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Kinetic Studies of Alkaline Phosphatase Extracted from Rabbit (*Lepus townsendii*) Liver

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ABSTRACT

Studies were carried out to ascertain some kinetic properties of alkaline phosphatase (ALP) extracted from *Lepus townsendii* liver. Incubation of ALP extract with 4-nitrophenylphosphate (4-NPP) in glycine-NaOH buffer mixture at 37°C for 30 min formed the basis for determination of enzyme activity. Spectrophotometric method was used to assay for the enzyme activity for 30 min and the kinetic constants-maximum enzyme velocity (V_{max}) and Michealis-Menten constant (K_m) were evaluated. The K_m and V_{max} values were 0.5×10^{-3} M and 20×10^{-6} M min^{-1}, respectively. Inhibition studies showed that ALP activity was competitively inhibited by 0.67 mM sodium hydrogen orthophosphate (NaH_2PO_4) and the inhibition constant (K_i) was 0.9×10^{-3} M. The optimum pH value for ALP activity was about 9.2 and optimum temperature registered 45°C. ALP activity exhibited linear Arrhenius relationship at temperature greater than 44.95°C with corresponding catalytic energy of activation (E_a) = 15.23 KJ $mole^{-1}$. The present study gave insights into characteristic catalytic properties of ALP extracted from *L. townsendii* liver.

Key words: Alkaline phosphatase, *Lepus townsendii,* 4-nitrophenylphosphate (4-NPP), Arrhenius relationship, michealis-menten constant

INTRODUCTION

Orthophosphoric monoester phosphohydrolase (E.C: 3.1.3.1), also referred to as Alkaline phosphatase (ALP) is a generic term that describes a group of catalytic proteins sharing the capacity to hydrolyse phosphate esters in alkaline medium (Zhang *et al*., 2004; Saini *et al*., 2005). The enzyme contains a zinc atom (Zn^{2+}) near the active site that is viewed to be responsible for its catalytic activity (Du *et al*., 2001; Dean, 2002). Also, activation of ALP activity is facilitated by divalent cations such as magnesium (Mg^{2+}) (Kim and Wyckoff, 1990; Du *et al*., 2001; Dean, 2002) and colbalt (Co^{2+}) (Arise *et al*., 2008). Conversely, ALPs are inactivated/inhibited by wide range of compounds, depending on the source or/and isoenzymic form of the enzyme (Hummer and Millan, 1991; Hoylaerts *et al*., 1992). For instance, whereas bone and kidney ALPs are inhibited by urea, those obtained from planceta and gastrointestinal are unaffected but inhibited by phenylalanine (Dean, 2002). Also, ALP extracted from *Escherichia coli* is not inhibited by phenylalanine (Hoylaerts *et al*., 1992). The wide distribution of ALP in biologic systems coupled with its broad specificity makes the enzyme a readily available parameter for diagnostic and research studies (Garba and Gregory, 2005).

Reports showed that ALP extracts from different tissue/organ and various biological systems exhibit variable but characteristic kinetic properties that serve as basis for distinguishing ALP isoforms (Mayer-Sabellek *et al.*, 1988; Dean, 2002). Therefore, the focus of the present study is to ascertain some kinetic parameters of ALP in liver extract of *L. townsendii*. The findings will contribute to the characterization of the enzyme.

MATERIALS AND METHODS

Animal handling: Five male rabbits between 8-9 weeks old were obtained between August and September, 2002, from the Experimental Animal House, Department of Biological Science, Usman Danfodiyo University, Sokoto, Nigeria. The animals were healthy and showed no sign of stress. They were fed *ad libitium* with hay and water for a period of 1 week for acclimatization before the animals were sacrificed. The institutional review board of the Department of Biochemistry, University of Port Harcourt, Port Harcourt, Nigeria, granted approval for this study in accordance with the guidelines of the National Institute for Animal Research, Nigeria.

