บทความปริทัศน์
(Review article)

สารพิษโบทูลินั่ม: โครงสร้าง คุณสมบัติ และพันธุศาสตร์

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บทคัดย่อ

Clostridium botulinum เป็นแบคทีเรียแกรมบวกที่ไม่ต้องการออกซิเจน สามารถสร้างสปอร์และผลิตสารพิษโบทูลินั่ม (Botulinum neurotoxin หรือ BoNT) ที่มีผลต่อระบบประสาท ทำให้เกิดโรคโบทูลิซึ่ม (Botulism) โดยสารพิษจะไปยับยั้งการหลั่งสารสื่อประสาทชนิด อะเซติลโคลีน (acetylcholine) ที่ปลายประสาทของระบบประสาทรอบนอก ทำให้กล้ามเนื้อเกิดการอ่อนแรง สารพิษโบทูลินั่มสามารถแบ่งเป็น 7 ซีโรไทป์ ได้แก่ ซีโรไทป์ A - G (serotype A-G) และซีโรไทป์ที่ก่อโรคในคน ได้แก่ ซีโรไทป์ A, B, E และ F โครงสร้างของสารพิษโบทูลินั่มประกอบด้วย 3 ส่วน โดยส่วนแรกคือ สายเบา (L-chain) ซึ่งอยู่ทางด้านปลาย N ของสายโปรตีน มีหน้าที่ในการตัดโปรตีน SNARE (soluble N-ethyl-maleimide-sensitive factor attachment protein receptor) ซึ่งเป็นโปรตีนที่เกี่ยวข้องในกระบวนการหลั่งสารสื่อประสาท และอีกสองส่วนอยู่ที่สายหนัก (H-chain) ซึ่งอยู่ทางด้านปลาย C ทำหน้าที่ในการจับกับโมเลกุลบริเวณผิวของปลายเซลล์ประสาท และช่วยในการส่งผ่านสายเบา (L-chain) เพื่อไปออกฤทธิ์ในไซโทพลาสซึมของเซลล์ประสาท โดยปกติแล้วสารพิษโบทูลินั่มถูกผลิตขึ้นในรูปของสารประกอบเชิงซ้อนที่เรียกว่า progenitor toxin complex (PTC) ซึ่งจะไปจับกับกลุ่มโปรตีนที่เรียกว่า neurotoxin-associated protein (NAPs) ซึ่งก็คือโปรตีน haemagglutinin (HA) และกลุ่มโปรตีน non- haemagglutinin (NTNH) กลุ่มโปรตีนนี้เกี่ยวข้องกับการปกป้อง BoNTs จากสภาวะกรดในระบบทางเดินอาหาร และช่วยในการดูดซึม BoNTs เข้าสู่ระบบหมุนเวียนผ่านทางเซลล์เยื่อบุลำไส้ ยีนที่สร้าง BoNT และ NAPs จะอยู่ร่วมกันเป็นกลุ่ม แบ่งได้เป็น กลุ่มยีน *ha* (*h*a operon) และกลุ่มยีน *orfX* (*orfX* operon) ซึ่งกลุ่มเหล่านี้สามารถพบได้บนโครโมโซมพลาสมิดขนาดใหญ่ หรือแบคเทอริโอฟาจ นอกจากนี้ กลุ่มของยีนนี้สามารถเคลื่อนย้ายส่งผ่านยีนในแนวราบไปยังแบคทีเรีย Clostridium สายพันธุ์อื่นได้

คำสำคัญ: คลอทริเดียมโบทูลินั่ม โบทูลิซึ่ม สารพิษโบทูลินั่ม

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Botolinum toxins:
their structure, properties, and genetics

