Asian Journal of Research in Biochemistry

5(4): 1-9, 2019; Article no.AJRB.51987
ISSN: 2582-0516

The Organ Distribution, Characterization and Modification of Acetylcholinesterase Activity in Adult African Grasshopper: *Zonocerus* sp Linn.

E. A. Fajemisin[1*], O. S. Bamidele[1], S. O. Ogunsola[1] and E. A. Aiyenuro[2]

[1]Department of Biochemistry, Federal University of Technology, Akure, Nigeria.
[2]Department of Microbiology, Federal University of Technology, Akure, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors EAF, OSB, SOO and EAA designed the study and supervised the practical. Authors EAF and OSB managed the analyses of the study and wrote the protocol. Authors EAA and OSB performed the statistical analyses and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2019/v5i430097
Editor(s):
(1) Dr. Héctor Manuel Mora Montes, Departamento de Biología, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Guanajuato, México.
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Reviewers:
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(2) R. K. Lal, CSIR-CIMAP, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/51987

Original Research Article

Received 02 September 2019
Accepted 08 November 2019
Published 19 November 2019

ABSTRACT

Aim: To determine the organ distribution and characterization of acetylcholinesterase in the adult African variegated grasshoppers – *Zonocerus variegatus* and *Zonocerus elegans*. (*Zonocerus* Sp. *Linn*)
Place and Duration of the Study: The insect model: African variegated grasshoppers are gotten from the Open green fields at the Federal University of Technology, Akure, Nigeria, and research was carried out between March and June, 2016 in the Enzymology laboratory, Biochemistry department, Federal University of Technology, Akure, Nigeria.
Methodology: Twenty (20) adults variegated grasshoppers were taken from the Open field in the University community, and taken to the Biology department for Identification. After identification, the specimen was weighed, freeze, dissected into fractions (Head, Thorax and Abdomen) and then

**Corresponding author: E-mail: Emmafajems16@gmail.com;*

homogenized to get the crude protein extract. The crude enzyme extract is further purified using the Ion-exchange chromatography with column bed packed with DEAE – Sephadex A_{50}. The protein content of the purified AChE was determined using the Lowry method while the Acetylcholinesterase activity was determined by the Ellman's assay procedures. The characterization of AChE was tested by modifying agent such as N-Bromo Succinamide (NBS) which confirms the presence of key aromatic proteins involve in catalysis at the active site of the enzyme.

Results: The protein concentration according to their fractions: Head (35.7%), Thorax (29.2%), and Abdomen (35.1%). The AChE activity according to their fractions: Head (38.6%), Thorax (23.7%), and Abdomen (37.7%). The specific activity which relates the AChE activity to protein content is given: Head (28.8%), Thorax (40.4%), and Abdomen (30.8%). From the Organ distribution and AChE activity, it was observed that the Head Fractions has the Highest protein content, and Enzyme activity. Comparatively, there are slight differences in the Enzyme activity of the Head and Abdominal fractions which represents the two peaks in the AChE chart. As well, the thorax has the highest specific activity. The modification by the chemical agent NBS shows a drastic decrease (about 50%) in Enzyme activity and characterize enzyme active site with aromatic proteins especially tryptophan residues.

Conclusion: Research findings shows the dominance of AChE protein in the Head region, hence high enzyme activity (useful for nervous coordination) as well as presence of tryptophan residues at the enzyme active site. The importance of research is useful in enzymology, neuroscience and public health.

Keywords: African grasshopper; acetylcholine; acetylcholinesterase; enzyme activity; Ion- exchange chromatography; N-BromoSuccinamide (NBS); protein concentration.

1. INTRODUCTION

Acetylcholine (ACh) was the first chemical substance proven to be a neurotransmitter in a living cell [1]. It was identified in *Ascaris* and other nematodes in 1955 by Helen Mellanby, and was subsequently shown to be an excitatory transmitter at nematode neuromuscular junction [2]. ACh is a simple molecule that is synthesized from choline and acetyl-coA through the action of the choline acetyltransferase. Neurons (brain cells) that synthesize and release acetylcholine are termed cholinergic neurons [3]. When an action potential reaches the terminal button of a presynaptic neuron, a voltage-gated calcium channel is opened. The influx of calcium ions, Ca^{2+} stimulates the exocytosis of the presynaptic vesicles containing ACh, which is thereby released into the synaptic cleft. Once released, ACh must be removed rapidly in other to allow for repolarization to take place; this step; hydrolysis is carried out by the enzyme acetylcholinesterase [4,5].

