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High hydrostatic pressure inactivation of murine norovirus and human noroviruses on green onions and in salsa

Robert F. Sido [1], Runze Huang [1], Chuhan Liu, Haiqiang Chen *

Department of Animal and Food Sciences, University of Delaware, Newark, DE 19716, USA

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ABSTRACT

In this study, high hydrostatic pressure (HHP) was evaluated as an intervention for human noroviruses (HuNoVs) in green onions and salsa. To determine the effect of water during HHP treatment on virus inactivation, a HuNoV surrogate, murine norovirus 1 (MNV-1), was inoculated onto green onions and then HHP-treated at 350 MPa with or without water at 4 or 20 °C. The presence of water enhanced HHP inactivation of MNV-1 on green onions at 4 °C but not at 20 °C. To test the temperature effect on HHP inactivation of MNV-1, inoculated green onions were HHP-treated at 300 MPa at 1, 4 and 10 °C. As the temperature decreased, MNV-1 became more sensitive to HHP treatment. HHP inactivation curves of MNV-1 on green onions and salsa were obtained at 300 or 350 MPa for 0.5–3 min at 1 °C. All three inactivation curves showed a linear relationship between log reduction of MNV-1 and time. *D* values of HHP inactivation of MNV-1 on green onions were 1.10 and 0.61 min at 300 and 350 MPa, respectively. The *D* value of HHP inactivation of MNV-1 in salsa at 300 MPa was 0.63 min. HHP inactivation of HuNoV GI.1 and GII.4 on green onions and salsa was also conducted. To achieve >3 log reduction of HuNoV GI.1, HHP treatments for 2 min at 1 °C should be conducted at 600 MPa and 500 MPa for green onions and salsa, respectively. To achieve >3 log reduction of HuNoV GII.4, HHP treatments for 2 min at 1 °C should be conducted at 500 MPa and 300 MPa for green onions and salsa, respectively.

1. Introduction

Human norovirus (HuNoV), a leading cause of foodborne illness in the United States, affects millions of people each year and is often linked to fresh produce (Hall et al., 2012). Green onions and fresh salsa (which typically contains green onions) are commonly consumed raw and without significant food safety interventions beyond washing (Wheeler et al., 2005). From 1973 to 2008, HuNoV accounted for 24% of foodborne disease outbreaks associated with either salsa or guacamole in the U.S. (Kendall et al., 2013). In 2003, green onions and fresh salsa containing green onions were identified as the source of an outbreak of hepatitis A virus (HAV) in Pennsylvania which led to 601 illness (Wheeler et al., 2005). In 2009, salsa was confirmed as the food vehicle for a HuNoV outbreak in California, leading to 14 illnesses (Centers for Disease control and prevention, 2016).

Currently no suitable cell culture systems or practical small animal models are available for HuNoV. The use of human subjects and gnotobiotic pig model for evaluating of a food processing technology on HuNoV inactivation is not practical due to the high cost involved (Leon et al., 2011; Lou et al., 2015). Recently an in vitro cell culture system for HuNoV was proposed by Jones et al. (2015) which still needs further development due to the modest viral output. There was an ex vivo model of human intestinal enteroids derived from stem cell recently developed for replication of HuNoV GII.3 and GII.4 strains. The study found that replication of HuNoV happened in enterocytes in human intestinal enteroids and some strains needed bile for replication (Ettayebi et al., 2016). Therefore, HuNoV surrogates, such as murine norovirus 1 (MNV-1), feline calicivirus (FCV) and Tulane virus (TV), have been commonly used to evaluate the efficacy of a food processing technology on HuNoV inactivation (Cannon et al., 2006; Chen et al., 2005; Li et al., 2013b). Li and Chen (2015) showed that a 2-min high hydrostatic pressure (HHP) treatment at 250 MPa and 21 °C reduce MNV-1 and FCV in cell culture media by 0.1 and 2.9 log, respectively. Chen et al. (2005) found that FCV in a cell culture medium was reduced by ~1.7 log with a 2-min HHP treatment at 250 MPa and 21 °C. These data demonstrated that MNV-1 was more resistant to HHP than TV and FCV. However, it is unclear which virus would be the best surrogate for HuNoV since direct comparison of surrogates with HuNoV is not possible. Molecular biology methods, mostly RT-qPCR, can be used for HuNoV quantification. However, these methods can only detect the presence of HuNoV RNA, but cannot distinguish between infectious and non-infectious virus particles. To overcome this limitation, Tian et al. (2007, 2008, 2010) pretreated HuNoV samples with porcine gastric mucin (PGM) before subjecting them to RT-qPCR quantification. PGM shares similar chemical and antigenic characteristics with histo-blood group antigens which are able to bind to HuNoV and are key factors in HuNoV infection

