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Bitter gourd proteinase inhibitors: potential growth inhibitors of *Helicoverpa armigera* and *Spodoptera litura*

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Abstract

Proteinase inhibitors (PIs) from the seeds of bitter gourd (*Momordica charantia* L.) were identified as strong inhibitors of *Helicoverpa armigera* gut proteinases (HGP). Biochemical investigations showed that bitter gourd PIs (BGPIs) inhibited more than 80% HGP activity. Electrophoretic analysis revealed the presence of two major proteins (BGPI-1 and-2) and two minor proteins (BGPI-3 and-4) having inhibitory activity against both trypsin and HGP. The major isoforms BGPI-1 and BGPI-2 have molecular mass of 3.5 and 3.0 kDa, respectively. BGPIs inhibited HGP activity of larvae fed on different host plants, on artificial diet with or without added PIs and proteinases excreted in fecal matter. Degradation of BGPI-1 by HGP showed direct correlation with accumulation of BGPI-2-like peptide, which remained stable and active against high concentrations of HGP up to 3 h. Chemical inhibitors of serine proteinases offered partial protection to BGPI-1 from degradation by HGP, suggesting that trypsin and chymotrypsin like proteinases are involved in degradation of BGPI-1. In larval feeding studies, BGPIs were found to retard growth and development of two lepidopteran pests namely *Helicoverpa armigera* and *Spodoptera litura*. This is the first report showing that BGPIs mediated inhibition of insect gut proteinases directly affects fertility and fecundity of both *H. armigera* and *S. litura*. The results advocate use of BGPIs to introduce insect resistance in otherwise susceptible plants.

Keywords: *Momordica charantia* L; Cucurbitaceae; Bitter gourd; Proteinase inhibitors; *Helicoverpa armigera*; *Spodoptera litura*

1. Introduction

Among various biotic stresses that plants face, insect attack has been a major challenge leading to severe losses in crop yields. Use of chemical pesticides has been the main insect controlling measure during recent decades. Due to indiscriminate use of pesticides and owing to the process of co-evolution and adaptation of insects, the pesticides are proving to be inadequate and ineffective (Lewis et al., 1997). Among the various approaches that are available today, the major focus is on developing pest-resistant transgenic plants (Jouanin et al., 1998; Schuler et al., 1998; De Leo and Gallerini, 2002). Biotechnology has opened an avenue where candidate protein from virtually any system can be expressed in desired plant tissue in a time specific manner (Boulter, 1993; Christeller et al., 2002). Hence, it is important to select appropriate proteins for expression that will strengthen the plant defense in a sustainable manner against the target pest (Giri and Kachole, 1998; Gurden et al., 1998; Jouanin et al., 1998; Harsulkar et al., 1999). One recent strategy that has been developed for insect control is the transfer of *Bacillus thuringiensis* (Bt) insecticidal crystal protein gene in plants like cotton, tomato, maize and potato (Barton et al., 1987;

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Schuler et al., 1998; Frutos et al., 1999; Kota et al., 1999). However, the reports of failure of this system (Forcada et al., 1996; Keller et al., 1996; Oppert et al., 1997) have prompted researchers to seek alternatives such as the enzyme inhibitors, lectins and secondary metabolites (Shade et al., 1994; Schroeder et al., 1995; Duffey and Stout, 1996; Birch et al., 1999; Foissac et al., 2000).

Proteinase inhibitors (PIs) have been established as plant defense agents against insect and microbial pests (Ryan, 1990; Boulter, 1993). Almost all plants are known to accumulate PIs in their storage organs in amounts much more than required to inhibit endogenous proteinases. This is apparent by their specific inhibitory activity towards pest proteinases and little if any against endogenous plant proteinases. (Lawrence and Koundal, 2002). Furthermore, it has been demonstrated that wounding of plant tissue by insect chewing or by microbial infection significantly enhances the level of PIs in local as well as in remote tissues (Green and Ryan, 1972; Schaller and Ryan, 1995). Among different types of inhibitors, serine PIs are extensively studied because they are ubiquitous in plants and most Lepidopteran pests like *Helicoverpa armigera* and *Spodoptera litura* largely depend on serine proteinases for digestion of food proteins. (Bown et al., 1997)

Our interest has been to identify effective inhibitors of insect gut proteinases, from different plant sources, that might prove alternative or complimentary to Bt strategy. Earlier we have demonstrated the potential of winged bean proteinase inhibitors in controlling *H. armigera* feeding on various crops like chickpea, cotton and okra (Harsulkar et al., 1999). In the present paper, we have shown that seeds of bitter gourd (*Momordica charantia* L.) contain small peptides possessing strong inhibitory activity against serine proteinases such as trypsin and elastase. Biochemical characterization of bitter gourd PIs (BGPIs) has revealed that they are squash-type serine PIs that are the smallest known polypeptides with inhibitory activity (Hara et al., 1989; Miura and Funatsu, 1995; Kamei et al., 2000; Leluk, 2000). These PIs are cysteine rich and have three disulfide bridges, which impart high stability and conformational rigidity (Ling et al., 1993). Although, biological functions of these PIs are still unclear, it is suggested that they are defense molecules against pests and also act as plant-growth stimulatory factors (Kamei et al., 2000). Owing to their small size, squash family PIs have proved to be ideal models for studying interaction with their target proteinases (Hara et al., 1989; Ling et al., 1993; Hernanadez et al., 2000; Kamei et al., 2000). In the present work, we have studied the potential of BGPIs against gut proteinases of *H. armigera*, and their growth inhibitory effects on *H. armigera and S. litura*, which are devastating pests of several crop plants.

