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Research Article

Proteinase Inhibitors of Pigeonpea cv. BSMR 736: Characterization and Bioefficacy against *Helicoverpa armigera(hübner)*

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ABSTRACT

Pigeonpea is an agriculturally important leguminous crop with high vulnerability to insect pest attack specifically, *Helicoverpa armigera*. The proteinase inhibitors (PIs) mediated host plant resistance against insect pests is a promising sustainable agricultural research practice. The current study was carried out to perceive biochemical characterization of proteinase inhibitors named PPTI in the pigeonpea (cv. BSMR 736). The purification of PPTI from crude protein seed extract was achieved by acetone precipitation, N-LP-IEF, and trypsin affinity chromatography. It was found to inhibit bovine trypsin and HaGPs *in vitro*. The optimal conditions for inhibition were pH 8 and temperature 40°C. The PPTI showed four isoinhibitors bands on native, non-reducing and reducing SDS-PAGE in the range of 26.7–19.3 KDa. On resolution on two-dimensional gel electrophoresis (2-DE), PPTI produced nine pI variant spots having isoelectric point (pI) 6.6, 6.6, 6.3, 6.1, 5.9, 5.8, 5.7, 5.6, and 5.6. An artificial diet containing PPTI reduced the *H. armigera* larval weight about 69%, with 25% mortality. For eco-friendly sustainable agricultural practices, natural compounds like PPTI could be expressed in transgenic crops to prevent the invasion of *H. armigera* in pigeonpea.

Keywords: Proteinase inhibitors, pigeonpea, PPTI, *helicoverpa armigera*, HaGPs

INTRODUCTION

Proteinase inhibitors (PIs) are plant proteins regulating endogenous proteinases and acting as defense molecules against biotic stresses.[1,2] They occur in tubers, seeds, leaves, and flowers.[3] With constitutive occurrence, enhanced expression was observed upon insect and pathogen attacks.[4] They are effective in the treatment of cancer, blood clotting, hemorrhage, and inflammation.[5] They are also useful as markers in studies of evolution and systematics.[6,7] Pigeonpea (*Cajanus cajan* L.) is a leguminous crop cultivated by farmers of subtropical and tropical regions. It is a major crop of protein-rich diet among others for the vegetarian population of the world. The range of biotic factors constrains the productivity of this crop. It is host to around 200 species of insects, among them *Helicoverpa armigera* is vivacious.[8] *Helicoverpa armigera* belongs to Lepidoptera species in the family Noctuidae. It is also named as cotton bollworm or corn earworm or scarce bordered straw.[9] The larvae of *H. armigera* feed on a wide range of plants, including significant no. of cultivated crops.

The pest account about US$ 317 million loss in semi-arid tropics in pigeonpea crop whereas above US$ 2 billion in other crops.[10] Farmers resort to the use of environmentally aggressive and ineffective pesticides to overcome these losses.[11] Hence, the probing and improving crop cultivars displaying resistance to *H. armigera* has huge potential in pest management.[12]

Host plant resistance could be attained through the use of PIs and is promising sustainable agricultural research practices.[13,14] Earlier insect *in vivo* feeding assays using artificial diet incorporated PIs and transgenic plants with

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PIs expressions have shown promising results.[15] The PIs are known to interfere with the digestive process of insects by inhibiting gut proteolytic enzymes. The arresting proteolytic activity causes limitation in the amino acids indispensable for the stages of development of insects.[16]

Several PIs have been identified from seed extracts of pigeonpea.[17] Pigeonpea encompasses predominantly PIs of the Bowman-Birk PIs (BBIs) family.[18] BBIs were found to be ineffectual against HaGPs.[19] The probable cause of feebleness lies in the dynamic gut protease expression system of *H. armigera*. The gut is found to express proteases that are PIs insensitive or degrading PIs.[20,21] Alongside BBIs, Kunitz PIs are also reported from pigeonpea.[22] Henceforth, the design of suitable PIs based defense strategies against *H. armigera* requires a broad understanding of various classes of PIs in pigeonpea as well as their effectiveness against *H. armigera*. The current study was commenced to perceive biochemical nature of PIs in the pigeonpea (cv. BSMR 736).

