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A preliminary study of the anti-inflammatory activities of the Japanese oak silk moth, *Antheraea yamamai*

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Abstract

The present study aimed to determine whether a hemolymph prepared from *Antheraea yamamai* larvae had the same biological activities using a *Bombyx mori* hemolymph prior to exposure to lipopolysaccharide (LPS) in order to induce an inflammatory response. The effects of the hemolymph were determined using a reverse transcription-quantitative polymerase chain reaction to assess the expression of pro-inflammatory molecules. The *A. yamamai* hemolymph exerted anti-inflammatory effects on LPS-activated human monocytic leukemia cells via Toll-like receptor (TLR) 4-mediated suppression, similar to the *B. mori* hemocyte extract. Treatment with the *A. yamamai* hemolymph significantly suppressed LPS-induced upregulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression at all tested concentrations compared with the control, similar to the *B. mori* immune-challenged hemolymph. Finally, the *A. yamamai* hemolymph, like the *B. mori* immune-challenged hemolymph, suppressed all of these concentrations in a dose-independent manner. These results demonstrate that the hemolymph of *A. yamamai* exhibited important biologically active substances. Further in-depth functional studies are required to fully understand the mechanisms underlying the biological activities of wild-type silkworm hemolymphs.

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Introduction

Traditionally, insects have been used by humans as food and oriental medicines for many years (Kim *et al*., 2019). Silk fiber is produced by two general insect groups: domestic (*Bombyx mori*) and wild-type (e.g., *Antheraea yamamai*). Around 5,000 to 10,000 years ago, *B. mori* was considered to have been domesticated from the wild-type silkworm (*Bombyx mandarina*) (Kim *et al*., 2017; Kwak *et al*., 2015; Reddy *et al*., 2012). *A. yamamai*, also known as the Japanese oak silk moth, is a wild species of silk moth (Kim *et al*., 2018). Unlike *A. yamamai*, which feeds on oak leaves, *B. mori* feeds on mulberry leaves. *A. yamamai* silk fiber has different characteristics when compared with common silk produced from the *B. mori*, such as thickness, compressive elasticity, and chemical resistance (Kim *et al*., 2018), and has been used as a valuable textile fiber due to its unique luster and color for more than 4,000 years (Jo *et al*., 2017). These unique characteristics have led to its use in biomedical science, and both the scientific and

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economic importance of the wild silk moth continues to gradually increase (Kim *et al*., 2018).

We previously published several studies using hemolymphs from normal and immune-challenged *B. mori*. Its hemocyte extract demonstrated anti-inflammatory effects via TLR4-mediated suppression of the nuclear factor-κB (NF-κB) signaling pathway (Kim *et al*., 2017). Immune-challenged hemolymph with *Lactobacillus* cell wall extracts were shown to cause antibacterial and anti-inflammatory activities on human phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 cells (Kim *et al*., 2019) and antioxidant activity on the human epithelial Caco-2 cell line (Kim *et al*., 2020). *A. yamamai* is native to Japan, however, it is also distributed throughout Korea, China and Taiwan (Nahirnic and Beshkov, 2015). This species was also introduced to Europe for silk production (Nahirnic and Beshkov, 2015). We have studied the general characteristics of its silk fiber in depth (Jo *et al*., 2017; Kweon and Park, 1994; Lee *et al*., 2015), however, sufficient functional data for the hemolymph of *A. yamamai* is still lacking.

In this present study, we examined the effects of the *A. yamamai* hemolymph on THP-1 cells and its anti-inflammatory activities. *A. yamamai* silkworm hemolymphs may have practical application as functional foods and preventive medicines. Our findings provide important insights into the application of *A. yamamai* as anti-inflammatory agents.

