Inactivating Effect of Heat-Denatured Lysozyme on Murine Norovirus in Bread Fillings

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In this study, we investigated the viability of murine norovirus strain 1 (MNV-1), a surrogate for human norovirus, in bread fillings used for making stuffed buns and pastries. The inactivating effect of heat-denatured lysozyme, which was recently reported to have an antiviral effect, on MNV-1 contaminating the bread fillings was also examined. MNV-1 was inoculated into two types of fillings (chocolate cream, marmalade jam) at 4.5 log PFU/g, and the bread fillings were stored at 4°C for 5 days. MNV-1 remained viable in the bread fillings during storage. However, addition of 1% heat-denatured lysozyme to the fillings resulted in a decrease of MNV-1 infectivity immediately after inoculation, in both fillings. On the fifth day of storage, MNV-1 infectivity was decreased by 1.2 log PFU/g in chocolate cream and by 0.9 log PFU/g in marmalade jam. Although the mechanism underlying the anti-norovirus effect of heat-denatured lysozyme has not been clarified, our results suggest that heat-denatured lysozyme can be used as an inactivating agent against norovirus in bread fillings.

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Key words: murine norovirus; heat-denatured lysozyme; inactivation; bread; food poisoning

Introduction

Norovirus is a non-enveloped virus belonging to the family Caliciviridae, and causes severe diarrhea, vomiting, abdominal pain, etc1. Over half of patients involved in food-poisoning outbreaks in Japan are infected with norovirus2. Further, norovirus is extremely infectious, and since it can infect humans with as little as 18 viral particles3, it can easily cause large outbreaks in hospitals, schools, restaurants, etc. Bivalves such as oysters are the primary food product responsible for norovirus outbreaks4–6. This is because when bivalves feed on plankton in seawater, they simultaneously ingest norovirus from seawater and concentrate it in their bodies.

On the other hand, many outbreaks of norovirus caused by transmission from infected humans have been reported in recent years. Indeed, cross-contamination of norovirus from food handlers in food manufacturing and processing has become a major problem, and in many cases of norovirus gastroenteritis, a food handler was thought to be the source of norovirus infection7,8. For example, in January 2014, a large outbreak of norovirus caused by bread provided as part of school meals occurred in Hamamatsu city, Shizuoka, Japan, involving 1,271 people, including elementary school children. As a result of on-site inspection by the Hamamatsu city public health center, norovirus GII was detected in the bread provided in the school meal and in workers at the bread factory. Thus, norovirus contamination of the bread from worker(s) at the bread factory was determined to be the cause of this outbreak8.

In the past 10 years, there have been 8 outbreaks of norovirus caused by bread in Japan9. There is also a report on an outbreak of norovirus caused by a baker in the Netherlands10. Although a recovery method of norovirus from bread products with real-time PCR has been developed in order to improve the detection of norovirus11, relatively little is known about the behavior of norovirus contaminating bread or its ingredients.

In a previous study, the viability of norovirus on bread products was investigated using murine norovirus strain 1 (MNV-1) as a surrogate for human norovirus. MNV-1 inoculated on bread remained viable at 20°C for up to 5 days, showing high survivability. Baking of the bread dough contaminated with MNV-1 led to MNV-1 inactivation by heat, but it was suggested that MNV-1 could survive at low heating temperatures. From these data, it was concluded that there is a risk of norovirus outbreaks due to contaminated bread products, even though such products are considered as having a low risk of bacterial food poisoning12.

In the present study, the viability of murine norovirus in bread fillings was investigated to understand the risk of norovirus contamination in the bread-manufacturing...
process. Fillings such as jam and fresh cream for pastries and the ingredients for stuffed buns are added after baking the bread dough, and there is no heating process after adding the fillings. Therefore, norovirus might remain viable if contamination occurred during the process of adding the filling.

In this study, we also evaluated whether heat-denatured lysozyme is effective for norovirus inactivation in bread fillings. Lysozyme hydrolyses peptidoglycans, which form the cell walls of gram-positive bacteria. Normally, it is used as a protective enzyme against gram-positive bacteria, and it is also used in manufacturing drugs and food additives. Our previous study showed that heat-denatured lysozyme has an inactivating effect against norovirus. In this study, therefore, we examined its inactivating effect against MNV-1 in bread fillings.

Materials and Methods

1. Bread fillings used in this study

Two types of bread fillings (chocolate cream, marmalade jam) were purchased from a retail store (Tokyo, Japan). The pH value and water activity of these fillings were measured by using pH meter (HM-25R, DKK-TOA Corp., Tokyo, Japan) and water activity meter (AQUALAB CX-3, Decagon Devices, WA, USA), respectively (Table 1).

