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Full Length Research Article

Purification Characterization and Inhibition Studies on Phospholipase C From *Opisthacanthus Capensis* (Black Scorpion) Venom

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ABSTRACT

Phospholipase C from *Opisthacanthus capensis* venom was partially purified and characterized. The enzyme was purified 105.2 fold with an overall yield of 13% using various purification steps involving ammonium sulphate precipitation, protamine sulphate treatment, sephadex G-75 fractionation and DE-52 anion exchange chromatography. The purified enzyme was homogeneous with a molecular weight of 29 kDa. The phospholipase C has pH and temperature optima of 7.2 and 60^0C, respectively with activation energy of 25KJ/mol and $t_{1/2}$ of 1.50 hr. Initial velocity studies on *O. capensis* venom phospholipase C revealed a K_M of 0.02 mM and V_{max} of 0.015 µmol/min. Studies on the effect of pH on K_M and V_{max} gave PKa_1 of 6.9 and PKa_2 of 7.4 with enthalpy of ionization of 20 KJ/mol suggesting histidine in the active site. The enzyme was positively modulated by Mg^{2+}, Zn^{2+} and Ca^{2+} and negatively by Fe^{2+}. While Hg^{2+} produced complete inhibition. Various concentrations of leaf aqueous extract of *Momordica charantia* also inhibited the activity of *O. capensis* venom phospholipase C *in vitro* with a competitive pattern. This study revealed the presence of phospholipase C in *O. capensis* venom and gave some scientific basis for the use of the plant in the treatment of scorpion envenomation.

Keywords: Phospholipase C; *Opisthacanthus capensis; Momordica charantia;* Venom

INTRODUCTION

Scorpion venom contains numerous toxins, biogenic amines, enzymes, salt, unidentified substances and water. The venom also contains many protein, peptides and other compounds, several of which are biologically active and found to be particularly useful in physiological and pharmacological research as investigatory tools (Petricevich, 2010]. Venom toxicity varies according to several factors such as genus, species, age, physiology, feeding state and geographical location of the scorpion (Emam *et al*., 2008). Hence, there is a strong demand to obtain venom from various species of scorpions for research purposes (Emam *et al*., 2008). Scorpion venom causes haemolysis. Some of the haemolytic activity caused by the venom is due to phospholipase C and other haemolytic enzymes such as phospholipase A_2. Phospholipase C (phosphatidylcholine phosphohydrolase, EC 3.1.4.3) is found in various toxin secreting organisms such as *P. aeruginosa* (Allan and Michell, 1974). Phospholipase C, a glycolipid and alkaline phosphatase is believed to function cooperatively in liberating inorganic phosphate from phospholipids (Bertognolo *et al*., 2007). The proposed mechanism involves the action of phospholipase C on glycolipid-solubilized phospholipids to liberate phosphorylcholine which can then be hydrolyzed by alkaline phosphatase to yield free phosphate (Bertognolo *et al*., 2007).

Phospholipase C comes in multiple forms and plays a key role in the signal transduction process for many receptors (Urbina *et al*., 2011). Its main function is to hydrolyze phosphatidylinositol diphosphate into diacylglycerol (DG) and inositol triphosphate (IP3) (Urbina *et al*., 2011). DG is necessary for further activation of protein kinase C while IP3 leads to the release of intracellular calcium (Urbina *et al*., 2011). DG and IP3 are the second messengers (Bertognolo *et al*., 2007). IP3 binds to ligand-gated Ca^{2+} channels on internal membranes leading to an influx of calcium ions into the cytoplasm (Bertognolo *et al*., 2007). High

intracellular Ca^{2+} can increase the risk of mitochondrial damage (Bertognolo *et al*., 2007). This can cause excitotoxicity, resulting in further activation of endonucleases and protease (calpain) leading to damage to the cell structures such as cytoskeleton, cell membrane and DNA (Bertognolo *et al*., 2007). Excitotoxicity may be involved in neurodegenerative disease of the central nervous system (CNS) such as multiple sclerosis (Kumar *et al*., 2009). Evidence suggests that phospholipase C (as alpha toxins) is the main virulent factor for gas gangrene in humans. The lipase activity assists toxin-producing organism to generate lipid signals in the host eukaryotic cell, and ultimately degrade the host cell membrane (Urbina *et al*., 2011).

