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Functional analysis of the antioxidant activity of immune-challenged *Bombyx mori* hemolymph extracts in the human epithelial Caco-2 cell line

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Abstract

Humans use insects as food and traditional medicine for many years. Hemolymph is the circulating fluid of insects and is a key component of their immune system. However, limited information is available regarding hemolymph identification, development, and differentiation, as well as the related cellular immune responses. In a previous study, hemolymph extracts prepared from *Bombyx mori* larvae were found to exert anti-inflammatory effects. In this study, we aimed to identify and compare the antioxidant activity of immune-challenged and unchallenged *B. mori* hemolymph extracts *in vitro*. For this purpose, human epithelial Caco-2 cells were first exposed to oxidative stress and then treated with various concentrations and incubation times of either immune-challenged or unchallenged *B. mori* hemolymph extracts. Next, we determined the effect of treatment on the relative expression of GPX-1, SOD-1, and SOD-2 antioxidant marker genes. We found that the expression rates of the three marker genes were markedly higher at a immune-challenged hemolymph extract concentration of 80 ppm compared to those at other concentrations, and the antioxidant effects were enhanced after treatment for 48 hr. Thus, *B. mori* hemolymph extracts showed antioxidant activity within the limited time and dose. Especially, the immune-challenged *B. mori* hemolymph extracts showed higher the antioxidant activities than unchallenged one. The activity of silkworm hemolymph extracts could facilitate the development of new types of functional foods, feed additives, and biomaterials with antioxidant properties.

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Introduction

To date, the traditional value of the mulberry silkworm, *Bombyx mori*, is its use as a source of silk. *B. mori* has been the most important commercial insect in the silk industry for thousands of years (Chen *et al.,* 1998; Künnapuu and Shimmi, 2010). However, its major role in the near future will most likely be that of a biomedical insect contributing to the production of biomedical proteins and biomaterials (Aono *et al.,* 1995; Künnapuu and Shimmi, 2010).

Insects constitute approximately 80% of the Earth. According to the Food and Agriculture Organization of the United Nations (FAO), insects are part of the common diet of at least two billion people worldwide (Mattia *et al.,* 2019; Van Huis *et al., 2013*).

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Recently, a new global interest in edible insects has emerged from the impellent necessity of preserving agricultural resources to feed the world's growing population, which is predicted to reach 9 billion by 2050 (Mason *et al.,* 2018; Mattia *et al.*, 2019; Pimentel and Pimentel, 2003). Insects provide a good alternative source of nutrient factors on a yearly basis.

Oxidative stress can be regarded as the excess generation of reactive oxygen species (ROS) (Felton and Summers, 1995; Oghenesuvwe and Paul, 2019; Pardini, 1995). ROS can cause oxidation of proteins, RNAs, DNAs, and peroxidation of membrane lipids, resulting in aging, as well as chronic diseases and cancer (Balakrishnan *et al.,* 2014; Baynes, 1991; Oghenesuvwe and Paul, 2019; Sosa *et al.,* 2013; Zhang *et al.,* 2016; Zielińska *et al.,* 2017). As defense mechanisms, organisms produce several endogenous antioxidants capable of scavenging these harmful free radicals, preventing an imbalance between pro- and anti-inflammatory status (Dhinaut *et al.,* 2017). However, under conditions of high oxidative stress, the ability of these antioxidants to eliminate free radicals is often exceeded, and therefore, dietary sources of antioxidants are required (Chew and Park, 2004). Antioxidant defense is primarily mediated by the enzymatic actions of glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (Barbehenn, 2002; Mittapalli *et al.,* 2007).

In our previous studies, we found that both *Lactobacillus plantarum* (*L. plantarum*) immune-challenged and unchallenged *B. mori* hemolymph extracts inhibit the mRNA expression of TLR4 and the induction of IL-1β, IL-6, IL-8, and TNF-α expression in response to lipopolysaccharide (LPS) in THP-1 cells. Treatment of phorbol myristate acetate (PMA)-differentiated THP-1 cells with *B. mori* hemolymph also inhibit the expression of genes encoding inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (Kim *et al.,* 2017; Kim *et al.,* 2019). In addition to the response to bodily injury, the inflammatory response is mainly associated with the host response to exogenous pathogens (Mookherjee *et al.* 2006; Munford and Pugin, 2001; Yoon *et al*., 2015). *B. mori* hemolymph has been shown to exert anti-inflammatory effects on LPS-induced THP-1 cells (Kim *et al.,* 2017; Kim *et al.,* 2019). Thus, *B. mori* hemolymph is expected to contain numerous hemolymph-derived bioactive peptides.

