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Purification of acethylcholinesterase from the mollusc *Mytilus galloprovincialis* Lam. and investigation of its kinetic properties

Servet DURANAY [1]*, Sezin ANIL [2], Gözde HASBAL [1], Nurten ÖZSOY [1]

[1] Department of Biochemistry, Faculty of Pharmacy, İstanbul University, İstanbul, Turkey.
[2] Department of Pharmacognosy, Faculty of Pharmacy, İstanbul University, İstanbul, Turkey.
* Corresponding Author. E-mail: servetduranay@gmail.com (S.D.); Tel. +90-212-440 00 00/13476.

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ABSTRACT: The role of acetylcholinesterase in terminating acetylcholine-mediated neurotransmission made it the focus of intense research. In this study the haemolymph Acetylcholinesterase (AChE) from the mollusc *Mytilus galloprovincialis* was purified to homogeneity by $(NH4)_2SO_4$ fractional precipitation and affinity chromatography on edrophonium-Sepharose 6B. The enzyme was purified approximatedly 13 fold over the crude extract and was obtained in 3 % yield. The specific activity of purified enzyme was 3 U/mg protein. It had an optimum pH of 7.5 and showed optimal activity at 35°C. Km and Vmax for acetylthiocholine iodide were 1.3 mM and 0.188 mM/mg/min, respectively. The purified enzyme migrated as a 51 000 dalton band during polyacrylamide gel electrophoresis under denaturing and reducing conditions. Three methoxyflavones were examined in order to evaluate their potential as anti-Alzheimer's Disease (AD) agents. All of the compounds were shown to be potent AChE inhibitors. Therefore, these compounds may have great value in the development of therapeutic and preventive agents for AD.

KEYWORDS: Acethylcholinesterase; *Mytilus galloprovincialis* Lam.; enzyme purification; kinetic properties; inhibition by methoxyflavones.

1. INTRODUCTION

Acetylcholinesterase (AChE) (acetylcholine acetylhydrolase, EC 3.1.1.7) functions in the central and peripheral nervous systems in the cholinergic neurotransmission by rapidly hydrolysing the neurotransmitter acetylcholine to choline and acetate [1]. In recent years there has been significant progress in the field of AChE research and special attention has been paid to the newly developed purification methods and characterization of AChE, to its possible biological function in the brain, and to its relationship to mental disorders. Much of these researches has been concentrated on gaining an understanding of its remarkable catalytic efficacy, developing therapeutic approaches in the management of Alzheimer's disease and organophosphate intoxication and discovering the new drugs [2].

Many research has been done on the purification of this enzyme from the brain of human [3-4], Japanese quail [5], fishs like *Oreochromis aurea* [6], *Pangasius pangasius* [7], electric eel [8-9] and *Periophthalmodon schlosseri [10]*, fetal bovine serum [11], sheep liver [12], mouse [13-14] the head and appendage of *Pardosa astrigera* L. Koch [15], cotton aphid *Aphis gossypii* Glover [16], nematodes [17-18], earthworm [19], cotton bollworm [20] and western corn rootworm, *Diabrotica virgiferavirgifera* [21].

This enzyme has been purified from several sources using conventional chromatographic procedures like DEAE cellulose [6-11-18] ion exchange chromatography, Sephadex G-100 [6] and G-200 [16] gel filtration. Inhibitor ligand affinity chromatography is a powerful technique for the purification of AChE, because of the potent and selective action of the inhibitors such as tacrin [9-12], procainamid [3-16], edrophonium [5-12, 22], *m*-trimetilaminofenilamin [13] and 9-[*N*-*beta*-(*epsilon*-aminocaproil)-*beta*-aminopropilamino]-akridinium bromid [17]. Sepharose gels served as the affinity matrix and afforded highly purified enzyme preparations. Of the affinity procedures, that employing edrophonium coupled to Sepharose 6B as described by Hodgson and Chubb [23] is effective and reliable and can be used to purify enzyme from a wide range of biological materials.

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Although AChE has been previously purified and investigated for certain kinetic characteristics there are still possibilities for further improving purification procedures in order to find a good enzyme source for a commercial and scientific need. Increasing knowledge on the kinetic properties of AChE has greatly increased the prospects of enzyme application as a specific biomarker for pesticide contamination and screening of environmental pollution [6].

Marine system produces richest and inexhaustible source of potential natural products with a nutraceutical and pharmaceutical activity [24]. The phylum Mollusca represents one of the largest and most diverse groups of marine animals. Marine mussels from the Mytilidae family, comprising genera such as Mytilus and Perna, are subject to many investigations for their possible use as a source for bioactive substances such as antimicrobial, anti-inflammatory as well as anti-cancer agents [24-25].

