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1-Naphthyl acetate as an alternative substrate of hemolysate cholinesterase: Direct visualization of enzyme activity within 10 minutes on polyacrylamide gels

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Hemolysate cholinesterase is currently recognized as the most preferred biomarker to detect acute organophosphorus poisoning. Direct visualization of cholinesterase activity on polyacrylamide gels is routinely practiced using acetylthiocholine as a substrate. Overnight incubation with the staining solution is required to understand the enzyme activity bands on gels. Therefore, the need arises to explore rapid detection methods, which can specifically detect hemolysate cholinesterase on polyacrylamide gels. Here, we have explored alternative substrates, such as 1-NA and 2-NA which might have the potential to behave as specific substrates for the detection of hemolysate cholinesterase activity on the gels. It is observed by the *in silico* studies that 1-NA bind at the active site of acetylcholinesterase akin to acetylcholine (ACh) with a better fitness score. Secondly, the hemolysate cholinesterase activity, as well as its inhibition by organophosphorus pesticides is understandable within 10 min using Fast Blue RR dye for the detection of 1-NA. The organophosphorus inhibited activity is regained in the presence of cholinesterase reactivator. Moreover, the enzyme activity bands formed using 1-NA proves the specificity of the substrate for hemolysate cholinesterase as in the presence of specific acetylcholinesterase inhibitors the band formation disappears. On the other hand, ATCh requires minimum 8-12 h staining time for detection of enzyme activity band following Karnovsky and Roots protocol. Our results prove that 1-NA is an alternative substrate of hemolysate cholinesterase which specifically detects the enzyme activity on gel rapidly. We recommend 1-NA for rapid detection of hemolysate cholinesterase activity on the gels.

Keywords: Acetylcholinesterase, Biomarker, Karnovsky staining, Naphthyl acetates, Organophosphorus pesticides

Acute organophosphorus pesticide poisoning is a common problem in the developing world[1]. Erythrocytes, known to contain acetylcholinesterase (AChE), are currently recognized as the most preferred biomarker of organophosphorus poisoning[2]. Detection of cholinesterase activity in hemolysate is generally done using acetylthiocholine (ATCh) as a substrate, which is cleaved by the enzyme to produce thiocholine. The thiocholine is measured using DTNB (Ellmans reagent). This method suffers from limitations, and several modifications have beene published. However, the thiocholine based cholinesterase assay is continuously getting evaluated with the help of advanced technologies[3-5].

Visualization of enzyme activity on poly-acrylamide gels is a popular method of assessing cholinesterase activity[6-9]. Karnovsky & Roots method is the common approach for detection of acetyl-cholinesterase on gel. It uses acetylthiocholine as substrate and requires more than 12 h to stain the enzyme activity band[10,11]. Therefore, it is necessary to search alternative substrates for quick detection of the enzyme activity on the gels.

1-Napthyl acetate (1-NA) has been used as a substrate for purified acetylcholinesterase detection[12]. However, in human umbilical vein cell lysate, 1-NA failed to stain AChE specifically[13]. In hemolysate, specificity of 1-NA for AChE is not investigated thoroughly. Assessment of specificity of 1-NA against hemolysate cholinesterase is crucial as other esterases also known to cleave 1-NA in erythrocytes[14]. It has been proved earlier by electrophoresis that plasma does not exhibit 1-NA esterase activity because of

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Abbreviation: ACh, acetylcholine; AChE, acetylcholinesterase; ATCh, acetylthiocholine; BW284c51, 1, 5-Bis (4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide; DTNB, 5, 5-dithio-bis-(2-nitrobenzoic acid); DPIP, 2, 6 dichlorophenol indophenol; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide; 1-NA, 1-naphthyl acetate; 2-NA, 2-naphthyl acetate; 2-PAM, 2-pralidoxime; PB, phosphate buffer.

carboxylesterases. In plasma butyrylcholinesterase, paraoxonase and albumin esterase are major players for 1-NA hydrolysis[6]. In hemolysate, it is not known whether carboxylesterases other than AChE can cleave 1-NA significantly.

AChE cleaves substrates other than acetylcholine or acetylthiocholine and the cleavage of such substrates is inhibited in the presence of cholinesterase inhibitors. Cleavage of aryl acylamides is also inhibited in the presence of cholinesterase inhibitors[15]. Moreover, recently the DNase activity of AChE is highlighted which suggests that biomolecules like DNA (plasmid or chromosomal) are cleaved by this enzyme even after inhibition of its cholinesterase function[16]. At the present moment, it is not clear that 1-NA cleavage action of AChE is exclusively due to its cholinesterase activity or not. The result of such investigation has profound toxicological relevance because if 1-NA is cleaved specifically by hemolysate cholinesterase, then it should be recognized as an alternative substrate for AChE, which has the potential to overcome the limitations of thiocholine based cholinesterase detection systems.

Therefore, in this work, we have explored 1-NA and 2-NA as candidate substrates for 'on the gels' detection of hemolysate cholinesterase.

