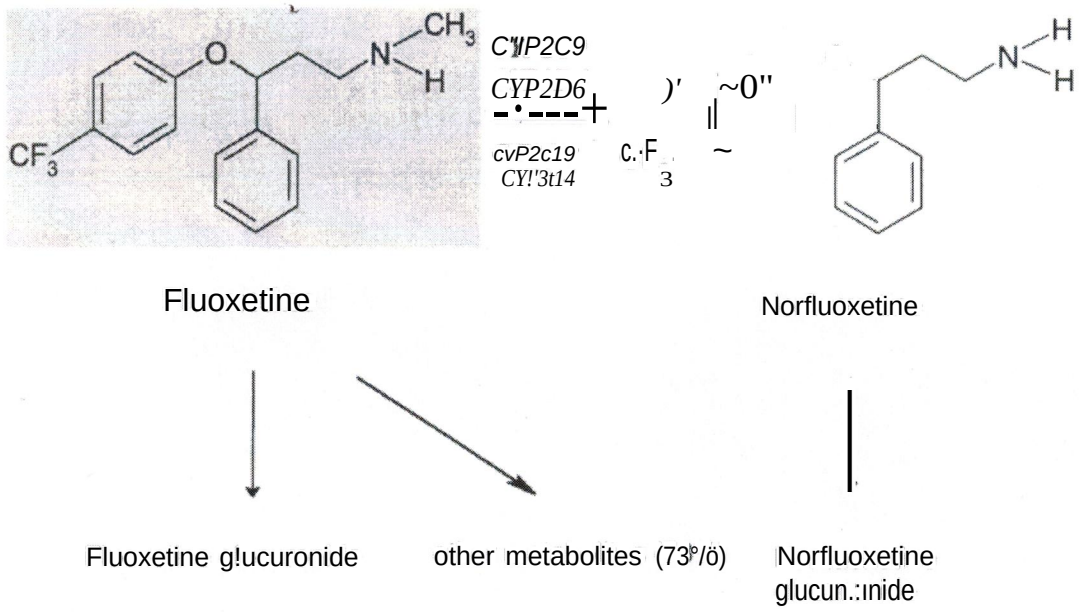


Figure 2.8. Metabolism of fluoxetine and cytochrome P-450 (GYP) isoenzymes, amine oxidase, and N-acetyltransferase, suggested to catalyze the Phase I reactions (Hiemke and Hartter, 2000).

After oral intake, fluoxetine is mainly excreted in urine with less than 10% excreted unchanged or as fluoxetine N-glucuronide (Benfield et al., 1986). Only a few studies have investigated the GYP isoenzymes responsible for the metabolism of fluoxetine and the results have been inadequate. Investigations of fluoxetine focused on the N-demethylation. Hamelin and co-workers reported a significant contribution of CYP2D6 in the N-demethylation of fluoxetine in healthy volunteers (Hamelin et al., 1996). It was suggested that CYP2C9 plays a leading role in the N-demethylation of fluoxetine *in vitro* with a possible assistance of the CYP2C19 and a CYP3A isoform. The assistance of CYP2D6 was found to be insignificant (von Moltke et al., 1995). Recent studies indicated that the CYP2D6 activity is responsible

for the clearance of R-, S-fluoxetine and S-norfluoxetine, but not R-norfluoxetine (Fjordside et al.,1999).

3. MATERIALS AND METHODS

3.1. Chemicals

Formaldehyde, 2-mercaptoethanol, Trizma base, acrylamide, **N,N'-**methylenebisacrylamide, qlycerol, ammonium persulfate, 5,5'-dithio-bis(2-nitrobenzoic acid), dimethyl sulphoxide, S-butyrylthiocholine iodide, **N, N,** N', N'-tetramethylethylenediamine, methanol, sodium thiosulfate, silver nitrate, glycine, potassium hydroxide, sodium azide, butyrylcholinesterase (from equine serum), sodium dodecyl sulfate, dithiooxamide, sodium carbonate, sodium sulphate, bovine serum albumin and bromophenol blue were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic acid and ethanol were obtained from Riedel-de Haen (Germany). Ammonium sulfate was obtained from Merck (Germany). Copper (II) sulfate pentahydrate and orthophosphoric acid were obtained from AppliChem (Darmstadt, Germany). Coomasie Brilliant Blue G-250 and Coomasie Brilliant Blue R-250 were obtained from Fluka (Steinheim, Germany). Roti-mark Standard was obtained from Carl Roth GmbH (Karlsruhe, Germany). Fluoxetine hydrochloride was purchased form LKT Laboratories (St. Paul, MN, USA).

3.2. Methods

3.2.1. Determination of Protein Concentration

Protein concentration of the butyrylcholinesterase enzyme purified from equine serum was carried out according to the Bradford protein assay. (Bradford, 1976). Coomassie Brilliant Blue G-250 is an acidic dye and specifically binds to positively charged groups (basic amino acids, e.g. arginine) in protein structure. Upon binding, the wavelenght at which Coomassie Brilliant Blue G-250-protein complex gives the maximum absorbance shifts from 470 nm to 595 nm. The absorbance of the dye-protein complex is measured at 595 nm and the amount of the protein is determined from the standard curve prepared by using bovine serum albumin.

Bradford reagent was prepared as follows: Twenty five mg of Coomassie Brilliant Blue G-250 was dissolved in 12.5 ml of absolute ethanol.

Twenty five ml of 85% orthophosphoric acid was added and the final volume was adjusted to 250 ml with distilled water. Reagent was filtered through Whatman No:1 filter paper and stored in dark bottle.

Bovine serum albumin (BSA) was used as a standart. 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml and 600 µg/ml of standart BSA concentrations were prepared by diluting 1 mg/ml stock BSA solution. Twenty µl of standart BSA solution was mixed with 1 ml of Bradford reagent and the absorbance was measured at 595 nm by using Perkin Elmer Lambda 25 UVNIS Spectrophotometer. In the same way, 20 µl of 1:50 diluted BChE enzyme was mixed with 1 ml of Bradford reagent and the absorbance was measured at 595 nm. Standards and samples were prepared in triplicates. BChE concentration was determined by using standart curve.

3.2.2. Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

Discontinious native-PAGE was used to determine the purity of BChE obtained from Sigma Aldrich. Visualization of the protein bands was achieved by Coomassie Brilliant Blue R-250, silver and activity stainings (Hames, 1998). Final acrylamide/bisacrylamide concentration in native gels to be stained with Coomassie Brilliant Blue were 7% and 4% for separ:.~~g and stacking gels, respectively. Gels prepared for silver and activity stainings consisted of 6% seperating and 4 % stacking gels.

Solutlons **used in discontinious native-PAGE**

-Separating gel buffer: 1 M Tris/HCI, pH 8.8

-Stacking gel buffer: 1.5 M Tris/HCI, pH 6.8

-10x Electrode (running) buffer: 25 mM Tris (Base), 192 mM glycine

-30% Acrylamide/Bisacrylamide solution (29.4% acrylamide / 0.6% N,N-methylenebisacrylamide)

-10% ammonium persulfate (APS), prepared daily.

-2x sample buffer: 1.25 ml of 0.5 M Tris/HCI pH 6.8, 4 ml of glycerol, 10 mg bromophenol blue and the volume was adjusted to 10 ml with distilled water.

-N,N,N',N'-tetramethylethylenediamine (TEMED)

Preparation of gel and sample loading

1.5 mm spacers were used for the preparation of gels. Spacer and plain glass were placed in casting stand vertically. Approximately 6.5 ml of gel was casted for separating gel and topped up with distilled water (dH_2O) to have a smooth surface then left for polymerisation about 1 hour. After polymerisation of separating gel excess dH_2O was thrown away. Stacking gel was casted on separating gel and immediately 10 well comb was placed in the gel. Gel polymerisation completed in about 1 hour. Gels were inserted to electrode assembly and transferred into electrophoresis tank. Tank was filled with running buffer and combs were removed. Wells were washed with buffer solution before loading the samples.

Table 3.1. Volumes used in gel preparation of Native-PAGE

	Separating Gel (6% or 7%)	**Stacking Gel (4%)**
1 M Tris/HCl, pH 8.8	5 ml	-
1.5 M Tris/HCl, pH 6.8	-	1.245 ml
30% **Acyrilamide/Bisacrylamide**	4 ml (for 6%) 4.6 ml (for 7%)	4ml
Distilled Water	10.690 ml (for 6%) 10.29 ml (for 7%)	9.665 ml
10% APS	100 µl	75 µl
TEMED	10 µl	15 µl
Total Volume	20 ml	15 ml

Ten µl of 1:10 diluted BChE was incubated at room temperature with 20 mM 2-mercaptoethanol (2-ME) for 1 hour. After incubation, according to the protein staining method to be used, BChE sample was diluted, then mixed with sample buffer by 1:2 ratio and loaded on gel. Electrophoresis was initiated with 150 V and when samples migrated into separating gel voltage was increased to 200 V. BIO-RAD Miniprotean Tetra Cell electrophoresis system was used. When bromophenol blue dye approaches to about 1 cm to

the end of the gel, electrophoresis completed and gels were transferred into petri dishes for staining protocols.

3.2.3. Sodium Oodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SOS-PAGE -was used to determine the purity and relative molecular weight (Mr) of BChE. Concentrations of separating and stacking gels were 10% and 4%, respectively (Laemmli, 1970).

Solutions used in discontinious SDS-PAGE

-Separating gel buffer: 1 M Tris/HCl, pH 8.8

-Stacking gel buffer: 1.5 M Tris/HCl, pH 6.8

-10x Electrode (running) buffer: 25 mM Tris(Base), 192 mM glycine, 0.1% SOS

-30% Acrylamide/Bisacrylamide solution (29.4% acrylamide / 0.6% N,N-methylenebisacrylamide)

-10% ammonium persulfate (APS), prepared daily

-2x sample buffer: 1,25 ml of 0,5 M Tris/HCl pH 6.8, 4 ml of glycerol, 10 mg bromophenol blue, 2 ml of 10% SOS and the volume adjusted to 10 ml with distilled water.

-10% SOS solution

-N,N,N',N'-tetramethylethylenediamine (TEMEO)

Preparation of gel and sample loading

1.5 mm spacers were used for preparing gels. Spacer and plain glass were placed in casting stand vertically. Approximately 6.5 ml of gel was casted for separating gel and topped up with dH_2O to have a smooth surface then left for polymerisation about 1 hour. After polymerisation of separating gel excess dH_2O was thrown away. Stacking gel was casted on separating gel and immediately 10 well comb was placed in the gel. Gel polymerisation completed in about 1 hour. Gels were inserted to electrode assembly and transferred into electrophoresis tank. Tank was filled with running buffer and

combs were removed. Wells were washed with buffer solution before loading the samples.