Little is known about the kinetic properties of the enzymes produced by marine organisms and whether or not they would confer technological advances in their use. Mussel hemolymph has also been employed as a target for measuring the alteration of AChE activity induced by contaminants. Several studies have emphasized AChE inhibition in bivalves such as *M. galloprovincialis* [26-27] and *M. edulis* [28] as a biomarker in species exposed to organophosphates, carbamates and some heavy metals.

Pharmacological inhibitors of AChE are important in controlling diseases that involve impaired acetylcholine-mediated neurotransmission [29]. The use of reversible acetylcholinesterase inhibitors has attracted particular attention for the treatment of Alzheimer's-type dementia and has been the subject of extensive research and clinical investigation [30]. Different classes of plant-derived natural products with AChE-I properties are used for treating symptoms of AD. The most potent AChE-I nowadays appear to be natural or synthetic alkaloids such as galantamine and rivastigmine [31]. Phenolics, the most abundant secondary metabolites of plants, were reported to exhibit neuroprotective effects and act as acetylcholinesterase inhibitors [30]. A limited amount of literatüre data are available on the pharmacological properties of methoxyflavones. Therefore, three of these compounds (hispidulin, 5-hidroxy-,6,7,8'4'-tetrametoxyflavon and gnafalin) were included in our study. Hispidulin (4',5,7-trihidroxy-6-metoxyflavon) was isolated from *Centaurea nerimaniae* [32], 5-hidroxy-6,7,3',4'-tetrametoxyflavon was isolated from *Artemisia austriaca* [33] and gnaphalin (5,7-dihidroxy-3,8-dimetoxyflavon) from *Gnaphalium luteo-album* L [34].

The aim of this study was to describe the purification of AChE from haemolymph of *M. galloprovincialis*, a mollusc species existing in the coast of Turkey, employing the affinity chromatography on edrophonium-Sepharose 6B. And to examine the inhibitory effects of some methoxyflavones on AChE, which are not so extensively studied previously.

2. RESULTS AND DISCUSSION

Mytilus galloprovincialis is the most frequently used organism in Mediterranean marine for monitoring the pollution. The measurement of AChE activity in haemolymph of *Mytilus galloprovincialis* mollusc is used as a specific biomarker of possible contamination in coastal waters [26].

In the present study we have purified the AChE from haemolymph of the *Mytilus galloprovincialis* by an efficient two-step procedure of ammonium sulfate precipitation and affinity chromatography on edrophonium-Sepharose 6B.

Ammonium sulphate precipitation proved to be very efficient in removing considerable amounts of other proteins prior to the affinity chromatography. It was found that AChE could be recovered mainly with a 45 % saturation in the supernatant, which is in accordance with Ma *et al*. [15] and Du and Dong [19].

The enzyme was purified approximatedly 13 fold over the crude extract and was obtained in 3 % yield. The final specific activity of the purified enzyme was 3 U/mg. The results of purification of AChE from the haemolymph of *M. galloprovincialis* Lam. are summarized on Table 1.

AChE is distinguished from other cholinesterases by its sensitivity to certain inhibitors, its relative substrate specificity, and its inhibition by high concentrations of the substrate acetylcholine. The success in the purification of AChE in many tissues depends on the selection of both the substrate and inhibitors [35]. Edrophonium-Sepharose 6B affinity chromatography was choosed because of its success in the separation of the AChE activity from other cholinesterases. The matrix which was synthesised by coupling the reversible cholinesterase inhibitor, edrophonium chloride, to epoxy-activated Sepharose had the novel property of selectively binding only one molecular form of acetylcholinesterase [23]. This form of acetylcholinesterase corresponded in molecular size and electrophoretic mobility to the unique form found in cerebrospinal fluid, i.e. secretory acetylcholinesterase (G_4H AChE). It cannot be used to isolate the amphiphilic AChE variants as

they fail to be bound by the matrix. Talesa et al. [22] reported that about 80% of ChE activity of specimens of *M. galloprovincialis* from distinct marine sites lies in the hemolymph as spontaneously soluble (SS) ChE.

Table 1. Purification of AChE of M. galloprovincialis Lam. from the haemolymph of 40 mussels.

Purification step	Volume (ml)	Protein (mg)	Total activity* (U)	Specific activity** (U/mg)	Recovery (%)	Purification fold
1. Crude extract	24	21.4	5.0	0.23	100	1
2. 45% Ammoniun sulphate fraction	2.5	2	1.4	0.7	28	3.0
3. Edrophonium-Sepharose 6B affinity chromatography	2	0.05	0.15	3	3	13

* µmol TNB
*** µmol TNB/mg protein

Elution of AChE with 10 mM potassium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride and 0.5 M NaCl resulted in an apparetly purified enzyme preparation. As can be seen in Figure 1 the affinity chromatography gave three protein containing peaks. The first and second peaks probably coresponds to other cholinesterases which was not bound to the matrix. The highest AChE activity was seen in the third peak, eluted with 10 mM potasium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride and 0.5 M NaCl. This result was in agreement with the reported purification procedure of other AChE's studied so far [5-12].