2. Results

2.1. Inhibition of HGP and trypsin by bitter gourd PIs

Inhibition of trypsin and HGP by BGPIs was studied by solution assays (Fig. 1). Low concentration of seed extract containing BGPIs (2.3 μg) inhibited up to 22% of HGP activity, for equivalent inhibition of trypsin nearly five times higher amount of extract (11.3 μg) was required. On titration, BGPIs could inhibit 100% of trypsin activity and maximum up to 84% of HGP activity, clearly indicating that 16% HGP activity was insensitive to the bitter gourd-inhibitors.

Electrophoretic profiles of trypsin inhibitors and HGPIs of bitter gourd were identical, suggesting that the trypsin inhibitor proteins are also inhibitors of HGP (Fig. 2). At least four trypsin inhibitors/HGPI proteins were detected; of which two slow-migrating proteins showed major activity (BGPI-1 and-2) and others had minor activity (BGPI-3 and -4). Although, these four PI proteins were well separated on native gel, a single broad band of approximately 4 kDa was observed on SDS–PAGE, suggesting that these proteinase inhibitors were charge variants (figure not shown). Analysis of four cultivars of bitter gourd for HGP inhibition in enzyme assays and proteinase inhibitors profiles on native electrophoretic gels revealed similarity. Bitter gourd variety White long was used for all further studies.

2.2. BGPIs differentially inhibit HGP activity of larvae fed on different diets

The inhibition by BGPIs, of HGPs from larvae grown on various host plants was investigated. About 2.3 mg protein of seed extract containing BGPIs that showed 84% inhibition of control–HGP activity and 100% inhibition of trypsin was used for this assay. At this concentration, 100% inhibition of HGP activity was observed in both cotton-field collected larvae and larvae grown on cotton bolls in the laboratory. On the other hand, less inhibition of HGP activity was seen in case of larvae grown on other host plants and those fed on artificial diet with added non-host PIs. For example, BGPIs inhibited 76% pigeonpea–HGP and 82% chickpea–HGP, however, showed only 54 and 62% inhibition of HGP from larvae fed on diets containing PIs of groundnut and winged bean, respectively (Table 1). Our earlier studies indicated significant proteinase activity in fecal matter of *H. armigera* (Giri et al., 1998) and differential expression of proteinases during various larval instars (Patankar et al., 2001). Evaluation of BGPIs towards HGP during various larval instars demonstrated that they could inhibit more than 82% of proteinase activity of 3rd, 4th and 5th instar larvae and inhibited up to 75% of the fecal matter proteinase activity.

