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Original Article

Phospholipase C Produced by *Clostridium botulinum* Types C and D: Comparison of Gene, Enzymatic, and Biological Activities with Those of *Clostridium perfringens* Alpha-toxin

Ni Nengah Dwi Fatmawati[a,b], Yoshihiko Sakaguchi[c], Tomonori Suzuki[a], Masataka Oda[d], Kenta Shimizu[e], Yumiko Yamamoto[a], Jun Sakurai[d], Osamu Matsushita[a], and Keiji Oguma[a]*

[a]Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700–8558, Japan, [b]Department of Microbiology, Faculty of Medicine, Udayana University, Jl. P.B. Sudirman Denpasar, Bali 80232, Indonesia, [c]Interdisciplinary Research Organization, Miyazaki University, Miyazaki 889–2192, Japan, [d]Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770–8514, Japan, and [e]Shin-Nakamura Chemical Co., Ltd., Wakayama 640–8390, Japan

Clostridium botulinum **type C and D strains recently have been found to produce PLC on egg yolk agar plates. To characterize the gene, enzymatic and biological activities of *C. botulinum* PLCs (Cb-PLCs), the *cb-plc* genes from 8 strains were sequenced, and 1 representative gene was cloned and expressed as a recombinant protein. The enzymatic and hemolytic activities of the recombinant Cb-PLC were measured and compared with those of the *Clostridium perfringens* alpha-toxin. Each of the eight *cb-plc* genes encoded a 399 amino acid residue protein preceded by a 27 residue signal peptide. The protein consists of 2 domains, the N- and C-domains, and the overall amino acid sequence identity between Cb-PLC and alpha-toxin was greater than 50%, suggesting that Cb-PLC is homologous to the alpha-toxin. The key residues in the N-domain were conserved, whereas those in the C-domain which are important in membrane interaction were different than in the alpha-toxin. As expected, Cb-PLC could hydrolyze egg yolk phospholipid, *p*-nitrophenylphosphorylcholine, and sphingomyelin, and also exhibited hemolytic activity; however, its activities were about 4- to over 200-fold lower than those of alpha-toxin. Although Cb-PLC showed weak enzymatic and biological activities, it is speculated that Cb-PLC might play a role in the pathogenicity of botulism or for bacterial survival.**

Key words: botulinum phospholipase C, botulinum toxin, phospholipase C activity, sphingomyelinase activity, hemolytic activity

Clostridium botulinum, a gram-positive spore-forming anaerobic bacterium, can be classified into seven distinct types (A–G) based on the immunological specificity of the neurotoxin (NTX) [1]. NTX is one of the most potent toxins among all bacterial, animal, and plant toxins as well as chemical compounds, and is considered as the essential virulence factor giving rise to food-borne botulism. *C. botulinum* types C, D, and E also produce hemolysin, named botulinolysin [2]. Furthermore, types C and D produce C2 toxin and C3 exoenzyme in addition to these toxins. C2 toxin exhibits an ADP-ribosyltransferase activity by which it ADP-ribosylates non-muscular G-actin, leading to a disruption of actin function, whereas the C3

Received July 26, 2012; accepted September 4, 2012.
*Corresponding author. Phone:+81-86-235-7158; Fax:+81-86-235-7162
E-mail:kuma@md.okayama-u.ac.jp (K. Oguma)

exoenzyme is a Rho-GTPase ADP-ribosylating enzyme [3, 4]. It has been reported that *C. botulinum* is a phospholipase C non-producing bacterium. Oguma *et al.*, however, found that the colonies of many type C and D strains, cultivated on GAM-egg yolk agar plates, demonstrated opacity around their colonies (Lecito-vitellin- or Nagler-reaction), suggesting that these cultures produce phospholipase C (PLC) [5].

Many studies have been conducted to characterize bacterial PLCs, and have shown that not all of them are toxic. *C. perfringens* alpha-toxin (alpha-toxin), *C. absonum* PLC (absonum-PLC), and *C. novyi* PLC (novyi-PLC) are toxic PLCs, but alpha-toxin is the most toxic among them. On the other hand, other clostridial PLCs such as *C. bifermentans* PLC (bifermentans-PLC) and *C. sordellii* PLC (sordellii-PLC) are considered to be of low toxicity compared with alpha-toxin and absonum-PLC [6–10]. Until now, only alpha-toxin has been widely investigated. Alpha-toxin, which is a zinc metallophospholipase C, is known as the key virulence factor in gas gangrene. It has PLC, sphingomyelinase (SMase), hemolytic, and myotoxic activities, and is toxic to humans and animals [7, 8]. The mature alpha-toxin is formed by 370 amino acid residues, and consists of 2 domains, the N- and C-domains [11]. The alpha helical N-domain is a catalytic domain containing the active site cleft that catalyzes phospholipid membrane hydrolysis with the preferred substrates phosphatidylcholine and sphingomyelin, which are the major components of the outer leaflet of the eukaryotic cell membrane, while the beta sandwich C-domain functions as a membrane-binding domain [7, 11, 12]. The C-domain is found to be an analog to the C2-like domain of the eukaryotic phospholipid-binding domain; therefore, it is thought that the C-domain plays an important role in membrane phospholipid recognition in a calcium-dependent manner [11]. The PLCs produced by the above-mentioned clostridia also have 2 domains like the alpha-toxin, although their amino acid sequences are somewhat different [6–10]. In contrast, the *Bacillus cereus* PLC (Bc-PLC), which is a non-toxic PLC, has only the N-domain [13].

