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Finding the Relationships between a Strain of *Bacillus thuringiensis*, Tannic Acid, and the Mid–gut Proteases of *Spodoptera exigua*

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A new strain of *Bacillus thuringiensis* exhibiting activity in *Spodoptera exigua* was isolated and identified. Synergistic effects appeared when it was mixed with tannic acid, which is a protease inhibitor that enhances insecticidal activities. Substrate reactions with different types of proteases were analyzed, and according to the results, trypsin showed proteolytic activity levels of 91.4% and 89.4% on BApNA and BPVApNA substrates, respectively. When trypsin was treated with tannic acid, proteolytic activity levels of 62.2% and 54.5% appeared on BApNA and BPVApNA substrates, respectively, and tannic acid suppressed the trypsin–substrate reactions by 29.2% and 34.9%, respectively. Colorimetric activity assays of trypsin identified slightly lower activity levels in larvae that ate a combination of *B. thuringiensis* KB100 strain and 40 mM of tannic acid, when compared to larvae that died after eating the positive control, control, or the *B. thuringiensis* subsp. *kurstaki* KB100. The results of trypsin gene expression level analyses conducted using the qRT–PCR analyses indicated that trypsin 1 gene expression levels were lower when 40 mM of tannic acid was added to the *B. thuringiensis* KB100 strain, compared to the *B. thuringiensis* KB100 strain alone. Trypsin 2 gene expression levels were also lower when 40 mM of tannic acid was added to the *B. thuringiensis* KB100 strain. The results of immunofluorescence antibody experiments indicated that the addition of 40 mM of tannic acid to the *B. thuringiensis* KB100 strain, during decomposition by the mid–gut proteases of *S. exigua*, led to differences in expression levels and image analyses.

Key words: *Bacillus thuringiensis*, Protease, Protease inhibitor, *Spodoptera exigua*, Synergistic effect, Tannic acid, Trypsin

INTRODUCTION

Spodoptera exigua, which is a major, omnivorous agricultural pest, has not only been widely reported in tropical, subtropical, and temperate regions (Mochida and Okada, 1974), but also shown to be harmful to a variety of plants, including vegetables, flower plants, fruit trees, whole crops, and special purpose crops. There are 52 suitable host plants in South Korea (Goh *et al*., 1991), and *S. exigua* is known to harm approximately 140 species of plants globally (Minamikawa, 1937; Mochida and Okada, 1974).

Control methods for *S. exigua* include chemical controls consisting of insecticides such as organophosphorus, cabamate, and pyrethroid (Eveleens *et al*., 1973), biological controls using natural enemies, and attracticide and mating disruption using sex pheromones (Yoo *et al*., 1995; Jung *et al*., 2003; Kim *et al*., 2004). This pest cannot be easily controlled during its third instar period or thereafter because of high resistance to organic synthetic insecticides, which has developed from its ecological characteristics (Meinke and Ware, 1978). Therefore, biological control was introduced to control cases of resistant and intractable pests.

Bacillus thuringiensis products are the most widely used in biological control. *B. thuringiensis* was first isolated in 1901 in Japan, from the bodies of infected silkworm larvae. It forms spores and protein (parasporal) crystals in its rod–shaped cells. *B. thuringiensis*' insecticidal crystal proteins appear in diverse forms, such as bipyramidal, spherical, and indeterminate forms. In general, the bipyramid type one form exhibits highly toxic activity on *Lepidoptera* larvae (Jin *et al*., 2009). Cry proteins that are toxins with insecticidal activities include 149 cry proteins (Cry1–Cry51) and nine cyt proteins (Cyt1–Cyt2), which were isolated by Raymond *et al*. (2010).

Insecticidal crystalline proteins (ICPs) produced by *B. thuringiensis* do not exhibit any insecticidal activity. Specific insect mid–gut proteases are essential for the production of *B. thuringiensis* toxins (i.e., ICPs). *B. thuringiensis* is divided into 55–70 kDa through proteolysis by mid–gut proteases. These toxins interact with the larvae's mid–gut epithelial cells to form holes in the cell membranes, eventually leading to the death of the larvae. ICPs, which are endotoxin proteins in *B. thuringiensis*, are decomposed by strong alkaline digestive enzymes generated in the mid–gut of susceptible insects.

Lepidoptera's proteinases are divided into three groups: (i) serine proteinases, (ii) cysteine proteinases, and (iii) aspartic proteinases. *S. exigua*'s proteases are known to be serine proteinases and cysteine proteinases (Jongsma *et al*., 1996). Proteases are the most important digestive enzymes for *B. thuringiensis*' activity. Trypsin

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and chymotrypsin, which are serine proteinases, are known to play major roles in the process of proteolysis (Tojo and Aizawa, 1983; Zhu *et al*., 2007) and are the most important digestive enzymes for activation of *B. thuringiensis*' protoxins in insect mid–guts, which are important for the decomposition of ICPs (Zhu *et al*., 2007; Oppert, 1999). In particular, trypsin is known to decompose cry 1 proteins that are highly active in *S. exigua* (Rukmini *et al*., 2000); however, mid–gut proteases may lead to toxin protein inactivation through continuous hydrolysis.

These changes in mid–gut proteases and activity may lead to resistance and tolerance to *B. thuringiensis*. Forcada *et al*. (1996) reported that *Heliothis virescens*' mid–gut fluid is resistant to *B. thuringiensis* and decomposes *B. thuringiensis* toxins in vitro faster than susceptible mid–gut fluids are able to. Furthermore, it has been reported that in the case of highly tolerant pests, such as *S. exigua*, *B. thuringiensis* is excessively decomposed by mid–gut proteases, leading to reduced insecticidal activities (Maclntosh *et al*., 1990). Excessive decomposition of *B. thuringiensis* protoxins by mid–gut fluids containing trypsin–, chymotrypsin–, and elastase–like breakdown enzymes in *H. armigera* larvae demonstrate this resistance (Zhu *et al*., 2007).