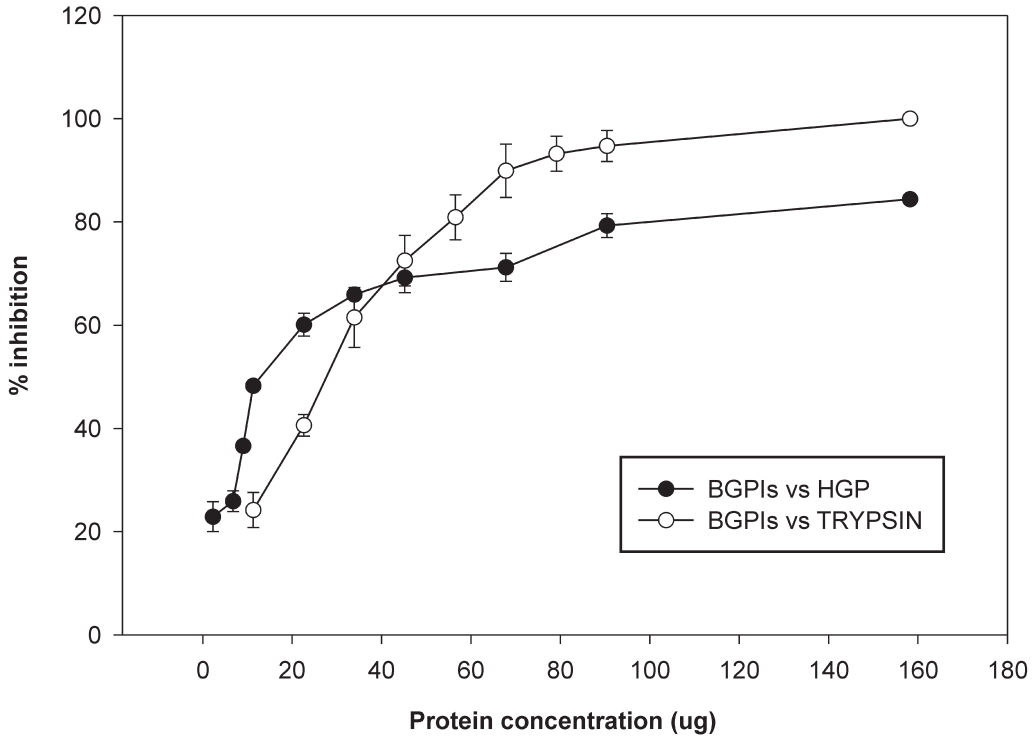

Fig. 1. Comparative inhibition of HGP and trypsin by BGPIs. Inhibition assays were conducted using Benzoyl-arginyl p-nitroanilide as a substrate. BGPIs were titrated against HGP and Trypsin. HGP was obtained from larvae fed on the control diet. The experiment was repeated at least three times. Pattern of inhibition curve obtained was similar. Standard error bars are shown.

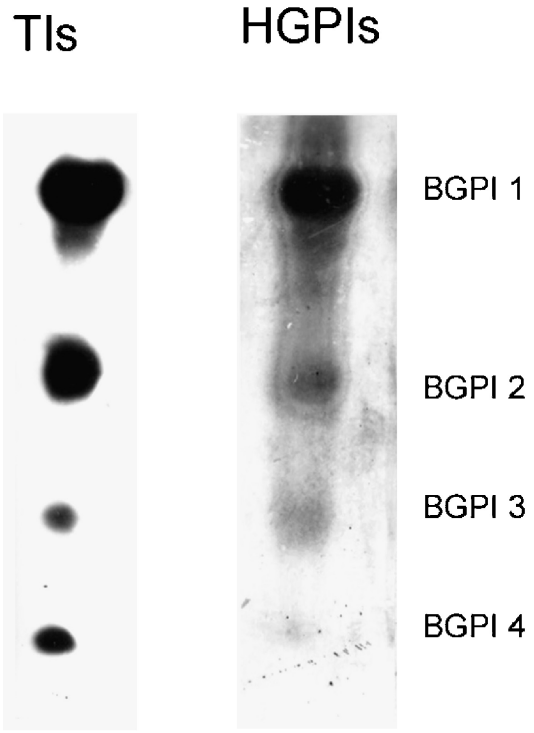

Fig. 2. Visualization of bitter gourd proteinase inhibitors. Equal units of inhibitor activity were loaded on 12% native polyacrylamide gels. TIs and HGPIs were visualized by gel X-ray film contact print technique by incubating gel strips in respective enzyme. For details, see Section 4.4.

Table 1
Inhibition of trypsin and various HGPs by BGPIs

Type of proteinase/source of HGP	% Inhibition by seed extract
Trypsin	100±0
Control HGP	84±1.02
Chickpea field HGP	82±0.1
Pigeonpea field HGP	76±2.5
Cotton field HGP	100±0
Lab grown cotton HGP	100±0
Groundnut fed HGP (diet incorporated)	54±2.1
Wing bean fed HGP (diet incorporated)	62±3.7

2.3. Purification of BGPIs and their identification as squash family inhibitors

BGPI-1 and -2 were purified and their homogeneity was confirmed on SDS–PAGE after silver staining (Fig. 3). BGPI-1 and BGPI-2 had molecular mass of about 3.5 and 3 kDa, respectively suggesting that they belong to squash family PIs. BGPI-1 and BGPI-2 were individually evaluated for their inhibition potential against HGP of larvae fed on different diets. BGPI-1 inhibited 80, 81 and 100% HGP activities and BGPI-2 inhibited 80, 82 and 94% HGP activities of larvae fed on control diet, pigeon pea and cotton, respectively (Fig. 4). No increase in inhibition of control or pigeon pea HGP was observed when BGPI-1 and BGPI-2 were

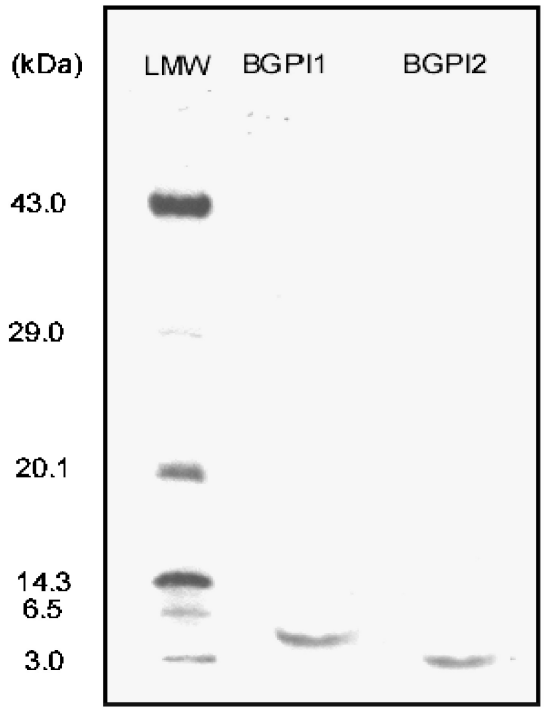

Fig. 3. Molecular weight determination of BGPI 1 and BGPI 2. Purified BGPI-1 and-2 were resolved on 15% SDS–PAGE and stained with silver staining protocol. Lane 1—Molecular weight marker of smaller range 43.3–3.0 kDa, Lane 2—BGPI-1, Lane 3—BGPI-2.

