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Rapid Detection of Proteinaceous Protease Inhibitor from Several Plants of North Maharashtra Region

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Abstract

Total 20 plants of north Maharashtra region were analysed for the presence of protease inhibitors in their seed. Protease inhibitors were detected from proteinaceous seed extracts by employing the rapid X-ray film method described in this investigation. Among studied plants, eleven plant seeds were reported to contain the protease inhibitor capable to inhibit various proteases like trypsin, pepsin, papain and metallo-protease. The testified results were authenticated by quantitative protease inhibition assay. The *Albizia amara, Albizia lebbeck, Cassia australis, Mimosa hamata, Peltophorum pterocarpum,* reported first time for the presence of trypsin inhibitor.

Keywords: Protease Inhibitor, Trypsin, Pepsin, Papain, Metallo-protease, X-ray Film

1. Introduction

Protease Inhibitor (PI) plays an essential role in plants by controlling proteolysis within the cell to maintain physiological regulatory cascade [1]. Various plants have been recognized as sources of protease inhibitors includes - soybeans, potatoes, squash, barley, wheat, millet, tomatoes, corn, kohlrabi and buckwheat [2]. Protease inhibitors effectively used in crop-protection [3] against attack by a large number of insects [4], fungi [5] and other phytopathogens [6].

The utility of protease inhibitor was also evaluated in the control of human diseases and pathological processes [7]. The role of proteases in physiological and several pathophysiological conditions made them an attractive pharmacological target and opened a new avenue in the development of future drugs. Synthetic protease inhibitors are popularly used as drugs in highly active anti-retroviral combination therapy, increasing life expectancy in HIV-positive patients [8]. Although, synthetic protease inhibitors are popularly used as drugs but natural protease inhibitors were not yet used commercially.

Several plant protease inhibitors were reported predominantly in plant seeds [9]. This investigation describes screening of several plants of north Maharashtra region (India) for the detection of protease inhibitor in their seeds by rapid X-ray film method described in this study.

2. Material and Methods

2.1 Plant Material

The seeds of 20 plants as were investigated for detection of protease inhibitor. The plant seeds of these plants were collected from several locations of north Maharashtra region (India) associated with the Satpura Mountain range during the month of April–May (2011–12).

2.2 Proteases Used in the Study

Trypsin (400 U/ml; EC 3.4.21.4, MP Bio USA; obtained from bovine pancreas), Papain (200 U/ml; EC 3.4.22.2, MP Bio USA, obtained from papaya latex), pepsin (480 U/ml; EC 3.4.23.1; MP Bio USA, obtained from the procaine stomach mucosa) and a purified alkaline

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metallo-protease of Pseudomonas aeruginosa MTCC 7926 (450 U/ml) [10] was employed in this study.

2.3 Extraction Procedure

The mature dried seeds (25 gm) of plants were soaked in 100 ml phosphate buffer (0.2 Mol/L, pH 7.2). A fine paste using grinder was obtained and further defatted by stirring for 30 min in a 2:1(v/v) mixture of ethanol and ether, the extraction process was repeated three times. The defatted seed paste was absorbed in phosphate buffer for overnight at 4°C. The seed suspension was further clarified by centrifugation at 5000 rpm for 10 min 4°C, the pellet was discarded and the suspension obtained after centrifugation was precipitated by adding ammonium sulphate (80%) and kept overnight at 4°C. The protein precipitate was dissolved in a minimum volume of 0.2 Mol/L phosphate buffer (pH 7.2) and dialyzed against same buffer. The dialyzed protein concentrates of seeds were further used for detection of protease inhibitor. Protein contents of plant seeds were measured as per Folin Lowry method [11] using bovine serum albumin (BSA) as a standard.

