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Marine Biotoxins in shellfish: Brevetoxin (A Review)

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ABSTRACT

Molluscan shellfish can accumulate natural poison produced by marine algae, one of which is brevetoxin (BTx). BTx is a toxin produced by marine dinoflagellates of the Karenia genus, especially Karenia brevis, which has the characteristics of being fat-soluble, lipophilic, colorless, tasteless, and very heat stable. Based on the backbone structure, BTx is classified into BTx-1 and BTx-2. BTx-2 is the most common type of toxin produced by K. brevis. BTx was synthesized from a polyketide synthase pathway that could potentially incorporate larger carbon units of acetate modified by the citric acid cycle. BTx poisoning causes neurologic shellfish syndrome (NSP), which is characterized by major symptoms in the neurological and gastrointestinal systems. NSP is due to the high affinity of BTx in binding to receptors on the cell wall, namely voltage-gated sodium channels (NaV). Based on the food safety risks posed, monitoring programs to detect the growth of harmful algae and the presence of brevetoxin in food should be implemented. Several detection methods have been developed, such as mouse bioassay, cytotoxicity assay, receptor binding assay, immunoassay and LC-MS/MS.

Keywords: Natural toxin, shellfish toxin, neurologic shellfish syndrome, karenia brevis, marine biotoxin

Introduction

Molluscan shellfish, such as mussels, oysters, scallops, and clams, are marine animals that can accumulate many toxins during their life cycle due to their filter-feeding nature (Turner et al., 2015; Watkins et al., 2008). One of the toxins that can accumulate is a natural poison produced by marine algae, such as brevetoxin (Abraham et al., 2018). Brevetoxin (BTx) is a toxin produced by marine dinoflagellates of the Karenia genus, especially *Karenia brevis* (formerly called *Ptychodiscus brevis* and *Gymnodinium breve* (Baden et al. 2005; Brovedani et al., 2016). This toxin was first identified in the Gulf of Mexico in 1947, when the BTx-producing algae caused the waters around the bay to turn red-brown. Besides K. brevis, several other algae species can also produce BTx-like toxins, such as *Chattonella antiqua*, *Chattonella marina*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* (FAO, 2004).

BTx is lipophilic, colourless, tasteless, and very heat stable (Abraham et al., 2018). Based on the molecular backbone structure, BTx is classified into two types, BTx type A (BTx-1) and type B (BTx-2) (EFSA, 2010). BTx-2 is the most common type of toxin produced by *K. brevis* (Plakas & Dickey, 2010). The two types of BTx are distinguished by the number of transfused cyclic rings, whereas BTx-1 and BTx-2 have 10 and 11 transfused cyclic rings, respectively. These differences in characteristics affect the structural flexibility of BTx (Brovedani et al., 2017). Based on this structure, BTx-1 and BTx-2 are parent toxins of other toxins that are also produced by *K. brevis*, such as hemi-brevetoxin and brevenals. This is because both toxins have a shorter ring structure and are considered biosynthetic products. incomplete BTx (Brovedani et al. 2017; EFSA, 2010).

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One of the main syndromes caused by BTx poisoning is neurologic shellfish syndrome (NSP) which is characterized by major symptoms in the neurological and gastrointestinal systems such as nausea, vomiting, diarrhea, paraesthesia, cramps, bronchoconstriction, paralysis, size and coma (FAO/IOC/WHO, 2004; Watkins et al., 2008). Until now, no cases of chronic effects or deaths in humans due to NSP syndrome are caused by consuming foods containing BTx (EFSA, 2010). However, several cases of BTx poisoning with high mortality rates were reported in some animals, such as fish, seabirds, and marine mammals (Flewelling et al., 2005; Plakas & Dickey, 2010; Shen et al., 2010). BTx-like production of *Chattonella marina* has even caused major fish mortality events in Australia, China, and Japan (Perrault et al., 2017).

Based on the food safety risks posed, several countries have implemented monitoring programs to detect the growth of harmful algae and the presence of BTx in food. This review will discuss the chemical properties, biosynthesis, toxicity and regulatory status, and detection methods of BTx in seafood.

