PURIFICATION OF TRYPSIN INHIBITOR FROM PIGEON PEA SEEDS

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ABSTRACT

Inhibitors of trypsin and chymotrypsin are involved in natural defense of plant against invading insect pests. These inhibitors are inducible in seeds of pigeonpea and accumulate sequentially during seed development. In this study, the trypsin inhibitors from pigeon pea were purified. The fractionation of trypsin inhibitors in crude extract was carried out with ammonium sulphate. The fraction showing more inhibitor activity was further purified by using ion-exchange (DEAE-cellulose) and affinity (Trypsin-agarose) column chromatography. Trypsin inhibitor activity in each step was assessed by trypsin solution assay using N-α- benzoyl-arginine-p-nitroanlide as synthetic substrate. The visualization of trypsin inhibitors was carried out by Gel-X-ray film contact print method (GXCP). The ammonium sulphate (25-60 %) fraction showed maximum inhibitor activity than other fractions. The fold of purification from ion-exchange and affinity column chromatography was found to 31.29 and 88.32 respectively. Two trypsin bands correspond to TI-6 and TI-8 were visualized on gelatin film in purified fraction from affinity chromatography. This study suggests that ion-exchange (DEAE-cellulose) and affinity (Trypsin-agarose) column chromatography are suitable for large and quick purification of trypsin inhibitors from pigeon pea.

Keywords: *purification, trypsin, inhibitors, pigeonpea seeds.*

Introduction

Protease inhibitors (PIs) are regulatory molecules found in microorganisms, plants, numerous animal tissues and fluids that control the activity of their target proteases (Bode and Huber, 1992; Ryan, 1990). Plant PIs [PPIs] have been involved in regulation of endogenous proteases, mechanisms of defense against infection by pathogens, act as antifeedant compounds and protect plants against herbivorous insects by inhibiting digestive proteases (Lawrence and Koundal, 2002; Valueva and Mosolov, 2004). PPIs accumulate in seeds, tubers and other plant-storage tissues; provide the sources of carbon, nitrogen, and sulfur required during germination (Mandal et al., 2002). The accumulation of these inhibitors in plant depends on maturation stage, tissue location, time of harvest and storage (Sels et al., 2008). High levels of PPIs are often found in plants belonging to the Solanaceae, the Leguminosae (Fabaceae), and the Gramineae (Poaceae) families (Brzin and Kidrič, 1996; Sin & Chye, 2004; Xu et al., 2001). Most of the plant PPIs are serine-protease inhibitors (Fan & Wu, 2005; Haq & Khan, 2003; Mello et al., 2001) and are classified into Kunitz, Bowman-Birk; Potato I and Potato II; and the superfamily of pumpkin, zucchini, and cereals. Plant serine-protease inhibitors have been isolated from the Solanaceae, Fabaceae, Euphorbiaceae, Poaceae, and Cucurbitaceae families (Haq et al., 2004). Kunitz and Bowman-Birk types are frequently found in members of the leguminous family. These inhibitors are classified on the basis of their cysteine-residue content and the number protein binding sites (Bendre et al., 2018). The Kunitz-type inhibitors exhibit a molecular mass of 18–24 kDa and contain 4 cystine residues with a single protein-binding site (Bendre et al., 2018). In contrast, the Bowman-Birk inhibitors are small proteins (molecular weight ca. 4–8 kDa) with 14 cystine residues and contain two protein-binding sites (Qi et al., 2005). PPIs are generally found in crop plants, and though displaying a particular abundance in legumes, are also present in cereals and tubers, where they form part of the plant's defense against pest attack. That defensive role of PPIs is based on their inhibition of

insects and pathogenic proteases, causing a critical shortage of essential amino acids (Hilder et al., 1993; Jongsma & Bolter, 1997). The high levels of PPIs are associated with plant resistance against insects and microbes (Dunse et al., 2010; Kim et al., 2009).