Materials and Methods

Silkworm collection and cell culture

The larvae of *A. yamamai* used in this study are preserved at the National Institute of Agricultural Sciences (NIAS), Republic of Korea. The insects were reared on an oak tree greenhouse to protect them from birds. The last stage larvae were obtained and then dissected to collect the hemolymph extracts. The samples were immediately frozen and stored in liquid nitrogen. The extracted samples were freeze-dried using an FD-1 freeze dryer (LABOGEN, Seoul, Korea) and stored at -70°C deep freezer until further use. The human monocyte cell line THP-1 was supplied by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics (all from WELGENE, Daegu, Korea) incubated at 37°C in a humidified 5% CO_2 atmosphere. For differentiation into macrophages, THP-1 cells were treated with 100 nM PMA (Sigma, Merck, Darmstadt, Germany) for 72 h. Following differentiation, unattached cells were removed by aspiration.

Treatment with LPS and freeze-dried hemolymph

THP-1 cells were pre-treated for 2 h in serum-free medium with freeze-dried hemolymph extract and then incubated with LPS (1 μg/ml) for 4 h for mRNA expression. At each time point, total RNAs were isolated from the cultured THP-1 cells.

cDNA synthesis and RT-qPCR

Total RNAs were purified from cultured cells using *TRIzo*l reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. For first-strand cDNA synthesis, 2 μg total RNAs were transcribed to cDNA using a reverse-transcription system with oligo-dT primer according to the manufacturer's protocol. Reverse transcription-quantitative polymerase chain reaction was performed on a StepOnePlus Real-Time PCR system with Power SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed with 1 μl cDNA in a 20 μl reaction mixture comprising 10 μl Power SYBR Green PCR Master Mix, 2 μl primers and 7 μl PCR-grade water. The PCR program was as follows: A denaturation step at 95˚C for 10 min, 40 cycles each of 95˚C for 15 sec and 60˚C for 1 min. Quantification of gene expression data was calculated using the $2^{-\Delta\Delta Cq}$ method the crossing point of the target genes with β-actin was calculated using the formula $2^{-(\text{target gene-}\beta\text{-actin})}$ and the relative amounts were quantified (Kim *et al*., 2017). The sequences of the gene-specific primers used (Bioneer Co, Daejeon, Korea) are listed in Table 1 (Kim *et al*., 2017).

Statistical analysis

Data are expressed as the mean ± standard error. Data were compared by one-way analysis of variance and Tukey's post hoc test. Statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table 1. Primer pairs used in qPCR\

Name	Sequences	Product Length (bp)
TLR2	5'-TCTCCCATTTCCGTCTTTTT-3' 5'-GGTCTTGGTGTTCATTATCTTC-3'	125
TLR4	5'-GAAGCTGGTGGCTGTGGA-3' 5'-TGATGTAGAACCCGCAAG-3'	213
iNOS	5'-TGGATGCAACCCCATTGTC-3' 5'-CCCGCTGCCCCAGTTT-3'	59
COX-2	5'-CAAATCCTTGCTGTTCCCACCCAT-3' 5'-GTGCACTGTGTTTGGAGTGGGTTT-3'	173
IL-1ß	5'-TGATGGCTTATTACAGTGGCAATG-3' 5'-GTAGTGGTGGTCGGAGATTCG-3'	140
IL-6	5'-GTGTTGCCTGCTGCCTTC-3' 5'-AGTGCCTCTTTGCTGCTTTC-3'	194
IL-8	5'-GACATACTCCAAACCTTTCCAC-3' 5'-CTTCTCCACAACCCTCTGC-3'	160
TNF-α	5'-ATCTTCTCGAACCCCGAGTG-3' 5'-GGGTTTGCTACAACATGGGC-3'	51
ß-actin	5'-GCGAGAAGATGACCCAGATC-3' 5'-GGATAGCACAGCCTGGATAG-3'	77

