Isolation and Purification of Acetylcholine Esterase from the Blood of Breast Cancer Female Patients in Baghdad City

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ABSTRACT
Acetylcholinesterase (AChE) breaks down acetylcholine to terminate synaptic transmission, and its regulation influences cancer progression. Blood samples from 20 healthy individuals and 69 breast cancer patients (30 pre- and 39 post-surgery) were analyzed. AChE was purified using techniques like precipitation with [$NH_4(SO_4)_2$], membrane dialysis, ion-exchange, and gel filtration. The highest enzyme activity (5.9 IU/mg) and yield (65.3%) were obtained by the precipitation with [NH4(SO4)2], while the highest specific activity (0.350 U/mg) was achieved using Sephadex G-100. Optimal enzyme conditions were 34 mM substrate, 28°C, pH 7.6, and 15 minutes incubation time. Kinetics showed Vmax of 5 ng/mL and Km of 20 mM. HPLC yielded 89.3% enzyme purity. Further research is needed to explore AChE's role in cancer.

Keywords: Acetylcholine Esterase (AChE), Breast Cancer, Enzyme Purification, Enzyme Kinetics.

1. INTRODUCTION

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are examples of choline esterase enzymes that are necessary for the breakdown of cholinergic neurotransmitters, primarily acetylcholine [1]. AChE, found in chemical synapses and red blood cells, hydrolyzes acetylcholine into choline and acetic acid (EC 3.1.1.7) [2]. Acetic acid is required for the contraction and relaxation of muscles. Through more efficient hydrolyzing of acetylcholine and butyrylcholine, BChE has a detoxifying effect [3]. Most of it exists in blood plasma (EC 3.1.1.8). Both enzymes control the quantity of neurotransmitters via serine residues, which are essential for neuromuscular activities. These enzymes are possibly inhibited by substances such as organophosphates with detrimental consequences including respiratory failure and muscular paralysis [4]

The nervous system breaks down the neurotransmitter acetylcholine (ACh) into choline and acetic acid for terminating synaptic transmission. In addition, the enzyme acetylcholinesterase (AChE) is known by its scientific name, acetylcholine acetylhydrolase (EC 3.1.1.7) which facilitates this process [5]. AChE prevents the incessant stimulation of muscles and neurons which allows for proper signaling and relaxation. It is usually at cholinergic synapses and neuromuscular junctions. Enzyme 334, histidine 447, and serine 203 stand for its catalytic triad [6]. This influential serine hydrolase possibly hydrolyzes up to 25,000 acetylcholine molecules in a second. In terms of clinic, AChE treats disorders like myasthenia gravis and Alzheimer's, but over-inhibition is a possible danger, which exposure to nerve agents or pesticides show [7].

Acetylcholinesterase (AChE) is significant in cancer because it regulates acetylcholine levels, influencing tumor growth and progression [8]. By hydrolyzing acetylcholine, AChE influences key cellular processes such as proliferation and apoptosis which are important in cancer development [9]. In certain cancers, line lung cancer, lower AChE activity makes acetylcholine persist which activates pro-survival pathways and promoting tumor growth [10]. In contrast, higher AChE activities in other cancers are caused by more aggressive tumor behaviors. AChE possibly act as a tumor suppressor by the promotion of apoptosis, in particular responding to chemotherapy [9]. Because it is abnormally expressed in different cancers, AChE is explored as a possible biomarker for cancer prognosis and treatment, with ongoing study focused on its mechanisms and therapeutic uses[11].

This study concentrates on isolating and purifying acetylcholine esterase from the blood of females with breast cancer by various purification techniques and for finding the optimal conditions which suits the purification processes, and studying the kinetics of the AChE enzyme.

2. MATERIALS AND METHODS

2.1. Patients and Blood Sample Collection

A. Control Group

Twenty female samples were selected randomly for serving as controls in the study for comparison.

B. Patients Group

From patients ranging in age from 33 to 68 years, blood samples were gathered. The period of sample collection was February 10, 2023 – May 20, 2023. Samples from patients were taken both prior to and following tumor removal surgery. 69 patients with breast cancer were among the samples; 30 samples were taken from patients before to surgery, and 39 samples were taken from patients who had had their tumors removed and were receiving treatment doses at various intervals. The samples were obtained following an accurate diagnosis by oncology doctors in cooperation with Al-Amal Cancer Hospital in Baghdad.

C. Blood Sample Collection

Using a single-use syringe (5 ml), blood was extracted from the vein and put into GEL tubes. Serum was extracted from the blood using a centrifuge running at 5000 rpm for five minutes after the blood was allowed to rest for thirty to fifty minutes. After that, the transparent serum was put into Eppendorf tubes and kept cold.

2.2.Procedures

2.2.1. Precipitation by Ammonium Sulfate

A gradient of ammonium sulfate concentrations was used to precipitate the enzyme from the serum until 70% saturation was reached. 15 mL of serum received 9 grams of ammonium sulfate in total over the course of 45–60 minutes, while the serum was continually agitated and stored in an ice bath. The precipitate that resulted was separated at 5000×g by centrifugation, and the supernatant was disposed of. The precipitate was then allowed to dissolve for 24 hours in the smallest volume of buffer solution (0.135 M Tris-HCl, pH 7.2). Then, to precipitate more proteins, more ammonium sulfate was added. Protein concentration and enzyme activity were assessed, and the specific activity was ascertained [12].