* Corresponding author.
E-mail address: haiqiang@udel.edu (H. Chen).
[1] Had approximately equal contribution to this manuscript.

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of human B cells (Jones et al., 2014; Marionneau et al., 2002). The pretreatment of HuNoV with PGM was used in their studies to bind and collect potentially infectious HuNoV, and exclude presumably inactivated virions unable to bind to PGM. After the pretreatment with PGM, Tian et al. (2007, 2008, 2010) used RT-qPCR to quantify the potentially infectious HuNoV bound by PGM. The utility of the PGM pretreatment followed by RT-qPCR assay (referred to as PGM/PCR assay) for discriminating potentially infectious HuNoV was further demonstrated by Dancho et al. (2012) who showed that thermal, UV, and HHP treatments result in loss of PGM binding consistent with inactivation of the virus by damage to its capsid. Li and Chen (2015) later compared HHP inactivation results of MNV-1 and TV obtained through the PGM/PCR assay and plaque assay and concluded that the PGM/PCR assay would be very likely able to estimate HHP inactivation of HuNoV at ≤2 log-reduction levels. They also showed that the order of HHP resistance for the virus strains used in their study was probably HuNoV GI.1 > MNV-1 > HuNoV GII.4 > TV, indicating that MNV-1 is probably a good surrogate for the GII.4 strain, but not a good surrogate for the GI.1 strain. A recent study by Lou et al. (2016) showed that even within the same GII genogroup, different HuNoV strains had different sensitivity to HHP with the order of GII.1 > GII.6 > GII.4, making it difficult to select an appropriate HuNoV surrogate for HHP.

HHP is a nonthermal processing technique which exposes foods to a high level of isostatic pressure, usually at 200–600 MPa (Cheftel, 1995). HHP has been used for a wide variety of food products, such as oysters, juice, jam, ready-to-eat meats, guacamole and salsa (Bermúdez-Aguirre and Barbosa-Cánovas, 2010; Oey et al., 2008; Torres and Velazquez, 2005). It has been shown that HHP can denature proteins while leaving small molecules, such as flavor and aroma compounds, intact (Bermúdez-Aguirre and Barbosa-Cánovas, 2010). HHP also has been identified as a potential intervention for HuNoV and HAV in various fresh produce foods, such as blueberries, strawberries and lettuce (Huang et al., 2014; Huang et al., 2016; Kingsley, 2013; Kingsley et al., 2005; Li et al., 2013b; Lou et al., 2011). Hirneisen and Kniel (2013) showed that a 2-min HHP treatment at 500 MPa and 20 °C could reduce MNV internalized in green onions and on the surface of green onions by >4.7 and >6.4 log, respectively. It was shown that a 5-min HHP treatment at 375 MPa could reduce 4.75 log of HAV on sliced green onions (Kingsley et al., 2005). Lou et al. (2011) showed that >5 log reduction of MNV-1 on lettuce was achieved with a 2-min HHP treatment at 400 MPa and 4 °C. In salsa, it was shown that a 1-min HHP treatment at 400 MPa and 9 °C could reduce MNV by >7.0 log in salsa (Hirneisen et al., 2014). Using the PGM/PCR assay, Li et al. (2013a), Ye et al. (2014) and Huang et al. (2016) demonstrated that HHP could inactivate HuNoV GI.1 and GII.4 in various foods. For example, Li et al. (2013a) demonstrated that a 2-min HHP treatment at 600 MPa and 2 min could inactivate HuNoV GI.1 by >3.0 log on blueberries with presence of surrounding water. Ye et al. (2014) showed that a treatment at 450 MPa for 5 min at 1 °C achieved a >4 log reduction of HuNoV GI.1 in both oyster and clam homogenates.

