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# Detection of organophosphorus compounds using AChE PVC strip colorimetric method

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**ABSTRACT**

*A 65KDa acetylcholinesterase enzyme was purified from hypocotyls of P. vulgaris. The purification was done with the help of Sephadex G-100 and the ion exchange chromatography on DEAE- Sephacel column. The active fractions were pooled and subjected to SDS PAGE for the confirmation of molecular weight. The purified enzyme was immobilized onto PVC strip for colorimetric detection. Immobilization was confirmed by SEM and FTIR. The effect of pH, temperature, time of incubation and effect of substrate concentration were also studied for both free and immobilized enzyme. The PVC enzymatic strip was also evaluated. The developed method can be exploited for the detection of OP compounds which are responsible for causing environmental pollution.*

**Keywords:** Acetylcholinesterase, ATCI, DEAE-Sephacel, Sephadex G-100.

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## INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is most efficient enzyme which is involved in the functioning of Central Nervous System (CNS) [1]. Histochemical studies showed that this enzyme is present in nerves. May be present in membrane bound, secretory and soluble forms. AChE is a serine hydrolase which belongs to the family of type B carboxylesterase in higher eukaryotes. The catalytic activity of the enzyme is very high hydrolyzing approximately 25000 molecules of acetylcholine per second. During the process of neurotransmission acetylcholine (ACh) is released into the synaptic cleft from the nerves and binds to the ACh receptor which is present on the post synaptic membrane. AChE is also present on the post synaptic membrane where it helps in the termination of signal which is passed through nerves. AChE splits the neurotransmitter acetylcholine by hydrolysis at the cholinergic synapses. AChE is inhibited by organophosphorus (OP) compounds such as Malathion, Carbamates etc., [2]. Due to this inhibition there is hindrance in process of neurotransmission finally leading to paralysis and even death [3]. This enzyme has been purified from the insect species which includes *Musca domestica* [4], *Leptinotarsa decemlineata* [5], *Blattella germanica*[6] and *Helicoverpa armigera* [7].Purified from plants such as *Zea mays* (Maize) [8] and from fresh electric organ tissue of Electric Eel [9]. The purification process of AChE enzyme involves the use of different gel filtration and chromatographic techniques. As the OP compounds are inhibiting the enzyme AChE, it is very necessary to develop the biosensors which are more accurate and reliable. The purified enzyme can be used for the development of the biosensors with the use of the nanomaterials for the betterment of the onsite detection of the OP compounds.

In this paper the AChE is purified from *P. vulgaris* by using gel filtration techniques. Then the purified enzyme is immobilized on PVC strip for the colorimetric detection method. Then the properties of free & immobilized AChE enzyme are also studied.

## MATERIALS AND METHODS

*2.1 Instruments and equipments*

UV spectrophotometer (Shimadzu corporation, Japan), digital pH meter (EUTECH), refrigerator (LG), microwave oven (LG),Sanyo MLR 350 incubator, rotatory incubator shaker (HICON), magnetic stirrer (HICON), refrigerated centrifuge (SIGMA),deep freezer (Voltas), Auto pipettes (Agile), Sephadex G-100 was purchased from MP bio-medicals (France). DEAE-Sepharose purchased from Sigma Chemical Co., USA.PVC Strip from local market and all other chemicals of AR grade.

*2.2 Plant Material*

In this study, seeds of *P. vulgaris* (*Dwarf bean*) were obtained from the local market, Rohtak. Mature dry seeds were germinated and hypocotyls were used as explants.

*2.2.1 Sterilization and Seed Germination of P. vulgaris*

The seeds were surface sterilized with 70 % ethanol for 1 minute and then with 0.1% mercuric chloride containing 0.01 % Tween 20 for 3 minutes. They were then washed with sterile water (at least five changes) for 15 minutes. The sterile seeds were germinated on MS basal medium (Murashige and Skoog 1962) without sucrose and then solidified with 0.8 % agar in 175 ml tissue culture vessels (Sigma- Aldrich). The seeds were incubated at 25°C, 12 h photoperiod and 100 µmole $m^{-2}s^{-1}$ light intensity in Sanyo MLR 350 incubator. All subsequent cultures were incubated under the same conditions unless specified.

*2.2.2 Extraction and Purification*

The *P. vulgaris* seedlings were harvested after 8 days and the hypocotyls were excised as explant. The above excised explants were homogenized in potassium phosphate buffer (10mM, pH 7) supplemented with EDTA (10mM) and ammonium sulphate in the ratio of 1:4 (W/V). After homogenization the slurry obtained was put in dark for 2 hours and the temperature maintained 10ºC. Then the homogenized slurry was filtered using a nylon net having pore size 60µm. The resulting extract was centrifuged at 30,000 x g in refrigerated centrifuge maintained at 4ºC for 20 minutes. This procedure was repeated and supernatant was collected. Supernatant from all the centrifuge tubes were then pooled together. Then the above pooled supernatant are saturated with the help of ammonium sulphate (70-80%) and left overnight in cold in order to precipitate the protein. The above material was subjected to centrifugation at 40,000 x g for 30 minutes (4ºC) and resuspended in 5ml of 10mM PBS (phosphate buffer saline) pH 7.5. Then it was subjected to dialysis using the PBS (pH 7.5). The precipitates were formed during dialysis these were removed by centrifugation (6000 x g, 30 minutes, 4ºC) and supernatant was collected.

*2.3 Gel Filtration*

Gel filtration was done using Sephadex G-100 and DEAE-Sephacel column chromatography.