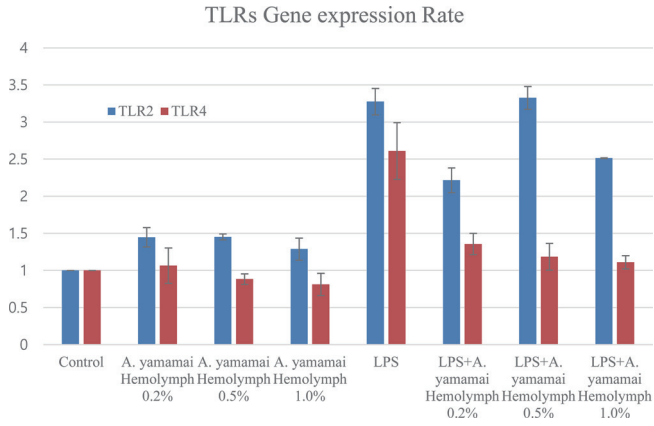

Fig. 1. Effects of the *A. yamamai* hemolymph on the LPS-induced increase in TLR mRNA expression in THP-1 cells. Cells were incubated for 2 h in serum-free medium containing different ratios of hemolymphs (0, 0.2, 0.5, and 1.0%) and were either left unstimulated or were stimulated with LPS (1 μg/ml) for 4 h. The mRNA levels of TLR2 and TLR4 were measured by RT-qPCR and normalized to β-actin. The data represent the mean ± standard deviation (SD) values of three independent samples. *$P < 0.05$, **$P < 0.01$ compared to stimulation with LPS alone.

Results and Discussions

We previously demonstrated that the *B. mori* hemocyte extract exhibited anti-inflammatory effects (Kim *et al*., 2017). In addition, a *B. mori* immune-challenged hemolymph with *Lactobacillus* cell wall extracts also possessed antibacterial and anti-inflammatory activities (Kim *et al*., 2019). Recently, we reported that a *B. mori* immune-challenged hemolymph exhibited antioxidant activity in the human epithelial Caco-2 cell line (Kim *et al*., 2020). In these previous studies, we verified that domesticated *B. mori*, which fed on mulberry leaves, possessed the most important biologically active substances. However, knowledge on biologically active substances for the *A. yamamai* remain insufficient. We predict that *A. yamamai* would have more important biologically active substances because this species grows outdoors and feeds on oak leaves. Further, *A. yamamai* is a wild-type insect frequently infected with outside microorganisms. We consider this to be the same effect compared with artificial immune-challenged hemolymph with Lactobacillus cell wall extracts. Therefore, we tested our hypothesis by studying the physiological activity effect for biologically active substances in *A. yamamai*.

TLRs play an important role in microbial detection and subsequently induce the release of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-12, and tumor necrosis factor-α (TNF-α) (Kim *et al*., 2019). To investigate the anti-inflammatory activity of the *A. yamamai* hemolymph, we measured TLR2 and TLR4 mRNA expression rate using RT-qPCR in THP-1 cells. THP-1 cells were treated with 100 nM PMA to cause differentiation to macrophages. Following LPS stimulation, TLRs mRNA expressions were compared in *A. yamamai* hemolymph using a dose-dependent manner. As shown in Fig. 1, the *B. mori* hemolymph suppressed the LPS-induced increase in the mRNA expression of TLR4 but not of TLR2. In previous data we also showed that the *B. mori* hemocyte extract exerted anti-inflammatory effects on LPS-activated human monocytic leukemia cells via TLR4-mediated suppression (Kim *et al*., 2017). In particular, the *B. mori* immune-challenged hemolymph suppressed the LPS-induced increase in TLR2 and TLR4 mRNA expression, unlike the unchallenged hemolymph (Kim *et al*., 2019). *A. yamamai* grows outside unlike *B. mori*, and wild-type insects have a higher risk of infection than domesticated insects. Therefore, we expected the *A. yamamai* hemolymph to possess the same effects as the *B. mori* immune-challenged hemolymph. However, further investigation for the defense mechanism of *A. yamamai* growing outdoors is required.

