Research Article
Journal of Research and Review in Science, 97–104
Volume 4, December 2017
DOI:10.36108/jrrslasu/7102/40(0141)

ORIGINAL RESEARCH

CHARACTERIZATION OF PARTIALLY PURIFIED CYSTEINE PROTEASE INHIBITOR FROM CANAVALIA ENSIFORMIS (WONDER BEAN)

Segun Adeola, Habeeb Adebodun Bankole, Kanmodi Rahmon Ilesanmi, Segun Osikoya

[1]*Department of Biochemistry,*
Faculty of Science,
Lagos State University, Ojo,
Lagos, Nigeria

email: segun.adeola@lasu.edu.ng

Correspondence
Segun Adeola,
Department of Biochemistry,
Faculty of Science,
Lagos State University, Nigeria.

Abstract:
Introduction: Cysteine protease inhibitors (CPIs) are widespread in plant seeds and are attractive candidates for use in the pharmaceutical industry. Wonder bean (*Canavalia ensiformis*) is a climbing perennial legume with fascinating pharmacological properties. A distinguishing characteristic of this plant is its ability to thrive under extreme environmental conditions such as nutrient-depleted, highly leached and acidic soil. Plants that survive in these extreme environmental conditions are known for high effective protease regulation by protease inhibitors which include CPI.
Aims: This study was therefore designed to isolate and characterize CPI from wonder bean.
Materials and Methods: CPI from *Canavalia ensiformis* was isolated and purified by simple methods consisting of ammonium sulphate precipitation, ion exchange chromatography and gel filtration. Mode of inhibition, optimum temperature and pH, as well as the effect of metals on the enzyme activity were determined using spectrophotometric method.
Results: The purified CPI was confirmed to be a competitive inhibitor against papain with the same Vmax = 78.59 x10^3µmol/min, Km=209µM, and Ki = 137 µM. The inhibitor exhibited maximal activity at temperature and pH of 40°C and 8.0 respectively. Metal cations such as, Pb^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} significantly inhibited CPI at a very low concentration (1.0mM). The SDS-PAGE analysis revealed two bands of molecular weight estimated to be 28 and 32 KDa.
Conclusion: CPI from *Canavalia ensiformis* was successfully isolated, and there was an indication that it is composed of two polypeptide chains that might have been denatured under reducing condition in the presence of β-mercaptoethanol. However, further investigation is required to verify this.
To Keywords: *Canavalia ensiformis*, cysteine protease inhibitor, papain, purification, characterization.

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Uncontrolled proteolysis resulting from an imbalance between cysteine proteases and their inhibitors play a critical role in a wide spectrum of pathological processes. Cysteine protease inhibitors thus have considerable chemotherapeutic potentials which can be exploited in a variety of disease states. Cysteine proteases (EC 3.4.22) are widespread among living organisms and are responsible for many biochemical and physiological processes. They play key roles in the regulation of mammalian programmed cell death [1]. They are responsible for major histocompatibility complex (MHC) class II immune responses [2], prohormone processing [3], and extracellular matrix remodeling important to bone development [4].

They are also capable of hydrolyzing important proteins of the extracellular matrix such as elastin [5, 6].They induce accelerated collagen and elastin degradation by macrophages at sites of inflammation in diseases such as atherosclerosis [7] and emphysema [8]. It has also been reported that proteases are implicated in tumor growth and progression, both at primary and metastatic sites [9, 10] and have been exploited as important molecular targets for the prevention and therapy of cancer. In plants, they are involved in the degradation and mobilization of storage proteins in seeds [11]. They are also involved in signaling pathways as well as response to biotic and abiotic stresses [12, 13]. Cysteine protease activity is dependent on cysteine and histidine residues, the order and spacing of which vary in different known families [14].

Stringent regulation and control of activities of these enzymes is an imperative function of cysteine protease inhibitors (CPIs) [15]. Proteolytic activities are generally regulated by protease inhibitors, which are usually proteins with domains that blocks a protease active site thereby making it inaccessible to substrate [16]. Plant CPIs that have been characterized successfully include cystatin [17], testican [18], lipocalin [19], tyropins and chagasins [20].

