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Isolation and purification of a papain inhibitor from Egyptian genotypes of barley seeds and its *in vitro* and *in vivo* effects on the cowpea bruchid, *Callosobruchus maculatus* (F.)

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ABSTRACT

The cysteine inhibitors that are known as cystatin have been identified and characterized from several plant species. In the current study, 44 barley (*Hordeum vulgare*) genotypes including 3 varieties and 41 promising lines were screened for their potential as protease inhibitors. The barley genotypes showed low inhibitory activity against trypsin and chymotrypsin enzymes with a mean of 4.15 TIU/mg protein and 4.40 CIU/mg protein. The barley variety, Giza 123, showed strong papain inhibitory activity of 97.09 PIU/mg proteins and was subjected for further purification studies using ammonium sulfate fractionation and DEAE–Sephadex A-25 column. Barley purified proteins showed two bands on SDS-PAGE corresponding to a molecular mass of 12.4–54.8 kDa. The purified barley PI was found to be stable at a temperature below 80 °C and at a wide range of pH from 2 to 12. Barley PI was found to have higher potential inhibitory activity against papain enzyme compared to the standard papain inhibitor, E-64 with an IC_{50} value of 21.04 μg/ml and 25.62 μg/ml for barley PI and E-64, respectively. The kinetic analysis revealed a non-competitive type of inhibition with a *Ki* value of 1.95×10^{-3} μM. The antimetabolic effect of barley PI was evaluated against *C. maculatus* by incorporating the F_{30-60} protein of the purified inhibitor into the artificial diet using artificial seeds. Barley PI significantly prolonged the development of *C. maculatus* in proportion to PI concentration. Barley PI significantly increased the mortality of *C. maculatus* and caused a significant reduction in its fecundity. On the other hand, barley PI seemed to have non-significant effects on the adult longevity and the adult dry weight. The *in vitro* and *in vivo* results proved the efficiency of the papain inhibitory protein isolated from barley as a tool for managing the cowpea bruchid, *C. maculatus*.

1. Introduction

Insect proteinases are a group of hydrolytic enzymes that are involved in digestive processes, proenzyme activation, liberation of physiologically active peptides, complement activation, and inflammation processes among others [1]. The proteinases are classified according to the presence of specific amino acid residues at their active side and their mechanism of catalysis in four groups: (1) serine proteinases; (2) cysteine proteinases; (3) aspartic proteinases, and (4) metalloproteinases [2,3]. Serine and cysteine proteinases are the two major proteinase classes in the digestive systems of phytophagous insects [4]. Serine proteases are known to dominate the larval gut environment and contribute to about 95% of the 190 total digestive activities in Lepidoptera [5], whereas the Coleopteran species were found to have a wider range of dominant gut proteinases [6]. Many coleopteran insects, such as the Colorado potato beetle, Western corn rootworm and cowpea bruchid, utilize cysteine proteinases as their major digestive enzymes for food protein degradation [7,8].

Inhibitors of proteinases have been known in plants for a long time and have been identified for each of the classes of proteinases with a large number of these inhibitors directed toward serine- and cysteine proteinases [9,10] while only a few inhibitors are known for aspartic- and metallo-proteinases [11,12]. The cysteine inhibitors that are known as cystatin have been identified and characterized from several plant species, including cowpea, potato, cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, Job's tears, sunflower, rice, wheat, maize, soybean and sugarcane [4,12,13]. The cysteine protease inhibitor, Oryzacystatin isolated from rice, is the first well defined phytocystein and has potential inhibitory activity against papain and several other cysteine proteases [14]. *In vivo* and *in vitro* inhibitions of cysteine from different coleopteran pests by plant cysteine inhibitors were reported by different authors.