<table>
<thead>
<tr>
<th>Fillings</th>
<th>pH</th>
<th>Water activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate cream</td>
<td>6.53</td>
<td>0.864</td>
</tr>
<tr>
<td>Marmalade jam</td>
<td>3.21</td>
<td>0.827</td>
</tr>
</tbody>
</table>

2. Viruses and cells

MNV-1 was used as a surrogate for human norovirus in this study. MNV-1 is a culturable virus belonging to the family Caliciviridae, and is genetically similar to human norovirus compared to other surrogates, so it is preferable for use as a surrogate virus.

Mouse macrophage cells (RAW264.7) were cultured at 37°C in 5% CO₂ 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 RAW264.7 cells had reached confluence, they were inoculated with MNV-1 at a multiplicity of infection (MOI) of 0.1 and then incubated for 3 days at 37°C in 5% CO₂. After confirmation of the cytopathic effect, the cells were subjected to freezing and thawing four times, and centrifuged at 8,000×g for 20 min. The supernatant was used as MNV-1 solution and was stored at −80°C until use.

3. Viability of MNV-1 in bread fillings

One milliliter of MNV-1 solution at a concentration of approximately 5.5 log plaque forming units (PFU)/mL was inoculated into 10 g of bread fillings (chocolate cream and marmalade jam). The bread fillings were then stored at 4°C for 5 days after stomacher treatment at 230 rpm for 30 s.

Samples were collected on day 0 (immediately after inoculation and stomacher treatment), days 1, 3, and 5. Phosphate-buffered saline (PBS) (90 mL) was added to each 10 g of bread filling, followed by stomacher treatment at 230 rpm for 30 s. The treated samples were centrifuged at 8,000×g for 10 min, and the supernatants were filtered with a CA filter the pore size of 0.20 μm. The resulting samples were serially diluted 10-fold in DMEM, and stored at −80°C until used for plaque assay (described in Section 5).

4. Treatment of MNV-1 in bread fillings with heat-denatured lysozyme

Heat-denatured lysozyme was prepared as previously described. Egg-white lysozyme (Kewpie Corporation, Tokyo, Japan) was dissolved in distilled water at a concentration of 5% (w/v, pH 6.5±0.2) and filtered using a 0.20 μm filter. The filtrate was heated in an oil bath at 100°C for 40 min and then cooled on ice to prepare the heat-denatured lysozyme.

After preparation, 5% heat-denatured lysozyme was added to 160 g of bread fillings (chocolate cream and marmalade) to give a final concentration of 1%. The prepared bread fillings were weighed, and 10 g of each filling was inoculated with 1 mL MNV-1 solution at an approximate concentration of 5.5 log PFU/mL, followed by storage at 4°C for 5 days after stomacher treatment at 230 rpm for 30 s.

Samples were collected on days 0, 1, 3, and 5 of storage. PBS (90 mL) was added to 10 g of the bread fillings, and the mixture was subjected to stomacher treatment, centrifugation, filtration, and serial dilution according to the procedure described above. The samples were stored at −80°C until used for plaque assay (Section 5).

5. Plaque assay

MNV-1 infectivity was measured by plaque assay as reported previously. RAW264.7 cells were seeded into 6-well plates ( Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ) at a final concentration of 6 log cells/mL and incubated for 18 h at 37°C in 5% CO₂.

Then, 500 μL of each of the prepared samples was inoculated into the incubated plates, and the plates were shaken at room temperature for 1 h. The virus samples were layered over the cells with 2 mL of 1.5% SeaPlaque Agarose (Lonza Japan, Tokyo, Japan)+DMEM, and the plates were further incubated for 48 h at 37°C in 5% CO₂.

After the incubation, 2 mL of 0.03% neutral red solution (Sigma-Aldrich Japan, Tokyo, Japan) was added to each well of the cultured plates. After further incubation for 1 h at 37°C in 5% CO₂, the plaques were counted.

6. Statistical analysis

All the experiments were performed in triplicate (n=3), and the results are shown as the mean value ± standard deviation. Experimental groups were
compared by sampling days, and the significance of differences was evaluated with Tukey’s test, conducted in Microsoft Excel.

Results and Discussion

1. Viability of MNV-1 in bread fillings

In response to the large outbreak of norovirus caused by contaminated bread in 2014 in Shizuoka, Japan, the viability of norovirus at the stage of bread consumption was investigated using MNV-1 as a surrogate for human norovirus\(^{12}\). Bread has been recognized as a low-risk of bacterial food poisoning because of its low water activity. However, our data suggested that norovirus contamination in bread products could result in food poisoning\(^{19}\).

In the present study, we focused on bread fillings that are added after the baking stage in the bread-manufacturing process. Two types of fillings (chocolate cream and marmalade jam) were inoculated with MNV-1 at 4.5 log PFU/g, and stored at 4°C for 5 days. We found that MNV-1 infectivity hardly decreased during this storage period in either filling, and the level of inoculation was maintained (\(p > 0.05\)) (Figs. 1, 2).