Whether 1) *C. botulinum* type C and D cultures produce PLCs, and 2) these PLCs (Cb-PLCs) possess toxic properties, has not yet been investigated. Therefore, we sequenced the predicted Cb-PLC genes (*cb-plc*) from eight strains of types C and D. Since the amino acid identities among these strains were high, only one representative gene, *i.e.*, the *C. botulinum* type D strain 1873 (D-1873) *cb-plc*, was cloned, and was used to construct the expression vector for the recombinant Cb-PLC (rCb-PLC). The primary structure, and the enzymatic and hemolytic activities of the rCb-PLC, as well as the PLC activity of the type C and D cultures, were analyzed and compared with those of alpha-toxin.

Materials and Methods

Bacterial strains, preparation of DNA, and sequence analysis. DNA was prepared from the *C. botulinum* type C and D strains C-Stockholm (C-St), C-468, C-203, C-CB19, C-6813, C-D6F, D-1873, and D-CB16, which demonstrated opacity around their colonies on egg yolk agar plates. These bacteria were cultivated overnight under an anaerobic condition at 37℃. The total DNAs of all eight strains were isolated using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To determine the nucleotide sequences of the internal region of *cb-plc*, the *cb-plc* genes were amplified with specific primers that recognize only the conserved region among clostridial PLC, PLC.IPCR-7f and PLC.IPCR-7r (Table 1, Fig. 1). Purified DNAs (1 μg) were digested separately by overnight incubation with *Mbo*I, *Nde*I, *Pst*I, *Pvu*II, and *Xba*I at temperatures recommended by the manufacturer. For circularization, each restriction fragment was subsequently self-ligated with a T4 DNA ligase (Toyobo, Osaka, Japan). Furthermore, the upstream and downstream flanking regions of unknown sequences were determined by a combination of inverse PCR (IPCR) and primer-walking methods using custom primers with sequences based on primer-walking analysis results (Table 1, Fig. 1). The PCR products were then sequenced with a Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and the dye-labeled extension products were analyzed with an ABI Prism 3130 × 1 DNA genetic analyzer (Applied Biosystems). The sequences were deposited in GenBank with the following Accession Numbers: AB699603, AB699604, AB699605, AB699606, AB699607, AB699608, AB699609, AB699612.

Construction of recombinant Cb-PLC (rCb-

PLC) and recombinant alpha-toxin (rAlpha-toxin) expression systems. The *cb-plc* of *C. botulinum* D-1873 without a signal peptide sequence was amplified by PCR using genomic DNA as a template with the set of PCR primers CPLC(EcoR)-1f GGAATTCCTGGGATGGAAAACCTGATGGTACA and DPLC(Xho)-1r CCGCTCGAGCGGTTATTTATTATTTATATAGAATGTTTTGTTATC. The amplified PCR products were digested with *Eco*RI and *Xho*I, and cloned into the pGEX-6P-3 plasmid vector (GE Healthcare, Piscataway, NJ). The resulting pGEX-6P-3 plasmid was then transformed into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA, USA). Transformants were selected on LB agar plates con-

