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Purification of trypsin inhibitor from*Pongamiapinnata* oil seed cake

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Abstract

A novel thermo stable trypsin inhibitor was isolated from *Pongamiapinnata*seed cake. The water and buffer crude inhibitor extract was found to exhibit 55% and 36% trypsin inhibition activity respectively. Trypsin inhibitor activity did not decrease drastically in presence of thiol reducing agents and metal chelating agent indicating that the inhibitor is stable. The trypsin inhibitor was purified from cure extract in single step purification by adapting affinity chromatography using trypsin agarose affinity matrix. The purity of inhibitor isolated was satisfactory and was found to be monomeric in nature as analysed by SDS-PAGE. The molecular weight of the trypsin inhibitor was found to be 24 KDa The purified trypsin inhibitor activity was measured and compared with crude inhibitor extract and found to be 61.49% and 39.4 % respectively.

Key Words: Trypsin inhibitor, *Pongamia*, seed cake,

Introduction

*Pongamiapinnata*seed cake is a low-value by-product resulting from biodiesel production. The seed cake is highly toxic, but it has great potential for biotechnological applications as it comprises of bioactive molecules that could be having beneficial application in agriculture, medicine, animal feed and other industries.

Proteinase inhibitors (PIs) have important mechanism that plants produce to offer protection against predators or from infection by pathogens. They are widely distributed in the plant kingdom and the plant families Leguminoseae, Solanaceae and Graminaceae are known to be rich in these inhibitors [1]. The PIs are small regulatory proteins and these proteins exhibit the presence of certain crucial active site amino acids such as serine, cysteine, aspartic, and metallo-PIs contain metal ion in their active site [2]. In specially, plant serine-PIs are grouped

into Bovine pancreatic trypsin inhibitor (Kunitz), Pancreatic secretory trypsin inhibitor (Kazal), Streptomyces subtilisin inhibitor, Soybean trypsin inhibitor (Kunitz), Soybean proteinase inhibitor (Bowman-Birk), Potato I inhibitor, Potato II inhibitor, Ascaris trypsin inhibitor and Other families [3].

The most popular classes of PIs are Kunitz and BBI (Bowman-Birk). The Kunitz inhibitors family is usually 18-24kDa heterogeneous proteins consisting of a number of isoinhibitors, with two disulfide linkages and a single trypsin reactive site (defined by an arginine residue) in one of the protein loops. The BBI are small cysteine-rich proteins in size (8-20kDa), with seven disulfide linkages, high cysteine content, and two independent reactive sites for trypsin and chymotrypsin [2]. These classes of proteins has been studied due to its application in the treatment of different pathologies as cancer [4], antifungal activity [5], inflammation and coagulation [6] or as bioinsecticides [7], demonstrating its importance as new therapeutic agents for the pharmaceutical industry. Despite there are abundantly studies of PIs in leguminous plants, little is known in cereals, such as amaranth [8], buckwheat [9]and buckwheat Seeds [10]. Since, in recent days hospital-acquired infection is the major cause of death in critically ill patients, both in developing and developed countries. Therefore much more effective new therapeutic agents that exhibit different mechanisms of action for treatment of infections caused by multidrug resistant bacteria are urgently needed[15]. In this direction many plants have been screened for numerous therapeutic compounds that could be used as antibacterial drugs with high efficacy and less toxicity can be developed to treat infectious diseases. To explore this potential we propose to isolate and characterise a novel trypsin inhibitor from *Pongamiapinnata*seed cake that could of greater use in future as therapeutic agent and as pest controlling agents in agriculture.

Methodology

Chemicals

BSA (bovine serum albumin), bovine pancreatic trypsin, bovine pancreatic a-chymotrypsin, 2-mercapto ethanol and casein were purchased from Sigma Aldrich. The standard molecular

weight marker, acrylamide, bis-acrylamide, DTT (Dithiothreitol) and other electrophoresis reagents were purchased from SRL Company.

Plant seed Cake

*Pongamia*seed cake was procured from GKVK Biofuel demonstration centre.

