Chinese Journal of Oceanology and Limnology
Vol. 25 No. 2, P. 227-234, 2007
DOI: 10.1007/s00343-007-0227-1

A sulfotransferase specific to N-21 of gonyautoxin 2/3 from crude enzyme extraction of toxic dinoflagellate *Alexandrium tamarense* CI01*

WANG Dazhi (王大志)**, ZHANG Shugang (张树刚), HONG Huasheng (洪华生)
(*State Key Laboratory of Marine Environmental Science/Environmental Science Research Center, Xiamen University, Xiamen 361005, China*)

Received May 25, 2005; revision accepted Nov. 24, 2006

Abstract Sulfotransferase (ST) is the first enzyme discovered in association with paralytic shellfish poisoning (PSP) toxin biosynthesis in toxic dinoflagellates. This study investigates the ST activity in crude enzyme extraction of a toxic dinoflagellate species, *Alexandrium tamarense* CI01. The results show that crude enzyme can transfer a sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to N-21 in the carbamoyl group of gonyautoxin 2/3 (GTX2/3) to produce C1/C2, but is inactive toward STX to produce GTX5. The crude enzyme is optimally active at pH 6.0 and 15°C. The activity is enhanced by Co^{2+}, Mg^{2+}, Mn^{2+} and Ca^{2+} individually, but is inhibited by Cu^{2+}. Moreover, the activity shows no difference when various sulfur compounds are used as sulfate donors. These results demonstrate that the ST specific to GTX2/3 is present in the cells of *A. tamarense* CI01 and is involved in PSP toxin biosynthesis. In addition, the ST from different dinoflagellates is species-specific, which explains well the various biosynthesis pathways of the PSP toxins in toxic dinoflagellates.

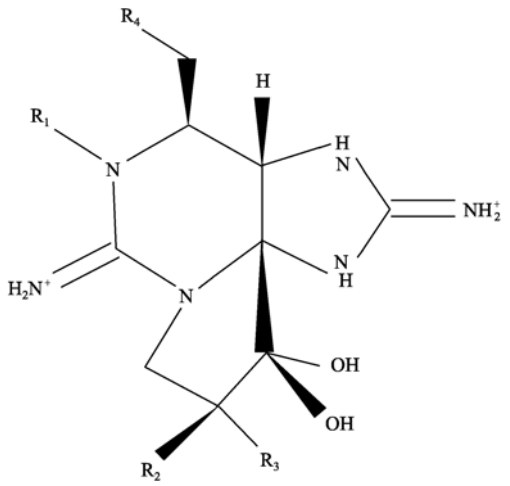
Key words: *Alexandrium tamarense* CI01; crude enzyme; sulfotransferase; paralytic shellfish poisoning

1 INTRODUCTION

The PSP toxins are a family of over 20 structurally related imidazoline guanidinium derivatives (Fig.1) produced by some marine dinoflagellates belonging to the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*, which are potent neurotoxin-blocking voltage-gated sodium channels on excitable cells (Kodama, 2000). These toxins vary in potency to hundreds of fold due to certain minor structural differences (Oshima, 1995a). In the past few decades, much work has been devoted to the bio-production of PSTs in toxic dinoflagellates (Shimizu, 1979; Hall, 1982; Boyer et al., 1987; Boczar et al., 1988; Anderson et al., 1990; Oshima et al., 1993; Flynn et al., 1996; Parkhill and Cembella, 1999; Hamasaki et al., 2001; Hwang et al., 2001; Wang et al., 2002). However, little is known about the biosynthesis of PSTs in toxic dinoflagellates at present (Plumley, 1997, 2001). Shimizu (1993, 1996) investigated the PSP toxins in cyanobacterium *Aphanizomenon flos-aquae* using the stable isotope method, and found that acetate, arginine, and methionine were the precursors of PSP toxins. However, the direct precursors were not elucidated and the enzymes involved in the toxin biosynthesis were not identified. More recently some studies indicate that ST, a sulfation enzyme, is involved in PSP toxin biosynthesis in some toxic dinoflagellates; it could transfer a sulfate group to N-21 in carbamoyl group of GTX2/3 or STX and produce various STX derivatives (Oshima, 1995; Yoshida et al., 1996, 1998; Sako et al., 2001).

