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Vol. 9(2), pp. 26-34, February, 2015
DOI: 10.5897/AJBR2014.0795
Article Number: 268ADA650301
ISSN 1996-0778
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African Journal of Biochemistry Research

Full Length Research Paper

Characterization of partially purified cysteine protease inhibitor from *Tetracarpidium conophorum* (African walnut)

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Received 23 August, 2014; Accepted 26 January, 2015

Cysteine protease inhibitors (CPIs) have been known to be present in a variety of seeds of plants, and have been intensively studied as useful tools for potential utilization in pharmacology. This study reports the isolation of CPI from *Tetracarpidium conophorum* by 65% ammonium sulphate saturation, followed by ion exchange chromatography; further purification was by gel filtration chromatography. The molecular weight of the partially purified protein inhibitor was analyzed by SDS-PAGE to be approximately 20 kDa. The inhibitor had an optimum pH and temperature of 8.0 and 40°C, respectively. The inhibitor competitively inhibited papain with the same V_{max} = 71.17×10^3 µmol/min, K_m = 166 µM, and K_i = 53.63 µM. Divalent metal ions such as, Mg^{2+}, Pb^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} had significant effect on inhibitory activity of CPI at concentration as low as 1 mM. Cysteine protease inhibitor of *T. conophorum* investigated in this study could serve as a template in biotechnology of herbal medicine to arrest the negative modulatory interactions of cysteine proteases in clinical pathogenic expressions.

Key words: *Tetracarpidium conophorum,* cysteine protease inhibitor, papain, purification, characterization.

INTRODUCTION

The interaction between cysteine proteases and their inhibitors is a requisite in preventing unwanted, potentially destructive proteolysis, which can be utilized in chemotherapy. Cysteine proteases (CPs) are widely distributed among living organisms, and are responsible for many biochemical processes occurring in living organisms, such as production of nutrients for cell growth and proliferation in *Rhizopus oligosoporus* (Lin et al., 2011). They play multi-faceted roles in plants, virtually in every aspect of their physiology and development, such as growth, senescence and apoptosis, in the accumu-lation and mobilization of storage proteins such as in seeds. They are also involved in signaling pathways triggered by protease-activated receptors (PARs), which are critical mediators of haemostasis, thrombosis, biotic and abiotic stresses, and have been implicated in cancer progressions, making this receptor class an important drug target (Chen et al., 2005). The *Carica papaya* enzyme papain (EC 3.4.22.2) was the first known proteolytic enzyme and its digestive properties were already being utilized in the

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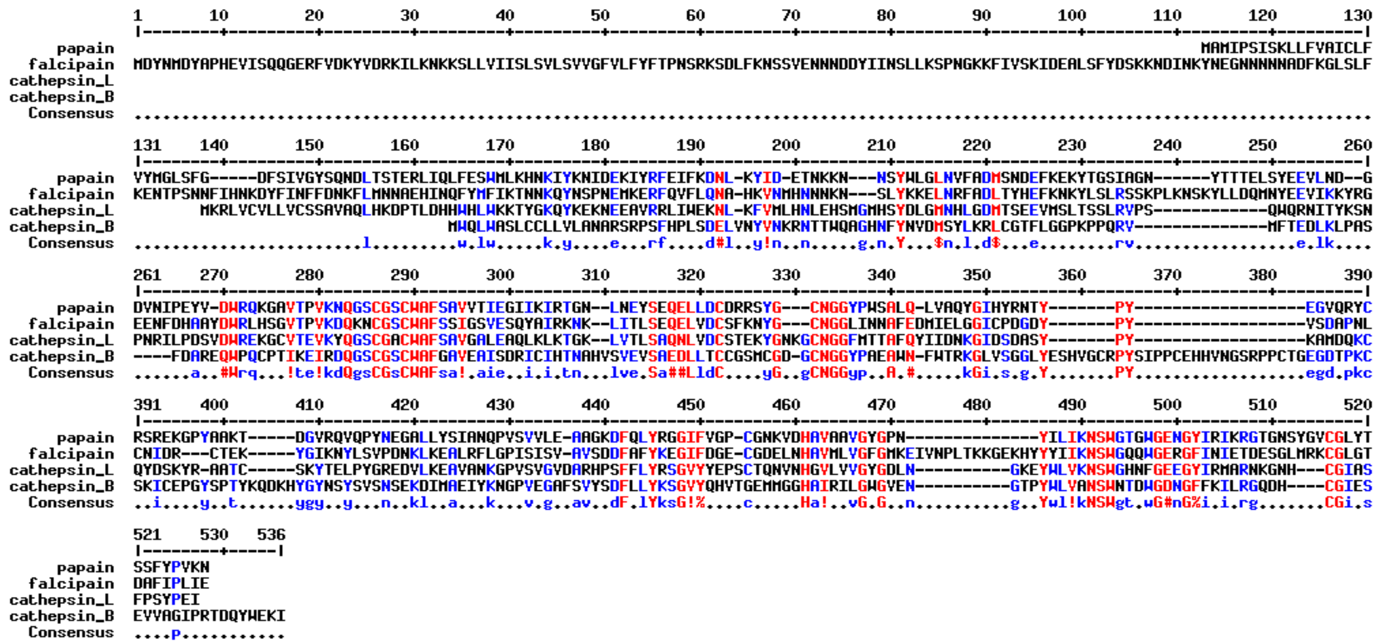

