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Protease purification and characterization of a serine protease inhibitor from Egyptian varieties of soybean seeds and its efficacy against *Spodoptera littoralis*

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Abstract: Serine inhibitors have been described in many plant species and are universal throughout the plant kingdom. Trypsin inhibitors are the most common type. In the present study, trypsin and chymotrypsin inhibitory activity was detected in the seed flour extracts of four Egyptian varieties of soybean (*Glycine max*). The soybean variety, Giza 22, was found to have higher trypsin and chymotrypsin inhibitory potential compared to other tested soybean varieties. For this reason, Giza 22 was selected for further purification studies which used ammonium sulphate fractionation and DEAE-Sephadex A-25 column. Soybean purified proteins showed a single band on SDS-PAGE corresponding to a molecular mass of 17.9 kDa. The purified inhibitor was stable at temperatures below 60°C and was active at a wide range of pH, from 2 to 12 pH. The kinetic analysis revealed a non-competitive type of inhibition against trypsin and chymotrypsin enzymes. The inhibitor constant (K_i) values suggested that the inhibitor has higher affinity toward a trypsin enzyme than to a chymotrypsin enzyme. Purified inhibitor was found to have deep and negative effects on the mean larval weight, larval mortality, pupation, and mean pupal weight of *Spodoptera littoralis*. It may be concluded, that soybean protease inhibitor gene(s) could be potential targets for those future studies which are concerned with developing insect resistant transgenic plants.

Key words: protease inhibitors, proteases, soybean, *Spodoptera littoralis*

Introduction

The most serious limiting factor in crop production is pest infestation that leads to massive crop damage. Such damage was estimated at 70% of worldwide crop production where pesticides were not used (Lawrence and Koundal 2002; Oliveira *et al*. 2007). Production of proteinaceous inhibitors that interfere with the digestive biochemistry of insect pests is one of the naturally occurring defense mechanisms in plants. Protease inhibitors reduce the digestive capability of insects by inhibiting proteases of the midgut, thereby arresting the growth and development of the insects (Broadway and Duffey 1986; Délano-Frier *et al*. 2008). Among the proteinaceous inhibitors, serine protease inhibitors are abundant in the Leguminosae (Ussuf *et al*. 2001). The most well known of the plant serine proteinase inhibitors is the soybean Kunitz trypsin inhibitor (SKTI). It is a seed specific protein that is expressed in high amounts during its development. The soybean Kunitz trypsin inhibitor has a molecular weight of 21 kDa. This inhibitor complexes with enzymes and there is a very high association constant (Laskowski and Kato 1980). The inhibitor originally isolated by Kunitz, is one of three active isoforms (Kim *et al*. 1985). There are soybean cultivars whose seeds lack this protein (Jofuko *et al*. 1989), but the soybean seed contains another serine protease inhibitor. This inhibitor is the Bowman-Birk inhibitor (BBI) that inhibits trypsin and chymotrypsin enzymes at independent reactive sites (Birk 1985).

Currently, the main emphasis of plant-PI studies is on identifying potential inhibitors of the target insect's digestive proteases. There is also an emphasis on understanding the dynamic nature of insect midgut proteases at the molecular level (Abe *et al*. 1980). Serine proteases is the major component of the digestive complement of Lepidoptera and among them, trypsin-and/or chymotrypsin-like are the most commonly found proteases (Srinivasan *et al*. 2006).

In the current study, four Egyptian soybean varieties were tested for their potential as trypsin and chymotrypsin inhibitors. Identification and partial characterization of the promising protease inhibitor from soybean was also conducted. The *in vivo* and *in vitro* effects on digestive proteases and the development of *Spodoptera littoralis* was evaluated.

Materials and Methods

Materials

Seeds of soybean (*Glycine max*) varieties were obtained from the Agriculture Research Center, Cairo, and the Faculty of Agriculture, Sohag University, Egypt. Bovine trypsin, chymotrypsin, standard substrates viz., N-a-benzoyl-

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-DL-arginine-p-nitroanilide (BApNA) and N-a-benzoyl-DL-tyrosine-p-nitroanilide (BTpNA), standard inhibitors (SKTI; chymostatine), protein molecular weight markers, acrylamide, bis-acrylamide, and DEAE-Sephadex A-25 were procured from Sigma Chemical Co. (St. Louis, MO, USA).

Purification of soybean protease inhibitor

Crude extract of different seeds was obtained according to Hajela *et al*. (1999) and Abe *et al*. (1980) with some modifications. Finely ground seeds were defatted by using ice-cold acetone (–20°C). After 1 h in acetone, the flour was separated using a Buchner funnel and vacuum. This process was repeated twice. The defatted flour was air dried overnight and then was extracted by homogenisation in a 0.01 M sodium-phosphate buffer (1 : 10 w/v) which had a pH 7.0, and which contained 0.15 M NaCl. Extraction took place for 10–15 min and was then stirred for 2 h at room temperature. The homogenate was then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant (crude extract) was passed through 2–3 layers of cheese cloth, diluted with extraction buffer, and used as the initial source for protease inhibitors as well as for protein estimation in all the screening studies.

