Use of spectrophotometry in enzyme kinetics

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An enzyme catalyses the conversion of a substrate to a product. The rate of the catalysed reaction or the activity of the enzyme can be determined by measuring either the decrease in substrate concentration or the increase in product concentration as a function of the reaction time. The development of an enzymatic reaction is monitored by the alteration in absorbance as a function of time – due to the differing absorbance values of the substrate and product. As absorbance changes correspond linearly with the changes in concentration, these changes can be calculated directly from the absorbance data (when provided with the absorption coefficients of the reacting species).

If there is no significant difference in absorbance values of the substrate and product, colour derivatives can be synthesized with chromophoric reporter groups. As such, a ‘ silent’ enzyme reaction with a colourless product can be linked with another enzyme reaction that uses the product of the initial enzymatic reaction for a conversion that initiates a change in absorbance. The introduction of an NADH-consuming or NADH-producing reaction is often employed as changes in NADH concentration are easily followed by the strong change in absorbance at 340 nm.

Alkaline Phosphatase

One of the first enzyme determinations to gain pervasive acquiescence in clinical medicine was the measurement of alkaline phosphatase activity and it is still widely used today. Alkaline phosphatase catalyses the cleavage of ester bonds in phosphoric acid (Figure 1).

Human serum alkaline phosphatase can hydrolyse natural phosphates found in bone, kidney and intestines. P-nitrophenylphosphate can be hydrolysed by

this enzyme to yield the alcohol p-nitrophenol, which absorbs strongly in alkali at 405nm. Two problems encountered in many past and currently used alkaline phosphatase methods are (1) the long incubation times required due to the extremely low concentration of alkaline phosphatase in serum; and (2) phosphate inhibition either from endogenous orthophosphate in the sample or from that released during the hydrolysis of the substrate. Therefore a new method should minimize the influence of these two factors, and ideally, any spectrophotometric procedure should be based upon the measurement of a highly absorbing reaction product at a wavelength where the substrate has negligible absorbance. Fortunately, hydrolysis of the colourless substrate p-nitrophenolphosphate in alkaline solution, to form the highly coloured yellow product p-nitrophenolate ion, fulfils these requirements.

In this experiment, the kinetics of hydrolysis of P-nitrophenolphosphate by alkaline phosphatase will be measured.

The activity of these enzymes can be measured by following the liberation of phosphate or of the other product released by hydrolysis. The assay can be simplified by using a substrate whose phosphate-free product is highly coloured. In this experiment, we will utilize p-nitrophenol phosphate as the substrate, which upon hydrolysis releases phosphate to generate p-nitrophenol under alkaline conditions. P-nitrophenol has a high molar absorptivity at 405 nm (e405 = 18. 8 x 103 M-1cm-1).

The purpose of this research endeavour was to determine the use of enzymes in biological systems, to use a spectrophotometer to assess the kinetics of the enzyme alkaline phosphatase, as well as to analyse the

effects of incubation time, enzyme concentration and also to determine the optimal pH at which alkaline phosphatase acts.

The success of such a method depends upon (1) the use of a suitable analytical procedure for the precise determination of small amounts of reducing sugar, and (2) a careful standardisation of the technique for mixing enzyme and substrate, withdrawing samples, and arresting the reaction in these samples.

Materials and Methods

Construction of Standard p-Nitrophenol Graph

The standard consisted of a series of test tubes of final volume 11. 1ml. Volumes 0. 1mM p-nitrophenol 0, 0. 5, 1. 0, 1. 5, 2. 5, 3. 5, 5. 0, 7. 0, 10. 0 were added to each test tube. 0. 02M NAOH sufficient enough to bring the final volume up to 11. 1ml was added. Test tubes were mixed by inversion and quantified against the blank sample using absorbance determinations conducted on a spectrophotometer at 405nm. Standard curve preparation is both labour intensive and error prone, with quantitative accuracy being dependent on both the accuracy of standard quantification and the quality of standard curve construction. http://www. ncbi. nlm. nih. gov/pmc/articles/PMC169985/

