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Storage stability of paralytic shellfish poisoning toxins

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Abstract

Variations in C toxins (C1- 2), GTX (gonyautoxin) 1-4, STX (saxitoxin) and NEO (neosaxitoxin) in scallop digestive glands and a mixture of purified paralytic shellfish poisoning (PSP) toxins were studied during storage at −35, 5 and 25°C and at different pH levels. Heated and unheated samples of homogenates and purified toxin mixtures were stored for 1 year and 4 months, respectively, and analyzed at different times by HPLC. C toxin levels decreased rapidly during storage at 25°C with a significant decrease after the first 2 months. GTX 2/3 in the unheated samples did not change initially at pH 3–4, but decreased at pH 6–7 with the fastest rate at 25°C followed by 5°C. There was no significant change in any toxin type stored at −35°C regardless of pH. GTX 1/4 decreased significantly after 4 months at 25°C. NEO and STX remained unchanged at all temperatures at low pH (pH 3), whereas NEO levels continued to decrease at higher pH (pH 6–7) at 25°C. All toxins were stable at low pH (pH 3–4) and −35°C. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Paralytic shellfish poisoning toxins are imidazoline derivatives which are accumulated by shellfish via the food chain and cause sporadic food poisoning in humans. These toxins have historically been a problem for the shellfish industry on both the Pacific and Atlantic coasts of Canada and the US (Lawrence, Maher & Watson-Wright, 1994). Several species of the dinoflagellate genus, *Protogonyaulax (Alexandrium)*, produce PSP toxins (Fig. 1) which are all related to STX. Structurally, they can be divided into carbamate, sulfomate and decarbamoyl toxins. Due to the nature of the differences in charge and substitutional groups to the basic saxitoxin structure, they bind with different affinities to site 1 of the sodium channel (Catterall, 1988) resulting in different toxicities. The stability of each toxin derivative may also change under different environmental conditions and storage.

Thermal stabilities of these toxins have been studied by many authors (Asakawa, Iida & Oishi, 1986; Asakawa & Takagi, 1983; Berenguer, Gonzalez, Jimenez, Legarda, Olmedo & Burdaspal, 1993; Chang, Shin, Cho, Park, Pylon & Park, 1988; Gill, Thompson & Gould, 1985; Indrasena & Gill, 1999; Lawrence et al., 1994) and some indicated that most of the PSP compounds are thermally stable at normal cooking temperatures (Gill et al., 1985; Indrasena & Gill, 1999; Nagashima, Noguchi, Tanaka & Hushimoto, 1991). However, some of these compounds can undergo structural changes especially at low pH, converting the less-toxic carbamoyl-*N*-sulfo PSP compounds such as B1 and B2 into highly toxic carbamoyl counterparts such as STX and NEO (Alfonso, Vieytes, Botana, Geonaga & Botana, 1993; Hall & Reichardt, 1984; Harada, Oshima & Yasumoto, 1982; Indrasena & Gill, 1999; Kobayashi & Shimizu, 1981; Nishio, Noguchi, Onoue, Maruyama, Hashimoto & Seto, 1982). Commercial canning reduced the PSP content when the shellfish were processed in brine water (Berenguer et al., 1993). Conventional canning at a neutral pH reduced the total toxicity by about 90–95% and the toxicity further decreased by another 36–65% during storage at room temperature for 1 year (Prakash et al., 1971).

Louzao, Medcoff & Tanent, et al. (1994) reported that lyophilization may render PSP toxins unstable during storage. They also indicated that lyophilized C toxins and GTXs lost toxicity during storage for 2 months at

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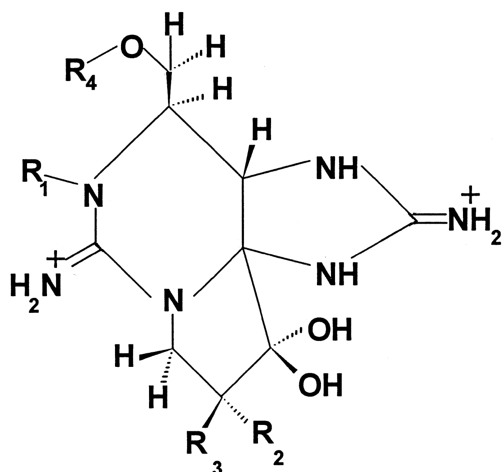