MATERIALS AND METHODS

Chemicals and Accessories

Immobilized Trypsin Agarose and porcine skin gelatin from porcine were procured from Sigma-Aldrich, USA. Protein ladders were procured from Genei Laboratories Pvt. Ltd, Banglore, India. Gelatin coated X-ray films were purchased from Fuji film, USA, and SELVAS photographic Ltd., Silvassa. IPG strips (11 cm, pH gradients 3-11), reagents and buffers required for 2D electrophoresis were obtained from GE Healthcare Bio-Sciences Corp., USA. The chemicals required for solution assays and 1D electrophoresis were procured from Sisco Research Laboratories Pvt. Ltd. (SRL), India. All chemicals and accessories utilized in this research were of the utmost purity available.

Pigeonpea Seeds Sample

The pigeonpea seeds (cv. BSMR 736) were acquired from the Agricultural research station, Badnapur (MS) India.

Procurement of Insects

The larvae (2nd and 4th instar) of *H. armigera* were obtained from chickpea and pigeonpea meadows at Maliwada, Aurangabad Maharashtra, India. *In vivo* insect feeding bioassays were performed with a chickpea flour-based synthetic diet.[23]

Extraction of PPTI from Pigeonpea

The seeds of pigeonpea were finely powdered in a mixer grinder. The solvents acetone and hexane were used for depigmentation and defatting respectively. The resulting dry defatted (fat removed) powder was adjourned in 1:6 (w/v) 100 mM Tris-HCl buffer with pH 7.8 comprising 1% polyvinylpyrrolidone with stirring conditions for 120 min. The resulting homogenate was further spin at 6,000 revolutions per minute (rpm) for 20 min at 4°C. The clear solution obtained by centrifugation was used for solvent precipitation of crude proteins by cold acetone. Acetone precipitated crude proteins were dialyzed against distilled water. The resulting precipitate was freeze-dried.

Helicoverpa armigera Gut Proteinases (HaGPs) Extraction

The *H. armigera* larvae were dissected at a lower temperature and stowed at –20°C. The gut is ruptured in alkaline buffer (100 mM glycine-NaOH buffer, pH 9.6) at 10°C. The resulting lysate was centrifuged at 10,000 rpm for 20 min at 4°C. The clear solution obtained was used as crude HaGPs.[3]

Native Liquid-phase Isoelectric Focusing (N-LP-IEF) of PPTI

The crude proteins extract was loaded on N-LP-IEF by Mini Rotofor™ cells (Bio-Rad Laboratories India Pvt. Ltd.). About 18 ml crude proteins extracts (0.4 g of proteins) were mixed along with 0.5% Triton X-100 and 20% glycerol. The 2% amphoteric molecules (40% ampholytes, BioLyte®, Bio-Rad) were selected/2000 μg of protein. Partitioning of crude proteins was carried out using pH 3–10 gradients at a continuous voltage of 12 W. The fractions obtained were further assessed for the presence of PIs using reveres zymography and solution assays. The fraction enriched with PIs activity was concentrated in 1M NaCl through an ultrafiltration membrane (Amicon Ultracell®-10K, Millipore). The resulting concentrated fraction (PPTI) was subjected to affinity chromatography.

Affinity Chromatography of PPTI

The concentrated protein fraction (no. 13) acquired by N-LP-IEF was subjected to affinity chromatography using immobilized trypsin agarose column (1.5 ml). The suspension contain (16) units/ml trypsin packed gel. The column was equilibrated with 100 mM Tris-HCl buffer with pH 7.8. Repeated elution of fractions of PPTI was eluted in 0.1N HCl with pH 3.0. The obtained fractions condensed and dialyzed using an ultrafiltration membrane.

