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Thermal inactivation kinetics of hepatitis A virus in spinach

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ABSTRACT

Leafy vegetables have been recognized as important vehicles for the transmission of foodborne viral pathogens. To control hepatitis A viral foodborne illness outbreaks associated with mildly heated (e.g., blanched) leafy vegetables such as spinach, generation of adequate thermal processes is important both for consumers and the food industry. Therefore, the objectives of this study were to determine the thermal inactivation behavior of hepatitis A virus (HAV) in spinach, and provide insights on HAV inactivation in spinach for future studies and industrial applications. The D-values calculated from the first-order model (50–72 °C) ranged from 34.40 ± 4.08 to 0.91 ± 0.12 min with a z-value of 13.92 ± 0.87 °C. The calculated activation energy value was 162 ± 11 kJ/mol. Using the information generated in the present study and the thermal parameters of industrial blanching conditions for spinach as a basis (100 °C for 120–180 s), the blanching of spinach in water at 100 °C for 120–180 s under atmospheric conditions will provide greater than 6 log reduction of HAV. The results of this study may be useful to the frozen food industry in designing blanching conditions for spinach to inactivate or control hepatitis A virus outbreaks.

1. Introduction

Hepatitis A virus (HAV) is the leading cause of acute viral hepatitis which may occasionally be fatal. Thus, it constitutes a serious concern for public health authorities (Coudray-Meunier et al., 2013). Even though the effectiveness of HAV vaccine is high and overall HAV cases have declined, outbreaks still continue to occur (Kingsley and Chen, 2009). HAV is able to replicate in the human gastro-intestinal tract and is dispersed by shedding in high concentrations into the stool. The stability of HAV with regard to several physical stresses, such as low pH and elevated temperatures, contributes significantly to its persistence in the environment (D'Souza et al., 2007). Transmission of these viruses occurs by the fecal–oral route, primarily through direct person-to-person contact, but they are also efficiently transmitted by ingestion of contaminated drinking water or contaminated food (D'Souza et al., 2007).

The foods most likely to be contaminated by HAV are leafy vegetables, fruits, shellfish and ready-to-eat foods (i.e., those with no lethality step prior to consumption) (CDC, 2014a). Gould et al. (2013) investigated foodborne disease outbreaks in the United States from 1998 to 2008, and found that among individual food categories, leafy vegetables were the second most commonly reported food vehicle associated with foodborne illness, accounting for 13% of outbreaks. Leafy vegetables are often consumed raw or mildly heated (e.g., blanched) and thus may become vehicles for viral transmission if contamination occurs anywhere from farm to fork (Brassard et al., 2011). Therefore, for mildly heated leafy vegetables, such as those blanched prior to freezing, the application of a precise thermal process to inactivate HAV would improve the microbiological safety of the products. Recent foodborne outbreaks of HAV in frozen berries and pomegranate kernels also underline the need to investigate proper means to inactivate this virus in food products that will be frozen (CDC, 2014b).

In the current literature, several studies have investigated the survival of HAV in leafy vegetables such as lettuce (Bidawid et al., 2000, 2001; Croci et al., 2002; Fino and Kniel, 2008; Fraisse et al., 2011), green onions (Fino and Kniel, 2008; Laird et al., 2011; Sun et al., 2012), and parsley (Butot et al., 2008). Until now, there are limited studies (Hida et al., 2013; Jones et al., 2009; Shieh et al., 2009) involving the survival of HAV in spinach. Among these studies, only Shieh et al. (2009) investigated the survival of HAV in spinach at refrigeration temperature (5.4 ± 1.2 °C) for up to 42 days. To our knowledge, thermal inactivation kinetics of HAV in spinach has not been reported so far.

The first step in designing any thermal process is defining the thermal resistance of the target pathogen (Solomon et al., 2002). A precise understanding of thermal inactivation kinetics is potentially useful for optimizing thermal treatments to eliminate the risk associated with foodborne pathogens while avoiding over-processing of the food material and thus resulting in optimal energy utilization. Thus, generation of correct thermal process data and establishment of proper thermal processes for inactivating HAV are important both for consumers and industry. Therefore, the objective of this study was to determine the thermal inactivation behavior of hepatitis A virus in spinach in order to provide insights on HAV inactivation for future studies and industrial applications.

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2. Materials and methods

2.1. Viruses, cell lines, and propagation of viruses

Hepatitis A virus (HAV: strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware). FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1× Anti–Anti (Antibiotic–Antimycotic; Invitrogen, Grand Island, NY) at 37 °C in an atmosphere containing 5% CO_2.

FRhK4 cells with ~90% confluence in cell culture flasks were washed twice with phosphate-buffered saline (PBS; pH 7.4) before adding HAV stocks to these cell monolayers. The infected cells were then incubated in a water-jacket CO_2 incubator at 37 °C until >90% cell lysis. All viruses were recovered by centrifugation at 5000 × *g* for 10 min (Eppendorf Centrifuge, model 5804R, USA), followed by filtration through 0.2-μm filters (Nalgene, Fisher Scientific), aliquoted, and stored at −80 °C until use.

