International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Isolation and Purification of Acetylcolinesterases (AchE) from Blood of Thalassimic Patient and Kinetic Studies for Purified Enzyme

Nazar Ahmed Naji[a*], Sayran Sattar Saleh[b], Galawesh Noori Taher[c]

[a]*Department of Chemistry / College of Science / Tikrit University, Iraq*

[b]*Department of Chemistry / College of Science / Kirkuk University, Iraq*

[c]*Department of Nursing/ Instituted Kirkuk Technical/ Northern Technical University, Iraq*

[a]*Email: nan.abed@yahoo.com,* [b]*Email: Sayran_university@yahoo,* [c]*Email: galawesh@yahoo.com*

Abstract

In this research to isolate and study the properties of the Acetylcolinesterase (Ec 3 .1.1.7) enzyme in the blood of a patient suffering from β- thalassemia major and study kinetic studies for purified AchE .The study involves taking(5ml) of crude serum of thalassemic patients and subjected to a series of purification processes including : precipitation by ammonium sulfate , filtration by centrifugation radiator , dialysis in presence of Tris-HCL,separation using the technology of gel and then Estimation approximate Molecular Weight of Partially Purified AchE using gel filtration technique and sodium dodecyl sulfate (SDS)-pag polyacrylamid gel electrophoresist.The result of the study indicates that AchE has an approximat molecular weight of 173720 Dalton.The kinetics of the enzyme were studied and the results showed that the maximum velocity was 7.8 µmol/min/mol and the K_m was 0.1 M whil the optimum temperature of the enzyme was(37℃)and the optimum pH was(7.4) .

Keywords : Acetylcolinesterase ; β- thalassemia ; Purification; Dialysis.

---

* Corresponding author.

1. Introduction

Thalassemia Syndrome is among the most common genetic disorders of hemoglobin Synthesis worldwide [1]. Ithas occurring more frequently in the Mediterranean Indian subcontinents, Africa and South East Asia, with a particular high occurrence in Arab world that including Iraq [2]. This disorder associated with reduction synthesis of alpha (α) or beta globin (β) poly peptide chains caused to chronic hemolytic anemia since birth [3].

β-thalassemia major is characterized by severe hemolytic anemia that entails regular blood transfusion. Cholinesterases, is a family of enzymes that present in the central nervous system , particuLarly in nervous tissue , muscle and red cells which catalyzing the hydrolysis of the neurotransmitter to choline and acetic acid [4].The reaction is necessary to allow a cholinergic neuron to return to its resting after activation. Cholinesterases is one of among many important enzymes that needed for the proper functing of the nervous system of human. It involves two types:

Acetylcholinesterase (AchE) , and pseudocholinesterase (PchE), the difference between the two types of (chE) is there relative preferences for substrates that AchE ehydrolyzes acetylcholin faster , while pchE hydrolyzes butyrylcholine faster [5].

Acetylcolinesterase (AchE), (Ec3 .1.1.7), the primary or major cholinesterase in the body that responsible for the hydrolytic metabolism of the neurotransmitter acetylcholine (Ach) to choline, and acetate .(AchE) is typically synthesized in nerve , muscle and certain hematopoietic cells , also found to be at neuromuscular junction and cholinergic synapses and also present in the membrance of erythrocytes and other organs such as liver and muscles [6]. AchE plays an important role in apoptosome formation, and it is translocated into the nucleus, that may be an essential event during apoptosis [7].

2. Materials and Methods

A- Isolation and Purification of Acetylcolinesterases Enzyme

The blood sample size of 7 ml is taken from thalassemic patient and allowed at 37℃ for 30 minutes and then centrifuged at 2500 xg for 15 min.The serum is separated and stored at -20℃ until its analysis. The analysis includes a series of purification steps including: salting out ,which is conduct at 4℃ by gradually adding of ammonium sulfate to the serum (70%)with a constant stirring by the magnetic stirrer until reaching the saturation point ,then the separation is done by the centrifugation radiator to move to the next step and the amount of protein and the effectiveness of the enzyme are all estimated[8]. Dialysis,the other process, is conductedat 4℃ after dissolving the precipitate yielding the least possible amount of distilled water and then it is transferred into a special plastic bag for dialysis . The plastic bag is transferred into a jar filled with (o.1 M Tris-HCL) pH (7.6).The plastic bag is completely covered with Tris –HCL to avoid any shrinkage and subsequently is losing its permeability .The external Tris –HCL is changed every four hours during the dialysis process until the enzyme solution volume inside the plastic bag reaches a constant one .The final size of the sample , the amount of protein and the effectiveness of the enzyme are all estimated after dialysis[9] gel – filtration chromatography , is a chromatographic that molecules in solution are separated by their size and molecular weight [10].