Preparation of liver extract of alkaline phosphatase: The extraction and purification of the liver enzyme was by the methods described by Malomo *et al.* (2003) and Arise *et al.* (2008), but with minor modifications. The rabbit was dissected and the liver carefully removed and washed in physiological saline solution. Two grams of the liver was homogenized in 40 mL of 0.1 M glycine-NaOH buffer (pH = 9.9) solution. The homogenate was centrifuged in a Shermond bench centrifuge at 5000x g for 40 min. The supernatant constituted the crude enzyme extract and was carefully harvested using a Pasteur pipette. To the supernatant, 4.17 M $(NH_4)_2 SO_4$ was gradually added while stirring until 30% saturation was achieved. The solution was stored at 4°C for 1 h to precipitate the enzyme (Hulett *et al.*, 1990). The precipitate was collected by centrifugation at 5,000x g for 20 min and re-dissolved in 5 mL of 0.1 M glycine-NaOH buffer (pH = 9.9) solution. The solubilized enzyme was subjected to dialysis at 2°C for 24 h to remove contaminating salt constituents (Ali-ul-Qader *et al.*, 2009) and further purified on a sephadex G-100 column to obtain a highly active ALP fraction. In total, ALP extracts from 5 rabbits were used. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. One unit of ALP activity (U) was expressed as 1 μM of 4-NP produced per min. The procedure for ALP purification is summarized in Table 1. The procedure for ALP purification is summarized in Table 1.

Determination of alkaline phosphatase activity: The enzyme activity represents the rate of hydrolysis of 4-Nitrophenylphosphate to 4-Nitrophenol described as follows:

Table 1: Purification protocol for liver ALP extract

Purification step	Total protein (mg)	Total unit (U)	Specific activity (U mg^{-1})	Purification fold
0.1 M glycine-NaOH buffer	19.8±2.43	9.702±3.01	0.49±2.91	1.00
4.17 M $(NH_4)_2 SO_4$	8.7±1.09	40.281±2.01	4.63±0.91	9.45
Dialysis at 2°C for 24 h	8.0±0.89	40.721±1.12	5.09±0.99	10.39
Sephadex G-100	5.6±1.56	52.808±0.98	9.43±1.09	19.24

The extract specific enzyme activity = 9.43±1.09 Unit mg^{-1} protein

ALP

4-Nitrophenylphosphate (4-NPP) + H_2O → 4-Nitrophenol (4-NP)+Phosphate ion

The enzyme assay was carried out by method described by Glogowski *et al.* (2002), but with minor modifications. Five test tubes containing 0.45 mL dilutions of 4, 3.2, 2.4, 1.6 and 0.8 mM of 4-NPP solutions were introduced into five corresponding test tubes containing 0.45 mL of 0.1 M glycine-NaOH buffer (pH = 9.9). The 4-NPP/glycine-NaOH buffer mixture was incubated at 37°C for 30 min. The enzyme reaction was started by adding 0.1 mL of enzyme extract into the various test tubes. At a regular time interval of 5 min for 30 min, 5.0 mL of 0.2 M NaOH was added to the assay mixture to terminate ALP activity. The mixture was transferred into a cuvette and absorbance was measured with a spectrophotometer (SPECTRONIC 20, Labtech-Digital Blood Analyzer®) at maximiun wavelength (λ_{max}) of 405 nm. The concentrations of 4-NP produced in the various tubes were interpolated from standard curves (not shown).

Inhibition studies using NaH_2PO_4 as inhibitor: The enzyme assay was carried out as described above. The glycin-NaOH buffer was substituted with 0.1 M glycine-NaOH buffer/2 mM NaH_2PO_4 mixture {ratio 2:1 (v/v)}. Final concentration of inhibitor = 0.67 mM.

Effect of pH on Enzyme Activity: The activity of ALP was investigated under varying pH values of 8.4, 8.8, 9.2, 9.6, 9.9, 10.0 and 10.4 of 0.1 M glycine-NaOH buffer. A substrate concentration of 4 mM 4-NPP was used for the enzyme assay.

Effect of temperature on enzyme activity: The enzyme assay was carried out by incubating the enzyme mixture containing 4 mM 4-NPP in varying temperature values of 5, 15, 25, 35, 45 and 55°C.