In this study, we aimed to identify and compare the antioxidant activity of immune-challenged and unchallenged *B. mori* hemolymphs *in vitro*. For this purpose, we analyzed the effect of hemolymph treatment on peroxide-induced cell damage. We used the Caco-2 cell line and studied the transcription profiles of GPX-1 and two SODs (SOD-1 and SOD-2) upon induction of oxidative stress followed by hemolymph treatment.

Materials and methods

Silkworm collection and cell culture

The three-days for the fifth instar larvae of *B. mori* (Baekokjam, Jam 123×Jam 124) used in this study are preserved at the National Institute of Agricultural Sciences (NIAS), Republic of Korea. The silkworms were reared on fresh mulberry leaves at 25°C, 65–75% relative humidity, using a 12-hr light:12-hr dark photoperiod. Fifth-instar larvae were obtained and then dissected to collect the hemolymph extracts. Insect immune-challenge manipulations were performed using general techniques described by Morath *et al.* (2001). Day 5 fifth instar larvae were used for immune challenge with *L. plantarum* cell wall extracts (Kim *et al.*, 2018). Fifty microliters of *L. plantarum* cell wall extracts dissolved in sterile saline solution was injected dorsolaterally into the hemocoel using 1 mL disposable syringes. The samples were immediately frozen and stored in liquid nitrogen. The extracted samples were freeze-dried using an FD-1 freeze dryer (EYELA, Tokyo, Japan) and stored at 4°C in a vacuum container until further use. The human epithelial cell line Caco-2 was supplied by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics (all from Gibco-BRL, Grand Island, NY, USA). For differentiation into macrophages, Caco-2 cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Treatment with H_2O_2 and hemolymph

Hydrogen peroxide (H_2O_2) was used as oxidant. To induce oxidative stress, H_2O_2 was prepared from a 30% stock solution. At 24 hr after seeding, Caco-2 cells were exposed to oxidative stress for 2 hr. H_2O_2 were diluted in DMEM with 10% FBS. Subsequent to treatment with H_2O_2, the cultures were rinsed twice with PBS and incubated in DMEM with 10% FBS. The oxidative stress induced Caco-2 cells were incubated with

hemolymph. We added various concentrations (0, 20, 40, 60, 80 ppm) of *B. mori* hemolymph extracts to check dose-dependence and different incubation time (24, 48, and 72 hr) of *B. mori* hemolymph extracts to check time-dependence of its activity. At each time point, total RNAs were isolated from the cultured Caco-2 cells.

cDNA synthesis and RT-PCR

Total RNAs were isolated from Caco-2 cells treatment with hemolymph using the TRIZOL reagent Solution (Life Technologies, Frederick, Maryland, USA) according to the manufacturer's instructions. The amounts of RNAs were measured spectrophotometrically by the absorbance of 260 nm. Total RNAs were treated with DNase I (Life Technologies) for 15 min at 37°C to remove the genomic DNA. After purification, oligo dT-primed cDNAs were made from total RNAs using the High-Capacity cDNA Archive kit (Applied Biosystems, CA, Foster city, CA, USA). The reaction was allowed to proceed for 2 hr at 37°C. To detect of antioxidant genes, RT-PCRs were performed using Top-Taq PreMix (CoreBioSystems, Korea) polymerase. Initial amplification using specific primer pairs were done with a denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and primer extension at 72°C for 1 min 20 sec. Upon completion of the cycling steps, a final extension at 72°C for 5 min was done and then the reaction was stored at 4°C. The primer sequences are shown in Table 1. To electrophoresis for evaluate quality of cDNA and antioxidant genes expression were used 1.0% and 2.0% agarose gel, respectively.

