

Biochemical Characterization of Alkaline Phosphatase (E.C. 3.1.3.1) from species of Crab (*Callinectes sapidus* and *Cardisoma carnifex*)

¹Raimi, O.G.^ϕ, ²Hammed, A.M., ¹Babatunde, O.T., Fajana, O.O. and ¹Bankole, H.A.

¹Department of Biochemistry, ²Department of Fisheries, Faculty of Science, Lagos State University, P.O.Box
0001, LASU Post office, Ojo, Lagos.

E-mail:wale.raimi@gmail.com **ϕ:corresponding author**

ABSTRACT

Alkaline phosphatase (EC 3.1.3.1), a metalloenzyme which catalyzes the nonspecific hydrolysis of phosphate monoesters was isolated from different tissue homogenates (Digestive gland, Muscle, Carapace and Appendages) of crabs (Callinectes sapidus and Cardisoma carnifex). The digestive gland was found to have the highest level of alkaline phosphatase. The K_m and V_{max} were estimated to be 2.756 mM and 2.838 mmole/min/ml respectively for Callinectes sapidus and 3.636 mM and 3.322 mmole/min/ml respectively for Cardisoma carnifex. The enzyme has an optimum pH of 10. Metals such as $MgCl_2$ gave an unstable activation effect on the enzyme. The highest activity of the enzyme as found in the digestive gland of crabs used in this study suggests that most of the organic phosphates in the species are hydrolyzed principally in this tissue.

KEY WORDS: Alkaline phosphatase, Crab, *Callinectes sapidus*, *Cardisoma carnifex*,
Metalloenzymes,

1. INTRODUCTION

A Phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolising phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. This action is directly opposite to that of Phosphorylases and kinases which attach phosphate groups to their substrate by using energetic molecule like ATP. The addition of a phosphate group may activate or de-activate an enzyme (e.g., kinase signalling pathways) [1], or enable a protein-protein interaction to occur (e.g., SH3 domains) [2]; therefore phosphatases are integral to many signal transduction pathways.

A common phosphatase in many organisms is alkaline phosphatase (ALP), although, we also have acid phosphatase. Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate group from many types of molecule including nucleotides, proteins and alkaloids to give inorganic phosphate and the corresponding alcohol, phenol and sugar [3].

Alkaline phosphatases are a group of enzymes found primarily in the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2) of mammals. There are also small amount produced by cells lining the intestines (isoenzyme ALP-3), the placenta and the kidney (in the proximal convoluted tubules). What is measured in the blood is the total amount of alkaline phosphatases released from these tissues into the blood. Alkaline phosphatases act by splitting off phosphorus (an acidic mineral) creating an alkaline pH. Alkaline phosphatase is a metalloenzymes containing zinc and magnesium ions and the structure of the eukaryotic ALP active site is similar to that of bacterial alkaline phosphatase [4]. The primary importance of measuring alkaline phosphatase is to check the possibility of bone or liver disease.

Crabs are crustaceans which are related to lobsters and shrimps [5]. They have evolved so that they walk or run sideways as well as burrow and swim. The abdomen serves as a brood pouch for the eggs. Crabs can possess complicated nervous system and are considered to be more advance and can live in more extreme areas because of their ability to adapt well to the changing environment.

Dietary phosphate availability has been reported to have strong impact on the somatic growth rate of zooplankton [6]. Therefore, efficient mobilization of phosphate from organic sources through catalysis by ALP may be crucial for growth in an environment where phosphate is limited.

The aim of this study is to isolate and characterize ALP from different tissue homogenates of *Collinectus sapidus* (CS) and *Cardisoma carnifex* (CC) the two major species of crab found in Nigerian waters.

2. MATERIALS AND METHODS

Species of *Callinectes sapidus* and *Cardisoma carnifex* were purchased from Fish mongers in Ojo area of Lagos state, Nigeria. RANDOX alkaline phosphatase kits produced by RANDOX Laboratories Ltd., United Kingdom, Biuret reagent, Sodium hydroxide, Magnesium chloride (MgCl_2), distilled water, Tris-HCl buffer, and phosphate buffer. All other reagents were of high quality and purity.

2.1 Tissue Extraction/Homogenization

The claws (Cheliped), appendages (legs) and the carapace were carefully removed to excise the tissues, (gut, digestive gland and muscle). The tissues were then homogenized differently in 10 ml of ice-cold 150 mM NaCl using mortar and pestle and the homogenate was then centrifuged at 5000 rpm for 10 mins. The supernatant was collected and kept in the freezer until the time of assay.