Chemical Characteristics

BTx is a fat-soluble cyclic polyether compound. In addition, BTx is lipophilic, colourless, tasteless and very heat stable (Abraham et al. 2018). Based on the molecular backbone structure, BTx is classified into two, BTx type A (BTx-1) and type B (BTx-2) (EFSA 2010). BTx-2 is the most common type of toxin produced by K. brevis (Plakas and Dickey 2010). The two types of BTx are distinguished by the number of transfused cyclic rings, whereas BTx-1 and BTx-2 have 10 and 11 transfused cyclic rings, respectively. BTx-1 has 10 transfused cyclic rings consisting of nine ether rings with 8, 6, 7, 9, 8, 8, 6, 6, and 6 atoms from rings B to J, and one terminal five-membered lactone in position A (Figure 1). Meanwhile, BTx-2 has 11 transfused cyclic rings by 6, 6, 6, 7, 7, 6, 6, 8, 6, 6, and 6 atoms from rings B to K but the lactone ring is in position A. fixed (Figure 1) (Ramsdell 2008). These differences in characteristics affect the structural flexibility of BTx (Brovedani et al., 2017). Based on this structure, BTx-1 and BTx-2 are considered parent toxins of other toxins produced by *K. brevis*, such as hemi-brevetoxin and brevenals. This is because the two toxins have a shorter ring structure, so they are considered products of incomplete BTx biosynthesis (Brovedani et al., 2017; EFSA, 2010).

Brevetoxin Biosynthesis

BTx is a secondary metabolite thought to be synthesized through the PKS (Polyketide Synthase) pathway. This assumption is because BTx has a backbone structure similar to the polyketide structure. However, the synthesis of BTx looks different because the structure of BTx has several methyl and oxygen groups, which are not characteristically generated from traditional polyketide synthesis (Shimizu, 2002). Another hypothesis reports that BTx is synthesized via the citric acid cycle. In this cycle, acetate is used in the PKS pathway or modified by the citric acid cycle. The intermediate products of this cycle can be reintroduced into the polyketide synthetic pathway, resulting in the addition of atypical carbon units. Previous studies of the citric acid pathway revealed three and four carbon units that could potentially explain the atypical condensation and oxidation patterns seen in BTx (Brovedani et al., 2017).

Based on several proposed biosynthetic pathways, it is concluded that the biosynthesis of BTx begins with the synthesis of traditional polyketides, which can potentially incorporate larger carbon units derived from acetate modified by the citric acid cycle. After the carbon backbone is synthesized, an oxidation process occurs, which produces the epoxide required for the closure of the multi-ring system. However, it is still unclear whether the methyl group present in the BTx structure is added after cyclization or during modification of the polybidide metabolite, but the methyl group may originate from sources other than acetate, such as S-adenosylmethionine (Brovedani et al., 2017).

Toxicity dan Regulatory Status

The potential toxicity of BTx depends on two main factors: the toxin's affinity level for its target and the binding efficacy for eliciting a response in target cells (Ramsdell, 2008). BTx has a high affinity for binding to receptors on the cell wall, namely voltage-gated sodium channels (NaV). This binding causes an uncontrolled influx of Na^+ into the cell and depolarization of the muscle and nerve cell membranes (Watkins et al., 2008). The study of Le Page et al. (2003) reported that NaV activation in mice could cause acute nerve injury and cell death, in which BTx-1 has a faster response than BTx-2. Other studies have also reported the effects of NaV activation such as depolarization of cell membranes, muscle contraction resulting in fasciculations or leaping, impaired immune cells leading to other biological responses such as cell proliferation, gene transcription, cytokine production and apoptosis, as well as induction of central depression of the heart and its function. respiratory tract (FAO/IOC/WHO, 2004; Dechraoui et al., 2007; EFSA, 2010).

