*J. Crop Prot.* **2016, 5 (3): 461-472**

Research Article

# Inhibition of *Chilo partellus* (Lepidoptera: Pyralidae) gut proteases with *Ipomoea batatas* inhibitors

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**Abstract:** *Chilo partellus* is a devastating sorghum and maize pest that exists worldwide. *C. partellus* gut proteases (CPGPs) were isolated, purified and characterized. We analyzed potential trypsin and chymotrypsin inhibitors (TIs) from tuber plants to identify the protease inhibitors (PIs) of CPGPs. PIs from *Ipomoea batatas* completely inhibited protease activity and growth and led to reduced *C. partellus* insect larvae weights. One PI was purified from *I. batatas* tubers and identified as a potent antagonist of CPGPs. In conclusion, we identified *Ipomoea batatas* inhibitors with significant potential applications in developing resistant transgenic sorghum and maize crops.

**Key words:** *Chilo partellus* insects, CPGPs, Host and non-host PIs, BA*p*NA, GXCP

## Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is used in food, feed, fuel, and fiber and is sometimes indispensable for the survival of humans and domestic animals in dry environments (Chandrashekar and Satyanarayana, 2006).

The spotted stem borer, *Chilo partellus,* is a polyphagous pest of sorghum and many other cereals (Glas *et al*., 2007).

Insect-resistant plants expressing genes that encode protease inhibitors (PIs) of plant origin may be incorporated into crop-production systems (Jouanin *et al*., 1998; Michaud, 1997). The use of PIs to imbue transgenic plants with insect resistance may be beneficial. Inhibiting insect gut proteases and preventing the proteolytic degradation of defense proteins can improve resistance (Michaud, 1997; Varshney *et al*., 2007). PIs inhibit insect gut digestive proteases and starve the insects of essential amino acids (Varshney *et al*., 2007; Ryan, 1990).

We investigated and identified potent inhibitors of *C. partellus* gut proteases (CPGPs) from a non-host tuber. Bioassays were performed to measure feeding, and the potencies of the *I. batatas* inhibitors against developing *C. partellus* insect larvae were determined.

## Materials and Methods

### Plant materials and chemical collection

Maize stem tissues and leaves were collected from fields. Casein, sucrose, bean powder, brewer's yeast, sorbic acid, ascorbic acid, methyl-p-benzoate, ascorbic acid, chloramphenicol and formaldehyde were purchased from a local market. The synthetic substrate n-α benzoyl-DL-arginine p-nitroanilide, N-succinyl-ala-ala-pro-phe p-nitroanilide, n-α-p-tosyl-L-lysine chloromethyl ketone, chymostatin (TLCK), 4-(2-aminoethyl-benzenesulfonyl fluoride-hydrochloride), Pefabloc, L-trans-Epoxysuccinyl-leucyl-amido-(4-guanidino) butane (E-64) and molecular

Handling Editor: Saeid Moharramipour

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Received: 7 March 2016, Accepted: 26 June 2016
Published online: 19 September 2016

weight marker were purchased from Merck (Germany). Bovine serum albumin was purchased from Sigma (USA). Trypsin and azo-casein were obtained from SRL chemicals (Mumbai, India), and X-ray films were obtained from Agfa (Silvassa, India).

**_C. partellus_ larvae reared on an artificial diet**

*C. partellus* were raised from insects collected in sorghum fields located near Aurangabad (Maharashtra), India. Larvae were sorted according to their instars and kept in plastic containers with a specific diet (Varshney *et al.*, 2007; Onyango and Oching-Oders, 1994). The food in the containers was replenished as needed until pupation. Pupae were removed from the containers and kept in other containers (14 cm in height and 12 cm in diameter) lined with moistened tissue paper for adult emergence (Varshney *et al.*, 2007). Twelve pairs of male and female moths were delivered in individual containers (14 cm in height and 12 cm in diameter) with their walls and open ends covered with butter paper to provide a soft surface for egg laying (Varshney *et al.*, 2007). A cotton wool swab soaked in distilled water was placed in each container to enable water intake by the moths (Varshney *et al.*, 2007). Blackhead-stage larvae were placed in the rearing containers with food and used for the detection, purification and characterization of CPGPs and for the interaction and feeding bioassays.

**Extraction of fresh _C. partellus_ gut proteases (CPGPs)**

*C. partellus* larvae reared on an artificial diet containing maize leaf powder were collected. The 3$^{rd}$-instar *C. partellus* larvae were dissected to remove the midgut, which was stored at -20 °C until further use. Gut proteases from twenty *C. partellus* gut samples were extracted by homogenization in 0.1-M glycine-NaOH buffer (pH 9.0) at a 1: 6 w/v ratio, and the samples were stored at 6 °C for 2 hr to allow the extraction to proceed. The suspension was then centrifuged at 10,000 rpm for 10 min at 6 °C. The resulting supernatant was collected and used for measuring the proteins (Lowry *et al.*, 1951; Panchal and Kachole, 2012) and purifying the proteases.

