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Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds

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Abstract

Dry mature seeds of winged bean (*Psophocarpus tetragonolobus* L., DC.) (WB) contain several proteinase inhibitors. Two-dimensional gel analysis of WB seed protein followed by activity visualization using a gel-X-ray film contact print technique revealed at least 14 trypsin inhibitors (TIs) in the range of 28–6 kD. A total of seven inhibitors (WBTI-1 to 7) were purified by heat treatment and gel filtration followed by elution from preparative native gels. Based on their biochemical characterization such as molecular mass, pI, heat stability, and susceptibility to inactivation by reducing agents, WBTI-1 to 4 are Kunitz type inhibitors while WBTI-5 to 7 are classified as Bowman–Birk type serine proteinase inhibitors. Although Kunitz type TIs (20-24 kD) of WB have been reported, the smaller TIs that belong to the Bowman–Birk type have not been previously characterized. Seven major TIs isolated from WB seed were individually assessed for their potential to inhibit the gut proteinases (HGP) of *Helicoverpa armigera*, a pest of several economically important crops, which produces at least six major and several minor trypsin/chymotrypsin/elastase-like serine proteinases in the gut. WBTI-1 (28 kD) was identified as a potent inhibitor of HGP relative to trypsin and among the other WBTIs; it inhibited 94% of HGP activity while at the same concentration it inhibited only 22% of trypsin activity. WBTI-2 (24 kD) and WBTI-4 (20 kD) inhibited HGP activity greater than 85%. WBTI-3,-5,-6 and-7 showed limited inhibition of HGP as compared with trypsin. These results indicate that WBTIs have different binding potentials towards HGP although most of the HGP activity is trypsin-like. We also developed a simple and versatile method for identifying and purifying proteinase inhibitors after two-dimensional separation using the gel-X-ray film contact print technique.

Keywords: Lepidoptera; Noctuidae; Leguminosae; *Helicoverpa armigera*; *Psophocarpus tetragonolobus*; Winged bean; Insect gut proteinases; Proteinase inhibitors; Two-dimensional gel electrophoresis; Activity visualization

1. Introduction

Plants are under constant attack by insect pests, and one potent defense response by plants is to produce inhibitors against the insect's gut proteinases (Ryan, 1973). Through evolutionary processes, insects adapt to plant proteinase inhibitors (PIs) by developing proteinase variants that are minimally affected by the plant defense proteins (Broadway, 1995, 1997; Jongsma et al., 1996; Giri et al., 1998). In addition, it appears that some polyphagous insects are capable of altering expression of different gut proteinase isoforms to maximize their resistance to the PIs produced by a particular plant species (Michaud, 1997; Wu et al., 1997; Girard et al., 1998; Patankar et al., 2001). While there is considerable interest in the use of plant PIs for engineering resistance to insects in crop species, for some insects the currently known PIs are ineffective (Jouanin et al., 1998; Schuler at al., 1998). A useful strategy for enhancing plant defense systems in the future is to identify PIs with high activity against the particular target insect (Koiwa et al., 1998). This will likely entail the examination of plant species beyond the host group (Jongsma et al., 1996).

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An example of an insect that can adapt to a range of plant PIs is *Helicoverpa armigera* (podborer), a polyphagous pest of many important crops, which often leads to heavy losses in the field (Manjunath et al., 1989). *H. armigera* larvae have an alkaline gut, which can produce at least six major, and several minor serine class proteinases that are able to overcome the native PIs of its host plants (Johnston et al., 1991; Bown et al., 1997, 1998; Giri and Kachole, 1998; Giri et al., 1998; Harsulkar et al., 1998, 1999; Patankar et al., 1999, 2001). Previously, we have observed that the PIs of winged bean (WB; *Psophocarpus tetragonolobus* L.), a non-host plant of podborer (*H. armigera*), can inhibit its gut proteinases (HGP) and larval growth (Harsulkar et al., 1999). Recently, a long-term effect of mustard trypsin inhibitor (TI) on *Spodoptera littoralis* larvae fertility has also been demonstrated (De Leo and Gallerani, 2002), and we observed ingestion of bitter gourd TIs by larvae dramatically affects the fertility of *H. armigera* and *S. litura* moths (Telang et al., 2003). These results point to the importance of screening defensive potential of PIs from plant species other than the host group of plants and their potential use to control pest outbreaks in the field via transgenic approach.

While the earlier study indicates that WBPIs may be good candidates for engineering resistance into *H. armigera* host plants (Harsulkar et al., 1999), the observed inhibition was a cumulative effect of several inhibitors present in WB seeds. Mature seeds of WB are known to contain several PIs, some of which are inhibitors of only trypsin or chymotrypsin while others inhibit both types of proteinase (Shibata et al., 1986). Although several studies on WBPIs are available (Yamamoto et al., 1983; Shibata et al., 1986, 1988; Peyachoknagul et al., 1989; Cladwell et al., 1990; Habu et al., 1992), the specific potentials of the individual inhibitors to inhibit insect gut proteinases have not been evaluated as has been done for the PIs of cowpea, soybean, potato and several other plants. In order to apply this knowledge towards strategies to engineer crop plants with WBPIs for *H. armigera* resistance, isolation and characterization of the individual PIs with reference to their inhibition potentials against HGP is essential.