Figure 1. Sequence alignment of structurally equivalent residues of some C1 family of cysteine proteases. Coloured red are residues that are conserved; blue are similar residues and black are residues that are different in all the groups.

19th century (Redzynia et al., 2009). Since then papain has been used as a model enzyme in many studies and is a founding member of a large C1 family of papain-like cysteine proteases (Rawlings et al., 2008), while calpain (EC 3.4.22.52) constitute the C2 family of calcium-dependent non-lysosomal cysteine proteases expressed ubiquitously in mammals and many other organisms (Ohno et al., 1984). C1 family of proteases are evolutionarily old and are found in both prokaryotes and eukaryotes and show activities that are indispensable for the organism (Redzynia et al., 2009). Some of the mammalian cysteine proteases are evolutionarily closely related to papain and hence belong to this family for example cathepsins B, H, L, S and K (Figure 1). These enzymes function in every cell as components of the lysosomal degradation system taking part in the turnover of proteins as well as participate in a number of proteolytic cleavages, activating pro-hormone and regulation of antigens (Redzynia et al., 2009).

Obligatorily, the activities of these enzymes need to be strictly regulated and controlled (Corrion et al., 2010) , by cysteine protease inhibitors. Activities of proteases are generally regulated by protease inhibitors, which are usually proteins with domains that enter or block a protease active site to prevent substrate access (Figure 2). A control mechanism of proteases involves interaction of the active enzymes with proteins that inhibit their activities. These proteins have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Outchkourov et al., 2003). C1 family of cysteine proteases are in equilibrium with protein inhibitors belonging to the cystatin family (Hartmann et al., 1997; Rawlings and Barrett, 1990; Lustigman et al., 1992; Rawlings et al., 2008). Most cystatins such as human cystatin B are single domain proteins of 100-120 residues with a characteristic wedge-like epitope consisting of the N-terminus and two β-hairpin loops which blocks the active site cleft of the target enzyme hence inhibiting the activity in a reversible manner (Abrahamson et al., 1986; Bode et al., 1988) (Figure 2).

The African walnut is an annual agricultural product found abundantly in Nigeria (Ayoola et al., 2011b). The plant, which belongs to the family of *Euphorbiaceae*, is a temperate fruit and nut crop, with a high fat content hence Walnuts are rich source of energy and contain health benefiting nutrients, minerals, antioxidants and vitamins that are essential for optimum health (Ayoola et al., 2011a). Walnuts are considered to be an herb in traditional Chinese medicine (Ganiyu and Mofoluso, 2004). The leaves, bark, root and fruit of *Plukenetia conophora* are considered to be medicinal (Enitan et al., 2014). The anti-microbial potential of *P. conophora* extracts and fractions against wide spectrum of bacteria (including *Staphylococci*, *Clostridia*, *Escherichia* and *Pseudomonas*) and some fungi like *Aspergillus niger* and *Candida albican* have been demonstrated (Ajaiyeoba and Fadare, 2006). The ethanol: water extract of the dried leaves had the best antioxidant activity; the broad range of antioxidant activity of this extract indicates the potential of the plant as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidetive stress and consequent health benefits (Amaeze et