Materials and Methods

Materials

DPIP (2,6 dichlorophenol indophenol), 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and AChE from human erythrocytes [buffered aqueous solution; ≥500U/mg protein (BCA); Sigma Catalogue no. C0663-50UN], ATCh and 1,5-Bis (4-allyldimethyl ammoniumphenyl) pentan-3-one dibromide (BW284c51) were purchased from Sigma Aldrich (St Louis, Missouri). Glycine, 1-naphthol, and glycerol were from Merck (Darmstadt, Germany). Chlorpyriphos (50% E.C., Trade name: Lara) was from Crystal Phosphates Ltd (Sonepat, Haryana). Dichlorvos (76% E.C. Trade name: Nuvan) was from Insecticides (India) Ltd (Jammu & Kashmir, India). 2-Pralidoxime (2-PAM) was from Samarth Life Sciences (Mumbai, Maharashtra) and rest all the chemicals namely; acrylamide, ammonium persulphate, bisacrylamide, copper (II) sulphate, pentahydrate, A.R, eserine salicylate, Fast Blue RR, maleic acid, naphthyl acetates, N,N,N,N, tetramethylethylene diamine, electrophoresis grade (TEMED), potassium ferricyanide, tris HClTris, base, and triton X-100 were purchased from HiMedia (Paris, France). The organic solvents (ethanol, acetone) used were of analytical grade.

Docking of acetylcholine (ACh), 1-NA and 2-NA with AChE

The 3-D structures of the substrates: ACh (CID: 187), 1-NA (CID: 13247), 2-NA (CID: 73709) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and the structure of recombinant human AChE (PDB 3LII) from Protein Data Bank (www.rcsb.org). The substrates were docked with AChE using GOLD[17] and goldscore was used as the scoring method. Docking was performed for each substrate with AChE using the default settings of GOLD. A maximum of 10 conformations for each enzyme-substrate complex was generated. Trp 86, present at the anionic site of the enzyme was chosen as the grid center for the docking purpose[18]. Goldscore of the best-ranking solution was taken into consideration for comparison of the fitness of the three substrates with AChE. Pymol (Schrodinger Inc. New York, US) was used for the visualization and analysis of the enzyme-substrate complexes (https://www.pymol.org).

Electrophoresis experiments

Preparation of stock solutions

1-NA (1 mg/mL), 2-NA (1 mg/mL), eserine salicylate (1 mM), and chlorpyriphos (143 µM) stocks were prepared in acetone. Dichlorvos stock solution (100 mM) was prepared in ethanol. ATCh stock (10 mM) was prepared in Phosphate buffer (PB) (pH 7.3). BW284c51 stock (0.03 M) was prepared in distilled water. ATCh stock was freshly prepared before use. The stock solutions were stored at 2-8°C.

Hemolysate preparation

The blood was collected in heparinized tubes from healthy individuals after obtaining informed consents. The study protocol was approved by the Institute Ethical Committee (Project no. NK/1221/Ph.D/20418, approved on 9 April 2014). The hemolysate (1:3 dilution) was prepared as described below. The collected blood was centrifuged at 2500 rpm for 5 min to separate out the plasma. The red cell pellet thus obtained was then washed three times with 0.9% normal saline followed by centrifugation at 2500 rpm for 5 min. The clear supernatant was discarded after washing. The lysis of the erythrocytes was done as per the procedure of Ravazzolo *et al.*[19].

Sample preparation

The hemolysate (1:3) was further diluted with the sample buffer [0.25 M Tris, pH 6.8, 20% glycerol (v/v)]

in the ratio of 1:11 (1 part hemolysate: 11 parts sample buffer). 50 µL of the sample was loaded into each well of the polyacrylamide gel before the electrophoresis. To understand the effect of enzyme concentration, varying volumes (1:3) of hemolysate (1, 10 and 25 µL) were diluted with sample buffer in different aliquots to make a final volume of 240 µL. From these dilutions, 50 µL was loaded in each well.

Ten microlitre (10 µL) of pure erythrocyte AChE solution (≥500 U/mg) was diluted in 490 µL of PBS (0.1 M, pH 7.5). This was further diluted with the sample buffer in the ratio of 1:1 to make a working enzyme solution. About 50 µL of the working enzyme solution was loaded into each well of the polyacrylamide gel before the run. 1U of AChE is defined as the concentration which will hydrolyze 1.0 µM of ACh iodide per min at pH 7.4 at 37°C.

Native PAGE of pure AChE and hemolysate:

PAGE was performed on vertical gel slab apparatus (GeneI, Bengaluru, Karnataka), and 12% resolving gel was prepared. It contained 30% bis-acrylamide-acrylamide solution, 1.5 M Tris (pH 8.8), distilled water, 10% ammonium persulphate and TEMED. 5% stacking gel was used which consisted of 30% bisacrylamide-acrylamide solution, 1 M Tris (pH 6.8), distilled water, 10% ammonium persulphate and TEMED. The gel was allowed to polymerize at room temperature (32-36°C). After loading the samples, a pre-run was given at 70 V for 15 min, followed by electrophoresis run at 100V for 2 h in glycine/Tris buffer pH 9.0. The apparatus was kept on ice during the run. After the completion of the electrophoresis run, the gels were subjected to staining using different substrates. Separate electrophoresis experiments were done for each substrate for several times with and without inhibitors as detailed below in the staining section.

Staining of gels for visualization of enzyme activity bands

The gels were stained for detection of enzyme activity using different substrates (ATCh, 1-NA and 2-NA). After the electrophoresis run, the gel was incubated for 45 min in 50 mL of solution, which consisted of ATCh (100 µM) and PB (pH 7.3). After 45 min the substrate solution was removed, and the gel was added with 50 mL of Karnovsky stain for detection of AChE activity and incubated overnight[10]. Throughout this time the gel was observed for the development of enzyme activity bands.

