

Purification of *Leptinotarsa decemlineata* (Say) Gut Specific Cysteine Protease Inhibitor(s) From Rapeseed

Sh. Ashouri¹, F. Zihnioglu², R. Farshbaf Pourabad^{1*}, and E. kocadag²

ABSTRACT

The aim of the present work was to purify cysteine protease inhibitors from rapeseed (*Brassica napus* L.), with potential activity on digestive protease of Colorado Potato Beetle (CPB), *Leptinotarsa decemlineata* (Say). Ammonium sulfate precipitated proteinaceous fractions; 30, 50, 70, and 100% showed 39.07, 57.03, 51.47, and 22.44% inhibition on the fourth instar larval gut general protease activity, respectively. Fraction 50% showed the highest inhibitory effect on digestive general protease activity of all developmental stages. Gel assays approved the inhibition of the enzyme activity. Fraction 50% was purified by using various chromatography techniques; ion-exchange using DEAE, gel filtration and affinity using SiO₂-CPB larval gut homogenate. Three peaks of protein were eluted from ion exchange chromatography using NaCl step gradient, also from gel filtration chromatography. When Z-Ala-Arg-Arg-4mbNA was used as cysteine protease substrate, the purification fold of second fraction of ion exchange chromatography was obtained 24.80, also the yield was 59.09%, the third fraction of gel permeation resulted in a 25.60 fold purification with 28.53% of recovery, and the fraction of affinity chromatography obtained a 22.72 fold purification and yielded 36.35%. In the SDS-PAGE, apparent molecular mass of purified proteins were 34 and 32 kDa by ion-exchange and 24 and 22 kDa by affinity. However, gel filtration was not an appropriate method in this study, because the purified protein band(s) were not observed on the gel. Consequently, these chromatography methods were appropriate methods to purification of inhibitor cystatins, specially affinity which was prepared by using CPB gut enzyme as ligand and obtained specific inhibitor proteins of CPB gut protease activity.

Keywords: Chromatography, Colorado potato beetle, Enzyme inhibition, Protein, Rapeseed.

INTRODUCTION

Proteolytic enzymes, also called proteases, catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins to supply free amino acids essential for normal growth and development. They are classified as serine, cysteine, aspartic and metalloprotease (Carlini and Grossi-de-Sa, 2002, Habib and Fazili, 2007). Cysteine proteases (EC 3.4.22), endopeptidyl hydrolases with a cysteine residue in their active center are generally identified based on the effect of their active site inhibitors and activation of the enzymes by thiol

compounds. Most cysteine proteases show acidic pH optima (Grudkowska and Zagdanska, 2004) and provide the major midgut proteolytic activity in the almost coleopteran larvae and adults because they have slightly acidic midguts (Novillo *et al.*, 1997).

Protease Inhibitors (PIs), broadly distributed in most organisms in nature; plants, animals and microorganisms, are small protein molecules that form very stable complexes with proteolytic enzymes and inhibit their activity (Aguirre *et al.*, 2004, Grudkowska and Zagdanska, 2004, Oliveira *et al.*, 2007). Plant cystatins or

¹ Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran

* Corresponding author; e-mail: rfpourabad@tabrizu.ac.ir

² Department of Biochemistry, Faculty of Science, Ege University, Izmir, Turkey.



phytochemicals are the second most studied class of inhibitors (after serine protease inhibitors) and have been identified and characterized from several plants (Fan and Wu, 2005). Production of these PIs is naturally occurring defense mechanisms of plants that interfere with the digestive biochemistry of insect pests (Abd El-Latif, 2015). PIs are often present in seeds and expressed in certain plant tissues in response to abiotic stress (wounding) or herbivorous attack (Macedo and Freire, 2011). *In-vitro* feeding assays using artificial diets containing the inhibitors have indicated that these proteins are responsible for the inhibition of a protease activity in the insect gut by reduction in the digestive capability of insects and have a protective role against several crop pests (Sharma, 2015). Bioassays have also shown detrimental retardation in the growth of insect pests fed on diets incorporating PIs or on transgenic plants expressing PIs and some of them also have insecticidal activities (Aguirre *et al.*, 2004, Kansal *et al.*, 2008, Abd El-Latif, 2015). In contrast to the successful examples of PIs conferring enhanced pest-resistance in plants, some failure cases have also been reported, because of either the ineffectiveness of some PIs on specific insect pests or the adaptation of insects to them (Fan and Wu, 2005). On the other hand, protease inhibitors have been recognized as potential candidates for the production of insect-resistant transgenic crops and their efficacy to reduce insecticide loads in the field has been documented (Schluter *et al.*, 2010). So far, the main emphasis of plant-PI studies is on identifying potential inhibitors of the major economic pest insect's digestive proteases (Karimi *et al.*, 2010; Abd El-Latif, 2015).

It is important to recognize and purify inhibitor proteins from natural sources and evaluate their insecticidal potential. To achieve a high level of purity in the purification of proteins, it is necessary to use several chromatographic steps including anion and cation exchange, gel filtration and affinity chromatography (Asenjo and

Andrews, 2009). So, the aim of the current investigation was to purify and characterize protease inhibitors from rapeseed, with potential for controlling the Colorado Potato Beetle [CPB; (*Leptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae)], by causing interruption in the gut protease activity of this economic interest pest.

MATERIAL AND METHODS

Materials and Equipment

Azocasein, Z-Ala-Arg-Arg 4-methoxy- β -naphthylamide acetate (Z-Ala-Arg-Arg-4m β NA) and silicon dioxide (nanopowder, 10 nm, 99.5%) were supplied by Sigma[®] (St Louis, MO, USA). DEAE (DiEthylAminoEthyl) cartridge (5 mL: 40 mm length \times 12.6 mm inner diameter) was supplied by Biorad[®] (Hercules, CA, USA). Ultrogel[®] column (AcA54, gel filtration, 5000-70000 Da, bead size 60-140 μ m, Serva) was purchased from Sepracor (France). 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).

Insect Rearing and Preparation of Enzyme Extract

The colony of Colorado potato beetle was maintained on potato foliage cv. "Agrida" at 27 \pm 1 $^{\circ}$ C, 60 \pm 5% relative humidity, under 16:8 hours (L:D) photoperiod and white fluorescent light. Insects were reared from egg hatch to adult in the clear plastic dishes containing fresh potato leaves. Enzyme samples from third instar larvae, fourth instar larvae and adults were prepared by

dissection of guts under a light microscope in the ice-cold phosphate buffer solution (pH: 7, Merck®; Darmstadt, Germany). About first and second instars, intact insects were used for grinding. The samples were homogenized and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was stored as an enzyme source at -20°C for subsequent analysis.

