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节织纹螺饲喂富集河豚毒素及相关差异蛋白表达的研究

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摘要: 我国东南沿海经常有食用含有河豚毒素节织纹螺的中毒事件发生,含有河豚毒素的动物对河豚毒素有很高的耐受能力,并对环境中的河豚毒素具有趋食性。织纹螺可以富集食物中的河豚毒素,但织纹螺体内的与河豚毒素富集相关的蛋白质还不清楚。为此我们收集了无毒的织纹螺,通过河豚毒素富集实验和蛋白质组学分析,发现在有毒织纹螺中有 4 种蛋白质表达量显著增多,飞行质谱(MALDI-TOF-MS)鉴定分析它们分别是 actin、actin2、beta-actin 、膜泡 ATP 合酶 B 亚基。分析表明这些蛋白可能与河豚毒素在织纹螺体内富集、传递有关。

关键词: 织纹螺; 河豚毒素; 富集; 双向电泳; 传递
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Tetrodotoxin Enrichment by Feeding Toxin to the Snail *Nassarius semiplicata* and Proteomic Analysis of Differentially Expressed Proteins

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Abstract: Nassarius poisoning was highly prevalent in the Fujian province of China. Snails contain tetrodotoxin (TTX) which is a potent neurotoxin. TTX-producing animals have higher resistance to the toxin. Snails contain TTX were like to eat diet with TTX. Consumption of TTX diet results in its accumulation in snails. The changes of protein expression profile in TTX-enrichment snail Nassarius semiplicata has not been explored yet. In this study, two-dimensional electrophoresis was used to investigate the protein profile in TTX-enrichment snail Nassarius semiplicata. The results showed that snail Nassarius semiplicata can be attracted by TTX and enrich TTX through food chain. Four proteins including actin, actin 2, β-actin, and vacuolar ATP synthase subunit B partial were observed in this snail. These four proteins may be related to TTX enrichment and transportation in Nassarius semiplicata.

Keywords: Nassarius; tetrodotoxin; enrichment; two-dimensional electrophoresis; transportation.

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Introduction

Nassarius semiplicata (Gastropoda, Nassariidae) are common snails mostly distributed in the southeastern part of China. It is a major cause of food poisoning in the Fujian province each year mainly because it is difficult to distinguish the toxic from the non-toxic Nassarius species through its appearance (Fig. 1). Tetrodotoxin (TTX) and its derivatives are the toxic components in the snails which cause the poisoning [1-3]. TTX is a small molecular weight guanidinium containing neurotoxinthat blocks the voltage-gated sodium channels in nerve and muscle tissues thereby blocking the excitatory conduction [4].

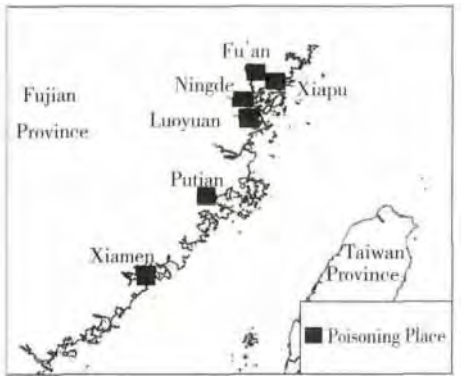

Fig. 1　Poisoning places in Fujian of China

TTX was firstly discovered in pufferfishand was lately reported in other animals like, newts (Triturus spp. and Cynops pyrrhogaster), frogs, nonvertebrates and bacteria [5-7]. TTX is used as a defensive biotoxin, defensive and predatory venom by animals [8]. Some species like the planocerid flatworm appears to use TTX to overcome larger preys like gastropod while some species are known to use it as a pheramone [9].