Wonder bean (*Canavalia ensiformis*) is a climbing perennial legume [21], which is native to Central America and West Indies, but widely cultivated in humid tropics of Asia and Africa [22]. A distinguishing characteristic of this plant is its ability to continuously grow under extreme environmental conditions such as nutrient-depleted, highly leached and acidic soil [23]. Historically, it was used by native tribes for food and forage in south-western United States, Mexico, Brazil, Peru, Ecuador and the West Indies [24].

Wonder bean displays desirable nutritional features such as rich source of essential amino-acids isoleucine, leucine and tyrosine as compared with some common other legumes [25] and high energy content (1470-1910 kJ/100g) [26]. In addition, it contains an ample amount of sodium, magnesium, phosphorus, and calcium [25].The seeds are also rich in palmitic acid and essential fatty acid; oleic and linoleic [27]. Despite these admirable features, raw unprocessed seed of the wonder bean is toxic which limit its use as human food or animal feed [28-30].

Wonder bean is also fascinating in terms of its pharmacological importance. In Nigeria, its seed is used as an antibiotic and antiseptic [31]. Cholesterol lowering effect of its seeds in hypercholesterolemia rats has also been demonstrated [32]. There is also a pharmaceutical interest in its use as a source of anti-cancer agents [33]. Since plants express protease inhibitors including CPI to modulate protease activities in response to stress conditions [34], This study sought to isolate, purify and characterize cysteine protease inhibitor from *Canavalia ensiformis* seeds.

2. MATERIAL AND METHODS

Canavalia ensiformis seeds were obtained at Ikorodu market in Ikorodu local Government Area of Lagos state, south-western part of Nigeria. The sample of the plant was deposited at the herbarium of the Botany Department, Faculty of Science, University of Lagos, Lagos state, Nigeria for proper identification and authentication, after which voucher number (LuH 6100) was issued. The 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 [35].

2.1 Extraction and isolation of cysteine protease inhibitor

Isolation of cysteine protease inhibitor was carried out according to the method of Benjakul et al; [36]. 40 g of the defatted sample was suspended in 250ml of 10mM phosphate buffer solution, pH 7.2 containing 0.13mM sodium and 0.1 % (v/v) β-mercaptoethanol with continuous stirring for 2 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. Total protein was then precipitated out from the resulting supernatant by ammonium sulphate precipitation according to the method described by Englard and Seifter [37].

2.1.1 Ammonium sulphate precipitation

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 7000g for 10 min. The precipitate was then re-dissolved in a small volume of buffer and dialyzed overnight against 100 mMTris buffer pH 7.8 that was changed every 6 h.

2.1.2 Protein determination

Protein concentration was determined using Bradford method and bovine serum albumin (BSA) as standard [38]. The absorbance was read at 595nm.

2.2 Inhibitory activity

Cysteine protease inhibitory activity of extracted protein was monitored using papain according to the modified method of Murachi using casein as substrate [39]. Papain (55 mg in 0.1m 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 2.0 ml of 0.5% casein (casein prepared in 0.1MTris-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 3.0 ml of 5% trichloroacetic acid (TCA).The absorbance was measured at 280nm 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 280nm at 37°C in a given assay volume. Percentage Inhibition was determined as shown in the equation below.

% Inhibition by each tube=
(Abs of standard-Abs of sample X 100)/(Abs of standard)

2.3 Protein purification

The crude protein after dialysis was purified by ion exchange chromatography as described by Rossomando [40], followed by size exclusion chromatography. 3.0 ml of the dialysate (with highest % inhibitory activity against papain) was loaded on DEAE-cellulose column previously equilibrated with 100mM 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 100mM 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.

2.4 Mechanism of inhibition

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

2.5 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.

2.6 Optimum pH

Estimation of the optimum pH was carried out using Tris-HCl buffer with varying pH (6.0 -11.0) differently in the reaction mixtures. Other steps were as described for inhibitory assay.

2.7 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 metal 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.