Measurements of pH yielded values of 6.53 for the chocolate cream and 3.21 for the marmalade jam (Table 1). MNV-1 was not inactivated in the marmalade jam despite its comparatively low pH (Fig. 2).

There are multiple reports on the viability of norovirus describing resistance to a wide pH range from 2 to 7\(^{10}\), and high stability in cut vegetables frozen at -21°C\(^{20}\) and in salads stored for five days at 4°C\(^{21}\). These reports indicate that norovirus is stable in various food products, and are consistent with the present findings.

2. Treatment of MNV-1 in bread fillings with heat-denatured lysozyme

Chocolate cream and marmalade jam added with 1% heat-denatured lysozyme were inoculated with MNV-1 and then stored at 4°C for 5 days. In chocolate cream, the infectivity of MNV-1 was decreased by 1.6 log PFU/g immediately after inoculation. By day 5, MNV-1 infectivity had decreased by 1.2 log PFU/g (\(p < 0.05\)) (Fig. 1). In marmalade jam, the infectivity of MNV-1 was decreased by 0.8 log PFU/g immediately after inoculation, and by day 5, it was decreased by 0.9 log PFU/g (\(p < 0.05\)) (Fig. 2).

Previous studies have confirmed that MNV-1 decreases by 4.5 log PFU/mL after exposure to heat-denatured lysozyme solution at 1%\(^{14}\). Further, a sufficient inactivation effect was obtained with 1% heat-denatured lysozyme in vegetable salads\(^{21}\). Therefore, we used 1% heat-denatured lysozyme in this study. Although a decrease in the infectivity of MNV-1 was observed in both chocolate cream and marmalade jam, the decrease was about 0.8–1.6 log, which was less than that seen in heat-denatured lysozyme solution (Figs. 1, 2).

In the previous study on salads\(^{21}\), we found that the infectivity of MNV-1 in vinaigrette salad and thousand-island salad was decreased to below the detection limit by 1% heat-denatured lysozyme, while in coleslaw salad, a maximum decrease of 3.0 log PFU/g was observed. On the other hand, the infectivity titer of MNV-1 hardly decreased in egg salad\(^{21}\). Thus, some ingredients in foods such as salads and bread fillings may protect MNV-1 from inactivation. In this study, heat-denatured lysozyme was added to bread fillings as an aqueous solution. However, in order to improve the effect further, it may be necessary to clarify the mechanism of inactivation and optimize the method of adding the heat-denatured lysozyme.

Previous reports have described norovirus inactivation using food-derived components, and grape seed extract, citric acid, etc. reportedly show a norovirus-inactivating effect\(^{20, 23}\). Compared to these food-derived components, heat-denatured lysozyme can be considered to have a potent inactivating effect, since exposure to 1% heat-denatured lysozyme solution at 1°C for 5 days. MNV-1 was inoculated into chocolate cream (4.5 log PFU/g) containing or not containing heat-denatured lysozyme, and the filling was stored at 4°C for 5 days. Values of MNV-1 titer are expressed as mean ± S.D. (n = 3).

![Fig. 1](image1.png) **Fig. 1.** Survival of MNV-1 in chocolate cream with (●) or without (○) heat-denatured lysozyme

MNV-1 was inoculated into chocolate cream (4.5 log PFU/g) containing or not containing heat-denatured lysozyme, and the filling was stored at 4°C for 5 days. Values of MNV-1 titer are expressed as mean ± S.D. (n = 3).

![Fig. 2](image2.png) **Fig. 2.** Survival of MNV-1 in marmalade jam with (●) or without (○) heat-denatured lysozyme

MNV-1 was inoculated into marmalade jam (4.5 log PFU/g) containing or not containing heat-denatured lysozyme and the filling was stored at 4°C for 5 days. Values of MNV-1 titer are expressed as mean ± S.D. (n = 3).
denatured lysozyme solution decreases MNV-1 by 4.5 log PFU/mL\textsuperscript{14}.

An advantage of using heat-denatured lysozyme as a viral inactivation agent is that it does not affect the flavor when added to food, because it is is flavorless and odorless\textsuperscript{15}. In addition, lysozyme is industrially extracted from albumin and is a safe substance approved for use as an additive in foods and medicines. Thus, heat-denatured lysozyme would be a practical inactivation agent against norovirus.

In conclusion, our results show that heat-denatured lysozyme has an inactivating effect on MNV-1 in bread fillings. Since it does not affect the flavor of food and is inexpensive, it could have wide applicability as a viral inactivating agent. Although it is currently added to foods as an aqueous solution, we are planning to investigate its efficacy when added in other forms, e.g., as a solid.

References