Alexandrium tamarense CI01, a toxic dinoflagellate strain isolated from the South China Sea, produces predominant C2 toxin (about 90% of total toxin) with a trace amount of C1 and GTX2+3 toxins (Wang et al., 2005), implying that there might exist a fast conversion between GTX2/3 and C1/2 with high activity of sulfotransferase in *A.*

* Supported by the National Natural Science Foundation of China (No.40376032) and the Ministry of Science and Technology of the People's Republic of China (No.2001CB409700).
**Corresponding author: dzwang@xmu.edu.cn



Toxins	R1	R2	R3	R4
STX	H	H	H	$OCONH_2$
neoSTX	OH	H	H	$OCONH_2$
GTX1	OH	H	OSO_3^-	$OCONH_2$
GTX2	H	H	OSO_3^-	$OCONH_2$
GTX3	H	OSO_3^-	H	$OCONH_2$
GTX4	OH	OSO_3^-	H	$OCONH_2$
GTX5	H	H	H	$OCOHNSO_3^-$
GTX6	OH	H	H	$OCOHNSO_3^-$
C1	H	H	OSO_3^-	$OCOHNSO_3^-$
C2	H	OSO_3^-	H	$OCOHNSO_3^-$
C3	OH	H	OSO_3^-	$OCOHNSO_3^-$
C4	OH	OSO_3^-	H	$OCOHNSO_3^-$
dcSTX	H	H	H	H
dcneoSTX	OH	H	H	H
dcGTX1	OH	H	OSO_3^-	H
dcGTX2	H	H	OSO_3^-	H
dcGTX3	H	OSO_3^-	H	H
dcGTX4	OH	OSO_3^-	H	H
11-α-hydroxySTX	H	H	OH	$OCONH_2$
11-β-hydroxySTX	H	OH	H	$OCONH_2$

Fig.1 Structure of paralytic shellfish poisoning (PSP) toxins
STX, saxitoxin; neoSTX, neosaxitoxin, GTX, gonyautoxin; dc, decarbamoyl

tamarense CI01 cells. To test the hypothesis, this study investigated sulfotransferase activity in the crude enzyme extraction of *A. tamarense* CI01, and discusses the biosynthesis pathway of the PSP toxin in *A. tamarense* CI01.

2 MATERIALS AND METHODS

2.1. Organism and culture conditions

Dinoflagellate *A. tamarense* CI01 was originally derived from a single cyst isolated from the sediments collected in Dapeng Bay, Shenzhen, China in 1992 (Wang and Hsieh, 2001). The algal culture was incubated in a modified K-medium under a set of optimal conditions: 23℃, 14/10 light/dark cycle, and 90 μE/m^2s light intensity. The cultured cells were harvested at the middle stationary phase by centrifugation. The cell pellets were washed with cold phosphate buffer (50 mmol/L KH_2PO_4-Na_2HPO_4, pH 7.0) and then stored at −80℃ until enzyme extraction.

2.2. Crude enzyme extraction

The thawed cell pellet of *A. tamarense* CI01 was suspended in 2 volumes of Tris buffer (50 mmol/L Tris-HCl, 4 mmol/L β-mercaptoethanol pH 8.0). The cell pellet was lysed for 20 min at 1 min intervals in ice bath by a microtip-probe sonifier. The sonicator was centrifuged at 100 000 g for 30 min at 4℃ and the supernatant containing enzymes was retained. Solid ammonium sulfate was added slowly to the supernatant to yield 20% saturation. After stirring for 30 min at 4℃, the sample was centrifuged at 50 000 g. The supernatant was added with solid ammonium sulfate to 80% saturation. After centrifugation, the precipitate was dissolved in Tris buffer and dialyzed over night against a 100-fold volume of the same buffer at 4℃. The dialyzed solution was the crude enzyme and stored at −80℃ before enzyme assay.

