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Characterization of pneumolysin from *Streptococcus pneumoniae*, interacting with carbohydrate moiety and cholesterol as a component of cell membrane

Jong Eun Lim [a], Seong Ah Park [a], Seoung Min Bong [b], Young Min Chi [b,*], Ki Seog Lee [a,*]

[a] Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 609-757, Republic of Korea
[b] Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea

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ABSTRACT

The cytolytic mechanism of cholesterol-dependent cytolysins (CDCs) requires the presence of cholesterol in the target cell membrane. Membrane cholesterol was thought to serve as the common receptor for these toxins, but not all CDCs require cholesterol for binding. One member of this toxin family, pneumolysin (PLY) is a major virulence factor of *Streptococcus pneumoniae*, and the mechanism via which PLY binds to its putative receptor or cholesterol on the cell membrane is still poorly understood. Here, we demonstrated that PLY interacted with carbohydrate moiety and cholesterol as a component of the cell membrane, using the inhibitory effect of hemolytic activity. The hemolytic activity of PLY was inhibited by cholesterol-MβCD, which is in a 3β configuration at the C3-hydroxy group, but is not in a 3α-configuration. In the interaction between PLY and carbohydrate moiety, the mannose showed a dose-dependent increase in the inhibition of PLY hemolytic activity. The binding ability of mannose with truncated PLYs, as determined by the pull-down assay, showed that mannose might favor binding to domain 4 rather than domains 1–3. These studies provide a new model for the mechanism of cellular recognition by PLY, as well as a foundation for future investigations into whether non-sterol molecules can serve as receptors for other members of the CDC family of toxins.

1. Introduction

Streptococcus pneumoniae is a major mucosal pathogen that is highly adapted to asymptomatically colonize the human upper respiratory tract, but in some hosts it produces localized respiratory tract infections such as otitis media, sinusitis, and bronchitis [1]. Particularly, *S. pnuemoniae* has a significant invasive potential and is a leading cause of bacterial pneumonia and meningitis [2]. Invasive *S. pneumoniae* infections still remain to be a major cause of morbidity and mortality worldwide [3] and thus, there is a growing need to understand the function of pneumococcal factors that underlie the invasive potential of this pathogen.

S. pneumoniae has many virulence factors that contribute to its ability to cause disease [4]. Among them, pneumolysin (PLY) is a 53 kDa protein toxin consisting of 471 amino acids [5] and one of the members of pore-forming toxins (PFTs), referred as thiol-activated cytolysins or cholesterol-dependent cytolysins (CDCs), that are a large family of PFTs [6], including perfringolysin O (PFO) from *Clostridium perfringens* [7], intermedilysin (ILY) from *Streptococcus intermedius* [8], and sterptolysin O (SLO) from *Streptococcus pyogenes* [9]. The CDCs are characterized by the presence of a highly conserved region (referred to as the Trp-rich loop) at the base of the 4th domain, which is thought to be responsible for membrane binding [10]. Membrane cholesterol is important for the mechanisms of eukaryotic [11,12] and prokaryotic toxins [13,14]. Cholesterol was thought to function as the sole CDC receptor until the discovery of ILY, which is active only on human cells [8], a feature seemingly inconsistent with the "cholesterol as receptor" paradigm. Giddings et al. [15] subsequently showed that ILY used human CD59 as its membrane receptor, but pore formation by ILY still required cholesterol [16]. Thus, whether cholesterol is bound directly by these proteins as a receptor or whether it indirectly influences the binding or activity of the protein at the membrane surface still remains to be determined. Recently, biological and biophysical studies have shed new light on the specific mechanisms used by these toxins for binding to, and for entering into cell membranes, as well as on their cooperative self-organizing oligomerization fostering pore formation and subsequent cell lysis [17,18].

Since most studies leading to membrane permeabilization are reproduced in artificial lipid bilayers consisting of phospholipids alone [19,20], non-lipid constituents of the membrane, such as proteins and carbohydrates, do not appear to be critically involved in the process. However, there is evidence indicating that glycoconjugates affect the pore-forming activity of cytolysins. For example, *Staphylococcus aureus* α-toxin is inhibited by GM1-ganglioside [21], and *Vibrio cholera* cytolysin is a lectin-like protein with pref-

* Corresponding authors. Fax: +82 2 921 3702 (Y.M. Chi), fax: +82 51 510 0568 (K.S. Lee).
E-mail addresses: ezeg@korea.ac.kr (Y.M. Chi), kslee@cup.ac.kr (K.S. Lee).