The soybean variety (Giza 22) that showed high inhibition activity toward trypsin and chymotrypsin activity, solid ammonium sulfate was added to the supernatant (crude extract) to obtain a precipitate formed at 0–30%, 30–60% and 60–90% saturation with respect to this salt. The pellet was collected in all fractions (F_{0-30}, F_{30-60}, and F_{60-90}) and was dissolved in minimal volume of extraction buffer and dialysed overnight with the same extraction buffer at 4°C, and then lyophilised. At each fraction, the trypsin and chymotrypsin inhibitory activity and protein content were estimated. The F_{30-60} fraction, which corresponds to a 30–60% saturation range, showed a high level of inhibitory activity against the trypsin and chymotrypsin enzymes. This fraction was applied to a DEAE-Sephadex A-25 column (50 × 2 cm column) according to Ramesh Babu and Subrhamanyam (2010), and equilibrated with several bed volumes of 20 mM Tris-HCl buffer (pH 8.0). Clear supernatant obtained after centrifugation, was applied to the column. Fractions of 5 ml were collected at an initial flow rate of 15 ml · h^{-1}. The column was washed with 20 mM Tris-HCl buffer (pH 8.0), with a flow rate of 30 ml · h^{-1}, and eluted by a linear gradient system in which a NaCl concentration was increased up to 0.4 M in 20 mM Tris-HCl (pH 8.0). The chromatography was monitored at 280 and 410 nm. The fractions that exhibited peaks of trypsin inhibitory activity were separately pooled, dialysed and lyophilised.

Estimation of proteases inhibitory activity

Trypsin and chymotrypsin activities were determined using synthetic substrates BApNA and BTpNA, respectively. For the trypsin assay, different volumes of inhibitor crude extracts were added to 20 µg of bovine trypsin in 200 µl of 0.01 M Tris-HCl (pH 8.0) containing 0.02 M $CaCl_2$. Incubation was done at 37°C in a water bath for 15 min. Residual trypsin activity was measured by adding 1 ml of 1 mM BApNA in pre-warmed (37°C) 0.01 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$. Incubation was done at 37°C for 15 min (Erlanger *et al*. 1961). Reactions were stopped by adding 200 µl of 30% glacial acetic acid. After centrifugation, the liberated *p*-nitroaniline in the clear solution was measured at 410 nm. Only 20 µg of trypsin in 200 µl of buffer without crude extract, was considered as the control. Inhibitor activity was calculated by the amount of crude extract required to inhibit 50% of trypsin activity, which is considered as one unit of trypsin inhibition and expressed as trypsin inhibitor units per mg seed protein. All assays were performed in triplicate. The chymotrypsin inhibitor activity was also measured in a similar way except that the substrate used was BTpNA (Bundy 1962, 1963). One millimolar BTpNA was prepared in 0.01 M Tris-HCl (pH 8.0) containing 40% ethanol (Hajela *et al*. 1999).

Protein determination

Protein was determined according to the method of Lowry *et al*. (1951) where bovine serum albumin was used as a standard.

Thermal and pH stability of soybean protease inhibitor

Thermal stability of the purified soybean protease inhibitor (PI) was determined by using 0.1 M Tris-HCl (pH 8.0). Incubation was done at various temperatures ranging from 20 to 100°C (±0.1°C) in a water bath for 45 min. After incubation at various temperatures, samples were cooled at 4°C for 10 min and centrifuged (Kamalakannan *et al*. 1984). The remaining protease inhibitor activity was measured as described previously.

The effect of pH on the inhibitory activities of soybean PI was investigated at different pHs which ranged from 2 to 12 using the following buffers at final concentrations of 0.1 M: glycine-HCl for pH 2 and 3; Na-acetate-acetic acid for 4 and 5; phosphate buffer for 6 and 7; Tris-HCl for 8; glycine-NaOH for 9 and 10, and CAPs buffer for pH 11 and 12. After a 24 h incubation at each pH at room temperature, the residual trypsin inhibitory activities were measured as mentioned earlier. All experiments were carried out in triplicate.

Polyacrylamide gel electrophoresis

A discontinuous buffer system of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 4% stacking gel and a 10% resolving gel, was done at room temperature using the method of Laemmli (1970). Bromophenol blue was used as the tracking dye. The molecular weight markers employed were α-lactalbumin (14.2 kDa) soybean trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 kDa). After electrophoresis, the gels were stained with coomassie brilliant blue R-250 staining solution (0.025% coomassie blue R-250, 40% methanol, 7% acetic acid). The gel was destained with solution I (40% methanol, 7% acetic acid, in distilled water) for 30 min. Next, the gel was placed in destaining solution II (7% acetic acid, 5% methanol in one liter distilled water) for 2 h with intermittent shaking. Destaining was continued until blue bands and a clear background were obtained and then a photograph was made. Molecular weights of unknown proteins were calculated from the standard graph using a regression equation.

Preparation of midgut homogenates of larvae

Gut enzyme extracts from 3rd instar of *S. littoralis* larvae was prepared according to the method of Johnston *et al*. (1993) with some modifications. The midguts were homogenised in ice-cold 0.2 M glycine-NaOH buffer, pH 8, containing 2 mM DTT and 10% PVP (10 guts/ml buffer) (Volpicella *et al*. 2003). The homogenates were kept for 2 h at 10°C and centrifuged at 10,000 rpm for 15 min at 4°C. The resultant supernatant was used as a source of gut proteases and stored at 20°C.