Seeds of pigeonpea are rich source of PIs, the member of Bowman-Burk inhibitor family and are accumulated sequentially in seed during developing stages of seeds as a part of natural defense against phytophagous insect pest (Tak et al., 2013). These PIs of pigeonpea are produced in response to mechanical wounding, insect chewing, fungal pathogenesis and application of salicylic acid in leaves and flowers (Padul et al., 2012). They are also involved in plant defense mechanism by inhibiting the digestive proteases in midgut of insect that leading their starvation and death (Gatehouse et al., 1999). Pigeon pea is susceptible to Helicoverpa armigera damage during seed development and accumulation of these inhibitors during seed development is important to develop natural resistance (Padul et al., 2012; Tak et al., 2013). Beside the role of PIs in natural defense, its antioxidant, anti-inflammatory and antimicrobial role was also investigated (Shamsi et al., 2016). Therefore, PIs could be utilized as pharmacological molecules and could be use along with medically important molecule. In this report, the pigeon pea trypsin inhibitor was extracted and purified by using ammonium sulphate precipitation followed by ion exchange chromatography and trypsin-agarose affinity column.

Material and method

Chemicals and Reagents

Dry seeds of pigeonpea (cultivar BDN2) were obtained from Mahatma Phule Krishi Vidyapeeth Rahuri, Dist. Ahmednagar, Maharashtra. Tris-buffer, Glycine, Acrylamide, Bis-acrylamide, Glycerol, Bromophenol blue, Coomassie blue R-250, Bovine serum albumin (BSA fraction V), Trypsin 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 purchased from Sigma-Aldrich. Other chemicals used were of the highest purity available

Extraction of Proteinase Inhibitors

Dry seeds of pigeonpea with seed coat were crushed into fine power usinghomogenizer. The obtained fine powder was defatted by soaking in hexane and washed with acetone. The completely fat free powder was dried at room temp. For extraction of trypsin inhibitors, the powder was soaked in 50 mMTris-HCL (pH 8.0) containing 1% PVP (1: 6 w/v) and kept for overnight extraction at 40C temperature. The mixed sample was centrifuged at 10,000 rpm for 15min at 40C. The supernatant was collected and preserved at -200C.

Protein Estimation

The total proteins concentrations in crude extract, ammonium sulfate fractions, eluted fractions from DEAE-celluloseand affinity columns were estimated by Folin-Ciocalteau method using bovine serum albumin as a standard protein (Lowry et. al., 1951).

Trypsin inhibitor assay

The presence of trypsin inhibitorsin crude extract, ammonium sulfate and chromatographic fractionswas assessed by trypsin assay using a synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanlide (BAPNA), as described by Erlanger et al. (1961). In a typical reaction containing 50µl of inhibitor solution, 10µl trypsin (1 mg/ml) and volume of reaction was adjusted with 0.1 M Tris-HCL buffers (pH 7.8). Each reaction mixer was incubated for10 min at 370C. After incubation 300µl substrate (1 mM) was added in each reaction and incubated for30 min at 370C. The reaction was terminated by the addition of 30% acetic acid and optical density of each solution was recorded at 410 nm. The unit of trypsin inhibitor was defined by the

reduction in absorbance of trypsin activity due to addition of inhibitor.

Purification of trypsin inhibitors (TIs)

Trypsin inhibitors form pigeon pea was purified using the procedure of Prasad et al. (2010) with some modifications. The crude protein extract was subjected to 0-25, 25-60 and 60-90% (NH4)2SO4 fractionation. Resulting solutions were centrifuged at 10000rpm for 10 min at room temperature. Supernatants were discarded and precipitates were dissolved in minimum quantity of water.Theprotein concentration and trypsin inhibitor activity was measured. The fraction showing maximum trypsin inhibitory activity was (25-60 % ammonium sulphate saturation) selected for dialysis against Tris-HCl buffer pH 8.0. Dialyzed solution was applied onto a DEAE-cellulose column (2.2 × 15 cm), pre-equilibrated with 50 mMTris-HCL buffer (pH 8.0). The column was washed with the above buffer, and bound protein was eluted with a linear gradient of 0.1-1 M NaCl in the same buffer at a flow rate of 0.5 ml/min. 2 ml of 60 fractions were collected. The fractions showing maximum TI activity were pooled and loaded (2 ml) onto a trypsin-agarose column (0.5× 10 cm), pre-equilibrated with 50 mMTris-HCL buffer (pH 8.0). After washing with the above buffer containing 100 Mm NaCl, the bound proteins were eluted with 0.01N HCL at a flow rate of 30 ml/h and neutralized with 2M Tris base. The fraction showing inhibitory activity was further loaded on native-PAGE for detection of PI profile. The 100µl from each fraction was loaded on 10% SDS-PAGE and the gel was stained by silver staining after electrophoresis.