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The potato multicystatin was found to inhibit the growth of the Western corn rootworm larvae [15] while Oryzacystatin I, a rice cystatin, could repress the growth of the red flour beetle (*Tribolium castaneum*) [16]. Koiwa et al. [7,17] reported that the soybean CPI soyacystatin N (scN) suppressed the digestive enzymatic activity of the Western corn rootworm and Colorado potato beetle, as well as the growth and development of these pests.

In the current study, 44 barley (*Hordeum vulgare*) genotypes including 3 varieties and 41 promising lines were screened for their potential as protease inhibitors. Identification and partial characterization of papain inhibitor of the promising barley genotype were also conducted and its *in vivo* and *in vitro* effects on digestive proteinases and the development of the cowpea bruchid, *Callosobruchus maculatus*, was evaluated.

2. Materials and methods

2.1. Materials

Seeds of barley (*Hordeum vulgare*) genotypes were obtained from the Faculty of Agriculture, Sohag University, Egypt. Bovine trypsin, chymotrypsin, papain, standard substrates; N-a-benzoyl-DL-arginine-p-nitroanilide (BApNA) and N-a-benzoyl-DL-tyrosine-p-nitroanilide (BTpNA) and N-α-benzoyl-arginine-p-naphthylamide (BANA), standard papain inhibitor (E-64), protein molecular weight markers, acrylamide, bis-acrylamide and DEAE–Sephadex A-25 were procured from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Purification of barley proteinase inhibitor

Crude extract of different seeds was obtained according to Hajela et al. [18] and Abe et al. [19] with some modifications. Finely ground seeds were defatted by using ice-cold acetone (–20 °C). After 1 hr in acetone, the flour was separated by Buchner funnel under vacuum and this process was repeated twice. The defatted flour was air dried overnight and then extracted by homogenization in 0.01 M sodium-phosphate buffer (1:10 w/v) pH 7.0 containing 0.15 M NaCl for 10–15 minutes and then stirred for 2 hr at room temperature. The homogenate was then centrifuged at 10,000 rpm for 30 minutes at 4 °C and the supernatant (crude extract) was passed through 2–3 layers of cheesecloth, diluted with extraction buffer and used as the initial source for proteinase inhibitors as well as for protein estimation in all screening studies.

The barley variety, Giza 123 showed high inhibition activity toward papain activity for which 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 a minimal volume of extraction buffer and dialyzed overnight with the same extraction buffer at 4 °C and lyophilized. At each fraction, the papain 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 papain enzyme. This fraction was applied to a DEAE–Sephadex A-25 column (50 cm × 2 cm column) according to Ramesh Babu and Subrahmanyam [20], equilibrated with several bed volumes of 20 mM Tris–HCl buffer, pH 8.0. Clear supernatant, obtained after centrifugation, was applied to the column and 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 540 nm. The fractions that exhibited peaks of papain inhibitory activity were separately pooled, dialyzed and lyophilized.

2.3. Estimation of proteinase inhibitory activity

2.3.1. Serine proteinases

Trypsin and chymotrypsin activities were determined using synthetic substrates BApNA and BTpNA respectively. For 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 $CaCl_2$ and incubated at 37 °C in a water bath for 15 min. Residual trypsin activity was measured by adding 1 ml of 1 mM BApNA in prewarmed (37 °C) 0.01 M Tris–HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and incubated at 37 °C for 15 min [21]. 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 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 [22,23]. One millimolar BTpNA was prepared in 0.01 M Tris–HCl (pH 8.0) containing 40% ethanol [18].

2.3.2. Papain enzymes

Different volumes of barley crude extracts were added to 10 microliters of papain enzyme (prepared by adding 10 µg papain to 1 mM HCl) and the volume was made to 300 µl by adding 0.1 M phosphate buffer pH 6.0 containing 2.5 mM EDTA and 3 mM DTT. After incubation at 37 °C for 15 minutes, the reaction was started by the addition of 100 µl of 1 mM BANA. After 15 minutes of incubation at 37 °C, the reaction was stopped by the addition of 1 ml of 2% HCl/ethanol and then the color was developed for 30 minutes by addition of 1 ml of 0.06% *p*-dimethyl amino cinnamaldehyde prepared in ethanol and the absorbance was taken at 540 nm. Only papain enzyme in 300 µl phosphate buffer was used as control. Inhibitor activity was calculated by the amount of crude extract required to inhibit 50% of papain activity, which is considered as one unit of papain inhibition and expressed as papain inhibitor units per mg seed protein.

