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Heat-denatured lysozyme could be a novel disinfectant for reducing hepatitis A virus and murine norovirus on berry fruit

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ABSTRACT

Hepatitis A virus (HAV) is well known worldwide as a causative virus of acute hepatitis. In recent years, numerous cases of HAV infection caused by HAV-contaminated berries have occurred around the world. Because berries are often consumed without prior heating, reliable disinfection of the raw fruit is important in order to prevent HAV outbreaks. Previous studies have found that murine norovirus strain 1 (MNV-1) and human norovirus GII.4 were inactivated in heat-denatured lysozyme solution. In this study, we investigated whether or not heat-denatured lysozyme is effective in inactivating HAV and whether it could be an effective disinfectant for berries contaminated with HAV or MNV-1.

We examined the inactivating effect of heat-denatured lysozyme on three strains of HAV and found that it reduced the infectivity of all three strains. We then immersed blueberries and mixed berries into solutions of HAV or MNV-1, and disinfected them by soaking them in 1% heat-denatured lysozyme for 1 min. Consequently, the infectious HAV and MNV-1 contaminating the berries were decreased by > 3.1 log units in all samples.

Our results demonstrate that heat-denatured lysozyme effectively inactivates HAV and suggest that heat-denatured lysozyme may be an effective disinfectant for berry fruit, which is a potential source of HAV food poisoning.

1. Introduction

Hepatitis A virus (HAV), a single-stranded RNA virus without an envelope, belongs to the genus *Hepatovirus* in the *Picornaviridae* family (Koopmans and Duizer, 2004). Its incubation period is around 14–28 days, and infection causes symptoms that include fever, vomiting, abdominal pain, and jaundice lasting from a few weeks to several months (Fiore, 2004). Although HAV does not cause chronic hepatitis, unlike the hepatitis B and C viruses, it may rarely cause transient fulminant hepatitis (Wenzel et al., 2014). There are 1.5 million cases of HAV infection yearly worldwide (Wasley et al., 2006), and although the number is declining in developed countries, relatively small-scale sporadic outbreaks do still occur (Cuthbert, 2001).

The main infection routes for HAV are fecal-oral transmission, contact with an infected person, and ingestion of contaminated water or food (Fiore, 2004; Hu and Arsov, 2014). The main foods causing HAV food poisoning are bivalves and other seafoods, vegetables, juices, and berries (Fitzgerald et al., 2014).

In recent years, numerous outbreaks of HAV infection caused by HAV-contaminated berries have occurred around the world (Fitzgerald et al., 2014; Wenzel et al., 2014). The outbreak that caused the greatest damage occurred in 2013, when 1444 people developed HAV food poisoning in 12 countries in the European Union. Investigation revealed that the main cause of this outbreak was frozen mixed berries that had been frozen and processed in Italy (Terio et al., 2015). According to the Rapid Alert System for Food and Feed notifications, the number of norovirus and HAV outbreaks caused by frozen berries over the past decade has been increasing annually (Tavoschi et al., 2015).

HAV contamination may conceivably occur at any stage of the berry supply chain, including production, harvesting, processing, and distribution. Maunula et al. investigated the prevalence of enteric viruses at each stage of the berry fruit supply chain in European countries (irrigation water, animal feces, swabs of the hands of food handlers, swabs of the toilets on farms, conveyor belts at processing plants, and raspberries or strawberries at points of sale) (Maunula et al., 2013). They found that the hands of food handlers and water during the

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production phase were particularly susceptible to contamination by enteric viruses (Maunula et al., 2013).

Berries and other fresh produce do not undergo heating or other processing that would inactivate viruses before shipment. Therefore, to prevent HAV outbreaks from berry ingestion, HAV contamination must be impermissible at any stage from production to consumption.

Previous studies have found that heat-denatured egg white lysozyme has a inactivating effect on human norovirus GII.4 and murine norovirus strain 1 (MNV-1) in solution (Takahashi et al., 2015). The fact that heat-denatured lysozyme inactivates noroviruses has raised hopes that it may also be an effective inactivating agent for foodborne and waterborne viruses, but as yet it has not been shown to inactivate other types of viruses.

In this study, we investigated whether or not heat-denatured lysozyme is effective in deactivating HAV. We also inoculated the foodborne and waterborne HAV and MNV-1 (used as a surrogate for the human norovirus) to berries, and investigated whether or not heat-denatured lysozyme acted as an effective disinfectant on the fruits.

2. Materials and methods

2.1. Viruses and cells used

Three strains of HAV, namely HM175/18f (ATCC VR-1402), HM175 uncloned (ATCC VR-2093), and HAS15 (ATCC VR-2281), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Additionally, MNV-1 was used as a surrogate for human norovirus.

HAV was propagated with FRhK-4 cells that were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS) and penicillin (100 U/mL)–streptomycin (100 μg/mL). After the FRhK-4 cells had reached confluency, they were inoculated with HAV at a multiplicity of infection (MOI) of 0.1 and incubated for 6 days at 37 °C in 5% CO_2. After confirmation of the cytopathic effect, the cells were subjected 4 times to freezing and thawing, followed by centrifugation at 8000 × *g* for 20 min. The supernatant was used as the HAV solution and stored at − 80 °C until use in the experiments.

