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Biochemical Characteristics of Immune-Associated Phospholipase A_2 and Its Inhibition by an Entomopathogenic Bacterium, *Xenorhabdus nematophila*

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An entomopathogenic bacterium, *Xenorhabdus nematophila*, induces an immunosuppression of target insects by inhibiting phospholipase A_2 (PLA_2) activity. Recently, an immune-associated PLA_2 gene was identified from the red flour beetle, *Tribolium castaneum*. This study cloned this PLA_2 gene in a bacterial expression vector to produce a recombinant enzyme. The recombinant *T. castaneum* PLA_2 ($TcPLA_2$) exhibited its characteristic enzyme activity with substrate concentration, pH, and ambient temperature. Its biochemical characteristics matched to a secretory type of PLA_2 ($sPLA_2$) because its activity was inhibited by dithiothreitol (a reducing agent of disulfide bond) and bromophenacyl bromide (a specific $sPLA_2$ inhibitor) but not by methylarachidonyl fluorophosphonate (a specific cytosolic type of PLA_2). The *X. nematophila* culture broth contained PLA_2 inhibitory factor(s), which was most abundant in the media obtained at a stationary bacterial growth phase. The PLA_2 inhibitory factor(s) was heat-resistant and extracted in both aqueous and organic fractions. Effect of a PLA_2-inhibitory fraction on the immunosuppression of *T. castaneum* was equally comparable with that resulted from inhibition of the $TcPLA_2$ gene expression by RNA interference.

***Keywords*:** *X. nematophila*, phospholipase A_2, *T. castaneum*, immunosuppression, RNA interference

An entomopathogenic bacterium, *Xenorhabdus nematophila*, is a Gram-negative bacterium carried in a vesicle attached to the intestinal tract of a dauer larval stage of the entomopathogenic nematode, *Steinernema carpocapsae* (Akhurst, 1982). When the nematodes enter target insect hemocoel via its natural openings (mouth, anus, and spiracles), they release the symbiotic bacteria, which in turn initiate lethal septicemia (Dunphy and Webster, 1991). The bacterial mutualism with *S. carpocapsae* in relation to pathogenic function in the infected host is mediated by a transcription factor σ^s of *X. nematophila* (Vivas and Goodrich-Blair, 2001). Thus, to be effective in the bacterial pathogenicity, the nematode host has been regarded as an indispensable agent for the symbiotic bacteria to be delivered into target insect hemocoel (Kaya, 1990). In the hemocoel, *X. nematophila* exhibits a high potency in the pathogenicity because only 100 bacterial cells were sufficient to kill the fifth instar larva of the beet armyworm, *Spodoptera exigua* within 16 h (Park and Kim, 2000).

Insects have innate immune systems consisting of cellular and humoral immune responses (Ratcliffe *et al*., 1991). To defend bacterial infection, phagocytosis, and nodule formation would be expressed by hemocytes for cellular immune responses while antimicrobial peptide produced by fat body would be used for humoral immune response (Gillespie *et al*., 1997; Lavine and Strand, 2002). Upon pathogen infection, non-self recognition by pattern recognition receptors initiates various immune responses via immune mediators, such as eicosanoids, biogenic monoamines or cytokines (Gillespie *et al*., 1997).

Eicosanoids are the group of oxygenated products of C20 polyunsaturated fatty acids, which actively mediate insect innate immune responses against various pathogens (Stanley, 2005). Eicosanoids are biosynthesized in insect immune tissues, in which several prostaglandins (PGA_2, PGE_2, PGD_2, and $PGF_{2\alpha}$) and 15-hydroxyeicosatetraenoic acid have been identified in fat body and hemocyte of *Manduca sexta* (Stanley-Samuelson and Ogg, 1994; Gadelhak *et al*., 1995). Phospholipase A_2 (PLA_2) catalyzes at *sn*-2 of phospholipids to release arachidonic acid to be oxidized into various eicosanoids (Schaloske and Dennis, 2006), which mediate cellular immune responses of various insect species (Stanley *et al*., 1999; Kim *et al*., 2005; Shrestha and Kim, 2007a, 2007b) and induces antimicrobial peptide production (Yajima *et al.*, 2003). It also mediates prophenoloxidase release from oenocytoids in *S. exigua* (Shrestha and Kim, 2008).

Inhibition of eicosanoid biosynthesis leads to immunosuppression in insects (Stanley, 2006b). This strategy is exploited by various pathogenic bacteria to avoid immune responses of the target insects (Kim *et al*., 2005). The suppression of eicosanoid biosynthesis by *X. nematophila* was first reported in *S. exigua* (Park and Kim, 2000). The inhibitory target of *X. nematophila* is PLA_2 of *S. exigua*, which is a secretory type due to its specific sensitivity to a secretory PLA_2 inhibitor (Park and Kim, 2003). Park *et al*. (2005) showed that the PLA_2 inhibitory factor(s) is synthe-

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sized and released into the bacterial culture broth. However, it was not clearly understood that the PLA_2 inhibited by the metabolite of *X. nematophila* is associated with immune responses of the insect. Also, any PLA_2-inhibitory metabolite has not been identified from the culture broth of *X. nematophila*.

This study investigated any inhibitory activity of *X. nematophila* against an immune-associated PLA_2 identified from the red flour beetle, *Tribolium castaneum*. It also determined the cultured extract containing PLA_2 inhibitor(s) synthesized by *X. nematophila*. To these ends, this study prepared an immune-associated PLA_2 of *T. castaneum* (Shrestha, 2008) using a bacterial expression system. To analyze the inhibitory metabolite(s) synthesized by *X. nematophila* against PLA_2 activity, bacterial culture broth was fractionated with organic solvents. Finally, the immunosuppressive activity of the active fraction was compared to the effect induced by RNA interference of the PLA_2 gene expression.

Materials and Methods

Insect rearing and bacterial culture

T. castaneum was reared in a dry and dark condition (a relative humidity 60±5%) at room temperature (25±1°C) with organic wheat flour (Pareve, USA). Fully grown late instar larvae (≥5 mm) were used in this study. *Escherichia coli* Top10 cells were cultured overnight in Luthia-Bertani (LB) medium at 37°C and *X. nematophila* in Tryptic Soy Broth (TSB, Difco, USA) at 28°C in shaking incubator (200 rpm). Heat-killed *X. nematophila* was prepared by incubating bacterial suspension in 70°C for 20 min. After heat treatment, dead *X. nematophila* was confirmed by streaking and culturing the suspension on tryptic soy agar medium.