Protease Inhibitory Assays

Azocasein and Z-Ala-Arg-Arg 4-methoxy- β -naphthylamide acetate (Z-Ala-Arg-Arg-4mBNA) were used as substrate for general protease, and cathepsin B (Cysteine protease) respectively. General protease assay was done according to Elpidina *et al.* (2001) and Gatehouse *et al.* (1999), with slight modification. General protease mixture contains 40 μ L universal buffer (0.02M, glycine, 2-morpholinoethanesulfonic acid and succinic acid disodium salt, pH: 5), 10 μ L enzyme extract and 50 μ L azocasein (2%). After addition of substrate the reaction mixture was incubated for 90 minutes at 37°C, and then 100 μ L of 30% trichloroacetic acid (TCA, Merck®) was added and kept at 4°C for 30 minutes. Finally the mixtures were centrifuged at 13,000 rpm for 20 minutes to precipitate non-hydrolyzed substrate and 100 μ L 1N NaOH was added to 100 μ L supernatant and the absorbance at 405 nm was recorded. Activity was expressed in Optical Density (OD) per minute (Unit) per mg of protein.

In the cathepsin B assay, reaction mixture consisted of 370 μ L universal buffer (pH: 5), 20 μ L 1 mM Z-Ala-Arg-Arg-4mBNA and 10 μ L of enzyme solution. After addition of substrate the reaction mixture was incubated at 37°C for 10 minutes. 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 per minute (Unit) per mg of protein. Appropriate blanks (no enzyme) were run for assays.

In the general protease and cathepsin B inhibition assay the mixture of enzyme extract and buffer was pre-incubated with 30 μ L and 100 μ L proteinaceous seed extracts, before addition of substrate, for 30 minutes at 37°C. All assays were performed in triplicate and each assay was repeated at least three times.

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 50% ammonium sulfate precipitated rapeseed protein extract and incubated for 30 minutes at 37°C. While in the control, enzyme solution was incubated with Tris-HCl buffer (0.02M 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 120V. The gel was rinsed with distilled water and washed by shaking gently by 2.5% (v/v) Triton X-100 for 45 minutes. 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.

Purification of Rapeseed Protein

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

For ammonium sulfate precipitation, rapeseed (*Brassica napus* L. cv. Karaj3) was grounded thoroughly using mortar grinder



Retsch® RM100 (Chino, California), and then 30 g of powdered seeds was mixed with 100 mL solution of 0.1 M NaCl and stirred for 2 hours, followed by filtration and centrifugation at 10,000 rpm for 30 minutes. Seed protein in the supernatant was extracted using a saturation of 0-30, 30-50, 50-70, 70-100% (as gradient; 30, 50, 70 and 100%) (w/v) ammonium sulfate (Merck®). Saturated ammonium sulfate was gradually added to the homogenate with constant stirring. Particularly, 70% saturation of ammonium sulfate precipitation was prepared for inhibitory pH tests. After 45 minutes, the crude extract was centrifuged in the same conditions. In every fraction extraction, the supernatant was brought to next saturation by further addition of ammonium sulfate and the pellet was dissolved in the minimal volume of the Tris-HCl buffer (0.02M and pH: 7) until the solid material dissolved completely, then dialyzed (using dialysis bag 1 kDa cutoff, 28 mm, Sigma®) against the distilled water for 20 hours with changing the dialysis water twice. Finally, this dialyzed solution was heated at 70°C for 20 minutes to inactivate endogenous enzymes within extract and after centrifugation in the same conditions it was used as inhibitors in enzymatic assays and further purification steps.

Purification with ion exchange chromatography (anion exchange) was done by means of DEAE cartridge. One milliliter of the ammonium sulfate precipitate (50%) was applied to single use filter unit (0.2 µm, Minisart® single use filter unit, Sartorius Stedim Biotech, Spain) and filtrate was loaded onto an ion exchange chromatography column previously equilibrated with 0.02M sodium phosphate buffer (pH: 7.5) (buffer A). The unbound proteins were washed out with the same buffer for 15 minutes. Subsequently, the inhibitor proteins were eluted from the column using increasing ionic strengths by step gradient of NaCl (0.3, 0.6 and 1M) in the buffer A at the flow rate of 1 mL min⁻¹ and each eluted fraction was 2 mL. The first two steps lasted 15 minutes and the last one

30 minutes. The chromatography was done and elution was monitored at 280 nm to detect the protein peak. Whole process was carried out at room temperature. The active fractions with the highest inhibitory activity from three replications were pooled, desalted (dialyzed as described above) and concentrated using ultrafiltration by centrifugal device (10 kDa cutoff, Macrosep®; Pall, Port Washington, NY, USA) to the suitable volume (the volume was recorded). Consequently, the protein concentration of the concentrated fractions was estimated and the molecular weight of them was determined by SDS-PAGE and finally the inhibitory activity of purified protein was tested.

Gel permeation chromatography was done by means of Ultrogel® as matrix. After removing sodium azide (bacteriostatic agent) from gel beads by vacuum pumping, the column (1.8×30 cm) was filled completely by mixture of beads and 0.05M sodium phosphate buffer containing 0.15M NaCl, pH: 7. Gravity flow was initiated to start packing and the procedure was continued until constant bed height was maintained. Packed column was allowed to swell at 4°C overnight. One milliliter of the ammonium sulfate precipitated (50%) rapeseed protein extract (7 mg protein mL⁻¹) was loaded onto the column and allowed to pass into the gel by running the column. Elution was carried out with the same buffer at a flow rate of 1 mL min⁻¹. Eluted fractions were collected at an interval of 2 min. The chromatography lasted 2 hours and was monitored at 280 nm. The fractions related to each peak with the highest inhibitory activity from three replications were pooled, dialyzed and concentrated. Further analysis of each fraction was carried out similarly as described above.

In the purification by affinity chromatography, silicon dioxide was used as matrix. For the preparation of affinity matrix CPB larval gut enzyme mix was chosen as ligand. Immobilization of larval gut enzyme was performed on silicon dioxide activated with glutaraldehyde (25%, Sigma®) (silica-

GA-enzyme): silicon dioxide (7 g) was incubated in 70 mL 0.1M acetate buffer (pH: 4) containing 1% glutaraldehyde at 25°C and 200 rpm for 1 hour. Excess of glutaraldehyde was removed using centrifugation at 6,000 rpm for 20 minutes and subsequently washed with distilled water. Activated silica was incubated in 2.5 mL enzyme extract (4 mg mL⁻¹) and 47.5 mL buffer A at 4°C and 200 rpm overnight. Finally, the immobilized enzyme preparation was washed with the same buffer at least twice and the protein concentration in washed parts was determined for estimating the immobilized protein concentration to the matrix. Subsequently, 50 mL of 1M glycine (Sigma®) was added to the enzyme immobilized matrix and after 10 minutes the extra glycine was washed by centrifugation. Finally, 2.5 mL ammonium sulfate precipitated 50% rapeseed protein extract (7 mg protein mL⁻¹) and 22.5 mL buffer A was added in the immobilized enzyme preparation and was shaken for 1 hour at room temperature. Then the extra unabsorbed inhibitor was removed by centrifugation for 20 minutes at 6,000 rpm and was again washed by 25 mL of the same buffer. Subsequently, 25 mL buffer A containing 0.1M NaCl was used to remove nonspecifically bound proteins. Finally, the matrix was eluted with 25 mL elution buffer, buffer A containing 0.1M NaCl, pH: 11.2 at least three times. These three fractions were pooled, dialyzed and concentrated. Further analysis of fraction was carried out similarly as described above.