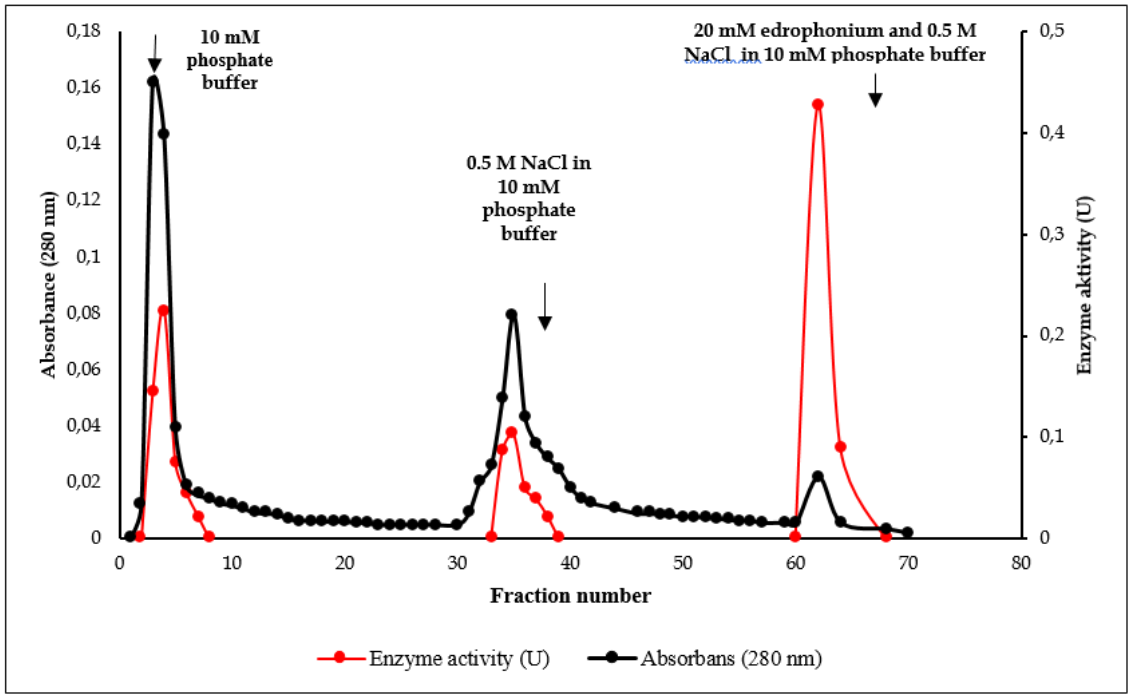

Figure 1. Purification of AChE from haemolymph of the mollusc *Mytilus galloprovincialis* L. by edrophonium-Sepharose 6B affinity chromatography. Protein elution was monitored at 280 nm. Desalted sample (2.5 ml) containing 2 mg protein precipitated by ammonium sulphate (45% salt saturation) was applied onto the column (1 x 10 cm) which had been previously pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.4). After extensive washing with the same buffer, followed by 10 mM potassium phosphate buffer (pH 7.4) containing 0.5 M NaCl until the protein content was below 0.01 absorbance at 280 nm, the elution was performed with 10 mM potassium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride and 0.5 M NaCl. Fractions of 1 ml were collected at a flow rate of 60 ml/h.

Depending on the source, AChE are known to exibit similarities and differences in properties which include pH and temperature dependence, molar mass, Michaelis constant (Km), and other physico-chemical parameters.

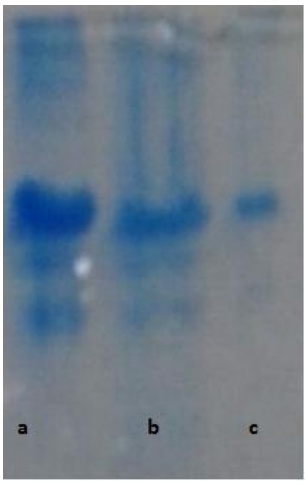

Figure 2. SDS-Polyacrylamide gel electrophoresis of the crude extract, 45% amonium sulphate fraction and purified AChE after affinity chromatography, from the haemolymph of the mollusc *Mytilus galloprovincialis* Lam. **a**. Crude extract, **b**. 45% Amonium sulphate fraction, **c**. Purified AChE after affinity chromatography.

