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Exosome isolation from hemolymph of white-spotted flower chafer, *Protaetia brevitarsis* (Kolbe) (Coleoptera: Scarabaeidae).

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Abstract

Exosomes are homogenous vesicles of 40-100 nm diameter produced endogenously. Exosomes are generated by inward budding into multi-vesicular bodies (MVB) and then released to extracellular space. Exosomes contain various nucleic acid and protein cargoes from their cells of origin and this endosomal cellular molecules are used for intracellular communication and for both promotion and suppression of immune responses. Recently, they are also considered as delivery vehicle for therapeutic proteins due to their characteristics of stability in body fluids and ability for target uptake. Also, they show less immune reactivity because the isolated exosome harboring therapeutic proteins can be from the same host. White-spotted flower chafer, *Protaetia brevitarsis* is one of the major insect commercially reared in Korea. There are bacterial and fungal pathogens causing diseases in the beetle, and these diseases incur economic loss to the larva-rearing farms. Due to their endosomal cargoes, exosomes are good candidates in use of disease diagnosis. In this study, we isolated insect exosome from the hemolymph of *P. brevitarsis*, and verified it by analysis of the exosome-specific surface proteins and RNA.

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Introduction

Exosome is one of the most highly studied extracellular vesicles, which is a homogenous vesicle of 40-100 nm diameter produced endogenously. Unlike other cellular organelles, exosomes do not reside within the cell but are released into the extracellular environment (Vlassov *et al*., 2012). By the time it was discovered from reticulocyte culture in 1983 by Pan and Johnstone, it was considered as waste product which will be removed from the cell, but in the past decade, the exosome researches have been increased exponentially. Exosomes are generated by inward budding into multi-vesicular bodies (MVB) and then released outside the cell, by fusion of MVB with plasma membrane or lysosome. (Kowal *et al*., 2014; Van Der Pol *et al*., 2012; Piper *et al*., 2007). Because of this inward budding, exosomes contain not only membrane proteins but also various important cellular components such as DNA, messenger

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RNA, microRNA, non-coding RNA, lipids, and proteins from their cells of origin (Sato-Kuwabara *et al*., 2015; Zhang *et al*., 2015; Koles *et al*., 2012). Through horizontal transfer of these cellular components, exosomes are deeply involved in intra and intercellular communication. Exosomes are secreted from almost all mammalian cell types including tumor cells, reticulocytes, dendritic cells, macrophages, B cells, T cells, mesenchymal stem cells, endothelial cells, and epithelial cells (Lai *et al.,* 2015; Skogberg *et al.,* 2015; Song *et al.,* 2014; Nolte *et al.,* 2009; Bhatnagar *et al.,* 2007; Clayton *et al.,* 2005; Thery *et al.,* 2002). Also they can be found in body fluid such as blood, urine, saliva, and breast milk (Mathivanan *et al*., 2010; Miranda *et al*., 2010; Simons *et al*., 2009). Exosomes contain endosomal proteins found in exosomal membrane, which are used as surface markers. These include proteins involved in membrane transport and exporting molecules such as annexins, RabGTPase, flotillin, alix and TSG101. Also there are heat shock protein, Hsp70 and Hsp90, and tetraspanins such as CD9, CD63, CD81, and CD82 (Belov *et al.,* 2016; Szatanek *et al.,* 2015; Lotval *et al.,* 2014; Pant *et al.,* 2012; Chaput *et al.,* 2012). These proteins are specifically enriched in exosomes and used as exosomal marker proteins. Exosome are also highly enriched in cholesterol, sphingomyelins, ceramide, and glycerophospholipids (Aalberts *et al*., 2013; Trajkovic *et al.,* 2008; Subra *et al*., 2007; Wubbolts *et al*., 2003). Because exosomes are fluent in almost all body fluids and contain cellular cargoes from their parent cells, they are considered as very promising biomarkers.