In preparing the purification table, for calculating the total inhibition ($U = \Delta Abs$ or $\Delta Intensity/min$), the inhibition rate for a total volume of fractions was calculated. Also, for calculating the specific inhibition ($U\ mg^{-1}$ protein), the total protein concentration of fractions was estimated. Yield of purification (%) was achieved by dividing the total inhibition value of obtained fraction to the total inhibition value of used fraction in chromatography (fraction 50%) and conversion to the percentage. For achievement of the purification fold, the

specific inhibition value of obtained fraction was divided to the specific inhibition value of used fraction in chromatography.

SDS-PAGE

Determination of molecular weight of purified rapeseed inhibitor proteins and monitoring their purity were carried out using Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) by the Laemmli (1970) method with 12% resolving and 4% stacking gels, under reducing conditions by using 2-mercaptoethanol (Sigma®) in the sample buffer and pre-boiled protein samples. Separated proteins were determined by staining with Coomassie brilliant blue R-250, the above-mentioned method. The molecular mass was estimated by comparison with the gel migration distances of the following standard molecular weight markers (Cat number: SDS7, Sigma®): bovine serum albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α -lactalbumin (14.2 kDa).

Protein Determination

Protein concentration was estimated according to the method of Bradford (1976), using bovine serum albumin (BSA, Sigma®) as standard.

Statistical Analysis

Analyses Of Variance (ANOVA) were employed on the data using a one-way ANOVA of the MSTAT-C statistical package. Means of the three replicates were tested by Tukey's test for significant differences.



RESULTS

Preliminary Analysis of Inhibitory Activities of Ammonium Sulfate Precipitated Fractions

The inhibition of *L. decemlineata* fourth instar larval gut general protease by 0-70% fraction of ammonium sulfate precipitated rapeseed protein extract was dependent on the pH value of the assay medium. The highest inhibitory effect by rapeseed extract was observed at pH 5, with a significant difference ($P < 0.05$) based on Tukey's test. The inhibitory values at different pHs, 4, 5, 6, 7 and 8 were measured 49.01, 59.51, 50.38, 42.78 and 39.75% respectively.

Four ammonium sulfate precipitated proteinaceous fractions; 30, 50, 70, and 100% saturation from rapeseed showed inhibitory effect with the percentage of 39.07, 57.03, 51.47, and 22.44%, respectively on the fourth instar larval gut general protease activity. Since the first three fractions of oilseed produced the greatest inhibition on enzyme activity with significant differences ($P < 0.05$) based on Tukey's test, these fractions were used for further analysis.

Comparison of developmental stages of

CPB (L_1 , L_2 , L_3 , L_4 and adults) showed differential inhibition of digestive protease activities only by 50% fraction (Figure 1). Namely, inhibition in L_3 and L_4 stages was significantly lower than inhibition in L_2 stage ($P < 0.05$).

In Gel Inhibition Assay

In complement to colorimetric inhibition assays, the enzymes were subjected to semi-denaturing native PAGE after the incubation of first instar larvae (Figure 2-a) and adult's (Figure 2-b) enzyme extracts with second ammonium sulfate precipitated proteinaceous fraction of rapeseed (Figure 2). The gel inhibition assays showed that protease of the larval and adult's gut was affected by the presence of the inhibitor and the intensity of protease bands decreased as compared with the control. The most relevant inhibition was seen for the larval gut enzyme.

Purification of Protease Inhibitor from Rapeseed

The crude protein extract from the rapeseed, 50% ammonium sulfate precipitated fraction which showed the highest inhibitory activity was selected and

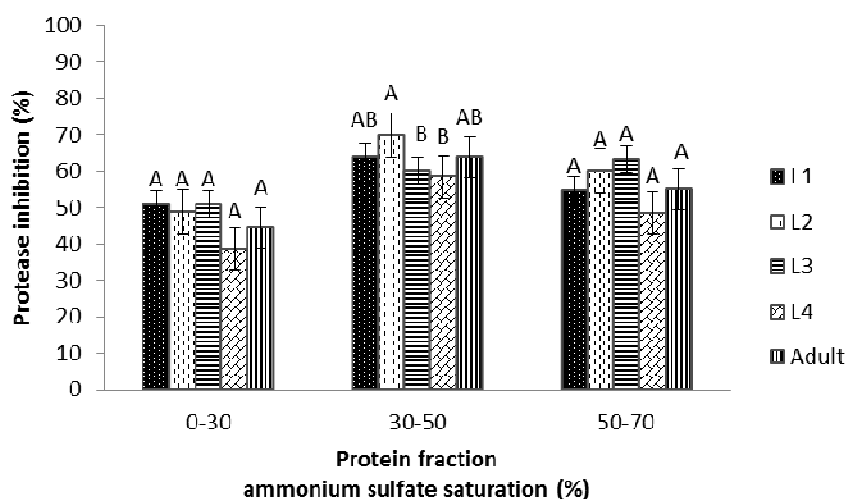


Figure 1. The inhibitory activities of effective ammonium sulfate precipitated proteinaceous fractions of rapeseed on the digestive protease of four Larval instars (L_1 - L_4), and adult's of CPB. Significant differences between the developmental stages are indicated by different letters (Tuckey's test, $P < 0.05$).

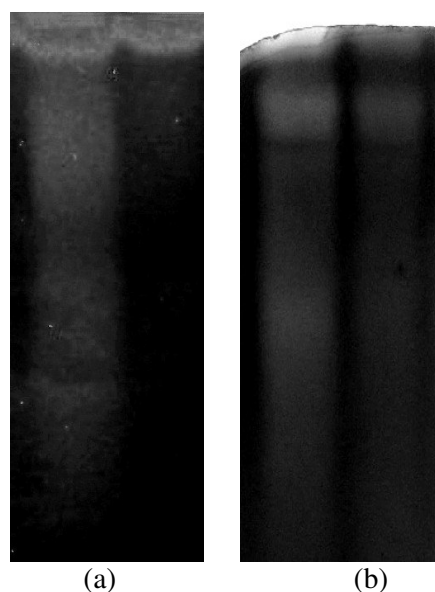


Figure 2. In gel inhibition assay of ammonium sulfate precipitated 50 % fraction of rapeseed on the first instar larvae (a) and adult's (b) digestive protease of CPB. Left columns are related to the general protease activity as control and right columns to the inhibited protease activity.

partially purified by using different chromatography steps; ion-exchange

chromatography, gel filtration chromatography, affinity chromatography as the materials and methods mentioned above. All pooled fractions related to protein peaks were tested for protease inhibition, and the fraction containing protease inhibitory activity was tested for its substrate specificity by using Z-Ala-Arg-Arg-4mBNA, as substrate.