In the present study, we report on the separation of seven major WBPIs with molecular mass ranging from 28 to 6 kD and evaluate the inhibition potentials of the individual PIs against HGP. Among the PIs identified are several small molecular weight TIs (6–9 kD), which might belong to the Bowman–Birk family. Bowman–Birk type inhibitors are well characterized to have small molecular masses, two active domains and high content of cysteine residues (Hammond et al., 1984; Garcia-Olmedo et al., 1987). Several of the individually purified WBTIs exhibited strong inhibitory activities against the HGP. We also demonstrated that TI activity can be visualized after two-dimensional gel electrophoresis using a simple and rapid gel-X-ray film contact print technique (GXCT), which greatly enhances the ability to separate and identify inhibitor proteins from co-migrating non-TI proteins.

2. Results

2.1. WBTIs detected by gel-X-ray film contact print technique after separation on SDS–PAGE and two-dimensional gels

At least seven TI bands between 28 and 6 kD were detected in WB seed protein extracts after SDS–PAGE using the GXCT technique originally described by Pichare and Kachole (1994) (Fig. 1). The 28 kD band shows up only with longer exposure (see Fig. 2) and is not clear in Fig. 1 because long exposures of the 1-D gels gives a large smear of all the other TIs. Because trypsin was used in the GXCT activity analysis, these inhibitors were designated WB trypsin inhibitors (WBTIs). We further extended the use of this technique to visualize inhibitor proteins on two-dimensional (2-D) gels, coupling high resolution of protein separation with high sensitivity of activity detection. After the WB seed protein was resolved on 2-D gels using a pH gradient of

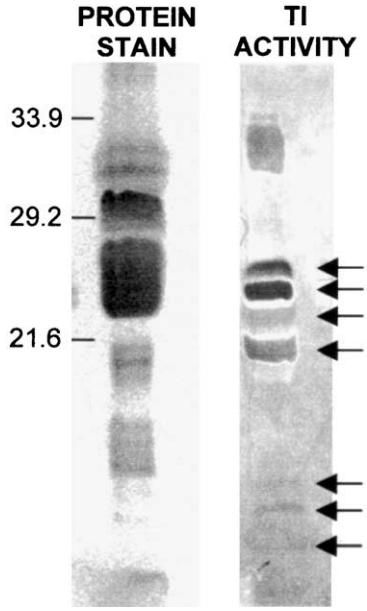

Fig. 1. SDS–PAGE profiles of proteins and TIs of WB seed extract. Protein and TI activity profiles of WB seed protein after separation on a 15% SDS–PAGE gel. Seed extract of about 20 μg proteins without β-mercaptoethanol was loaded in each lane. After electrophoresis, the gel was washed in 2.5% Triton X-100 and then equilibrated in 0.1 M Tris–HCl, pH 7.8. The gel was subsequently incubated in trypsin solution (1 mg/ml) and overlaid on an X-ray film for 7 min. The gel was removed and the X-ray film was washed with water to visualize the inhibitor bands. The same gel was then stained for total protein. Arrows indicate TI bands in the molecular mass range of 30–5 kD.

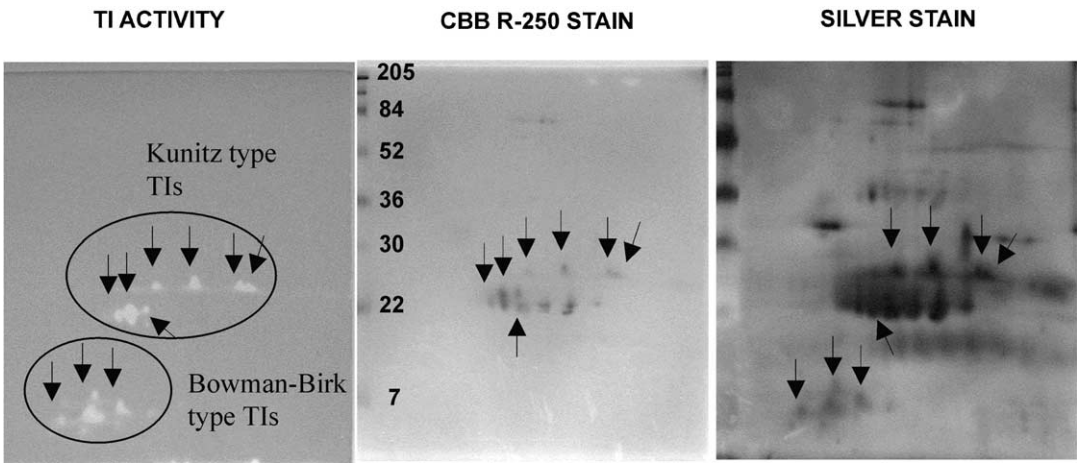

Fig. 2. Two-dimensional gel analysis of WB seed proteins for three different staining on same gel. The same gel is shown after TI activity visualization, Coomassie Brilliant Blue (CBB R-250) and silver staining. A number of TI proteins were identified, as indicated by arrows. WB seed protein (27μg) was first resolved on an IEF gel with a pH gradient of 4.2–7.5 and then separated on a 15% SDS–PAGE gel. After separation, the gel was processed for trypsin activity visualization (see Fig. 1 legend) followed by protein staining with CBB and silver staining, respectively. The inhibitor profile shown was from a short contact (2 min) of the gel to X-ray film in order to obtain maximum resolution. A 28 kD inhibitor with an apparent pI of 5.4 band was detected only after a longer contact time (e.g. 5 min). CBB stained gel image shows molecular mass markers in kD.