Haemoglobinuria, acute tubular necrosis, interstitial nephritis and haemolytic–uraemic syndrome have been documented in human victims of scorpion envenomation (Kumar *et al*., 2009).. The haemolytic activity can be related to the venoms effects on disrupting the phospholipid bilayer (Kumar *et al*., 2009). It is therefore, of interest to screen scorpion venoms for the specific enzymes (such as phospholipase C) believed to be responsible for carrying out this activity and to, evaluate the inhibitory effect of *Momordica charantia* (*Guna*, in Hausa language and *Tumbul fari* in kanuri language).

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used in this study were of analytical grade and were purchased from various sources. *Opisthacanthus capensis* were collected in Bam Bam village of Balanga local government, Gombe State, Nigeria and was authenticated by Dr. A. M. Kokori of Biological Sciences Department, University of Maiduguri, Maiduguri, Nigeria.

Methods

Plant Extract Preparation by Maceration

Exactly 20 g of the ground sample from the leaf of *Momordica charantia* were mixed with about 100 ml of distilled water in a conical flask stirred, covered with foil paper, and allowed to stand overnight with occasional shaking. The supernatant formed was then carefully decanted into a conical flask (100 ml). This process was repeated until the supernatant became colorless. The average room temperature during the extraction was 27 Celcius. The fraction obtained was then filtered using a clean filter paper.

Venom Extraction

Venom was extracted by crushing the scorpion tail (telson) into a 50mM Tris-HCl buffer pH 7.2 at 40°C in a cleaned centrifuge tube. After centrifugation at 10,000 x g, the supernatant was collected and used as crude enzyme.

Total Protein Estimation and Enzyme Assay

The UV spectrophotometric method was used to estimate total protein content (Stoscheck, 1990). Phospholipase C (PLC) assay was carried out as described by Kurioka and Matsuda, (1976). The assay was carried out at 35°C in a cuvette with a 10 mm optical path length. This method was based on the principle that phospholipase C hydrolyses *p*-nitrophenylphosphorylcholine (pNPPC) to phosphorylcholine and p-nitrophenol which is, monitored spectrophotometrically at 410 nm. The buffer used was Tris HCl pH 7.2 in 60% sorbitol. Exactly 200 µL substrate, 200 µL crude (from each centrifuged fraction) and 100 µL 60% sorbitol in Tris-Hcl buffer pH 7.2 was used for the assay.

Phospholipase C Purification and Molecular Weight Estimation

The Purification of Phospholipase C was conducted as follows: precipitation with Ammonium Sulphate The crude PLC (3 ml for each grade) was precipitated using gradient solutions of ammonium sulphate saturation (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% and 90%). Each fraction was centrifuged at 10,000 x g for 20 min after standing overnight at 4°C. Phospholipase C activity and total protein were estimated in the precipitate as well as the supernatant for each fraction. The ammonium sulphate fraction that gave the highest specific activity was treated with

3 mL 2% protamine sulphate (pH adjusted to 7.2) and was allowed to stand for 10 min at 37^{o}C then centrifuged at 10,000 x g for 15 minutes. The precipitate was then discarded and the supernatant was subjected to gel purification using sephadex G-75. The gel was pre-equilibrated with 0.05 M Tris-HCl buffer pH 7.2 for 6 hours. The column (2 x 50 cm) was then loaded with 1.5 mL solution obtained after protamine sulphate treatment and eluted with the same equilibration buffer. Five milliliter (5 mL) fraction was collected at a flow rate of 0.3 ml/min. Each fraction was assayed for phospholipase C activity and total protein. Subsequently, phospholipase C active fractions obtained were pooled together, concentrated by dialysis and 5 mL was applied to a DEAE-Cellulose, DE- 52 column (2 x 30 cm). The column was eluted with a linear gradient of NaCl (0.025- 0.50 M) prepared in 0.05 M Tris HCl buffer. The flow rate was maintained at 0.2 ml/min. Molecular weight of the partially purified enzyme was carried out using SDS- PAGE as described by Laemmli (1970).

