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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 $1.95 \times 10^{-3}$ 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