Bowman−Birk Proteinase Inhibitor from *Cajanus cajan* Seeds: Purification, Characterization, and Insecticidal Properties

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A red gram proteinase inhibitor (RgPI) was purified from red gram (*Cajanus cajan*) seeds by using ammonium sulfate precipitation and ion-exchange, affinity, and gel filtration chromatography. SDS-PAGE under nonreducing condition revealed two protein bands with molecular masses of ~8.5 and ~16.5 kDa corresponding to monomeric and dimeric forms of RgPI, respectively. Similarly, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry also confirmed the presence of dimer as well as other oligomeric forms: trimer, tetramer, and pentamer. Reduction of RgPI with dithiothreitol (DTT) led to the dissociation of the dimeric and oligomeric forms. Native-PAGE and two-dimensional gel electrophoresis indicated the existence of isoinhibitors with p*I* values of 5.95, 6.25, 6.50, 6.90, and 7.15, respectively. The MALDI-TOF-TOF mass spectrum and N-terminal sequence 'DQHHSSKACC' suggested that the isolated RgPI is a member of the Bowman−Birk inhibitor family. RgPI exhibited noncompetitive type inhibitory activity against bovine pancreatic trypsin and chymotrypsin, with inhibition constants of 292 and 2265 nM, respectively. It was stable up to a temperature of 80 °C and was active over a wide pH range between 2 and 12. However, reduction with DTT or 2-mercaptoethanol resulted in loss of inhibitory activity against trypsin and chymotrypsin. It also decreased the activity of larval midgut trypsin-like proteinases in *Manduca sexta*. Its insecticidal property was further confirmed by reduction in the growth and development of these larvae, when supplemented in the diet.

KEYWORDS: *Cajanus cajan*; protein purification; self-association; Bowman−Birk inhibitor (BBI); *Manduca sexta*; midgut proteinases

INTRODUCTION

In the majority of the higher plants, seed proteins and in particular enzyme inhibitors exhibit specialized functions in the defense against pathogenic microorganisms and pests (*1*−*3*). The enzyme inhibitors comprise amylase and proteinase inhibitors (PIs) (*4*). Seeds usually accumulate PIs during maturation, and the concentrations of these inhibitors vary between 1 and 10% of total seed proteins (*5*). The PIs are generally small, stable, and abundant proteins showing specificity for serine, cysteine, aspartic, and metallo-proteinases (*6*). Among them, serine-PIs are most common and best characterized (*1*). Serine-PIs comprise several protein families including Kunitz and Bowman−Birk inhibitor (BBI) families, which are abundant in various Leguminosae seeds (*7*). Kunitz-type inhibitors are usually ~20 kDa proteins with four cysteine residues forming two disulfide bridges and possess a single reactive site (mostly against trypsin) (*7*). BBIs are 6−9 kDa proteins with 14 cysteine residues forming seven disulfide bridges and possess two reactive sites (*8*,*9*). The exposed surface loop of the inhibitor adopts a characteristic canonical peptidase inhibitory conformation and binds to the active site of the proteinase, and the resulting noncovalent complex inactivates the proteinase (*6*,*9*). The two heads located at the opposite sides of the molecule inhibit trypsin and chymotrypsin independently or simultaneously (*10*).

Several in vitro and in vivo studies have indicated that the PIs are active on proteinases of larval guts, which in turn impairs digestion and absorption of amino acid, causing retarded larval growth and development (*11*), leading to a decline in the fertility and fecundity of the adult moths (*12*). *Manduca sexta* is a well-known economic pest on the tobacco plants in the southern United States, especially the gulf coast. Larvae are defoliators, which feed on blossoms as well as green fruits, and about 90% of the foliage consumption occurs during the final instar. The PI isolated from *Hyptis suaveolens* (L.) moderately inhibited the trypsin-like gut proteinases of *M. sexta* (*13*). Transgenic plants expressing tomato or potato inhibitor II significantly retarded the larval growth of *M. sexta* (*14*). The present study further evaluates the usage of PI-based strategy in the control of *M. sexta*.

Red gram (pigeonpea) [*Cajanus cajan* (L.) Millsp.] is an important pulse crop in subtropical and semiarid tropical areas of the world including India. The presence of trypsin and

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chymotrypsin inhibitors was reported in the seeds of *C. cajan* (*15−17*). Although PIs from *C. cajan* have been purified before, the family or type of inhibitor to which they belong was not clear. Godbole et al. (*18*) 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. However, BBI-type PIs are known to be small proteins, having molecular masses around 6−9 kDa (*9*). Conversely, Haq and Khan (*19*) purified 14 kDa PI from the PUSA-33 variety of *C. cajan* and suggested that it belongs to the Kunitz inhibitor family on the basis of its N-terminal sequence. Furthermore, Osowole et al. (*20*) showed the presence of 18.2 kDa PI in *C. cajan*, whereas Norioka et al. (*21*) reported the presence of only BBI type of PIs in *C. cajan* based on the gel filtration peaks. To unveil this discrepancy in molecular mass and biochemical properties and to investigate the significance of these PIs against biotic stress, we have purified and characterized PI from *C. cajan* cv. Abhaya (ICP 14770) and examined its insecticidal potential against the lepidopteran pest *M. sexta*.

MATERIALS AND METHODS

Materials. Red gram (*C. cajan*) cv. Abhaya (accession no. ICP 14770) seeds were obtained from International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad, India. Bovine serum albumin (BSA, fraction V), bovine pancreatic trypsin and α-chymotrypsin, and 2-mercaptoethanol (2-ME) were procured from Sisco Research Laboratory (Mumbai, India). DEAE-cellulose, cyanogen bromide-activated-Sepharose 4B, Sephadex G-50, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *n*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), 1,2-cyclohexanedione (CHD), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N*-acetylimidizole (NAI), soybean Kunitz trypsin inhibitor (SKTI), tricine, gelatin, and Coomassie Blue R250 were purchased from Sigma (St. Louis, MO). Immobiline dry strips (pH 3−11 nonlinear, 11 cm), IPG buffer (pH 3−11 nonlinear), dithiothreitol (DTT), and iodoacetamide (IDA) were procured from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Sequence grade modified porcine trypsin was obtained from Promega (Madison, WI). Gypsy moth diet was purchased from M. P. Biomedicals, Inc. (Illkirch, France). All other chemicals and reagents used were of analytical grade.