**CPGP and PI activity assays**

CPGP activity was estimated using the chromogenic substrate BApNA, (Telang *et al.*, 2005) which was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM in 1.0 ml of glycine-NaOH buffer (pH 9). Assays were conducted at 38 °C for 30 min (Telang *et al.*, 2005). The reaction was terminated by the addition of 200 µl of acetic acid (30%) (Telang *et al.*, 2005; Patankar *et al.*, 2001; Telang *et al.*, 2003). The supernatant (0.5 ml) was added to 1-M NaOH (0.5 ml), and the absorbance of this solution was measured at 410 nm (Telang *et al.*, 2005). One protease unit is defined as the amount required for increasing the absorbance by 1 OD/min.

An azocasein assay was performed using a 1% (w/v) solution of the substrate in buffer (Telang *et al.*, 2005). CPGPs (15 µl) were added to the substrate (200 µl) and incubated at 38ºC for 30 min. The reaction was terminated by the addition of 5% trichloro-acetic acid (300 µl). The precipitated protein was centrifuged at 10,000 rpm for 10 min. Then, 0.5 ml of the supernatant was added to 1-M NaOH (0.5 ml), and the absorbance of the solution was measured at 450 nm (Kumar, 1997). In the inhibitor bioassay, a suitable volume of inhibitor was mixed with CPGPs and incubated at 38 ºC for 15 min. A PI unit was defined as the amount of inhibitor required to inhibit one unit of protease activity under the assay conditions (Kumar, 1997).

**Purification of the CPGPs and tuber PIs**

DEAE-Sepharose fast-flow anion-exchange chromatography was used for the partial purification of CPGPs (Kumar, 1997). For the CPGPs, 50-mM NaOH buffer (pH 9) was used to equilibrate the column. The gut homogenate was also equilibrated with 50-mM NaOH buffer (pH 9). The buffer was loaded onto the column, and elution was achieved using a 0-0.6-M NaCl gradient (Kumar, 1997). Two-milliliter fractions

were collected and analyzed for proteolytic activity using BApNA and SAAPFpNA. The fractions were dialyzed against distilled water and concentrated for further analysis. The protein concentrations in the gut homogenate and fractions were determined (Lowry *et al.*, 1951). Ammonium sulfate was precipitated (60-90% saturation), and *I. batatas* extracts (200 TI units, 40 mg proteins) were loaded onto a 50-ml-capacity DEAE ion-exchange column (1.6 × 12.4 cm, bed volume 25 ml) and equilibrated with 50-mM Tris-HCl buffer (pH 8.0). The column was washed with 50-mM Tris-HCl (pH 8.0, 200 ml), and 2-ml fractions (fractions 1 to 30) were collected. A solution of 0.25-M NaCl in 50-mM Tris-HCl (pH 8, 30 ml) (fractions 31 to 60) was then passed through the column, followed by a gradient of 0.25-0.4-M 50-mM Tris-HCl (pH 8, 60 ml) (fractions 61 to 91). Finally, the column was washed with 25 ml of 0.4-M NaCl in 50-mM Tris-HCl (pH 8.0) (fractions 92 to 123). Each collected fraction was 2 ml (Tamhane et al., 2005). The fractions were analyzed at 280 nm using a PC-based spectrophotometer (JASCO 500 series). Dot-blot assays of each fraction were conducted to determine the presence of the inhibitory activity using X-ray film (Pichare and Kachole, 1994). The concentrated fractions were further separated on a native gel, followed by trypsin inhibitor (TI) activity visualization (Tamhane et al., 2005).

To identify the optimum pH for the CPGPs, the gut proteases were assayed in different buffers with pH values ranging from 5 to 11. For this purpose, 0.1-M Na-acetate buffer was used for pH 5.0 and 6.0, 0.1-M Tris-HCl buffer was used for pH 7.0 and 8.0, and 0.2-M glycine-NaOH buffer was used for pH 9.0 to 11.0. BApNA and SAAPFpNA acted as CPGP substrates (Kumar, 1997).

**CPGP visualization by the gel X-ray film contact print (GXCP) method**

Gel electrophoresis was conducted using a previously reported buffer system (Davis, 2006). The electrophoresis gel was processed for protease activity detection using the GXCP method (Harsulkar *et al.*, 1998). After electrophoresis, the gel was incubated in 0.1-M glycine-NaOH buffer (pH 9) for 7 to 8 min and then placed on an X-ray film (Padul *et al.*, 2012). The gel was removed from the X-ray film after 45 min depending on the extent of gelatin hydrolysis. The X-ray film was washed with warm water to observe the CPGP bands as hydrolyzed gelatin against the gelatin background (Padul *et al.*, 2012). The X-ray film was then developed. After development, gut proteases were visible as light bands against the dark opaque background (Padul *et al.*, 2012).