Inhibitory potential of soybean PI against gut extracts from *S. littoralis* larvae

Three to four different concentrations of protease inhibitor from the selected soybean variety, standard SKTI and chymostatin were used to determine the IC_{50} values against proteases of *S. littoralis* midgut extract. All the inhibitors were mixed with 20 µl of *S. littoralis* gut extract. To start the reaction, the mixture was incubated at 37°C for 15 min, before the addition of the substrate (Lee and Anstee 1995). Residual activity was determined spectrophotometrically at 410 nm. The results were expressed as IC_{50} or % inhibition relative to the controls without inhibitor. The enzyme activity was expressed as µmol of *p*-nitroaniline released/min/mg protein. All *in vitro* assays were carried out in triplicate.

Kinetics of inhibitory activity against *S. littoralis* from soybean PIs

The mechanism of inhibition (competitive or non-competitive) against the gut enzymes of *S. littoralis*, was determined at different substrate concentrations and at a fixed concentration of inhibitor. Using Lineweaver-Burk plots, in which the inverse of the initial velocity was plotted against the inverse of the substrate concentration in the absence of inhibitor and in the presence of inhibitor, the Michaelis constant (K_m), the maximum rate of reaction (V_{max}), and the inhibitor constant (K_i) were calculated. The reaction velocity was expressed as (µmol pNA released/min/mg protein).

In vivo effect of soybean PI on larvae of *S. littoralis*

For feeding studies, the protease inhibitors from the selected soybean variety (Giza 22), partially purified by ammonium sulfate saturation (at 30–60%), was incorporated into the artificial diet at three concentrations (w/w) of 0.1%, 0.5%, and 1.0%, as suggested by Johnston *et al*. (1993). A diet without added PI was used as the control diet. The tested protease inhibitor was dissolved in a small amount of distilled water before incorporation into the diet. All diets were incubated overnight at 4°C before being offered to the larvae. Starved third instar larvae were released into the rearing trays containing either the control diet (or) an inhibitor-containing diet. Larval weights were recorded at the same time every day. Fresh diet was added when the larvae required it or on alternate days. Data on larval mortality, pupation, and pupal weight were recorded. Three replications of ten larvae each were used for each treatment and data were statistically analysed.

Statistical analysis

All data were examined using analysis of variance (ANOVA). Comparisons of the means of the larval weight and other parameters were made using Duncan's multiple range test (DMRT) at a 5% level of probability.

Results

Trypsin inhibitory activity

The inhibitory potential of the soybean varieties' crude extracts against the trypsin and chymotrypsin standard enzymes were estimated and presented in table 1. All the soybean varieties were found to contain trypsin inhibitor activity with inter-varietal variation with a mean of 178.74 TIU/mg proteins. The highest trypsin inhibitor activity; 338.72 TIU/mg protein, was observed in the Giza 22 variety. The lowest trypsin inhibitor activity was observed in the Giza 111 variety (40.87 TIU/mg protein).

The chymotrypsin inhibitory potential was detected in all tested varieties with a mean CIU/mg protein of 40.06. The soybean variety, Giza 22, exhibited the highest chymotrypsin inhibitory activity of 52.94 CIU/mg protein. The Giza 111 variety had the lowest chymotrypsin inhibitory activity (25.89 CIU/mg protein).

Purification of soybean PI

The soybean variety, Giza 22, showed the high trypsin and chymotrypsin inhibition activities. For this reason, it was selected for further purification steps. A summarisation of the yield of protease inhibitor activity and the fold of purification of the seed of the soybean variety, Giza 22, can be found on table 2. It was found that $F_{30\text{-}60}$ $(NH_4)_2SO_4$ (w/v) saturation was efficient for precipitating the protease inhibitor in both varieties compared to other fractions for which the $F_{30\text{-}60}$ $(NH_4)_2SO_4$ was then applied to ion exchange chromatography, DEAE-Sephadex A25 column. The fold of purification obtained for $F_{30\text{-}60}$ $(NH_4)_2SO_4$ was 2.63 times that of the crude extract and the recovery percentage was 72.32%. The specific activity of the purified fraction was 3.46 times that of the crude extract and the recovery percentage was 47.76%. The DEAE-Sephadex column yielded different peaks in which only three peaks (PI, PII, PIII) exhibited high inhibitor activity against bovine pancreatic trypsin with a 5.47, 5.18, and 6.62 times fold of purification for PI, PII, and PIII, respectively, compared to the crude extract (Fig. 1).

