Mechanism of the Decrease of Tetrodotoxin Activity in Modified Seawater Medium

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This study was designed to clarify the mechanism of the decrease of tetrodotoxin (TTX) toxicity during storage in a modified seawater medium (MSWM). When TTX was added to sterilized MSWM, the toxicity of TTX in the medium markedly decreased within 1 day, as determined by a mouse bioassay. HPLC (high-performance liquid chromatography) analysis showed that the peak of TTX was reduced and new unidentified peaks were observed. Omission of the P-1 metal solution from MSWM suppressed the decrease in TTX toxicity and the disappearance of TTX. Further studies indicated that boric acid in the P-1 metal solution triggers this toxicity decrease, indicating that TTX is chemically, not microbiologically, converted to unknown compounds in MSWM.

Key words: boric acid; activity loss; tetrodotoxin; medium

Introduction

Puffer fish is commercially important and is consumed as a popular dish by Japanese because of its distinctive taste and texture. However, food poisoning incidents caused by its ingestion are reported every year in Japan, since it contains a potent neurotoxin called tetrodotoxin (TTX). In food poisoning cases, respiratory paralysis and reduction in blood pressure generally occur in victims, and stop-gap measures are used for treatment. Some drugs have been used to counteract TTX toxicity in mice. For example, cysteine was reported to be effective1. Shimada et al. reported that sodium salts of mono- and dicarboxylic acids progressively decrease TTX activity with increasing molecular weight2. Amino acids, carbohydrates, sodium chloride and sodium bicarbonate were also reported to counteract TTX toxicity3-5. However, the mechanisms involved have not been established yet.

We have recently investigated TTX-degrading bacteria in a Japanese traditional food, fermented puffer fish ovary6,7. In the manufacturing process, raw puffer fish ovaries are first salted for at least half a year. Then, the salted ovaries are pickled in rice bran for more than 2 years. This product is safely consumed as a local Japanese delicacy. To our knowledge, no food poisoning cases due to fermented puffer fish ovary have been reported. Migita and Hashimoto8 and Ozawa9 found that the toxicity of this product decreases during the preparation process, and suggested that TTX in these ovaries diffuses out and become diluted within the fermentation container. Moreover, they suggested that some microorganisms may possibly decrease the toxicity during the manufacture of pickled ovary. However, our recent studies6,7 showed no evidence of a distinct toxicity decrease caused by rice-bran-paste microorganisms, implying the involvement of chemical detoxification but not microbiological detoxification. Furthermore, in preliminary experiments in vitro we found that the toxicity of TTX in some broth media incubated under sterile and acidic conditions was reduced during enrichment culture for the purpose of screening TTX-decomposing bacteria in fermented ovary.

In this study, we examined the factors responsible for this unique decrease in toxicity, and found that boric acid is responsible for the toxicity decrease of TTX in broth media.

Materials and Methods

Reagents

The TTX used in this study was purchased from Wako Corporation (Tokyo, Japan). All other reagents were of analytical grade.

Toxin assay and pH measurement

The toxin bioassay was performed according to the Japanese standard method for TTX determination9. In all experiments, an aliquot of each broth medium was diluted to 1/40 with distilled water, which is enough to kill a mouse in approximately 10 min after intraperitoneal injection. A one-mL aliquot of each sample was injected into male ddY mice weighing approximately 20 g, and five mice were sacrificed for each test. Median death time was determined to calculate the toxicity score as mouse unit (MU). One MU is defined as the amount of toxin required to kill a mouse of 20 g
Then, only one component, either H₃BO₃, MnCl₂, ZnCl₂, 1metal solution was removed from the TTX-MSWM. the toxicity of each medium was examined. First, the P- components were removed from the TTX-MSWM and the basal medium containing TTX, several medium conditions to approximately 6.3 using PIPES bu

The changes of TTX toxicity were examined in TTX-MSWM as determined by the mouse bioassay, although the pH of the medium at each step was maintained at weakly acidic levels, being almost the same as that of the distilled water control. The initial toxicity of the TTX-MSWM was determined to be 69 MU/mL. After 1-day storage, the mouse death time was more than 30 min and the toxicity was calculated to be less than 40 MU/mL, which was the detection limit in this experiment.

The HPLC chromatograms are shown in Fig. 1. Initially, TTX in the TTX-MSWM was eluted at a retention time of 13.8 min and calculated to be equivalent to 27 MU/mL, although the others remain unidentified. The chromatograms of TTX-MSWM stored for 1 and 4 weeks were similar to that of TTX-MSWM stored for 1 day. HPLC analysis suggested the conversion of TTX to unidentified substances with presumably lower toxicity.