Elution of the proteinaceous materials was carried out at a flow rate (24) ml / hour, using phosphate buffer solution , as eluant, The fractions were collected , approximately 2 mL of fractions were collected each 5 min. The protein concentration and AchE activity of each fraction was measured.The proteinaceous compounds in each fraction collected were detected by following the absorbance at wave length (280) nm using UV/Visible Spectrophotometer,whie the follow –up of AchE enzyme , that we are looking for , occurs by measuring the activity in all the separated parts [11].

Gel filtration technique is used to Estimation approximate Molecular Weight of Partially Purified enzyme .Number of compounds (standards).Known as molecular weight (204-2000000 Dalton), are passing through the separation column with the dimension (1.5 cm × 50 cm)filled by the gel type Sephadex G- 200 to calculate the elution volume for each compound ,and then attended standard curve by drawing logarithm molecular wight against elution volume.

Approximate Molecular Weight of AchE enzyme is found from this curve [12] SDS-PAGE poly acrylamide gel electrophoresis is the most widely used method for qualitatively analyzing protein mixtures, by which a mixture of charged molecules is separated and migrated according to size under the influence of an electric filed at the pH used polyacrylamide gel electrophoresis in a medium containing sodium dodeyl sulfate (SDS – PAGE) , is commonly used [13].

To determine the molecular weight of AchE , the relative distance of the standard proteins are determined and a graph of log molecular weight vs. distance is plotted .

The distance of the AchE is then is determinedand the log molecular weight (and hence molecular weight) is determined from this graph.

B-Estimation of the AchE Activity

AChE activity was measured in human serum using the modified Ellman method based on a colorimetric procedure [14].

As follows: (50 µl) of DTNB solution (0.001 M) was added to 2.25 ml of phosphate buffer solution pH=7.3, 0.2 M, then (10µl) of serum was added, mixed well and (2 ml) of the mixture was transferred to a measuring cell (3 mm), then (34 µl) of ASChI (0.06M) was added, the change in absorbency was measured before and after adding the substrate at (430 nm) for (3 min).

The enzyme activity was calculated as the concentration in µmole of the substrate hydrolyzed to each (ml) of sample in (3 minute) and expressed as (µmole/ 3 min/ml). One unit of activity is defined as the amount of enzyme required to convert 1 µmol of ASChI to the product under assay condition.

C- Estimation of the Total Protein

The amount of total protein is determined by the modified Lowry's Method [15].Using bovine serum albumin

(BSA) as a standard. The absorbance of blue colored complex was monitored at 620nm.

D- Kinetic studies for AchE was purified from serum of thalassemic patient[16].

1-Effect of substrate concentration [S] (S-Acetyl thiocholin iodide)

The kinetic parameters Michaelis– Menten constant (km) and maximum velocity (Vmax), were estimated by assaying the enzyme activity using varying substrates concentrations (acetylthiocholine iodide, 0.02,0.04,0.05,0.06,0.08,0.1,0.15,0.2)M were measured .

2- Effect of Enzyme concentration [E]

AchE enzymatic reaction was carried out using different concentration of enzyme by adding different volume of purified sample (0.01,0.022,0.034,0.046,0.058,0.06,0.062)ml distllied water was used to complete mixtures volume .

3- The effect of pH

In order to test the pH effect on AchE activity, the activity was carried out using different pH levels (6,6.5,7,7.5,8,8.5,9), 1M HCl was used to adjust the pH of phosphate buffer (0.2 M). The rate of the reaction was plotted versus the pH to determine the optimum pH for AchE reaction.

4- The effect of Temperature

The enzyme activity of AchE were done at temperature ranges (7 ,17 ,27 , 37 , 47,57 ,67) ℃, Enzymatic reaction proceeds by using phosphate buffer PH (7.4) and (2mM) substrate concentration . The rate of the reaction was plotted versus the Tm to determine the optimum Tm for AchE reaction.

3. Statistical analysis

Statistical analysis of data was performed using Graph Pad Prism version 6 for windows. All values were expressed as mean standard deviation of 3 observations.

4. Results

4.1 Results of the purified Enzyme

The purification of the AchE enzyme includes a series of steps summarized in Table 1 ,where we try to purify the AchE enzyme by gel - filtration technique .The draw which represent absorbance ,at a wavelength of 280 nm ,against elution volume using SephadexG-200 we noted a single peak of enzyme for thalassemic patients as shown in Figure 1

Table 1: Purification Step of AchE Enzyme.