In previous studies, *B. thuringiensis*, which is highly active in *S. exigua*, and tannic acid were selected as protease inhibitors, which prevent excessive decomposition of *B. thuringiensis*' toxin proteins due to the strong digestive activity of the pests' mid–gut fluids. The fact that *B. thuringiensis* showed high activity when tannic acid was added to it was identified (Jin *et al*., 2009, 2015). In addition, the secondary metabolites of plants are protease inhibitors that play important roles in pest resistance. It is known that in cotton, many secondary metabolites, such as terpenes, flavones, and tannis, act on pests as feeding deterrents, anthelminthics, growth inhibitors, and toxins (Rhoades and Cates, 1976). Tannins inhibit or weaken the growth of larvae and pupae (Chan *et al*., 1978) and have been reported to reduce larval feeding and enhance the effects of *B. thuringiensis* (Lord and Undeen, 1990; Navon *et al*., 1993). According to a report by Salama *et al*. (1984), tannic acid increased the efficacy of δ–endotoxin by two to four times, and after eating tannic acid, the pH in insect mid–guts rose, increasing the effects of *B. thuringiensis* (Schnepf *et al*., 1998). However, the mechanism of interaction between *B. thuringiensis* and tannic acid has not yet been identified.

In the present study, attempts were made to find the cause of the synergistic effects that appear when *B. thuringiensis* and tannic acid are mixed. Since the acting mechanism of tannic acid as a protease inhibitor has not been identified, experiments were conducting focusing on the determination of the relationship between tannic acid and proteases.

MATERIALS AND METHODS

Experimental insects

The *Plutela xylostella* used in the present experiment were collected from an outdoor population and successively bred in the insect breeding room in the biological pest control laboratory of Chungnam National University, using Chinese cabbage as a feed. *Spodoptera* litura and *S. exigua* were purchased from the National Institute of Agricultural Science and Technology of the Rural Development Administration and used while being successively bred with artificial feeds (Goh *et al*., 1990). As a feed for imagoes, 10% sugar water was supplied to the spawning box (plastic, diameter: 20 cm, height; 15 cm). All the insects were bred under the same breeding conditions as follows: temperature 25±°C, lighting condition 16 L:8 D, and relative humidity 50–60%.

Plasmid DNA extraction

The plasmid DNA of the *B. thuringiensis* strain was extracted using a Qiagen midi kit to identify the plasmid DNA. First, 10 ml of *B. thuringiensis* was inoculated into luria–bertani (LB) media and cultured for 16 hours at 28°C and centrifuged at 200 rpm. Thereafter, 1 ml of the culture fluid was inoculated into 100 ml of a SPY medium (0.2% $(NH_4)_2PO_4$; 1.4% K_2HPO_4; 0.6% KH_2PO_4; 0.1% sodium citrate; 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract; and 0.1% glucose) to culture the bacteria for approximately four hours until the measured value of OD_{600} became 0.7–1.0. All the cultured bacteria were subjected to centrifugation at 10,000 rpm for 5 minutes at 4°C. Thereafter, the supernatant was discarded and only the microbial cells were collected. Then, 4 ml of P1 solution (50 mM tris · Cl, pH8; 10 mM EDTA; and 100 μg/ml RNaseA) and 50 μl of lysozyme (50 mg/ml) were added to the microbial cells, and the mixture was kept at 37°C for approximately 30 minutes to induce reactions. Thereafter, 4 ml of the P2 solution (200 mM NaOH, 1% SDS) was added to the mixture, and reactions were induced in ICE for approximately 15 minutes. The mixture was then subjected to centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant fluid was moved to a new tube and subjected to centrifugation at 15,000 rpm for 30 minutes at 4°C. A Qiagen–tip 100 was installed on the tube, and 4 ml of QBT solution (750 mM NaCl; 50 mM MOPS; 15% isopropanol; and 0.15% Triton) was added, followed by 5 ml of a QC buffer (1 M NaCl; 50 ml MOPS; and 15% isopropanol). The tube below the Qiagen–tip 100 was replaced and 5 ml of a QF buffer (1.25 M NaCl; 50 mM Tris · Cl; and 15% isopropanol) was added. Then, 1 ml of each fluid gathered in the tube was placed in a 2 ml Eppendorf tube, and 0.7 ml of isopropyl alcohol (IPA) was added. The solution was subjected to centrifugation at 13,000 rpm for 30 minutes at 4°C. The supernatant fluid was discarded, 70% alcohol was added to the tube, and the solution was subjected to centrifugation again. Then, the fluid was discarded and the Eppendorf tube was placed on a clean bench until moisture in the tube disappeared. After drying, 10 μl of sterilized water was placed in the tube to dissolve any residue, the solution was subject to electrophoresis in 0.7% agarose gel for 60 minutes at 50 V, and the results were checked.

PCR analysis

Gene–specific primer sets (Table 1) were used to identify cry–type genes among the endotoxin genes of *B. thuringiensis*. A Thermal Cycle C1000TM (BIO–RAD) was used for PCR amplification. The final volume of the sample used in the analysis was 20 μl and created by mixing the premix (Bioneer) containing buffer solution components and dNTP with the template DNA (1.0 μl), the primer sets (1.0 μl each), and distilled water (17 μl). PCR was implemented for 30 cycles at 95°C and 5 minutes, 94°C and 1 minute, 57°C and 1 minute, 72°C and 1 minute, and 72°C and 5 minutes to produce PCR products. Cry genes were identified through electrophoresis in 1.2% agarose gel (Yang *et al*., 2011; Abdullah *et al*., 2009; Ye *et al*., 2009).

Western blotting analysis

Western blotting analysis was conducted using the methods presented by Xue *et al*. (2008) and Isabel *et al*. (2014), with some modifications. A nitrocellulose membrane was sufficiently wetted with a blotting buffer (25 mM Tris, pH 8.3; 192 mM glycine; and 20% v/v methanol) for 10 minutes. The gel that underwent SDS–PAGE was blotted to the membrane for two hours at 70 V using a blotting tank. After the blotting, the membrane was immersed in a 5% skimmed milk solution diluted with a TBS–T buffer and stirred for one hour at room temperature. After the initial stirring, the membrane was stirred in a wash buffer (TBS–T buffer) three times for five minutes each time. The primary antibody [anti–*Bacillus thuringiensis* cry1Ab toxin antibody (Ca.ab51586)] was diluted to a ratio of 1:1000 with the TBS–T buffer, the membrane was immersed in the solution, and the solution was stirred for three hours at room temperature. The membrane was immersed in a new wash buffer, which was stirred three times for five minutes each time. The secondary antibody [goat anti–rabbit IgG (HL)–HRP conjugate (Ca.170–6515)] was diluted to a ratio of 1:10,000 with a TBS–T buffer, the membrane was immersed in the solution, and the solution was stirred for one hour at room temperature. Again, the membrane was immersed in a new wash buffer, and the wash buffer was stirred three times for five minutes each time. Films were developed from the membrane using the chemiluminescence detection method and X–ray films.