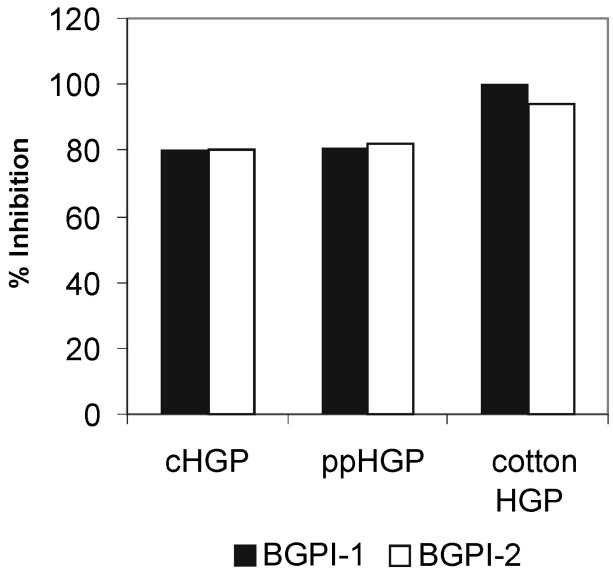

Fig. 4. Maximum % inhibition of HGP by purified BGPIs. Inhibition assays were conducted using Benzoyl-arginyl *p*-nitroanilide as a substrate. Purified BGPI-1 and -2 were assayed against HGPs from larvae fed on the control diet or larvae collected from fields of pigeon pea or cotton.

used in combination indicating that their activities were not synergistic.

2.4. Tolerance of BGPIs towards proteolysis by HGP

While selecting candidate PIs for use in developing insect resistant plants, their stability against digestion by insect gut proteinases is very important. When seed extract containing BGPIs was incubated with HGP (0.2U) for 3 h, inhibitory activity of BGPIs remained unaffected. These samples were further resolved on native gels to determine the integrity of the individual PI proteins. Degradation of BGPI-1 was observed with progressive accumulation of BGPI-2 like peptide, which remained stable and active even after 3 h of incubation with HGP (Fig. 5a) Purified BGPI-1 was also treated with HGP and then resolved on native gel. It showed complete conversion to BGPI-2, which supports our earlier observation (Fig. 5b). BGPI-2 like peptide was also found to be tolerant to HGPs from cotton, chickpea and pigeonpea fed larvae and also from larvae fed on artificial diet with added winged bean and groundnut PIs.

To identify the types of proteinases responsible for proteolysis of BGPI-1 and subsequent accumulation of BGPI-2 like peptide, we used chemical inhibitors to selectively block serine proteinases in HGP. Pretreated HGP (0.3 U) was incubated with BGPIs (1.4 U) for 3 h at 37 °C. When used in combination, phenylmethylsulfonyl fluoride (general serine PI) and elastatinal (inhibitor of elastase) showed limited protection of BGPI-1 from proteolysis by HGP. On the other hand, *N*-tosyl-L-phenylalanine chloromethyl ketone (chymotrypsin inhibitor) or *N*α-*p*-tosyl-L-lysine chloromethyl ketone (trypsin inhibitor) offered better protection of BGPI-1 (Fig. 6). This indicates that chymotrypsin and trypsin like proteinases from HGP might play a major role in proteolysis of BGPI-1. However, none of these inhibitors could completely protect BGPI-1 from degradation, as accumulation of BGPI-2 was observed in all the cases. Interestingly, commercial chymotrypsin (bovine) could not produce the same effect, but excess of commercial trypsin (bovine) was able to produce BGPI-2 like peptide (figure not shown). This supports our observation that HGP is a complex mixture of proteinases in which some serine proteinases are insensitive to chemical inhibitors like phenylmethylsulfonyl fluoride and 4(amidinophenyl) methanesufonylfluoride (Harsulkar et al., 1998; Patankar et al., 2001).