Purification Step	Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Enzyme Activity U/ml	Total Activiy (U)*	Specific Activity U/mg	Fold of Purification	Yield 100%
Serum	**5**	**4.1**	**20.5**	**9.3**	**46**	**2.2**	**1**	**100%**
precipitation by Ammonium sulphate 70%	**3.5**	**0.71**	**2.5**	**7.4**	**25.9**	**10.4**	**4.7**	**56.3%**
Dialysis	**2.5**	**0.6**	**1.5**	**10.8**	**27**	**18**	**8.18**	**58.7 %**
Gel Filtration Sephadex G – 200	**3**	**0.006**	**0.198**	**10.7**	**32.1**	**162.1**	**73.6**	**69.8%**

(U)*One unit of activity is defined as the amount of enzyme required to convert 1 µmol of ASChI to the product under assay condition.

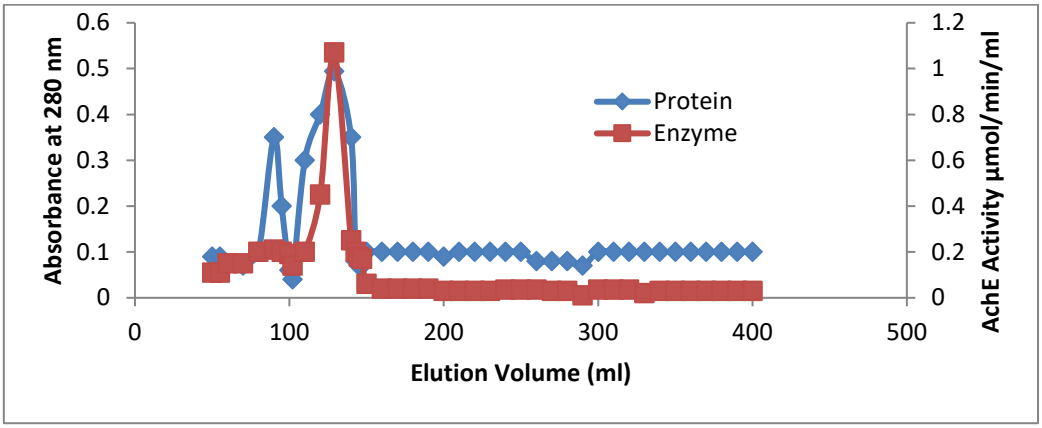

Figure 1: The Elution Profile of AchE From Thalassemic Patients by Using Gel Fltration Method on Sephadex G - 200

5. Estimation approximate Molecular Weight of the AchE

1-By gel- Filtration Technique:

As shown in figure 1, the elution volume of AchE solution, collected from gel filtration separation column ,is 129 ml and this volume correspond to a molecular weight of (173720)Da when using the standard curve shown in figure 2.

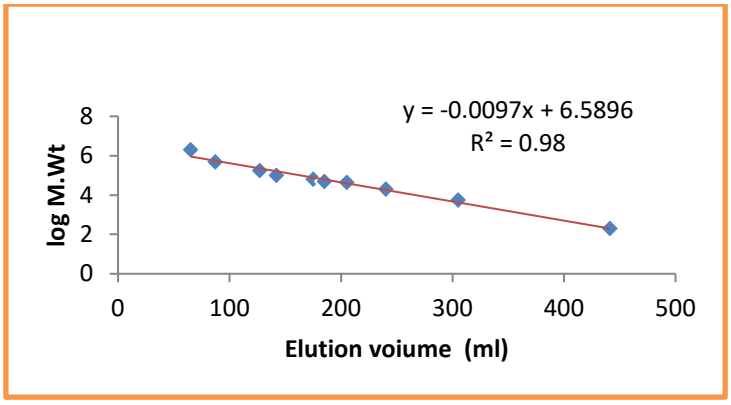

Figure 2: Calibration Curve for Estimation the Approximate Molecular Weight of Partially Purified AchE Using Gel Filtration on Sephadex G-200 .

2- By SDS-PAGE Gel Electrophoresis

As shown in figure 3, the pure enzyme obtained shows a single band when analyzed by acrylamide electrophoresis in the presence of 0.1% sodium dodecyl sulfate.

The relative distance of the AchE is 4.9 cm from the point of origin, was calculated by estimating the approximate molecular weight of the(67000) Dalton and using the standard curve shown in figure 4 .