Evaluation of kinetic parameters: The kinetic parameters were evaluated with the plot of the reciprocal of initial velocities (V_I) against corresponding reciprocal of substrate concentrations [4-NPP], i.e., $1/V_i$ vs 1/[S] (Lineweaver and Burk, 1934).

Statistical analyses: The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the Least Significance Difference (LSD) incorporated in the Statistical Analysis System (SAS) package of 9.1 version.

RESULTS

The activity (V_i) of ALP measured in the absence and presence of inhibitor ([I] = 0.67 mM NaH_2PO_4) is represented by the Lineweaver-Burk plot (Fig. 1).

By inspection, the Lineweaver-Burk plot (Fig. 1) showed that ALP activity was competitively inhibited by 0.67 mM NaH_2PO_4. Also, the plots representing the presence and absence (control) of the inhibitor intersected the x-axis at -1.1 and -2, respectively. Furthermore, the two plots converged at a common point of intersection on the y-axis: 5×10^4 $\mu M^{-1}/min^{-1}\times10^4$. The kinetic properties of ALP deduced from the Lineweaver-Burk plot above are summarized in Table 2.

The activity of ALP investigated in varied alkaline pH environment is presented in Table 3.

A cursory look at Table 3 shows that the activity of ALP exhibited two phase profile within the range of values of pH = 8.4 to 10.4. The first phase showed increasing ALP activity as pH values

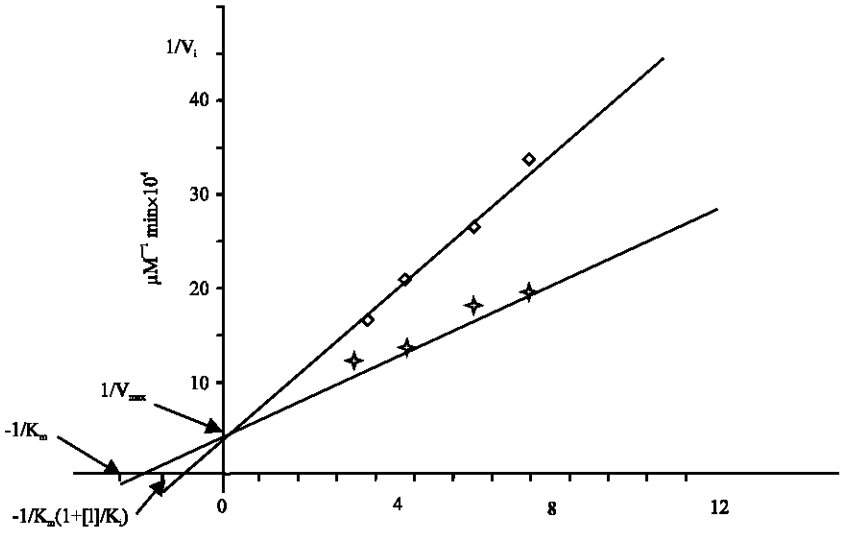

Fig. 1: Double reciprocal plot of ALP assay in the presence of inhibitor NaH_2PO_4 ( ) and the control ( ) analysis. [I]: Concentration of inhibitor = 0.67 mM

Table 2: Kinetic parameters of ALP

K_m (mM)	K_i (mM)	V_{max} (μM min^{-1})
0.5±0.25	0.9±0.33	20±0.63

Values are Mean±S.D of five (n = 5) determinations

Table 3: Levels of ALP activity with corresponding pH values

Buffer pH	8.4	8.8	9.2	9.6	10.0	10.4
[V_i] (μM min^{-1})	4.60±0.38^a	7.23±0.99^b	11.20±0.76^d	8.96±0.73^c	8.80±0.34^c	7.36±0.66^b

Values are Mean±S.D of five (n = 5) determinations. Means in the rows with the same letter are not significantly different at $p<0.05$ according to LSD

Table 4: Levels of ALP activity with corresponding temperatures (T°C)

Temperature (°C)	5	15	25	35	45	55
[V_i] (μM min^{-1})	2.00±0.46^a	2.83±0.41^b	4.00±0.36^c	4.67±0.79^d	5.01±0.56^e	4.53±0.83^{c,d}

