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ASSAY OF ACETYLCHOLINE ESTERASE ENZYME ACTIVITY BY TITRIMETRIC METHOD

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ABSTRACT

Acetylcholine esterase (EC 3.1.1.7) is an enzyme, actively involved in cleaving the ester bond found in the molecule acetylcholine (a neurotransmitter). The enzyme plays an important role in detecting a wide array of neurodegenerative disorders like Parkinson's disease, Alzheimer's disease and Huntington's disorders. An easy and effective assay is required to monitor the enzyme activity; which we have achieved by establishing a titrimetric assay that helps in determining the activity of the enzyme. The assay involves the use of an alkali to neutralize the carboxylic acid moiety released by the activity of the esterase enzyme on the substrate. A chromogenic indicator is used to detect the end point.The enzyme activity values were checked in comparison with the values obtained from Ellman's method. The values obtained from both Ellman's and the titrimetric methods are in agreement when produced under similar lab conditions. The percentage difference between the values form the Ellman's method and Titrimetric method for Zebrafish brain acetylcholine esterase enzyme was found to be 1.53% and for the RBC acetylcholine esterase it was found to be 1.60%.

KEY WORDS: **esterase enzyme, titrimetric assay, neutralize the carboxylic acid, enzyme activity**

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INTRODUCTION

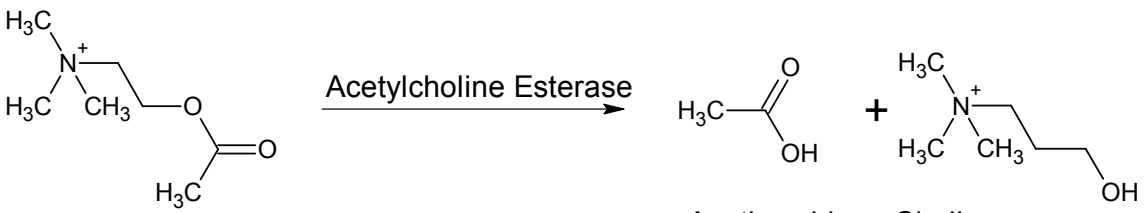
A series of assay have been established like the Ellman's method and Assessment of Acetylcholinesterase Activity Using Indoxylacetate require several steps to establish the activity of an esterase enzyme from an enzyme homogenate [7]. Though the protocols used provide a good estimate of the enzyme activity they require the addition of certain enzyme inhibitors that can hamper the optical density of the medium and in turn reducing the precision of the values while employing spectrophotometric methods. Therefore, an easier method is required to determine the enzyme activity. In situ performance of a lot of the previously established procedure is inconvenient keeping in mind the degree of instrumentation required. Another common set back of the previously established protocols is that they employ the use of certain reagents that are expensive and require solicit maintenance; this does not favour small labs in performing the assay. Our assay stands apart from the previously established assay as it employs minimum instrumentation and the cost of the reagents used is several times less when compared to a lot of the previously established protocols. The procedure is so adjusted that labs with minimum facilities or small wet labs can easily perform the assay with a good degree of accuracy and precision.