**CPGP treatment with PIs**

To study the interactions between the PIs and CPGPs, PIs were incubated with CPGPs at ratios of 1: 1 and 1:2 (inhibitor:protease [unit:unit]) at 37 °C for 3 hr (Laura *et al.*, 2005). TI activity was estimated as described in the CPGP and PI activity assay section. PI-treated CPGPs were resolved on native polyacrylamide gel electrophoresis (PAGE) gels and processed for TI activity visualization (Tamhane et al., 2005).

**CPGP inhibition by host and non-host tuber PIs**

The GXCP method was used to measure CPGP inhibition by plant PIs (Panchal and Kachole, 2012). The appropriate volumes of gut extracts and PIs from plant extracts (5 µg of gut extracts and 15 µg of plant extracts) were incubated with host and non-host tuber PIs for 30 min at room temperature. After 30 min, the suspension was loaded onto a native PAGE gel (Panchal and Kachole, 2012). Upon completion of the electrophoresis, the gel was placed in 0.1-M glycine-NaOH buffer (pH 9) for 10 min for equilibration. The gel was then placed on an X-ray film for 35 to 45 min, and the hydrolysis of the gelatin was monitored visually (Panchal and Kachole, 2012). The X-ray film was washed with warm water, dried at room temperature, and scanned.

**Feeding assay**

The *in vivo* efficacies of the host and non-host tuber PIs belonging to different families were

investigated by feeding bioassays using 130 second-instar *C. partellus* larvae. An artificial diet was prepared as described (Ryan, 1990). The feeding assay was conducted by adding plant PIs to the artificial diet: 0.63 g of food containing PIs was mixed with 0.57 g of yeast, 0.06 g of ascorbic acid, 0.03 g of sorbic acid, 0.05 g of methyl-p-hydroxy benzoate, 0.05 g of vitamin E capsules, and 0.88 g of sucrose (Panchal and Kachole, 2012). This mixture and 0.32 g of agar were dissolved in 25 ml of water (50 to 60°C). Then, 0.05 ml of formaldehyde (5%) was added, and the solution was mixed thoroughly and poured into trays. Cubes of food (1.0 g) were cut and used for the feeding experiments (Panchal and Kachole, 2012). The control group received an artificial diet without PIs. The larvae were reared on an artificial diet, and weight loss or gain was recorded each day until pupation (11 to 12 days). In the feeding assay, inhibitors were used at a concentration of 20 mg per gram of artificial diet. Ten days after the feeding experiment, some insects were dissected and kept at -20 °C for further experimental use. Gut proteases from the CPGP gut tissues were extracted by mixing the tissue with 0.1-M glycine-NaOH buffer (pH 9) at a 1: 6 w/v ratio and storing them at 6 °C for 2 hr. The suspension was centrifuged at 10,000 rpm at 6 °C for 10 min (Panchal and Kachole, 2012). The resulting supernatant was used for the enzymatic bioassays.

**Statistical analysis**

Statistical analyses were conducted using SPSS for Windows (version 15). The significance of the statistical tests comparing the treatments was calculated at the 5% level of probability. A general linear model analysis of variance (ANOVA) was used for the data analysis.

**Results**

**Rearing of *C. partellus* insects**

An artificial diet is necessary for rearing because it produces more uniform *C. partellus* insects than their natural diet. Such artificial diets are produced following standardized methods, and cleaning is more easily performed when an artificial diet is used. The concentrations were 20 and 40 mg/g for the first generation of *C. partellus*. In the second generation, concentrations of 20, 30, 40 and 50 mg/g were used, and their effects on *C. partellus* larval growth and development were studied (Fig. 1). When *C. partellus* insects were fed a prepared diet, the *C. partellus* pupal period was 9-10 days, and the eggs hatched in 4-5 days. The eggs hatch in the early morning (6-7 hr) 6-8 days after being oviposited. The mean durations of the 1st, 2nd, 3rd, 4th and 5th instars were 3, 5, 6, 8 and 8 days, respectively. The young larvae eat the whole egg shell during hatching. Insect mortality is very low in an artificial environment. The pupae were cylindrical and dark brown, with an average weight of 211 mg. The male pupae lasted 7 days, and the female pupae lasted 8 days. The oviposition period was 4 days. The adult male and female moths lived for 4-8 days and 4-7 days, respectively.