Values are Mean±S.D of five (n = 5) determinations. Means in the rows with the same letter are not significantly different at $p<0.05$ according to LSD

were adjusted from 8.4 to 9.2. Noteworthy, the pH value of about 9.2 corresponded to the maximum enzyme activity attained within the experimental pH range. Therefore, about pH = 9.2 represented the optimum pH value of ALP extracted from *L. townsendii* liver. However, ALP activity at pH 10.0 was not significantly different ($p<0.05$) from the activity at optimum pH. Further increases in pH values beyond the optimum engendered decreasing level of ALP activity. The relationship between ALP activity and experimental incubation temperature of the enzyme assay is presented in Table 4.

The pattern ALP activity in relation to changes in experimental temperature showed two phase profile. The results in Table 4 showed the optimum temperature of ALP activity was about 45°C. The maximum enzyme activity attained at optimum temperature was 5.01±0.56 μM min^{-1}. ALP

activity at 35°C (4.67±0.79 μM min^{-1}) was not significantly different ($p<0.05$) compared to activity at 55°C (4.53±0.83 μM min^{-1}). Using Arrhenius plot, log *V* versus 1/T, the activation energy (E_a) of catalysis was evaluated thus:

Evaluating for E_a: Log V = E_a/2.3RT where, R = 8.31447 J K^{-1} mol^{-1}: molar gas constant But gradient of plot: -800 = E_a/2.3R, Therefore, E_a= 15.23 KJ $mole^{-}$.

DISCUSSION

This study demonstrated the activity of ALP in liver extract of *L. townsendii*. The occurance and derversity of ALP in biological systems has been reported by several authors (Coburn *et al.*, 1998; Zhang *et al.*, 2004). For instance, Weissig *et al.* (1993), reported on sequence comparisons between different ALP indicated that about 25-30% homology exist between mammalian and *Escherichia coli* ALP. Despite differences in amino acid sequences and three-dimensional structures, all ALP catalyze the hydrolysis of almost any phosphomonoester with release of inorganic phosphate and alcohol. In addition, although ALP exhibits broad specificity for wide range of phosphorylated substrates, the enzyme displays variable affinity for these substrates depending on the molecular nature of the substrate, isoform and source of the enzyme. Szalewicz *et al.* (2003) noted that the equivalent enzyme, acid phosphatase, of frog *Rana esculenta* liver displayed higher affinity for peptidic substrates than for small phosphate esters. Also, Coburn *et al.* (1998), from their findings stated that in the presence pyridoxal phosphate as substrate, bovin kidney ALP K_m was 0.42±0.04 μmol L^{-1}, whereas enzyme extracted from the intestine gave 1.6±0.2 μmoL^{-1} and placenta gave 0.42±0.08 μmoL^{-1}. Comparatively, the present investigation showed that ALP liver extract of L. townsendii exhibited higher affinity for 4-NPP (K_m = 0.5±0.25 mM) (Table 2) than enzyme extracts from rat Rattus novergicus kidney (K_m = 6.41 mM; Arise *et al.*, 2008) and human placental isoenzyme (K_m = 5.55 mM; Saini *et al.*, 2005). Comparatively, the present investigation showed that ALP liver extract of *L. townsendii* exhibited higher affinity for 4-NPP (K_m = 0.5±0.25 mM) (Table 2) than enzyme extracts from rat *Rattus novergicus* kidney (K_m = 6.41 mM; Arise *et al.*, 2008) and human placental isoenzyme (K_m = 5.55 mM; Saini *et al.*, 2005). These findings serve to highlight and confirm the occurance of isoforms of ALP amongst the mammalian genera.