Figure 3 shows the ion-exchange chromatographic profile of the rapeseed proteinaceous extract. Three peaks of protein were eluted from ion exchange chromatography using DEAE column with 0.02M sodium phosphate buffer (pH: 7.5) with 300, 600 and 1,000 mM salt step gradient (NaCl) were designated as peak A, B and C. When active inhibitory fractions were separated on DEAE-column, first peak was eluted by salt concentrations of 300 mM, the major second peak was eluted by 600 mM and the third one by 1,000 mM NaCl. By using azocasein as a general substrate, first pooled fraction (14-17), second fraction (22-27), and third fraction (29-31) resulted 0.8, 4.16, 2.67-fold purification, representing a yield of 8.52,

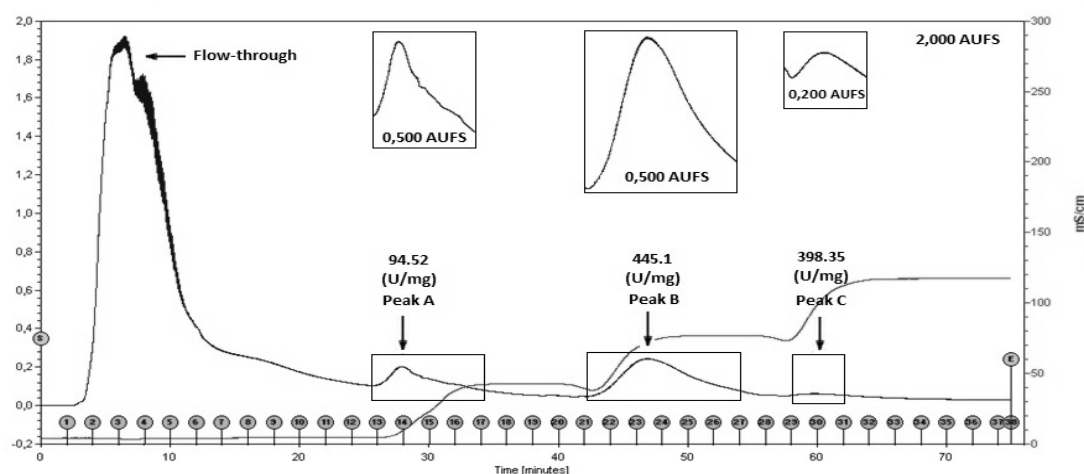


Figure 3. Elution profile from ion exchange chromatography of 50 % ammonium sulfate precipitated rapeseed proteins. Three protein peaks are shown with higher detection (0,500 and 0,200 AUFS= Absorbance Units Full Scale), and their specific cysteine protease inhibition (U mg^{-1} protein) is presented above the arrows. The step line is related to three step gradient of salt (NaCl, 300, 600 and 1,000 mM) by conductivity (mS cm^{-1}). The first peak shows flow-through elution. Chromatogram shows a fraction number ranging from 1 to 38 and 75 minutes elution time. The detector measures absorbance at 280 nm (expressed in Absorption Units, AU).



9.89 and 3.61%, respectively. Besides when Z-Ala-Arg-Arg-4mβNA were used as cysteine protease substrate, the purification folds of these fractions were obtained 5.27, 24.80 and 22.20, also the yield was 56.44, 59.06 and 30.13%, respectively. Peak B fraction showed the highest specific cysteine protease inhibition as compared to the ammonium sulfate fraction (17.94 U mg⁻¹ protein), it suggests the improvement of the purification by ion-exchange chromatography. Therefore, the results of the purification procedure for second peak by using azocasein and Z-Ala-Arg-Arg-4mβNA are summarized in Table 1.

When active inhibitory fractions were separated by gel filtration, three peaks were eluted from the column which the chromatographic profile was shown in

Figure 4. The purification of protease inhibitor by using azocasein as general substrate, pooled fraction of peak A (fractions 13-17), B (fractions 24-32) and C (fraction 35-48) resulted 2.05, 3.14 and 3.29-fold purification, representing a yield of 1.65, 3.59 and 3.66%, respectively. Moreover by using Z-Ala-Arg-Arg-4mβNA as cysteine protease substrate, purification folds were found 4.51, 7.44 and 25.60 with 3.65, 8.51 and 28.53% of recovery using pooled fractions of protein peak A, B and C, respectively. In the cysteine protease inhibition, ammonium sulfate precipitation lead to a 28.08 U mg⁻¹ inhibition, while the third protein peak showed the highest specific inhibition, designated as peak C. So the results of the purification showing the yield, fold purification and the specific

Table 1. Purification of protease inhibitor from rapeseed by ion exchange chromatography.

Purification step	Total protein (mg)	Total inhibition (U)	Specific inhibition (U mg ⁻¹ protein)	Yield (%)	Purification fold
(NH ₄) ₂ SO ₄ precipitation (50%)	21	2.55 ^a 376.8 ^b	0.12 ^a 17.94 ^b	100	1
DEAE fraction (Peak B)	0.5	0.25 ^a 222.6 ^b	0.5 ^a 445.1 ^b	9.9 ^a 59.1 ^b	4.2 ^a 24.8 ^b

^a General protease inhibitory activity; Azocasein as substrate. ^b Cystein protease inhibitory activity Z-Ala-Arg-Arg-4mβNA as substrate.

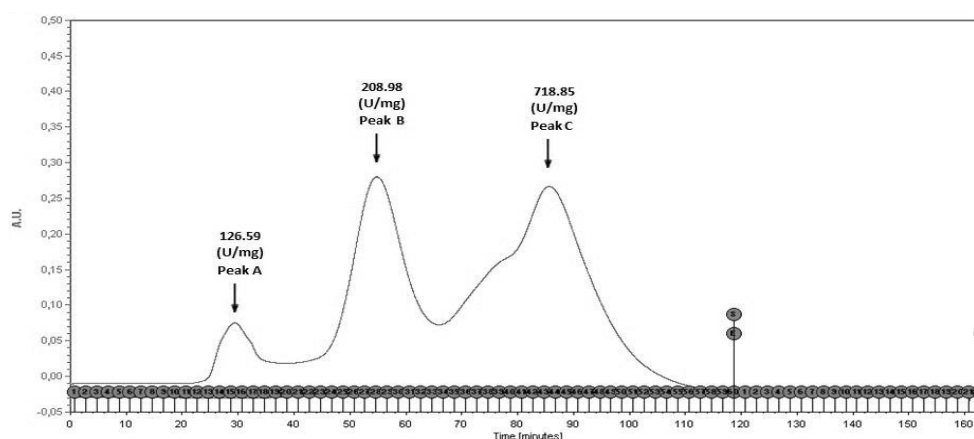


Figure 4. Gel filtration chromatogram of 50% ammonium sulfate precipitated rapeseed proteins. Three protein peaks can be noticed and their specific cysteine protease inhibition (U mg⁻¹ protein) is presented above the arrows. Chromatogram shows a fraction number ranging from 1 to 60 in 120 minutes, and again elution time was continued for 45 minutes. The detector measures absorbance at 280 nm (expressed in Absorption Units, AU).

inhibition activity at purification procedures of protease inhibitor using azocasein and Z-Ala-Arg-Arg-4mßNA as substrate from the ammonium sulfate precipitated 50% by the third fraction obtained from gel filtration chromatography is given in Table 2.