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Abstract

Clostridium botulinum is Gram positive, spore-forming anaerobic bacteria, which can produce botulinum neurotoxins (BoNTs). The toxins block the release of neurotransmitter, acetylcholine, at peripheral cholinergic nerve terminal and cause flaccid paralysis of muscle in human and animals, a condition known as botulism. BoNTs are classified into seven different serotypes (designated as BoNT/A-BoNT/G), in which serotype A, B, E, and F cause botulism in human. BoNTs are comprised of one domain of light chain (L-chain) at N-terminus and two domains of heavy chains (H-chain) at C-terminus. The function of L-chain is to cleave SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins that involve in the exocytosis of neurotransmitter whereas H-chain is responsible for binding of toxin with nerve terminal and translocating of L-chain into cytosol from synaptic vesicle. The BoNTs are usually produced as complexes called progenitor toxin complex (PTC). They bind together with neurotoxin-associated proteins (NAPs), which are haemagglutinin (HA) and non-toxin non-haemagglutinin (NTNH). The NAPs can protect BoNTs from gastrointestinal environment and facilitate the absorption and translocation of neurotoxin into main circulation. The genes encoding BoNTs and NAPs are arranged as gene cluster, which are organized in two operons: *ha* and *orfX* operons. Mostly, they are located on the chromosome, large plasmid, or bacteriophage at the specific location and can be transferred horizontally to other clostridia strains.

Keywords: *Clostridium botulinum*, botulism, botulinum toxin, BoNT

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Introduction

Clostridium botulinum is Gram positive, rod-shaped, spore-forming and anaerobic bacteria contaminating in the environments such as soil, aquatic sediment, and intestine of various animals. These bacteria produce endospore, which can survive in harsh environments (presence of oxygen, high pressure, UV light, heat treatment and nutrient starvation) and their endospores can germinate during favorable conditions (humidity, nutrient source, and anaerobic condition). The spores then contaminate in environments and food chains leading to intoxication of animals and human. All *C. botulinum* strains also produce botulinum neurotoxins (BoNTs) that cause a disease known as botulism by inhibiting the release of acetylcholine, which cause flaccid paralysis of muscle in human and animals[1]. Botulism forms by ingestion of food contaminated with *C. botulinum*, its spores, or its toxins followed by absorption of BoNTs from the digestive system. Clinical features of botulism can be varied, ranging from headache, blurred vision, dizziness, flaccid paralysis of respiratory system and death. Botulism can be classified into 5 forms according to the entry route of BoNTs[2], which are (1) food-borne botulism; (2) infant botulism; (3) wound botulism; (4) iatrogenic botulism; (5) inhalation botulism.

BoNTs are produced by six distinctive *Clostridium* sp. groups (Group I-VI) with seven different serotypes (BoNT/A-BoNT/G). Recently, a novel BoNT namely BoNT/H has been reported[3]. However, after genetic analysis and serological characterization, it has been found that the novel BoNT is in fact a hybrid toxin between serotype A and serotype F, specially classified as serotype F5 (BoNT/FA)[4]. The BoNTs are initially synthesized as inactive toxin with molecular mass of 150 kDa. To yield mature toxin, protease cleavage of disulfide bond is required resulting in light chain (L-chain) and heavy-chain (H-chain) with molecular mass of 50 and 100 kDa, respectively. The L-chain contains Zn^{2+} metalloprotease activity, which cleaves SNARE proteins mediating exocytosis of neurotransmitter of the cholinergic nerve terminal[5]. The H-chain comprises of two domains responsible for binding of toxin and translocation of L-chain into cytosol via the route of endocytosis in the nerve terminal. The BoNTs are usually produced as complexes (progenitor toxin complex; PTC)[6] with sizes up to 900 kDa including neurotoxin-associated proteins (NAPs), which are haemagglutinin (HA) and non-toxin non-haemagglutinin (NTNH). The binding of NTNH and HA complex to the BoNT holotoxin can protect the toxin from low pH and proteolytic enzymes in the stomach, thereby assisting the absorption and translocation of neurotoxin into the body[7,8].