2.5. Effects of bitter gourd PIs on growth and development of two lepidopteran insects H. armigera and S. litura

Effects of BGPIs on insect growth and development were investigated by incorporation of four different doses of inhibitors in artificial diet (3, 6, 9 and 12 trypsin inhibitor units/g of feed). Development of pupae and adults was monitored to evaluate the effect of BGPIs on fertility and fecundity. These results are summarized in Tables 2 and 3. In case of *H. armigera*, larvae fed on diet containing the highest dose of BGPIs (12 trypsin inhibitor units/g) showed reduction in weight by about

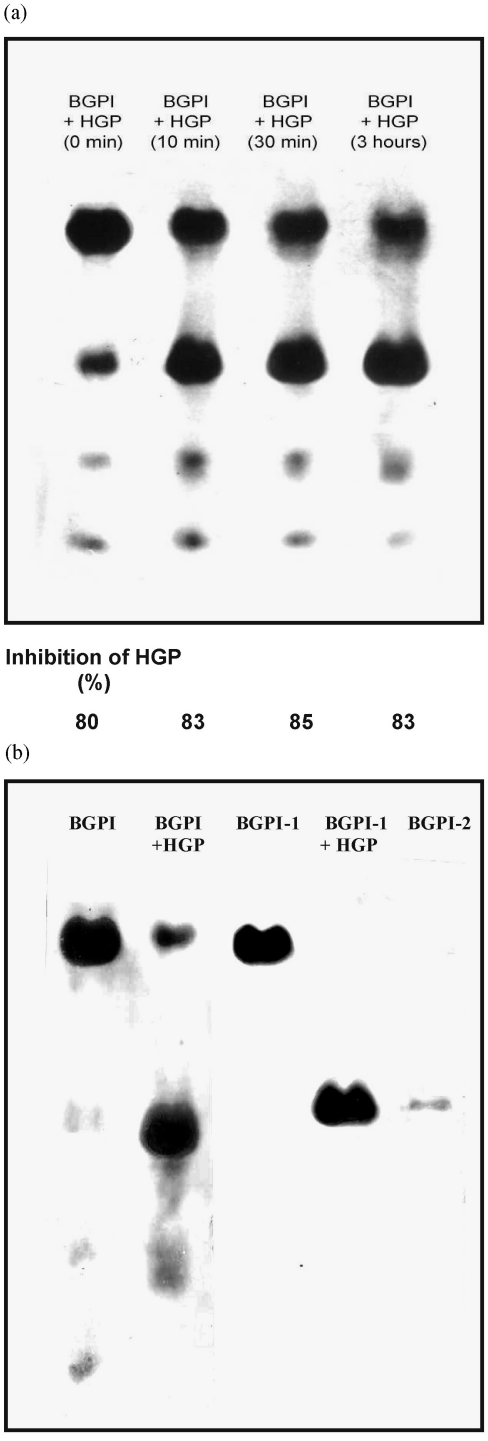

Fig. 5. Stability of bitter gourd PIs towards control HGP. (a) BGPIs (1.4 U) were pre-incubated with HGP (0.2 U) at 37 °C and resolved on 12% native polyacrylamide gel. Treatment time is indicated above the lane. TI bands were visualized as described in Section 4.4. (b) Purified BGPI-1 was treated with HGP as in (a).

43% after 10 days of feeding as compared with larvae fed on control diet. But the loss in larval weight was not consistently dose dependent. Highest larval mortality of 30% was observed in larvae fed on diet containing 6-trypsin inhibitor units/g of feed whereas it reduced to

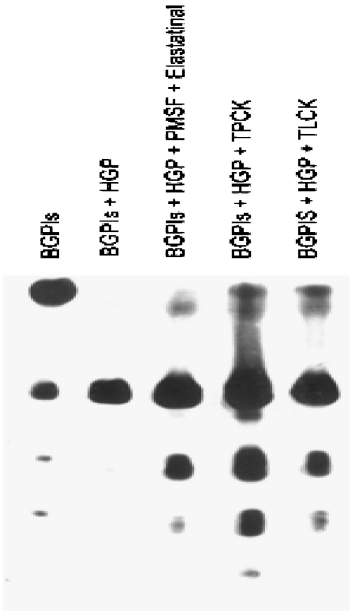

Fig 6. Effect of chemical proteinase inhibitors on proteolysis of BGPIs by HGP. BGPIs (1.4 U) were pre-incubated with HGP (0.3 U) treated with chemical inhibitors for 3 h at 37 °C and resolved on native 12% polyacrylamide gel. Different chemical inhibitors used are indicated on the top of the lane. The TI bands were visualized as described in the Section 4.4.

10% at higher doses of BGPIs (Table 2). Pupal weight was reduced in a dose-dependent manner by about 26% at the highest dose of BGPIs as compared with control. Larval and pupal periods were marginally reduced. Interestingly, fertility and fecundity were adversely affected in the adults emerging from PI fed larvae. Egg laying capacity of adult females reduced significantly from an average of 357 eggs (control) to 113 eggs (PI-fed). Hatching of larvae from eggs was also reduced in a dose dependent manner from 88% in control to 58% (3 trypsin inhibitor units/g feed), 25% (6 trypsin inhibitor units/g feed) and 0% (9 and 12 trypsin inhibitor units/g feed).

