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Research Article

Efficient Method for the Rapid Purification of *Nosema ceranae* Spores

Dong-Jun Kim, Hwi-Geon Yun, In-Hui Kim, Won-Seok Gwak and Soo-Dong Woo*

Department of Agricultural Biology, College of Agriculture, Life & Environment Science, Chungbuk National University, Cheongju 28644, Korea

Abstract *Nosema ceranae* is an obligate intracellular fungal parasite that causes mortality in honey bees and enhances the susceptibility of honey bees to other pathogens. Efficient purification of *Nosema* spores from the midgut of infected honey bees is very important because *Nosema* is non-culturable and only seasonably available. To achieve a higher yield of spores from honey bees, in this study, we considered that the initial release of spores from the midgut tissues was the most critical step. The use of 2 mm beads along with enzymatic treatment with collagenase and trypsin enhanced the homogenization of tissues and the yield of released spores by approximately 2.95 times compared with the use of common 3 mm beads alone. The optimal time for the enzyme treatment was determined to be 1 hr as measured by the yield and viability of the spores. A one-step filtration using a filter paper with an 8–11 μm pore size was sufficient for removing cell debris. This method may be useful to purify not only *N. ceranae* spores but also other *Nosema* spp. spores.

Keywords Honey bee, *Nosema ceranae*, Purification, Spore

Honey bees (Hymenoptera: Apidae) are an important pollinator to humans in both agricultural and wild ecosystems. However, a significant decline of honey bee populations has been observed over the last 50 years. The decline has been caused by a complex set of interacting stresses from pests, parasites, pesticides, and pathogens. Among these, honey bee disease has become more serious and has been suggested as one cause of colony collapse disorder [1]. Although there are many environmental stressors implicated in honey bee disease, there has been intensifying focus on the role of microbial infections, such as *Nosema apis* and *Nosema ceranae* belonging to the phylum Microsporidia Balbiani 1882, on honey bee health [2].

Microsporidia are eukaryotic, obligate intracellular fungal parasites that infect a wide range of vertebrates and invertebrates and cause economically important losses in animal species. To date, two microsporidian species are known to infect *Apis mellifera* worldwide. The microsporidian species *N. ceranae* and *N. apis* can cause individual mortality and enhance the susceptibility of honey bees to other pathogens [1]. Once *Nosema* spores are ingested by honey bees, the spores germinate in the midgut lumen, thereby extruding their infection apparatus, the polar tube [3]. The polar tube penetrates the host cell membrane, enabling the sporoplasm to be transferred into the host cell. Subsequently, the proliferation of the parasite in midgut epithelial cells begins, and characteristically spindle-shaped meronts, then sporonts, and finally new environmental spores are produced and released via cell lysis [4, 5]. Infection causes a shortened life span in adult bees and changes in bee behavior; heavily infected bees might develop dysentery (nosemosis), which might lead to death of the individual bee and rarely death of the entire colony [6, 7].

The only known reliable treatment for *Nosema* in honey bees is the antibiotic fumagillin, which is derived from *Aspergillus fumigatus* and has been widely used to treat colonies infected with *N. apis* since the 1950s [8, 9]. Although fumagillin can control *N. ceranae* and *N. apis*, its effect has been in doubt continuously and residual antibiotic in honey has become a serious problem. *N. ceranae* is the dominant microsporidium infecting honey bees in many countries [10-12].

One limitation of studying *Nosema* infections in honey bees is that the pathogens are non-culturable, and thus, artificially infecting honey bees with spores requires extracting

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*Corresponding author
E-mail: sdwoo@cbnu.ac.kr

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them from infected bees, which are only seasonably available, typically during the spring in temperate countries. A constant supply of viable and infective *N. ceranae* spores requires a reliable technology to purify spores from infected honey bees. The methods for preservation and purification of *Nosema* spores, however, have not been well established. The efficient purification of spores from honey bees is fundamental to the study of *Nosema* diseases. The purpose of purifying a spore suspension is to remove unwanted host tissues and microbial contaminants that may confound experimental data. A high purity of spores can facilitate accurate microscopic counting and is an important quality assurance parameter in pathological studies. The purification of *Nosema* spores is performed by either centrifugation or filtration of a spore suspension [5, 13, 14]. A method using both filtration and centrifugation was introduced to purify spores, but the yield of purified spores from honey bees was insufficient. In addition to the purity of the spores, the yield of spores is also very important because of the difficulties of *Nosema* production and storage. To enhance the yield of purified spores, therefore, we suggest the modified efficient method for the purification of *N. ceranae* spores from honey bees described herein.