Visualization of trypsin Inhibitors (PIs) by Gel-X-ray Film Contact Print Method (GXCP)

For the visualization of PIs in the seed crude extract, ammonium sulphate fractions and eluted chromatographic fraction containing various concentration of protein 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 (Pichare and Kachole 1994). 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 with 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. 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 on scanner.

Results and Discussion

Determination of protein

Pigeon pea is an important crop, cultivated as rich source of dietary proteins and other important nutrients. The extracted protein in crude extract from dry seeds was found to 91.5mg/gm. Sekhon et al., (2017) reported that total soluble protein content in 50 different cultivars of pigeonpea varied from 170.37 to 251.16 mg/g. The estimated protein was lower as compared to protein reported in earlier study; this difference may be utilization of different extraction procedure. The protein content in ammonium sulfate fractionations such as 0-25%, 25-60% and 60-90% fraction were observed 11, 16.5 and 12.5 mg/ml respectively. The high protein content (90.65 ± 0.25%) when isolated through alkaline isoelectric precipitation technique has been reported in pigeon pea (Olawuni et. al., 2012). Similarly 91.83% is proximate composition of protein, recovered in water extract has been reported by Adenekan et al., (2018).

Purification of trypsin inhibitors

The crude extract of C. cajan was passed through various steps of purification. It was observed that 25-60% (NH4)2SO4 saturated protein fraction has maximum trypsin inhibitory activity when compared with other (NH4)2SO4 saturated fractions (Table-1)

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Table 1.: Purification of trypsin inhibitor from pigeon pea cultivar (BDN2)

Purification step	Total protein (mg)	Total activity (TI units)	Yield recovery (%)	Specific activity (TI units/mg of protein)	Purification fold
Crude extract	345	10,619	100	30.78	1
$(NH4)_2SO_4$ (25-60) %	82.2	2552.75	24.03	30.94	1
Ion-exchange chromatography	2.067	1984.32	18.68	963.2	31.29
Trypsin-Agarose	0. 642	1745.32	16.43	2718.56	88.32

The maximum trypsin inhibitory activity in same fraction was found in the previous study of Prasad et al. (2010). This fraction was loaded on DEAE-cellulose column and the profile of trypsin inhibitory activity in eluted fractions as shown in figure 1.

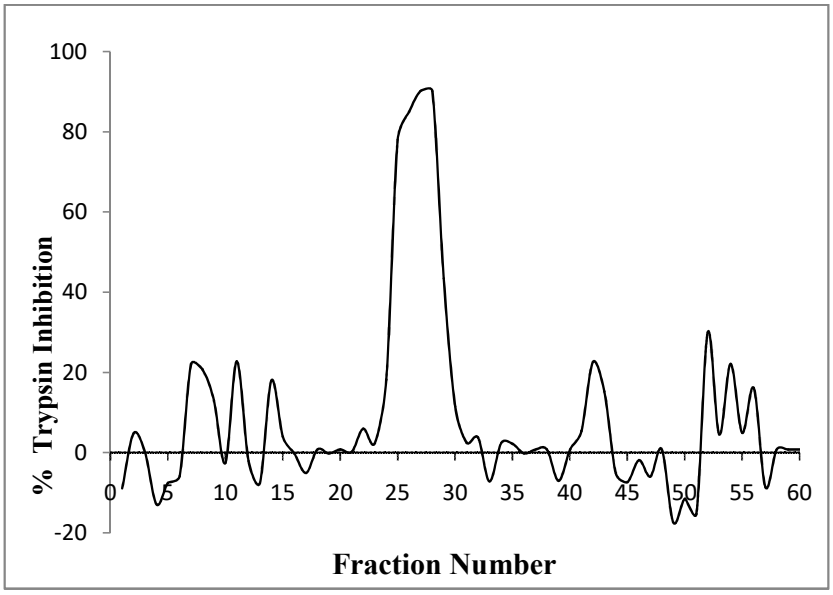

Fig. 1. **Trypsin inhibitor assay profile**. Ion-exchange (DEAE-Cellulose) column loaded with 25-60% $(NH4)_2SO_4$ fraction. Trypsin (10ug) treated with 50ul solution of each 2 ml fraction and incubated for 10 min. after incubation trypsin activity was measured by using BAPNA.