Characterization of Partially Purified PLC

Optimum Temperature of Phospholipase was determined by incubating the mixture of enzyme and substrate at varying temperatures ranging from 30^{o}C - 80^{o}C for 10 minutes and the activity assayed. The operational stability of PLC was determined by transferring aliquots every hour to ready reaction mixture after incubating the enzyme in a water bath at 60^{o}C. Residual activity which is directly related to enzyme stability was determined at constant pH, temperature, and ionic strength using the same buffer in all determinations. Half-life of the enzyme was calculated using the formula: $t_{1/2} = 0.693/k_D$ where k_D is the decay constant and is given by $K_D = 2.303/t - \log (E_O/E)$, where (E_O/E) is the fraction of enzyme activity remaining after incubation after time t. Activation energy (Ea) was determined by preincubating the enzyme and substrate at 5ºC interval range from 20ºC - 80ºC for 10 minutes before assaying for activity. Arrhenius plot was used to determine Ea. The pH optimum of phospholipase C was determined by assaying enzyme activity at varying pH ranging from pH 4-9 (acetate buffer for pH range 4- 5; phosphate buffer for pH range 6-6.9 and Tris-HCl buffer for pH range 7- 9). A plot of activity against pH was used to determine pH optimum.

Kinetic Studies

An initial velocity study was done by incubating 100 µL enzyme in 100 µL each of 0.1, 0.2, 0.3, 0.4 and 0.5 mM of the substrate and data obtained was used for double reciprocal plot from which K_m and V_{max} were extrapolated. The effect of pH on kinetic parameters was estimated from initial velocity of PLC at varying pH of 4, 6, 7.2, 8 and 9. The K_m and V_{max} were determined at each pH and Dixon-Webb plot was used to determine the likely ionisable groups in the active site of PLC. Effects of presence and absence of Ca^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, and Cu^{2+} on PLC activity was also assayed using a working standard of 10 mM concentration of each ion in the assay medium.

Inhibition Study using aqueous extract of *Momordica charantia*

For this purpose, initial velocity study was carried out in the presence and absence of three different concentrations of *M. charantia* (8 mg/mL, 4 mg/mL, and 2 mg/mL). Lineweaver-Burk plot was used to deduce type of inhibition.

Statistical Analysis

The data were presented as mean ± standard deviation and Prism® version 3.05 (GraphPad, USA) was used to determine the level of significance at $p<0.05$

RESULTS

Table 1 presents purification profile of phospholipase C from *O. capensis* venom. It showed a progressive increase in enzyme specific activity from crude sample to the final step of DE-52 anion exchange chromatography. The crude PLC was purified 1.2 folds after undergoing ammonium sulphate precipitation with a percentage yield of 92%. Further purification of the ammonium sulphate treated PLC with protamine sulphate resulted in a 3.6 fold

purification with a yield of 56%. Subsequent purification on sephadex G-75 column chromatography produced a purification fold of 47.6 with a yield of 11%. Finally, purification on DE-52 anion exchange chromatography produced a purification fold of 105.2 with a yield of 13%.

Table 1. Summary of Purification Profile of Phospholipase C from the Venom of *Opisthacanthus capensis.*

Fraction	Total Protein (mg/ml)	Activity (µmol/min)	Specific Activity (µmol/min/mg)	Fold Purification	Yield (%)
Crude Protein	0.70581	1.492×10^{-2}	2.1134×10^{-2}	1.0	100
Ammonium Sulfate Precipitation (30 - 60%)	0.52268	1.3725×10^{-2}	2.6259×10^{-2}	1.2	92
Protamine Sulfate precipitation	0.1100	3.2679×10^{-3}	2.9700×10^{-2}	3.6	56
Sephadex G-75	0.00161	6.405×10^{-4}	3.9700×10^{-1}	47.6	11
DE-52 anion Exchange	0.00088	7.712×10^{-4}	8.7640×10^{-1}	105.2	13

Effects of pH and Temperature on Phospholipase C Activity

Figure 1 shows the effect of pH on the activity of phospholipase C. A plot of phospholipase C activity against varying pH revealed that the optimum pH for the purified phospholipase C was 7.2. Similarly, Figure 2 shows the effect of temperature on the activity of phospholipase C. A plot of phospholipase C activity against varying temperature revealed that the optimum temperature for the purified phospholipase C was 60°C.

