Formation of intermediate conjugates in the reductive transformation of gonyautoxins to saxitoxins by thiol compounds

Original Article

INTRODUCTION

Paralytic shellfish toxins (PSP toxins) are potent neurotoxins of dinoflagellate origin which accumulate in shellfish via food webs. More than 20 components, analogs of saxitoxin (STX), are known to occur naturally.1 Paralytic shellfish toxins accumulated in shellfish have been reported to suffer biotransformation in the shellfish in which chemical and enzymatic processes are involved.1–3 In contrast, some bacteria are also involved in the transformation of toxin components.4 These indicate that the factors involved in toxin transformation occur widely in the ecosystem. Asakawa et al. reported that glutathione (GSH), a biological reductant occurring widely in organisms, transforms gonyautoxins (GTX) to STX.5 During the investigation on transformation of PSP toxins by bacterial extracts, we found that thiol compounds such as 2-mercaptoethanol (2-ME) as well as GSH convert the mixtures of GTX2,3 and GTX1,4 to STX and neosaxitoxin (neoSTX), respectively. We report here that the conjugates of PSP toxins and thiol compounds are formed in the course of the reaction to transform GTX to STX.

SUMMARY: The activity of bacterial extracts to transform gonyautoxins to saxitoxins was found to be due to glutathione. This activity was also found in another thiol compound, 2-mercaptoethanol. These thiol compounds did not reduce 1-N-OH of gonyautoxin 1 and 4. Interestingly, the stable intermediate conjugates of paralytic shellfish toxins and thiol compounds were formed in the course of the reaction, which demonstrates that this is a two-step reaction consisting of formation of intermediate and degradation of the intermediate to saxitoxins.

KEY WORDS: 2-mercaptoethanol, conjugate, glutathione, gonyautoxin, intermediate, paralytic shellfish toxin, saxitoxin, thiol compound.

MATERIALS AND METHODS

Gonyautoxins

Mixtures of GTX 1 and 4 (described as GTX1,4 hereafter) and GTX2,3 were purified by chromatographies on Bio-Gel P-2 and Bio-Rex 70 from toxic digestive glands of scallop Patinopecten yessoensis collected from Ofunato Bay, Japan, during a bloom of Alexandrium tamarense in June 1998 as described previously.6 As shown in Fig. 1, GTX2,3 are epimers of sulfated esters of 11-hydroxySTX, and GTX1,4 are those of 11-hydroxyneoSTX which easily form an equilibrium mixture of epimers under mild conditions. Therefore, GTX1,4 and GTX2,3 in 0.03 M acetic acid were kept in a refrigerator for several weeks, respectively, to obtain equilibrium mixtures of epimers in which the molar ratio of GTX1,4 or GTX2,3 was 3:1.3 Therefore, obtained GTX1,4 and GTX2,3 were used for the following experiments. Standard mixtures of PSP toxins for high-performance liquid chromatography (HPLC) analysis were kindly supplied by Dr Oshima, Professor of Tohoku University.

Conversion of gonyautoxins to saxitoxins by bacterial extracts

Cells of Moraxella sp.7 cultured in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) at 30°C for 3 days were harvested by centrifugation (5000 g, 30 min).
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each homogenate during incubation was also analyzed by the method of Oshima.8

Transformation of gonyautoxins to saxitoxins with thiol compounds

GTX1,4 (4 nmol) and GTX2,3 (4 nmol) were mixed with 1 mL of 8 mM GSH in 0.1 M phosphate buffer, pH 7.4, respectively. These mixtures were incubated at 70°C for 2 h. During incubation, an aliquot of reaction mixtures was taken out and analyzed for toxin components by HPLC according to Oshima.8 The same mixtures of toxins treated with 1 mL of 8 mM 2-ME were also analyzed in the same way.

Thin layer chromatography analysis of the reaction mixture

GTX 1,4 (100 nmol) mixed with 1 mL of 8 mM GSH was incubated at 70°C for 2 h and analyzed by thin layer chromatography (TLC) (Silica gel 60 precoated plates) with pyridine–ethylacetate-acetic acid–water (75 : 35 : 15 : 30 v/v). Two TLC plates run separately were visualized by spraying ninhydrin and Weber reagents, respectively.

Confirmation of saxitoxin and neosaxitoxin converted from gonyautoxins with thiol compounds

GTX1,4 (1 μmol) and GTX2,3 (1 μmol) were mixed with 2 mL of 90 mM GSH in the phosphate buffer described above, respectively. These mixtures were incubated at 70°C for 40 min to convert GTX to corresponding STX. Saxitoxin and neoSTX appeared in the reaction mixtures after incubation and were purified by chromatographies on Bio-Gel P-2 and Bio-Rex 70 as reported previously.6 Saxitoxin and neoSTX obtained from the reaction of GTX (1 μmol) and 2 mL of 1 M 2-ME were also purified as described above. The isolated toxins were analyzed by fast atom bombardment mass spectrum (FAB-MS, Jeol SX-102, positive mode) using glycerol as a matrix.