Table 1. Oligonucleotide PCR primers for antioxidant-specific and internal reference genes

Oligonucleotide name	Primer sequences
human GAPDH-F	5'- AGAAGGCTGGGGCTCATTTG-3'
human GAPDH -R	5'- AGGGGCCATCCACAGTCTTC-3'
GPx-1-F	5'- AACGATGTTGCCTGGAACTTTG -3'
GPx-1-R	5'- GAAGCGGCGGCTGTACCT -3'
SOD-1-F	5'- TGGTGTGGCCGATGTGTCTA -3'
SOD-1-R	5'- TTCATGGACCACCAGTGTGC -3'
SOD-2-F	5'- GCCTGCACTGAAGTTCAATGG-3'
SOD-2-R	5'- GCTTCCAGCAACTCCCCTTT -3'

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on a StepOnePlus Real-Time PCR system with Power SYBR Green PCR Master Mix (Takara, Japan). PCR was carried out using the following protocol: 40 cycles of denaturation at 95°C for 5 sec and annealing and elongation at 60°C for 35 sec. Fluorescence was detected at the end of every 60°C extension phase. Quantification of gene expression data was performed using the $2^{-\Delta\Delta Ct}$ method, and the crossing point of the target genes with *β-actin* was calculated using the formula $2^{-(\text{target gene}-\beta\text{-actin})}$ for quantification of relative expression. The sequences of the gene-specific primers used (Bioneer Corporation, Daejeon, Korea) are listed in a Table 1.

Results and Discussion

An initial control RT-PCR was performed on the isolated RNA using the human internal control primer pair to test for genomic DNA contamination in the RNA isolated from *B. mori* hemolymph extract-treated Caco-2 cells and assess the quality of the synthesized cDNA. We confirmed that the isolated RNA contained no genomic DNA and that the synthesized cDNA was suitable for RT-PCR analysis of the target genes. RT-PCR was used to examine target gene expression in total RNAs isolated from Caco-2 cells treated with different concentrations of immune-challenged and unchallenged *B. mori* hemolymph extracts. The qRT-PCR method was used to determine the expression profile of antioxidant target mRNA expression according to the type of hemolymph extract (i.e., immune-challenged or unchallenged). The expression levels of the three marker genes were normalized to those of the endogenous human GAPDH gene, which was used as an internal reference control gene. The C_T values obtained indicated differential expression. Specifically, we determined the relative expression of GPx-1 and two SODs (SOD-1and SOD-2) target mRNAs from Caco-2 cells treated with either immune-challenged or unchallenged hemolymph (data not shown).

To verify the antioxidant effects of the target cells, we used the quantitative real-time RT-PCR method. The expression levels of three antioxidant marker genes (SOD-1, SOD-2, and GPx1) were normalized to those of the endogenous human GAPDH gene, which was used as an internal reference. The

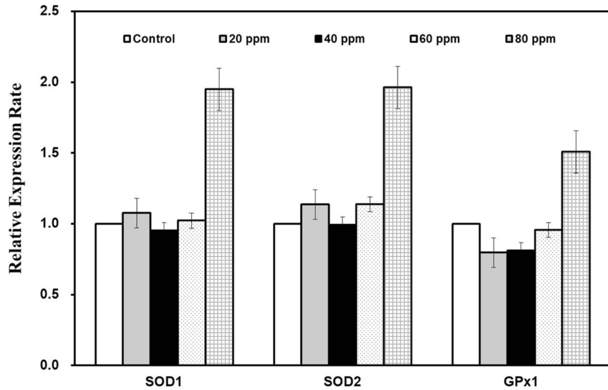

Fig. 1. Dose-dependent antioxidant activity. Hydrogen peroxide (H_2O_2) was used as the oxidant. Caco-2 cells were seeded for 24 hr, following which they were exposed to H_2O_2 diluted in DMEM with 10.0% FBS for 2 h. The oxidative stress-induced Caco-2 cells were then incubated with various concentrations (0, 20, 40, 60, and 80 ppm) of immune-challenged *B. mori* hemolymph extracts to assess dose dependence. All experiments were performed at least twice.

mRNA expression rate of each antioxidant marker gene isolated from the target cells was compared with that of the control cells. The C_T values for mRNA expression of SOD-1, SOD-2, and GPx1 indicated their expression in the antioxidant-specific markers that were examined. We added different concentrations of immune-challenged *B. mori* hemolymph extracts to assess the dose-dependence of its activity. Cells were treated with various concentrations (0, 20, 40, 60, and 80 ppm) of *B. mori* hemolymph extracts. As shown in Fig. 1, *B. mori* hemolymph extracts showed antioxidant activity, reaching its maximum activity at the highest concentration used (80 ppm).