In the case of *S. litura*, larvae fed on diet containing BGPIs showed reduction in weight that was dose-dependent, as compared with those fed on control diet. Larval weight was reduced by a maximum of 70% at the highest dose of inhibitor (12 trypsin inhibitor units/g) after 10 days of feeding. Larval mortality ranged from 10 to 20% of the total larval population (Table 3). Reduction in pupal weight by 30%; and 35% pupal mortality were also observed with the highest dose of BGPIs. About 12% larval–pupal intermediates were recorded with higher doses of BGPIs. Larval period was not affected but pupal period was delayed by up to 3 days. Malformed adults (14–20%) were recorded as an effect of abnormal development. Fertility was drastically affected as the egg-laying incidence reduced from 790 eggs per adult female (control) to 260 eggs (PI fed); however, emergence of larvae from eggs was not affected.

Table 2
Effect of bitter gourd proteinase inhibitors on growth and development of *Helicoverpa armigera* (Hubner)

Parameters	Control	3TIU[a]/g	6TIU/g	9TIU/g	12TIU/g
Larval weight (mg/larvae)					
6DAT[b]	5.6±0.32	4.0±0.16	3.0±0.27	2.0±0.16	3.0±0.16
8DAT	25.1±1.75	22.5±1.13	19.6±1.23	19.3±5.75	20.6±2.31
10DAT	36.3±3.40	32.4±1.93	30.6±5.10	22.8±2.49	21.0±2.35
Larval period	19.0±1.02	18.0±0.62	17.0±0.58	17.0±0.60	17.0±0.80
Larval pupal intermediate (%)	0.0	20.0	10.0	0.0	0.0
Larval mortality (%)	0.0	0.0	30.0	10.0	10.0
Pupal weight (mg/pupa)	320.0±1.74	308.4±2.51	280.2±1.64	259.3±2.83	236.4±2.04
Pupal period	16.0±0.72	13.0±0.6	16.0±0.9	15.0±1.0	15.0±0.70
Pupal mortality (%)	0.0	0.0	20.0	28.6	0.0
Malformed adults (%)	0.0	0.0	0.0	0.0	0.0
Fertility (eggs/female)	357.0±9.42	307.0±7.04	156.0±4.50	113.0±2.20	122.0±2.90
Fecundity (hatching%)	87.8	58.6	25.6	0.0	0.0

[a] TIU, trypsin inhibitor units
[b] DAT, days after treatment.

Table 3
Effect of bitter gourd proteinase inhibitors on growth and development of *Spodoptera litura* (Fab.)

Parameter	Control	3TIU[a]/g	6TIU/g	9TIU/g	12TIU/g
Larval weight (mg/larvae)					
6DAT[b]	9.9±2.08	9.4±0.72	8.5±2.28	7.8±0.42	6.8±0.47
8DAT	60.4±1.81	58.0±4.38	52.0±5.78	51.4±1.1	49.8±2.94
10DAT	141.3±27.0	113.9±18.02	112.4±22.84	105.4±32.78	42.4±19.52
Larval period in days	18.0±0.62	18.0±0.60	18.0±0.69	18.0±0.90	18.0±0.70
Larval pupal intermediate (%)	0.0	0.0	11.1	11.8	12.5
Larval mortality (%)	0.0	10.0	10.0	15.0	20.0
Pupal weight (mg/pupa)	314.4±55.0	239.6±43.80	238.3±9.00	229.3±26.00	220.7±26.55
Pupal period in days	15.0±0.46	17.0±1.20	17.0±1.01	18.0±0.90	18.0±0.70
Pupal mortality (%)	15.0	11.8	25.0	33.3	50.0
Malformed adults (%)	0.0	0.0	0.0	20.0	14.0
Fertility (eggs/female)	790.0±17.0	584.0±58.0	298.0±51.8	260.0±26.2	302.0±42.1
Fecundity (hatching%)	76.8	68.4	97.3	81.5	96.0

[a] TIU, trypsin inhibitor units.
[b] DAT, days after treatment.

3. Discussion

The major challenge for PI based strategy is to combat the constitutive and induced complexity of gut proteinases. In Lepidoptera, complex multigene families encode digestive proteinases. About 27 different genes encoding trypsin-like proteinases alone are reported in *H. armigera* (Bown et al., 1998). Being derived from this genetic compliment, HGP is a complex mixture of serine proteinases, predominantly trypsin-like with marginal chymotrypsin like activity. Minor variations in the amino acid sequences near the binding or active site might render proteinases entirely insensitive towards PIs (Bown et al., 1998). In this context, our results on BGPIs are significant because, despite the variability observed in HGPs from larvae fed on different diets, BGPIs inhibit more than 80% of HGP activity.

Insects also secrete proteinases that are able to digest the inhibitor protein (Michaud 1997; Giri and Kachole 1998; Giri et al., 1998; Girard et al., 1998a, b). Therefore, it is essential that candidate inhibitors should have high tolerance to insect proteinases (Harsulkar et al., 1999). It is noteworthy that BGPIs remain stable and active even after incubation with gut proteinase for 3 h, which is an average food retention time in the Lepidopteran larvae (Fig. 5 a). It has been revealed that BGPI-2 is a highly stable form. Upon proteolysis of BGPI-1 by HGP, BGPI-2 like peptide is generated and gets accumulated in large amount. However, overall inhibitory activity of BGPIs is not affected in spite of this degradation. Thus, BGPI-1 is probably cleaved at a site different from the active domain, and the resultant fragment retains full activity. A similar scenario is expected in the insect gut environment, where action of the gut proteinases leads to proteolysis of BGPI-1 and the resultant BGPI-2 like peptide effectively inhibits digestive action.