The genes encoding BoNTs and NAPs are located on the chromosome, large plasmid[9,10] and bacteriophage[11]. They seem to be located on the mobile genetic at the specific location, which can be horizontally transfer to other bacterial strains[12]. There are two BoNT cluster types, which are *ha* cluster and or *orfX* cluster[13]. The bont is next to *ntnh* whereas the *ha* or *orfX* genes are located upstream with opposite direction with *bont-ntnh* genes[14]. Mostly, *C. botulinum* contains one copy of toxin serotype on the genome. However, variations were also observed, in which more than one toxin genes have been discovered[15,16].

In the past decade, BoNTs received much attention in many aspects, including bioweapon for terrorism and therapeutic purposes. This review will focus on BoNTs from *C. botulinum* for a better understanding regarding their structure, properties and genetics.

Clostridium botulinum, Botulism, and Botulinum neurotoxin

BoNTs are produced mainly by *C. botulinum* and other related *Clostridium* species (*C. barati, C. argentinense* and *C. butyricum*). They acts mainly at neuromuscular junction by inhibition of neurotransmitter release[5] caused botulism. The clostridial strains producing BoNT are divided into six groups (Group I - VI) according to their genetics and physiology. *C. botulinum* strains producing BoNTs are belonged in Group I - IV. BoNTs associated with most cases of foodborne botulism are produced from Group I (proteolytic *C. botulinum*) and Group II (non-proteolytic *C. botulinum*). The proteolytic *C. botulinum* (Group I) produces a single active neurotoxin type A, B or F. The BoNTs serotype B, E and F are produced by non-proteolytic *C. botulinum* (Group II). The strains of Group III produce toxin serotypes C, D, mosaic C/D or D/C toxin. Group IV refers as *C. argentinense* or *C. botulinum* G, which produce neurotoxin serotype G. Group V and Group VI, which are *C. butyricum* and *C. baratii*, produce neurotoxin serotype E and serotype F, respectively.

Botulism can be classified into 5 categories according to the entry route of toxins[2]. First, foodborne botulism is caused by consumption of foods typically canned food contaminated with BoNT absorbed in the intestine. Second, infant (adult) botulism is caused by ingestion of food contaminated with *C. botulinum* spore, which then germinates in the intestine. It can compete microbiota in the infant intestine leading to outgrowth and colonization of the bacteria. Third, wound botulism including injection of drug users that tissues are contaminated with *C. botulinum* spores. Forth, iatrogenic botulism causes by cosmetic or therapeutic treatment, which patients are exposed to excessive level of BoNT. Finally, inhalational botulism occurs by inhalation of toxin via respiratory system but it is rarely since the delivery of toxin through this system is inefficient[17].

BoNTs are synthesized from vegetative cell and released by autolysis of bacterial cell. Seven serotypes of botulinum toxin exist in nature (BoNT/A to BoNT/G). The serotype A, B, E, and F can cause botulism in human in which rare cases were observed from serotype F 1. Moreover, each serotype can be classified into subtypes as BoNT/A1-A10; BoNT/B1-B7; BoNT/E1-E11; and BoNT/F1-F7[2]. The dose of BoNTs is subnanogram level, which is less than mouse lethal dose (LD_{50}) of 0.1 - 1 ng per kg leading to physiological dysfunctions[18]. Due to high toxicity to humans and animals and lack of immunization protection, BoNTs are considered to be potential terrorism bioweapon[19]. In contrast, BoNT/A and BoNT/B are widely used for neurological disorder treatments for human diseases such as hyperexcitability of peripheral nerve terminals and hypersecretory syndromes[20].

BoNT Structures

BoNTs are synthesized as an inactive single polypeptide chain with molecular mass of 150 kDa. They are activated by proteolytic cleavage of disulfide bridge at a loop to yield mature toxin consisting of light chain (L-chain; 50 kDa) and heavy chain (H-chain; 100 kDa). They were bound together by a loop protein segment surrounding L-chain and attaching with H-chain called peptide belt and by a disulfide bond[5].