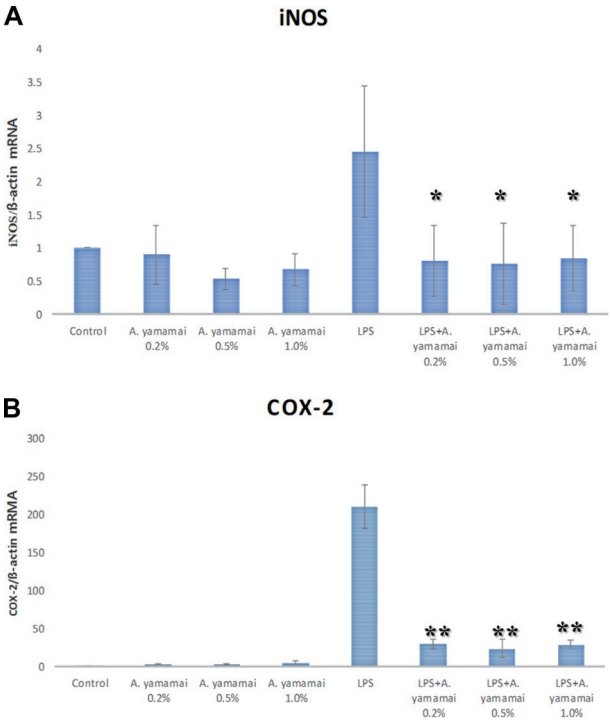

Fig. 2. Effects of the *A. yamamai* hemolymph on the expression of iNOS and COX-2 genes in THP-1 cells. Cells were cultured with different ratios of hemolymph for 2 h and were then either left unstimulated or were stimulated with LPS (1 μg/ml) for 4 h (for mRNA expression). iNOS (A) and COX-2 (B) mRNA levels were measured by RT-qPCR and normalized to β-actin. The data is expressed as the normalized expression ratio and represent the mean ± SD values of three independent samples. **P* < 0.05, ***P* < 0.01 compared to stimulation with LPS alone.

To compare the functional effect, we examined the effects of the *A. yamamai* hemolymph on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression levels. THP-1 cells were treated with various concentrations of hemolymph and LPS. Next, iNOS and COX-2 mRNA expression levels were measured using RT-qPCR. Treatment with the hemocyte extract significantly suppressed LPS-induced upregulated iNOS and COX-2 (Fig. 2.A and B) mRNA expression at all tested concentrations compared with the control (P<0.05 and P<0.01, respectively) (Fig. 2). The *B. mori* hemocyte extract significantly suppressed only the iNOS mRNA expression level (Kim *et al*., 2017). However, *B. mori* immune-challenged hemolymph suppressed both (Kim *et al*., 2019). The same results were exhibited with the *A. yamamai* hemolymph.

To analyze whether the *A. yamamai* hemolymph also affects inflammatory cytokine mRNA expression level, we tested the transcription level of genes encoding inflammatory cytokines using RT-qPCR. As shown in Fig. 3, LPS treated cells increased cytokine mRNA expression level more than controls. *A. yamamai* hemolymph suppressed the LPS-induced increases in IL-6, TNF-α, IL-8, and IL-1β (Fig. 3.A-D) mRNA expression levels. For the *B. mori* hemocyte extract, mRNA expression level declined in all cytokine mRNA transcripts in a dose-dependent manner (Kim *et al*., 2017). However, *A. yamamai* hemolymph, similar to the *B. mori* immune-challenged hemolymph, suppressed all of these in a dose-independent manner (Kim *et al*., 2019).

In our previous full-genome sequencing study, we determined that *A. yamamai* possessed unique characteristics, making it valuable in various fields (Kim *et al*., 2018). However, it has not been completely domesticated like the *B. mori*, making artificial mass breeding of larvae infeasible. Understanding the functional effects of hemolymphs is also essential for mass production using biotechnology. Therefore, further in-depth functional studies are required to understand the mechanisms underlying the bioactive effects of the *A. yamamai* hemolymph.

