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Purification of protease inhibitors from *Cajanus cajan* (L) Millsp. by preparative gel electrophoresis

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Abstract
Protease inhibitors (PIs) of *Cajanus cajan* (L) Millsp. are known for its inhibitory action against trypsin, chymotrypsin and *Helicoverpa armigera* gut proteinase. These inhibitors were extracted in 1% polyvinyl pyrrolidone, separated by preparative polyacrylamide gel electrophoresis and visualized using gel-X-ray film contract print method. Individual PIs were excised from gel and precipitated in acetone. The purity and inhibitor potency against trypsin were confirmed by sodium dedocyl sulphate-polyacrylamide gel electrophoresis and trypsin inhibitory assay using synthetic substrate Nα-Benzoyl-DL-Arginine-p-Nitroanilide. 100 ug of protein was sufficient for visualization of all inhibitor (PI-1 to PI-9) in 10% polyacrylamide gel solution with high resolution. PI-6, PI-7 and PI-8 showed 22.22 %, 21.77% and 37.77 % inhibition potential against trypsin. The solubility properties of PIs may be altered due to acetone precipitation method. Therefore, use alternate method for purification of PI-1 to PI-5 and PI-9 is needed for further characterization of *Cajanus cajan* PIs.

Keywords: protease inhibitors; *Cajanus cajan* (L) Millsp; preparative gel electrophoresis

1. Introduction

Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants [1]. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes [2, 3]. These proteins have diverse biochemical functions, some of which include elimination of unwanted proteolysis [4]. Pigeonpea [*C. cajan* (L) Millsp.] is a multipurpose, hardy grain legume crop grown by resource poor farmers of many developing countries in semiarid tropics and subtropics. It occupies an important position in human diet as a protein source especially in the vegetarian population [5]. However, pre-harvest damage due to insect pests on developing seed and post-harvest losses due to storage pests are severe [6]. The plant proteinases inhibitors of serine proteinases play a dominant role in natural plant defense and infection processes [7]. The PIs are generally small, stable, and abundant proteins showing specificity for serine, cysteine, aspartic, and metallo-proteinases [8]. Among them, serine-PIs are most common and best characterized and comprise several protein families including Kunitz and Bowman-Birk inhibitor (BBI) families, which are abundant in various Leguminosae seeds [9]. Pigeonpea is an important pulse crop in subtropical and semiarid tropical areas of the world including India. The presence of trypsin and chymotrypsin inhibitors was reported [10]. Although PIs from pigeonpea have been purified before, the family or type of inhibitor to which they belong was not clear. Godbole *et al.* (1994) [11] purified two inhibitors having molecular masses around 15 and 10.5 kDa from C. cajan cv. TAT-10 and suggested that the inhibitors belong to the BBI family [11]. However, BBI-type PIs are known to be small proteins, having molecular masses around 6-9 kDa [12]. Conversely, Haq and Khan (2003) [13] purified 14 kDa PI from the PUSA-33 variety and suggested that it belongs to the Kunitz inhibitor family [13] and (Prasad *et al.*, 2010) [14] have been purified two protein bands with molecular masses of ~8.5 and ~16.5 kDa and N-terminal sequence 'DQHHSSKACC' suggested that the isolated RgPI is a member of the Bowman-Birk inhibitor family [14]. These reports showed the presence of only two inhibitors bands. But actually mature and developed seed contain 9TI-CTIs [10]. In present study we have described the purification of individual proteinase inhibitor from seed extract pigeonpea.

2. Materials and Methods

2.1 Materials

Dry seeds of pigeonpea were obtained from Mahatma Phule Krishi Vidyapeeth Rahuri. Tris-buffer, Glycine, Acrylamide, Bis-acrylamide, Glycerol, Bromophenol blue, Coomassie blue R-250, Bovine serum albumin (BSA fraction V), Trypsin 3x cryst and Chymotrypsin 3x cryst were obtained from Sisco Research Laboratories (SRL), Pvt. Ltd., India. DEAE-cellulose, Trypsin-agarose, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), PVP (Polyvinyl pyrrolidone (k-30) were obtained from Sigma-Aldrich. Other chemicals used were of the highest purity available.

2.2 Extraction of pigeonpea proteinase inhibitors (PPI)

Dry seeds with seed coat were crushed into fine powder by grinder mixer. The powder was defatted in hexane. The completely fat free powder was dried at room temp. For proteinase inhibitors, the powder was soaked in 1% PVP (1:6 w/v) and kept for overnight extraction at 4^0C. This solution was centrifuged at 10,000 rpm for 15 min at 4^0c. The supernatant was collected and preserved at -20^0C for further experiment.

2.3 Protein Estimation

The total protein concentration was estimated by the methods of Lowry *et al.* (1951) [15], using bovine serum albumin as a standard [15].