4.2–7.5 for IEF in the first dimension and a 15% SDS–PAGE gel for the second dimension, several inhibitor proteins could be detected. With a short contact time of the gel to the X-ray film to obtain maximum resolution, at least four isoforms of TIs with apparent molecular mass of 24 kD and three TI isoforms with a molecular mass of about 20 kD were detected by GXCT (Fig. 2). In addition, seven small TIs of molecular masses in the range of 6–9 kD could be detected on 2-D gels (Fig. 2). With a longer contact time to the X-ray film, a 28 kD inhibitor with an apparent pI of 4.5 was detected (Fig. 2), which is not seen in the shorter contact time shown in Fig. 1. Thus, the major classes of TIs as originally revealed by single dimension on SDS–PAGE were further separated with a high resolution by 2-D method. Overall, the 2-D gel separation followed by GXCT proved to be a very sensitive method for detection of TIs and extended the usefulness of this technique over the typical SDS–PAGE gels. This technique was able to detect not only highly active TIs but also several inhibitors having moderate and low activities. The sensitivity of the GXCT is further demonstrated by observations of TI activity in protein spots which could not be detected by Coomassie Brilliant Blue R250 (CBB) staining and were barely visible after silver staining (Fig. 2).

One unique advantage of this method is that to detect inhibitors of low activity or low abundance, the gel can be exposed to X-ray film several times at increasing time intervals. Initially only strong TI activity bands will appear on the film while for the same gel exposed to another film for longer time the weaker activity bands will show up. Moreover, the same gel can be used for protein staining either by CBB and/or by silver staining (Fig. 2). To the best of our knowledge, this is the first attempt to visualize the activity of TI proteins after 2-D gel separation. It is also clear from our results that TIs remain active after exposure to several detergents and urea used in 2-D gels. However, inclusion of reducing agent such as β-mercaptoethanol or dithiotheritol (DTT) in the samples irreversibly inactivates the inhibitor activity of the Bowman–Birk type TIs but gives only partial inactivation of the Kunitz type TIs (see Table 2).

2.2. Purification of individual WBTIs

For assessing the inhibition potentials of the individual TIs against HGP, several methods were employed to attempt to further resolve the WBTIs. Heat treatment of WB seed protein at 60 °C for 30 min removed some of the non-TI proteins, resulting in an approximately two-fold increase in specific inhibitory activity (Table 1). Heat treatment did not cause any significant loss in inhibitor activity as detected by enzyme assay and by GXCT. Heat-treated WBTI extract was further resolved by gel filtration using Superdex G-75, which clearly separated two pools of TIs based on size but did not resolve individual TIs. Using GXCT after SDS–PAGE, it was found that pool I contains TIs in the molecular mass range from 20 to 28 kD and pool II contained three TIs of 7–9 kD. Protein from both pools gave 100% inhibition of trypsin activity; however, there was a significant difference between the two preparations in their inhibition against HGP. Pool I protein inhibited 100% HGP activity while a maximum 70% inhibition of HGP was obtained with pool II protein (Table 1).

Preparative gel electrophoresis was used to further separate individual TIs from the mixture of partially purified WBTIs. Pool I and pool II fractions from gel

filtration were concentrated and loaded separately onto 10% native polyacrylamide preparative tube gels (Prep-Cell unit, BioRad, USA). From pool I, three TIs with molecular masses of approximately 28, 24 and 20 kD were resolved by this method. From pool II, one TI each of 7, 8 and 9 kD was detected. While the separated TIs were still contaminated with other non-TI proteins, these preparations allowed us to assess their individual inhibition potentials against trypsin and HGP since the contaminating proteins do not appear to interfere in the in vitro inhibitor activity assay and in the detection of activity on gels.