Table 1. Serine inhibitory activity and protein content of soybean (*Glycine max*) cultivars

Cultivar	Protein content [mg/g seed]	TIU/g seed	TIU/mg protein	CIU/g seed	CIU/mg protein
Giza 22	41.8	14,158.69	338.72	2,212.89	52.94
Giza 111	79.8	3261.09	40.87	2,066.08	25.89
Hybrid 30	42.4	10,086.91	237.90	1,990.36	46.94
Hybrid 32	89.4	8,712.81	97.46	3,080.88	34.46
Mean	63.35	9,054.88	178.74	2,337.55	40.06

Table 2. Purification steps of protease inhibitors from soybean (*Glycine max*), Giza 22

Step	Total protein [mg]	Total trypsin inhibitory unit [TIU]	Specific activity [TIU/mg protein]	% recovery	Fold purification
Crude extract	16,720.92	5,425,102.49	324.45	100.00	1.00
$F_{30\text{-}60}$ $(NH_4)SO_{4\ ppt}$	4,597.95	3,923,434.12	853.30	72.32	2.63
DEAD-Sepadex A-25	603.29	1,873,832.14	3,106.01	47.76	3.46
PI	24.42	414,866.44	16,989.87	22.14	5.47
PII	35.17	565,897.31	16,089.13	23.02	5.18
PIII	12.72	261,586.97	20,561.79	13.96	6.62

ppt – purified protein; PI, PII, PIII – peaks I, II, III

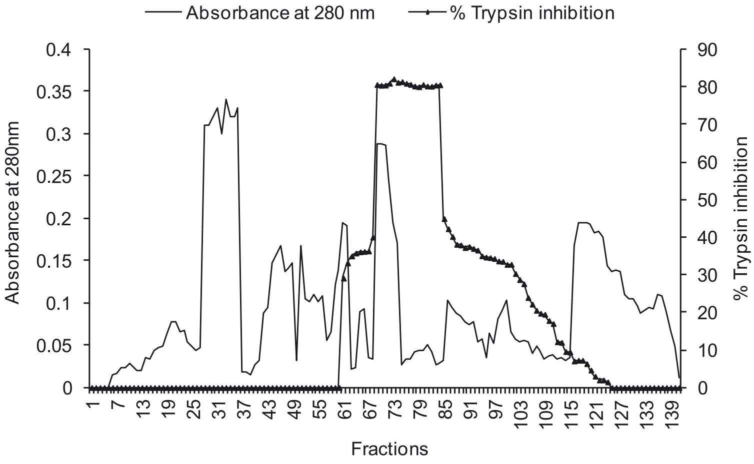

Fig. 1. Elution profile of DEAE-Sephadex A-25 of $F_{30–60}$ from seeds of the soybean cultivar, Giza 22

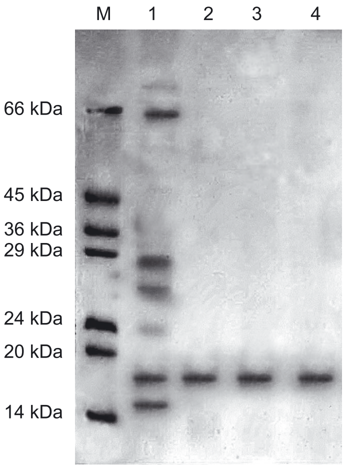

Fig. 2. SDS-PAGE analysis of soybean PI fractions, stained with coomassie blue: M – molecular weight marker; 1 – $F_{30–60}$ fraction; 2 – PI; 3 – PII; 4 – PIII

Molecular weight

The $F_{30\text{-}60}$ $(NH_4)_2SO_4$ fraction and DEAE-Sephadex products of soybean inhibitor proteins were resolved in 10% SDS-PAGE (Fig. 2). The $F_{30\text{-}60}$ $(NH_4)_2SO_4$ fraction was resolved into six protein bands ranging from 14.9 to 64.3 kDa. The PI, PII, and PIII fractions were resolved in a single band of 17.9 kDa.

Thermal and pH stability

The results for a 45 min incubation of soybean PI at temperatures varying from 20 to 100°C, are illustrated in figure 3. The inhibitor activity of soybean PI against both the trypsin and chymotrypsin enzymes, was found to be stable at temperatures below 60°C while the inhibitor lost 45% of its activity at 80°C. It was at 100°C, that soybean PI fully lost its inhibitory potential.

The stability of soybean PI at a pH level which ranged from 2 to 12 is presented in figure 4. It was found, that soybean PI is active over a wide range of pH. The range was from 2 to 11. Maximal inhibitory activity was recorded at a pH of 8. The inhibitor lost around 50% of its activity at a pH of 2 (highly acidic) and at a pH of 11 (highly alkaline). The inhibitor was unstable at a pH of 12 and totally lost its inhibitory potential against both the trypsin and chymotrypsin enzymes.

In vitro effect of soybean PI on serine proteases from *S. littoralis* midgut

Different concentrations (1–20 μg/ml) of soybean PI, the standard SKTI and the standard chymotrypsin inhibitor (chymostatin) were used to determine the IC_{50} of prote-

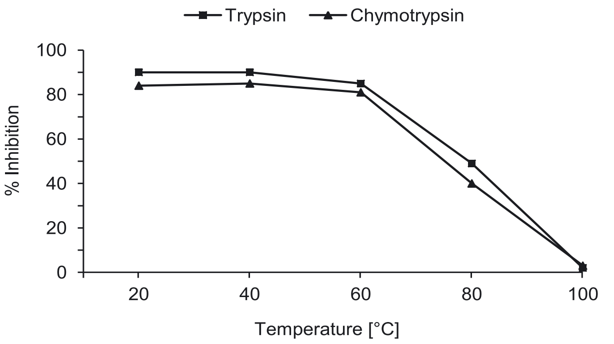

Fig. 3. Thermal stability profile of the soybean PI (Giza 22) against trypsin-like and chymotrypsin-like enzyme activity