2.2.2. Dialysis with Membranes

One of the first methods for purifying enzymes is membrane dialysis, which removes any remaining ammonium sulfate that was added during protein precipitation. A dialysis bag containing the dissolved protein was submerged in a buffer solution (0.135M Tris-HCl, pH 7.2). For eighteen hours, the buffer solution was changed on a regular basis. At 4 ± 1 °C, this step was performed to keep the cholinesterase activity constant. The enzyme concentration was assessed following dialysis [12].

2.2.3. Ion-Exchange Chromatography

Buffer solution (0.135M Tris-HCl) was used: made by adjusitng the pH to 7.4 by dissolving (19.7 g) of Tris-HCl in one liter of deionized water.

DEAE: Ion-exchange cellulose resin: prepared by combining 400 mL of the buffer solution with 5 milligrams of resin.

Sodium chloride solution (200 mM): prepared by adding (1 L) of the buffer solution (0.125M TrisHCl, pH 7.4) to (11.7 g) of NaCl. Additionally, a number of NaCl solutions were made in different concentrations (25, 50, 75, and 100 mM).

Hydrochloric acid (0.25 N): prepared by diluting the solution to a final volume of (500 mL) with distilled water after adding (10.4 mL) of concentrated HCl (12N) to (400 mL) of distilled water.

Procedure [13]

1- To stop gel particle leakage, a little amount of glass wool was inserted at the bottom of a glass column measuring 1.5 cm in diameter and 30 cm in length. To prevent air bubbles that could interfere with the separation process, the gel solution was added to the column gradually and evenly until it reached a height of 12 cm. After that, enough buffer solution (0.125M Tris-HCl, pH 7.4) was added to the column to wash it down to a flow rate of 1 mL/min.

2- The sample of ammonium sulfate-treated serum (5mL) that had been centrifuged in accordance with the previous steps, was introduced to the column and given five minutes to absorb into the gel. The same buffer solution was used for the separation, but with progressively higher NaCl concentrations (25, 50, 75, and 100 mM).

3- After fractions eluted from the column, absorbance at 412 nm was measured and collected in 3 mL quantities. Protein concentration was quantified, and the enzyme activity for each peak that emerged from the separation was also assessed. For use in later investigations, the separated fractions were kept in storage at -20°C.

2.2.4. Gel Filtration

One important method for separating substances based on their size and molecular weight is gel filtration. Smaller proteins enter the gel beads and are eluted later, while larger proteins travel outside the gel with the solvent—typically distilled water or buffer—and are eluted first. Proteins are isolated by measuring absorbance at 280 nm or by using a protein assay kit. Fractions are collected using a fraction collector. The purification gel is Sephadex G-100 [14].

Solutions used

A. Buffer Solution (Tris-HCl at 0.125M): pH was adjusted to 7.4 by dissolving (19.7 g) of Tris-HCl in (1 liter) of deionized water.

B. Gel Filtration Suspension for Sephadex G100: Prepare by adding 200 milliliters of (0.125M) TrisHCl buffer, pH 7.4, to (2.5 g) of Sephadex G100 column packing material. Let the mixture remain at 4°C for 20 to 24 hours. Replace the buffer solution multiple times throughout this time to get rid of any fine particles that can lower the column's flow rate.

Procedure

1. To stop the leaking of gel beads, a little piece of glass wool was inserted at the bottom of a glass column that was 1.5 cm in diameter and 30 cm in length. After that, the gel suspension was gradually added to the column until the gel height of 12 cm, being sure to pour it gently and evenly to prevent air bubbles that could impede separation. After that, the column was sufficiently cleaned with 0.125M Tris-HCl buffer (pH 7.4) to reach a flow rate of 1 ml/min.
2. After adding a 5 ml sample to the column, the gel was given 5 minutes to absorb the sample. 150 ml of 0.125M Tris-HCl buffer, pH 7.4, was used as the eluent to start the separation process.
3. The absorbance was measured at 412 nm after (3 mL) of eluted parts were removed from the column. Enzyme activity was evaluated for each peak that emerged from the separation, and protein content was also calculated.

2.2.5. Acetylcholinesterase Assay

A. Preparation of pH 7.2-7.4 Buffer: (0.25 g) of KH_2PO_4 and (0.55 g) of K_2HPO_4 were dissolved in 90 mL of distilled water.

B. Preparation of DTNB Solution (0.001 mmol): (0.01 g) of DTNB was dissolved in 25 mL of distilled water. To ensure complete dissolution, a small amount of $NaHCO_3$ has been added.

C. Preparation of S-Acetyl Thiocholine Iodide (S-AchEI): (0.017 g) of S-AchEI was dissolved in 1 mL of distilled water.

D. Blank Preparation: A blank solution was prepared for each sample. The absorbance for each blank has been recorded at 412 nm [15].

Procedure

1. pH buffer (2.25 mL) was added to the test tube.
2. Followed by the addition of (50 µL) of DTNB solution.
3. Then (10 µL) of serum was also added.
4. The absorbance has been recorded at 412 nm.

Assay Procedure

1. A new test tube was used.
2. Two milliliters of the blank solution were added to the test tube.
3. substrate solution (34 µL) was also added, noticing the color change to yellow.
4. The absorbance was recorded at 412 nm.

Determination of AChE Activity

The following equation has been employed to determine the enzyme activity:

Enzyme activity = (**Absorbance of Blank – Absorbance of Substrate**) **25 µmol/mL @3minute**s

2.2.6. Assessment of Purification efficiency via HPLC

Purification efficiency was evaluated via HPLC as follows; C18 column (150 mm × 4.6 mm ID, 5 µm particle size) has been used for reverse-phase columns. The mobile phase flow rate is a (1:1) combination of acetonitrile (solvent B) and distilled water (solvent A). Using samples at a wavelength of 319 nm, the UV-Vis spectrophotometer was used as the detector. At 37°C as the temperature [16].

