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Kinetic Studies of Alkaline Phosphatase from the Liver of *Agama agama* Lizard

M.K. Abubakar, *R.S.U. Wasagu and M.I. Umar
Department of Biochemistry, Usmanu Danfodiyo University, P.M.B. 2346, Sokoto Nigeria
[*Corresponding Author, E-mail: rsuwasagu282@gmail.com; ☎: +2348031599039]

ABSTRACT: Kinetic studies were carried out on alkaline phosphatase (ALP) extracted from the liver of *Agama agama* lizard. Incubation of ALP extract with para – nitrophenyl phosphate formed the basis for the determination of enzyme activity. Spectrophotometric method was used to assay the enzyme, and the kinetic constants: Maximum velocity (V_{max}) and Michaelis Menten constant (K_m) were calculated. The K_m and V_{max} values were 2.5mM and 1.538 X 10^{-3} µmol/min respectively. Inhibition studies show that the enzyme activity was competitively inhibited by 0.67mM sodium hydrogen orthophosphate (NaH_2PO_4) with inhibition constant (K_i) of 2.27mM. The optimum pH of the enzyme was 9.9 and the optimum temperature was 35^{O}C. The enzyme exhibited linear Arrhenius relationship with corresponding catalytic energy of activation (E_a) of 1.44KJmol^{-1}. This study gives an insight of the catalytic properties of ALP extracted from the liver of *Agama agama* lizard.

Keywords: Alkaline phosphatase, *Agama agama*, para – nitrophenyl phosphate (p - NPP), Arrhenius relationship, Michaelis Menten constant.

INRODUCTION

The term "alkaline phosphatase (ALP)" was applied to group of catalytic proteins (enzymes) that have the ability to catalyse or hydrolyse phosphate esters in an alkaline environment/medium (Zhang *et al.,* 2004). The enzyme is also referred to as orthohosphoric monoester phosphorylase as its systemic name, with enzyme commission number as (E.C. 3.1.3.1).

The ALP are zinc metallo enzymes and it is known that zinc has a functional role in their catalysis. Magnesium or cobalt is also required for enzyme activity (Clark *et al.,* 1965; Fishman *et al.,* 1967 and Dean 2002). The enzyme activation is facilitated by divalent cations such as magnesium (Mg^{2+}) (Dean, 2002) and cobalt (Co^{2+}) (Arise *et al.,* 2008). The type of inhibition exhibited by ALP depends on the nature of the inhibitor (Fishman *et al.,* 1967) and on the source of the enzyme for instance, bone and kidney ALP are inhibited by urea, while placental and gastrointestinal ALP are not inhibited by urea but by L – phenylalanine (Dean, 2002).

Several reports also showed ALP extracted from different organ/tissue and different biological systems to exhibit variable but characteristic kinetic properties serving as the basis for distinguishing the enzyme isoforms (Dean, 2002). This work was aimed at evaluating some kinetic parameters of ALP in liver extract of *Agama agama* lizard.

MATERIALS AND METHODS

All the chemicals and reagents used in this work were of analytical grade.

Sample (Animal)

Agama agama lizard was caught at the back of the laboratory of Biochemistry Department, Usmanu Danfodiyo University, Sokoto. The lizard was then taken to the Zoology unit Biological Sciences Department, of the same University for identification. After identification standard procedure was employed to collect the liver, it was then washed with ice – cold normal saline ready for homogenization.

Preparation of Liver Extract of Alkaline Phosphatase

The extraction of the liver enzyme was by the method described by Arise *et al.,* (2008), with little modification. The *Agama agama* was dissected and the liver carefully removed and washed with ice – cold normal saline solution. One gram of the liver was homogenized in 20 ml of 0.1M Glycine – NaOH buffer (pH 9.9) solution. The homogenate was then centrifuged in a shermond bench centrifuge at 4000 x g for 50min. The supernatant constituted the crude enzyme extract and was carefully harvested using Pasteur pipette and this was used for the kinetic studies. Protein concentration was determined by the method of Biuret (Gornall *et al.,* 1949) using bovine serum albumin as the standard.