MATERIALS AND METHODS

Purification of *Nosema* spores. *Nosema ceranae* spores were provided from the Sericulture and Apiculture Division of the Rural Development Administration, Republic of Korea. To produce infective spores for experiments, honey bees were held in plastic cages and inoculated with 10^6 spores of *N. ceranae* in a sucrose solution (50% v/v in water). To obtain purified *Nosema* spores, after 10 days, the midgut tissues of heavily infected honey bees were separated individually using forceps and washed in phosphate buffered saline [13]. The isolated midgut was crushed in 200 μL of sterile distilled water in a Bullet Blender Homogenizer (Scientific Instrument Services Inc., Ringoes, NJ, USA) set at speed 8 or 10 with 3 mm or 2 mm diameter tungsten carbide beads (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. The homogenate increased the volume up to 1 mL, which was filtered through a nylon cell strainer (SPL, Pocheon, Korea) using a 100 μm mesh diameter or an Qualitative filter paper No. 2 (Advantech MFS Inc., Dublin, Canada) with an 8–11 μm pore size to remove tissue debris. The filtered suspension was centrifuged at 400 ×g for 5 min, and the supernatant was discarded. The pellet was resuspended in 1 mL of sterile water, overlaid very gently on a discontinuous 25%, 50%, 75%, and 90% Percoll (Sigma-Aldrich) gradient from the top to the bottom of a 50 mL centrifuge tube and centrifuged twice at 15,000 ×g for 30 min at 20°C using a Mega21R ultracentrifuge angle rotor (Hanil Scientific Inc., Gimpo, Korea) [15]. The small but dense band just above the bottom of the tube was collected and resuspended in sterile distilled water. After a final centrifugation at 15,000 ×g for 10 min at 20°C, the spore pellet was resuspended in sterile distilled water and stored at room temperature (RT) until used. The spore concentration was determined by counting with a hemocytometer [16], and the suspension was freshly prepared before use. Five honey bees were used to each treatment. All experiments were performed in triplicate.

Enzyme treatment. The homogenization solution was prepared with a mixture of 0.5% (w/v) collagenase (Sigma-Aldrich) and 0.05% (w/v) trypsin (Sigma-Aldrich). An isolated midgut was crushed in 200 μL of enzyme solution. The homogenate was adjusted to a volume of 1 mL with enzyme solution and kept at RT for different periods of time. The number of spores released by the enzyme solution treatment was calculated using a hemocytometer. The effect of enzyme solution on the spores was evaluated using an *in vitro* germination assay. Each treatment was performed in triplicate.

***In vitro* germination assay.** Aliquots of purified spores (10 μL; 1×10^3 spores) were spotted into glass slide reaction cells (12 well; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and air dried at RT for 2 hr. Germination was triggered by the addition of 1.5 μL of 0.1 M sucrose in distilled water to the air-dried spores [17], a procedure that mimics the natural conditions for the germination of environmental spores. Covered glass slides were kept at RT for 6 hr, and then the germinated spores were observed under light microscopy (magnification, 400×; Nikon Instech Co., Ltd., Tokyo, Japan). Depending on the formation of polar tube, germination was determined. About two-hundred spores were observed randomly to each well. The germination rate was calculated as the percentage of the total observed spores that had germinated. Each assay was performed in triplicate.

RESULTS

Enhancement of initially released spore yield. The efficiency of the initial release of spores from the midgut tissue affects the final spore yield. The use of a smaller bead and enzyme treatments were introduced to enhance the release of *Nosema* spores from the honey bee midgut tissues. Use of 2 mm beads, instead of 3 mm, homogenized the midgut tissues more thoroughly and increased the yield of released spores from the midgut by approximately 1.2-fold (Fig. 1). To enhance the efficiency of homogenization and release, an enzyme solution consisting of collagenase and trypsin was prepared and used in the homogenization step. Enzyme treatment further increased the homogenization of tissues, and the yield of released spores was increased approximately 2.45 times compared with the untreated homogenate (Fig. 1). Although the enzyme treatment enhanced the yield of released spores, the enzymes may affect the viability of the *Nosema* spores. To determine the optimal time of enzyme treatment, the yield and viability