Chemicals

Benzylideneacetone [trans-4-phenyl-3-buten-2-one], dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione], arachidonic acid [5,8,11,14-eicosatetraenoic acid], 4-bromophenacyl bromide (BPB), methyl arachidonyl fluorophosphonate (MAFP), and dithiothreitol (DTT) were purchased from Sigma-Aldrich Korea (Korea). All these chemicals were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich). Ethylene glycol-bis (2-amino-ethyl ether)- N,N,N',N'-tetraacetic acid (EGTA) and bovine serum albumin were purchased from Sigma-Aldrich Korea and dissolved in sterilized water. For enzyme activity, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine was purchased from Molecular Probes, Inc. (USA) and dissolved in high grade ethanol (Sigma-Aldrich).

Extraction of *X. nematophila* metabolites

X. nematophila was cultured in TSB at 28°C. The bacterial solution was centrifuged at 7,000 rpm for 15 min using A6.14 rotor (Kontron Instrument, Italy) at 48 h after culture. The collected supernatant (1 L) was transferred to separation funnel and mixed with 330 ml of hexane. After vigorous shaking and incubation for 1 h at room temperature, the supernatant was collected. This hexane extraction was repeated two more times. The resulting 1 L hexane extract was concentrated into 5 ml by using a rotary evaporator (Ayala, Japan). This concentrated solution of hexane extract was sterilized by using membrane filter (0.22 μm pore size) and stored at 4°C. The remaining culture broth after the hexane extraction was subjected to diethyl acetate extraction, which followed the same procedure described in the hexane extraction. The final remaining culture broth was taken as an aqueous fraction. To compare these three extraction fractions, both hexane and diethyl acetate extracts were diluted at 200× with DMSO to make original volume before extraction. Two microliter of each extract was used in total reaction mixture (see below) in analysis of enzyme activity, while 200 nl was injected into hemocoel to analyze effects of immunosuppression and pathogenicity on *T. castaneum*.

Nodulation assay

Nodulation assay to measure immune capacity was performed by injecting 1.28×10^4 cells of heat-killed *X. nematophila* in a 60 nl volume into hemocoel of late instar *T. castaneum* using a nanoliter injector (WPI, Inc., USA) under a microscope (S730, Olympus, Japan). After 4 h incubation at room temperature, melanized black nodules were counted externally on its ventral side of transparent body cuticle under a stereomicroscope (SZX9, Olympus) at 50× magnification.

For analysis of time-course of hemocytic nodule formation, the larvae were injected with 6.25×10^5 cells of *X. nematophila.* Nodules were counted at 0, 0.5, 1, 2, 4, 6, and 8 h post-injection. At each time point, a treatment was replicated three times. Numbers of nodules were counted as described above.

Preparation of recombinant $TcPLA_2$ protein

Open reading frame of $TcPLA_2$ (GenBank accession no.: FJ768719) was cloned into an expression vector, pBAD-TOPO containing polyhistidine tail and V5 epitope (Invitrogen, USA) according to the manufacturer's instruction. After transformation into an electro-competent *E. coli* Top10 cells, colonies in frames were chosen for bacterial expression. A recombinant colony was cultured at 37°C with shaking. At 0.6~0.9 absorbance at 600 nm, an inducer L-arabinose (Sigma-Aldrich) was added in 0.008% final concentration to over-express the inserted PLA_2 gene. The recombinant bacterial cells were harvested by centrifugation at 5,000 rpm using A6.14 rotor (Kontron Instruments, Italy) and resuspended in a double detergent buffer [50 mM Tris, 30 mM NaCl, 0.1% sodium dodecyl sulfate (Sigma-Aldrich), 0.02% NaN_3, 0.01% Igepal, 0.005% phenylmethylsulfoxide (Sigma-Aldrich), 0.005% protease inhibitor cocktail]. Then an ultrasonicator (Bandelin Sonoplus, Germany) was used for subsequent bacterial cell wall lysis with 30 sec at each cycle for 20 min. After the cell lysate was centrifuged at 14,000 rpm using A8.24 rotor (Kontron Instruments, Italy), the supernatant containing $TcPLA_2$ protein was collected and used as enzyme source for enzyme activity assay.

Pathogenicity

Bioassay test was performed using *T. castaneum* larvae after intra-hemocoelic injection of freshly cultured *X. nematophila* or its culture extracts. Late instar larvae (>5 mm in length) were injected with 200 nl of each test solution using nano-

liter injector (Nanojet II, WPI, Inc., USA). Live larvae were counted after 48 h post-injection.

Enzyme activity measurement

PLA_2 activity was measured by spectrofluorometry using a pyrene-labeled phospholipid [1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycerol-3-phosphatidyl choline] as the substrate in the presence of bovine serum albumin (Radvanyi *et al.*, 1989). The fluorometric phospholipid was dissolved in ethanol to prepare 10 mM stock solution and 10% bovine serum albumin was prepared in sterilized distilled water. The reaction mixture (2 ml) was prepared in a cuvette by sequentially adding 50 mM Tris-HCl buffer (pH 7.0), 10% of bovine serum albumin, 1 M $CaCl_2$, and enzyme extract. The reaction was initiated by addition of 10 mM pyrene substrate and subsequently fluorescence intensity was monitored with an Aminco Bowmen Series 2 luminescence spectrometer (FA257, Spectronic Instruments, USA) using excitation and emission wavelengths of 345 and 398 nm, respectively. The specific enzyme activity was calculated by the method described by Radvanyi *et al.* (1989). For pH preference, different pH buffers were prepared from pH 4.5 to pH 10.0 with sodium phosphate. To check inhibition of its activity, 2 μl of test chemical was used in above described reaction mixture.

RNA interference (RNAi)

Double stranded RNA (dsRNA) of $TcPLA_2$ was prepared with gene specific primer; 5'-CAAAAGCTACACAGGGAGG AGGG-3' and 5'-CTTTGAAGGAAATTGCAACGGGGC-3' according to manufacturer's instruction of Megascript RNAi kit (Ambion, USA). The PCR product was cloned into the pCR2.1 cloning vector (Invitrogen) and their orientations were analyzed by PCR using a combination of vector- and $TcPLA_2$-specific primers. Sense and antisense strands were transcribed *in vitro* using T7 RNA polymerase. Both RNA strands were annealed at 37°C for 4 h and then digested with DNase I and RNase to remove DNA and any single-stranded RNA, respectively.