3. RESULTS AND DISCUSSION

Table 1. Purification table of cysteine protease inhibitor (CPI) from *Canavaila ensiformis*

The purification fold of cysteine protease inhibitor from ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography increased significantly.

Sample	Total protein (mg)	Inhibitory activity (units)	Specific inhibitory activity (unit/mg)	Yield (%)	Activity yield	Purification fold
Crude extract	0.90	13.30	14.7	100	100	1.00
65% ammonium sulphate	0.46	9.90	22.0	50	74.4	1.4
DEAE-Ion exchange	0.25	12.2	48.8	27.8	91.7	3.3
Sephadex G-100	0.11	11.1	99.1	12.2	81.0	6.7

Figure 1 shows the mode of inhibition of CPI isolated from *Canavaila ensiformis* seed. 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 (Figure 1). The double reciprocal plot shows that inhibition is competitive having the same Vmax of 78.59µmol/min, different Km =209 µM and Ki= 137 µM. [V_i]= with inhibitor and [V_o] = without inhibitor.

Figure 2(a) and 2(b) shows the effect of temperature and pH on purified cysteine protease inhibitor from *Canavalia ensiformis.* 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. The optimum pH of purified cysteine protease inhibitor is pH 8.0. Cysteine

protease inhibitor was almost inactive at low pH values, however, as the pH increases, the inhibitor activity increased gradually but decreased at above pH 8.

Figure 3 shows that the inhibitory activity of CPI from *Canavaila ensiformis* was modulated by divalent metal ions. At concentration as low as 1mM, divalent metal ; Pb^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}decreased the residual cysteine protease inhibitory activity . However, the inhibitory activity of the CPI was increased as the concentration of Zn^{2+}, Cu^{2+} and Mg^{2+} increases.

Figure 4 shows the SDS-PAGE gel profile of purified CPI isolated from *Canavaila ensiformis.* The gel pattern of the cysteine protease inhibitor fraction when subjected to SDS-PAGE yielded a double polypeptide band with a molecular weight of 28 and 32kDa

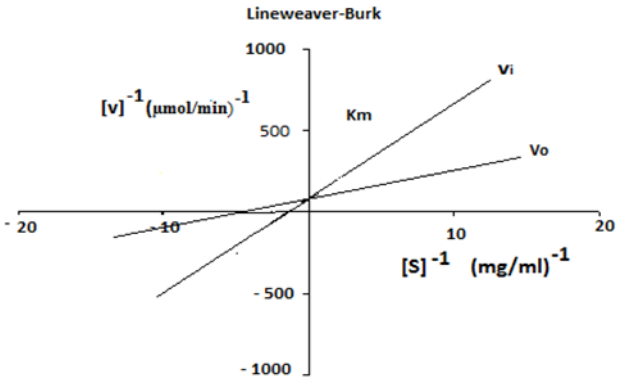

Fig 1; Mode of inhibition of CPI isolated from *Canavaila ensiformis* determined using Lineweaver-Burk plot

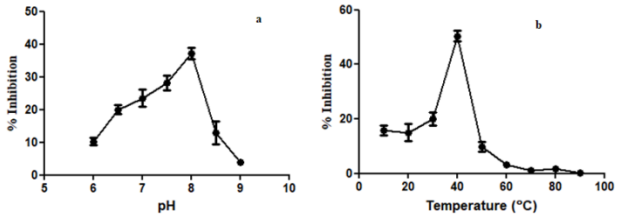

Fig 2; Effect of temperature and pH on the activity of purified CPI isolated from *Canavaila ensiformis*

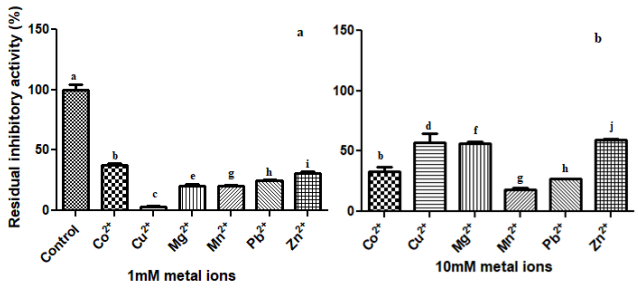

Fig 3; Effect of divalent metal ions on the activity of purified CPI isolated from *Canavaila ensiformis* at different concentrations (a) effect of ions at 1mM concentration (b) effect of ions at 10mM concentration. Each bar represent mean ± SEM, bar with same superscripts are not statistically different $p \leq 0.05$.

