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# Extraction and purification of protease inhibitor(s) from seeds of *Helianthus annuus* with effects on *Leptinotarsa decemlineata* digestive cysteine protease

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ABSTRACT

The aim of the current investigation was to purify cysteine protease inhibitors from sunflower (*Helianthus annuus* L. cv. Record) (Asteraceae) seeds with potential activity on gut protease of Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). Ammonium sulfate precipitated proteinaceous fractions; 0–30%, 30–50%, 50–70%, and 70–100% showed 41.03%, 48.66%, 41.53%, and 28.83% inhibition on the last instar larval gut general protease activity, respectively. Also, fraction 30–50% showed the highest inhibitory effect on digestive general protease activity of all developmental stages. This fraction was purified using two different chromatography techniques; ion-exchange using DEAE and affinity using $SiO_2$-CPB larval gut enzyme mix. Four peaks of protein were eluted from ion-exchange chromatography using NaCl step gradient. When used Z-Ala-Arg-Arg-4mßNA as cysteine protease substrates, the purification fold of first fraction was obtained 28.31, also the yield was 66.89%. Fraction of affinity chromatography obtained 26.98 purification fold and yielded 53.96%. Both of the purified protein fractions from two methods showed two similar bands in SDS-PAGE with apparent molecular mass of 14 and 15 kDa. Consequently, the high level of purification fold and yield suggest that both methods were appropriate for purification, but affinity was more specific because CPB gut enzyme was used as ligand.

## 1. Introduction

Proteases are assumed to assert physiological functions in the regulation of protein synthesis and supply of free amino acids essential for normal growth and development (Lawrence and Koundal, 2002). Cysteine proteinases, endopeptidyl hydrolases with a cysteine residue in their active center are usually identified based on the effect of their active site inhibitors and activation of the enzymes by thiol compounds (Macedo and Freire, 2011). Proteolysis as a key process in all insect pests must be strictly regulated and controlled; otherwise it could be hazardous to their natural environment (Carlini and Grossi-de-Sa, 2002; Habib and Fazili, 2007).

Protease inhibitors (PIs) comprise one of the most abundant classes of proteins in plants (Ussuf et al., 2001) with a great proportion being small proteins of only 8–20 kDa and enriched in cysteine residues in amino acid composition, which are significant in the formation disulfide bridges and in conferring stability to heat, pH changes, and proteolysis (Chye et al., 2006). Each protease inhibitor interacts with its target protease at the catalytic domain, forming a stable protease-inhibitor complex, thus renders the protease inactive (Lawrence and Koundal, 2002). They are specific for each of the four mechanistic classes (serine, cysteine, aspartyl and metallo) of proteolytic enzymes. These inhibitors are distributed among different families of plants, particularly abundant in storage tissues such seeds and tubers (Ussuf et al., 2001; Oliveira et al., 2007). PIs are naturally occurring defense mechanisms of plants against the attack of insect pests that interfere with the digestive biochemistry of them by inhibition of a protease activity in their gut (Aguirre et al., 2004; Abd El-latif, 2015). Following inhibition, the target proteases can no longer cleave peptide bonds, which cause a detrimental disruption of dietary protein assimilation in herbivorous pests leading to significant growth and development delays (Schluter et al., 2010). Bioassays have also shown such retardation in

*Abbreviations:* CPB, Colorado potato beetle; DEAE, Diethylaminoethyl; PIs, Protease inhibitors; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; U, Unit; Z-Ala-Arg-Arg-4mßNA, Z-Ala-Arg-Arg 4-metoxy-β-naphtylamide acetate

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the growth of insect pests fed on diets incorporating PIs or on transgenic plant expressing PIs and some of them also have insecticidal activities (Aguirre et al., 2004; Kansal et al., 2008; Abd El-latif, 2015).

Determination of specificity of inhibition is the main step towards the discovery of an inhibitor (Franco et al., 2002) and it is important to biochemically characterize the PIs from various sources and evaluate their insecticidal potential (Kansal et al., 2008). Since the economically important classes of pests like Lepidoptera, Diptera and Coleoptera use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food, efforts have been focused to use these classes of PIs for developed transgenic plants (Ussuf et al., 2001). Most of the PIs (serine and cysteine type) are isolated and purified by ammonium sulfate precipitation, ion-exchange chromatography, gel filtration chromatography, and affinity column chromatography along with reverse phase HPLC (high performance liquid chromatography) and FPLC (fast protein liquid chromatography) (Abd El-latif, 2015). PI purification can be carried out specifically by the use of affinity chromatography techniques, where the binding agents are particular proteases (Oliveira et al., 2007). Several plant PIs such as soybean trypsin inhibitor have been successfully used for the affinity purification of their inhibited proteases from a wide variety of sources (Fan and Wu, 2005).

The aim of this study was to purify protease inhibitors by ion-exchange and affinity chromatography techniques from sunflower seeds, with potential for the control of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae), by causing inhibition on digestive cysteine protease activity of this economic interest pest.

## 2. Material and methods

### 2.1. Chemicals and devices

Azocasein, Z-Ala-Arg-Arg 4-metoxy-β-naphtylamide acetate (Z-Ala-Arg-Arg-4mßNA) and silicon dioxide (nanopowder, 10 nm, 99.5%) were supplied by Sigma® (St Louis, MO, USA). DEAE (Diethylaminoethyl) cartridge (5 ml: 40 mm length x12.6 mm inner diameter) was supplied by Biorad® (Hercules, CA, USA). Biorad® BioLogic™ LP System (Low-Pressure Liquid Chromatography, Milan, Italy) was used for chromatography. Spectrophotometric measurements were made using ELISA reader, BioTek® ELx800 (Winooski, Vermont, USA) and Thermo Scientific™ Multiskan™ FC microplate photometer (Rockford, IL, USA). Fluorometric measurements were done using fluorescence spectrophotometer (Varian, Cary Eclipse®, Palo Alto, California).