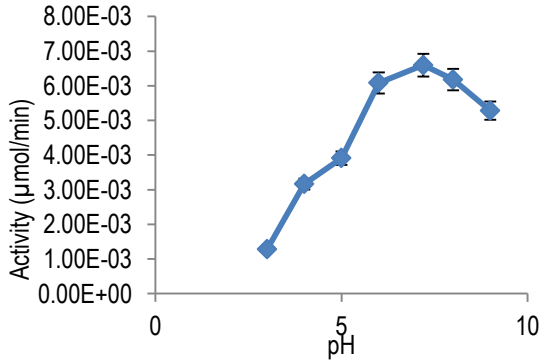

Figure 1: Effects of pH on Phospholipase C Activity

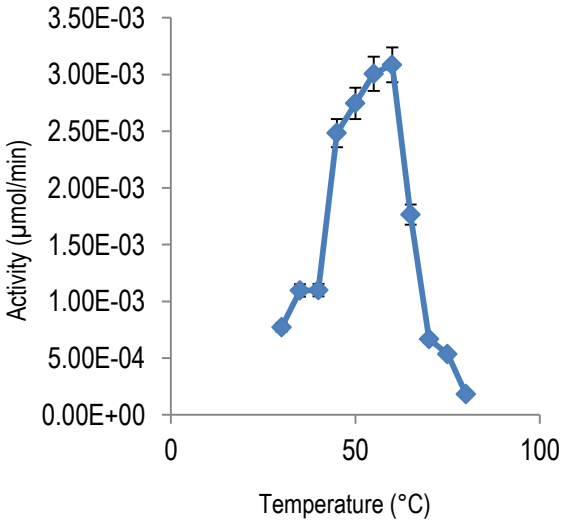

Figure 2: Effects of Temperature on Phospholipase C Activity.

The activation energy deduced from Arrhenius plot (Figure 3) was calculated as 25 kJ/mol while the operational stability of Phospholipase C at 37ºC after 12 hours incubation was 43% with a $t_{1/2}$ of 1.50 hours (Figure 4).