Measurement of protease activities in *S. exigua*

To measure protease activities, experiments were conducted using the method presented by Bradford (1976), with some modification. To measure the activities of individual digestive enzymes, Nα–Benzoyl–DL–arginine 4–nitroanilide hydrochloride (BApNA) and N–Benzoyl–Phe–Val–Arg–p–nitroanilide hydrochlorid (BPVApNA) were used as trypsin protease substrates; N–Benzoyl–L–tyrosine p–nitroanilide (BTpNA), N–Succinyl– Ala–Ala–Pro–Phe p–nitroanilide (SAAPPpNA), and Ala–Ala–Val–Ala p–nitroanilide (AAVApNA) were used as chymotrypsin protease substrates; and N–Succinyl–Ala–Ala–Ala–p–nitroanilide (SAAApNA) and N–Succinyl–Ala–Ala–Pro–Leu p–nitroanilide (SAAPLpNA) were used as elastase protease substrates. To identify the inhibitory activity of tannic acid on the mid–gut fluid of *S. exigua*, an azocasein substrate was used at different concentrations (10, 20, 40, and 80 mM) during analyes. *S. exigua*'s mid–gut fluid diluted with distilled water and tannic acid were mixed to a ratio of 1:1 and kept at 37°C for 15 minutes to induce reactions. After mixing 300 μl of each substrate with 100 μl of a digested sample, the mixture was kept at 37°C for 15 minutes to induce reactions. Thereafter, 200 μl of 10% TCA was added to the mixture to stop the reactions. After subjecting the sample to centrifugation for 30 minutes at 15,000 rpm and 4°C, the supernatant fluid was mixed with 1 M of NaOH to a ratio of 1:1 to condense proteins, and the protein concentration was measured at an optical density of 405 nm (Jin *et al*., 2015).

Table 1. Crystal protein gene–specific primers for PCR analysis

Cry genes	Primer sequences	Size of product (bp)
cry1Aa	5'GAGCCAAGCGACTGGAGCAGTTTACACC3'	782
cry1Ab	5'TCGAATTGAATTTGTTCC3'	238
cry1Ac	5'GTCCAACCTTATGAGTCACCTGGGCTTC3'	550
cry1B	5'GTCCAACCTTATGAGTCACCTGGGCTTC3'	902
cry1C	5'CAACCCTATTTGGTGCAGGTTC3'	288
cry1D	5'GGTACATTTAGATGTTCACAGCCAC3'	465
cry1E	5'CTTAGGGATAAATGTAAGTACAG3'	961
cry1F	5'CCGGTGACCCATTAACATTCCAATC3'	383
cry13'	5'ATCACTGAGTCGCTTCGCATCTTTGACTTTCTC3'	–
cry1G5'	5'ATATGGAGTGAATAGGGGG3'	235
cry1G3'	5''TGAACGGCGATTACATGC3'	–
cry1I5'	5'GCTGTCTACCATGATTCGCTTG3'	1584
cry1I3'	5'CAGTGCAGTAACCTTCTCTTGC3'	–

Trypsin activity analysis

The activity of trypsin among *S. exigua* mid–gut proteases was analyzed using a trypsin activity colorimetric assay kit (Biovision, catalog #K771–100). Live *S. exigua*, those larvae that died after eating the *B. thuringiensis* KB100 strain, and those larvae (10–100 mg) that died after eating the *B. thuringiensis* KB100 strain and 40 mM tannic acid were cut to small pieces, and a trypsin assay buffer was added at 4 X–6 X volumes. The solutions were kept on ice. While observing the cells under a microscope, the lyses were completed and the solutions were homogenized using a homogenizer (10–15 repetitions), while iced. After the samples were subjected to centrifugation, only the supernatant fluids were collected and inoculated into 96–plate wells. When the final volume was smaller than 50 μl, an equal amount of assay buffer was added (50 μl). Each sample was treated with 1 μl of a chymotrypsin inhibitor (TPCK) solution and kept at room temperature for 10 minutes to induce reactions. To prepare a positive control, a trypsin buffer (45 μl) and a positive control solution (5 μl) were mixed together and 2 mM of a p–NA standard in a micro–centrifuge tube were used to prepare a standard curve. The standard diluted solution (50 μl) was added to each 96–plate well to produce a sufficient quantity of the 2 × duplicates × 50 μl /well. Then, 50 μl of the reaction mixture was prepared for each well by mixing the assay buffer (48 μl) and trypsin substrate (2 μl). Each well that contained p–NA standards, positive controls, test samples, or the trypsin inhibitor control was added to the 50 μl of the reaction mixture. The solutions were cultured for one to two hours (or longer when trypsin activity was low) at 25°C, and protein concentrations were measured at an optical density of 405 nm using a microplate reader system (VersaMax).

***S. exigua's* total RNA extraction and cDNA synthesis**

Total RNAs were extracted from untreated larvae, those larvae that died after eating the *B. thuringiensis* KB100 strain, and those larvae that died after eating the *B. thuringiensis* KB100 strain and 40 mM of tannic acid, using Qiagen RNeasy mini kits (Qiagen, Korea) pursuant to the protocol. Then, cDNAs were synthesized using LA TaqTM DNA polymerase (Takara, Japan).