*2.4 Determining the Activity and Protein Content*

*2.4.1 Assay for AChE*

The purified enzyme obtained above was diluted by mixing 100 µL of AChE in 200 µL of the double distilled water (DDW). Then ATCI (12.5mM) mixed with sodium phosphate buffer (100mM, pH 7) followed by incubating at 30ºC for 5 minutes, 200 µL of this was added to the diluted enzyme and again incubated at 30ºC for 90 minutes. This reaction mixture taken into fresh vial and 100 µL of DTNB (100mM sodium phosphate buffer, pH 7) was added. Now the final volume was raised to 2 mL by adding 1400 µL of 100 mM sodium phosphate buffer pH 7. The spectrophotometric readings were taken at $\Delta A_{412}$ after 5 minutes along with the blank (reaction mixture without enzyme and the total volume compensated with the sodium phosphate buffer).Thionitrobenzoic Acid imparts yellow color and show activity at 412nm (Fig. 1).

*2.4.2 Protein Determination*

Lowry [10] method was used for the determination of total protein content of the enzyme purified. Bovine Serum Albumin (BSA) was used as standard. The purified protein reacts with Folin Ciocalteau (FC) Reagent and imparts a colored complex. FC is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric *in vitro* assay. This color formation is a result of reaction between the peptide part of protein with Copper (Alkaline) followed by reduction reaction in which tyrosine and tryptophan reduces phosphomolybdate.

*2.4.3 Polyacrylamide gel electrophoresis of purified AChE*

The gel electrophoresis was done for purified enzyme for the determination of molecular weight.

*2.5 Preparation of Reusable AChE Immobilized PVC Strip*

The rectangular strips of 10 × 1 cm size were cut from a PVC plastic sheet (thickness 0.5mm). The purified AChE was immobilized covalently onto activated PVC strip surface using our earlier reported method [11]. To confirm immobilization of enzymes, the PVC strip was studied by scanning electron microscope at Department of Chemistry, M. D. University, Rohtak.

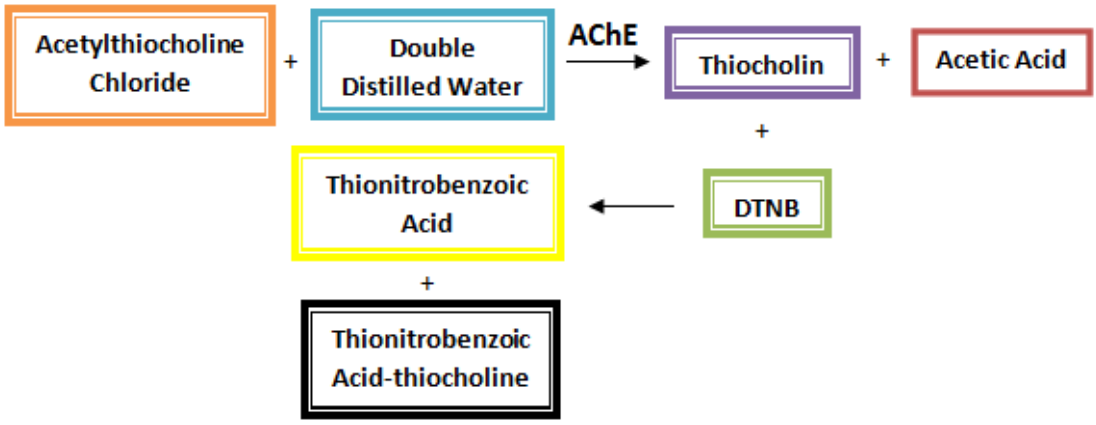


**Fig. 1 Chemical Reaction Involves**

*2.6 Kinetic properties of free and immobilized AChE enzyme*

For the determination of optimum pH conditions of the free enzyme, pH of reaction buffer was varied from 6-10 using three buffers: sodium succinate (5.0 and 5.5), sodium phosphate (pH 6-7.5) and borate buffer (pH 8.0 to 10.0) each at a final concentration of 50 mM. *Effect of incubation temperature***:** For the determination of incubation temperature for optimum activity, the reaction mixture was incubated and subjected to different temperatures ranging from 20$^{o}$C to 50$^{o}$C at an interval of 5$^{o}$C. *Effect of time of incubation:*Incubation time was studied from 2 min to 12 min with regular interval of 2 min. *Effect of substrate concentration:*The effect of substrate (ATCI) concentration on the enzyme activity was studied up to 600 µM in the reaction mixture at an interval of 50 µM. *Determination of $K_m$ and $V_{max}$:*$K_m$ and $V_{max}$ values for free AChE enzyme were determined from Lineweaver-Burk plot between reciprocal of substrate concentration [1/S] and reciprocal of initial velocity of the reaction [1/V].

*2.7 Evaluation of Enzyme immobilized PVC Strip*

To evaluate the enzyme immobilized PVC Strip the linearity, detection limit, recovery, precision and accuracy were studied.

## RESULTS AND DISCUSSION

*3.1 Preparation of AChE from P. vulgaris (Dwarf bean) seedlings*

The crude enzyme was extracted from 250gms of frozen *P. vulgaris* hypocotyls and showed an activity of 0.87 µmolesthiocholine/mL/min.