For visualization of enzyme activity bands using 1-NA and 2-NA, Fast Blue RR was used. It is a diazonium salt used for detection of naphthols[13]. The gels were first incubated in 50 mL solution containing 10 mL of 1-NA and 2-NA and 40 mL PB (pH 7.3) for 45 min in separate runs. The final concentration of the substrates was 100 µM in the 50 mL mixture. After 45 min of incubation, the substrate solution was removed and the gel was added with 50 mL of 0.03% Fast Blue RR solution prepared in PB (pH 7.3) and incubated for overnight. While the gels were on incubation, they were observed for development of bands.

Inhibition of AChE activity

Eserine salicylate (also known as physostigmine) is a classical inhibitor of cholinesterase (AChE and butyrylcholinesterase)[20]. On the other hand, BW284c51 is known to inhibit specifically AChE[21]. We used both of these inhibitors to understand the specificity of the substrates for AChE. After electrophoresis of hemolysate, gels were first incubated for 30 min in a solution containing PB (pH 7.3) and the desired concentration of eserine salicylate (2, 50 and 100 µM, respectively) or BW284c51 (450 µM). It was followed by incubation with the substrates (ATCh, 1-NA, 2-NA, respectively; 100 µM each) for 45 min in the same reaction mixture containing the inhibitor with the final volume of 50 mL and stained overnight as mentioned above. For each substrate, a separate run was performed.

Inhibition with chlorpyriphos and reactivation by 2-PAM

Chlorpyriphos is a commonly used organo-phosphorus pesticide, which inhibits both neuronal and erythrocyte AChE[22]. 2-PAM is used for regeneration of AChE activity inhibited by organophosphorus pesticides[23]. After electrophoresis of hemolysate, the gels were incubated with a solution containing PB (pH 7.3) and 72 µM of chlorpyriphos for 30 min; followed by incubation with 720 µM of 2-PAM for 30 min in the same reaction mixture of a total volume of 40 mL. Finally, different gels were incubated with the substrates (ATCh, 1-NA, 2-NA; 100 µM each) to make 50 mL as the final volume, and stained for AChE activity as mentioned above.

Comparison of 1-NA and 2-NA staining protocols with Karnovsky staining

After the electrophoresis of pure erythrocyte AChE, gel was incubated with ATCh, 1-NA and 2-NA,

respectively (100 µM each) in 50 mL [5 mL 1-NA, 2-NA (1mg/mL stock) diluted in 5 mL of acetone plus PB (pH 7.3); 500 µL of ATCh (10 mM stock) diluted in PB (pH 7.3)] for 45 min following separate runs. It was followed by staining with Fast Blue RR for detection of naphthols and Karnovsky staining for detection of thiocholine produced by AChE. The similar staining procedure was followed for enzyme activity detection in hemolysate electrophoresis. The gels were incubated with the staining solutions for 12 h and observed for band formation.

Time-dependent staining of hemolysate enzyme activity using Fast Blue RR and DTNB staining methods

After the electrophoresis of hemolysate, the gel was incubated with ATCh (6 mM) and staining was performed using the Ellmans reagent (DTNB) (3 mM) following the published protocol [7]. In this experiment, the substrate and the stain were added together. The same experiment was repeated with 100 µM ATCh as a substrate. In another set of hemolysate gels, 1-NA was used for the detection of enzyme activity. In a separate set of experiments, 1-NA (100 µM) and Fast Blue RR (0.03%) were added together for the detection of enzyme activity. To understand the inhibition with organophosphorus pesticide, hemolysate (1:3) was incubated with dichlorvos (116 µM) for overnight. Electrophoresis of the dichlorvos-preincubatedhemolysate was performed and staining for the detection of enzyme activity bands was done following the above-mentioned procedures. The gels were examined for appearance of enzyme activity bands for 12 hours.

Staining for detection of enzyme activity in hemolysate using DPIP and ATCh

Another staining procedure was used to understand the enzyme activity utilizing ATCh as a substrate. After the electrophoresis run, the gels were incubated with ATCh (800 µM) and stained by DPIP (1 mM) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) (10 mM) following the published protocol[19]. The same protocol was repeated by using 100 µM of ATCh. The gels were examined for appearance of enzyme activity bands for 12 h.

Densitometric analysis of electrophoresis experiments

The bands obtained in different electrophoresis experiments were subjected to densitometric analysis using AlphaView software following manufacturer's instructions (CellBiosciences, Santa Clara, CA), version 1.2.0.1. The values of band density are represented as mean±SD (n=6) of Integrated Density Value (IDV) of the band after subtraction of the background values and statistical significance was calculated using unpaired Student t-test (GraphPad Software, La Jolla, CA). A P value <0.05 was taken as significant.

Results

Interactions of ACh, 1-NA and 2-NA with AChE

ACh, 1-NA & 2-NA showed interaction with AChE via H-bond formation and stacking interaction with the anionic site residues of AChE akin to earlier reports[18] (Suppl. Fig. 1. *All supplementary figures are available only online along with the respective paper at repository. http://nopr.res.in*). The comparison of the goldscore of the best ranked structures indicates that 1-NA (41.50) has the highest goldscore with AChE followed by 2-NA (36.74) in comparison to ACh (31.99) docked with AChE.