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erence for the terminal β1-galactosyl moiety of glycoprotein [22], and *Aeromonas hydrophila* hemolysin is inactivated by erythrocyte membrane glycopeptides [23]. These observations imply that specific carbohydrates might regulate the interaction of the cytolysins with the membrane bilayer as well as their oligomerization behavior. However, the possible existence of a carbohydrate-dependent regulatory mechanism for a primarily lipid-mediated process has never before been identified.

In this study, we have constructed full-length and truncated PLYs in order to analyze the structure–function relationship, and investigate the role of the fourth domain interacting with carbohydrate moieties and cholesterol as a component of the cell membrane, using inhibition of hemolytic activity. Our results provide evidence that a specific carbohydrate moiety on the cell membrane may play an important role in the interaction of PLY with the membrane bilayer, although it is not essential for membrane binding.

2. Materials and methods

2.1. Cloning, plasmids, and protein expression

The gene encoding the full-length (PLY-F) and two truncated PLYs (PLY-L containing domain 1–3, and PLY-S containing domain 4) were amplified by PCR from a *S. pneumoniae* genomic DNA using the following primers: PLY-F_fwd, 5′-GCA ACC **CAT ATG** GCA AAT AAA GCA GTA AAT-3′; PLY-F_rev 5′-ACA ATA **CTC GAG** CTA GTC ATT TTC TAC CTT-3′; PLY-L_fwd 5′-GCA ACC **CAT ATG** GCA AAT AAA GCA GTA AAT-3′; PLY-L_rev 5′-ATA GAC **CTC GAG** CTA GTT TCT GTA AGC TGT-3′; PLY-S_fwd 5′-ACA ACA **CAT ATG** GGA GAT TTA CTG CTG GAT-3′; PLY-S_rev 5′-ACA ATA **CTC GAG** CTA GTC ATT TTC TAC CTT-3′. The primers contained modifications to add appropriate restriction endonuclease for insertion into the vector, where *Nde*I site in the forward primer and the *Xho*I site in the reverse primer are shown in bold. The amplified DNA fragments and the expression vector pET-28a vector (Novagen) were digested with *Nde*I and *Xho*I, and were then ligated with T4 ligase (Enzynomics), to generate six consecutive histidines (His_6)-tagged at the N-terminus of protein. The recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) cells (Novagen). Transformants were grown in Luria–Bertani medium with 50 μg/ml kanamycin at 37 °C to an optical density of 0.6 at 600 nm. Protein expression was induced by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubating at 18 °C for a further 18 h. The cells were then harvested by centrifugation at 5000*g* for 30 min at 4 °C.

2.2. Protein purification

The harvested cell pellets were suspended in lysis buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, and 5 mM imidazole, 1 mM phenylmethylsulfonylfluoride) and were disrupted by sonication at 4 °C. The crude lysate was then centrifuged at 25,000*g* for 30 min at 4 °C. The supernatant was loaded onto an Ni^{2+}-chelated HiTrap chelating HP column (GE Healthcare, USA) equilibrated in buffer *A* (20 mM Tris–HCl pH 7.9, 500 mM NaCl, and 5 mM imidazole). The bound protein was eluted with a linear gradient of buffer *B* (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 1 M imidazole). Fractions containing PLYs were identified by SDS–PAGE and were subsequently purified to their final state by gel filtration chromatography on a HiLoad 16/60 Superdex 200 (GE Healthcare, USA), which had been equilibrated with gel buffer (20 mM Tris–HCl pH 7.9, 50 mM NaCl). The soluble fractions containing protein were pooled together and were concentrated to 13 mg/ml using an Amicon Ultra-15 centrifugal filter device (Millipore, USA). The protein concentration was estimated using the Bradford assay and the purity was confirmed by 15% SDS–PAGE to be >95%.