*3.2 Purification of AChE from the P. vulgaris hypocotyls*

The crude enzyme AChE was subjected to purification by ammonium sulfate precipitation (0-80%), Sephadex G-100 columnchromatography and ion exchanger chromatography on DEAE-Sephacel column. 0-80% ammonium sulphate of crude enzyme gave 7.72-fold purification with 69.88% yield (Table 1). Then dissolved ammonium sulphate precipitate subjected to Sephadex G-100 column, five peaks of enzyme activity were obtained (Fig. 2). First and secondminor peaks comprised of fractions 5 to 8 and 8 to 11 respectively, which showed some activity of enzyme. The third major peak, comprised of fractions 16 to 23showed maximum activity (Fig. 2). The fourth and fifth minor peak, were of fractions 28 to 30 and 30 to 33 respectively, which showed activity of enzyme. There were seven peaks of protein. Maximum protein peak were between fractions 18 to 21, whereas six minor peaks were also observed for protein from fractions 2 to 4, 4 to 7, 8 to 10, 14 to 16, 28 to 30 and 30 to 34. Then these active fractions from Sephadex G-100 were pooled, which showed overall 33.08-fold purification with 18.58% yield (Table 1). The pooled fractions from Sephadex G-100 were subjected to DEAE-Sephacel column; it showed six minor peaks and one major peak of enzyme activity. Fractions 7 to 12, 12 to 17, 19 to 22, 22 to 24, 32 to 34 and 34 to 36 comprised of minor peaks, while the major peak was noticed from 25 to 30. Maximum protein was obtained in a single major peak from fractions 25 to 30 (Fig. 3).The active fractions which showed high specific activity from DEAE-Sephacel were pooled and treated as purified enzyme. The enzyme was finally purified by 39.37-fold purification with 17.93% yield. The purified enzyme had a specific activity of 31.54 U/mg (Table 1).

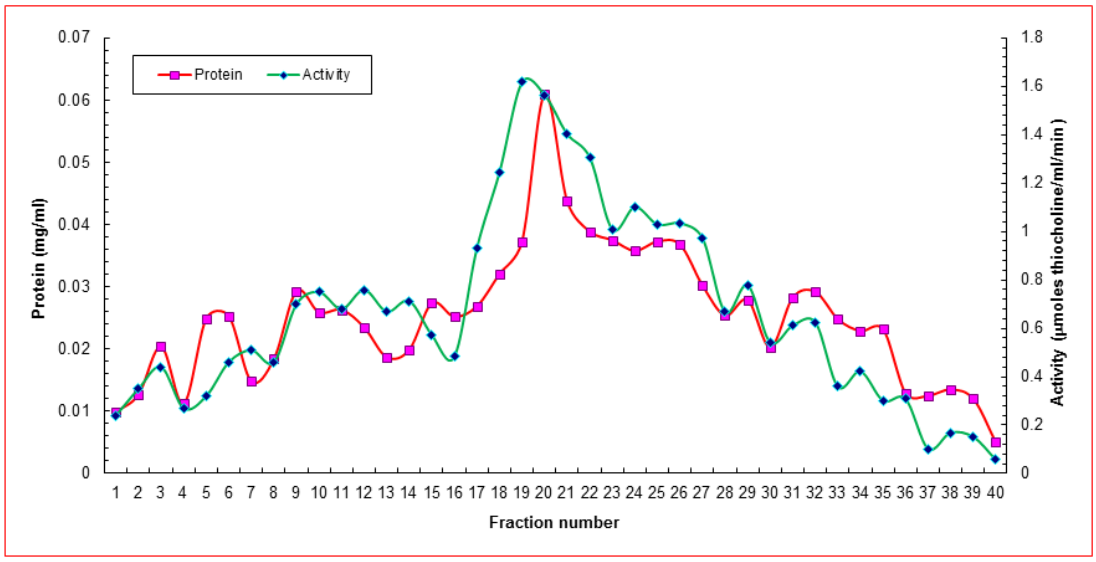


Fig.2. Gel filtration of 0-80% $(NH_4)_2SO_4$ fraction of *P. vulgaris* seedlings acetylcholinestrase) on Sephadex G-100 column chromatography [size (2.0 cm ×20 cm)].One enzyme unit activity is defined as 1 µmoles of thiolcholine formed/mL/min at pH 7.0 and 30°C. Activity was assayed using standard assay procedure, while protein was estimated by Lowry method.

**Table 1: Purification of AChE from *P. vulgaris***

| Fraction | Total volume (ml) | Protein (mg/ml) | Activity (Unit/ml) | Specific activity (Unit/mg) | Total protein (mg) | Total activity (Units) | Purification fold | % yield |
|---|---|---|---|---|---|---|---|---|
| **Crude** | 150 | 1.085 | 0.87 | 0.801 | 162.75 | 130.5 | 1 | 100 |
| **$(NH_4)_2SO_4$ precipitate** | 12 | 1.227 | 7.6 | 6.19 | 14.72 | 91.2 | 7.72 | 69.88 |
| **Sephadex G-100** | 15 | 0.061 | 1.617 | 26.50 | 0.91 | 24.25 | 33.08 | 18.58 |
| **DEAE-Sephacel** | 14 | 0.053 | 1.672 | 31.54 | 0.74 | 23.40 | 39.37 | 17.93 |

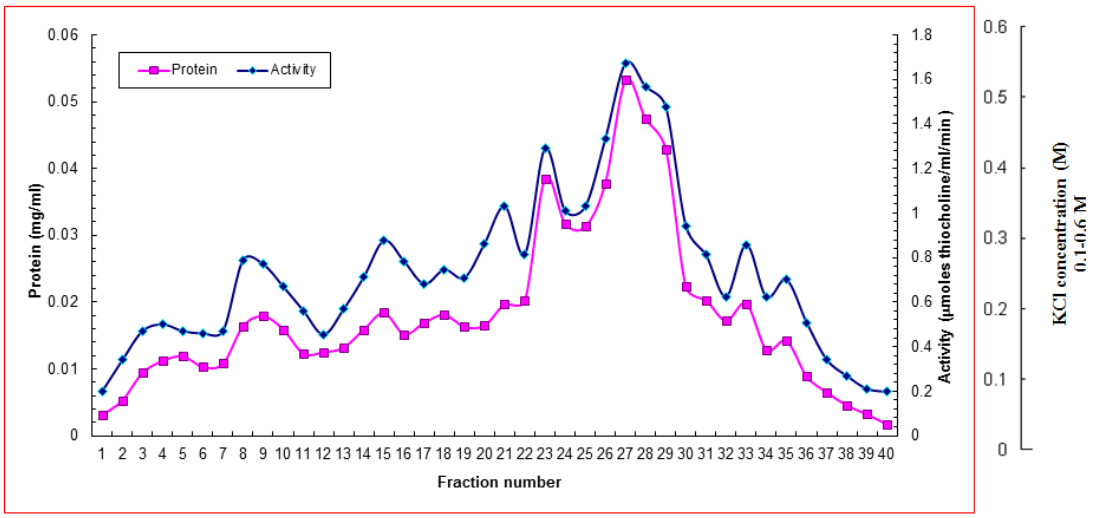


Fig.3. Ion-exchange chromatography of pooled fractions of Sephadex G-100 of *P. vulgaris* seedlings acetylcholinestrase on DEAE-Sephacel column in linear gradient of 0.1 M to 0.6 M KCl in 0.05 M potassium phosphate buffer (pH 7.0). Activity was assayed using standard assay procedure and the protein was estimated using Lowry method.