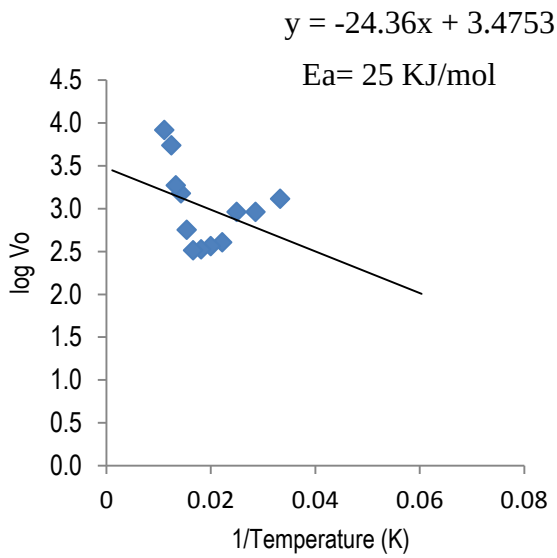

Figure 3: Arrhenius Plot for Phospholipase C

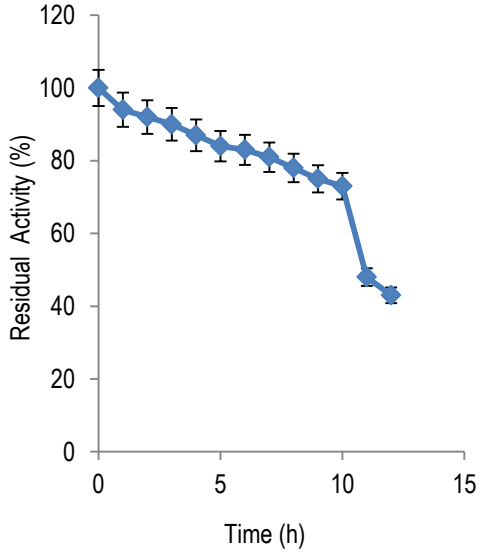

Figure 4: Operational Stability of Phospholipase C at 37°C

Double reciprocal and Dixon plot of Partially Purified Phospholipase C are presented in Figures 5 and 6 respectively. The K_m and V_{max} were 0.02 mM and 0.015 (µmol/min); while the pKa_1 and pKa_2 values were approximately estimated to be 6.9 and 7.4 respectively (Figure 6).

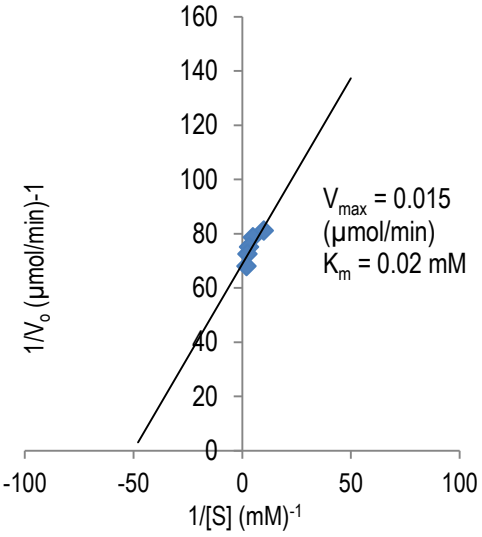

Figure 5: Double Reciprocal (Lineweaver- Burk) Plot of Purified Phospholipase C

Effect of divalent cations on the Activity of Phospholipase C as presented in Figure 7a and 7b show the effect of different divalent cations on phospholipase C activity. It represents phospholipase C activity in the presence of ions and control.

Inhibition Study on Phospholipase C Activity

Figure 8 Depicts the Lineweaver-Burk plot of competitive inhibition of phospholipase C by aqueous extract of *Momordica charantia.* The set of data showing a series of lines crossing the Y (1/V) axis at the same point that is V_{max} is unchanged, but with increase in K_m as the concentration of *Momordica charantia* was increased.

SDS-PAGE Electrophoregram of Phospholipase C

Figure 9 shows the SDS-PAGE Banding Pattern of Phospholipase C. Lane 1- marker proteins (from top 150 kDa, 80 kDa, and 25 kDa), lane 2- crude phospholipase C, lane 3-partially purified phospholipase C after ammonium sulfate fractionation, lane 4-partially purified phospholipase C after protamine sulfate treatment, lane 5-partially purified phospholipase C using Sephadex G-75 column chromatography,

and lane 6-partially purified phospholipase C using DE-52 anion exchange chromatography.