Table 1 PCR primer sets for amplifying cb-plc

No.	Primer name	Sequence (5'→3')	Position (bp)*	Aim
1.	PLC.IPCR-1f	GTTCTACTTATCCAGATTATGATCC	232 to 256	IPCR and walking primer
2.	PLC.IPCR-2f	GCAACATGGCTTTTAGGACAAGG	429 to 451	IPCR and walking primer
3.	PLC.IPCR-3f	CAGCAGGAACTAATAGTGTAAAGG	577 to 600	IPCR and walking primer
4.	PLC.IPCR-4f	AGTATCAGGAACAATAAATACAACTG	806 to 831	IPCR and walking primer
5.	PLC.IPCR-5f	TTTCAAGTACATCACCTAAAG	−454 to −474	Walking primer
6.	PLC.IPCR-6f	CATACAAAAATGTTAACAATAAG	−224 to −253	Walking primer
7.	PLC.IPCR-7f	TAGTGCAGTATTATCATTCAC	32 to 52	IPCR, walking primer, and internal sequence
8.	PLC.IPCR-8f	ATGAGTGGTTTGGAGATAAC	1153 to 1172	IPCR and walking primer
9.	PLC.IPCR-13f	CATTGTAACTTCTTTTGCATC	1782 to 1802	Walking primer
10.	PLC.IPCR-14f	GATCCAAAATTATTAAGGTACATCCC	−1701 to −1726	Walking primer
11.	PLC.IPCR-15f	TATTTTAATGTTTGGACTTCC	2079 to 2099	Walking primer
12.	PLC.IPCR-16f	CTGCAGCTGGTAAGAGAACTAAG	−2842 to −2864	Walking primer
13.	PLC.IPCR-17f	AAGACTTCTTATTAGGAGAAG	−2511 to −2531	Walking primer
14.	PLC.IPCR-18f	TCATCACGATCATGATCATG	−2155 to −2174	Walking primer
15.	PLC.IPCR-19f	TTATCTATTGCAGGAGATGC	1462 to 1481	Walking primer
16.	PLC.IPCR-1r	TTCTTTTACACTTAAAGGTGATGTAC	163 to 188	IPCR and walking primer
17.	PLC.IPCR-2r	TTGACTAATTGCATATGATAGATACC	331 to 356	IPCR and walking primer
18.	PLC.IPCR-3r	CCTTTACACTATTAGTTCCTGCTG	577 to 600	IPCR and walking primer
19.	PLC.IPCR-4r	TAGCTGCTATTTCCCAATCTTTCC	721 to 744	IPCR and walking primer
20.	PLC.IPCR-5r	ATCCTATATTTCCATCTTATTC	−83 to −104	IPCR and walking primer
21.	PLC.IPCR-6r	CTACAAGTTCTATTTAAACATAC	−313 to −335	Walking primer
22.	PLC.IPCR-7r	TTAATTCTATAACTATCTTCTTG	987 to 1009	IPCR, walking primer, and internal sequence
23.	PLC.IPCR-8r	TATTATAGCAAGTGCAGCTC	2102 to 2121	Walking primer
24.	PLC.IPCR-14r	GGAGGATAATATATGTGATG	1945 to 1964	Walking primer
25.	PLC.IPCR-16r	AAAAAGGACAACTCTATTCC	−2341 to −2360	Walking primer
26.	PLC.IPCR-17r	AATTACCCTTAGGTTCTTTAG	−2535 to −2555	Walking primer
27.	PLC.IPCR-18r	TTCCTATCTTCTGTAATGTAC	1350 to 1370	IPCR and walking primer

*Position 0 (zero) refers to the first nucleotide of the ATG start codon of *cb-plc*.
IPCR, inverse PCR.

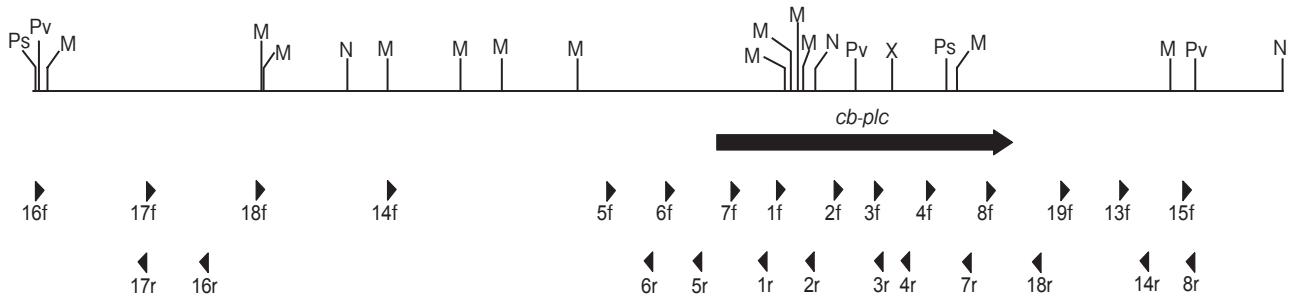

Fig. 1 Schematic representation of the *cb-plc* sequencing map. The details of the primer sequences are described in Table 1. Arrowheads represent the primer positions and their directions. The names of primer are labeled as numbers, and "f" and "r" indicate forward and reverse primers. Abbreviations of the restriction enzymes: M, *Mbo*I; N, *Nde*I; Ps, *Pst*I; Pv, *Pvu*II; X, *Xba*I. Primers PLC. IPCR-7f and PLC.IPCR-7r were used to amplify the internal sequence of *cb-plc*.

taining 50 μg/ml ampicillin, and plasmid DNA was extracted from the *E. coli* cell cultures. Sequencing reactions were performed with vector-specific primers. The construction of recombinant alpha-toxin (rAlpha-toxin) was conducted as reported by Nagahama *et al.* [14]. Briefly, the 1.3-kb *Ssp*I*Hind*III fragment containing the alpha-toxin gene was subcloned from pUAT into the pHY300PLK (*E. coli-Bacillus subtilis shuttle* vector) *Sma*I site and transformed into *B. subtilis* ISW1214.