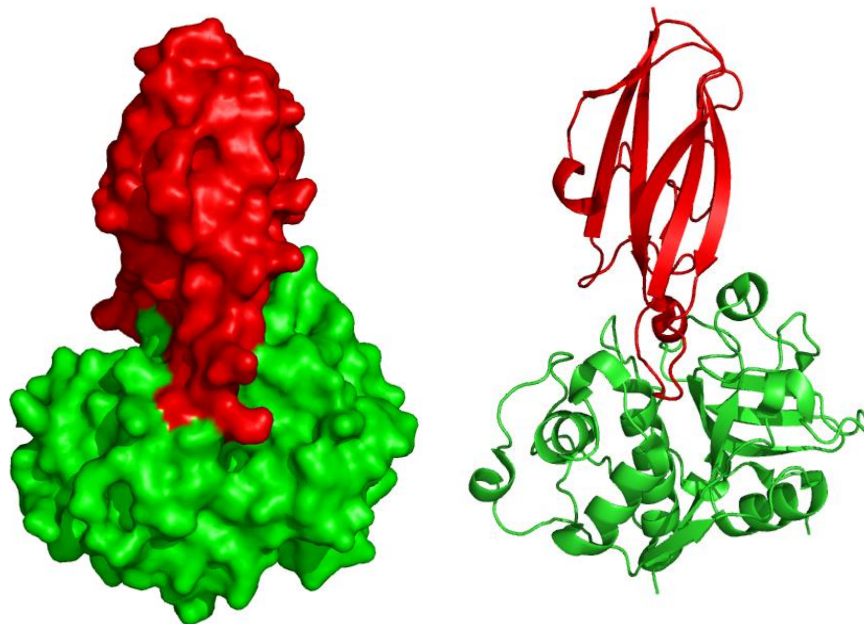

Figure 2. Surface and cartoon representations of the crystal structure of Chagasin-papain complex. The chagasin molecule is coloured in red and papain in green. The view is along the catalytic cleft of papain and corresponds to the standard orientation used for cysteine proteases (Redzynia et al., 2009; pdb: 3E1Z).

al., 2011). Antibacterial activity of methanolic leaf extract of *P. conophora* Mull. arg. against selected bacteria isolated from urinary tract infection has also been demonstrated (Enitan et al., 2014). The aim of the study was to isolate, purify and characterize cysteine protease inhibitor (CPI)from *Tetracarpidium conophorum* seeds.

MATERIALS AND METHODS

Sample collection and preparation

T. conophorum seeds and leaves were obtained at Ketu market in Lagos State, Southwestern Nigeria. The leaves were deposited at the herbarium of the Botany Department, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria for proper identification and authentication. 1000 g of *T. conophorum* seeds were de-shelled, dried and ground to powder with a grinding machine. The powdered sample was then defatted with n-hexane using the soxhlet apparatus, according to the method of Franz von Soxhlet (1879).

Extraction and isolation of cysteine protease inhibitor

Cysteine protease inhibitor was isolated as described by Benjakul et al., 1998. 40 g of the defatted sample was extracted in 500 ml ice-cold 100 mM phosphate buffer pH 7.2 containing 130 mM NaCl and 0.1% β-mercaptoethanol, stirred thoroughly for 1 h. The mixture was filtered using clean white piece of cloth. The filtrate was centrifuged at 7000 *g* for 10 min to collect cell debris. Cysteine protease inhibitor was then isolated from the resulting supernatant by ammonium sulphate precipitation according to the method described by Englard and Seifter (1990). Ammonium sulphate required to precipitate the protein was optimized by adding varying concentrations, (35, 55, 65, 75 and 90%) to the crude extract independently.

After each precipitation the precipitate was collected by centrifugation at 7000 *g* for 10 min. The precipitate was then re-dissolved in a small volume of buffer and dialyzed overnight against 100 mm Tris buffer pH 7.8 that was changed every 6 h.

Protein determination

Protein concentration was determined by Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard. The absorbance was read at 595 nm.