Determination of alkaline Phosphatase Activity

The enzyme assay was carried out by method described by Glogowski *et al.,* (2002), with minor modification. The enzyme activity was monitored by monitoring rate of hydrolysis of para – nitrophenylphosphate to para – nitrophenol as follows:

$$p\text{-nitrophenylphosphate}\,(p\text{-NPP}) + H_2O \longrightarrow p\text{-nitro phenol}\,(p\text{-NP}) + \text{phosphate}$$

Set of six test tubes were labelled. To each of the test tubes, 0.45ml of the buffer Glycine – NaOH (0.1M pH 9.9) were added and 0.45ml of the different substrate concentration (4mM, 3.2mM, 2.4mM, 1.6mM, 0.8mM) respectively and to the blank 0.00ml of substrate and 0.9ml of buffer were added. The test tubes were then incubated for 5 minutes at 37^{O}C. The crude enzyme extract (0.1ml) solution was added into the various test tubes to start the reaction at interval of 5 min for 30 min. The reaction was terminated by addition of 5ml of 0.2M NaOH solution to each test tube. The absorbance was taken spectrophotometrically at 405nm against the solution containing no substrate serving as the blank and the amount of para – Nitrophenol formed were extrapolated from the standard curve.

Inhibition studies using NaH_2PO_4 as inhibitor

The enzyme assay was carried out as described above. The glycine – NaOH buffer was substituted with 0.1M glycine – NaOH/2mM inhibitor mixture in the ratio 2:1 (v/v). Final concentration of inhibitor was 0.67mM.

Effect of pH on ALP Activity

The activity was monitored under varying pH values of 8.4, 8.8, 9.2, 9.6, 9.9, 10.0 and 10.4 of the Glycine – NaOH buffer. 4mM substrate concentration was used.

Effect of temperature on ALP Activity

Assay mixtures were prepared, incubated at different temperatures ranging from 15, 25, 30, 35, 40, 50, 60, 70 and 80 ^{O}C for 5 min. followed by addition of the enzyme extract.

Determination of temperature stability of ALP

The assay was carried out by incubating the enzyme at different temperatures ranging from 30, 35, 40, 50, 60, 70 and 80 ^{0}C for 5 min. followed by addition of substrate and the buffer.

RESULTS AND DISCUSSION

The activity of ALP in the presence and absence of inhibitor is presented in Fig. 1 (Lineweaver Burk plot). The result shows that the inhibitor (NaH_2PO_4) competitively inhibits the activity of the enzyme, as the V_{max} (1.538 X 10^{-3} μmolmin^{-1}) was not altered in the presence of inhibitor. The current result is consistent with previous reports (Coburn *et al.,* 1998, Njoku *et al.,* 2010 and So *et al.,* 2007).

The K_m was found to be 2.5 mM and K_i was 2.27 mM. Therefore, in comparison the ALP extract from *Agama agama* lizard was of higher affinity to 4 –NPP than the ALP extract from rat (*Rattus novergicus*), kidney (K_m = 6.41mM) (Arise *et al.,* 2008) and the human placental isoenzymes (K_m = 5.55 mM) (Saini *et al.,* 2005), but was lower than that of rabbit (*Lepus townsendii*) (Njoku *et al.,* 2010).

The effect of pH on the activity of ALP is shown in Fig. 2. The result indicate that the enzyme has an optimum pH of 9.9. This indicates the presence of ionizable groups in the active site of the enzyme (Saidu *et al.,* 2005). Enzymes posses these groups that dictate the three dimensional structure of the active site and the enzyme in general.

The extract specific activity = 2.144 X 10^{-3} unit/mg protein. One unit of enzyme activity was expressed as 1 μM of para – Nitrophenol (4-NP) produced per min.