RNase–free H2O (7 μl), 5 × RT buffer (4 μl), dNTP (2 μl), RNase inhibitor (1 μl), primer (forward: 5' TCCACCTGGGCCAACAGC 3', reverse: 5' ACGATGCCGTTGTGGTAGAG 3'; 0.5 μl), RNA (4 μl), and Revertra Ace (1 μl) were mixed together and treated at 42°C for 20 minutes and at 99°C for five minutes to synthesize the second strand. Primers were made using the trypsin RNA sequences for four Lepidoptera species (*S. litura*, *Heliothis armigera*, *H. virescens*, and *Spodoptera frugiperda*) because the trypsin RNA sequence for *S. exigua* was not registered in the

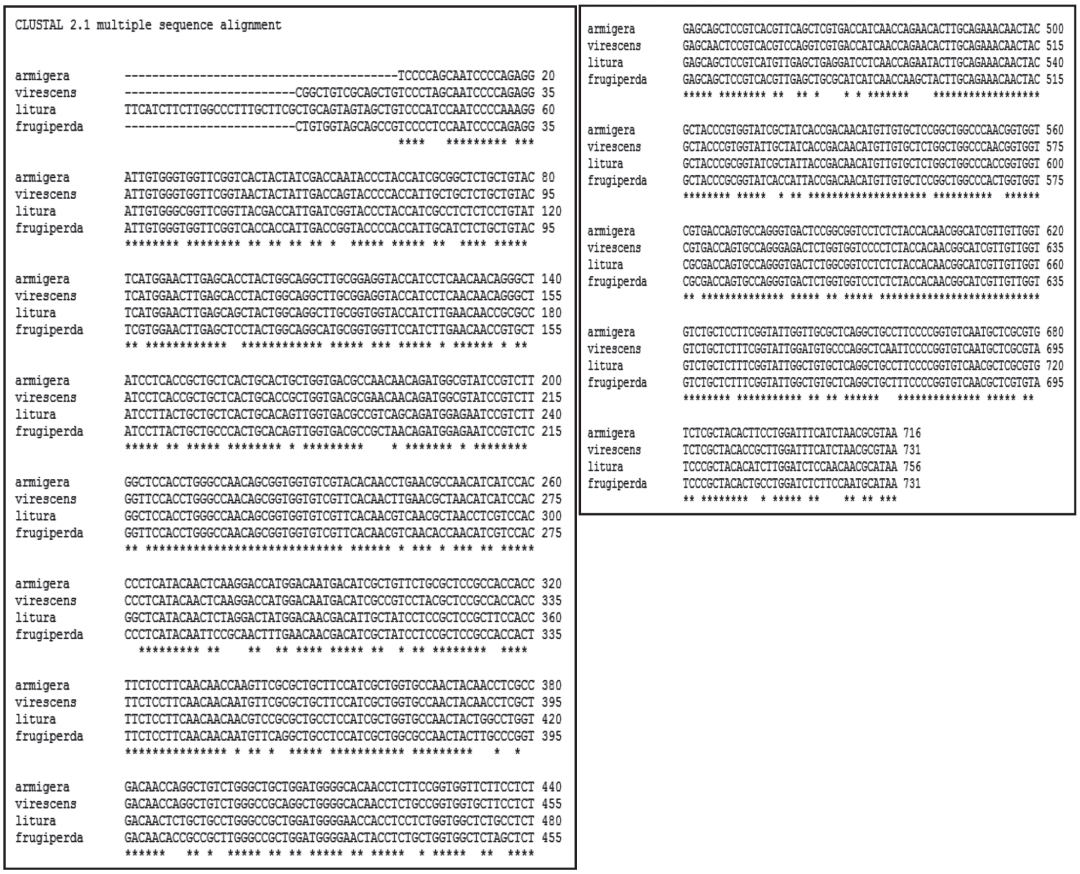

Fig. 1. *S. exigua* trypsin using a trypsin mRNA sequence analysis of the few species of lepidoptera in Genbank (primer production).

Table 2. Oligonucleotide primers for qRT–PCR

Gene	Primer sequences	Length	Position	PCR product (bp)
Trypsin 1	Sense: ACGACATCGCTATCCTCC	18	86	85
Trypsin 1	Antisense: GAAGTAGTTGGAACCAGCAA	20	171	85
Trypsin 2	Sense: CATCGTTCACGGTTCATACA	20	48	93
Trypsin 2	Antisense: GCGGACATTGTTGTTGAAG	19	141	93
Actin	Sense: ATCGTGCGTGACATCAAG	18	687	110
Actin	Antisense: GTCGGGAAGTTCGTAGGA	18	797	110

Genbank. After checking with the Genbank, the primers were made assuming that *S. exigua* should also have a partially common trypsin RNA sequence (Fig. 1). Actin (forward: 5'CCCAGAGCAAGAGAGGTATC 3', reverse: 5'ACCCCTCTCGGTGAGGATCT 3') was used as a positive control. After synthesizing, cDNA was subjected to electrophoresis in 0.8% agarose gel at 60 V for 33 minutes.

S. exigua trypsin gene sequencing and qRT–PCR analysis

The qRT–PCR method presented by Kaminska *et al.* (2014) was used with some modification to analyze the expression level of trypsin genes among the mid–gut proteases of *S. exigua*. Primers for qRT–PCR were made to check the levels of expression of the trypsin genes and actin of *S. exigua* (Table 2). First, cDNA (5 μl) of each of the untreated larvae, larvae that died after eating the *B. thuringiensis* KB100 strain, and larvae that died after eating the *B. thuringiensis* KB100 strain and 40 mM of tannic acid were mixed with a 2 × SYBR mix (10 μl), primer (0.5 μl), and distilled water (4 μl) in each 96–plate well, and reactions were induced. The qRT–PCR condition was 32 cycles at 25°C for 10 minutes, 37°C for 12 minutes, 85°C for 5 minutes, and 4°C for 5 minutes. The trypsin gene expression level analysis was conducted using the method presented by Pfaffl (2001).

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Immunofluorescence antibody method and confocal laser scanning microscopy

Immunofluorescence experiments were conducted to identify the activity of trypsin in the body of *S. exigua* using the method presented by Ruiz *et al.* (2004), with some modification. A spore–crystal mixture solution (50 μl) diluted with 40 mM of tannic acid (15 μl) was sprayed on 0.2 g of an artificial feed. The primary trypsin antibody (Santa Cruz Biotechnology, Cat.sc–137077) was diluted to 1:500 with sterilized water and the secondary mouse IgG2a antibody (Bethyl Laboratory, Cat. A90–107F) diluted to 1:100, before both were inoculated into the feed. Two third–instar larvae, starved for 12 hours, were put into each petri dish and allowed to eat feed for 30 minutes. After eating, each larva was put into each well of a 96–well black immunoplate, and the trypsin expression levels of the larvae were identified using an immunofluorescence microplate reader (Hidex Sense, Cat.425–301). After identifying the expression levels, images of trypsin expressed in the bodies of the pests were taken using a confocal laser scanning microscope (Leica, TCS SP8 STED).