Inhibitory activity

The inhibitory activity of cysteine protease inhibitor on papain was monitored according to the modified method of Murachi, 1970, using casein as substrate. Papain (6 mg in 100 mM Tris-HCl buffer, pH 7.8, 0.5 mM cysteine, 0.2 mM EDTA) and the inhibitor extract (50 µl) were pre- incubated at 37°C for 15 min. The reaction mixture (150 µl) was then added to tubes containing 1.0 ml of 0.5% casein (casein prepared in 100 mM Tris-HCl pH 7.8 containing 0.2 mM EDTA and 0.5 mM cysteine) at 37°C. The assay was incubated for 30 min at 37°C and the reaction terminated by the addition of 1.0 ml of 5% trichloroacetic acid (TCA). The absorbance of the supernatant was measured at 280 nm after 30 min. One unit of inhibitor activity was defined as the decrease by one unit of absorbance of tricholoroacetic acid-soluble casein hydrolysis product liberated by protease action at 280 nm at 37°C in a given assay volume. Percentage Inhibition was determined as shown below:

Table 1. Purification table of cysteine protease inhibitor (CPI) from *T. conophorum.*

Sample	Total protein (mg)	Inhibitory activity (units)	Specific inhibitory activity (units/mg)	Protein yield (%)	Activity yield (%)	Purification fold
Crude extract	363	4000	11.02	100	100	1
Ammonium sulphate Fraction (65%)	126	1500	11.9	34.7	37.5	1.1
Ion Exchange chromatography (DEAE-cellulose)	46	3500	76.1	12.67	87.5	6.9
Gel filtration (Sephadex G-100)	26	3330	128.1	7.16	83.25	11.61

The purification fold of cysteine protease inhibitor from ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography were 1.1, 6.9 and 11.6 respectively.

$$\text{\% Inhibition by each tube} = \frac{\text{Abs of Std} - \text{Abs of Sample}}{\text{Abs of Std}} \times 100$$

Protein purification

The crude protein after dialysis was purified by ion exchange chromatography according to the method of Rossomando (1990), followed by size exclusion chromatography. 3.0 ml of the dialysate (with highest % inhibitory activity against papain) was then loaded on DEAE-cellulose column previously equilibrated with 100 mM Tris-HCl buffer pH 7.8. 5 ml fractions were collected into 60 test tubes using an increasing linear gradient of NaCl concentration from 0 to 0.3 M in the same buffer (Tris-HCl). Total protein and inhibitor activity were carried out on each fraction as earlier described. Fractions with highest inhibitory activity were pooled together concentrated and loaded on sephadex G-100 column previously equilibrated with 100 mM Tris-HCl buffer pH 7.8 and eluted using the same buffer. Fractions with highest inhibitory activity were pooled together and analyzed further by SDS-PAGE for molecular weight estimation.

Mechanism of inhibition

Mode of inhibition of the purified CPI was carried out as described for inhibitory assay above but now varying the concentration of casein. This was carried out both in the absence and presence of the inhibitor, respectively.

Optimum temperature

The optimum temperature for the inhibitor was determined by incubating the reaction mixtures at varying temperatures ranging from 10 - 100°C with 10-unit increase.

Optimum pH

Estimation of the pH optimum was carried out using Tris-HCl buffer with varying pH (6-11) differently in the reaction mixtures. Other steps were as described for inhibitory assay.

Effect of metals on inhibitory activity

Effect of different metal ions on protease inhibitory activity was carried out by incubating the protease inhibitor with different concentrations of various metals ions for 30 min followed by measuring inhibitory activity as described above. The metals that were investigated included Mg^{2+},Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+},and Pb^{2+}. Each with concentration between 1 to 10 mM.

RESULTS AND DISCUSSION

Plant CPIs have been researched extensively, but this study however is the first at attempting to characterize cysteine protease inhibitor from *T. conophorum. T. conophorum* is a temperate fruit and nut crop which possess an elevated level of fat content. The seed is an annual agricultural product, which is found abundantly in Nigeria (Ayoola et al., 2011).

CPI was isolated by ammonium sulphate precipitation, using different concentrations of ammonium sulphate (35, 55, 65, 75 and 90%) and maximal inhibitory activity was observed at 65% saturation and this was used in further studies. The increase in hinbitory activity observed at 65% might be due to an increase in concentration of the salt ions having a greater charge density, thus increasing its ionic strength through the decline in solvent molecules being used to solvate the salt ions for optimum saturation, stressing the role of hydrophobic aggregation of proteins in molecular interaction.