Table 3.2. Volumes used in gel preparation of SOS-PAGE

	Separating Gel (10%)	Stacking Gel (4%)
1 M Tris / HCl, pH 8.8	3.75 ml	-
1.5 M Tris / HCl, pH 6.8	-	1.245 ml
30% Acyrilamide/Bisacrylamide	5 ml	1.95 ml
Distilled Water	6.0175 ml	11.565 ml
10% APS	75 µl	75 µl
TEMED	7.5 µl	15 µl
10% SDS	150 µl	150 µl
Total Volume	15 ml	15 ml

Ten µl of 1:10 diluted BChE was incubated at room temperature with 20 mM 2-ME for 1 hour. Fifty µl of 2-ME was mixed with 950 µl of sample buffer before use. After incubation, BChE sample was diluted, then mixed with sample buffer by 1:2 ratio and further incubated at 100°C for~,~minutes. Sample and standard were loaded on gel in sequence of 2 µl of Roti-mark Standard, 10 µl of BChE, 2 µl of Roti-mark Standard. Electrophoresis was started with 150 V and when samples migrated into separating gel voltage was increased to 200 V. BIO-RAD Miniprotean Tetra Cell electrophoresis system was used. After electrophoresis gels were transferred into petri dishes for silver staining protocol.

3.2.4. Coomassie Brilliant Blue (CBB) R-250 Staining

After native-PAGE, CBB staining protocol was applied to determine protein bands on gel. Staining solution consisted of 40% methanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250. Gel was stained with this solution for 30 minutes, then transferred into destaining solution for 2 hours. Destaining solution consisted of 40% methanol and 10% acetic acid.

Every 30 minutes destaining solution was replaced with fres\ ~~e. After destaining process gel was stored in 5% acetic acid (Wilson, 1979~.

3.2.5. Silver Nitrate Staining

After completing native and SOS-PAGE procedures, gels were silver stained according to the method described by Blum et al. with slight modifications (Blum et al., 1987). Gels were fixed with 50% methanol, 12% glacial acetic acid and 0.005% formalin solution for 2 hours. After fixation step, gels were washed three times with 50% ethanol for 20 minutes. Gels were sensitized with 0.02% sodium thiosulfate ($Na_2S_2O_3$) for 2 minutes and then washed with distilled water three times for 20 seconds. Gels were stained with 0.2% silver nitrate ($AgNO_3$) and 0.076% formalin solution for 20 minutes. After staining step gels were washed with distilled water two times for 20 seconds. Gels were kept in 6% sodium carbonate (Na2C03), 0.05% formalin and 0.0004% sodium thiosulfate solution until the appearence of bands. When the bands were observed, gels were washed with distilled water two times for 2 minutes. Staining was terminated by the addition of 40% methanol, 10% glacial acetic acid and gels were kept in this solution for 20 minutes. Gels were stored in 1% glacial acetic acid solution after staining protocol ended. >~

3.2.6. Activity Staining

After completing native-PAGE procedure, gels were incubated in 10 mM glycine, 2 mM copper(II) sulfate (CuS04), 30 mM sodium sulfate (Na_2SO_4) solution for 30 minutes at 25°C. After incubation, 3.2 mM butyrylthiocholine was added to 2 mM copper(ll) sulfate (CuS04), 30 mM sodium sulfate (Na_2SO_4) solution and further incubated for 15 minutes at 25°C . Gels were transferred into 3 M ammonium sulfate solution and kept at 4°C for 24 hours. Then gels were transferred into 3 M ammonium sulfate solution saturated with dithiooxamide and kept at 4°C for 24 hours. Finally gels were stored in 7% acetic acid solution (Juul, 1968).

3.2.7. Measurement of the BChE Enzyme Activity

Kinetic studies were carried out for the determination of *Km* and Vmax values. BChE activity was determined by the method of Ellman et al. by using Perkin Elmer Lambda 25 UV/VIS Spectrophotometer (Ellman et al., 1961). BChE activity was measured by following the increase of absorbance at 412 nm due to the reaction of thiocholine formed by the enzymatic hydrolysis of butyrylthiocholine with 5,5-dithio-2-bis-nitrobenzoate (DTNB) ion resulting the production of yellow 5-thio-2-nitrobenzoate (TNB) ion. Reaction catalyzed by butyrylcholinesterase is shown below:

Butrylthiocholine + H20 ——• Butyrate + Thiocholine

Thiocholine + 5,5'-Dithiobis-2-Nitrobenzoic acid ——• TNB

Eight different BTC concentrations (0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM) were tested at 37°C. Total measurement time was 20 sec. The reaction conditions were: 250 µl of 200 mM MOPS pH 8.5, 50 µl of 2.5 mM DTNB, 165 µl of dH2O, 10 µl of BTC prepared in different concentrations (as a stock solution) and 25 µl of BChE. The reaction was always initiated by the addition of the enzyme. Each activity measurement was repeated three times. Average activity (U/L) values were ~cin~rted to specific activity (U/mg protein) and specific activity was used to draw optimum pH, optimum temperature, Michealis-Menten, Lineweaver-Burke, Dixon and other plots. (Segel 1975).

The definition of one unit of the enzyme activity was the amount of the enzyme catalyzing the production of 1 µmol of product per minute at pH 8.5 and 37°C. Formula used for the calculation of the enzyme activity is shown below:

$$\text{Specific Activity (UniUmg protein)} = \frac{\text{Mbs412} \cdot \text{XVI X 1000}}{13.6 \times \text{Vs} \times [\text{Protein}]}$$

LI Abs 412 I min : Absorbance change per minute at 412 nm

Vt : Volume of total activity mixture (500 µl)

V_s : Sample volume (µl) used to measure enzyme activity

13.6 : Extinction coefficient of 5-thio-2-nitrobenzoic acid (mM)

1000 : A factor used to convert ml to liter.

3.2.8 Determination of Optimum pH

BChE enzyme activity was measured by using 200 mM MOPS buffer prepared at seven different pH values in order to understand the effect of pH on enzyme activity. The pH values varied between 6.5-9.5. Measurements were carried out according to Ellman's method (Ellman et al., 1961). Reaction mixture consisted of 250 µl of 200 mM MOPS buffer, 10 µl of 50 mM BTC, 50 µl of 2.5 mM DTNB, 165 µl of dH_2O and 25 µl of BChE enzyme. Each pH value was tested in triplicates at 37°C and reaction was followed at 412 nm for 20 sec. Average enzyme activity was calculated for each pH value and specific activity vs. pH plot was depicted.

3.2.9 Determination of Optimum Temperature

BChE enzyme activity was measured at different temperature points in order to understand the effect of temperature on enzyme activity. The measurements were carried out according to Ellman's method (Ellrı:ı~ et al., 1961). Reaction was followed for 20 sec at 412 nm. Reaction mixture consisted of 250 µl of 200 mM MOPS pH 8.5, 10 µl of 50 mM BTC, 50 µl of 2.5 mM DTNB, 165 µl of dH_2O and 25 µl of BChE. In each assay, reaction medium was heated to different temperatures (20°C, 25°C, 30°C, 35°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C) and at each temperature, activity was measured in triplicates. Average enzyme activity was calculated for each temperature point and specific activity vs. temperature plot was depicted. Using the same data Arrhenius plot was depicted for the calculation of energy of activation (Ea) and temperature coefficient (Q10).

3.2.10. Effect of Fluoxetine on BChE Enzyme Activity

Fluoxetine was dissolved in dimethyl sulphoxide (DMSO). Enzyme activity was measured according to Ellman's method at 412 nm for 20 sec

(Ellman et al., 1961). Reaction mixture consisted of 250 µl of 200 mM MOPS pH 8.5, 50 µl of 2.5 mM DTNB, 155 µl of dH_2O, 10 µl of 50 mM BTC, 25 µl of BChE and 10 µl of fluoxetine prepared in different concentrations. Final fluoxetine concentration in the. reaction medium was: 0.03125 mM, 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM. BChE activity was determined in triplicates for each fluoxetine concentration.

3.2.11. Inhibitory Kinetic Experiments with Fluoxetine

Inhibitory kinetic studies were carried out to determine *Km*, Ki and Vmax values of BChE in the presence of fluoxetine. Five different fluoxetine concentrations (5 µM, 10 µM, 20 µM, 40 µM and 80 µM) and six different BTC concentrations (0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM) were used in inhibition kinetic studies. For each fluoxetine concentration, BChE activity was determined in triplicates. Reaction mixture consisted of 250 µl of 200 mM MOPS pH 8.5, 50 µl of 2.5 mM DTNB, 155 µl of dH_2O, 10 µl of BTC and 10 µl of fluoxetine. BTC and fluoxetine were prepared as a stock solution with different concentrations and 10 µl of each was added to the reaction medium for each measurement. Increase in the absorbance at 412 nm was followed for 20 sec (Ellman et al., 1961).

3.2.12. Statistical Analysis

SPSS version 22 was used for the calculation of kinetic parameters and estimation of the inhibition type.

4.RESULTS

4.1. Chracterization of Equine Serum Butyrylcholinesterase

4.1.1. Purity Control of Equine Serum Butyrylcholinesterase

Butyrylcholinesterase purified from equine serum was purchased from Sigma Aldrich and used in further experiments. First of all protein content of the commertially "obtained enzyme was determined by Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) standards were prepared at a final concentration of 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml and 600 µg/ml. Standard BSA and sample solutions (20 µl) were mixed with Bradford reagent (1 ml) and after incubation at dark for 5 minutes, absorbances were measured at 595 nm.

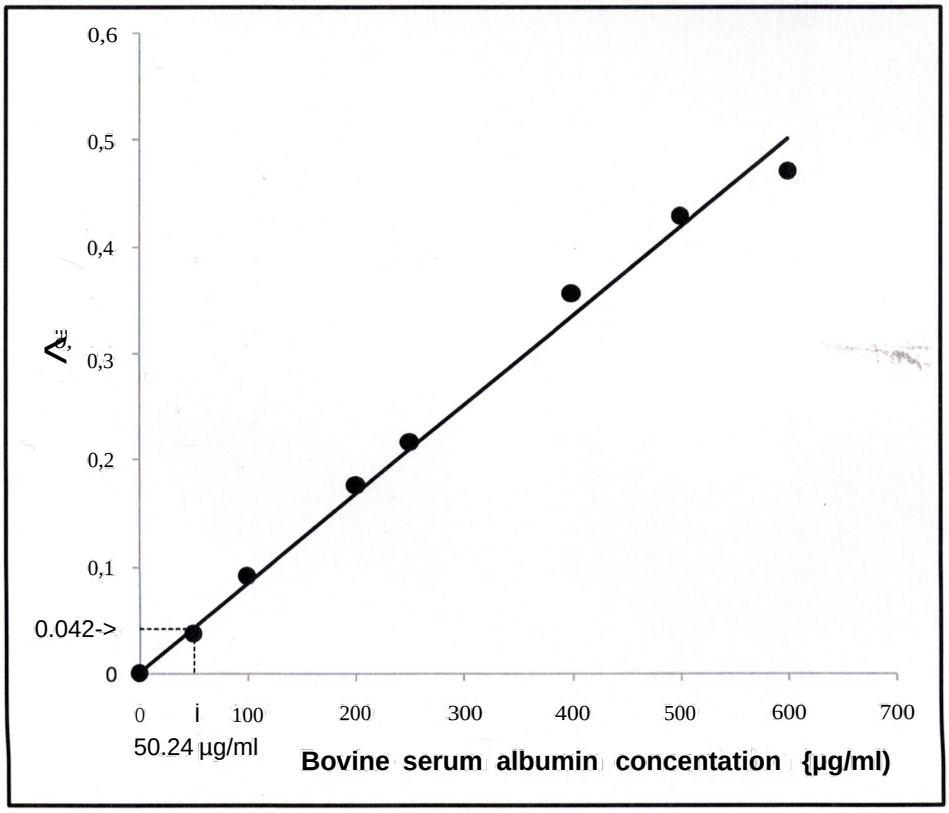


Figure 4.1. Standard curve

Concentration of BChE was found to be 50.24 µg/ml. Since the enzyme was diluted by 50 times before use, stock BChE concentration was calculated as 2.5 mg/ml.