TTX-producing animals have high resistance to the toxin, with minimal lethal dose in the range of 3.0 to 7.5 mg TTX·kg^{-1} body weight in while it ranges from 3 to 42 μg TTX·kg^{-1} body weight in non-producers [10]. TTX-accumulating snails and pufferfish (Polinices didyma, Natica lineata, Natica vitellus, Zeuxis sufflatus, Niotha clathrata, Oliva miniacea, Oliva mustelina, Oliva hirasei, T. rubripes) are also found to get attracted to the presence of TTX in its diet, while non TTX snails do not respond to the presence of the toxin in diet [11-12].

TTX-binding proteins were found in puffer fish and toxic snails which are likely to contribute their resistance to TTX. Saxitoxin and tetrodotoxin binding protein (PSTBP) bind to TTX in pufferfish plasma and transport TTX to toxin-accumulation tissue, such as liver and egg [13-15]. Two proteins homologous to PSTBP were also found in the plasma of non-toxic cultured specimens of the pufferfish (Takifugu rubripes) suggesting that PSTBP may not be the only proteins related to TTX reserve [16]. TTX-binding high molecular weight substance (HMWS) in the toxic gastropods is also likely to play a role in the transfer and transport of the toxin [17].

Although many studies on TTX-binding proteins have been carried out, TTX enrichment and proteomic analyses after enrichment have not been studied so far. In the present study, The Nassarius semiplicata snails were fed with toxin containing feed and the protein profiles of the muscle were analyzed using the 2D electrophoresis. Differentially expressed proteins were identified using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry(MALDI-TOF-MS). The purpose of this study was to investigate the proteins involved in TTX enrichment and molecular mechanism of tolerance to TTX toxicity.

1　Materials and methods

1.1　Toxin reaction experiment

Fresh Nassarius semiplicata samples were collected from Ningde city, Fujian province in June 2011. The snails´weight and length were 0.99-0.09 g and 1.98-0.15 cm. The maximum toxicity of snails detected were 150 MU·g^{-1}. They were separated into two groups, with 100 snails in each group, and cultured in two water tanks with simulated seawater.

Sliced flesh sea bass (20 g) were soaked in 200 mL TTX solution (150 MU·mL^{-1}) overnight to make feed with high toxin content. The Nassarius semiplicata snails were fed with toxin containing feed or normal feed, and photos were taken within 2 minutes.

1.2 Attraction of snails to Toxin

The diet of Nassarius semiplicata was prepared as above. The equipment used for the attracting test is shown in Figure 2. The height of seawater was about 20 cm. The TTX diet was applied to sites B and the control diet was applied to sites A, and the distance between site A and site B was about 25 cm, and 50 specimens of snail species were put into the bottom of the seawater. Photos were taken every 2 minutes and numbers of snails in sites A and sites B were counted. The number of snails that had migrated into area A or B compared with the total number of snails tested gave the percentage attracted.

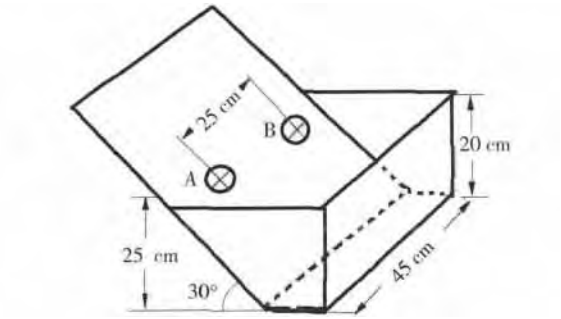

Fig. 2 The equipment for the attracting test

1.3 Toxin content analysis

20 grams of the edible part of snails were homogenized with 20 mL 0.1% (V/V) acetic acid solution, centrifuged and the supernatant were incubated in boiling water for 10 min and used for toxicity assay. The assay was performed by Japanese standard method for TTX analysis. Shortly, the supernatant was injected into three male ICR mouse (body weight 18-20 g) by intra peritoneal route. Toxicity was calculated using the median death time of mice and the result was expressed as MU · g^{-1}. One MU is defined as the amount of toxin required to kill a mouse in 30 min after injection.