Detection of PPTI by GXCP Technique and Reverse Zymography

Pigeonpea crude PIs and affinity chromatography purified PPTI were qualitatively analyzed by gel-X ray-film contact print (GXCP) technique[24] plus reverse zymography. PIs were applied on 1-dimensional native PAGE (Polyacrylamide gel electrophoresis).[25] The PIs were electrophoresed at a constant current (20 mA) until the tracing BPB (bromophenol blue) dye touched the end of the gel. For the GXCP technique, the gel was submerged in 100 mM Tris-HCl buffer, pH 7.8, and then immersed in 100 μg/ml trypsin for 10 min. After removing excess trypsin, the gel overlapped on the X-ray film for 10 min at 37°C. After incubation, hydrolyzed gelatin from X-ray film was removed by washing with distilled water. The PIs bands were seen as unhydrolyzed gelatin. The X-ray film was then photographed. The resulting gel, after washing with water stained for protein bands using coomassie brilliant blue R 250 (CBB R 250) dye. The identical protocol was employed for detecting PIs of HaGPs using 100 mM glycine-NaOH, 0.3 M $CaCl_2$ buffer, pH 9.6. The experimentation was repeated thrice with 3 repeats. To analyze PIs by reverse zymography, the native PAGE gel was immersed in 100 mM Tris–HCl buffer, pH 7.8 for 10 min and further incubated in 1% casein (made in 100 mM Tris–HCl buffer with pH 7.8) for 20 min. Subsequently,

the gel was immersed in trypsin (0.1%) solution for 10 min at 37°C. Afterward, the gel was washed with distilled water and stained with CBB R 250 dye. The polypeptide content of PPTI was explored by 12% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[26]

PPTI Visualization by 2-DE (2-Dimensional Electrophoresis)

Pigeonpea crude PIs and affinity chromatography purified PPTI were further analyzed by 2-DE and 2-D reverse zymography using 7 cm IPG strip with 3–10 pH gradients (Bio-Rad, USA). Rehydration of IPG strips was carried out using buffer comprising 2% CHAPS (w/v), 0.2% (v/v) IPG buffer, 2M thiourea, 7M urea, pigeonpea PIs, and trace of BPB dye. The IEF (Isoelectric Focusing) was carried out at 20ºC on the Protean® IEF system (Bio-Rad, USA) according to manufactures protocol (Catalog # 163-2099). After IEF, strip was equilibrize for 30 minutes in equilibration buffer (2% SDS, 30% glycerol), and 50 mM Tris–HCl buffer with pH 8.8 at 37°C. The IPG-strip was further exposed to 12% SDS PAGE and the gel subsequently was subjected to reverse zymography by the following methodology mentioned above. The resulting protein spots were visualized by CBB R-250 and silver stain. Using 2D-SDS PAGE standards, isoelectric points (pIs) of PPTI were determined.

Solution Assay of PIs

PIs activity was assessed using method reported earlier by Kakade *et al*.[27] The reaction mixture comprising PIs and trypsin in HCl and 100 mM Tris–HCl buffer, pH 7.8, 300 mM $CaCl_2$ were incubated for 10 min. To this 300 µl, BApNA (1 mM in Dimethyl sulfoxide) was added. The reaction mixture was adjusted to 1.2 ml by the buffer. After end of reaction time, that is, 30 min, 300 µl 30% acetic acid was added to terminate the reaction. The blank and enzyme control tubes were prepared concurrently. The optical density was computed at 410 nm. The PIs activity against HaGPs was calculated by mixing the PIs with HaGPs for 20 min at 37°C in 100 mM glycine-NaOH buffer with pH 9.6 containing 300 mM $CaCl_2$. One unit enzyme activity is defined as the release of 1.0 µM of *p*-nitroaniline per minute under standard conditions whereas one PIs unit could be inhibition of one enzyme unit.

Effects of pH on PPTI

The buffer systems, that is, 100 mM glycine-HCl (pH 2), 100 mM sodium citrate (pH 4 and 6), 100 mM Tris-HCl (pH 8), and 100 mM glycine-NaOH (pH 10, 12) were used to study effect of H^+ ion concentrations on PPTI activity using standard assay parameters.

Effects of Temperatures on PPTI

Temperatures range 20°C–80°C (at 10°C intervals) in dry water bath was used to study effect of temperature on PPTI activity using standard assay parameters.

Insect Bioassay of PPTI

The larvae of *H. armigera* (2^{nd} instar) were nurtured on a synthetic chemical diet comprising altered dosages of PPTI maintaining 28 ± 2°C and 16:8 light/dark day length conditions. The experiment was performed in three replicates using a 100 ml synthetic chemical diet.[23] The PIs was integrated into the synthetic chemical diet at 3 altered dosages, that is, 5, 10, and 15 µg/g of food, w/w). The diet deprived of PI fraction was considered as the control diet. All the experimental food was solidify at 4°C for 24 h before offered to the *H. armigera*. All larvae were distributed in individual vials for feeding experiment and nourished daily. The food in vials were changed daily and not permitted to dry or be fully consumed. The weights of *H. armigera* were noted at identical time daily.