RESULTS

Transformation of gonyautoxins to saxitoxins by bacterial extracts

Moraxella sp. used in the present study is a PSP toxin-producing bacterium isolated from a toxic dinoflagellate Alexandrium tamarense, although its toxin productivity was very low.12,13 No toxin peak was detected in the bacterial extract in HPLC analysis, which indicates that the toxin production of Moraxella sp. was under a detectable level in the conditions of the present study.

Soluble protein fraction was prepared by extraction of the cells (3.2 g, wet weight) with 15 mL of 0.1 M phosphate buffer, pH 7.4. Membrane bound protein fraction was prepared from the residue of cell extracts with 4 mL of the same buffer containing 1% Triton X-100 and 0.2 mM 2-ME at 4°C overnight. Each 1 mL extract was mixed with GTX1,4 (10 nmol) and GTX2,3 (10 nmol), respectively, and then incubated at 30°C for 24 h. Before and after the incubation, toxin components in the mixtures were analyzed by HPLC according to Oshima.8 The extracts without adding GTX, and GTX in the phosphate buffer were also incubated in a same way and analyzed as controls.

In order to examine the stability of GSH in bacterial cells during extraction, cells (3.2 g, wet weight) were homogenized with 15 mL of the phosphate buffer described above and 15 mL of 0.1 M borate buffer, pH 7.4, containing 100 mM l-serine which inhibits γ-glutamyl transpeptidase,9 a membrane-bound enzyme, which functions in the degradation of GSH.10 These homogenates were incubated at 30°C for 24 h. During incubation, GSH level in the extracts prepared from aliquots of each homogenate by centrifugation (15 000 g, 30 min) was monitored by the method of Tietze.11 At the same time, the change of GTX2,3 (10 mM) added to

![Fig. 1 Structures of gonyautoxins (GTX)1-4, neosaxitoxin (neoSTX), and saxitoxin (STX).](image)
The extract prepared with the phosphate buffer often converted GTX slightly to STX. However, this transformation activity was not always observed, suggesting that the activity of the extract is not stable. In contrast, GSH which is known to distribute in a variety of organisms including bacteria, is reported to transform GTX to STX under mild conditions. It is also reported that bacteria possess a membrane-bound enzyme which degrades GSH. Thus the stability of GSH during extraction was examined under the absence and presence of the inhibitor of GSH-degrading enzyme.

Figure 2 shows the change of GSH level in the extracts prepared from the homogenates with and without the inhibitor. Glutathione level in the extract in the absence of the inhibitor was 0.21 mM when the extract was prepared just after the cells were homogenized (Fig. 2, upper). However, this level decreased gradually when the homogenate was incubated at 30°C. At 24 h incubation, the level was decreased to 0.06 mM. In contrast, GSH levels in the extract under absence of the inhibitor were 0.36 mM when the extract was prepared just after the cells were homogenized (Fig. 2, lower). This level also decreased slightly, but 0.21 mM of GSH remained in the extract even when the homogenate was incubated for 24 h. These results show that GSH in bacterial cells is easily decomposed by the GSH degrading enzyme during extraction unless the extract was prepared under presence of the inhibitor of the enzyme.

Figure 2 also shows the change of GTX2,3 added to these homogenates during incubation. When GTX2,3 was incubated with the cell homogenate with the inhibitor, more than 80% of GTX2,3 disappeared within 0.5 h incubation, although no STX was detected (Fig. 2, lower). At 1 h incubation, most of GTX2,3 disappeared and a trace amount of STX appeared. The level of STX increased gradually during further incubation and reached 2.2 mM after 24 h incubation, showing that about 20% of GTX2,3 added to the homogenate was transformed to STX. This phenomenon also suggests the formation of intermediate compound which cannot be detected by HPLC in the transformation reaction of GTX to STX.

When GTX2,3 was added to the homogenate without the inhibitor, about 20% of toxins also disappeared within 0.5 h incubation (Fig. 2, upper). However, the level of GTX2,3 did not change during further incubation. No STX nor other known PSP toxin components were detected during incubation. These results show that the bacterial activity to transform GTX to STX is mainly due to GSH. Unstable activity of bacterial extract prepared under absence of the inhibitor seems to be due to degradation of GSH during extraction by GSH degrading enzyme.

Membrane bound protein fraction showed a significant activity to transform GTX to STX. This activity was stable under heating and protease treatment (EC3.4.24.31; Sigma, St Louis, MO, USA), thus showing that it is not enzymatic. The activity was observed also in the extraction buffer, suggesting that it is due to 2-ME contained in the buffer.