Expression and purification of rCb-PLC and rAlpha-toxin. rCb-PLC was expressed as a GST fusion protein after adding isopropyl-β-D(-) thiogalactopyranoside (IPTG) (Wako, Osaka, Japan) to a final concentration of 0.1 mM to *E. coli* BL21-CodonPlus® (DE3)-RIL (Stratagene, La Jolla, CA, USA) transformant cell cultures. Cells were harvested by centrifugation at 8,000 × g, 4℃, for 20 min, and then resuspended in phosphate-buffered saline (pH 7.3) (PBS). The cells were sonicated on ice and centrifuged at 24,000 × g for 30 min at 4℃, and then the supernatant was collected and applied to Glutathione Sepharose 4B affinity chromatography (GE Healthcare) at a flow rate of 0.5 ml/min. After washing the column with PBS, the protein was eluted with 10 mM reduced glutathione (Wako) in 50 mM Tris-HCl (pH 8.0). The eluted fractions were pooled and dialyzed against cleavage buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 1 mM DTT without EDTA). After dialysis, GST was removed by treatment with PreScission Protease (GE Healthcare). The solution was loaded onto the affinity chromatography column, and the pass-through fractions containing rCb-PLC were collected, dialyzed against 10 mM phosphate buffer (pH 7.5), and applied to a MonoQ HR5/5 anion-exchange chromatography column (GE Healthcare) equilibrated with the same buffer. The proteins were eluted with a sodium chloride gradient (0–1 M) in 50 mM phosphate buffer (pH 7.5). The eluted proteins were dialyzed against 0.02 M Tris-HCl (pH 7.5). The expression and purification of rAlpha-toxin was performed as described in a previous study [14]. Briefly, transformants were cultured in LB broth at 37℃ for 12 h. The culture supernatant was collected by centrifugation, precipitated by ammonium sulfate, and incubated at 4℃ overnight. The precipitate was collected by centrifugation and was dissolved in 20 mM Tris-HCl buffer (pH 7.5). The ammonium sulfate fraction was applied to a Cu-chelate Sepharose affinity column. Fractions containing rAlpha-toxin were dialyzed against 20 mM bis Tris- HCl buffer (pH 6.2) at 4℃ for 12 h and were then loaded onto a Mono-Q HR5/5 column (Pharmacia, Uppsala, Sweden) preequilibrated with 20 mM bis Tris-HCl buffer (pH 6.2). The recombinant Alpha-toxin was eluted with a sodium chloride gradient (0–0.5 M) in 20 mM bis Tris-HCl (pH 6.2). The protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Detection of enzymatic and biological activities. To determine the PLC activity on solid medium, *C. botulinum* C-St, D-1873, and *C. perfringens* NCTC 8237 were inoculated on GAM-egg yolk agar plates, and incubated under appropriate conditions as reported previously [5]. The activity was observed as clearly marked opacity spreading from the colony edge.

To examine PLC activity using 20% egg yolk solu-

tion, the solution was prepared as described elsewhere [15] with slight modifications. One freshly obtained egg yolk diluted in 500 ml of 0.02 M Tris-HCl (pH 7.5) containing 0.9% NaCl (TBS) was mixed with 20 g kaolin. The egg yolk solution was collected from the supernatant after centrifugation at 11,000 × g for 20 min at 4℃.

C. botulinum C-St, D-1873, and *C. perfringens* NCTC 8237 were cultivated in LYG medium [5] under an anaerobic condition at 37℃ for 16 h. Ten-fold serially diluted filtered-bacterial cultures (100 μl) were mixed with 200 μl of egg yolk solution, and were incubated at 37℃ for 30 min. To measure the egg yolk hydrolysis activity of the recombinant proteins, two-fold serially diluted rCb-PLC and rAlpha-toxin (protein concentration 0.04–10 μg/ml) were pre-incubated with $CoCl_2$ (final concentration: 1 mM) at 37℃ for 20 min, and diluted with TBS containing 0.25% gelatin (G-TBS) to a final volume of 100 μl. Egg yolk solution (200 μl) was then added into the protein solution, and the incubation was continued at 37℃ for 30 min. All egg yolk hydrolysis assay measurements were made at 655 nm. The concentration of protein which showed 50% turbidity was calculated. The *p*-NPPC assay was conducted as described previously [16]. Recombinant proteins were mixed with 20 mM *p*-NPPC, 0.25 M Tris-HCl (pH 7.2), 60% w/v sorbitol, and 0.5 M $CoCl_2$, and then incubated at 35℃ for 90 min. The amount of *p*-nitrophenol product was calculated by using a molar extinction coefficient of 1.54×10^4.