Native polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SOS-PAGE) were carried out to confirm the purity and also molecular weight of the enzyme. Coomassie Brilliant Blue R-250 (Figure 4.2), silver (Figure 4.3.A) and activity (Figure 4.3.B) stainings were performed on gels used in native-PAGE. Gel used in SOS-PAGE was silver stained (Figure 4.4).

Although native-PAGE stained with Coomassie Brilliant Blue R-250 revealed more than single protein band, migration pattern overlapped in gels stained with silver nitrate and activity staining.

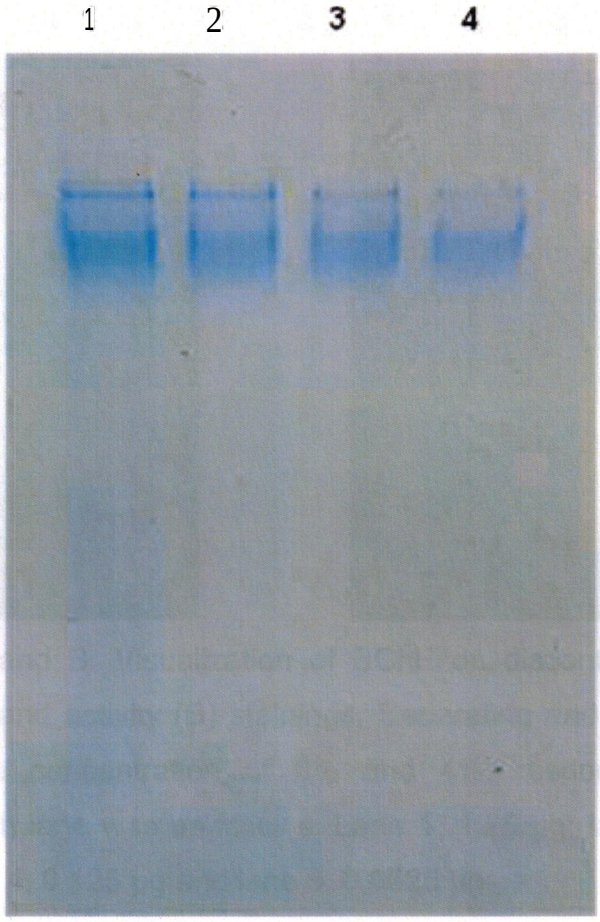


Figure 4.2. Visualization of BChE on discontinious native-PAGE by Coomassie Brilliant Blue R-250 staining. Separating and stacking"'ğ~~ were casted in the concentration of 7% and 4%, respectively. BChE concentration in each lane was as follows: Lane 1, 50 µg; Lane 2, 20 µg; Lane 3, 10 µg and Lane 4, 5 µg.

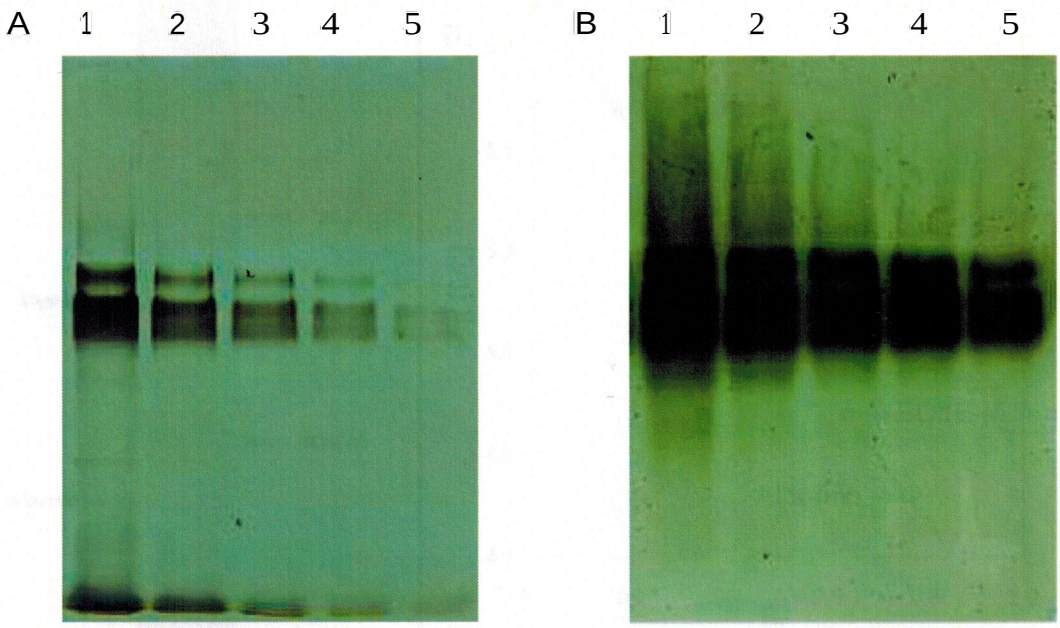


Figure 4.3.A and B. Visualization of BChE on discontinious native-PAGE by silver (A) and activity (B) stainings. Separating and stacking gels were casted in the concentration of 6% and 4%, respectively. BChE concentration in each lane was as follows: Lane 1, 1.25 µg; lane 2, 0.5 µg; lane 3, 0.25 µg; lane 4, 0.125 µg and lane 5, 0,0625 µg.

SOS-PAGE was carried out in order to determine the "~lecular weights of the subunits of the BChE enzyme. For this purpose 2-mercaptoethanol was used as a reducing agent. Five µg of enzyme sample was applied to the gel and migration pattern of standarts and enzyme sample was evaluated by visualization of the bands by silver staining. Figure 4.4.A shows that 2-mercaptoethanol was unable to separate BChE enzyme into its monomers. Therefore, three bands, belonging BChE-tetramer (ChE-T), BChE-dimer (ChE-D) and BChE-monomer (ChE-M) were detected. Considering the molecular weights and Rt values of the standard proteins (myosin and albumin), Log Mr versus Rt plot was drawn and the molecular weights of the tetrameric, dimeric and monomeric forms were found to be in correlation (Figure 4.4.B). Molecular weight of the tetrameric, dimeric and monomeric forms was calculated as 380 kDa, 190 kDa and 95 kDa, respectively.

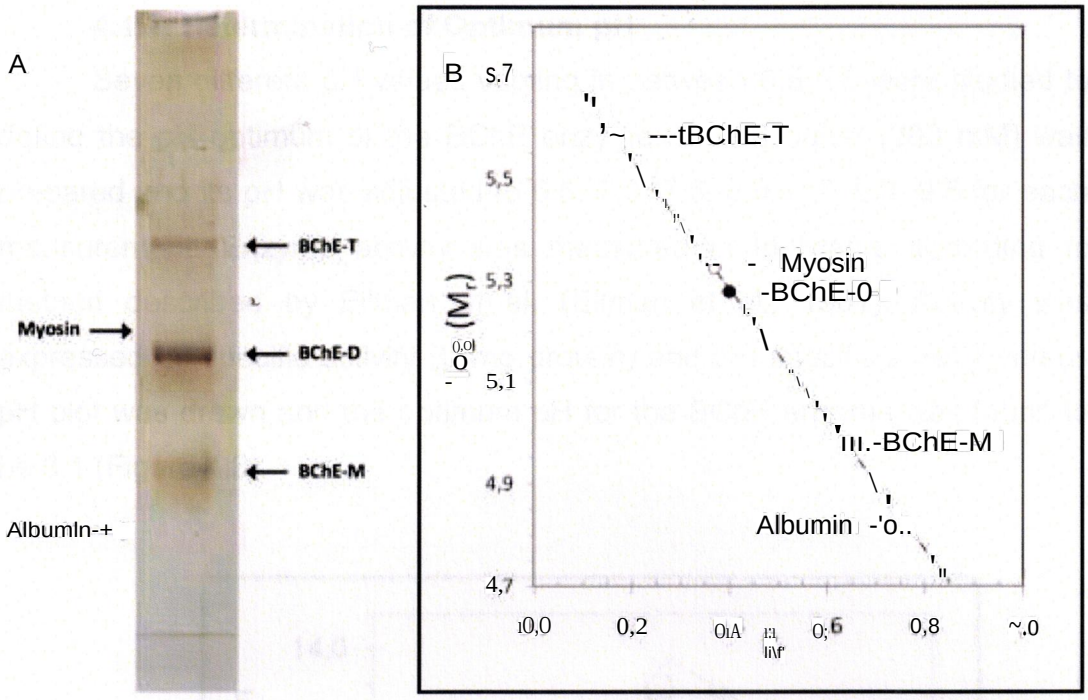


Figure 4.4.A. Visualization of BChE on discontinious SOS-PAGE by silver staining. Separating and stacking gels were casted in the concentration of 10% and 4%, respectively. Concentration of BChE was 5 µg. Molecular weights of the myosin and albumin were 212 and 66 kDa, respectively. BChE-T (Butyrylcholinesterase tetramer), BChE-D (Butyrylcholinesterase dimer), BChE-M (Butyrylcholinesterase monomer). Figure 4.4.B. Log'4Mr) vs. Rt plot.

4.1.2. Determination of Optimum pH

Seven different pH values varying in between 6.5-9.5 were studied to define the pH optimum of the BChE enzyme. MOPS buffer (200 mM) was prepared and its pH was adjusted to 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 for each measurement. Enzyme activity was measured in triplicates according to method described by Ellman et al. (Ellman et al., 1961). Activity was expressed as specific activity (U/mg protein) and the specific activity versus pH plot was drawn and the optimum pH for the BChE enzyme was found to be 8.1 (Figure 4.5).