Although most exosome researches are focused on mammalian cells, plant-derived exosome-like vesicles are reported recently. Regente *et al*. isolated plant exosomes from sunflower seed in 2009, and observed a protein similar to Rab GTPase involved in vesicular trafficking and Wang et al. reported the exosome-like vesicle, EXPO (exosome positive organelle) from *Arabidopsis thaliana* and tobacco (Regente *et al*., 2012; Wang *et al*., 2010; Regente *et al*., 2009). Although their functions are not cleared as in mammal, same as in mammalian exosomes, these vesicles contain specific protein released into the extracellular environment for further functions. For plant exosome, many studies are related with interspecific communication, especially when attacked by pathogen. It was reported that exosome-like vesicles were secreted from barley and *A. thaliana* upon pathogen attack (Micali *et al*., 2011; An *et al*., 2007; An *et al*., 2006), and several defense-related molecules including a leaderless aspartyl protease and phospholipid phosphatidylinositol 4-phosphate were found in those vesicles (Gonorazky *et al*. 2012). Also, there are some reports that exosome-derived from plants communicates with mammalian cells. Exosome-like vesicles isolated from edible plants such as grape, grapefruit, ginger, and carrot were taken up by mouse intestinal stem cell after they were eaten by mouse, orally (Mu *et al*., 2014; Ju *et al*., 2013). Those edible fruits and vegetables are known for their abundant nutrients and phytochemicals, and their effects on various human diseases. Because exosomes can transfer their molecular cargoes to the recipient cell and play an important roles, this interspecific communication between edible plants and mammal may suggest a better therapeutic strategy.

Recently, exosome are also isolated from insects. In fruit fly, *Drosophila melanogaster*, insect exosome was isolated from cultured insect cell, and the signaling molecules inside are involved in intracellular communication (Koles and Budnik, 2012; Koles *et al.,* 2012; Koppen *et al.,*2011; Korkut *et al.,*2009). Plant-infecting *Rice dwarf virus* was detected in insect exosome isolated from cultured leafhopper cell (Wei *et al.,* 2009; Wei *et al.,* 2008). The virus engulfed in insect exosome was transported and released from the cell of insect vector.

In 2015, we isolated insect exosome from the hemolymph of Korean rhinoceros beetle, *Allomyrina dichotoma*. The exosome isolation was verified by the exosomal proteins analysis and exosomal RNA profiling (Kim *et al.,* 2015). The market size of Korean insect industry is rapidly increasing every year, and currently there are about 800 local farms are related in this business. White-spotted flower chafer, *Protaetia brevitarsis* is one of the strongest candidates for insect industry as medicinal purpose and recently it has been listed as a food ingredient by the Korean Ministry of Food and Drug Safety. In Korea, *P. brevitarsis* has been traditionally used as oriental medicine for various liver diseases and it is known as suppressing liver cancer. However, many larva-rearing farms are suffered by insect diseases occurring in *P. brevitarsis* larva. The typical diseases are white muscardine and green muscardine disease when infected by fungi *Metarhizium anisopliae* and *Beauveria bassiana*, and bacterial diseases caused by *Bacillus thuringiensis* and *Serratia marcescens*.

Therefore, early and accurate diagnosis of these diseases is extremely important for the local farmers. In this study we isolated exosome from the hemolymph of *P. brevitarsis* larva and verified the exosome isolation by analyzing exosomal proteins and RNA. Further study of identification of nucleic acids and amino acids from the pathogens may give a strong foundation for the development of a new diagnosis method or future exosome research in insects.

Materials and Methods

Experimental insect

Third-instars larvae of *P. brevitarsis*, were purchased from a commercial seller in Siheung-si, Korea. The beetles were reared at 25°C and 40% relative humidity.

Hemolymph preparation and exosome isolation

Hemolymph was extracted by clipping a leg of *P. brevitarsis* larva and collecting the hemolymph in a microtube. Ten μL of 10% propylthiouracil was added in the tube immediately as an anticoagulant. After that, 5 μL of thrombin (1000 u/mL) was mixed with 500 μL of hemolymph and the mixture was incubated for 5 min at 37°C. Then, the hemolymph was centrifuged at 20,000 x g for 10 min at 4°C, and the supernatant was transferred to a new tube. The supernatant was centrifuged again for three times at 20,000 x g for 30 min at 4°C, and after the last repeat, it was filtrated with 0.22 μm pore.