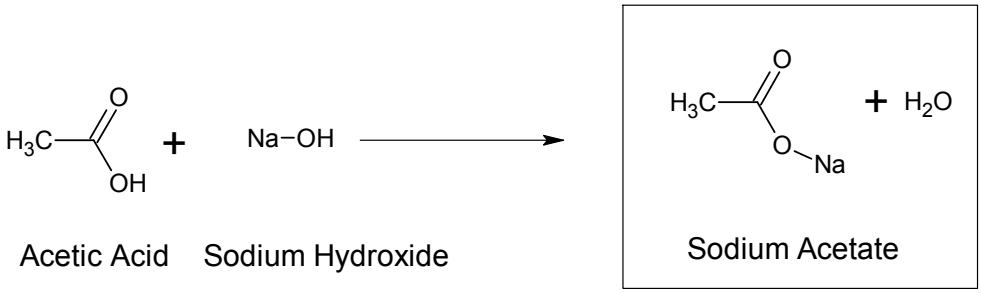
REVIEW OF LITERATURE

Esterase enzymes are commonly found on all floors of the living world [3]. The enzyme is employed by various life forms in several ways. These esterase enzymes are chiefly involved in cleaving an ester bond resulting in formation of alcohol and carboxylic acid as chief products. Acetyl choline esterase is one such enzyme that is found in the central nervous system of animals.[1, 8] This enzyme was first isolated from electric eels by Walo Leuzinger[6]. Later in 1991 Joel Sussman configured the 3D structure of the enzyme [9]. The activity of enzyme plays a major role in diagnostic purposes to establish normal cognitive and motor functions in C.N.S related diseases [5, 7]. A common protocol has been established that can help find the activity of all the esterase enzymes irrespective of their source (with appropriate substrates or substrate analogues).One of the common titrimetric methods followed by far to assay the enzyme is the one formulated by Schwartz M and Myers TC[10] Their assay is based on similar principle except for the use of the chromogenic indicator, a pH meter coupled with an electrode which makes the stirring of the reaction mixture while the addition of the alkali a clumsy process resulting in improper results because of improper stirring or mixing.

PRINCIPLE OF TITRIMETRIC APPROACH

Acetylcholine esterase enzyme hydrolyses the ester bond found in Acetylcholine molecule. The hydrolysis of this bond results in the formation of acetic acid and choline[2]; the acetic acid liberated by the hydrolytic action of the enzyme can be estimated by titrating it against an alkali (NaOH in this case) which in the presence of phenolpthaline gives a pale pink colour when all the acetic acid liberated is neutralized. Therefore the assay provides the amount of the product formed; which is instrumental in determining the enzyme activity.





The mixture turns pink in the presence of Phenolpthaline indicator as the Acetate liberated is completely neutralized as the alkaline content increases indicating the end point of the reaction. (Reactions drawn using Chem-sketch free ware)

MATERIALS

1. Ellman's reagent(5-(3-Carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid), Acetylcholine iodide were purchased from sigma.
2. Zebrafish were procured from a local supplier. The fish were stored in a 20L aquarium and fed regularly for a span of two weeks before the commencement of experiments.

METHODS

Euthanization of Zebrafish

Zebrafish were euthanized in accordance to guidance laid down by NIH by using 5 parts ice and one parts water and leaving the fish in the ice bath for instant euthanization and exposure was continued for 5-10 minutes to ensure death by hypoxia [4, 5]. Once the fish showed complete cease of opercula movement, the blood and brain were isolated for further experimental purpose.[4, 5]

1. Preparation of enzyme suspension from Zebrafish Brain

The brain isolated from the fish were homogenised with 0.01M Phosphate Buffer at pH 7.2 and centrifuged at 5000rpm for 10min at 4^0C. Supernatant was used to perform the assay of acetylcholine esterase enzyme by Ellman's method and the titrimetric assay we have established [5].

2. Isolation of Blood from Zebrafish

Euthanized fish were placed on a cavity slide, a cut was made beneath the operculum with a sterile surgical blade and the blood was carefully pooled into the cavity. Using a sterile micropipette tip the blood pooled in cavity was transferred into a sterile Eppendorf tube per-coated with Heparin. The blood was used to perform assay of acetylcholine esterase by Ellman's method and the titrimetric assay we have established [4].

3. Assay of acetyl choline esterase by Ellman's method

Ellman's assay of acetyl choline esterase was performed by adding 200µl of the prepared enzyme suspension to 100 µl of 3.3mM DTNB [5,5'-Dithiobis(2-nitrobenzoic acid)] prepared in 0.1M pH 7.2 Potassium phosphate buffered solution with 6mM Sodium Bicarbonate. The mixture was allowed to stand at 25^0C for a span of 20 minutes[2, 5]. To this mixture 100µl of acetylcholine iodide was added and the change in absorbance was monitored using a spectrophotometer at 412nm at 1min intervals. A blank was prepared using 100 µl of 3.3mM DTNB [5,5'-Dithiobis(2-nitrobenzoic acid)] prepared in 0.1M pH 7.2 Potassium phosphate buffered solution with 6mM Sodium Bicarbonate and 100µl of acetylcholine iodide[2].The same procedure was followed for blood by adding 10µl (of the RBC layer instead of 200µl of the prepared enzyme suspension) and 190µl 0.01M Phosphate Buffer at pH 7.2. The enzyme activity was calculated by using molar extinction coefficient of acetylcholine [2].

4. Assay of acetylcholine esterase enzyme by titrimetric method [a new assay established for assessment of Acetylcholine esterase enzyme]

For the titrimetric assay, 5ml of 10mM acetylcholine iodide was taken in a 50ml conical flask and to this 200µl of the prepared enzyme suspension was added. To perform the assay with RBC, 10µl of the RBC was added into a conical flask containing 5ml of 10mM acetylcholine iodide. Both of the mixtures were incubated at room temperature

for a span of 5 minutes, and the reaction was stopped by placing the conical flask in a boiling water bath for a 2 minutes (optimum time required to completely cease the enzyme activity {established by multiple trials}). The mixture was rapidly cooled and to these 2drops of phenolpthaline indicator was added and titrated against 0.05M NaOH taken in a micro-burette till a persistent pale pink colouration was formed; indicating the end point of the reaction.