2.3. Enzyme assay

The ST activity was assayed as previously described by Sako et al. (2001). Briefly, all assays were conducted by adding the crude enzyme to a preheated reaction mixture (10 μmol/L GTX2/3 or STX, 1 mmol/L PAPS, 125 mmol/L phosphate buffer pH 6.0, and 5 mmol/L $MgCl_2$) in a final volume of 100 μmol/L. The reaction mixture was incubated at 15℃ for an hour, and the reaction was terminated by adding 0.5 mol/L acetic acid. The solution was filtered by centrifugation (25 000 g, 30 min) through a membrane filter with a 10 000 molecular weight cutoff. The reaction product was analysed by the HPLC with post-column derivatization and fluorescence detection (Oshima, 1995b), and identified by comparison with standard PSP toxins. To study the effects of crude enzyme volume on ST activity, 3 volumes (30, 40, and 50 μl) were used in the reaction mixture at 15℃ and 25℃. To examine the effects of reaction time on ST

activity, 4 time ranges were used (0.5, 1, 2, and 3 h) at 15℃. To test the effects of temperature on the ST activity, the reaction was conducted at different temperatures: 10, 15, 20, 25, and 30℃. To determine the effects of pH on the activity, different ranges of phosphate buffers were used: pH 5, 6, 7, and 8. To investigate the roles of divalent cations in the enzyme activity, 5 cations (Mg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Ca^{2+}) were used at a final concentration of 5 mmol/L in the reaction mixture. To determine the sulfate donor specificity of ST, we tested DMSO, PAPS and $MgSO_4$. Duplicate determinations of N-ST activity assay did not differ by more than 5%. All the reactions were conducted at a temperature of 15℃ except otherwise in test of temperature.

3 RESULTS

3.1. Presence of sulfotransferase activity in the crude enzyme

The HPLC chromatograms of PSP toxins in different samples are shown in Fig.2. Good resolutions of GTX2/3, C1 and C2 shown in Fig.2a indicate the adequacy of analytical power for the enzyme substrates and possible enzyme products in question. Fig.2b reveals that there was no PSP toxin in the crude enzyme extraction to interfere with the enzyme assay. The large peak in the chromatogram of the supernatant of the saturated $(NH_4)_2SO_4$ solution, as shown in Fig.2c indicates the presence of C2 as a predominant PSP toxin with trace amounts of C1 and GTX2/3. Fig.2d shows the presence of GTX2/3 alone as the substrate in the reaction mixture containing no crude enzyme extract. In the reaction mixture containing the crude enzyme extract, C1/2 appeared as a reaction product as shown in Fig.2e, indicating the presence of the ST activity in the crude enzyme extract. No new toxin peak was found in the reaction mixture using STX as the sole substrate (chromatogram not shown), indicating that the ST was not active towards STX, but was specific to GTX2/3 as the substrate.

3.2 Characterization of the crude enzyme

At the optimum incubation temperature of 15℃, the reaction rate (product produced in 1 h) is proportional to the amount of crude enzyme extract used (Fig.3a), demonstrating the presence of enzyme in the cell extract. The dose-dependency of the reaction rate was not evident when incubation was at sub-optimal temperature (25℃) (Fig.3a). In a 3-h incubation, the enzymatic reaction rate declined with time (Fig.3b), suggesting that either the enzyme was unstable or certain co-factors were being exhausted. The optimal condition for the enzyme activity was at pH 6.0 and 15℃ (Fig.3c and 3d). Deviating from the optimal, the enzyme activity declined sharply, suggesting that the enzyme is pH and temperature sensitive. The crude enzyme was active without adding any divalent cations. However, the enzyme activity was enhanced by adding Co^{2+}, Mg^{2+}, Mn^{2+}, or Ca^{2+}individually, and was inhibited by Cu^{2+} (Fig.3e). No difference was seen when PAPS, DMSO, or $MgSO_4$, was used as a supplementary sulfate donor (Fig.3f), indicating that the enzyme was not specific to the sulfate donor.