Acetylcholinesterase (ACis the primary cholinesterase in the body which catalyzes the breakdown of acetylcholine (ACh) and other choline esters that functions as neurotransmitter in the living cell [6]. It belongs to the carboxyl esterase (hydrolase) family of enzyme which are found mainly at the neuromuscular junction (point of contact between the nervous system and the muscles) and in chemical synapses of the cholinergic types [7,8]. ACh hydrolyses and inactivates acetylcholine, thereby regulating the concentration of the transmitter at the synapse [9]. Termination of Ache activation is usually dependent on dissociation of acetylcholine from the receptor and its subsequent diffusion and hydrolysis, except in a diseased state where acetylcholine levels are limiting or under conditions of acetylcholinesterase (AChE) inhibition which increases the duration of receptor activation [10].

Grasshoppers comprises of a group of short horned insects that belongs to the superfamily *Acridoidea*, suborder *Caelifer*a within order *Orthoptera*. They are characterized by gradual metamorphosis, mouthparts made for chewing, and two pairs of wings, the anterior part being thick and leathery and covering the folded second pair [11,12]. Grasshoppers have a typical insect body plan of head, thorax and abdomen. Ion exchange chromatography is a process that allows the separation of ions and polar molecules based on their charge [13]. It can be used for almost any kind of charged molecules including large proteins, small nucleotides and amino acids [14]. The solution to be injected is usually called a sample, and the individually separated components are called analytes [15]. The basic

theory of separation in ion exchange chromatography depends upon irreversible adsorption of charged solute molecules to immobilized ion exchanged groups of opposite charge. It is often used in protein purification, water analysis and quality control [16].

Most studies carried on AChE have used human serum, electric eel and bovine red blood cell (erythrocytes) as enzyme source. *Zonocerus variegatus* as a source of AChE was, however selected and used here for this study [17]. The isolation of the enzyme, purification using ion exchange chromatography, determination of enzyme protein concentration and enzyme activity will be elucidated.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Twenty adult grasshoppers were gotten from an open field in the university community and taken to the biology department for identification as African Variegated Grasshopper (*Zonocerus variegatus*) and migratory elegant Grasshopper (*Zonocerus elegans*). They were kept inside the freezer to lower their body temperature, hence lowering their activities.

2.2 Immobilization and Decapitation of Specimen

The weight of 20 grasshoppers was determined using a weighing balance after which they were kept back in the freezer. Each grasshopper was dissected into three main fractions: Head, Thorax, and Abdomen.

2.3 Preparation of Crude Enzyme

Each fraction was placed in different bottle tubes and weighed using a weighing balance after which each fraction was homogenized in 50 Mm Phosphate buffer (1 g sample in 3 ml buffer). The homogenate was centrifuged at 10,000 rpm for 20 minutes at 4ºC. The supernatant gives the crude protein (crude enzyme) homogenate of each fraction. Each of the fractions are kept inside the freezer to preserve activity.