2.4. Protein determination

Protein was determined according to the method of Lowery et al. [24] where bovine serum albumin was used as a standard.

2.5. Thermal and pH stability of barley PI

Thermal stability of the purified barley PI was determined by using 0.1 M phosphate buffer, pH 6.0 incubated 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 [25]. The remaining papain inhibitor activity was measured as described previously.

The effect of pH on inhibitory activities of barley PI was investigated at different pHs ranging 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 24 h incubation at each pH at room temperature, residual papain inhibitory activities were measured as mentioned earlier. All experiments were carried out in triplicate.

2.6. 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 by the method of Laemmli [26] at room temperature. Bromophenol blue was used as tracking dye. The molecular weight markers employed were a-lactoalbumin (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 and then the gel was placed in destaining solution II (7% acetic acid, 5% methanol in 1 l distilled water) for 2 h with intermittent shaking. Destaining was continued until blue bands and a clear background were obtained and was then photographed. Molecular weights of unknown proteins were calculated from the standard graph using a regression equation.

2.7. Preparation of larvae midgut homogenates

Guts of the 4th instar larvae of *Callosobruchus maculatus* were collected as described by Kitch and Murdock [27], homogenized in dissection buffer (100 mM sodium acetate, 1 mM EDTA, pH 5.5) and centrifuged at 10,000 *g* for 10 min. The resultant supernatant was collected and used as a source of papain and stored at –20 °C.

2.8. Inhibitory potential of barley PI against gut extracts from C. maculatus larvae

Five concentrations of proteinase inhibitor from the selected barley variety and the standard papain inhibitor (E-64) were used to determine the IC_{50} values against proteinases of *C. maculatus* midgut extract. All the inhibitors were mixed with 30 µl of *C. maculatus* gut extract. It was incubated at 37 °C for 15 min, before addition of substrate to start the reaction. Papain residual activity was determined spectrophotometrically as described early and results were expressed as IC_{50} or % inhibition relative to controls without inhibitor.

2.9. Kinetics of inhibitory activity against C. maculatus from barley PI

The mechanism of inhibition (competitive or non-competitive) against papain of *C. maculatus* 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 an inhibitor and in the presence of an inhibitor, *K*m, *V*max and *K*i were calculated. The reaction velocity was expressed as $1/V\ (O.D.540/h/ml)^{-1}$.

2.10. In vivo effect of barley PI on, C. maculatus

For feeding studies, artificial seeds made of chickpea flour were prepared according to Subba [28] and Macedo et al. [29]. Different concentrations (w/w) of 0.25, 0.5 and 1.0% of the barley PI partially purified by ammonium sulfate saturation (at 30–60%) were added to the chickpea flour before preparing the artificial seeds. Initially the mixture was conditioned at 28 °C and 70% RH for 3 days. The mixture was filled compactly in gelatin capsules (0.5 g/capsule). After a 48 hour period for adjustment in the growth chamber, the artificial seeds were presented to three 2–3 days-old fertilized females for oviposition and incubated for 3 days at 28 °C and 70% RH. The excess eggs were removed from the seeds leaving four eggs per seed to ensure the uniformity of seed infestations among treatments. The infested artificial seed was incubated at 28 °C and 70% RH and the following biological characteristics were evaluated according to Murdock et al. [30]: 1) Within seed developmental time – defined as the duration of larval + pupal + unemerged adult stages beginning when larvae borings were observed in 50% of the viable eggs and ending at the time of adult emergence from the seeds; 2) Mortality – the percentage of hatched eggs not producing adults; 3) Mean adult longevity (males and females) – defined as the period from adult emergence from the seed to death; 4) Fecundity – the total number of eggs per female produced by five pairs of adults emerging from each treatment and ovipositing on cowpea seeds and 5) Adult dry weight.

