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Cloning and expression of immunogenic *Clostridium botulinum* C2I mutant proteins designed from their evolutionary imprints

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ABSTRACT

C2 toxin produced from *Clostridium botulinum* serotypes C and D has a potential role in many pathophysiological mechanisms in birds and animals. It has encompassed an ADP ribosyltransferase subunit (C2I) and a translocation/binding subunit (C2II). In the present study, we intended to produce C2I mutant proteins as recombinant subunit vaccines by using glutathione-S-transferase-gene fusion system. The mutants of this study were previously evaluated from their evolutionary imprints and suggested as suitable candidates for subunit vaccines. A synthetic C2 gene was inserted in a pGEX-2T vector, cloned and expressed in *Escherichia coli* BL21 host. The expressed mutant proteins were purified by using glutathione-agarose column and then examined for their ADP ribosyltransferase activity and vaccinogenic characteristics. The pGEX-2T-C2I constructs with Y298F, S347A and S350A substitutions have shown effective transformation efficiencies in *E. coli* XL10 competent cells but their mutagenesis efficiency was relatively low. Gene expression analysis indicated the rate of gene expression was depended on the fused mutant genes. A high-level expression was achieved for Y298F, S347A and S350A mutant proteins. All purified protein exhibited a molecular mass of 49 kDa. C2I mutant proteins exhibited a reduced ADP ribosyltransferase activity with retained immunogenic and vaccinogenic characteristics compared to the wild-type C2I subunit. The overall analysis of our study suggested the recombinant C2I proteins (S197A and Y298F) are the most promising candidates for the development of subunit vaccine or immunogen for C2 mutants mediated diseases in birds and animals.

1. Introduction

Subunit vaccine research is dependent on the efficient production of protein antigens, and recombinant strategies are today overlooking worldwide. There are several approaches to design and optimize the production and purification systems for recombinant subunit vaccines [1]. Vaccination combined with antigenic or immunogenic proteins from pathogenic microorganisms trigger protective immune responses against one or several diseases [2,3]. However, the heterologous host used may affect the immunogenicity and protective efficacy of antigenic proteins. A protein antigen can be expressed at high levels in bacterial expression systems, but may only fold partially into its native conformation [4]. Even if several bacterial systems are available, *Escherichia coli* is the best characterized heterologous host, when no post-translational modification of a protein is required [5]. Many well-defined affinity tags have been described in the literature that will make effective recovery of expressed genes. Therefore, recombinant subunit vaccines, specifically for animal botulism have the potential to decrease the manufacturing process with the substantial use of animals required for the determination of vaccine efficacy for the production on a commercial scale [6].

Apart from botulinum neurotoxins, *Clostridium botulinum* serotypes C and D strains are capable of producing C2 toxin, a member of binary toxin A family in the ADP-ribosylation superfamily. C2 toxin composes C2I, an enzymatic subunit with 431 residues and C2II, a binding/translocation subunit with 721 residues. Both subunits are expressed as separate proteins contributing in many pathophysiological functions [7]. C2 toxin induces necrotic-hemorrhagic lesions, vascular permeability, and hypertension. It also instigates to accumulate a lethal fluid in the lungs and intestinal tracts of birds and animals [8,9]. C2I modifies β/γ-non-muscle actin and γ-smooth muscle actin at the side chain of Arg177 [10,11]. C2II is cleaved at Lys181 with protease and yielded C2IIa that binds to a receptor on the cell surface. Active C2I is released from an acidic endosome to the cytosol by the action of endosomal H^+-ATPase and a host cell chaperone Hsp90 and peptidyl-prolyl cis/trans isomerases cyclophilin A [12,13], cyclophilin 40 [14], and FK506 binding protein 51 (FKBP51) [15]. C2I binds to A_1B_5 heterohexamer of C2IIa, inserts into the membranes and forms pores under

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acidic endosomal pH. This toxin-receptor complex allows translocation of C2I into the cytosol wherein C2I transfer of an ADP-ribose moiety of NAD^+ to G-actin [16]. C2I-mediated ADP-ribosylation turns actin into a capping protein that binds to the barbed ends of actin filaments, leading to disruption of the cytoskeleton architecture [9].