Protein Determination

The total protein was quantified using method devised by Lowry *et al*. using bovine serum albumin (BSA) as the standard protein.[28]

Statistical Analysis

The experimental data reported in the present research were performed in triplicate. The statistical analysis, mean ± S.D, and ANOVA (One-way analysis of variance) were performed in Microsoft Excel 2010. The statistical significance examined with $p \leq 0.001$.

RESULTS AND DISCUSSION

PIs act as the plant's endogenous arsenals to counterbalance insect pest activities. Overexpression of PIs in the most vulnerable target tissues and/or raising transgenic plants expressing PIs is the current strategy against insect pests.[29,30] The exploration of PIs based *H. armigera* resistance in pigeonpea may prove a vital component in IPM (Integrated Pest Management). The current investigation was aimed at exploring the biochemical nature and characteristics of PPTI from pigeonpea cv. BSMR 736.

Pigeonpea PIs Extraction and Detection

As per the literature review, most of the pigeonpea cultivars including BSMR 736 exhibits monomorphic PIs proteins pattern on the electrophoretic gel.[19] Here in accordance, we are also reporting monomorphic PIs bands from the crude proteins extract of pigeonpea cv. BSMR 736 by GXCP technique against bovine trypsin [Figure 1]. The GXCP facilitates simple and rapid analysis of the proteinases and their inhibitors from seeds and other samples.

Prefractionation of PIs by N-LP-IEF

Several research groups employed various strategies for the prefractionation of proteins before electrophoretic characterization and preparative isolation of desired protein and N-LP-IEF was found to be convenient.[31,32] The N-LP-IEF allows the separation of protein even if it is present in low abundance in the sample based on their isoelectric point (pI). Conventional fractionation strategies such as $(NH_4)_2SO_4$ precipitation, subsists traces of salts in the sample which creates trouble in further purification and identification of molecules. Hence, to overcome these issues, we applied acetone precipitated protein onto N-LP-IEF. About 20 fractions

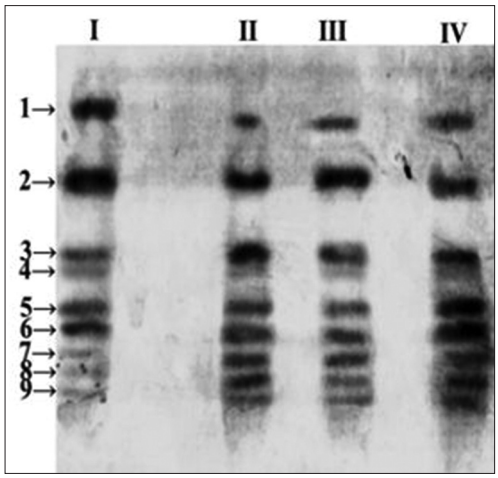

Figure 1: Detection of TIs of pigeonpea by GXCP technique. The sample (100 μg) was loaded in each lane (I-IV) on 10% native PAGE, PIs activity bands were detected by immersing the gel in bovine trypsin and overlapping gel on the X-ray film

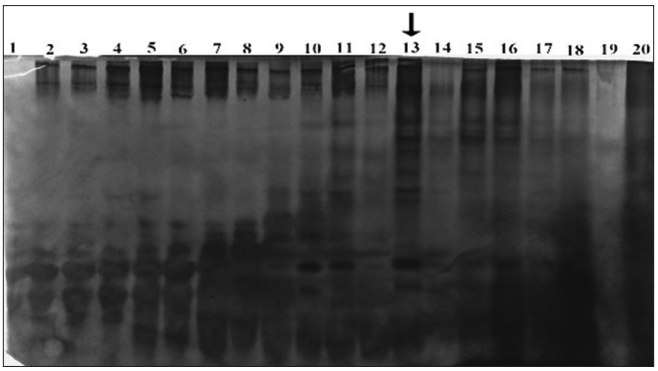

Figure 2: Isoelectric point (pI) based fractionation of acetone precipitated protein N-LP-IEF was carried out between pH 3-10. An equal volume of each fraction was loaded and visualized by SDS-PAGE GXCP. Arrow (↓) indicates fraction (13) used for subsequent analysis

were collected after N-LP-IEF ranging in pH from 3 to 10. The prefractionation by N-LP-IEF revealed that PIs was restricted to acidic to neutral fractions with maximum at pIs 6.6, 6.6, 6.3, 6.1, 5.9, 5.8, 5.7, 5.6, and 5.6. (Fraction no.13). Earlier PIs showing activity in the acidic to neutral pIs were reported in the pigeonpea cv. Abhaya and ICP 7118.[18,33] This prefractionation also showed the presence of more number of PI bands in fraction 13 on X-ray films suggesting active fractionation of low abundant PIs.

