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Oxidative stress induced by chlorine dioxide as an insecticidal factor to the Indian meal moth, *Plodia interpunctella*

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ABSTRACT

A novel fumigant, chlorine dioxide (ClO_2) is a commercial bleaching and disinfection agent. Recent study indicates its insecticidal activity. However, its mode of action to kill insects is yet to be understood. This study set up a hypothesis that an oxidative stress induced by ClO_2 is a main factor to kill insects. The Indian meal moth, *Plodia interpunctella*, is a lepidopteran insect pest infesting various stored grains. Larvae of *P. interpunctella* were highly susceptible to ClO_2 gas, which exhibited an acute toxicity. Physiological damages by ClO_2 were observed in hemocytes. At high doses, the larvae of *P. interpunctella* suffered significant reduction of total hemocytes. At low doses, ClO_2 impaired hemocyte behaviors. The cytotoxicity of ClO_2 was further analyzed using two insect cell lines, where Sf9 cells were more susceptible to ClO_2 than High Five cells. The cells treated with ClO_2 produced reactive oxygen species (ROS). The produced ROS amounts increased with an increase of the treated ClO_2 amount. However, the addition of an antioxidant, vitamin E, significantly attenuated the cytotoxicity of ClO_2 in a dose-dependent manner. To support the oxidative stress induced by ClO_2, two antioxidant genes (superoxide dismutase (SOD) and thioredoxin-peroxidase (Tpx)) were identified from *P. interpunctella* EST library using ortholog sequences of *Bombyx mori*. Both SOD and Tpx were expressed in larvae of *P. interpunctella* especially under oxidative stress induced by bacterial challenge. Exposure to ClO_2 gas significantly induced the gene expression of both SOD and Tpx. RNA interference of SOD or Tpx using specific double stranded RNAs significantly enhanced the lethality of *P. interpunctella* to ClO_2 gas treatment as well as to the bacterial challenge. These results suggest that ClO_2 induces the production of insecticidal ROS, which results in a fatal oxidative stress in *P. interpunctella*.

1. Introduction

The Indian meal moth, *Plodia interpunctella*, is a lepidopteran insect pest undergoing larval diapause as a pre-pupa [1,2]. Low temperature and short photoperiod during the early larval period induce the diapause development [3]. During the growing larval stages, the larvae feed on food products stored in households, groceries, and warehouses in most of the world except the Antarctic region [4]. Especially, the larval stage gives serious damage on the stored products by penetrating foil, polyester, and polypropylene film covering the stored food [5]. In addition, the larvae secrete lots of threads to tangle stored products and produce an allergic material causing asthma and skin disease, which lead to aversion from consumers and it makes difficult to manufacture processes [6].

To control *P. interpunctella*, chemical sprays have been applied using neurotoxic synthetic insecticides [7]. To be effective, methyl bromide (MB) fumigation treatment has been widely applied. As an alternative fumigation method to replace MB treatment, which is under phase-out due to environmental issue, ozone fumigation was tested. However, it was effective only to pupae and had no effect on eggs and larvae of *P. interpunctella* [8]. Phosphine treatment was also considered, but it needed an additional oxygen condition to obtain optimal control efficacy [9]. Ionizing irradiation is used as a phytosanitary treatment against quarantine pests. However, a relatively high dose was required to control *P. interpunctella* and proved to be not applicable [10]. To develop an environmentally friendly control technique, a sex pheromone has been tested to disrupt their mating signals. Indeed, a mating disruption using a single major component of the sex pheromone was successful to suppress *P. interpunctella* population in small-scale plot experiments [11]. An

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attract-to-kill strategy using the sex pheromone lure was used to control the pest populations in warehouses [12]. Application of insect growth regulators to replace highly toxic MB treatment was proposed to control *P. interpunctella* [13]. *Bacillus thuringiensis* (Bt) insecticides are also effective to control *P. interpunctella*. However, development of Bt resistance was reported in the species in a processing step from protoxin to toxin and a subsequent binding of active toxin to midgut receptor [14]. Biological control of *P. interpunctella* using a parasitoid wasp, *Brecon hebetor*, was evaluated and recommended to introduce the wasps to the pest population in a periodic and iterative manner [15]. *Trichogramma* egg parasitoids have been proposed as augmentative biological control agents for *P. interpunctella* in stored product systems due to ovicidal action to prevent the advent of the damaging larval stage, commercial availability, and their small sizes making them unlikely to be noticed by consumers [16]. On the other hand, a behavioral modification strategy was developed to minimize the attack of *P. interpunctella* on the commercial food products. Cinnamon oil was demonstrated to have a significant repellent activity against *P. interpunctella* larvae [17,18]. Kim et al. [19] developed a coating technique of cinnamon oil on polyethylene (PE) package film. Indeed, *P. interpunctella* larvae are able to infest and digest PE film via their enteric microbes [20].

Chlorine dioxide (ClO_2) is a disinfectant against bacteria, fungi, and viruses with its strong oxidizing property [21]. It oxidizes the sulfydryl groups in the microbial membrane enzymes to form disulfide analogs, which make the enzymes functionless [22]. Especially, ClO_2 gas has high penetration and solubility, which allow it to be applied to disinfect pathogenic microbes contaminating food surfaces [23]. Furthermore, ClO_2 has been demonstrated to have an insecticidal activity. In an aquatic system, ClO_2 completely kills chironomid larvae [24]. In a human-dwelling habitat, ClO_2 gas was shown to be effective in controlling bedbugs [25]. However, the insecticidal mechanism of ClO_2 has not been understood.