The purified AChE was resolved as a single band by denaturing polyacrylamide electrophoresis. In contrast to previous findings concerning the subunit molecular weight (68 000) of highly purified enzyme preparation from the hemolymph of *Mytilus galloprovincialis* collected in sites of the Adriatic sea [22], in this study the relative molecular mass of purified AChE was estimated to be 51 kDa. It was lower than molecular mass reported for Japanese quail brain (62.5 kDa) [5], human brain (66 kDa) [36] and *Torpedo californica* (71–82 kDa) [37], and similar to that of electric organ of the electric eel (25–59 kDa) [38]. SDS-PAGE under reducing conditions is shown in Figure 3.

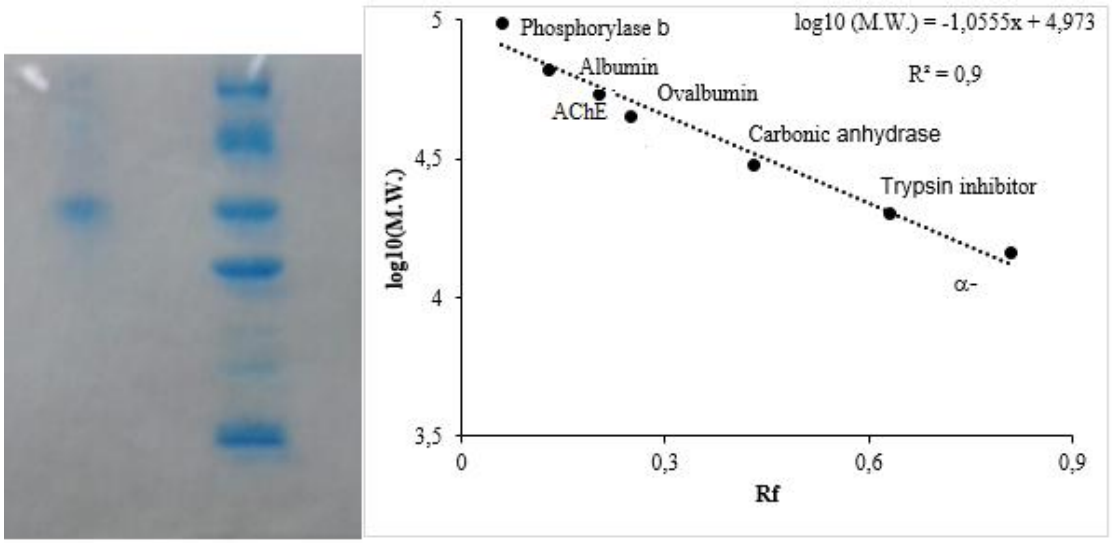

Figure 3. Molecular weight determination based on SDS-polyacrylamide gel electrophoresis under reducing conditions. Standards used were phosphorylase b (M_r=97000), albumin (M_r=66000), ovalbumin (M_r=45000), carbonic anhydrase (M_r =30000), trypsin inhibitor (Mr=20000) and α-lactalbumin (M_r = 14400).

Optimal activity was observed at 35°C (Figure 4), which was similar to that reported for AChE from freshwater fish [6]. The AChE showed a pH optimum at approximately pH 7.5 (Figure 4), which is within the range reported for most invertebrate AChE's.

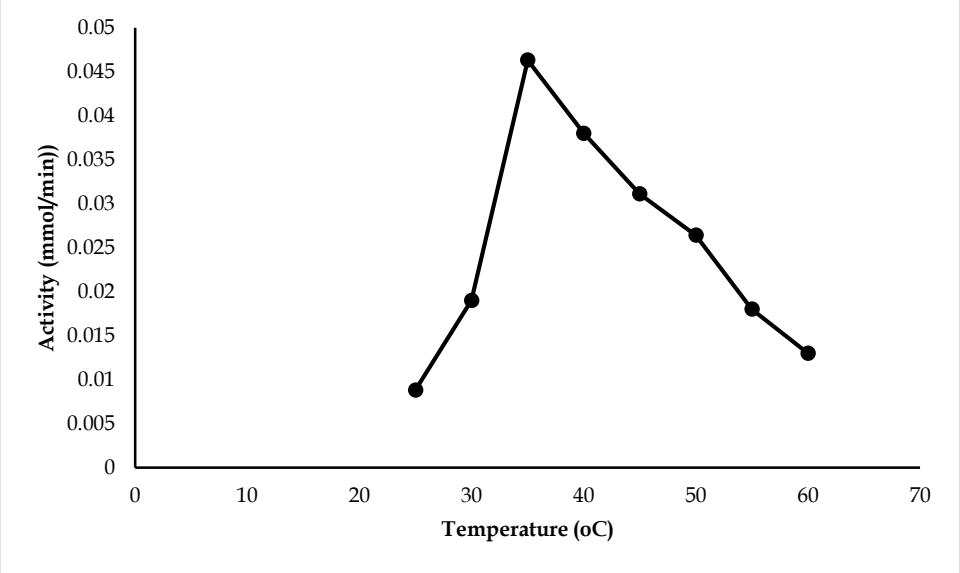

Figure 4. The effect of temerature on the purified AChE from the haemolymph of the mollusc *Mytilus galloprovincialis* Lam.