C2I includes two-domain structure viz. N-terminal adapter domain and a C-terminal catalytic domain. N-terminal adapter domain, particularly a segment 29–86 (α1-helix), is essential for binding to the prepore C2IIa [17,18]. A central quartet of β-strands with two middle strands in C-terminal catalytic domain harbors its catalytic function [19]. C2I shares a highly conserved serine-threonine-serine motif [20,21]. Conserved Arg299 is most likely involved in NAD-binding and/or stabilization of its active site structure [16,22]. Glu389 is a catalytic site of C2I and Glu387 is important for its transferase reaction, but not for the NAD-glycohydrolase activity of C2I [21,23,24]. Site-directed mutagenesis studies reported that ADP-ribosyltransferase activity was drastically decreased by S174A, R299K, R300K, S348A and T349V mutants of C2I [16,22].

It is imperative to discover site-directed mutagenesis targets from the sequence-structure-function-virulence link of active C2I subunit for the development of recombinant subunit vaccines or immunogens [25–29]. Thus, the present study was intended to clone, express and purify five recombinant C2I proteins retaining immunogenicity with no toxic enzymatic activity, as it is in the active C2I subunit. Our experimental mutants were screened, selected and evaluated from their evolutionary blueprints, as published earlier [28,29]. Heavy chain subunits of various botulinum neurotoxins are evaluated as immunogens in monovalent and bivalent formulations [30]. Several recombinant vaccines have been produced to provide active immunity against human and animal botulism [31,32]. A recombinant subunit vaccine consisting of non-catalytic botulinum neurotoxin and non-catalytic ADP-ribosyltransferase was developed to provoke innate immunity in mammals [33]. Hence, our study addresses the challenges associated with utilizing non-catalytic C2I subunit in developing an immunogenic protein for *C. botulinum* intoxicated avian and cattle.

2. Materials and methods

2.1. Gene synthesis and cloning

A synthetic gene encoding C2I subunit (UniProt ID: CAA11969) was synthesized by GeneScript (USA) with optimal codon usage for *E. coli*. Gene-on-Demand® gene synthesis platform was employed to mutation-free DNA synthesis for C2I gene. This synthetic gene was inserted in a pGEX-2 T plasmid (Agilent Technologies, USA) digested with BamHI and EcoRI using CloneEZ® seamless cloning technology (GeneScript, USA) according to the manufacturer's instructions. It was also inserted in a pUC57 plasmid digested with SalI and SacI and subcloned in *E. coli* JM101. After digestion, bands on the agarose gel (1%) representing the fragments encoding proteins of interest and expression vectors were purified from the gel or digestion reactions. After purification, the inserts and vectors were quantified and ligated with T4 DNA ligase (Sigma Aldrich, USA). The ligation products were used to transform into *E. coli* JM101 cells, which were cultured overnight on Luria-Bertani (LB) agar plates with 100 μg/ml ampicillin. Bacterial clones were screened on LB-ampicillin agar containing isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal (Sigma Aldrich, USA) for the recombinants. Plasmid DNA was extracted from the selected recombinants using the alkaline lysis method and then recombinant clones were characterized by BamHI and EcoRI digestion [34].

2.2. Construction of recombinant C2I genes

C2I gene and its mutants (S197A, Y298F, S347A, R300K, and S350A) were constructed by site-directed mutagenesis with the pGEX2T-C2I plasmid as a template using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer's instructions. We used two complementary synthetic oligonucleotides for the generation of each mutant gene as represented in Table 1. The pWhitescript and pUC18 plasmids were used as mutagenesis and transformation controls, respectively. The mutated plasmids were transformed into *E. coli* XL-1 Blue super-competent cells. The clone was inoculated into 100 ml of LB Broth (HiMedia Laboratories, India) supplemented with 20 mM glucose and 100 μg/ml ampicillin and grown overnight at 37 °C. The culture was diluted 1:50 in fresh medium and grown for 4 h at 37 °C (OD_{600} = 0.8). Following transformation, colonies were screened for recombinants by using X-gal assay. Bacterial transformants were also confirmed by standard colony PCR procedure using C2I forward and SP6 primers whereas T7 and C2I reverse primers [34]. The presence of respective mutations was further confirmed by DNA sequencing.

