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Isolation of botulinolysin, a thiol-activated hemolysin, from serotype D *Clostridium botulinum*: A species-specific gene duplication in *Clostridia*

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A B S T R A C T

Botulinolysin (BLY) is a toxin produced by *Clostridium botulinum* that belongs to a group of thiol-activated hemolysins. In this study, a protein exhibiting hemolytic activity was purified from the culture supernatant of *C. botulinum* serotype D strain 4947. The purified protein displayed a single band by sodium dodecyl sulfate polyacrylamide gel electrophoresis with a molecular mass of 55 kDa, and its N-terminal and internal amino acid sequences exhibited high similarity to a group of thiol-activated hemolysins produced by gram-positive bacteria. Thus, the purified protein was identified as the BLY. Using the nucleotide sequences of previously cloned genes for hemolysins, two types of genes encoding BLY-like proteins were cloned unexpectedly. Molecular modeling analysis indicated that the products of both genes displayed very similar structures, despite the low sequence similarity. *In silico* screening revealed a specific duplication of the hemolysin gene restricted to serotypes C and D of *C. botulinum* and their related species among thiol-activated hemolysin-producing bacteria. Our findings provide important insights into the genetic characteristics of pathogenic bacteria.

1. Introduction

Clostridium botulinum is taxonomically defined as a species that produces the botulinum neurotoxin (BoNT). Based on the serotypes of the BoNT produced by the bacteria, *C. botulinum* is serologically classified into seven serotypes (A–G) (Collins and East, 1998). Some strains of serotypes C and D produce not only BoNT but also other toxins, such as C2 toxin, alpha-toxin, and epsilon-toxin, among others (Knapp et al., 2016; Popoff and Bouvet, 2013). Of the toxins produced by the serotype C and D strains, botulinolysin (BLY) is a member of thiol-activated hemolysins produced by gram-positive bacteria, including *Streptococcus*, *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Clostridium*, *Listeria*, and *Arcanobacterium* (Alouf, 2003; Sekiya et al., 1998). In contrast to BoNT, which has been studied intensively because of its extraordinarily potent toxicity, the pathogenic role of BLY is still unclear. In 1992, Haque et al. (1992) established a method to purify the BLY protein with molecular mass of 58 kDa from the culture supernatant of *C. botulinum* serotype C. Subsequently, the activity of BLY in cells, including erythrocytes, was investigated using BLY from *C. botulinum* serotype C. BLY forms a ring-shaped structure on the erythrocyte membrane and consequently lyses the cells (Sekiya et al., 1998). Moreover, BLY exhibits lethal toxicity when administered to animals *in vivo* (Sugimoto et al., 1995).

During the purification of the BoNT complex from *C. botulinum* serotype D (Hasegawa et al., 2004), we obtained a fraction that showed significant hemolytic activity. In this study, we purified a protein from this fraction that exhibited hemolytic activity. Additionally, two types of genes, each encoding the BLY-like protein in the *C. botulinum* serotype D strain were partially cloned.

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2. Materials and methods

2.1. Purification of BLY from *C. botulinum* strain 4947 (serotype D)

C. botulinum serotype D strain 4947 (D-4947) was cultured at 37 °C for 5 days using the cellophane tube method, as described by Hasegawa et al. (2004). The proteins in the culture supernatant were precipitated with saturation of ammonium sulfate at 60%, dialyzed against 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl, and then applied to a TOYOPEARL SP-650S column (1.6 × 40 cm; Tosoh, Tokyo, Japan) equilibrated with the same buffer used for the dialysis. Proteins not bound to the resin were washed out with the same buffer. The bound proteins were then eluted with a linear gradient of NaCl (0.2–0.8 M) in 50 mM acetate buffer (pH 4.0). The fractions showing hemolytic activity were collected and then applied to a HiLoad 16/60 Superdex 200 pg column (GE Healthcare, Buckinghamshire, UK) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. The proteins were eluted with the same buffer. The purity of the BLY was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. Hemolysin assay

A mixture of 500 μL hemolysin solution diluted 2-fold with 5 mM Tris/HCl buffer (pH 7.2) containing 0.85% NaCl and 5 mM cysteine plus an equal volume of a 1% suspension of horse erythrocytes that had been washed in the same buffered saline was incubated at 37 °C for 60 min. After incubation, hemolysis was confirmed visually.

2.3. SDS-PAGE and N-terminal amino acid sequencing

SDS-PAGE was performed as described by Laemmli (1970) using a 15% polyacrylamide gel in the presence of 2-mercaptoethanol. The molecular size markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), egg ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibiter (20.1 kDa), and α-lactalbumin (14.4 kDa). The separated protein bands were detected with Coomassie Brilliant Blue R-250. The protein bands developed on the SDS-PAGE were transferred onto a PVDF membrane as previously described (Hirano and Watanabe, 1990), and the N-terminal amino acid sequences of V8-protease derived fragments were determined using an automated protein sequence analyzer (model 492HT; Applied Biosystems, Foster City, CA, USA).