The SMase activity of the recombinant protein was examined by incubating rPLCs (1.0 μg/ml) with 1.0 mM sphingomyelin from bovine brain (Nacalai Tesque, Kyoto, Japan) solubilized in 1.0% sodium cholate containing $CaCl_2$ (final concentration: 0.5 mM), and incubated at 37℃ for 3 h. The turbidity of the reaction mixture was measured at 620 nm. The standard curves are described by the turbidity of ceramides from bovine brain (Avanti Polar Lipids, Alabaster, AL, USA).

The hemolysis assay was performed as described elsewhere [17] with some modifications. Two-fold serially diluted recombinant proteins (the concentrations of rAlpha-toxin were 0.01–0.5 μg/ml, while those of rCb-PLC were 0.4–50 μg/ml) in G-TBS (100 μl) were pre-incubated with $CoCl_2$ (final concentration: 4 mM) at 37℃ for 20 min. The solution was incubated with 1% washed rabbit erythrocytes (100 μl) in TBS containing $CaCl_2$ (final concentration: 3 mM) and G-TBS to give a total volume of 300 μl, at 37℃ for 30 min, and subsequently chilled at 4℃ for 10 min. Unlysed cells were removed by centrifugation at 1,750 × g, 4℃ for 5 min, and the absorbance of the supernatant was measured at 570 nm. The concentration of protein which showed 50% hemolytic activity was calculated. All the assays above were performed in triplicate.

Results

Cb-PLC nucleotide sequence and deduced amino acid sequence. The predicted *cb-plc* genes were successfully identified in all eight strains of types C and D employed. Each of these genes consisted of 1,200 nucleotides and encoded a 399 amino acid residues protein. The amino acid sequence identity among these strains was over 98%; therefore, we used *C. botulinum* D-1873 as the representative of all strains employed to construct the expression vector of rCb-PLC. A previous study reported that the *C. perfringens* alpha-toxin is post-translationally processed into a mature protein [18]. Therefore, the deduced amino acid sequence of the Cb-PLC was analyzed using the SignalP (version 3.0) program [19], which predicted that the N-domain of the precursor protein had characteristics of a signal peptide with a probable cleavage site between Ala27 and Trp28 (Fig. 2). The mature Cb-PLC had 372 amino acid residues with a calculated molecular mass of ~43 kDa. The alpha-toxin is known to consist of 2 domains, the N- and C-domains, which contain the active site and membrane binding site, respectively [11]. Multiple sequence alignments of Cb-PLC and alpha-toxin suggested that Cb-PLC was also composed of an N-domain and a C-domain, with an overall amino acid sequence identity of 52.6%. The amino acid identity of the N-domain between Cb-PLC and alpha-toxin was 60.8%. The identities of the N-domains among Cb-, bifermentans-, sordellii-, novyi-, and absonum-PLCs ranged from 55.9% to 63.7% (Table 2). However, the identity of the N-domain between Cb-PLC and the non-toxic Bc-PLC was low (less than 25%). Between Cb-PLC and alpha-toxin, the amino acid identity of the C-domain was 44.9%, and the identity between Cb-PLC and other clostridial PLCs was 43.5–49.5% (Table 2).