RESULTS AND DISCUSSION

Analysis of the cry genes of the *B. thuringiensis* subsp. *kurstaki* KB100

The *B. thuringiensis* subsp. *kurstaki* HD–1 has been reported as having cry1Aa, cry1Ab, cry1Ac, and cry1I genes (Jung *et al.*, 2010), and several *B. thuringiensis* subspecies, including *aizawai* and *entomocidus*, have been reported as having cry1Aa, cry1Ab, cry1E, and cry1F genes and showing activity in *Lepidoptera* (Sanchis *et al.*, 1989; Visser *et al.*, 1988, 1990). Cry1 genes having insecticidal activities on *S. exigua* are assumed to include cry1Aa, cry1Ab, cry1C (de Maagd *et al.*, 2003), cry1D (Bravo *et al.*, 1998; Porcar *et al.*, 2000; Lee *et al.*, 2001), and cry1F (Luo *et al.*, 1999; Hernández and Ferre, 2005) genes. In addition, cry1 genes have been reported as showing activity on *Lepidoptera* and Coleoptera (Tamez–Guerra *et al.*, 2004), and cry2 genes have been reported as having activity on *Lepidoptera* and Diptera (Donovan *et al.*, 1994).

According to the results of examination, the *kurs-*

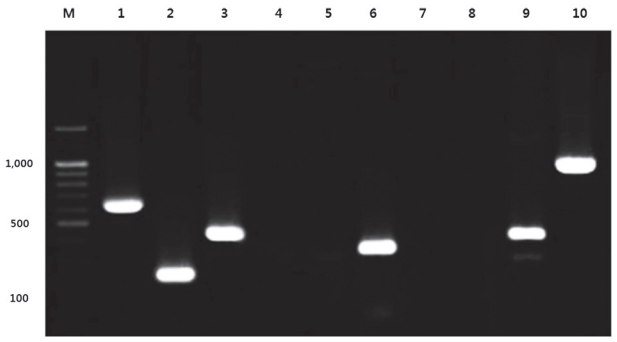

Fig. 2. Agarose gel (1.2%) electrophoresis of PCR products obtained with specific primers for the cry1 genes of *B. thuringiensis* subspecies *kurstaki* KB100. M: 100 bp ladder marker; lane 1: cry1Aa; lane 2: cry1Ab; lane 3: cry1Ac; lane 4: cry1B; lane 5: cry1C; lane 6: cry1D; lane 7: cry1E; lane 8: cry1F; lane 9: cry1G; lane 10: cry1I.

taki KB100 strain that was selected for the present study has cry1Aa, cry1Ab, cry1Ac, cry1D, cy1G, and cry1I genes (Fig. 2) and could be assumed to have high insecticidal activities in *S. exigua* because of cry1Aa, cry1Ab, and cry1D genes. In particular, compared to the cry genes of the *B. thuringiensis* KB100 and HD–1 strains, which both showed high activity in *S. exigua*, the *kurstaki* KB100 strain had cry1Ab genes that were not identified in other strains.

B. thuringiensis subsp. *kurstaki* KB100 strain: Western blotting analysis

In the results from the western blotting method experiments, it was identified that the cry genes of the *kurstaki* KB100 strain showed 100% insecticidal activity in *S. exigua* compared to the *B. thuringiensis* subspecies *kurstaki* HD–1 strain, which was the reference strain.

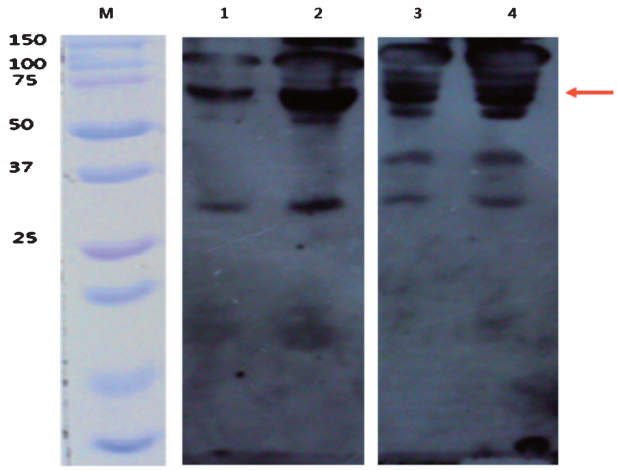

Fig. 3. Western blotting analysis of the *B. thuringiensis* subspecies *kurstaki* KB100 (Cry1Ab protein). M: standard marker; lane 1: *B. thuringiensis* subspecies *kurstaki* KB100 (3 μl); lane 2: *B. thuringiensis* subspecies *kurstaki* KB100 (5 μl); lane 3: *B. thuringiensis* subspecies *kurstaki* HD–1 (3 μl); lane 4: *B. thuringiensis* subspecies *kurstaki* HD–1 (5 μl)

The existence of cry1Ab proteins was checked using an antibody (anti–*Bacillus thuringiensis*, CRY1Ab toxin antibody (Ca.ab51586)) with the endotoxin proteins. According to results, 130 kDa and 60 kDa bands could be identified (Fig. 3). Because it is known that trypsin plays important roles in the decomposition of cry 1 proteins, according to Rukmini *et al.* (2000), the possibility for tannic acid to suppress the excessive decomposition of the cry 1 proteins of the *B. thuringiensis* KB100 strain by trypsin among *S. exigua* proteases could be predicted.