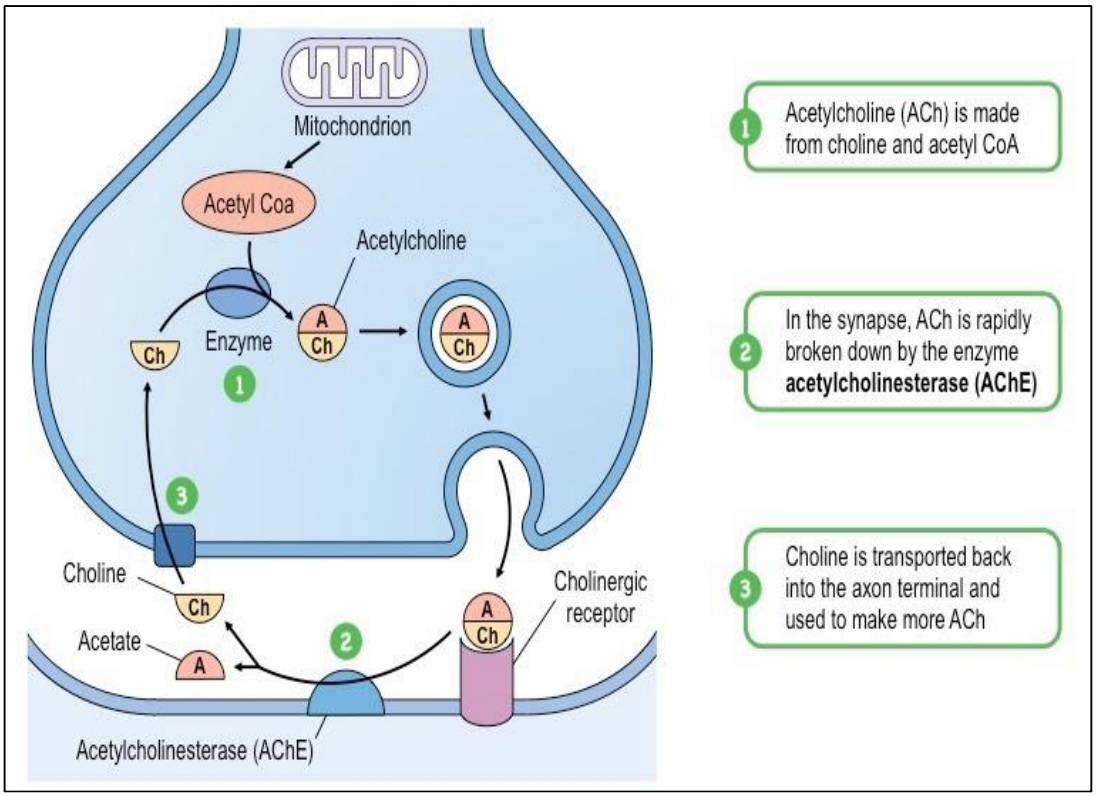

Fig. 1. Acetylcholine reaction in the synapse (source https://ib.bioninja.com.au)

2.4 Determination of Protein Concentration

The total protein content of each of the fractions was determined using Lowry method. 10 ml standard protein sample (having serum albumin) was used and 100l crude homogenate. To 20l of diluted protein, 480l of distilled water was added and 2.5 ml of reagent C. The reaction mixture was allowed to stand for 10 mins. Then 250 ml of reagent D (folin) was added. The resulting mixture was allowed to stand for 30 min and absorbance readout at 600 nm using a spectrophotometer.

2.5 Enzyme Separation and Purification

The separation and partial purification technique employed in this work was ion-exchange chromatography. The ion-exchange chromatography (1.5 * 25 cm) column was carefully packed with a bed of DEAE Sephadex A_{50} (diethyl amino ethyl Sephadex) and equilibrated with 25 mM phosphate buffer, pH 7.0. 5 ml fractions were collected at a flow rate of 2 ml/min. crude enzyme was applied gently unto the surface of the gel, the eluent was then collected 5 ml at a time. Afterwards, sodium chloride solutions (NaCl) were introduced in the bed to help elute the bound proteins. Buffers were also continually added to help move the extract down and prevent the gel from cracking. The absorbance of the eluent was read using the double beam UV/Visible spectrophotometer at 280 nm; the graph of absorbance against the tube number was plotted. Distinct peaks of slopes were combined to form pools. The protein content of the partially purified AChE was quantified using the Lowry method (7).

2.6 Determination of Acetylcholinesterase Activity

The acetylcholinesterase activity of each fraction (Head, Thorax, and Abdomen) was determined using Elman method (5). The mixture was placed in a water bath at 40ºC for 3 minutes after which DTNB was added. A blank sample was prepared in a similar way but without addition of DTNB. The absorbance was read out at 410 nm using a spectrophotometer.

2.7 Chemical Modification of Acetylcholinesterase

Acetylcholinesterase modification was carried out using the chemical agent, N-bromosuccinamide (NBS). Purified acetylcholinesterase from *Zonocerus variegatus* head was incubated at room temperature with different concentration of NBS (20M – 100M) in 50 mM sodium acetate buffer at pH 4.5, from freshly prepared stock solutions of NBS (1 mM). Aliquots were withdrawn at regular intervals of time for the assay of enzyme activity. Enzyme incubated in buffer in the absence of modifying agent served as control. The titration of accessible tryptophan residues in acetylcholinesterase with NBS was followed spectrophotometrically at 410 nm. The control cuvette had 50 Mm Sodium acetate buffer, pH 4.5 and the experimental had acetylcholinesterase in a total volume of 1.0 ml of 50 mM sodium acetate buffer.