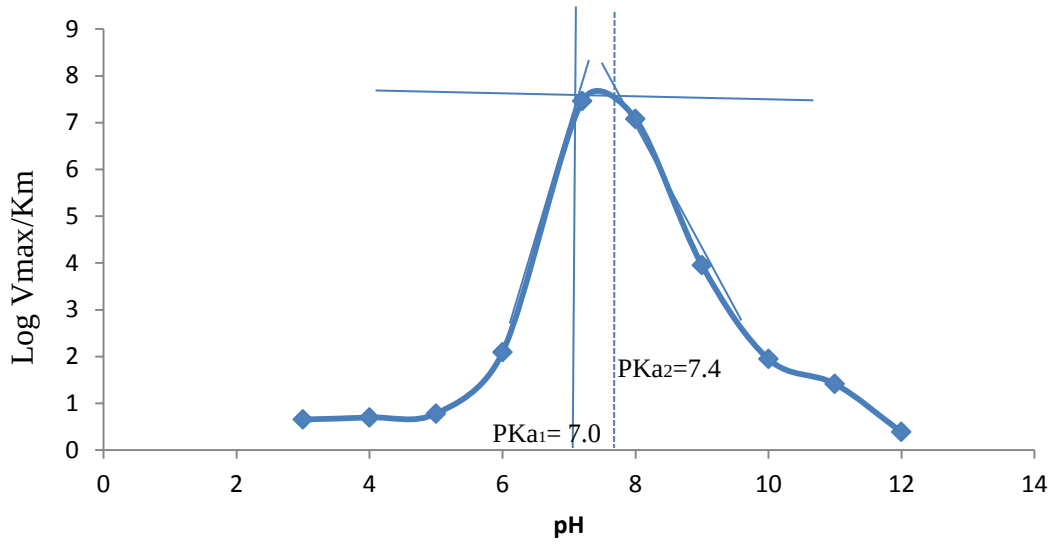

Figure 6: Dixon plot for *Opisthacanthus capensis* Venom Phospholipase C. pka_1 and pKa_2 values are deduced from the pH values corresponding to the tangent points

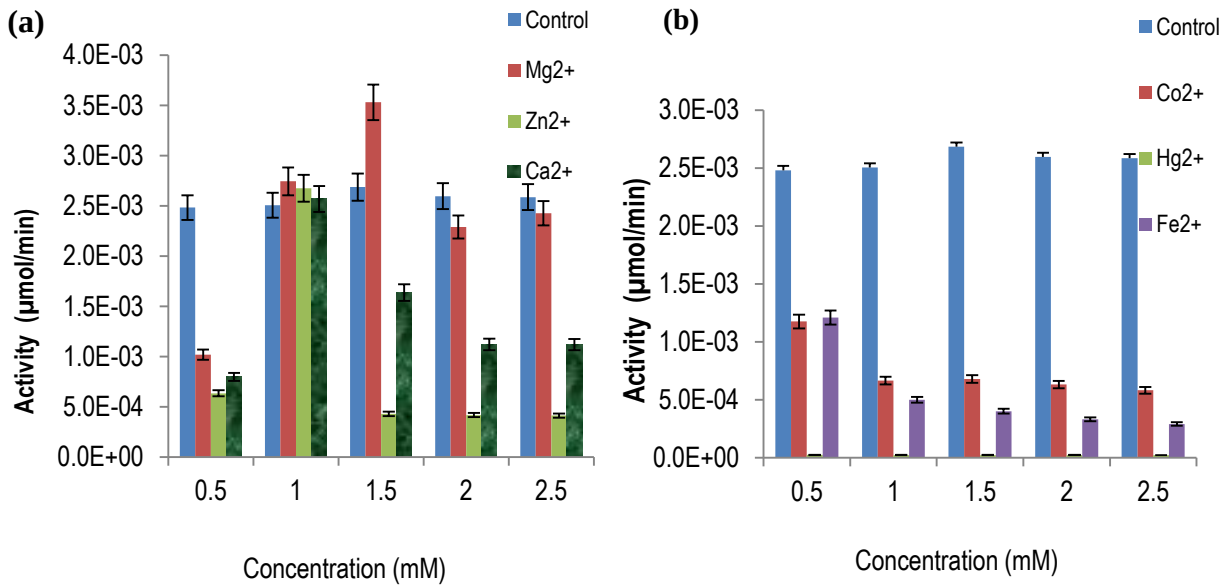

Figure 7: Effect of Divalent Cations on Phospholipase C Activity. (a) effect of Mg^{2+}, Zn^{2+}, and Ca^{2+} on phospholipase C activity and (b) effect of Co^{2+}, Hg^{2+}, and Fe^{2+} on phospholipase C activity.