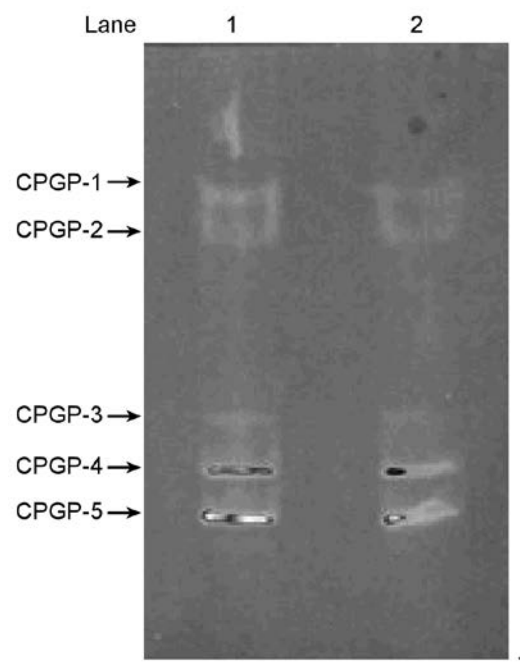


**Figure 1** Identification of *Chilo partellus* gut proteases (CPGPs). Electrophoresis was conducted on a 10% native PAGE gel. Approximately 10 µg and 20 µg of protein were loaded in lanes 1 and 2, respectively.

### Visualization of gut proteases of *C. partellus* (CPGPs)

The *C. partellus* gut extract contained five major protease isoforms that were detected on a native PAGE gel (Fig. 2). Four bands corresponding to CPGP proteases were identified and showed high activity, and one band exhibited low activity. The CPGP-1 band was the largest and was much larger than those of CPGP-3 and 4. Thus, CPGP-1 was present at higher concentrations in the gut than CPGP-3 and 4. CPGP-5 existed at a low concentration compared to CPGP-2. Thus, five CPGP isoforms were found in the investigation. The molecular weights of CPGPs-1 to 5 ranged from approximately 27.23 kD to 95.23 kDa (Fig. 3).

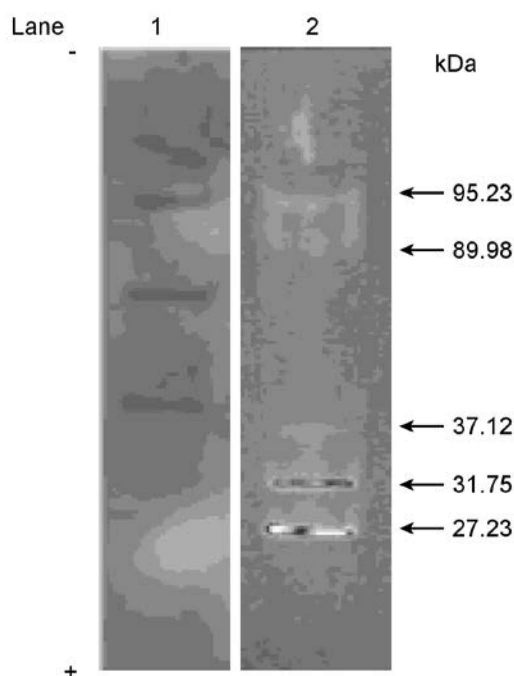


**Figure 2** Determination of *Chilo partellus* gut protease (CPGP) molecular weights by SDS-PAGE (12%) stained with CBB R-250. CPGP activity bands were visualized using an X-ray film contact print technique. Lane 1 contains the molecular weight marker, and Lane 2 contains 10 µg of larval CPGP proteins.

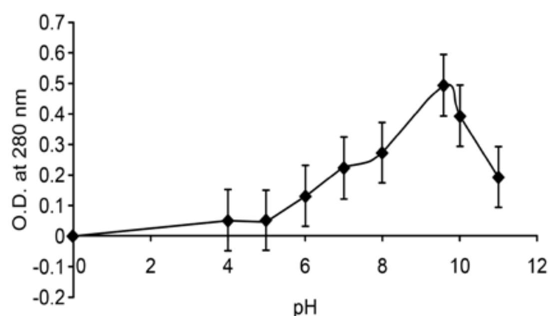


**Figure 3** Effect of pH (4 to 11) on CPGP activity. An enzymatic assay was conducted in different buffers with pH values ranging from 4 to 10.5 using BApNA as a substrate.

In the enzyme assays, TLCK (50 mM) inhibited 75.38 ± 0.57% of CPGP activity. Chymostatin (50 mM) inhibited 36.04 ± 0.90% of CPGP activity. The serine PI Pefabloc (50 mM) inhibited 83.21 ± 0.82% of CPGP activity. Pefabloc caused an average of 89.63 ± 0.75% inhibition against semi-purified CPGPs, whereas TLCK showed 80.02 ± 0.60% inhibition, and chymostatin showed 25.47 ± 1.21% inhibition (Table 1). The PI mixture revealed that the major CPGP constituents were serine proteases.