3. RESULTS

3.1 Organ Distribution of Acetylcholinesterase

The acetylcholinesterase protein concentration and the activity of various organs of adult *Zonocerus variegatus* was determined respectively; the head was found to have 3.50 mg/ml and 0.055 mol/min/ml; the thorax was found to have 2.15 mg/ml and 0.045 mol/min/ml; and the abdomen was found to have 3.425 mg/ml and 0.055 mol/min/ml. it was observed that the head has the highest AChE protein concentration and hence the highest AChE activity. This is closely followed by the abdomen and lastly the thorax. Specific activity gives an indication of the ratio of the activity to its concentration and this is highest in the thorax.

Table 1. Table showing the acetylcholinesterase protein concentration and the activity of various organs of adult *Zonocerus variegates*

Organ	ACHE activity	Protein	Specific activity
Head	0.055	3.5	0.015
Thorax	0.045	2.15	0.021
Abdomen	0.055	3.425	0.016

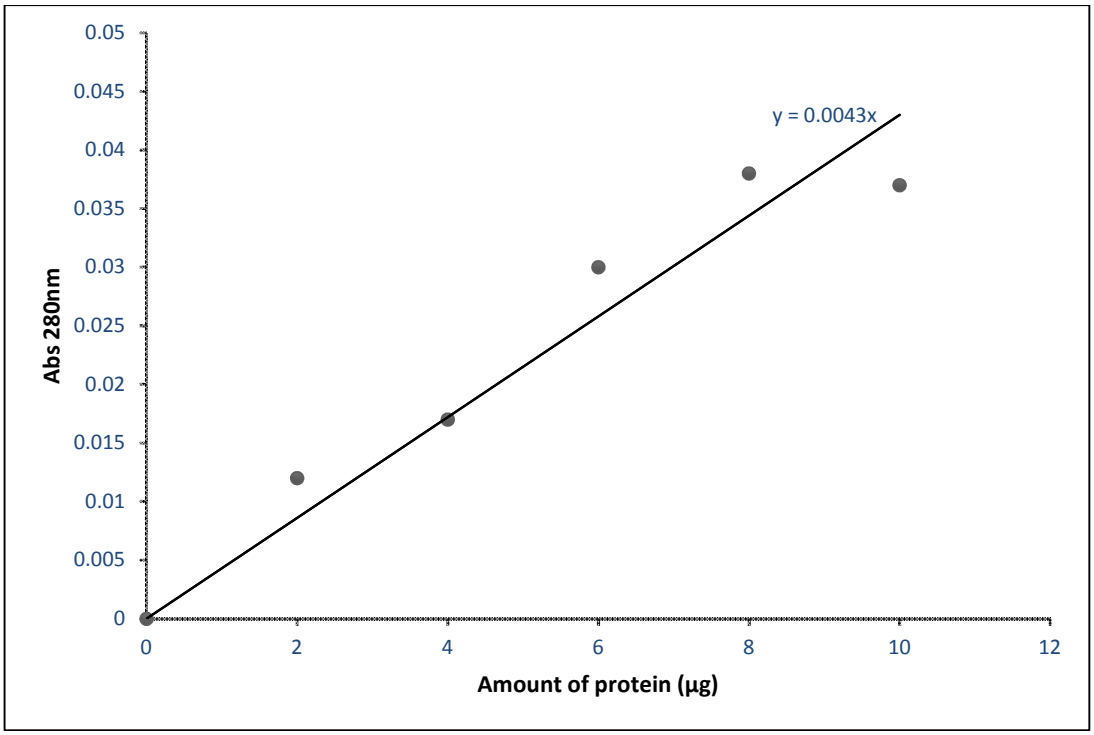

Fig. 2. Standard protein curve

3.2 Purification of Acetylcholinesterase

The graph shows the elution profile of the enzyme acetylcholinesterase gotten from the head of adult *Zonocerus variegatus* through the use of ion exchange chromatography using a column packed with DEAE sephadex A50. From the elution profile, the results indicate that after washing with 25 mM phosphate buffer at pH 7.0, 5 peaks were observed by reading the absorbance at 280 nm, indicating presence of proteins especially aromatic amino acids such as tryptophan. Also, acetylcholinesterase activity was carried out by pooling at two major peaks (8-16; 20-29) and presence of acetylcholinesterase activity was found to flow through the fractions as there are presence of peaks indicating AChE activity. When sodium chloride was used to wash the column, a major protein peak was observed which also contain acetylcholinesterase activity.