2.4 Detection of Protease Inhibitor using X-ray Film

Preliminary detection of protease inhibitor was performed by using gelatin coated commercial X-ray film (thickness 0.18 mm). A mixture of dialyzed protein concentrates obtained from seed and respective proteases viz. trypsin, papain, pepsin and a purified alkaline *metallo-protease* were incubated separately with X-ray strip (5 × 30 mm), at 30°C for 1 hour. No hydrolysis of the gelatin layer of the X-ray film, confirms the presence of protease inhibitors in extract while hydrolysis of the gelatin layer indicate that no protease inhibitor was detected (Fig.1).

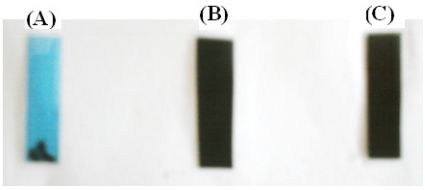

Fig. 1. X-ray gelatin film incubated (A) with protease (B) without protease and (C) Protease and protease inhibitor

2.5 Protease Inhibition Assay

Protease inhibition activity was analysed by modified Kunitz method [12] by using N-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The aliquots of proteases (trypsin, papain, pepsin and metallo-protease) and respective seed extract were prepared in 1mM HCL.

The 0.5 ml protease (trypsin, papain, pepsin and metallo-protease) solution and 0.5 ml proteinaceous seed extract (10 times diluted) were mixed and incubated at 25°C for approximately 15 minutes. The solution was further diluted 1:10 with 1mM HCl. The 0.2 ml diluted solution (containing protease and protease inhibitor) was added to the 3.0 ml of 0.25 mM BAEE solution, prepared in phosphate buffer (pH 7.2) with mixing and immediately transferred to a cuvette. The increase in absorbance at 253 nm per unit time was recorded for approximately 5 minutes by using spectrophotometer UV 2450 (Shimadzu). The ΔA253/minute was obtained over the linear portion of curve. Similarly, the blank sample was prepared without a proteinaceous seed extract. The assay was carried out in triplicate. One unit of protease activity was defined as the amount of enzyme causing an increase in absorbance at 253 nm of 0.001 per minute at 25°C and pH 7.2, using N-α-Benzoyl-L-arginine ethyl ester (BAEE) as substrate, while one unit of protease inhibition activity was defined as the amount of inhibitor which will inhibit one unit of protease activity.

3. Results and Discussion

Plants investigated in this study were identified as - *Acacia arabica* (Fabaceae), *Acacia nilotica* (Fabaceae), *Aegle marmelos* (Rutaceae), *Albizia amara* (Fabaceae), *Albizia lebbeck* (Fabaceae), *Alpinia purpurata* (Zingiberaceae), *Butea monosperma* (Fabaceae), *Calotropis procera* (Asclepiadaceae), *Carica papaya* (Caricaceae), *Cassia australis* (Fabaceae), *Cassia occidentalis* (Fabaceae), *Datura inoxia* (Solanaceae), *Delonix regia* (Fabaceae), *Lawsonia inermis* (Lythraceae), *Mimosa hamata*(Fabaceae), *Peltophorum pterocarpum* (Fabaceae) *Sesbania grandiflora* (Fabaceae), *Tamarindus indica* (Fabaceae), *Terminalia bellirica* (Combretaceae, and *Ziziphus mauritiana* (Rhamnaceae). The identified plants were authenticated by Dr. D. A. Patil (Botanist). A voucher specimenshave been deposited in the

herb-arium of the department of Botany, SSVPS's Dr. P. R. Ghogrey Science College, Dhule.

The total protein content of plant seed extract was evaluated, each plant seed possesses different concentrations of protein as summarized in Table 1. By employing X-ray gelatin film method described in this investigation, total eleven plant seeds were confirmed to contain the protease inhibitor. Qualitatively analysed extracts were further investigated for determining protease inhibition units (Table 1).

Among eleven plants, two plants *Tamarindus indica* and *Lawsonia inermis* seed extract shown protease inhibition against trypsin, pepsin, papain and metallo-protease. A trypsin inhibiting Kunitz-type proteinase inhibitor from *Tamarindus indica* seeds was reported previously [13]; this study revealed the inhibition of pepsin, papain and metallo-proteaseby seeds extract of *Tamarindus indica.* Thispredicts the presence of several iso-forms of protease inhibitors active against these proteases. *Lawsonia inermis* seed contains a broad spectrum protease inhibitor [14].