2.11. Statistical analysis

All data were examined using analysis of variance (ANOVA) and the comparisons of the means of the larval weight and other parameters were made by the Duncan's Multiple Range Test (DMRT) at a 5% level of probability.

3. Results

3.1. Proteinase inhibitory activity

In this study the proteinase inhibitory activity was studied in the crude extract of 44 Egyptian genotypes of barley seeds (see Appendix: Supplementary material). The barley genotypes showed low inhibitory activity against trypsin and chymotrypsin enzymes with a mean of 4.15 trypsin inhibitory unit (TIU)/mg protein and 4.40 chymotrypsin inhibitory unit (CIU)/mg protein. All the tested barley genotypes were found to have papain inhibitory activity with wide inter-varietal variation with a mean of 11.23 papain inhibitory unit (PIU)/mg proteins. Among the tested genotypes, Giza 123 showed high papain inhibitory activity of 97.09 (PIU)/mg proteins. On the other hand Giza 123 showed low trypsin and chymotrypsin inhibitory activity of 4.67 TIU/mg proteins and 4.86 CIU/mg protein.

3.2. Purification of barley PI

As the barley variety, Giza 123 showed high papain inhibition activity; it was selected for further purification steps. A summarization of the yield of protease inhibitor activity and the fold of purification from the seed of the barley variety, Giza 123, can be found in Table 1. It was found that $F_{30–60}\ (NH_4)_2\ SO_4$ (w/v) saturation was efficient for precipitating the protease inhibitor compared to other fractions for which the $F_{30–60}\ (NH_4)_2SO_4$ was then applied to ion exchange chromatography, DEAE–Sephadex A25 column. The fold of purification obtained for $F_{30–60}\ (NH_4)_2\ SO_4$ was 3.02 times of

Table 1
Purification steps of protease inhibitors from barley (*Hordeum vulgare*), Giza 123.

Step	Total protein (mg)	Total trypsin inhibitory unit (TIU)	Specific activity (TIU/mg protein)	% Recovery	Fold purification
Crude extract	8400.56	813,846.25	96.88	100.00	1.00
$F_{30–60}\ (NH_4)SO_{4ppt}$	2117.93	619,662.54	292.58	76.14	3.02
DEAE–Sephadex A-25	262.66	336,600.69	1281.50	54.32	4.38
PI	14.20	131,341.59	9252.43	39.02	7.22

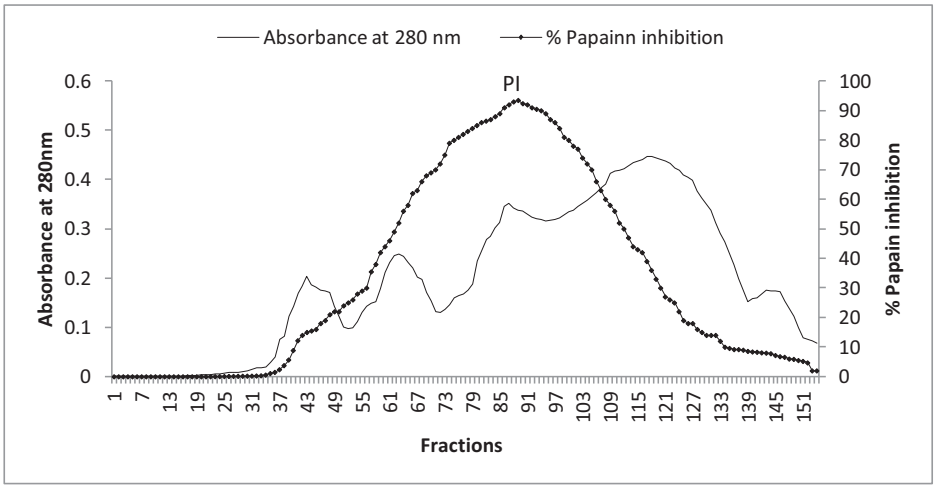

Fig. 1. Elution profile of DEAE–Sephadex A-25 of F_{30-60} from seeds of the barley cultivar, Giza 123.