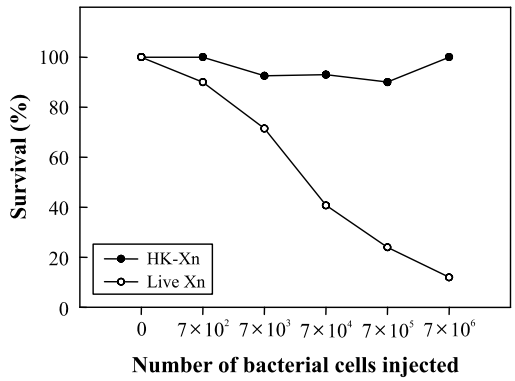

Fig. 1. Pathogenicity of *X. nematophila* ('Xn') against late instar larvae (>5 mm in length) of *Tribolium castaneum*. In heat-killed Xn, ('HK-Xn'), the bacteria were treated at 70°C for 20 min. The bacterial suspension (200 nl) was microinjected into the hemocoel and incubated at 25°C for 48 h. Each treatment tested fifty larvae.

Transfection of dsRNA was carried out with Metafectene PRO transfection reagent (Biontex, Planegg, Germany) in a 1:1.5 volume ratio and incubated for 20 min at 25±1°C. Twenty five nanoliters of the dsRNA solution were injected into larval hemocoel by the micro-injection method described above. However, one hundred nanoliters of dsRNA was injected for their survival test. Knockdown was evaluated by RT-PCR at 48 h after the microinjection.

RT-PCR

Total RNA was extracted using Trizol reagent (MRC, USA) according to manufacturer instruction. First strand cDNA was synthesized by reverse transcription using RT-premix (Intron Biotechnology, Korea) containing an oligo dT primer; 5'-C CAGTGAGCAGAGTGCGAGGACTCGAGCTCAAGCTT TTTTTTTTTTTTTT-3'. Ten times diluted cDNA was used

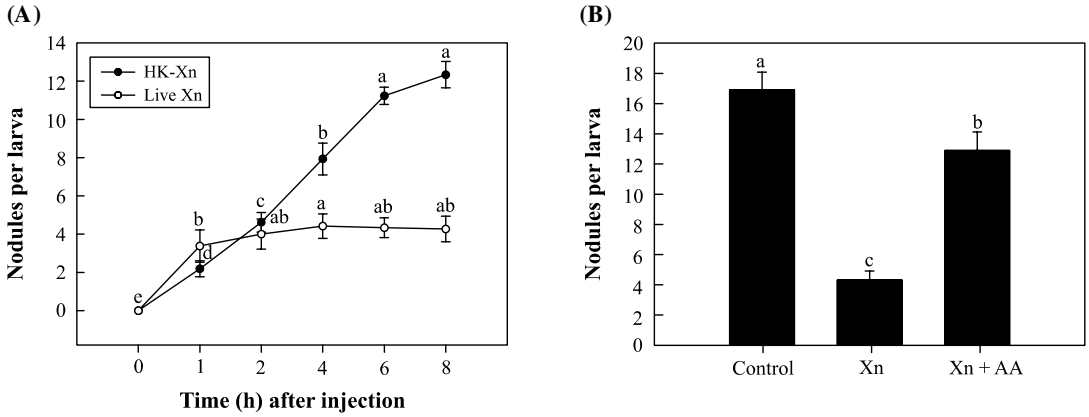

Fig. 2. Immunosuppressive effect of *X. nematophila* ('Xn') on nodule formation of late instar larvae (>5 mm in length) of *T. castaneum* in response to the bacterial challenge. In heat-killed Xn ('HK-Xn'), the bacteria were treated at 70°C for 20 min. (A) Time-course experiment, where the bacterial suspension (200 nl) was microinjected into the hemocoel and incubated at 25°C for different time periods. (B) Rescue effect of exogenous arachidonic acid (2.2 μg/larva, 'AA') on the suppressive nodule formation by Xn infection. Each treatment consisted of at least 10 larvae. Different letters above standard deviation bars represent significant difference among means at Type I error=0.05 (LSD test).

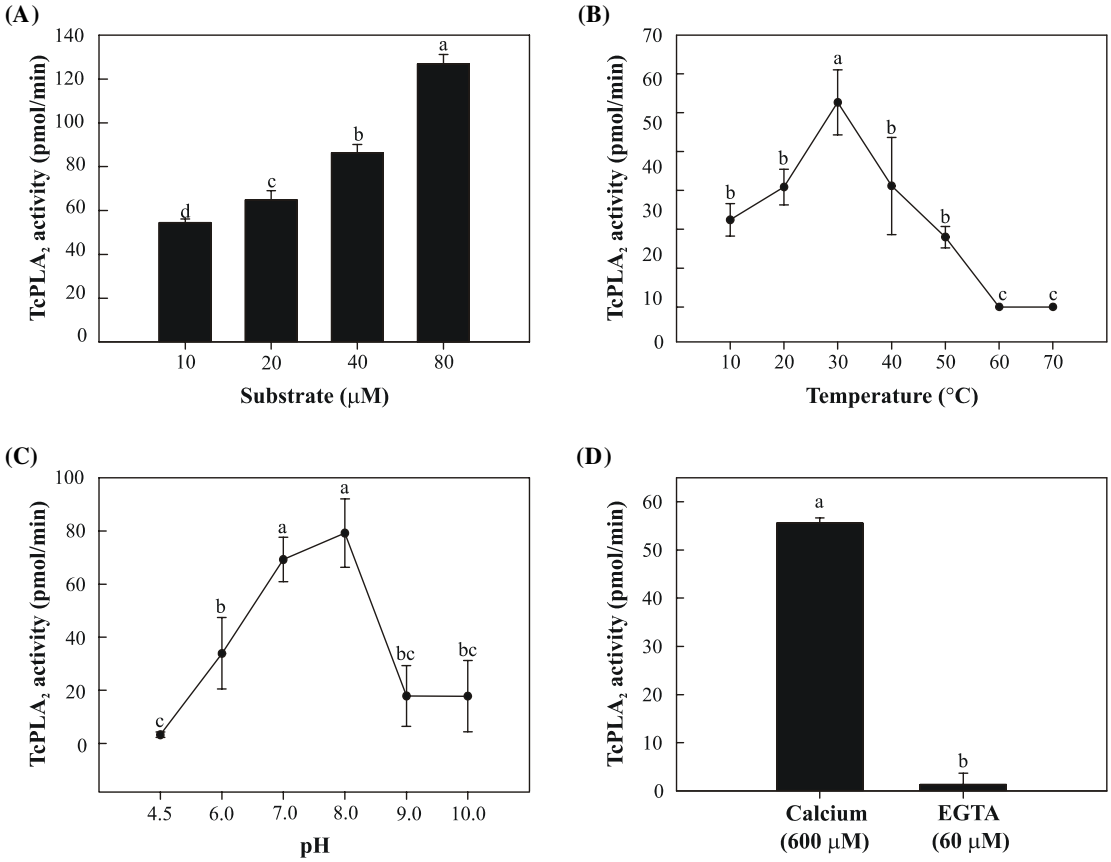

Fig. 3. Biochemical characteristics of a recombinant phospholipase A_2 of *T. castaneum* ($TcPLA_2$). The PLA_2 gene was over-expressed in *E. coli* and the resulting extract was used for catalytic activity analysis. Effects of substrate (A), temperature (B), pH (C), and EGTA (D) on $TcPLA_2$ enzyme activity. Each treatment was replicated three times. Different letters above standard deviation bars represent significant difference among means at Type I error=0.05 (LSD test).