Purification of Red Gram (*C. cajan*) Proteinase Inhibitor (RgPI). The crude protein extract from mature dry seeds was prepared using 50 mM Tris-HCl, pH 8.0, containing 1% polyvinylpyrrolidone (1:6 w/v) as described in Prasad et al. (*17*). The crude protein extract was subjected to 0−25, 25−60, and 60−90% $(NH_4)_2SO_4$ fractionation. The fraction showing maximum trypsin inhibitory activity (TI activity) was applied onto a DEAE-cellulose column (2.2 × 20 cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed with the above buffer, and bound protein was eluted with a linear gradient of 0.1−1.0 M NaCl in 50 mM Tris-HCl, pH 8.0, at a flow rate of 30 mL/h. The fractions (1 mL) showing TI activity were pooled and loaded onto a trypsin−Sepharose 4B column (1.3 × 15 cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0. After a washing with the above buffer containing 100 mM NaCl, the bound proteins were eluted with 0.01 N HCl at a flow rate of 30 mL/h and neutralized with 2 M Tris base. The fractions (1 mL) with TI activity were pooled, dialyzed against 50 mM Tris-HCl, pH 8.0, and concentrated using Amicon filters (Millipore, 3 kDa cutoff). The partially purified protein was finally subjected to gel filtration chromatography on a Sephadex G-50 column (1.5 × 100 cm), using the above buffer at a flow rate of 15 mL/h. The fractions (1 mL) with TI activity were pooled and concentrated using Amicon filters for further use. Protein content was determined according to the Folin-Ciocalteu method using BSA as a standard (*22*) or by UV absorbance at 280 nm.

Assay of Proteinase Inhibitory Activity. TI activity or chymotrypsin inhibitory activity (CI activity) was determined by using an appropriate volume of crude extract or purified protein that results in 40−60% decrease in the activity of trypsin or chymotrypsin. The assay mixture consisted of inhibitor in assay buffer, 50 mM Tris-HCl containing 20 mM $CaCl_2$, at pH 8.2 for trypsin or at pH 7.8 for chymotrypsin. Trypsin (10 μg) or chymotrypsin (80 μg) was added to the assay mixture and incubated for

15 min at 37 °C. Residual trypsin or chymotrypsin activity was determined after incubation for 45 min at 37 °C with 1 mM BAPNA (*23*) or GLUPHEPA (*24*), respectively. The reaction was terminated with 30% (v/v) acetic acid, and the absorbance at 410 nm was recorded. One inhibitor unit was defined as the amount of inhibitor required to inhibit 50% of the BAPNA or GLUPHEPA hydrolysis by trypsin or chymotrypsin, respectively.

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE (18%) was performed as described by Schägger and von Jagow (*25*). The acrylamide−bisacrylamide mixture consists of 49.5% T and 3% C. The protein molecular mass standards (Bangalore Genei, India) were ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), and insulin (3 kDa). Native-PAGE was performed as described by Laemmli (*26*) excluding SDS in gel, electrode, and sample buffers, respectively. Inhibitor bands against trypsin (TI bands) and chymotrypsin (CI bands) were visualized by performing gelatin-SDS-PAGE (*27*). In two-dimensional (2-D) electrophoresis, isoelectric focusing (IEF) was performed with Immobiline dry strips pH 3−11 (nonlinear) using an Ettan IPG Phor 3 IEF system (GE Healthcare) following the manufacturer's instructions. The second dimension was performed by SDS-PAGE as described above. Proteins were detected by staining with either Coomassie Blue R250 (0.1%) or silver nitrate according to standard procedures.

Mass Spectrometry. The molecular masses of the native RgPI and tryptic digested isoinhibitors were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF, Autoflex II Bruker Daltonics, Bremen, Germany). RgPI (1 μL) mixed with 1 μL of saturated solution of sinapinic acid matrix in 50% ACN and 0.1% TFA in water was spotted on a MALDI target plate, and its mass was analyzed by linear mode in the detection range of 1−66 kDa. For in-gel digestion, the isoinhibitors of RgPI separated on 2-D gel were excised, destained with acetonitrile/25 mM NH_4HCO_3 (1:1), and incubated for 5 min in 100% acetonitrile. After drying, the spots were incubated with 10 mM DTT at 56 °C for 1 h followed by 55 mM IDA at room temperature in the dark for 45 min. Furthermore, after the spots had been washed and dried with 25 mM NH_4HCO_3 and 100% acetonitrile, respectively, they were incubated with 10 ng of trypsin (sequencing grade) at 37 °C overnight. The digested peptides were extracted by adding acetonitrile/trifluoroacetic acid/water (50:1:49) and vortexing the tubes for 5 min. The extracted peptides were mixed with an equal volume of HCCA matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA/CH₃CN, 2:1 ratio), and 2 μL of this mixture was applied onto a MALDI target plate. The peptide mass fingerprint of the isoinhibitors was analyzed by reflectron mode in the detection range of 500−3500 Da. External standards [Bruker's protein calibration standard: Pepmix II containing bradykinin, 757.399 Da; angiotensin-II, 1046.541 Da, and ACTH (18−39), 2465.198 Da] were used for calibration.

N-Terminal Sequencing. RgPI resolved in 2-D gel was electro-blotted onto a PVDF membrane (sequence grade, 0.22 μm from Millipore) using 10 mM CAPS buffer (pH 11.0). The membrane was stained with 0.1% Coomassie Blue R250 in methanol/acetic acid/water (40:1:59) and destained with 50% methanol in water. The isoinhibitor spot with p*I* 5.95 was excised from the membrane and transferred into a 1.5 mL vial and washed three times alternatively with Milli-Q water and 50% methanol in water. The sequence of the N-terminal region was determined by Edman degradation in an Applied Biosystems model 494 Procise protein sequencing system facilitated by Proteomics International Pty Ltd., East Perth, Australia. The sequence was read by analysis software program model 610. All sequences were confirmed by a second operator.

Inhibition Constant (K_i) Determination. The inhibition constant of RgPI against trypsin and chymotrypsin was determined by preincubating the respective enzyme with increasing concentrations of RgPI for 15 min followed by 45 min of incubation at 37 °C with different concentrations (0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mM) of BAPNA or GLUPHEPA, respectively. The K_i value was estimated from the Lineweaver-Burk plot using Sigma Plot 10, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, CA).

Stability Studies. RgPI was incubated at different temperatures between 37 and 100 °C for 30 and 60 min, and the residual TI and CI activities were determined after the samples had cooled to room temperature.

To analyze the effect of pH, RgPI was incubated for 1 h at 37 °C in the following buffers: glycine−HCl (pH 2−3), sodium acetate−acetic acid (pH 4−5), sodium phosphate buffer (pH 6), Tris-HCl (pH 7−9), and glycine−NaOH (pH 10−12) at a final concentration of 50 mM. To analyze the effect of reducing agents, RgPI was incubated with different concentrations of DTT (0.05−1.0 mM) and 2-ME (1−10 mM) for 15 min at 56 °C. In the presence of DTT, the reaction was terminated by adding IDA at twice the amount of each DTT concentration before the residual TI and CI activities were analyzed. All of the incubations were done in triplicate along with appropriate controls.

Circular Dichroism (CD) Spectroscopy. CD spectral measurements were performed at a scan speed of 50 nm/min with three accumulations on a JASCO J-810 spectropolarimeter, equipped with a peltier-type temperature controller and a thermostated cell holder. The entire instrument, including the sample chamber, was constantly flushed with nitrogen gas during the operation. The secondary structure of RgPI at far-UV (195−250 nm) was recorded in a 1 cm path length at a concentration of 0.3 mg/mL in 10 mM phosphate buffer, pH 7.4. Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis. RgPI was incubated at 25, 60, and 90 °C using the peltier (thermostat), and far-UV CD spectra were recorded. Similarly, RgPI was incubated at pH 2.0, 8.0, and 12.0 for 1 h before far-UV CD spectra were recorded. The effect of disulfide bond reduction and alkylation on secondary structure of RgPI was determined by incubating RgPI with 1 mM DTT for 1 h at 56 °C followed by treatment with 2 mM IDA for 45 min in the dark at room temperature.