Figure 2 displays the SDS-PAGE stained gel map of the total fractionated PIs (20 fractions) collected from the N-LP-IEF with equal volume loaded on the gel. It indicates the distribution of PIs in different fractions, and the relative numbers of PIs of each fraction are also different. However, it was observed that most of the PIs had diffused into several neighboring fractions. This may be due to the diffusion of PIs in the solution since it is not isoelectrically focused as it would be in a gel.[34] Fraction 13, showed more activity, and numbers of PIs was used for further purification purpose.

Trypsin-agarose Affinity Chromatography

The N-LP-IEF fraction was further applied to the trypsin-agarose affinity column. A trypsin-agarose resin is selectively purifying an inhibitor of trypsin. The PIs bound to the matrix were eluted in 0.1N HCl with pH 3.0. These PIs were collected, condensed and dialyzed using membrane ultrafiltration. The unbound fraction did not show any TI activity. The concentrated fraction was eluted from the trypsin-agarose column showing TI activity at 280 nm was named as PPTI and used in further characterization study.

One Dimensional Electrophoretic Characterization of PPTI

On affinity purification, the PPTI displayed separation of 4 PIs bands, namely, PI1, PI2, PI3, and PI4 on native as well as non-reducing SDS-PAGE as compared to PI pool present in the crude seed extract. This might be due to the self-association tendency of PIs during purification stages. Earlier such a phenomenon of self-association was observed in BBI like PIs.[35] Such PIs were also reported from two cultivars of pigeonpea.[20,21] The PPTI showed inhibitory activity against bovine pancreatic trypsin and HaGPs checked by native reverse zymography [Figure 3]. Treatment of PPTI with β-mercaptoethanol slightly alters the migration of the PI4 band on the gel indicating its reduction [Figure 4]. The four PIs on non-reducing and reducing SDS-PAGE with β-mercaptoethanol exhibit molecular weights in the range of 26.7–19.3 kDa. This finding is in agreement with earlier studies that reported high molecular weights of PIs from various legume seed extracts.[36] The self-association of PI molecules might result in the overestimation of molecular mass as reported for several legumes.[37,38] Furthermore, the occurrence of PPTI in a group of four isoinhibitors may be attributed to the particularly individual narrow range masses and high molecular weights of these PIs may also be associated with the different degrees of glycosylation.[39] Other possibilities like oxidation of cysteine residues have been reported earlier.[40] Similar findings of purification of TI with a group of three isoinhibitors were reported from *Acacia victoriae* Bentham seeds.[41] The functional role of occurrence of many isoinhibitors is not clear; it seems to be a part of approaches adopted by the host plant to ensure its existence.

Inhibition Assay of PPTI

The trypsin and HaGPs activities were reduced in the presence of crude PIs as well as an affinity-purified PPTI *in vitro* [Figure 5]. Previously, moderate to low inhibition potential against HaGPs was reported from several pigeonpea cultivars by *in vitro* and *in vivo* bioassays.[19,23] In agreement with the above studies, our finding advocates that pigeonpea (cv. BSMR 736) could be a potent source of PIs.

Optimum pH of PPTI

In general, legumes PIs are identified to be stable under extremes of pH. The PPTI was found to be active at a varied range of pHs 3–12 with optimum inhibition at pH 8 [Figure 6]. This finding is in agreement with earlier studies where optimum PIs activity was retained between pH 4 and 10.[16,36,37] The optimum activity of PI from other legumes such as mungbean (*V. radiate* L. Wilczek) seeds was also observed at pH 8.0.[41] The gut content of *H. armigera* is highly alkaline

