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RAPID COMMUNICATION

Effects of specific inhibitors on the gene expression of a digestive trypsin in *Pieris brassicae* L. (Lepidoptera: Pieridae)

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Abstract

Protein digestion in insects relies on several groups of proteases, among which trypsin plays a prominent role. In the current study, larvae of *Pieris brassicae* L. were fed radish leaves treated with 1 mM concentrations of three specific inhibitors of trypsin: AEBSF.HCl [4-(2--aminoethyl)-benzenesulfonyl fluoride, monohydrochloride], TLCK (N-a-tosyl-l-lysine chloromethyl ketone) and SBTI (Soybean Trypsin Inhibitor) to find their potential effects on gene expression of trypsin. Initially, RT-PCR analysis revealed a gene of 748 bp responsible for synthesizing the digestive trypsin in *P. brassicae* larvae. Also, qRT-PCR data indicated a statistically greater expression of trypsin gene in the larvae fed 1 mM concentrations of AEBSF.HCl, TLCK and SBTI than the control. Results of the current study indicated that synthetic inhibitors can not only negatively affect the gene expression of *P. brassicae* trypsin, but also the insect can activate a compensatory mechanism against interruption of protein digestion by inducing more expression of the gene and producing more trypsin into the midgut lumen.

Key words: gene expression, *Pieris brassicae*, synthetic inhibitors, trypsin

Introduction

Amino acids are some of the most important components of insect foods and are divided into two groups: non-essential and essential amino acids. The latter should be derived from the diet and are utilized in several physiological processes (Klowden 2007; Nation 2008). Insects, both herbivorous and carnivorous insects are able to process the proteins found in their diets to obtain the amino acids they need. For this purpose, insects have a set of protease enzymes which is classified according to molecular structure and biochemical properties (Nation 2008; Terra and Ferreira 2012). Initially, the digestive proteases of insects are divided into two groups of exopeptidases and endopeptidases. The exopeptidases include two subgroups of aminopeptidase and carboxypeptidase, which separate amino acids from the amino- and carboxy-terminals of protein, respectively (Terra and Ferreira 2012). However, endopeptidases are divided according to the nature of their active site and the acidity in which they are active. The first subgroup is serine proteases that contain serine, histidine and aspartic acids in their active site and digest proteins in alkaline pH. These enzymes include trypsin, chymotrypsin astase. The second subgroup contains cysteine proteases that are active under acidic conditions and include catepsins L, B and D. Other endopeptidases are known as aspartic acid proteases and metalloproteases, which are usually active under acidic and neutral conditions although metalloproteases require metal ions in their active site (Terra and Ferreira 2012).

In herbivorous butterflies, serine proteases, and especially trypsin (EC 3.4.21.4), are some of the most important enzymes that are effective in digesting proteins. After ingestion of a meal, trypsin is secreted

into the small vesicles within the cytoplasm of midgut cells, moved to the microvilli of the columnar cells then released into the gut lumen as an exocytotic process (Nation 2008). In the lumen, the enzyme digests protein chains on the carboxyl side of basic l-amino acids like arginine and lysine (Terra and Ferreira 2012). The presence of the enzyme in midgut lumen of insects can be identified using benzoyl-arginine p-nitroanilide (B-R-pNA, often referred to as BAp-NA) or benzoylarginine 7-amino-4-methyl coumarin (B-R-MCA) as substrates and the specifically synthetic inhibitors of N-a-tosyl-l-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP) (Terra and Ferreira 2012). Gene sequences of trypsins have been reported in several insect orders by typical properties of the conserved N-terminal residues IVGG, the catalytic amino acid triad of serine proteinase active sites (His 57, Asp 102, and Ser 195), three pairs of conserved cysteine residues for disulfide bonds, and the residue Asp 189 (Peterson *et al*. 1994; Zhu *et al*. 2000; Terra and Ferreira 2012). In our previous study, we determined trypsin activity in the alimentary canal of *Pieris brassicae* L. (Lepidoptera: Pieridae) and its enzymatic properties via purification processes and *in vivo* experiments. The enzyme showed a higher activity in the anterior larval midgut rather than in the posterior larval midgut. Also, the purified enzyme had a specific activity of 21 U · mg^{-1} protein, a recovery of 22%, a purification fold of 28-fold and molecular weight of 25 kDa. The specific inhibitors such as AEBSF.HCl [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], TLCK (N-a-tosyl-l-lysine chloromethyl ketone) and SBTI (Soybean Trypsin Inhibitor) significantly decreased the activity of the purified trypsin and the nutritional indices of the larvae (Sharifloo *et al*. 2017). The current study was conducted to identify the gene responsible for trypsin synthesis in the midgut of *P. brassicae* larvae and to find possible changes in its expression in the larvae fed a diet containing specific synthetic inhibitors, ABESF.HCl, TLCK and SBTI.