Bijina et al. (2011), reported 30 - 60% ammonium sulphate saturation for *Moringa oleifera* protein. The protein was separated from the salt ions by dialysis, which allows ammonium sulphate salts and small metabolites to diffuse across a semi-permeable membrane. This step of purification yielded a low purification fold close to the crude protein, which suggests the presence of impurities (Table 1). Further purification of cysteine protease inhibitor by chromatographic methods, using DEAE cellulose an anion exchanger, yielded multiple peaks with a single peak of maximum inhibitory activity and a slight increase in purification fold (Table 1).

The single peak with the maximum inhibitory activity obtained after ion exchange chromatography was purified further by gel filtration yielded multiple peaks with two

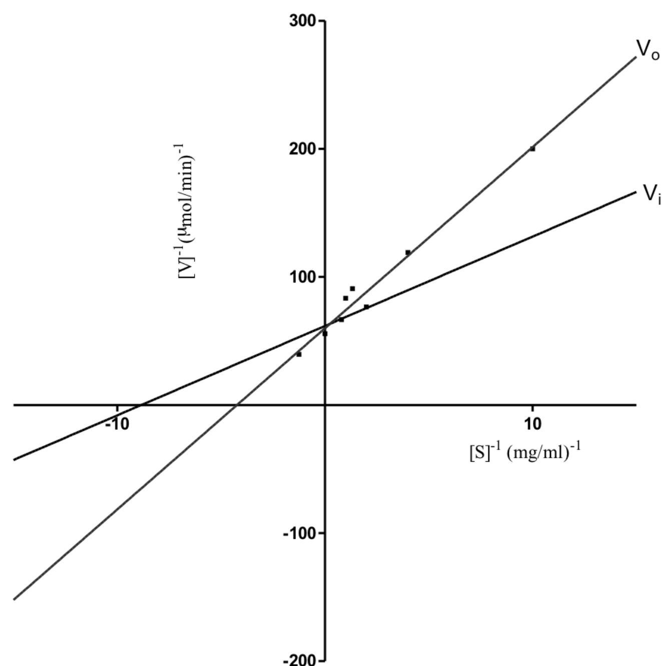

Figure 3. Line weaver-Burk plot of purified CPI. In the absence and presence of purified inhibitor extract, the reciprocal of the varying amount of substrate concentration [1/S] used and the reciprocal of the absorbance (1/V) at 280 nm were plotted against each other. The double reciprocal plot shows that inhibition is competitive having the same V_{max} of 71.17×10^3 μmol/min, different K_m =166 μM and K_i = 53.63 μM. $[V]^{-1}$ = with inhibitor and $[V_0]^{-1}$ = without inhibitor.

peaks having maximum inhibitory activity. This suggests that a splitting of the polypeptide chain might have occurred or there might be two different cysteine protease inhibitors present. The gel filtration chromatography using sephadex G-100, resulted in the highest purification fold of 11.61 and protein yield of 83.3% (Table 1). Bijina (2006), in his work, purified cysteine protease inhibitor from *Moringa oleifera,* using ammonium sulphate precipitation (30 - 90%) and ion exchange chromatography, and obtained purification fold of 1.5 and 2.5%, respectively. This could be accounted for by the fact that each purification step reduced the level of impurities associated with the inhibitor, which suggests that the fold can be increased by repetitive purification steps using advanced purification methods.

CPI from *T. conophorum,* demonstrated a competitive mode of inhibition on papain (Figure 3) with the same V_{max} = 71.17×10^3 μmol/min, K_m = 166 μM, and K_i = 53.63 μM. The result obtained was contrary to that reported by Bijina (2006), who reported the mechanism of protease inhibition of *Moringa oleifera* as uncompetitive. Kinetic studies between chagasin and papain (Figure 2) revealed the interaction to be a very strong reversible interaction with a K_i of 36 pM with chagasin blocking the active site cleft of papain (Redzynia et al., 2009) (Figure 2).

