Effect of *Citrullus colocynthis* L. (Cucurbitaceae) agglutinin on gene expression of caspases in *Ectomyelois ceratoniae* Zeller (Lepidoptera: Crambidae)

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Abstract

The larvae of *Ectomyelois ceratoniae* Zeller were reared on the artificial diets containing 2% of *Citrullus colocynthis* L. (Cucurbitaceae) lectin and control to find any changes in gene expression of caspases. Specific primers were designed to amplify genes of six found caspases including *Caspase 1*, *2*, *3*, *4*, *5* and *6*. RNA of both treatments were extracted and cDNA molecules were synthesized prior to gene expression. Results of quantitative real-time polymerase chain reaction revealed that expression of all caspases in the fed larvae by 2% of lectin increased in comparison with control except for caspase 1 as 2.99, 5.7, 17.13, 23.18, 10.44-fold. These results are consistent with our previous studies on the lower activities of digestive enzymes that are due to death of midgut cells followed by binding of lectin molecule.

Introduction

Apoptosis is a programmed cell death or suicide which play the critical roles in various physiological processes of insects such as immunity, development, reproduction, homeostasis, DNA damage, *etc*. (Courtiade *et al.,* 2011). Cells engaged in the process have several characteristic marks such as membrane blebbing, chromatin condensation, dismantling of cellular contents, packaging of dead cell into apoptotic bodies prior to be phagocytized by immune-involved cells (Bryant, 2002). A group of cysteine-rich proteases called caspases are involved in apoptosis. Studies on ancestral origin of these enzymes revealed metacaspases in plants and fungi, paracaspases in slime molds and animals and true caspases in animals like insects (Koonin & Aravind, 2002). Distribution of these enzymes is different within Animalia kingdom but all of them were synthesized as inactive form to prevent their damage to normal tissues (Courtiade *et al.,* 2011). Five caspases have been reported in lepidoptera based on phylogenetic analyses through genomic, structural and functional studies (Courtiade *et al.,* 2011). During apoptotic process, initiator caspases are activated initially leading to activate the effector caspases, which are responsible for cleavage of many cellular components as DNA fragmentation and membrane blebbing (Bryant, 2002).

Lectins are the carbohydrate-binding proteins in many organisms that bind reversibly to mono or oligosaccharides on the surface of cells (Peumans & van Damme, 1995). Different studies have shown reduced performance of insects after adding lectins to their diets that may confirm lectins as the entomotoxic proteins. Histofluorescence studies revealed that lectins bind to epithelial cells of insect midgut and cause total loss of cell viability. In details, Hamshou *et al.* (2010) demonstrated that these molecules bind to specific carbohydrate moieties on the cell membrane proteins and start a signaling transduction cascade leading to death of the midgut epithelial cells.

Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae) is a serious pest of pomegranate in Iran. Larvae utilize inner parts of fruit so control procedures have no suitable efficiencies against the pest. In our previous studies, we determined effects of a lectin from *Citrullus colocynthis* on life table, larval weight, survival, digestive physiology and intermediary metabolism of *E. ceratoniae* larvae (Ramzi & Sahragard, 2013; Ramzi *et al.,* 2014, 2015). Different concentrations of the lectin led to significant decrease of larval survival, weight, activity of digestive enzyme and intermediary metabolism. The current study was conducted to determine expression changes of caspase genes in the larvae of *E. ceratoniae* because lectins can harm epithelial cell of larval midgut because of their entomotoxic properties.

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Materials and methods

Insect rearing

Larvae of *E. ceratoniae* were collected from pomegranate orchards

and fed on artificial diet containing wheat bran (100 g), yeast (3 g), sugar (10 g), glycerine (40 mL) and water (40 mL) for at least 5 generations to have a homogeneous stock population at 28±2°C, 85% of relative humidity and 16:8 h. L:D (Ramzi *et al.,* 2014).

Treatment of *Ectomyelois ceratoniae* larvae by *Citrullus colocynthis* lectin

Artificial diets containing 0 (as control) and 2% of *C. colocynthis* agglutinin (CCA) were prepared and 30 newly laid eggs of *E. ceratoniae* were put on each diet. Hatched larvae were allowed to feed on control and CCA diets to the fifth larval instars.

Larval dissection and RNA extraction

Fifth larval instars of control and treated diets were dissected in diethylpyrocarbonate (DEPC) water and 50 mg of their midguts were used for RNA extraction. Kit of Cynacolon Company (Cat #RN7713C; Tehran, Iran) was used to extract RNA from larvae. Larval midguts (50 mg) were separately homogenized in 1 mL of RNX™ (plus) (CinnaGen Co., Teheran, Iran) solution and incubated for 5 min in room temperature prior to be vortexed. Then, 200 μL of chloroform was added, shaked and incubated on ice for 5 min. After 15 min of centrifugation, four phases were determined that the first one contained RNA that transferred to new tube. Equal volume of isopropanol alcohol was added to samples and those were centrifuged at 12,000 rpm for 15 min. Then, 1 ml of alcohol 75% was added and centrifugation was made at 7500 rpm for 8 min. Finally, samples were dried and 50 μL of DEPC water was added abd quality of extracted RNA was evaluated in Agarose gel (1%).