2.3. Hemolytic activity of recombinant PLYs

The hemolytic activity of full-length and truncated PLYs was measured as follows. Human type O erythrocytes were washed three times with PBS buffer (20 mM sodium phosphate buffer, pH 7.4, 145 mM NaCl) and were added to yield a final concentration of 0.5% (*v*/*v*) for the assay of hemolytic activity. Each recombinant PLY was serially diluted to the equal volume in hemolytic reaction, and was incubated at 37 °C for 30 min. To remove the unlysed erythrocytes, the reactants were centrifuged gently at 800*g* for 10 min. Supernatants were transferred to clean tubes and the absorbance of released hemoglobin was determined at 540 nm using a spectrophotometer (UV/VIS Spectronic Genesys5, USA). The hemolytic dose (HD_{50}) was defined as the amount of toxin required for 50% hemolysis of 1 ml of 0.5% (*v*/*v*) human erythrocytes, and was calculated using a nonlinear sigmoidal dose–response curve fit equation [24]. The control, containing 0.5% (*v*/*v*) erythrocytes and deionized water, and which was considered as a complete hemolysis, was used to determine the percentage of hemolytic activity. All experiments were carried out in triplicate.

2.4. Binding of recombinant PLYs to erythrocytes, cholesterol, and carbohydrates

To determine the binding ability of recombinant PLYs to erythrocytes, the inhibitory effect of truncated PLYs on hemolysis caused by PLY-F was performed with the same manner of hemolytic activity assay as previously mentioned. Each truncated PLY was pre-incubated at the concentration of 200 nM with 0.5% (*v*/*v*) human erythrocytes in PBS buffer at 25 °C for 15 min. PLY-F was then added to result in a 1 nM of final concentration in the reaction mixture. The percentage of hemolytic activity was calculated by comparing with 100% hemolysis induced by PLY-F.

In the binding of cholesterol or carbohydrate moieties to PLY-F, various concentrations of cholesterol-methyl-β-cyclodextrin (cholesterol-MβCD, Sigma), deoxycholic acid or carbohydrate were pre-incubated with 1 nM of PLY-F in PBS buffer at 20 °C for 15 min, and were then added to 0.5% (*v*/*v*) human erythrocytes in the reaction mixture. All mixtures were incubated at 37 °C for 30 min. Unlysed erythrocytes were removed through centrifugation, and the absorbance of released hemoglobin was determined at 540 nm. The residual hemolytic activities of PLY-F by inhibition of cholesterol or carbohydrates were calculated as follows: The residual activity (%) = [absorbance of hemolytic activity by adding various concentration of cholesterol or carbohydrates]/[absorbance of hemolytic activity of 1 nM PLY-F] × 100. The binding constant (K_D) between PLY-F and cholesterol was calculated using a nonlinear least squares fit equation [25] $Y = B_{max}X/(K_D + X)$ for one site binding, where B_{max} is the maximal binding and K_D is the concentration of ligand necessary to achieve half-maximal binding. The Hill coefficient (n_H) of cholesterol interacted with PLY-F was calculated using the Hill plot. The EC_{50} (effective dose, 50%) value for carbohydrate was calculated using a nonlinear sigmoidal dose–response (variable slope) curve fit equation [24] $Y = \mathrm{Min} + [\mathrm{Max} - \mathrm{Min}]/[1 + 10^{(\log EC_{50} - X) \times \mathrm{Hill\ slope}}]$, where Min and Max are the minimal and maximal binding, respectively, and Log-EC_{50} is the concentration of ligand when the response is halfway between Min and Max, and the Hill slope describes the Hill coefficient (n_H). Data analysis was carried out using SigmaPlot 10.0 software.

2.5. Binding of mannose to recombinant PLYs by pull-down assay

The binding effects of full-length PLY and truncated PLYs with mannose were performed using the affinity chromatography on a nickel–nitrilotriacetic acid (Ni–NTA) column (Protino®, USA). His_6-tagged recombinant PLYs (1 nM) were mixed with 50 mM mannose in PBS buffer. After incubation at 37 °C for 15 min, the mixtures were loaded onto a Ni–NTA column equilibrated in reaction buffer (20 mM Tris–HCl pH 7.9, 300 mM NaCl). The unbound mannose was measured by the Somogyi–Nelson method [26], in order to calculate bound mannose to PLYs from the initial concentration of mannose. The absorbance of a colored complex compound, which is formed between a copper-oxidized sugar and arsenomolybdate, was measured at 500 nm using a spectrophotometer (UV/VIS Spectronic Genesys5, USA). The amount of mannose was determined by the standard calibration curve. All experiments were carried out in triplicate.