The Km and Vmax of the enzyme for ACTI were 1.3 mM and 0.183 mM/min/mg (Figure 5, 6).

This value was higher than that reported for AChE from the brain tissues of *Oreochromis aurea*(0.183 mmol/L) [6], *Nebia albiflora* muscle (0.10 mmol/L) [39], brain tissue of *Scomberomorus niphonius* (0.311 mmol/L) [36], and *Pseudosciaena crocea* muscle (0.125 mmol/L) [40].

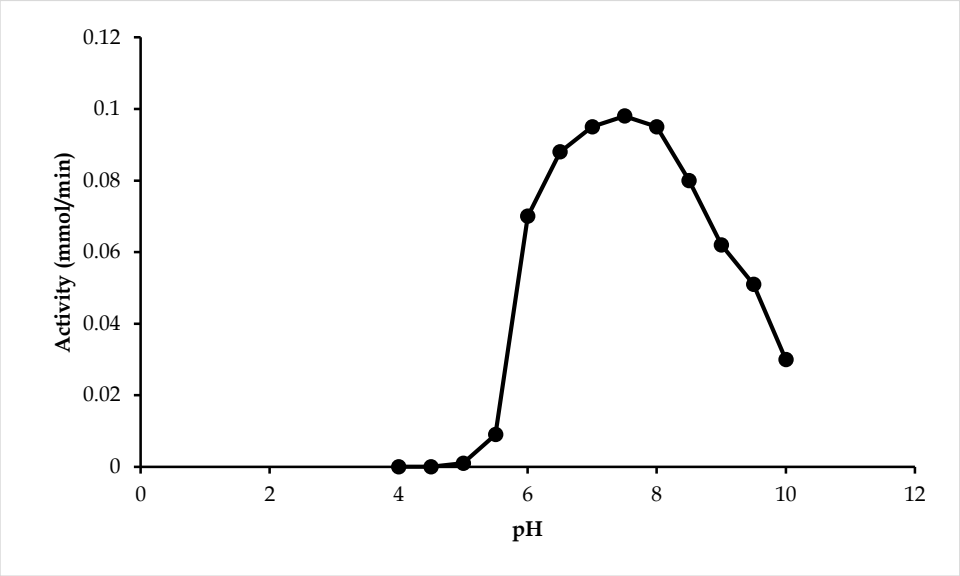

Figure 5. The effect of pH on the purified AChE from the haemolymph of the mollusc *Mytilus galloprovincialis* Lam.

Hispidulin, 5-hidroxy-6,7,3',4'-tetrametoxyflavon and gnaphalin were tested for their *in vitro* AChE inhibitory activities using galantamine as a positive control. The results obtained with three concentrations of the compounds and expressed as IC_{50} values, are summarized in Table 2. All the three compunds exhibited potent AChE inhibitory activity, and thus may be beneficial in the treatment of AD. The potency of AChE inhibition was mainly influenced by the number of $-OCH_3$ groups. 5-hidroxy-6,7,3',4'-tetrametoxyflavon with

three –OCH_3 groups was the most potent AChE inhibitor, while the relatively less potent inhibitors hispidulin (4',5,7-trihidroxy-6-metoxyflavon) and gnaphalin (5,7-dihidroxy-3,8-dimetoxyflavon) have one and two –OCH_3 groups respectively suggesting the critical role of numbers of methoxy groups in AChE inhibition.

Table 2. Acethylcholinesterase inhibitory activity of hispidulin, 5-hidroxy-6,7,3'4'-tetramethoxyflavon and gnaphalin

Substrate	IC_{50} (µg/ml)	K_i (µg/ml)	Type of inhibition
Hispidulin	30.17 ± 2.45[a]	28.15 ± 3.89[a]	Non-competitive
5-OH,6,7,8'4'-tetrametoxyflavon	10.67 ± 1.70[b]	7.029 ± 0.84[b]	Non-competitive
Gnaphalin	13.74 ± 0.70[c]	16.76 ± 2.72[c]	Non-competitive
Galanthamin	0.63 ± 0.09[d]	0.2826 ± 0.02[d]	Competitive

Values were the means of three replicates ± standard deviation. Values with different letters in the same column were significantly (p<0.05) different.