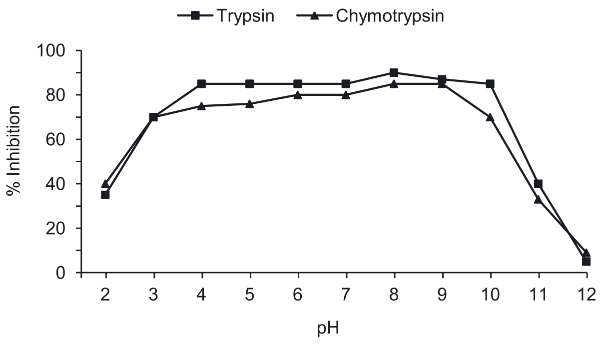

Fig. 4. Stability profile of soybean PI (Giza 22) at different pH against trypsin-like and chymotrypsin-like enzyme activity

Table 3. The *in vitro* effect of different protease inhibitors on activity of serine proteases extracted from the midgut of third instar larvae of *Spodoptera littoralis*

Inhibitor	IC_{50} [μg/ml] trypsin (BA*p*NA)	IC_{50} [μg/ml] chymotrypsin (BT*p*NA)
Soybean (Giza 22)	2.84	5.12
SKTI (trypsin specific)	2.98	> 100
Chymostatin (chymotrypsin specific)	> 100	2.68

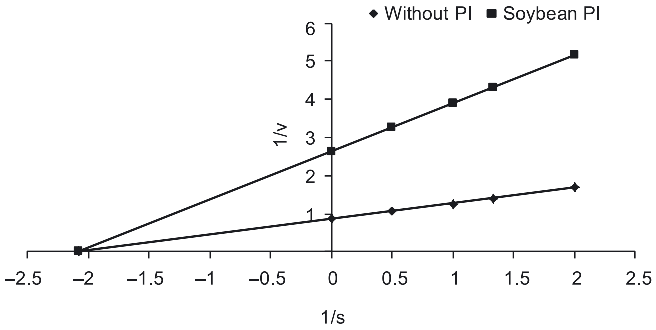

Fig. 5. Kinetic studies on the inhibition of trypsin-like activity of *Spodoptera littoralis* larval gut by soybean (Giza 22) PIs

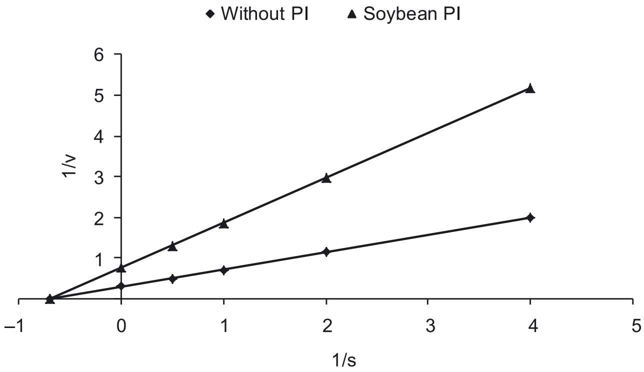

Fig. 6. Kinetic studies on the inhibition of chymotrypsin-like activity of *Spodoptera littoralis* larval gut by soybean (Giza 22) PIs

ases of *S. littoralis* midgut extracts. All the assayed results showed linear inhibition of proteolytic activity with an increasing of the inhibitor, until saturation was achieved. Calculated values of IC_{50} are presented in table 3. The inhibitory potential of the soybean (Giza 22) PI was higher against trypsin-like enzyme with IC_{50} of 2.84 μg/ml than chymotrypsin-like enzyme with IC_{50} of 5.12 μg/ml. The inhibitory potential of soybean against the trypsin-like enzyme was almost equal to that of the standard trypsin inhibitor, SKTI (IC_{50} = 2.98 μg/ml). The chymotrypsin inhibitor activity of soybean PI was half that of the chymotrypsin standard inhibitor, chymostatin (IC_{50} = 2.68 μg/ml).

Table 4. Kinetic analysis of the midgut proteases of the third instars larvae of *Spodoptera littoralis* against synthetic serine protease substrates

Proteinase inhibitor	Trypsin-like enzyme			Chymotrypsin-like enzyme		
	K_m [mM]	V_{max} [μmol pNA released/ min/mg protein]	K_i [μM]	K_m [mM]	V_{max} [μmol pNA released/ min/mg protein]	K_i [μM]
Without inhibitor	0.48	1.15	–	1.47	3.24	–
Soybean (Giza 22) PI	0.48	0.38	1.87×10^{-4}	1.47	1.32	7.48×10^{-4}

PI – protease inhibitor; K_m – the Michaelis constant; V_{max} – the maximum rate of reaction; K_i – the inhibitor constant

Table 5. *In vivo* effects of soybean (Giza 22) PI on the growth and development of the third instar larvae of *Spodoptera littoralis*

