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Selective isolation of gonyautoxins 1,4 from the dinoflagellate *Alexandrium minutum* based on molecularly imprinted solid-phase extraction

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ABSTRACT

Gonyautoxins 1,4 (GTX1,4) from *Alexandrium minutum* samples were isolated selectively and recognized specifically by an innovative and effective extraction procedure based on molecular imprinting technology. Novel molecularly imprinted polymer microspheres (MIPMs) were prepared by double-templated imprinting strategy using caffeine and pentoxifylline as dummy templates. The synthesized polymers displayed good affinity to GTX1,4 and were applied as sorbents. Further, an off-line molecularly imprinted solid-phase extraction (MISPE) protocol was optimized and an effective approach based on the MISPE coupled with HPLC-FLD was developed for selective isolation of GTX1,4 from the cultured *A. minutum* samples. The separation method showed good extraction efficiency (73.2–81.5%) for GTX1,4 and efficient removal of interferences matrices was also achieved after the MISPE process for the microalgal samples. The outcome demonstrated the superiority and great potential of the MISPE procedure for direct separation of GTX1,4 from marine microalgal extracts.

Harmful algal blooms are a global threat to coastal marine ecosystems, with consequences for fisheries and shellfish production. *Alexandrium* is among the most common bloom forming toxic dinoflagellate genera and is generally held responsible for the outbreak of paralytic shellfish poisoning (PSP) (Harrison et al., 2016; Waal et al., 2015). More specifically, *A. minutum* has been observed worldwide with increasing bloom frequency (Diercks et al., 2008). PSP toxins are a group of neurotoxic compounds, including saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins (GTX), and their *N*-sulfocarbamoyl variants the B- and C-toxins (Costa et al., 2016; Wang et al., 2016). Structurally, they are perhydropurine skeletons fused with a five-membered ring and two guanidinium groups. They also possess a hydrated ketone, a unique structural feature stabilized by the electron-withdrawing guanidine moieties linked to the carbonyl α-carbon (Jansson and Åstot, 2015). These potent neurotoxins can accumulate in the food chain through the filter-feeding of toxin-producing algae by bivalvemollusc shellfish (Burrell et al., 2016).

Currently, the mouse bioassay (MBA) is internationally accepted for monitoring of PSP in shellfish (Etheridge, 2010), however, this method faces increasing ethical concerns. As an alternative to the mouse bioassay method, methods based on high-performance liquid chromatography-fluorescence detection (HPLC-FLD) have been developed and approved by the Association of Analytical Communities (Lawrence et al., 2005; Rey et al., 2017). The basis for the method is the ability of PSP toxins to be easily converted into fluorescent derivatives. Regardless of the detection method, sample extraction plays a significant role. An improper extraction method can inadvertently alter the original toxin profiles due to the easily biological and chemical interconversion of paralytic shellfish toxins. In addition, taking into account that the developed separation method should answer the regulatory need, the extraction procedure should be easy, rapid and reproducible.

Solid phase extraction (SPE) has been the most widely used separation technique in the past decades. The commercially available SPE cartridges mainly are packed with silica, C8, C18, florisil and Al_2O_3. A C18 cartridge has been used to separate the derivates of PSP toxins and one of the main disadvantages of the classical SPE sorbents is low selectivity to the targets. Matrix interferences can be retained on SPE sorbents and co-extracted unavoidably, which can seriously affect the accurate quantitation. Fortunately, molecularly imprinted solid-phase extraction (MISPE) is an efficient approach for purification and preconcentration of analytes from complex matrices, and it is gaining considerable interest in environmental, clinical, and food analysis (Lian and Wang, 2016; Miranda et al., 2016; Nestora et al., 2016). Molecular imprinting is known as a technique for creation of tailor-made binding sites with memory of the shape, size and functional groups of the template molecules. Hence, molecularly imprinted polymers (MIPs) bear molecular recognition sites that resemble immunosorbents, and they can specifically rebind to a target molecule or other closely related

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compounds. The molecular recognition properties, combined with high stability, mechanical robustness and low cost make MIPs extremely attractive as selective capture materials. Compared to traditional SPE, MISPE presents the high resolving power for separation (He et al., 2016; Vlakha et al., 2016).