Real-time polymerase chain reaction

A kit from Thermo Fisher Scientific Co. (Waltham, MA, USA) was used to synthesize cDNA from control and treated larvae. Briefly, 5 μL of RNA was incorporated by 0.5 μg of Oligo dT and 11 μL DEPC. The mixture was incubated at 60°C for 5 min. After cooling, 4 μL of reaction buffer, 2 μL of deoxynucleotriphosphate (10 μM) and 20 u of RNase inhibitor were added to 19 μL of DEPC and incubated for 5 min at 37°C. Then, 200 u of Revert Aid M-Mulv was added and the reaction was incubated at 42°C for 60 min prior to final incubation at 70°C for 10 min.

Quantitative real-time polymerase chain reaction

Reaction mixture contained 10 μL of SYBR®Premix EXTaqTM (Takara Bio Inc., Shiga, Japan), 0.8 μL of forward and reverse primers (Table 1), 0.4 μL of dye and 2 μL of cDNA. Amplification program was adopted as Table 1. Quantification of gene expression was calculated based on $2^{-\Delta\Delta Ct}$ proposed by Schmittgen and Livak (2008). Data were normalized by using *18SrRNA* gene.

Results and discussion

Results of the current study revealed feeding of *E. ceratoniae* larvae on the diet containing 2% of *C. colocynthis* lectin affect expression of caspase genes. Figure 1 shows quality of the extracted RNAs from control and treated larvae indicating 28S, 18S and 5S RNAs (Figure 1). The samples were used in polymerase chain reaction (PCR) of synthesized cDNA and results revealed specific amplification of caspase genes (Figure 2). Results of quantitative real-time-PCR revealed that expression of all caspases in treated larvae by 2% of lectin were increased in comparison with control except for caspase 1 as 2.99, 5.7, 17.13, 23.18, 10.44-fold (Table 2).

Suicide pathways of cells via apoptosis are the crucial phenomena in the life of living organisms. These paths are involved in the several physiological processes by the main roles of proteases with conserved domains called caspases (Accorsi *et al.,* 2015). In lepidopterans, apoptosis is the main mechanism in metamorphosis on defence against pathogens (Courtiade *et al.,* 2011). It has been reported 66 sequences out of 27 lepidopterous species with emphasis on presence of at least 5 caspases in insects although role of caspase 4 has not been yet eluci-

Table 1. Primers and polymerase chain reaction programs used in amplification of caspase genes.

Primers	Amplification program	Gene 5'-3'
Caspase 1	Step 1: 95°C, 2 min Step 2: 95°C, 30 s Step 3: 57.2°C, 30 s Step 4: 72°C, 20.0 s Step 5: Repeat steps 2-4 29 more times Step 6: 72°C, 5 min Step 7: 4°C, forever	R: TGTCTAGCGCCCTTTATGCT F: CATCTCCACCATTGGCTCTT
Caspase 2	-	R: CAATCTGGGGTCTTTGAGGA F: CACAGTCTTTTCCAGTGCCA
Caspase 3	-	R: ACCTACGGAAAACCAAGCCT F: CAGGTACTTTTTCCCCGACA
Caspase 4	-	R: CGTTCCATTAACGAATCGGAG F: AGCCTGTCTATTTCCTCGCA
Caspase 5	-	R: ACATTCAATCCCATTCCCAA F: TGAACTTGGTGCTCTTGACG
Caspase 6	-	R: GACCGTGGTATGCCAAAAGT F: TGCAGTGGCCCAATATATCA
18SrRNA	-	F: CACGGGAAATCTCACCAGG R: CAGACAAATCGCTCCACCAACTA

dated (Courtiade *et al.,* 2011). In our study, caspase 5 showed the highest expression rate in comparison with other caspases except for caspase 1 with no expression. Shahidi-Noghabi *et al.* (2010) found that fed larvae of *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) on lectin I and II from *Sambucus niger* L. induced caspase 3 activity versus control. In details, SNA-I causes cell death via their wrinkling, condensing of nucleus and DNA fragmentation. Effect of the lectin was dose-dependent and killed the cells after four days. Similar results were observed followed by feeding on SNA-II. Hamshou *et al.* (2013) demonstrated that an agglutinin from *Rhizoctonia solani* de Bary bound to microvillar brush-border of the midgut in *Spodoptera littoralis* Hubner (Lepidoptera: Noctuidae) larvae, induced cell death, DNA fragmentation and increased activity of caspase 3. Also, Sprawka *et al.* (2013) showed that a phytohemagluttinin from fabaceae had a cytotoxic effect in the midgut of *Sitobion avenae* Fabricius (Hemiptera: Aphididae). The authors observed DNA fragmentation and increased activity of caspase 3 in the treated aphids.