Materials and Methods

Insect rearing

A laboratory cohort of *P. brassicae* was reared on radish leaves at 25±2°C, 85% of relative humidity (RH) and 16 L : 8 D h photoperiod. The larvae were reared till 5th instars to use in the experiments. Every day, fresh radish leaves were provided and the rearing containers were cleaned to avoid pathological infection (Sharifloo *et al*. 2017).

In vivo assay of specific inhibitors

Based on our previous results (Sharifloo *et al*. 2017), 1 mM concentrations of SBTI, TLCK, AEBSF.HCl were separately prepared in Triton X-100 solution (0.02%). The radish leaves were dipped into the prepared solution separately for 30 s and put on filter paper (Whatman No. 1) to be dried at laboratory temperature for 60 min. Control leaves were dipped only into Triton X-100 solution (0.02%). The fourth instar larvae of *P. brassicae* were weighed and fed control and treated leaves (already weighed) separately for 72 h. At the end of the given time, the live larvae were dissected for molecular studies. Thirty larvae in three replicates were used for each treatment of AEBSF.HCl, TLCK, SBTI and control, separately.

Gene expression of trypsin

RNA extraction

Guanidine/phenol solution in a RNX-PLUS kit provided by Cynaclone Company (Tehran, Iran) was used to extract total RNA from the midgut of *P. brassicae* larvae fed leaves treated by with AEBSF.HCl, TLCK, SBTI and Triton X-100 (Control). The midguts of treated larvae were homogenized in 1 ml of ice cold RNX-PLUS solution, vortexed for 10 s and incubated at room temperature for 5 min. Then, 200 µl of chloroform was added to the mixture and shaken for 15 s. After 15 min of incubation on ice, the mixture was centrifuged at 20,000 rpm at 4°C for 15 min. The aqueous phase of the tubes was transferred to other tubes and an equal volume of isopropanol was added to the new tubes which were cooled for 15 min on ice. This mixture was centrifuged at 25,000 at 4°C for 15 min. Supernatants were discarded and 1 ml of 75% ethanol was added and the tubes were re-centrifuged at 9500 rpm at 4°C for 8 min. The supernatants were discarded and the pellets were kept at room temperature for a few minutes to be dried. Finally, 50 µl of DEPC (diethylpyrocarbonate) treated water was added to the pellets and kept at –20°C for cDNA synthesis.

RNA quantity and quality determination

The quantity of the extracted RNA was assayed using 50 µl of DEPC treated water and 1 µl of the extracted RNA at an optical density (OD) of 260 nm to calculate the ratio of OD 260/280 as the quality, OD 260/240 or OD 260/320 as the purity and the extraction performance. The value of 1.8 is a fine indicator for RNA quality.

cDNA synthesis

A Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was used to synthesize cDNA of the extracted RNA. Briefly, 1 µl of RNA was added to a mixture containing 4 µl of Reaction Buffer 5×, 20 µl

of RiboLock, 2 µl of dNTP 10 mM, 1 µl of Revert Aid, 1 µl of Oligo-dT primer and 11 µl of DEPC water. The mixture was incubated at 42°C for 60 min. The procedure was terminated at 70°C for 10 min and the synthesized cDNA was kept at –20°C for specific amplification.

cDNA amplification

The cDNA was amplified by polymerase chain reaction (PCR) using a specific forward primer 5'-TCGAATTCATTGTGACCGCCGCTCAYTG-3' and a specific reverse primer 5'-TATCTAGATGGGCCACCG GARTCNCCYTG-3'. PCR program used for amplification included 30 cycles at a denaturing temperature of 95°C for 2 min, 95°C for 30 s, at an annealing temperature of 51°C for 30 s, 72°C for 30 s and at an extending temperature of 72°C for 5 min in a tube with 25 µl containing 1 µl cDNA templates, 2.5 µl reaction buffer, 0.5 µl dNTPs, 0.75 µl $MgCl_2$, 0.25 µl Taq polymerase, 19 µl DEPC treated water and 0.5 µl of forward and reverse primers in a thermocycler.