3. Results and discussion

3.1. Hemolytic activity and binding of recombinant PLYs to human erythrocytes

The full-length PLY (PLY-F, residue no. 1–471) was truncated to be PLY-L (residue no. 1–360) and PLY-S (residue no. 361–471), which represented domain 1–3 and domain 4 of PLY, respectively, in order to analyze the interaction of PLY with the components of cell membrane regarding the structure–function relationship. To confirm the cytolytic activity caused by recombinant PLYs, the hemolytic activities of PLY-F and two truncated PLYs were measured against type O human erythrocytes. The complete hemolysis was observed by adding a final concentration of 1 nM PLY-F, whereas PLY-L and PLY-S was not observed through hemolytic activity. The HD_{50} of PLY-F was estimated to be 0.125 ± 0.002 nM (Fig. 1A).

To investigate the binding ability of each recombinant PLY to human erythrocytes, the hemolytic activity of PLY-F was measured using the inhibition of hemolysis induced by binding each truncated PLY. Human erythrocytes were pre-incubated with 200 nM of PLY-L (domain 1–3) or PLY-S (domain 4), and PLY-F was then added to a final concentration of 1 nM in the reaction mixture. The hemolytic activity of PLY-F was clearly inhibited by pretreatment with PLY-S, while PLY-L did not exert any inhibitory effect on hemolysis induced by PLY-F (Fig. 1B). These results indicate that domain 4 (PLY-S) is capable of binding to erythrocyte membrane by itself and is essential for the membrane binding, whereas domain 1–3 (PLY-L) affect the consequence of hemolytic process, but not binding.

3.2. Selective binding and stoichiometry of PLY to cholesterol in aqueous solution

Cholesterol has been known to be equally effective in the inhibition of CDCs activity, but coprostanol and 3-epicholesterol are slightly less or not effective [27,28]. Interestingly, the inhibition of CDCs activity by sterols depends on whether the position of the C-3 hydroxyl group is in a 3α or in 3β configuration [28]. To investigate the selective binding of PLY to different sterols containing 3α or 3β hydroxyl groups at C-3 atom, the inhibition of PLY by cholesterol-MβCD and deoxycholic acid (Fig. 2A and B) was measured using a hemolytic assay. PLY was pre-incubated with graded doses of cholesterol-MβCD and deoxycholic acid ranging from 0.05 to 2.0 μM, and was then incubated with 5% (*v*/*v*) human erythrocytes for 30 min at 37 °C. When PLY was titrated with cholesterol-MβCD, the complete binding was observed by adding >0.5 μM of cholesterol-MβCD. The dissociation constant (K_D) between PLY and cholesterol-MβCD was estimated to be