At present, there have been some researches on the clean-up or isolation of marine biotoxins using the molecular imprinting technology. Kubo et al. (2006) developed an effective molecularly imprinted polymer for domoic acid. The polymer was packed with a LC column for separation of domoic acid in the extract from blue mussels, and the effective chromatographic separation was achieved. Zhou et al. (2011) showed that MISPE-HPLC method could be applied to direct determination of domoic acid from seafood samples, and good linearity was obtained in range of 0.5–25 mg L^{-1} with a quantitation limit of 0.1 mg L^{-1}. Mei et al. (2016) synthesized a kind of new imprinted polymers by bulk polymerization using guanosine as dummy template molecule for clean-up of PSP toxins in cultured seawater samples, and the analyte was successfully detected under optimizing MISPE conditions with low limit of detection. In our previous studies (Lian and Wang, 2013), a molecularly imprinted material was prepared by suspension polymerization using caffeine as the dummy template molecule. A highly selective MISPE procedure was developed for the isolation of GTX2,3 from *A. tamarense* sample, which contained C toxins and GTXs. The MIP successfully carried out for the isolation and clean-up of GTX2,3 and there is no interferences (C1,2 and GTX1,4) detected in the elution after MISPE. After that, the imprinted material was employed as sorbents to selectively recognize and purify GTX2,3 from *A. minutum* extract (Lian et al., 2016).

On the basis of these reports described above, the aim of this study was to develop an efficient isolation method of GTX1,4 from *A. minutum* samples using MISPE technology. Molecularly imprinted polymer microspheres (MIPMs) were synthesized by double-templated imprinting involvement of both caffeine and pentoxifylline (PTX) as the pseudo co-templates. The morphology and structure information of imprinted microspheres were observed and the selective recognition ability was investigated by adsorption experiments. Afterwards, MIPMs were applied as a special MISPE sorbent for selective recognization of GTX1,4 in marine microalgal extract samples.

MIPMs were synthesized using caffeine and PTX as double templates by aqueous suspension polymerization with methacrylic acid (MAA) as function monomer, ethylene glycol dimethacrylate (EGDMA) as cross-linker and polyvinyl alcohol (PVA) as dispersive reagent. Typically, caffeine (1 mmol), PTX (1 mmol), MAA (8 mmol) and EGDMA (40 mmol) were dissolved in 15 mL of chloroform in a 250 mL borosilicate glass bottle. The mixture were added into 150 mL water solution containing 6 g PVA, and rotated at 150 rpm for 30 min at 25 °C to form a complex of template molecules and monomers. After adding 100 mg of 2,2-azoisobutyronitrile, the solution was purged with dry nitrogen for 15 min and polymerization was done overnight in a water bath at 60 °C by stirring vigorously. Subsequently, the obtained microspheres were sieved to collect particles with sizes of 75–125 μm and extracted with pure methanol using a Soxhlet apparatus. Finally, MIPMs were washed with methanol thrice and dried under vacuum at room temperature. As a control, the non-imprinted polymer microspheres (NIPMs) were prepared using the same formulation but without the addition of the double templates.

To evaluate the static adsorption capacity of the microspheres to GTXs, 20 mg MIPMs (or NIPMs) were dispersed in 2 mL of aqueous solution of GTX1,4 with various concentrations from 80 to 640 μg L^{-1}. Then the mixture was sealed and shaken for 24 h at 25 °C to ensure the equilibrium. After centrifugation and filtration with 0.22 μm cellulose acetate membrane filters, the solution containing the free GTX1,4 was measured by HPLC. The amount of GTX1,4 bound to the polymer was calculated by subtracting the amount of free GTX1,4 from the initial amount added to the mixture.