### 2.2. Insect rearing and preparation of enzyme source

The colony of Colorado potato beetle was maintained on potato foliage cv. “Agria” at 27 ± 1 °C, 60 ± 5% relative humidity, under 16:8 h (L:D) photoperiod and white fluorescent light. Insects were reared from egg hatch to adult in a clear plastic dishes contain fresh potato leaves. Guts from adults, fourth and third instar larvae were isolated by dissection under a light microscope in the ice-cold phosphate buffer solution (pH 7, Merck®; Darmstadt, Germany). For first and second instar larvae, intact insects were grounded. The samples were homogenised in cold distilled water and the mixtures were centrifuged at 17,000×*g* for 30 min at 4 °C. The supernatant was stored as an enzyme source at −20 °C before analysis. The protein content of the enzyme source was adjusted to 2 mg/ml.

### 2.3. Protease inhibitory assays

Azocasein and Z-Ala-Arg-Arg 4-metoxy-β-naphtylamide acetate (Z-Ala-Arg-Arg-4mßNA) were used as substrate for general protease, and cathepsin B (Cysteine protease) respectively. General protease assay was conducted according to Elpidina et al. (2001) and Gatehouse et al. (1999), with slight modification. General protease mixture contains 40 µl universal buffer (0.02 M, glycine, 2-morpholinoethanesulfonic acid and succinic acid disodium salt, pH 5), 10 µl enzyme extract and 50 µl azocasein (2%). After addition of substrate the reaction mixture was incubated 90 min at 37 °C, and then 100 µl of 30% trichloroacetic acid (TCA, Merck®) was added and the incubation mixture was kept at 4 °C for 30 min. Finally the mixtures were centrifuged at 17,000×*g* for 20 min to precipitate non-hydrolysis substrate and 100 µl 1 N NaOH was added to 100 µl supernatant and the absorbance at 405 nm was recorded. One unit of protease activity was defined as the amount of enzyme required to produce an increase of one absorbance unit at 405 nm per minute in the assay conditions. Specific activity was expressed in absorbance per min (Unit) per mg of protein.

In the cathepsin B assay, reaction mixture was consisted of 370 µl of universal buffer (pH 5), 20 µl 1 mM Z-Ala-Arg-Arg-4mßNA and 10 µl of enzyme solution. After addition of substrate the reaction mixture was incubated at 37 °C for 10 min. The absorbance of the resulting mixture was then measured fluorometrically at an excitation of 335 nm and emission of 425 nm. Activity was expressed in emission intensity (Arbitrary unit, a.u.) per min (Unit) per mg of protein.

In the general protease and cathepsin B inhibition assay the mixture of enzyme extract and buffer was pre-incubated with 30 µl and 100 µl proteinaceous seed extracts, before addition of substrate, 30 min at 37 °C. Appropriate blanks (reaction without enzyme extract as control) were run for all assays. Tests were performed in triplicate, and each of them was repeated three times.

### 2.4. Zymography

Proteolysis was qualitatively assayed by semi-denaturing native polyacrylamide gel electrophoresis (PAGE). To test the effect of inhibitor on protease activity, adult's gut extract was mixed with 30–50% ammonium sulfate precipitated sunflower seed protein extract and incubated for 30 min at 37 °C. While in the control, enzyme solution was incubated with Tris-HCl buffer (0.02 M and pH 7). Electrophoresis was performed in 10% (w/v) gel for separating gel and 5% for stacking gel, with a 1% gelatin as substrate. Electrophoresis was conducted at 4 °C and a voltage of 120 V (10 V/cm) until the dye front reaches the margin of the gel. The gel was rinsed with distilled water and washed by shaking gently by 2.5% (v/v) Triton X-100 for 45 min. Then, the gel was incubated in MES buffer solution (2-morpholinoethanesulfonic acid, pH 5, Merck®) at room temperature overnight. Finally, the gel was stained in 50% methanol, 10% acetic acid and 0.05% Coomassie brilliant blue R 250 and destained by 40% methanol and 10% acetic acid until proteolytic activity bands were seen as a light band against the dark background of the gel.

### 2.5. Purification of CPB protease inhibitor from sunflower seeds protein

Purification of inhibitor proteins was carried out by using various combinations of ammonium sulfate precipitation and chromatographic methods including ion-exchange and affinity.

For ammonium sulfate precipitation, sunflower (*Helianthus annuus* L. cv. Record) (Asteraceae) was grounded thoroughly using mortar grinder Retsch® RM100 (Chino, California), and then 30g of powdered seeds was mixed with 100 ml solution of 0.1 M NaCl and stirred for 2 h, followed by filtration and centrifugation at 12,000×*g* for 30 min. Seed protein in the supernatant was extracted using a saturation of 0–30%, 30–50%, 50–70%, 70–100% (w/v) ammonium sulfate (Merck®). Saturated ammonium sulfate was gradually added to the homogenate with constant stirring. Particularly, 0–70% saturation of ammonium sulfate precipitation was prepared for testing inhibition at various pHs. After 45 min, the crude extract was centrifuged at same condition. In every fraction extraction, the supernatant was brought to