Table 1: Properties of ALP extracted from *Agama agama* lizard in a single purification step

Purification	Total protein (mg)	Total activity (x10^{-4})	Specific activity (U/mg) X10^{-3}	Purification fold
0.1M glycine – NaOH buffer	447	9.585	2.144	1

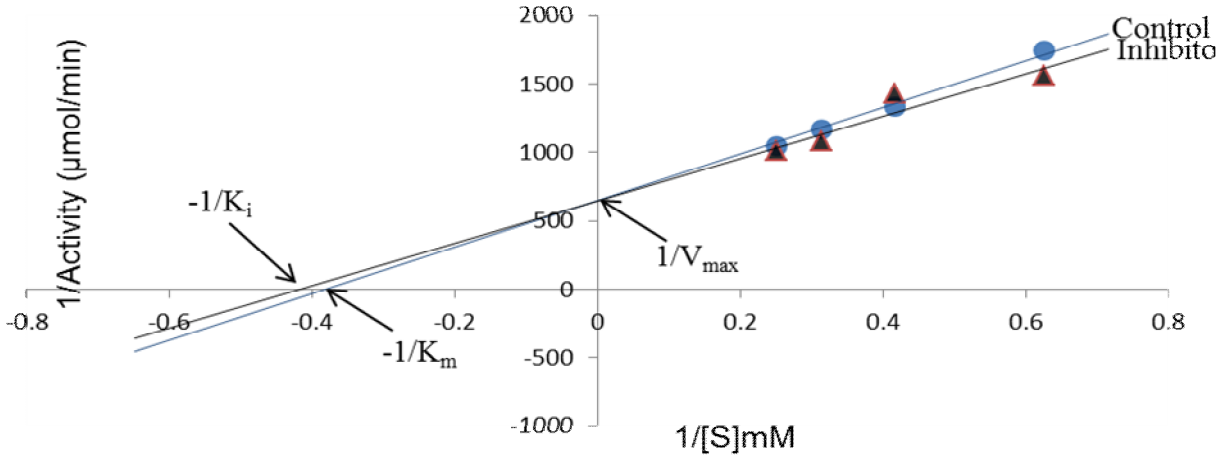

Figure 1: Lineweaver Burk plot (A) of ALP assay in the presence and absence of the inhibitor.

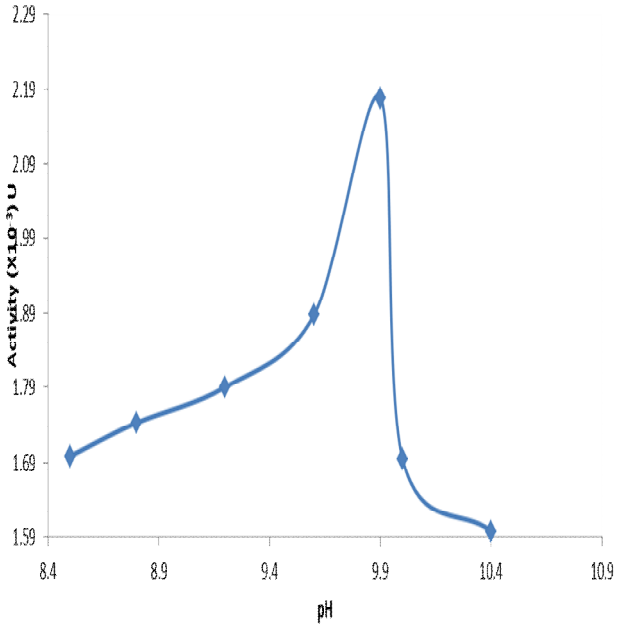

Figure 2: Effect of pH on the activity of ALP extracted from the liver of *Agama agama* lizard.