The active BoNTs comprise three domains, which are one domain of L-chain at N-terminal and two domains of H-chain at C-terminal (translocation domain at N-terminus; HN and receptor bind-

ing domain; HC at C-terminus with molecular mass of 50 kDa each)[21]. The N-terminus of L-chain is Zn^{2+} metalloprotease acting specifically to SNAREs, which mediate exocytosis of neurotransmitter into the synaptic cleft[5]. The HN domain (N-terminus of H-chain) is involved in the translocation process of L-chain across endocytic vesicle membrane into the cytosol of targeted neuron[22]. HC domain (C-terminus of H-chain) can be confined into 2 subdomains with different folding and binding properties: a β-sheet jelly roll fold, HC_N (25 kDa), and a β-tree foil fold, HC_C (25 kDa), located at N-and C-terminus of HC, respectively[22]. The HC_N domain is suggested to interact with the head group of phosphatidylinositides and facilitate interactions of HN domain with membrane of the nerve terminal[23]. The HC_C domain contains two binding sites, in which one domain binds specifically to the oligosaccharide moiety of polysialogangliosides enriched in neuron of presynaptic membrane as first receptor[22] and the other binds to intravesicular segments of a synaptic vesicle (SV) protein receptor appeared transiently during exocytosis[24].

Typically, BoNTs are released in the form of holotoxin together with one or more accessory proteins to form PTC[6]. The accessory proteins, neurotoxin-associated proteins (NAPs) are comprised of haemagglutinins (HA) and non-toxin non-haemagglutinin (NTNH). There are 3 types of HA with molecular weight of 15-17 kDa (HA17), 33-35 kDa (HA33), and 71-76 kDa (HA70) and one type of NTNH with molecular weight of 140 kDa[6,8]. The PTC complexes have different mass sizes known as 12S (300 kDa), 16S (500 kDa), and 19S (900 kDa) complexes[25]. The NTNHA/A1 adopts similar structure with that of BoNT/A1 and can also form heterodimer together with BoNT/A1 (known as minimally functional PTC; M-PTC), which shields the toxin from the gastrointestinal (GI) digestion[7]. The HA complex binds to the M-PTC via little protein-protein contact with NTNH/A suggesting that the function of HA complex is to facilitate translocation of BoNT/A rather than protecting of BoNT/A from the proteolytic environment of the GI tract[8]. The HA complex can form multivalent interactions with carbohydrate receptors on the several cells including the mucus layer, epithelial cell or other cells in the intestinal layer[2]. Taken together, the NAPs can associate with certain serotypes of BoNTs and protect them from proteolytic environment of mammalian gastrointestinal tract and also play a role in entry mechanism of neurotoxin through the membrane.

Translocation of toxin across the intestinal epithelial barrier

BoNTs are released as progenitor toxin complex (PTC), in which NAPs can protect them from proteolytic degradation by trypsin and pepsin in GI tract[7] and also facilitate the entry mechanism of neurotoxin through the epithelial barrier[8]. To date, at least two models have been proposed for penetration of BoNTs across the epithelial barrier. First, the toxin itself (holotoxin) can bind and pass across epithelium cell from apical side through basalateral side reaching general circulation system known as transcytosis[26]. Second, the HA complex binds to the epithelial cell and disrupts the tight junction, facilitating the translocation of toxin across the intestinal epithelial barrier known as a paracellular mechanism[27].

The assembly of HA protein with the M-PTC showed greater oral toxicity than that of BoNT holotoxin[25]. The HA complex decreases transepithelial electrical resistance (TER) of

Caco-2 cell causing the reduction in the integrity of the epithelial cell layer[8]. The HA complex also showed glycan binding sites interacting with monosaccharide and oligosaccharide molecules in which plays an important role during the initial absorption of PTC in the intestinal lumen and also facilitates the transport of toxin across the basalateral surface of epithelial cell via paracellular route[28]. The blockage of these binding sites by mimicking carbohydrate molecules could prevent the intestinal absorption of BoNT/A *in vivo* however the neurotoxicity of BoNT/A is not affected[8]. Nevertheless, the details of translocation mechanism of BoNT toxin across the epithelial barrier is still unknown.