Chemical Modification of Amino Acid Residues. Arginine residues of RgPI were modified according to the method of Patthy and Smith (*28*) using CHD. RgPI in 50 mM borate buffer, pH 9.0, was incubated with a 15-fold molar excess of CHD, and the reaction tube was flushed with nitrogen and kept at 37 °C for 2 h. The reaction was terminated by the addition of 5% acetic acid. Lysine residues of RgPI were modified using TNBS (*29*). RgPI in 50 mM phosphate buffer, pH 7.6, was incubated with a 10-fold molar excess of TNBS at 40 °C for 2 h. The reaction was stopped by adding 10% SDS followed by 0.2 mL of 1 N HCl. Tyrosine residues of RgPI were modified using NAI (*30*). RgPI in 50 mM Tris-HCl, pH 7.5, was incubated with a 60-fold molar excess of NAI at 37 °C for 2 h. The reaction was terminated by adding excess NAI and dialyzing for 5 h at 4 °C against 50 mM Tris-HCl, pH 7.5. The residual TI and CI activities were estimated as described above. The activity staining for TI and CI bands was done by gelatin-SDS-PAGE (*27*).

Preparation of *M. sexta* Midgut Proteinases. The midgut from fifth-instar larvae of *M. sexta* were dissected, and the gut contents were collected. The gut contents were diluted (1:1) with the assay buffer (50 mM Tris-HCl, pH 8.2, containing 20 mM CaCl₂) and vortexed for 5 min. After centrifugation at 12000 rpm for 20 min at 4 °C, the supernatant was collected and further diluted as required with assay buffer for use.

Effect of RgPI on Midgut Proteinase Activity of *M. sexta*. The effect of RgPI on trypsin-like proteinase activity of midgut extract was measured by using BAPNA as a substrate. RgPI (0.5−4 μg) was incubated with midgut extract in 50 mM Tris-HCl, pH 8.2, containing 20 mM CaCl₂ at 37 °C for 15 min before the addition of BAPNA. After incubation with 1 mM BAPNA for 45 min at 37 °C, the reaction was stopped with 30% acetic acid (v/v), and absorbance at 410 nm was recorded. All of the assays were done in triplicates along with appropriate controls.

Effect of RgPI on Growth and Development of *M. sexta*. Artificial diet was prepared by mixing 2 g of agar powder with 100 mL of boiling water, followed by the addition of 16.6 g of gypsy moth diet. The mixture was allowed to cool to 50 °C, and 24 mg of chloramphenicol suspended in 0.1 mL of ethanol was added to block bacterial growth on the diet. RgPI in two different concentrations, 0.001 and 0.01% (w/w), respectively, was added to the trays along with diet and stored at 4 °C until further use. Seven newly hatched larvae were allowed to grow on the artificial diet with and without RgPI, and the difference in the weight of the larvae was monitored after 5 days.

Statistical Analysis. All of the experiments were carried out at least three times each with three replications, and the mean ± SE was reported. Statistical differences were determined by one-way ANOVA followed by Student−Newman−Keuls method at a significance level of $p < 0.05$ using SigmaStat 3.1 software (Systat Software Inc., San Jose, CA).

RESULTS

Purification of Red Gram Proteinase Inhibitor (RgPI). The 25−60% (NH₄)₂SO₄ fraction, which showed maximum TI activity, was initially loaded onto a DEAE-cellulose column. The proteins eluted into two peaks (**Figure 1A**), and peak I fractions, which showed TI activity, were pooled and further applied onto a trypsin−Sepharose 4B column. The proteins bound to this affinity matrix eluted into a single peak (**Figure 1B**). The active fractions from this affinity column, which showed high molecular mass contaminants in SDS-PAGE, were finally subjected to gel filtration chromatography (**Figure 1C,D**). The proteins eluted into two peaks, and peak II fractions (RgPI), which showed TI activity, were used for further characterization studies. The protocol resulted in 66.5-fold purification of RgPI with a recovery of 55% yield (**Table 1**).

Molecular Mass and Self-Association Behavior of RgPI. The presence of two bands with molecular masses of ∼8.5 and ∼16.5 kDa indicates that RgPI exists in both monomeric and dimeric states (**Figure 1D**). The multimeric nature of RgPI was further evident in the MALDI-TOF mass spectrum, which showed several peaks with molecular masses of 8376.8, 16745.9, 24529.4, 32844.7, and 41193.3 Da, respectively, under native condition (**Figure 2A,B**). In contrast, reduction and alkylation of RgPI with DTT and IDA, respectively, yielded only one peak at 9189.4 Da in the MALDI-TOF mass spectrum (**Figure 2C**). Furthermore, RgPI existed as five different isoinhibitors as shown by native-PAGE (**Figure 3A**) as well as 2-D electrophoresis (**Figure 3B**) with p*I* values of 5.95, 6.25, 6.50, 6.90, and 7.15, respectively.

Mass Spectrometry and N-Terminal Sequencing of RgPI. Tryptic digestion of the five isoinhibitors of RgPI with different p*I* values showed several common peaks (ions) closer to 1638, 1851, 1993, 2150, 2501, and 2705 *m/z* in the MALDI-TOF mass spectrum (Figure 1 of the Supporting Information). Further ionization of all these peaks in MALDI-TOF-TOF and MS/MS ion search in Mascot search engine (www.matrixscience.com) revealed that LIFT spectrum of the peak 1638.8 *m/z* of RgPI isoinhibitor (p*I* of 6.90) matched with BBI-type PI from *Glycine max* (Figures 2 and 3 of the Supporting Information). The N-terminal sequence of amino acid residues of RgPI isoinhibitor (p*I* of 5.95) was as follows: Asp-Gln-His-His-Ser-Ser-Lys-Ala-Cys-Cys (DQHHSSKACC). This sequence showed up to 70% similarity with inhibitors from the BBI family (**Figure 4**).

Inhibitory Activity and K_i Determination. RgPI showed TI and CI activities (**Figure 5A**). However, inhibitory activity against trypsin was higher, compared with chymotrypsin. Trypsin lost 55% of its activity when the molar ratio of RgPI to trypsin was 0.5 and further lost ∼100% of its activity when the molar ratio was increased to 1.0. A linear extrapolation to obtain 100% inhibition indicated that RgPI binds to trypsin at a 1:1 molar ratio. In contrast, RgPI showed no obvious stoichiometry with chymotrypsin (**Figure 5A**). A Lineweaver−Burk plot showed noncompetitive type of inhibition against both trypsin ($K_i = 292$ nM) and chymotrypsin ($K_i = 2265$ nM) (**Figure 5B,C**).