2.3. Biochemical properties of WBTIs indicate presence of both Kunitz and Bowman–Birk type serine PIs in WB seeds

The molecular mass determination of the seven purified WBTIs showed that there are at least two types of serine PIs, 20–28 kD TIs (TI-1 to TI-4) and 7–9 kD TIs (TI-5 to TI-7) (Table 2). Their isoelectric points were in the range of 4.8–6.6 for TI-1 to TI-4 and 6.6–6.8 for TI-5 to TI-7 (Table 2). Upon heat treatment at 90 °C for 15 min, 20–28 kD TIs (TI-1 to TI-4) showed inactivation of inhibitor activity while the smaller TIs (7–9 kD, TI-5 to TI-7) remained active indicating that smaller TIs were heat stable. In contrast, when these TIs were treated with β-mercaptoethanol, the smaller TIs (TI-5 to TI-7) were inactivated whereas TI-1 to TI-4 remained partially active or stable. It is well documented that Bowman–Birk TIs are rich in the sulfur containing amino acid cystine, which makes them stable to heat treatments. However, these TIs lost their activity upon treatment with reducing agent such as β-mercaptoethanol and DTT. It has also been reported earlier that Kunitz type WBTIs (>20 kD) with acidic isoelectric point (pI 5.1) are heat labile (Kortt, 1979). Thus these results suggested that TI-1 to

Table 1
Summary of purification of WBTIs by various methods

Step	Protein (mg/g seed)	Total TI (Units/g seed)	Specific activity (Units/mg protein)	% Inhibition against	
				Trypsin	HGP
Crude protein extract	31.20	130.0	4.16	100	100
Heat treatment	12.26	98.2	8.0	100	100
Gel filtration					
PooI I (20–28 kD TIs)	2.30	60.0	26.1	100	100
Pool II (7–9 kD TIs)	2.05	34.0	16.5	100	70

Different concentrations of crude seed protein extract or partially purified inhibitor preparations were used to check the activities of the WBTIs against trypsin and HGP. The experiment was repeated at least three times and yielded similar results. Maximum inhibition obtained by the respective inhibitor preparations is presented in the last column.

Table 2
Biochemical properties of WBTIs

WBTIs	Apparent molecular mass (kD[a])	Apparent pI[b] (pH)	Effect of treatment on TI activity	
			Heat	β-mercaptoethanol
Kunitz-type				
TI-1	28	4.8	Inactivated	Highly Stable
TI-2	24	5.4	Inactivated	Active
TI-3	24	5.7	Inactivated	Active
TI-4	20	6.6	Inactivated	Active
Bowman–Birk type				
TI-5	9	6.8	Stable	Inactivated
TI-6	8	6.6	Stable	Inactivated
TI-7	7	6.8	Stable	Inactivated

TI activity bands were visualized using GXCT after SDS–PAGE or two-dimensional gels. The presence or absence of inhibitor of band/spot was considered for inferring the results. Active indicates TI bands were detected on the X-ray film but with lesser intensities. The experiments were repeated at least three times with similar results.

[a] Apparent molecular masses of various PIs were calculated after resolving the proteins by SDS–PAGE followed by detection of activity by GXCT. The mobility values of the PIs were compared with those of broad range molecular mass standards (BioRad).

[b] The isoelectric points of the PIs were estimated by measuring pH gradient (pH 4.2–7.5) on IEF gels after focusing using a surface pH electrode and compared with the mobilities of the inhibitor activity spots on the gels using GXCT. Heat or β-mercaptoethanol treated WBTIs were run on the gel and visualized for presence or absence TI bands.

TI-4 are of Kunitz type while TI-5 to TI-7 are of Bowman–Birk type serine PIs.

N-terminal sequencing of WBTI (28 kD) was carried out after eluting the protein from 2-D gel. The N-terminal sequence information obtained from a spot of 28 kD TI (LALXLVSAI) protein showed homology with deduced amino acid sequence (LALFLVSAI) of cloned winged bean proteinase inhibitor genes (Peyachoknagul et al., 1989; Habu et al., 1992; our unpublished results). No sequence data could be obtained for the remaining TIs due to their N-terminal blockage. Cladwell et al. (1990) found that that winged bean Kunitz type TIs have N-terminal pyroglutamic acid obstructing the N-terminal sequencing, while Yamamoto et al. (1983) were able to generate sequences from two winged bean TIs, and Shibata et al. (1983) for a chymotrypsin inhibitor from winged bean. It is possible that the N-terminal blockage we encountered was introduced during preparation, although we took all the customary precautions to avoid this.

MALDI-TOF analysis of smaller TIs (7–9 kD) further revealed more accurate molecular masses. The molecular masses for TI-5 and TI-6 are 7.92 and 7.6 kD, respectively, which is in close agreement with the molecular masses of Bowman–Birk type TIs from other plants (Hammond et al., 1984; Birk, 1985; Garcia-Olmedo et al., 1987). Upon cyanogen bromide cleavage TI-5 and TI-6 showed appearance of several peptides of which major were 3.2 and 4.2 kD. This is consistent with reported cleavage patterns of Bowman–Birk type TIs in other plant species (Birk, 1985; Prakash et al., 1996).