2.2 Protein Determination

Protein concentration of tissue homogenates was determined using Biuret method [7]. 1.0 ml of the homogenate was mixed with 3.0 ml of Biuret reagent and incubated for 10 min at 37°C. Absorbance was taken at 540 nm using BSA as standard.

2.3 Enzyme Assay

Alkaline phosphatase activity in tissue homogenates was determined by a modified method of Bowers and McComb [8] as described earlier by Raimi *et al.* [9]. The method involves the incubation of 0.1 ml of each tissue homogenate in 80 mM Tris-HCl buffer (pH 10.4) with 0.5 ml p-nitrophenyl phosphate (pNPP, 10 mmol/l) at 37°C for 30 min. The reaction was terminated by adding 5.0 ml of 0.02 M NaOH after which absorbance was taken at 405 nm against reagent blank.

2.4 Enzyme Kinetics

The K_m and V_{max} of the tissue homogenate with the highest enzyme activity was determined. The reaction mixtures contain 0.1 ml of the tissue homogenate (digestive gland), 80 mM Tris-HCl buffer, (pH 10.4) and varying concentration of p-nitrophenylphosphate (10 mmol/l). The reaction mixtures were incubated for 10 min at 37°C after which 5.0 ml of 0.02 M NaOH was added and the corresponding absorbance taken at 405 nm.

2.5 Effect of Mg^{2+}

MgCl₂ was used for this assay. A range of 0.1 to 1.5 mol/l metal concentrations were added to 0.1 ml of tissue homogenate in the test tubes and 0.1 ml 80 mM Tris-HCl buffer, (pH 10.4) and 2.0 ml of p-nitrophenylphosphate was added. Other steps were as described for enzyme assay.

2.6 Effect of EDTA

A range of 5.0 to 30.0 mmol/l of EDTA concentration was added to 0.1ml of tissue homogenate and 0.1 ml 80 mM Tris-HCl buffer, (pH 10.4) and 2.0 ml of p-nitrophenylphosphate was added. Other steps were as described for enzyme activity.

2.7 Effect of pH

The reaction mixture contains 2.0 ml of p-nitrophenylphosphate and 0.1 ml of tissue homogenate in 80 mM Tris-HCl buffer of pH range of between 7.0 and 11.0.

2.8 Effect of Temperature

The reaction mixtures were incubated at a temperature range of between 20°C and 50°C in a temperature regulated water bath after which 5.0 ml of 0.02 M NaOH was added and the corresponding absorbance was read at 405 nm.

TABLE 1: Concentration of protein in tissue homogenates

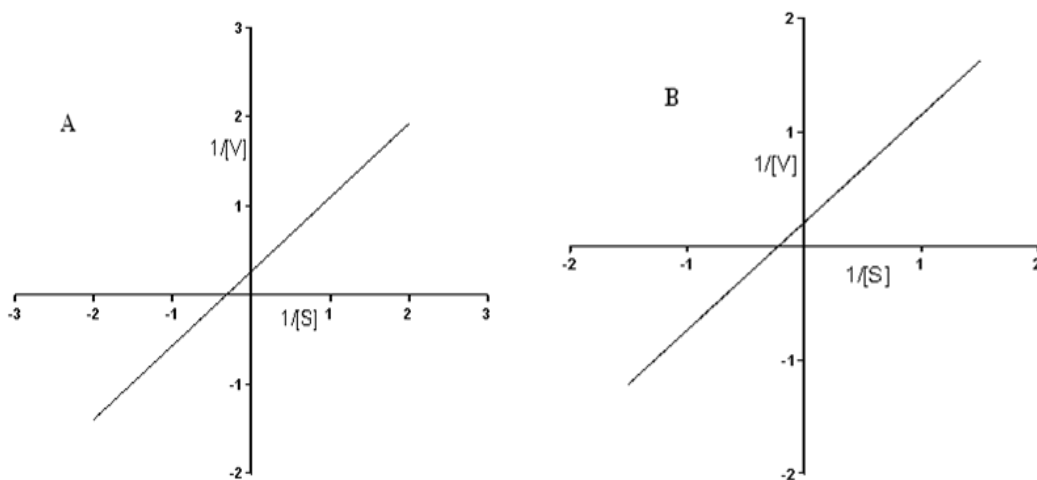
Tissues	Protein Concentration (mg/ml)	
	<i>Callinectes sapidus</i>	<i>Cardisoma carnifex</i>
Gut	9.915 ± 0.021	10.125 ± 0.177
Muscle	6.735 ± 0.049	9.035 ± 0.049
Appendages	6.310 ± 0.014	8.085 ± 0.021
Carapace	4.595 ± 0.134	6.705 ± 0.078