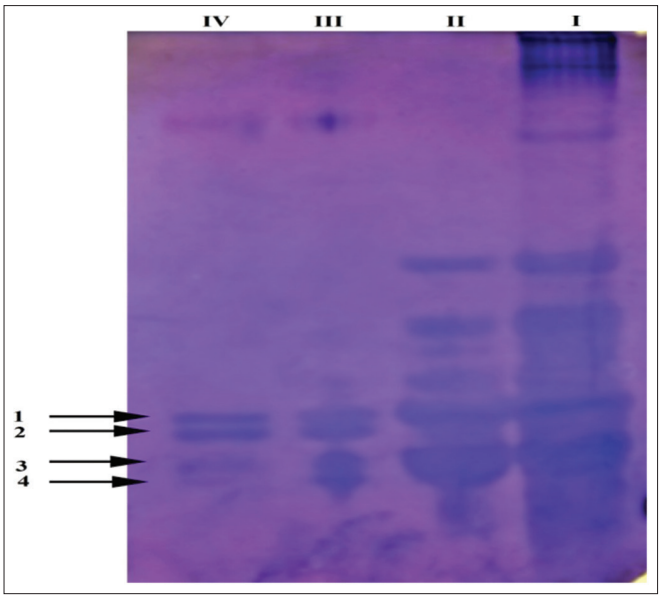

Figure 3: Electrophoretic analysis (10% native PAGE reverse zymography) of PPTI. Lanes I-IV crude protein extract (100 µg), the active fraction (Fraction-13) from N-LP-IEF (50 µg), affinity column pooled fraction (15 µg), and membrane filtration fraction (10 µg; 10 kDa cut-off), respectively

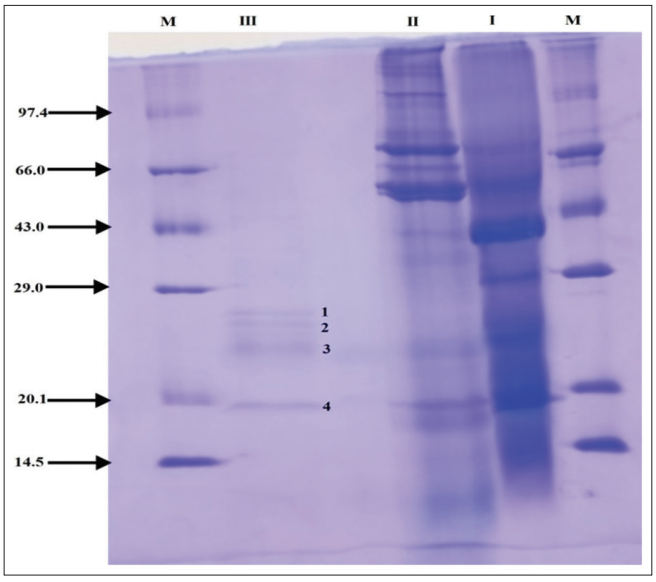

Figure 4: 12% SDS-polyacrylamide electrophoresis of PPTI. Lane M, molecular weight standards; lane I Crude extract, lane II N-LP-IEF fraction no. 13, lane III PPTI with β- mercaptoethanol; proteins were detected by dye CBB R-250

with maximum HaGPs activity at pH 10.0–11.0.[42] This result with PPTI indicated that it is suitable against HaGPs, which work in the alkaline gut of *H. armigera*.

Optimum Temperature of PPTI

The optimum temperature of PPTI was found to be 40°C and showed considerable activity even at 80°C [Figure 7], suggesting that PPTI is cysteine-rich which imparts high stability and conformational rigidity at high temperatures.[43]

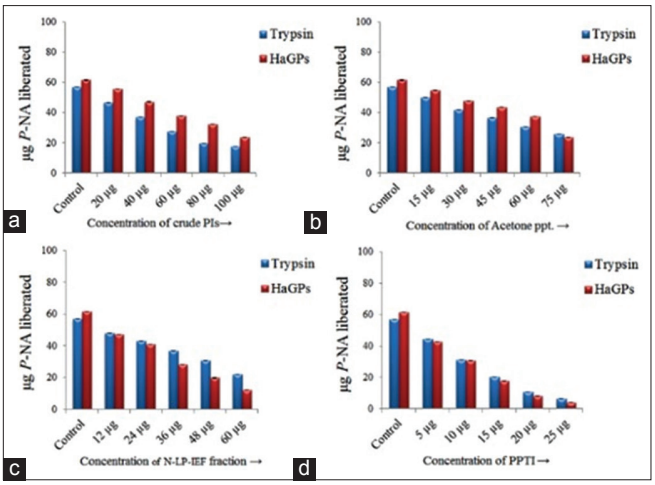

Figure 5: Trypsin and HaGPs inhibition assays by (a) crude seed protein, (b) acetone ppt. protein, (c) N-LP-IEF fraction, and (d) PPTI. The enzyme activity was assayed using BApNA. The results were reported as mean ± SD, $n = 3$