	R_1	R_2	R_3	R_4
STX	H	H	H	$CONH_2$
B1 (GTX5)	H	H	H	$CONHSO^-_3$
GTX 2	H	OSO^-_3	H	$CONH_2$
C1 (epi GTX8)	H	OSO^-_3	H	$CONHSO^-_3$
GTX 3	H	H	OSO^-_3	$CONH_2$
C2 (GTX8)	H	H	OSO^-_3	$CONHSO^-_3$
NEO	OH	H	H	$CONH_2$
B2 (GTX6)	OH	H	H	$CONHSO^-_3$
GTX 1	OH	OSO_3^-	H	$CONH_2$
C3	OH	OSO^-_3	H	$CONHSO^-_3$
GTX 4	OH	H	OSO^-_3	$CONH_2$
C4	OH	H	OSO^-_3	$CONHSO^-_3$
dc-STX	H	H	H	H
dc-GTX 2	H	OSO^-_3	H	H
dc-GTX 3	H	H	OSO^-_3	H
dc-NEO	OH	H	H	H
dc-GTX 1	OH	OSO^-_3	H	H
dc-GTX 4	OH	H	OSO^-_3	H

Fig. 1. Structures of PSP toxins (STX-saxitoxin, NEO-neosaxitoxin, GTX-gonyautoxin, dc-decarbamoyl).

−80, −20, 4 and 35°C with the fastest rate of degradation observed at 35°C. Alfonso, Louzao, Vieytes and Botana (1994) demonstrated that STX is more stable than NEO in acidic solutions and the toxins in solution were more stable than in the lyophilized form. They also suggested that STX could be stored at temperatures below 5°C in acidic solutions for up to 2 years without loss of toxicity. Shimizu (1988) also indicated that STX can be stored in dilute hydrochloric acid for years without loss of potency and the hydrolysis of the carbamoyl ester can only occur in concentrated acid solutions such as 7.5 N hydrochloric acid at 100°C. According to Louzao, et al. (1994), B2 was the most unstable toxin under any condition and at temperatures above 0°C whereas GTX 2 and 3 were the most stable in acidic solutions, especially at 4°C.

A detailed study of the nature of PSP toxicity changes during the storage at different pH levels has not been published to date. Hence, the objective of this study was to examine the stability of purified PSP toxins and PSP compounds in toxic scallop digestive glands during storage at different pH values and temperatures.

2. Materials and methods

2.1. Preparation of homogenates

Toxic scallops provided by Fisheries and Oceans Canada Inspection Branch (Digby and Yarmouth), were transported in ice to the laboratory in Halifax and were frozen immediately at −35°C. They were thawed at 5°C prior to homogenization. Pieces of shells, sand and other debris were removed and tissue was washed gently. The digestive glands were carefully removed from the rest of the tissues and were collected in a container surrounded by ice. They were homogenized in a Commitrol 3600 flake cutter (Urshel Corp., Valparaiso, Indiana), to produce a finely-divided paste. The homogenate was thoroughly mixed so that all sub-samples taken from the homogenate would contain identical amounts of toxins.

The proximate analysis, amino acid composition, mineral composition, pH and salinity of the homogenate were determined as additional information.

2.2. Buffering, heating and storage

Six-milliliter homogenate samples were mixed with 6 ml aliquots of 1.2 M citrate/phosphate buffer adjusted to pH's ranging from 3 to 7 and stored under nitrogen in sealed screw-cap tubes at 5, 25 and −35°C for 1 year. Samples prepared in the same manner were heated for 60 min in screw cap culture tubes in a 120°C thermostatically controlled oil bath and temperatures were monitored using thermocouples inserted through the screw caps. Control samples (with no buffer, NB) were heated in the presence of distilled water. After heating, all tubes were immediately transferred into an ice bath. Heated/cooled homogenates were also stored under similar conditions. Three samples of both heated and unheated homogenate from each pH and temperature combination were removed for the extraction and analysis every four months. Homogenates stored at 25°C were extracted only once after one month.

Mixtures of partially purified toxins containing STX, NEO, GTX 1, GTX 2, GTX3, GTX 4, C1, C2, and B1 with the pH levels ranging from 3 to 7 were also prepared and heated in the same manner. Both heated and unheated samples were stored under nitrogen at 5, 25 and −35°C for four months and 2–3 samples from each pH and temperature were analysed monthly for four months.