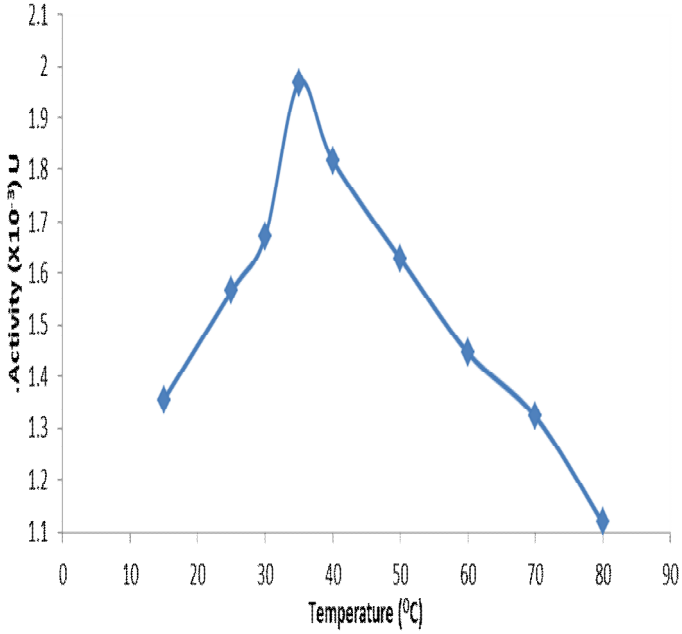

Figure 3: Effect of temperature on the activity of ALP extracted from the liver of *Agama agama* lizard.

The role of an enzyme depends on the precise orientation of these groups, often affected significantly by optimum pH of the environment (Saidu *et al.,* 2005). The pH of the present work was close to that of intestinal ALP of Harp seal (9.8) reported by Fernley (1971). A lower value of 8.0 was reported for *E.coli* (Garen *et al.,* 1960) and bovine ALP 8.5 (Harada *et al.,* 1986).

The effect of temperature on the enzyme activity is presented in Fig. 3. The optimum temperature is 35^{O}C. Enzymes are proteins, whose three dimensional structures are stabilized by weak forces (Saidu *et al.,* 2005), because of the weak nature of these forces they are disrupted at high temperatures (Saidu *et al.,* 2005). The two major effects of temperature observed on the activity of the enzyme were increase in the rate of the reaction at lower temperatures 15 – 30 ^{O}C, as the enzyme gains kinetic energy and a decrease in the rate of activity due to increase in the rate of denaturation of the enzyme at temperatures greater than 35^{O}C. The optimum temperature obtained was

lower than that of bovine alkaline phosphatase which was reported as 47^{O}C (O` Keefe and Kinsella, 1979) and that of rabbit liver 45^{O}C (Njoku *et al.,* 2010).

Fig.4 presents the plot of $Log_{10}V$ against the reciprocal of the corresponding absolute temperature (T) in Kelvin for the reaction catalysed by ALP. The slope of the right hand side of the curve was used for the calculation of activation energy (E_a) of the reaction. According to the Arrhenius equation, the slope (M) was related to the activation energy by the equation:

M = - E_a/2.303R (where R is the universal gas constant which is 8.314Jmol^{-1}).

Enzymes bring about increase in reaction by reducing the activation energy of the reaction (Saidu *et al.,* 2005). The activation energy (E_a) of the ALP in this study is1.44 KJmol^{-1} which is significantly different compared to that of rabbit (*Lepus townsendii*) liver E_a 15.23KJmol^{-1} (Njoku *et al.,* 2010), but still showed linear Arrhenius relationship in concord with the studies by Copeland *et al.* (1985).