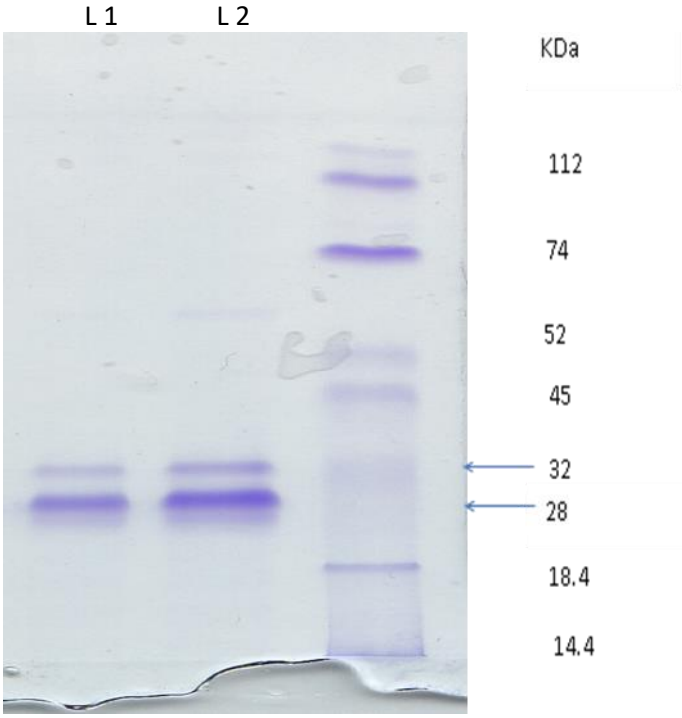

Fig 4. SDS-PAGE analysis of purified cysteine protease inhibitor isolated from *Canavaila ensiformis.* M= marker, L1 and L2 = sample of purified CPI in duplicate.

4. Discussion

Plant cysteine protease inhibitors (CPIs) have been studied extensively [12, 41, 42], but this study is the first at attempting to characterize CPI from *Canavalia ensiformis*. *Canavalia ensiformis* is an annual legume which grows on fertile soil and sunny or warm climate [43] The seed which possesses attractive nutritional properties is found abundantly in Nigeria [44]. However, its use is not maximized because of the toxic nature of raw unprocessed seeds. Therefore, there is the need to analyze the plant as potential source for biomolecules that can be of great use in medical and pharmaceutical industries.

Activities of cysteine protease inhibitor were enhanced as a consequence of an upsurge in concentration obtained from its crude extraction. It seems the stabilizing influence of phosphate buffer, and the effect of 2-mercaptoethanol in reconfiguring the disulfide linkage of the cysteine protease inhibitor provided an ideal condition for optimum extraction of cysteine protease inhibitor from its crude extract in this study. It was reported that 0.1M phosphate buffer at pH 7.6 is most suitable for the maximal extraction of protein from pigeon pea seeds (*Cajanus cajan*) with high protease inhibitor activity [45].

Isolated cysteine protease inhibitor (CPI) from *Canavalia ensiformis* seeds were purified using different concentrations of ammonium sulphate (35, 55, 65, 75 and 90%). Maximum inhibitory effect was observed with 65% ammonium sulphate precipitation and this was used for further studies. Several protease inhibitors extracted from plants have been reported to be more effective after precipitating with high ammonium sulphate concentration. 50 - 70% ammonium sulphate precipitation of protease inhibitors from seeds of *Helianthus annuus* gave the highest yield and inhibitory effect when incubated with larva gut proteases [46]. 65% ammonium sulphate saturation of CPI that exhibited the maximum inhibitory effect has also been reported [47].