The objectives of this study was to determine the HHP inactivation effect of MNV-1, HuNoV GI.1 and GII.4 strains on green onions and in salsa and to identify practical HHP parameters for processing green onions and salsa.

2. Material and methods

2.1. Virus and cell lines

Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA kindly provided the HuNoV GII.4 strain in fecal suspension used in this study. The information of the HuNoV GI.1 strain (8FIIb) used in this study was described by Leon et al. (2011). Fecal suspensions were centrifuged at 4000 × *g* for 20 min, filtered through a 0.22-μm filter, aliquoted, and stored at −80 °C until use.

MNV-1 and murine macrophage cell line RAW 264.7 were generously provided by Dr. Jianrong Li at the Ohio State University. RAW 264.7 cells were cultured in DMEM (Gibco, ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS (Gibco, ThermoFisher Scientific, Waltham, MA) at 37 °C under a 5% CO_2 atmosphere. To prepare MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h incubation at 37 °C under a 5% CO_2 atmosphere, 25 ml of DMEM supplemented with 2% FBS was added. MNV-1 was harvested 2 days after post-inoculation by three freezing–thawing cycles and centrifugation. The virus was stored at −80 °C until use.

2.2. Inoculation of MNV-1 and HuNoVs on green onions and in salsa

Fresh green onions were obtained from local markets, washed and dried before use. The green part of the green onions was removed using a sterile knife and the remaining white part was sliced into 3-mm sections. The cut surfaces of white part sections (3 g) were inoculated with 100 μl of MNV-1 stock (~10^7 PFU/ml) or 50 μl of HuNoV GI.1 (~10^6 genome copies/ml) or GII.4 (~10^7 genome copies/ml) stocks. The inoculated samples were dried in a biosafety hood for 2 h for viral attachment and then placed in sterile stomacher bags (3 g of sample/bag) with or without 1 ml of sterile dd-H_2O in the bag. The bags were sealed and double-bagged. To prepare salsa for HHP treatment, Tostitos restaurant style salsa (medium heat; major ingredients are diced tomatoes in tomato juice, water, unpeeled ground tomatoes in tomato puree, onions, jalapeno peppers) was bought from local market, supplemented with 5% (w/w) 3-mm sections of washed green onions and stomached at 260 RPM for 2 min to reduce the size of vegetable chunks. pH of the salsa samples was ~4.0 and water activity of salsa samples was ~0.967. Salsa samples (2.5 g) were inoculated with 100 μl of MNV-1 stock or 50 μl of HuNoV GI.1 or GII.4 stocks. Samples were placed in sterile stomacher bags, sealed and double-bagged.

2.3. HHP treatment

Pressure treatments were conducted using an Avure PT-1 pressure unit (Avure Technologies, Kent, WA) with temperature control and water as a hydrostatic medium. A circulating water bath surrounded the pressure vessel (50 ml) to control the temperature. The pressure come-up rate was approximately 22 MPa/s. The pressure-release was almost immediate (<4 s). Pressurization hold time reported in this study did not include the pressure come-up or release times. To determine whether the presence of water surrounding green onions would affect the HHP inactivation of MNV-1, inoculated green onions samples with and without water in the bags were pressurized at 350 MPa for 2 min at initial temperatures of 4 and 20 °C. To determine the temperature effect on HHP inactivation of MNV-1 on green onions, green onion samples with water in the bags were treated at 300 MPa for 2 min at initial temperatures of 1, 4 and 10 °C. To obtain the HHP inactivation curves of MNV-1 on green onions and in salsa, samples were treated at 300 or 350 MPa for 0.5–3 min at an initial temperature of 1 °C. The *D* value (decimal reduction time; time required at a given condition to reduce target microorganism by 1 log) of MNV-1 under difference conditions were also calculated. To determine the HHP inactivation of HuNoV GI.1 and GII.4 on green onions and in salsa, samples were treated at 100–600 MPa for 2 min at an initial temperature of 1 °C. MNV-1 and HuNoVs were extracted from the pressurized samples and untreated samples as described in Section 2.4. MNV-1 was quantified by plaque assay as described in Section 2.5 and HuNoVs were quantified by PGM-MBs (PGM-magnetic beads) binding assay and RT-qPCR as described in Sections 2.6 and 2.7.