The filtered supernatant was then ultracentrifuged at 100,000 x g for 70 min at 4°C with Beckman Optinal LE-80K. The supernatant was discarded and 200 μL of phosphate-buffered saline (PBS) was added to exosome pellet. The pellet was votexed briefly and PBS was added fully into the tube. The suspension was ultracentrifuged again at 100,000 x g for 70 min at 4°C, and the pellet was resuspended in PBS. Exosome can be stored at -70°C or at 4°C for a month.

In case for the ultracentrifugation with sucrose, 2 mL of 30% sucrose in 20 mM Tris buffer, pH 8.6 was added in the ultracentrifuge tube, before adding slowly the filtered supernatant of hemolymph on top of sucrose solution. Then the tube was ultracentrifuged at 100,000 x g for 3 h at 4°C. Same as above, the exosome cushion was resuspended in PBS added fully into the tube. The suspension was ultracentrifuged again at 100,000 x g for 70 min at 4°C, and the pellet was resuspended in PBS added fully into the tube. This PBS wash step was repeated 3-4 times before final resuspension in PBS.

Exosome protein analysis

Western blotting analysis was performed as standard procedures. The isolated exosome was lysed by adding lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, protease inhibitor cocktail; Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO), and then boiled with 2 x sample buffer. The lysate solution was chilled on ice for 20 min and then centrifuged at 13,000 x g for 10 min at 4°C. After measuring the protein concentration, the supernatant was run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 100 V, and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% dried skim milk in 0.1% Tween 20 PBS (PBST) and incubated with primary antibodies for overnight at 4°C. Then, the membrane was washed three times with PBST and incubated with secondary antibodies. The exosome proteins attached with secondary antibodies was examined with enhanced chemiluminescence (ECL).

RNA isolation and analysis

Exosome RNA was prepared using the SeraMir Exosome RNA Amplification Kit (System Biosciences, Mountain View, CA), based on standard protocol. The lysis of exosome was conducted with 350 μL of lysis buffer by vortexing for 15 s, and then incubated for 5 min at room temperature. The lysate was put on a spin column and centrifuged at 13,000 x g for 1 min. The column was washed twice with 400 μL of wash buffer by centrifugation at 13,000 x g for 1 min. The column was centrifuged one more time without buffer at 13,000 x g for 2 min. Then, 30 μL of elution buffer was added to the column placed on an RNase-free tube. To soak the membrane with elution buffer, the column was centrifuged slowly at 2,000 x g for 2 min, and exosome RNA was eluted by centrifugation at 13,000 x g for 1 min. Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to analyze the electrophoresis and flow cytometry patterns of exosome RNA.

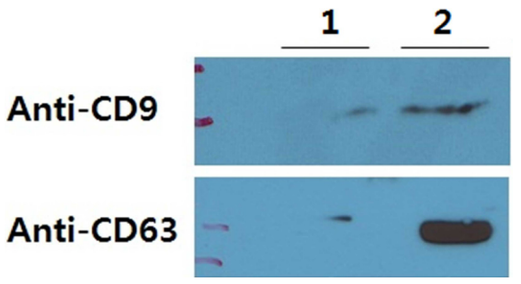

Fig. 1. Western blotting of the exosome-specific surface proteins, CD9 and CD63. In lane 1, the exosome-specific protein level in whole hemolymph is shown, and the protein level in isolated exosome is in lane 2. The exosome-specific protein CD9 and CD63 are more enriched in isolated exosome.