3. RESULTS AND DISCUSSIONS

3.1.Partial Purification of Acetylcholinesterase from Breast Cancer Patients and Study of Molecular Weight Estimation

Protein concentration is typically measured in the early stages of enzyme purification to remove a significant amount of water and achieve a degree of purity. Table (1) shows the results of AChE enzyme purification.

Table 1. Acetylcholine esterase purification results.

Purification Stages	Vol. (mL)	Activity (IU/mg)	Total Activity	Protein Conc. (mg/mL)	Specific Activity (U/mg)	Yield %	Purification Folds	Total Protein Conc.
Crude Serum	15	6.5	97.5	65	0.188	100	1	975
Precipitation with 70% $[NH_4(SO_4)_2]$	13	5.9	63.7	40	0.147	65.3	0.64	520
Membrane Dialysis	10	4.3	43	21	0.204	44.1	1.08	430
Ion-Exchange (DAEA-Cellulose)	7	3.5	24.5	16	0.218	25.1	1.15	112
Gel Filtration (Sephadex G-100)	5	2.45	12.25	7	0.350	12.5	1.86	35

Acetylcholinesterase (AChE) from breast cancer patients' sera was partially purified using ammonium sulfate (70%), which neutralizes protein charges and decreases solubility. This approach lowers the volume of the extract, improves purifying efficacy, and helps remove proteins and water that could interfere with enzyme function. Reagents are introduced gradually to remove precipitated proteins. It is preferred because it has a minimal impact on enzyme performance and is economical. The results will be compared to these given by reference [17], which studied the purification of AChE in diabetic individuals as follows:

According to Table (1), low molecular weight materials such as salts, amino acids, cofactors, and small carbohydrates were removed from larger molecules like proteins and nucleic acids during the purification of acetylcholinesterase (AChE) from breast cancer patient serum using dialysis. Dialysis, which separates molecules by size using semi-permeable membranes, increased AChE's specific activity from 0.147 U/mg (after precipitation) to 0.204 U/mg, with a protein concentration of 21 mg/mL and an activity of 4.3 IU/mg, yielding 44.1% and a purification fold of 1.08. In comparison to [17], AChE's specific activity slightly increased from 28.48 IU/mg (after precipitation) to 28.82 IU/mg, with a protein concentration of 0.37 mg/mL and an activity of 84 IU/mg, yielding 97.14% and a purification fold of 10.2. Some enzyme activity loss during dialysis may occur due to instability or loss of co-factors, while trace metal contamination from unpurified water is a common issue. The crude serum had the highest initial activity of 97.5 IU/mg and a total protein concentration of 65 mg/mL, with a specific activity of 0.188 U/mg. Following precipitation with 70% [NH4(SO4)2], activity dropped to 63.7 IU/mg and specific activity to 0.147 U/mg. Whereas the precipitation with 70% [NH4(SO4)2] in [17] has shown activity dropped from 84 to 81.6 IU/mg and specific activity increased from 12.72 to 28.48 U/mg. Further purification via membrane dialysis reduced activity to 43 IU/mg, and Ion-Exchange (DEAE-Cellulose) decreased it further to 24.5 IU/mg, with a specific activity of 0.218 U/mg compared to [17] increased activity to 123.2 IU/mg, with a specific activity of 91.3 U/mg. The final gel filtration step (Sephadex G-100) resulted in the lowest activity of 12.25 IU/mg but the highest specific activity of 0.350 U/mg compared to [17] which showed activity of 18.84 IU/mg and specific activity of 29.34 U/mg. The greatest yield of 65.3% achieved after the 70% ammonium sulfate precipitation step compared to 97.14% achieved by [17]. To improve purity, excess salt was eliminated by dialysis using Tris-HCl buffer (pH 7.4).

Table (1) also shows that most proteins during the purification process increased the specific activity of acetylcholinesterase (AChE), which increased from 0.204 U/mg after precipitation to 0.218 U/mg after the third purification step. At 16 mg/mL of protein and 3.5 IU/mg of enzyme activity, the protein yielded 25.1% and a purification fold of 1.15. A DEAE-cellulose column was directly treated with the partially purified enzyme solution (obtained after ammonium sulfate precipitation) in this investigation. Three protein peaks are shown to be eluted with increasing NaCl concentration in (Figure 1). AChE was purified to almost homogeneity, as seen by the second active peak, which corresponds to AChE eluted by NaCl, between tubes 10 and 11. The first peak, or elution peak, between tubes 9 and 10 shows the elimination of denatured proteins. The purified AChE peak displayed a specific activity of 0.218 IU/mg with a 1.2-fold

purification, suggesting that this peak is a reliable source of the enzyme for additional purification. Since cellulose is hydrophilic, it is an efficient tool for enzyme purification when used in conjunction with a DEAE-cellulose column following ammonium sulfate concentration. These exchangers are made to work with biological materials including proteins, carbohydrates, and nucleic acids and have no effect on denaturation of proteins. The results agree with the literature [8][18][19].