Proteinaceous seed extracts of *Terminalia bellirica* shown inhibition against trypsin and metallo-protease. *Terminalia bellirica* was described to promote the healing of infected full-thickness dermal wound [15], in light of this description there is scope to assess the role of protease inhibitor in wound healing of this plant. Our purification study is underway which will explore that the proteinaceous extract might contain a broad spectrum protease inhibitor or whether several iso-forms are present in proteinaceous extracts.

Acacia nilotica and *Aegle marmelos* shown metallo-protease inhibition, signifies the clinical importance of these protease inhibitors to be used effectively against *Pseudomonas aeruginosa* and other proteolytic pathogens. The seeds of *Acacia arabica* corroborated for the presence of trypsin inhibitor in this study, this in accordance to previous report of reduced protease activities of periodontal pathogens (*Porphyromonas gingivalis and Prevotella intermedia*) in the presence gum of *Acacia arabica* [16].

The *Albizia lebbeck, Albizia amara, Mimosa hamata, Peltophorum pterocarpum, Cassia australis* revealed the presence of trypsin inhibitor. The presence of protease inhibitors in these plants was revealed first time in this investigation.

The plant seed extract in which protease inhibitor was not detected were *Alpinia purpurata, Butea frondosa (Butea monosperma*), *Calotropis procera, Carica papaya,* Cassia *occidentalis* (*Senna occidentalis*), *Delonix regia, Datura inoxia, Sesbania grandiflora, Ziziphus mauritiana*. The plant seed extract probably contains a protease inhibitor in a too smaller amount which is not detected by X-ray film method described in this work. Several proteinaceous seed extracts like *Carica papaya* and *Calotropis procera* shown enhanced proteolysis this due to the presence of endogenous protease present in their seeds.

Table 1: Protein yield and protease inhibition activity

Plant seeds	Seed protein content (gm%)	Protease inhibition activity (U/mg)			
		Trypsin	Papain	Pepsin	Metallo-protease
1. *Acacia arabica*	19.15	nd	nd	nd	15.9± 0.2
2. *Acacia nilotica*	27.78	nd	nd	nd	8.1± 0.1
3. *Aegle marmelos*	2.46	nd	nd	nd	145.9 ± 7.2
4. *Albizia amara*	12.78	110.9 ± 2.0	nd	nd	nd
5. *Albizia lebbeck*	38.53	35.3 ± 0.5	nd	nd	nd
6. *Cassia australis*	3.87	311.2 ± 8.2	299.2 ± 6.6	127.8 ± 1.3	136.9 ± 3.6
7. *Lawsonia inermis*	3.28	192.3 ± 3.8	176.4 ± 1.3	162.8 ± 5.4	188.2± 2.4
8. *Mimosa hamata*	3.72	40.9 ± 3.5	nd	nd	nd
9. *Peltophorum pterocarpum*	10.79	108.1 ± 4.1	nd	nd	nd
10. *Tamarindus indica*	18.98	75.1 ± 0.8	53.7 ± 0.4	51.7 ± 0.5	26.2 ± 0.3
11. *Terminalia bellirica*	14.87	67.8 ± 1.4	nd	nd	59.4 ± 0.3

nd = No protease inhibitor was detected

4. Conclusion

The discovery of novel selective protease inhibitors is currently progressing by exploration of natural compounds. The diversity of protease inhibitors in plants makes them outstanding sources for discovering novel protease inhibitors. The competent method to detect the protease inhibitor from plant seed is endorsed in this study. The precision of method is validated with standard enzyme inhibition assay. By using a rapid X-ray film method, the proteinaceous protease inhibitors were reported for the first time from seeds of *Albizia lebbeck, Albizia amara, Mimosa hamata, Peltophorum pterocarpum, Cassia australis* in this investigation.

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