To date, data on the acute toxicity of BTx toxin are still limited. EFSA (2010) reported that no lethal dose (LD50) data were available for the BTx-1. In BTx-2, the LD50 value found is 200 mg/kg b.w. Another study by Selwood et al. (2008) reported intraperitoneal LD50 for BTx-B2 toxin and its BTx-B2 derivative S-desoxy BTX-B2 of 400 and 250 mg/kg b.w, respectively. Selwood et al. (2008) also reported that in an in vivo test using mice, in which the toxin was injected intraperitoneally, it showed signs of poisoning 15 minutes after injection, including respiration paralysis, immobility, echophthalmia, and hind limb shaking before death. Meanwhile, in experiments using sub-lethal doses, rats showed symptoms such as complete paralysis of the limbs and rapid abdominal breathing followed by a decrease in respiratory rate. In the in vivo test given to rats orally, the LD50 value was 6600 mg/kg b.w for BTx-2 toxin and 52- mg/kg b.w for BTx-3 toxin. In contrast to intravenous and intraperitoneal treatment, oral toxin administration occurs after a 5-hour interval.

Despite having a fairly high food safety risk, several countries, especially European countries, do not yet have regulations regarding toxins from the BTx group found in shellfish or fish. (EFSA, 2010). However, several other countries such as the United States have set a maximum level or ac- tion level for BTx in clams at 20 mouse units (MUs/100 g) or the equivalent of 0.8 mg/kg mussels (U.S. FDA, 2001). New Zealand and Australia have also set a maximum level of BTx in shellfish, which is 20 MUs/100 g (NZFSA, 2006; FSANZ, 2010).

Table 1. Acute toxicity of several types of BTx toxin after intravenous (*i.v.*) and intraperitoneal (*i.p.*) administration in mice

Toxin types	Route	Toxicity
BTx-2	*i.v.*	LD_{50} 200
BTx-2	*i.p*. 24 hours	LD_{50} 200
BTx-3	*i.v.*	LD_{50} 94
BTx-3	*i.p.* 24 hours	LD_{50} 270
BTx-B2	*i.p.* 24 hours	LD_{50} 400
S-deoxcy-BTx-B2	*i.p.* 24 hours	LD_{50} 211
BTx-B3	*i.p.* 24 hours	MLD >300
BTx-B4	*i.p.* 6 – 24 hours	MLD 100
BTx-B5	*i.p.*	MLD 300 – 500

LD50: lethal lecturer resulting in 50% death of the mice; i.v. : intravenous; i.p.: intraperitoneal; MLD: minimum lethal dose (Source: Plakas & Dickey, 2010)

Detection methods of Brevetoxin

The complex metabolism of various metabolites with different polarities and hydrophobicity has become a major challenge for monitoring and developing BRx detection methods. Several methods have been developed to detect and measure BRx, such as cytotoxicity test, receptor

binding assay, immunoassay, and LC-MS/MS (Abraham et al., 2018). If grouped, the cytotoxicity test method and receptor binding assay is pharmacology-based, while the immunoassay and LC-MS/MS methods are structure-based (Van Dolah et al., 2001). In general, the detection method using LC-MS/MS and ELISA has a better correlation than the toxicity test method and receptor binding assay.