Preparation of the protein extract

Aqueous (10%) and buffer extracts (10%) was prepared from acetone defatted *Pongamia* seed cake flour in 50mM TrisHCl containing 25mM ascorbic acid and 10mM β-mercaptoethanol, pH-7.5, by constant stirring at 4^0 C for 1 hour. The extract was centrifuged at 10,000 rpm, 4 ^{0}C for 20 minutes. The clear supernatant obtained after centrifugation used as crude trypsin inhibitor extract. The protein content and trypsin inhibitory activity was analysed in the crude extract and same extract further used for purification of the trypsin inhibitor. Supernatant of the buffer extract was subjected to thermal denaturation at 70^0 C for 10 min; snap chilled for 30min on dry ice, centrifuged at 10,000rpm for 20min at 4^0C to remove the precipitated proteins. The clear supernatant having heat stable proteins was assayed for trypsin inhibitory activity (TIA).

Trypsin inhibition assay

TIA was carried out using 1% casein as substrate[4] and absorbance of TCA soluble products was measured at 280nm. The inhibitory activity was calculated as the difference between the proteolytic activity with and without inhibitor.

Protein determination

The protein content crude inhibitor extract and the column effluent fractions were measuring absorbance at 280 nm and the protein standard calibration curve was developed using bovine serum albumin (BSA) [11].

Purification of trypsin inhibitor

Trypsin-Sepharose affinity column was saturated with repeated washing in wash buffer (40mM Tris, 10mM $CaCl_2$, pH 7.6). The column was washed thoroughly to remove unbound proteins. The inhibitor was decoupled with 0.2N HCl (pH 3.0). The pH of the eluent was adjusted to 7.6 with 2N NaOH immediately, assayed for TIA and analysed on SDS-PAGE.

Molecular mass determination of trypsin inhibitor

The column eluted fraction that exhibits the trypsin inhibitor activity was subjected for SDS-PAGE analysis along with standard protein markers to access the molecular mass of the inhibitor[12].

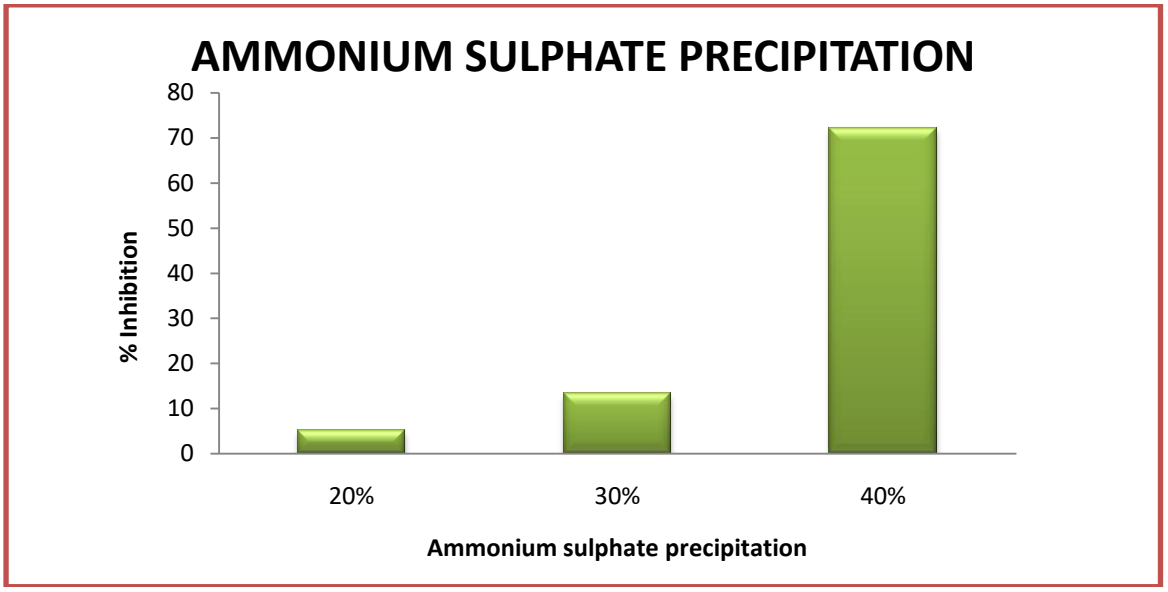
Result and discussion

Protease inhibitors were found in plants belonging to a variety of systematic groups.Protease inhibitors in plants are usually considered to work as storage proteins (nitrogen source) and as a defence mechanism.Trypsin inhibitors(TIs) exhibits insectisidal, anticancer and anitimicrobial properties.

