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

Purification of soybean protease inhibitor

Crude extract of different seeds was obtained accord-
ing 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 homogeni-
sation 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 super-
natant (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 pro-
etin estimation in all the screening studies.

The soybean variety (Giza 22) that showed high inhi-
bition activity toward trypsin and chymotrypsin activity,
solid ammonium sulfate was added to the supernatant
(cruude 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 (F0-30, F30-60, and
F60-90) 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 F30-60 fraction, which corre-
sponds to a 30–60% saturation range, showed a high level
of inhibitory activity against the trypsin and chymotryp-
sin enzymes. This fraction was applied to a DEAE-Sepha-
dex A-25 column (50 × 2 cm column) according to Ra-
mesh Babu and Subramaniam (2010), and equilibrated
with several bed volumes of 20 mM Tris-HCl buffer (pH
8.0). Clear supernatant obtained after centrifugation, was
applied to a DEAE-Sephadex A-25 column (50 × 2 cm column) according to Ramesh Babu and Subramaniam (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 exhib-
ted 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, respec-
tively. For the trypsin assay, different volumes of inhibi-
tor 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
CaCl₂. Incubation was done at 37°C in a water bath for
15 min. Residual trypsin activity was measured by add-
ing 1 ml of 1 mM BAPNA in pre-warmed (37°C) 0.01 M
Tris-HCl buffer (pH 8.0) containing 0.02 M CaCl₂. Incuba-
tion 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-nitroani-
line 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 cal-
culated 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
tripleic. 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 Low-
ry 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 inhibi-
tor (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 mea-
sured 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 CAP’s buffer for pH 11
and 12. After a 24 h incubation at each pH at room tem-
perature, the residual trypsin inhibitory activities were
measured as mentioned earlier. All experiments were car-
ried 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
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), trypsin-
ogen (24 kDa), carbonic anhydrase (29 kDa), glyceralde-
hydes-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 0°C and centrifuged at 10,000 rpm for 15 min at 4°C. The 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 IC₅₀ 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 IC₅₀ 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 (*Kₘ*), 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 F₃₀₋₆₀ (NH₄)_2SO₄ (w/v) saturation was efficient for precipitating the protease inhibitor in both varieties compared to other fractions for which the F₃₀₋₆₀ (NH₄)_2SO₄ was then applied to ion exchange chromatography, DEAE-Sephadex A25 column. The fold of purification obtained for F₃₀₋₆₀ (NH₄)_2SO₄ 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

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

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein [mg]</th>
<th>Total trypsin inhibitory unit [TIU]</th>
<th>Specific activity [TIU/mg protein]</th>
<th>% recovery</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>16,720.92</td>
<td>5,425,102.49</td>
<td>324.45</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>F30-60 (NH4)2SO4 ppt</td>
<td>4,597.95</td>
<td>3,923,434.12</td>
<td>853.30</td>
<td>72.32</td>
<td>2.63</td>
</tr>
<tr>
<td>DEAE-Sephadex A-25</td>
<td>603.29</td>
<td>1,873,832.14</td>
<td>3,106.01</td>
<td>47.76</td>
<td>3.46</td>
</tr>
<tr>
<td>PI</td>
<td>24.42</td>
<td>414,866.44</td>
<td>16,989.87</td>
<td>22.14</td>
<td>5.47</td>
</tr>
<tr>
<td>PII</td>
<td>35.17</td>
<td>565,897.31</td>
<td>16,089.13</td>
<td>23.02</td>
<td>5.18</td>
</tr>
<tr>
<td>PIII</td>
<td>12.72</td>
<td>261,586.97</td>
<td>20,561.79</td>
<td>13.96</td>
<td>6.62</td>
</tr>
</tbody>
</table>

Molecular weight

The F30-60 (NH4)2SO4 fraction and DEAE-Sephadex products of soybean inhibitor proteins were resolved in 10% SDS-PAGE (Fig. 2). The F30-60 (NH4)2SO4 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.

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 IC50 of prote-
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 soybean against 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).

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

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

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*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>trypsin (BA(_{p})NA)</th>
<th>chymotrypsin (BT(_{p})NA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean (Giza 22)</td>
<td>2.84</td>
<td>5.12</td>
</tr>
<tr>
<td>SKTI (trypsin specific)</td>
<td>2.98</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Chymostatin (chymotrypsin specific)</td>
<td>&gt; 100</td>
<td>2.68</td>
</tr>
</tbody>
</table>

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

![Fig. 6. Kinetic studies on the inhibition of chymotrypsin-like activity of *Spodoptera littoralis* larval gut by soybean (Giza 22) PIs](image)
weight reduction was obtained when larvae were fed soybean diet containing soybean PI at any level. The highest larval weight reduction (398±24.58 mg) was significantly higher than those fed an artificial diet without an inhibitor (the control), gained 135.15±33.42 mg. On the ninth day of treatment, the larvae fed the artificial diet 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 first day of treatment, the larvae fed the artificial diet containing soybean PI resulted in maximum mortality of 86.66%. The difference between the two levels. After day nine, the control larvae and the larvae fed soybeans at a 0.5 and 1.0% level. The maximum reduction in the availability of amino acids necessary for protein synthesis was observed 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 soybean 0.1% were pupated, while pupation was delayed for larvae that were fed soybeans at a 0.5 and 1.0% level.

**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 larval digestive system. In vivo effects of soybean PI on the growth and development of the third instar larvae of Spodoptera littoralis

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mean fresh weight of the larvae (mg ±SD)</th>
<th>Larval mortality [%]</th>
<th>Pupation [%]</th>
<th>Mean pupal weight (mg ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The control (Chick pea only)</td>
<td>24.80 ±0.95</td>
<td>3.33</td>
<td>96.67</td>
<td>260.25 ±10.42</td>
</tr>
<tr>
<td>Soybean PI (0.1%)</td>
<td>25.24 ±0.76</td>
<td>20.00</td>
<td>80.00</td>
<td>200.64 ±13.88</td>
</tr>
<tr>
<td>Soybean PI (0.5%)</td>
<td>25.10 ±0.78</td>
<td>228.38</td>
<td>46.66</td>
<td>146.62 ±19.36</td>
</tr>
<tr>
<td>Soybean PI (1.0%)</td>
<td>25.10 ±0.78</td>
<td>228.38</td>
<td>46.66</td>
<td>146.62 ±19.36</td>
</tr>
</tbody>
</table>

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
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 bicos, 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 inter-varietal 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), Acaia senegal (Ramesh Babu and Subhramanym 2010), and A. nilotica (Ramesh Babu et al. 2012). The Kunitz type inhibitor was isolated from soybean. Extraction was done using HCI and NaOH and DEAE-cellulose (Rakkis 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 Pls from soybean. Most of the Pls (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 F30-60 (NH4)2SO4 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 Subhramanym 2010), and A. nilotica (Ramesh Babu et al. 2012). The F30-60 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 Subhramanym 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: 498×, 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 soy 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 F30-60 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 inhibitor 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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References


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