The inhibition consants were determined from initial velocity measurements at various concentrations of substrate ACTI (10 mM to 0.1 mM) and at three different concentrations of metoxyflavones. From the Lineweaver and Burk plot, the common intercept on 1/V axis indicated that the inhibition of the enzyme by methoxyflavones was of the non-competitive type (Figure 7, 8, 9).

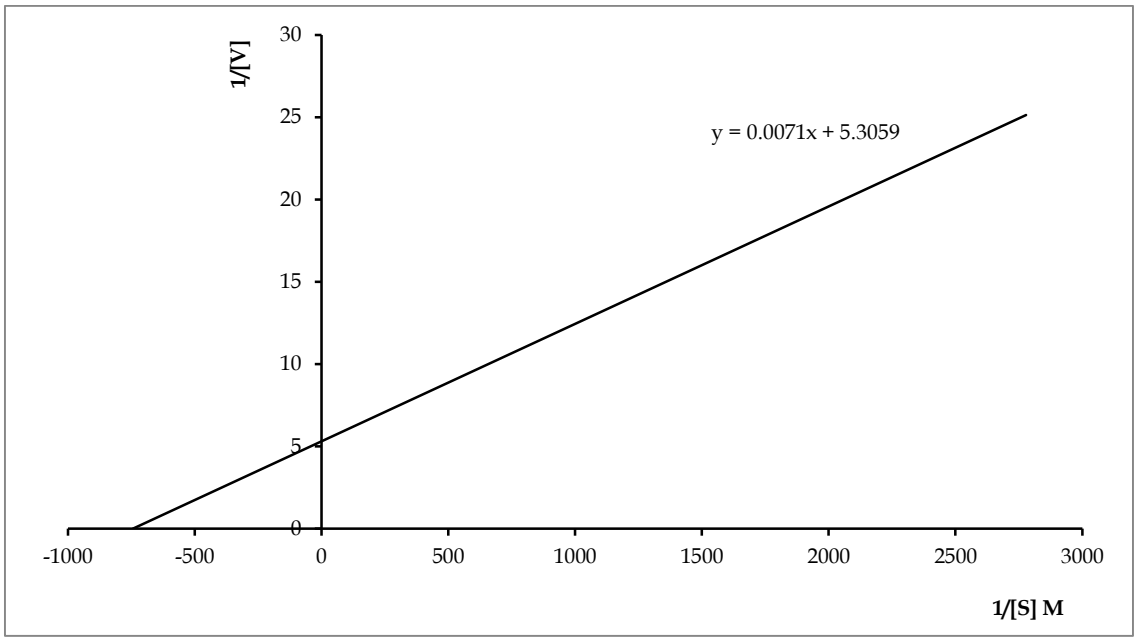

Figure 6. Michaelis constant (Km) of AChE for acetylthiocholine iodide.

The Ki values obtained for hispidulin, 5-hidroxy-6,7,3'4'-tetrametoxyflavon and gnaphalin were 28.15 ± 3.89, 7.029 ± 0.84 and 16.76 ± 2.72 µg/ml, respectively (Table 2).

Galanthamine acts as a competitive inhibitor of AChE, while hispidulin, 5-hidroxy-6,7,3'4'-tetrametoksiflavon and gnaphalin were determined as non-competitive inhibitors. Competitive inhibition takes place by blocking substrate at the active site (tacrine, edrophonium), while non competitive inhibition occurs by binding to the peripheral site (propidium, gallamine) of the enzyme [41].

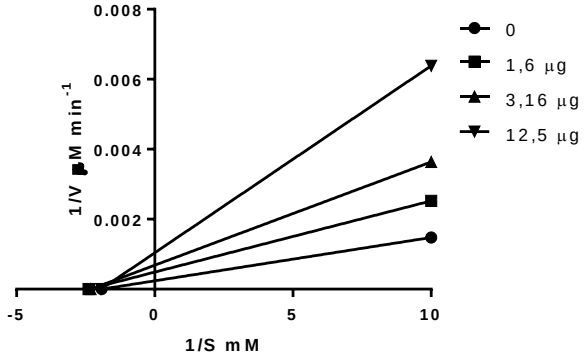

Figure 7. Lineweaver-Burk plot of the inhibitory effect of 5-hidroxy-,6,7,8′4′-tetrametoxyflavon on AChE.

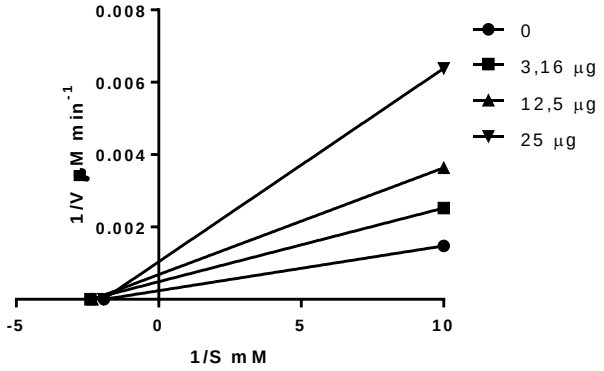

Figure 8. Lineweaver-Burk plot of the inhibitory effect of hispidulin on AChE.