Gene expression

Expression of trypsin gene was analyzed using real--time quantitative PCR (qRT-PCR) in the control, AEBSF.HCl-, TLCK- and SBTI-treated larvae, separately. A Maxima SYBR Green/ROX Kit (FERMENTAS Co) was used in the three replicates and reaction mixture containing 12.5 µl reaction volumes containing 6.25 µl the SYBR Green PCR Master Mix, 0.5 µl of forward (5'-AACCAAGTTGTCTGGGC-3') and reverse primer (3'- GACACCAGGGAAGCG-5') designed from the gained sequence of trypsin, 3.3 µl Nuclease-Free Water and 1 µl of cDNA. Thermal cycling was carried out at 95°C for 2 min, at 95°C for 30 s, at 51°C for 30 s, at 72°C for 30 s and finally at 72°C for 5 min. The control gene in qRT-PCR was 18 srRNA which was amplified using forward primer 5'-CACGGGAAATCTCACCAGG-3' and reverse primer 3'-CAGACAAATCGCTCCAC-CAACTA-5', as suggested by Lu *et al.* (2013).

Relative gene expression analysis calculation using a ΔΔCt method

The relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). First, Ct values of technical replicates were averaged and ΔCt was calculated by normalizing Ct (Target gene) to Ct (Reference gene) for control and treatments (AEBSF.HCl, TLCK and SBTI). Then, ΔΔCt was calculated by normalizing ΔCt from Cq of Target gene to Cq of Reference gene to average ΔCt for the treatment control. Relative gene expression was calculated (Step 5) as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Sequence analysis and homology modeling

A BLAST program, available from the National Center for Biotechnology Information (NCBI), NIH, Bethesda, MD, USA, was used to sequence analyses. The phylogenetic analysis was performed using Gene-Dock and Mega software.

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey test using SAS software (Version 9.3, SAS Inc.). Differences between samples were considered statistically significant at $p < 0.05$ and marked in figures and tables by letters.

Results and Discussion

Gene expression of trypsin

The trypsin gene of *P. brassicae* larvae was partially identified by a pair of specific primers as a product length of 748 bp (Fig. 1). The sequence showed a significant

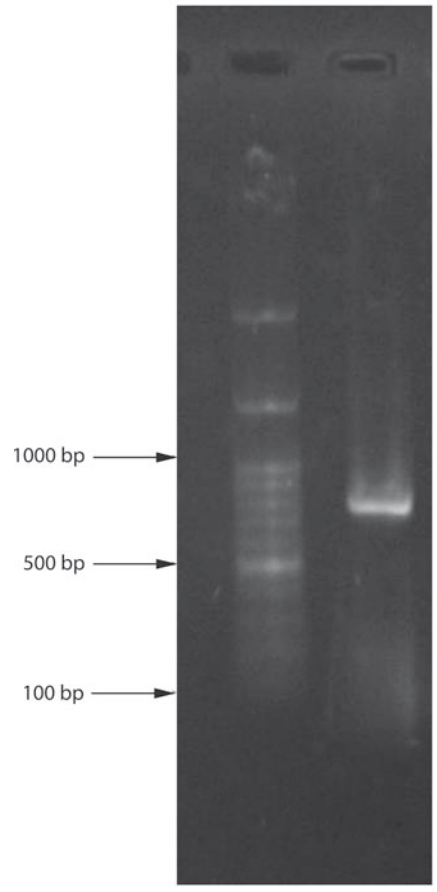

Fig. 1. PCR products of the extracted total mRNA from *Pieris brassicae* larvae. Determination of cDNA goodness using specific primers for trypsin gene amplification. PCR product shows the relative length between 600 and 700 bp of ladder