3.3 Chemical Modification of Acetylcholinesterase by N-BromoSuccinamide (NBS)

This plot (Fig. 4) shows the modifying roles of N-BromoSuccinamide (NBS) on acetylcholinesterase activity taken at absorbance 410 nm. The relative activity of unmodified acetylcholinesterase activity is 100% using different concentration of NBS (20M to 100M), the relative activity was found to drastically reduce to half (i.e. 50%) or less. Although, there are various peaks depending on the concentration of NBS used on the acetylcholinesterase showing the different rate of modification, it can be concluded that this modification can lead to drastic reduction and/or loss of enzymatic activity.

3.3.1 Equations

The results obtained from the protein standard curve measures the protein concentration from the absorbance of each solution using spectrophotometer. Beer – Lamberts law states that absorbance of a solution is directly proportional to the concentration of the absorbing medium and to the sample pathlength. Mathematically,

$$A = £CL$$

Where A is the absorbance (has no units), £ is the extinction co-efficient (a constant of a compound having units of $Lmol^{-1}cm^{-1}$), C is the Concentration (units in M), and L is the pathlength (units in cm).

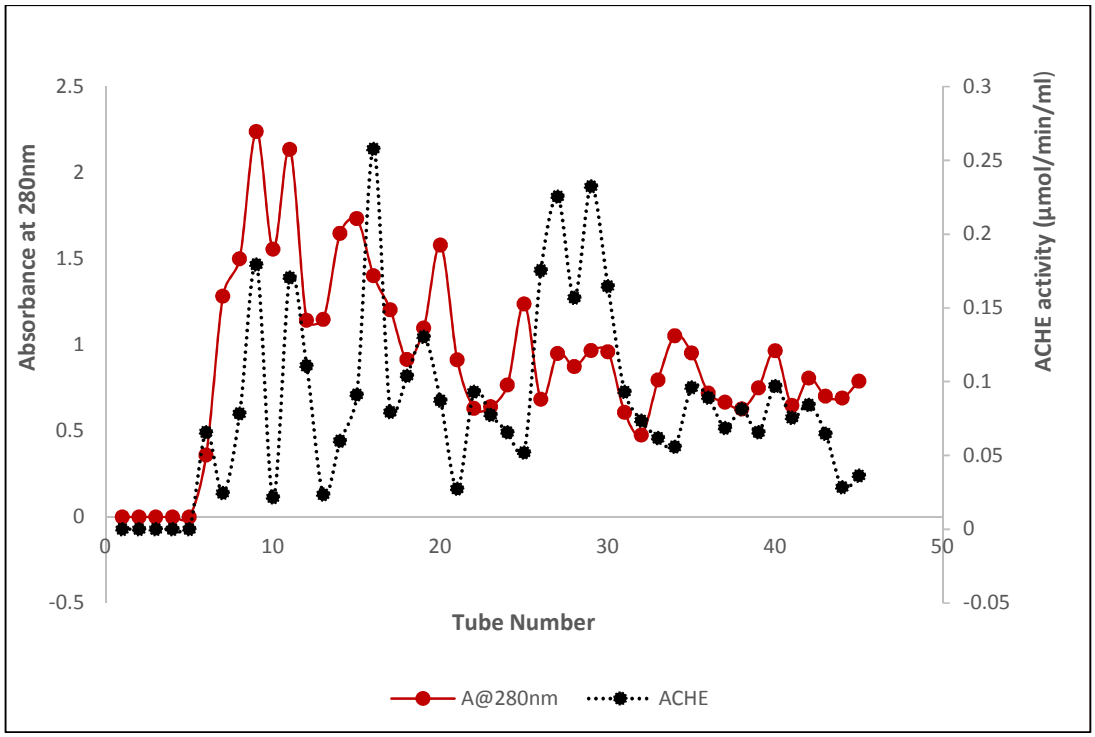

Fig. 3. Graph showing the elution profile of the enzyme acetylcholinesterase gotten from the head of adult *Zonocerus variegatus* through the use of ion exchange chromatography using a column packed with DEAE sephadex A50