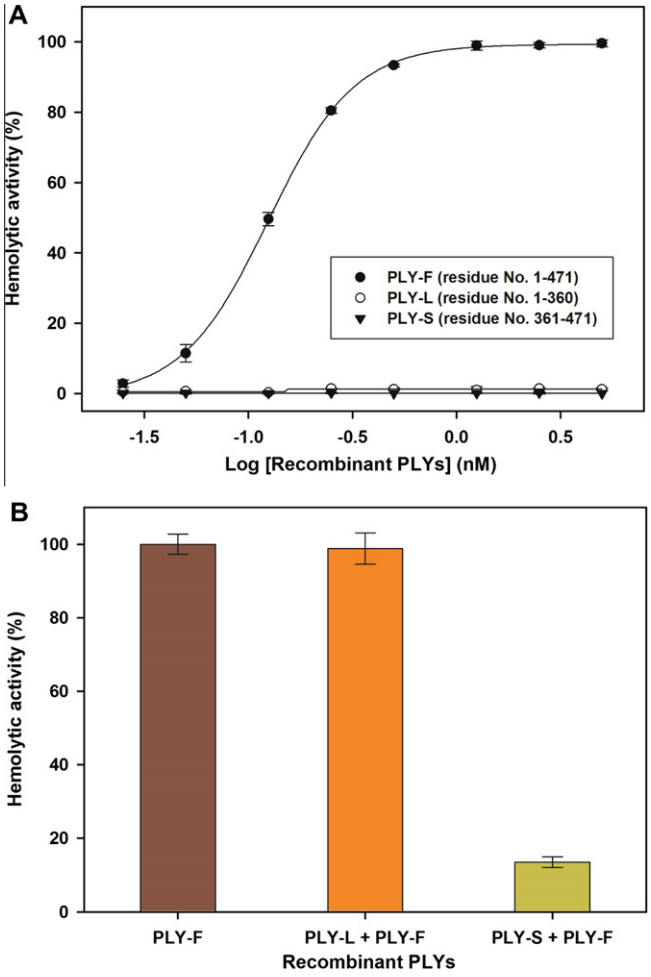

Fig. 1. (A) Hemolytic activities of full-length and truncated PLYs. Recombinant PLYs were mixed with 0.5% (*v*/*v*) human erythrocytes in PBS buffer and were incubated at 37 °C for 30 min. After incubation of the mixture, hemolytic activity was measured for PLY-F (full-length; closed circles), PLY-L (domain 1–3; open circles) and PLY-S (domain 4; closed inverted triangles) by absorbance at 540 nm, respectively. (B) The inhibitory effects of truncated PLYs on hemolysis caused by PLY-F. Each truncated PLY was pre-incubated at a concentration of 200 nM with 0.5% (*v*/*v*) human erythrocytes in PBS buffer at 25 °C for 15 min. PLY-F was then added to a final concentration of 1 nM in the reaction mixture. The hemolytic activity was performed in the same manner as that previously mentioned. The percentage of hemolytic activity was calculated by comparing with 100% hemolysis induced by PLY-F.

68.9 ± 6.7 nM by the nonlinear least squares fit equation (Fig. 2C). Furthermore, a representative binding curve shown in Fig. 2C fitted well to the Hill equation ($\log B/B_{max} - B$ vs $\log F$) with a Hill coefficient (n_H) of 1.06 ± 0.07 (Fig. 2D), and it was possible to establish that the functional stoichiometry of the PLY-cholesterol complex was 1:1. However, no significant binding was detected when PLY was titrated with deoxycholic acid (Fig. 2C), which slightly differs from cholesterol in that the C-3 hydroxyl group is directed axially instead of equatorially. These results indicate that PLY binds selectively to cholesterol with 3β hydroxyl group at the C-3 atom, and it is consistent with the fluorescence change resulted from a selective interaction between the domain 4 loop of PFO and the cholesterol molecules [28].

3.3. Interaction of PLY with the carbohydrate moiety

Several lines of evidence have recently indicated that the carbohydrate moiety of glycoprotein or glycolipid can be regulated in the interaction between the cell membrane and CDCs [21–23].

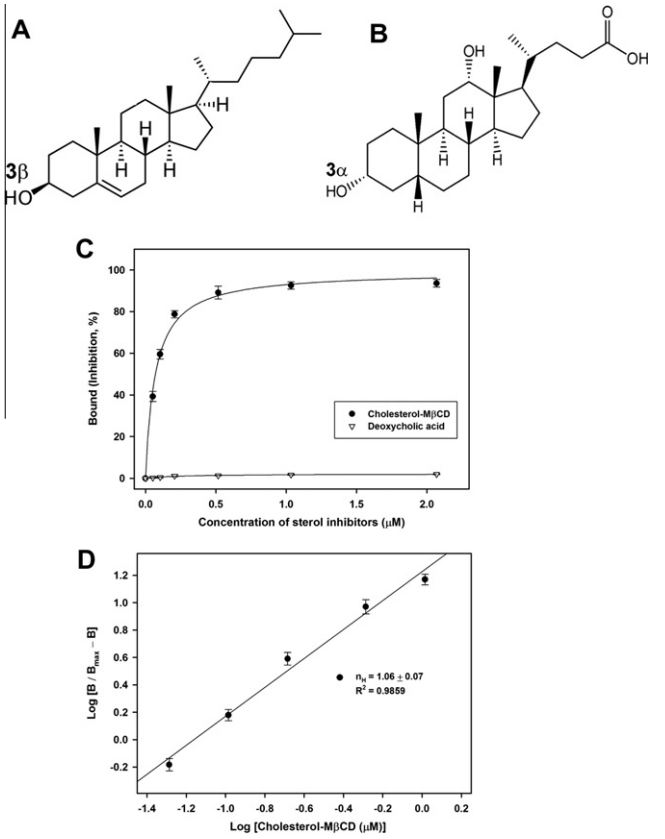

Fig. 2. Selective binding and stoichiometry of PLY to cholesterol in aqueous solution. Chemical structures of (A) cholesterol and (B) deoxycholic acid. (C) Nonlinear least-square fit plot and (D) Hill plot of cholesterol binding to PLY. PLY-F was mixed with varying concentrations (0.05–2.0 μM) of cholesterol-MβCD or deoxycholic acid and 0.5% (*v*/*v*) human erythrocytes. After incubation at 37 °C for 30 min, hemolytic activity was measured by absorbance at 540 nm. The cholesterol binding to PLY was calculated with inhibition of PLY hemolytic activity. Residual activity was measured in comparison with the inhibitor-free control, and was expressed as mean ± standard deviation (SD).

