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A combined treatment of UV-assisted TiO_2 photocatalysis and high hydrostatic pressure to inactivate internalized murine norovirus

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ABSTRACT

Human norovirus (HuNoV) is a major cause of foodborne illness associated with shellfish consumption. A solidified agar matrix (SAM) was experimentally prepared using agar solution for inactivation of murine norovirus (MNV-1) as a surrogate for HuNoV in a simulation model approach. MNV-1 was injected inside the SAM for virus internalization, and the effects of single and combined UV-assisted TiO_2 photocatalysis (UVTP) and high hydrostatic pressure (HHP) treatments were determined. The internalized MNV-1 were reduced by 2.9-$\log_{10}$ and 3.5-$\log_{10}$, respectively, after single treatments of UVTP (4.5 mW/cm^2, 10 min) and HHP (500 MPa, 5 min, ambient temperature). However, the internalized MNV-1 was reduced by 5.5-$\log_{10}$ (below the detection limit) when UVTP was followed by HHP, indicating a synergistic inactivation effect. Analysis of viral morphology, proteins, and genomic RNA allowed elucidation of mechanisms involved in the synergistic antiviral activity of combined treatments, which appeared to disrupt the MNV-1 structure and damage both the capsid protein and genomic RNA.

Industrial relevance: HHP treatment of raw oysters has proved commercially successful, but there is a less evidence available regarding the potential of HHP for inactivation of localized viruses present inside foods. A sequential combination of UV-assisted TiO_2 photocatalysis (UVTP) and high hydrostatic pressure (HHP) achieved significantly higher inactivation of localized virus compared to individual treatments due to a synergistic mechanism. An experimentally prepared model food system was found useful to simulate foods with morphological variations and unpredictable viral internalization patterns. This UVTP-HHP combined treatment for inactivation of localized MNV-1 can be useful for disinfection of raw oysters and other similar foods.

1. Introduction

Human norovirus (HuNoV) remains the leading cause of acute non-bacterial gastroenteritis outbreaks, causing approximately 60% of all foodborne illnesses reported annually (Lou, Neetoo, Chen, & Li, 2011; Moore, Goulter, & Jaykus, 2015). HuNoV is transmitted primarily through a fecal-oral route via consumption of contaminated water and food or aerosolization of the virus and subsequent contamination of surfaces (Lee, Zoh, & Ko, 2008). HuNoV is extremely contagious and fewer than twenty viral particles can cause an infection (Moore et al., 2015). HuNoV is the most common foodborne pathogen in Korea and there have been 542 documented foodborne outbreaks of HuNoV from 2002 to 2015 (Ministry of Food and Drug Safety, 2015).

Research regarding HuNoV has been hampered by lack of a robust cell culture system and a small animal model. Jones et al. (2014) reported development of an in vitro infection model for HuNoV in human B cells, but this cell culture system is complex with specific requirements. Ettayebi et al. (2016) recently reported the successful cultivation of multiple HuNoV strains in enterocytes in stem cell–derived, non-transformed human intestinal enteroid monolayer cultures. Studies of HuNoV rely on a proper alternative candidate, such as murine norovirus (MNV), feline calicivirus, or Tulane virus (Predmore, Sanglay, Li, & Lee, 2015). MNV not only has genetic and pathological features similar to HuNoV, but also is environmentally stable and persistent over a wide pH range (Cannon et al., 2007). MNV can serve as a surrogate for HuNoV.

Shellfish is a common transmission vector for different human enteric pathogens because they can accumulate pathogenic microorganisms through filtering of marine and estuarine waters, and are particularly susceptible to HuNoV contamination (Polo et al., 2014). Some viruses are internalized within cells of digestive organs, the mucous of the gills, and other tissues of oyster (McLeod, Hay, Grant, Greening, & Day, 2009; Polo et al., 2014). Viral titers were 400 times

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higher in oysters than in surrounding water and elimination of virus particles taken into oyster cells may take a long period of time (Richards, McLeod, & Le Guyader, 2010). The elimination of internalized viruses is difficult using traditional decontamination strategies because these techniques usually target the pathogens on the surface of the food. Alternative processing methods are, therefore, needed to improve the safety of foods with respect to internalized pathogens (Hirneisen & Kniel, 2013).

Nonthermal processing technologies provide effective microbial inactivation while maintaining safety, quality, and freshness of foods (Palgan et al., 2011; Shahbaz et al., 2016). HHP has shown potential for inactivation of pathogenic *Vibrio* spp. in bivalve shellfish, such as oysters and clams, and has been used for separation of raw shellfish meat from shells, known as shucking (Richards et al., 2010). HHP is effective in elimination of microorganisms, but there is a less information available regarding the potential of HHP for inactivation of internalized enteric viruses in foods, such as shellfish (Ye et al., 2015). UVTP is another non-thermal technique that can degrade pathogenic microorganisms via generation of strong oxidizing agents (Thiruvenkatachari, Vigneswaran, & Moon, 2008; Kim et al., 2013). Combining non-thermal treatments can enhance microbial inactivation efficiencies while allowing use of lower individual treatment intensities (Palgan et al., 2011). Hence, an effective microbial inactivation strategy is use of a combined treatments or hurdle technology for enhanced microbial safety and quality of foods (Chai, Lee, Lee, Na, & Park, 2014; Shahbaz et al., 2016). A combined application of HHP with UVTP has been reported to achieve synergistic inactivation of microorganisms in food products (Shahbaz et al., 2016; Yoo et al., 2015a).