Results and Discussion

Insect exosome was isolated from the hemolymph of *P. brevitarsis* larva by ultracentrifugation method. To examine the presence of exosomal surface protein, exosome-specific tetraspanins CD9 and CD63 was used for Western blotting in this study. Also, exosomal RNA was detected by comparing RNA migration patterns using Bioanalyzer (Agilent Technologies). As shown in Fig. 1, the quantity difference of exosome-specific proteins CD9 and CD63 present in whole hemolymph and isolated exosome solution was compared. The exosome-specific tetraspanins were detected much higher in purified exosome solution than whole hemolymph sample, especially for CD63. This comparison result of exosomal protein level looks less significant than our previous report of exosome isolation from third-instars larva of *A. dichotoma*. It is because the size of *P. brevitarsis* larva is much smaller than *A. dichotoma*, and the quantity of extractable hemolymph of *A. dichotoma* is also ten to twenty times more than *P. brevitarsis* larva. Thus the isolation and purification of exosome from *P. brevitarsis* was much difficult and some of the exosomal proteins might have been degraded or lost during the process. Further study for another isolation method might be needed to improve the quality and quantity of the isolated exosome from *P. brevitarsis*.

The presence of exosomal RNA was also analyzed to confirm the successful exosome isolation from insect hemolymph. The RNA migration pattern was compared between whole hemolymph sample and isolated exosome by using Bioanalyzer in quality check mode. As shown in Fig. 2, the RNA migration patterns were clearly different between them. Also, their electrophoretic patterns of exosomal RNA corresponded to

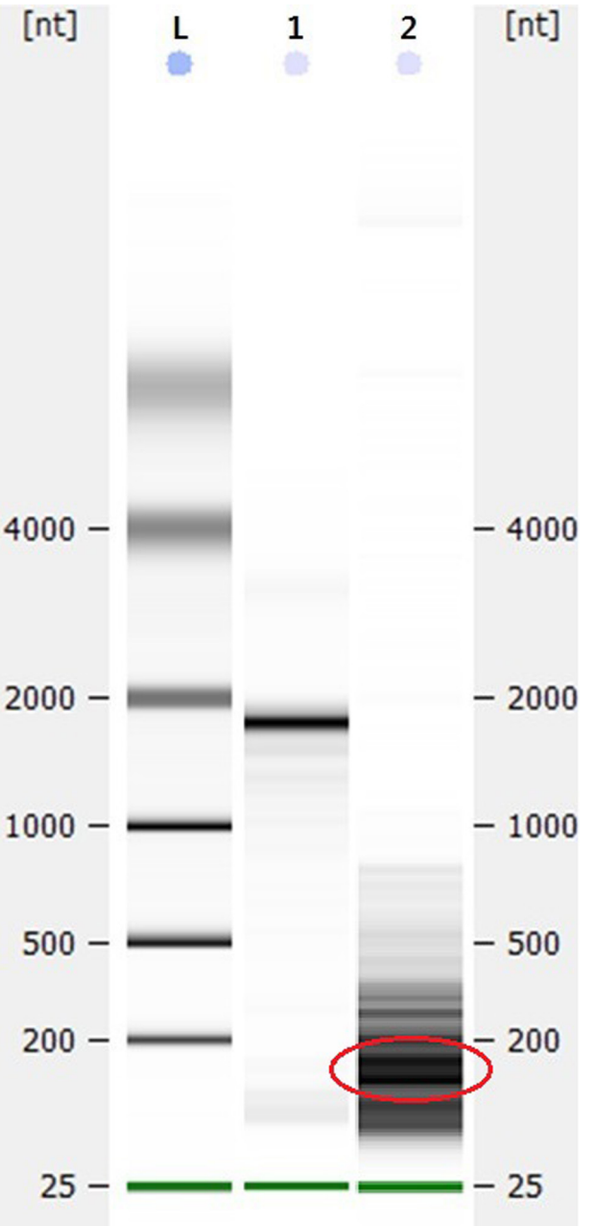

Fig. 2. Comparison of RNA migration pattern. The migration pattern of total RNA from whole hemolymph is shown in lane 1, and the exosomal RNA from isolated exosome is in lane 2. The short exosomal RNA fragments are indicated by red circle, while the thick band from whole hemolymph is approximately same size of combined 18s and 28s rRNA. L stands for the RNA marker.