Temperature and pH Stability of RgPI. TI and CI activities of RgPI were stable even after heating to 100 °C for 30 min. However, marginal losses in TI and CI activities (10−20%) were observed after 60 min of heating at 90 and 100 °C (**Figure 6A**). Increasing the temperature from ambient to 90 °C resulted in marginal changes in the far-UV CD spectra of RgPI (**Figure 6B**). However, on cooling to 25 °C, the temperature-induced conformational changes in RgPI were reversed. The TI and CI activities of RgPI were stable at different pH values tested between 2 and 12, with marginal loss (10%) in inhibitory activity at pH 6.0 (**Figure 6C**). The far-UV CD spectral studies displayed structural
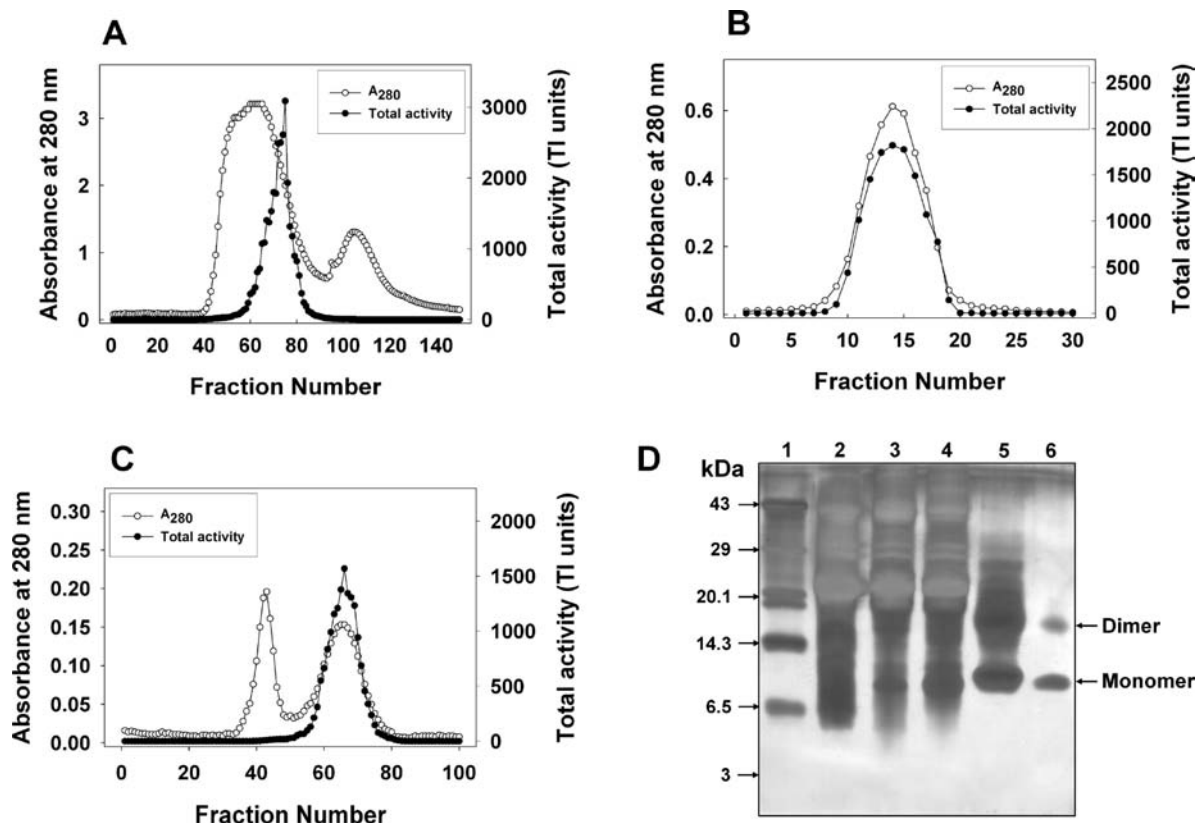
Figure 1. (**A**) Elution profile of ion-exchange (DEAE-cellulose) column loaded with 25−60% (NH$_4$)$_2$SO$_4$ fraction. (**B**) Elution profile of affinity (trypsin−Sepharose 4B) column loaded with peak I fraction pool of the ion-exchange column. (**C**) Elution profile of gel filtration (Sephadex G-50) column loaded with active fraction pool of the affinity column. Peak II fractions from gel filtration column, which showed TI activity, were pooled and named RgPI. (**D**) SDS-PAGE (18%) under nonreducing condition showing different fractions of the purification: lane 1, molecular mass standards; lane 2, crude extract; lane 3, 25−60% (NH$_4$)$_2$SO$_4$ fraction; lane 4, peak I fraction of ion-exchange column; lane 5, affinity column; lane 6, peak II fractions of gel filtration column.

Table 1. Purification of Proteinase Inhibitor from Red Gram (RgPI) Seeds

purification step	total protein (mg)	total activity (TI units)[a]	yield recovery (%)	specific activity[b] (TI units/mg of protein)	purification (fold)
crude extract	11426	217962	100	19	1
(NH$_4$)$_2$SO$_4$ fraction (25−80%)	3548	144773	66	41	2.16
DEAE-cellulose column	1933	134052	62	69	3.63
trypsin−Sepharose 4B column	118	126430	58	1071	56.37
Sephadex G-50 column	95	120118	55	1264	66.52

[a] One TI unit is defined as the amount of RgPI required to inhibit 50% of BAPNA hydrolysis by trypsin. [b] Specific activity is defined as the number of TI units/mg of protein.
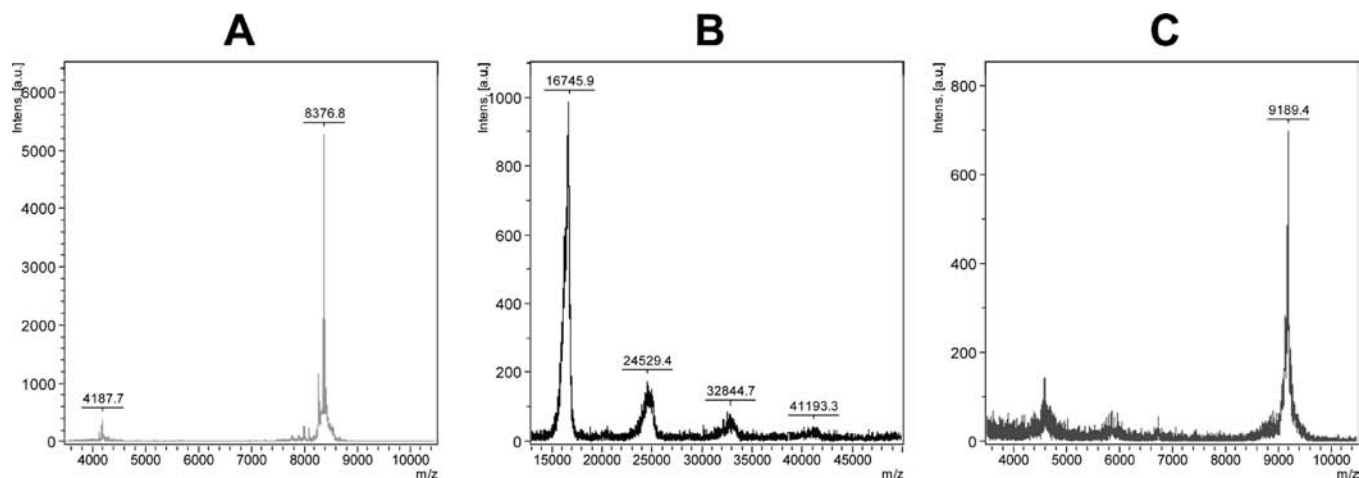
Figure 2. MALDI-TOF mass spectrum of RgPI in native condition up to 10000 m/z (**A**) and 50000 m/z (**B**) showing the oligomeric peaks. (**C**) MALDI-TOF mass spectrum of RgPI after reduction and alkylation with DTT and IDA.