that of the crude extract and the recovery percentage was 76.14%. The specific activity of the purified fraction was 4.38 times of that of the crude extract and recovery percentage was 54.32%. The DEAE–Sephadex column yield revealed the presence of five peaks through the lectures at 280 nm and the papain enzyme was strongly inhibited with elutes of the 80 up to 97 fractions (Fig. 1). There was only one peak (PI) that exhibited high inhibitor activity against papain enzymes obtained with elute of fraction 91 with 7.22 fold of purification and a recovery percentage of 39.02% compared to the crude extract.

3.3. Molecular weight

The F_{30-60} $(NH_4)_2$ SO_4 fraction and DEAE–Sephadex products of barley inhibitor proteins were resolved in 10% SDS-PAGE (Fig. 2). The F_{30-60} $(NH_4)_2$ SO_4 fraction was resolved into 4 protein bands ranging from 12.4 to 54.8 kDa. The PI fraction was resolved in two bands of 12.4 and 13.9 kDa.

3.4. Thermal and pH stability

Incubation of barley PI at various temperatures from 20 to 100 °C for 45 min gave the result illustrated in Fig. 3. The inhibitor activity of barley PI against papain was found to be stable at temperatures below 80 °C while the inhibitor lost 15% of its activity at 100 °C.

The stability of barley PI at pH ranging from 2 to 12 is presented in Fig. 4. It was found that the barley PI is stable over a wide range of pH 2–12 recording maximal inhibitory activity at pH 7. The inhibitor lost about 20 and 15% of its activity against papain enzyme at pH 2 and 12 respectively.

3.5. In vitro *effect of barley PI on papain enzyme from* C. maculatus *midgut*

Different concentrations (5–50 µg/ml) of barley PI and the standard papain inhibitor (E-64) were used to determine the IC_{50} of proteases of *C. maculatus* midgut extracts. All the assayed results showed linear inhibition of papain activity with increase of inhibitor until saturation was achieved. Barley PI was found to have higher potential inhibitory activity against papain enzyme compared to the standard papain inhibitor, E-64 with IC_{50} value of 21.04 µg/ml and 25.62 µg/ml for barley PI and E-64, respectively.

3.6. Kinetic analysis of the C. maculatus midgut papain enzyme

Figure 5 shows the Lineweaver–Burk double reciprocal plots of the inhibition of the midgut papain by barley 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

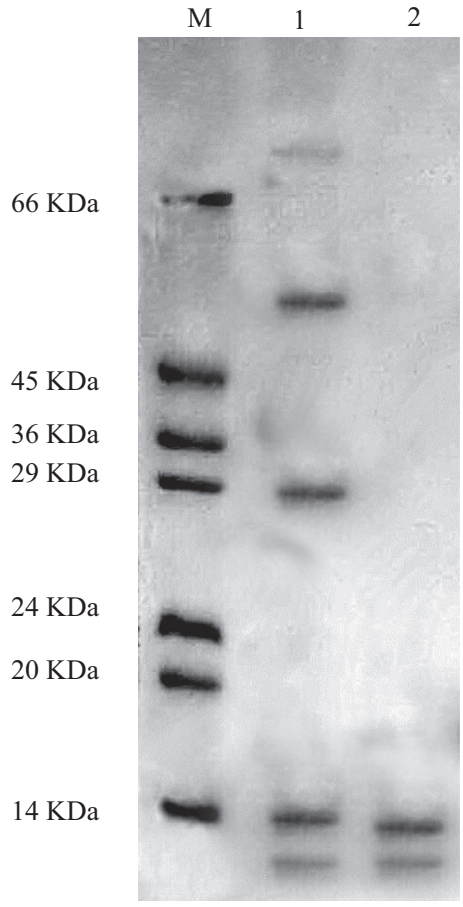

Fig. 2. SDS-PAGE analysis of barley PI fractions, stained with coomassie blue. M = molecular weight marker; 1 = F_{30-60} fraction; 2 = PI.