similarity (67–82%) to the previously reported lepidopteran serine proteases of *P. brassicae*, *Papilio xuthus* L. (Lepidoptera: Papilionidae) and *P. polytes* L. as well as trypsins of the two isolates of *Antheraea assama* Helfer (Lepidoptera: Saturniidae) (KF471009.1, AK402734.1, AK404896.1, DQ872511.1, FJ217713.1) (Fig. 2). The phylogenetic tree drawn by mega software showed that the serine and trypsin genes identified in lepidopterans may be divided into two main groups based on the maximum likelihood tool (Fig. 3). The first group contained the genes from moths but the second group had the serines and trypsins identified in butterflies (Fig. 3). Results on the qRT-PCR revealed a higher expression of trypsin gene in *P. brassicae* larvae fed leaves treated with AEBSF, TLCK and SBTI than the control so that the highest expression ratio was obtained for SBTI as 95-fold (Fig. 4).

Serine proteases are the predominant proteases in lepidoptera because of the alkali pH in their midgut (Applebaum 1985). The biochemical and molecular properties of these enzymes have been determined in several lepidopterous insect pests (Terra and Ferreira 2012). Among serine proteases, trypsins are of interest because of their important role in protein digestion and interaction with plant compounds, mainly inhibitors. The partially determined gene responsible for synthesis of trypsin in *P. brassicae* larvae had more than 67% similarities to some other lepidopteran trypsins mainly butterflies. In fact, the phylogenetic tree using the maximum likelihood tool revealed two groups of trypsin genes: one belongs to moths, the other to butterflies. These results revealed a genetic divergence of trypsin in these two groups based on environmental and habit variations. Lazarevic and Jankovic-Tomanic (2015) believe differences of amino acid sequences and the number of trypsin genes may undergo the different molecular mechanisms highlighting species variations in trypsin activity (as we found in the phylogenetic