2.4. Potential of WBTIs to inhibit HGP activity

Inhibition capacity of individual WBTIs towards trypsin and HGP was evaluated by enzyme assays. Different concentrations of inhibitor protein were used to obtain maximum inhibition of trypsin and trypsin-like activity of HGP. Fig. 3 summarizes the potentials of the individual WBTIs to inhibit the activity of HGP versus trypsin. The results clearly demonstrate that WBTIs exhibit differential inhibitory activity toward HGP and trypsin. Most interesting and important is the 28 kD WBTI (TI-1) that shows at least three-fold greater inhibition of HGP than that of trypsin. The 20 kD TI (TI-4) showed 86% inhibition of HGP whereas it inhibited 100% of the trypsin activity at equivalent concentration. The smaller TIs (TI-5 to TI-7) were able to inhibit HGP activity only up to 64%, even though very high concentrations of the inhibitors were used. On the other hand, at these concentrations, trypsin activity was totally inhibited by the small TIs (Fig. 3). The two 24 kD TI isoforms (TI-2 and TI-3) showed variation in inhibition of trypsin and HGP activity. For example, the 24 kD TI-2 was able to inhibit HGP activity up to 92% whereas at the same concentration it inhibited 100% of the trypsin activity. Another 24 kD TI isoform (TI-3) inhibited total trypsin activity, however, only 67% of the HGP activity was inhibited even at high concentration suggesting the remaining 33% proteinase activity is insensitive to the inhibitor (Fig. 3).

To confirm that the inhibition of HGP obtained by WBTI-1 (28 kD) preparation was due to the inhibitor protein and not because of a potential co-migrating protein, we further resolved the 28 kD WBTI protein preparation by preparative SDS–PAGE. After electrophoresis, the protein band corresponding to TI activity was cut out, electro-eluted, dialyzed and its activity assessed against HGP and trypsin. WBTI-1 gave more than three-fold inhibition of HGP versus trypsin. Other proteins from this preparation showed neither trypsin nor HGP inhibitory activity. In view of the strong inhibition of HGP exhibited by WBTI-1, -2, and -3, they are promising candidates for engineering *H. armigera* resistance in crop plants.

2.5. Application of 2-D gel analysis and visualization of TI activity in proteomic studies

Prep-Cell eluted protein fraction which contains the 24 kD WBTI was further resolved by SDS–PAGE. This TI preparation contained two co-migrating major proteins of similar molecular masses as detected by CBB staining (Fig. 4A). However, only one protein band showed TI activity (Fig. 4A). Therefore, 2-D IEF/SDS–PAGE followed by TI activity visualization using GXCT was used for further separation of the inhibitor from other proteins (Fig. 4B). After activity visualization, the same gel was stained for total protein using

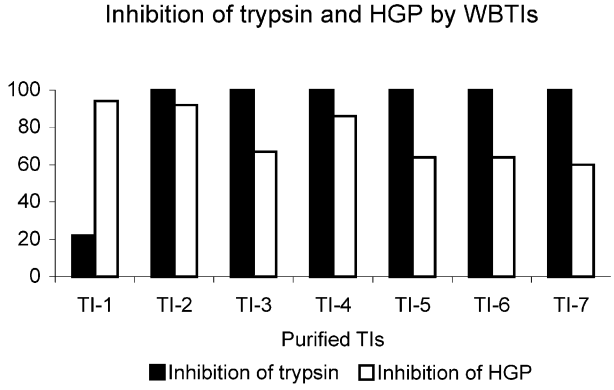

Fig. 3. Inhibitory potentials of WBTIs against HGP. To check the potential of individual inhibitors against trypsin and HGP, various concentrations of the purified inhibitors were used. Maximum inhibition obtained for each inhibitor is presented. The experiment was repeated at least thrice with similar results. Concentration of bovine trypsin used was 6 μg per reaction, which gives 0.4 OD at 410 nm using BApNA as substrate under given assay condition. Equivalent amount of HGP activity, which produces 0.4 OD at 410 nm, was used with same assay conditions.

CBB. The protein eluted from the 24 kD activity band showed at least four protein spots on 2-D gels. However, only one of the four proteins had TI activity (cf. Fig. 4A and B). This demonstrates the advantage of using 2-D gel electrophoresis for identifying TI proteins. This strategy was followed to isolate the TI to homogeneity. The technique of detecting TI activity after high-resolution 2-D gel electrophoresis developed in this paper will facilitate the isolation of other TIs and their isoforms for further characterization using a proteomics approach.

3. Discussion

PIs are among the best-studied candidates for genetic engineering of plant resistance to insect pests. There is a growing interest in identification of novel PIs and their use in developing resistance in otherwise susceptible plants (Jouanin et al., 1998; Schuler et al., 1998). Here we report identification of several PIs from winged bean seed that are potent inhibitors of the gut proteinases of *H. armigera* and also describe a powerful technique of 2-D GXCT, which will be useful for studying PI activity and their characterization in general. *H. armigera* is a polyphagous pest of several important crops (e.g. chickpea, pigeonpea, cotton, tomato, okra, sunflower and corn), which can cause damage ranging from heavy reduction in yield to complete crop failure (Manjunath et al., 1989). Chemical control is no longer a lasting solution for pest control in the field due to capabilities of insects to develop quick resistance against the pesticides, apart from their ecological problems (Armes et al., 1996). Recombinant DNA technology offers the potential to enhance the native resistance of the host plants against this pest; however, success of this strategy relies on the identification of target proteins such as the winged bean TIs identified in this report that can provide selective resistance properties.