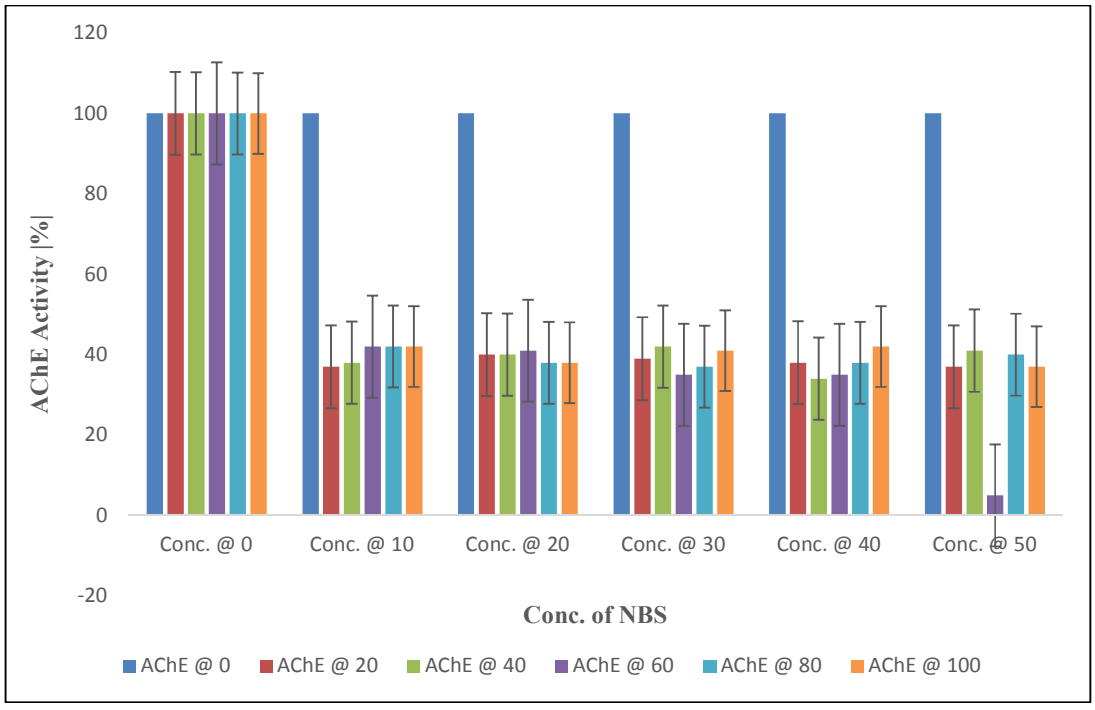

Fig. 4. Chart showing the modifying roles of N-BromoSuccinamide (NBS) on acetylcholinesterase activity taken at absorbance 410 nm