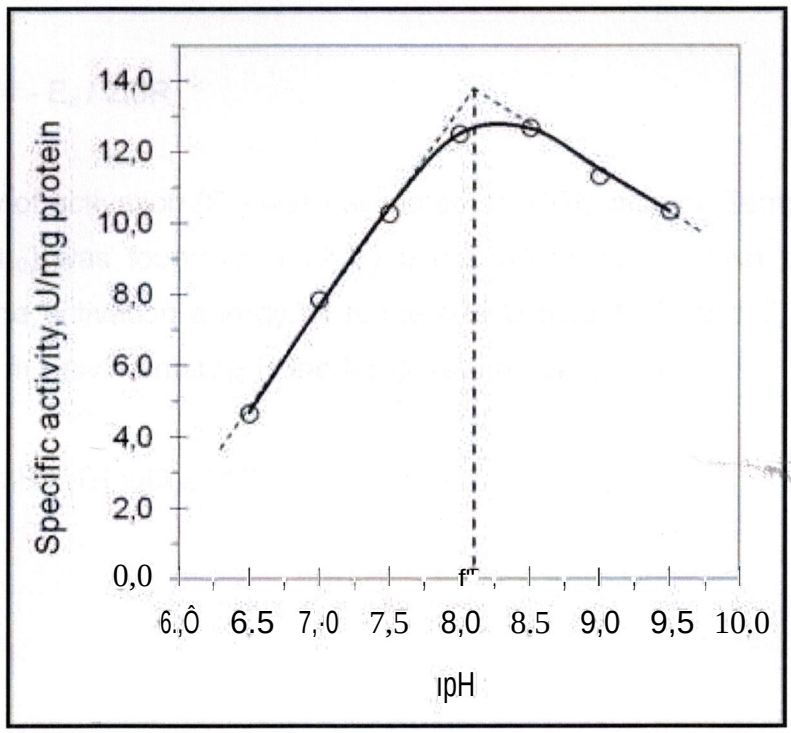


Figure 4.5. Specific activity (U/mg protein) vs. pH plot

4.1.3. Determination of Optimum Temperature

The effect of temperature on BChE activity was studied by the measurement of enzyme activity after incubating the reaction medium at different temperatures (2'ü°C, 25°C, 30°C, 35°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C). Activity was expressed as specific activity (U/mg protein) and the specific activity versus temperature plot was drawn (Figure 4.6.A). Optimum temperature was found to be 36.4°C. Logarithm of the rates versus reciprocal of temperature (in Kelvin) was plotted (Figure 4.6.B). Slope of the plot was calculated and placed in a formula shown below in which Ea is the activation energy, R is the gas constant.

-slope= - Ea / 2.3R

Energy of activation (Ea) was calculated as 1526 cal/mol. Temperature coefficient (010) was found as 1.19 by using the formula shown below in which Ea is the activation energy, R is the gas constant, T_1 and T_2 are the temperatures in Kelvin and O10 is the temperature coefficient.

$$E_a = 2.3RT_1T_2\log Q_{10} / 10$$

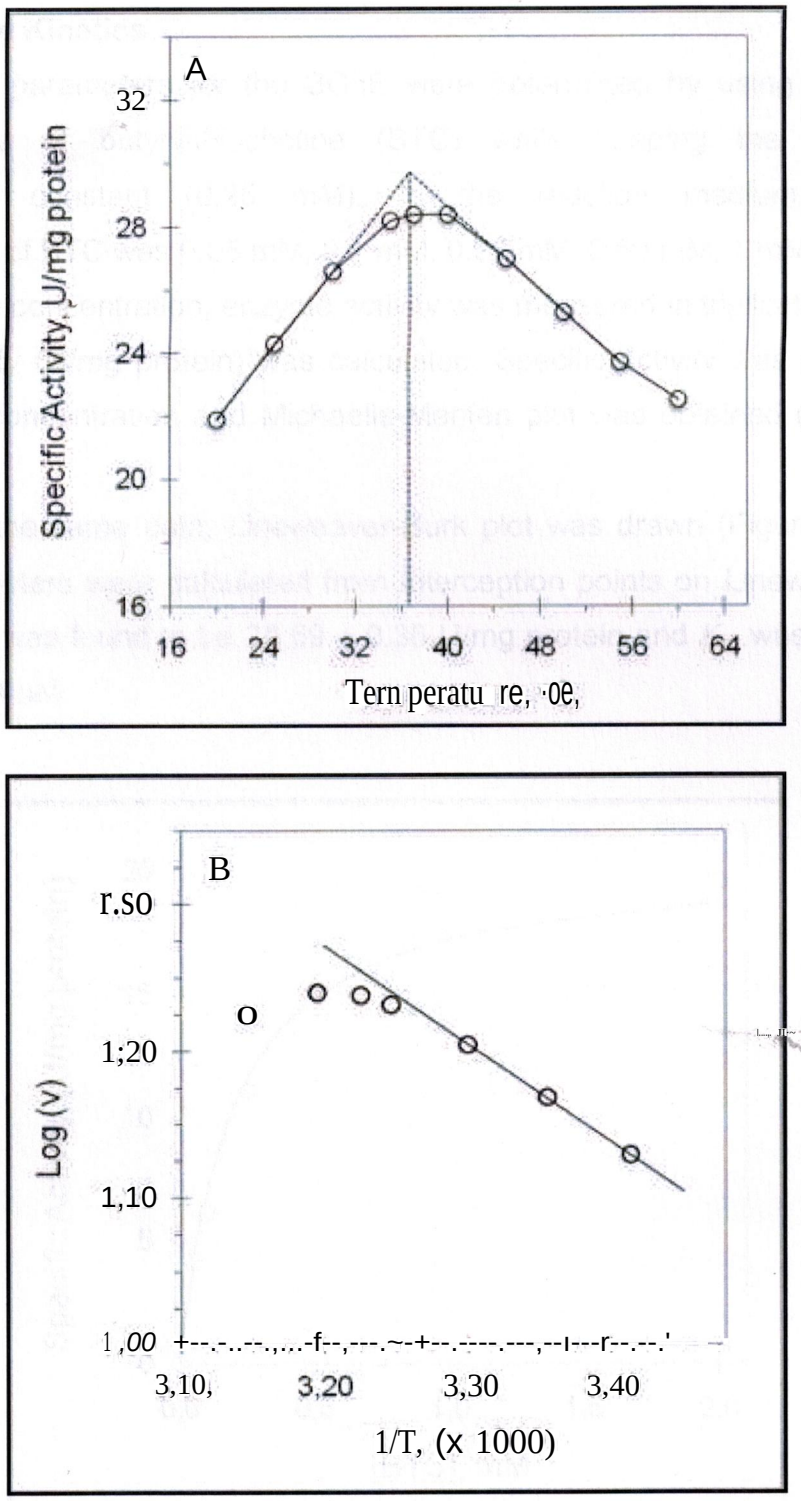


Figure 4.6.A. Specific activity vs. temperature plot. B. Log (V) vs. 1/T (Kelvin) plot.

4.2. Substrate Kinetics

Kinetic parameters for the BChE were determined by using varied concentrations of butyrylthiocholine (BTC) while keeping the DTNB concentration constant (0.25 mM). In the reaction medium, final concentration of BTC was 0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM. For each concentration, enzyme activity was measured in triplicates and specific activity (U/mg protein) was calculated. Specific activity was plotted versus BTC concentration and Michaelis-Menten plot was obtained (Figure 4.7).

Using the same data, Lineweaver-Burk plot was drawn (Figure 4.8). Kinetic parameters were calculated from interception points on Lineweaver-Burk plot. *Vm* was found to be 20.59 ± 0.36 U/mg protein and *Km* was found to be 194 ± 14 µM.

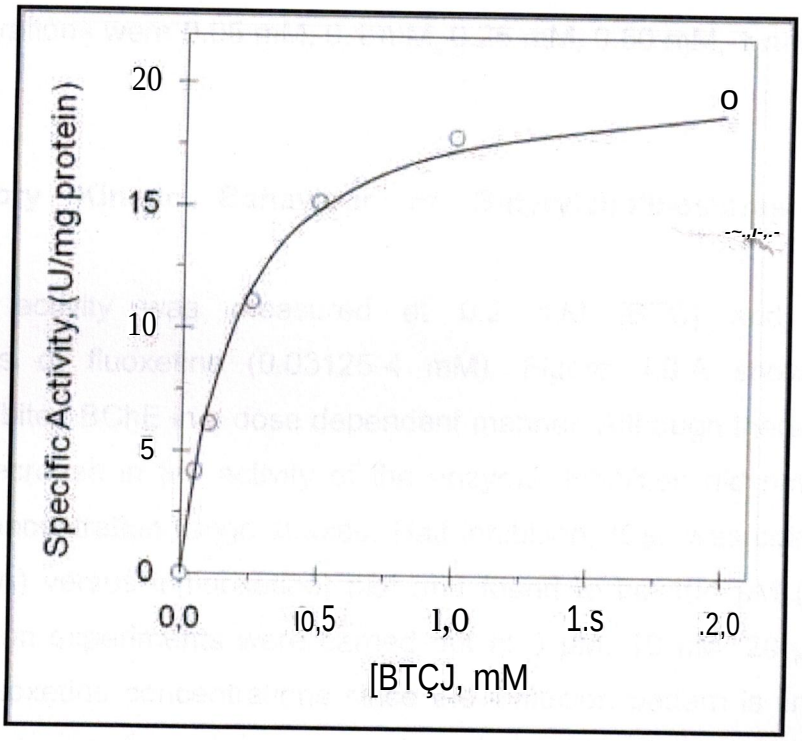


Figure 4.7. Michaelis-Menten plot of butyrylcholinesterase enzyme. BTC concentrations were 0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM.