Also, AChE was purified from *MacroptiliumatropurpureumUrb*. With the help of Sephadex G-200, ion-exchange column, Poros HS/20 column and gel Superdex 200 HR showing 134.8-fold purification along with 33.4% yield and specific activity of 0.957 U/mg [12] from the infective juveniles of the heterorhabditid nematode strain using ammonium sulfate precipitation, gel filtration on Sephacryl S-200 and DEAE-Sepharose showing 17.5-fold purification along with 34% yield and having specific activity of 1378.1 U/mg protein [13], heads of adult *Musca domestica*a Korean housefly strain (KNIH) by affinity chromatography on trimethyl (m-aminophenyl) ammonium chloride resin showing 400-fold along with 8.8% yield and having specific activity of 290 U/mg [14],erythrocyte of mouse by affinity chromatography in edrophonium-Sepharose showing 4800-fold purification and with specific

activity of 7700 U/mg [15],Japanese quail brain with the help of affinity chromatography on a concanavalin A–Sepharose and edrophonium–Sepharose showing 10416-fold purification, with yield of 4.3% and a specific activity of 2500 U/mg protein [16].

*3.3 Testing the homogeneity of purified enzyme*

The purified enzyme was subjected to native PAGE it showed an enzyme as a single band. This protein band showed that the enzyme is having the molecular weight of 65 KDa when compared to the standard ladder. After this the purified enzyme was subjected to SDS-PAGE and showed the two bands one of 32 KDa and the other is 33 KDa when compared with the ladder. This confirmed that the purified enzyme is a dimer.

*3.4 $M_r$ and subunit $M_r$ Determination by PAGE and SDS-PAGE*

The molecular weight of purified enzyme was determined by plotting a graph between Log $M_r$ Vs $R_f$values. The molecular weight of the subunits is determined in same manner by SDS PAGE. The enzyme has Mr 65KDa (Fig. 4) with native PAGE and subunits 32 KDa and 33 KDa with SDS PAGE (Fig. 5).