Table 1
Analysis of transformation and mutagenesis efficiency of C2I gene and its mutants.

utant genes	Transformation efficiency (Transformants/μg DNA)	Mutagenesis efficiency (ME cfu/μg DNA)	Mutagenesis efficiency (%)
C2I wild-type	1.67	–	–
S197A	2.64	1.4×10^3	57 ± 3.4
Y298F	4.43	8.4×10^2	34 ± 2.8
R300K	2.64	1.4×10^3	57 ± 3.4
S347A	4.04	9.1×10^2	37 ± 2.1
S350A	4.56	8.1×10^2	32 ± 3.9

2.3. Expression of recombinant C2I genes

The pGEX-2T-C2I and pGEX-2T-C2I-mutant plasmids were isolated from the selected bacterial clones and then purified using the alkaline lysis method [34]. C2I and its mutant genes were expressed as recombinant glutathione S-transferase (GST)-fusion proteins in *E. coli* BL21 (Agilent Technologies, USA) according to manufacturer's instructions with modifications by Harper and Speicher [35]. The pGEX-2T-C2I and pGEX-2T-C2I-mutant plasmids were transformed into *E. coli* BL21 by heat shock method (Green and Sambrook, 2012). Bacteria transformed with each recombinant vector were grown in LB broth supplemented with 100 μg/ml of ampicillin in an orbital shaker (37 °C, 200 rpm) until the mid-log growth phase (OD_{600} = 0.8). Heterologous protein expression was induced at 37 °C for 6 h by the addition of IPTG to a final concentration of 0.2 mM. The cells were harvested by centrifugation (14,000 rpm, 10 min, 4 °C), suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1% Triton-X100, 1 mM phenylmethylsulfonyl fluoride, 2 mM $MgCl_2$, 3U DNase), incubated with 100 μg/ml of lysozyme for 1 h at 4 °C, and centrifuged again (10,000 rpm, 30 min, 4 °C). The supernatant was collected and used for further purification of fused C2I protein.

2.4. Purification of recombinant C2I proteins

Glutathione-Agarose powder (Sigma Aldrich, USA) was swelled in distilled water (50% v/v), and then washed with equilibration buffer (pH 7.8) containing 1% Triton X-100 (Sigma Aldrich, USA). The culture supernatant was loaded into Glutathione-Agarose column, incubated for 30 min at 4 °C and centrifuged (5000 rpm, 5 min, 4 °C). The column was again washed with equilibration buffer (pH 7.8) and then GST-C2I fusion protein recovered four times with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.0). To remove the GST-tag, the immobilized protein was incubated with thrombin (80 NIH units/L culture; Agilent Technologies, USA) for 16 h at 25 °C. The supernatant containing C2I was obtained by centrifugation at 14,000 rpm for 2 min.

It was further incubated with benzamidine beads (Agilent Technologies, USA) for 10 min to eliminate thrombin. Finally, the purified fraction of C2I protein was collected and analyzed for homogeneity by SDS-PAGE on 12% gels according to the method of Laemmli [36]. Protein identity was confirmed by Western blot analysis with use of an anti-GST antibody (Merck, USA). The concentration of protein from each purification task was quantified by using the Branford protein assay kit (Merck, USA) was used for protein quantification according to the manufacturer's instruction.

2.5. Mono ADP-ribosyltransferase assay

All fluorescence measurements were carried out using an LS45 Fluorescence Spectrophotometer (Perkin Elmer, USA), as a method described by Armstrong and Merrill [37]. A reaction mixture consisted of 500 μM ε-NAD^+, 23 μM actin and 20 mM Tris-HCl buffer (pH 7.8) in a microcuvette. Following temperature equilibration for 10 min, the reaction was initiated by the introduction of crude or purified fraction of C2I mutant proteins (20 μl). The reaction was monitored by recording the increase in fluorescence intensity with time (excitation 300 nm, emission 410 nm-cutoff filter).