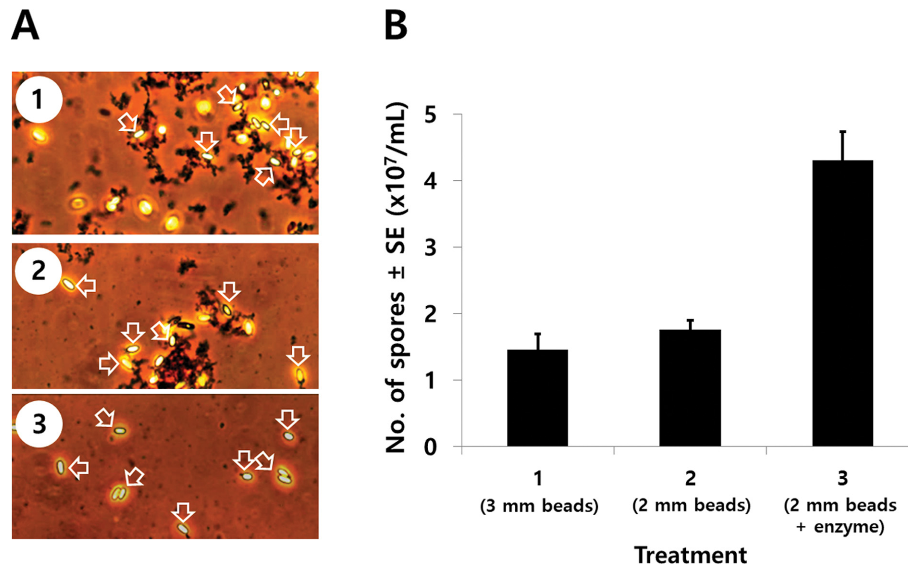

Fig. 1. Homogenized midgut epithelial cells (A) and released spore yield (B) from *Apis mellifera* infected with *Nosema ceranae*. Cells and debris were stained with trypan blue. Arrows indicate spores. Vertical bars correspond to standard error. 1, homogenization with 3 mm beads; 2, homogenization with 2 mm beads; 3, homogenization with 2 mm beads and enzyme treatment for 1 hr.

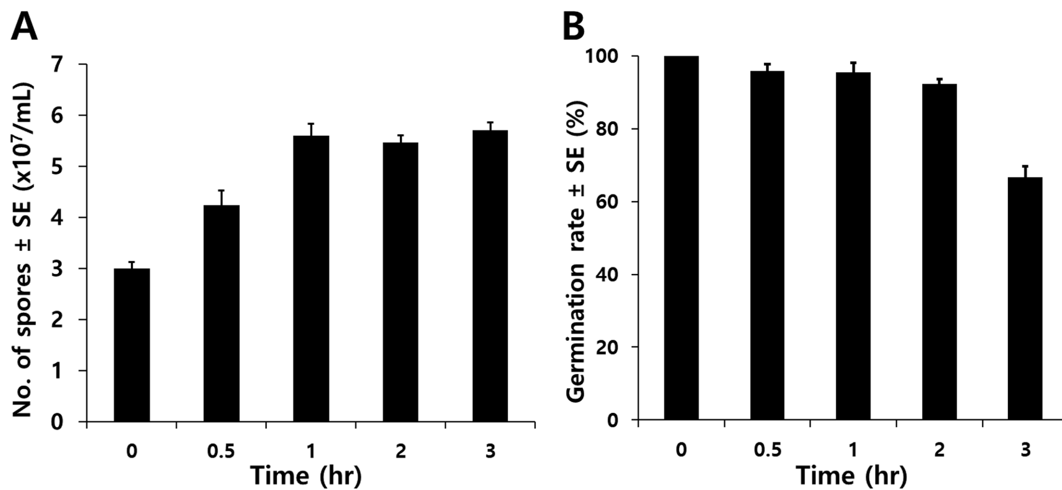

Fig. 2. Yield (A) and viability (B) of *Nosema ceranae* spores according to the enzyme treatment times. The homogenized midgut cells were treated with an enzyme solution consisting of collagenase and trypsin. The number of released spores was calculated using a hemocytometer. The viability of the spores was evaluated by an *in vitro* germination assay. Vertical bars correspond to standard error.

of spores were evaluated across different treatment times. The yield of spores reached and maintained a maximum after 1 hr of treatment (Fig. 2A). The viability of the spores was slightly reduced by approximately 5% by the 2-hr enzyme treatment (Fig. 2B). From these results, a 1-hr enzyme treatment was determined to be the most appropriate to release spores.