2.3. Extraction of toxins

The thawed homogenates were stirred well for about 2 min and the contents transferred into a 25 ml beaker. The tubes were rinsed with double distilled, de-ionized water and the contents were added to the beaker,

macerated with a Polytron homogenizer for 10 min at the high speed setting (8). The macerated homogenates were centrifuged in plastic tubes for 30 min at 6000 rpm (4266 *g*), the supernate from each tube was transferred into a 150 ml separatory funnel and de-fatted with dichloromethane:water (2:1). The aqueous layer of each funnel was carefully transferred into Teflon tubes, centrifuged and frozen at −35°C until used for subsequent toxin analyses by high performance liquid chromatography.

2.4. Analysis of toxins by high performance liquid chromatography

All samples were ultrafiltered through 1000 da MW cutoff membranes (MSI, MICRON Separation Inc., West Bord, MA 01581) to remove proteins prior to HPLC injection. PSP's were determined with a Waters high performance liquid chromatograph equipped with two Model 510 pumps, a WISP auto injection system controlled via a system interface module and a Shimadzu Model Rf 535 fluorescence detector (338 nm excitation, 400 nm emission). Samples were analysed on a Whatman PRP-1 column (15 cm×4.1 mm) packed with 10 μm beads using a binary elution gradient (Mobile phase A: water with 1 mM hexane and heptane sulphonic acid, Mobile phase B: acetonitrile with hexane and heptane sulphonic acid) according to Sullivan and Wekell (1987). The HPLC was equipped with a dual reagent post column reaction system. Post-column derivatization was carried out by sequential mixing with nitric acid (0.75 M) and periodic oxidant (5 N sodium hydroxide, 5 mM periodic acid and 0.5 M ammonium hydroxide) prior to the fluorescence detection. Individual toxins were identified by running a standard mixture of PSP toxins and were externally quantified using standard purified natural PSP toxins obtained from the National Research Council of Canada, Institute for Marine Biosciences, Halifax, Nova Scotia. Theoretical specific toxicities for HPLC data obtained for scallop homogenates were calculated using toxicity factors for individual compounds according to Hall and Reichardt (1984).

2.5. Statistical analysis

The toxin levels ($\log_{10}$) at each pH and time interval were compared by Analysis of Variance (ANOVA) with general linear model via multiple regression using indicator variables for log transformed data, and in some cases the rates of change in the amounts of individual toxin components were compared by analysis of covariance (ANCOVA) via multiple regression using indicator variables, in MINITAB Version 12 in the Dalhousie University main frame computer.

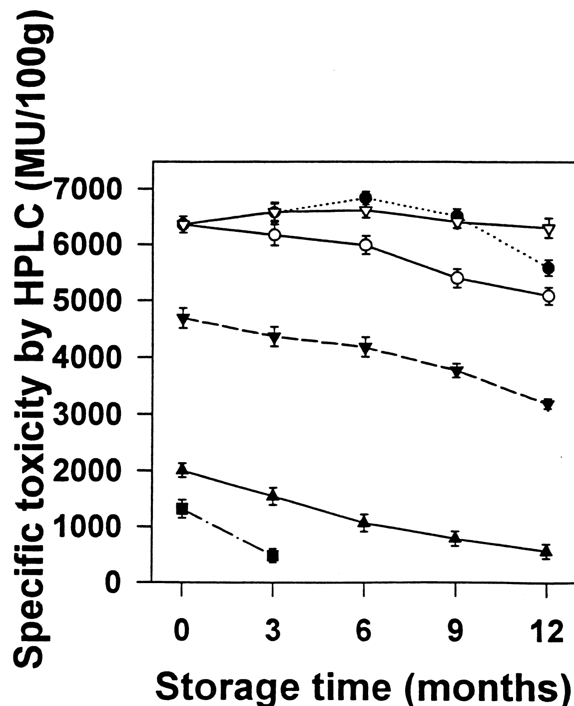

Fig. 2. Variation in the calculated specific toxicities of scallop homogenate during storage [∇ unheated (pH = 3,−35°C), ● unheated (pH = 3, 5°C), ○ unheated (pH = 7, 5°C), ▼ heated (pH = 3, 5°C), ▲ heated (pH = 7, 5°C), ■ heated (pH = 7, 25°C); MU = mouse units; vertical lines show standard error of the mean, *n* = 3].