2.6. Prediction of vaccinogenic characteristics

Sites that produce an immunogenic response against C2I and its mutants were predicted from their sequences by Antibody epitope prediction tool [38]. Hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and the antigenic propensity of polypeptides chains are considered as the parameters for prediction of their immunogenic properties. The location of a linear B-cell epitope from their protein sequences by a combination of a hidden Markov model and a propensity scale method. Virulence characteristics of these mutants were predicted by the VICMpred server using support vector machine algorithm having patterns, amino acid and dipeptide composition of their sequences [39]. Vaccinogenic characteristics of these mutants were predicted with VaxiJen on the basis of their physicochemical properties without recourse to sequence alignment [40]. Allergenic properties of them were predicted by AllergenFP v.1.0 using fingerprint descriptor (http://www.ddg-pharmfac.net/AllergenFP/).

3. Results

The synthetic gene (C2I) of this study has 1296 nucleotides and coding protein has 431 amino acids in length. It has ADPrib_exo Tox domain located in the amino acid position of 227-427. It was inserted into a pGEX-2T plasmid, cloned and expressed in *E. coli* BL21. Restriction digestion analysis indicated that C2I gene was properly inserted into a pGEX-2 T plasmid and also pUC57 plasmid. After insertion of this gene, a molecular mass of pGEX-2T-C2I construct was found to be 6244 bp whereas a molecular mass of pUC57-C2I was 4006 bp (Fig. 1). In this study, we generated five pGEX-2T-C2I-mutant constructs from the pGEX-2T-C2I construct by mutating a residue that was functionally essential for toxic enzymatic activity (mono ADP-ribosyltransferase).

All mutant constructs were transformed into *E. coli* XL10 and *E. coli* JM101 competent cells (Table 1). The results of transformation efficiency indicated that pGEX-2T-C2I harboring Y298 F, S347 A, and S350 A substitutions were effectively transformed into *E. coli* JM101. *E. coli* XL10 was more effective for increased transformation efficiency of pGEX-2T-C2I and mutant constructs (S197 A and R300 K). The pGEX-2T-C2I-S347 A construct has yielded more recombinant clones on X-gal agar plates (**Fig. S1**). The wild-type C2I and its mutant genes have shown considerable recombinant clones in *E.coli*. As similar to that, we observed the mutagenesis efficiency of pGEX-2T-C2I and its mutant constructs in the respective competent cells. Mutation efficiency was raised for pGEX-2T-C2I constructs harboring S197 A and R300 K