4 DISCUSSION

4.1 Roles of ST in toxin biosynthesis of dinoflagellates

Sulfotransferase (ST) is an important enzyme found in mammals and some other organisms, which can transfer a sulfate group to various hydroxy or amine groups, resulting in lessening the bioactivity, increasing the water solubility and accelerating the excretion of these compounds (Matsui and Homma, 1994; Saidha and Schiff, 1994). However, little is known about ST in PSP-producing dinoflagellates. Oshima (1995a) reported that the whole-cell extracts of toxic dinoflagellate *Gymnodium catenatum* could transfer the 21-NH_2 to 21-NSO_3 in the presence of PAPS. He postulated that ST might be present in the cells of PSP-producing dinoflagellates and involved in toxin conversion. Several studies have proved this point. Yoshida et al. (1996) obtained two STs specific to N-21 of STX and GTX2/3 (N-ST), and O-22 of 11-α, β-hydroxy STX (O-ST) from *G. catenatum*. Yoshida et al. (1998) found a ST in *Alexandrium catenella* Acko5, which could produce C1/2 and GTX5 from GTX2/3 and STX, respectively. Sako et al. (2001) purified and characterized N-ST from *G. catenatum*, which transferred the sulfate group of PAPS to N-21 of STX and GTX2/3 to give rise to GTX5 and C1/2, respectively. Our results indicated that the ST