as a template for RT-PCR with gene specific forward; 5'-C AAAAGCTACACAGGGAGGAGGG-3' and reverse; 5'-C TTTGAAGGAAATTGCAACGGGGC-3' primers. β-Actin gene was used as an internal control with forward; 5'-TG GCACCACACCTTCTAC-3' and reverse; 5'-CATGATCTG GGTCATCTTCT-3' primers. PCR was performed with 35

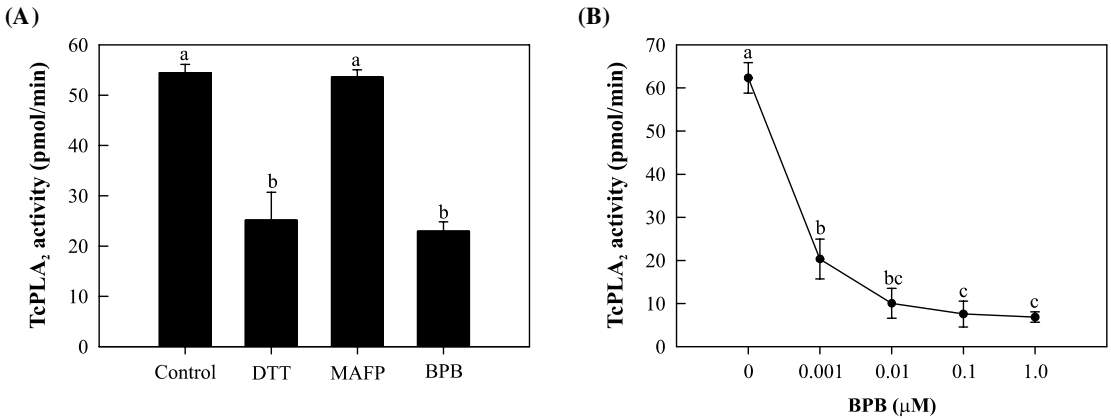

Fig. 4. (A) Specific inhibitory effects of different inhibitors on a recombinant phospholipase A_2 of *T. castaneum* ('$TcPLA_2$'). The PLA_2 gene was over-expressed in *E. coli* and the resulting extract was used for catalytic activity analysis. DTT, MAFP, and BPB represent dithiothreitol, methyl arachidonyl fluorophosphonate, and *p*-bromophenacyl bromide, respectively. (B) Dose effect of BPB on $TcPLA_2$ activity. Each treatment was replicated three times. Different letters above standard deviation bars represent significant difference among means at Type I error=0.05 (LSD test).

cycles under a condition of denaturation at 95°C for 1 min, extension at 72°C for 1 min and at specific annealing temperature for each gene for 1 min.

Statistical analysis

All studies were performed in three independent replicates and plotted by Mean±SD using Sigma plot. Means were compared by a least squared difference (LSD) test of one way ANOVA using PROC GLM of SAS program (SAS Institute, 1989) and discriminated at Type I error=0.05.

Results

Influence of *X. nematophila* on pathogenicity of *T. castaneum* larvae

An entomopathogenic bacterium, *X. nematophila*, is highly pathogenic to most lepidopteran larvae; however its effect on other insects including coleopteran insects was not clear. *X. nematophila* showed pathogenicity at only several hundred cell dose against late larval stage of *T. castaneum* by hemocoelic injection (Fig. 1). With increase of injection dose of *X. nematophila*, larval mortality significantly increased, while no significant mortality was found in heat-killed bacterial treatment.

To explain the pathogenic effect of *X. nematophila* on *T. castaneum*, the larvae were analyzed by their capacity to form hemocytic nodules in response to bacterial challenge. The larvae infected with heat-killed *X. nematophila* formed several round black nodules around the trachea, gut, and fat body. The nodules were easily visible externally due to its transparent cuticle. Nodule formation in *T. castaneum* began after an hour of bacterial injection and increased with incubation time up to 4 h after the injection (Fig. 2A). When live *X. nematophila* was injected to *T. castaneum*, the infected larvae formed significantly less number of nodules compared to the heat-killed *X. nematophila*. The suppression of the nodule formation was clearly recovered by an additional injection of arachidonic acid, a precursor of eicosanoid biosynthesis (Fig. 2B). These results indicated immunosuppressive activity of *X. nematophila* on *T. castaneum* by inhibiting PLA_2.

Recombinant PLA_2 of *T. castaneum* biosynthesis and its biochemical properties

An immune-associated PLA_2 gene was chosen and cloned into an expression vector, pBAD-TOPO. The $TcPLA_2$ gene was over-expressed to prepare a recombinant PLA_2 protein. The enzyme activity of the recombinant $TcPLA_2$ increased with substrate concentration (Fig. 3A) and exhibited optimal activity at 30°C (Fig. 3B). The $TcPLA_2$ protein preferred neutral pH for their enzymatic activity (Fig. 3C) and showed calcium dependency because a calcium chelator, EGTA, significantly inhibited the enzyme activity (Fig. 3D). The enzyme activity of $TcPLA_2$ was inhibited by BPB, a specific inhibitor to secretory type PLA_2 and by DTT, a reducing agent of disulfide bond. However, there was no inhibition of $TcPLA_2$ with MAFP (a specific inhibitor to cytosolic type PLA_2) (Fig. 4A). Its enzyme activity was inhibited in a dose dependent manner with BPB (Fig. 4B).