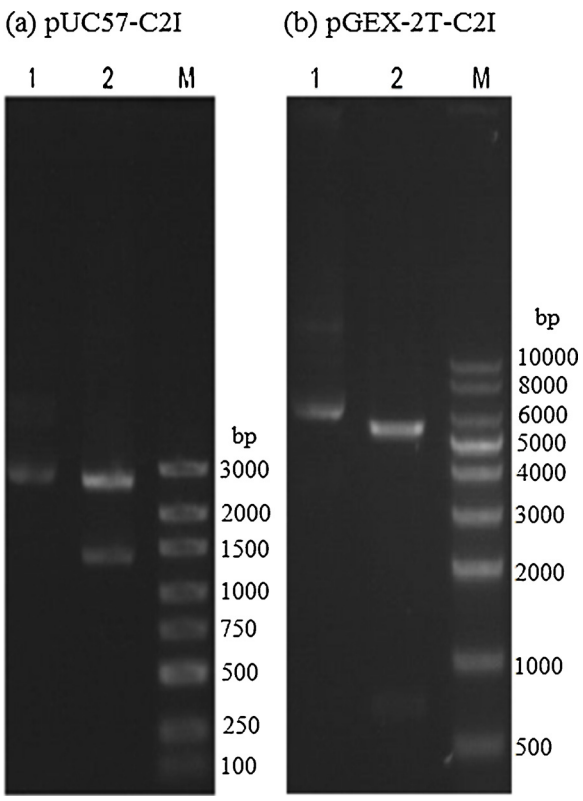

Fig. 1. Molecular cloning and restriction enzyme digestion of pUC57-C2I plasmid (a) and pGEX-2T-C2I plasmid (b).
Lane 1: pUC57-C2I plasmid; Lane 2: pUC57-C2I plasmid digested by SacI and SalI; Lane 3: DL3000 marker
Lane 1: pGEX-2T-C2I plasmid; Lane 2: pGEX-2T-C2I plasmid digested by BamHI; Lane 3: 1 Kb marker

mutations and it was found to be 57% (1.4×10^3 cfu/μg DNA) (**Fig. S2a**).

The entire mutant constructs were transformed into *E. coli* BL21, screened on LB agar containing X-gal and IPTG and then transformants confirmed by colony PCR (**Fig. S2b**). It indicated that all mutated C2I genes were transformed altogether into heterologous expression host. Results of colony PCR show the dense bands for S197 A and Y298 F mutant colonies representing the transformation efficiency of them higher than other mutants. However, the expression rate of these recombinant clones was different and therefore, the selected recombinants subjected for further gene expression studies. Gene expression rate of fused C2I and its mutated genes was calculated from GST activity on 1-chloro-2, 4-dinitrobenzene (**Fig. S2c**). GST activity of S197 A and Y298 F mutant proteins in cell lysates and purified fractions were measured to be more compared to other mutant proteins. Gene expression rate of C2I with R300 K and S350 A mutations was relatively low when compared to other. GST activity in the crude fraction of wild-type C2I protein was significantly increased, which was slightly more than the purified fraction of GST-C2I fusion proteins.

Bacterial lysates were prepared and C2I mutant proteins purified by using GST-gene fusion system. The purity of expressed proteins in each purification task was analyzed by SDS-PAGE (Fig. 2a). All C2I mutant proteins exhibited the expected molecular mass of 49 kDa. After cleavage with thrombin, we observed a relative protein quantity (40 μg/mL) of wild-type C2I protein and Y298 F, S347 A and S350 A mutants, which was rather than other mutants (Fig. 2b). It represented a high-level expression of these mutant proteins in *E.coli*. It also observed more quantity of proteins in S350 A mutant proteins, when it was in the cell lysate. It may be resulted due to the expression of some host proteins. Shown by our analysis, SDS-PAGE and quantitative protein assay