Dissociation of BoNTs from the complex to release 150 kDa free neurotoxin is controlled by pH, which is neutral to basic pH in the intestine. The releasing step of free BoNT toxin is required for entering into the GI tract of host cells. The holotoxin only travels in the blood circulation and reaches the target at the neuromuscular nerve terminal. Although the toxins are active at nerve terminal especially at skeletal and autonomic cholinergic nerves, they cannot enter the central nervous system (CNS) via circulation system[29].

BoNT genetics

The BoNTs are encoded by *bont* genes (~3880 bp) exhibiting 34-97% similarity of the amino sequences among serotypes[30]. The genes are mostly located on the chromosome however some botulinum toxin genes are encoded from large plasmid[9,10] and from bacteriophage[11]. The genes show high level of genetic variation in terms of gene location (chromosome, plasmid, or phage) and spreading of gene among *Clostridium* species (*C. botulinum, C. argentinense, C. butyricum, C. baratii*)[12]. Moreover, the toxin gene loci are found at specific location that sometimes located on the mobile genetic element, which can be transferred horizontally to non-toxigenic strains and become toxigenic clostridia strains[12,16].

Most of *C. botulinum* strains produce a single toxin serotype. However, some *C. botulinum* isolates can produce mixture of toxin serotypes (bivalent) such as Ab, Af, Ba, and Bf serotypes[16]. Investigation by Dover et. al has shown that certain *C. botulinum* strains contain more than one *bont* genes located on different locations (*bonta2* and *bontf4* gene on chromosome, and *bontf5* gene on plasmid)[15]. Some *C. botulinum* strains harbor more than one toxin genes and one of those gene is silent, such as BoNT/A(B), which indicates BoNT serotype A with a silent of *bontb* gene. Furthermore, some BoNTs can be the chimeric toxin, for example BoNT/CD and BoNT/DC that consist of domains from both toxins[2].

The progenitor toxin complex (PTC) is encoded from gene cluster on chromosome or plasmid[9,10]. There are two major conserved BoNT cluster types; the *ha* plus/*orfX* minus cluster (or *ha* cluster) and the *ha* minus/*orfX* plus cluster (or *orfX* cluster)[13]. The *bont* is located immediately next to *ntnh*. Since NTNH exhibits similar protein folding to that of BoNTs and forms heterodimer with BoNT, this might indicate that both genes result from gene duplication, in which NTNH functions as protective protein of BoNTs[12,16]. The upstream of the *ntnh-bont* genes are *ha* (*ha33, ha17, ha70* genes) or *orfX* genes, which are transcribed in opposite direction[14]. The *ha* cluster comprises *bont* under regulation of *botR* encoding a sigma 70 factor. The arrangement of the genes in *orfX* cluster is *orfX3-orfX2-orfX1-(botR)-p47-ntnh-bont*[31]. The structure and function of the proteins

encoded from *orfX* cluster are still unknown. The proteolytic strains of *C. botulinum* mostly contain one neurotoxin cluster in a single copy on the genome producing a single toxin serotype (monovalent). Isolates producing bivalent toxin serotypes (bivalent) such as Ab, Af, Ba, and Bf is resulted of having two neurotoxin clusters with a single neurotoxin gene per cluster. However, they produce neurotoxin in different portions[12].

Conclusion remarks

There are many studies of BoNTs from *C. botulinum* based on in their structures, properties and genetics, which can encompass a piece of knowledge into a whole story of botulinum toxins. Together with modern technique and knowledge in molecular biology, these knowledge can be applied for development in the methods and the effective molecules for prevention and treatment of botulism. The use of BoNTs for further therapeutic treatment such as neurological disorder treatments for human diseases is also remarked.

Conflict of interest

The authors declare no competing interests.

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