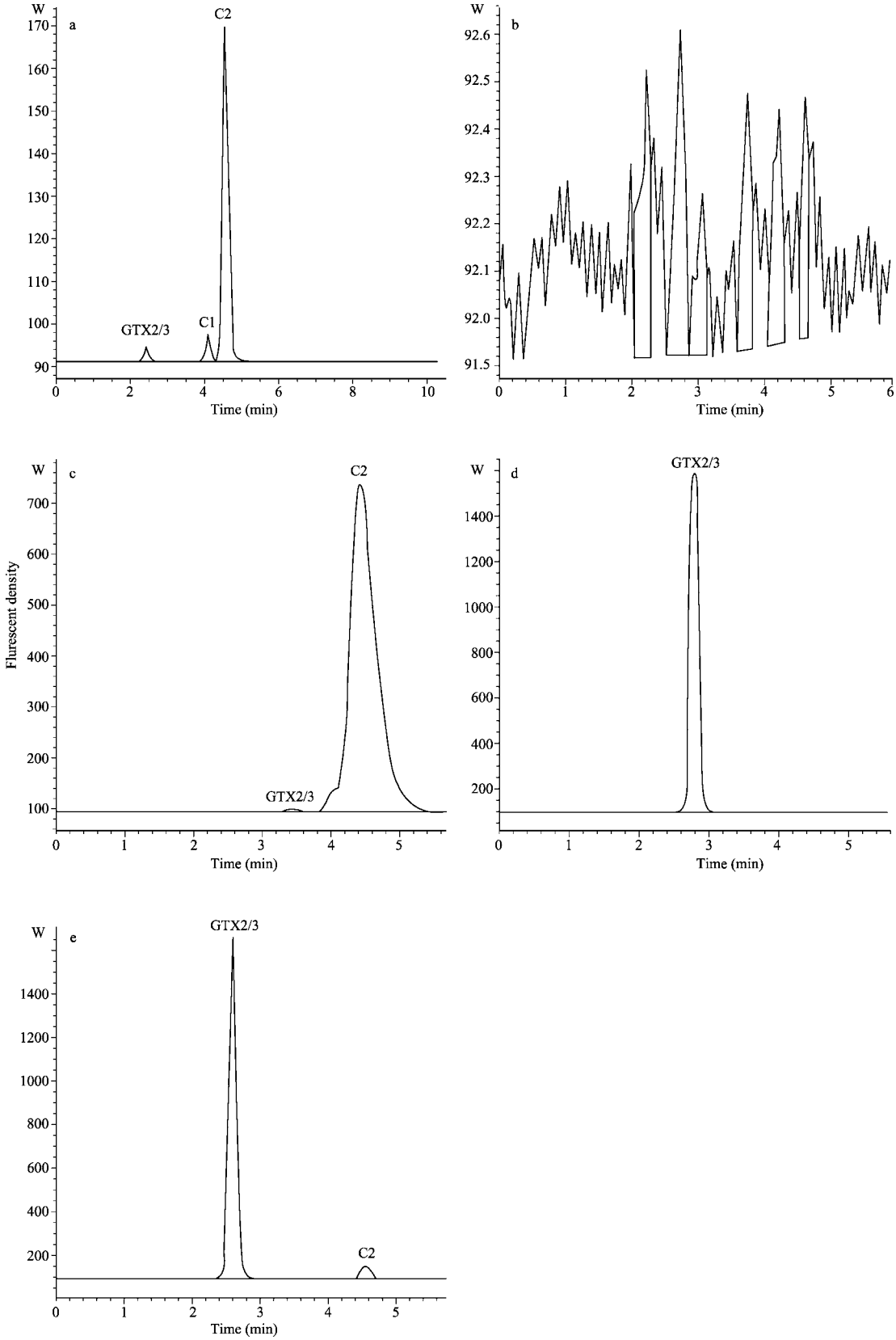


Fig.2 HPLC chromatogram of different samples

a. Standard PSTs; b. Crude enzyme extraction; c. Supernatant of saturated $(NH_4)_2SO_4$; d. Reaction mixture without crude enzyme; e. Reaction mixture with crude enzyme