In current study a novel thermo stable trypsin inhibitor was isolated from *Pongamiapinnata*seed cake. The water and buffer crude inhibitor extracts were determined and was found to be 55% and 36% inhibition respectively (Table-1). The inhibitor activity of buffer crude extract was reduced by 19% in comparison with water crude extract indicating that trypsin inhibitor was quite stable even upon heat denaturation. Trypsin inhibitor activity did not decrease drastically even in the presence of thiol reducing agents and metal chelating agent indicating that the inhibitor is stable under all these harsh conditions .

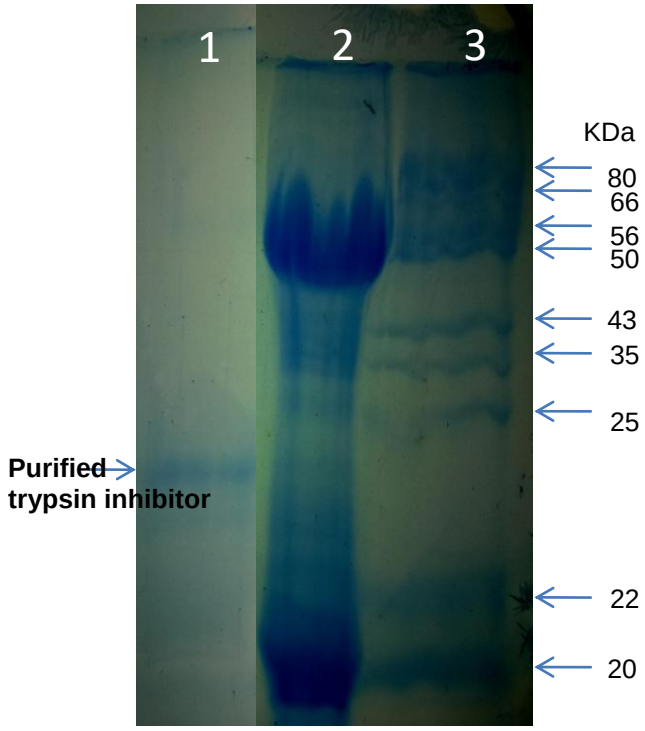
The water crude extract was precipitated by 20, 30 and 40% ammonium sulphate saturation out of which the 40% saturated pellet exhibit maximum inhibition of trypsin activity (70% inhibition) suggesting that trypsin inhibitor was precipitated maximum at this saturation (Figure-1).

Figure-1: Ammonium sulphate precipitation of trypsin inhibitor.



The trypsin inhibitor was purified by trypsin agarose affinity column chromatography. The purity was satisfactory and found to be monomeric protein as it showed a single protein band in SDS-PAGE analysis and the molecular weight of the trypsin inhibitor was found to be 24 KDa (Figure-2).

Figure-2: SDS-PAGE profile of crude and affinity purified trypsin inhibitor



Lane1- Affinity purified trypsin inhibitor
Lane 2- Crude inhibitor extract
Lane-3 – Molecular weight marker

The trypsin inhibitor activity was measured and compared with crude inhibitor extract and found to be 61.49% and 39.4 % respectively indicating the enrichment of inhibitor in the affinity purified sample (figure-3). The elution profile for affinity chromatography demonstrated one peak (figure-4) which after subjecting to SDS-PAGE analysis shown that the trypsin inhibitor is a monomeric protein.