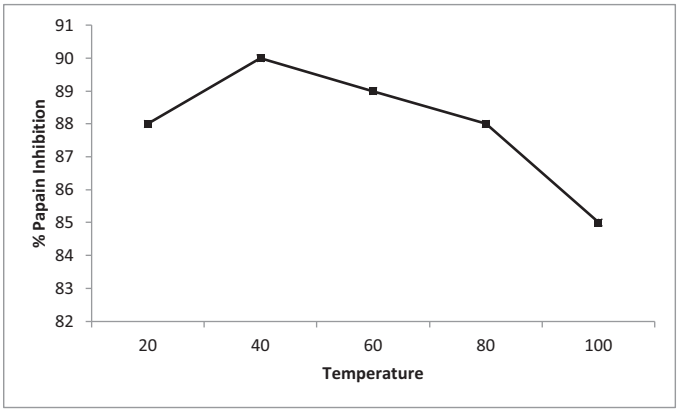

Fig. 3. Thermal stability profile of the barley PI (Giza 123) against papain enzyme activity.

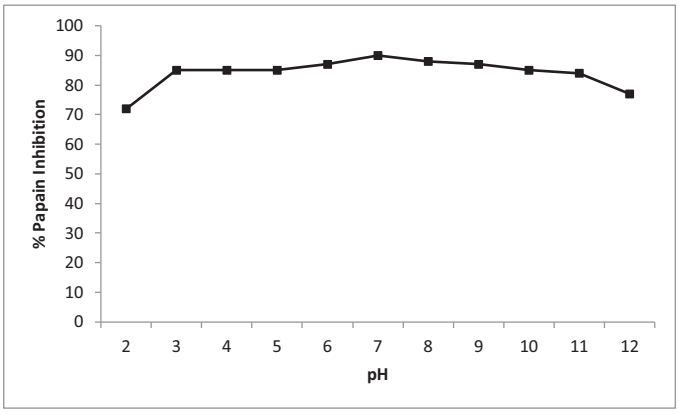

Fig. 4. Stability profile of barley PI (Giza 123) at different pH against papain enzyme activity.

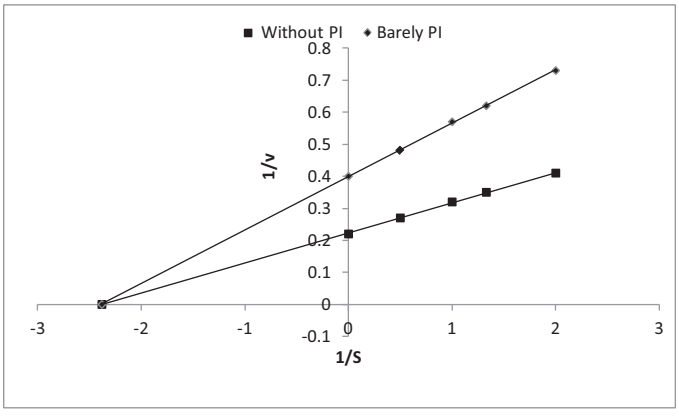

Fig. 5. Kinetic studies on the inhibition of papain enzyme of *C. maculatus* larval gut by barley (Giza 123) PIs.

Table 2
Kinetic analysis of the midgut papain of *C. maculatus* larvae against synthetic papain substrate.