It has been reported that strong inhibitors of gut proteinases, in vitro, do not necessarily retard larval growth and development (Edmonds et al., 1996). Insect feeding assays were therefore performed to assess the

antibiosis exerted on *H. armigera* and *S. litura* by BGPIs. BGPIs caused deformities in the developing larvae and also had adverse effects on pupae and adults. Apart from inhibition and disruption of molting, larval–pupal intermediates and malformed adults were also observed. The malformed adults were short-lived and infertile. Adult insects emerging from PI-fed larvae had impaired fertility and fecundity. In case of *H. armigera,* both fertility and fecundity were reduced and in case of *S. litura* fertility was repressed. The female moth of *H. armigera* typically lays about 700 eggs and this process requires a major input of proteins. Since the adults feed only on plant nectar, a poor protein source, it is apparent that the pool of reserve proteins is generated during actively feeding larval stage. Female larvae of some Lepidopteran insects, such as the Gypsy Moth, *Lymantria dispar,* are known to undergo an additional larval stage so as to feed and accumulate more proteins (Telang et al., 2001). Any disturbance in protein metabolism at larval stage is thus expected to reflect on the number and quality of eggs. Ingestion of BGPIs adversely affected the protein intake, at the larval stage, which caused developmental abnormalities and also reduced fertility and fecundity of the adult. Thus, our observations are in agreement with these recent studies that accumulation of proteins during the larval stage is critical to vitellogenesis (Telang et al., 2001). It has also been reported that oryzacystatin-I affects fertility and fecundity of *Perillus bioculatus* (Ashouri et al., 1998)

In conclusion, we have identified serine PIs from bitter gourd seeds, which can cause deleterious effects on growth and development of *H. armigera* and *S. litura*. Biochemical analysis has shown that they are strong and stable inhibitors of insect proteinases. For the first time, experimental data is provided which clearly shows reduction in fertility and fecundity of insect larvae due to antifeedant properties of BGPIs. Being small peptides, it would be easy to express BGPIs in plants to confer protection against devastating pests such as *H. armigera* and *S. litura*.

4. Experimental

4.1. General

Bitter gourd seeds of cv. White-Long were obtained from Navlakha Seeds, Pune, India, seeds of cv. Long-Green, Trailing and Green-Glory were procured from Damani seeds, Pune. Bovine trypsin and chymotrypsin were obtained from Sisco Research Laboratory, Mumbai, India. Elastase, benzoyl-arginyl *p*-nitroanilide (BA*p*NA) and chemical inhibitors *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF) and elastatinal were obtained from Sigma Chemicals, St Louis, USA. X-ray films and Kodak-163DA developer were purchased from Kodak, Chennai, India.

4.2. Extraction of bitter gourd PIs and Helicoverpa armigera gut proteinase (HGP)

Dry seeds of bitter gourd were dekernelised, ground to a fine powder and then defatted and depigmented with several washes of hexane and acetone, respectively. The solvents were filtered off and the seed powder was recovered after air-drying. Protein in seed powder was extracted at 27 °C for 2 h in distilled water (1:10 w/v) with continuous stirring. The suspension was centrifuged twice at 10,000 rpm for 30 min at 4 °C. Supernatant was collected and stored frozen in aliquots, or freeze-dried. Protein content was determined by Bradford's method (1976).

H. armigera larvae were reared on an artificial diet as described earlier (Gupta et al., 2000). For feeding studies, the artificial diet was supplemented with seed extracts of known PI activity while diet without added PIs was used as control diet. The larvae were also collected from fields of chickpea, pigeonpea and cotton or reared in the laboratory by feeding them on developing bolls of cotton. Mid-guts of the fifth instar larvae were removed by carefully dissecting them and stored at −20 °C until further use. Proteinases from the midgut tissue were extracted in equal volume of 0.2 M glycine–NaOH buffer (pH 10) for 2 h at 4 °C and then centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting supernatant was collected, frozen in aliquots, analyzed for proteinase activity and used as proteinase source in subsequent solution assays. Excreted matter of different larval stages was collected, washed with acetone, extracted and treated similarly as the midgut extracts.

4.3. Proteinase and proteinase inhibitor assay

Trypsin and HGP activities were estimated using the chromogenic substrate BA*p*NA. For the inhibitor assay, a suitable volume of BGPIs was mixed with commercial bovine trypsin (24 μg) or with HGP of trypsin-equivalent activity and incubated at 27 °C for 15 min and the assays were conducted at 37 °C for 10 min. Residual proteinase activity was then estimated as described earlier (Giri et al., 1998). One proteinase unit was defined as the amount of enzyme that increased absorbance by 1 OD/min at 410 nm and one PI unit was defined as inhibition of 1 unit of proteinase activity under the given assay conditions.