Decontamination methods for internalized viral particles are difficult to evaluate in shellfish due to an inconsistent degree of contamination, morphological irregularities and unpredictable localization sites in digestive track tissues (McLeod et al., 2009). A standard food model approach simulator needs to be developed for investigation of the efficiency of disinfection methods (Hirneisen et al., 2014; Yoo et al., 2015b). Agar matrix has been reported for evaluation of the efficiency of different disinfection treatments, such as photodynamic inactivation of *E. coli* (Caminos & Durantini, 2006) and atmospheric pressure plasma inactivation of *L. monocytogenes* (Lee et al., 2011). In this study, an artificially substituted matrix was prepared using an agar matrix and simulation of actual virucidal effects of different decontamination treatments was investigated.

The objectives of this study were i) to evaluate the efficacy of UVTP and HHP combined and individual treatments for inactivation of MNV-1 experimentally internalized in a solidified agar matrix and ii) determination of the mechanism of MNV-1 inactivation using four different assay methods.

2. Materials and methods

2.1. Sample preparation

2.1.1. Virus and cell line

The murine norovirus (MNV-1) CW1 strain was propagated in the RAW 264.7 mouse leukemic macrophage cell line (Korean Cell Line Bank, Seoul, Korea). RAW 264.7 cells were cultured in 75-cm^2 tissue culture flasks and maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For preparation of MNV-1 stock, the growth medium was removed and confluent cells were infected with MNV-1 at a multiplicity of infection of 0.1, followed by incubation for 48 h. When significant cytopathic effects were observed, infected cells were subjected to a freeze/thaw cycle three times to lyse cells and release viral particles. Cell debris was removed via centrifugation at 1500 ×*g* for 10 min at 4 °C. For further concentration of MNV-1, the supernatant was ultrafiltered using Amicon Ultra-15 (Millipore, MA, USA) at 5000 ×*g* for 30 min at 4 °C. The supernatant fluid was collected and stored at −80 °C until further use.

2.1.2. Purification of MNV-1

Purification of MNV-1 was carried out following the protocol of Huhti et al. (2010) with minor modification. Viruses were purified via ultracentrifugation on a discontinuous sucrose density gradient (10–60%) in a SW-41 Ti swing bucket rotor (BeckmanCoulter, CA, USA) at 120,000 ×*g* for 2 h 30 min at 4 °C. Fractions were collected and the presence of the VP1 protein was confirmed using SDS-PAGE. VP1-positive fractions were pooled, dialyzed overnight to remove sucrose, and concentrated using ultrafiltration (Amicon MWCO 100 kDa, Millipore). Viral particles were re-suspended in deionized HPLC-grade water and 1× phosphate-buffered saline (pH 7.4) (PBS, Gibco) (both microfiltered at 0.2 μm, Sartorius, Goettingen, Germany). Concentrations were estimated based on measurement of absorbance values at 280 nm using a Nanodrop instrument (NanoDrop ND-1000, ThermoFisher, DE, USA) and an extinction coefficient of $E_{280} = 72.560\ cm^{-1}\ M^{-1}$ for MNV-1. The purified virus was suspension and stored at −80 °C.

2.1.3. Preparation of a solidified agar matrix (SAM)

SAM was prepared following the method of Yoo et al. (2015b). Briefly, SAM was prepared via casting a Bacto™ agar (Becton, Dickinson and Company, MD, USA) solution (2.5%) in a ellipsoidal mold after sterilization at 121 °C for 15 min, followed by rapid cooling in a deep freezer (−80 °C) for 30 min for hardening. The SAM surface and inner texture were both observed using scanning electron microscopy (FE-SEM S-800, Hitachi Ltd., Tokyo, Japan) Yoo et al., 2015b). SAM was prepared to be porous for stable virus adhesion with homogenous morphological characteristics analyzed by scanning electron microscopy (Fig. 1). SAM were irradiated with UV light on a clean bench (VS-1400LS, Vision Scientific, Daejeon, Korea) for 30 min to ensure sterilization prior to inoculation. Inoculation was achieved based on a 10-hole injection of 10 μL/hole of viruses (approximately 6.7-$\log_{10}$ PFU/sample) at a depth of 9 mm using pipetting, followed by 30-min air-drying on a clean bench (Fig. 1). Two SAM samples were used for each experiment.

2.2. Inactivation treatments

2.2.1. UV-assisted TiO_2 photocatalysis

A lab-scale UV-assisted TiO_2 photocatalytic reactor was customized following the method of Yoo et al. (2015a). The reactor consisted of a 30-L. stainless steel vessel containing six low pressure mercury UV lamps (254-nm wavelength, 35 W, Sankyo Denki, Tokyo, Japan) in TiO_2-coated quartz tubes of 38-cm length with a 24.5-mm outer diameter (a TiO_2 coating thickness of 0.7 to 0.9 mm, Taekyeong UV Co., Namyangju, Korea). UV lamps were fitted into non-coated quartz tubes for UV single treatment. The UV intensity was measured before treatment based on placement of the sensor of a UV radiometer (Sentry Optronics Corp., New Taipei City, Taiwan). The assembly was submerged in water inside the reactor. An air pump was placed at the bottom of the reactor to create turbulent flow for achieving random rotation and movement of SAM. A washing treatment with air bubbles in water was carried out as a dark control with disconnected UV lamps. Inoculated SAM samples were immersed in a vessel containing 28 L of deionized water, and UVTP and UV single treatments were applied for 0, 5, 10, 15, and 20 min.

2.2.2. High hydrostatic pressure

Inoculated SAM samples were transferred to individual sterile polyethylene terephthalate bags. Each bag was sealed leaving no headspace and the PBS was ten times the SAM sample volume. HHP treatments were conducted in a HPP-600 lab-scale unit (Baotou Kefa Co., Ltd., Inner Mongolia, China) of 5-L capacity using water as a pressure transmission fluid. The pressure come-up rate was approximately 25 MPa/s and pressure-release was rapid at <4 s. The pressure-holding time in