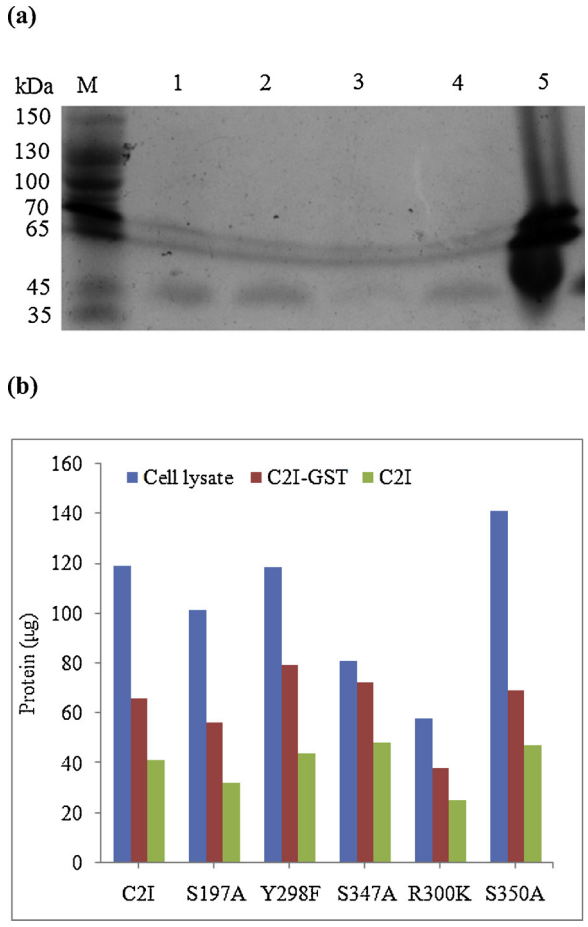

Fig. 2. Identification and purification of expressed mutant C2I proteins. Proteins were expressed as GST fusion proteins in *E. coli* (Lane 5) and cleaved with thrombin from glutathione-agarose beads (Lane 1–4). Proteins were run in a 12% SDS-PAGE and subsequently stained with Coomassie brilliant blue (a). Expressed C2I and its mutant proteins were purified using glutathione-agarose column (b).

confirmed the purity status and expression rate of C2I protein and its mutant proteins in *E.coli*.

ADP-ribosyltransferase activities of C2I mutant proteins were tested by fluorometric assay (**Table S2**). We measured high fluorescence intensity for mono-ADP ribosyltransferase activity in the cell lysate of wild-type C2I protein. After the removal of other proteins and impurities, mono-ADP ribosyltransferase activity was raised in both wild-type C2I protein and mutant proteins. It has shown that there was no significant fluorescence intensity observed for mutant proteins compared to wild-type C2 protein. It indicated that mono ADP-ribosyltransferase activity was relatively reduced from the mutant proteins compared to wild-type C2I protein. Exchange of the serine residues at the position of 347 and 350 with alanine showed in the reduction of ADP-ribosyltransferase activity by about 70–75%. Exchange of arginine residues at the position of 300 with lysine was retarded its effect on the ADP-ribosyltransferase activity up to 80%. It suggested that our C2I mutant proteins have a low catalytic function with different immunogenic and vaccinogenic features.

The results of immunogenic characteristics of C2I mutant proteins predicted a linear B-cell epitope (349-TSLKSTPSSFS-359) with S197 A, Y298 F, and R300 K mutations (Table 2; **Fig. S3**). The sites that produce an immunogenic response against expressed C2I mutants were considerably exerted in S197A and R300K mutations. Other mutant proteins found to show no immunogenicity at the mutated sites. Interestingly, C2I mutant protein with Y298F exhibited significant virulence

Table 2
Prediction of immunogenic characteristics of expressed C2I and its mutant proteins with no catalytic activity.

Mutant proteins	Virulence	Vaccine	Allergen
C2I wild-type	−1.7965	0.3072	0.82 ± 0.76
S197A	−1.6199	0.3070	0.82 ± 0.62
Y298F	−2.0936	0.3068	0.83 ± 0.34
R300K	−1.7113	0.3039	0.82 ± 0.55
S347A	−1.8361	0.3000	0.83 ± 0.51
S350A	−1.2633	0.3008	0.82 ± 0.86

compared to wild-type C2 protein. The Tanimoto coefficients calculated for all mutant proteins were statistically significant and have more prediction accuracy, reflected that there were no allergenic characteristics in these proteins. It may be resulted due to no structural flexibility and conformational changes in the mutated site of understudied mutant proteins. C2I mutant protein with S197 A and Y298 F mutations exhibited favorable vaccinogenic characteristics, suggestive of their suitability as an immunogen.

4. Discussion

C. botulinum C2 toxin affects eukaryotic cell function by the ADP-ribosylation of regulatory proteins. It consists of two independent polypeptides, which correspond to the catalytic subunit and binding subunit. BINARYTOXINA (PRINT ID: PR01390) is an 8-element fingerprint that provides a signature for C2I. Conserved serine-threonine-serine motif and Arg299 are most likely involved in NAD-binding and catalytic function of C2I structure [16,22]. Glu387 and Glu389 in C2I structure are important for its ADP ribosyltransferase reaction, which is highly conserved within ADP-ribosyltransferase superfamily [24]. Site-directed mutagenesis studies indicated that C2I mutant protein with S197 A and Y298 F substitutions almost have reduced the ADP-ribosyltransferase activity and prevented the cytotoxic effects on the hosts. Both enzyme and cytotoxic effects can be blocked by introducing respective mutations at the functional sites of C2I protein, which was agreed to the previous investigations [16,22].