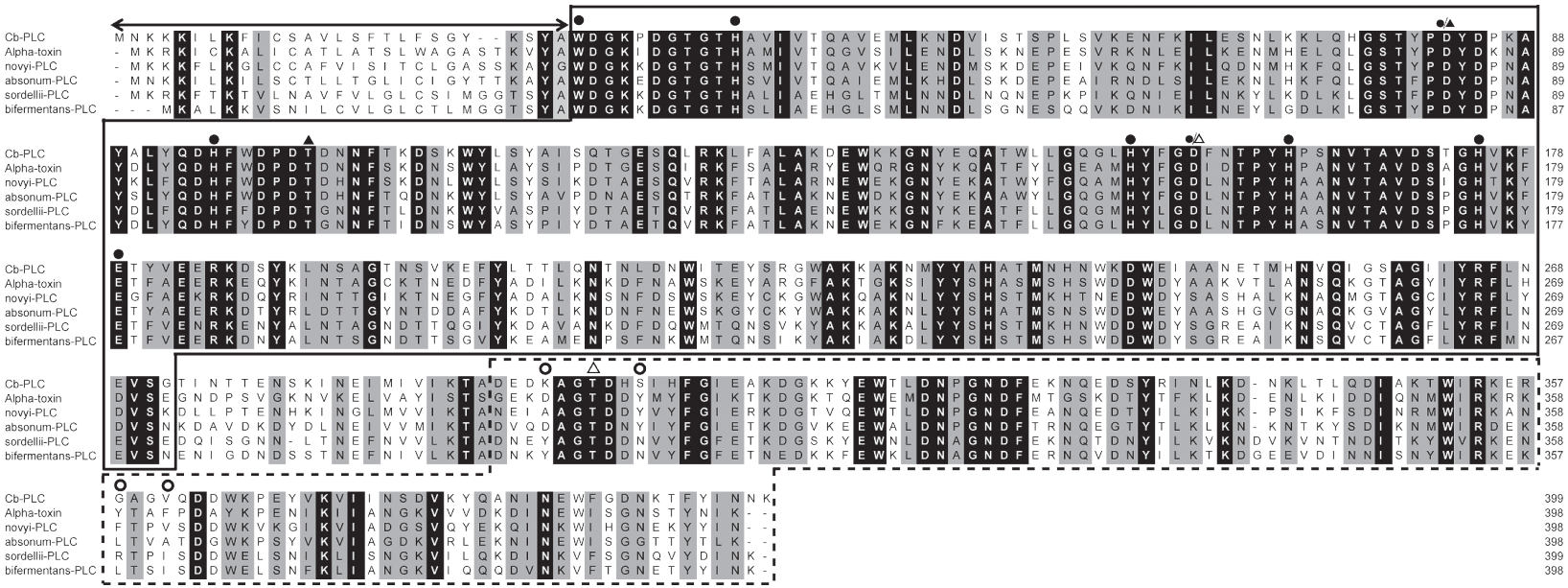

Fig. 2 Amino acid sequence alignment of Cb-PLC, alpha-toxin (accession no. ABA64093), novyi-PLC (accession no. BAA06851), absonum-PLC (accession no. AY159815), sordellii-PLC (accession no. BAB83263), and bifermentans-PLC (accession no. BAB83265). The sequences indicated by an arrow are the signal peptides. Clostridial PLC signal peptide sequences were predicted using the SignalP program [19]. The amino acid residues for zinc-coordination (●) that are important for the enzymatic activity, for catalyzing membrane phospholipid hydrolysis (▲), and for maintaining the conformational structure of PLC (Δ) are conserved, whereas those of membrane recognition (○) are found to be not conserved in Cb-PLC. The N- and C-domains are indicated by the solid- and dotted-line boxes, respectively. The N-domains among clostridial PLCs showed more homologues than those of the C-domains.

Table 2 Comparison of amino acid sequences of Cb-PLC, alpha-toxin, novyi-, absonum-, sordellii-, and bifermentans-PLCs[a]

Phospholipase C	% Identity (N-domain/ C-domain)					
	Cb-PLC	alpha-toxin	novyi-PLC	absonum-PLC	sordellii-PLC	bifermentans-PLC
Cb-PLC		52.6 (60.8/44.9)	55.5 (60.4/49.5)	56.9 (63.7/47.7)	51.5 (55.9/47.2)	50.4 (56.3/43.5)
alpha-toxin			60.6 (69.4/52.4)	58.3 (64.1/56.2)	51.4 (58.3/45.8)	50.9 (58/46.7)
novyi-PLC				61.3 (69.4/53.3)	54.8 (59.6/53.3)	52 (59.6/48.6)
absonum-PLC					52.6 (58.8/46.7)	52.3 (59.6/42.5)
sordellii-PLC						76 (82/70.1)
bifermentans-PLC						

[a]The data for Cb-PLC is from this study. The data for the other clostridial PLC sequences are registered at the GenBank database under the following accession numbers: alpha-toxin, accession no. ABA64093; novyi-PLC, no. BAA06851; absonum-PLC, no. AY159815; sordellii-PLC, no. BAB83263; and bifermentans-PLC, no. BAB83265.

Construction and expression of rCb-PLC and rAlpha-toxin. The *C. botulinum* type D-1873 PLC gene (*cb-plc*) was cloned and expressed as a representative of the PLCs produced by *C. botulinum* types C and D, and alpha-toxin was also expressed as a recombinant protein as described in the Materials and Methods. The purified recombinant proteins were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol (Fig. 3). The results showed that the molecular masses of proteins were about 43 kDa and both proteins seemed to be homogenous.

The enzymatic and biological activities of Cb-PLC. *C. perfringens* NCTC 8237, *C. botulinum* C-St, and D-1873 produced opacity around their colonies on GAM-egg yolk agar plates (Fig. 4A). Moreover, the serially diluted cultures of C-St and D-1873 successfully hydrolyzed egg yolk phospholipid in solution, though their titers were significantly lower than that of *C. perfringens* (data not shown). The rCb-PLC and rAlpha-toxin also produced opacity on egg yolk agar plates (Fig. 4B), although a higher concentration of rCb-PLC (50 μg/ml) was required to produce opacity similar to that induced by rAlpha-toxin (5 μg/ml).