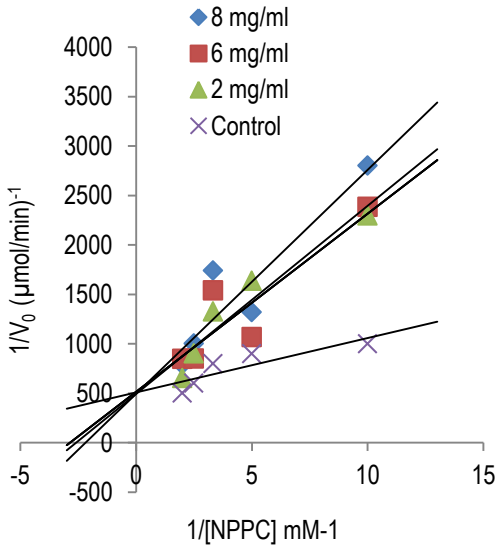

Figure 8: Lineweaver-Burk plot showing Competitive Inhibition of Phospholipase C by Aqueous Extract of *Momordica charantia.*

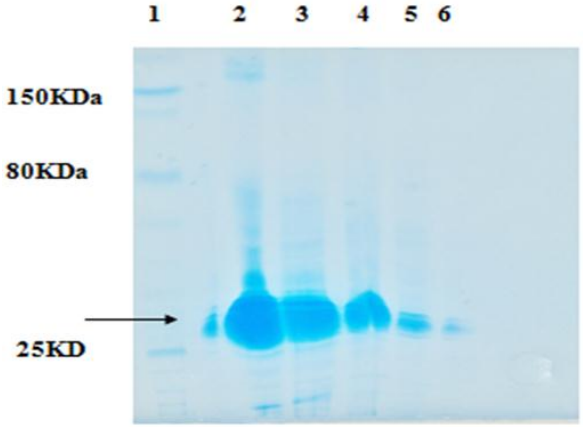

Figure 9: SDS-PAGE Electrophoregram of phospholipase C.

Lane 1= molecular weight marker; lane 2 = ammonium sulphate fraction; lane 3 = protamine sulphate fraction;lane 4 = sephadex G75 fraction; lane 5 = DE-52 anion exchange fraction

DISCUSSION

The purification steps of phospholipase C from *O. capensis* venom indicated both the crude and purified samples exhibited phospholipase C activity. The purified enzyme also exhibited appreciable degree of operational stability at 37°C which might be one of the factors that the microbial source of the enzyme is used for the enzymatic degumming of phospholipids in addition to its thermal stability (Dayton *et al*., 2010).

Scorpion venom contains small and larger molecular weight components (Rodolfo *et al*., 2013). In this study, *O. capensis* venom phospholipase C was purified 105.2 fold with overall yield of 13% and a specific activity of 8.784×10^{-1} µmol/min/mg. Hirata *et al*., (1995) reported that phospholipase C (in the supernatant of *Clostridium perfringens* homogenate) was recovered at 60% ammonium sulphate saturation, supporting the present study.

The estimated molecular weight of 29 KDa from SDS polyacrylamide gel electrophoresis was similar to the molecular weight of phospholipase C, 29 KDa and 24 KDa from *Listeria monocystogens* (Geoffroy *et al.,* 1990) and *L. ivanovii* (Jürgen *et al*., 1984) respectively. The same enzyme from *Clostridium perfringens* gave a molecular weight of 43 KDa (Hale and Stiles, 1999). However, phospholipase C isolated from cells of haematopoietic origin in humans gave a molecular weight of 133.7 KDa (Bertognolo *et al*., 2007).

The optimum pH of 7.2 indicated that the enzyme could operate optimally at physiological pH. This report is in agreement with a previous report by Kurioka and Liu, (1967). Patricia *et al* (2009) reported a pH optimum of 7.0 for phospholipase C from *C. perfringens*.

The enzyme was found to be active at a wide range of temperatures from 30°C to 75°C with an optimum at 60°C (Fig. 2.), but lost its activity at 80°C probably due to denaturing at elevated temperature (Mandels and Andreotii, 1976). A previous study found that the optimum temperature of phospholipase C from *Bacillus mycoides* was 60°C (Chang *et al.*, 2010). The activation energy of phospholipase C using *p*-nitrophenyl phosphorylcholine as a substrate was found to be 25.00 KJ/mol (Figure 3). Enzymes usually operate by effectively lowering the amount of energy required to start a reaction. Sometimes this happens because enzymes might weaken a covalent bond within a substrate molecule (Temple, 2012). In other

cases this lowering of activation energy seems to happen because the enzyme holds the substrate molecule in a particular orientation that increases the likelihood hood that the molecules are going to react (Temple, 2012). Without the enzyme much more activation energy is required to get the reaction to take place (Temple, 2012).
The K_M of 0.02 mM indicates high substrate affinity. The stability at 60^oC makes phospholipase C a potentially suitable biocatalyst for industrial processes especially for degumming of phospholipids in the food industries. The differences in organism/species might contribute to the differences in K_m and V_{max} found in other organisms/species (Hafiz *et al.,* 2011).