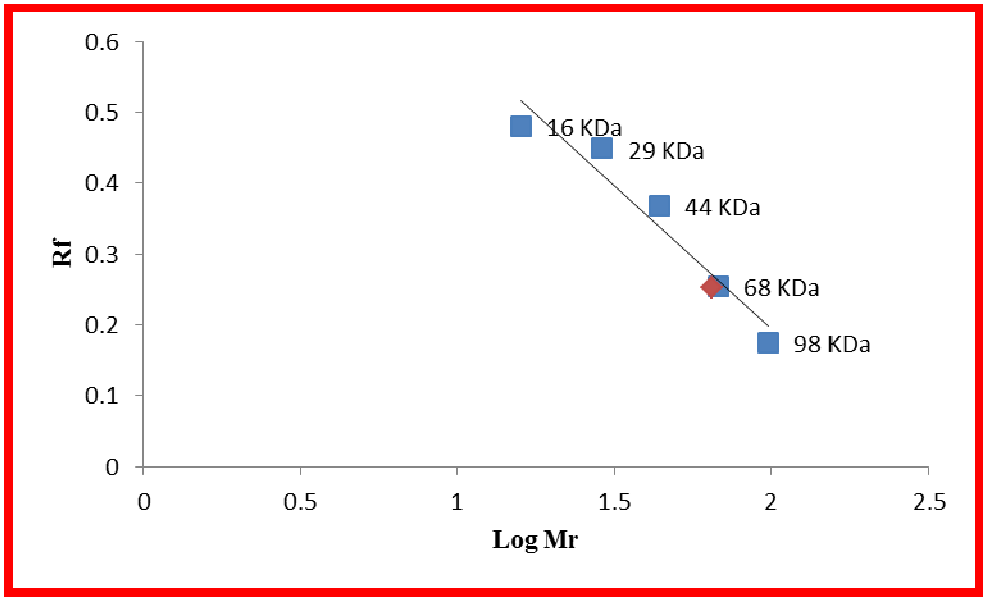


**Fig.4. Apparent molecular weight determination by PAGE of AChE purified from *P. vulgaris* seedlings**

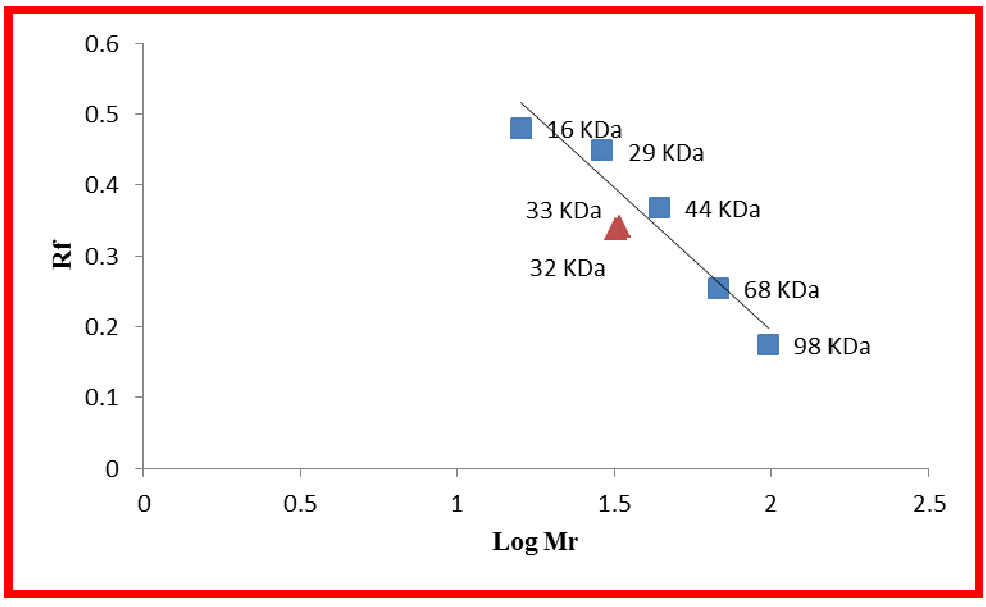


**Fig.5.Apparent molecular weight determination by SDS-PAGE of AChE purified from *P. vulgaris* seedlings**

The Mr. of present enzyme (AChE) is lower than adult rat brain (328 KDa) [17], fruit fly *(Drosophila melanogaster)* (180 KDa) [18], *Eurytemoraaffinis* (140 KDa) [19], mouse erythrocyte (66 KDa) [15], and *Bactroceradorsalis (Hendel)* (116 KDa) [20].

*3.5 SEM Images PVC strip without enzyme and with enzyme*

The morphology of bare PVC and AChE immobilized onto PVC Strip was characterized by SEM studies.

The SEM image of the bare PVC strip (Fig.6a) showed a smooth and featureless morphology. However, the covalent immobilization of purified AChE modified the surface of PVC strip (Fig.6b) showed the difference in morphology.

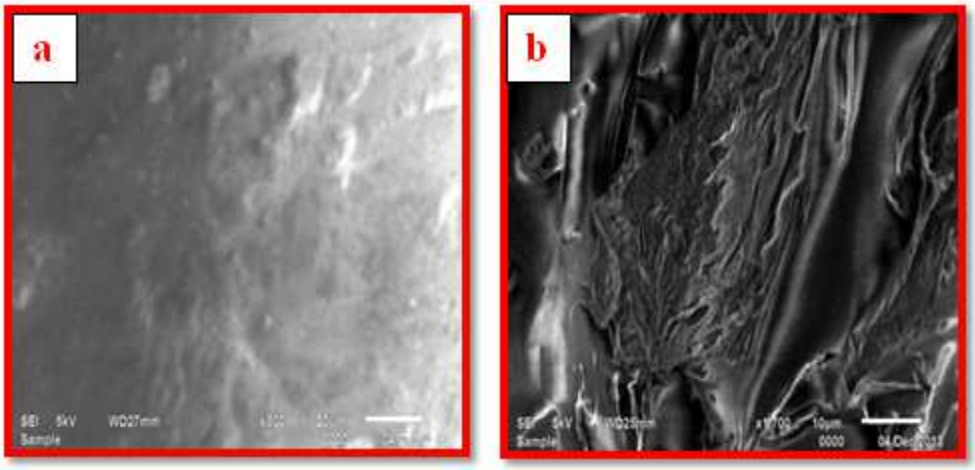


**Fig.6.SEM images of PVC strip without (a) and with (b) enzyme**

The covalent immobilization of AChE on the PVC surface was also confirmed by the FTIR analysis of the bare PVC and the PVC with enzyme. Figure 7 curve 'A' shows the FTIR spectra of PVC without AChE. There are three regions in which the bands of PVC can be classified. The C-Cl stretch, C-C stretch and CH modes ranging from 550-850cm$^{-1}$, 900-1200cm$^{-1}$ and 1250-2970cm$^{-1}$ respectively. The curve 'B' showing immobilization of AChE onto PVC strip. The C-Cl bond is formed at 720cm$^{-1}$, C-C bond is at 1130cm$^{-1}$ and CH modes are at 2750cm$^{-1}$. The peak at 1650cm$^{-1}$ incurve 'B' shows the presence of aldehyde bond (-C-O-) while no peak in curve 'A'. This shows the binding of –NH2 (amide group) with the –C O- group of glutaraldehyde and formation of covalent bond.

