CHARACTERISATION OF AN ASSAY FOR KINETIC STUDIES OF ACETYLCHOLINESTERASE

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SUMMARY

The effect of different enzyme and substrate preparation on the parameters (akm, aVm) of acetyl cholinestrase (AChE) (EC. 3.1.1:7) were estimated. Time for preincubating enzyme with 5,5 dithio-2-nityrobenzoic acid (DTNB) were also assessed. The effect of storage on activity of AChE was determined. Moreover the unsatisfactory result of storage of substrate and DTNB on ice during assays were also reported.

INTRODUCTION

The AChE of human erythrocyte is described as an elongated structure, however in the membrane, the molecules of the enzyme appears to be spherical in shape and dimeric[1,2]. They are presumed to be present in the other lipid bilayer of the membrane[3] with their active centres exposed outwards[4], either as individual or in chains or a large aggregates[5], Nevertheless the enzyme may still be called a peripherointegral glycolipoprotein[6].

The molecular and kinetic properties of the erythrocyte AChE are known to depend on cell intactness and on the nature of the microenvironment of the enzyme in the membrane.[6,7,8] There seems to be a correlation between membrane changes and the structure and the behavior of the enzyme. The kinetic properties of the human erythrocyte AChE are not immutables, they could be used to follow changes in the integrity and dynamics of the intact membranes and isolated membranes both in vitro and in vivo as well as in the organisation of the enzyme.[6,8]

MATERIAL AND METHODS

For the study, the method of Ellman et al[9] was chosen and assays were run at the physiological pH of 7.4 and tempera 30C. The substrate acetylthiocholine lodide (ATChI) the colour reagent 5,5-dithiobis -(2 nitrobenzoate) (DTNB) were obtained from E.Merck(Germany). All other chemicals (A grad) were either from Sigma (U.S.A.) or BDH (U.K.).

Enzyme Preparations

Blood was collected by sterile venipuncture and added to acid-citrate-dextrose solution 0.44% citric acid (w/v) 1.32% sodium citrate (w/v) and 1.47% glucose (w/v) in the proportion of four parts of blood to one part of the solution. The plasma was separated immediately after collection of samples and the erythrocytes were washed three times with 10 vol. of ice cold 0.9% (w/v) NaCl.

Stock haemolysate was prepared by adding 0.04% packed washed erythrocytes to ice cold water. After 15 minutes the stock was diluted with an equal volume of ice cold phosphate