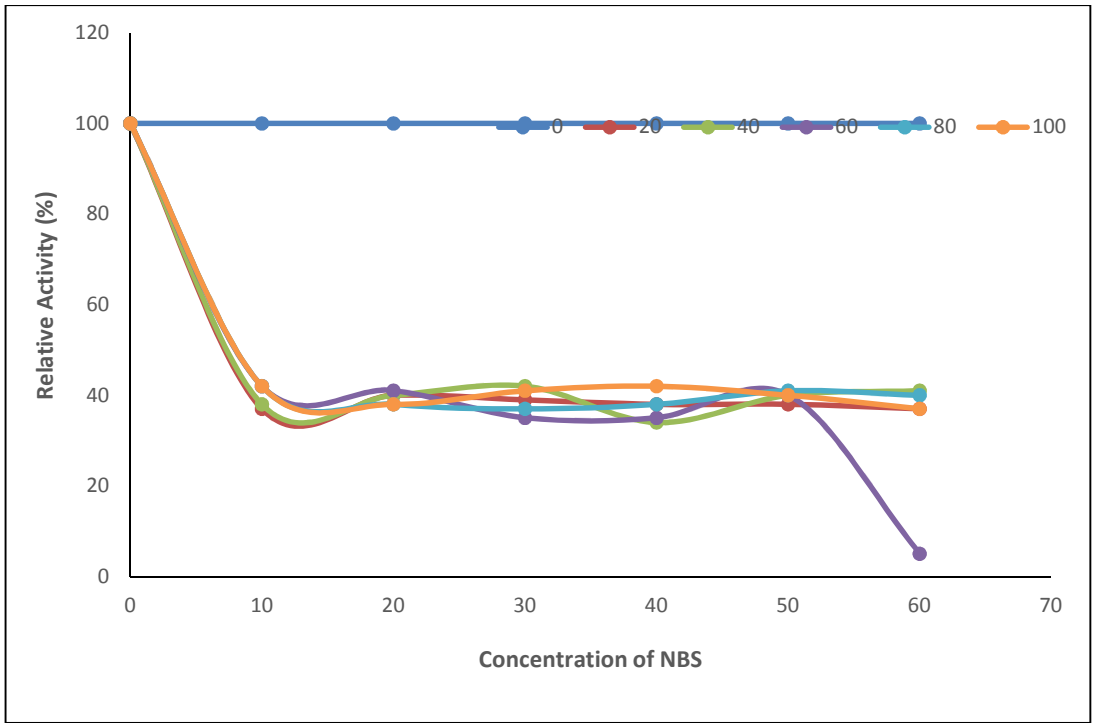

Fig. 5. Chart of N-Bromosuccinamide on acetylcholinesterase activity

4. DISCUSSION

The straight-line graph that pass through the origin obeys beer-lamberts law [18]. The slope of the graph on extrapolation is 0.0043 and the protein concentration in 1000 µL of the purified Acetylcholinesterase (AChE) was calculated to be 3.50 mg/ml. Furthermore, the results gotten shows the AChE activities in the Head, Thorax, and Abdomen Regions to be 0.055, 0.045, and 0.055 µmol min^{-1} mg^{-1} protein respectively. The organ distribution and characterization of the enzyme through graphical plots and extrapolation has showed protein concentration and enzyme activity dominance in the head region and abdominal regions. The dominance in the head and thorax can be attributed to the link in neuro-muscular actions that takes place between the brain and the body part of the specimen [19]. This is also a true case for most models ranging from insects to mammals.

The fractions of purified acetylcholinesterase with the major and minor peaks pooled separately at 410 nm were read. The graph shows that the activity of the enzymes is in the two pooled peaks. The major peak in the elution profile shows acetylcholinesterase activity of 0.280 M/min/ml. This shows considerable elution of the pure enzyme from its crude homogenate under considerable standard conditions [20].

The reaction of NBS on the enzyme acetylcholinesterase is rapid as observable in the sharp downward curve and the indole side chain of the tryptophan residues that absorbs at 410 nm is converted to an oxindole, a much weaker chromophore at this wavelength. The enzyme activity in the presence of varying concentration of NBS was found to drastically reduce (to approximately 50%) due to the modification of the tryptophan residues present at the active sites of Acetylcholinesterase [21]. This is very significant as observable with most chemical agents like organophosphates and carbamides on enzymes like Acetylcholinesterase (ACHE) as intense exposure can lead to denaturation [22]. This can be visible in cases of neurodegenerative dysfunction like Alzheimer's disease and Schizophrenia accompanied by rapid lack of psychomotor coordination of the body in larger animate models like mammals especially humans [23].

5. CONCLUSION

From the experiment, acetylcholinesterase purified from Zonocerus variegatus was found to

have high enzyme content and activity in the head as found in other insects. This suggests that Z. variegatus is a good source of acetylcholinesterase for assays; and the organ serve as a control center for insect coordination and other metabolic processes. It can also be inferred that this organ is the primary target of most chemical agents that target acetylcholinesterase used in the control of organism, usually when it is a pest. The reduction in enzymatic activity was due to the modification of tryptophan residues at or near the enzyme active site, therefore it can be inferred that tryptophan residues and other aromatic amino acids are present at the active sites of the acetylcholinesterase enzyme functioning both structurally and catalytically to enhance neuromuscular coordination.

Acetylcholinesterase is a vital enzyme in the organs of the living organism, Zonocerus variegatus, most especially the head. It should however, be further or completely purified using more viable methods of purification like SDS – PAG electrophoresis. Likewise, further analysis using the required methods should be used in determining the functions of each isoforms of acetylcholinesterase in the head of the adult insect. Furthermore, researches should be carried out on other potential chemical agents that modifies acetylcholinesterase activity and analyze considerable health impacts which will be beneficial for further research in the field of toxicology, neuroscience, and environmental health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/51987