The purification fold after dialyzing the protein to separate it from the salt ion was low when compared to the crude extract. This is an indication that the protein still has some impurities. Further purification of the CPI using DEAE cellulose ion exchange chromatography yielded multiple peaks. The eluent from DEAE cellulose anion exchange chromatography with the highest inhibitory activity was purified further by gel filtration. This yielded multiple peaks with two peaks having maximum inhibitory activity. This suggests that splitting of the polypeptide chain might have occurred or there might be two different cysteine protease inhibitors present. Activities of cysteine protease inhibitors were reported to be enhanced after purification with DEAE ion exchanger [48]. However, extracted CPI from the wonder bean gave the highest purification fold of 6.7 and percentage activity of 81% after gel filtration chromatography using sephadex G-100.

The cysteine protease inhibitor from *Canavalia ensiformis* seed was active at pH range of 6.5 to 8 with an optimum pH at 8.0. Extreme pH condition will affect the state of ionization of acidic or basic side chain of amino acid thereby changing the chemical feature (e.g. via covalent bond formation) [49]. If the state of ionization of amino acid in a protein is modified then the ionic bond that helps determine the 3-D structure of the protein is also reformed which leads to the alteration in protein recognition or the protein becomes inactive. Change in pH would not only affect the configuration of the protein, but also influence the charge of the substrate limiting its binding abilities to the active site of the protein. This is a schematic template that can be explored in drug targets for protease pathologies. Change in pH causes modification in protein folding resulting in the deactivation of the inhibitor, causing irreversible proteolysis [50].The optimum pH of cysteine protease inhibitors isolated in this study is similar to that reported for *M.oleifera* leaves (pH 7.0) [48].

The optimal temperature of the cysteine protease inhibitor isolated from the *Canavaila ensiformis* seeds was found to be 40˚C. Most plant's cysteine protease inhibitors are active from this temperature up to 50°C [48].Thermal inactivation of the cysteine protease inhibitor at temperature below or above the optimum temperature resulted in radical loss of activity. The thermal inactivation could be due to folding and unfolding of protein due to change in temperature resulting in modulation of covalent and non-covalent interaction and consequent loss of inhibitory activity of the protease inhibitor [51].

Interactions of metal ions such as Mg^{2+}, Pb^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} with cysteine protease inhibitor from *Canavalia ensiformis* showed that some metals modulates the inhibitory activity of cysteine protease inhibitor. Zn^{2+}, $Cu^{2+}$and Mg^{2+}at concentration of 10 mM enhanced the activity of the CPI with residual activities of 59%, 57% and 56% respectively as compared to the concentration at 1mM. This is similar to the findings of Bijina *et al.,* who reported that Zn^{2+} at 10mM enhanced the activity of cysteine protease inhibitor isolated, purified and characterized from *Moringa oleifera* [52].

These ions could play a major role in maintaining the structural integrity of the reaction by binding with enzyme or substrate for the inhibitor to exhibit the anti-protease activity. However at concentration of 1.0 mM, metal ions (Pb^{2+}, $Cu^{2+,}$ Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}) reduced the residual inhibitory activity of CPI compared to the control. It may be due to alteration of the protease inhibitor and deactivating it due to its sulphur-philic nature. As a result, there is a conformational change in the three-dimensional structure of the protein and is denatured, thereby inhibiting its activity [51].

The mode of inhibition of the purified cysteine protease inhibitor from *Canavalia ensiformis*, is competitive, yielding the same Vmax of 78.59 x 10^3µmol/mim, Km of 209 µM and Ki of 137 µM. This suggests that the inhibitor altered the rate of cysteine protease reaction by competitively inhibiting the enzyme or probably directs its effect on the substrate as substrate ligand, making it inaccessible to the active site of the protease. A regulatory mechanism of cysteine proteases involve interaction with exosite-binding inhibitors which directly block the active site by binding adjacent to it and covering it partially in a substrate-like manner [53] . On the other hand, the cysteine protease inhibitor might have reacted with the substrate to create a conjugate molecule which can no longer be recognized by the active site of the enzyme.