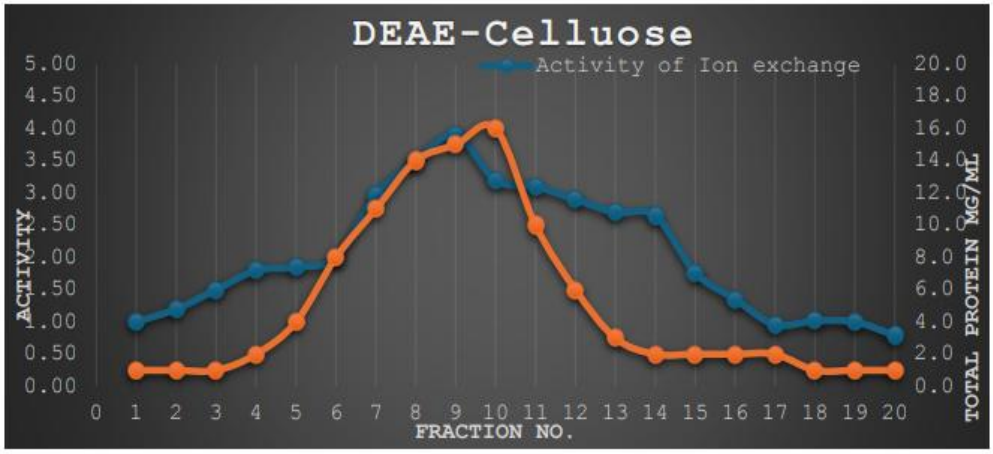

Figure 1. Purification of AChE using (DEAE-Cellulose) ion-exchange chromatography.

Table (1) displays the results of the analysis process for the majority of proteins. The specific activity of acetylcholinesterase (AChE) increased from 0.218 U/mg during precipitation to 0.350 U/mg after the fourth purification step, with a purification fold of 1.86 achieved at a protein concentration of 7 mg/mL, an activity of 2.45 IU/mg, and a yield of 12.5%. The first and second peaks had elution volumes of 8 and 7, respectively. Enzyme activity was shown to be more concentrated at the first peak (Peak A) by monitoring AChE activity, as seen in Figure (2). The findings also showed that women with breast cancer had lower AChE activity, as shown by the two peaks in Figure (2). According to the literature [20][21], the activity of AChE also decreases in breast cancer patients, which confirms the results of our current study.

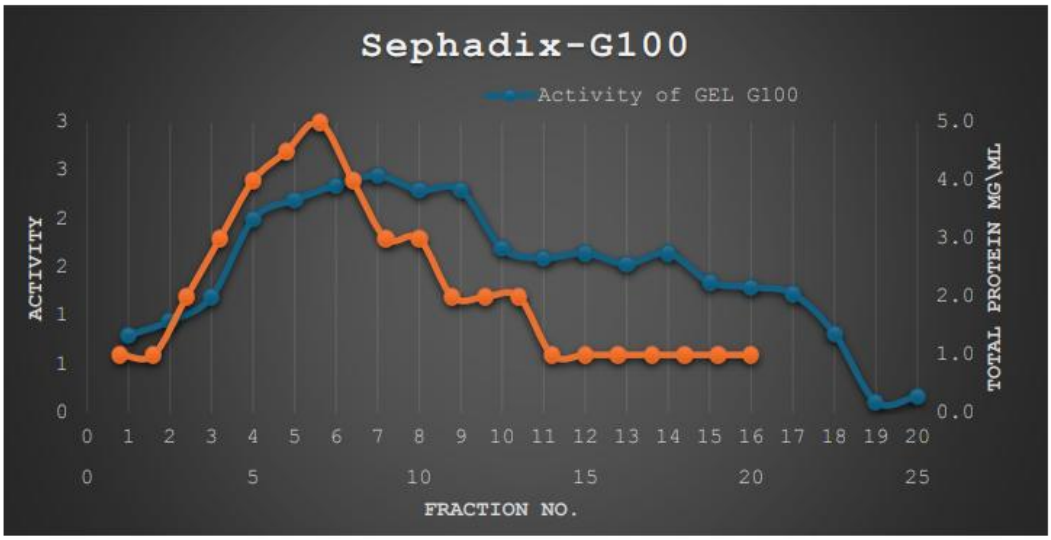

Figure 2. Purification of AChE using gel-filtration chromatography.

3.2.Estimating the Molecular Weight of AChE Enzyme via Electrophoresis

Using SDS-PAGE, the molecular weight of acetylcholinesterase (AChE) was ascertained by denaturing the enzyme and creating negatively charged compounds. These protein chains moved toward the positive electrode during electrophoresis, with molecular weight having the biggest effect on migration. According to Figure (3), AChE, which was mostly isolated from the serum of breast cancer female patients, had a single, distinct band and a molecular weight of about 65.60 kDa. No contamination was found. Other studies found that the AChE molecular weight estimated to be 51 kDa measured for Mytilus galloprovincialis and the molecular weight of AChE from human brain achieved 66 kDa [18].

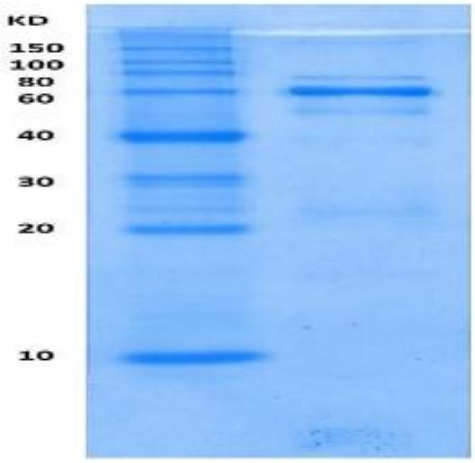

Figure 3. Electrophoresis of purified acetylcholinesterase (AChE) to estimate molecular weight in comparison to standard solutions.