### Semi-purification and characterization of CPGPs

The semi-purification steps used in this work to purify the CPGPs are shown in Table 2. Semi-purification of the CPGPs was achieved by subjecting the gut extracts to DEAE ion-exchange chromatography. CPGP-1 was eluted by DEAE ion-exchange chromatography. The gut protease activity bands of the fractions were visualized with a X-ray film contact print technique (Harsulkar *et al.*, 1998). CPGPs readily hydrolyzed BApNA and caused considerable hydrolysis of the synthetic substrate SAAPFpNA for chymotrypsin. The CPGP-1 yield exceeded 30% of the total gut protease activity with a specific activity of 2.82 U/mg. The trypsin activities of the crude and purified CPGPs were 3.4 ± 0.1 and 2.11 ± 0.0, respectively. The chymotrypsin activities of the crude and purified CPGPs were 4.9 ± 0.3 and 3.05 ± 0.2, respectively (Table 3). Thus, more CPGP-1 was present in the *C. partellus* insect gut.

**Table 1** Comparison of synthetic inhibitors inhibition with *Chilo partellus* gut proteases (CPGPs) activity of crude gut extract and partially purified CPGP.

| Inhibitors | Specificity | Inhibitor conc. | Inhibition CPGPs (%) | Inhibition purified CPGP (%) |
|---|---|---|---|---|
| Pefabloc | Serine proteinase | 50 mM | 83.21 ± 0.8 | 89.63 ± 0.7 |
| TLCK | Trypsin | 50 mM | 75.38 ± 0.5 | 80.02 ± 0.6 |
| Chymostatin | Chymotrypsin | 50 mM | 36.04 ± 0.9 | 25.47 ± 1.2 |

Each value is an average of three replicates ± SE.

**Table 2** Partial purification of *Chilo partellus* gut proteases (CPGPs).

| Fractions | Total Protein (mg) | Total Activity U | Specific Activity (U/mg) | Purification (Fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude CPGPs | 16.5 ± 0.3 | 24.31 ± 0.0 | 1.48 ± 0.1 | 0 | 100 |
| Purified CPGP | 0.62 ± 0.7 | 1.75 ± 0.0 | 2.82 ± 0.1 | 1.9 ± 0.2 | 10.6 ± 0.1 |

Each value is an average of three replicates ± SE.

**Table 3** Substrate specificity of crude and partially purified *Chilo partellus* gut proteases (CPGPs).

| Substrates | Specificity | Crude CPGPs | Purified CPGP |
|---|---|---|---|
| Casein | General | 1.7 ± 0.0 | 3.23 ± 0.4 |
| BApNA | Trypsin | 3.4 ± 0.1 | 2.11± 0.0 |
| SAAPFpNA | Chymotrypsin | 4.9 ± 0.3 | 3.05 ± 0.2 |

Each value is an average of three replicates ± SE.

**Biochemical properties of the CPGPs**

CPGPs showed activity over a broad range of pH from 7 to 10 but were relatively inactive below pH 5.0; maximum activity was observed at pH 9. The proteases were found to be active in the pH range from 9 to 10. The optimum pH for the CPGPs was alkaline, with maximum protein hydrolysis occurring at alkaline pH. The results for the *C. partellus* larvae revealed a major involvement of alkaline proteases in insect protein digestion. These findings confirmed that serine proteases (especially trypsin) are the predominant proteases in the *C. partellus* gut.

**Semi-purification and characterization of *I. batatas* tuber PIs**

Semi-purified *I. batatas* fractions showed trypsin and chymotrypsin-specific activities. The purifications are summarized in Table 4. TIs were detected in the crude and purified fractions from *I. batatas* extracts. The purified and crude TI profiles from tuber plants are shown in Fig. 4. Eight TIs were present in the crude *I. batatas* extracts. Eight inhibitors could be identified after very brief contact between the gel and the X-ray film. The purified 84-kDa inhibitor band exhibited very little activity. Trypsin was used to analyze the GXCP activity. The molecular weight of the *I. batatas* purified inhibitor was determined by sodium dodecyl sulfate (SDS)-PAGE in the absence of a reducing agent.