The purity and homogeneity of single fraction with maximum inhibitory activity obtained after gel filtration using Sephadex G100 were analyzed by SDS page. The result of SDS page analysis revealed two polypeptide bands with molecular weight of 28 and 32 KDa. the sds-page analysis was done in the presence of β-mercaptoethanol, which is a reducing agent. This suggests that *Canavalia ensiformis* cysteine protease inhibitor is probably composed of two polypeptide chains linked by one or more disulphide bonds. Double chain structured protease inhibitors was reported to be isolated from seeds of *Adenanthera pavonia*, *Acacia elata*, A*lbizia julibrissin* and *Prosopsis julijlora* [54]. Also the double band of cpi witnessed in the SDS-PAGE analysis could be the same protein with the lower molecular-weight band having a minor modification during purification. this is in line with what was reported for cpi isolated from native soy bean [55] as well as that reported for lima bean [56].

5. Conclusion

The cysteine protease inhibitor from *Canavalia ensiformis* was successfully isolated and purified. There was an indication that the CPI is composed of two polypeptide chains that might have been denatured under reducing condition in the presence of β-mercaptoethanol or single protein which some have probably been modified. However, further investigation is required to verify these.

COMPETING INTERESTS

The authors declared that no competing interests exist.

References

1. Eckelman B P, G S Salvesen, and F L Scott, Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO reports, 2006. **7**(10): p. 988-994.
2. Zavašnik-Bergant T and B Turk, Cysteine cathepsins in the immune response. Tissue antigens, 2006. **67**(5): p. 349-355.
3. Yasothornsrikul S, D Greenbaum, K F Medzihradszky, T Toneff, R Bundey, R Miller, et al., Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. Proceedings of the National Academy of Sciences, 2003. **100**(16): p. 9590-9595.
4. Lutgens S P, K B Cleutjens, M J Daemen, and S Heeneman, Cathepsin cysteine proteases in cardiovascular disease. The FASEB Journal, 2007. **21**(12): p. 3029-3041.
5. Kelly E, C M Greene, and N G Mcelvaney, Targeting neutrophil elastase in cystic fibrosis. Expert opinion on therapeutic targets, 2008. **12**(2): p. 145-157.
6. Piña-Vázquez C, M Reyes-López, G Ortíz-Estrada, M De La Garza, and J Serrano-Luna, Host-parasite interaction: parasite-derived and-induced proteases that degrade human extracellular matrix. Journal of parasitology research, 2012. **2012**.
7. Liang J, E Liu, Y Yu, S Kitajima, T Koike, Y Jin, et al., Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits. Circulation, 2006. **113**(16): p. 1993-2001.
8. Maclay J D, D A Mcallister, R Rabinovich, I Haq, S Maxwell, S Hartland, et al., Systemic elastin degradation in chronic obstructive pulmonary disease. Thorax, 2012: p. thoraxjnl-2011-200949.
9. Yang Y, H Hong, Y Zhang, and W Cai, Molecular imaging of proteases in cancer. Cancer growth and metastasis, 2009. **2**: p. 13.
10. Eatemadi A, H T Aiyelabegan, B Negahdari, M A Mazlomi, H Daraee, N Daraee, et al., Role of protease and protease inhibitors in cancer pathogenesis and treatment. Biomedicine & Pharmacotherapy, 2017. **86**: p. 221-231.
11. Jinka R, V Ramakrishna, S K Rao, and R P Rao, Purification and characterization of cysteine protease from germinating cotyledons of horse gram. BMC biochemistry, 2009. **10**(1): p. 28.
12. Grudkowska M and B Zagdanska, Multifunctional role of plant cysteine proteinases. ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-, 2004: p. 609-624.
13. Vaseva I, J Sabotič, J Šuštar-Vozlič, V Meglič, M Kidrič, K Demirevska, et al., The response of plants to drought stress: the role of dehydrins, chaperones, proteases and protease inhibitors in maintaining cellular protein function. Droughts: new research, 2012: p. 1-45.
14. Mcgrath M E, The lysosomal cysteine proteases. Annual review of biophysics and biomolecular structure, 1999. **28**(1): p. 181-204.
15. Corrion K, S Scoffier, C Gernigon, F Cury, and F D'arripe-Longueville, Development and factorial validity of a moral disengagement in sport short scale. L'Encephale, 2010. **36**(6): p. 495-503.
16. Dos Santos A L S, Protease expression by microorganisms and its relevance to crucial physiological/pathological events. World journal of biological chemistry, 2011. **2**(3): p. 48.
17. Shah A, Studies on Thiol proteinase inhibitors (cystatins) from goat liver. 2011.
18. Bocock J P, C J S Edgell, H S Marr, and A H Erickson, Human proteoglycan testican-1 inhibits the lysosomal cysteine protease cathepsin L. The FEBS Journal, 2003. **270**(19): p. 4008-4015.
19. Van't Hof W, M F Blankenvoorde, E C Veerman, and A V N Amerongen, The salivary lipocalin von Ebner's gland protein is a cysteine proteinase inhibitor. Journal of Biological Chemistry, 1997. **272**(3): p. 1837-1841.
20. Rzychon M, D Chmiel, and J Stec-Niemczyk, Modes of inhibition of cysteine proteases. Acta Biochim. Pol, 2004. **51**(4): p. 861-873.
21. Ekanayake S, E Jansz, and B M Nair, Literature review of an underutilized legume: Canavalia gladiata L. Plant foods for human nutrition, 2000. **55**(4): p. 305-321.
22. Malviya N, S Jain, and S Malviya, Antidiabetic potential of medicinal plants. Acta Pol Pharm, 2010. **67**(2): p. 113-118.
23. Udedibie A and C Carlini, Questions and answers to edibility problem of the Canavalia ensiformis seeds–a review. Animal Feed Science and Technology, 1998. **74**(2): p. 95-106.
24. Sauer J and L Kaplan, Canavalia beans in American prehistory. American Antiquity, 1969: p. 417-424.
25. Mohan V and K Janardhanan, The biochemical composition and nutrient assessment of less known pulses of the genus Canavalia.