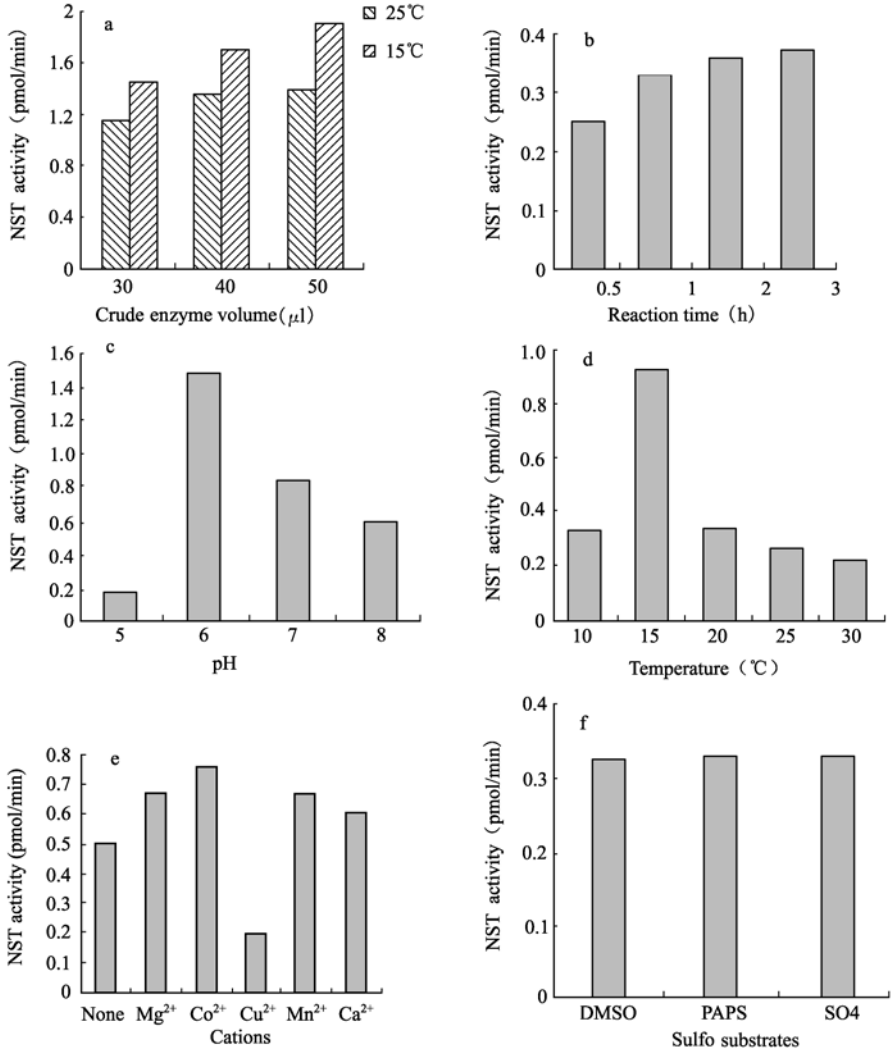


Fig.3 Properties of the crude enzyme from *A. tamarense* CI01

A. Crude enzyme volume; B. Reaction time; C. pH; D. Temperature; E. Divalent cations; F. Sulfate donors

specific to GTX2/3 was present in the crude enzyme extraction of *A. tamarense* CI01, which could transfer the sulfate group of PAPS to N-21 of GTX3 to produce C1/2 but was not active towards STX to produce GTX5. However, the ST from different species showed diverse characterizations (Table 1). The ST of *A. tamarense* was not active towards STX, whereas it was active to STX in *G. catenatum* and *A. catenella*. The optimal reaction temperature of ST from *A. tamarense* and *A. catenella* was 15℃, and 25℃ for *G. catenatum*. The ST activity of *A. catenella* was inhibited by divalent cations except Mg^{2+}, and it was enhanced by Mg^{2+} and Co^{2+}, and inhibited by other divalent cations in *G. catenatum*. However, the ST activity was enhanced by Mg^{2+}, Co^{2+}, Mn^{2+}, and Ca^{2+} but inhibited by Cu^{2+} in *A. tamarense*. Therefore, it means that Cu^{2+} was more toxic to NST than other cations. Moreover, the ST from *A. tamarense* showed no specific preference on sulfate donors, whereas only PAPS showed specific activity in *A. catenella* and *G. catenatum*. These results suggest that the properties and functions of N-ST from different species are species specific.

One interesting finding is that the optimal growth temperature of ATCI01 ranged from 20 to 28℃; however, the highest ST activity occurred at 15℃. The reason remains unclear at present. It is known that at low temperatures, the growth rate would decrease resulting in nutrient accumulation in cells, such as ammonium, which would cause toxicity to cells. High ST activity of ATCI01 at low temperatures might convert more ammonium to

Table 1 Comparison of ST from different PSP-producing dinoflagellate species

	G. catenatum GC21V	*A. catenella* Acko5	*A. Tamarense* CI01
Substrate	GTX2/3, STX	GTX2/3, STX	GTX2/3
Products	C1/C2, GTX5	C1/C2, GTX5	C1/C2
Sulfate donor	PAPS	PAPS	PAPS, DMSO, SO SO_4^{2-}
Optimal pH	6	6	6
Optimal temperature	25℃	15℃	15℃
Cation requirement	Mg^{2+}, Co^{2+}	None	None

toxin C2/C1, which could reduce the ammonium toxicity to cells. Further study is suggested.

4.2 Biosynthesis of PSTs

Toxin biosynthesis pathway has been an active research area in PSP-producing dinoflagellates in the past few decades. So far, little is known about it due to the specialty of dinoflagellates and the limitation of investigating methods (Plumley, 1997, 2001; Cembella, 1998). Our current understanding on the biosynthesis of saxitoxin and its related PSP toxins is largely derived from studies of PSP-producing cyanobacterium, *A. flos-aquae* (Shimizu, 1993, 1996). This species is able to use arginine, acetate and S-adenosylmethionine to synthesize saxitoxin as the parent PSP compound, which was modified subsequently by addition or removal of hydroxyl, carbamyl, and/or hyroxysulfate moieties to yield 21 derivatives. However, the direct precursors and enzymes involved in PSP toxin biosynthesis have not been identified. Taroncher-Oldenburg et al. (1997) proposed a biosynthesis pathway of PSP toxins in dinoflagellate, *Alexandrium fundyense*, based on the study on cell cycle. They postulated that the C2 toxin was the parent compound synthesized in *A. fundyense*, and C2 toxin was subsequently desulfated to GTX2/3, which was further desulfated to STX. Sako et al. (2001) gave a different biosynthesis pathway of PSP toxins according to their study on ST in *G. catenatum*. In their hypothesis, STX was the parent compound synthesized by cells, and was converted predominantly to 11-α, β-hydroxy STX by C-11 oxidation and partially to GTX5 by N-21 sulfation. The 11-α, β-hydroxy STX is immediately sulfated and converted to GTX2/3 by O-ST. Finally, GTX2/3 is sulfated at N-21by N-ST or oxidized at N-1 and converted to C1/2 and GTX1/4. Our results on toxin composition and *in vivo* conversion of PSP