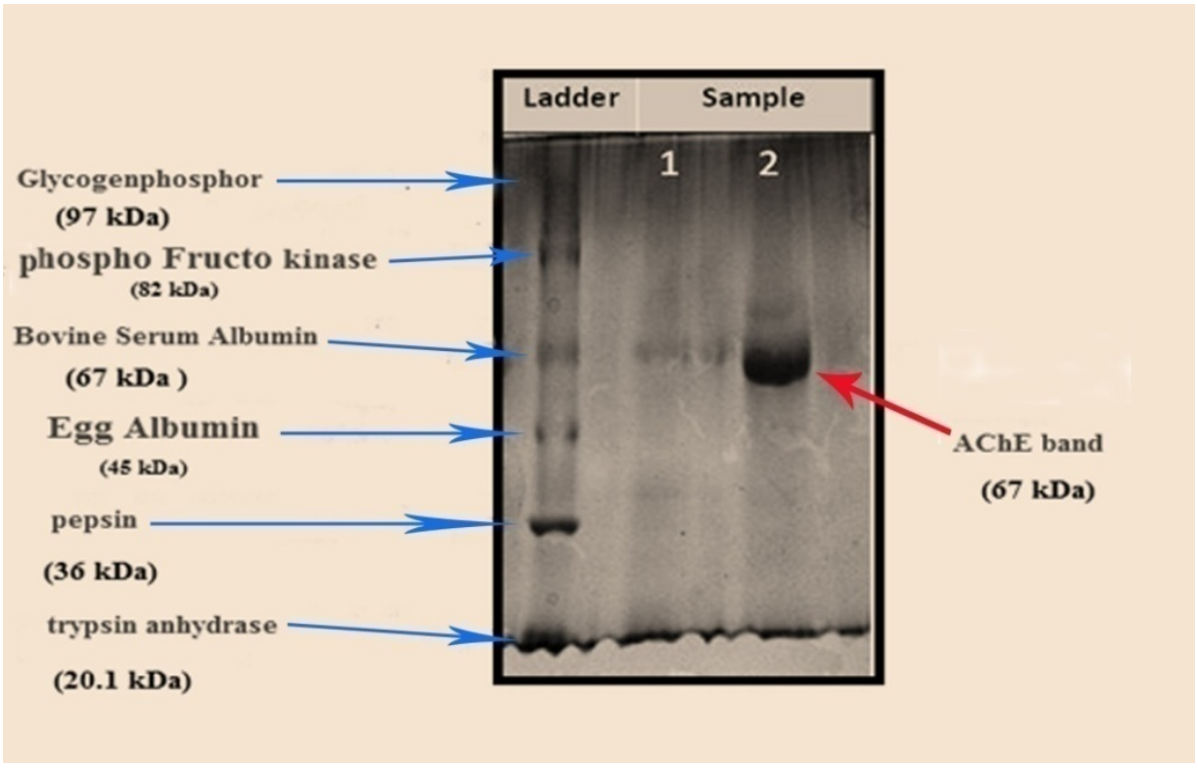

Figure 3: SDS-PAGE Analysis Bands of the Purified AchE and the Standard Proteins

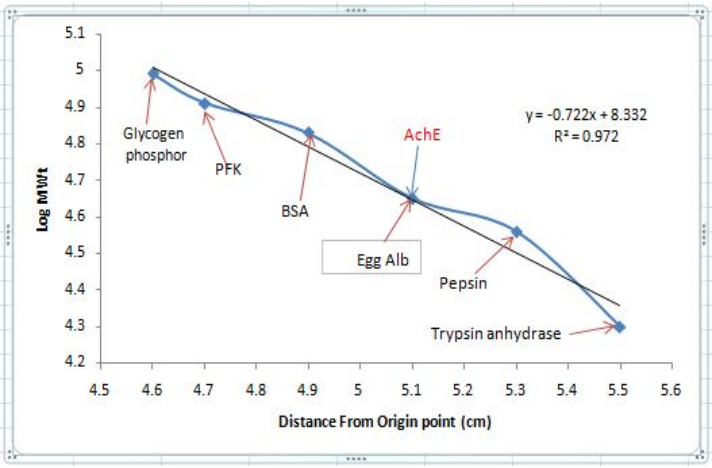

Figure 4: Calibration Curve Used to Determine the Approximate Molecular Weight of AchE Using SDS-Page Polyacrylamid

6. Electrophoresis

B- Results of - Kinetic studies for AchE

1- **Effect of[S] with Determination of Km and Vmax**

It was found that the velocity of AchE reaction was increased depending on the substrate concentration until reaching the (V_{max}) at the optimum concentration Figure shows that the enzyme saturation of substrate was at the concentration of (0.35 M)of (S-Acetyl thiocholine iodide) .Using the drawing of (LineWeaver-Burk plot) found that the value of V_{max} and K_m is (7.8 µmol/min/ml)and (o.1 M) respectively ,as shown in fig (5)and (6).