International journal of food sciences and nutrition, 1994. **45**(4): p. 255-262.
26. Sridhar K and S Seena, Nutritional and antinutritional significance of four unconventional legumes of the genus Canavalia–A comparative study. Food Chemistry, 2006. **99**(2): p. 267-288.
27. Arun A, K Sridhar, N Raviraja, E Schmidt, and K Jung, Nutritional and antinutritional components of Canavalia spp. seeds from the west coast sand dunes of India. Plant Foods for Human Nutrition, 2003. **58**(3): p. 1-13.
28. Udedibie A, Nutritional evaluation of jackbean (Canavalia ensiformis) for the Nigerian poultry industry. Ambio (Sweden), 1990.
29. Herrera F, M Gutierez, S Cupul, M Ferrioro, J Carabano, and J Montilla, The effect of incorporation of Canavalia ensiformis seed into a ration of laying hens at 10% and 20%. Tropical Animal Production, 1981. **6**: p. 775-776.
30. D'mello J and A Walker, Detoxification of jack beans (Canavalia ensiformis): studies with young chicks. Animal Feed Science and Technology, 1991. **33**(1-2): p. 117-127.
31. Olowokudejo J, A Kadiri, and V Travih, An ethnobotanical survey of herbal markets and medicinal plants in Lagos State of Nigeria. Ethnobotanical leaflets, 2008. **2008**(1): p. 116.
32. Marfo E, P Wallace, G Timpo, and B Simpson, Cholesterol lowering effect of jackbean (Canavalia ensiformis) seed protein. General Pharmacology: The Vascular System, 1990. **21**(5): p. 753-757.
33. Morris J B, Legume genetic resources with novel “value added” industrial and pharmaceutical use. Perspectives on new crops and new uses, 1999: p. 196-201.
34. Kidrič M, J Kos, and J Sabotič, Proteases and their endogenous inhibitors in the plant response to abiotic stress. Bot Serb, 2014. **38**: p. 139-58.
35. Soxhlet F, Die gewichtsaiialytische Bestimmung des Milchfettes; von. 1879.
36. Benjakul S, W Visessanguan, and H An, Screening and isolation of cysteine proteinase inhibitors from Thai legume seeds. Prince of Songkla University, Thailand. Unpublished data, 1998.
37. Englard S S S, Precipitation techniques. Methods Enzymol. 1990. **182**(11): p. 285-300.