The protein inhibitor has an optimum pH of 8.0 (Figure 4) and observed to be inactive at extreme acidic and alkaline pHs. This indicates that extreme pH conditions could alter the electrostatic interactions between charged amino acids such as aspartate, lysine, arginine, and glutamate, thereby disrupting the structure of the cysteine protease inhibitor*,* making it loose its activity partially or completely. The optimum pH of cysteine protease inhibitors isolated in this study is in accordance with those reported for other isolated plants cysteine protease inhibitor such as *Dimorphandra mollis* seeds (pH 8.0) (Mello et al., 2002) and *M. oleifera* leaves (pH 7.0) (Bijina, 2006). *T. conophorum* CPI has a temperature optimum of 40°C (Figure 5). Most plant's cysteine protease inhibitors are active from this temperature up to 50°C (Bijina, 2006). However, the inhibitory activity declined at temperatures lower than its optimum temperature, and was totally inactive at temperatures above the optimum temperature.

The probable precursor for the modulating activities of cellular actino-regulatory proteases is a consequence of thermal influence on violently disrupted bonds of cysteine protease inhibitor from elevated kinetic energy (Bein and Simons, 2000). This result shows that cysteine protease inhibitor might undergo thermal denaturation when exposed to temperatures higher than its optimum temperature.

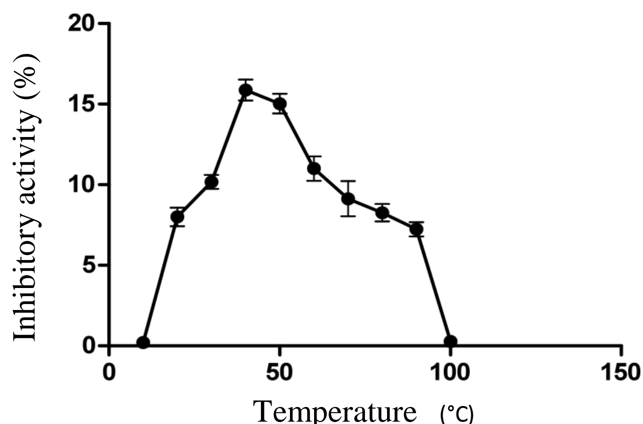

Figure 4. Optimum temperature of crude cysteine protease inhibitor. The result shows the optimal temperature of the cysteine protease inhibitor is 40°C. Cysteine protease inhibitor was almost inactive at low temperature, however as the temperature increases, the inhibitory activity increased gradually but decreased at temperatures above 40°C.

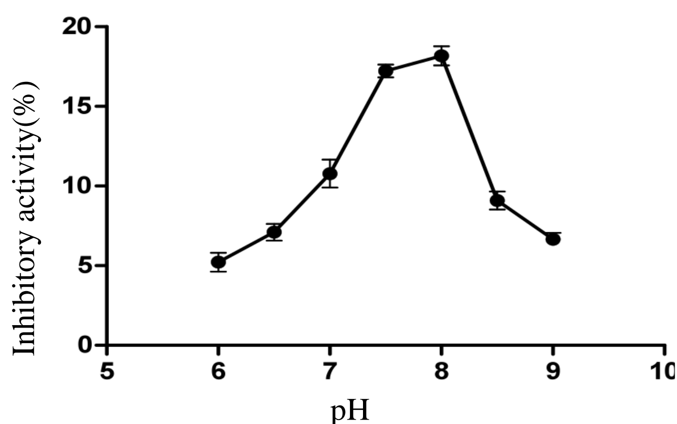

Figure 5. Optimum pH of purified cysteine protease inhibitor. The result shows the optimum pH of purified cysteine protease inhibitor at pH 8.0. Cysteine protease inhibitor was almost inactive at low pH values, however, as the pH increases, the inhibitory activity increased gradually but decreased at above pH 8.0.

This result is in agreement with those reported for other isolated plants cysteine protease inhibitor such as *Dimorphandra mollis* seeds (40°C) (Mello et al., 2002) and *M. oleifera* leaves (40°C) (Bijina, 2006).