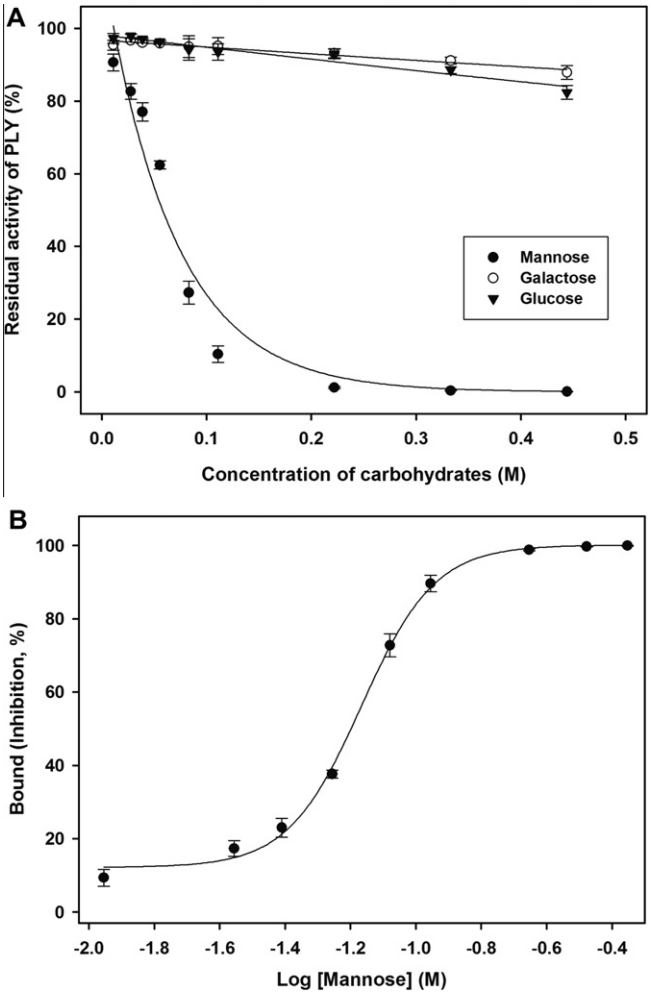

Fig. 3. Interaction of PLY with the carbohydrate moiety. (A) Inhibition of PLY hemolytic activity by carbohydrates. (B) The sigmoidal dose–response curve of mannose binding to PLY. PLY was mixed with various carbohydrates in the range of 0.01–0.45 M (mannose; closed circles, galactose; opened circles, glucose; closed inverted triangles), and 0.5% (*v*/*v*) human erythrocytes was then added to each mixture. After incubation at 37 °C for 30 min, hemolytic activity was measured by absorbance at 540 nm. Residual activity was measured in comparison with the inhibitor-free control and was expressed as mean ± SD.

The mechanism by which cholesterol affects the cytolytic activity of the CDCs remains ambiguous, even though the defining property of these toxins is their dependence on the presence of membrane cholesterol. Hence, the binding analysis of CDCs, which considers the importance of the carbohydrate moiety in the interaction between cell membrane and bacterial toxins, was required in order to reflect the actual interactions occurring on the cell membrane.

To elucidate the interaction between PLY and carbohydrate moiety on the cell membrane, the dose–response relationship was estimated by the inhibitory effect of PLY hemolytic activity induced by various concentration (0.01–0.45 M) of monosaccharides such as mannose, galactose, and glucose. The residual hemolytic activity of PLY was obtained by measuring the absorbance of released hemoglobin at 540 nm. As shown in Fig. 3A, the inhibitory effect by galactose and glucose showed a negligibly slight decrease in the hemolytic activity of PLY, but mannose showed a dose-dependent increase in the inhibition of PLY hemolytic activity. Moreover, in order to assess the interaction between PLY and mannose, the EC_{50} value and Hill coefficient (n_H) for the mannose binding to PLY were calculated by a nonlinear sigmoidal dose–response (variable slope) curve fit equation, involving the Hill slope (Fig. 3B). The EC_{50} value for mannose binding to PLY was estimated to be $6.8 \pm 0.2 \times 10^{-2}$ M, and the Hill coefficient (n_H) was 3.86 ± 0.31, which made it possible to establish that the functional stoichiometry of the PLY-mannose complex was approximately 1:4.