3. Results and discussion

The toxic scallop homogenate contained 71.12% (±2.34) water, 3.68% (±0.82) lipids, 10.52% (±2.42) ash, 8.82% (±1.23) protein. Glutamic acid, alanine and leucine were the most predominant amino acids whereas cysteine and glycine were present only in small amounts. Triglycerides were the main lipid component in the homogenate followed by free fatty acids and other unknown oxidized compounds. It also contained a wide array of minerals including iron (200 mg/kg), aluminum (84 mg/kg) and cadmium (54 mg/kg).

The toxin levels in heated samples varied widely depending on the pH and the storage temperature. Composition of both heated and unheated homogenates containing mainly GTX 2/3, NEO, STX and C toxins as well as the partially purified toxin mixture consisting of C1/2, GTX 1/4, NEO, B1 and STX changed at different rates during the storage at all temperatures except at −35°C.

Theoretical specific toxicities calculated for HPLC data obtained for some homogenate samples stored at different pH levels and temperatures in the present study are shown in Fig. 2. The toxicities did not change significantly during the storage at −35°C at any pH or at 5°C and at pH 3, whereas there was a slight decrease

at 5°C and at pH 7. Heating for 60 min at 120°C had a far greater effect than storage with regard to total PSP destruction. The rate of thermal destruction of PSP toxins in toxic homogenates was 2-fold at pH 7 compared to that at pH 3, although subsequent storage for 12 months at 5°C showed no comparable pH effect. Specific toxicities determined using the mouse neuroblastosoma cell bioassay (Jellet, Marks, Stewart, Dorey, Watson-Wright & Lawrence, 1992) were positively correlated ($P \leqslant 0.05$) with the theoretical integrated toxicities calculated from data obtained from Sullivan and Wekell (1987) HPLC method (Indrasena & Gill, 1999).

C toxins with relatively low specific toxicity, can be readily converted into highly toxic carbamates such as GTX 2 and 3 under certain conditions (Shimizu & Yoshioka, 1981). However, the C toxins in the purified toxin mixture did not change significantly ($P \leqslant 0.05$) during storage at −35°C for 4 months (not shown). Although they did not change significantly at 5°C at low pH values, there was a significant decrease ($P \leqslant 0.05$) at pH 7 after 2 months (Fig. 3A and B). The C toxin levels also decreased significantly after one month of storage at pH 6–7 and 25°C. There was about a 25% decrease at pH 5 samples stored at 5°C and about a 47% decrease at 25°C after 4 months. The decrease of C toxins at low pH (3–5) may possibly be due to the conversion of C1 and C2 toxins into GTX 2 and 3. However, it is difficult to conclude that this conversion is indeed significant since levels of GTX 2/3 may also be increasing or decreasing by other mechanisms, simultaneously. Although there was about a 40% decrease at pH 7 at 25°C after 2 months, the C toxin levels (pH 6–7) increased after 3 months. This apparent increase may either be due to co-elution of some degraded compounds or to the conversion of some other toxin counterparts to C toxins or by both mechanisms. However at low pH, the toxin degradation was less severe. C toxin levels in the scallop homogenates began to decrease significantly at all pH levels after one month at 25°C, however at −35°C, remained almost unchanged for 12 months.

GTX 2/3 levels in the purified toxin mixture did not change significantly ($P \geqslant 0.05$) at −35°C. However, GTX 2/3 levels decreased slightly after 4 months and were significantly ($P \geqslant 0.05$) reduced after 3 months at 25°C and pH 7. Heated toxin mixtures followed a similar pattern with nearly complete GTX 2/3 destruction in unbuffered and pH 7 samples during storage at 5°C. GTX 2/3 in the unheated homogenates remained more or less constant at 5°C for approximately 3 months and then gradually decreased with the greatest change at the highest pH (Fig. 4A) whereas in heated homogenates they decreased dramatically with the greatest reduction at high pH (Fig. 4B). The consistent decrease of toxin levels may be due to chemical degradation or the conversion of GTX 2/3 into STX by reductive elimination of the sulfonyl group (Shimizu & Yoshioka, 1981) or both. Louzao et al. (1994) indicated that GTX 2/3 in lyophilized samples containing C1-4 toxins increased initially, and decreased during the storage at −80, −20,