The out of 60 fractions, the fraction 25-29 were showed maximum trypsin inhibitory activity (Table 2). The elution of trypsin inhibitors in less number of fractions (25-29) was observed. The earlier reported study in which the purification of trypsin inhibitor by DEAE-cellulose column required more number of fractions (20-63) for elution of trypsin inhibitors (Swathi et al., 2016).

Table 2: Separation and distribution of trypsin inhibitors in eluted fractions of DEAE-cellulose chromatography based on their percentage of trypsin inhibition:

Fraction Number	% Inhibition	Fraction Number	% Inhibition	Fraction Number	% Inhibition	Fraction Number	% Inhibition
1	-8.92	16	-0.19	31	2.48	46	-1.882
2	4.86	17	-5.05	32	3.76	47	-5.95
3	0	18	0.79	33	-7.14	48	0.79
4	-12.89	19	-0.19	34	2.38	49	-17.36
5	-7.53	20	0.79	35	2.18	50	-11.50
6	-5.85	21	0.19	36	-0.19	51	-15.27
7	22.12	22	5.95	37	0.79	52	30.05
8	20.73	23	2.38	38	0.79	53	4.56
9	12.99	24	18.15	39	-7.04	54	22.12
10	-2.57	25	78	40	0.79	55	4.96
11	22.72	26	85	41	5.45	56	16.07
12	-2.08	27	90.17	42	22.61	57	-8.53
13	-7.53	28	90.17	43	15.27	58	0.79
14	17.95	29	43.45	44	-5.15	59	0.79
15	3.96	30	11.50	45	-7.34	60	0.79

The reason in vary in number of fractions requirement for elution of trypsin inhibitor may be based on application of different protein concentration on column. Our study gives quite confirmation about the low concentration of proteins is suitable for effective purification of trypsin inhibitors. The low trypsin inhibitory activity also found in same other fractions as shown in table 2. The fractions 9, 14, 22, 24, 30, 41, 43 and 56 showed lowest inhibition (10-20%). The moderate inhibitions (20-50%) have been shown by fractions 7, 8, 11, 29, 42, 52 and 54. The fractions 25 and 26 showed maximum inhibition (70-90%).

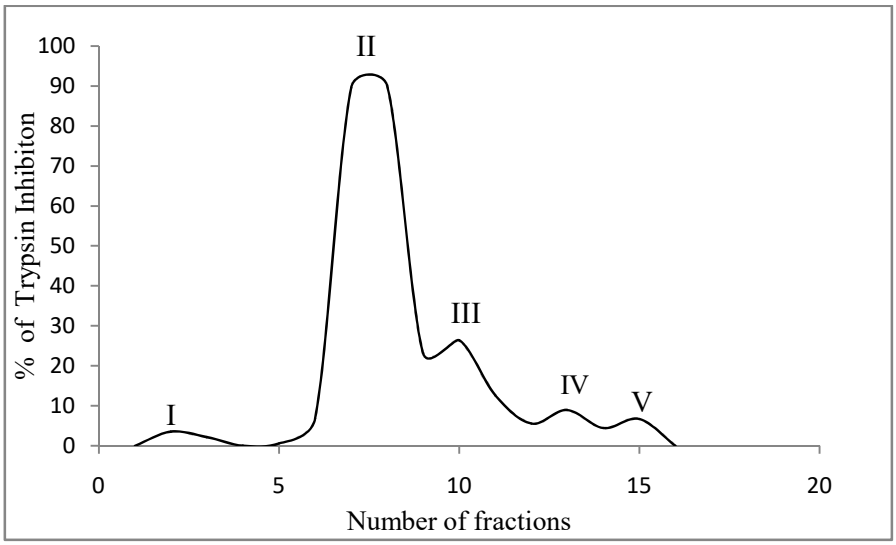

Fig 2: **Trypsin inhibitor assay profile**: Ion exchange chromatography (trypsin-agarose column). Bound protein eluted with 0.01 M HCL and neutralized with 2N Tris base and 40 ul used for trypsin assay.