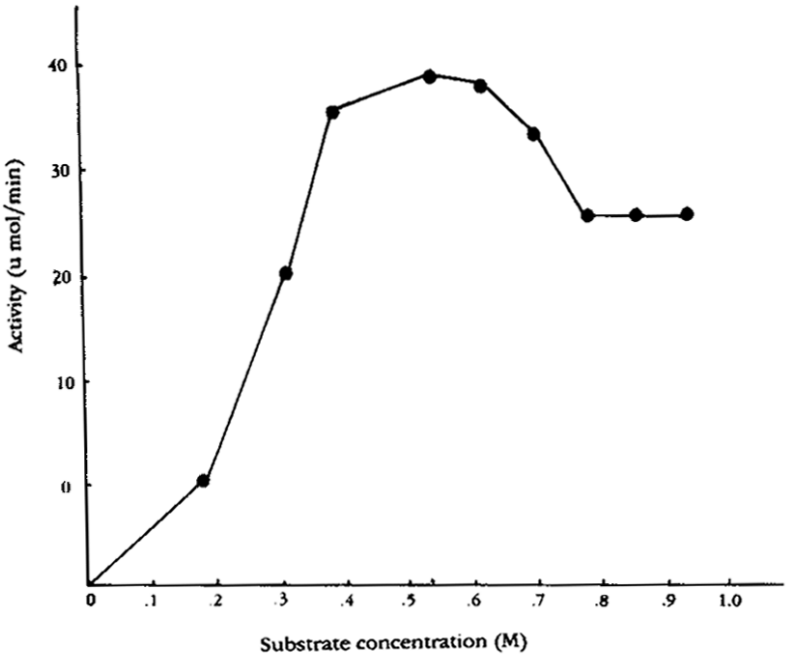

Fig 1. Effect of substrate concentration

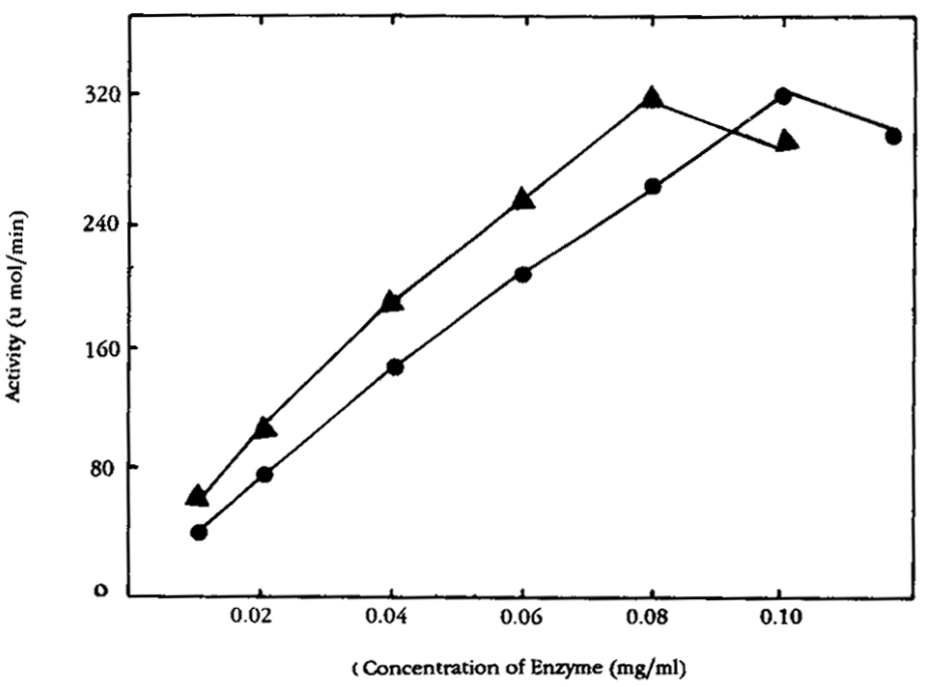

Fig. 2 Relationship between activity and enzyme concentration on of haemolysate (▲) and whole suspension (●)

buffer (0.2M,pH 7.4). The whole cell suspension was prepared by suspending 0.02% packed washed erythrocytes in ice cold phosphate buffer (0.1 M, pH 7,4).

Estimation of Enzymic Activity

The activity of AChE was assayed in replicate at 30C and pH 7.4 with ATChI (160 M) as colour reagent, using spectrophotometer at 412 nm. wave length. All the assays were run at two concentrations of substrate one much lower (S_1 = 10 u mol/L) and the other much higher (S_2 = 200 u mol/L). The enzyme parameters aKm and aVm were calculated by the following equation, derived from s/v versus S plot.

$$aKm = \{(Sv1/V_{s1})(S_2 - S_1)/(S_2/Vs_2) - (S_1/Vs_1)\} - S_1$$

$$\text{and } aVm = 1/\{(S_2/Vs_2) - (S_1/Vs_1)/(S_2 - S_1)\}$$

Where Vs_1 and Vs_2 represent absolute activities at S_1 and S_2 respectively.

RESULTS

1. Effect of substrate concentration upto 1 ml on rate of reaction. (Fig. 1) It was found that the rate increased with the concentration of the substrate up to 300 mM, it was of zero order between 10-300 mM, and declined when the concentration of the substrate was increased further. However on increasing the concentration of substrate up to 1 M, the rate again became steady. It is concluded that routine working assays should be run up to 300 mM substrate.

2. Effect of enzyme concentration on rate of reaction (Fig. II) The activity of the haemolysate increased linearly up to an equivalent of 0.04% of packed erythrocytes (0.08 mg/ml of HbO_2). When the concentration of the enzyme preparation were increased further, the activity declined rapidly. These findings suggest that a high concentration of the enzyme preparation, such as haemolysate (0.08 mg/L of HbO_2) or an erythrocyte suspension with more than 0.04% of packed cells, could diminish the apparent absorbance of the coloured compound (5, thio- 2-nitrobenzoate anion) at. It is therefore, an optimum concentration of the enzyme preparation (equivalent to about 0.03% cells). Beyond this concentration there were slight changes in the apparent Michaelis Menten parameters (Kapp. and Vapp.) of the enzyme.

3. Effect of preincubating enzyme with DTNB. When DTNB was allowed to react with haemolysate at 30C in the absence of substrate and absorbance was followed till it reached to a plateau (Table I). It showed that the reaction was essentially complete after 10-22 minutes. When the same experiment was repeated with ghosts and whole cell suspensions prepared from freshly washed erythrocytes, there was no observable reaction, suggesting that these preparations contained no free thiol groups. It was therefore, concluded that prior to the addition of the substrate, DTNB must be preincubated with haemolysate for at least 15 minutes at 30C, in order to allow it to react with the free thiols.

Table

DTNB (M)	Reaction time to plateau (min)
16	22
32	18
80	14
160	12
320	10

Effect of pre-incubating haemolysate with DTNB. DTNB was added to the haemolysate and the absorbance was followed till it reached to a plateau at 30C.

4. Effect of storage on enzyme, substrate and DTNB during assay: The stability of the enzyme was tested for 24 hours. The result suggested that the activity of the enzyme slightly decreased when stored at room temperature, where as the activity was normal for 8 hours if kept on ice. In contrast, storage of substrate and DTNB on ice during assays gave unsatisfactory results done, slight crystallization.

DISCUSSION

For the study of advance kinetics, the assay preparations were thoroughly investigated. The results showed that the erythrocyte AChE is a suitable enzyme for detailed kinetic studies because it is able for at least 8 hours at 0C thereby allowing many replicate measurements to be made. It does however, have some disadvantages, the effect of a high concentration of the enzyme preparation (haemolysate) on the absorption coefficient of the coloured product, at a high concentration of the enzyme, activity is not linear. The presence of DTNB in the assay because preincubation is secondly required and where as extended preincubation leads to inhibition of the enzyme.

The concentration of DTNB is also important because DTNB can inhibit the enzyme and yet if the concentrations were too low the reaction rate would be under estimated. The concentrations chosen was therefore, also controlled, being high enough to render the coupling reaction rapid and yet low enough not to inhibit the enzyme.

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