Transformation of gonyautoxins to saxitoxins with thiol compounds

Figure 3 shows the change of toxin components in the mixture of GTX1,4 and GSH during incubation at 70°C in the phosphate buffer. GTX1,4 decreased markedly and disappeared from the mixture within 2 h. In contrast, neoSTX appeared at 30 min and gradually increased to 35% of the amount of GTX1,4 added to the reaction mixture at 2 h. In contrast, GTX1,4 in the phosphate buffer decreased slightly after 12 h incubation, but no neoSTX was detected by HPLC. Similar results were obtained when GTX 2,3 was incubated with GSH. In this case, however, STX was formed instead of neoSTX (data not shown).

Figure 4 shows the change of toxin components in the reaction mixture of GTX 1,4 and 2-ME during incubation at 70°C in the phosphate buffer. In this case also, most of GTX1,4 disappeared within 2 h while a small
Formation of thiol conjugates of PSP toxins

The formation of thiol conjugates of PSP toxins was investigated using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The results showed that thiol compounds transformed GTX to STX. However, the sum of GTX and STX in the reaction mixtures was less than half of the original amount of GTX, indicating that conjugates of GTX and GSH formed in the course of the transformation. Thus, the 2 h incubation mixtures of GTX and GSH in which GTX disappeared from the mixtures were heated in 1 M 2-ME at 100°C for 10 min. High-performance liquid chromatography analysis showed that almost 100% of the added GTX was recovered as STX. This indicates that GTX and thiol compounds form conjugates and that additive molecules of thiol compounds are necessary to release STX from the conjugates.

Confirmation of the conjugates of gonyautoxins and thiol compounds

Table 1 summarizes the results of TLC of the reaction mixtures of GTX and GSH before and after the incubation. After incubation, spots of GTX disappeared and those of STX appeared instead. In addition to these spots, spots different from those of toxin components and GSH appeared at Rf 0.10 (mixture of GTX1,4 and GSH) and 0.04 (mixture of GTX2,3 and GSH), respectively, indicating formation of conjugates of GTX and GSH.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>TLC of GTX, STX and reaction intermediates</th>
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<tbody>
<tr>
<td>Rf</td>
<td>Color</td>
</tr>
<tr>
<td>GTX1</td>
<td>0.89</td>
</tr>
<tr>
<td>GTX4</td>
<td>0.84</td>
</tr>
<tr>
<td>GSH</td>
<td>0.41</td>
</tr>
<tr>
<td>neoSTX</td>
<td>0.71</td>
</tr>
<tr>
<td>A*</td>
<td>0.10</td>
</tr>
<tr>
<td>GTX2</td>
<td>0.77</td>
</tr>
<tr>
<td>GTX3</td>
<td>0.69</td>
</tr>
<tr>
<td>STX</td>
<td>0.55</td>
</tr>
<tr>
<td>B*</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*A and B were the intermediates obtained from GTX1,4 and GTX 2,3, respectively. GTX, gonyautoxin; STX, saxitoxin; GSH, glutathione.
A spot corresponding to GSH became very faint after incubation, suggesting that excess amounts of GSH were oxidized to GSSG during incubation which could not be detected in this system.

**Confirmation of saxitoxins converted from gonyautoxins with thiol compounds**

Figure 5 shows the chromatograms of the mixtures of GTX (0.5 mM) and GSH (90 mM) before and after the incubation at 70°C for 40 min, respectively. (a) Before incubation; (b) after incubation.

Most GTX added to the bacterial homogenate with serine-borate complex disappeared within 1 h, which seems to be due to the first step of reaction of GTX and GSH, although GSH level in the homogenate was significantly low (Fig. 2). Disappearance rate of GTX in the bacterial homogenate was comparable with those in the reaction mixture in which 30 mM GSH was mixed with 10 μmol/L GTX and incubated at the same temperature. This may suggest the occurrence of unknown factor(s) which enhances the first step of the reaction in bacterial cells. In the homogenate with phosphate buffer, the GSH level of which was lower than that of serine-borate buffer, GTX decreased slightly within 1 h. This seems to be also due to the first step of reaction between GTX and GSH.

Asakawa et al. indicated for the first time that GSH transforms GTX to STX. However, they described that GSH transforms GTX1 as well as GTX2,3 to STX. The present results showed that 1-N-OH of GTX1,4 is not reduced by thiol compounds. The discrepancy between these data can be explained by formation of the intermediate conjugates in the course of the reaction. Failure of neoSTX detection in the reaction mixture of GTX and GSH by Asakawa's group might not derive from the reduction of GTX1 at 1-N-OH, but from the formation of GSH conjugate which could not be detected by cellulose acetate membrane electrophoresis used by them. A small portion of GTX1 (2%) in their substrate toxins might be another reason for misinterpretation of the experiment.

Although more than 20 analogues of STX are known to occur naturally, no derivatives bound with thiol compounds is known. The conjugate of PSP toxins with thiol compounds, especially with GSH, a biological reductant distributed widely in various organisms, is important in the metabolism of PSP toxins accumulated in various organisms as well as that of toxin-producing ones. Further study on the chemical structures and detail formation mechanism of the intermediate conjugates is under progress.

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REFERENCES