Enhancement of purified spore yield. After tissue homogenization, a filtration method is generally used to remove larger pieces of tissue materials. The use of a filter paper with an 8–11 µm pore size instead of 100-µm-sized mesh increased the purity of the spore suspension, but the yield of spores was reduced to approximately 60% (Fig. 3A). To overcome this reduced spore yield, the optimal volume of spore suspension for the filtering with filter paper was determined. Increasing the volume of the spore suspension to more than 2 mL was sufficient to recover the yield of filtered spores to approximately 95% (Fig. 3A). The spore yield after the final purification using Percoll was approximately 88% of the initial spore yield released from the midgut (Fig. 3B).

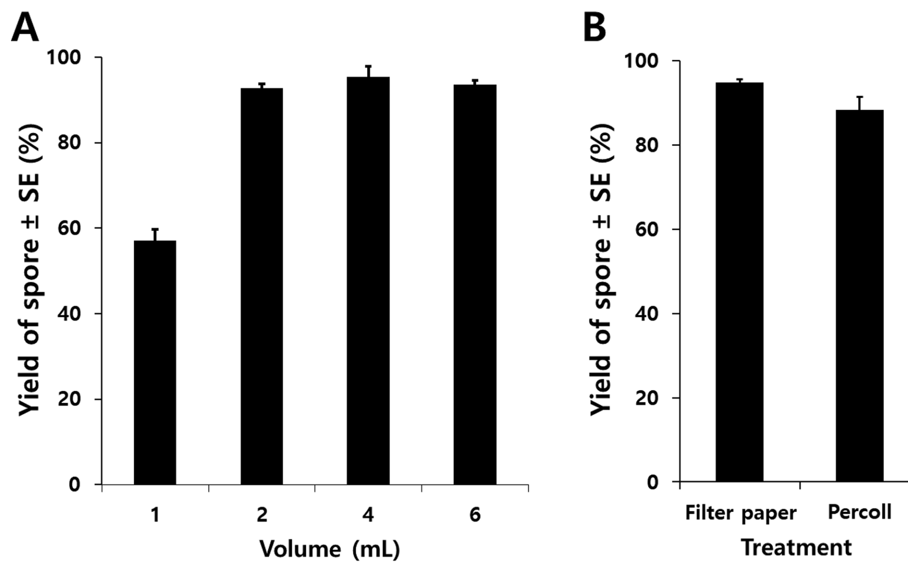

Fig. 3. Relative yield of spores according to the suspension volume for filtering (A) and filtering process (B) from infected honey bee midgut epithelial cells. The yield was calculated relative to the yield of initially released spores after homogenization with 2 mm beads. Vertical bars correspond to standard error. Filter paper, spore yield after filtering with a filter paper; Percoll, final spore yield after Percoll gradient purification.

DISCUSSION

Because *N. ceranae* is an obligate intracellular parasite, it only grows efficiently in honey bees, typically during the spring in temperate countries. Additionally, the preservation of spores is very difficult due to the high sensitivity of *N. ceranae* to low temperatures [18]. When spore suspensions are stored, the viability of the spores is rapidly lost over time in the refrigerator and almost completely lost after freezing [1, 19]. A sufficient supply of viable *N. ceranae* spores from infected honey bees, therefore, is very important to prepare inocula and perform various experiments. To obtain a sufficient quantity of spores, in this study, we focused on increasing the efficiency of the initial release of spores from the honey bee midgut. The use of an enzyme solution with collagenase and trypsin and 2 mm beads increased the yield of released spores from the midgut tissues by approximately 3 times compared with the use of common 3 mm beads alone. Collagenase is used to break the peptide bonds in collagen, which is the main structural protein in the connective tissues. Trypsin is a serine protease and functions to hydrolyze peptide bonds, which breaks down proteins into smaller peptides. Therefore, the homogenization of the midgut and release of spores might be accelerated by the disconnection of midgut tissues and hydrolysis of cells owing to the enzyme treatment.