Conclusions

Caspases are the unique enzymes involved in programmed cell death caused during metamorphosis or defend against a xenobiotic factor. Since lectins bind to epithelial midgut cells of insects, programmed cell death by means of caspases remove dead cell and protect other cells. Our results showed as the first time increased expression of 5 out of 6 identified caspases in lepidopteran followed by feeding on CCA. Based on the earlier studies, if we consider main role of caspase 3, it can be found expression rate of 5.7-fold versus control. Also, our results highlighted previous studies on the cytotoxic effects of lectins on epithelial cells of insects and apoptosis via caspase activities. These results may be helpful to better elucidation of lectin entomotoxic mechanism followed by ingestion by an insect which may be helpful to find whether lectins cause similar results on non-target organisms or not.

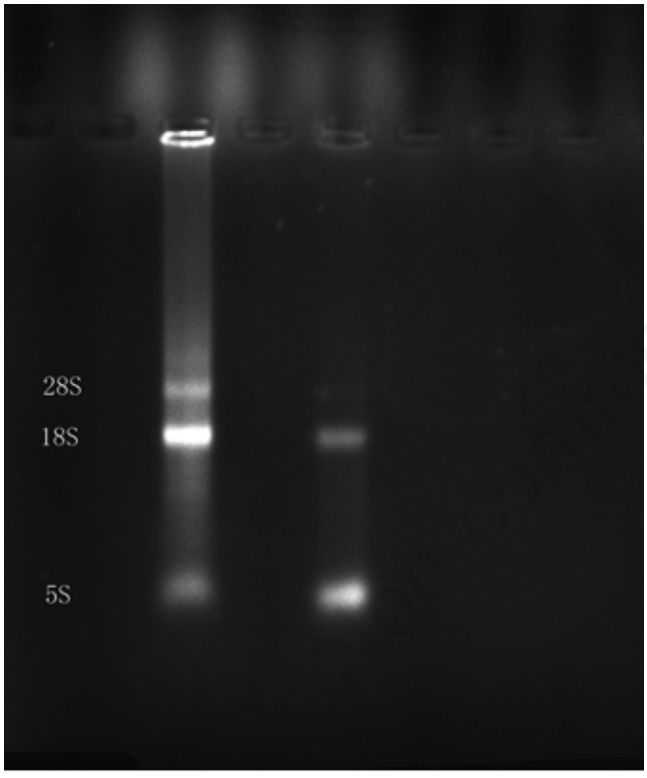

Figure 1. Quality of extracted RNA from control and treated larvae of *Ectomyelois ceratoniae* by *Citrullus colocynthis* lectin.

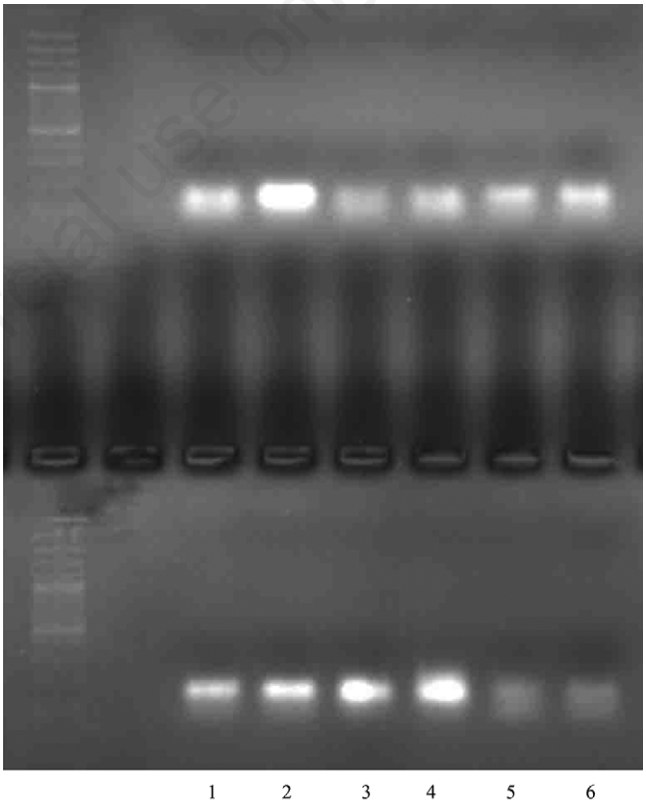

Figure 2. Amplification of caspase genes by specific primers. Top: treatment larvae; Down: control larvae.

Table 2. Gene expression of caspases in control and treated larvae of *Ectomyelois ceratoniae* fed on *Citrullus colocynthis* lectin.

	Caspase 1	*Caspase 2*	*Caspase 3*	*Caspase 4*	*Caspase 5*	*Caspase 6*
Treatment	15.12	18.48	23.16	17.45	12.21	10.28
Control	15.11	16.98	26.01	26.02	23.80	15.51
ΔΔCt	0.0066	1.49	2.85	8.56	11.59	5.22
Expression ratio	0.0013	2.99	5.7	17.13	23.18	10.44

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