Following ammonium sulfate precipitation, rapeseed extract and enzyme extract was applied in affinity chromatography using silicon dioxide. A summary of CPB gut protease inhibitor purification using affinity chromatography is given in Table 3 in which azocasein and Z-

Ala-Arg-Arg-4mßNA were used as substrate.

SDS-PAGE

Analysis of the purified proteins by SDS-PAGE is shown in Figure 5. Using SDS-PAGE, the purified proteins from each ion exchange and affinity chromatography indicated two bands. The bands of protein were compared to the standard molecular mass of marker to determine their molecular

Table 2. Purification of protease inhibitor from rapeseed by gel filtration chromatography.

Purification step	Total protein (mg)	Total inhibition (U)	Specific inhibition (U mg ⁻¹ protein)	Yield (%)	Purification fold
(NH ₄) ₂ SO ₄ precipitation (50%)	21	3.35 ^a 589.66 ^b	0.16 ^a 28.08 ^b	100	1
Gel permeation fraction (Peak C)	0.23	0.12 ^a 168.21 ^b	0.52 ^a 718.85 ^b	3.7 ^a 28.5 ^b	3.3 ^a 25.6 ^b

^a General protease inhibitory activity; Azocasein as substrate. ^b Cystein protease inhibitory activity Z-Ala-Arg-Arg-4mßNA as substrate.

Table 3. Purification of protease inhibitor from rapeseed by affinity chromatography.

Purification step	Total protein (mg)	Total inhibition (U)	Specific inhibition (U mg ⁻¹ protein)	Yield (%)	Purification fold
(NH ₄) ₂ SO ₄ precipitation (50%)	17.5	2.51 ^a 314.02 ^b	0.14 ^a 17.94 ^b	100	1
Affinity chromatography fraction	0.28	0.21 ^a 114.14 ^b	0.74 ^a 407.64 ^b	8.3 ^a 36.3 ^b	5.2 ^a 22.7 ^b

^a General protease inhibitory activity; Azocasein as substrate. ^b Cystein protease inhibitory activity Z-Ala-Arg-Arg-4mßNA as substrate.

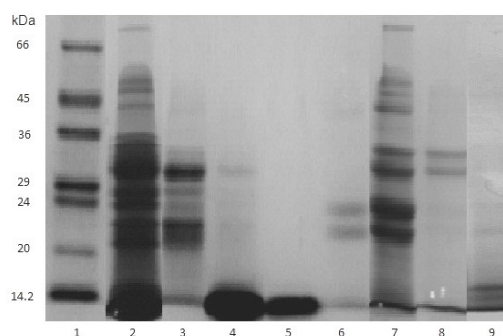


Figure 5. SDS-PAGE analysis for the purified rapeseed proteins by different chromatography methods. Coomassie brilliant blue was used to localize the protein bands. Lane 1: Molecular mass marker; Lane 2: Ammonium sulfate precipitated (50%); Lanes 3-5: Fractions A, B, C after gel filtration chromatography; Lane 6: After affinity chromatography, and Lanes 7-9: Fractions A, B, C after ion-exchange chromatography.



weight. Apparent molecular mass of the purified proteins from ion exchange were estimated approximately 34 and 32 kDa and affinity chromatography was about 24 and 22 kDa. Meanwhile, third fraction of ion-exchange chromatography showed double purified bands with approximately 15 and 16 kDa, as shown in lane 9. It is obvious that all these purified proteins have different molecular weights. However, the purified protein band(s) were not observed by using gel filtration chromatography (lanes 3-5). However, the single major band of apparent molecular mass of 14 kDa, visualized at lane 5, can be considered as a potential purified inhibitor, but this major band can consist of more than one purified protein.

DISCUSSION

In the search for various proteins with potential effects on the defense mechanism of plants, which are induced in response to insect attack, it has been essential to investigate different plant species to identify several protease and amylase inhibitors. Plant PIs are the most extensively studied group of plant-defense proteins whose primary site of action is the insect digestive system. The inhibition of proteases by PI causes a reduction in the availability of essential amino acids for the growth and development of the insect (Aguirre *et al.*, 2004, Abd El-Latif, 2015). The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access of substrates to the enzyme active site (Fan and Wu, 2005). Seeds are rich in defense proteins, so in the present report, protease inhibitors were purified and characterized from the seeds of rapeseed.

It is important that *in vivo* conditions may modulate enzyme specificity. For example, the acidic optimum pH for inhibition by inhibitors may be responsible for their inhibition of enzymes in Coleoptera, whose intestinal contents are acidic (Franco *et al.*, 2002). For this purpose, the inhibitory pH of

proteinaceous extract of rapeseed on digestive protease of CPB was studied in the current paper. In our previous study (Ashouri *et al.*, 2015), the optimum pH of digestive protease activity of CPB was found acidic (pH: 5). In accordance to the optimum pH of enzyme activity, the maximum inhibitory effect of extract was found at pH 5. It was said that interaction between enzyme and seed extract is also pH dependant. Saberi Riseh *et al.* (2014) investigated optimal trypsin activity of digestive protease in the date palm fruit stalk borer (*Oryctes elegans* Prell) larvae and obtained pH 11, also the highest inhibition of trypsin activity by inhibitor from the broad bean (*Vicia faba* L.) occurred at pH: 11.

The results showed that ammonium sulfate is an effective and simple means for protein separation. Ammonium sulfate precipitation is a common method for purifying proteins; Ammonium sulfate can "salt out" proteins in the solution, causing their precipitation at selective concentrations. This is due to the high solubility of ammonium sulfate which allows aggregation and precipitation of proteins in aqueous solutions at different salt concentrations (Wenk and Fernandis, 2007). This provides a convenient method to fractionate complex protein mixtures. In this study, after achievement of optimum inhibitory pH by a general protein fraction (0-70%), four selective fractions (30, 50, 70 and 100%) were prepared and their inhibitory activity on fourth larval gut protease was compared. Results indicated that the first three fractions had high inhibitory activity on digestive protease of L₄, significantly. When the sensitivity of all developmental stages of CPB digestive protease to these inhibitors was compared, it is obvious that all stages were affected significantly and there is a significant difference between stages in enzyme inhibition just by second fraction (50%). As a whole statement, the fourth instar larva of CPB is the least sensitive developmental stage. The gel assay showed more than ten proteolytic bands in the insect gut. Also, gel

assays confirmed the meaningful inhibition of protease by inhibitor and the intensity of the bands significantly decreased.