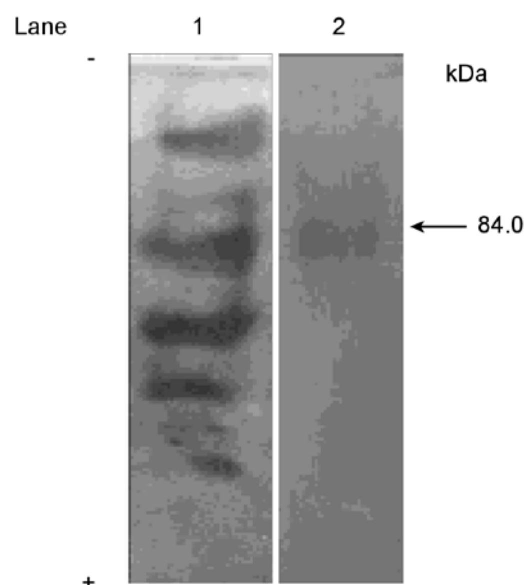


**Figure 4** Scanned X-ray film of a gel used to detect trypsin inhibitors (TIs). Electrophoresis was conducted on a 10% native-PAGE gel. TI activity bands were visualized using a gel X-ray film contact print technique. Lane 1 contains the crude *Ipomoea batatas* extract (25 µg). Lane 2 contains the purified *Ipomoea batatas* extract (25 µg).

**Inhibition of CPGPs by host and non-host PIs from plants**

The CPGP interactions of *Z. mays* host and different non-host tuber plants were analyzed, and *I. batatas* non-host plant PIs exhibited total inhibition. CPGP inhibition was investigated at pH 9 because gut proteases showed activity at pH 9 (Table 5). PIs from the host plant *Z. mays* caused 0% inhibition. At pH 9, *Z. mays* PIs did not inhibit CPGP activity. PIs from *I. batatas* showed complete inhibition of CPGP activity, resulting in inhibition of up to 80 ± 1.21%. Thus, *I. batatas* PIs can inhibit the total protease activity in *C. partellus*.

**Table 4.** Purification of trypsin (TI) and chymotrypsin (CTI) inhibitors from *Ipomoea batata*.

| Purification steps | Total protein (mg) | TI units | CTI units | Specific activity | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 2300 ± 0.2 | 12000 ± 0.1 | 10000 ± 0.1 | 20.3 ± 0.0 | 1.00 ± 0.1 |
| Ammonium sulphate fraction | 890 ± 0.2 | 7000 ± 0.1 | 63000 ± 0.1 | 67.4 ± 0.2 | 3.32 ± 0.3 |
| Sephadex G-75 | 60 ± 0.1 | 2500 ± 0.3 | 21000 ± 0.2 | 412.0 ± 0.1 | 20.29 ± 0.1 |

Each value is an average of three replicates ± SE.

**Table 5** Inhibition potential of non-host plant PIs against gut proteinase activity of *Chiilo partellus*. BApNAase activity assays were performed at pH 9. The maximum possible inhibition of CPGPs respective PIs is shown as follows.

| Plant PIs | Inhibition of CPGPs activity at pH 9 (%) |
|---|---|
| Zea mays | 0 |
| *Daucus carota* tubers extracts | 37 ± 1.0 |
| *Raphanus sativum* tubers extracts | 25 ± 0.8 |
| *Beta vulgaris* tuber extracts | 45 ± 1.0 |
| *Ipomoea batatas* tubers extracts | 80 ± 1.2 |
| *Esculent root* tuber | 25 ± 1.5 |
| *Solanum tuber*s extracts | 50 ± 0.6 |

CPGP: *Chilo partellus* gut proteases.
Each value is an average of three replicates ± SE.

**Effect of *I. batatas* PIs on the development and growth of *C. partellus***

PI bioassays revealed a remarkable reduction in *C. partellus* larval weight when they were fed PIs from *I. batatas* (Table 6). The effects of different parameters on *C. partellus* were recorded, including larval mortality, pupation rate, reduction in pupal weight, malformed pupae, pupal mortality and malformed adults. Larval mortality was observed 10 days after ingestion, and the mortality was 70 ± 0.58% when they were fed *I. batatas* proteases. In contrast, no mortality was recorded in control larvae. The larvae fed PIs from *I. batatas* formed blackish, malformed pupae, whereas the normal pupae were dark brown. The pupation rate was lower in the population fed *I. batatas* PIs than in the control population. The population fed *I. batatas* PIs contained 30 ± 0.67%, malformed pupae. The control pupae exhibited only 10% mortality. *I. batatas* PIs also exerted adverse effects on adult emergence, and after emergence, the adults were found to be malformed.

***In vivo* inhibition of larval CPGPs in larvae reared on PIs**

The CPGP activity was estimated after *C. partellus* larvae were fed *I. batatas* or *Z. mays* PIs for ten days (Table 7). The CPGP activity in control larvae was considered to be 100%, and the gut protease activities of PI-fed larvae were calculated. The larvae fed non-host tuber PIs showed 39% caseinolytic activity. BA*p*NAase activity, which reflects the activities of trypsin-related proteases, was

lowest (29%) in the *I. batatas* PI-fed larvae. Thus, *I. batatas* PIs inhibit proteases and larval growth in *C. partellus*.