The specific activities of rCb-PLC were then quantitatively compared with those of rAlpha-toxin. First, the effects of $CoCl_2$, $MgCl_2$, $CaCl_2$, and $ZnCl_2$ on the enzymatic and biological activities of the recombinant protein were conducted. We found that

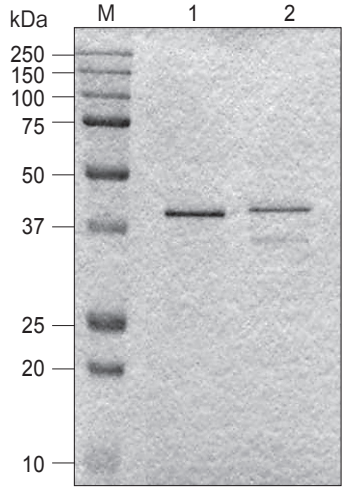

Fig. 3 SDS-PAGE analysis of purified rPLC. Electrophoresis was performed on a 12.5% polyacrylamide gel in the presence of 2-mercaptoethanol. Lane 1: rCb-PLC; Lane 2: rAlpha-toxin. M: standard molecular mass markers (kDa). The molecular mass of either rCb-PLC or rAlpha-toxin is approximately 43kDa.

the egg yolk phospholipid hydrolysis, *p*-NPPC hydrolysis, and hemolytic activities were most activated by $CoCl_2$, while SMase activity was activated by $CaCl_2$. As summarized in Table 3, the egg yolk phospholipid hydrolysis and *p*-NPPC hydrolysis activities of rCb-PLC were 23.3% and 1.6% those of rAlpha-toxin, respectively. Furthermore, the SMase and hemolytic

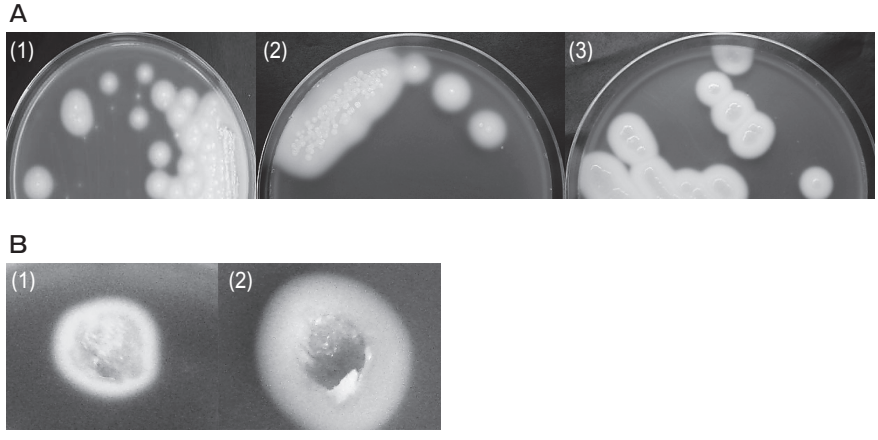

Fig. 4 **A**, The PLC reaction of *C. botulinum* C-St (1), D-1873 (2), and *C. perfringens* NCTC 8237 (3) on GAM-egg yolk agar plates. The reaction resulted in opacity around the colonies; **B**, The PLC activity of the rCb-PLC (1) and rAlpha-toxin (2) (incubation time: 3 h) was represented as the opacity observed on GAM-egg yolk agar plates (concentration of rCb-PLC: 50 μg/ml; concentration of rAlpha-toxin: 5 μg/ml). These figures confirm that the *C. botulinum* type C and D strains produce PLC.

Table 3 Comparison of the enzymatic and biological activities of rCb-PLC and rAlpha-toxin

Phospholipase C	Egg yolk-phospholipid hydrolysis (ng/300 μl) (% activity relative to alpha-toxin)	*p*-NPPC hydrolysis (nmol/mg/min) (% activity relative to alpha-toxin)[a]	Sphingomyelinase (U/mg/min) (% activity relative to alpha toxin)[a]	Hemolysis (ng/300 μl) (% activity relative to alpha-toxin)
rCb-PLC	490.2 (23.3)	3.75 $\pm$ 0.19 (1.6)	0.24 $\pm$ 0.09 (9.4)	1,719 (0.5)
rAlpha-toxin	114.4 (100)	235.21 $\pm$ 4.02 (100)	2.55 $\pm$ 0.25 (100)	8.7 (100)

[a]Data are the mean values $\pm$ SD (n = 3).

activities of rCb-PLC were approximately 10- and 200-fold lower than those of rAlpha-toxin.