PIs are of particular interest for engineering resistance because they are a part of the plant's native defense tools and impose no long-term toxic effects on insects, other predators or humans (Ryan, 1990; Boulter, 1993; Lewis et al., 1997). PIs retard insect growth and development by inhibiting digestion and forcing the feeding insect to produce alternative proteinases if such a genetic plasticity exists in the particular insect pest. This process is responsible for growth retardation of insects because the insect utilizes essential amino acids and energy for secretion of new PI-insensitive proteinases

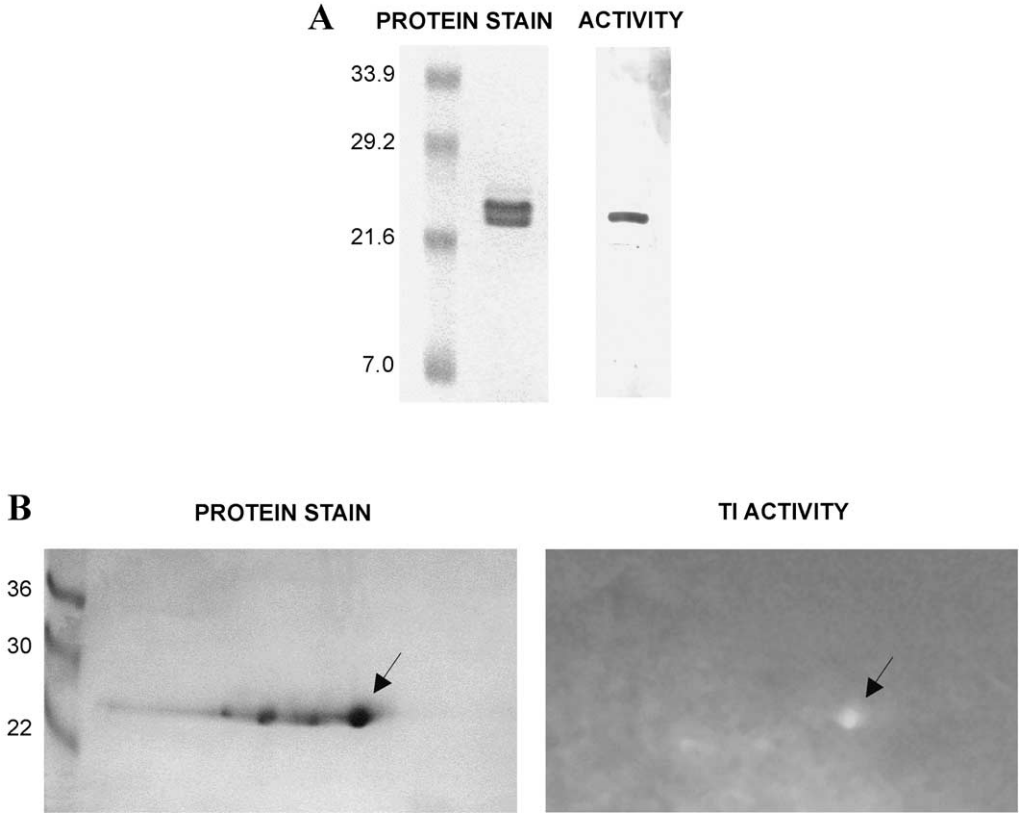

Fig. 4. SDS–PAGE and 2D analysis of 24 kD WBTI protein. (A) SDS–PAGE analysis of Prep-Cell eluted protein fraction containing the 24 kD WBTI. After separation, the gel was processed for TI activity visualization followed by CBB-250 protein staining. SDS–PAGE shows two proteins of about 24 kD but only one of these proteins has TI activity as shown in the activity gel assay by GXCT. (B) Two-dimensional gel electrophoretic analysis of 24 kD WBTI. Protein staining detects four distinct spots but activity visualization shows only one (arrow) TI activity spot.

while at the same time amino acid availability is reduced in general (Broadway and Duffey, 1986; Hilder et al., 1993). With retardation of growth, the destructive potential for the insect is greatly reduced, ameliorating the loss in crop yield. Since the effect of PIs is non-toxic and does not dramatically increase insect mortality, there are less chances of developing resistance in the descendant insect populations (Harsulkar et al., 1999).

Several reports are available demonstrating increased resistance to a particular insect pest in transgenic plants expressing high levels of heterologous PIs (Jouanin et al., 1998; Schuler et al., 1998). A few studies, however, have also observed that insects can grow normally on PI-expressing transgenic plants (Jongsma and Boulter, 1997; Cloutier et al., 2000). This is probably because of low inhibition potential of the selected inhibitor against the pest proteinases and/or low levels of expression of the PIs in the target tissues (Jongsma and Boulter 1997; De Leo et al., 1998). In some of these instances, detailed biochemical analysis of interaction of PIs with the insect gut proteinases revealed that the insect produces new proteinases, which are insensitive or resistant to the expressed PI (Jongsma et al., 1995; Broadway, 1995; 1997; Wu et al., 1997; Girard et al., 1998; Giri et al., 1998). In such cases the expressed PIs are of little use in protecting the plant from the pest.