1.4 Toxin enrichment and detoxification

Diet was prepared as described earlier, with different concentrations of TTX solutions ranging from 0 MU·mL^{-1}, 20 MU·mL^{-1}, and 150 MU·mL^{-1}. Groups of 100 snails were fed with 20 grams food, twice a week for 2 months. Samples were collected every 15 days to detect toxin content. Experiments were repeated twice.

1.5 Proteomic Analysis

1.6 Protein extraction

The edible part of specimen was dried and powdered using liquid nitrogen and then was homogenized and lysed using an ultrasonic disrupter in 1 ml of 20% TCA/acetone (wt/vol) buffer with 20 mmol·L^{-1} dithiothreitol (DTT). The supernatant was removed by centrifugation at 17,000 × g for 30 min at 4 ℃, and the pellet was washed twice with 80% acetone (vol/vol) and twice with ice-cold acetone with 20 mmol·L^{-1} DTT. The pellet was recovered by centrifugation at 17,000 × g for 30 min at 4 ℃ each time. Residual acetone was removed in a Speed Vac for about 5 min. The pellet was dissolved in 100 μL rehydration buffer containing 7 mol·L^{-1} urea, 4% CHAPS, 2 mol·L^{-1} Thiourea. The solution was centrifuged at 20,000 × g for 30 min at 4℃ and the supernatant was collected for two-dimensional electrophoresis (2-DE) analysis. The protein content was quantified using the 2-D Quant kit (GE Healthcare, San Fransico, CA).

1.7 Two-dimensional electrophoreses analysis

Hundred micrograms of each protein sample was mixed with 340 L buffer containing 2 L IPG buffer, 0.0034 g DTT, and rehydration buffer then loaded onto IPG strips of linear pH gradient 4-7 (GE Healthcare). Rehydration and subsequent isoelectric focusing were conducted using the Ettan IP Gphor III Isoelectric Focusing System (Amersham Biosciences, San Fransico, CA). After rehydration, isoelectric focusing was performed in the following manner: 6 h at 40 V, 6 h at 100 V, 30 min at 500 V, 1 h at 1 000 V, 1 h at 2 000 V, and 1.5 h at 10 000 V. 60 kVh at 10 000 v. After the first dimension was run, each strip was equilibrated with about 10 ml equilibration buffer containing 50 mmol·L^{-1} Tris (pH 8.8), 6 mol·L^{-1} urea, 30% glycerol, 2% SDS, 1% DTT, and a trace amount of bromophenol blue for 17 min. The strip was then placed in fresh equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 17 min. Subsequently, a 12.5% SDS-PAGE second dimension was

performed. Electrophoresis was carried out at 25 mA·gel^{-1} for 30min, followed by a 4.5 h run at 50 mA·gel^{-1} until the bromophenol blue front reached the edge of the gels. The gels were then visualized by silver staining.

1.8　Silver staining

Silver staining was performed following the method of Wang [18]. Briefly, the gel was fixed for 2 h initially in a fixation solution containing 40% ethanol and 10% acetic acid. It was then sensitized in a solution containing 30% ethanol, 0.2% sodium thiosulphate, 6.8% sodium acetate, and 0.125% glutaraldehyde, followed by three Milli-Q water washes (5 min each time). Then, the gel was stained for 20 min in 0.25% silver nitrate with 0.015% formaldehyde and washed twice with Milli-Q water (0.5 min each time). The gel was developed in 2.5% sodium carbonate containing 0.0074% formaldehyde. The reaction was stopped with 1.5% EDTA, disodium salt. Finally, the gel was washed three times with Milli-Q water.

1.9　Image capture and analysis

Gel images were captured using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad and 2-DE electrophoretogram matching software. Images were saved in TIFF format before analysis. Computerized 2-DE gel analysis (spot detection, spot editing, pattern matching, and database construction) was performed using the Image Master 2D Elite (GE Life Science) and Melanie IV. There were three gels for samples from control or toxic sample. After spot detection and matching, specific spot in toxic sample were selected for identification by mass spectrometry.