Detection of AChE activity using ACh, 1-NA and 2-NA as substrates from pure enzyme

A lower concentration of ATCh (100 µM) with pure AChE produced a faint band indicating esterase activity. With a higher concentration of ATCh (400 µM), diffused pattern of esterase activity bands were observed when stained by Karnovsky method (Suppl. Fig. 2). A higher concentration of ATCh showed a significant difference in esterase activity bands when compared to the tested lower concentration (Fig. 1B). Similar results were seen with 1-NA and 2-NA. However, even with the tested lower concentration (20 µM) of the naphthyl acetates, the esterase activity band was visible. In the case of 1-NA, the band observed was prominent in comparison to 2-NA. The colours of the enzyme activity bands were different for the three substrates. With 1-NA, brown colored bands were observed and with 2-NA pink colored bands appeared. ATCh produced brownish bands (Fig. 1B & Suppl. Fig. 2).

Detection of hemolysate cholinesterase activity using ACh, 1-NA and 2-NA as substrates

Study of enzyme activity with varying substrate concentrations

With the increase in ATCh concentration, a significant increase in band intensity was observed [Fig 1A & Suppl. Fig. 2 (A-C)]. However, no significant difference in IDV was observed between the blank gel and the lowest ATCh concentration tested (20 µM) [Suppl. Fig 2 (Blank-A)]. With the higher concentrations of ATCh (25 and 50 µM), a

clear band pattern was observed after overnight incubation of the gel with the staining solution (Karnovsky stain) [Suppl. Fig. 2 (B-C)].

The intensity of the bands significantly increased with the increasing concentrations of 1-NA and 2-NA [Fig. 1A & Suppl. Fig. 2 (D-F)]. Bands of enzyme activity were observed with all the tested concentrations of 1-NA and 2-NA after incubation with the Fast Blue RR staining solution. The pattern of band appearance with 1-NA and 2-NA is similar to bands formed with ATCh (Suppl. Fig. 2). Moreover, band colours with 1-NA and 2-NA in hemolysate are akin to band colour appeared in pure erythrocyte AChE electrophoresis. Similar is the case when ATCh is used as substrate (Suppl. Fig 2).

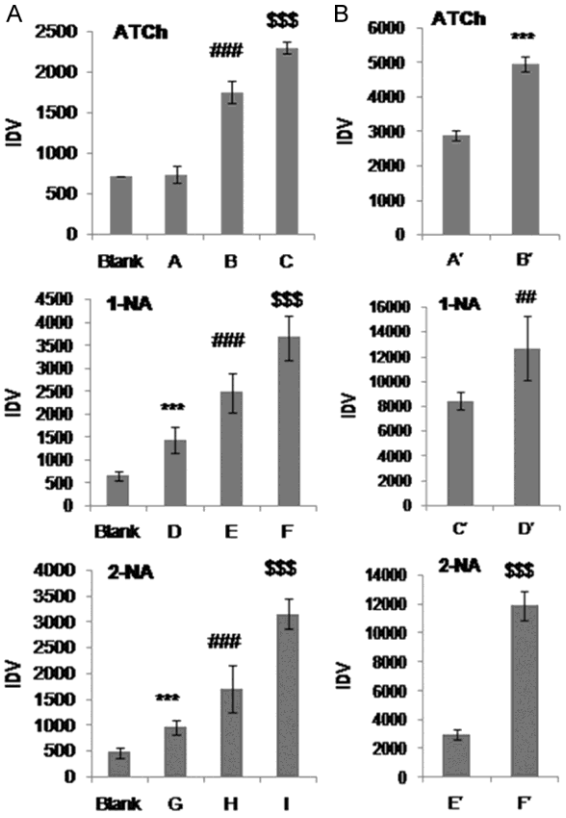

Fig. 1— Bar diagram representing mean±SD (n=6) values of band densities (IDV) following Native PAGE of (A) haemolysate; and (B) pure erythrocyte AChE with increasing substrate concentrations (ATCh, 1-NA and 2-NA, respectively) after staining. [The amount of enzyme (hemolysate and erythrocyte AChE) are kept constant. Corresponding representative gel pictures are shown in suppl. Fig. 2. Blank: 0 uM of the substrate; A-C: incubation with 20, 25, 50 uM ATCh, respectively; D-F: incubation with 20, 25, 50 uM 1-NA respectively; G-I: incubation with 20, 25, 50 uM 2-NA, respectively; A', B': incubation with 100, 400 uM ATCh, respectively; C', D': incubation with 20, 100 uM 1-NA, respectively; E', F': incubation with 20, 100 uM 2-NA, respectively. ATCh stained by Karnovsky method and 1-NA, 2-NA are stained by Fast Blue RR. *: Blank *vs.* A, D, G, and A' *vs.* B'; #: Blank *vs.* B, E, H, and C' *vs.* D'; $: Blank *vs.* C, F, I, and E' *vs.* F' where ***P <0.0001; $^{###}P$ <0.0001; $^{\$\$\$}P$ <0.0001; $^{\#\#}P$ <0.01]

Study of enzyme activity with different volumes of hemolysate

With the increase in hemolysate (1, 10 and 25 µL, respectively), the intensity of the bands increased with the tested substrates (ATCh, 1-NA; 100 µM each). Further, a significant difference in band intensity was observed between ATCh and 1-NA with all the dilutions of hemolysate (1, 10, 25 µL, respectively) (Fig. 2).