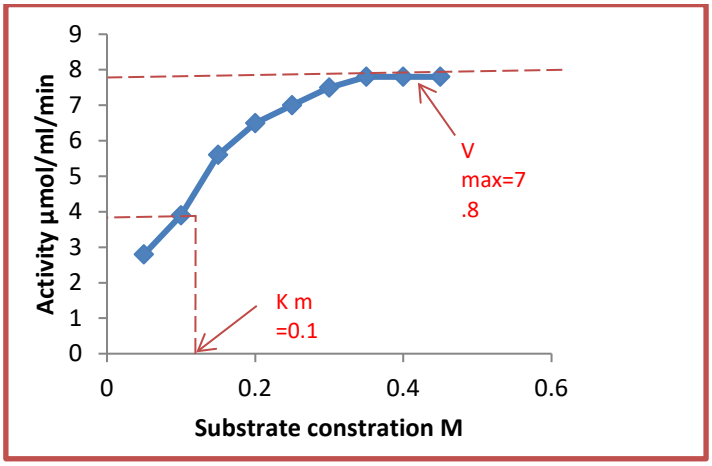

Figure 5: The effect of the substrate (S-Acetyl thiocholine iodide) concentration on the velocity of the purified AchE

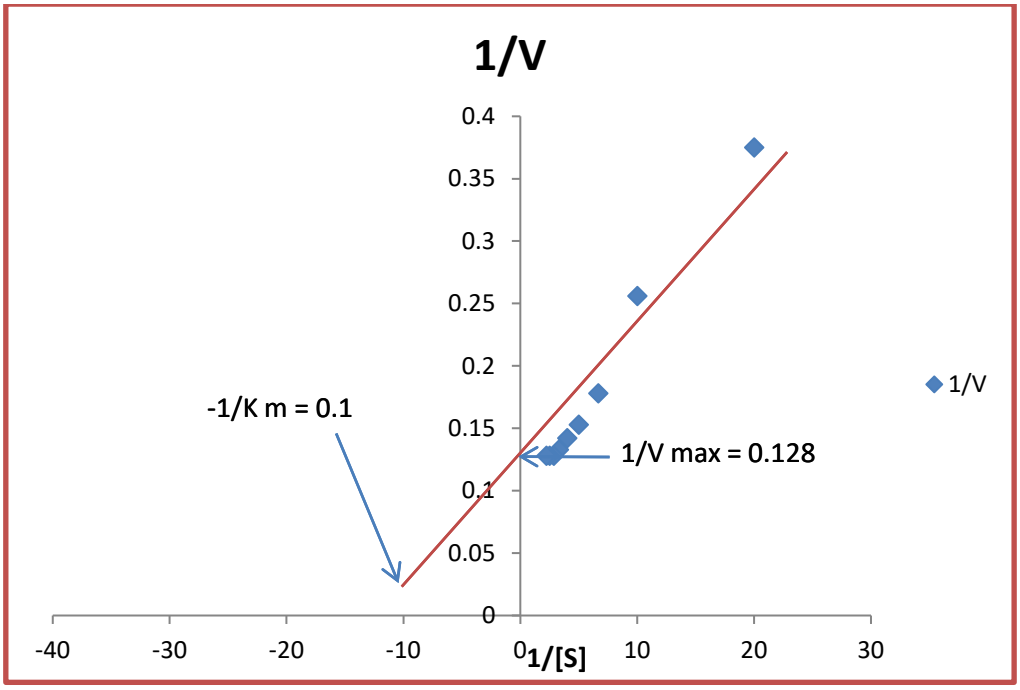

Figure 6: Lineweaver –Burk Plot for Michaelis -Menten Km measurement of AchE

2- Effect of[E] Concentrations on AchE Activity

It was observed that the enzyme velocity increased by increasing the concentration of the enzyme as shown in