the pattern of insect ribosomal RNA and exosomal RNA, as Winnebeck *et al*. and Palanisamy *et al*. showed in 2010. In Fig. 2, the migration pattern of total *P. brevitarsis* RNA was shown in the lane 1, whose thick band is approximately same size of combined 18s and 28s rRNA. This is a unique pattern of the insect RNA profile (Winnebeck *et al*., 2010). While, exosomal

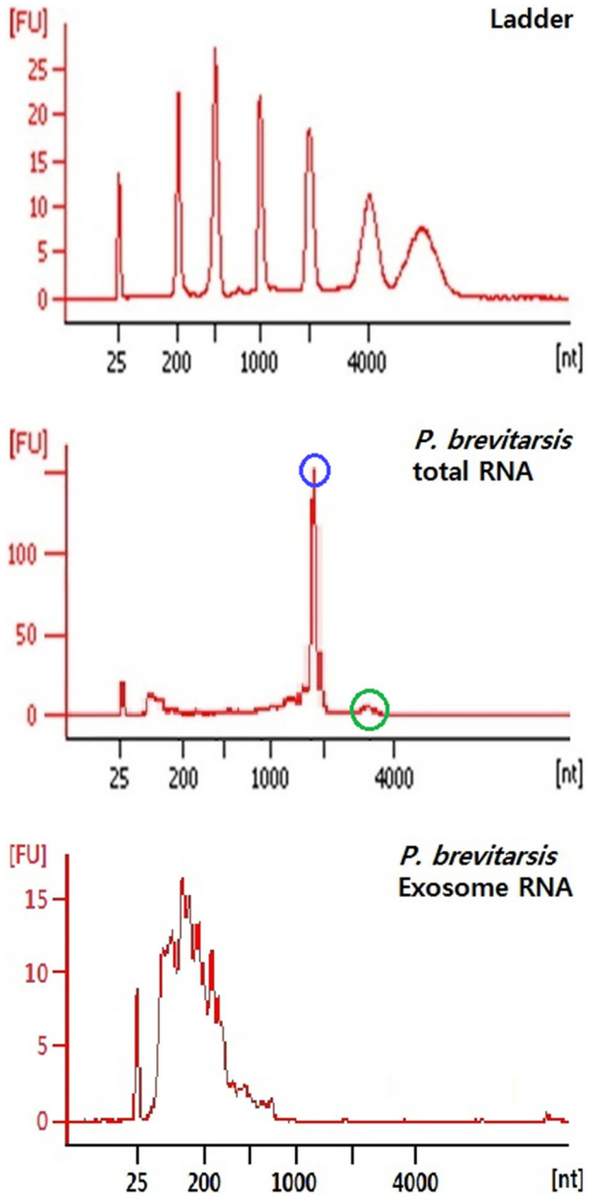

Fig. 3. Spectrum analysis of total RNA from whole hemolymph and exosomal RNA. Blue and green circles in *P. brevitarsis* total RNA indicates 18s and 28s rRNA, respectively, while in exosomal RNA, these RNA were removed during exosome isolation.

RNA was shown in short bands in lane 2, which are typical pattern of exosomal RNA. The spectrum analysis of total RNA of hemolymph and exosomal RNA was also performed. In total *P. brevitarsis* RNA, those 18s and 28s rRNA exists while in exosomal RNA, does not. Therefore, it was concluded the short exosomal RNA fragments indicate that insect exosome was successfully isolated from hemolymph of *P. brevitarsis* larva through ultracentrifugation.

Exosome isolated from insect hemolymph is an excellent candidate for diagnosis method with high accuracy, because exosomal nucleic acid and protein are derived both from the parent cells and pathogens. Besides, due to the characteristics of insects including a high reproducibility, short life cycle, easy manipulation with light regulation unlike animal or human research, exosome research with insect holds great promise for future.

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