More than five decades ago, Hilliard *et al.* (1965), demonstrated that endogenous phosphate interferes with the determination of ALP in urine and suggested that the wide variation in serum inorganic phosphate concentrations in diseases such as uremia or renal tubular disease might interfere with ALP measurements in serum. From inspection of the double reciprocal plot (Fig. 1), V_{max} of the enzyme was not altered in the presence of inhibitor. (Therefore, the present kinetic studies showed that phosphate salt, NaH_2PO_4, is a competitive inhibitor of ALP activity. This finding is in concord with previous research reports by Fox (1978), Coburn *et al.* (1998) and So *et al.* (2007), in which they noted that ALP is competitively inhibited by physiological concentrations of inorganic phosphate. Therefore, the present kinetic studies showed that phosphate salt, NaH_2PO_4, is a competitive inhibitor of ALP activity. This finding is in concord with previous research reports by Fox (1978), Coburn *et al.* (1998) and So *et al.* (2007), in which they noted that ALP is competitively inhibited by physiological concentrations of inorganic phosphate. They further averred that inorganic phosphate is an important physiologic regulator of extracellular ALP

activity. Incongrously, the possible effects of endogenous phosphate on phosphatase activity with natural substrates under physiological conditions have not been rigorously investigated (Coburn *et al*., 1998).

Although, the pH optimum of an enzyme often reflects the pH of its normal environment, the optimum value may not be precisely the same in every biological system. Noteworthy, as far back as five decades ago, Garen and Levinthal (1960), reported optimal pH for ALP activity of *E. coli* to be about 8.0 while, the bovine enzyme optimum pH was slightly higher at about 8.5 (Harada *et al*., 1986). Furthermore, study of ALP from human hydatidiform by Aberomand *et al*. (2008), gave pH optimum of 10.8. The current study reported optimum pH value of ALP liver extract of L. townsendii to be about 9.2. These findings are consistent with prevoius studies by Zappa *et al*. (2001), The current study reported optimum pH value of ALP liver extract of *L. townsendii* to be about 9.2. These findings are consistent with prevoius studies by Zappa *et al*. (2001), in which they observed that mammalian ALPs possess higher pH optima than procaryotes (*E. coli*) enzyme and proposed that substitution of two amino acid residues with corresponding histidine molecules in the mammalian enzymes are responsible for this increase in optimum pH (Holtz and Kantrowitz, 1999; Murphy *et al*., 1995). These variations in ALP activity which are consequence of adjustments in K_m or V_{max} values or both, suggest that pH/activity relationship of ALP may be a factor in the intracellular regulation of its activity.

Enzyme activity/temperature linked properties reported elsewhere (Suzuki *et al*., 2005; Lee *et al*., 2007; Copeland *et al*., 1985; Aberomand *et al*., 2008) defined the so-called enzyme "temperature optimum" of ALP activity. The present report suggests that at experimental temperature of about 45°C, ALP extract of L. townsendii exhibited maximum conformational flexibility to accommodate the substrate and enhanced conformational changes required for maximum catalysis. Contrary to the present suggestion, Daniel *et al*. (2001), proposed that temperature optimum is not an intrinsic property, being derived from a complex mixture of both activity and thermal stability effects.

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The Arrhenius plot (Fig. 2) conformed to the pattern previously described by Copeland *et al*. (1985). They averred that all enzymes of human origin exhibited similar linear Arrhenius relationships over the range of 20-37°C with corresponding E_a values between 30 and 36 $KJmol^{-1}$. In the present study, ALP activity exhibited linear Arrhenius relationship at values of temperature greater than 44.95°C with corresponding E_a= 15.23 $KJ\ mol^{-1}$ (Fig. 2). Although, ALP obtained from human and L. townsendii showed linear Arrhenius relation within variable temperature range, E_a values of catalysis were significantly different. Again, in concord with the present investigations, studies by Copeland *et al*. (1985) reported that porcine kidney enzyme obeyed an Arrhenius relationship that was slightly but significantly different from the isoenzymes of human origin.