**Effect of purified *I. batatas* PIs on the growth and development of *C. partellus***

An artificial diet containing purified *I. batatas* PIs was fed to *C. partellus* insects for two generations. In the 1st generation, two concentrations were used (0.5 × and 1×). In the 2nd generation, four concentrations were used (0.5 ×, 1 ×, 3 × and 6 ×). The effects of the different concentrations on *C. partellus* larval growth were studied (Table 8). In the 1st generation, a 13 to 22% reduction in weight was observed in the larvae fed purified *I. batatas* PIs. Additionally, the pupation of 29 to 45% of *C. partellus* insects was delayed by 4 days. Egg hatching and adults' egg laying were reduced by 35 ± 0.0 and 71 ± 0.25%, respectively. In the 2nd generation, the larval weight reduction was 55 ± 0.12% for *I. batatas* PIs. The effect of the PIs on larval growth was found to be dose dependent, excluding the 0.5 × and 1× purified *I. batatas* PIs, for which no difference was evident. The 3× concentrated *I. batatas* PIs caused a reduction in pupal weight of 57 ± 0.17%. Approximately 70 ± 0.1% larval mortality was observed with the 6× purified *I. batatas* PI diet, whereas 70 ± 0.1 and 19 ± 0.18% larval mortality were observed for the 3× and 1 × purified *I. batatas* PI diets, respectively. Pupal mortality was 9 ± 0.28% for the 3× purified *I. batatas* PIs. Malformed pupae were observed at a rate of 30 ± 0.67% for the 3× purified *I. batatas* PIs. The results revealed that pupa formation was delayed by 4 to 9 days. An increased rate of malformed pupae was also detected in the population fed purified *I. batatas* PIs. Additionally, fewer eggs were laid by female moths compared to the control moths, and egg hatching was dramatically reduced in the groups fed purified *I. batatas* PIs. Increased larval mortality was also evident.

**Table 6** Mortality, malformed pupae and pupation in *Chilo partellus* larvae fed on different host and non host tuber plant PIs.

| Name of fed diet samples | Mortality (%) | Malformed pupae (%) | Pupae (%) |
|---|---|---|---|
| *Zea mays* | 0 ± 0.0 | 20 ± 0.1 | 80 ± 0.2 |
| *Ipomoea batatas* tuber extract | 70 ± 0.5 | 30 ± 0.6 | 0 ± 0.0 |
| *Daucus carota* tubers extract | 40 ± 0.5 | 40 ± 0.4 | 20 ± 0.3 |
| *Raphanus sativum* tubers extract | 50 ± 0.4 | 50 ± 0.2 | 0 ± 0.1 |
| *Beta vulgaris* tubers extracts | 60 ± 0.8 | 30 ± 0.5 | 10 ± 0.4 |
| *Esculent root* tubers extracts | 50 ± 1.0 | 30 ± 1.0 | 20 ± 1.0 |
| *Solanum tuberosum* tubers extract | 50 ± 0.9 | 30 ± 0.8 | 20 ± 0.7 |

Each value is an average of three replicates ± SE.

**Table 7** In vitro stability of host and non-host tuber plant PIs against CPGPs. Inhibitors were incubated with CPGPs for 30min at 37 °C and then assayed for their inhibitory activity toward CPGPs.

| Plant PIs | Inhibition of CPGPs activity after 30min (%) |
|---|---|
| *Zea mays* | 0 |
| *Daucus carota* tubers extracts | 35 ± 0.4 |
| *Raphanus sativum* tubes extract | 22 ± 0.9 |
| *Beta vulgaris* tuber extracts | 42 ± 1.0 |
| *Ipomoea batatas* tubers extracts | 78 ± 1.0 |
| *Esculent root* tuber extract | 24 ± 0.6 |
| *Solanum tuberosum* tubers extract | 41 ± 1.2 |

Each value is an average of three replicates ± SE.