The findings varied when compared to a number of research that used the same method to estimate the molecular weight of the enzyme from various sources. The molecular weight of acetylcholinesterase (AChE) isolated from lymphocytes of breast cancer patients was determined to be 96 kDa using Sepharose [20]. Furthermore, the molecular weight of the enzyme isolated from tobacco cells using a DEAE-Sephadex column was calculated to be 30 kDa [22]. In comparison, the enzyme isolated from frog ovary cells using a phosphocellulose column had a molecular weight of 165 kDa [15]. The discrepancies in molecular weights observed among research can be due to changes in purifying procedures, the species and tissues employed as enzyme sources, the underlying concepts of each method, and the use of detergents. Furthermore, longer purification processes in some experiments may cause enzyme breakdown, resulting in lower molecular weights than expected [15][20].

3.3. Kinetic Properties of Partially Purified Enzyme from Breast Cancer Patients

3.3.1. Effect of Substrate Concentration

The enzymatic reaction rate of acetylcholinesterase (AChE), partially isolated from female breast cancer patients using a Sephadex G100 column, increased with increasing substrate concentration, peaking at 0.1 ng. The highest AChE activity was recorded with dipeptidyl peptidase (DPP) as the substrate at 34 mM as shown in Figure (4). Studies showed that AChE activity does not necessarily increase with the increase of the substrate concentration [20].

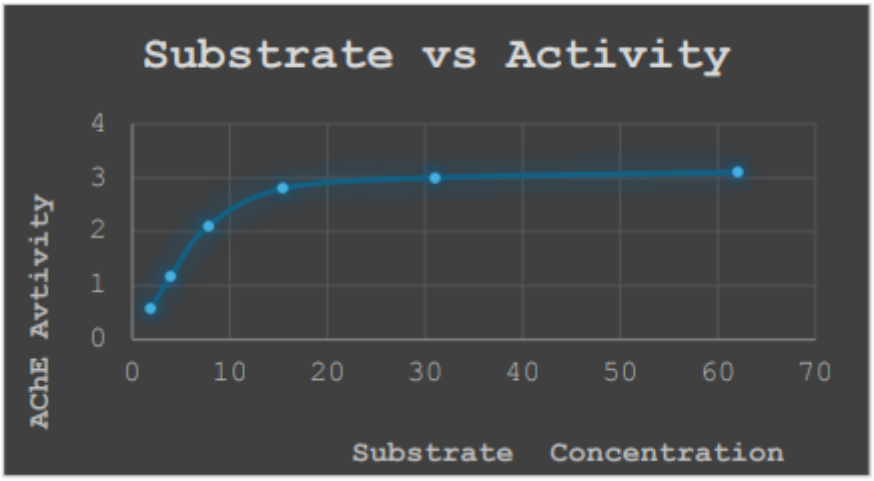

Figure 4. The effect of substrate concentration on the purified AChE activity.

3.3.2. The Temperature influence on AChE Enzyme Activities

The temperature impact on acetylcholinesterase (AChE) and has been studied at different temperatures (7-57°C), which Figure (5) shows, and 28°C was the best temperature for AChE activities. Enzyme

activities are in positive core relationship with temperature until it peaked, then dropped. This decrease is due to enzyme denaturation occurring when high temperatures distort the enzyme's tertiary structures and break bonds between active amino acids which losses activities. In addition, high temperatures disrupt the ionization states on the enzyme's surfaces which affect its actions. Another scholar showed that the optimum is 35°C for achieving the maximum activities of the AChE enzymes [18].

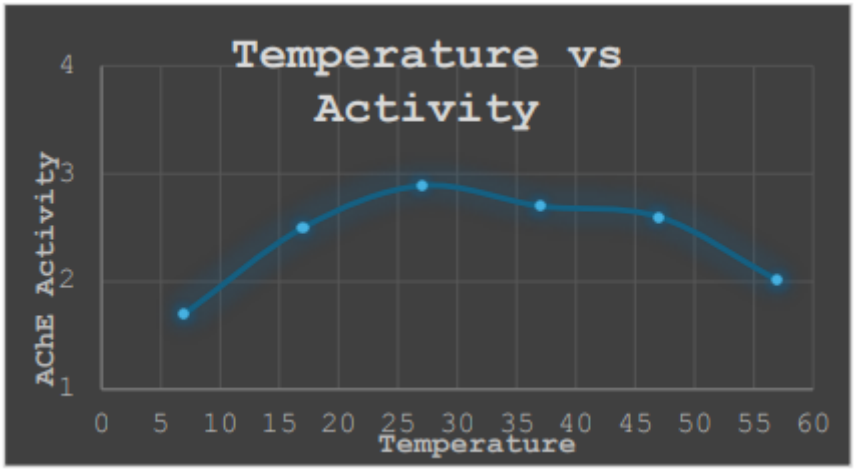

Figure 5. The effect of temperature on AChE Activity.

3.3.3. The pH impact on AChE Enzyme Activity

The pH impact on acetylcholinesterase (AChE) activity was studied which shows that enzyme activity relies on pH because of the differences in enzyme structure and ionic groups. The enzymes have an optimal pH when exhibiting maximum activities, which typically show a bell-shaped curve of reaction rate versus pH. For AChE, the optimal pH was 7.6, as in Figure (6). Beyond this pH, activity declines due to protein denaturation and loss of enzyme-substrate complex formation. Extremely high or low pH values can lead to complete loss of enzyme activity, affecting ionic groups at the active site or altering enzyme-substrate interactions. Literatures have confirmed that maximum AChE activity was obtained at pH between 7.0 to 8.0 [15][18].