Proteinase inhibitor	Papain enzyme		
	*K*m (mM)	*V*max (μmol pNA released/min/mg protein)	*K*i (μM)
Without inhibitor	0.42	4.50	1.95×10^{-3} μM
Barley (Giza 123) PI	0.42	2.50	15.6×10^{-3}

the reaction in the absence of inhibitors. Barley PI showed high affinity toward the midgut papain enzyme c with *K*i values of 1.95×10^{-3} μM (Table 2).

3.7. *In vivo effects of barley PI on larvae of* C. maculatus

The antimetabolic effects of barley PI isolated from the barley variety, Giza 123, was tested against *C. maculatus* by incorporating the F_{30-60} $(NH_4)_2$ SO_4 protein into the artificial diet at levels of 0.25, 0.5 and 1.0% using artificial seeds and results are presented in Table 3. Barley PI prolonged the development of *C. maculatus* in proportion to PI concentration. The significant effect on the within seed development time of *C. maculatus* was observed when barley PI was incorporated in the weevil artificial diet at a concentration of 0.5 and 1.0%. At a concentration of 1.0% within seed development time of *C. maculatus* was prolonged by 166% of that of the control bruchid. Feeding *C. maculatus* on a diet containing barley PI caused larval mortality ranging from 10.03 ± 2.34 to 29.08 ± 4.02%. Maximum mortality of 29.08 ± 4.02% was achieved when *C. maculatus* was fed on a diet containing 1.0% of barley PI.

The barley PI was found to have a non-significant effect on the mean adult longevity of both females and males of *C. maculatus*. Significant reduction in the fecundity of *C. maculatus* was noticed in 0.5 and 1.0% barley PI treatments while barley PI was found to have a non-significant effect on the adult dry weight.

4. Discussion

Cysteine proteinase inhibitors have been detected in seeds of plants of all botanical families so far examined, from gymnosperms to angiosperms [31]. They are mainly isolated from seeds of the Gramineae and Leguminosae families. Papain inhibitors showed almost constant low concentrations ($0.3–0.6 \times 10^{-2}$%) of the several seeds examined [32]. Hines et al. [33] made a screening for papain inhibitors in 44 vegetables and papain was inhibited by soybean hypocotyls and cotyledon extracts.

In the current study, 44 Egyptian barley genotypes were examined for their protease inhibitory activity. The tested genotypes showed low trypsin and chymotrypsin inhibitory activities. The barley variety, Giza 123, showed high papain inhibitory activity for which it was selected for further purification by ammonium sulfate

Table 3
In vivo effects of barley (Giza 123) PI on the growth and development of *C. maculatus*.

Treatment	Mean within seed development time (day)	Mortality (%)	Mean adult longevity (day)		Fecundity (no. of eggs/female)	Adult dry weight (mg)
			Female	Male		
Control (chick pea only)	29.60 ± 1.78[a]	5.12 ± 1.14[a]	11.25 ± 0.50[a]	14.20 ± 0.65[a]	78.60 ± 3.70[a]	2.10 ± 0.13[a]
Barley PI (0.25%)	34.25 ± 2.05[a]	10.03 ± 2.34[b]	10.50 ± 0.75[a]	13.95 ± 0.92[a]	76.54 ± 3.82[a]	2.11 ± 0.15[a]
Barley PI (0.5%)	40.40 ± 2.34[b]	18.12 ± 4.82[c]	10.20 ± 0.85[a]	13.25 ± 1.15[a]	68.86[c] ± 5.54[c]	1.98 ± 0.26[a]
Barley PI (1.0%)	49.15 ± 4.86[c]	29.08 ± 4.02[d]	9.75 ± 1.05[a]	12.90 ± 1.70[a]	47.52[c] ± 7.88[d]	1.96 ± 0.22[a]
L.S.D.	5.12	4.51	1.76	2.84	4.82	0.23

Means in a column followed by the same letter are not significantly different.