a. *Mouse Bioassay*
   The mouse bioassay method is a standard method to detect toxins that cause NSP in the United States (Abraham et al., 2017). According to APHA (1970), the value of 20 MU/100 g or equivalent to 0.8 mg/kg of shellfish tissue is a guidance level of shellfish that is safe for harvesting and human consumption. This is the most internationally approved method for detecting NSP toxins (Abraham et al., 2018). This method injects crude fat extract from shellfish (extracted with acetone or diethyl ether) via intraperitoneal rats. The mouse bioassay method has advantages such as not requiring complex and sophisticated instruments and equipment and a toxicity measure based on the animal's biological response to the toxin. However, this method also has several disadvantages, such as using inefficient solvents, cannot be automated, requiring special animal facilities and expertise, the variability of different animals requires validation, having very low throughput, and the results of the analysis tend to be subjective and less specific and cannot be used to detect polar metabolites. However, polar metabolites may play a significant role in the NSP syndrome (Abraham et al., 2018; EFSA, 2010).
b. C*ytotoxicity Assay*
   The cytotoxicity test method is based on the mechanism of action of BTx toxin on voltage-gated sodium channels of nerve cells (Bottein et al., 2010). In this assay, veratridine was administered to neuroblastoma cells to induce the open state transition of voltage-gated sodium channels and block the activity of the sodium-potassium pump. The study of Manger et al. (1993) reported that this method has a limit of detection (LOD) value for BTx-1 of 0.25 mg/kg. The cytotoxicity test is a very sensitive test method and is helpful as a screening method for the activity of compounds present in shellfish extracts. However, this method has some disadvantages, such as low yield, low specificity and interference between the extracted shellfish compounds and the test response (Bottein et al., 2007).
c. *Receptor Binding Assay*
   This assay method is a pharmacologically based method based on the affinity of BTx for sodium channel receptors (Abraham et al., 2017). In the test process, BTx in shellfish and fish extracts was determined based on the binding competition between radioactive 3H-BTX-3 and native BTx to the receptor site (Fire et al., 2008). FAO/IOC/WHO (2004) reported that this method has a limit of quantification (LOQ) value of 30 g BTx equivalent/kg oyster homogenate. This method has several advantages, such as the process being simple, fast, and sensitive enough to be used as a monitoring tool. However, the disadvantage of this method is that it uses radioactive compounds, so it requires trained operators and licenses. McCall et al. (2014) developed fluorescent ligands to replace radioactive ones, but these ligands have low specificity.
d. *Immunoassays*
   This method is a structure-based method used to assess the level of BTx based on the epitope specificity of the Incorporated antibody. Currently, most of the immunoassays for NSP toxin assay use antibodies with high specificity, in which the BTx-2 and BTx-1 backbone structures do not have cross-reactions. This method has also been proven to measure BTx in a wide variety of matrices such as shellfish, fish, seawater and clinical samples, with minimum sample clearance and excellent sensitivity of 0.025mg/kg (Plakas et al., 2008, Zhou et al., 2010). Another study by Baden et al. (2005) reported the development of a competitive ELISA immunoassay method using a native anti-BTx antibody with high specificity for type BTx-2. This method has a LOD of 25 g/kg for BTx on spiny oysters. The Immunoassays method provides

high precision, sensitivity, and specificity and is available in a high-sample yield format (Abraham et al., 2018). Meanwhile, the weakness of this method is the use of radioactive materials in the radioimmunoassay, the test results do not provide any information about the toxin profile, and this method has not been validated (EFSA, 2010).

e. LC-MS/MS

The LC-MS/MS method is a structure-based method widely used to measure and characterize BTx in shellfish, fish and algae (Wang & Cole, 2009). EFSA (2010) reported that ESI (electrospray ionization) in positive and negative ion modes was used to determine the BTx group toxin in shellfish. Abraham et al. (2014) reported that this method had been used successfully in characterizing various BTx strains in several matrices such as K. brevis blooms, epidemiological outbreak investigations, metabolites in shellfish, and clinical samples. Several studies indicated that the LC-MS/MS method had LOQ of 2, 0.2, 0.3 and 0.4 g/kg shellfish for BTx-2, BTx-3, BTx-B5, and BTx-B1, respectively (Ishida et al., 2004). The LC-MS/MS method offers high specificity and sample throughput for detecting BTx in various matrices. However, this method has several disadvantages, such as the complex sample extraction process, the minimal need for BTx analytical standards, the need for expensive LC-MS/MS instruments and highly trained analysts, and no published inter-laboratory validation studies (Abraham et al., 2014; EFSA, 2010).

Conclusion

The characteristics of BTx, which are lipophilic, colorless, tasteless, and very heat stable, and symptoms of neurologic shellfish syndrome poisoning have become a concern and a potential food safety risk, especially in seafood. Several countries have set the action or maximum level of BTx in shellfish at 20 MUs/100 g or the equivalent of 0.8 mg/kg shellfish. In vitro and in vivo BTx detection methods such as mouse bioassay, cytotoxicity assay, receptor binding assay, immunoassay, and LC-MS/MS have also been developed. Each method has its advantages and disadvantages. Extensive method assessment and additional data are needed for this purpose.

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