Study of enzyme activity with different concentrations of eserine salicylate (*non-specific cholinesterase inhibitor*)

The gels after electrophoresis of hemolysate (as described in the Methods) were incubated with increasing concentration of eserine salicylate (a non-specific cholinesterase inhibitor) followed by detection of enzyme activity by Karnovsky stain using ATCh [Suppl. Fig. 3(I)][20]. It resulted in the significant decrease in the band intensity even with the tested lowest concentration of eserine salicylate (2 µM) (Fig. 3) when compared to the control without eserine. With 100 µM of eserine, a further decrease in band intensity was recorded. Similar results were obtained with naphthyl acetates [Fig. 3A & Suppl. Fig. 3(I)].With 1-NA and 2-NA diminished enzyme activity bands were observed in the presence of the inhibitor in a dose dependent manner. This signifies that the enzyme activity detected with 1-NA and

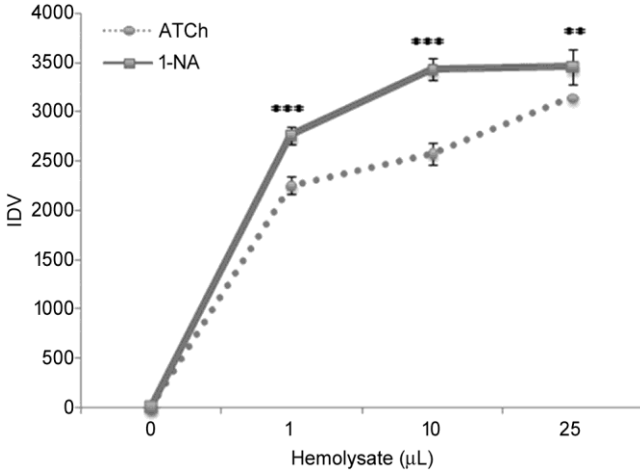

Fig. 2 — Graphical representation of mean±SD (n=6) values of band intensities (IDV) obtained after Native PAGE of hemolysate (increasing volumes in different wells) followed by incubation with a fixed concentration of substrate (ATCh and 1-NA, respectively) and staining. [The gels were stained for 1-NA with Fast Blue RR and Karnovsky method for ATCh. The X-axis represents the volumes of hemolysate (1:3) in microliter and Y-axis represents the band intensity (IDV). *: 1-NA vs ATCh where **P <0.01; ***P <0.0001]

2-NA is majorly due to cholinesterase present in hemolysate.

Study of enzyme activity in the presence of specific AChE inhibitor (*BW284c51*)

Similarly, after electrophoresis of hemolysate the gels that were incubated with BW284c51 (a specific inhibitor of AChE) showed a significant decrease in band intensity when 1-NA and 2-NA were used as substrates [Fig. 3A &, Suppl. Fig. 3(II)] in comparison to the control without the inhibitor[21]. This confirms that the observed bands are due to AChE activity [Suppl. Fig. 3(II)].

Inhibition of enzyme activity with chlorpyriphos and its reactivation by 2-PAM

The gels (as described in the Methods) incubated with chlorpyriphos showed significant decrease in band intensity when stained for enzyme activity using 1-NA, 2-NA, and ATCh in comparison to the control gels (Fig. 4 & Suppl. Fig. 4). However, in the presence of 2-PAM, a significant increase in band