The selectivity of the obtained imprinted polymers was also investigated and GTX2,3 was selected as its structural analog. Briefly, 2 mL of toxins mixed standard (GTX1,4 and GTX2,3) at the concentration of 80 μg L^{-1} was equilibrated with 20 mg MIPMs or NIPMs. The mixture was treated as mentioned in the static adsorption experiment. The concentration of each toxin in the supernatant was determined by HPLC-FLD, according to the Lawrence et al. (1996) method. Pre-column oxidation was performed using the oxidation of periodate for GTX1,4 and hydrogen peroxide for GTX2,3.

Toxic strains of *A. minutum* AM-1 isolated from Taiwan, China were selected for this work because the predominant toxins produced by this strain are GTX1,4, while low levels of GTX2,3 have also been reported (Lian et al., 2016). Cultures were maintained in f/2 modified medium at 20 °C, 4000 lx on 12 L/12 D cycle. Microalgal samples were harvested with approximately density of $\sim 1 \times 10^5$ cells mL^{-1} by centrifugation. The supernatant was discarded and the precipitate was transferred to a 10 mL glass bottle using 5 mL of 0.1 M acetic acid. The mixture was immediately sonicated with a probe sonicator for 10 min and the supernatant was collected, filtered through 0.22 μm membrane filter. The crude extract was stored at − 20 °C and diluted to the required concentration with Milli-Q water just prior use.

The MISPE procedure was performed in off-line cartridge mode and an empty glass syringe (1.0 mL, 2 cm × 0.9 cm i.d.) was filled with 100 mg of MIPMs (or NIPMs) absorbents. Before analyte loading, the MISPE cartridge was pre-conditioned by 2 mL of pure water and 2 mL of methanol. For the MISPE procedure of GTX1,4 toxins, 1 mL of the diluted extract samples were passed through the MISPE cartridge at a flow rate of 0.25 mL min^{-1}. The cartridge was successively rinsed with 0.5 mL of methanol-water (95:5, v/v) and 0.5 mL of pure water. Then, the analyte retained on the sorbent was eluted with 1 mL of 0.1 M acetic acid and collected for subsequent HPLC analysis.

There are many methods, such as bulk polymerization, suspension polymerization and multi-step polymerization for the preparation of imprinted materials (Fayazi et al., 2015; He et al., 2016; Yang et al., 2015). In the study, MIPMs were prepared through suspension polymerization. The polymerization can obtain desired copolymer with a narrow, homogeneous and spherical particles, promoting the collection of smaller particles and increasing surface area, which are more suitable as stationary phase in chromatography. On account of the high toxicity and expensive cost of GTXs, dummy imprinting is employed to synthesize MIPMs. By the imprinting method, the structural difference between the pseudo template and the analyte is very likely to cause impact on the performance of the imprinted material, such as reduction of adsorption capacity and selectivity (Liu et al., 2015). In our previous study (Lian and Wang, 2013), the caffeine was chose as the pseudo template imprinting GTXs, however, it could not offer enough adsorption capacity due to smaller spatial configuration of caffeine molecule. Thus, double-templated imprinting strategy was used to prepare the imprinted material in this study. Caffeine and PTX were chosen as double templates, which both belong to the alkaloids containing purine ring and have similar chemical property with GTXs. At the same time, acidic compound MAA was selected to be functional monomer and its carboxylic functional group is considered as an excellent hydrogen bond donor acceptor group which can participate in the formation of hydrogen bonding interactions with the template.

The shape and surface morphology of the polymer were investigated and shown in Fig. S1 by cold field emission scanning electron microscope (SEM, Hitachi S-4800, Japan). It was found that spherical particles with uniformly size were obtained by suspension polymerization from Fig.S1-A. The average particle diameter was approximately 100 μm and it is suitable as stationary phase in chromatography. MIPMs have more dense and homogenous micropores in the surface (Fig. S1-B). Obviously, the uniform and more open structure favored the embedding of the target molecule. It is well known that not only porogenic solvent but also template molecule would affect the surface morphology and particle sizes of imprinting materials (Miura et al., 2016; Valero-Navarroa et al., 2011). Moreover, specific surface area was also