Two fractions (27 and 29) showed more than 90% inhibition (Table 2). From this data it was probability that separation of trypsin inhibitor isoform more in fractions 25-29. The fold trypsin inhibitors purification in these fractions was found as 31.29 with yield recovery 18.68% as shown in table 1. These fractions were pooled together and loaded on trypsin-agarose as affinity column chromatography. The fraction containing trypsin inhibitor activity was found to 88.32 fold purification as shown in table 1 and figure 2. The fold purification of inhibitors by DEAE-cellulose and trypsin-agarose columns was found comparatively more than fold purification in earlier reported studies (Khan et al., 2003; Prasad et al., 2010). This drastic change in the purification fold and trypsin inhibitor unit activity may be due to extractability of trypsin inhibitors and quite improvement in purification method based on utilization of low protein concentration.

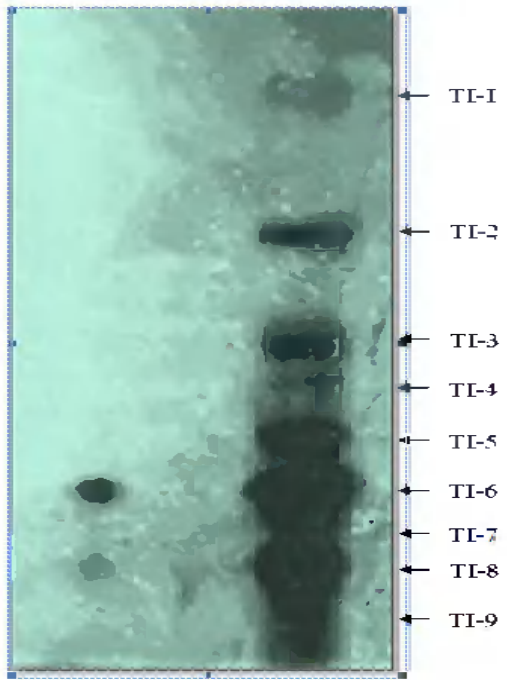

Fig. 3. X-ray film showing trypsin inhibitor profile. The 10 ul solution of PI showing inhibitory activity fraction eluted from affinity column. PIs detected by GXCP method. The pooled fractions further applied for Trypsin-agarose column and total unbound and bound protein 40 fraction were collected. The fraction no. 25 and 26 showed trypsin inhibitory activity shown in figure. The TI-6 and TI-8 were observed on X-ray film from each fraction only in 10µl solution figure no. The silver stained band profile corresponding to TI-6, TI-7, TI-8 and TI-9 were observed by SDS-PAGE.

Visualization of Proteinase Inhibitors

The isoforms of inhibitors in fractions eluted from trypsin-agarose column were detected on GXCP. Figure 3 and 4 show that separation of total nine inhibitors (TI-1, TI-2, TI-3, TI-4, TI-5, TI-6 TI-7, TI-8 and TI-9) from crude extracts and only two (TI-6 and TI-8) in fractions eluted from trypsin-agarose column. Low concentration was loaded on electrophoresis during experiment; hence the other inhibitors were not detected. It was high possibility that purified fraction contain almost all inhibitors but in less amount because the same fraction showed maximum purification fold. Prasad et al (2010) detected two protein bands (monomeric and

dimeric forms) on SDS-PAGE under non-reducing condition with molecular masses correspond to 8.5 and 16.5 kDa from purified fraction.

Fig. 4. SDS-PAGE with silver staining technique. Lane1: crude extract, Lane 2: 100 ul solution form DEAD-Cellulose fraction, Lane 3: 100 ul solution from fraction 6 and Lane 4: 100 ul solution from fraction 7 of affinity column.

Conclusion

The result of this revealed that protease inhibitors could be purified by ion-exchange (DEAE-Cellulose) and (trypsin agarose) affinity chromatography from C. cajan. The protease inhibitor available in C. cajan are very low. The purification method of this study is useful for effectively purification of same inhibitors. Use of low protein concentration of C. cajan during purification could increase the purification fold.

Acknowledgments

This work was supported by grants from University Grants Commission (UGC), New Delhi, India [No. F 38-15/ 2009 (Sr)]. We acknowledge the facilities of DST-FIST to Department of Chemistry, Dr. John Barnabas Post Graduate School for Biological Studies, B. P. H. Education Society's Ahmednagar College, used for the present study. Seeds of pigeonpea provided by Mahatma Phule Krishi Vidyapeeth, Rahuri-413722, Dist. Ahmednagar, Maharashtra, India, are gratefully acknowledged.

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