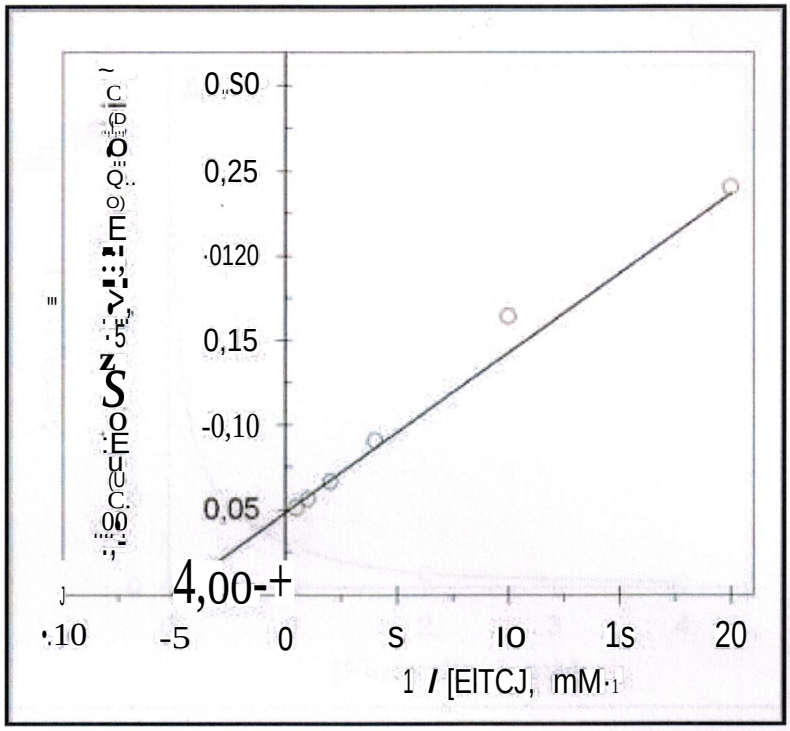
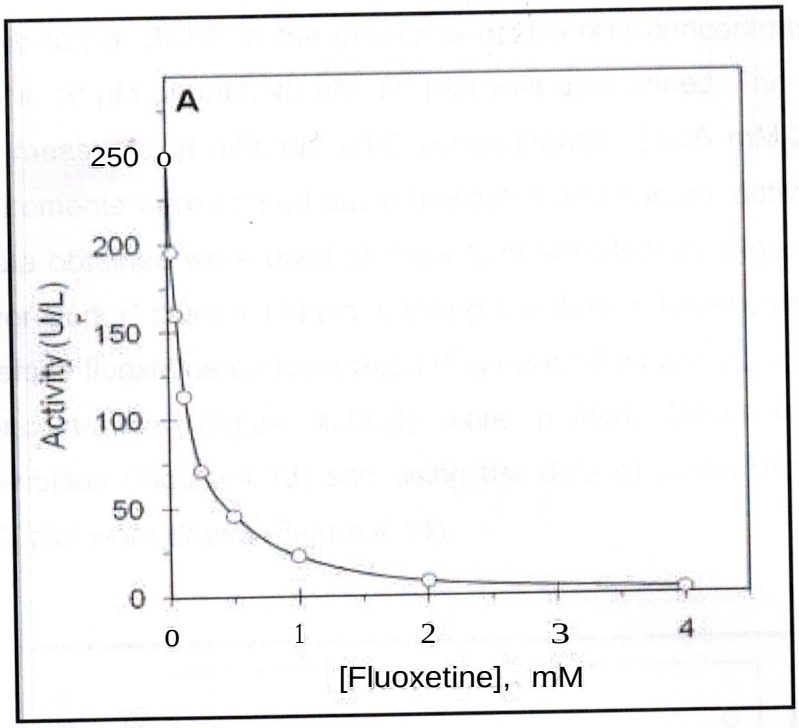


Figure 4.8. Lineweaver-Burk plot of butyrylcholinesterase enzyme. BTC concentrations were 0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM.

4.3. Inhibitory Kinetic Behaviour of Butyrylcholinesterase with Fluoxetine

BChE activity was measured at 0.2 mM [BTC] and varied concentrations of fluoxetine (0.03125-4 mM). Figure 4.9.A shows that fluoxetine inhibited BChE in a dose dependent manner. Although there was a continuous decrease in the activity of the enzyme, inhibition did not go to zero in the concentration range studied. Half inhibition, IC_{50}, was calculated from logit (v/v_0) versus ln[fluoxetine] plot and found to be 104 µM (Figure 4.9.B). Inhibition experiments were carried out at 5 µM, 10 µM, 20 µM, 40 µM, 80 µM fluoxetine concentrations since the inhibition pattern is linear at this range.



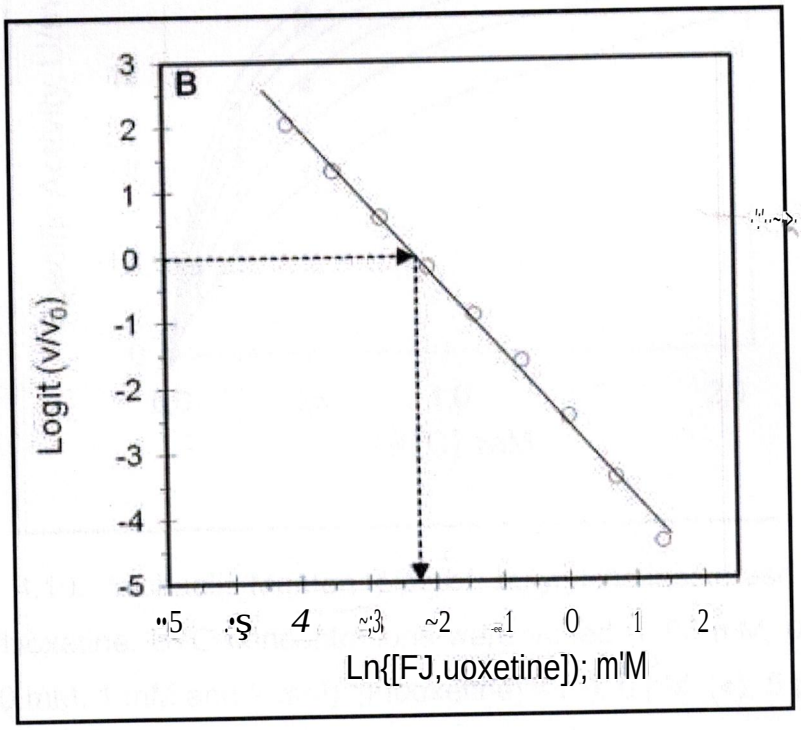


Figure 4.9.A. Dose dependent inhibition of butyrylcholinesterase by fluoxetine. B. Logit (v/v_0) vs. ln[fluoxetine] plot. [BTC] = 0.2 mM; [Fluoxetine] = 0.03125 mM, 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM.

The inhibition of BChE in the presence of different concentrations of fluoxetine (5 µM, 10 µM, 20 µM, 40 µM, 80 µM) was determined. The activity of BChE was measured at different BTC concentrations (0.05 mM-2 mM). Activity measurements were carried out in triplicates and specific activity was calculated. Data obtained were used to draw Michalis-Menten (Figure 4.10) and Lineweaver-Burk (Figure 4.11) plots. Using the data in Lineweaver-Burk plot, slopes versus fluoxetine concentration (Figure 4.12.A) and *Kmapp* versus fluoxetine concentration (Figure 4.12.B) were plotted. Dixon for pure competitive inhibition (Figure 4.13) and using the data of Dixon plot, slope versus 1/[BTC] plot were drawn (Figure 4.14).

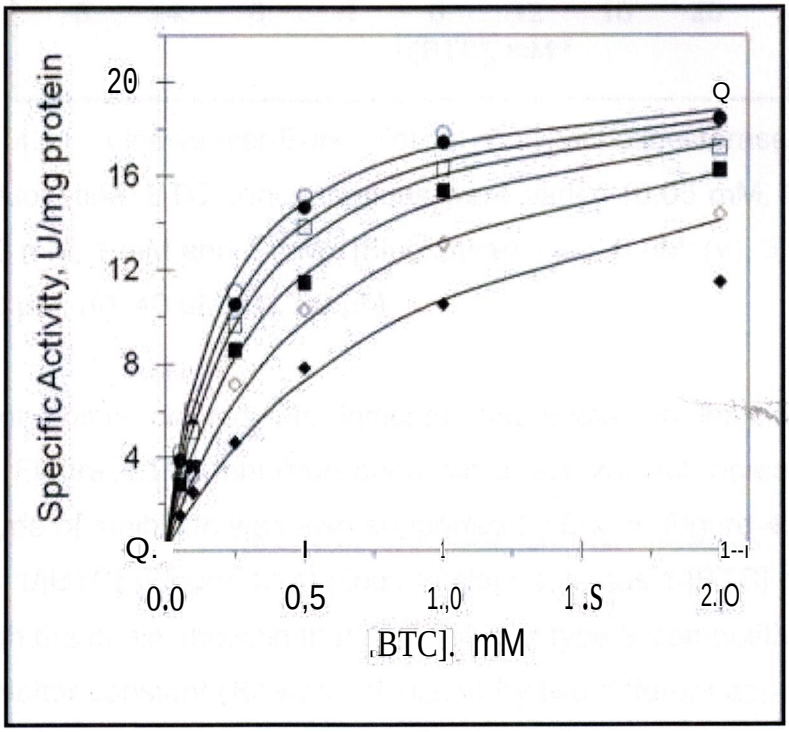


Figure 4.10. Michaelis-Menten plot of butyrylcholinesterase in the presence of fluoxetine. BTC concentrations were varied (0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM). [Fluoxetine] = (o), 0 µM; (•), 5 µM; (o), 10 µM; (•), 20 µM; (O), 40 µM; (+), 80 µM.

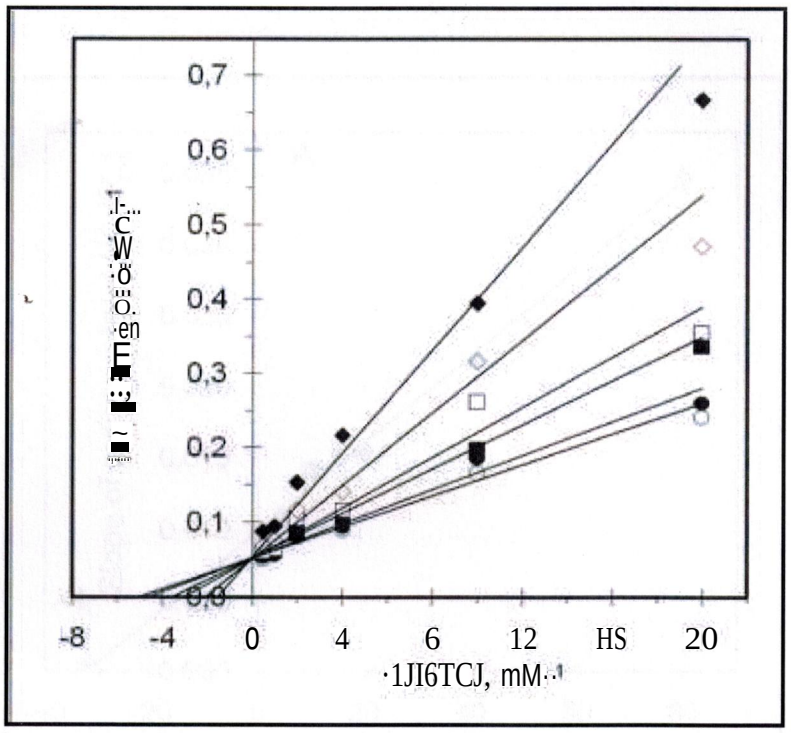


Figure 4.11. Lineweaver-Burk plot of butyrylcholinesterase in the presence of fluoxetine. BTC concentrations were varied (0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM). [Fluoxetine] = (o), 0 µM; (•), 5 µM; (o), 10 µM; (•), 20 µM; (O), 40 µM; (+), 80 µM.

Intercept points on ordinate indicate that fluoxetine irih'ra.its BChE competitively (Figure 4.11). Inhibition does not affect Vm, but increases *Km*. Competitive type of inhibition was also supported by Dixon (Figure 4.13) and slopes versus 1/[BTC] (Figure 4.14) plots. In slopes versus 1/[BTC] plot, line passes through the origin showing that the inhibition type is competitive.

The inhibitor constant *(Ki)* was calculated by two different approaches. First approach is the evaluation of data by using statistical analysis (SPSS, version 22). *K;* was calculated as 21.33 ± 2.7 µM by SPSS. The second approach is the estimation of *K;* from interception points on slope versus [fluoxetine] (Figure 4.12.A), Kmapp versus [fluoxetine] (Figure 4.12.B) and Dixon (Figure 4.13) plots. *K;* was found as 32.145 µM, 35.408 µM and 41.255 µM from Figure 4.12.A, Figure 4.12.B and Figure 4.13, respectively. Average *K;* was calculated as 36.3 ± 4.7 µM.