Identifying inhibition of trypsin, a *S. exigua* mid–gut digestive enzyme, by tannic acid

S. exigua's mid–gut proteases consist of serine proteases, such as trypsin, chymotrypsin, and elastase (Oppert, 1999). Using highly specific substrates to determine the characteristics of the digestive enzymes of insects is known to be useful (Law *et al.*, 1977). *S. exigua*'s mid–gut proteases were induced to react with specific substrates, and the activity of the proteases was measured. When the protease activity was measured using azocasein, which is a serine substrate, a value of 100% was indicated, and when BApNA and BPVApNA, which are trypsin substrates, were used, values of 91.4 ± 1.8% and 89.4 ± 0.7% were shown, respectively. BTpNA, SAAPPpNA, and AAVApNA were among the chymotrypsin substrates used, and the protease activity levels were shown to be 55.4 ± 0.6%, 56.5 ± 3.9%, and 52.7 ± 1.5%, respectively. SAAApNA and SAAPLpNA were the elastase substrates used, and activity levels of 48.5 ± 1.6% and 38.7 ± 4.9% were shown, respectively. High protease activity levels, reaching approximately 90%, were shown when measured using trypsin–specific substrates, while protease activity levels of approximately 55% were shown when chymotrypsin substrates were used. When elastase substrates were used, the activity levels were approximately 44%, which were the lowest among those shown by the three types of proteases.

Based on these results, it could be assumed that among *S. exigua*'s mid–gut proteases, trypsin exhibits the highest activity. To identify the degree of inhibition of proteolytic activity in *S. exigua* mid–gut protease–specific substrate reaction tests, *S. exigua* mid–gut fluids were treated with tannic acid at four concentrations

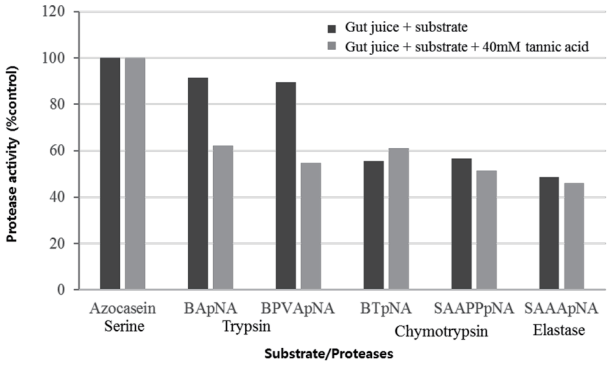

Fig. 4. Comparison of proteolytic activity when using 40 mM of tannic acid with the substrates of the proteolytic enzyme.

(10, 20, 40, and 80 mM), separately, and protease activity levels were measured. When *S. exigua* mid–gut fluid was treated with 40 mM of tannic acid and the levels of protease activity on different substrates were measured, BApNA and BPVApNA, which are trypsin substrates, showed activity levels of 62.2 ± 0.3% and 54.5 ± 1.1%, respectively, indicating that 40 mM of tannic acid inhibited proteolytic activity on these substrates (Fig. 4). Unlike the results for trypsin, the extent to which tannic acid inhibited proteolytic activity on serine, chymotrypsin, and elastase substrates were insignificant. In comparison, the activity of trypsin in *S. exigua* mid–gut fluid on different substrates was suppressed by approximately 30–40% when treated with 40 mM of tannic acid. Tannic acid could be expected to effectively inhibit the activity of trypsin among serine proteases.

Analysis of the activity of trypsin among *S. exigua* mid–gut proteases and identification of the inhibitory activity of tannic acid

A colorimetric analysis for trypsin activity was conducted to identify the inhibitory activity of tannic acid on the activity of trypsin among *S. exigua* proteases. For accurate analysis of trypsin activity, a standard curve was drawn using p–nitroaniline, which is a trypsin substrate (Fig. 5). The results of the analyses of untreated larvae, those larvae that died after eating the *B. thuringiensis* KB100 strain, and those larvae that died after eating the *B. thuringiensis* KB100 strain and 40 mM of tannic acid are shown in Fig. 6. It was identified that trypsin activity slightly decreased in larvae that ate the *B. thuringiensis* KB100 strain and 40 mM of tannic acid, compared to the positive control, the control, and those larvae that ate the *B. thuringiensis* KB100 strain (1.05×10^7, 1.05×10^6). Similar to the results of SDS–PAGE, these results indicated that 40 mM of tannic acid inhibits trypsin activity in *S. exigua*.

S. exigua's trypsin gene sequencing and qRT–PCR analysis

In the qRT–PCR analyses, differences in trypsin gene expression levels were identified in untreated larvae, those larvae that died after eating the *B. thuringiensis* KB100 strain, and those larvae that died after eating the *B. thuringiensis* KB100 strain and 40 mM of tannic acid. The trypsin RNA sequence was identified by synthesizing and amplifying cDNA (Fig. 7). The partial sequence is indicated by B, and the bands were identified through electrophoresis. The sequence of the actin RNA of *S. exigua*, which is a positive control, was identified in the Genbank (accession no. AY507963) and primers were made to identify the bands through electrophoresis (Fig.

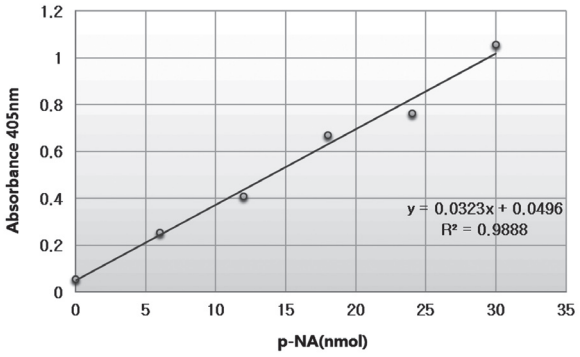

Fig. 5. The p–NA standard curve, performed according to the assay protocol.

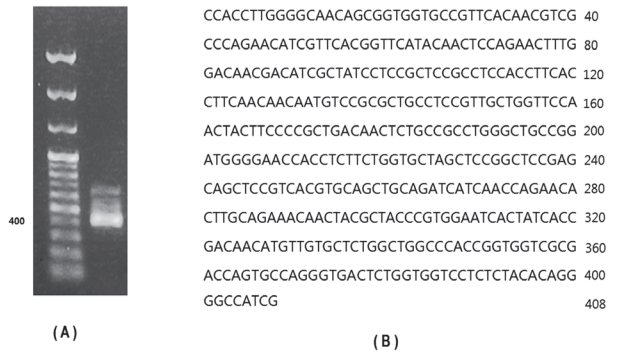

Fig. 7. An electrophoresis pattern (A) of the partial sequence (B) of an *S. exigua* trypsin gene.