Time-dependent analysis of the antioxidant activity was performed based on specific incubation times (0, 24, 48, and 72 hr), and all experiments were performed at least twice. The antioxidant effects of immune-challenged *B. mori* hemolymph extracts showed its maximum activity after 48 hr of treatment at this concentration and an enhancement of antioxidant gene expression by approximately 30% after 48 hr, at a minimum concentration of 20 ppm (Fig. 2).

In this study, we used immune-challenged hemolymph extracts derived from silkworm larva by injecting *L. plantarum* extracts dorsolaterally into the hemocoel. By comparing the antioxidant activity of hemolymph extracts from immune-challenged and unchallenged larvae, we measured the mRNA expression of these targets using RT-qPCR. Following H_2O_2 stimulation, immune-challenged *B. mori* hemolymph extracts suppressed the H_2O_2-induced increase in SOD-1and GPX-1 mRNA expression

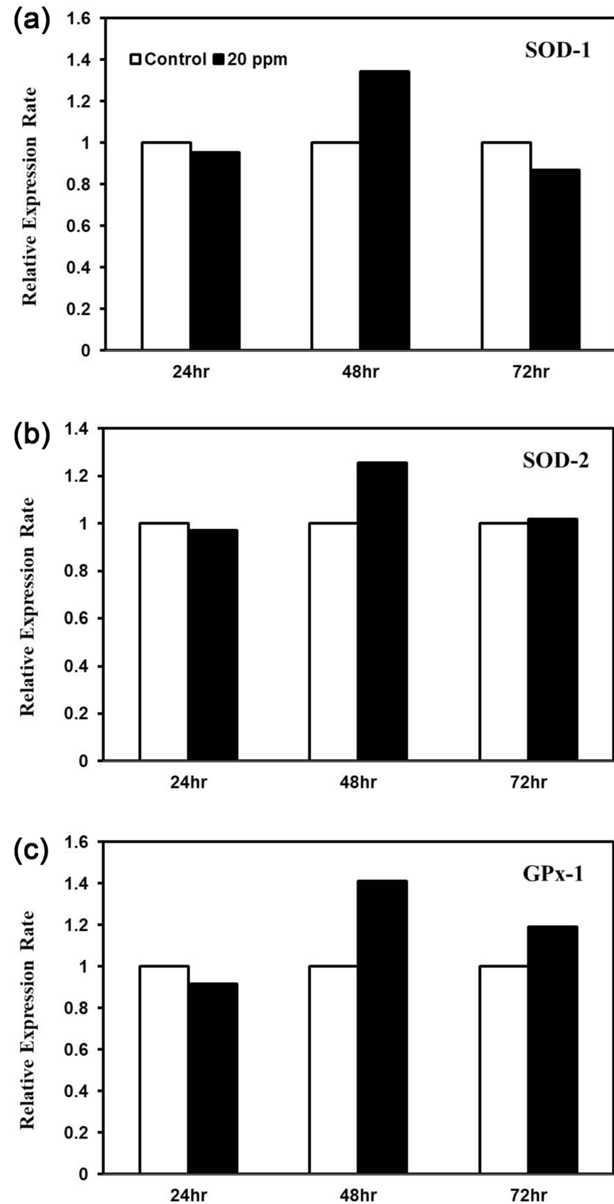

Fig. 2. Time-dependent antioxidant assay results. The oxidative stress-induced Caco-2 cells treated with H_2O_2 were incubated with immune-challenged *B. mori* hemolymph extracts for different lengths of time (24, 48, and 72 hr) to assess the time-dependence of the antioxidant activity. All experiments were performed at least twice.

(Fig. 3A and C). Contrary to our expectations, the antioxidant activity of both immune-challenged and unchallenged *B. mori* hemolymph extracts showed the similar activity in SOD-2 mRNA expression (Fig. 3B).