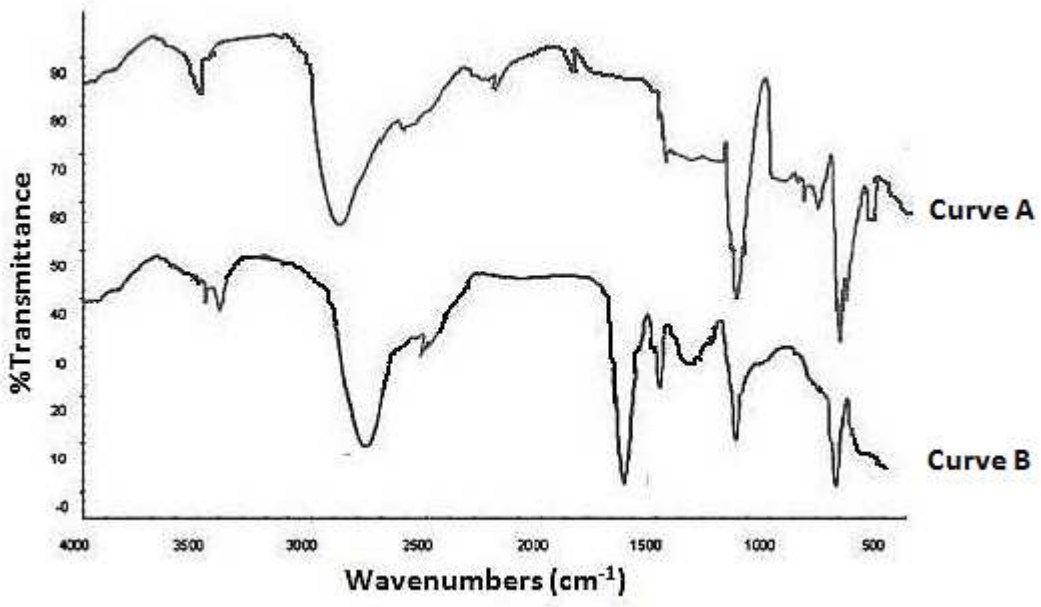


**Fig.7. FTIR of PVC without Enzyme (Curve A) PVC with bound enzyme (Curve B)**

The developed enzymatic strip was used for the testing of presence of pesticide in the samples. In absence of the OP compounds the strip will give yellow color which is directly visible to naked eyes. In presence of OP Compound

(Malathion) the reduction in the intensity of yellow was observed. Reduction in color is directly proportional to concentration of OP compound present.

*3.6 Studying the Kinetic Properties of Free and Immobilized Purified Enzyme*

*3.6.1 Effect of pH*

For determination of effect of pH on the activity of purified free enzyme AChE, the reaction mixture was subjected to different pH ranging from 6.0 to 10 with a regular increase of 0.5 the free and immobilized enzyme showed maximum activity at pH 7.5 as shown in Fig. 8.

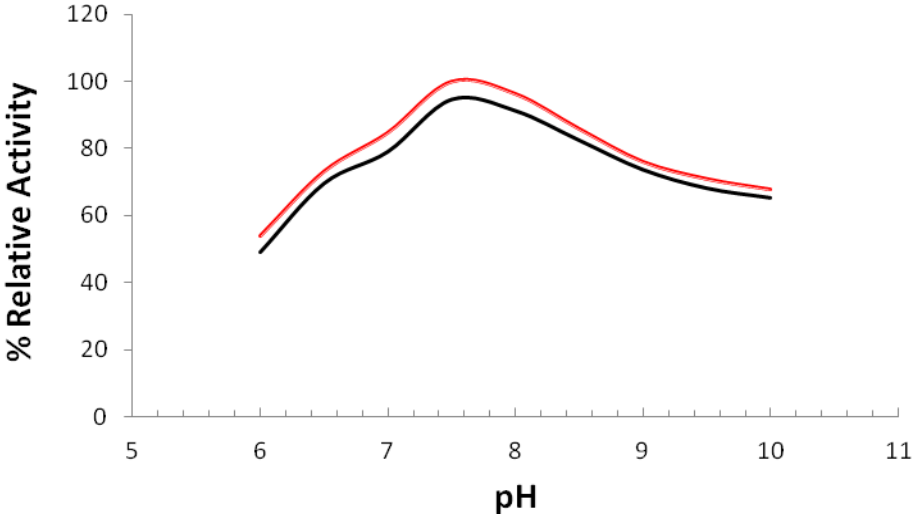


**Fig.8. Shows relationship between Percent Relative Activity and pH**

*3.6.2 Effect of Temperature*

For determination of effect of temperature on the activity of purified enzyme, the reaction mixture was subjected to temperature ranging from 10ºC to 50ºCthe optimum temperature for free AChE was 25ºC to 35ºC and immobilized enzyme showed maximum activity at 30ºC as shown in Fig. 9.

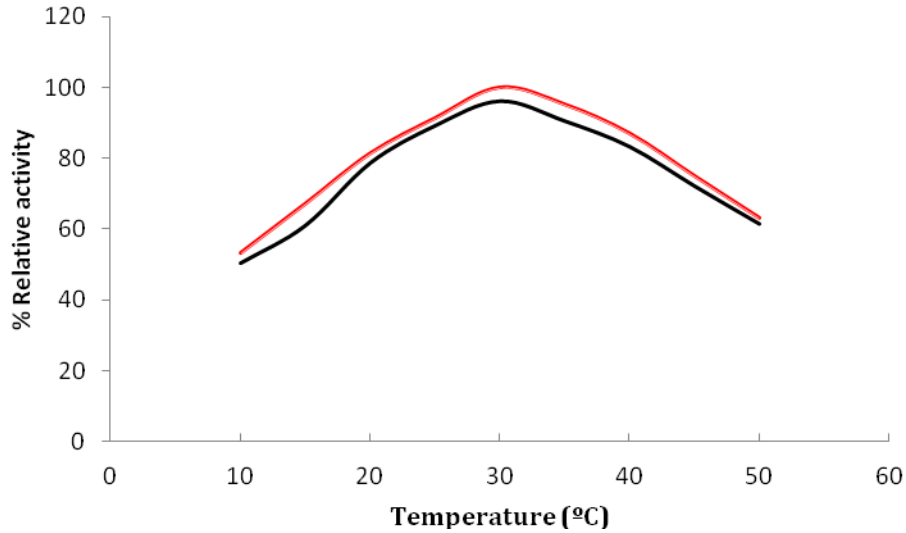


**Fig.9. Shows relationship between Percent Relative Activity and Temperature**

*3.6.3 Effect of Incubation Time*

The activity increased linearly upto 7 minutes when the reaction mixture having free purified enzyme AChE was subjected to time ranging between 2 to 12 minutes at a regular interval of 2 minutes. After 8 minutes no increase was observed for free enzyme and after 10 min no increase was observed for immobilized enzyme as shown in Fig. 10.