Data represent Mean \pm SD (n=2). Concentration expresses total protein in mg/ml

TABLE 2: Alkaline phosphatase activity in tissue homogenates

Tissues	Alkaline Phosphatase activity(U/l)	
	<i>Callinectes sapidus</i>	<i>Cardisoma carnifex</i>
Gut	4844.733 \pm 37.664	5618.033 \pm 417.439
Muscle	4575.467 \pm 189.282	4493.267 \pm 166.429
Carapace	3096.1 \pm 61.634	2500.533 \pm 103.886
Appendages	2508.8 \pm 326.276	1480.867 \pm 61.371

Data represent Mean \pm SD (n=2). Activity is expressed in Units per liter (U/l)



$K_m = 2.756 \text{ mM}$, $V_{max} = 2.838 \text{ } \mu\text{mole/min/ml}$, Slope = 0.971 $K_m = 3.636 \text{ mM}$, $V_{max} = 3.322 \text{ } \mu\text{mole/min/ml}$, Slope = 1.095

Figure 1: Reciprocal plot of absorbance against substrate concentration: **A**; (*Callinectes sapidus*) and **B**; (*Cardisoma carnifex*)

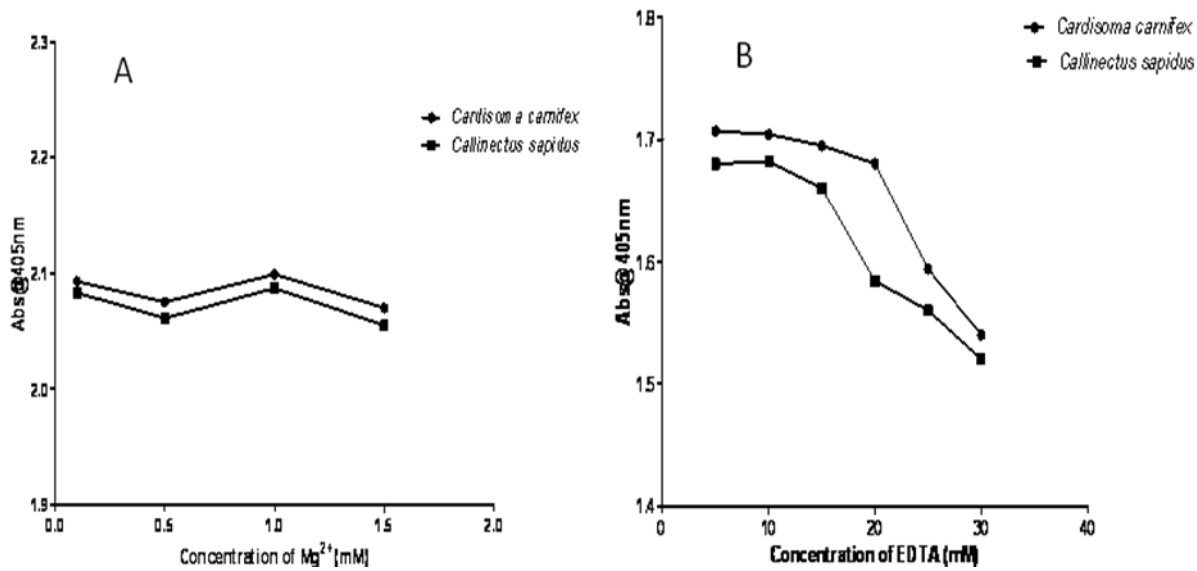
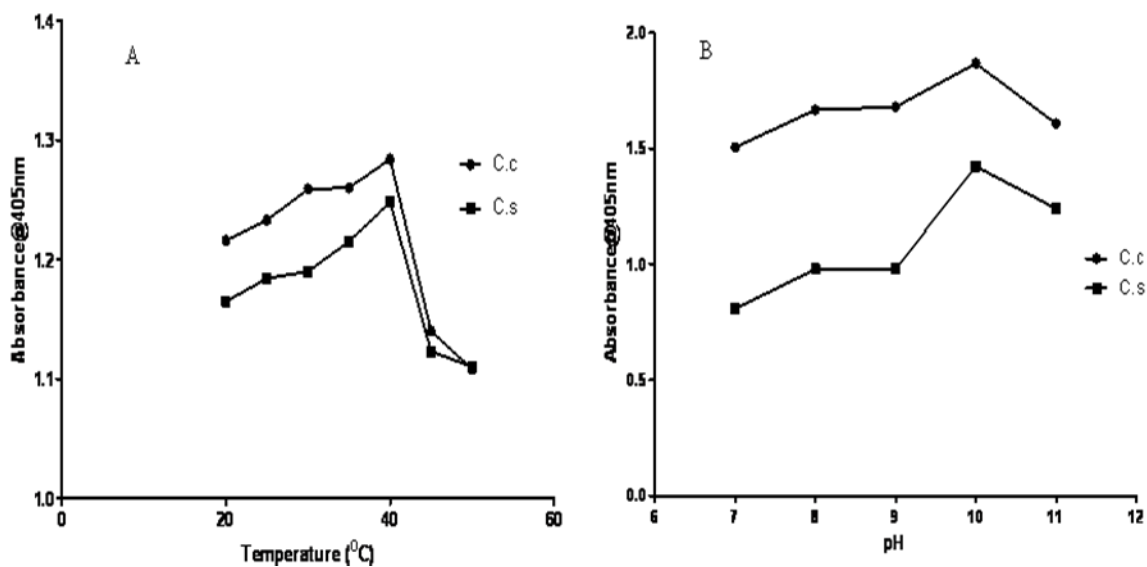