2.2. Inoculation of spinach

Samples of frozen chopped spinach were purchased from a local grocery store. The chopped spinach samples were homogenized (Waring Blender, Model 1063, Waring Commercial, USA). Five-milliliters of HAV stock with initial titers of 7.34 ± 1.28 log plaque forming units/ml (PFU/ml) were added to 25 ml of homogenized spinach sample in a sterile beaker and held at 4 °C for 24 h.

2.3. Thermal treatment

An aliquot (6 ml) of the homogenized inoculated spinach was added to moisture barrier plastic vacuum bags (13 cm × 19 cm). The inoculated samples were vacuum sealed in to −100 kPa (Multivac A300/16 vacuum-packaging unit, Sepp Haggemuller KG, Wolfertschwenden, Germany) and then the bags were flattened. The internal temperature of the spinach was monitored with a thermocouple placed at the geometric center of an uninoculated package of spinach (temperature control). The holding unit with the bags was immersed in a thermostatically controlled (±0.1 °C) circulating water bath (Haake model V26, Karlsruhe, Germany). The temperature of the water bath was monitored with T-type thermocouples (Omega Engineering, Inc., Stamford, CT), and also confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). The thermocouples were connected to a MMS3000-T6V4 type portable data recorder (Commtest Instruments, New Zealand) to monitor internal temperature of the control spinach sample. The samples were subjected to heating at 50, 56, 60, 65, and 72 °C for 0–6 min. The treatment time began when the internal temperature reached the desired target temperature. Triplicate bags were used for each time-point. After the thermal treatment, sample bags were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The bags were washed with ethanol before removal of the contents, placed in a biosafety cabinet and aseptically cut with sterilized scissors. Bag contents were removed and the inside of the bags were washed with elution buffer (15 ml) to remove the remaining sample. The unheated virus suspensions from spinach were used as controls and enumerated.

2.4. Virus extraction

Virus extraction was performed as described by Baert et al. (2008) with some modifications. Inoculated and thermally treated spinach was washed with 15 ml of elution buffer pH 9.5 (0.1 M Tris–HCl, 3% beef extract powder, 0.05 M glycine). The pH was adjusted to 9.5 using 10 M NaOH (Sigma-Aldrich, USA). The sterile beakers with the samples were kept on a shaking platform (120 rpm) for 20 min at 4 °C, and then transferred into a sterile stomacher bag with a filter compartment and homogenized at high speed for 60 s (Seward Stomacher 80, Biomaster, Seward Inc., FL). The filtrate obtained was centrifuged (10,000 ×*g* for 15 min at 4 °C, Eppendorf Centrifuge, model 5804R, USA), and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl (Sigma-Aldrich). Polyethylene glycol (PEG) 6000 (10%) and NaCl (0.3 M) were added. Samples were placed on a shaking platform (120 rpm) overnight at 4 °C and then centrifuged (10,000 ×g for 30 min at 4 °C). The supernatant was discarded and the pellet was dissolved in 1 ml PBS. Extracts containing the virus were stored at −80 °C until the HAV plaque assay was performed.

2.5. Enumeration of survivors by plaque assays

HAV plaque assays were conducted using confluent FHRK cells in 6-well plates. Ten-fold serial dilutions of treated and untreated samples in Dulbecco's Modified Eagle Medium (DMEM-F12) containing 2% fetal bovine serum were prepared and 0.5 ml of the dilution was inoculated into each well. Viruses were allowed to adsorb for 3 h at 37 °C in a CO_2 incubator. Cells were then overlaid with 2 ml of DMEM containing 1% agarose, 2% FBS and 1% Anti–Anti. Plates were incubated for 7 days and overlaid with a second overlay media containing 0.01% neutral red (Sigma). Plaques were counted after incubation at 37 °C for 7 days. Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

2.6. Modeling of inactivation kinetics

The traditional approach to describe the change in number of survivors over time for first-order kinetic model can be written as follows:

$$\ln N_{(t)} = \ln N_0 - kt \tag{1}$$

where $N_{(t)}$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml), and k as the first-order rate constant (1/s). This equation is then rearranged into:

$$\log_{10} \frac{N(t)}{N_0} = \frac{t}{D} \tag{2}$$

where D is the decimal reduction time (D = 2.303/k, units in min or s) and is thus actually a reciprocal first-order rate constant. The resulting semi-logarithmic curve when $\log N(t)/N_0$ is plotted vs. time is frequently referred to as the survival curve.

The definition of z-value is the number or degrees (F or °C) to cause 90% change in log D. According to this concept, when the survival curve is constructed by plotting logarithm of D-values versus exposure temperatures, z value will be equal to the slope of survival curve (Bigelow, 1921):

$$z = \frac{T_1 - T_2}{\log D_2 - \log D_1} \tag{3}$$

where D_2 and D_1 are the D-values corresponding to T_2 and T_1 respectively.The inactivation rate is primarily influenced by temperature, and the temperature dependence of the rate constant is typically described by the Arrhenius equation:

$$k = A \exp\left(-\frac{E_\alpha}{RT}\right) \tag{4}$$

where A is a frequency factor which is constant, E_a is the activation energy (J mol), R is the universal gas constant (8.314 J mol^{-1} K^{-1}), k is the rate constants (1/min), T is the absolute temperatures (K). The construction of ln $k_{(T)}$ versus *1/T*, the slope of the curve will be a straight line which equals to activation energy. This concept has been used to calculate activation energy of microbial inactivation (Corradini et al., 2005).