

## High-Pressure Inactivation of Hepatitis A Virus within Oysters

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Previous results demonstrated that hepatitis A virus (HAV) could be inactivated by high hydrostatic pressure (HHP) (D. H. Kingsley, D. Hoover, E. Papafragkou, and G. P. Richards, *J. Food Prot.* 65:1605–1609, 2002); however, direct evaluation of HAV inactivation within contaminated oysters was not performed. In this study, we report confirmation that HAV within contaminated shellfish is inactivated by HHP. Shellfish were initially contaminated with HAV by using a flowthrough system. PFU reductions of >1, >2, and >3 log<sub>10</sub> were observed for 1-min treatments at 350, 375, and 400 megapascals, respectively, within a temperature range of 8.7 to 10.3°C. Bioconcentration of nearly 6 log<sub>10</sub> PFU of HAV per oyster was achieved under simulated natural conditions. These results suggest that HHP treatment of raw shellfish will be a viable strategy for the reduction of infectious HAV.

Bivalve shellfish readily bioconcentrate microbial pathogens from marine and estuarine waters. While fecal coliforms and other pathogenic bacteria from human and animal wastes do not persist within shellfish tissues beyond a few days, enteric viruses such as hepatitis A virus (HAV) can persist in estuarine waters and within shellfish tissues for periods of several weeks or more (1, 4, 15, 28, 38). In fact, HAV and other viruses are readily identified in shellfish harvested in certain European regions (3, 10, 17, 29, 31). HAV-contaminated shellfish have caused significant outbreaks of human disease (5, 11, 22). Furthermore, global trade of virally contaminated shellfish has resulted in outbreaks and dissemination of HAV and other exotic virus strains to geographic areas where they are not endemic (5, 19, 27, 35, 36).

Once virus contaminated, there are limited postharvest options for inactivating infectious virus within shellfish while retaining the raw characteristics and high market value of the product. A commercial process called depuration, in which live shellfish stock are placed in tanks of clean seawater for periods of up to several days, is generally recognized as inadequate for purging HAV and other enteric viruses (10, 18, 21, 28, 38). Irradiation is of limited utility, because inactivation of enteric viruses requires relatively high levels of radiation that can negatively affect shellfish taste, appearance, and shelf life (16, 23). Presently, cooking is generally recognized as the only reliable mitigation method to sanitize shellfish potentially contaminated with HAV (13, 32). However, many shellfish consumers insist on eating raw shellfish or at least minimally cooked oysters, because cooking alters the organoleptic qualities of shellfish.

Recently, an alternate technology, high hydrostatic pressure

(HHP), has come to the forefront as a potential means for mitigating pathogens within raw shellfish. HHP is utilized commercially on the United States gulf and western coasts (Gold Seal Oysters Inc., Homa, La., and Nisbet Oyster Inc., Bay Center, Wash.) at pressures of up to 275 megapascals (MPa), principally because it can facilitate the oyster-shucking process and extend the shelf-life of raw oysters due to the reduction of spoilage bacteria (24). Organoleptic evaluations have shown that HHP-treated oysters are acceptable to consumers at treatment pressures as high as 400 MPa or approximately 60,000 lb/in<sup>2</sup> (30).

Experimental evidence suggests that HHP can inactivate some important bacterial pathogens. Berlin and coworkers (2) demonstrated that *Vibrio vulnificus*, *V. parahaemolyticus*, and *V. cholerae* were inactivated in artificial seawater by 15-min treatments at 250 MPa. In this same study, a 10-min, 200-MPa treatment of homogenized raw oysters inoculated with 10<sup>7</sup> CFU of either *V. vulnificus* or *V. parahaemolyticus*/g reduced the bacteria to levels of <10 CFU/g. Cook (12) has recently extended this work to demonstrate that naturally occurring *V. vulnificus* in both whole oysters (*Crassostrea virginica*) and oyster meat homogenates were reduced by ~5 log after pressure treatment at 241 MPa for 120 s.