Figure3- Trypsin activity of purified inhibitor and crude extract

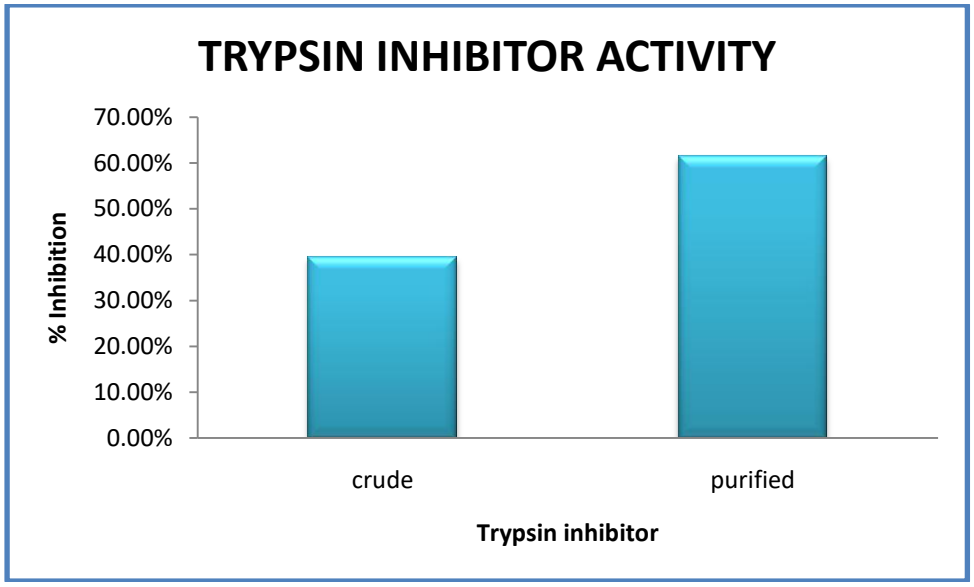
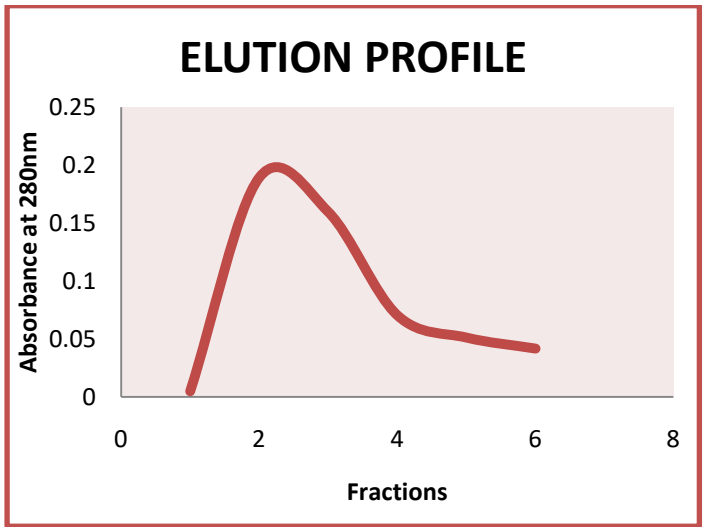


Figure-4 Elution profile of inhibitor by affinity chromatography



Trypsin inhibitor has wide biotechnological application including therapeutic agent. It also has insecticidal activity that can be of great commercial value. The low value by product of biofuel industries such as oil seed cake can be used as a source of trypsin inhibitor.

The literature has demonstrated the existence of numerous isoinhibitors in plants such as in Cajanuscajan, Vignaunguiculata and Fagopyrumtataricum seeds [2]. The nature of a large number of isoforms could be explained by the existence of multiple genes and the possibility of hydrolysis [13]. New forms of PIs and proteinases had been developed in plants and insects to combat against each other's in coevolution system [13]. The *Pongamia* seed cake

trypsin inhibitor did not show any isoinhibitor which was evident by SDS-PAGE analysis of affinity purified inhibitor as it is demonstrated by a single protein band.

JcTI-I presented antibacterial activity againstthe human pathogenic bacteria *Salmonella enterica subspecies entericaserovarcholeraesuis*and *Staphylococcus aureus*, with minimum inhibitory concentration (MIC)less than 5 µg/mL[14]. To understanding the biochemical basis of the antiproteolytic and antimicrobial activities of protease inhibitor there is a need for theelucidation of structure-function relationship. Trypsin inhibitor play a vital role in plant defence responses, use of these inhibitor could be a new ecofriendly strategies to protect plants against pests and pathogens. This strategy is currently one of the most dynamic areas of research in plant science. In addition, they may have the potential drug and used as a noncytotoxic clinical agents.

Table -1:Trypsin inhibitor activity of water and buffer crude extract

Extract sample	Enzyme: Inhibitor ratio	Percentage activity(%)	Percentage inhibition(%)
Buffer extract	1:2	74	36
Water extract	1:2	45	55

Conclusion

The trypsin inhibitor from water crude extract was precipitated by different ammonium sulphate saturation out of which the 40% saturated pellet exhibit maximum inhibition of trypsin activity (70% inhibition) suggesting that trypsin inhibitor was precipitated maximum at this saturation.The trypsin inhibitor was purified by trypsin agarose affinity column

chromatography and purity was found to be satisfactory. Affinity purified inhibitor exhibited 61.49% inhibition. A single protein band was observed by SDS-PAGE analysis demonstrating the inhibitor was a monomeric protein and the molecular weight of the trypsin inhibitor was found to be 24KDa.

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