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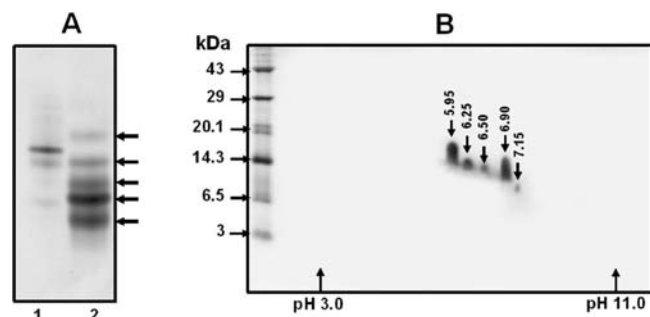
Figure 3. (**A**) Isoinhibitors of RgPI separated on native-PAGE (12.5%). The samples in lanes 1 and 2 are soybean BBI and RgPI, respectively. (**B**) Two-dimensional gel showing the isoinhibitors of RgPI. The proteins were separated in IEF using 3–11 pH nonlinear strips having maximum resolution at pH 4–7. The second dimension was performed in SDS-PAGE (18%).

Figure 4. Comparison of N-terminal sequence of the RgPI isoinhibitor (p*I* of 5.95) with known following BBIs from NCBI database using ClustalW2 alignment. CmTI$_2$, *Cratylia mollis* BBI (*43*); CLTI-II, *Canavalia lineata* BBI (*37*); TBPI, *Phaseolus acutifolius* BBI (*41*); PcBBI$_1$, *Phaseolus coccineus* BBI (*46*); KBPI, *Phaseolus vulgaris* BBI (*36*); SBTI, *Glycine max* BBI (*35*); DE-3 and DE-4, *Macrotyloma axillare* BBI (*32*); AB-I, *Vigna angularis* BBI (*34*); HGI-3, *Dolichos biflorus* BBI (*38*); MBTI-F, *Vigna radiata* BBI (*33*). Identical amino acid residues are in shaded blocks.

changes on both the acidic (pH 2.0) and basic sides (pH 12.0) (**Figure 6D**).

Effect of DTT and 2-ME on RgPI. Incubation of RgPI with 0.25 mM DTT resulted in significant decrease in the TI activity (90%) and CI activity (80%). With further increase of the concentration of DTT to 1.0 mM, complete loss in TI and CI activities was observed (**Figure 7A**). In contrast, incubation of RgPI with 2-ME resulted in a gradual decrease in TI and CI activities (**Figure 7B**). At 1.0 mM 2-ME, there was a negligible loss in TI and CI activity. Furthermore, when the concentration of 2-ME was increased to 10 mM, TI activity (74%) and CI activity (48%) were decreased significantly. Nevertheless, the loss in inhibitory activity was more pronounced with DTT than with 2-ME, which was also evident from activity gels in gelatin-SDS-PAGE (**Figure 7C**). RgPI existed as both monomeric and dimeric active forms in nonreducing condition, and reduction with 2-ME showed the loss of dimer formation by retaining the monomer activity. However, on reduction with DTT complete loss of monomer and dimer activities was observed. Reduction of RgPI with DTT also showed the decline in ellipticity at 203 nm associated with marginal changes in its overall structure. However, alkylation of reduced RgPI with IDA led to further decrease in ellipticity at 203 nm followed by complete loss in structural conformation (**Figure 7D**).
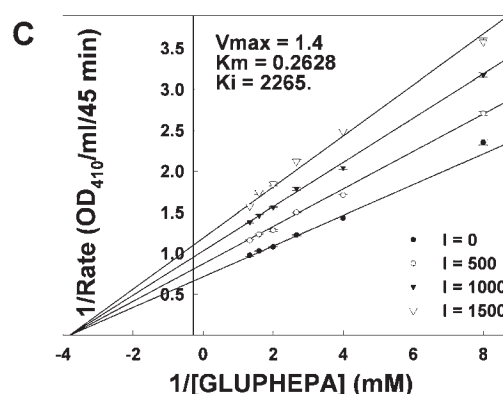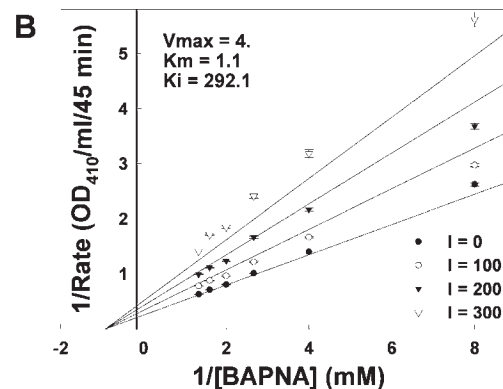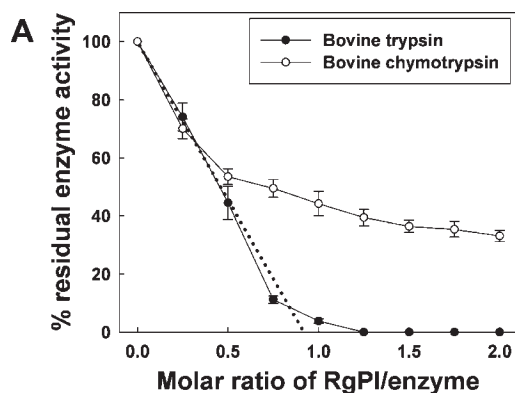

Figure 5. Titration curves of trypsin and chymotrypsin inhibition by RgPI. Increasing concentrations of RgPI were added to a fixed concentration of enzyme (1 μM). The residual trypsin or chymotrypsin activity was determined as described under Materials and Methods. The molar ratio of the inhibitor to the trypsin or chymotrypsin was the intercept of the *x*-coordinate, when the tangent was extrapolated to zero activity (*31*). (**B**) Lineweaver–Burk plot showing noncompetitive nature of trypsin inhibition by RgPI at various concentrations of BAPNA (0.125, 0.250, 0.375, 0.500, 0.625, and 0.750 mM). (**C**) Lineweaver–Burk plot showing noncompetitive nature of chymotrypsin inhibition by RgPI at various concentrations of GLUPHEPA (0.125, 0.250, 0.375, 0.500, 0.625, and 0.750 mM). Each value is the mean ± SE of three independent assays with three replicates.