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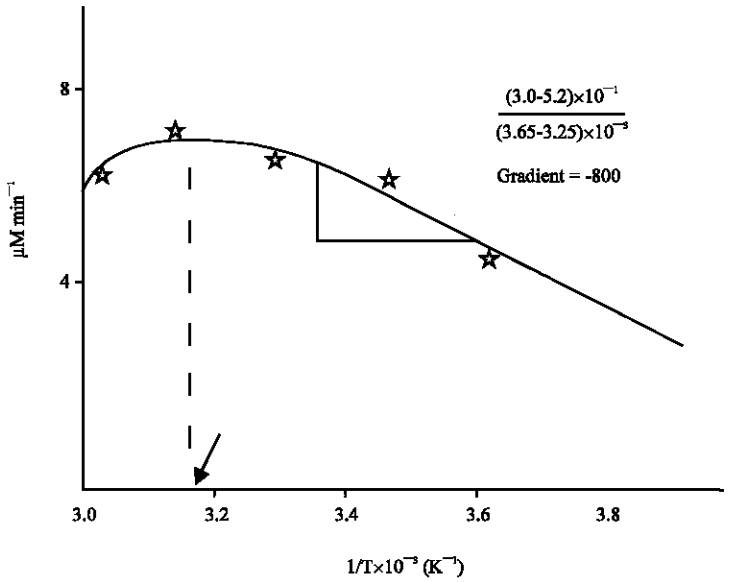

Fig. 2: Arrhenius Plot: Effect of Temperature on ALT Activity. Where $1/T_{opt}$: Reciprocal of Optimum Temperature = 3.144×10^{-3} K^{-1}. ALP exhibited linear Arrhenius relationship within the temperature range: 35-55°C

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REFERENCES

Aberomand, M., F. Rahim and S.A. Hosseini, 2008. Study of alkaline phosphatase from human hydatidiform mole Pak. J. Med. Sci., 24: 471-474.

Ali-ul-Qader, S., S. Iqbal and Z. Niazi, 2009. Partial purification and characterization of intracelluar alkaline phosphatase from newly isolated strain of *Bacillius subtilis* KIBGE-HAS. Internet J. Microbiol. Vol. 7.

Arise, R.O., F.F. Davies and S.O. Malomo, 2008. Cobalt reverses vanadate inhibition of rat kidney alkaline phosphatase. Sci. Res. Essay, 3: 613-620.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254.

Coburn, S.P., J.D. Mahuren, M. Jain, Y. Zubovic and J. Wortsman, 1998. Alkaline Phosphatase (EC 3.1.3.1) in serum is inhibited by physiological concentrations of inorganic phosphate. J. Clin. Endocrinol. Metab., 83: 3951-3957.

Copeland, W., D.A. Nealon and D. Rej, 1985. Effect of temperature on measurement of alkaline phosphatase activity. Clin. Chem., 31: 185-190.

Daniel, R.M., M.J. Danson and R. Eisenthal, 2001. The temperature optima of enzymes: a new perspective on an old phenomenon. Trends Biochem. Sci., 26: 223-225.

Dean, R.L., 2002. Kinetic studies with alkaline phosphatase in the presence and absence of inhibitors and divalent cations. Biochem. Mol. Biol. Educ., 30: 401-407.

Du, M.H.L., T. Stigbrand, M.J. Taussig, A. Ménez and E.A. Stura, 2001. Crystal structure of alkaline phosphatase from human placenta at 1.8 Å resolution: Implication for a substrate specificity. J. Biol. Chem., 276: 9158-9165.

Fox, I.H., 1978. Degradation of Purine Nucleotides. In: Uric Acid, Kelley, W.N. and I.M. Weiner (Eds.). Springer, Berlin, pp: 93-124.

Garba, I.H. and U. Gregory, 2005. Serum alkaline phosphatase activity as a potential biomarker for the intergrity of the hepatic drainage system in acute *Falciparum* malaria infection. Internet J. Infecious. Dis., 4: 1-5.

Garen A. and C. Levinthal, 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. Biochim. Biophys. Acta, 38: 470-483.

Glogowski, J., D.R. Danforth and A. Ciereszko, 2002. Inhibition of alkaline phosphatase activity of boar semen by pentoxifylline, caffeine and theophylline. J. Androl., 23: 783-792.

Harada, M., N. Udagawa, K. Fukasawa, B.Y. Hiraoka and M. Mogi, 1986. Inorganic pyrophosphatase activity of purified bovine pulp alkaline phosphatase at physiological pH. JDR, 65: 125-127.

Hilliard, S.D., J.F. O'Donnell and S. Schenker, 1965. On the nature of the inhibitor of urinary alkaline phosphatase. Clin. Chem., 11: 570-574.