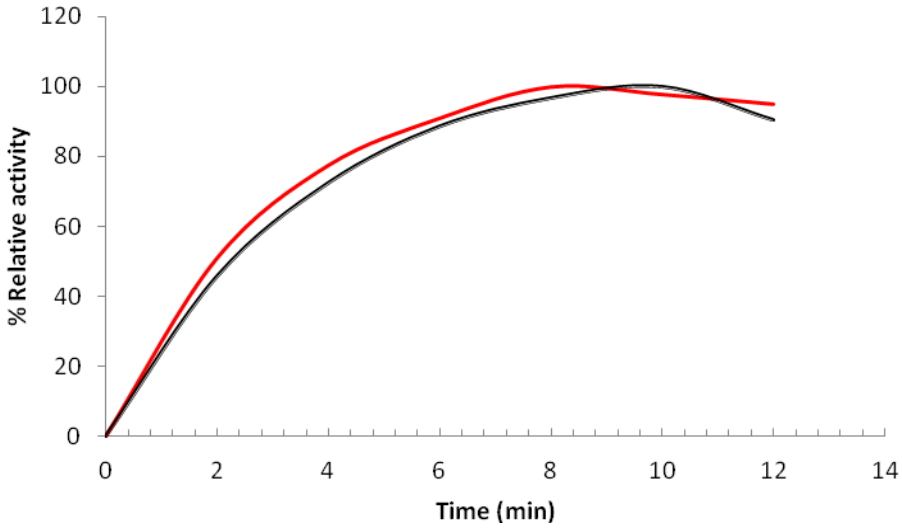


**Fig.10. Shows relationship between Percent Relative Activity and Incubation Time**

*3.6.4 Effect of Substrate Concentration*

The concentration of substrate ATCI was optimized for its effect on the activity of free and immobilized enzyme in the concentration range upto 600µM. Free AChE to showed a hyperbolic relationship between activity and substrate concentration final concentration of 550µM and immobilized enzyme upto 500µM(Fig. 11) after which no increase was observed.

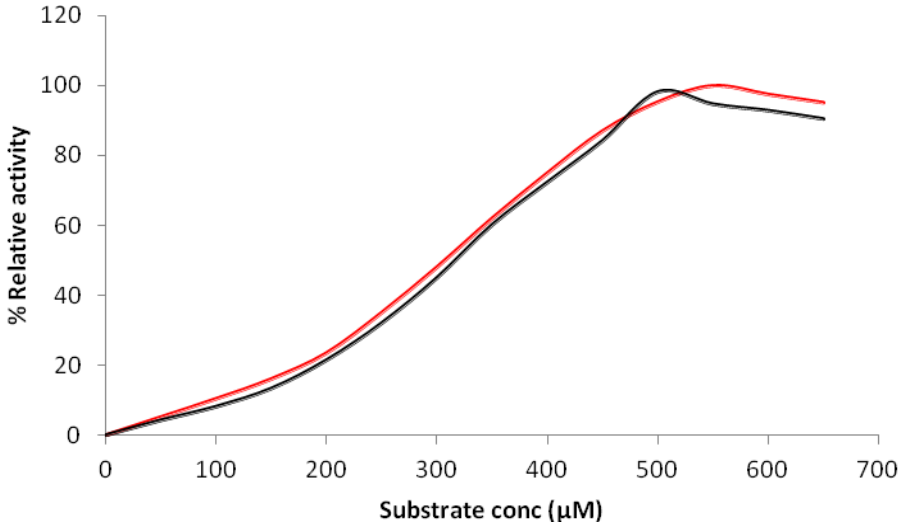


**Fig.11.Shows relationship between Percent Relative Activity and Substrate Concentration**

*3.6.5 Determination of $K_m$ and $V_{max}$*

The Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) values of free AChE enzyme for ATCl as substrate were 3.8 mM and 2.9 µmol/min/mg respectively (Fig. 12) and for immobilized AChE were 3.5 mM and 2.7 µmol/min/mg respectively (Fig. 13).

*3.7 Evaluation of the Enzymatic PVC strip*

*3.7.1 Linearity:*A linear relationship was obtained between substrate concentrations and absorbance at 410 nm ranging from 10 µM to 300 µM.

*3.7.2 Minimum Detection limit:*The minimum detection limit of the present method was 1 µM.

*3.7.3 Analytical Recovery:*In order to check the reliability of the present method, the analytical recovery of added pesticide was determined (Table 2). The mean analytical recovery of added pesticide (5 µM and 10 µM) was 98.6% and 99.2% respectively, which is better than recently reported method.