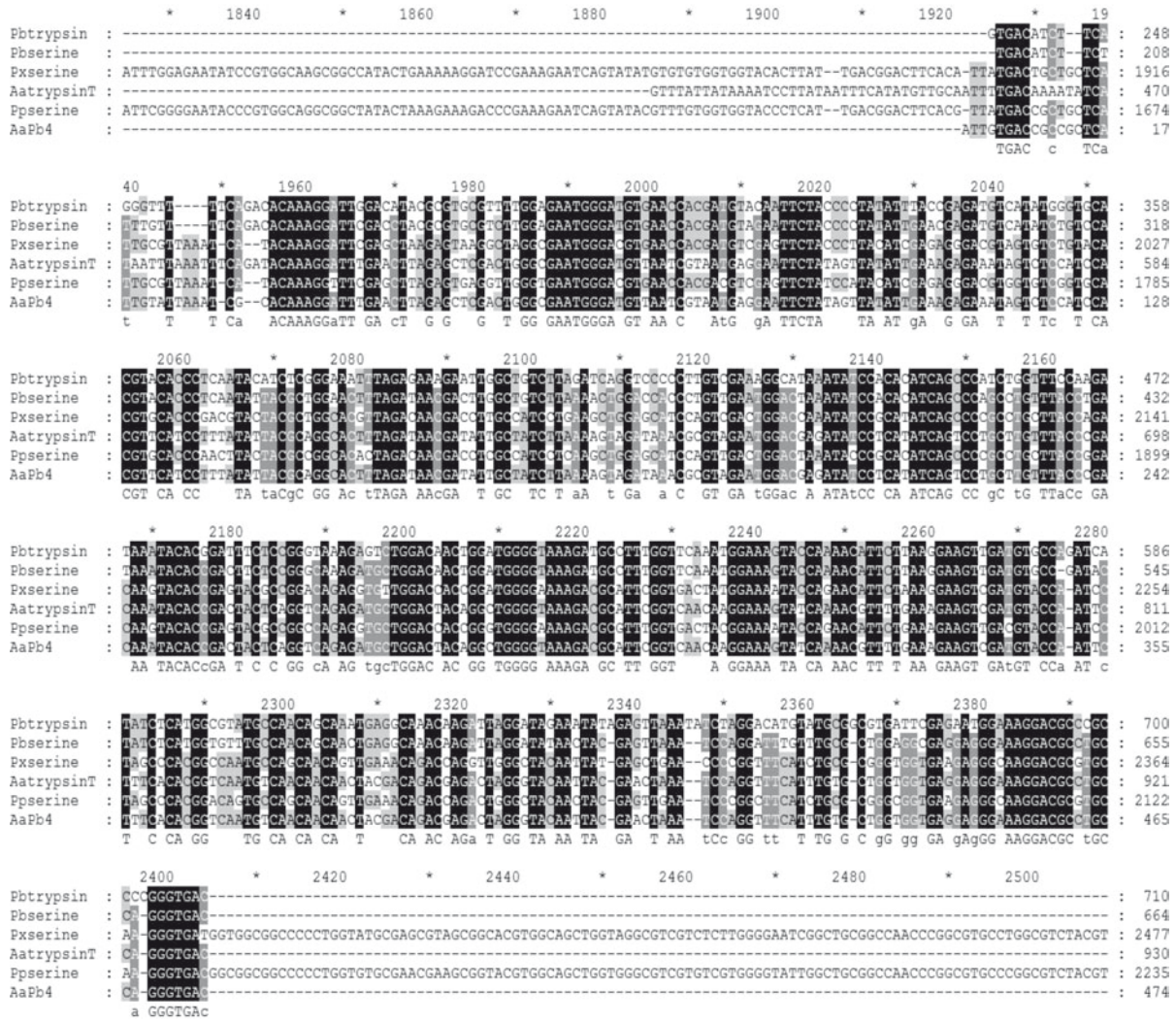

Fig. 2. Sequencing results and alignment of PCR products proved to be trypsin of *Pieris brassicae* larvae by GENEDOC software. The given sequence was aligned with the serines of *P. brassicae* and *Papilio xuthus* as the most similar sequences based on NCBI database. Pbserine – *P. brassicae* trypsin, Pxserine – *Papilio xuthus* serine, AatrypsinT933 – *Antheraea assama* T933, Ppserine – *Papilio polytes* serine, AaPb4 – *Antheraea assama* clone AAPb4