Holtz, K.M. and E.R. Kantrowitz, 1999. The mechanism of the alkaline phosphatase reaction: insights from NMR, crystallography and sitespecific mutagenesis. FEBS Lett., 462: 7-11.

Hoylaerts, M.F., T. Manes and J.L. Millan, 1992. Molecular mechanism of uncompetitive inhibition of human placental and germ-cell alkaline phosphatase. Biochem. J., 286: 23-30.

Hulett, F.M., C. Bookstein and K. Jensen, 1990. Evidence for two structural gene for alkaline phosphatase in *B. subtilis*. J. Bacteriol., 172: 735-740.

Hummer, G. and J.L. Millan, 1991. Gly429 is the major determinant of uncompetitive inhibition of human germ cell alkaline phosphatase by L-leucine. Biochem. J., 274: 91-95.

Kim, E.E. and H.W. Wyckoff, 1990. Structure of alkaline phosphatases. Clin. Chim. Acta, 186: 175-188.

Lee, C.K., R.M. Daniel, C. Shepherd, D. Saul and S.C. Cary *et al*., 2007. Eurythermalism and the temperature dependence of enzyme activity. FASEB J., 21: 1934-1941.

Lineweaver, H. and D. Burk, 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc., 56: 658-666.

Malomo, S.O., F.J. Olorunniji, R.O. Arise, J.O. Adebayo, O.T. Adedosu and A.A. Odutuga, 2003. Synergistic interaction between two linear inhibitors on a single enzyme: Vanadate and L-phenylalanine inhibition of partially purified rat liver alkaline phosphatase. Biokemistri, 13: 23-30.

Mayer-Sabellek, W., P. Sinha and E. Köttgen, 1988. Alkaline phosphatase laboratory and clinical implications. J. Chromatography B Biomed. Sci. Appl., 429: 419-444.

Murphy, J.E., T.T. Tibbitts and E.R. Kantrowitz, 1995. Mutations at positions 153 and 328 in *Escherichia coli* alkaline phosphatase provide insight towards the structure and function of mammalian and yeast alkaline phosphatases. J. Mol. Biol., 253: 604-617.

Saini, D., M. Kala, V. Jain and S. Sinha, 2005. Targeting the active site of the placental isozyme of alkaline phosphatase by phage-displayed scFv antibodies selected by a specific uncompetitive inhibitor. BMC Biotechnol., 5: 33-33.

So, P., W.L.F. Tsui, R. Vieth, J.H. Tupy and P.H.K. Pritzker, 2007. Inhibition of alkaline phosphatase by cysteine: implications for calcium pyrophosphate dihydrate crystal deposition disease. J. Rheumatol., 34: 1313-1322.

Suzuki, Y., T. Mizutani, T. Tsuji, N. Ohtani and K. Takano *et al*., 2005. Gene cloning, overproduction and characterization of thermolabile alkaline phosphatase from a psychrotropic bacterium. Biosci. Biotechol. Biochem., 69: 364-373.

Szalewicz, A., B. Strzelczyk, M. Sopel and A. Kubicz, 2003. The 35 kDa acid metallophosphatase of the frog *Rana esculenta* liver: studies on its cellular localization and protein phosphatase activity. Acta Biochimica Polonica, 50: 555-566.

Weissig, H., A. Schildge, M.F. Hoylaerts, M. Iqbal and J.L. Millan, 1993. Cloning and expression of the bovine intestinal alkaline phosphatase gene: biochemical characterization of the recombinant enzyme. Biochem. J., 290: 503-508.

Zappa, S., J.L. Rolland, D. Flament, Y. Gueguen, J. Boudrant and J. Dietrich, 2001. Characterization of a highly thermostable alkaline phosphatase from the euryarchaeon *Pyrococcus abyssi*. Applied Environ. Microbiol., 67: 4504-4511.

Zhang, L., R. Buchet and G. Azzar, 2004. Phosphate binding in the active site of alkaline phosphatase and the interactions of 2-nitrosoacetophenone with alkaline phosphatase-induced small structural changes. Biophys. J., 86: 3873-3881.