Effect of Chemical Modification of Amino Acids on Inhibitory Activity of RgPI. Modification of the guanidium group of arginine residues or the phenolic group of tyrosine residues of RgPI had not shown any effect on TI or CI activity (**Figure 8A**), also evident from gelatin-SDS-PAGE, which showed activity against trypsin and chymotrypsin without any loss in dimer formation (**Figure 8B,C**). The modification of lysine residues of RgPI resulted in complete loss of TI activity and partial loss in CI activity (**Figure 8A**). RgPI modified for lysine residues showed complete loss in dimer activity and marginal loss in monomer

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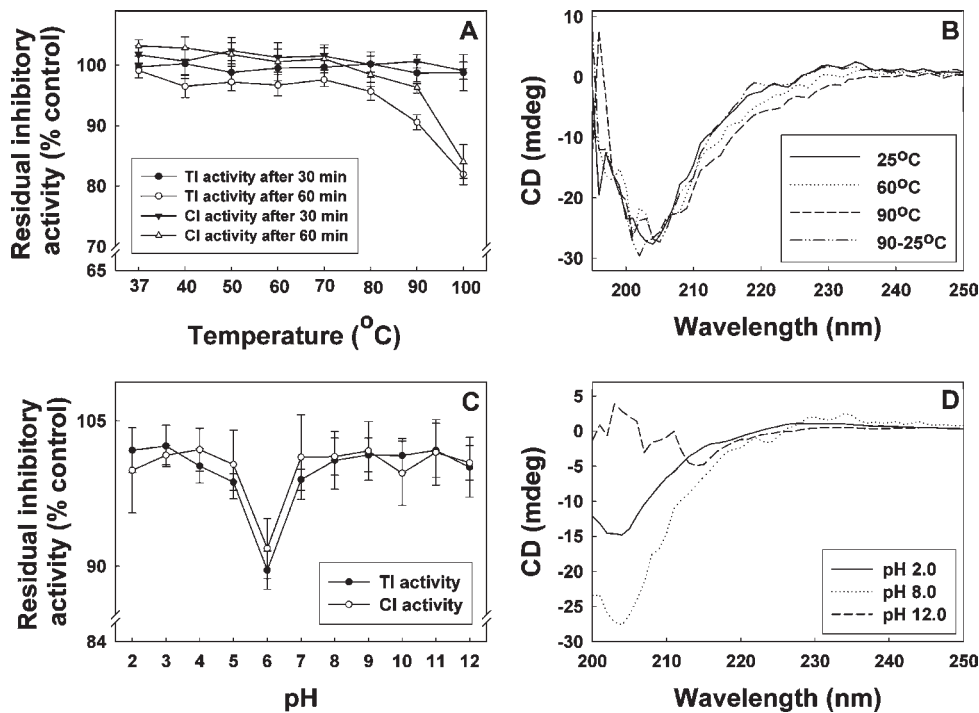
Figure 6. (**A**) Temperature stability of RgPI after incubation for 30 and 60 min at the indicated temperatures. (**B**) Effect of temperature on far-UV CD spectra of RgPI. The required temperature (°C) was induced by using the thermostat (peltier, Jasco). (**C**) Stability of RgPI after incubation at various pH values ranging from 2 to 12 for 1 h. (**D**) Effect of pH on the far-UV CD spectra of RgPI. The RgPI was incubated at the respective pH for 1 h before the spectra were taken. The residual inhibitory activity of RgPI against trypsin and chymotrypsin was determined as described under Materials and Methods. Each value is the mean $\pm$ SE of three independent assays, each with three replicates.
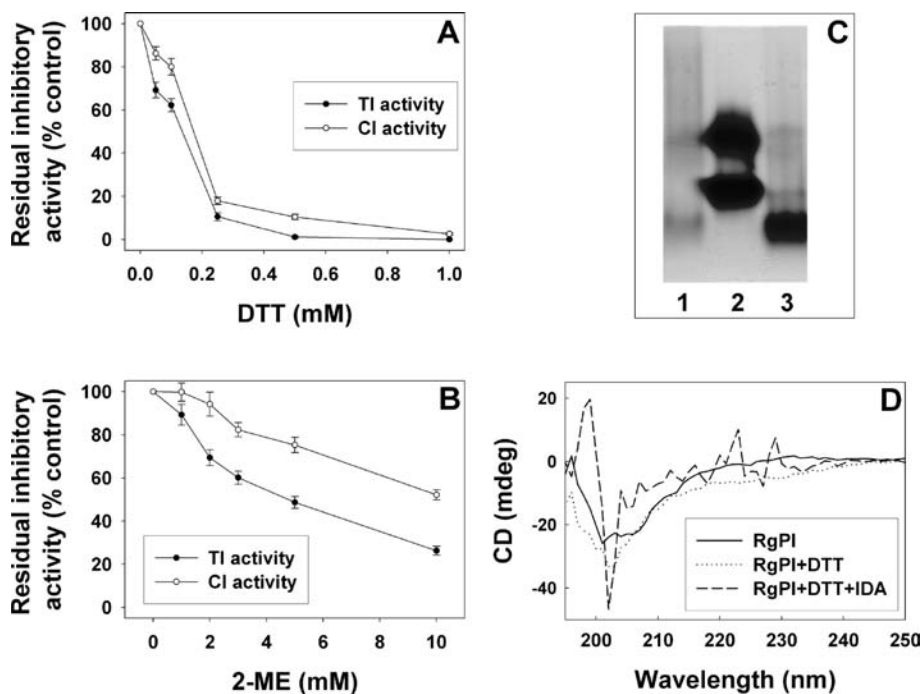
Figure 7. Effect of DTT (**A**) and 2-ME (**B**) reduction on trypsin and chymotrypsin inhibitory activity of RgPI. The residual inhibitory activity of RgPI against trypsin and chymotrypsin was determined as described under Materials and Methods. Each value is the mean $\pm$ SE of three independent assays with three replicates. (**C**) Gelatin-SDS-PAGE (12.5%) showing the trypsin inhibitory activity of reduced RgPI: lanes 1 and 3, RgPI treated with DTT and 2-ME, respectively; lane 2, nonreduced RgPI. (**D**) Far-UV CD spectra of native RgPI and reduced RgPI with DTT (1 mM) followed by alkylation with 2 mM IDA. Other details were as described under Materials and Methods.

activity when incubated with trypsin (**Figure 8B**). However, when incubated with chymotrypsin, loss in dimer activity was observed, whereas monomer activity was retained (**Figure 8C**).