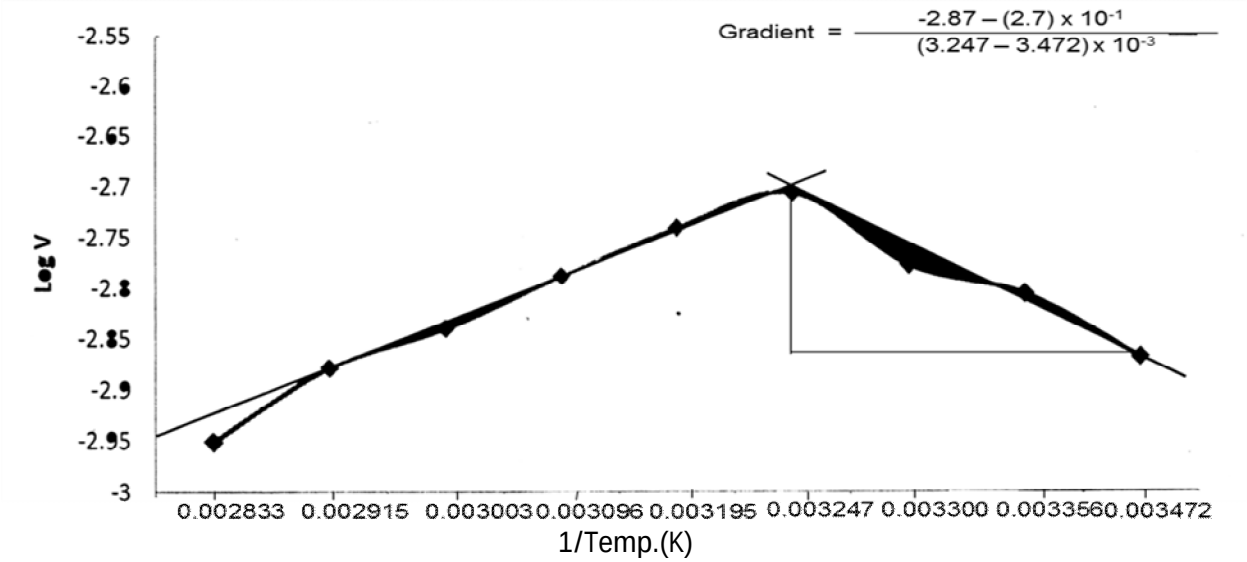

Figure 4: Plot of LogV against reciprocal of absolute temperature.

Fig. 5 shows the temperatures at which the enzyme was found to be stable. At the temperature ranges of 35 – 50^{O}C the enzyme remained stable. This indicates that at temperatures below 35 ^{O}C the enzyme operate maximally while at temperature above 50 ^{O}C the enzyme get denatured and hence becomes unstable. The range of temperature stability for calf and seal ALP was reported to be 55 – 60 ^{O}C (Asgeirsson *et al.,* 1991), which was slightly high than ALP of the current study. This is possible due to different organ as well as experimental conditions (Morton *et al.,* 1957).

The organism`s liver was exploited for this work despite the fact that it is a cold blooded animal. This shows the occurrence and diversity of ALP in the biological system as reported by several authors (Coburn *et al.,* 1998; Zhang *et al.,* 2004).

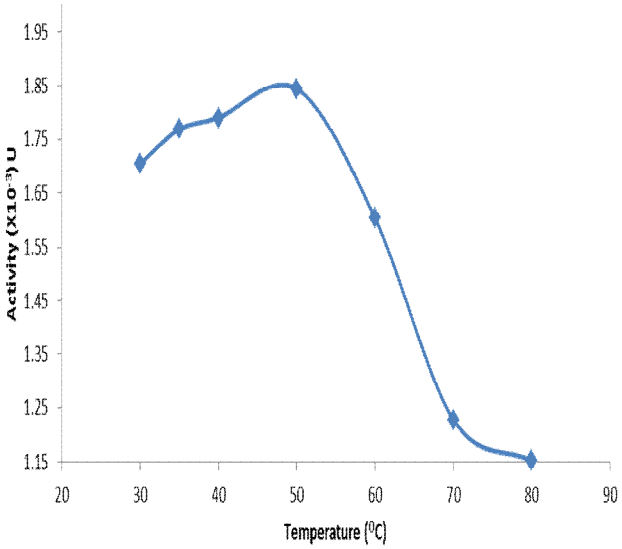

Figure 5: Determination of temperature stability of ALP

This revealed the nature of the enzyme catalysed reaction therefore, this study suggests that at experimental temperature of about 35^{O}C and pH of 9.9 ALP extract of *Agama agama* exhibited maximum conformational flexibility to accommodate the substrate and enhanced conformational changes required for maximum catalysis.

CONCLUSION

The kinetic parameters obtained in this work may reflect some of the physiological properties of the enzyme in Agama lizard in line with its nutritional requirements

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