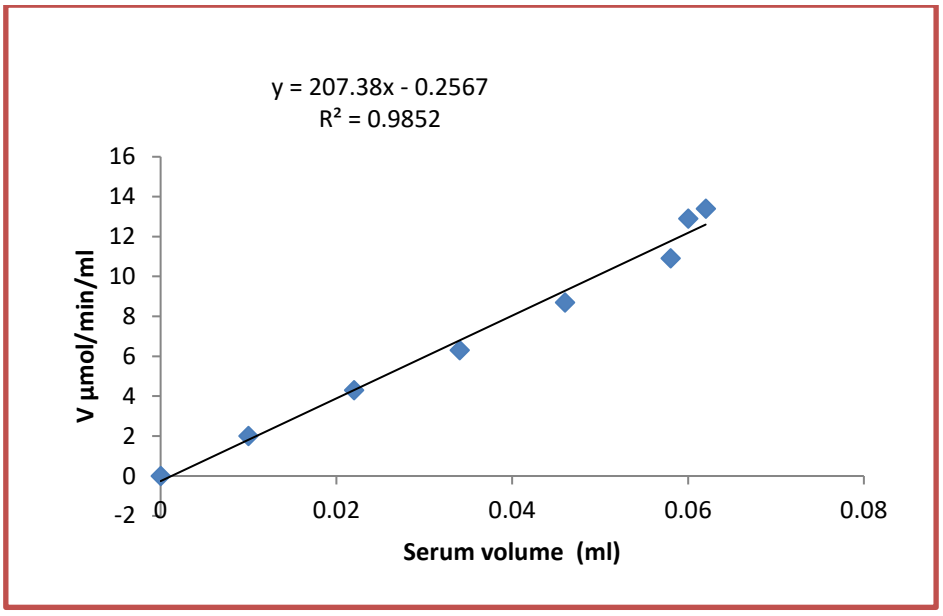

Figure 7: The Effect of the Enzyme Concentration on the Velocity of the Purified AchE

3-Effect of Temperature on the Rate of Reaction for AchE

The velocity of the reaction is increased by temperature increasing and reaches the maximum at 37℃ followed

by gradual loss in its activity after increasing temperature and decreasing in the rate of interaction due to denaturation. As shown in fig (7).

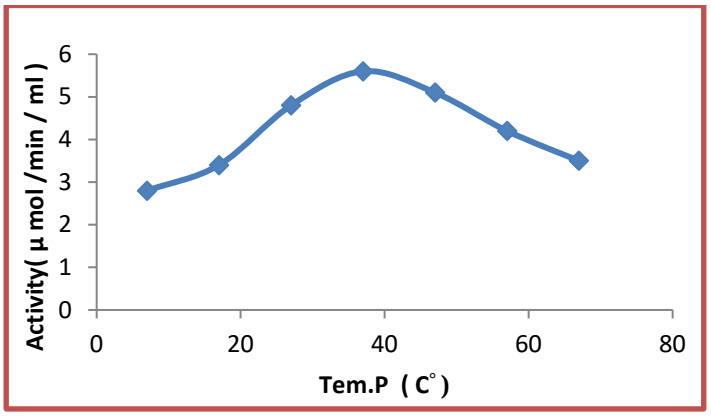

Figure 7: The effect of the Temperature on the velocity of the purified AchE

4- Effect of the pH on the Rate of Reaction for AchE

The effect of different degrees of pH on the velocity of AchE reaction was increased with increasing of the pH until it reached the Vmax on the optimum pH (7.4), as shown in fig (8).

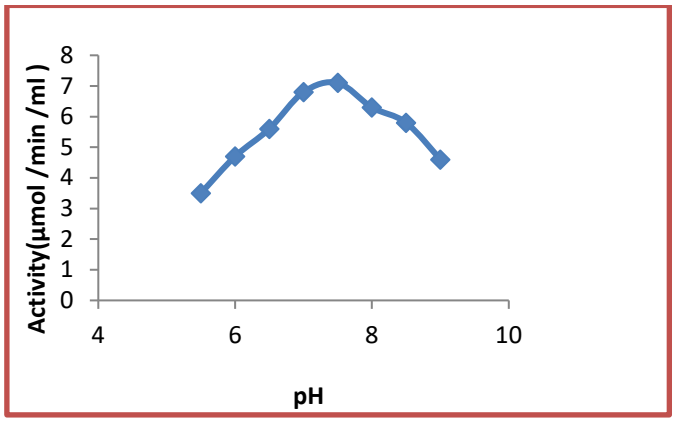

Figure 8: The effect of the pH on the velocity of the purified AchE

7. Discussion

The current study shows that the specific activity of the crude serum in patients sample equal to (2.2 IU/mg), after adding ammonium sulfate equal to (10.4 IU/mg), after dialysis equal to (18 IU/mg) and finally after gel filtration equal to (162.1 IU/mg), while the fold of purification were equal to (4.7,8.18 and 73.6) and the yield % equal to (56.3, 58.7 and 69.8) % respectively . These results can attributed to the fact that the presence of inflammatory factors in patients sample may affect the purifie AchE [17]. AchE is found in many cell types, in the nerves, muscles, central and peripheral tissues ,sensory fibers, amygdale, valleys and different regions of the body where there are jaunction points between cells [18] . And its vital importance has been isolated from many

tissues of the body where isolated from the human brain using the affinity chromatography and electrophoreses by researcher.Also,the enzyme was isolated from red blood cell walls using different resins where the percentage of yiled 23.5% and the Specific activity 9.22 IU/mg .[19]The enzyme isolated from red blood cells was used as avital indicator of lead neurotixinemia especially in people exposed to lead [4].