The partially purified phospholipase C was found to be positively modulated by Mg^{2+} ion at concentration of 1.5 mM, but at higher concentration, it inhibited the enzyme activity. The metal may reside at sites other than the active site and change the conformation of phospholipase C or it may bind near the active site and effect some conformational changes. In this way, Mg^{2+} ion may facilitate binding of the substrate *p*-nitrophenyl phosphorylcholine to the enzyme. However, Ca^{2+} and Zn^{2+} slightly show positive modulatory effect on the enzyme, but Co^{2+} and Fe^{2+} inhibited phospholipase C. The catalytic activity of phospholipase C depends on Ca^{2+} as a cofactor and the activity increases with a rise of Ca^{2+} concentration within the physiological range (0.01-10µm) (Mara *et al*, 1998). Certain bacteria phospholipase C requires Mg^{2+} for maximum catalysis (Richard, 1993). Hg^{2+}, completely inhibited phospholipase C. These ions may have also occupied the same site on phospholipase C as Mg^{2+} but induce a conformational change such that substrate no longer fits into the active site. Since Hg^{2+} has strong affinity for sulfhydryl -SH, it can irreversibly bind to the (~SH) group of phospholipase C and completely inhibits its activity (Angelovicovâ *et al*., 2014). The decreasing order of effectiveness of these divalent cations is as follows; Mg^{2+}> >Ca^{2+}> Zn^{2+}> Co^{2+}> Fe^{2+}> Hg^{2+}. This shows that phospholipase C is a metalo enzyme (Kurioka and Matsuda, 1976).

It is evident that the phospholipase C from *Opisthacanthus capensis* venom was competitively inhibited by the aqueous extract of *Momordica charantia* with an inhibition constant (Ki) of 4.2 mM (Figure 8). This indicates that the constituents of the aqueous extract of *Momordica charantia* binds reversibly to the active site of the enzyme and occupies it in a mutually exclusive manner with the substrate forming enzyme – inhibitor complex. This can be reversed by increasing the concentration of p-nitrophenyl phosphorylcholine. The values of V_{max} were found to be significantly not altered, while the K_M values were significantly altered. This is because V_{max} is a function of all enzyme molecules uniting with the substrate (Johnson *et al*., 2010). The Michaelis constant $K_{M,}$ on the other hand, will be altered with changes in the concentration of *Momordica charantia* because it requires larger concentration of the substrate, p nitrophenyl phosphorylcholine to overcome the direct competition by *Momordica charantia* for the active site.

The study on the ionisable groups in the active site of phospholipase C revealed the presence of groups with enthalpy of ionization of 20 KJ/mol. This result is suggestive of histidine in or around the active site of the enzyme. Ionisable groups at the active site of plant phospholipase C have been shown include histidine residue (Amerjeet *et al.,* 2015). This residue is well conserved in eukaryotic phospholipase C (Amerjeet *et al.,* 2015). Therefore, substrate recognition and mechanism of catalysis are likely to be similar in all eukaryotic phospholipase C (Marchler-Bauer *et al*., 2015).

CONCLUSION

From the characteristics of the enzyme observed, *Opisthacanthus capensis* can serve as a potential source of phospholipase C and the use of *Momordica charantia* extract by local population especially in North Eastern part of Nigeria as anti-scorpion venom have some scientific basis. Therefore, the active ingredients in *Momordica charantia* responsible for the *in vitro* inhibition of phospholipase C should be isolated and studied. Also, the gene for phospholipase C from *Opisthacanthus capensis* should be isolated, cloned and expressed.

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