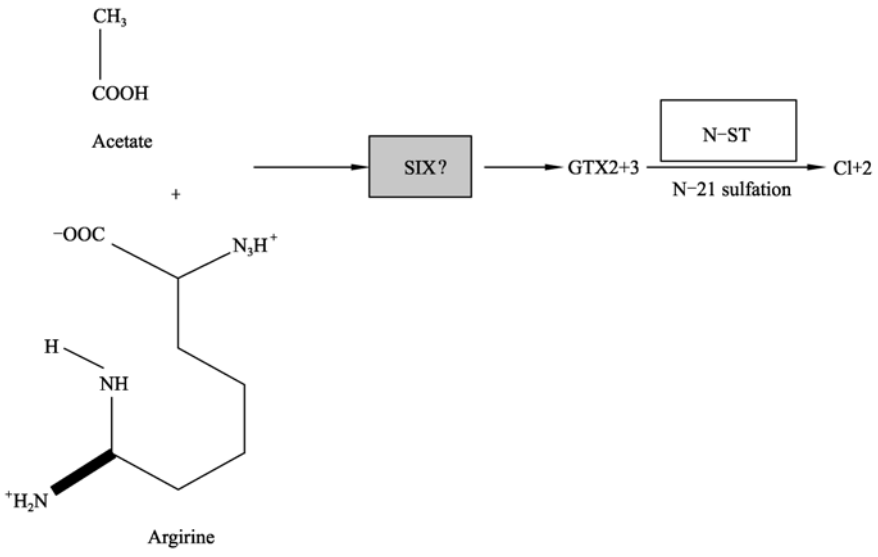


Fig.4 Hypothesis of PSP toxin biosynthesis in *A. tamarense* CI01

toxins in *A. tamarense* CI01 indicated that another parent compound more than STX was synthesized; this compound was sulfated and converted to GTX2+3 by certain enzymes. Finally, GTX2+3 was sulfated at N-21 by ST and converted to C1+2. (Fig.4).

In summary, our findings and other studies have revealed that ST is present in PSP-producing dinoflagellates and is involved in the PSP toxin biosynthesis. However, ST from different toxic dinoflagellates shows species-specific in physical and chemical properties, which results in various conversion pathways of PSP toxins in toxic dinoflagellates. Further work should investigate extensively the presence and functions of ST in PSP-producing and non-PSP-producing dinoflagellates, compare ST regulating mechanisms, and clone and sequence the N-ST gene, to elucidate the biosynthesis and conversion of PSP toxins in toxic dinoflagellates.

References

Anderson, D. M., D. M. Kulis, J. J. Sullivan and S. Hall, 1990. Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. *Toxicon* **28**: 885-893.

Boczar, B. A., M. A. Beitler, J. Liston, J. J. Sullivan and C. A. Cattolico, 1988. Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. Plant. *Physiol.* **88**: 1 285-1 290.

Boyer, G. L., J. J. Sullivan, R. J. Anderson, P. J. Harrison and F. J. R. Taylor, 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. *Mar. Biol.* **96**: 123-128.

Cembella, A. D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. *In*: DM Anderson, AD Cembella and GM Hallegraeff ed., Physiological Ecology of Harmful Blooms. Springer-Verlag, Berlin Heidelberg, p. 381-403.

Flynn, K. J., K. J. Jones and K. J. Flynn, 1996. Comparisons among species of *Alexandrium* grown in nitrogen- or phosphorus-limiting batch culture. *Mar. Biol.* **126**: 9-18.

Hall, S., 1982. Toxins and Toxicity of *Protogonyaulax* from the Northeast Pacific. Ph.D. Thesis. University of Alaska.

Hamasaki, K., M. Horie, A. Tokimitsu, A. Toda and S. Taguchi, 2001. Variability in toxicity of the dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions. *J. Plank. Res.* **23**: 271-278.

Hwang, D. F. and Y. H. Lu, 2001. Influence of environmental and nutritional factor on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. *Toxicon* **38**: 1 491-1 503.