Polyphagous insects in particular have a pronounced capacity to produce alternative proteinases as a provision for adaptation to the broad range of PIs they encounter during their feeding (Broadway, 1995, 1997). The larvae of *H. armigera* are known to feed on more than 180 plant species (Manjunath et al., 1989). Our strategy has been to characterize the biochemical interaction of PIs with the insect's gut proteinases (HGP) so as to select candidate PIs that are capable of inhibiting the dynamic combination of gut proteinases of this insect (Giri and Kachole, 1998; Giri et al., 1998; Harsulkar et al., 1998, 1999; Patankar et al., 1999, 2001). Our results show that the PIs from WB seeds together are able to inhibit more than 70% of the altered proteinase activity in *H. armigera* fed on WBPIs (Harsulkar et al., 1999). Identification of the individual WBPIs with a demonstrated potency specifically against HGP will allow for expression of the right combination of several PIs ideal for developing and maintaining resistance in the host plants of this pest. The WB PIs identified in this study provide a step toward developing a rationalized strategy for long-term resistance to *H. armigera* via a molecular engineering approach based on sound biochemical principles. Although PIs show high sequence homology of over 80% among themselves, they can still vary considerably in their potential to bind and inhibit a particular proteinase (Zhao et al., 1996). Small variation near the active domain of the inhibitor proteins may potentially alter their biological property and specificity. This variation is essential to inhibit the complement of proteinases present in the insect's gut, as is the case of *H. armigera*, and to withstand proteinases, which have the capability to degrade the inhibitor proteins. The issue is further complicated by the insect's capability to produce alternative proteinases in response to the inhibitor(s) in its diet (Bown et al., 1997, 1998; Gatehouse et al., 1997; Harsulkar et al., 1999; Patankar et al., 2001). After extensive screening of PIs from several host and non-host plants in our earlier work we have identified PIs from WB seeds as the potential candidates based on inhibition of HGP and feeding studies.

In this study we have purified seven WBTIs of which four resemble Kunitz type serine PIs and three showed similarity with Bowman–Birk type serine PIs. Several aspects of their biochemical properties such as their molecular masses, heat stability and susceptibility to inactivation by reducing agents suggest that three of the low molecular mass PIs (TI-5 to TI-7) belong to Bowman–Birk type serine PIs. Kunitz type PIs of WB seeds have been studied earlier but presence of Bowman–Birk type serine PIs in WB has not been reported. MALDI-TOF analysis of the small native WBTI forms and cyanogen bromide cleavage patterns also support that the small TI group (TI-4 – TI-7) which we have identified in WB seed are similar to Bowman–Birk type TIs reported from other plant species (Birk, 1985; Prakash et al., 1996). N-terminal amino acid sequence of 28 and 24 kD TI proteins has confirmed their identity as Kunitz type serine PIs.

Another novelty of the present investigation is that we have described a new protocol using GXCT for identification of inhibitor proteins from crude extract after 2-D gel separation. In proteomics, it is customary to directly use protein spots detected on 2-D gels for their identification by peptide sequencing. Thus this method will facilitate specific identification and characterization of major PI proteins as well as of minor isoinhibitors directly from their mixtures present in plant extract, avoiding tedious purification steps especially from seed proteins.

In summary, we have characterized the inhibitory potential of 7 individual TIs from winged bean against gut proteinase of *H. armigera*. It has been shown that even in a non-host plant such as winged bean not all PIs have equal or strong inhibitory potential against HGP. Of particular significance in this study is the identification of a 28 kD WBTI, which shows at least three fold higher inhibitory activity against HGP than with bovine trypsin. We suggest that such PIs are a good target for future work aimed at enhancing resistance of crop plants to *H. armigera*. Work is now in progress to further characterize isolated WBPI gene products specifically for their in vitro and in vivo effectiveness against *H. armigera* and their subsequent use in developing transgenic chickpea for *H. armigera* resistance.

4. Experimental

4.1. Extraction of WBPIs

Seeds of winged bean (*Psophocarpus tetragonolobus* cv. iiHp Sel 21) were obtained from the National Bureau of Plant Genetic Resources, Akola, Maharashtra, India. Dry seeds were ground to fine powder, defatted and depigmented with several washes of hexane and acetone. The solvents were filtered off and the seed powder was recovered after air-drying. Protein in seed powder was extracted overnight in six volumes of distilled water at 4 °C. The extract was centrifuged twice at 7820 *g* for 30 min at 4 °C, and the final supernatant was collected and stored frozen in aliquots, or freeze-dried.