Treatments	Mean fresh weight of the larvae (mg ±SD)						Larval mortality [%]	Pupation [%]	Mean pupal weight [mg ±SD]
	D1*	D3	D5	D7	D9	D11			
The control (Chick pea only)	24.80 a ±0.95	135.15 a ±1.35	246.30 a ±1.34	338.6 a ±8.70	398.25 a ±24.58	pupated	3.33 ±0.34	96.67 ±2.78	260.25 a ±3.15
Soybean PI (0.1%)	24.82 a ±0.78	133.88 a ±7.34	210.52 b ±10.62	222.54 b ±16.82	276.85 b ±22.76	pupated	20.00 ±2.24	80.00 ±3.02	200.64 b ±13.88
Soybean PI (0.5%)	25.24 a ±0.76	129.88 a ±8.12	148.54 c ±14.34	168.86 c ±23.54	198.46 c ±33.42	228.38a ±35.22	53.34 ±4.34	46.66 ±2.42	146.62 c ±19.36
Soybean PI (1.0%)	25.10 a ±0.78	130.48 a ±10.22	140.45 c ±18.90	175.52 c ±31.88	205.14 c ±29.34	229.68 a ±37.86	86.66 ±7.02	13.34 ±1.78	130.44 c ±22.76
LSD	1.52	10.42	20.46	31.22	30.06	29.88	–	–	19.06

Means in a column followed by the same letter are not significantly different
*the first day of treatment
PI – protease inhibitor; LSD – low significant difference

Kinetic analysis of the midgut trypsin-like and chymotrypsin-like enzymes

Figures 5 and 6 show the Lineweaver-Burk double reciprocal plots of the inhibition of the midgut protease by soybean PI. The inhibition was of the non-competitive type for both enzymes as there was a decrease in V_{max} values with no change in K_m values compared to the reaction in the absence of inhibitor. Soybean PI showed higher affinity toward the midgut trypsin-like enzyme compared to the chymotrypsin-like enzyme with K_i values of 1.87×10^{-4} and 7.48×10^{-4} μM for trypsin-like and chymotrypsin-like enzymes, respectively (Table 4).

In vivo effects of soybean PI on *S. littoralis* larvae

The antimetabolic effects of soybean PI isolated from the soybean variety, Giza 22, was tested against the third instar larvae of *S. littoralis* by incorporating the $F_{30\text{-}60}$ $(NH_4)_2SO_4$ protein (at levels of 0.1, 0.5, and 1.0%) into the artificial diet. The mean larval weight, larval mortality, pupation, and mean pupal weight were recorded and presented in table 5. Feeding larvae a diet containing soybean PI resulted in a reduction in larval weight, compared to the control. The larval weight reduction caused by soybean PI was noticed only after five days of treatment. On the ninth day of treatment, the larvae fed the artificial diet without an inhibitor (the control), gained 398±24.58 mg which was significantly higher than those fed an artificial diet containing soybean PI at any level. The highest larval weight reduction was obtained when larvae were fed soybean PI at a level of 0.5 and 1.0%, with no significant difference between the two levels. After day nine, the control larvae and the larvae fed on soybean 0.1% were pupated, while pupation was delayed for larvae that were fed soybeans at a 0.5 and 1.0% level.

Feeding larvae a diet containing soybean PI caused larval mortality which ranged from 20.00 to 86.66%. The maximum mortality of 86.66% was achieved when larvae were fed a diet containing 1.0% of soybean PI.

The soybean PI treatment caused reduction in pupation and pupal weight. The pupal weight was reduced in a dose dependent manner. The highest weight reduction was observed when larvae were fed a diet containing 1.0% of maize PI.

Discussion

In response to insect attack, plants synthesise various proteinaceous and non-proteinaceous compounds, amongst these, PIs are the most-studied class of plant-defense proteins. The primary site of action of PIs is the insect larva digestive system (Ghoshal *et al*. 2001). The defensive capacities of plant PIs rely on the inhibition of proteases present in the gut of an insect. This process causes a reduction in the availability of amino acids necessary for the growth and development of the insect (De Leo *et al*. 2000). Protease inhibitors do not pose a direct problem for humans, because the foods that contain high levels of these proteins are cooked, which inactivates the inhibitors (Ryan 1990).

Serine proteases were found to dominate the lepidopteran insect's larval gut environment, and contribute to 95% of their total (Srinivasan *et al*. 2006). Inhibitors of these serine proteases have been described in many plant species and the inhibitors are universal throughout the plant kingdom. The trypsin inhibitor is the most common type (Lawrence and Koundal 2002). The serine protease inhibitors are found in almost all plants. In the bicots, the family Leguminosae and Solanaceae have the largest number of species with serine inhibitors. Gramineae has the largest number of species with these inhibitors in monocots (Mendoza-Blanco and Casaretto 2012). In the constant search for new sources of protein with inhibition potential against insect proteases, the current study evaluated the serine inhibitor activity in the seed flour extracts of four Egyptian varieties of the soybean (*G. max*). All the tested varieties were found to have trypsin and chymotrypsin inhibitor activity with significant intervarietal variation. The chymotrypsin inhibitor activity was low when compared to the trypsin inhibitor activity of different varieties. The soybean variety, Giza 22, was found to have higher trypsin and chymotrypsin inhibitory potential compared to other tested varieties. For further purification studies, Giza 22 was selected. Kollipara *et al.* (1994) isolated and characterised trypsin and chymotrypsin inhibitors from the wild perennial *Glycine* species of soybean. Significant variations were found between different species of *Glycine*. The conclusion was that most of the trypsin inhibitors found in the wild perennial species had weak chymotrypsin inhibitor activity.