1.10　Mass spectrometric analysis

Differentially expressed protein spots in edible part of snail were manually excised from 2-DE gels. The gel pieces were washed twice with 200 mmol·L^{-1} ammonium bicarbonate in 50% acetonitrile/water (20 min at 30 ℃), dehydrated using acetonitrile, and spun dried. Trypsin digestion was performed by adding 20 ng·μL^{-1} trypsin (Promega, Madison, WI) in 25 mmol·L^{-1} ammonium bicarbonate buffer overnight at 37 ℃. For MALDI-TOF-MS analysis, (4800 Proteomics Analyzer; Applied Biosystems, Foster City, CA) 1 μL of the digest mixture was mixed on-target with 1 μL of 100 mol·L^{-1} α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid on the target plate. MALDI-TOF-MS and TOF/TOF tandem MS were performed, and data were acquired in the positive MS reflector mode with a scan range from 900 to 4 000 Da, and five monoisotopic precursors (S/N > 200) were selected for MS/MS analysis. F peptide fragmentation patterns were used for protein identification in an NCBI non-redundant database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm.

One missed cleavage site was allowed for trypsin digestion, cysteine carbamidomethylation was assumed as a fixed modification, and methionine was assumed to be partially oxidized. Results with confidence interval% (CI%) values greater than 95% were considered to be a positive identification for the interpretation of the mass spectra.

2　Results

2.1　Reaction of Nassarius semiplicata to TTX

After feeding flesh containing TTX, snails appeared activated, energetic and moved towards the diet, however the response of snail to diet without TTX was relatively slow. When 66 snails moved towards the diet containing the toxin in 2 minutes, only 31 moved towards diet without TTX (Fig. 3).

2.2　Toxin attraction

The TTX diet was applied to sites B and the control diet was applied to sites A. Photos were taken every two minutes and the number of snails

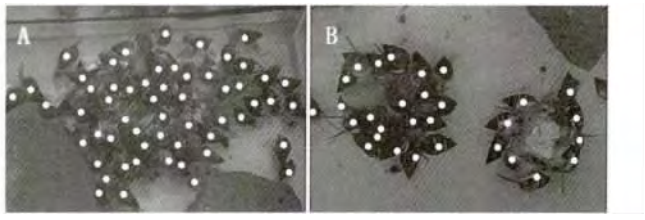

Fig. 3　The effects of feed attraction of flesh with TTX (A) or flesh without TTX (B) in Nassarius semiplicata within 2 minutes (snails were counted by white spots)

that had migrated into area A or B were compared with the total number of snails tested and the percentage of animals attracted to the toxin was calculated (Fig. 4). The number of snails attracted by the diet containing TTX gradually increase and reached to peak value 74% at 3rd minute, while the attraction to the diet without toxin did not show any increase until the toxin containing diet was consumed.

2.3 Toxin enrichment experiment

The non-toxic snails were fed with a diet containing 20 MU·g^{-1} or 150 MU·g^{-1} TTX and the toxic snails were fed with diet containing 0 MU·g^{-1} or 20 MU·g^{-1}. When snails given a diet with high toxin content, they could enrich tetrodotoxin, but the toxin content fluctuated with time when the snails were given a low toxin diet (Fig. 5). The toxin content slowly declined in snails which were given a low or non-toxin containing diet, with the rate of decline higher with no toxin diet compared to low toxin diet.