When using the standard curve shown in figure 2,it elutes as a single peak from gel- Filtration Technique column .These results are close to the findings of the previous studies of this enzyme [19].These results were approximate to the molecular weight of the enzyme isolated from red blood cell walls of 70,000 Dalton for each subunit isolated by using electrophoreses. . It also correlate with the study of Lelas F. B in [20]. Which obtained the molecular weight of the enzyme isolated from Cerebrospinal Fluid was 163,606 Dalton by using gel filtration.

The pure enzyme obtained shows a single band when analyzed by acrylamide electrophoresis in the presence 0f 10% sodium dodecyl sulfate The relative distance of the AchE is 4.9 cm from the point of origin ,was calculated by estimating the approximate molecular weight of the(67000) Dalton when using the standard curve shown in figure 4 .

These results were approximate to the molecular weight of the enzyme isolated from red blood cell walls of (70,000) Dalton for each subunit isolated by using electrophoreses [19]. Researchers also noted the molecular weight of the enzymes subunit using electrophoreses technique was about (60,000) Dalton [18].The researchers found that the enzyme is in different forms and that the shape in the human brain is a quadruple, so the approximate molecular weight of the enzyme ranges (240000-280000)Dalton [17].

Several investigators have given 260000 as the molecular weight of the solubilized enzyme from several source. In addition, detergents and other disaggregating agents have been used to demonstrate the existence of a 50000-80000- mol.wt. subunit, and evidence has been presented which indicates that the 260000-mol.wt. Species contains four to six subunits [21]. Other Results suggest that the form of the enzyme in the membrane is a monomer of molecular weight approx. 75000 and that multiple forms of the enzyme observed in solubilized preparations are aggregates of this monomer [22]

The hyperbolic curve obtained shows a positive correlation between the activity and the increase in the substrate concentration. The K_m value for any enzyme is a measure of its affinity towards substrate. In present study, the K_m value for serum thalassemic patients AChE was found to be 0.1 M. The results are in agreement with the study done by Firas T.M(2017) [23]which obtained that the value reported for the enzyme from diabetic patients 0.66M . The enzyme was found to be optimally active at pH 7.4 ,and it has maximum activity at 37°C .The results are in agreement with those of Vivek K .G and his colleagues [24,25] which obtained that This enzyme exhibited maximum temperuature at (37℃) and pH(7.4)from rat heart and brain tissue,and Firas T.M(2017) [23] which obtained that This enzyme exhibited maximum temperuature at (37℃) and PH(7.4) from diabetic patients. Finding not agreed with the results found by Ahmed. M and his colleagues [26] which obtained that This enzyme exhibited maximum temperuature at (45℃) and pH(8.5) from Bungarus sindanus snake venom .

8. Conclusion

1- AchE was isolated and partially purified from serum by gel filtration chromatography, the approximate molecular weight of the AchE was estimated by using gel filtration which was (173720)Dalton, and SDS-PAGE gel electrophoresis which was (67000)Dalton.

2- The kinetic studies on purified AchE , showed that this enzyme obeys Michaelis - Menton equation, it also showed that substrate concentration,enzyme concentration, pH and temperature had an effect on the rate of the reaction. The Kmand Vmax value for AchE was determined using the lineweaver - Burk which was (0.1 M),(7.8 µmol/min/ml)respectively .

9. Recommendations

1- Isolation of other enzyme such as heme oxygenase-1 which have antioxidant benefits and its role in β-thalassemia pathophysiology.

2- Further study to isolate the AchE in other body fluids in patients with β-thalassemia major .

References

[1]– Weatherall, D.J. (2010) . "The inherited diseases of hemoglobin are an emerging global health burden" J.; Blood, 115 .pp: 4331,36.

[2]- S. Sultan, S. M. Irfan, R. Zeeshan, J. Kaker, and A. Kidwai.(2014). “Zinc status and its correlation with basic parameters in transfusion dependent thalassemic patients: a Pakistani perspective,” Iranian Journal of Blood and Cancer, vol. 6, no. 3, pp. 113–118.

[3]- Nasiri, m.; Hosseini,S.H.;and shah Mohammadi,S.(2014).Mental health status in patiemts with Thalassemia major in Iran,Journal of Pediatrics Review office ,2 (1):55-56 .

[4]- Gupta, V.K, Pal,NJ. Siddiqi andB.Sharma,(2015).Acetylcholineserase From human erythrocytes as a surrogate biomarker of lead induced neurotoxicity .Enzyme Res, 10.1155/2015/370705.

[5]- Jean P.Dzoyem ,Victor Kuete ,Jacobus N.(2014).Toxicological Survey of African Medicinal plants , p 659-715.