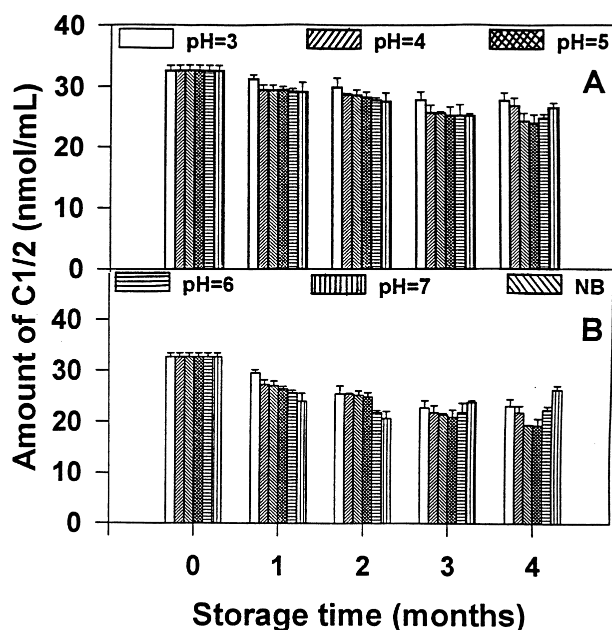

Fig. 3. Changes of C1/2 toxins in the unheated purified toxin mixture (A) storage 5°C, (B) storage at 25°C. (Vertical bars show the variation of toxins at different pH and the symbols show the corresponding pH).

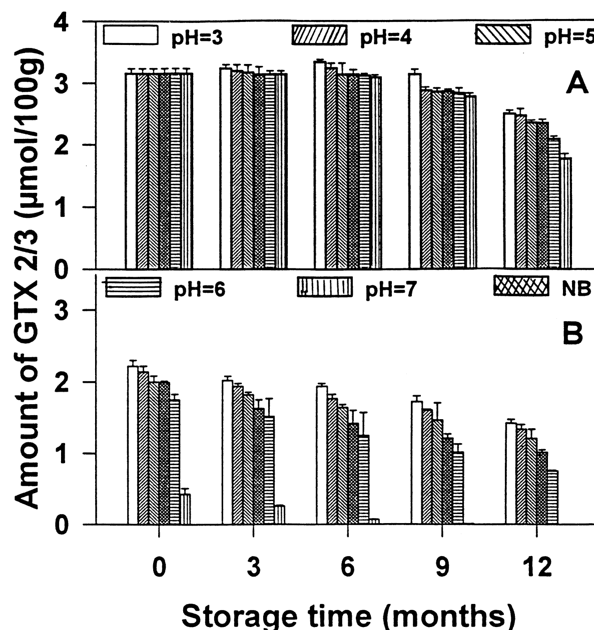

Fig. 4. Changes of GTX 2/3 toxins in scallop homogenate (A) Unheated samples stored at 5°C, (B) heated samples stored at 5°C. (Vertical bars show the variation of toxins at different pH and the symbols show the corresponding pH).

4 and 37°C over a period of 2 years. Louzao et al. (1994) also noticed that all GTXs were stable for almost 2 years when stored at −80 and −20°C in mild acidic (pH 5) solutions whereas the GTX 2/3 increased at 4 and 37°C initially due to interconversions and then gradually declined. However, these changes were only studied at low pH (pH value not given), and whether or not the toxin profiles were significantly different from the initial samples, was difficult to judge since their data were not statistically analyzed. The present study clearly demonstrated that at −35°C and at pH 3–4, GTX 2/3 levels in homogenates as well as in the purified toxin mixture were stable throughout the study period.