Discussion

In the present study we successfully cloned the *cb-plc* gene, constructed and purified rCb-PLC, and determined the enzymatic and hemolytic activities of rCb-PLC. The multiple sequence alignment between Cb-PLC and alpha-toxin showed that Cb-PLC is a homolog of the alpha-toxin and consists of 2 domains, the N- and C-domains. The N-domain is a catalytic domain containing the active site cleft of the PLC protein, within which 3 zinc cations are coordinated. Several amino acid residues in the N-domain that are important for 1) zinc binding coordination of Zinc 1 (Trp1, His11, Asp130), Zinc 2 (His136, His148, Glu152), and Zinc 3 (His68, His126, Asp56, Asp130), 2) catalytic activity (Asp56 and Thr74) and 3) maintaining the conformational structure of PLC (Asp130 and Thr272), were found to be conserved in Cb-PLC compared with those of alpha-toxin and other clostridial PLCs [11, 14, 20–23]; thus, it is speculated that Cb-PLC has an N-domain structure that corresponds to a zinc metallophospholipase C. In contrast, compared with the alpha-toxin, several important residues in the C-domain of Cb-PLC were not conserved. Residues Asp269, Tyr275, Tyr331, and Phe334 in alpha-toxin [24, 25], which lie on the protein surface and have been proposed to be involved in membrane binding, were found to be replaced by Lys268, Ser 275, Gly330, and Val333 in Cb-PLC, respectively (Fig. 2).

We confirmed that *C. botulinum* type C and D produced PLC. We measured the activities of rCb-PLC and compared them with those of rAlpha-toxin. In the

present study, we used a GST-fusion protein in preparing rCb-PLC, whereas for rAlpha-toxin we used an *E.coli-B.subtilis* expression system. Although we used a GST-fusion protein for rCb-PLC preparation, we successfully removed the tag from the recombinant protein, thereby avoiding any GST-tag-interference in the recombinant protein activities. In addition, we successfully measured the enzymatic and biological activities of rCb-PLC, although these activities were somewhat weaker than those of the alpha-toxin. The specific activities of rCb-PLC reported in the present study seemed to be comparable to those of bifermentans- and sordellii-PLCs [9]. In that study, Karasawa *et al.* reported both bifermentans- and sordellii-PLCs that were expressed as His-tag recombinant proteins showed weaker enzymatic and hemolytic activities compared with those of alpha-toxin.

As described above, the key residues of the N-domain (catalytic domain) in Cb-PLC and other clostridial PLCs were fully conserved, as in the alpha-toxin. However, 4 key residues in the C-domain of Cb-PLC that are important for membrane binding were not conserved, just as they were not conserved in bifermentans- and sordellii-PLCs [9]. In contrast, absonum-PLC, a toxic PLC, showed only 2 mutations in 4 four residues, Leu331 and Ala334 [10]. Although we have not yet performed experiments for the enzymatic and binding activities using the isolated N-domain or C-domain, or the binding experiment with whole rCb-PLC, we speculate that the relatively low enzymatic activity of Cb-PLC may be due to the differences of amino acid residues, except the important ones, in the N-domain. Since the C-domain has structural analogy with the C2-like domain of eukaryotic phospholipid-binding proteins, which is involved in membrane phospholipid interaction, it is thought that the quite low hemolytic activity may be influenced by low cell binding ability through the C-domain, which is caused by differences of amino acid residues, including the key residues.

Alpha-toxin is considered to be the important virulence factor in causing gas gangrene. As previously reported, a large amount of alpha-toxin can directly cause membrane hydrolysis [26]. In the case of types C and D of *C. botulinum*, these bacteria rarely cause botulism in humans, but are known to be responsible for big outbreaks in wild birds and domestic animals such as cattle, even though they are much harder to grow in media than other types of botulinum organisms. In our preliminary experiments, large amounts of type C and D NTXs (more than 10^6 LD_{50} in total) were needed to kill the cattle, when they were orally administered. As mentioned above, the amount of rCb-PLC required for complete hemolysis was high compared with the amount of alpha-toxin required, but it was still about 7.5μg protein/300μl, a volume that might be produced during the cell growth of types C and D in the animal intestine or in the environment. Therefore, we speculate that organisms may produce enough Cb-PLC to disrupt the intestinal cell membranes to facilitate the passage of NTX across the intestinal barrier into the blood circulation. In addition, since *C. botulinum* types C and D also produce botulinolysin (Bly), which is a cytolytic toxin, it is suggested that Cb-PLC and Bly might have a synergistic effect in membrane disruption. It is also considered that Cb-PLC might participate indirectly in *C. botulinum* pathogenicity by obtaining the nutrients needed for bacterial growth and multiplication from lysed host cells. We are now studying these points.

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