**Table 8** Effect of purified *I. batatas* on the growth and development of *C. partellus* insects.

| Growth and development parameters | *Ipomoea batatas* PIs | | | | *Zea mays* PIs |
|---|---|---|---|---|---|
| | 0.5× | 1× | 3× | 6× | |
| Larval mortality (%) | 12 ± 0.5 | 19 ± 0.1 | 70 ± 0.5 | 70 ± 0.1 | 0 ± 0.0 |
| Larval wt. reduction (%) | 10 ± 1.2 | 15 ± 1.9 | 55 ± 0.1 | 54 ± 0.3 | 0 ± 0.1 |
| Reduction in pupal wt. (%) | 9 ± 0.3 | 20 ± 0.2 | 57 ± 0.1 | 54 ± 1.2 | 0 ± 0.9 |
| Malformed pupae (%) | 4 ± 0.2 | 9 ± 0.8 | 30 ± 0.6 | 31 ± 0.3 | 0 ± 0.0 |
| Pupal mortality (%) | 1 ± 0.1 | 3 ± 0.3 | 9 ± 0.2 | 9 ± 1.0 | 0 ± 0.8 |
| Malformed adult (%) | 2 ± 0.0 | 8 ± 0.2 | 25 ± 0.1 | 24 ± 2.4 | 0 ± 0.4 |

Each value is an average of three replicates ± SE.

## Discussion

In this study, *C. partellus* insects were reared on an artificial diet. Other researchers have investigated pupation time in *C. partellus* (Mailafiya et al., 2011).

According to our results, the protease activity in *C. partellus* gut extracts includes trypsin and chymotrypsin-like activities, and trypsin-like activity was predominant. Proteases have been investigated in extracts of the digestive tracts of insects from many families, particularly Lepidoptera (Broadway, 1989). Research on insect proteases has revealed that trypsin-like activities are commonly observed in insect guts (Baker, 1977) and that chymotrypsin-like activity is sometimes found (Gooding, 1975).

PIs were purified from *I. batatas* tuber extracts. PIs have been reported in many tropical root crops, including taro, giant taro and sweet potato, and in the temperate tuber crop potato (Yan, 1990; Sasi Kiran and Padmaja, 2003). PIs play a regulatory role in plant metabolism, helping to preserve proteins and acting as defenses against insects (Sasi Kiran and Padmaja, 2003). Harsulkar *et al.*, (1999) suggested that the structural similarity between plant PIs and insect gut proteases determines the level of inhibitory activity against the proteases. Indeed, pests and plants frequently develop new forms of enzymes and inhibitors to counteract one another's defense mechanisms (Shimoto and Chrispeels, 1996; Bown et al., 1997). In a stabilized host-pest complex, insects have adapted to overcome the host plant's inhibitors (Broadway, 1995, 1996, 1997; Bolter and Jongsma, 1995; Jongsma *et al.*, 1996). Insect gut proteases neutralize the effects of PIs by degrading them; for example, multicystatin from potato tubers is degraded by *Diabrotica* larval gut proteases (Orr *et al.*, 1994). Other researchers have observed growth retardation and mortality in insect larvae fed inhibitors (Bhavani *et al.*, 2007; Tamhane et al., 2005; Kranthi et al., 2001; Shukla et al., 2005).

This study shows that *I. batatas* PIs are natural agents that control *C. partellus* insects. A feeding bioassay will help to identify the inhibitors responsible for decreasing the activity of *C. partellus* larvae. Our results indicate that *I. batatas* PIs are suitable candidates for the development of insect-resistant transgenic sorghum crops.

## Acknowledgements

This research was financially supported by Department of Biochemistry, Dr. B. A. M. University, Aurangabad -431004 (MS), India.

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# بازدارندگی پروتئاز معده ساقه‌خوار *Chilo partellus* (Lepidoptera: Pyralidae) توسط بازدارنده‌های سیب‌زمینی شیرین *Ipomoea batatas*

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دریافت: ۱۷ اسفند ۱۳۹۴؛ پذیرش: ۶ تیر ۱۳۹۵

**چکیده:** ساقه‌خوار *Chilo partellus* یکی از آفات مخرب سورگوم و ذرت در دنیا می‌باشد. پروتئاز ساقه‌خوار *C. partellus* (CPGPs) جداسازی و خالص‌سازی شد. در این بررسی تأثیر بازدارندگی پروتئازی سیب‌زمینی شیرین روی بازدارندگی آنزیم‌های تریپسین و کیموتریپسین مورد مطالعه قرار گرفت. بازدارنده‌های پروتئازی سیب‌زمینی شیرین فعالیت پروتئازی و رشد ساقه‌خوار را مهار نمود و منجر به کاهش وزن لارو شد. بنابر این یک پروتئاز بازدارنده از سیب‌زمینی شیرین خالص‌سازی شد و به‌عنوان یک آنتاگونیست مؤثر برای CPGPs تشخیص داده شد. در نتیجه بازدارنده پروتئازی شناسایی شده از سیب‌زمینی شیرین می‌تواند برای تولید سورگوم و ذرت‌های مقاوم ترانسژنیک مورد استفاده قرار گیرد.

**واژگان کلیدی:** *Chilo partellus*، CPGPs، پروتئازهای بازدارنده میزبان و غیرمیزبان، BA*p*NA، GXCP