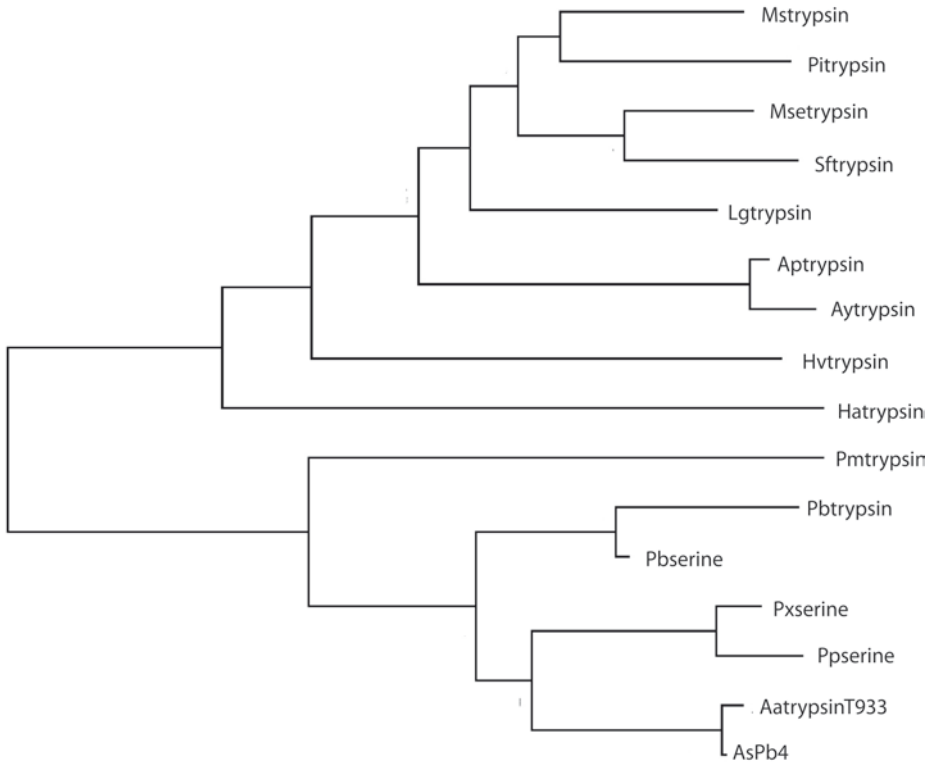

Fig. 3. Phylogenetic tree of trypsin from *Pieris brassicae* (Pbtrypsin) with other lepidopterans. Mstrypsin – *Manduca sexta*, Pitrypsin – *Plodia interpunctella*, Msetrypsin – *Mythimma separata*, Sftrypsin – *Spodoptera frugiperda*, Lgtrypsin – *Leguminivora glycinivorella*, Aptrypsin – *Antheraea pernyi*, Aytrypsin – *Antheraea yamami*, Hvtrypsin – *Heliothis virescens*, Hatrypsin – *Helicoverpa armigera*, Pmtrypsin – *Papilio machaon*, Pbserine – *P. brassicae* serine, Pxserine – *Papilio xuthus* serine, Ppserine – *Papilio polytes* serine, AatrypsinT933 – *Antheraea assama* T933, AaPb4 – *Antheraea assama* clone AAPb4

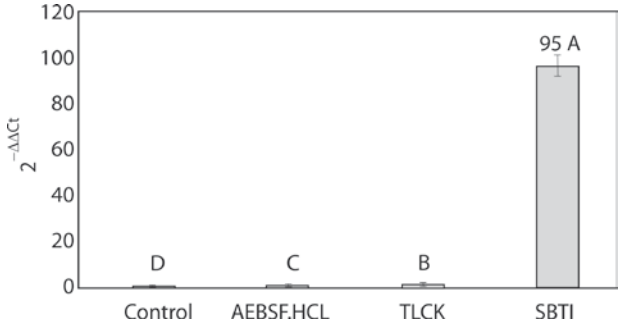

Fig. 4. Gene expression of trypsin in control and treated *Pieris brassicae* larvae by specific inhibitors. Means ± SE are shown for each item. Statistical differences are shown by different letters (Tukey test, $p \leq 0.05$)

tree, see above). On the other hand, adaptation to specific diets or environment may cause mutations in structural genes related to the activity and stability of trypsin, as well as mutations in regulatory genes of adaptive expression patterns (Lazarevic and Jankovic-Tomanic 2015).

The higher expression of trypsin gene in larvae fed the synthetic inhibitors indicates the presence of a compensatory mechanism in *P. brassicae* overcoming nutrient deficiencies due to biochemical trypsin inhibition. Such a mechanism can be considered from an evolutionary perspective. Nation (2008) believes there is a relationship between the type of inhibitors in food and the evolutionary selection for secretion of type and number of digestive proteases. The majority of inhibitors affect serine proteinases, mainly trypsins, which can be chemically synthesized or derived from plants, e.g. SBTI, which are used in plant breeding programs leading to resistant host plants. In response to the consumption of a trypsin inhibitor, insects secrete additional trypsin-like enzyme(s) to continue digestion of food (Broadway 1995; Broadway and Villani 1995). Such hyperproduction has been observed in our study by the higher expression of trypsin gene in the *P. brasicae* larvae treated with the synthetic inhibitors.

Concerns about using synthetic insecticides have encouraged the use of non-chemical or safer control procedures to decrease yield losses caused by insect pests. Nature-based molecules are promising alternatives to chemical insecticides, for example, plant protease inhibitors which are used in several molecular approaches to provide host plant resistance to a specific pest. However, first, it is imperative to fully understand the biochemical and molecular nature of the target enzyme and its potential interaction with

inhibitors. Our previous study revealed that, SBTI, a plant protease inhibitor, had the highest inhibition on the purified trypsin of *P. brassicae* via a different inhibitory mechanism than AEBSF.HCl and TLCK (Sharifloo *et al.* 2017). Since a significant increase of trypsin expression was found in the larvae fed SBTI it may be concluded that SBTI can be a more appropriate inhibitor against *P. brassicae* not only for its significant inhibition of trypsin but also for imposingcost of trypsin overexpression in the treated larvae.

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