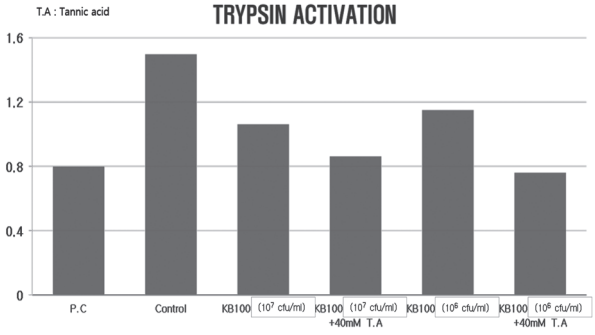

Fig. 6. Trypsin activity of the third larvae after being fed the *B. thuringiensis* KB100 single treatment and *B. thuringiensis* KB100 and 40 mM of tannic acid feed.

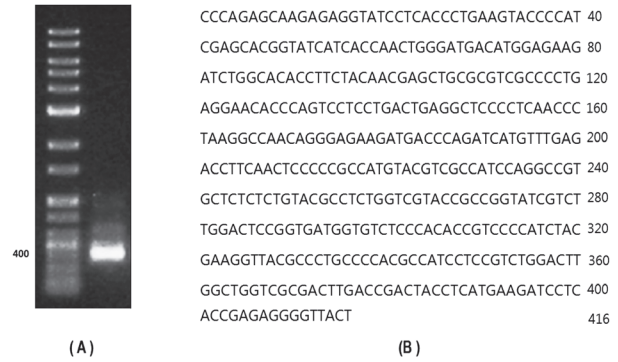

Fig. 8. An electrophoresis pattern (A) of the partial sequence (B) of an *S. exigua* actin (Genbank accession no. AY507963) gene.

Table 3. Mean cycle threshold (Ct) values of qRT–PCR for trypsin and actin genes

Pest	Target gene	Target sample	Revelation value (Ct value)			Average (Mean±SD)
			Rep. 1	Rep. 2	Rep. 3	
S. exigua	Trypsin 1	KB100	35.12	34.8	34.93	34.95±0.83
		KB100 + T.A	32.53	32.73	32.15	32.47±0.32
		Non	30.52	30.07	30.25	30.28±0.24
	Trypsin 2	KB100	30.48	30.52	30.23	30.41±0.18
		KB100 + T.A	28.53	27.25	28.12	27.96±0.84
		Non	25.45	25.85	25.23	25.51±0.34
	Actin	KB100	22.56	22.53	23.12	22.73±0.39
		KB100 + T.A	22.72	22.11	22.17	22.33±0.39
		Non	22.89	22.63	22.73	22.75±0.14

Values represent by mean ± SD, *: $P<0.05$; **: $P<0.01$; Completely randomized one–way analysis of variance, ANOVA, Post Hoc Test by Duncan in SPSS version 18.0 NS; statistically not significant.

8). The results of the qRT–PCR indicated that the expression level of the trypsin 1 gene was lower when the larvae were fed the *B. thuringiensis* KB100 strain and 40 mM of tannic acid, compared to larvae fed only the *B. thuringiensis* KB100 strain. Likewise, the expression level of the trypsin 2 gene was also lower when the larvae were fed the *B. thuringiensis* KB100 strain and 40 mM of tannic acid (Table 3). The expression level of actin, which is a housekeeping gene, was not significantly different among the untreated larvae, those larvae that died after eating the *B. thuringiensis* KB100 strain, and those larvae that died after eating the *B. thuringiensis* KB100 strain and 40 mM of tannic acid.

Immunofluorescence and confocal laser–scanning microscopy

Immunofluorescence experiments were conducted to detect trypsin, which is a target protein of *S. exigua* proteases. The detected amounts and images of trypsin were analyzed under an immunofluorescence microplate reader and a confocal laser–scanning microscope, using antibodies bound to fluorophores. Fluorophores absorb light with certain wavelengths and show colors according to the wavelength range. Fluorescein–labelled antibodies that are specifically bound to trypsin existing in *S. exigua* were induced to react with the trypsin, and the detected amounts of trypsin were measured. The amount of trypsin detected from untreated larvae was approximately 3,505 amol, and the amount of trypsin detected from the larvae treated with fluorescein–labelled antibodies was approximately 15,959 amol. The amount of trypsin detected from the larvae treated with 40 mM of tannic acid was approximately 11,754 amol (Table 4). After identifying the detected amounts of trypsin in numerical values, the images were identified using a confocal laser scanning microscope (Fig. 9). Trypsin activity was identified in the gut of the insect using fluores-

Table 4. Revelation value of immunofluorescence of trypsin (D–1): sc–137077 and a mouse IgG2a antibody after a 30 minute feeding

Treatment	Revelation value (Average)
Fluorescein–labeled antibody	3,505 ± 155
Fluorescein–labeled antibody + 40 mM tannic acid	15,959 ± 580
None	11,754 ± 250

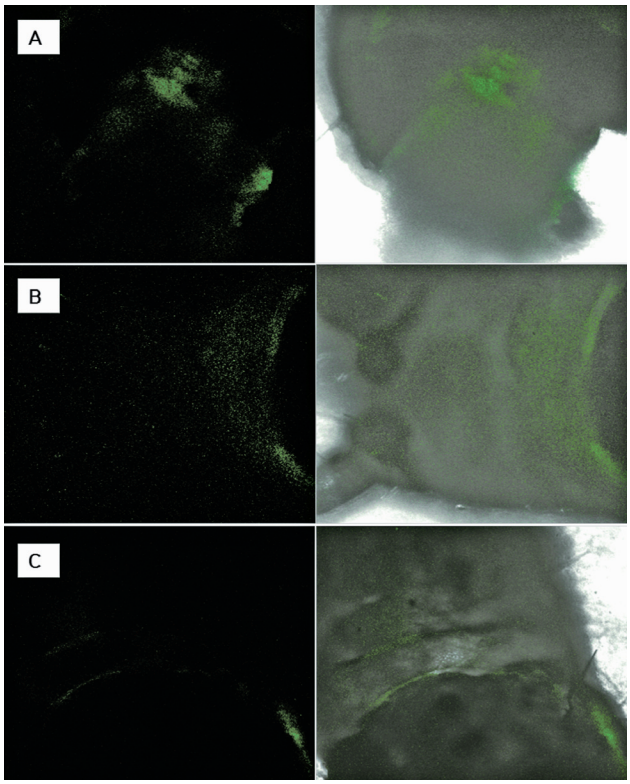

Fig. 9. Immunofluorescence and microscopic imaging (Carl Zeiss Axiovert, 135 M microscope) of Trypsin (D–1): sc–137077 and a mouse IgG2a antibody after a 30 minute feeding; (A) and (B): Antibody treatment; (C): Antibody and 40 mM of tannic acid.