.
38. Bradford M M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 1976. **72**(1-2): p. 248-254.
39. Murachi, Bromelain enzymes. Methods Enzymol. 1970. **19**: p. 274-283. .
40. Rossomando, Ion-exchange chromatography. Methods Enzymol. 1990. **182**: p. 309-317.
41. Harrak H, S Azelmat, E N Baker, and Z Tabaeizadeh, Isolation and characterization of a gene encoding a drought-induced cysteine protease in tomato (Lycopersicon esculentum). Genome, 2001. **44**(3): p. 368-374.
42. Habib H and K M Fazili, Plant protease inhibitors: a defense strategy in plants. Biotechnology and Molecular Biology Reviews, 2007. **2**(3): p. 68-85.
43. Fao., Grassland species index. Canavalia ensiformis.. 2012.
44. Chee Y K H, J. B. ; Ramirez, L. ; Chen, C. P.,, Canavalia ensiformis (L.) DC.. Record from Proseabase. Mannetje, L.'t and Jones, R.M. (Editors). PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia. 1992.
45. Bijina B, S Chellappan, S M Basheer, K Elyas, A H Bahkali, and M Chandrasekaran, Protease inhibitor from Moringa oleifera leaves: Isolation, purification, and characterization. Process Biochemistry, 2011. **46**(12): p. 2291-2300.
46. Ashouri S, R F P Abad, F Zihnioglu, and E Kocadag, Extraction and purification of protease inhibitor (s) from seeds of Helianthus annuus with effects on Leptinotarsa decemlineata digestive cysteine protease. Biocatalysis and Agricultural Biotechnology, 2017. **9**: p. 113-119.
47. Obayomi A, S Adeola, H Bankole, and O Raimi, Characterization of partially purified cysteine protease inhibitor from Tetracarpidium conophorum (African walnut). African Journal of Biochemistry Research, 2015. **9**(2): p. 26-34.
48. Bijina B and M Chandrasekaran, Isolation, purification and characterization of Protease Inhibitor from Moringa oleifera Lam. 2006, Cochin University of Science & Technology.
49. Faber K and K Faber, Biotransformations in organic chemistry. Vol. 4. 1992: Springer.
50. Volkin D B, H Mach, and C R Middaugh, Degradative covalent reactions important to protein stability. Molecular biotechnology, 1997. **8**(2): p. 105-122.
51. Greenwood I A, N Leblanc, D V Gordienko, and W A Large, Modulation of I Cl (Ca) in vascular smooth muscle cells by oxidizing and cysteine-reactive reagents. Pflügers Archiv European Journal of Physiology, 2002. **443**(3): p. 473-482.
52. Bijina B, S Chellappan, J G Krishna, S M Basheer, K K Elyas, A H Bahkali, et al., Protease inhibitor from Moringa oleifera with potential for use as therapeutic drug and as seafood preservative. Saudi J Biol Sci, 2011. **18**(3): p. 273-81.
53. Stubbs M T, B Laber, W Bode, R Huber, R Jerala, B Lenarcic, et al., The refined 2.4 A X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. The EMBO journal, 1990. **9**(6): p. 1939.
54. Ee K Y, J Zhao, A-U Rehman, and S O Agboola, Purification and characterization of a Kunitz-type trypsin inhibitor from Acacia victoriae Bentham seeds. Journal of agricultural

and food chemistry, 2009. **57**(15): p. 7022-7029.
55. Hines M, C Osuala, and S Nielsen, Isolation and partial characterization of a soybean cystatin cysteine proteinase inhibitor of coleopteran digestive proteolytic activity. Journal of agricultural and food chemistry, 1991. **39**(8): p. 1515-1520.
56. Lawrence J C and S S Nielsen, Partial isolation and characterization of a cysteine proteinase inhibitor from lima bean (Phaseolus lunatus). Journal of agricultural and food chemistry, 2001. **49**(2): p. 1020-1025.