Kodama, M., 2000. Ecology, classification and origin. *In*: LM Botana ed., Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection. Marcel Dekker, Inc. USA, p.125-150.

Matsui, M. and H. Homma, 1994. Biochemistry and molecular biology of drug-metabolizing sulfotransferase. *Int. J. Biochem.* **26**: 1 234-1 247.

Oshima, Y., 1995a. Chemical and enzymatic transformation of paralytic shellfish toxins in marine organisms. *In*: P Lassus ed., Harmful Marine Algal Blooms, Lavoisier, New York, p. 475-480.

Oshima, Y., 1995b. Post-column derivatization liquid chromatographic method for paralytic shellfish toxins. *J. AOAC Int.* **78**: 528-532.

Oshima, Y., S. J. Blackburn and G. M. Hallegraeff, 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. *Mar. Biol.* **116**: 471-476.

Parkhill, J. P. and A. D. Cembella, 1999. The effects of salinity, light and inorganic nitrogen on the growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense*. *J. Plank. Res.* **21**: 939-955.

Plumley, F. G., 1997. Marine algal toxins: biochemistry, genetics, and molecular biology. *Limnol. Oceanogr.* **42**: 1 240-1 251.

Plumley, F. G., 2001. Purification of an enzyme involved in saxitoxin synthesis. *J. Phycol.* **37**: 926-928.

Saidha, T. and J. A. Schiff, 1994. Purification and properties of a phenol sulfotransferase from Euglena using L-tyrosine as substrate. *J. Biochem.* **298**: 45-50.

Sako, Y., T. Yoshida, A. Uchida, O. Arakawa, T. Noguchi and Y. Ishida, 2001. Purification and characterization of a sulfotransferase specific to N-21 of saxitoxin and gonyautoxin 2+3 from the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.* **37**: 1 044- 1 051.

Shimizu, Y., 1979. Dinoflagellate toxin. *In*: PJ Scheuer ed, Marine Natural products-Chemical and Biological Perspectives. Academic Press, New York, p. 1-42.

Shimizu, Y., 1993. Microalgal metabolite. *Chem. Rev.* **93**: 1 685-1 698.

Shimizu, Y., 1996. Microalgal metabolites-a new perspective. Annu. *Rev. Microbiol.* **5**0: 431-465.

Taroncher-Oldenbugr, G., D. Kulis and D. M. Anderson, 1997. Toxin variability during the cell cycle of the

dinoflagellate *Alexandrium fundyense*. *Limnol. Oceanogr*. **42**: 1 177-1 188.

Wang, D. Z and D. P. H. Hsieh, 2001. Dynamics of C2 toxin and chlorophyll-a formation in the dinoflagellate *Alexandrium tamarense* during large-scale cultivation. *Toxicon* **39**: 1 533-1 536.

Wang D Z, AYD Ho and DPH Hsieh, 2002. Production of C2 toxin by *Alexandrium tamarense* CI01 using different culture methods. *J. Appl. Phycol.* **14**:461-468.

Wang, D. Z., T. J. Jiang and D. P. H. Hsieh, 2005. Toxin composition variations in cultures of *Alexandrium* species isolated from the coastal waters of Southern China. *Harmful Algae* **4**: 109-121.

Yoshida, T., Y. Sako, A. Uchida, Y. Ishida, O. Arakawa and T. Noguchi, 1996. Purification and properties of paralytic shellfish poisoning toxins sulfotransferase from toxic dinoflagellate *Gymnodinium catenatum*. *In*: T Yasumoto, Y Oshima and Y Fukuyo eds., Harmful and Toxic Algal Blooms. IOC (UNESCO), Paris, p.499-502.

Yoshida, T., Y. Sako, T. Kakutani, A. Fujii, A.Uchida, Y. Ishida, O. Arakawaand and T. Noguchi, 1998. Comparative study of two sulfotransferase for sulfation to N-21 of *Gymnodinium catenatum* and *Alexandrium catenella* toxins. *In*: B. Reguera, J. Blanco, M. L. Fernandez and T. Wyatt eds., Harmful Algae. Xunta de Galicia and IOC of UNESCO, Vigo, p. 366-369.