With respect to viral pathogens, it was demonstrated that feline calicivirus, a norovirus surrogate, can be inactivated by HHP at pressures of 275 MPa (26) and that San Miguel sea lion virus 17 (SMSV-17), a second norovirus surrogate, was inactivated by HHP. In oyster homogenate, the titer of SMSV-17 was reduced by 0.04, 1.57, 3.35, and >3.97 log<sub>10</sub> PFU/ml at 200, 250, 275, and 300 MPa, respectively, when pressurized for 1 min (7).

For HAV, previous work using 5-min treatments has shown limited inactivation at 300 MPa in cell culture media. Treatments of 460 MPa resulted in a 7 log<sub>10</sub> reduction of HAV to nondetectable levels (26). In this study, we investigated the potential of HHP to inactivate HAV directly within shucked

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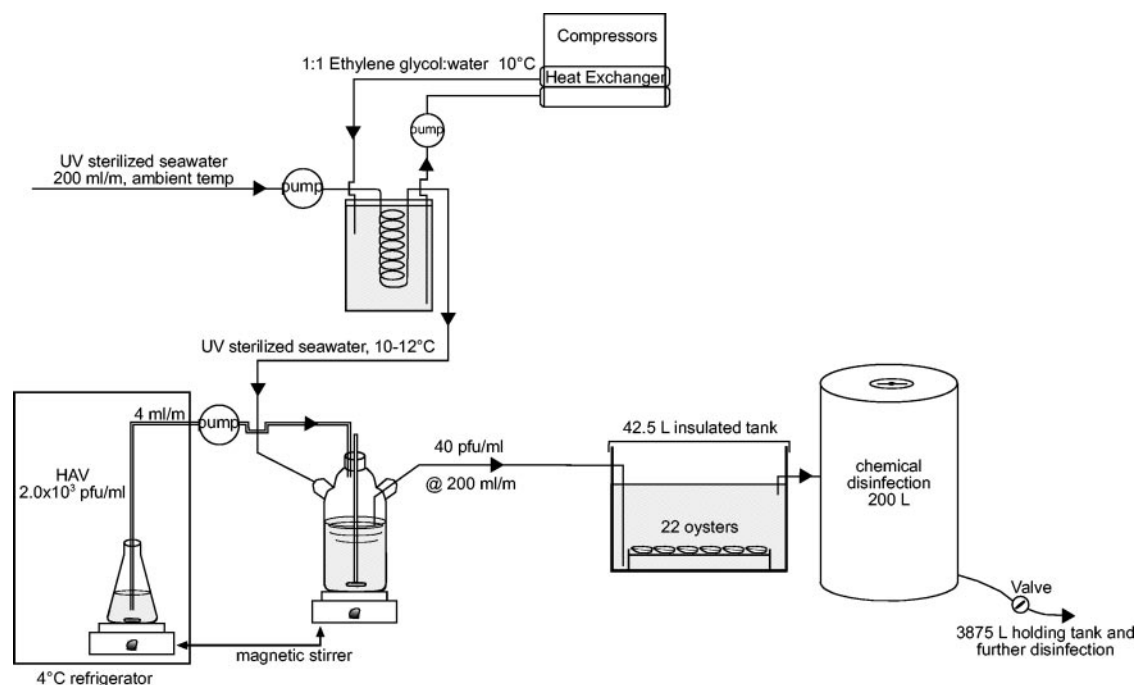


FIG. 1. Diagram of a seawater flowthrough unit designed for contamination of oysters with HAV. Oysters were exposed to HAV in groups of 22 oysters per trial. Three oysters were selected for each pressure treatment and assay.

oyster meats after contamination with HAV in a flowthrough natural seawater system.

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#### MATERIALS AND METHODS

**HAV and oysters.** HAV virus strain, HM-175, was propagated in fetal rhesus monkey kidney (FRhK-4) cells (14). Working stocks were propagated on confluent FRhK-4 cells in Eagle's minimum essential medium (MEM) supplemented with 15% fetal bovine serum, 15 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.11% sodium bicarbonate, 0.1 mg of kanamycin/ml, and 0.05 mg of gentamicin/ml (Invitrogen Corp., Carlsbad, Calif.). Cells were infected (multiplicity of infection [MOI] = 1) in medium containing 2% fetal calf serum, and after propagation for 2 weeks, lysates were obtained after three freeze-thaw cycles. This was followed by an extraction with equal volumes of chloroform. One-milliliter portions supplemented with 2% fetal bovine serum were frozen at  $-80^{\circ}\text{C}$ .