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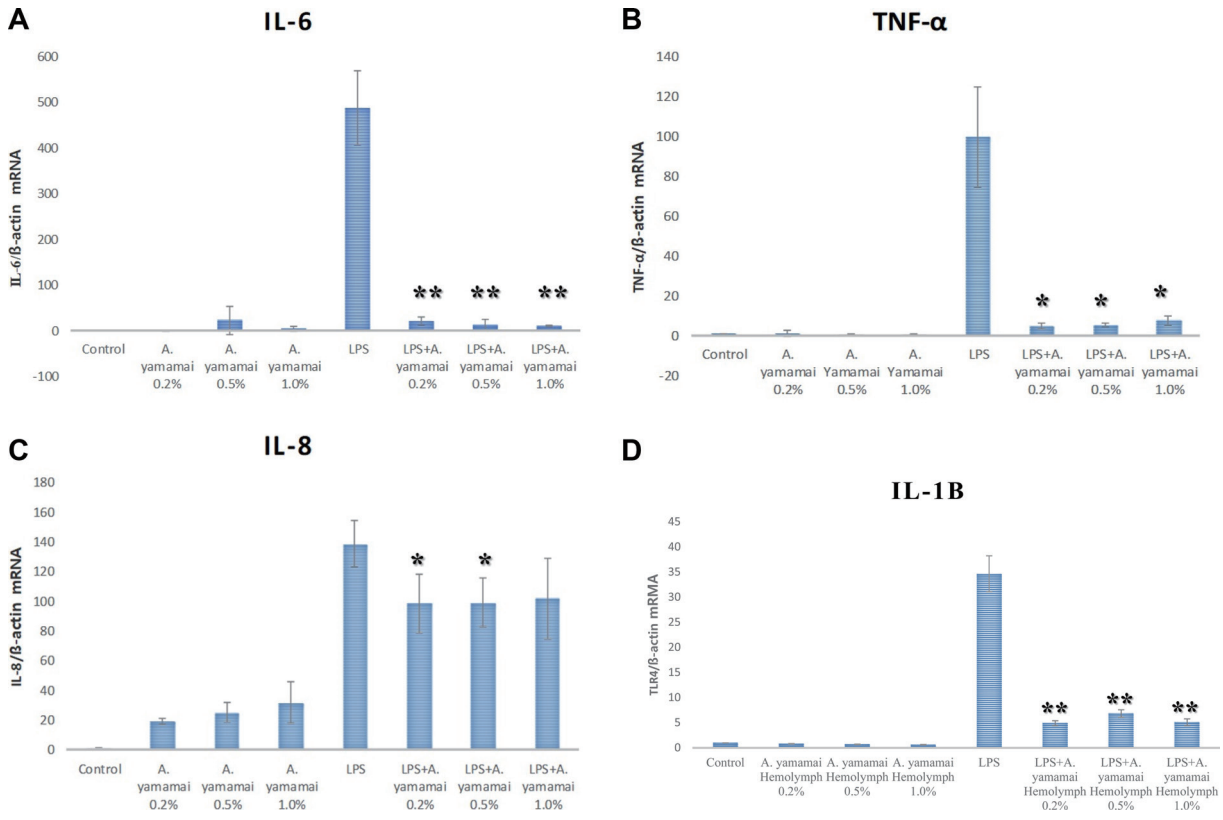

Fig. 3. Effects of the *A. yamamai* hemolymph on the LPS-induced increase in cytokine mRNA expression in THP-1 cells. Cells were treated for 2 h with hemolymph and were either left untreated or were treated with LPS (1 μg/ml) for 4 h. The mRNA levels of IL-6 (A), TNF-α (B), IL-8 (C), and IL-1β (D) were measured by RT-qPCR and normalized to β-actin. The data represent the mean ± SD values of three independent samples. *$P < 0.05$, **$P < 0.01$ compared to stimulation with LPS alone.

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