Effect of RgPI on Midgut Trypsin-like Proteinases, Growth, and Larval Development of *M. sexta*. RgPI showed significant reduc- tion in the activity of midgut trypsin-like proteinases with
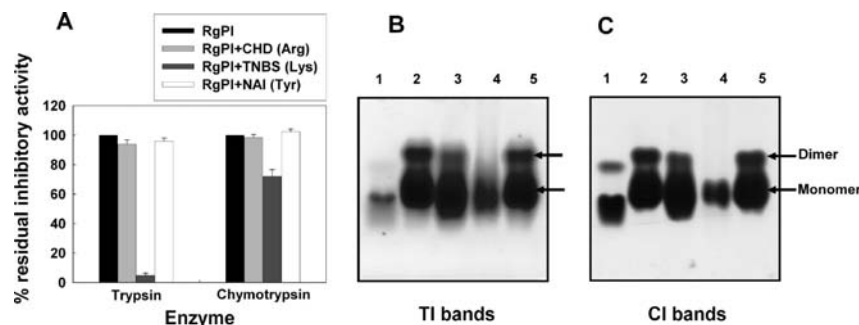
Figure 8. (**A**) Effect of amino acid residue modification on inhibitory activity of RgPI against trypsin and chymotrypsin. The residual inhibitory activity was determined as described under Materials and Methods. Each bar is the mean ± SE of three independent assays with three replicates. Gelatin-SDS-PAGE (12.5%) showing the TI bands (**B**) and CI bands (**C**) of control and modified RgPI using amino acid specific modifying agents: lane 1, loaded with soybean BBI; lanes 2–5, loaded with control RgPI and RgPI treated with CHD (Arg), TNBS (Lys), and NAI (Tyr), respectively. The arrows indicate monomer and dimer forms of RgPI.
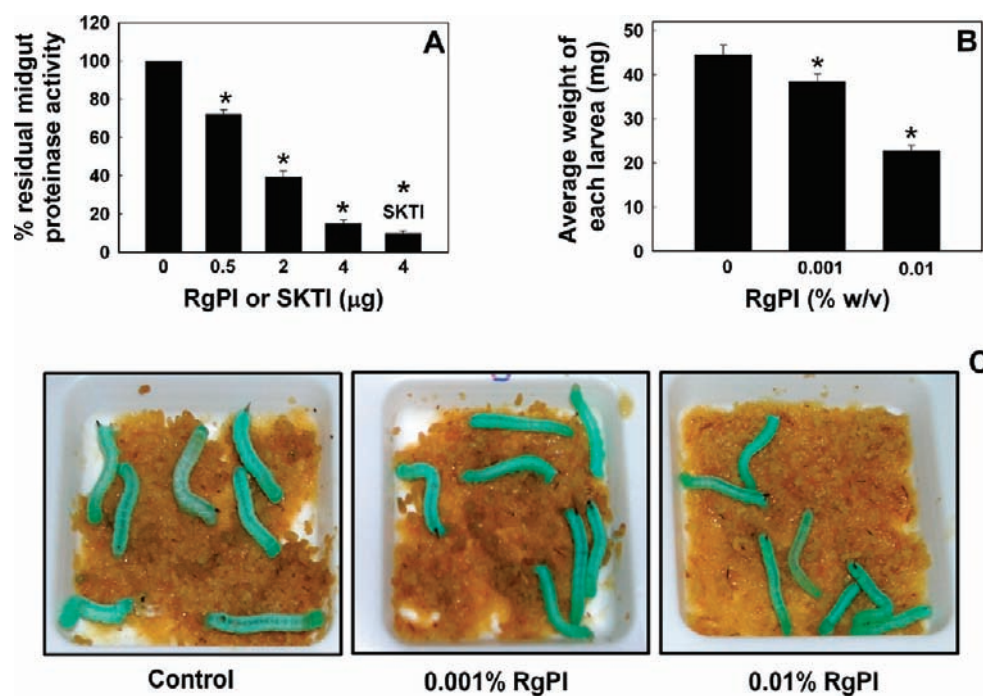
Figure 9. (**A**) Effect of RgPI on the activity of larval midgut trypsin-like proteinases of *M. sexta*. Residual activity of the trypsin-like proteinases was examined in the presence of BAPNA as described under Materials and Methods. Each bar represents the mean ± SE of three independent sets of experiment each with three replicates. (**B**) Growth and development of newly hatched larvae of *M. sexta* reared on artificial diet containing RgPI. Each vertical bar represents the average weight of seven larvae after 5 days, grown on different concentrations of RgPI in the diet. The experiment was repeated five times using different batches of purified RgPI and different sets of newly hatched larvae. (**C**) Photograph of newly hatched larvae of *M. sexta* showing stunted growth with increasing concentration of RgPI in the artificial diet. Asterisk indicates treatments that were statistically significant from controls (Student–Newman–Keuls method, $p < 0.05$).

increasing concentrations, in the presence of trypsin-specific substrate BAPNA (**Figure 9A**). At 4 μg of RgPI, there was 85% reduction in trypsin-like proteinase activity, and the effect was comparable to the well-known SKTI. After 5 days of feeding the diet containing 0.001 and 0.01% RgPI, the body weight of the newly hatched larvae decreased by 15 and 50% respectively, compared with controls (**Figure 9B**). The larvae fed on the diet supplemented with RgPI showed stunted growth, as compared to larvae fed on the control diet (**Figure 9C**).

DISCUSSION

Serine PIs, particularly of the BBI family, have been purified and characterized from a variety of plant sources (*32–49*). The role of PIs in combating insect pests is well established. On this

basis, several research groups have purified PIs from different leguminosae members and examined their in vitro effect against digestive enzymes and in vivo effect toward growth and development of lepidopteran insect pests (*11–14*). The present study reveals the biochemical properties exhibited by RgPI and their insecticidal potential against *M. sexta*.

The protocol used in the present study resulted in higher fold purification of RgPI than PI purified from PUSA-33 variety of *C. cajan* (*19*). The existence of multimeric forms and a monomer molecular mass of 8.5 kDa are the characteristic features of BBI-type PIs, and results from the present study indicate that RgPI belongs to the BBI family. BBIs are well-known to undergo self-association in solution to form homodimers or trimers or more complex oligomers (*40*), which results in overestimation of

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molecular mass in SDS-PAGE and gel filtration chromatography for several legume BBIs (*18*, *36*, *38*). The presence of hydrophobic surface patches in the monomer and a strong hydrogen-bonded network in the dimer structure are responsible for the existence of BBIs in higher ordered multimeric conformations (*47*). Kumar et al. (*42*) reported that Lys[24] (P$_1$ residue) at the amino terminal of one monomer and Asp[76] at the carboxy terminal of another monomer are responsible for self-association and the formation of dimers. However, reduction and alkylation of RgPI resulted in the disappearance of oligomeric peaks (**Figure 2C**), indicating that native structural conformation is essential for self-association of RgPI. Furthermore, the increase in mass by 812.8 Da demonstrates that RgPI possesses 14 cysteine residues (for each cysteine residue 57 Da mass was increased), and they participate in the formation of seven disulfide bonds (*8*). Similar findings were observed in BBIs from *Cratylia mollis* (*43*), *Dolichos biflorus* (*10*), *Glycine max* (*35*), *Lupinus albus* (*48*), *Medicago scutellata* (*40*), *Phaseolus acutifolius* (*41*), and *Phaseolus coccineus* (*46*).