This study analyzed the insecticidal activity of ClO_2 by its oxidizing activity, which would induce a fatal oxidative stress to the target larvae of *P. interpunctella*. First, this study showed an acute insecticidal activity of ClO_2 gas to the larvae of *P. interpunctella*. Second, the changes in expressions of two antioxidant genes were monitored to test the oxidative stress of the larvae exposed to ClO_2 gas. Third, using a cytotoxic activity of ClO_2 against hemocytes of *P. interpunctella* as well as two insect cell lines, a direct effect of ClO_2 on inducing oxidative stress was tested by quantifying the amounts of reactive oxygen species (ROS). Finally, an antioxidant, vitamin E was added to the ClO_2 treatment to test whether it rescued the cell death by attenuating oxidative stress.

2. Materials and methods

2.1. Test insect rearing

A laboratory colony of *P. interpunctella* was originated from some dried vegetable commodities stored in a warehouse located in Taegu, Korea in 1994. Larvae of *P. interpunctella* were fed artificial diets (800 g rice bran, 200 g yeast extract, 500 mL glycerol, 2 g sorbic acid and 2 g methyl *p*-hydroxybenzoate) and reared in a growth chamber at 28 ± 1 °C temperature with 65–75% relative humidity and a photoperiod of 16:8 (L:D) h. To prevent random genetic drift, more than 100 male and females were mated in a cage (25 × 25 × 25 cm) and the subsequent mated females laid eggs on the diet.

2.2. Bacterial culture

Escherichia coli Top10 (Invitrogen, Carlsbad, CA, USA) was cultured in Luria–Bertani broth at 37 °C with shaking at 270 rpm prior to use in immune challenge assay. Each larva was injected with 5×10^4 cells of *E. coli* in culture broth by a glass capillary syringe operated with a nanoliter injector (World Precision Instruments, Sarasota, FL, USA) under a stereo-microscope (Olympus S730, Olympus, Tokyo, Japan). For control, 100 mM phosphate buffer saline (PBS, pH 7.4) used for dilution of the bacteria was injected.

2.3. Identification of SOD and Tpx genes in P. interpunctella

Superoxide dismutase (SOD) and thioredoxin peroxidase (Tpx) sequences were obtained from *Bombyx mori* genome with GenBank accession numbers of NP001037084 and AHK05704, respectively. These sequences were used as queries to search homologous gene(s) from EST libraries of *P. interpunctella* using the NCBI-BlastN tool. The predicted amino acid sequences were aligned with homologous sequences using ClustalW program (DNAStar, Madison, WI, USA).

2.4. Chlorine dioxide treatment

ClO_2 gas was generated by a chlorine dioxide generator (PurgoFarm Co. Ltd., Hwasung, Korea) using an electrochemical method [26]. Briefly, aqueous $NaClO_2$ was electrolyzed and the cleaved sodium ion was migrated to the cathode through patented multi-porous membrane electrode assembly leaving a high pure ClO_2 (>99%) in the anode chamber. The evaporating ClO_2 gas was blown out through a vent into a test chamber (54 × 44 × 46 cm). Gas entry to the chamber was manually controlled depending on the preset concentration of ClO_2, which was monitored at every 10 min by PortaSens II gas leak detector (Analytical Technology, Collegeville, PA, USA). Larvae of *P. interpunctella* were put in a 9 cm petri dish containing a layer of brown rice granules. The petri dish was covered with mesh cloth (196 mesh counts, Saatilene Hitech, Somers, NY, USA) to allow the fumigant to enter the dish. Each petri dish was an experimental unit containing 30 individuals and was replicated three times. After exposure to ClO_2 gas, the treated dish was transferred to normal air and incubated at 25 °C for 72 h. Autonomous movement of individuals was the criterion for being categorized as alive.

2.5. Hemocyte classification and counts

The fifth instar larvae were surface-sterilized in 70% ethanol for a few seconds and rinsed with sterile water. After cutting off one of the abdominal prolegs with sterile scissors, the exuded hemolymph was collected with a glass capillary. To avoid hemocyte aggregation, the hemolymph was mixed with anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na_2EDTA and 41 mM citric acid, pH 4.5) in a microfuge tube immediately after collection. Hemocytes were counted using a hemocytometer (Superior, Germany) under a phase contrast microscope (Olympus BX41, Japan). Total hemocyte count (THC) was expressed as cell number per mL. For measurement of differential hemocyte count (DHC), hemocyte types were classified into granulocyte, plasmatocyte, oenocytoid, and spherulocyte by morphological characters [27]. Granulocytes were round cells with granules in their cytoplasm and exhibited a spreading shape around all entire peripheries with a similar size extension. Plasmatocytes looked similar to granulocytes except that they had much less number of granules and exhibited a directional extension of the cytoplasm during spreading. Oenocytoids were characterized by a large cytoplasm without granules. Spherulocytes contained big granules in the cytoplasm.

2.6. In vitro *hemocyte-spreading behavior assay*

Bioassays were performed in 96-well culture plates (SPL, Pocheon, Korea), where 50 μL of reaction mixture in each well consisted of a plasmatocyte-spreading peptide (1 μL, donated from Dr. Kevin