In our study, the trypsin inhibitors from the soybean variety, Giza 22, was purified by ammonium sulfate precipitation and ion exchange chromatography on DEAE-Sephadex A-25. These techniques are identical to that followed for the purification of the trypsin inhibitor and chymotrypsin inhibitor in other plant species: *Crotalaria pallida* (Gomes *et al*. 2005), *Acacia senegal* (Ramesh Babu and Subrhamanyam 2010), and *A. nilotica* (Ramesh Babu *et al*. 2012). The Kunitz type inhibitor was isolated from soybean. Extraction was done using HCl and NaOH and DEAE-cellulose (Rackis *et al*. 1959). The Bowman-Birk type inhibitor was purified by using 60% ethanol, CM-cellulose, and DEAE-cellulose chromatography (Birk 1961). Odani and Ikenaka (1977) used 60–80% ethanol for isolating PIs from soybean. Most of the PIs (serine and cysteine type) are isolated and purified by ammonium sulfate precipitation, ion-exchange chromatography (DEAE and CM-cellulose column), gel filtration chromatography (Sephadex column), and affinity column chromatography (Trypsin-Sepharose or agarose) along with reverse phase HPLC (high performance liquid chromatography) and FPLC (fast protein liquid chromatography) (Abe *et al*. 1987; Misaka *et al*. 1996; Srinivasan *et al*. 2006; Rai *et al*. 2008).

The crude soluble protein extracts obtained from the Giza 22 seeds were initially precipitated using ammonium sulphate at 30, 60, and 90% saturation. Then, the three fractions were tested for their trypsin and chymotrypsin inhibitor activity. The $F_{30\text{-}60}$ $(NH_4)_2SO_4$ protein exhibited strong inhibitory activity against trypsin-like and chymotrypsin-like enzymes, compared to other fractions. The same results were also reported in other plant species e.g. chickpea (Kansal *et al*. 2008), *A. senegal* (Ramesh Babu and Subrhamanyam 2010), and *A. nilotica* (Ramesh Babu *et al*. 2012). The $F_{30\text{-}60}$ protein was then applied to ion exchange chromatography, DEAE-Sephadex A-25 column, and the retained peak was assayed against the trypsin enzyme. Three peaks assigned as PI, PII, and PIII showed high inhibitory activity against bovine pancreatic trypsin. This purification procedure resulted in a purification fold which ranged from 5.18 to 6.62 folds and a recovery percentage which ranged from 13.69 to 23.02%. The fold of purification obtained in this study was in the same range as that obtained by using the same procedure in other plant species (Odei-Addo 2009; Ramesh Babu and Subrhamanyam 2010; Ramesh Babu *et al*. 2012). Protease inhibitor purification methods reported in the research on legumes, achieved purification levels which ranged from 19 to 489, and yield which ranged from 1.3 to 69.7%. The purification levels and yields were: 489×, 4.16% for kidney bean (Godbole *et al*. 1994), 19×, 1.3% for *P. mungo* (Hajela *et al*. 1999), 116.2× , 7.6% for *Dimorphandra mollis* (Macedo *et al*. 2002), 246×, 95×, 2.9% for *Prosopis juliflora* (Oliveira *et al*. 2002); and 29×, 3.24% for *Terminalia arjuna* (Rai *et al*. 2008). The low level of purification achieved in this study may be due to the high concentration of the inhibitor in the seed, as suggested by Prabhu and Pattabiraman (1980) and Ramesh Babu *et al*. (2012).

The purification steps of the soybean protein extracts were observed in 10% SDS-PAGE and resolved in protein bands ranging from 14.9 to 64.3 kDa. The PI, PII, and PIII fractions were resolved in a single band of 17.9 kDa. The size of plant protease inhibitor proteins varied from 4 to 85 kDa with the majority in the range of 8–20 kDa (Macedo and Freire 2011). Different molecular mass was reported for protease inhibitors from different plant resources e.g. cowpea (18.5 kDa), soybean (19 kDa), mustard seed (20 kDa), and *Cajanus cajan* (14 kDa) (Lawrence and Nielson 2001; Mandal *et al*. 2002; Haq *et al*. 2004).

The thermostability of the purified inhibitors was also studied. According to Hamato *et al*. (1995), most of plant protease inhibitors are active at temperatures up to 50°C. In the present study, the soybean PI were found to be stable at temperatures below 60°C while the inhibitors lost 45% of their activity at 80°C. The inhibitors totally lost their activity at 100°C. Soybean Kunitz trypsin inhibitor was reversibly denatured by short heating to 80°C and irreversibly denatured by heating at 90°C while BBI from soybean showed no loss in activity at 105°C and the inhibitor was stable in acid medium (Kunitz 1945). It was reported by DiPietro and Liener (1989), that soy extract lost inhibitory activity most rapidly, but purified BBI was heat stable as compared to purified SKTI in soya extract. Similarly, Babu *et al*. (2012) found that inhibitor extracted from *A. nilotica* was stable up to 60°C. The stability of the inhibitor at high temperatures may be attributed to its rigid and compact protein structure stabilised by a number of disulfide linkages, as suggested for protease inhibitor from pea seeds (Sierra *et al*. 1999).