Eastern oysters (*C. virginica*) were harvested from an approved area in Mobile Bay, Ala. After culling and sorting, 200 commercial-size oysters were placed into a depuration flume at the U.S. Food and Drug Administration (FDA) Gulf Coast Seafood Laboratory, Dauphin Island, Ala. Oysters were maintained for more than 3 weeks prior to being transferred to a flume which utilized single-pass UV-treated natural seawater. Salinities ranged from 5 to 20 ppt.

Three days before virus accumulation, 22 oysters were placed in the accumulation tank to acclimatize to  $10^{\circ}\text{C}$ . This tank received UV-treated seawater which had been refrigerated to  $10^{\circ}\text{C}$  at the rate of 200 ml/min in laminar flow (Fig. 1). Temperature and flow conditions that optimize natural virus accumulation were previously determined by Burkhardt and Calci (6). HAV ( $7.1 \log_{10}$  PFU) was added to 6 liters of sterile RO (reverse osmosis) water and was continually mixed at  $4^{\circ}\text{C}$ . Peristaltic pumps (Cole-Parmer, Vernon Hills, Ill.) combined 4 ml of virus suspension/min with 200 ml of UV-treated seawater/min in a cytotir vessel (Kimble, Vineland, N.J.). The calculated overall concentration within the accumulation tank was 40 PFU/ml. After 24 h, the oysters were divided into six groups of three oysters each and were shucked into sterile cups. The total weight for each group was approximately 25 g.

**High-pressure treatment.** Shucked oyster samples were transferred into 4.5-ml Scotchpak pouches (Kapak 500, Minneapolis, Minn.) and heat sealed using an

Impulse Food Sealer (American International Electric Co., Whittier, Calif.) according to the manufacturer's instructions. An overpack 2-mm pouch was sealed over the inner pouch. Refrigerated, shucked oyster samples were packed in accordance with International Air Transport Association Dangerous Goods Shipping Regulations in a biohazard shipping container (STP 100; SAF-T-PAK, Alberta, Canada) and enclosed in an insulated carton with blue-ice packs to insure that the temperature remained at  $<10^{\circ}\text{C}$  during shipping. This was verified by including a continuous recording digital thermometer (RD-temperature; Omega, Stamford, Conn.) in several shipments. Shipments were by overnight carrier to the U.S. FDA, National Center for Food Safety and Technology, Summit Argo, Ill., for processing. Pressurization of oyster samples was carried out for 1 min using a Quintas Model QFP-6 high-pressure food processor (ABB Autoclave Systems, Inc., Columbus, Ohio). Samples were pressurized at 300, 325, 350, 375, and 400 MPa for 1 min at approximately  $9^{\circ}\text{C}$  using a 50/50 mix of water and ethylene glycol medium. The come-up times to reach final pressures and temperature parameters for each sample group are shown in Table 1. Pressure release time was almost immediate ( $<3$  s). After processing, the refrigerated samples were shipped overnight to the USDA Microbial Food Safety Research Unit at Dover, Del., for virus extraction and assay.

**Virus extraction and plaque assays.** Virus-contaminated shellfish (three shellfish per group) were removed from pressurized sealed pouches, placed in 50-ml conical tubes, and briefly centrifuged in a table top centrifuge to facilitate separation of oyster meat from oyster liquor. Uncontaminated (negative) and nonpressurized (0 MPa) HAV-contaminated (positive) controls were also tested.