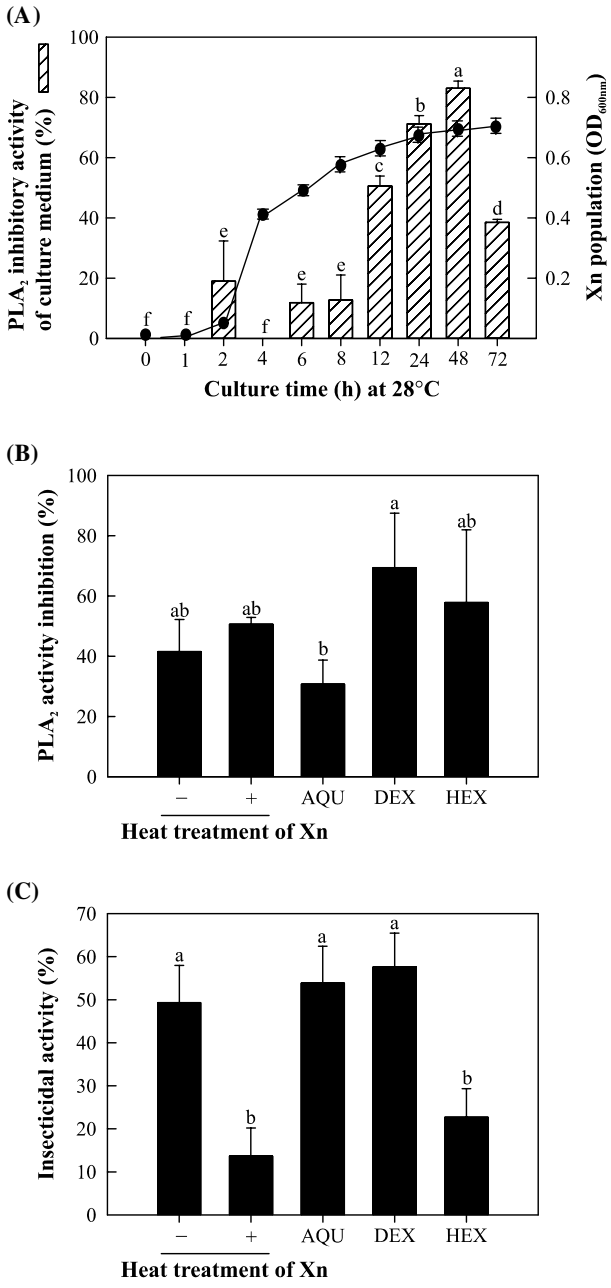

Fig. 5. Inhibitory effect of culture media of *X. nematophila* ('Xn') on a recombinant phospholipase A_2 ('PLA_2') of *T. castaneum*. The PLA_2 gene was over-expressed in *E. coli* and the resulting extract was used for catalytic activity analysis. (A) Effects of Xn culture media for different time periods on PLA_2 activity inhibition (bar). In each culture medium sample, the bacterial population was monitored by measuring absorbance at 600 nm (line). Effect of different extraction fractions of Xn on inhibition of PLA_2 activity (B), and insecticidal activity (C). Heat treatment used 70°C for 20 min. The 48 h culture medium was fractionated into hexane ('HEX'), diethylacetate (DEX'), and remaining aqueous ('AQU') phases. For insecticidal activity analysis, 100 nl of extract was injected into late instar larva (>5 mm in length) of *T. castaneum*, and incubated at 25°C for 48 h. Each treatment was replicated for three times. Different letters above standard deviation bars represent significant difference among means at Type I error=0.05 (LSD test).

Screening of PLA_2-inhibitory metabolite(s) from *X. nematophila*

Culture broth samples of *X. nematophila* were taken at different times according to the bacterial growth phases and monitored in their inhibitory effects on $TcPLA_2$ activity. After 12 h culture time, the bacterial population reached to a stationary growth phase and showed significantly high PLA_2-inhibitory activities (Fig. 5A). The 48 h culture medium contained heat-resistant PLA_2 inhibitor(s), which was contrasted with high susceptibility of its insecticidal activity to the heat-treatment (Fig. 5B). The 48 h culture broth was further separated into hexane, diethyl acetate, and remaining aqueous fractions. Most PLA_2-inhibitory activities were found in diethyl acetate and hexane fractions, while most insecticidal activities were in aqueous and diethyl acetate fractions (Fig. 5C).

Immunosuppressive effect of diethyl acetate extract was comparable to that induced by RNA interference of $TcPLA_2$

Immunosuppression due to a specific RNA interference against $TcPLA_2$ was confirmed (Fig. 6). Transfection of dsRNA of $TcPLA_2$ was analyzed by RT-PCR at 48 h after the injection (Fig. 6A). The mRNA expression of $TcPLA_2$ was completely inhibited (Fig. 6A). The knockdown of $TcPLA_2$ expression clearly suppressed nodule formation of *T. castaneum* in response to bacterial challenge (Fig. 6B). This immunosuppressive effect was also found in the larvae treated with the diethyl acetate extract prepared from 48 h culture medium. To link this immunosuppression to the bacterial pathogenicity, a low dose of *X. nematophila* was injected to the late instar larvae (Fig. 6C). Additional treatment with diethyl acetate extract could significantly increase the bacterial pathogenicity. Similar enhanced pathogenicity was found in the larvae treated with dsRNA specific to $TcPLA_2$.

Discussion

A whole genome sequence of *T. castaneum* was reported and annotated (Tribolium Genome Sequence Consortium, 2008). Furthermore, an EST database study supported the validation of the genome annotation (Park *et al*., 2008). Comparisons of the annotated genes of *T. castaneum* with those of *Drosophila melanogaster*, *Bombyx mori*, and *Anopheles gambiae* provided putative functional genes, which would be subjected to be proved in their physiological functions in *T. castaneum* (Schröder, 2003; Bonneton *et al*., 2008; Tan and Palli, 2008). By this reverse physiological strategy, five PLA_2 genes of *T. castaneum* have been identified to be associated with its immune responses (Shrestha, 2008). This study chose a $TcPLA_2$ that was known as '$TcPLA_2B$' encoding an amino acid sequence with 261 residues and putative five disulfide bonds as well as calcium-binding and active site domains (Shrestha, 2008).

Injection of live *X. nematophila* gave significant pathogenicity to *T. castaneum* in a dose-dependent manner. However, its pathogenicity to *T. castaneum* was much lower than that to a lepidopteran insect, *S. exigua*, where only 100 live cells were enough to kill the fifth instar larvae (Park and Kim, 2000) while at least 10^4 times more cells were required to give similar mortality in *T. castaneum*. This pathogenic difference may be due to a host preference of *X. nematophila*, not to a phase variation of *X. nematophila* (Boemare *et al*., 1997) because we used a primary form in both assays. On the other hand, other nematode-symbiotic entomopathogenic bacterium, *Photorhabdus temperata* subsp. *temperata*, showed