2.4 Polyacrylamide gel electrophoresis (PAGE)
The proteins profile was carried out by using vertical slab Polyacrylamide gel electrophoresis unit by using method of Davis (1994) [16]. The extract containing various concentration of protein such as 50µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, 350 µg, 400 µg, 450 µg and 500 µg were mixed with sample buffer (0.08M Tris-HCL Ph 6.8, 30% glycerol, 0.01% Bromophenol blue). The mixed samples were loaded on 10% polyacrylamide gel electrophoresis and electrophoresis was carried out at room temperature with constant 25 mA current. After electrophoresis gel was removed, washed with distilled water and stained by Coomassie blue R-250 (50% methanol and 8% acetic acid). The gel was destained by destaining solution (30% methanol and 10% acetic acid) and protein bands were observed visually and image of gel was taken by scanner.

2.5 Visualization of Proteinase Inhibitors (PIs) by Gel-X-ray Film Contact Print Method (GXCP)
For the visualization of PIs, the seed extract containing various concentration of protein such as 50µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, 350 µg, 400 µg, 450 µg, 500 µg and 550 µg were mixed with sample buffer and loaded on gel electrophoresis (same as discussed in PAGE section). After electrophoresis the gel was processed for proteinase inhibitory activity by the gel-X-ray film contact print method [17]. The gel was washed in 0.1 M Tris-HCl buffer (pH 7.8) for 15 min, followed by incubation in 0.1% trypsin solution for 15 min at room temperature. The gel was then briefly rinsed in Tris buffer to remove the excess trypsin. Immediately the wet gel was overlaid on X-ray film in a tray and incubated at 37 °C. The hydrolysis of the gelatin on X-ray film was monitored visually and after extent hydrolysis of gelatin, the gel was removed from X-ray film. The X-ray film was washed with either tap water or warm water and kept at room temperature for drying. The same gel was overlaid three to four times with different pieces of X-ray films. Occasionally the X-ray film was also rubbed gently to remove the hydrolyzed gelatin. Protease inhibitor bands appeared as unhydrolyzed gelatin against the background of hydrolyzed gelatin. The inhibitory bands were observed by visually and image of X-ray film was taken using scanner (Fig 1).

2.6 Separation of Proteinase Inhibitor (PI) on different concentration of polyacrylamide gel
For the improved resolution of PIs on gel electrophoresis we have prepared different concentration gel using acrylamide solution such as 7.5%, 10%, 12.5% 15% and 17.5%. The crude seed extract was loaded on these gels with 50µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, 350 µg, 400 µg, 450 µg and 500 µg of proteins. After electrophoresis GXCP was performed by using previously mentioned method (Fig 2).

2.7 Purification of Proteinase inhibitors (PI) by preparative page
A native 10% polyacrylamide gel electrophoresis with Davis buffer system was used for the purification of individual proteinase inhibitor (PI) from pigeonpea seed extract. The native gel was prepared without wells, only stacking gel layer was prepared above the resolving gel and seed extract (containing 100-150mg proteins) was mixed with sample buffer and loaded on gel electrophoresis. The electrophoresis was carried out at room temperature with a constant current of 30 mA. After electrophoresis the gel was removed from glass slides and ¼th part of total gel was cut vertically into small strip by gel cutter. The small strip was processed for GXCP and remaining gel was stained by staining solution. The proteinase inhibitory bands were visually observed on X-ray film and arranged the X-ray film parallel to stained gel. The stained bands corresponding to trypsin inhibitors were excised from the gel, crushed, and eluted in distilled water and concentrated by acetone precipitation method. The precipitate was dissolved in 50 mM Tris-HCL buffer pH 7.8 and reanalyzed by electrophoresis and GXCP assay to visualize active TI fragments.

2.8 Trypsin inhibitor assay
The trypsin inhibitor assay from crude extract and purified protein were carried out by using a synthetic substrate benzoyl-arginyl-p-nitro-anilide (BAPNA), as described by Erlanger *et al.* (1961) [18]. In a typical reaction containing 20µg of proteins of each purified bands were mixed with 10µl trypsin (1 mg/ml) and volume of reactions were adjusted with 0.1 M Tris-HCL buffers (pH 7.8). Each reaction mixer was incubated 10 min at 37^0c and 300µl substrate (1 mM) was added in each reaction and incubated 30 min at 37^0C. The reaction was terminated by the addition of 30% acetic acid. Thereafter optical density of each sample was recorded at 410 nm. Simultaneously one aliquot was kept without inhibitor protein for control (Trypsin activity).

2.9 Separation of protease inhibitor on SDS-PAGE
The fraction no. 6 and 8 containing inhibitor activity collected from preparative electrophoretic gel were pooled together and 50µg protein used for SDS-PAGE. Simultaneously, crude extract also separated on same gel. After electrophoresis gel was washed by 2% triton X-100 for removal of SDS and after washing GXCP was carried out for identification of PIs.