TABLE 1. Physical parameters during HHP treatment

| Pressure (MPa) | Trials (n) | Mean temp, $^{\circ}\text{C}$ (SE) |            |            | CUT <sup>a</sup> (SE) |
|----------------|------------|------------------------------------|------------|------------|-----------------------|
|                |            | Initial                            | Maximum    | Final      |                       |
| 300            | 3          | 9.9 (0.3)                          | 17.7 (0.6) | 17.6 (0.5) | 79 (4.5)              |
| 325            | 3          | 9.3 (0.1)                          | 17.8 (0.3) | 17.7 (0.4) | 80 (1.2)              |
| 350            | 3          | 9.6 (0.7)                          | 18.5 (0.8) | 18.5 (0.8) | 83 (0.9)              |
| 375            | 3          | 9.1 (0.3)                          | 18.8 (0.5) | 18.8 (0.5) | 89 (1.5)              |
| 400            | 3          | 9.0 (0.3)                          | 19.6 (0.9) | 19.5 (0.9) | 94 (2.7)              |

<sup>a</sup> CUT, Come-up time or length of time in seconds needed to reach set pressure.

TABLE 2. Viable HAV detected after HHP treatment

| Pressure (MPa) | Average log <sub>10</sub> PFU (SE) | Log <sub>10</sub> PFU (SE) for trial no.: |             |             |
|----------------|------------------------------------|---|-------------|-------------|
|                |                                    | 1   | 2           | 3           |
| 0              | 5.82 (0.48)                        | 5.71 (0.07)                               | 5.29 (0.03) | 6.45 (0.01) |
| 300            | 5.58 (0.26)                        | 5.42 (0.19)                               | 5.37 (0.10) | 5.95 (0.02) |
| 325            | 5.04 (0.19)                        | 4.78 (0.09)                               | 5.16 (0.08) | 5.20 (0.07) |
| 350            | 4.54 (0.06)                        | 4.62 (0.06)                               | 4.49 (0.13) | 4.51 (0.03) |
| 375            | 3.50 (0.14)                        | 3.63 (0.14)                               | 3.3 (0.26)  | 3.56 (0.25) |
| 400            | 2.67 <sup>a</sup> (1.12)           | 2.82 (0.45)                               | <1.5        | 3.69 (0.12) |

<sup>a</sup> HAV was not detected in one trial, so the detection limit 1.5 log<sub>10</sub> was assumed.

Virus extractions were performed as described by Kingsley and Richards (28). Two milliliters of extract or 2 ml of 10-fold serial dilutions were made in Earle's balanced salt solution, and plaque assays were performed in triplicate using FRhK-4 cells as described by Richards and Watson (33).

**RT-PCR.** The glycine, polyethylene glycol, trireagent, poly(dT) magnetic bead (GPTT) method for viral RNA extraction was adapted from Kingsley and Richards (25). Essentially, 0.15 M Na<sub>2</sub>HPO<sub>4</sub> (pH 9.5) was substituted for glycine buffer for extraction of oyster meats, with the remainder of the procedure being the same. For reverse transcription-PCR (RT-PCR) amplification of HAV sequences, primers originally described by Robertson et al. (34), (+) 2949 5'TATTTGTCTGTACAGACAATCAG3' and (B) 3192 5'AGGAGGTG GAAGCACTTCATTGA5', were used. Conditions for RT-PCR and the use of heat-denatured HAV virions as positive RT-PCR controls were the same as those previously described (25). The 267-bp major amplification product was observed for HAV-contaminated samples in all groups after treatment. All uncontaminated oyster samples tested negative by RT-PCR for HAV.

**Analysis of data.** Data from three 1-min trials with HAV-contaminated shellfish (Table 2) were plotted as groups at 300, 325, 350, 375, and 400 MPa as a function of the logarithmic reduction in HAV titer compared to that of untreated samples (Fig. 2). Trend lines were determined using the curve-fitting graphing program Sigma Plot, version 8.02, for Windows (SPSS Inc., Chicago, Ill.). Analysis of slope covariance was performed using the SAS program (SAS Institute, Cary, N.C.). HAV titers were determined in triplicate. The average PFU obtained was determined, with standard error expressed logarithmically (Table 2).