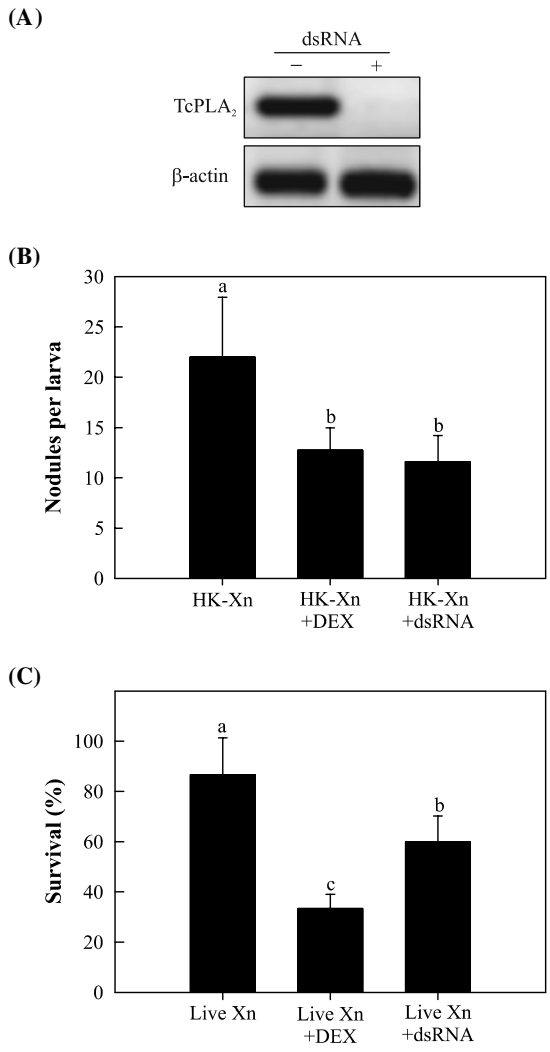

Fig. 6. Immunosuppression induced by a culture extract using diethyl acetate ('DEX') of *X. nematophila* was compared with an effect of RNA interference of an immune-associated phospholipase A_2 ('$TcPLA_2$') in *T. castaneum*. (A) Effect of double strand RNA ('dsRNA') injection on expression of $TcPLA_2$. The dsRNA (25 nl) was injected into late instar larva (>5 mm in length) and incubated for 48 h at 25°C. Then RNA was extracted and used for RT-PCR of $TcPLA_2$. β-Actin gene was used as a positive control of cDNA preparation. (B) Xn was heat-killed ('HK- Xn') at 70°C for 20 min. HK-Xn (10^5 cells/larva) was injected with DEX (100 nl/larva) or dsRNA (50 nl/larva). Nodulation was analyzed after 12 h of injection at 25°C. (C) Live Xn (7×10^3 cells) was injected with DEX (100 nl) and dsRNA (50 nl). Larval survival was analyzed 48 h after the injection treatment at 25°C. Each treatment was replicated three times. Different letters above standard deviation bars represent significant difference among means at Type I error=0.05 (LSD test).

high pathogenicity against *T. castaneum* (unpublished data). Thus this differential pathogenicity may come from variation in target insect susceptibilities to bacterial virulence factors. Another source of the pathogenicity variation may be due to difference in immunity between *T. castaneum* and *S. exigua* to defend the infection of *X. nematophila*. Host immunosuppression by *X. nematophila* significantly affects its pathogenicity by assessment of clonal variation of the bacteria (Park *et al*., 2006). The causative link between bacterial pathogenicity and host immune capacity was further supported because prior immunization enhances tolerance against the entomopathogenic bacteria and RNA interference of the antimicrobial peptide expression enhances the bacterial pathogenicity (Eleftherianos *et al*., 2006). However, it looks difficult to explain the low pathogenicity of *X. nematophila* in terms of differential immunosuppression because the bacterial infection significantly inhibited the immune responses of *T. castaneum* as they did in *S. exigua* (Park and Kim, 2000). This immunosuppression was due to inhibition of PLA_2 because the addition of arachidonic acid significantly rescued the suppressed state. The inhibition of PLA_2 activity by *X. nematophila* was first reported in *S. exigua* (Park and Kim, 2000, 2003). This was further confirmed in other lepidopteran insect, *M. sexta* (Park *et al*., 2004). In turn, PLA_2 is known to be the common inhibitory target of these nematode-symbiotic bacteria (Kim *et al*., 2005). The reason to inhibit PLA_2 activity by the bacteria is likely to shutdown biosynthesis of eicosanoids. Eicosanoids are well known immune mediators in various insect orders (Stanley, 2006a). Especially, in relation to bacterial infection, eicosanoids mediate hemocyte-spreading behavior (Kwon *et al*., 2007), phagocytosis (Shrestha and Kim, 2007a), and nodule formation (Stanley, 2006a) as well as antimicrobial peptide production (Morishima *et al*., 1997). However, due to lack of molecular information on immune-associated PLA_2 in insects, it was difficult to make a direct link between each immune response and specific PLA_2 activities. Thus, molecular information on the target PLA_2 of *X. nematophila* was needed.

Based on the identified five immune-associated PLA_2 genes in *T. castaneum*, this study used a $TcPLA_2$ after preparing its recombinant protein using a bacterial expression system. The recombinant $TcPLA_2$ showed specific conditions for optimal its PLA_2 activity depending on substrate concentrations, pH, temperature, and calcium. Generally, PLA_2 has been classified into 15 Groups comprising four main types including secretory ($sPLA_2$), cytosolic Ca^{2+}-dependent ($cPLA_2$), cytosolic Ca^{2+}-independent ($iPLA_2$), and PAF acetyl hydrolase/oxidized lipid ($LpPLA_2$) PLA_2 (Burke and Dennis, 2009). They differ in molecular size, number of disulfide bonds, and calcium dependency as well as nucleotide sequence. The only known insect PLA_2s in amino acid sequence are the secretory types of *Apis mellifera* (Kuchler *et al*., 1989) and *D. melanogaster* (Ryu *et al*., 2003), which are classified into Group III PLA_2. $TcPLA_2$ analyzed in this study was $sPLA_2$ because it showed sequence similarity with other $sPLA_2$ (Shrestha, 2008) and was susceptible to a $sPLA_2$ specific inhibitor, BPB, as shown in this study. Furthermore, its calcium dependency and essential disulfide bond(s) for optimal activity reflect its biochemical nature of $sPLA_2$. Since $TcPLA_2$ has ten cysteines in its amino acid sequences forming putative five disulfide bonds, inhibition of the enzyme activity by DTT might be due to reducing the disulfide bonds. Park and Kim (2003) showed that immune-associated PLA_2 is likely to be a $sPLA_2$, which would be a target of *X. nematophila*.