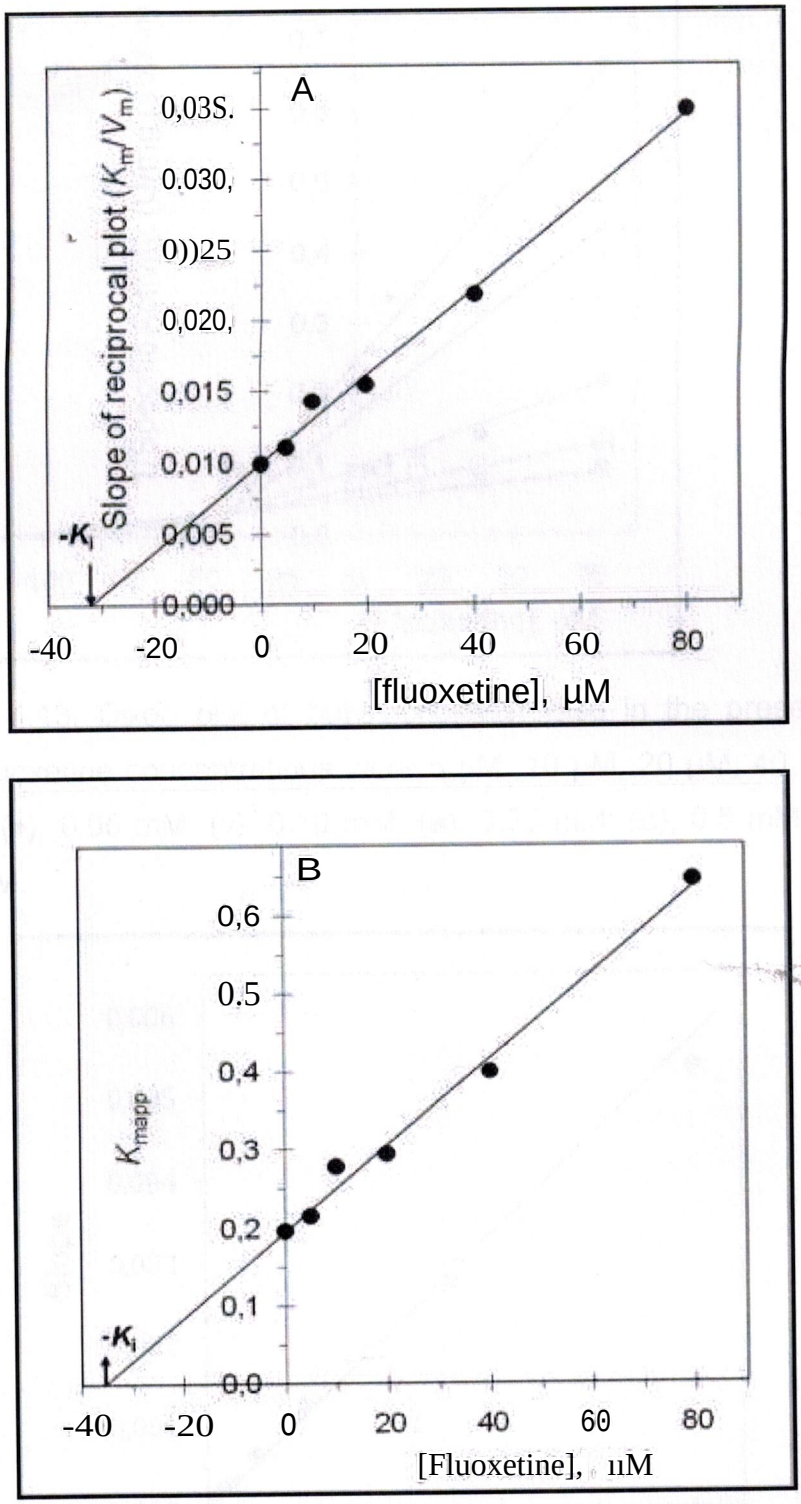


Figure 4.12.A. Slope of reciprocal plot (Km/Vm) (obtained from Figure 4.11) vs. [fluoxetine]; B. *Kmapp* vs. [fluoxetine]. [Fluoxetine] = 5 µM, 10 µM, 20 µM, 40 µM, 80 µM.

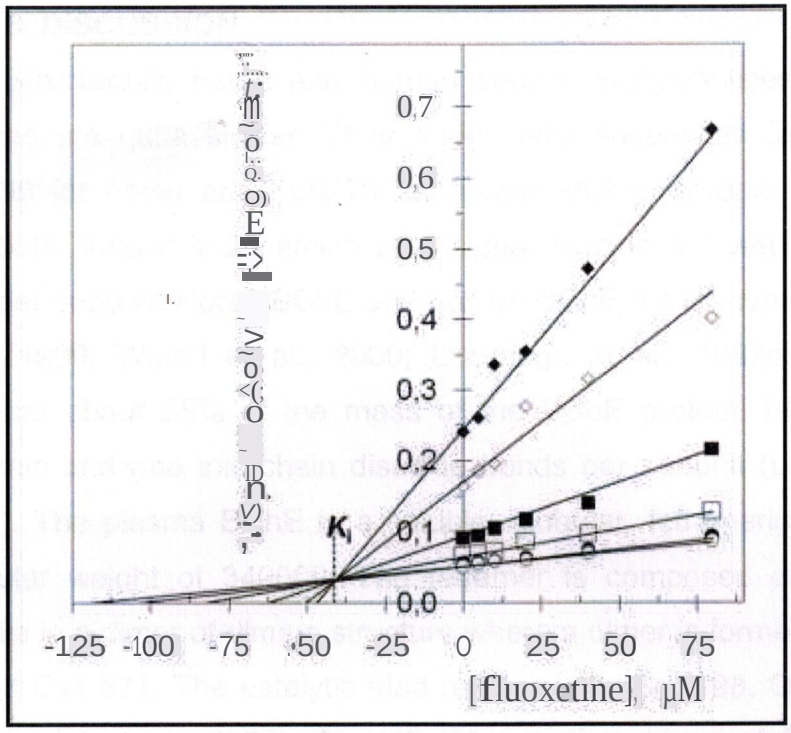


Figure 4.13. Dixon plot of butyrylcholinesterase in the presence of fluoxetine. Fluoxetine concentrations were 5 µM, 10 µM, 20 µM, 40 µM, 80 µM. [BTC] = (+), 0.05 mM, (0), 0.10 mM; (•), 0.25 mM; (o), 0.5 mM; (•), 1 mM; (o), 2 mM.

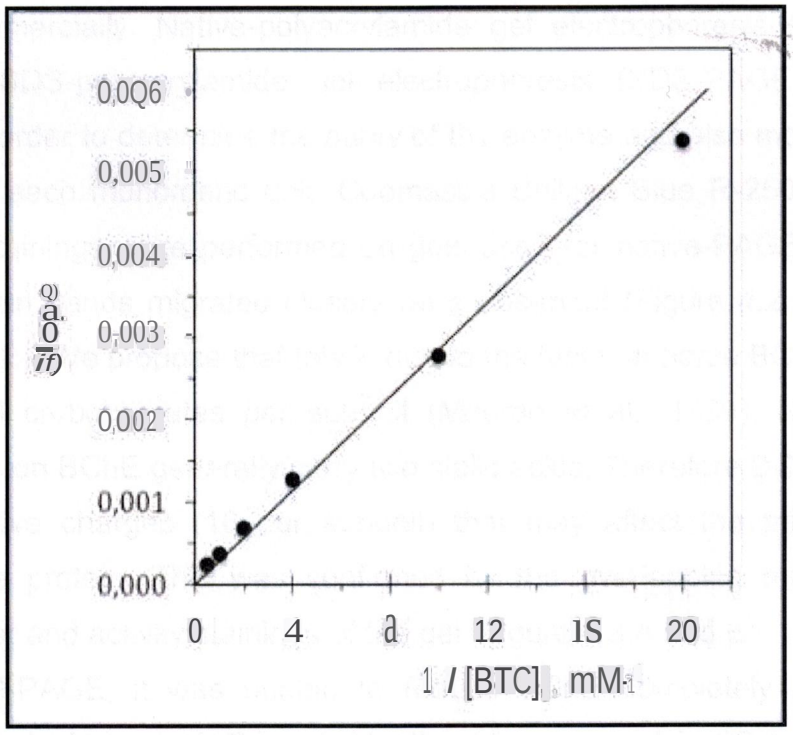


Figure 4.14. Slope (obtained from Figure 4.13) vs. 1 / [BTC] plot.

5. DISCUSSION

Structurally, horse and human serum butyrylcholinesterase (BChE) enzymes are quite similar. Their amino acid sequences (accession code Q9N1N9 for horse and P06276 for human BChE) reveals 90% similarity. They both contain a 28 amino acid signal peptide followed by 574 amino acids per subunit. Horse BChE and human BChE are glycoproteins (Moorad et al., 1999; Wierdl et al., 2000; Lockridge et al., 1987b). The glycans constitute about 25% of the mass of the BChE protein. BChE has three intrachain and one interchain disulfide bonds per subunit (Lockridge et al., 1987a). The plasma BChE is a soluble, globular, tetrameric protein with a molecular weight of 340000. The tetramer is composed of four identical subunits in a dimer of dimers structure where a dimer is formed by a disulfide bond at Cys 571. The catalytic triad residues are Ser 198, Glu 325 and His 438 (Nicolet et al., 2003). The 40 C-terminal residues of BChE form the tetramerization domain (Blong et al., 1997; Altamirano et al., 1999). Series of praline-rich peptides derived from lamellipodin function as tetramer-organizing peptides for both human and horse serum BChE (Li et al., 2008).

In our study, equine serum butyrylcholinesterase enzyme was obtained commercially. Native-polyacrylamide gel electrophoreşi (native-PAGE) and SOS-polyacıylamide gel electrophoresis (SOS-PAGE) were carried out in order to determine the purity of the enzyme and also molecular weight of the each monomeric unit. Coomassie Brilliant Blue R-250, silver and activity stainings were performed on gels used for native-PAGE. In all gels two protein bands migrated closely were observed (Figure 4.2, Figure 4.3.A and 4.3.B). We propose that this is due to the fact that horse BChE has eight N-linked carbohydrates per subunit (Moorad et al., 1999). N-linked carbohydrates on BChE generally carry two sialic acids. Therefore BChE has several negative charges (16 per subunit) that may affect the migration pattern of the protein. This was confirmed by the overlapping migration pattern in silver and activity stainings of the gel (Figure 4.3.A and B).