The toxicity of the initial TTX-MSWM was determined to be 107 MU/mL by the HPLC method and 69 MU/mL by the mouse bioassay. This difference in toxicity is thought to be due to the effect of coexisting substances in the injected samples. As reported previously, the death time of mice is prolonged by the coexistence of substances such as mineral salts, glucose, carboxylic acids, and amino acids; hence, the toxicity is underestimated. It is considered that TTX absorption onto capillaries in the abdominal cavity is

Table 1. Changes in the Toxicity Determined by Mouse Bioassay and in the pH of Various Media during Storage under Laboratory Conditions

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<tbody>
<tr>
<td>TTX-DW</td>
<td>107</td>
<td>82</td>
</tr>
<tr>
<td>pH</td>
<td>6.25</td>
<td>6.26</td>
</tr>
<tr>
<td>TTX-MSWM</td>
<td>69</td>
<td>ND</td>
</tr>
<tr>
<td>pH</td>
<td>6.36</td>
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<tr>
<td>TTX-(MSWM without P-1 metal solution)</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>pH</td>
<td>6.37</td>
<td>6.35</td>
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<tr>
<td>H₃BO₃-TTX-(MSWM without P-1 metal solution)</td>
<td>43</td>
<td>ND</td>
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<tr>
<td>pH</td>
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</tr>
<tr>
<td>H₃BO₃-TTX-DW</td>
<td>67</td>
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ND: Not detected (<40 MU/mL)
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Table 1 shows the changes in the toxicity and pH of the TTX-MSWM under laboratory conditions. First, we observed a marked toxicity decrease during storage in TTX-MSWM as determined by the mouse bioassay, although the pH of the medium at each step was maintained at weakly acidic levels, being almost the same as that of the distilled water control. The initial toxicity of the TTX-MSWM was determined to be 69 MU/mL. After 1-day storage, the mouse death time was more than 30 min and the toxicity was calculated to be less than 40 MU/mL, which was the detection limit in this experiment.

The HPLC chromatograms are shown in Fig. 1. Initially, TTX in the TTX-MSWM was eluted at a retention time of 13.8 min and calculated to be equivalent to 107 MU/mL (Fig. 1(A)). On the other hand, the chromatogram of the sample stored for 1 day showed three peaks at retention times of 8.3, 14.3 and 17.5 min (Fig. 1 (B)). The middle peak coincided with that of authentic TTX and was equivalent to 27 MU/mL, although the others remain unidentified. The chromatograms of TTX-MSWM stored for 1 and 4 weeks were similar to that of TTX-MSWM stored for 1 day. HPLC analysis suggested the conversion of TTX to unidentified substances with presumably lower toxicity.

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Medium for study of TTX toxicity decrease

The changes of TTX toxicity were examined in MSWM, which is a modified mineral medium based on seawater. MSWM was prepared using modified artificial seawater. The modified artificial seawater contained high salt concentrations, because fermented puffer fish ovary is a high-salt food product. This medium contained 11.6 mg of Na₂EDTA, 170 mg of NaNO₃, 0.227 mg of ZnCl₂, 1.19 mg of CuCl₂, and 2 mL of P-1 metal solution containing 3 mol/L NaOH. The pH of the media was directly measured with a glass electrode (Model D-12, Horiba, Kyoto).

Changes in toxicity during storage under various storage conditions

To clarify the mechanism of the toxicity decrease in the basal medium containing TTX, several medium components were removed from the TTX-MSWM and the toxicity of each medium was examined. First, the P-1 metal solution was removed from the TTX-MSWM. Then, only one component, either H₃BO₃, MnCl₂, ZnCl₂, CoCl₂ or CuCl₂, was added to the TTX-MSWM without the P-1 metal solution. Finally, TTX and H₃BO₃ were dissolved in sterilized water to investigate the effect of H₃BO₃.

Results and Discussion

Table 1 shows the changes in the toxicity and pH of the TTX-MSWM under laboratory conditions. First, we observed a marked toxicity decrease during storage in TTX-MSWM as determined by the mouse bioassay, although the pH of the medium at each step was maintained at weakly acidic levels, being almost the same as that of the distilled water control. The initial toxicity of the TTX-MSWM was determined to be 69 MU/mL. After 1-day storage, the mouse death time was more than 30 min and the toxicity was calculated to be less than 40 MU/mL, which was the detection limit in this experiment.

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inhibited by inorganic salts. If the inhibition of TTX absorption onto capillaries is primarily associated with the toxicity decrease, the toxicity decrease should not depend on storage time. However, in this study, a toxicity decrease of TTX-MSWM with time was observed, and could not have been due to biological effects because the experiments were performed under sterile conditions.