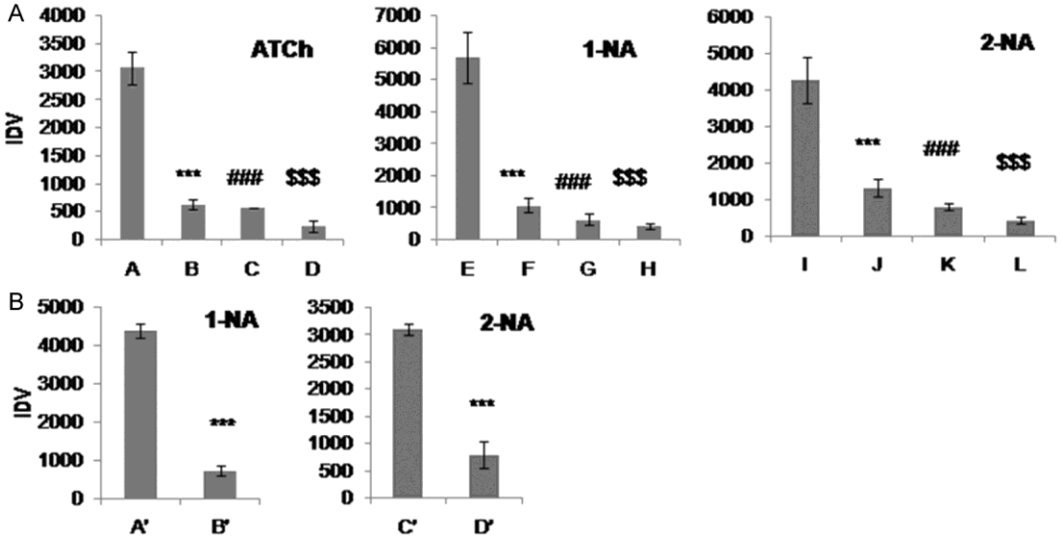

Fig. 3 — Graphical representation of mean±SD (n=6) values of band densities (IDV) of hemolysate (fixed volume) treated with (A) eserine salicylate (cholinesterase inhibitor); and (B) BW284c51 (a specific inhibitor of AChE) followed by incubation with ATCh (for eserine incubated gels only), 1-NA and 2-NA (100 uM each) respectively; and stained with Karnovsky for ATCh; Fast blue RR for 1-NA and 2-NA. Corresponding representative gel pictures are shown in Suppl. Fig. 3. [A: ATCh (100 uM) without eserine, B-D: incubation with 2, 50 and 100 uM eserine, respectively followed by ATCh incubation; E: 1-NA (100 uM) without eserine, F-H: incubation with 2, 50 and 100 uM eserine, respectively followed by 1-NA incubation; I: 2-NA (100 uM) without inhibitor, J-L: incubation with 2, 50 and 100 uM eserine, respectively followed by 2-NA incubation; A': 1-NA (100 uM) without BW284c51, B': 450 uM of BW284c51 followed by 1-NA incubation; C': 2-NA (100 uM) without BW284c51, D': 450 uM of BW284c51 followed by 2-NA incubation. *: A *vs*. B, E *vs*. F, I *vs*. J, A' *vs*. B', and C' *vs*. D'; #: A *vs*. C, E *vs*. G, and I *vs*. K; $: A *vs*. D, E *vs*. H, and I *vs*. L where ***P <0.0001; $^{###}P$ <0.0001; $^{\$\$\$}P$ <0.0001]

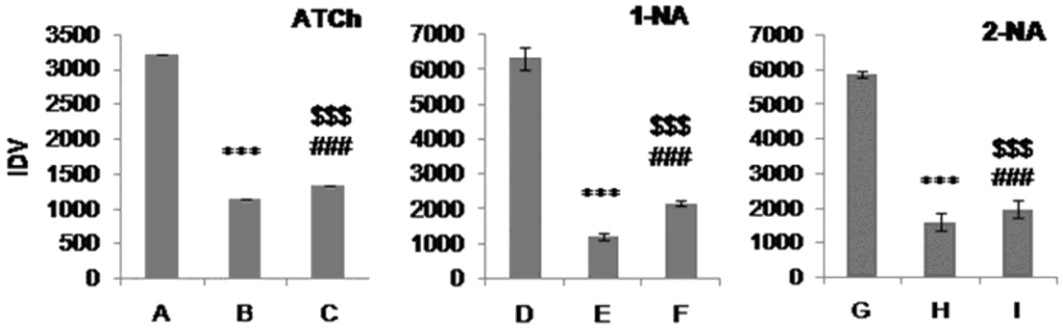

Fig. 4—Bar diagram representing mean±SD (n=6) values of band densities (IDV) of a fixed volume of hemolysate treated with chlorpyriphos (organophosphorus pesticide) followed by incubation with 2-PAM (a cholinesterase reactivator). [A, D, G: ATCh, 1-NA, 2-NA, respectively (100 uM each). B, E, H: incubation with 72 uM of chlorpyriphos followed by incubation with ATCh, 1-NA, 2-NA, respectively. C, F, I: incubation of gels first with 72 uM of chlorpyriphos followed by incubation with 2-PAM (720 uM) and staining for ATCh, 1-NA and 2-NA, respectively. Staining method for ATCh is Karnovsky staining and for 1-NA, 2-NA is Fast Blue RR. *: A *vs*. B, D vs E and G *vs*. H; #: A vs C, D *vs*. F and G *vs*. I; $: B *vs*. C, E *vs*. F and H *vs*. I. ***P <0.0001; $^{###}P$ <0.0001; $^{\$\$\$}P$ <0.0001. Corresponding representative gel pictures are shown in Suppl. Fig. 4]

intensity was observed with the three substrates. It indicates that organophosphorus (chlorpyriphos) inhibited AChE is reactivated on gels where ATCh, 1-NA, and 2-NA are used as substrates.

Comparison of staining time using different substrates

With pure erythrocyte AChE, after incubation with substrates (ATCh, 1-NA and 2-NA, 100 µM each) and staining with respective methods, the activity bands appeared within 10 min in gels stained with Fast Blue RR. However, with ATCh incubated gels (Karnovsky staining), clear bands were not observed within this period (Fig. 5).

In hemolysate with 1-NA and 2-NA bands appeared within 10 min (Fig. 5). 1-NA produced more prominent bands in comparison to 2-NA. On the other hand, Karnovsky staining did not produce any visible AChE activity bands within this time span (Fig. 5). The band formation takes 8-12 h. The background staining was minimal in all the three cases.

It was observed that with DTNB (3 mM), ATCh (6 mM) produced a yellow band within 10 min. The background of the gel was yellowish. However, after 12 h, the background of the gels became darker and a significant reduction in the band intensity was observed. 1-NA when detected by Fast Blue RR also produced prominent sharp bands within 10 min, and the bands were stable for 12 h (Fig. 6 & Suppl. Fig. 5). When the stain and the substrate (1-NA) were added together a high background staining was observed although sharp bands were visible (Suppl. Fig. 5). The band formation was completely inhibited in case of dichlorvos pre-incubated hemolysate with both the substrates (Fig. 6 & Suppl. Fig. 5).

With the tested concentrations of ATCh (100 and 800 µM), DPIP plus MTT (staining reagent) produced a high background of the gels after overnight incubation. No clear bands were understandable (Suppl. Fig. 5).