After homogenization, a cell strainer with a 100-μm-sized mesh was used to filter the spore suspensions to eliminate larger pieces of tissue. This filtration method was not sufficient to acquire a high purity solution of spores, even if further purification is performed using a Percoll gradient. Filters with smaller pore sizes have been used to enhance the purity of spores, but these steps reduced the yield of spores. In this study, a filter paper with 8–11 μm pore size, which is twice the size of *N. ceranae* spores, was sufficient to remove tissue and cell debris because the midgut tissues and cells were thoroughly homogenized by the 2 mm beads and enzymes.

Considering that the spore yield was increased approximately 3 times when using 2 mm beads and enzymes compared with 3 mm beads alone (Fig. 1B), the spore yield from our method was increased at least 2.94 times after the final Percoll purification. This method may be useful for purifying not only *N. ceranae* spores but also other *Nosema* spp. spores.

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REFERENCES

1. Fries I. *Nosema ceranae* in European honey bees (*Apis mellifera*). J Invertebr Pathol 2010;103 Suppl 1:S73-9.
2. Adl SM, Simpson AG, Lane CE, Lukeš J, Bass D, Bowser SS, Brown MW, Burki F, Dunthorn M, Hampl V, et al. The revised classification of eukaryotes. J Eukaryot Microbiol 2012;59:429-93.
3. Bigliardi E, Sacchi L. Cell biology and invasion of the microsporidia. Microbes Infect 2001;3:373-9.
4. Cali A,Takvorian PM. Developmental morphology and life cycles of the microsporidia. In: Wittner M, Weiss LM, editors. The microsporidia and microsporidiosis. Washington, DC: ASM Press; 1999. p. 85-128.
5. Gisder S, Möckel N, Linde A, Genersch E. A cell culture

model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. Environ Microbiol 2011;13:404-13.
6. Anderson DL, Giacon H. Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. J Econ Entomol 1992;85:47-51.
7. Kralj J, Fuchs S. *Nosema* sp. influences flight behavior of infected honey bee (*Apis mellifera*) foragers. Apidologie 2010; 41:21-8.
8. Pajuelo AG, Torres C, Bermejo FJ. Colony losses: a double blind trial on the influence of supplementary protein nutrition and preventative treatment with fumagillin against *Nosema ceranae*. J Apic Res 2008;47:84-6.
9. Williams GR, Shutler D, Little CM, Burgher-MacLellan KL, Rogers RE. The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B(R), and western honey bee (*Apis mellifera*) colony strength. Apidologie 2011;42:15-22.
10. Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J Invertebr Pathol 2007;96:1-10.
11. Chen Y, Evans JD, Simith IB, Pettis JS. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J Invertebr Pathol 2008;97:186-8.
12. Emsen B, Guzman-Novoa E, Hamiduzzaman MM, Eccles L, Lacey B, Ruiz-Pérez RA, Nasr M. Higher prevalence and levels of *Nosema ceranae* than *Nosema apis* infections in Canadian honey bee colonies. Parasitol Res 2016;115:175-81.
13. Gisder S, Hedtke K, Möckel N, Frielitz MC, Linde A, Genersch E. Five-year cohort study of *Nosema* spp. in Germany: does climate shape virulence and assertiveness of *Nosema ceranae*? Appl Environ Microbiol 2010;76:3032-8.
14. Fries I, Chauzat MP, Chen YP, Doublet V, Genersch E, Gisder S, Higes M, McMahon DP, Martín-Hernández R, Natsopoulou M, et al. Standard methods for *Nosema* research. J Apic Res 2013;52:1-28.
15. Chen YP, Evans JD, Murphy C, Gutell R, Zuker M, Gundensen-Rindal D, Pettis JS. Morphological, molecular, and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. J Eukaryot Microbiol 2009;56:142-7.
16. Shimanuki H, Knox DA. Diagnosis of honey bee diseases. Agriculture Handbook No. AH-690. Washington, DC: U.S. Department of Agriculture; 2000.
17. Olsen PE, Rice WA, Liu TP. *In vitro* germination of *Nosema apis* spores under conditions favorable for the generation and maintenance of sporoplasms. J Invertebr Pathol 1986;47:65-73.
18. Forsgren E, Fries I. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. Vet Parasitol 2010;170:212-7.
19. Fenoy S, Rueda C, Higes M, Martín-Hernández R, del Aguila C. High-level resistance of *Nosema ceranae*, a parasite of the honeybee, to temperature and desiccation. Appl Environ Microbiol 2009;75:6886-9.