The fraction that contained the highest inhibitory activity (50%) was selected and applied in further purification procedures. As can be seen ion-exchange chromatography by DEAE column and affinity chromatography by silicon dioxide were able to separate the crude extract of rapeseed into active fractions which resulted in the observed increase in specific inhibition activity and purification fold. To sum up, purification fold and yield in all pooled fractions of each chromatography were not similar. The highest level of purification (5.2 fold) and specific inhibition activity (0.743 U mg^{-1} protein) was observed after affinity chromatography by using azocasein as a substrate as compared to ion exchange and gel filtration chromatography. This suggests that affinity chromatography would be a more appropriate method, because larval gut enzyme was used as ligand of protease inhibitor, therefore purification was more specific compared to the other methods. Strong cysteine inhibitory activity was detected in the fractions B and C of ion-exchange chromatography, fraction C of gel permeation, also the single fraction of affinity chromatography. The purification fold in all cysteine inhibition tests was similar but specific inhibition activity of gel filtration fraction was high (718.85 U mg^{-1} protein) as compared to the others. However, gel filtration was not an appropriate inhibitor purification method from rapeseed extract because the precisely purified protein band(s) were not observed on the gel. Purified proteins by ion-exchange and affinity chromatography appeared as double bands in SDS-PAGE under reducing conditions, and all of them had low molecular weights.

Menegatti *et al.* (1992) isolated a serine protease inhibitor with molecular weight of about 7 kDa from white mustard (*Sinapis alba* L.) seed by affinity chromatography and reverse phase HPLC which inhibited the

catalytic activity of bovine trypsin and chymotrypsin. Ceciliani *et al.* (1994) isolated trypsin inhibitor protein from rapeseed (*B. napus* var. Oleifera) which inhibited the catalytic activity of bovine beta-trypsin and bovine alpha-chymotrypsin, corresponding to a molecular weight of about 6.7 kDa. Ascenzi *et al.* (1999) isolated a low-molecular-mass (about 7 kDa) serine protease iso-inhibitor from oil-rape (*B. napus* var. Oleifera) seed. In accordance, the cysteine protease inhibitors were purified from this oilseed in the present study.

In other literatures about purification of insect digestive enzyme inhibitor from the plant seeds with different methods Oliveira *et al.* (2002) purified a 20 kDa protein with high inhibitory activity against papain (cysteine protease) from seeds of the algarroba tree (*Prosopis juliflora* Sw.) using Sephacryl S-200 gel filtration. This was followed by reverse-phase high-performance liquid chromatography, with effects on digestive proteases of the bean weevil (*Acanthoscelides obtectus* Say) and the cowpea weevil (*Callosobruchus maculatus* Fabricius), moderately active toward midgut proteases from the pod weevil (*Mimosestes mimosae* Fabricius) and the Mexican bean weevil (*Zabrotes subfasciatus* Bohemann). Giri *et al.* (2003) purified seven serine proteinase inhibitors from winged bean (*Psophocarpus tetragonolobus* L.) by heat treatment and gel filtration, which had inhibitory effects on gut serine protease of the podborer (*Helicoverpa armigera* Hübner). Aguirre *et al.* (2004) purified a novel trypsin inhibitor from Chan seeds (*Hyptis suaveolens* L.) with apparent molecular mass of 8.7 kDa and highly specific inhibitory activity toward all trypsin-like proteases from the gut of the maize pest (*Prostephanus truncatus* Horn). They claimed that this inhibitor has potential to enhance the defense mechanism of maize against the attack of *P. truncatus*. Oliveira *et al.* (2007) purified a trypsin papain inhibitor from the seeds of a leguminosae tree (*Pithecellobium dumosum* Benth.) with molecular weight of 18.1 kDa and a



competitive inhibition mechanism. They stated that it was effective against digestive protease of some insect pests; the Mexican bean weevil, the cowpea weevil, the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann), the Indian meal moth (*Plodia interpunctella* Hübner) and the cotton leafworm (*Alabama argillacea* Hübner), with 74.5, 70.0, 70.3, 48.7, and 13.6% inhibition, respectively. Kansal *et al.* (2008) purified trypsin inhibitor up to 60.46 fold and 29.20% recovery from chickpea (*Cicer arietinum* L.) seeds using ammonium sulfate fractionation, DEAE-Sephadex A-25 and Sephadex G-75, with a single band 30 kDa molecular mass, which showed inhibitory activity against gut trypsin protease of the *H. armigera*, *in-vitro* and *in-vivo*. Shamim *et al.* (2011) isolated a cysteine protease inhibitor from the seeds of jackfruit (*Artocarpus heterophyllus* Lam.) by precipitation with ammonium sulfate, DEAE-cellulose and gel filtration (Sephadex G-100) chromatography that strongly inhibited papain and midgut proteases of yellow stem borer (*Scipophaga incertulas* Walker) larvae with the maximum activity between pH 4 and 10 and the molecular mass of 14.5 kDa. Babu *et al.* (2012) isolated proteinase inhibitor of *H. armigera* gut trypsin from *Acacia nilotica* (L.) by ammonium sulfate precipitation and chromatography on DEAE-Sephadex A-25 and found a single band with molecular weight of 18.6 kDa, in a purification of 10.68-fold with a 19.5% yield. Aghaali *et al.* (2013) found that protease inhibitor extracted from grass pea (*Lathyrus sativus* L.) was able to inhibit 34.72 and 100% of the fall webworm (*Hyphantria cunea* Drury) gut general protease and trypsin activities, respectively. They used ammonium sulfate precipitation and gel filtration methods for purification and determined the molecular mass of the purified inhibitor as 45 kDa. Abd El-Latif (2015) detected trypsin and chymotrypsin inhibitory activity in the flour seed extracts of four Egyptian varieties of soybean (*Glycine max* L.) with non-competitive type of inhibition and used ammonium sulfate fractionation and DEAE-

Sephadex A-25 column for purification, also purified protein showed a single band on SDS-PAGE (17.9 kDa). In these literatures, all purified insect gut enzyme inhibitors have low molecular weight in accordance with obtained results for purified proteins in this study.

Due to dependence on protease for survival, this enzyme can be a good target candidate for bio-insecticides via enzyme inhibitors. Whereas herbivore insects are highly complex organisms and will express any ability to avoid PI effects, inactivate them or compensate for any nutritional stress as it was seen with the Colorado potato beetle larvae (Cloutier *et al.*, 2000). For instance, oryzacystatin I and II (OCI and OCII) on transformed plants have potential in controlling pests that use cysteine proteinases for food digestion, however; expression of a single *OC* gene in potato exhibited a minimal or no effect on CPB fitness traits and larval survival (Cloutier *et al.*, 2000, Cingel *et al.*, 2016). Moreover, co-expression of *OC* genes reduced the development time, also general and cysteine protease activities, thus significantly decreased plant damage caused by CPB larvae (Cingel *et al.*, 2016). Consequently, in trying to find more effective PI proteins for plant protection, we need to isolate inhibitors from various plant sources and assess their efficiency against CPB proteases *in-vitro* and *in-vivo*. The current study provides a biochemical analysis of purified proteins of rapeseed on CPB digestive protease. These data revealed that rapeseed proteinaceous molecules that can interfere with digestive protease of the Colorado potato beetle may present an interesting potential for the development of insect-resistant transgenic potato that express heterologous protease inhibitors. Many of these kinds of plants are now being tested in field conditions or awaiting commercialization. Further studies on the characterization of these proteins with protease inhibitory potential are in progress.