The GTX 1/4 levels in the purified toxin mixture changed at all pH levels with time of storage and, as with the other toxins, the changes were more pronounced at neutral pH and at the highest storage temperature. GTX 1/4 levels in unheated samples stored at 25°C decreased significantly ($P \leqslant 0.05$) after 3 months with the maximum destruction of 88% at pH 7 after 4 months (Fig. 5A). However, these toxins remained almost unchanged at pH 3–4 and at 5°C. There was only a 17% decrease in GTX 1/4 at pH 7. GTX 1 and 4 are epimeric 11-hydroxyneosaxitoxin sulphates which can be converted to NEO by reductive cleavage of the O-sulphate group (Shimizu & Yoshioka, 1981) and the decrease of GTX 1/4 on storage may possibly be due to the conversion of these toxins into NEO. Samples stored at −35°C did not show any change for 4 months. The GTX 1/4 content in the heated toxin mixtures also had the same general pattern of change during the storage (Fig. 5B).

The levels of NEO in both heated and unheated homogenates as well as in the purified toxin mixture did not change at any pH during storage at −35°C. Although there was no significant change in NEO in the purified toxin mixture at 5°C at pH 3–5, it degraded slowly at pH 7 with the maximum degradation after 4 months. NEO in unheated purified toxin mixtures decreased by about 33% at pH 7 after 4 months at 25°C.

It was apparent that the toxin degradation observed was primarily due to heating rather than due to storage. NEO in the scallop homogenate at 5°C (pH 3–5) also did not change significantly throughout the study period whereas, at pH 7, NEO levels declined with the maximum loss of 29% after 12 months (Fig. 6A). However, it is interesting to note that NEO degraded much faster at 25°C with the maximum decrease of 25% at pH 7 after 1 month whereas a significant change was not observed at pH 3–5 and in control samples. Alfonso et al. (1994) indicated that lyophilized NEO as well as NEO in mild acidic solutions decreased after 6 months at −80°C and they almost disappeared at −20°C after 1 year.

STX in the frozen (−35°C) homogenate and purified toxin mixture did not change during storage at −35°C at any pH. STX in both heated and unheated homogenates did not change significantly at pH 3–5 and NB samples for 12 months at 5°C whereas it decreased by

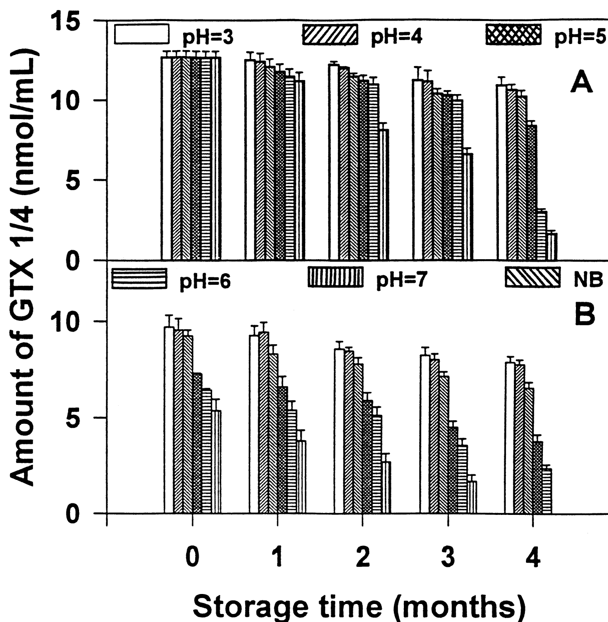

Fig. 5. Changes of GTX 1/4 toxins in the purified toxin mixture (A) unheated samples stored at 25°C, (B) Heated samples stored at 25°C. (Vertical bars show the variation of toxins at different pH and the symbols show the corresponding pH).