To determine the cause of the toxicity decrease of the TTX-MSWM, we performed omission and addition tests of several components in MSWM. When TTX-MSWM without the P-1 metal solution was stored, the toxicity remained constant irrespective of storage time, although the initial toxicity of this medium was 59 MU/mL in the mouse bioassay. HPLC results showed that TTX is not affected during storage in the TTX-MSWM without the P-1 metal solution (Fig. 2). This result indicates that the P-1 metal solution triggers this toxicity decrease. Then, either $\text{H}_3\text{BO}_3$, MnCl$_2$, ZnCl$_2$, CoCl$_2$ or CuCl$_2$ was added to TTX-MSWM without the P-1 metal solution to examine which factor is responsible for the toxicity decrease. As shown in Table 1, the addition of $\text{H}_3\text{BO}_3$ to the medium resulted in a significant decrease in toxicity in the mouse bioassay, although the other four salts showed results similar to those without the P-1 metal solution.

Figure 3 shows the HPLC results for TTX-MSWM without the P-1 metal solution, but with one of the five inorganic salts. The medium containing $\text{H}_3\text{BO}_3$ yielded a considerable amount of a substance with a peak at a retention time of 7.9 min, besides TTX with a retention time of 14.4 min (Fig. 3(B)). In contrast, the other four salts did not affect TTX on the chromatograms (Fig. 3(C), (D), (E) and (F)). Therefore, these results revealed that $\text{H}_3\text{BO}_3$ in the P-1 metal solution is the main cause of the toxicity decrease during storage of the TTX-MSWM, and that it critically affects TTX activity.

Finally, we examined if only $\text{H}_3\text{BO}_3$ affects TTX toxicity. The initial toxicity of $\text{H}_3\text{BO}_3$-TTX-distilled water was 81 MU/mL and the final toxicity (after 4 weeks) was 63 MU/mL. The toxicity decrease of the $\text{H}_3\text{BO}_3$-TTX-distilled water during storage was less than...
that of the TTX-MSWM without the P-1 metal solution, to which H₃BO₃ was added. The HPLC chromatograms in Fig. 4 support the result of the toxicity assay and show less degradation of TTX.

As shown in this experiment, the TTX toxicity was reduced by H₃BO₃ in TTX-MSWM. To our knowledge, this is the first report of such an effect of H₃BO₃, although there have been previous reports on TTX-counteracting substances such as bicarbonate⁴,⁵, cysteine¹, etc. The decrease of TTX activity by bicarbonate is applied in the manufacture of Japanese fermented puffer fish ovary, and is assumed to be due to alkalization¹². Fujii et al.¹¹ reported that cysteine adds to the lactone ring in the TTX molecule to decrease the toxicity of TTX, it also decreases hydroxyl radical formation. Cysteine was reported to decrease the TTX activity to approximately 70% of the initial toxicity².

From our results, we can consider two possible reasons for the toxicity decrease of the TTX-MSWM. One is a chemical transformation of TTX to TTX derivatives that exhibit a weaker toxicity, or the degradation of the TTX structure. The other is interaction between TTX and H₃BO₃ or its ions in the medium. Negative ions may electrostatically interact with TTX to neutralize TTX toxicity. However, there are several negative ions in MSWM, so it is not clear why the decrease of
TTX toxicity was specific to H₃BO₃, if this explanation is the case. Moreover, there is as yet no clear explanation as to why H₃BO₃ alone less effectively decreased TTX toxicity. The synergistic actions of a variety of coexisting inorganic salts may be necessary to inhibit the lethal toxicity of TTX against mice.

Recently, we have performed experiments to clarify the toxicity decline during the manufacture of fermented puffer fish ovary. Our results demonstrated that the decrease of toxicity during the manufacture of the product is not a result of microbiological effects. However, the mechanism involved is not yet fully understood. The process appears to be slow, because the toxicity of sterilized fermented ovary decreases gradually during storage for 24 weeks in vitro. According to previous reports, large amounts of low-molecular-weight substances such as organic acids, salts, amino acids and fatty acids are detected in fish products fermented in rice bran, so that the components of these fermented products are more complicated than those of MSWM. Therefore, low-molecular-weight substances that possess a function similar to that of H₃BO₃ or its ions, may be important in reducing TTX toxicity in fermented puffer fish ovary. These results might contribute to the development of a strategy for medical treatment of puffer fish poisoning in the future. Further studies of the toxicity decrease are underway, to identify the so-far unidentified substances by HPLC, to examine the relationship between H₃BO₃ quantity and decrease of TTX toxicity, and to look at the synergistic actions of various coexisting inorganic salts.

References