ACKNOWLEDGEMENTS

This project was funded by a grant from University of Tabriz. The authors are thankful to the Biochemistry Department, Ege University, Izmir, Turkey, where a big part of the study was conducted and to Dr. Serap Evran. Thanks to the Seed and Plant Improvement Institute of Karaj (Iran) for supplementation of seeds.

REFERENCES

1. Abd El-Latif, A. O. 2015. Protease Purification and Characterization of a Serine Protease Inhibitor from Egyptian Varieties of Soybean Seeds and Its Efficacy against *Spodoptera littoralis*. *J. Plant Prot. Res.*, **55**: 16-25.
2. Aghaali, N., Ghadamyari, M., Hosseiniaveh, V. and Saberi Riseh, N. 2013. Protease Inhibitor from the Crude Extract of Plant Seeds Affects the Digestive Proteases in *Hyphantria cunea* (Lep.: Arctiidae). *J. Plant Prot. Res.*, **53**: 338-346.
3. Aguirre, C., Valdes-Rodriguez, S., Mendoza-Hernandez, G., Rojo-Dominguez, A. and Blanco-Labra, A. 2004. A Novel 8.7 kDa Protease Inhibitor from Chan Seeds (*Hyptis suaveolens* L.) Inhibits Proteases from the Larger Grain Borer *Prostephanus truncatus* (Coleoptera: Bostrichidae). *Comp. Biochem. Physiol. B*, **138**: 81-89.
4. Ascenzi, P., Ruoppolo, M., Amoresano, A., Pucci, P., Consonni, R., Zetta, L., Pascarella, S., Bortolotti, F. and Menegatti, E. 1999. Characterization of Low-Molecular-Mass Trypsin Inhibitors from Oilrape (*Brassica napus* var. *Oleifera*) Seed. *Eur. J. Biochem.*, **261**: 275-284.
5. Asenjo, J. A. and Andrews, B. A. 2009. Protein Purification Using Chromatography: Selection of Type, Modelling and Optimization of Operating Conditions. *J. Mol. Recognit.*, **22**: 65-76.
6. Ashouri, S., Farshbaf Pourabad, R., Bandani, A. and Dastranj, M. 2015. Inhibitory Effects of Barley and Wheat Seed Protein on Digestive α -Amylase and General Protease Activity of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *Turk. J. Entomol.*, **39**: 321-332.
7. Babu, S. R., Subrahmanyam, B., Rinivasan, S. and Santha, I. M. 2012. *In vivo* and *In vitro* Effect of *Acacia nilotica* Seed Proteinase Inhibitors on *Helicoverpa armigera* (Hübner) Larvae. *J. Biosci.*, **37**: 269-276.
8. Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.*, **72**: 248-254.
9. Carlini, C. R. and Grossi-de-Sa, M. F. 2002. Plant Toxic Proteins with Insecticidal Properties: A Review on Their Potentialities as Bioinsecticides. *Toxicon.*, **40**: 1515-1539.
10. Cecilian, F., Bortolotti, F., Menegatti, E., Ronchi, S., Ascenzi, P. and Palmieri, S. 1994. Purification, Inhibitory Properties, Amino Acid Sequence and Identification of the Reactive Site of a New Serine Proteinase Inhibitor from Oil-Rape (*Brassica napus*) Seed. *FEBS Lett.*, **342**: 221-224.
11. Cingel, A., Savić, J., Lazarević, J., Ćosić, T., Raspor, M., Smigocki, A. and Ninković, S. 2016. Co-Expression of the Proteinase Inhibitors Oryzacystatin I and Oryzacystatin II in Transgenic Potato Alters Colorado Potato Beetle Larval Development. *Insect Sci.*, Doi: 10.1111/1744-7917.12364.
12. Cloutier, C., Jean, C., Fournier, M., Yelle, S. and Michaud, D. 2000. Adult Colorado Potato Beetles, *Leptinotarsa decemlineata* Compensate for Nutritional Stress on Oryzacystatin I Transgenic Potato Plants by Hypertrophic Behavior and Over-Production of Insensitive Proteases. *Arch. Insect Biochem. Physiol.*, **44**: 69-81.
13. Elpidina, E. N., Vinokurov, K. S., Gromenko, V. A., Rudenskaya, Y. E. and Zhuzhikov, D.P. 2001. Compartmentalization of Proteinases and Amylases in *Nauphoeta cinerea* Midgut. *Arch. Insect Biochem. Physiol.*, **48**: 206-216.
14. Fan, S. and Wu, G. 2005. Characteristics of Plant Proteinase Inhibitors and Their Applications in Combating Phytophagous Insects. *Bot. Bull. Acad. Sin.*, **46**: 273-292.
15. Franco, O. L., Rigden, D. J., Melo, F. R. and Grossi-de-Sa, M. F. 2002. Plant Alpha-Amylase Inhibitors and Their Interaction with Insect Alpha-Amylases, Structure, Function and Potential for Crop Protection. *Eur. J. Biochem.*, **269**: 397-412.
16. Gatehouse, A. M. R., Norton, E., Davison, G. M., Babbe, S. M., Newell, C. A. and Gatehouse, J. A. 1999. Digestive Proteolytic