Trace metal ions such as Co^{2+} and Zn^{2+} at low concentrations reduced the activity of CPI with a residual activity of 58 and 88%, respectively (Figure 6). However, when present in high concentrations, they are toxic and can denature proteins. Heavy metal ions such as Cu^{2+}, Mg^{2+}, Pb^{2+} and Mn^{2+} at 1 mm decreased the residual inhibitory activity of cysteine protease inhibitor up to 47.5, 51, 55 and 61.4%, respectively, compared to that of control. This suggest that these heavy metal ions interacted with the sulfhydryl groups of the cysteine protease inhibitor, thereby deactivating the inhibitor due to its sulphur-philic nature. The sulfhydryl group is respon-sible for the stabilization of tertiary and quaternary structure ofproteins. As a result, there is a conformational change in the three-dimensional structure of the protein and is denatured, thereby inhibiting their activity (Greenwood et al., 2002).

The heavy metal ions functioned in a concentration dependent manner, as their concentration increases, their inhibitory effect increases. The result obtained is in agreement with that of Jack et al. (2004), which confirmed that some divalent metal ions could play an important role in sustaining the structural integrity of protease inhibitors, such as Zn^{2+} and Mg^{2+}, that maintains the secondary and tertiary structure of cysteine protease inhibitor. The side chain carboxylates of glutamate and aspartate residues can however, participate in binding of divalent cations to metalloproteins, thereby resulting in amino acid modifications of the structure leading to a decrease in inhibitory activity, whereby the inhibitor is unable to bind to the active site of the enzyme or substrate to exhibit inhibitory activity. Most metalloproteases require zinc, but some use cobalt (Szeto et al., 2008).

The purified CPI was further analyzed by SDS-PAGE to determine its molecular weight. The molecular weight of cysteine protease inhibitor from *T. conophorum* was estimated to be 20 kDa (Figure 7). This result indicates that *T. conophorum* CPI is composed of a single polypeptide chain. Martinez-Vicente et al. (2005), reported a molecular weight of 23.1 kDa, for cysteine protease inhibitor obtained from *Fragaria x ananassa*. Cysteine protease inhibitor from black gram (*Vigna mungo Hepper)* and rice bean (*Vigna umbellata Thunb),* have molecular weight of 12 kDa by Tricine-SDS-PAGE (Benjakul and Visessanguan, 2000). Most of these protein inhibitors are small molecules with relative molecular masses ranging from 5 - 25 kDa (Singh and Rao, 2002; Lustigman et al., 1992).

The inhibition of papain and not trypsin, a serine protease (data not shown) reveals the specificity of this inhibitor against cysteine protease, which could be explored in biotechnology for the *in-vitro* synthesis and development of target drugs for pathogenesis of atherosclerosis, dementia and cancer. The inhibition of calpain has been shown to prevent NMDA-induced cell death and β-amyloid-induced synaptic dysfunction in hippocampal slice cultures (Nimmrich et al., 2010).

Conclusion

Cysteine protease inhibitor was isolated and partially purified from *T. conophorum*. The protein inhibitor was demonstrated to inhibit papain a representative of the C1 family of cysteine proteases in a competitive manner. The biological, specificity and physicochemical properties exhibited by this inhibitor certainly indicate its likely suitability for application in biotechnology as pharmaceutical agents in the treatment of some pathological conditions such as