Insects are often consumed as a whole, and although some people still view entomophagy as a disgusting practice, it is becoming a popular human nutritional trend in many countries

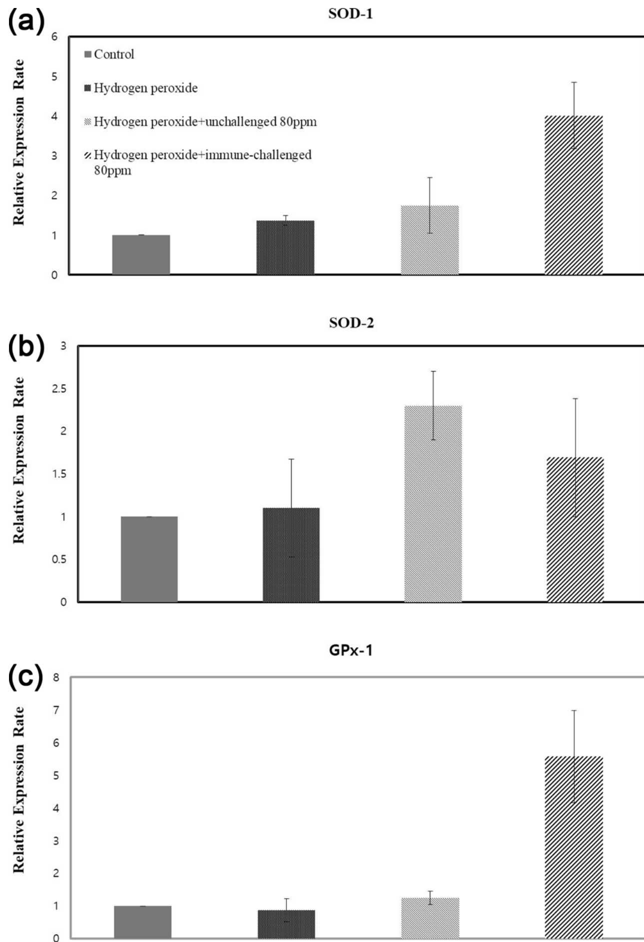

Fig. 3. Comparison of the antioxidant activity of immune-challenged and unchallenged *B. mori* hemolymph extracts. To compare the antioxidant activity of hemolymph extracts from immune-challenged and unchallenged larvae, we measured the mRNA expression of these targets using RT-qPCR. All experiments were performed at least twice.

(Mattia *et al.,* 2019). Consumption of antioxidant-rich foods, such as insects, plays an important role in the prevention of oxidative stress-related diseases, including cardiovascular disease, diabetes, and cancer (Mattia *et al.,* 2019; Magrone *et al.,* 2013). The *in vivo* efficiency of antioxidant-rich food is highly dependent on its bioavailability and the presence of an ongoing oxidative stress (Lettieri-Barbato *et al.,* 2013; Mattia *et al.,* 2019; Serafini *et al.,* 2003; Serafini *et al.,* 2011). The antioxidant defense is primarily mediated by the enzymatic actions of GPX, CAT, SOD, and ascorbate peroxidase (Barbehenn, 2002; Mittapalli *et al.,* 2007). GPXs reduce H_2O_2 and hydroperoxides, thereby playing an important role in protecting biological membranes against oxygen toxicity. SODs are characterized by the presence of metal prosthetic groups and can be classified into two major families: SOD-1, located mainly in the cytosol, and SOD-2, which is found in the mitochondria (Landis and Tower, 2005; Mittapalli *et al.,* 2007). SOD converts O_2^- to molecular O_2 and H_2O_2 (Fridovich, 1978; Mittapalli *et al.,* 2007).

In conclusion, we examined here the dose- and time-dependent antioxidant activity of immune-challenged and unchallenged *B. mori* hemolymph extracts. We determined the relative expression of GPx-1 and two SOD target mRNAs from Caco-2 cells treated with either immune-challenged or unchallenged hemolymph. The expression rates of the three marker genes were considerably higher at the 80 ppm concentration of *B. mori* hemolymph extract compared to those at other concentrations, and the antioxidant gene expression was enhanced by approximately 30% after 48 hr of treatment at this concentration. And also, immune-challenged *B. mori* hemolymph extracts suppressed the H_2O_2-induced increase in SOD-1 and GPX-1 mRNA expression. However, SOD-2 mRNA expression rate in Caco-2 cells treated with either immune-challenged or unchallenged *B. mori* hemolymph extracts were not statistically significant. Further functional studies are required for an in-depth analysis of the antioxidant activity of the *B. mori* hemolymph extracts prepared in this study.

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