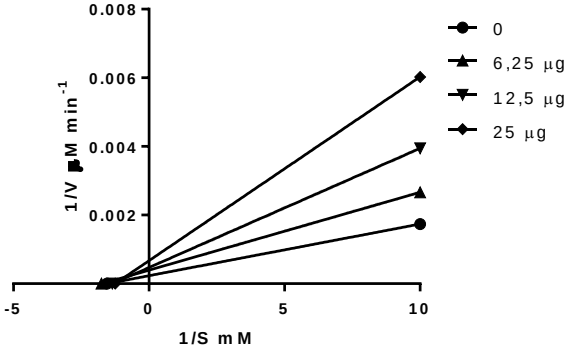

Figure 9. Lineweaver-Burk plot of the inhibitory effect of gnaphalin on AChE.

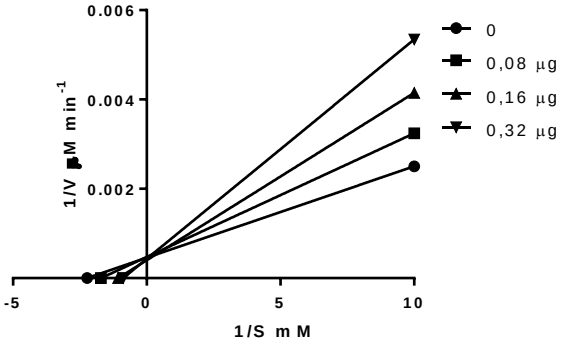

Figure 10. Lineweaver-Burk plot of the inhibitory effect of galanthamin on AChE.

3. CONCLUSION

In this study the usefulness of affinity chromatography for rapidly purifying inhomogeneous enzyme preparations is demonstrated by isolation of haemolymph acethylcholinesterase. The preparation obtained appears to be a homogeneous protein. We have also determined the kinetic parameters of the enzyme purified by the current method.

Three methoxyflavones were examined fort he first time in order to evaluate their potential as anti-AD agents. All of the compounds were shown to be potent AChE inhibitors; in particular, 5-OH,6,7,3'4'-tetrametoxyflavon possessed the most potent inhibitory activity. Reversible inhibitors, competitive or noncompetitive, mostly have therapeutic applications and they could be considered for further studies in the treatment of AD.

4. MATERIALS AND METHODS

4.1. Chemicals and materials

Acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid), edrophonium chloride, epoxy-activated Sepharose 6B, acrylamide, sodium dodecyl sulfate, molecular weight marker proteins were purchased from Sigma Chemical Co. All other chemicals were of the analytical grade.

4.2. Sample collection

40 mussels belonging to the species *Mytilus galloprovincialis* L. were collected from the Yenikapı coastal region of Istanbul.

4.3. Purification of haemolymph AChE

4.3.1. Isolation of haemolymph

Step 1, Preparation of crude extract:

Using a sterile 1 ml syringe, haemolymph was extracted from the posterior adductor muscle and placed in Eppendorf tubes, than centrifuged at 13000 rpm for 10 minutes at 4°C. Aliquots of the resulting supernatant were frozen at −80°C until analysis.

Step 2, Ammonium sulphate precipitation:

The supernatant was precipitated with solid ammonium sulphate at 45% saturation. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes, dissolved in 2 ml of 10 mM potassium phosphate buffer, pH 7.4, and dialysed against 10 mM potassium phosphate buffer, pH 7.4. All of these operations were accomplished at 0°-4°C.

Step 3, Affinity chromatography:

Edrophonium–Sepharose 6B affinity gel was prepared as described by Son et al. [5].

The 45% NH_4SO_4 fraction containing 2 mg protein was applied to the affinity column (1 x 11 cm). After extensive washing with 10 mM potassium phosphate buffer (pH 7.4), followed by 10 mM potasium phosphate buffer (pH 7.4) containing 0.5 M NaCl until the protein content was below 0.01 absorbance at 280 nm, the enzyme was eluted with 10 mM potasium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride and 0.5 M NaCl. The flow rates throughout the experiments were 60 ml/h.

The eluates was collected in 1 ml fractions and assayed for protein content at 280 nm and enzyme activity, those containing AChE activity were pooled and concentrated by ultrafiltration with a stirred cell (model; Amicon, Inc., Beverly, Mass.) equipped with a PM 10 membrane under nitrogen pressure of 20 lb/in^2. After dialysis against 10 mM potassium phosphate buffer (pH 7.4) the preparation was used in enzyme characterization experiments.