2.4. Nucleotide sequence of the bly gene

Total genomic DNA was prepared from D-4947 as previously described (Takeshi et al., 1996). The polymerase chain reaction (PCR) primers and inverse PCR primers for the *bly* gene were designed based on the nucleotide sequences of the tetanolysin O gene (sequence accession numbers: CDI50028 and AAO36403), as shown in Table 1. For inverse PCR, *Hind*III and *Ase*I digests of genome DNA were used as template DNA. The PCR conditions were as follows: 94 °C for 5 min; 35 cycles of 92 °C for 1 min, 55 °C for 1 min, and 74 °C for 1 min; and a 5 min extension at 74 °C. The conditions for inverse PCR were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 54 °C for 2 min, and 74 °C for 1 min; and a 5 min extension at 74 °C. The 1,117-bp partial *bly1* gene and 2,151-bp sequence containing the whole *bly2* gene were determined by direct sequencing of the amplified products. A fluorescence-based cycle sequencing reaction was performed to sequence DNA using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequencing primers used were the same as those used for PCR; however, the concentration was one-tenth that used for PCR. The sequencing reaction conditions were as follows: 25 cycles of 95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The DDBJ/EMBL/GenBank accession number for the nucleotide sequences of *bly1* and *bly2* are LC149614 and LC149615, respectively.

Table 1
PCR primers for amplifying the *bly1* and *bly2* genes.

Primer	Sequence	Target gene
bly 1F	5′-GAGACAACATCTAAGAGTGG-3′	*bly1*
bly 1R	5′-TTCTCCACCATTCCCATGC-3′	
bly 2F	5′-GTTAAAGCAGCTTTTAAAGCAC-3′	*bly2*
bly 2R	5′-GCCACATATGCTCCTCTATG-3′	
bly 3F	5′-ATTTTGATTCCATATTTAAAGGTG-3′	*bly1*
bly 3R	5′-GCAAGTTGTATAGCACCAGG-3′	
bly 4F	5′-ATTTCAGATTCCACAGCAGAC-3′	*bly1*
bly 4R	5′-GCAAGTTGTATAGCACCAGG-3′	
bly 5F	5′-GGATTATCCTATGATCCACG-3′	*bly2*
bly 4R	5′-GCAAGTTGTATAGCACCAGG-3′	
bly IP1[a]	5′-CAAACCCTTCAGCAGGTACA-3′	*bly2*
bly IP2[a]	5′-ATCTCCTCGTATACACCGAC-3′	
bly IP3[a]	5′-AGACATTTCCGTTCCCTCAC-3′	*bly2*
bly IP4[a]	5′-AGCTCGTGGACTTGATGTTG-3′	

[a] Primers used for inverse PCR.

2.5. Southern blot analysis

Southern blot analysis was performed as described previously (Sagane et al., 2003). Purified genomic DNA was digested with restriction enzymes (*Eco*RI, *Hind*III, and *Xba*I, 100U each). The digoxigenin (DIG)-labeled probe was prepared using DIG labeling mix (Roche, Mannheim, Germany) and the following primers: 5′-TTCTCCACCATTCCCATGC-3′ and 5′-GAGACAACATCTAAGAGTGG-3′; this sequence has homology among hemolysisn genes of *Clostridium* bacteria.

2.6. Homology modeling

Three-dimensional (3D) models of the *C. botulinum* strain DC5 BLY structures (sequence accession numbers: KGN01966 and KGN00720) were predicted by utilizing the structure of perfringolysin O as a template (PDB ID: 1PFO), using the homology modeling server http://swissmodel.expasy.org/(Arnold et al., 2004; Biasini et al., 2014). In this study, we could not determine the complete sequence of *bly* genes. Therefore, strain DC5 BLY structures were predicted as an alternative structure showing high similarity with D-4947 BLY. The template structure was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Structural images were generated using the Discovery Studio Visualizer (available at http://accelrys.com/products/collaborative-science/biovia-discovery-studio/). The models exhibited good geometry according to Ramachandran plots generated using Swiss-PdbViewer (available at http://spdbv.vital-it.ch/). In both structures, the most favored region contained over 83% of the dihedral angle pairs, and the additionally allowed region contained another over 13% of the pairs.

2.7. *In silico* screening of hemolysin genes in the genome database of gram-positive bacteria

Hemolysin genes in the genome database of gram-positive bacteria were obtained from the Ensemble Bacteria database. The nucleotide sequences of the perfringolysin O gene or the related genes tetanolysin O, botulinolysin, novylysin, streptolysin O and cereolysin (sequence accession numbers: ABG82518, KGI39567, KGN01966, KGN00720, KEH89269, KEH89490, EFM32745, and AAS42222, respectively) were employed as a query sequence in the BLAST search.