- Activity in Larvae of Tomato Moth, *Lacanobia oleracea*, Effects of Plant Protease Inhibitors *In vitro* and *In vivo*. *J. Insect Physiol.*, **45**: 545-558.
17. Giri, A. P., Harsulkar, A. M., Ku, M. S., Gupta, V. S., Deshpande, V. V., Ranjekar, P. K. and Franceschi, V. R. 2003. Identification of Potent Inhibitors of *Helicoverpa armigera* Gut Proteinases from Winged Bean Seeds. *Phytochem.*, **63**: 523-32.
18. Grudkowska, M. and Zagdanska, B. 2004. Multifunctional Role of Plant Cysteine Proteinases. *Acta Biochim. Pol.*, **51**: 609-624.
19. Habib, H. and Fazili, K. M. 2007. Plant Protease Inhibitors: A Defense Strategy in Plants. *Biotechnol. Molec. Biol. Rev.*, **2**: 68-85.
20. Kansal, R., Kumar, M., Kuhar, K., Gupta, R.N., Subrahmanyam, B., Koundal, K. R. and Gupta, V. K. 2008. Purification and Characterization of Trypsin Inhibitor from *Cicer arietinum* L. and Its Efficacy against *Helicoverpa armigera*. *Braz. J. Plant. Phys.*, **20**: 313-322.
21. Karimi, J., Haubruge, É. and Francis, F. 2010. Development of Entomotoxic Molecules as Control Agents: Illustration of Some Protein Potential Uses and Limits of Lectins (Review). *Biotechnol. Agron. Soc. Environ.*, **14**: 225-241.
22. Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of Bacteriophage T4. *Nature*, **227**: 680-685.
23. Macedo, M. L. R. and Freire, M. D. G. M. 2011. Insect Digestive Enzymes as a Target for Pest Control. *Invert. Surv. J.*, **8**: 190-198.
24. Menegatti, E., Tedeschib, G., Ronchib, S., Bortolotti, F., Ascenzi, P., Thomasd, R. M., Bolognesi, M. and Palmieri, S. 1992. Purification, Inhibitory Properties and Amino Acid Sequence of a New Serine Proteinase Inhibitor from White Mustard (*Sinapis alba* L.) Seed. *FEBS Lett.*, **301**: 10-14.
25. Novillo, C., Castanera, P. and Ortego, F. 1997. Characterization and Distribution of Chymotrypsin-Like and Other Digestive Proteases in Colorado Potato Beetle Larvae. *Arch. Insect Biochem. Physiol.*, **36**: 181-201.
26. Oliveira, A. S., Pereira, R. A., Lima, L. M., Morais, A. H. A., Melo, F. R., Franco, O. L., Bloch, J. C., Grossi-de-Sa, M. F. and Sales, M. P. 2002. Activity toward Bruchid Pest of a Kunitz-Type Inhibitor from Seeds of the Algaroba Tree (*Prosopis juliflora* D.C.). *Pest Biochem. Physiol.*, **72**: 122-132.
27. Oliveira, A. S., Migliolom L., Aquino, R. O., Ribeiro, J. K. C., Macedo, L. L. P., Andrade, L. B. S., Bemquerer, M. P., Santos, E. A., Kiyota, S. and Sales, M. P. 2007. Purification and Characterization of a Trypsin-Papain Inhibitor from *Pithecellobium dumosum* Seeds and Its *In vitro* Effects towards Digestive Enzymes from Insect Pests. *Plant Physiol. Biochem.*, **45**: 858-865.
28. Saberi Riseh, N., Ghadamyari, M., Hosseinnaveh, V., Moetamedinia, B. and Aghaali, N. 2014. Effect of Inhibitors from Plant Seeds on Digestive Proteolytic Activities in Larvae of the Date Palm Fruit Stalk Borer, *Oryctes elegans* Prell (Coleoptera: Scarabaeidae). *J. Agr. Sci. Tech.*, **16**: 981-992.
29. Schluter, U., Benchabane, M., Munger, A., Kiggundu, A., Vorster, J., Goulet, M., Cloutier, C. and Michaud, D. 2010. Recombinant Protease Inhibitors for Herbivore Pest Control: A Multitrophic Perspective. *J. Exp. Bot.*, **15**: 4169-4183.
30. Shamim, M. D., Khan, N. A. and Singh, K. N. 2011. Inhibition of Midgut Protease of Yellow Stem Borer (*Scirpophaga incertulas*) by Cysteine Protease-Like Inhibitor from Mature Jackfruit (*Artocarpus heterophyllus*) Seed. *Acta Physiol. Plant*, **33**: 2249-2257.
31. Sharma, K. 2015. Protease Inhibitors in Crop Protection from Insects. *Int. J. Curr. Aca. Rev.*, **3**: 55-70.
32. Wenk, M. R. and Fernandis, A. Z. 2007. *Manuals in Biomedical Research: A Manual for Biochemistry Protocols*. World Scientific Publishing Co., Pte. Ltd., Singapore, **3**: 2-7.

خالص سازی مهار کننده سیستمین پروتئاز گوارشی *Leptinotarsa decemlineata* (Say) از بذور کلزا

ش. عاشوری، ف. ذیهنی اوغلو، ر. فرشاف پورآباد، و ا. کوجاداغ

چکیده

هدف از این تحقیق، خالص سازی مهار کننده های سیستمین پروتئاز از بذور کلزا (*Brassica napus* L.) با فعالیت بالقوه روی پروتئاز گوارشی سوسک کلرادو سیب زمینی (*Leptinotarsa decemlineata* Say) می باشد. فرکشن های پروتئینی رسوب یافته با سولفات آمونیوم ۳۰، ۵۰، ۷۰ و ۱۰۰٪ به ترتیب ۳۹/۰۷، ۵۷/۰۳، ۵۱/۴۷ و ۲۲/۴۴٪ مهار در فعالیت پروتئاز تام روده لارو سن چهار نشان دادند. فرکشن ۵۰٪ بالاترین اثر مهار را روی فعالیت پروتئاز تام گوارشی تمام مراحل رشدی نشان داد. سنجش در ژل نیز مهار فعالیت آنزیم را تأیید کرد. فرکشن ۵۰٪ با استفاده از روش های مختلف کروماتوگرافی؛ تعویض یونی با استفاده از DEAE، ژل فیلتراسیون و تمایلی با استفاده از SiO_2 و هموژنات روده لارو سوسک کلرادو خالص سازی شد. سه پیک پروتئینی از کروماتوگرافی تعویض یونی با استفاده از شیب نمک NaCl و نیز از کروماتوگرافی ژل فیلتراسیون به دست آمد. زمانی که Z-Ala-Arg-Arg-4mβNA به عنوان زیرنشت سیستمین پروتئاز استفاده گردید، میزان خالص سازی فرکشن دوم کروماتوگرافی تعویض یونی ۲۴/۸۰ برابر و بازده آن ۵۹/۰۹٪ به دست آمد، فرکشن سوم ژل فیلتراسیون ۲۵/۶۰ برابر خالص سازی و ۲۸/۵۳٪ بازده داشت و برای فرکشن تمایلی ۲۲/۲۷ برابر خالص سازی و ۳۶/۳۵٪ بازده محاسبه شد. در SDS-PAGE وزن مولکولی ظاهری پروتئین های خالص سازی شده توسط تعویض یونی ۳۲ و ۳۴ کیلو دالتون و توسط تمایلی ۲۴ و ۲۲ کیلو دالتون تعیین شدند. با این حال، ژل فیلتراسیون روش مناسبی در این مطالعه به شمار نیامد، زیرا که نوار پروتئینی خالصی در ژل مشاهده نشد. در کل، روش های کروماتوگرافی مورد استفاده در این تحقیق روش های موفق در خالص سازی پروتئین مهار کننده به شمار می آیند، به ویژه کروماتوگرافی تمایلی که در آن از آنزیم روده سوسک کلرادو سیب زمینی به عنوان لیگاند استفاده شد و بنابراین پروتئین های به دست آمده دارای خواص مهار کنندگی مختص پروتئاز روده این حشره آفت بودند.