The pGEX-2T plasmid is commonly used for successful expression of C2 toxin in *E. coli* [41–43]. *E. coli* can produce recombinant proteins with an average range of 40 mg/L, but the yield of subunit vaccine production for botulism is varied in many studies [44]. In our study, it was expected to produce a maximum of 36 mg of recombinant protein per liter of culture. It pointed out the high level of expression efficiency for S437A and S350A mutant proteins. *E. coli* BL21 was a suitable host for efficient expression of C2I toxin as reported earlier [41–43] *E. coli* BL21 (DE3) was used as a heterologous host for a high-level expression of C2I protein [16]. In this study, recombinant subunit vaccines composed with the purified recombinant C2I proteins with no toxic enzymatic activity. These vaccine candidates could induce with more effective immunogenic response compared to the wild-type C2I toxin. It suggested the C2I mutant proteins able to produce the antibody against C2 toxin in the *C. botulinum* intoxicated birds and animals.

A linear B-cell epitope (349-TSLKSTPSSFS-359) was predicted with S197A, Y298F, and R300K mutations in C2I toxin. Interestingly, the structure of many mutant proteins was stabilized their transition states to retain near-native-like conformation and immunogenicities by reordering the local structural environments. A mutation that increases the catalytic activity is likely to decrease the global stability of a virulence protein [45,46] and often to exert a new substrate specificity or catalytic activity [47]. Molecular virulence of one or two mutant proteins of C2I subunit was increased as a result of their physiochemical properties of exchanged amino acid. Vaccinogenic characteristics of C2I mutant proteins with S197A and Y298F mutations were also provoked significantly as compared to the wild-type C2 protein. It is most likely to be achieved by evolutionary constraints in sequence-structure-function-

virulence link in accordance to our previous works [27–29].

Generally, catalytic sites consist of a handful of residues that are important for the catalytic reaction. The surrounding residues around catalytic sites are important for ensuring proper substrate- and cofactor-binding in the active site cavity. Catalytic residues were often highly conserved in evolution, suggesting that point mutation of such residues was very detrimental in C2 toxin. It implied that various mutational effects influenced in the active site and binding pockets of C2I subunit, which may be amenable to mutations with optimal structural stability. The mutational effect of its sequence-structure-function-virulence link has shown a good agreement with the earlier hypotheses [48,49]. Compensation mutations might also have imposed for optimal stability of such relationship, as described previously [27,50,51]. During organismal evolution, compensatory adaptation (cryptic epistasis) is more likely to be fixed in the genome than neutral mutations, which could compensate for destabilizing mutations in protein-binding interfaces [52].

5. Conclusions

Proteins evolve under the law of natural selection and undergo conservative evolution. Extensive analysis of our study is expected to reveal that the introduced point mutation at the defined site can fine-tune the function of C2I protein and subsequently fixed in the genome of *C. botulinum*. However, such evolutionary constraints may depend on the residue position in the three-dimensional structure and fitness of the expressed mutant proteins (phenotype). Finding evolutionary imprints in its sequence-structure-function-virulence link are valuable informative measures for screening, selection, and evaluation of the stable mutants from C2 toxin. Our study would provide an insight into the evaluation of the mutable sites that are very important to design a recombinant subunit vaccine or immunogen against *C. botulinum* intoxicated birds and animals. The purified fractions of C2I mutant proteins exhibit no toxic enzymatic activity, but retained immunogenicity and vaccinogenic properties compared to the wild-type C2I subunit. Several recombinant vaccines have been developed for human and animal botulism, not for C2 toxin-mediated disorders. Hence, our study suggests that C2I mutant protein is one of the recombinant vaccine candidates holding great promise to emphasis immunotherapeutic development against the cytotoxic effects of C2 toxin.

Declarations of interest

The authors confirm that this article's content has no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cimid.2019.01.012.

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