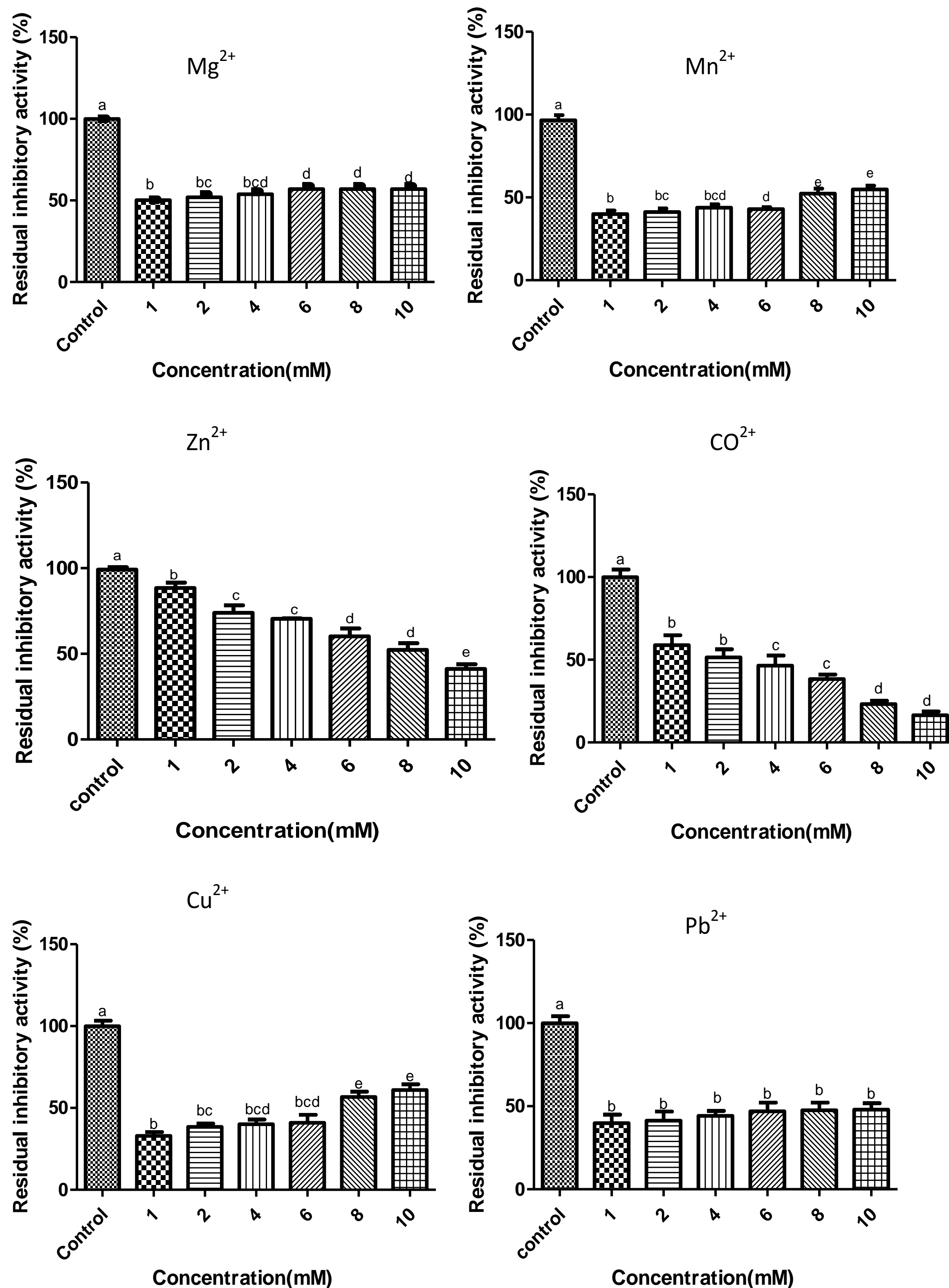

Figure 6. Effect of metal on CPI activity. The bars with the same superscripts are not statistically different (P<0.05).

cancer, atherosclerosis, and neurodegenerative diseases. However, further research is still required in determining its structure in other to elucidate its function and absolute specificity.

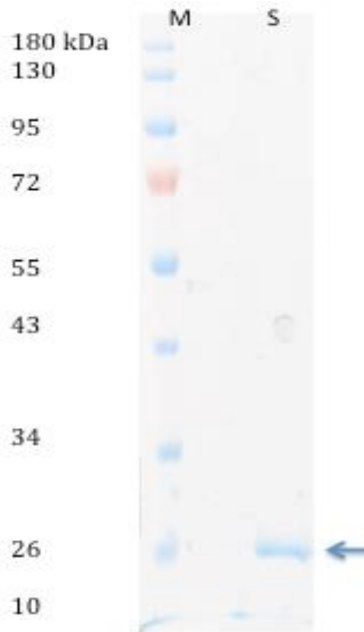

Figure 7. SDS-PAGE analysis of purified cysteine protease inhibitor. The gel pattern of the cysteine protease inhibitor fraction when subjected to SDS-PAGE yielded a single polypeptide band with a molecular weight of 20 kDa. M=marker; S=sample.

Conflict of interests

The authors did not declare any conflict of interest.

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