The pH stability-results indicated that inhibitors were active at a wide range of pH: from 2 to 11 with maximum activity recorded at pH 8, while the inhibitors were unstable at extreme acidic pH (pH 2) and extreme alkaline pH (pH 11 and 12). Soybean Kunitz trypsin inhibitor retains its activity from pH 1 to 12 (Hamato *et al*. 1995) In general, all the protease inhibitors isolated from plants have a wide pH range of from 2 to 10 (Bijine *et al*. 2011). Many enzyme inhibitors in seeds are presented in multiple molecular forms which may differ considerably in their PI values. The amino acids composition of plant protease inhibitors is enriched in cysteine residues that are significant in the formation of disulfide bridges and in conferring stability to heat, pH changes, and proteolysis (Macedo and Freire 2011). In the current study, the stability of the inhibitors isolated from soybean over a wide range of pH, might suggest efficiency in controlling a variety of phytophagous insects that have a gut environment variation e.g. the acidic condition in Homoptera and Coleoptera and the alkaline condition in Lepidoptera.

The inhibitor assays of purified protein extracted from soybeans against the midgut crude extract of *S. littoralis* revealed a linear inhibition of proteolytic activity when increasing the inhibitor until saturation. Soybean PI was more active against the trypsin enzyme than against the chymotrypsin enzyme.

For a better understanding of soybean inhibitor interaction with trypsin enzymes, a study of trypsin kinetic was performed, using the midgut extracts as source of enzymes. The kinetic analysis revealed a non-competitive type of inhibition for both inhibitors against both enzymes. Soybean PI was found to have a higher affinity towards the trypsin enzyme than towards the chymotrypsin enzyme. Similar inhibition patterns were also reported for inhibitor from other plant species e.g. *A. sengal* (Babu and Subrhamanyam 2010) and *A. nilotica* (Ramesh Babu *et al*. 2012). Inhibitors from capsicum demonstrated promising *in vitro* inhibition of the gut protease activity of *Helicoverpa armigera*, exhibiting more affinity towards the trypsin-like protease than towards the chymotrypsin-like protease (Tamhane *et al*. 2005).

Disruption of amino acid metabolism by inhibition of protein digestion has been a key target for use in insect control (Hilder and Boulter 1999). Protease inhibitors have been evaluated as natural control agents against herbivorous insects, and have been shown to reduce the digestive proteolytic enzyme activity and/or larval development in different species of Coleoptera and Lepidoptera (Macedo *et al*. 2002; Gomes *et al*. 2005; Chen *et al*. 2007; Ramesh Babu *et al*. 2012; Aghaal *et al*. 2013). However, pests are able to adapt to the presence of an inhibitor. The pests are able to modify the composition of digestive proteases. The concentration can be altered or the expression of novel proteases induced (Jongsma *et al*. 1995). Therefore, we studied, the antimetabolic effect of soybean PI against the 3rd instar larvae of *S. littoralis*, by incorporating the $F_{30\text{-}60}$ protein of the purified inhibitor into an artificial diet. The results indicated that soybean PI has deep and negative effects on the mean larval weight, though this effect could not be observed until five days after treatment. The mean weight of larvae fed a standard chick pea diet, was significantly greater than the mean weight of larvae fed a diet containing 0.2 or 0.5% (w/v) SKTI (McManus and Brugess 1995). Similarly, Broadway (1986) observed a significant reduction in the growth and development of the larvae of *Helicoverpa zea* and *Salix exigua* when larvae were fed with soybean trypsin inhibitor and potato protease inhibitor II. Johnston *et al*. (1993) found that after 14 days, the biomass of larvae fed SKTI (0.047 mM), was more than 50% lower than that of the control larvae. In this study, the soybean inhibitor caused a two-day delay in the larval period when a high concentration of 0.5 and 1.0% were used. McManus and Brugess (1995) reported a delayed larval period of *Spodoptera litura*, by one day, when larvae were fed soybean trypsin inhibitor at 0.2 and 0.5% (w/v). The delay of the larval period caused by the soybean inhibitor in this study was accompanied by an increased larval mortality, reduced pupation percentage and reduced pupal weight. Faktor and Raviv (1997) reported that larvae of *S. littoralis* fed a diet containing 2% soybean Bowman-Birk inhibitor (SBBI) exhibited slower biomass production, when compared to the controls. However, these larvae reached normal weight, and delay in growth was not accompanied by mortality. All the tested larvae pupated properly. The mean pupal weight was similar to the controls.

In conclusion, the in vivo and in vitro results of the current study uniquely demonstrate that protease inhibitory proteins isolated from the soybean variety, Giza 22, are very effective in inhibiting the development of *S. littoralis*, and also the gut proteases of *S. littoralis*. The current study suggests that soybean PI gene(s) could be potential targets for future studies which concern the development of insect resistant transgenic plants.

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