In SOS-PAGE, it was unable to reduce BChE completely into its monomers. Reducing agent, 2-mercaptoethanol, was used in 20 mM final

concentration and Figure 4.4.A shows the separation of tetrameric, dimeric and monomeric forms and by using myosin and albumin as standard proteins the molecular weight of the each protein fragment was calculated as 380 kDa, 190 kDa and 95 kDa, respectively (Figure 4.4.B). Similar results were obtained by Biberoglu et. al. In nondenaturating PAGE, activity and CBB staining gave two protein bands. They also performed SOS-PAGE by using 50 mM dithiothreitol which is a stronger reducing agent than 2-ME. CBB R-250 staining of the SOS-PAGE indicated dimeric and monomeric forms of BChE (Biberoglu et al., 2012).

Characterization of BChE was achieved by the determination of optimum pH and temperature. Enzyme activity was measured by using MOPS buffer with different pH values ranging in between 6.5-9.5 and optimum pH was found as 8.1 (Figure 4.5). Optimum temperature, energy of activation (Ea) and temperature coefficient (Q_{10}) of the BChE were calculated as 36.4°C, 1526 cal/mol and 1.19, respectively (Figure 4.6.A and B). Our findings are highly consistent with the literature. Lee and Harpst purified BChE from horse serum and the enzyme exhibited highest activity at an optimum pH of 8 and the temperature optimum was around 36°C (Lee and Harpst, 1971).

Determination of kinetic parameters were carried out by using varied concentrations of butyrylthiocholine (BTC). Final BTC concentrations were 0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM and V_m and K_m was found to be 20.59 ± 0.36 U/mg protein and 194 ± 14 µM, respectively (Figure 4.7, Figure 4.8).

In our study, we mainly focused on potential butyrylcholinesterase inhibitiors that may be beneficial for the treatment of diseases in which elevated BChE activity is the causative factor of the pathology of the disease. Therefore we investigated the kinetic behaviour of BChE in the presence of fluoxetine.

Fluoxetine is a widely used selective serotonin reuptake inhibitor (Gram, 1994). SSRIs were reported to inhibit AChE activity when added to serum samples suggesting that apart from direct activation of muscarinic

receptors, fluoxetine may enhance cholinergic signalling by elevating the *levels* of ACh through AChE inhibition (Müller et al., 2002).

Activity measurements were performed at varied fluoxetine concentrations (0.03125-4 mM) when BTC concentration was constant (0.2 mM). Although fluoxetine inhibited BChE in a dose dependent manner (Figure 4.9.A), inhibition did not go to zero in the concentration range studied. Half inhibition, IC_{50}, was calculated as 104 µM (Figure 4.9.B). For further studies, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM fluoxetine concentrations were chosen since the inhibition pattern was linear at this range. Enzyme activity was measured at these fluoxetine concentrations and also BTC concentration was in the range of 0.05-2 mM. Fluoxetine inhibited BChE competitively. Inhibitor constant (K_i) were calculated by using both interception points and statistical analysis (SPSS ver. 22). Interception points on graphs (Figure 4.12.A and B and Figure 4.13) *gave* an *average* K_i of 36.3 ± 4.7 µM. According to statistical analysis K_i was calculated as 21.33 ± 2.7 µM. Discrepency in K_i *values* can be explained by combination of competitive and partly mixed type inhibitions. At low fluoxetine concentrations, active site of the enzyme is occupied by inhibitor, but at high concentrations it is possible that apart from the active site fluoxetine also binds other parts in the enzyme structure.

The effect of antidepressants (fluoxetine, sertraline and amitriptyline) on BChE and AChE activities was investigated. Human serum and erythrocyte membrane were used as the sources of BChE and AChE, respectively. Fluoxetine has the lowest potency in the inhibition of BChE with an IC_{50} and K_i values of 32 µM and 106 µM, respectively. Difference in these values can be explained by the utilization of acetylthiocholine as a substrate (MOiieret al., 2002).

Among all BChE-related diseases, Alzheimer's disease (AD) has an additional importance. It is a progressive neurodegenerative disease and its symptoms are thought to be associated with decreased levels of synaptic or neuronal acetylcholine (ACh) brought about by the loss of cholinergic neurons from the basal forebrain (Lane et al. 2006), cortex and

hippocampus (Darreh-Shori and Soininen, 2010). In AD, while the level of AChE decreased, the level of BChE is increased. High levels of BChE are found to be associated with neuritic plaques and neurofibrillary tangles (Guillozet et al., 1997; Mesulam and Geula, 1994, Op Den Velde and Stam, 1976; Perry et al., 1978). BChE activity is reported to be significantly elevated (41-80% in brains of patients with advanced AD (Perry et al., 1978). Oppositely, there is a decrease of 10-60% of AChE in affected brain regions of AD patients (Davies and Maloney, 1976). There are several factors indicating that BChE might be considered as a potential target in AD treatment: (1) BChE has a higher half-life than AChE due to its higher number of glycation sites that are important for stability and clearance (Darvesh et al., 2003). (2) BChE G1 form, which is predominant in the developing brain, is increased in AD patients (Kasa et al., 1997). (3) AChE is inhibited by high concentrations of ACh, while BChE remains unaffected (Kasa et al., 1997). (4) Inhibition of BChE induces a dose-dependent increase in brain levels of ACh (Darvesh et al., 2003) (5) Toxic effects are observed after treatment of mice lacking AChE with BChE inhibitors (Xie et al., 2000). Thus, BChE has a regulatory role in ACh hydrolysis, being an alternative to AChE, whereby therapeutic agents that specifally inhib~BChE could provide therapeutic benefits in AD.

Wang et al. showed that fluoxetine improved spatial memory, learning and emotional behaviors of APP/PS1 mice, a well characterized model of AD and also it effectively prevented the protein loss of synaptophysin (SYP) and microtubule associated protein 2 (MAP2). Although it was unable to prevent plaque formation, but significantly lowered high levels of soluble P-amyloid (AP) in brain tissue, cerebrospinal fluid (CSF) and blood sera. Fluoxetine also effectively inhibited the phosphorylation of amyloid precursor protein (APP) at T668, which may be a possible mechanism of the reduced AP production in APP/PS1 mouse after treatment (Wang et al., 2014).

6. CONCLUSION

In this study, inhibitory effect of fluoxetine on horse serum butyrylcholinesterase (BChE) was investigated. Native (native-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SOS-PAGE) were carried out to confirm the purity of the enzyme. BChE was further characterized in terms of its optimum pH and optimum temperature. Kinetic experiments were performed by using different concentrations of butyrylthiocholine (BTC) to calculate V_m and K_m of the enzyme. Finally the inhibitory potency of fluoxetine was tested by different fluoxetine concentrations.

Although Coomassie Brilliant Blue R-250 staining of the native gel gave two closely migrated bands, protein bands obtained in activity and silver staining of the same gel revealed overlapping migration pattern. SOS-PAGE was used to estimate the molecular weight of the BChE. It was unable to convert tetrameric form to monomeric form by using 20 mM 2-mercaptoethanol. Instead, we obtained three different protein bands equivalent to tetrameric, dimeric and monomeric forms and molecular weights were calculated as 380 kOa, 190 kOa and 95 kOa, respectively.

Optimum pH and temperature of the enzyme was found to be 8.1 and 36.4°C, respectively. Energy of activation (E_a) and temperature coefficient (Q_{10}) were 1526 cal/mal and 1.19, respectively.

In kinetic studies, V_m and K_m were calculated as 20.59 ± 0.36 U/mg protein and 194 ± 14 µM, respectively. Competitive type of inhibition was observed when varied concentrations of fluoxetine was used. Half maximal inhibitory concentration, IC_{50}, and K_i were found to be 104 µM and 36.3 ± 4.7 µM, respectively.

Further studies on the molecular mechanism of the BChE inhibition by fluoxetine and its clinical use should be performed since BChE is responsible for the appearance of the pathologic hallmarks of the some diseases such as plaque formation in Alzheimer's disease.

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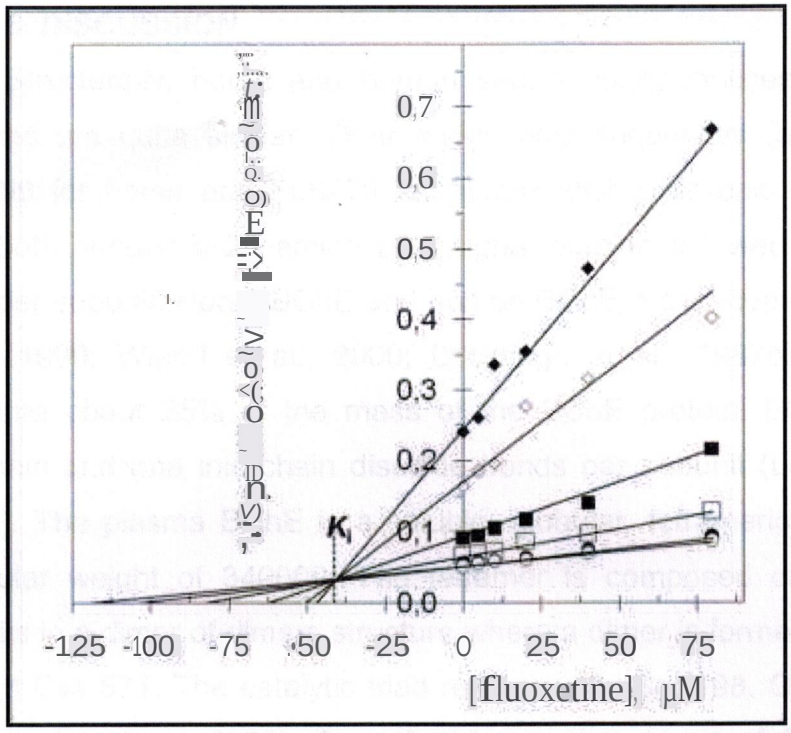

Figure 4.13. Dixon plot of butyrylcholinesterase in the presence of fluoxetine. Fluoxetine concentrations were 5 µM, 10 µM, 20 µM, 40 µM, 80 µM. [BTC] = (+), 0.05 mM, (0), 0.10 mM; (•), 0.25 mM; (o), 0.5 mM; (•), 1 mM; (o), 2 mM.

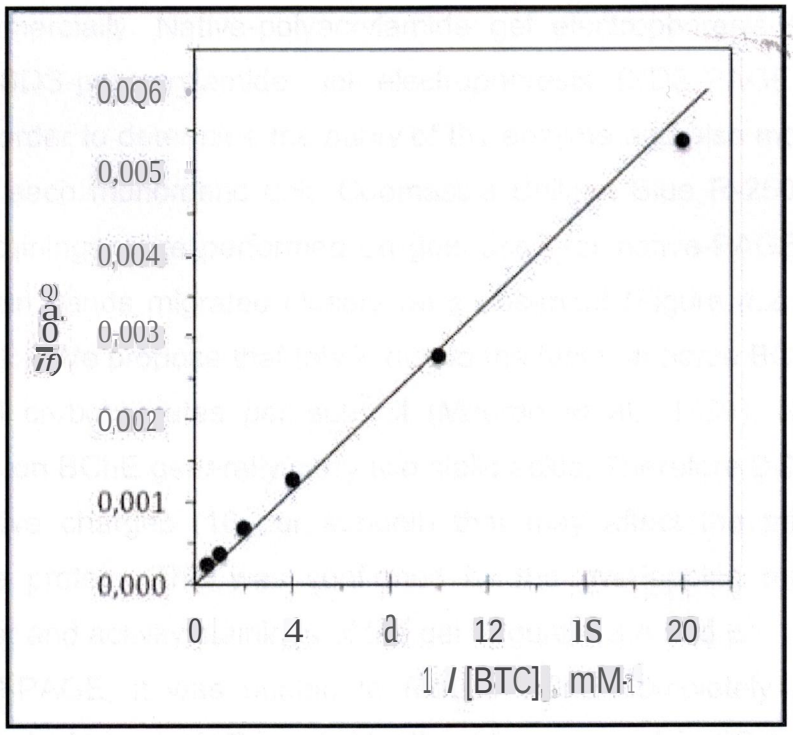

Figure 4.14. Slope (obtained from Figure 4.13) vs. 1 / [BTC] plot.