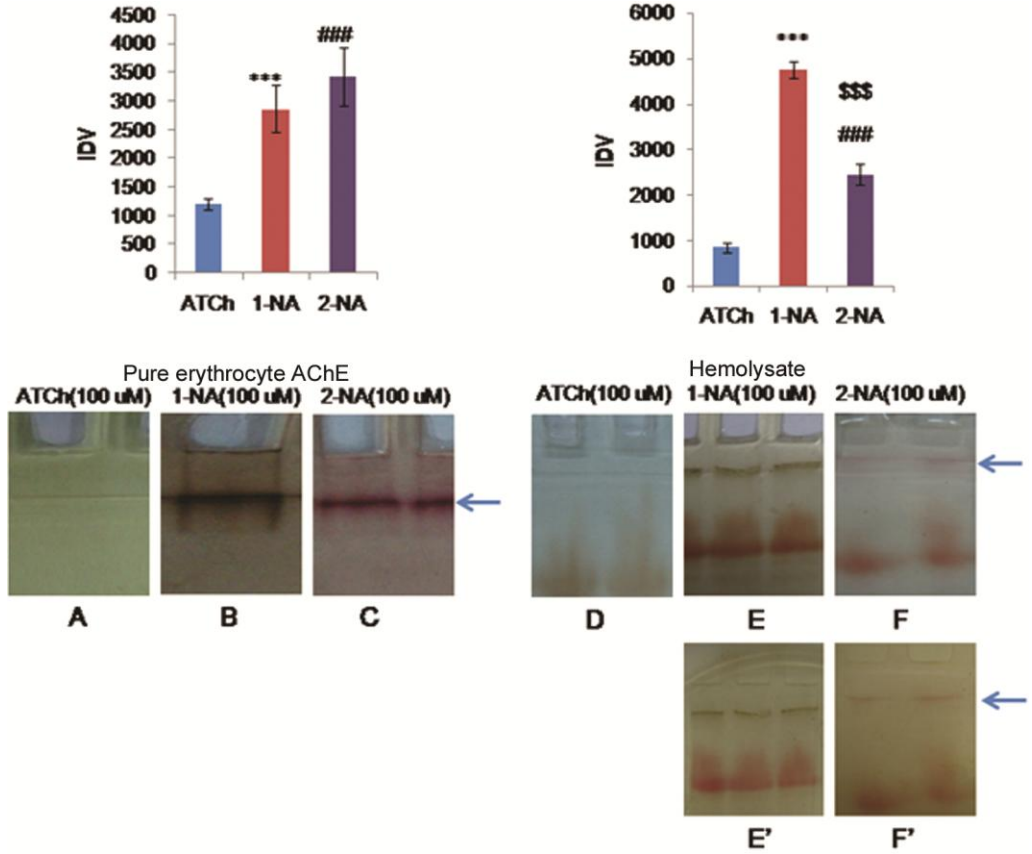

Fig. 5 — Bar diagram representing mean±SD (n=6) values of band densities (IDV) obtained with pure erythrocyte AChE and hemolysate after Karnovsky and Fast Blue RR staining (10 mins staining time) with respective substrates. A, D: ATCh; B, E: 1-NA; C, F: 2-NA; E', F': 1-NA (E') and 2-NA (F') incubated gels in the staining solution. The pictures are taken at 10 minutes of addition of staining solution. . Band formation is indicated by *: ATCh *vs.* 1-NA; #: ATCh *vs.* 2-NA; $: 1-NA *vs.* 2-NA where $^{###}P$ <0.0001; ***P <0.0001; $^{\$\$\$}P$ <0.0001.

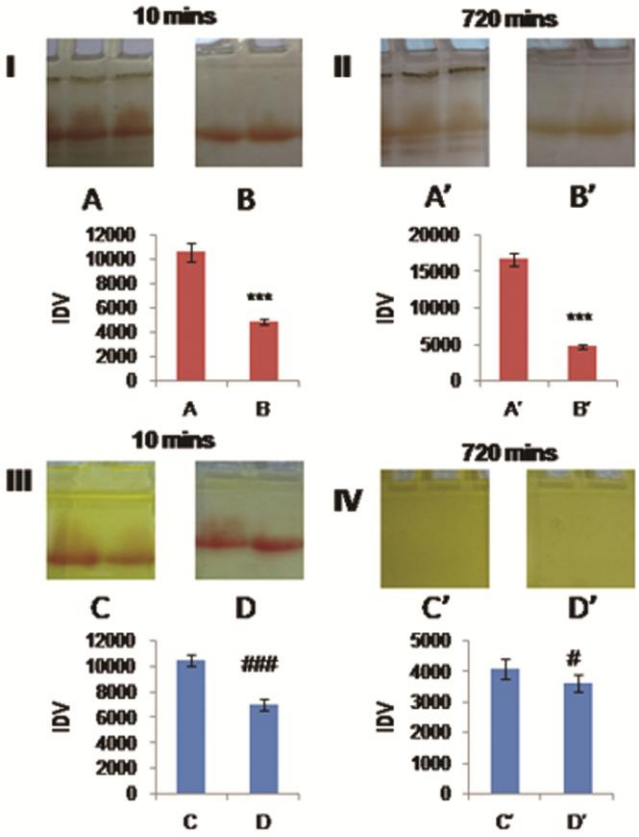

Fig. 6 — Bar diagram representing mean±SD (n=6) values of band densities (IDV) at different time points after Native PAGE of hemolysate without (A, A' C, C') and with (B, B', D, D') dichlorvos (116 uM) pretreatment followed by substrate incubation and staining. 1-NA (100 uM) incubation and staining with Fast Blue RR (0.03%) in A, A', B, B'. ATCh (6 mM) incubation and staining with DTNB (3 mM) in C, C', D, D'. The IDV values were taken at 10 mins (I, III) and 720 mins (II, IV). Corresponding representative gel pictures are also shown. *: A *vs.* B, and A' *vs.* B'; #: C *vs.* D, and C' *vs.* D' where ***P <0.0001; ###P <0.0001; #P <0.05.