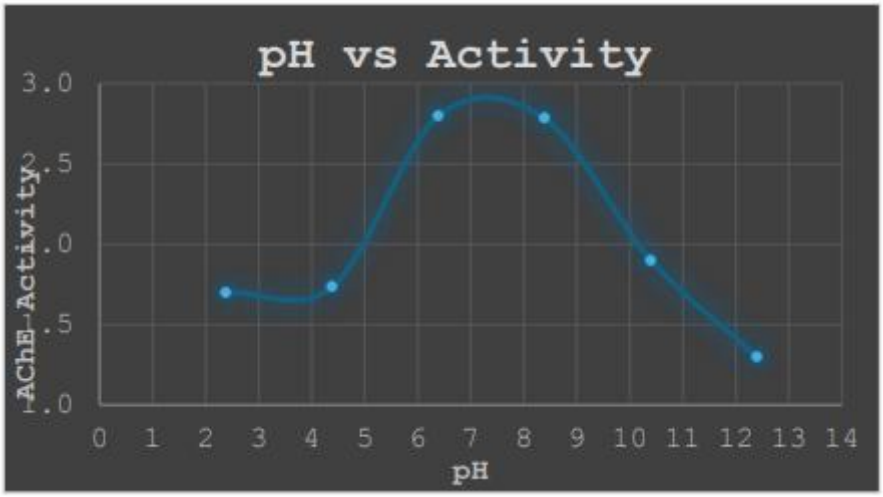

Figure 6. The effect of pH on AChE Activity.

3.3.4. The Effect of Incubation Time on AChE Enzyme Activity

Figure (7) depicts the AChE enzyme activity versus incubation period (0, 5, 10, 15, 20, 25 minutes). Throughout the investigation, an incubation duration of fifteen minutes (15 minutes) was used as the standard for acetylcholine esterase activity because it corresponds to a linear section of the curve and produces consistent absorbance readings. This could be linked to the enzyme's temperature sensitivity, as extended incubation can cause the breaking of amino acid bonds owing to heat. The incubation time of 15 minutes has always been the ideal duration in determining the AChE enzyme activity [23][24].

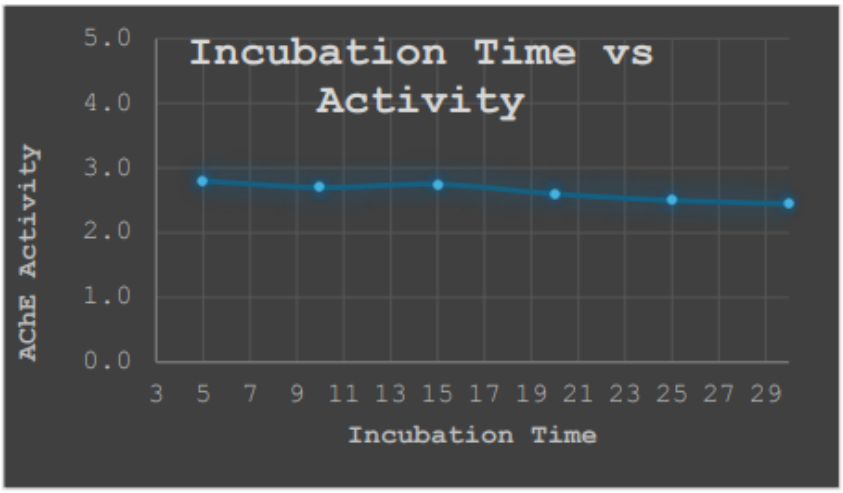

Figure 7. The Effect of incubation time on AChE activity

3.3.5. Acetylcholine Esterase AChE Kinetics (Michaelis Constant Km and Maximum Velocity Vmax)
Acetylcholinesterase (AChE)'s maximum velocity (Vmax) and Michaelis constant (Km) were found using the Lineweaver-Burk plot analysis used in this study. This plots the reciprocal of the initial reaction rate against the reciprocal of substrate concentration. The results showed a linear relationship. The Vmax was 5 ng/ml, while the Km value was 20 mM. AChE follows the MichaelisMenten using the Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax}$$

Resulting in a hyperbolic curve, according to the Lineweaver-Burk plot (Figure 8), which also shows that the enzyme exhibits an increased reaction rate with growing substrate concentration. The enzyme-substrate affinity is reflected by the Km value, which shows that lower Km values correspond to stronger affinity and higher Km values correspond to lower affinity. Because it is applicabile, simple, and precise in testing enzyme efficiency and comprehending the impact of activators and inhibitors on enzyme activities, the Lineweaver-Burk approach is advised. With one substrate concentration, the enzyme maximum velocity (Vmax) the gel filtrated purified by was 5 ng/ml and its Km value was 20 mM. The outputs resemble the values of Km and Vmax which Gerczei and Pattison achieve [25]. To understand these kinetic features is important to comprehend the substrate affinity and AChE catalytic effectiveness. Bigger affinity for the substrate is usually shown by a lower Km value, but the enzyme capability of catalyzing at substrate saturating levels is shown by the Vmax values.