4.2. Purification of WBTIs

Crude protein extract of WB seeds was heated at 60 °C for 30 min and immediately chilled. The protein precipitated by heat treatment was removed by centrifugation and the supernatant and pellet were analyzed for activity by enzyme assay (inhibition of *H. armigera* gut proteinases and trypsin) and run on SDS–PAGE gels to check for TI activity. The supernatant fraction, which contained most of the PIs present in the crude protein extract, was concentrated and loaded (30 mg) on a Superdex G-75 (Amersham Pharmacia Biotech, Sweden) gel filtration column (80×1 cm). The column and protein were pre-equilibrated with 10 mM Tris–HCl, pH 7.8, which was also used for protein elution with 6 ml/h flow rate. Fractions were analyzed for TI activity by dot-blot assay on X-ray film (Pichare and Kachole, 1994), enzyme assay and on 15% SDS–PAGE gels for TI activity and protein profiles. Two pools were made, of which pool I contained 20–28 kD TI proteins and pool II contained 6–9 kD inhibitors. Pool I or II (1.0 mg protein) was loaded on a 10% native preparative gel for electro-elution (Prep Cell Unit, BioRad, USA). The gel was run at 50 mA for 20 h and fractions of 5.0 ml each were collected at a flow rate of 0.4 ml/min. The eluted protein fractions were analyzed for inhibitor activity against HGP in in vitro assays and for TI activity on gels. Protein in the aliquots was determined by Bradford's method (1976).

4.3. Preparation of HGP

H. armigera larvae were reared on an artificial diet as described earlier (Giri and Kachole, 1998; Harsulkar et al., 1999). Mid-guts isolated by dissecting the larvae were stored at −20 °C until further use. For extraction, the gut tissue was mixed with 1 volume of 0.2 M glycine–NaOH buffer (pH 10) and allowed to stand for 2 h at 4 °C. The gut luminal contents were removed by centrifugation at 7820 *g* rpm for 15 min at 4 °C. The resulting supernatant was analyzed for proteinase activity.

4.4. Proteinase and PI assays

Trypsin and HGP activity was estimated using the chromogenic substrate benzoyl-arginyl *p*-nitroanilide (BApNA) (Erlanger et al., 1964). For the inhibitor assay, a suitable volume of seed protein extract or purified inhibitors was mixed with the HGP or purified bovine trypsin (Sigma, USA) and incubated at 27 °C for 15 min. The residual proteinase activity was then estimated by the method of Erlanger et al. (1964) as described previously (Giri et al., 1998; Harsulkar et al., 1999). One proteinase unit was defined as the amount of enzyme that increases absorbance by 1 OD/min, and one PI unit was defined as the amount of inhibitor that causes inhibition of 1 unit of proteinase activity under the given assay conditions.

4.5. Visualization of TIs

Seed protein extract or purified inhibitors were separated on a 15% SDS–PAGE gel according to the method of Laemmli (1970) without β-mercaptoethanol in the protein sample and also in the gel. Two-dimensional IEFSDS–PAGE was run as described by O'Farrell (1975) with a pH range 4.5 to 7.5 in the IEF gels without adding β-mercaptoethanol. Visualization of TIs after SDS–PAGE or 2-D gel electrophoresis was carried out using the gel-X-ray film contact print technique (Pichare and Kachole, 1994; Mulimani et al., 2002). After electrophoresis, the gel was washed with 2.5% Triton X-100 in 0.1 M Tris–HCl (pH 7.8) for 20 min for three times to remove SDS. The gel was incubated in 0.1% trypsin solution for 10 min, and excess trypsin was rinsed off and the gel was overlaid on an X-ray film for 2–7 min. For weakly active bands, the overlay time was extended, but this will also give rise to a loss of resolution of the more highly active bands. The film was then washed with tap water and inhibitor activity bands were visualized as unhydrolyzed gelatin. In some cases the image polarity was reversed so the active bands appeared dark against a light background (Fig. 1), rather than light against a dark background. The same gel was then washed and stained for protein using Gelcode (Pierce, USA) according to the manufacturer's instructions, or CBB R-250 in 40% methanol and 10% acetic acid, or by silver staining (Rabilloud et al., 1988).

4.6. N-terminal sequencing and MALDI-TOF analysis of WBTIs

WBTIs were separated by 2-D gel electrophoresis and blotted on PVDF membrane. The membrane was stained for protein using Gel-code (Pierce, USA). The

protein spots corresponding to TI activity was identified carefully, the membrane was cut accordingly and used to obtain N-terminal sequence by automated Edman degradation on sequencer (Applied BioSystems, USA).

MALDI-TOF analyses of smaller molecular mass TIs were carried out after eluting individual TI spots from polyacrylamide gel. The gel was crushed using a tissue grinder in ultra-pure sterile water and kept at 4 °C overnight. The suspension was centrifuged and supernatant was collected and dried in a speed-vac. These preparations were used for MALDI-TOF analyses for determination of accurate molecular mass and identification of the protein. Further, these proteins were treated with 0.4 M cyanogen bromide in 70% formic acid in dark for 15 h. The samples were dried in speed-vac, dissolved in 10 μl water and treated with DTT (2 μl of 10 mg/ml) by incubation at 70 °C for 1–2 min. Samples were concentrated using Ziptip C-18 (Millipore, USA) using water, acetonitrile and α-cyano-4-hydroxycinnamic acid as solvents and applied on MALDI-gold plate. The peaks were accumulated and used to generate the data on a PE BioSytems Voyager System 2025.

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