[6]- Gupta, V.K, Pal,NJ. Siddiqi andB.Sharma,(2016).Carbofuran modulating functions of acetylcholinesterase from rat brain in vitro Adv. Biol,10.1155/2016/3760967.

[7]- Zhang ,X.J., Greenbreg,D. S.(2012). Acetyl cholinesterase involvement in apoptosis. Front Mol Neuro Sci.,5:40.

[8]- Moscoso,F.,Deive,FJ.,Esperanca,JS.and Rodriguez,A.2013.Pesticide Removal from Aqueous Solution by Adding Salting Out Agents Int.J.Mol,Sci.,14:20954- 20965 .

[9] - Plummer,T.D. (1978). "An Introdaction of Practical Biochemistry". 2nd ed .; Mcgraw – Hill Book. ; Co . V. K.pp: 48, 52, 174, 270, 274.

[10]- Garrett, Reginald H.; Grisham, Charles M. (2013). Biochemistry (5th ed.). Belmont, CA: Brooks/Cole, Cengage Learning. p. 108. ISBN 9781133106296.

[11]- Burgess, R . R .and Dustscher,M . P.(2013).Guid to protein(purification and characterization),Method in Enzymology.,(463):320 .

[12]- Berg, J. M., Tymoczko, J. L., Stryer, L. (2012). Biochemistry. 17th ed. W. H. Freeman and Company. New York. USA. pp. 68,69,138, 139, 145, 146.

[13]- A.DrabikA.Bodzoń-KułakowskaJ.Silberring Proteomic Profiling and Analytical Chemistry (Second Edition) 2016, Pages 115-143.

[14]- Ellman G. L.. Courtney K. p., Andres V. and Feather stone R. M., Bioche , Pharmacol, 1961, 7, 88-91

[15]- Lowry, O. H., Rosbrough, N.J.,Farr, A. L.and Randall, R.J.1951. Protein measurement with the afolin phenol reagent , J Biol. Chem.;193:265.

[16]- Murrqy,RK.,Bender,DA.,Botham,KM.,Kennelly,PJ.,Rodell,VW.,Weil, PA.2012.Harber Illustrated Biochemistry.30th ed.,The Mcgraw-Hill Companies,China.Chapter 8.p.209.

[17]- Xi, H. J., Wu, R. P., Liu, J. J., Zhang, L. J., Li, Z. S.Role of acetyl cholinesterase in lung cancer. Thorac Cancer, 2015; 6(4): 390-398.

[18]- Tripathi, A., Srivastava ,U.C. (2008). Acetylcholinesterase : Aversatile enzyme of enzyme of nervous system .Annals of Neurosclences ,Vol. No.4.

[19]- Kaya, H. B. Özcan, B. Şişecioğlu, M. Ozdemir, H.(2013). Purification of acetylcholinesterase by 9-Amino-1,2,3,4-tetrahydroacridine from human erythrocytes .Applied Biochemistry and Biotechnology.170(1):198–209.

[20]- Lelas F. B.(2017). Studying of SomeBiochemical Parameters in

Cerebrospinal Fluid for Children with Acute Lymphoblastic Leukemia and Hydrocephalus Ph. D. Thesis Submitted to College of Science. University of Mosul.

[21]- McIntosh, C. S. H. & Plummer, D. T. (1973) Biochem. J. 133, 655-665.

[22]- Levinson S. R. and Ellory J. C.(1974). The Molecular Form of Acetylcholinesterase as Determined by Iradiation Inactivation Biochem. J. 137, 123-125.

[23]- Firas T.M .(2017).Biochemical study of novel phosphate paracetamol prodrug invivo and invitro. Journal of university of Anbar for pure Science.,vol 11(2) ,p 12-29 .

[24]- Vivek Kumar Gupta, Abhishek Kumar1, Nikhat Jamal Siddiqi and Bechan Sharma.(2016)." Rat Brain Acetyl Cholinesterase as a Biomarker of Cadmium Inducecity" Journal of Toxicology Volume (1) Issue (1).

[25]- Vivek Kumar Gupta,Abhishek Kumar, Sarita H. Yadav, Rashmi Pandey and Bechan Sharma.(2017)." Acetylcholinesterase as a Biomarker of Arsenic Induced Cardiotoxicity in Mammals"DOI: 10.17311/sciintl..142.149.

[26]- Ahmed M , Latif N , Khan RA , Ahmad A , Rocha JBT , Mazzanti CM , Bagatini MD ,Morsch VM , Schetinger MRC .(2012). Enzymatic and biochemical characterization of Bungarus sindanus snake venom acetylcholinesterase.J Venomous Animals and Toxins including Tropical Diseases ., volume 18 | issue 2 | pages 236-243.

.