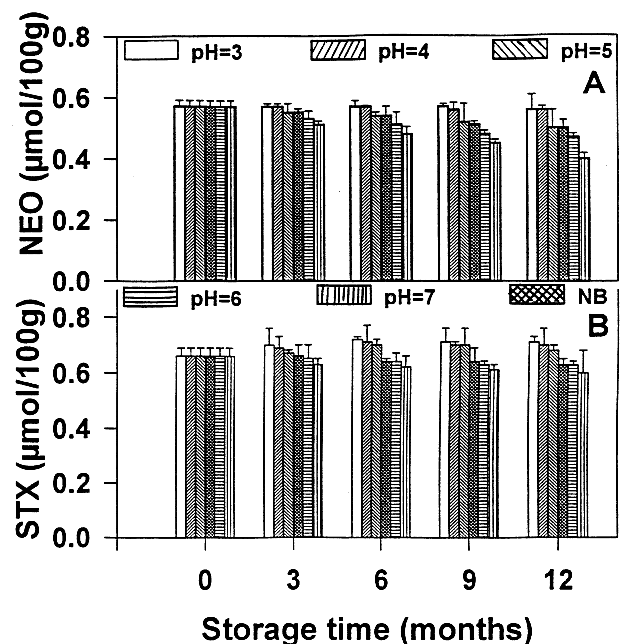

Fig. 6. (A) Changes of NEO in scallop homogenate stored at 5°C, (B) changes of STX in scallop homogenate stored at 5°C (Vertical bars show the variation of toxins at different pH and the symbols show the corresponding pH).

4.5% and 12% at pH 6 and 7, respectively (Fig. 6B). However, at 25°C, STX content at pH 6 and 7, increased by 1.4 and 2.5% after 1 month although there was no significant change at low pH. STX in the heated homogenates decreased to a maximum of 61% after 12 months at 5°C and 41% after 1 month at 25°C. STX in the purified toxin mixture also remained unchanged at 5°C (pH 3–5) whereas there was about a 13% decrease at pH 7 after 4 months. Even at 25°C, STX levels at pH 4–5 as well as NB sample did not change significantly ($P \geqslant 0.05$) (Fig. 7A). It is also interesting to note that STX in the heated purified toxin mixture also followed the same general pattern as unheated samples with the maximum destruction at pH 6–7 at 25°C after 4 months (Fig. 7B).

It is clear that regardless of whether purified or in a shellfish matrix, PSP toxins are most stable at low pH levels. Indrasena and Gill (1999) previously reported that these toxins remain unchanged or increase slightly during heating at low pH, and the present study confirms that toxins retain their potency under acidic conditions during the storage even at room temperature for 4 or 12 months at 5°C. However, the toxicity rapidly decreased at high pH (pH 7) during the storage at room temperature after heating at a conventional canning temperature, as also indicated by Prakash et al. (1971). Thus, selection of proper storage conditions is important. pH 3 was judged as the best pH and low temperature storage at $\leqslant -35$°C is recommended to prevent toxin interconversions and/or degradation.

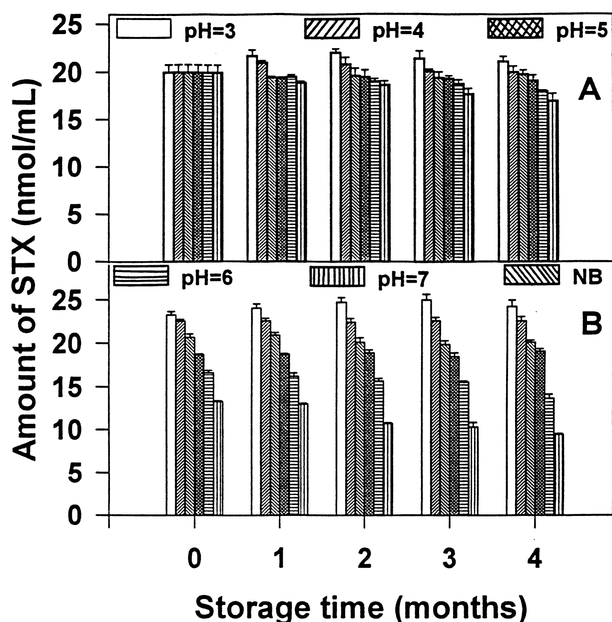

Fig. 7. (A) Changes of STX in the unheated purified toxin mixture stored at 25°C, (B) Changes of STX in the heated purified toxin mixture stored at 25°C. (Vertical bars show the variation of toxins at different pH and the symbols show the corresponding pH.).

4. Conclusions

All toxins were most stable at −35°C and at pH 3–4. C toxins were the most sensitive followed by GTX 1/4 for changes at all pH levels and at 5 and 25°C. STX followed by NEO were the most stable at −35 and 5°C, especially at pH 3–4. Although previous reports indicated further decreases in toxicity during storage subsequent to canning, these decreases are dependent at least in part upon the pH of the matrix in which the toxins are found. Thus, purified toxins used as primary analytical standards as well as partially purified toxins and toxins in a homogenized matrix, sometimes used as secondary standards, can be stored safely at pH 3 and at $\leqslant -35$°C.

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