X. nematophila synthesized PLA_2 inhibitor(s) and secreted into culture broth. The inhibitory activity of *X. nematophila* culture broth was found as early as 2 h after culture. But, maximal effects of the culture broth on PLA_2 inhibition were found in samples obtained at the stationary bacterial growth phase. This suggests that *X. nematophila* can produce PLA_2 inhibitor(s) at early infection stage in *T. castaneum* and increase its concentration with bacterial population density. The PLA_2 inhibitor(s) of *X. nematophila* was heat-tolerant, which was compared with heat-susceptible insecticidal factor(s) in the culture broth. The insecticidal toxins are identified in *X. nematophila* as *Xenorhabdus protein toxins* (*xpt*) that are heat-labile and show high similarity to the well-known toxin genes of *Photorhabdus* (Morgan *et al*., 2001). The insecticidal toxic genes of *X. nematophila* have been identified and their pathogenic interactions have been evaluated in terms of target insect spectrum (Morgan *et al*., 2001; Sergent *et al*., 2003). Heat-labile nature of insecticidal activity found in this study may be explained by presence of the *xpt* protein.

This study showed that PLA_2 inhibitor(s) of *X. nematophila* in its 48 h culture broth could be fractionated into both aqueous and organic phases while most insecticidal factor(s) was extracted in polar phases. This suggests that there may be more than one type of PLA_2 inhibitors synthesized and secreted by *X. nematophila*. For insecticidal factors other than *xpt*, *X. nematophila* secretes the outer membrane vesicles in its culture media, which exhibit cytotoxicity to insect cells and oral insecticidal activity (Khandelwal and Benerjee-Bhatnagar, 2003). These metabolites would be dissolved in organic solvent, such as diethyl acetate. This study showed a significant insecticidal activity of the diethyl acetate extract. In addition, *X. nematophila* has been known to release antimicrobial compounds such as indoles (Paul *et al*., 1981), dithiolopyrrolones (McInerney *et al*., 1991), xenocoumacins (Sundar and Chang, 1993), and monoterpene (Ji *et al*., 2004) to keep monogenic state in the infected insect hemocoel.

Among active fractions, diethyl acetate fraction contained large amount of PLA_2 inhibitor(s) and was further analyzed in its effect on immunosuppression in *T. castaneum*. The inhibitory effect of the diethyl acetate fraction was equally comparable with that of RNA interference of the $TcPLA_2$ expression in *T. castaneum*. We already showed that the diethyl acetate extract could inhibit $TcPLA_2$ activity. Thus the inhibitory activity of the diethyl acetate against $TcPLA_2$ led to immunosuppression of *T. castaneum* and enhanced the bacterial pathogenicity. Here, we do not avoid a possibility that the extract also inhibited other $TcPLA_2$s, which would give synergistic effects on the immunosuppression. Thus these results suggest that the immunosuppressive effect of *X. nematophila* on *T. castaneum* can be explained by the PLA_2 inhibitor(s) action of the bacteria against $TcPLA_2$ associated with the immune response. It also suggests that more than

one type of PLA_2 inhibitors may be synthesized by *X. nematophila* and released into culture broth. These molecules need to be identified in order to understand pathogenic interaction between *X. nematophila* and insect immune system.

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References

Akhurst, R.J. 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *J. Gen. Microbiol*. 128, 3061-3065.

Boemare, N., A. Givaudan, M. Brehelin, and C. Laumond. 1997. Symbiosis and pathogenicity of nematode-bacterium complexes. *Symbiosis* 22, 21-45.

Bonneton, F., A. Chaumot, and V. Laudet. 2008. Annotation of *Tribolium* nuclear receptors reveals an increase in evolutionary rate of a network controlling the ecdysone cascade. *Insect Biochem. Mol. Biol*. 38, 416-429.

Burke, J.E. and E.A. Dennis. 2009. Phospholipase A_2 structure/function, mechanism and signaling. *J. Lipid Res*. 50, S237-S242.

Dunphy, G.B. and J.M. Webster. 1988. Interaction of *Xenorhabdus nematophilus* subsp. *Nematophilus* with the haemolymph of *Galleria mellonella*. *Int. J. Parasitol*. 30, 883-889.

Dunphy, G.B. and J.M. Webster. 1991. Antihemocytic surface components of *Xenorhabdus nematophilus* var. Dutki and their modification by serum of non-immune larvae of *Galleria mellonella*. *J. Invertebr. Pathol*. 58, 40-51.

Eleftherianos, I., P.J. Milichap, R.H. ffrench-Constant, and S.E. Reynolds. 2006. RNAi suppression of recognition protein mediated immune response in tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. *Dev. Comp. Immunol*. 30, 1099-1107.

Gadelhak, G.G., V.K. Pedibhotla, and D.W. Stanley-Samuelson. 1995. Eicosanoid biosynthesis by hemocytes from tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol*. 25, 743-749.

Gillespie, J.P., M.R. Kanost, and T. Trenczek. 1997. Biological mediators of insect immunity. *Annu. Rev. Entomol*. 42, 611-643.

Ji, D., Y. Yi, G.H. Kang, Y.H. Choi, P. Kim, N.I. Baek, and Y. Kim. 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. *FEMS Microbiol. Lett*. 239, 241-248.

Kaya, H.K. 1990. Soil Ecology, p. 215-231. *In* R. Gaugler and H.K. Kaya (eds.) Entomopathogenic nematodes in biological control. CRC, Boca Raton, Florida, USA.

Khandelwal, P. and N. Benerjee-Bhatnagar. 2003. Insecticidal activity associated with the outer membrane vesicles of *Xenorhabdus nematophila*. *Appl. Environ. Microbiol*. 69, 2032-2037.

Kim, Y., D. Ji, S. Cho, and Y. Park. 2005. Two groups of entomopathogenic bacteria, *Photorhabdus* and *Xenorhabdus*, share an inhibitory action against phospholipase A_2 to induce host immunodepression. *Arch. Insect Biochem. Physiol*. 59, 230-244.

Kuchler, K., M. Gmachl, M.J. Sippl, and G. Kreil. 1989. Analysis of the cDNA for phospholipase A_2 from honeybee venom glands. The deduced amino acid sequence reveals homology to the corresponding vertebrate enzymes. *Eur. J. Biochem*. 184, 249-254.

Kwon, H.S., D.W. Stanley, and J.S. Miller. 2007. Bacterial challenge and eicosanoid act in plasmatocyte spreading. *Entomol. Exp. Appl.* 124, 285-292.

Lavine, M.D. and M.R. Strand. 2002. Insect hemocytes and their roles in immunity. *Insect Biochem. Mol. Biol*. 32, 1295-1309.

McInerney, B.V., R.P. Gregson, M.J. Lacey, R.J. Akhurst, G.R. Lyons, S.H. Rhodes, D.R.J. Smith, L.M. Engelhardt, and A.H. White. 1991. Biologically active metabolites from *Xenorhabdus* spp. Part 2. Benzopyran-1-one derivatives with gastroprotective activity. *J. Nat. Prod*. 54, 785-795.