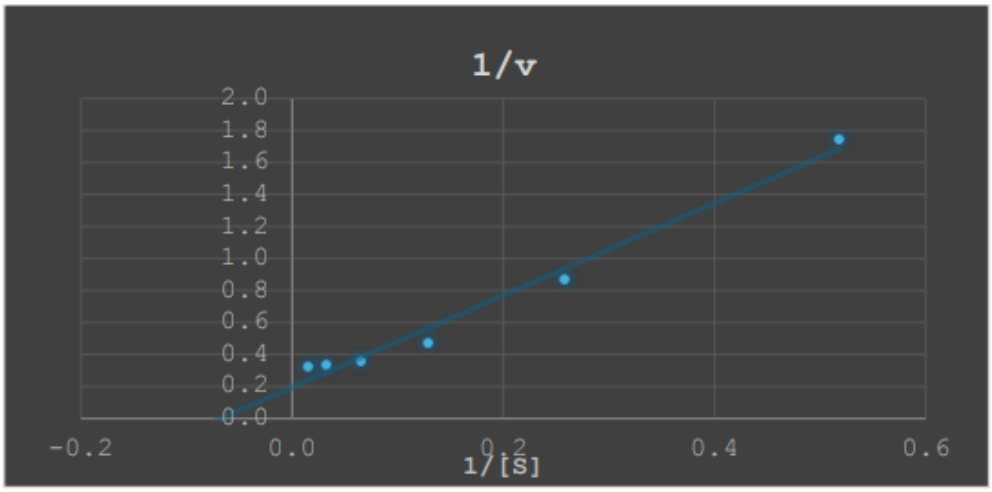

Figure 8. Lineweaver-Burk plot of the purified AChE enzyme.

3.3.6. AChE Enzyme Purification Efficiency Assessment Sesults via HPLC

Table (2) and Figure (9) from the HPLC technique at a wavelength of 319 nm showed the findinfs.

Table 2. AChE Enzyme Purification Efficiency Results by HPLC.

Acetylcholine Esterase						
Peak	Retention Time (mm:ss)	Start Time (mm:ss)	End Time (mm:ss)	Area (mAs)	Height (mA)	Quantity (mg/L)
1	04:42.7	04:31.5	04:51.9	4.2	0.5	N/A
2	05:07.8	04:54.9	05:35.8	35.2	2.0	N/A

The acetylcholinesterase (AChE) purity assay results by HPLC revealed an 89.3% purity. The examination of reaction kinetics and product generation, HPLC is reliable to calculate the molecular weight and evaluate AChE enzyme activities. The method sensitivity, specificity, and adaptability for the intricate biological matrices are well-known. HPLC produces High enzyme purity based on the literature [15][18] which confirms our study.

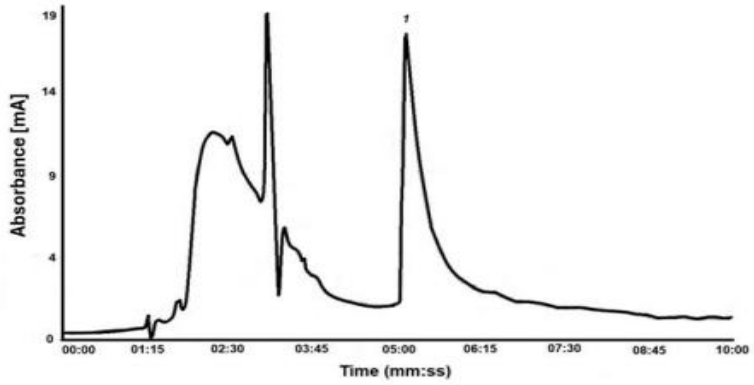

Figure 9. Results of AChE given by HPLC.

4. CONCLUSION

Acetylcholinesterase (AChE) has the ability of regulating acetylcholine levels being critical to develop cancer. By many purification methods for separating the enzyme, the AChE activity was tested in breast cancer patients and healthy controls. Ammonium sulfate precipitation yielded the maximum enzyme activities while Sephadex G-100 filtration made the best specific activities. Calculating kinetic parameters (Vmax and Km) and determining optimal conditions for enzyme activity indicated enzyme performance. HPLC purification proved the AChE purity at 89.3%. These results stress the need for more studies of the AChE function in cancer.

REFERENCES

[1] De Boer, D., Nguyen, N., Mao, J., Moore, J., & Sorin, E. J. (2021). A comprehensive review of cholinesterase modeling and simulation. Biomolecules, 11(4), 580. https://doi.org/10.3390/biom11040580

[2] Patočka, J., Kuča, K., & Jun, D. (2004). Acetylcholinesterase and butyrylcholinesterase–important enzymes of human body. Acta Medica (Hradec Kralove), 47(4), 215-228.

[3] Colovic, M. B., Krstic, D. Z., Lazarevic-Pasti, T. D., Bondzic, A. M., & Vasic, V. M. (2013). Acetylcholinesterase inhibitors: pharmacology and toxicology. Current neuropharmacology, 11(3), 315-335.