The reason for the existence of several isoinhibitors of PIs in the same plant and their physiological roles is still not very clear. BBIs are the products of multigene families, and the derivation of multiple isoforms has been associated with protein processing at both the amino and carboxylic ends (*50*). Harsulkar et al. (*51*) proposed that in a coevolving system, plants and insects had evolved with new forms of PIs and proteinases to combat against each other's defense mechanism. Hence, it is not surprising that the plants are capable of producing numerous isoinhibitors. The BBIs isolated from various leguminous plants such as *C. cajan* (*17*), *C. mollis* (*43*), *D. biflorus* (*38*), *Inga umbratica* (*45*), *Lens culinaris* (*44*), *P. acutifolius* (*41*), *Phaseolus vulgaris* (*36*), *Pisum sativum* (*50*), *Vigna radiata* (*33*), and *Vigna unguiculata* (*47*) also revealed the presence of isoinhibitors. Despite their pronounced microheterogeneity, the isoinhibitors share a relatively high degree of sequence homology, especially in the inhibitory domains as well as in highly conserved disulfide bridge network (*44*). Hence, the mass spectrum of tryptic digested isoinhibitors of RgPI showed the presence of peaks with identical masses. The N-terminal sequence, DQHHSSKACC, showed 70% similarity with BBI from *C. mollis* (*43*) and 60% similarity with BBIs from *Canavalia lineata* (*37*), *P. acutifolius* (*41*), *P. coccineus* (*46*), and *P. vulgaris* (*36*). These results further confirm that RgPI belongs to the BBI family.

Stoichiometric studies showing a 1:1 molar ratio with trypsin concorded well with previously reported BBIs (*39*, *52*), and the stoichiometric pattern of chymotrypsin agrees with BBI isolated from *Apios americana* tubers (*49*). The RgPI showed noncompetitive type of inhibition against both trypsin (K_i = 292 nM) and chymotrypsin (K_i = 2265 nM). Generally, the BBIs from different Leguminosae plants showed K_i values for trypsin in the range between 0.1 and 5 nM (*43*, *44*, *48*, *49*), except a few in which K_i values was slightly higher, that is, 0.21–52 μM (*39*, *52*). The lower K_i value observed for trypsin compared with chymotrypsin (7.75-fold) could be due to the differential rates of binding of RgPI to the enzymes (*19*), and this difference resembled well with the BBI (13.4-fold) isolated from *L. culinaris* (*44*). Despite having a higher K_i value for trypsin, RgPI showed significant inhibitory activity against midgut trypsin-like proteinases of *M. sexta*.

The presence of many cysteine residues that form disulfide bonds may account for the striking stability in structure and activity of BBI-type PIs in the presence of various denaturants such as temperature, pH, and reducing agents. The reversal of temperature-induced conformational changes suggests that RgPI exhibited some degree of flexibility and retained its structural conformation as well as activity after thermal stress. The RgPI

showed the predominance of β-sheets (68%) and random coil (25%) over α-helix (11%). Similar reports were observed for BBIs isolated from *G. max* (*53*), *I. umbratica* (*45*), and *V. unguiculata* (*47*). Whereas the marginal loss in inhibitory activity at pH 6.0 could be due to isoelectric precipitation, the structural changes observed at acidic pH could be attributed to the loss of native confirmation caused by electrostatic repulsion. In fact, the observed stability in activity over a wide pH range might be possible due to reversal of active structural conformation in assay buffer at pH 7.8 and 8.2. The results obtained in the present study corroborate well with the properties of BBIs isolated from *D. biflorus* (*10*), *C. mollis* (*43*), *L. albus* (*48*), and *P. coccineus* (*46*). We further evaluated the importance of disulfide bonds for maintaining the inhibitory activity of RgPI. The loss of inhibitory activity in the presence of reducing agents could be due to the loss in conformation of the reactive site loop (nine residues), which is formed by disulfide bonds and responsible for maintaining its activity (*8*). Reduction of RgPI with 2-ME led to the more pronounced loss of inhibitory activity against trypsin compared with chymotrypsin, and this could be due to the existence of more disulfide bonds near the trypsin inhibitory site than chymotrypsin inhibitory site (*10*). RgPI was more susceptible to DTT reduction, similar to PI purified from *C. cajan* variety PUSA-33 (*19*). However, the BBI from *D. biflorus* was stable even after reduction with 1.0 mM DTT (*54*). Thus, it appears that the stability of some of the inhibitors is apparently unrelated to the presence of disulfide bonds.

Chemical modification studies were performed to elucidate the amino acids responsible for inhibitory activity against trypsin and chymotrypsin. The loss of inhibitory activity against trypsin could be due to the presence of lysine at the P$_1$ position in the trypsin inhibitory reactive site of the RgPI, which is a characteristic feature of BBI-type PIs (*8*). Lysine modification also resulted in the complete loss of dimer activity and marginal loss of monomer activity against trypsin. Similar results were observed in BBI isolated from *D. biflorus* (*42*), which suggests that lysine residues play an important role in self-association of monomer. The partial loss of inhibitory activity against chymotrypsin emphasizes the role of lysine even in the chymotrypsin reactive site of RgPI.

Serine peptidases are the enzymes responsible for most of the proteolytic activity in the lepidopteran gut with pH optima between 9 and 11. Among the serine peptidases, trypsin and chymotrypsin are the major digestive enzymes (*55*). In the present study, we examined the in vitro effect of RgPI on the activity of midgut trypsin-like proteinases from *M. sexta*. The significant inhibitory activity of RgPI could be because RgPI is a nonhost PI to *M. sexta*. In general, during host−insect interaction, insects develop inhibitor-resistant or inhibitor-degrading gut proteinases against host PIs (*51*, *56*). In such instances, it would be advantageous to screen the nonhost PIs over host PIs to combat insect pests. We further investigated the in vivo effect on the growth and development of *M. sexta* larvae by feeding experiments. Feeding of the newly hatched larvae on 0.01% RgPI in the diet resulted in a 50% decrease of their body weight. The RgPI showed moderate effect on the growth and development of *M. sexta* when compared to strong inhibitory activity against gut trypsin-like proteinases. These results suggest that the trypsin-like gut proteinases are not solely responsible for the growth and development of *M. sexta*.

ABBREVIATIONS USED

BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; BBI, Bowman−Birk inhibitor; BSA, bovine serum albumin; CD,

circular dichroism; CHD, 1,2-cyclohexanedione; DTT, dithiothreitol; GLUPHEPA, *n*-glutaryl-ʟ-phenylalanine-*p*-nitroanilide; IDA, iodoacetamide; IEF, isoelectric focusing; K_i, inhibition constant; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; 2-ME, 2-mercaptoethanol; NAI, *N*-acetylimidizole; PI(s), proteinase inhibitor(s); RgPI, red gram proteinase inhibitor; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SKTI, soybean Kunitz trypsin inhibitor; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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