4.4. Determination of protein content

Protein concentration was determined either by the Bradford method [42] using BSA as a standard or measurement of absorbance at 280nm.

Five dilutions of protein standard (128 μg/ml), which covered the linear range from 8 to 64 μg/ml were prepared. 100 μl of each standard or sample solution were pipette into separate microplate wells. 150 μl of dye reagent concentrate were subsequently added to each well. The samples and reagent were mixed thoroughly using a microplate mixer and incubated at room temperature for 10 minutes. The absorbance was read at 595 nm using a multiplate ELISA reader (BioTek Eon) within one hour of the assay.

4.5. Enzyme activity

The samples were screened for their AChE inhibitory activity through the modified Ellman's spectrophotometric method [43]. Briefly, 100 mM sodium phosphate buffer at pH 7.5 containing 1.5 mg NaH_2CO_4, 75 mM acetylthiocholine iodide and 10 mM DTNB (3.96 mg/ ml) in 100 mM sodium phosphate buffer was mixed at a ratio of 150: 2: 5 to give final concentrations of 1 mM for acetylthiocholine iodide and 0.3 mM for DTNB. 50 µl of the samples and 10 mM potassium phosphate buffer, pH 7.4 were transferred to the experimental and blank wells, respectively, in a 96 well microplate. 250 µl of the Ellmans assay solution were pipette to all the wells. The increase in absorbance of the produced yellow 5-thio-2-nitrobenzoate was measured at 412 nm every minute for 10 min using a microplate reader (Biotek, Winooski, VT, USA). Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme. Enzyme activity was expressed as µmol/min/ml of formation of TNB by using extinction coefficient of 13.6 mM^{-1} cm^{-1}.

4.6. Electrophoresis

Polyacrylamide gel (10%) was used to determine the apparant molecular weight (Mr) and the degree of purity of the acethylcholinesterase.

4.7. Subunit molar mass determination

SDS-polyacrylamide gel electrophoresis followed the method of Laemmli [44], modified with 4 % polyacrilamide stacking gel in 0.125 mol/L Tris-HCl buffer, pH 6.8, with 0.1 % SDS and 10 % polyacrilamide separating gradient gel in 0.25 mol/L Tris-HCl buffer, pH 8.8, with 0.1 % SDS. The electrophoresis buffer consisted of 0.05 mol/L Tris, 0.192 mol/L glycine, pH 8.3, with 0.1% SDS. Subunit molar mass was analysed under reduced conditions. Before application to the gel the samples were boiled for 90 s with sample buffer containing 0.125 mol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol and 5% 2-mercaptoethanol at a ratio 1:1. After a run at 5 mA/gel at room temperature, the gels were stained overnight with 0.2% Coomassie Brilliant Blue R 250 in 45 : 45 : 10 (methanol : water : acetic acid). The destaining mixture contained 10 ml of acetic acid, 25 ml of methanol and 65 ml of water.

4.8. Kinetic studies

4.8.1. Effect of pH on enzyme activity

The pH optimum was determined performing the assay in the pH range of 3.0-7.0 potassium phosphate buffer and pH range of 8.0-9.0 using Tris-HCl buffer.

4.8.2. Effect of temperature on enzyme activity

The enzyme activity was measured in the range of 10-70°C using the standard activity assay procedure at related temperature.

4.8.3. Substrat specificity

To determine the substrate specificity of the enzyme, we assayed the hydrolysis of the substrate acetylthiocholine iodide. The Michaelis constant value (Km) was determined at concentrations ranging from 0.1 to 10 mM.

4.9. Inhibition of AChE by 5-hydroxy-6,7,8'4'-tetrametoksiflavon, hispudiline and gnaphaline

In order to elucidate the type of inhibition of the compounds, the enzyme activity was measured in the presence of an increasing concentrations of ACTI (0.1 M – 0.01 M), and in the presence or absence of four concentrations of hispidulin, 5-hydroxy-6,7,8'4'-tetrametoksiflavon and gnaphaline (final 1,6; 3,16; 12,5 and 25 µg/ml). Incubation of enzyme preparation with methoxyflavones for 30 minutes was performed prior to determination of residual activities.
Determination of enzyme kinetic parameters was perform using GraphPad Prism version 7.00. The kinetic constant Km and Vmax were determined by double-reciprocal Lineweaver-Burk plot analysis of the data.

4.10. Statistical analysis

All measurements were made in triplicate. The results were statistically analyzed using GraphPad Prism version 7.00. Results were considered significant at $p < 0.05$.

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Conflict of interest statement: The authors declared no conflict of interest.

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