[4] Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical pharmacology, 7(2), 88-95. https://doi.org/10.1016/0006-2952(61)90145-9

[5] Trang, A., & Khandhar, P. B. (2019). Physiology, acetylcholinesterase. PMID: 30969557

[6] Zhou, Y., Wang, S., & Zhang, Y. (2010). Catalytic reaction mechanism of acetylcholinesterase determined by Born– Oppenheimer ab initio QM/MM molecular dynamics simulations. The journal of physical chemistry B, 114(26), 8817-8825. https://doi.org/10.1021/jp104258d

[7] Mehta, M., Adem, A., & Sabbagh, M. (2012). New Acetylcholinesterase Inhibitors for Alzheimer' s Disease. International Journal of Alzheimer's disease, 2012(1), 728983. https://doi.org/10.1155/2012/728983
[8] Pérez-Aguilar, B., Marquardt, J. U., Muñoz-Delgado, E., López-Durán, R. M., Gutiérrez-Ruiz, M. C., GomezQuiroz, L. E., & Gómez-Olivares, J. L. (2023). Changes in the acetylcholinesterase enzymatic activity in tumor development and progression. Cancers, 15(18), 4629. https://doi.org/10.3390/cancers15184629
[9] Ye, X., Zhang, C., Chen, Y., & Zhou, T. (2015). Upregulation of acetylcholinesterase mediated by p53 contributes to cisplatin-induced apoptosis in human breast cancer cell. Journal of Cancer, 6(1), 48. https://doi.org/10.7150/jca.10521
[10] Xi, H. J., Wu, R. P., Liu, J. J., Zhang, L. J., & Li, Z. S. (2015). Role of acetylcholinesterase in lung cancer. Thoracic cancer, 6(4), 390-398. https://doi.org/10.1111/1759-7714.12249
[11] Ahmed, G. Y., Osman, A. A., & Mukhtar, A. (2024). Acetylcholinesterase enzyme among cancer patients: a potential diagnostic and prognostic indicator a multicenter case–control study. Scientific Reports, 14(1), 5127. https://doi.org/10.1038/s41598-024-55604-6
[12] Whitaker, J. R., & Bernhard, R. A. (1972). Experiments for: an introduction to enzymology.
[13] Erum, S. (2010). Production of Peroxidases from Indigenous Fungal Strains and Their Application for Azo Dye Decolorization (Doctoral dissertation, Quaid-i-Azam University Islamabad, Pakistan).
[14] Segrest, J. P., & Jackson, R. L. (1972). Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In Methods in enzymology (Vol. 28, pp. 54-63). Academic Press.
[15] Li, S., Li, A. J., Zhao, J., Santillo, M. F., & Xia, M. (2022). Acetylcholinesterase inhibition assays for highthroughput screening. In High-Throughput Screening Assays in Toxicology (pp. 47-58). New York, NY: Springer US. https://doi.org/10.1007/978-1-0716-2213-1_6
[16] Sinha, S. N., Ungarala, R., Kumar, D., Sangaraju, R., & Kumar, S. (2022). A novel RP-HPLC method for quantification of cholinesterase activity in human blood: An application for assessing organophosphate and carbamate insecticide exposure. Plos one, 17(12), e0279287. https://doi.org/10.1371/journal.pone.0279287
[17] Mohammad, S. A., Maher, F. T., & Slman, S. A. (2022). Purification and Isolation of Choline Esterase and Study of Some Chemical Variants for Diabetics. Biochemical & Cellular Archives, 22(1). https://connectjournals.com/03896.2022.22.2215
[18] Duranay, S., Anil, S., Hasbal, G., & Özsoy, N. (2019). Purification of acethylcholinesterase from the mollusc Mytilus galloprovincialis Lam. and investigation of its kinetic properties. J Res Pharm, 23(4), 759-769. https://doi.org/10.12991/jrp.2019.185
[19] Carroll, R. T., Grimm, J. L., Hepburn, T. W., & Emmerling, M. R. (1995). Purification of acetylcholinesterase by tacrine affinity chromatography. Protein expression and purification, 6(4), 389-393. https://doi.org/10.1006/prep.1995.1051
[20] Ruiz-Espejo, F., Cabezas-Herrera, J., Illana, J., Campoy, F. J., Muñoz-Delgado, E., & Vidal, C. J. (2003). Breast cancer metastasis alters acetylcholinesterase activity and the composition of enzyme forms in axillary lymph nodes. Breast cancer research and treatment, 80, 105-114. https://doi.org/10.1023/A:1024461108704
[21] Ye, X., Zhang, C., Chen, Y., & Zhou, T. (2015). Upregulation of acetylcholinesterase mediated by p53 contributes to cisplatin-induced apoptosis in human breast cancer cell. Journal of Cancer, 6(1), 48. https://doi:10.7150/jca.10521
[22] Sagane, Y., Nakagawa, T., Yamamoto, K., Michikawa, S., Oguri, S., & Momonoki, Y. S. (2005). Molecular characterization of maize acetylcholinesterase. A novel enzyme family in the plant kingdom. Plant Physiology, 138(3), 1359-1371. https://doi.org/10.1104/pp.105.062927
[23] Caeiro, L., Novais, F., Saldanha, C., e Melo, T. P., Canhão, P., & Ferro, J. M. (2021). The role of acetylcholinesterase and butyrylcholinesterase activity in the development of delirium in acute stroke. Cerebral Circulation-Cognition and Behavior, 2, 100017. https://doi:10.1016/j.cccb.2021.100017
[24] Asen, N. D., Okagu, O. D., Udenigwe, C. C., & Aluko, R. E. (2022). In vitro inhibition of acetylcholinesterase activity by yellow field pea (Pisum sativum) protein-derived peptides as revealed by kinetics and molecular docking. Frontiers in Nutrition, 9, 1021893. https://doi:10.3389/fnut.2022.1021893
[25] Gerczei, T., & Pattison, S. (2014). Biochemistry Laboratory Manual for Undergraduates: An Inquiry-based Approach. Berlin: De Gruyter Open. ISBN: 978-3-11-041132-4