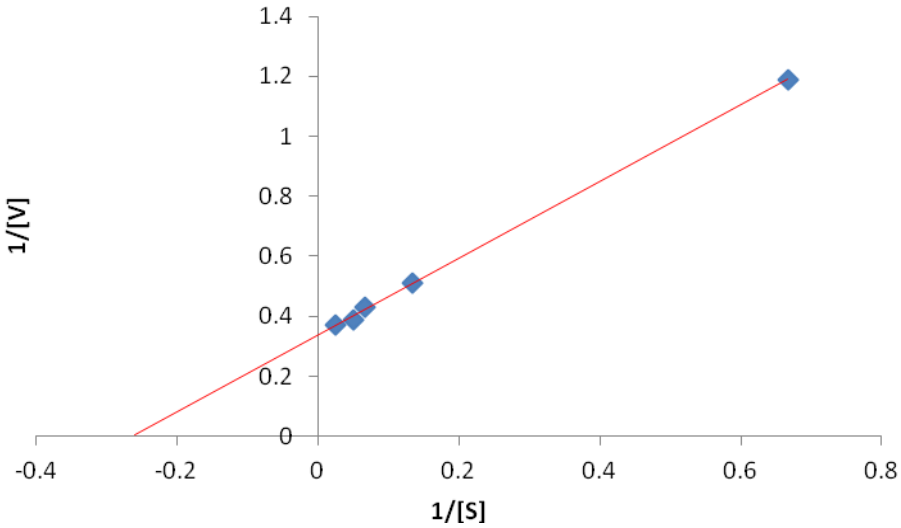


**Fig.12. Shows Lineweaver–Burk plot between *1/[S]* and *1/[V]* for free AChE enzyme**

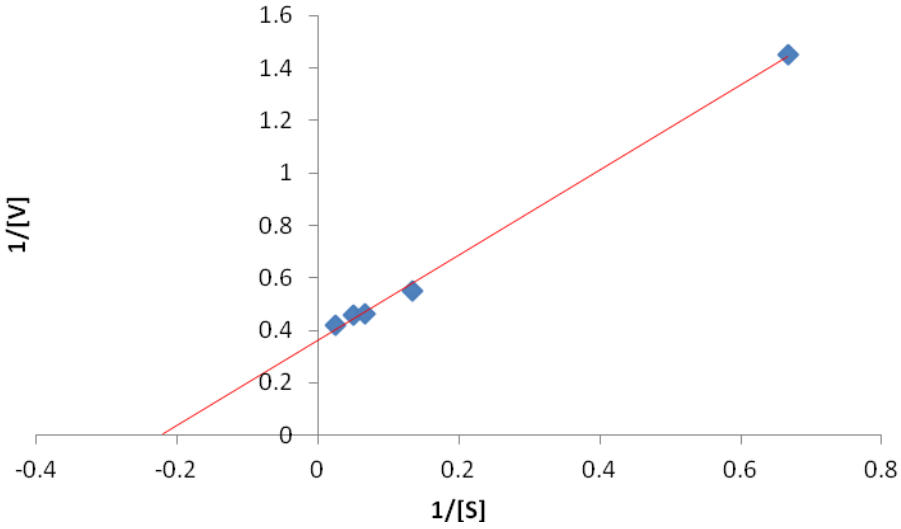


**Fig.13. Shows Lineweaver–Burk plot between *1/[S]* and *1/[V]* for Immobilized AChE enzyme**

**Table 2: Analytical recovery of added organophosphorus compounds**

| Pesticide added (µM) | Pesticide found (µM) Mean (n=3) ±SD | % Recovery |
|---|---|---|
| Nil | - | - |
| 5 | 4.93±0.04 | 98.6 |
| 10 | 9.92±0.01 | 99.2 |

*3.7.4 Precision:*To check the reproducibility and reliability of the present method, the level of pesticide in one run (within a batch) and after storage at -20 °C for one week (between batches) were determined (Table 3). The pesticide values obtained by these determinations agreed with each other and the result of within batch and between batch coefficients of variation (CVs) were < 1.5% and < 0.8%, which are better than earlier reported methods.
*3.7.5 Accuracy:*To study the accuracy of the present method, the level of pesticide was determined by the standard method (x) and by the present method (y). The pesticide values obtained by both the methods matched with each other with a good correlation (0.987) (Fig. 14). Evaluation studies showed that the method was fairly reliable with good recovery and in agreement with the standard method.

**Table 3: Within and between assay coefficients of variation for determination of pesticide by using enzyme immobilized PVC strip.**

| n | Pesticide(µM) Mean ± S.D | CV (%) |
|---|---|---|
| Within assay (6) | 9.92 ±0.15 | 1.5 |
| Between assay (6) | 9.87 ±0.07 | 0.8 |

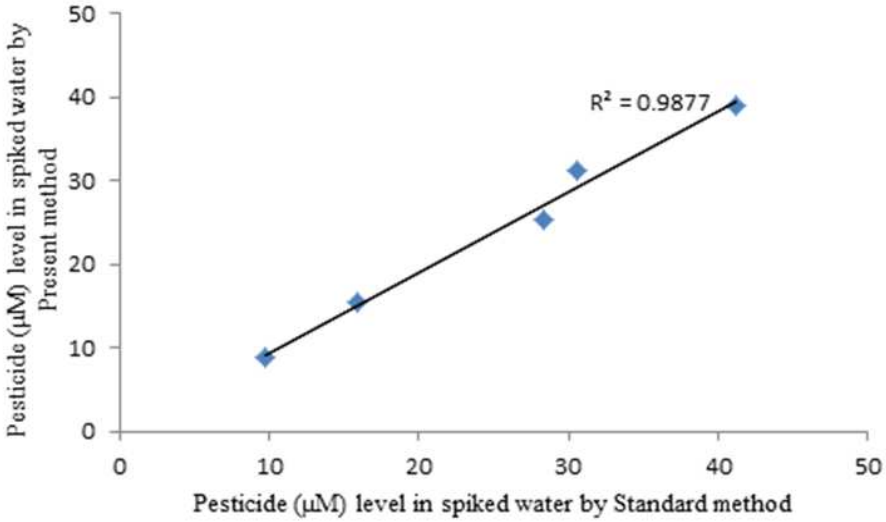


**Fig.14. Correlation between pesticide levels in spiked water determined by standard method (x-axis) and by the present method (y-axis)**

## CONCLUSION

Acetylcholinesterase (AChE) had been purified from the hypocotyls of 8 day old germinated seedlings. The gel filtration methods were used which included Sephadex G-100 followed by DEAE-Sephacel column chromatography. With Sephadex G-100, 18.58% yield was obtained with 33.08 fold purification and specific activity of 26.50 Unit/mg.DEAE Sephacel provided a yield of 17.93% with purification fold 39.37 and specific activity of 31.54 Unit/mg. The molecular weight was 65KDa as determined by SDS. The working conditions were also optimized for free and immobilized AChE. The optimum pH for free and immobilized AChE was 7.5, optimum temperature at which immobilized enzyme showed maximum activity was 30ºC, time of incubation for free and bound AChE were 8 minutes and 10 minutes respectively and the substrate concentration at which maximum activity showed was 550µM (for free enzyme) and 500µM (for immobilized enzyme). The developed PVC enzymatic strip can be used for the detection of the presence of the OP compounds in the fruit juices, soil samples, water sources etc.

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