RESULTS

Live oysters were contaminated with HAV to >10<sup>5</sup> PFU/oyster in three separate experimental trials. During each of three trials, HHP, ranging from 300 to 400 MPa in 25-MPa increments, was administered to five treatment groups of three oysters each for 1 min. In this study, maximal and final adiabatic temperature increases during pressure treatments were recorded and are shown in Table 1. Results indicate that there was an average of 10.6°C adiabatic heating effect at 400 MPa with maximal temperature not exceeding 20°C. As HAV is highly thermostable, readily resisting temperatures of >60°C, these results confirm that inactivation observed for HAV is not due to adiabatic heating during pressurization.

Virus was extracted from all groups by using phosphate buffer. Extracted HAV was assayed by plaque assay. Results for all three trials are shown in Table 2. Levels of HAV achieved within oyster meats, as measured by HAV extraction from three non-pressure-treated oyster samples per trial, averaged 5.82 log<sub>10</sub> extractable PFU. The average titer obtained at each pressure is displayed in Table 2 (three oysters/group) and as a log<sub>10</sub> reduction plot in Fig. 2. The detection limit for the cell culture assay was approximately 33 PFU/oyster group or 1.5 log<sub>10</sub>, as 6 ml of the original 200 ml of extract was assayed for viable virus. This value was assumed for virus extractions which yielded no detectable virus when constructing Table 2 and Fig. 2.

Viral RNA extraction via the GPTT procedure was performed on all shellfish extracts. All samples exposed to HAV tested positive by RT-PCR. All noncontaminated oysters tested negative for HAV by RT-PCR, indicating that oysters were originally free of HAV. It was previously demonstrated that pressure-treated HAV maintains the integrity of its RNA (26).

DISCUSSION

Although previous results demonstrated the potential effectiveness of HHP against HAV in cell culture media (26), it was necessary to directly confirm that HAV can be inactivated within the context of a contaminated oyster, because food matrix composition, such as fat content, water activity, and salt concentration, can affect HHP pathogen inactivation rates (9, 26, 37). Conceivably, HAV could be found in a number of different anatomical structures within the shellfish, such as being bound to shellfish mucus membranes, within the digestive gland contents, encased within phagocytic cells, or free floating within the open circulatory system of the bivalve. Results reported here with oysters contaminated with >10<sup>5</sup> PFU demonstrate that the virus is inactivated by HHP within these environments, as judged by a 3 log<sub>10</sub> reduction of virus at 400 MPa for 1 min. Results from experiments using oysters contaminated with ~10<sup>3</sup> to 10<sup>4</sup> PFU (data not shown) yielded no infectious virus after 400-MPa treatment, indicating that lesser concentrations of HAV can be completely inactivated within shellfish.

HHP inactivation results for HAV within oysters are similar to results obtained previously with 5-min treatments with a high-titer HAV stock in DMEM with 10% fetal bovine serum (26). The previous inactivation curve obtained is plotted as a

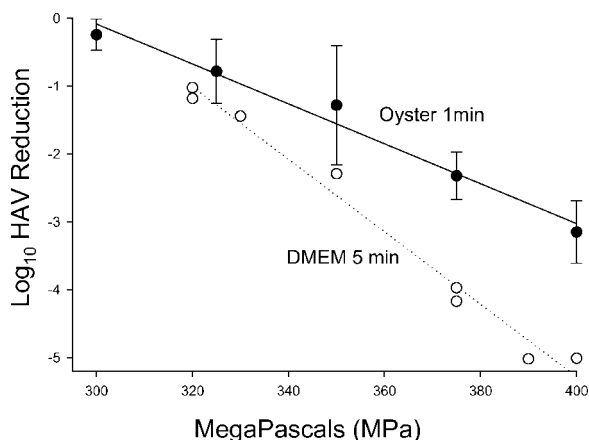


FIG. 2. Effect of pressure treatments on HAV within contaminated oysters. HAV-contaminated oysters were administered 1-min pressure treatments at pressures ranging from 300 to 400 MPa (dark circles, solid line). Average extractable HAV reductions are displayed graphically as a logarithmic reduction from three trials. HAV error bars represent standard errors for titer reductions from three trials. Reductions within oyster tissues are graphically compared with previously published results (26) for 5-min treatments of HAV in DMEM with 10% fetal bovine serum (dashed line, open circles). The two slopes displayed were determined to be significantly different (*P* < 0.05).