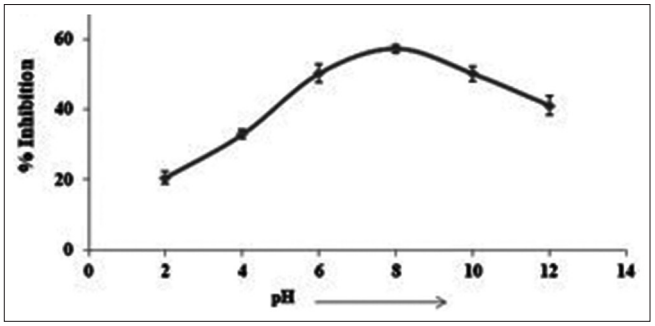

Figure 6: Effect of pH on PPTI activity. An assay was executed in altered buffer solutions, as revealed in the methodology section. The results are mentioned as mean ± SD, $n = 3$

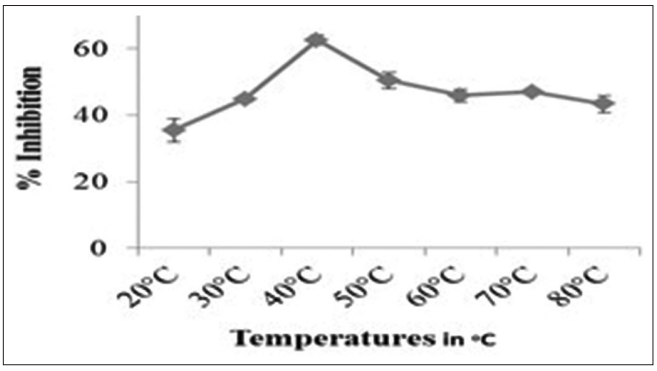

Figure 7: Effect of temperature on PPTI. An assay was executed at altered temperatures for 10 min as mentioned in the methodology section. The results were presented as mean ± SD, $n = 3$

This study is in agreement with earlier finding where PIs with stability up to a temperature of 80°C was reported from pigeonpea.[16,37]

Analysis of pI Variant Isoforms of PPTI

The isoinhibitors complexity of affinity-purified PPTI was further resolved by 2-DE. Figure 8 illustrates 2-DE analyses

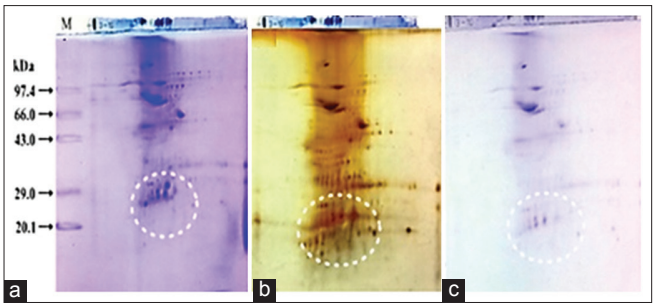

Figure 8: Two-dimensional electrophoretic analyses of pigeonpea (BSMR 736) seed proteins. (a) CBB R-250, (b) silver staining, and (c) PIs activity visualization by reverse zymography

of pigeonpea seed proteins. The pigeonpea cv. BSMR 736 is a rich source of proteins hence several proteins with varied MW and pI were seen on the gel [Figure 8a] when stained with CBB R-250. Subsequently, staining of the gel with silver stain increased the quantity of proteins bands on the gel corroborating the sensitivity of strain [Figure 8b]. When the gel was digested with trypsin in reverse zymography, most of the proteins disappeared due to the hydrolytic action of trypsin [Figure 8c]. Several trypsin inhibitors or trypsin-resistant proteins could be detected on the lower and upper panels of the gel. The lower panel of the gel is shown by the circle corresponding to the affinity purified PPTI.