Many studies, primarily those performed with perfringolysin O (PFO), have shown that membrane binding is sensitive to the loss of cholesterol [29], and that derivatives of PFO have been used as probes for membrane cholesterol [30]. However, studies on other members of the CDC family suggest that the loss of membrane binding may not account for the extreme sensitivity of these toxins to membrane cholesterol levels. As mentioned earlier, the intermedilysin (ILY) from *S. intermedius*, exhibits an exquisite specificity for human cells, suggesting that cholesterol cannot be responsible for membrane recognition by ILY, yet its mechanism still seems to be sensitive to cholesterol [8]. Also, Jacobs et al. [31] have shown that the addition of exogenous cholesterol inhibited the activity of the CDC listeriolysin O (LLO), but did not prevent its binding to the membrane. Our results show that mannose is able to inhibit the hemolysis induced by PLY, and also provide evidence to infer a possible mechanism of PLY in selectively interacting with glycoproteins or glycolipids conjugated with mannose as a specific receptor for the recognition of the target cell.

3.4. Pull-down assay for mannose binding to recombinant PLYs

Domain 4 of the CDCs is important for membrane binding [30]. Therefore, we tested whether domain 4 is also responsible for the

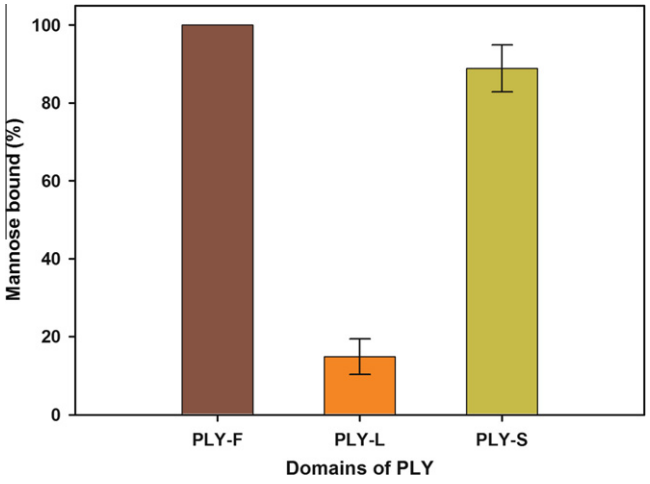

Fig. 4. Pull-down assay for mannose binding to recombinant PLYs. His_6-tagged recombinant PLYs (1 nM) were mixed with 50 mM mannose in PBS buffer. After incubation at 37 °C for 15 min, the mixtures were loaded onto a Ni–NTA column and were equilibrated in reaction buffer containing 20 mM Tris–HCl pH 7.9, 300 mM NaCl. The unbound mannose was measured by the Somogyi–Nelson method [26], in order to calculate mannose bound to PLYs from the initial concentration of mannose.

recognition and binding of mannose by PLY, or whether other domains are involved. To determine which domains of PLY contributed to the recognition of mannose, the binding effects of His_6-tagged PLY-F and truncated PLYs (PLY-L and PLY-S) with mannose were performed with pull-down assay using affinity chromatography on a Ni–NTA column. The unbound mannose was measured by the Somogyi–Nelson method [26], in order to calculate bound mannose to PLYs from initial concentration of mannose. The percentage of mannose binding for each truncated PLY was calculated by comparing with the mannose bound to PLY-F based on 100%. As shown in Fig. 4, the percentages of mannose binding with PLY-L (domain 1–3) and PLY-S (domain 4) were estimated to be 14.9 ± 4.6% and 88.9 ± 6.0%, respectively. These results show that the mannose may prefer binding to domain 4 rather than domain 1–3 of PLY, although it is still unclear which amino acid residues of domain 4 participate in these interaction, due to the lack of structural information on PLY.

In conclusion, the results of the present studies show that PLY, as a member of CDC family, may target human cells by interacting with specific carbohydrate moieties on the cell membrane. Moreover, these results provide a foundation for future investigations into whether non-sterol molecules can serve as receptors for other members of the CDC family of toxins. Further study may elucidate an atomic structure of PLY in order to understand a more detailed molecular interaction between PLY and carbohydrate moiety or cholesterol as a component of cell membranes.

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