5. DISCUSSION

Structurally, horse and human serum butyrylcholinesterase (BChE) enzymes are quite similar. Their amino acid sequences (accession code Q9N1N9 for horse and P06276 for human BChE) reveals 90% similarity. They both contain a 28 amino acid signal peptide followed by 574 amino acids per subunit. Horse BChE and human BChE are glycoproteins (Moorad et al., 1999; Wierdl et al., 2000; Lockridge et al., 1987b). The glycans constitute about 25% of the mass of the BChE protein. BChE has three intrachain and one interchain disulfide bonds per subunit (Lockridge et al., 1987a). The plasma BChE is a soluble, globular, tetrameric protein with a molecular weight of 340000. The tetramer is composed of four identical subunits in a dimer of dimers structure where a dimer is formed by a disulfide bond at Cys 571. The catalytic triad residues are Ser 198, Glu 325 and His 438 (Nicolet et al., 2003). The 40 C-terminal residues of BChE form the tetramerization domain (Blong et al., 1997; Altamirano et al., 1999). Series of praline-rich peptides derived from lamellipodin function as tetramer-organizing peptides for both human and horse serum BChE (Li et al., 2008).

In our study, equine serum butyrylcholinesterase enzyme was obtained commercially. Native-polyacrylamide gel electrophoreşi (native-PAGE) and SOS-polyacıylamide gel electrophoresis (SOS-PAGE) were carried out in order to determine the purity of the enzyme and also molecular weight of the each monomeric unit. Coomassie Brilliant Blue R-250, silver and activity stainings were performed on gels used for native-PAGE. In all gels two protein bands migrated closely were observed (Figure 4.2, Figure 4.3.A and 4.3.B). We propose that this is due to the fact that horse BChE has eight N-linked carbohydrates per subunit (Moorad et al., 1999). N-linked carbohydrates on BChE generally carry two sialic acids. Therefore BChE has several negative charges (16 per subunit) that may affect the migration pattern of the protein. This was confirmed by the overlapping migration pattern in silver and activity stainings of the gel (Figure 4.3.A and B).

In SOS-PAGE, it was unable to reduce BChE completely into its monomers. Reducing agent, 2-mercaptoethanol, was used in 20 mM final

concentration and Figure 4.4.A shows the separation of tetrameric, dimeric and monomeric forms and by using myosin and albumin as standard proteins the molecular weight of the each protein fragment was calculated as 380 kDa, 190 kDa and 95 kDa, respectively (Figure 4.4.B). Similar results were obtained by Biberoglu et. al. In nondenaturating PAGE, activity and CBB staining gave two protein bands. They also performed SOS-PAGE by using 50 mM dithiothreitol which is a stronger reducing agent than 2-ME. CBB R-250 staining of the SOS-PAGE indicated dimeric and monomeric forms of BChE (Biberoglu et al., 2012).

Characterization of BChE was achieved by the determination of optimum pH and temperature. Enzyme activity was measured by using MOPS buffer with different pH values ranging in between 6.5-9.5 and optimum pH was found as 8.1 (Figure 4.5). Optimum temperature, energy of activation (Ea) and temperature coefficient (Q_{10}) of the BChE were calculated as 36.4°C, 1526 cal/mol and 1.19, respectively (Figure 4.6.A and B). Our findings are highly consistent with the literature. Lee and Harpst purified BChE from horse serum and the enzyme exhibited highest activity at an optimum pH of 8 and the temperature optimum was around 36°C (Lee and Harpst, 1971).

Determination of kinetic parameters were carried out by using varied concentrations of butyrylthiocholine (BTC). Final BTC concentrations were 0.05 mM, 0.1 mM, 0.25 mM, 0.50 mM, 1 mM and 2 mM and V_m and K_m was found to be 20.59 ± 0.36 U/mg protein and 194 ± 14 µM, respectively (Figure 4.7, Figure 4.8).

In our study, we mainly focused on potential butyrylcholinesterase inhibitiors that may be beneficial for the treatment of diseases in which elevated BChE activity is the causative factor of the pathology of the disease. Therefore we investigated the kinetic behaviour of BChE in the presence of fluoxetine.

Fluoxetine is a widely used selective serotonin reuptake inhibitor (Gram, 1994). SSRIs were reported to inhibit AChE activity when added to serum samples suggesting that apart from direct activation of muscarinic

receptors, fluoxetine may enhance cholinergic signalling by elevating the *levels* of ACh through AChE inhibition (Müller et al., 2002).

Activity measurements were performed at varied fluoxetine concentrations (0.03125-4 mM) when BTC concentration was constant (0.2 mM). Although fluoxetine inhibited BChE in a dose dependent manner (Figure 4.9.A), inhibition did not go to zero in the concentration range studied. Half inhibition, IC_{50}, was calculated as 104 µM (Figure 4.9.B). For further studies, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM fluoxetine concentrations were chosen since the inhibition pattern was linear at this range. Enzyme activity was measured at these fluoxetine concentrations and also BTC concentration was in the range of 0.05-2 mM. Fluoxetine inhibited BChE competitively. Inhibitor constant (K_i) were calculated by using both interception points and statistical analysis (SPSS ver. 22). Interception points on graphs (Figure 4.12.A and B and Figure 4.13) *gave* an *average* K_i of 36.3 ± 4.7 µM. According to statistical analysis K_i was calculated as 21.33 ± 2.7 µM. Discrepency in K_i *values* can be explained by combination of competitive and partly mixed type inhibitions. At low fluoxetine concentrations, active site of the enzyme is occupied by inhibitor, but at high concentrations it is possible that apart from the active site fluoxetine also binds other parts in the enzyme structure.

The effect of antidepressants (fluoxetine, sertraline and amitriptyline) on BChE and AChE activities was investigated. Human serum and erythrocyte membrane were used as the sources of BChE and AChE, respectively. Fluoxetine has the lowest potency in the inhibition of BChE with an IC_{50} and K_i values of 32 µM and 106 µM, respectively. Difference in these values can be explained by the utilization of acetylthiocholine as a substrate (MOiieret al., 2002).

Among all BChE-related diseases, Alzheimer's disease (AD) has an additional importance. It is a progressive neurodegenerative disease and its symptoms are thought to be associated with decreased levels of synaptic or neuronal acetylcholine (ACh) brought about by the loss of cholinergic neurons from the basal forebrain (Lane et al. 2006), cortex and

hippocampus (Darreh-Shori and Soininen, 2010). In AD, while the level of AChE decreased, the level of BChE is increased. High levels of BChE are found to be associated with neuritic plaques and neurofibrillary tangles (Guillozet et al., 1997; Mesulam and Geula, 1994, Op Den Velde and Stam, 1976; Perry et al., 1978). BChE activity is reported to be significantly elevated (41-80% in brains of patients with advanced AD (Perry et al., 1978). Oppositely, there is a decrease of 10-60% of AChE in affected brain regions of AD patients (Davies and Maloney, 1976). There are several factors indicating that BChE might be considered as a potential target in AD treatment: (1) BChE has a higher half-life than AChE due to its higher number of glycation sites that are important for stability and clearance (Darvesh et al., 2003). (2) BChE G1 form, which is predominant in the developing brain, is increased in AD patients (Kasa et al., 1997). (3) AChE is inhibited by high concentrations of ACh, while BChE remains unaffected (Kasa et al., 1997). (4) Inhibition of BChE induces a dose-dependent increase in brain levels of ACh (Darvesh et al., 2003) (5) Toxic effects are observed after treatment of mice lacking AChE with BChE inhibitors (Xie et al., 2000). Thus, BChE has a regulatory role in ACh hydrolysis, being an alternative to AChE, whereby therapeutic agents that specifally inhib~BChE could provide therapeutic benefits in AD.

Wang et al. showed that fluoxetine improved spatial memory, learning and emotional behaviors of APP/PS1 mice, a well characterized model of AD and also it effectively prevented the protein loss of synaptophysin (SYP) and microtubule associated protein 2 (MAP2). Although it was unable to prevent plaque formation, but significantly lowered high levels of soluble P-amyloid (AP) in brain tissue, cerebrospinal fluid (CSF) and blood sera. Fluoxetine also effectively inhibited the phosphorylation of amyloid precursor protein (APP) at T668, which may be a possible mechanism of the reduced AP production in APP/PS1 mouse after treatment (Wang et al., 2014).

6. CONCLUSION

In this study, inhibitory effect of fluoxetine on horse serum butyrylcholinesterase (BChE) was investigated. Native (native-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SOS-PAGE) were carried out to confirm the purity of the enzyme. BChE was further characterized in terms of its optimum pH and optimum temperature. Kinetic experiments were performed by using different concentrations of butyrylthiocholine (BTC) to calculate V_m and K_m of the enzyme. Finally the inhibitory potency of fluoxetine was tested by different fluoxetine concentrations.

Although Coomassie Brilliant Blue R-250 staining of the native gel gave two closely migrated bands, protein bands obtained in activity and silver staining of the same gel revealed overlapping migration pattern. SOS-PAGE was used to estimate the molecular weight of the BChE. It was unable to convert tetrameric form to monomeric form by using 20 mM 2-mercaptoethanol. Instead, we obtained three different protein bands equivalent to tetrameric, dimeric and monomeric forms and molecular weights were calculated as 380 kOa, 190 kOa and 95 kOa, respectively.

Optimum pH and temperature of the enzyme was found to be 8.1 and 36.4°C, respectively. Energy of activation (E_a) and temperature coefficient (Q_{10}) were 1526 cal/mal and 1.19, respectively.

In kinetic studies, V_m and K_m were calculated as 20.59 ± 0.36 U/mg protein and 194 ± 14 µM, respectively. Competitive type of inhibition was observed when varied concentrations of fluoxetine was used. Half maximal inhibitory concentration, IC_{50}, and K_i were found to be 104 µM and 36.3 ± 4.7 µM, respectively.

Further studies on the molecular mechanism of the BChE inhibition by fluoxetine and its clinical use should be performed since BChE is responsible for the appearance of the pathologic hallmarks of the some diseases such as plaque formation in Alzheimer's disease.

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