3. Results & Discussion
3.1 Visualization of Proteinase Inhibitors (PIs) from crude extract
The crude extract of seed was separated on native-gel and gel was processed for GXCP. The total nine protease inhibitors were observed on X-ray film in figure no.1. The pattern of protease inhibitors on X-ray film was similar to previously described from seed extract of C. cajan [10,19]. At 50µg protein concentration only PI-3, PI-6 and PI-8 were observed and 100µg protein concentration was sufficient for detection of all PI from crude extract (Fig 1). We have performed six electrophoretic gels with various acrylamide percentages for good resolution of PI. This result proved that 10% acrylamide concentration is good for detection of total PIs with high resolution (Fig 2).

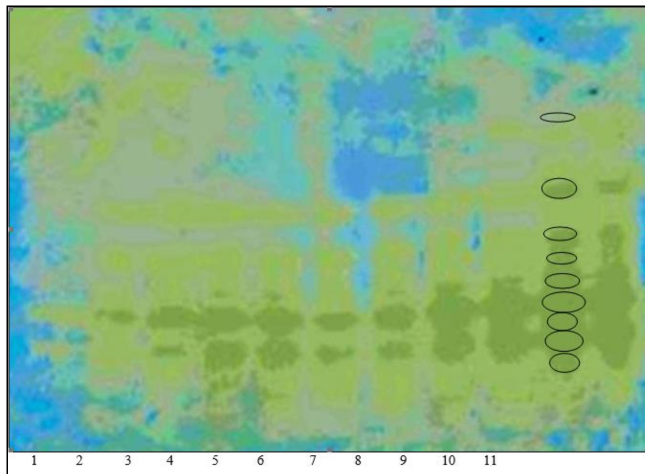

Fig 1: Gel-X-ray film contact print photograph. The crude extract containing various concentration of protein such as 50 ug, 100 ug, 150 ug, 200 ug, 250 ug, 300 ug, 350 ug, 400 ug, 450 ug, 500 ug and 550ug separated on native PAGE.

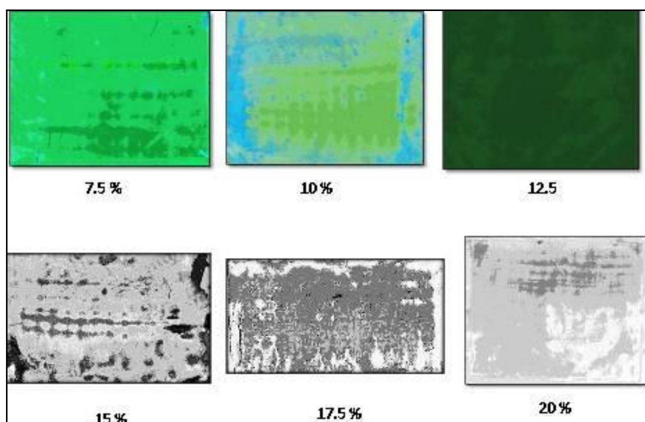

Fig 2: Gel-X-ray film contact print photograph. The crude extract containing various concentration of protein such as 50 ug, 100 ug, 150 ug, 200 ug, 250 ug, 300 ug, 350 ug, 400 ug, 450 ug, 500 ug and 550ug separated on different concentration of actrylamide (7.5 % to 20 %) for standardization of CXCP method for high resolution.

3.2 Purification of trypsin inhibitor (PI)

Trypsin inhibitors were purified by preparative gel electrophoresis. We observed very low concentration of elicited protein form gel, therefore extracted proteins from each fraction was concentrated by acetone precipitation. The total twelve fractions containing individual inhibitors were eluted from gel and after acetone precipitation; completely soluble proteins were estimated by UV spectrophotometry. Same soluble protein was analyzed for individual inhibitors by GXCP method qualitatively and inhibitory assay were carried for quantitative analysis (Only soluble acetone fraction was used for this study). Only two inhibitory bands (PI-6 and PI-8) were detected from 50µg protein in the fraction no. 6, 7 and 8 on X-ray film. The remaining PIs (PI-1, PI-2, PI-3, PI-4, PI-5, PI-7 and PI-9) were not detected on X-ray film. Undetected PIs on the X-ray film may be due to alteration of PIs property during acetone precipitation or may be the amount of these PIs was very low in gel and they were not extracted (eluted) completely from the gel after separation. Hence, we concluded that preparative electrophoresis technique was not good for purification of individual PIs and total number (all) of trypsin inhibitors.

3.3 Trypsin Inhibitor (TI) assay

Trypsin inhibitors assay was performed by using BAPNA as a synthetic substrate and 20µg protein from each fraction was used for trypsin inhibition study. The out of nine fractions only fraction no. 6, 7 and 8 showed trypsin inhibitory activity and their percent inhibitions were 22.22%, 21.77% and 37.77% resp. According to X-ray film observation the inhibitory activity in fraction no. 6, 7 and 8 is due to the presence of TI-6 and TI-8. The remaining fractions were not showed inhibitory activity.

4. Conclusions

Our result shows that the electrophoretic method is not suitable for purification of protease inhibitor from *C. cajan* seed extract. The amount of PIs in seed extract is very low and electrophoretic method only suitable for purification PI-6 and PI-8. The solubility property of protein may be altering due to acetone precipitation.

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