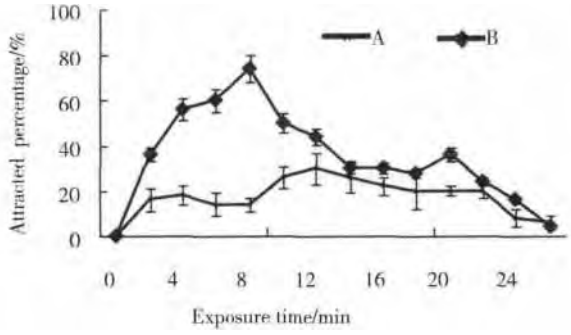

Fig. 4 The relationship between the percentage attracted and exposure time when snails were exposed to the tetrodotoxin diet (150 MU·g^{-1}, site B) and the control diet (site A)

The results showed that snail Nassarius can enrich and exhaust tetrodotoxin.

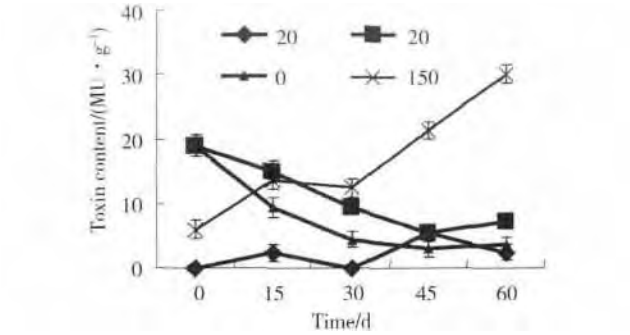

Fig. 5 The toxin enrichment and discharge experiment in Nassarius cultured by toxic and non-toxic diet.

2.4 Proteomic Analysis

Non-toxic snails had been given a toxin containing diet for 2 months, the content of toxin in muscular tissue detected by two-dimensional electrophoresis, silver staining, and scanning, electrophoresis. Snails fed with non-toxin diet were used as control Fig. 6. Proteins (100 μg per sample) were separated by 2-DE and detected by silver staining. Spot numbers refer to the numbers given in Table 1.

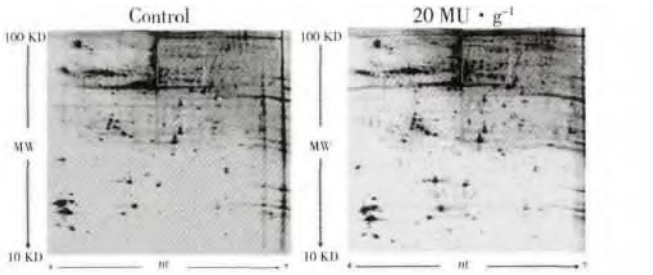

Fig. 6 Protein expression patterns of muscular tissue of Nassarius semiplicata from Ningde

Table 1 List of spots/proteins identified by MS + MS/MS analysis from Nassarius semiplicata after 2D electrophoresis

Spot ID	Accession No.	Protein name	Theoretical pI	Theoretical MW	No. of matched peptides	Protein score	Protein score C. I. %
1	223016073	actin (Todarodes pacificus)	5.22	42022.9	16	554	100
2	18565104	actin 2 (Crassostrea gigas)	5.3	42001.9	9	500	100
3	159507454	β-actin (Crassostrea ariakensis)	5.3	42055.8	10	126	100
4	119874561	vacuolar ATP synthase subunit B partial (Leptochiton sp. SJB-2006)	4.81	21958	5	309	100

Four altered protein spots were submitted for identification using MALDI-TOF-MS analysis and searches in the NCBI nr database (Tables 1). They are actin, actin 2, β-actin and vacuolar ATP syntheses subunit B partial.

3 Discussion

TTX stimulates the gustatory nerve of fish at a fairly low concentration [19]. TTX may serve as an anti-predator defense, an offensive weapon (venom), or for inter-species communication related to location of mates/eggs, potential food sources, or threat [18-19]. TTX has been shown to be produced by bacteria, including Vibrio alginolyticus, Vibrio parahaemolyticus, Pseudomonas sp. and Aeromonas sp. [22-23], but no direct relationship between the total count or viable count and the toxicity of the shellfish [24]. The function of TTX in any microbial producers has not been tested. Many TTX-accumulating snails (Polinices didyma, Natica lineata, Natica vitellus, Zeuxis sufflatus, Niotha clathrata, Oliva miniacea, Oliva mustelina, and Oliva hirasei) are attracted to the toxin, while non-toxin producing species are not attracted to TTX [11]. The toxin producing snails appear to prefer foraging on TTX containing food items.