4.4. Visualization of TIs and HGPIs

Seed protein extract or partially purified inhibitors were separated on native polyacrylamide gel electro-

phoresis (PAGE) in a vertical slab gel unit using Davis buffer system (Davis, 1964) or by denaturing sodium dodecyl sulfate (SDS)–PAGE according to the method of Laemmli (1970). β-Mercaptoethanol was excluded for all TI activity visualizations after SDS–PAGE and was added in the protein samples for molecular mass determination. PIs were visualized after native PAGE or after SDS–PAGE by using the gel-X-ray film contact print technique as described by Pichare and Kachole (1994). After electrophoresis, native gels were equilibrated in proteinase assay buffer followed by incubation in respective proteinase solution and then overlaid on X-ray film. After SDS–PAGE, the gels were washed thrice, for 20 min each, in 2.5% Triton X-100 in 0.1 M Tris–HCl (pH 7.8) to remove SDS. The gels were then washed with assay buffer as described earlier and incubated in 0.1% trypsin solution or HGP of equivalent activity for 10 min. The excess proteinase was rinsed off and the gel was overlaid on an X-ray film for 5 min. The films were then washed with water and inhibitor activity bands were visualized as unhydrolyzed gelatin on the X-ray film. The X-ray film was developed using Kodak 163A developer and contact printed to obtain the photographs.

4.5. Treatment of PIs with HGP

To confirm the stability of bitter gourd PIs against HGP, 1.4U of inhibitor was incubated with 0.2U of HGP at 37 °C for 0, 10, 30 min and 3 h. PI activity was estimated by solution assay using Benzoyl-arginyl *p*-nitroanilide as substrate, as described earlier. HGP-treated BGPIs were also resolved on the native gel to check the stability of different iso-inhibitors by processing the gel for TI activity visualization.

4.6. Purification of BGPIs

Bitter gourd seed extract was electrophoresed on 12% preparative gel (Davis, 1964). A thin strip corresponding to approximately one lane was cut from the gel and processed for TI visualization. Segments corresponding to the TI bands were excised from the remaining gel, crushed and eluted in distilled water. Inhibitor activity of these preparations against trypsin and HGP was determined. Individual inhibitor preparation was resolved by SDS–PAGE (Laemmli, 1970) in the presence of β-mercaptoethanol and stained with silver nitrate (Blum et al., 1987). Molecular mass markers were included in the gel to determine molecular weight of BGPIs.

4.7. Bioassays with H. armigera and S. litura larvae on diet containing BGPIs

Laboratory cultures of *H. armigera* and *S. litura* were maintained in environmental simulation walk-in chamber (27±1 °C, 60±5%RH, 16:8 h scoto/photophase) for several generations to ensure genetic homogeneity. The artificial diet as suggested by Nagarkatti and Satyaprakash (1974) for *H. armigera* and by Okamoto and Okada (1968) for *S. litura* was modified for our laboratory conditions (Gupta et al., 2000). Composition of the diet for *H. armigera* was as follows (for 650 ml distilled water): 77.7 g chickpea seed meal, 5.6 g of wheat germ, 19.2 g of dried yeast powder, 12.8 g of casein, 4.6 g of ascorbic acid, 1.5 g of methyl-*p*-hydroxybenzoate, 0.8 g of sorbic acid, 0.2 g of streptomycin sulfate, 0.2 g of cholesterol, one capsule of vitamin B complex (approx. 0.2 g), 1.0 ml formaldehyde (40%), 0.8 ml multivitamin drops, 0.8 ml vitamin E and 12.0 g of agar–agar. The diet ingredients for *S. litura* consisted of (for 600 ml distilled water): 65.0 g of kidney bean flour, 65.0 g of wheat bran, 10.0 g of agar–agar, 25.0 g of dried yeast powder, 3.0 g casein, 4.0 g of ascorbic acid, 0.4 g of methyl-*p*-hydroxybenzoate, 0.9 g of sorbic acid, 0.1 g of streptomycin sulfate, 0.3 g of cholesterol, one capsule of vitamin B complex (approx. 0.2 g), 2–3 drops of olive oil and 0.8 ml multivitamin drops. These artificial diets were composed to support the larval growth, development and reproduction of the test insects. The basic diet was supplemented with appropriate amount of seed extract of bitter gourd so as to give the final concentration of BGPIs to 3, 6, 9 and 12 trypsin inhibitor units/g (TIU/g) of feed. The bioassays were conducted on neonate larvae of *H. armigera* and *S. litura*, in three sets, each consisting of 30–40 larvae. Larval weight was recorded after 6, 8 and 10 days of feeding. Pupal weight, larval and pupal period, larval–pupal intermediate, pupal mortality, malformed adult emergence and total developmental period were also recorded. After the emergence of adults, their fertility (number of eggs/adult female) and fecundity (number of larvae hatched) was recorded and this data was compared with that of the control population (larvae fed on artificial diet without added BGPIs).

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