Figure 2: Effect of Mg²⁺ on alkaline phosphatase activity (A) and (B) EDTA on alkaline phosphatase activity in *Callinectes sapidus* and *Cardisoma carnifex*



Legend: Cc (*Cardisoma carnifex*), Cs (*Callinectes sapidus*)

Figure 3: Effect of temperature (A) and pH (B) on alkaline phosphatase activity

3. RESULTS AND DISCUSSION

In this study, alkaline phosphatase was isolated and characterized from different tissues (carapace, appendages, digestive gland (gut) and muscle) of crabs (*Cardisoma carnifex* and *Callinectes sapidus*). Of all these tissues examined, the digestive gland contains the highest concentration of protein 9.915 ± 0.021 and 10.125 ± 0.177 mg/ml followed by the muscle 6.735 ± 0.049 and 9.035 ± 0.049 mg/ml, the carapace 6.31 ± 0.014 and 8.1 ± 0.021 mg/ml and the appendages 4.595 ± 0.134 and 6.705 ± 0.078 mg/ml for *Callinectes sapidus* and *Cardisoma carnifex* respectively (Table 1).

Alkaline phosphatase activity was highest in the digestive gland homogenates of the two crab species with CS having an enzyme activity of 4844.73 ± 37.664 U/l and that of CC as 5618.033 ± 417.439 U/l. These are followed by activities in muscles, appendages and the carapace homogenates respectively (Table 2). The highest activity of this enzyme in the digestive gland of these two species suggests that most organic phosphates are hydrolyzed in this tissue. This corroborates our earlier findings that most hydrolyses of organic phosphate of big African snail occur in the digestive gland [9]. However, ALP activity has been reported to vary using different buffer. ALP activity from shrimp increased significantly when the homogenate buffer was change to AMP buffer however, there was no increase in activity when Tris-Hcl buffer was used [10].

The K_m and V_{max} of the digestive gland ALP (DGALP) from CS and CC were 2.756 mM and 3.636 mM, and $2.838 \mu\text{mole/min/ml}$ and $3.322 \mu\text{mole/min/ml}$ respectively (Figures 1a and 1b). The lower K_m of CS for PNPP might be an indication that the enzyme from the CS has a higher affinity for the substrate.

Alkaline phosphatase is a divalent metalloenzymes, both induction and inhibition of phosphatases have been reported to take place depending on metal concentration [11]. Our result showed that MgCl_2 had an unstable activating effect on the DGALP (Figure 2a). EDTA (10 mmole/l) however had an inhibitory effect on the enzyme activity (Figure 2b) [11], studied the effect of copper on oxygen consumption and phosphatase activity in *S. serrata* and concluded that there was decrease in alkaline phosphatase activity in muscle, hepatopancreas and haemolymph. Similar observations were noted in crab in response to naphthalene [12]. There are few reports describing the behaviour of alkaline phosphatase activity when challenge by some compounds. These reports include the effect of MgCl_2 and EDTA [13, 14, 15].