cence (Fig. 9A, B), and when the larvae were treated with 40 mM of tannic acid, trypsin activity decreased (Fig. 9C). Trypsin activity was shown to be lower following the treatment with tannic acid, which is a protease inhibitor.

Diverse attempts to increase the insecticidal activity of *B. thuringiensis* have been made. Heimpel (1967) reported that when the use of 35 types of agricultural chemicals in combination with *B. thuringiensis* was tested, insecticidal activity increased in six types of agricultural chemicals. These results have been seen not only with insecticides but also with many other compounds used to increase the insecticidal activity of *B. thuringiensis*. Salama *et al*. (1984) examined the addition of inorganic compounds or organic compounds to *B. thuringiensis* and evaluated the insecticidal activities of the mixtures on *S. littoralis*. They mixed *B. thuringiensis* with compounds using three methods: (i) they added compounds, such as sodium carbonate (Na_2CO_3) and calcium carbonate ($CaCO_3$) to *B. thuringiensis* to enhance the alkalinity of insects' mid–guts to accelerate the dissolution of crystals, (ii) they added compounds, such as calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$), to *B. thuringiensis* to increase concentrations of Ca^{++} ions and Mg^{++} ions to accelerate conversion of *B. thuringiensis* pre–toxins into active toxin, and (iii) they added compounds, such as borax and tannic acid, to *B. thuringiensis* to increase the activity of *B. thuringiensis* in insects. According to the experimental results, the *B. thuringiensis* subspecies entmocidus HD–635 combined with calcium carbonate remarkably increased activity on *S. littoralis*. The synergistic effects of the addition of calcium chloride and magnesium chloride were directly proportional to the concentrations of the added calcium chloride and magnesium chloride, and the *B. thuringiensis* added with borax and tannic acid also showed remarkable increases in insecticidal activity (Salama *et al*., 1984).

In a previous study by the current researchers, a new *B. thuringiensis* strain was isolated and identified, tannic acid was selected and mixed with the *B. thuringiensis* strain to increase its insecticidal activity to show synergistic effects, and diverse experiments were conducted to determine the causes of the results. According to the results of a colorimetric assay of trypsin activity, activity decreased slightly in larvae that ate the *B. thuringiensis* KB100 strain and 40 mM of tannic acid. A trypsin gene expression level analysis conducted using qRT–PCR analysis and immunofluorescence antibody method experiments revealed that the *B. thuringiensis* KB100 strain was decomposed by *S. exigua* mid–gut proteases and that the addition of 40 mM of tannic acid changed expression levels and the image analysis.

Trypsin is composed of three substances: cationic trypsinogen, anionic trypsinogen, and chymotrypsinogen and is decomposed by enterokinase into trypsin 1, 2, and 3. According to Rukmini *et al*. (2000), cry1A (a), cry1A (b), and cry1A (c) proteins are quickly decomposed by trypsin. Trypsin decomposition of the cry toxins of the *B. thuringiensis* subspecies *kurstaki* LB1 and *kurstaki* HD251 was checked, and the results indicated that there

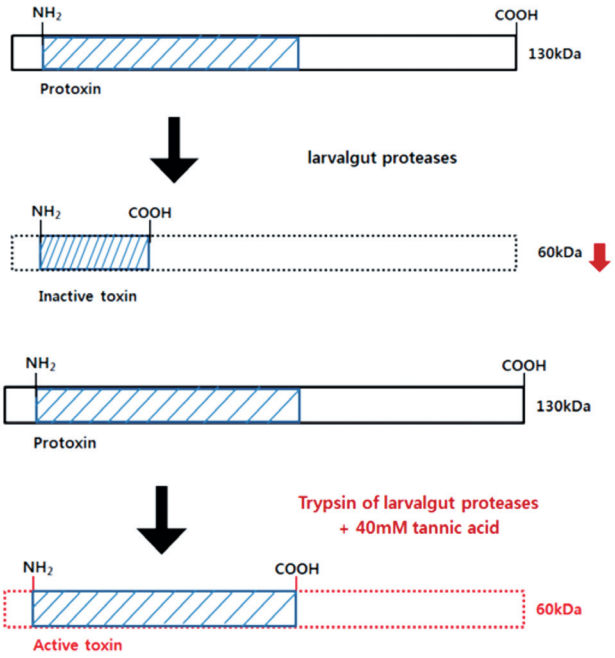

Fig. 10. The role of tannic acid in mid-gut trypsin (diagram of *S. exigua*).

were differences in digestion time (Rukmini *et al*., 2000). It could be assumed that in the case of the *kurstaki* KB100 strain, tannic acid effectively suppresses the activity of cry1Aa, cry1Ab, and cry1Ac proteins when these proteins are hydrolyzed by trypsin (Fig. 10).

Therefore, for environmentally friendly control of *S. exigua*, which is an intractable pest, tannic acid added to *B. thuringiensis* products exhibits high activity, and the mechanism through which tannic acid effectively suppresses trypsin among diverse protease inhibitors can be identified. Future studies that attempt to enhance the toxicity of existing components with insecticidal activities of biological pesticides to control intractable pests should be conducted. The present study showed that the effects of microbial pesticides can be enhanced by appropriate improvement of formulations to relieve problems, such as narrow host ranges and low activity, which are disadvantages of biological pesticides. Research and development are necessary to enhance the effects of diverse methods for control of domestic intractable pests.

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