dashed line in Fig. 2. In both experiments, a linear inactivation curve was obtained when the  $\log_{10}$  of virus titer was plotted with pressure treatments above 300 MPa. For the HAV-contaminated oysters, the inactivation rate was not as great as that obtained previously with HAV in DMEM. Beyond the obvious oyster matrix and salinity differences, there may be a number of potential explanations for this. First, pressure treatments performed in the present study were for 1 min rather than 5 min. Although the pressure levels are the predominating factor determining the degree of inactivation of virus, it has been shown that increased treatment time at a given pressure will enhance the amount of virus inactivation observed (9, 26). Second, in this study oysters were pressurized at an initial temperature of 9°C for 1 min. In contrast to previous experiments with HAV (26), pressurization of samples was performed in an oil-based pressure unit at room temperature (21 to 22°C). The influence of temperature on HHP effectiveness against viruses has not been defined in this study.

For oysters, it is conceivable that physiological or environmental factors, such as water salinity, may alter HHP effectiveness. Shellfish, unlike marine vertebrates, do not osmoregulate, hence their intracellular ionic strength mimics that of the estuarine or marine environment. *C. virginica* oysters are indigenous to areas with salinities ranging from 5 to 30 ppt, and salt content can vary considerably depending on the harvest area or can even vary within the same harvest area due to fluctuations in rainfall. Oysters tested here were from Mobile Bay, Ala., a low-salinity estuary, and they were maintained in approximately 5- to 20-ppt-salinity seawater.

Unlike experiments performed here, commercial high-pressure processes use whole shellfish (within shell) rather than shucked product. It was not possible to use whole HAV-contaminated oysters due to the potential of the sharp shell edges to puncture the containment bags. No appreciable differences of inactivation between shucked meats and whole-shell oysters are envisioned, because HHP is uniformly applied and the bivalve's shells cannot form a protective airtight seal.

Although the molluscan bivalve's ability to concentrate viruses and bacteriophages from contaminated water is well documented, use of the flowthrough unit with natural estuarine water confirms that oysters can and do concentrate HAV to quite high levels (above 5  $\log_{10}$ ) in a relatively short period of time (24 h). In fact, it has been suggested that virus bioconcentration rates from contaminated water to shellfish tissues can be as high as 1,000-fold on a per-gram basis (8, 20). Actual levels of HAV and other viruses achieved during natural contamination events would be a function of virus concentration within the contaminated water, duration of exposure, shellfish pumping, and metabolic rates, as well as virus inactivation rates within oyster tissues and the water column.

The quantity of virions, or PFU, that constitute an infectious dose or the amount of HAV in shellfish typically associated with outbreaks have not been determined. It would seem probable that shellfish legally harvested from approved growing areas would not be grossly contaminated. Presumably, a logarithmic reduction in PFU after HHP treatment would result in a concomitant reduction in infectious dose. Therefore, high-pressure treatment capable of reducing infectious doses 1,000-fold (3  $\log_{10}$ ) would probably be sufficient to render all but the most highly contaminated shellfish safe for consumption with-

out cooking. Human fecal viruses do not replicate within shellfish tissues, therefore virus levels cannot increase as a result of temperature abuse after harvest or HHP treatment.

While we view application of HHP technology to raw shellfish as an important potential means of inactivating HAV and perhaps other pathogens, we do not envision HHP as a direct alternative to proper shellfish harvest water classification under the U.S. National Shellfish Sanitation Program or the European Union shellfish fecal coliform meat standard. Rather, we suggest that HHP, applied in addition to present sanitation standards, could provide an added measure of safety to shellfish designated for raw consumption and/or cooking.

In summary, this study demonstrates that HAV can be inactivated within the environmental context of the oyster and suggests that this technology has strong potential as an intervention strategy for shellfish sporadically contaminated with HAV. Additional studies evaluating HHP effectiveness of several types of shellfish from different water salinities, physiological states, and seasons may be necessary. Lastly, a means of directly demonstrating HHP effectiveness against norovirus would be especially beneficial.

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Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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