The DGALP has an optimum temperature of 40°C (Figure 3a) and optimum pH of 10.0 (Figure 3b). Any alterations in the activity of alkaline phosphatase affect the organisms in a variety of ways. Alkaline phosphatase is a brush border enzyme that splits various phosphorus esters at an alkaline pH and mediates membrane transport [16].

4. CONCLUSION

Our findings therefore suggest that ALP activity is highest in the digestive gland of these invertebrates which further confirms our earlier findings that most organic phosphates are hydrolyzed in this tissue in invertebrates as against the highest ALP activity in bones of some vertebrates and other higher animals.

5. REFERENCES

1. R. Seger and E.G. Krebs "The MAPK signaling cascade". *FASEB J.* 9 (9): 726–35, 1995
2. J.E. Ladbury "Measurement of the formation of complexes in tyrosine kinase-mediated signal transduction". *Acta Crystallogr. D Biol. Crystallogr.* 63 (Pt 1): 26–31, 2007
3. M.J., Martins, M.R. Negrao and C. Hipolito-Reis "Alkaline phosphatase from rat liver and kidney is differentially modulated" *Clinical Biochemistry* 34: 463-468, 2001
4. E.E. Kim and H.W. Wyckoff "Structure of alkaline phosphatases" *Clin. Chem. Acta* 186: 175-188, 1991
5. Sammy De Grave, N. Dean Pentcheff, Shane T. Ahyong *et al.* "A classification of living and fossil genera of decapod crustaceans". *Raffles Bulletin of Zoology Suppl.* 21: 1–109, 2009
6. R.W. Sterner and J.J. Elser "Ecological stoichiometry: the biology of elements from molecules to the biosphere" Princeton University Press, Princeton, New Jersey, USA, 2002
7. E. Layne "Spectrophotometric and Turbidimetric Methods for Measuring Proteins" *Methods in Enzymology* 10: 447-455, 1957.
8. G.N. Bomers and R.B McComb "Measurement of total alkaline phosphatase activity," *Clinical Chemistry*, vol. 21, pp. 1988-1995, 1975.
9. O.G. Raimi, A.A. Fatai, H.A. Bankole, S.N. Olaitan, O.O. Fajana, M.I. Kazeem, and K.O. Akobada "Characterization of alkaline phosphatase from giant African snail" *J. Cell Tissue Res.* 11(1): 2485-2489, 2011
10. K. Puttige and K.R. Nooralabettu "Alkaline phosphatase activity during homogenization of hepatopancreatic tissue of shrimps using sodium acetate, KCl solution, Tris-HCl and Glycine-NaOH buffer" 2(10):1-7, 2011
11. M.R. Ahmed, M. Elumalai, S.E. Balasubramanian and M.P. Balasubramanian "Individual and Combined Effect of Copper and Chromium on Oxygen Consumption and Phosphatases of a Marine Edible Crab, *Scylla serrata*" *Biomed. Lett.* 55: 147-152, 1997
12. M. Elumalai, S.E. Balasubramanian, M.P. Balasubramanian "Influence of Naphthalene on Protein, Carbohydrate and phosphatases System during the Vitellogenesis Marine Edible Crab, *Scylla serrata*" *Bull. Environ. Contam. Toxicol.* 60: 25-29, 1998

13. H. van Belle "Kinetics and inhibition of rat and avian alkaline phosphatases" *Gen. Pharmacol.*, 7: 53-58, 1976
14. J. Fraki, O. Ruuskanen and K. Kouvalainen, "Biochemical characterization of alkaline phosphatase in guinea pig thymus" *Biochim. Biophys Acta*, 482: 370-378, 1977
15. F.J. Vanneuville and J.G. Leroy "Properties of alkaline phosphatase in crude homogenates of human diploid fibroblasts" *Arch. Internal. Physiol. Biochim.*, 88: 155-161, 1980
16. S.E. Goldfisher, E. Esser and A.B. Novikoff "Use of Histological and Histochemical Assessment in the Prognosis of the Effects of Aquatic Pollutants" *Amer. Soc. Test. Mat. Philadelphia*. 194 -208, 1964