Discussion

Direct visualization of AChE activity on polyacrylamide gels is still routinely practiced[6-9, 24]. It is in this context; our observed results hold importance.

Our *in silico* experiments have shown that 1-NA and 2-NA interact with the critical active site residues of AChE similar to ACh. 1-NA and 2-NA have interacted with anionic site residues (Trp 86, Glu 202, Tyr 133) (Suppl. Fig. 1). Studies suggest that the residue present at these sites hold a significant role in guiding the substrate to the esteric site of AChE for an efficient catalytic process[18,25]. There is evidence that goldscore is a determinant of fitness of small molecules at the ligand binding site[26]. Here, the goldscore (fitness score) of the best-ranked solution of 1-NA and 2-NA docked with AChE are comparatively higher than the best-ranked ACh-AChE complex. Higher goldscore indicates that 1-NA and 2-NA have better fitness at the active site of AChE in comparison to ACh. Therefore, the *in silico* results suggest that 1-NA and 2-NA can be considered as potential substrates for detection of AChE activity.

Pure AChE from human erythrocytes had shown enzyme activity bands with 1-NA and 2-NA. The enzyme activity bands formed with naphthyl acetates were prominent in comparison to ATCh (Fig. 1 & Suppl. Fig. 2). Moreover, 100 µM of 1-NA and 2-NA produced visible enzyme activity bands with pure erythrocyte AChE on polyacrylamide gels within 10 min while 100 µM of ATCh failed to visualize the enzyme activity prominently up to 12 h (Fig. 5). These results indicate that lower concentration (100 µM) of 1-NA and 2-NA can detect erythrocyte AChE activity on polyacrylamide gels. Therefore, 1-NA and 2-NA are more sensitive substrates in comparison to ATCh for the detection of erythrocyte AChE activity on polyacrylamide gels. This correlates with the *in silico* results where we have observed that 1-NA & 2-NA have better docking fitness score at the active site of AChE. Furthermore, our result is supported by the fact that pure AChE obtained from housefly is screened for esterase activity on the polyacrylamide gel using 1-NA as a substrate and stained to detect the enzyme activity using Fast Blue B[9]. In such experiment, similar to our study, in 10 min enzyme activity band was visualized.

The results obtained with hemolysate were analogous to the results observed with pure erythrocyte AChE. 1-NA and 2-NA (100 µM each) produced prominent enzyme activity bands with hemolysate AChE within 10 min of the addition of Fast Blue RR in comparison to the same concentration of ATCh stained by Karnovsky method. The band formation with ATCh was visible after overnight incubation with the staining solution. The enzyme activity bands were specific for AChE because it was observed that the bands were significantly inhibited in the presence of BW284c51 (specific AChE inhibitor) [21], eserine salicylate (non-specific cholinesterase inhibitor)[20] (Fig. 3) and organophosphorus pesticides (chlorpyriphos and dichlorvos; inhibits cholinesterase) (Fig. 4)[22,27,28]. It is further supported by the fact that chlorpyriphos inhibited enzyme activity was regenerated after the

oxime (2-PAM) treatment since oximes are known to reactivate cholinesterase (Fig. 4)[1,23].

It is known that acetylcholinesterase has DNase activity which is not inhibited in presence of specific cholinesterase inhibitors[16]. However, the naphthyl acetate esterase action of hemolysate is observed to be completely inhibited in the presence of cholinesterase inhibitors. The observations made in the reactivation study coupled with the results of the inhibition study proved that under the chosen experimental conditions the naphthyl acetates had produced enzyme activity bands specifically for AChE (Figs. 3 & 4). It may be argued that hemolysate contain esterases other than acetylcholinesterase (like esterase D) that cleaves 1-NA. However, within our chosen experimental condition at low 1-NA concentration (100 µM) esterase D is not expected to significantly cleave 1-NA because the Km of 1-NA with esterase D is high (around 2.1 mM)[29]. Consistent with the above fact, 215 µM of 1-NA has failed to stain esterase D activity on polyacrylamide gels[14]. Therefore, under the chosen experimental condition 1-NA behaved as a specific substrate for hemolysate cholinesterase and by our method, it is possible to stain hemolysate cholinesterase specifically. Further, the result of our inhibition study proved that akin to plasma, in hemolysate carboxylesterase induced cleavage of 1-NA is not significant[6].

It is noteworthy that 100 µM of ATCh did not form enzyme activity bands with the DTNB and DPIP[7,19]. However, 6 mM of ATCh with 3 mM of DTNB produced a single band with a dark background, which was not stable for 12 h[7]. High concentration of ATCh has the potential to be cleaved by thiolesterases widely distributed in all the human tissues[30]. Moreover, a high concentration of DTNB is known to inhibit AChE[1]. Therefore, the DTNB based approach is not recommended for routinely visualizing the AChE activity on polyacrylamide gels.

Our study has proved that 1-naphthyl acetate can act as a specific substrate for hemolysate cholinesterase and lower conentration (100 µM) of the substrate is required to stain cholinesterase activity within 10 min on the polyacrylamide gels.

It is particularly noteworthy that unlike hemolysate obtained from healthy individuals organophosphorus pesticide exposed hemolysate does not show enzyme activity band within 10 min when stained with the naphthyl acetates. Therefore, we propose that our developed method (particularly 1-NA) can be used for detection of cholinesterase activity of hemolysate on polyacrylamide gels.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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