precipitation and ion exchange chromatography on DEAE–Sephadex A-25. The barley crude extracts were initially precipitated using ammonium sulfate at 30, 60 and 90% saturation and the three fractions were tested for their papain inhibitor activity. The $F_{30–60}$ $(NH4)_2$ SO_4 protein exhibited strong inhibitory activity against papain compared to other fractions. Oliveira et al. [34] reported that the $F_{30–60}$ protein fraction of algarroba tree showed a strong inhibitory activity against papain while the other fractions had weak inhibitory activity. The barley $F_{30–60\ protein}$ fraction was then applied to ion exchange chromatography, DEAE–Sephadex A-25 column and the retained peak was assayed against papain enzyme. Five peaks were obtained in which only one peak (PI) was found to have high inhibitory activity against papain. Monti et al. [35] purified papain inhibitor from papaya tree by filtration in sephadex G-25 and the filtration revealed four peaks and only one peak was found to have papain inhibitory activity.

The purification steps of the barley protein extracts were observed in 10% SDS-PAGE and resolved in four protein bands ranging from 12.4 to 54.8 kDa. The PI fraction was resolved in two bands of 12.4 and 13.9 kDa. Rele et al. [36] isolated four papain inhibitors from cowpea, *Vigna unguiculata* seed with a molecular weight ranging from 5 to 12 kDa. Silva et al. [37] suggested that leguminous plant seeds contain at least three sizes of papain inhibitors: a low molecular weight class (5–12 kDa); an intermediate size class (20–30 kDa) and a high molecular weight class (60–80 kDa). Richardson [38] isolated a papain inhibitor of 9.2 kDa from maize endosperms.

The purified barley PI was found to be stable at a temperature below 80 °C and at a wide range of pH from 2 to 12 that suggest its efficiency in controlling a variety of phytophagous insects that have variation in their gut environment.

The inhibitor assays of purified protein extracted from barley against the midgut papain extract of *C. maculatus* revealed linear inhibition of proteolytic activity with increase of inhibitor until saturation. The barley PI showed higher papain inhibitory activity compared to the standard papain inhibitor, E-64. The kinetic analysis revealed a non-competitive type of inhibition with a *Ki* value of 1.95×10^{-3} μM. Barret [9] reported that cystatins are apparently reversible competitive inhibitors of papain. However, Zhao et al. [39] reported noncompetitive inhibition of papain by soybean Cys PI with *Ki* values ranging from 21 to 57×10^{-3} μM.

The antimetabolic effect of barley PI was evaluated against *C. maculatus* by incorporating the $F_{30–60}$ protein of the purified inhibitor into the artificial diet using artificial seeds. Barley PI significantly prolonged the development of *C. maculatus* in proportion to PI concentration. Barley PI significantly increased the mortality of *C. maculatus* and caused a significant reduction in its fecundity. On the other hand, barley PI seemed to have non-significant effects on the adult longevity and the adult dry weight. High doses of soyacystatin N (scN) caused high bruchid mortality, while low doses caused only delayed insect growth and development [17]. Zhu-Salzman et al. [8] demonstrated that the feeding inhibition and the growth retardation of the cowpea bruchid caused by scN only occurred during earlier developmental stages and the larvae were later able to recover their normal feeding and growth in the presence of scN in diet. The papain inhibitor, E-64 prolonged the developmental time and increased mortality of the cowpea weevil, *C. maculatus* [30] and the Mexican bean beetle [40]. E-64 was also found to delay the pre-ovipositional period and decrease the fecundity of the Mexican bean beetle [40].

In conclusion, the *in vivo* and *in vitro* results of the current study uniquely proved the efficiency of the papain inhibitory protein isolated from barley variety, Giza 123 as a tool for managing the cowpea bruchid, *C. maculatus.* However, further studies on the possibility of using barley PI gene(s) in developing insect resistance transgenic plants are required.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.pestbp.2014.10.016.

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