The 2-DE analysis showed 9 *pI* variant isoforms of PPTI with *pI* range 5.6–6.6, matching their isoinhibitors migration on the gel [Figure 9]. For instance, they were collected from alkaline regions earlier only very slight *pI* dependent variations were observed among them with molecular weights nearly in same the range as observed in 1-D SDS PAGE with β-mercaptoethanol [Figure 9a]. The PPTI remained active after exposure to several detergents and urea used in 2-D gels assessed by reverse zymography [Figure 9b]. The *pI* variant isoforms showing identity toward BBI like PIs were reported from the seeds of pigeonpea cv. Abhaya and cv. ICP 7118.[16,19] The advent of different PIs classes in the same plants with the potential to inhibit the same enzyme is due to convergent evolution.[44]

Bioefficacy of PPTI

The Kunitz, BBI, cysteine, Knottin PIs, and lectins are the most studied PIs showing future potential to use them in insect resistance strategies.[45,46] The faith of the PI strategy can largely depend on the nature of the insect response to the ingested PIs. Hence, the effectiveness of affinity-purified PPTI against HaGPs was assessed by integrating 3 altered dosages (i.e., 5, 10 and 15 µg/gm w/w of diet). Here artificial diet containing PPTI reduced the *H. armigera* larval weight by 69%, with 25% mortality. The average larval weight was reduced in the control from 689 ± 17.45 mg to 214 ± 12.45 mg when fed a 15 µg/gm diet, at the end of the feeding assay period (10 days), indicating a substantial decline in the growth and development of *H. armigera* with $P \leq 0.001$ [Figure 10].

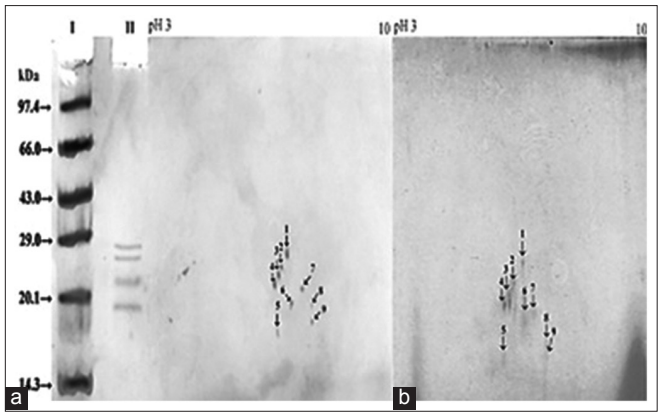

Figure 9: Two-dimensional electrophoretic analyses of PPTI isoforms. (a) PPTI protein spots (b) Reverse zymogram. Un-digested proteins signify PIs. Black arrow specify the location of PIs. Lane I, Protein ladder in kDa; Lane II, purified PIs (PPTI) from affinity column. The pHs range is shown on the gel

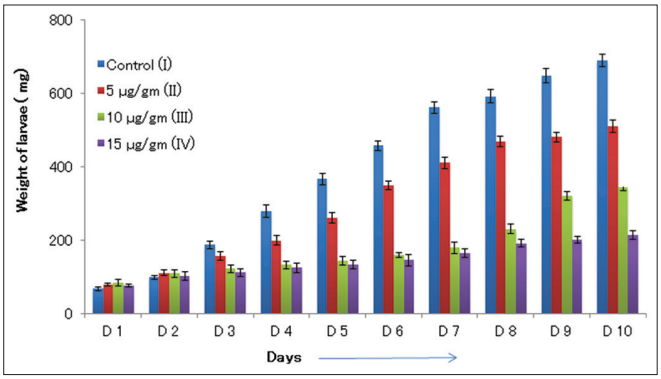

Figure 10: Effect of PPTI on the development of *H. armigera* larvae. The larvae were permitted to feed on a synthetic chemical diet with PPTI and without PPTI. Larval weight was noted every 24 h. Each mean signifies three replicates ± SD, and a difference among analyses of $P \leq 0.001$ was considered significant

CONCLUSIONS

In a nutshell, the above results indicate that the PPTI is responsible for the reduction in the growth of *H. armigera* with low mortality. Further research in understanding the proteomic nature of isoinhibitors variant isoforms of PPTI is required. Comprehensive knowledge of the occurrence of isoinhibitors of PPTI in host plants like pigeonpea might provide insect resistance modules with resistance against *H. armigera* invasion.

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