Amount of acetic acid liberated by the enzyme's esterase action was found using the formula:

$N_{CH3COOH}\, V_{CH3COOH} = N_{NaOH}\, V_{NaOH}$.

The enzyme activity was calculated using the formula [11]:

$$\text{Enzyme Activity} = \frac{(\text{amount of product formed}) \times 2 \times (df)}{(\text{Molecular weight of substrate}) \times T}$$

df - Dilution factor

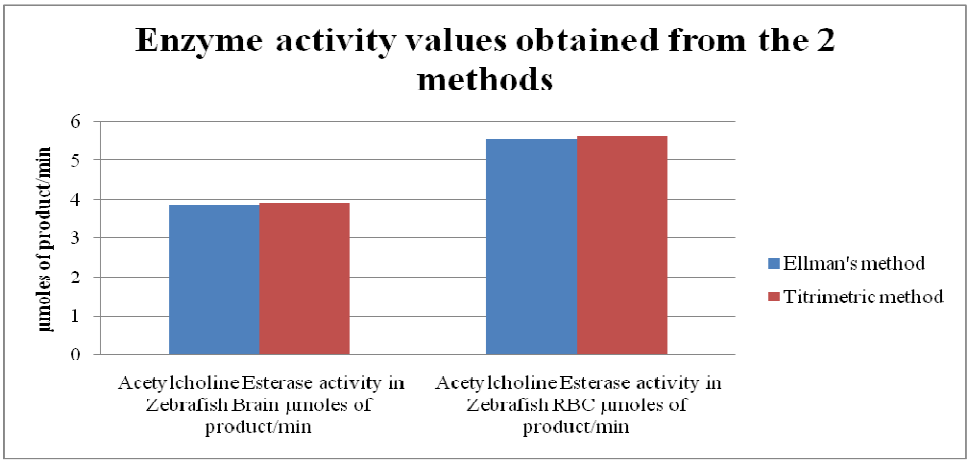
T - Time of assay in minutes or seconds

Note: The values were obtained when the experiment was run at room temperature and incubation time was 5min. Triplicates were run simultaneously and the concordant titre value was considered for calculating the enzyme activity.

RESULTS & DISCUSSION

The rate of acetylcholine hydrolysis was determined using the Ellman's method and the titrimetric method. Both the methods gave values well in agreement with each other. The mean value of the two methods with the SEM is discussed in the tabular matrix given below. (The data tabulated in the matrix are a mean of 25 sampling units)

Enzyme activity values obtained from the 2 methods		
SAMPLE	Ellman's Method µmoles of product/min± SEM	Titrimetric Method µmoles of product/min± SEM
Zebrafish Brain Acetylcholine Esterase	3.87 ± 0.049	3.93 ± 0.074
Zebrafish Red Blood Cell Acetylcholine Esterase	5.54 ± 0.097	5.63 ± 0.072



Graph 1

A bar graph showing the values of enzyme activity in Zebrafish Brain and RBC samples obtained by Ellman's method and the Titrimetric method. (Values represented in the graph are mean of 25 sampling units)

The assay is reproducible and at standard lab conditions gives values similar to those obtained from Ellman's Method. The cost of performing the assay using the titrimetric method was found to be reduced by 63% with no compromise with the level of accuracy or precision. Therefore, the titrimetric assay we have established is reliable, reproducible and cost effective. Owing to the simplicity, accuracy and reproducibility of the assay students, researchers, scientists and industries can employ the assay and benefit from it. Difference between the Titrimetric method and Ellman's method for Zebrafish brain acetylcholine esterase enzyme activity was 1.53% and for the RBC acetylcholine esterase it was found to be 1.60%.

CONCLUSION

The assay established in here is useful in estimating the activity of a wide range of esterase enzyme. Assay is easy and convenient to perform and involves a lesser degree of instrumentation. The assay is not just limited to Acetyl choline esterase enzyme but can be performed with all enzymatic reactions which involve the release of a carboxylic acid as one of the end product. The values of enzyme activity obtained by following the titrimetric method are in concordance with the values obtained from other similar assays, therefore the titrimetric method stands as an effective assay for the monitoring of enzyme activity.

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