MNV-1 was propagated with RAW 264.7 cells that were cultured at 37 °C in 5% CO_2 in DMEM containing 10% FBS and penicillin (100 U/mL)–streptomycin (100 μg/mL). After the RAW264.7 cells had reached confluency, they were inoculated with MNV-1 at an MOI of 0.1 and then incubated for 3 days at 37 °C in 5% CO_2. After confirmation of the cytopathic effect, the cells were subjected 4 times to freezing and thawing, and then centrifuged at 8000 × *g* for 20 min. The supernatant was used as the MNV-1 solution and stored at − 80 °C until use.

2.2. Berry samples

In this study, fresh blueberry (*Vaccinium corymbosum*) and a mixture of berries containing *V. corymbosum*, strawberry (*Fragaria* × *ananassa*), and raspberry (*Rubus idaeus*) were used which were purchased from a grocery store.

2.3. Preparation of heat-denatured lysozyme

Heat-denatured lysozyme was prepared as previously reported (Takahashi et al., 2016). Egg white lysozyme (Kewpie Corporation, Tokyo, Japan) was dissolved in distilled water at a concentration of 1% and 2% (w/v, pH 6.5 ± 0.2) and filtered using a 0.20-μm filter. The resulting filtrate was heated in an oil bath at 100 °C for 40 min and then cooled on ice for use as the working solution of heat-denatured lysozyme.

2.4. Inactivation of HAV with heat-denatured lysozyme

First, the inactivating effect of heat-denatured lysozyme was tested on HAV HM175/18 f. Thirty microliters of the HAV solution (approximately 5 log MPN/mL) was mixed with 30 μL of the heat-treated lysozyme solution at 1% (w/v) or 2% to obtain the final concentration of 0.5% or 1%, and the mixtures were allowed to stand for 1 or 60 min. Following this, 60 μL of samples were diluted 10-fold with DMEM and the infectivity was then measured using the most probable number (MPN) method (described in Section 2.6).

Second, the inactivating effects of heat-denatured lysozyme against various strains of HAV were compared. Thirty microliters of HAV solution (HAV HM175/18f, HM175 uncloned, or HAS15) at approximately 5 log MPN/mL was mixed with 30 μL of heat-denatured lysozyme at 2% to obtain a final lysozyme concentration of 1%. The mixtures were allowed to stand for 1 or 60 min. Following this, they were diluted 10-fold with DMEM and subjected to the MPN method.

2.5. Inactivation of foodborne virus-contaminated berries with heat-denatured lysozyme

The blueberries (10 g) or mixed berries (20 g) were soaked for 2 min in 200 mL of HAV HM175/18f (at 6.1 log MPN/mL) or MNV-1 solution (at 6.2 log PFU/mL). After viral contamination of the berry samples, the contaminated berries were soaked for 1 min in 100 mL each of distilled water, or sodium hypochlorite at an effective chlorine concentration of 100 ppm, or 1% of heat-denatured lysozyme. After the soaking period, the contaminants were washed out with 10 mL of phosphate-buffered saline in a stomacher bag. The treated samples were centrifuged at 8000 × *g* for 10 min, and the supernatants were passed through a filter with a pore size of 0.20 μm. The resulting samples were immediately subjected to the determination of infectivity (described in Sections 2.6 and 2.7).

2.6. Determination of HAV infectivity by the most probable number method

The viral infectivity was determined by the MPN method with 95% confidence limits, using the 3-tube method. In brief, samples were serially diluted in 10% FBS-containing DMEM and then inoculated into 96-well plates at 200 μL/well in which FRhK-4 had been cultured until confluency. Then the inoculated plates were incubated at 37 °C in 5% CO_2 until development of cytopathic effect. The incubation period varied depending on the strain used, HM175/18f was incubated for 14 days, HM175 was for 28 days, and HAS15 for 21 days. After the incubation, the plates were washed with 100% methanol and the cells were stained with 5% crystal violet. Unstained cells were considered to be dead, indicating the presence of HAV. From the number of HAV-positive wells among three wells at the appropriate dilution, the MPN was determined according to a conversion table.

2.7. Determination of MNV-1 infectivity by plaque assay

The infectivity of MNV-1 was measured by plaque assay, using a method described previously (Gonzalez-Hernandez et al., 2012). RAW264.7 cells were seeded into 6-well plates (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to obtain a final concentration of 6 log cells/mL. The cells were then incubated for 18 h at 37 °C in 5% CO_2. Next, 500 μL of each of the prepared samples was inoculated into the plates containing RAW 264.7 cells, and the plates were rocked at room temperature for 1 h. Subsequently, the virus samples were layered with 2 mL of 1.5% SeaPlaque Agarose (Lonza Japan, Tokyo, Japan) in DMEM. These plates were incubated at 37 °C for 48 h in 5% CO_2.

Next, 2 mL of 0.03% neutral red solution (Sigma-Aldrich Japan, Tokyo, Japan) was added to each well of the culture plates, and the plates were incubated for 1 h at 37 °C in 5% CO_2. The plaques formed