The results of TTX enrichment experiment showed that Nassarius could enrich TTX when fed with toxic diet, and could balance the toxin on low or non-toxin diet. We believe that TTX had a significant ecological function for Nassarius. There are some mechanisms which contribute to TTX enrichment and transport in the Nassarius.

Tetrodotoxin-bearing animals usually have higher resistance to the toxicity of TTX than normal animals. For example, the toxic xanthid crabs Zosimus aeneus and Atergatis floridus have a high resistance to TTX [25-26]. Shiomi et al. reported that the mechanism of resistance was related to neutralization of the toxin by TTX-binding high molecular weight substances from crab body fluid [27]. These TTX-binding proteins in the animal system seem to be biologically significant with respect to defense against the toxins or bioaccumulation of the toxins [28]. It is likely that absence of these TTX-binding proteins make non-toxic snails rejecting the TTX diet. Further investigations are being carried out at the Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, concerning the TTX-binding protein in toxic snails.

The differentially expressed proteins related to TTX enrichment in toxic Nassarius were determined to be actin and ATP synthase subunit B partial. Actin is the most abundant protein in most eukaryotic cells, accounting for 10% of the total protein. Actin participates in many important cellular processes, including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Actin contributes the intracellular transport of vesicles and organelles as well as muscular contraction and cellular migration.

We earlier found higher levels of TTX in muscle, as reported in the new gastropods Olive maniacal, O. mustering and O. nausea [17]. Muscle tissues are rich in actins (about 10%), so altered actins may be connected with Nassarius toxin enrichment.

Vacuolar ATP synthase is also a highly conserved evolutionarily ancient enzyme with remarkably diverse functions in eukaryotic organisms. V-ATPase´s couple the energy of ATP hydrolysis to proton transport across intracellular and plasma membranes of eukaryotic cells and are important to form a proton gradient in various intracellular organelles. Subunit B of vacuolar ATPase´s, pump protons at the expense of ATP hydrolysis. V-ATPases contain more than 10 subunits. There are three copies of subunit A, at the site of ATP hydrolysis. Three copies of subunit B form an alternating heterohexamer with subunit A. V-ATPase have actin binding sites that mediate interactions between the intact enzyme and filamentous-actin [29]. The actin-binding site in subunit B is physically located in the region of the intact enzyme furthest away from the associated membrane [30]. Vacuolar H + -ATPase (V-ATPase) is a fundamentally important enzyme in eukaryotic cells

that is responsible for acidification of endocytic compartments, which is important for cellular material transportation. The B subunits are regulating switch of V-ATPase [31]. We speculate that the higher expression of B subunits of V-ATPases may be related for TTX enrichment and transportation.

As mentioned above, it can be concluded that the snail Nassarius gets attracted to TTX and enriches TTX through food chain and keeps a balance of toxin by discharge of TTX. We found four altered proteins related to toxin enrichment by two-dimensional electrophoresis and MALDI-TOF-MS analysis. Actin, actin 2, β-actin, and vacuolar ATP synthase subunit B partial, were differentially expressed and may be related to enrichment and transportation of TTX in Nassarius snails.

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ISO 发布关于纺织品中的邻苯二甲酸酯的国际标准

2014 年 10 月 10 日 来源: 国际标准化组织

国际标准化组织(ISO)发布了一项关于纺织品中的邻苯二甲酸酯的全球标准。这一编号为 EN ISO 14389:2014 的标准涵盖用四氢呋喃使用超声波提取法测定涂覆或印花纺织品中 10 种邻苯二甲酸酯的含量。

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