Morgan, J.A.W., M. Sergent, D. Ellis, M. Ousley, and P. Jarrentt. 2001. Sequence analysis of insecticidal genes from *Xenorhabdus nematophilus* PMF1296. *Appl. Environ. Microbiol*. 67, 2062-2069.

Morishima, I., Y. Yamano, K. Inoue, and N. Matsuo. 1997. Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*. *FEBS Lett*. 419, 83-86.

Park, Y., J. Aikins, L.J. Wang, R.W. Beeman, B. Oppert, J.C. Lord, S.J. Brown, M.D. Lorenzen, S. Richards, G.M. Weinstock, and R.A. Gibbs. 2008. Analysis of transcriptome data in the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol*. 38, 380-386.

Park, Y., A.R.N. Aliza, and A. Stanley. 2005. A secretory PLA_2 associated with tobacco hornworm hemocyte membrane preparations acts in cellular immune reactions. *Arch. Insect Biochem. Physiol*. 60, 105-115.

Park, Y., E.E. Herbert, C.E. Cowles, K.N. Cowles, M.L. Menard, S.S. Orchard, and H. Goodrich-Blair. 2006. Clonal variation in *Xenorhabdus nematophila* virulence and suppression of *Manduca sexta* immunity. *Cell Microbiol*. 9, 645-656.

Park, Y. and Y. Kim. 2000. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. *J. Insect Physiol*. 46, 1469-1476.

Park, Y. and Y. Kim. 2003. *Xenorhabdus nematophila* inhibits *p*-bromophenacyl bromide (BPB)-sensitive PLA_2 of *Spodoptera exigua*. *Arch. Insect Biochem. Physiol*. 54, 134-142.

Park, Y., Y. Kim, H. Tunaz, and D.W. Stanley. 2004. An entomopathogenic bacteria, *Xenorhabdus nematophila*, inhibits hemocytic phospholipase A_2 (PLA_2) in tobacco hornworm, *Manduca sexta*. *J. Invertebr. Pathol*. 86, 65-71.

Paul, V.J., S. Frautschy, W. Fenical, and K.H. Nealson. 1981. Antibiotics in microbial ecology, isolation and structure assignment of several new antibacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* spp. *J. Chem. Ecol*. 7, 589-597.

Radvanyi, F., L. Jordan, F. Russo-Marie, and C. Bon. 1989. A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. *Annu. Biochem*. 177, 103-109.

Ratcliffe, N.A., J.L. Brookman, and J.F. Rowley. 1991. Activation of the phonoloxidase cascade and initiation of nodule formation in locust by bacterial polysaccharides. *Dev. Comp. Immunol*. 5, 33-40.

Ryu, Y., Y. Oh, J. Yoon, W. Cho, and K. Baek. 2003. Molecular characterization of a gene encoding the *Drosophila melanogaster* phospholipase A_2. *Biochem. Biophys. Acta* 31, 4654-4662.

SAS Institute, Inc. 1989. SAS/STAT User's Guide, release 6.03 ed. SAS Institute, Cary, NC, USA.

Schaloske, R.H. and E.A. Dennis. 2006. The phospholipase A_2 superfamily and its group numbering system. *Biochem. Biophys. Acta* 1761, 1246-1259.

Schröder, R. 2003. The genes *orthodenticle* and *hunchback* substitute for *bicodal* in the beetle, *Tribolium*. *Nature* 422, 621-625.

Sergent, M., P. Jarett, M. Ousley, and J.A.W. Morgan. 2003. Interactions of insecticidal toxin gene products from *Xenorhabdus nematophila* PMF1296. *Appl. Environ. Microbiol*. 69, 3344-3349.

Shrestha, S. 2008. MS thesis. Andong National University, Andong,

Korea.

Shrestha, S. and Y. Kim. 2007a. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits hemocyte phagocytosis of *Spodoptera exigua* by inhibiting phospholipase A_2. *J. Invertebr. Pathol*. 96, 64-70.

Shrestha, S. and Y. Kim. 2007b. Factors affecting the activation of hemolymph prophenoloxidase of *Spodoptera exigua* (Lepidoptera: Noctuidae). *J. Asia-Pacific Entomol.* 10, 131-135.

Shrestha, S. and Y. Kim. 2008. Eicosanoid mediates prophenoloxidase release from oenocytoids in the beet armyworm, *Spodoptera exigua*. *Insect Biochem. Mol. Biol*. 38, 99-112.

Stanley, D.W. 2005. Eicosanoids, p. 307-339. *In* L.I. Gilbert, K. Latrou, and S. Gill (eds.). Comprehensive Molecular Insect Science. 4th ed. Elsevier, Oxford, UK.

Stanley, D.W. 2006a. Prostaglandins and other eicosanoids in insects: biological significance. *Annu. Rev. Entomol*. 51, 25-44.

Stanley, D.W. 2006b. The non-venom insect phospholipase A_2. *Biochem. Biophys. Acta* 1761, 1383-1390.

Stanley, D.W., W.W. Hoback, J.C. Bedick, H. Tunaz, R.L. Rana, A.R.N. Aliza, and J.S. Miller. 1999. Eicosanoid mediate nodulation reactions to bacterial infections in larvae of the butterfly, *Colias eurytheme*. *Comp. Biochem. Physiol*. *C* 123, 217-223.

Stanley-Samuelson, D.W. and C.L. Ogg. 1994. Prostaglandin biosynthesis by fat body from the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol*. 24, 481-491.

Sundar, L. and F.N. Chang. 1993. Antimicrobial activity and biosynthesis of indole antibiotics produced by *Xenorhabdus nematophilus*. *J. Gen. Microbiol*. 129, 3139-3149.

Tan, A. and S.R. Palli. 2008. Identification and characterization of nuclear receptors from the red flour beetle, *Tribolium castaneum*. *Insect Biochem. Mol. Biol*. 38, 430-439.

Tribolium Genome Sequencing Consortium. 2008. The genome of the model beetle and pest *Tribolium castaneum*. *Nature* 452, 949-955.

Vivas, E.I. and H. Goodrich-Blair. 2001. *Xenorhabdus nematophilus* as a model for host-bacterium interactions: *rpoS* is necessary for mutualism with nematodes. *J. Bacteriol*. 183, 4687-4693.

Yajima, M., M. Takada, N. Takahashi, H. Kikuchi, S. Natori, Y. Oshima, and S. Kurata. 2003. A newly established *in vitro* culture using transgenic *Drosophila* reveals functional coupling between the phospholipase A_2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (Imd) pathway in insect immunity. *J. Biochem*. 371, 205-210.