Effect of Incubation Time on Enzyme Activity

Buffered substrate (5. 5mM p-nitrophenylphosphate in 0. 05M glycine buffer containing 0. 5mM MgCl2 at pH 9. 5) was equilibrated at 370C for 10 minutes before being added to 10. 1 ml 0. 02M NaOH. Upon addition of the enzyme,

the tube was capped, inverted and incubated at 370C. After 5, 10, 20, 30 and 40 minute intervals, 1. 0ml of the reaction mixture was transferred to a tube containing NaOH and was measured spectrophotometrically at 405nm.

Effect of Enzyme Concentration on Substrate Hydrolysis

Varying concentrations of distilled water and enzyme solution were added to test tubes filled with buffered substrate solution and incubated at 370C for 30 minutes. After 30 minutes, 10. 0ml NaOH was added to inactivate the enzyme and the absorbances were read at 405nm.

Effect of pH on Enzyme Activity

0. 5ml of buffer (at varying pH) were added to 0. 5ml of 11mM unbuffered substrate solution and incubated at 370C. After 5 minutes, 0. 1ml of selected enzyme solution was added and reaction mixtures were left to incubate for 30 minutes. After the incubation period, 10. 0ml NaOH was added to each test tube and the absorbance was read at 405nm.

Discussion

Effect of incubation time on Enzyme Activity

The effects of different incubation times on the kinetics of alkaline phosphatase are summarized in Table 2. The rate of reaction for each time period was calculated and results were displayed in Table 3. Upon examination of results, it is clear that the rate of product generation slowed as time went on. The linear relationship is lost and a clear polynomial relationship is seen between incubation time and production of p-nitrophenol. During the initial 5 minutes, 5. 57 umoles of p-nitrophenol was

generated at a rate of 1. 114 umol/ min, producing over 2-fold more product than in any other time-bracket. The rate of reaction of alkaline phosphatase decreased during the period 0-10 minutes to that of 0. 223 umoles/ min. There is little variation in the amount of end-product created in the 10-40 minutes and the graph plateaus (SD= 0. 06). One possibility for the loss of linearity could be that the substrate concentration has decreased significantly after the first 5 minutes of the reaction, resulting in the slower reaction rate. Overall, the product concentration decreases as incubation time increases. This " falling-off" in rate is a common hinderance in enzyme kinetic studies and to overcome this, many measures are often only conducted in the initial period.

Effect of Enzyme concentration on Substrate hydrolysis

The effects of different enzyme concentrations on substrate hydrolysis of alkaline phosphatase are summarized in Table 4. A clear linear relationship can be observed from Figure 5. As increasing concentrations of alkaline phosphatase are added to the reaction mixture, the velocity of substrate hydrolysis increases exponentially. According to the Michaelis theory, the reaction velocity would be expected to be proportional to enzyme concentration so long as the concentration of enzyme remains small compared with that of the substrate. The relationship seen in Figure 5 is in close agreement with that predicted by the Michaelis theory. Additionally, Eadie [1926]'s work highlighted the relationship between the level of activity of the enzyme (i. e. the reaction velocity with high substrate concentration) and amount of substrate concentration required for the reaction to occur. Therefore, in order to further the understanding of alkaline phosphatase

kinetics, the author suggests the introduction of measures investigating the level of activity of alkaline phosphatase. In summary, the enzymatic hydrolytic power increases progressively during the period of increasing enzyme concentration.

Effect of pH on Enzyme Activity

The effects of pH on enzyme activity are summarised in Table 5. Figure 6 highlights the rate of product generated in varying alkali conditions. As stated before, alkaline phosphatase, as its name suggests, hydrolyses its substrate at alkali conditions. However, it is clear from Table 5 that the optimal pH for alkaline phosphatase to hydrolyse p-nitrophenolphosphate is 10. 4, with 3. 81 umol p-nitrophenol being generated. Decreased concentrations of product were observed for pH values lower and higher than 10. 4.