Antigenicity of Partial Fragments of Recombinant *Pasteurella multocida* Toxin

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***Pasteurella multocida* serogroup D strain, which produces *P. multocida* toxin (PMT), is a widespread and harmful pathogen of respiratory diseases such as pneumonia and progressive atrophic rhinitis (PAR) in swine. Vaccination has been considered the most desirable and effective approach for controlling the diseases caused by toxigenic *P. multocida*. To investigate the antigenicity and immunogenicity of partial fragments of recombinant PMT, recombinant proteins of the N-terminal (PMT-A), middle (PMT-B), C-terminal (PMT-C), and middle-C-terminal (PMT2.3) regions of PMT were successfully produced in an *Escherichia coli* expression system. The molecular masses of PMT-A, PMT-B, PMT-C, and PMT2.3 were ca. 53, 55, 35, and 84 kDa, respectively, purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography. All the recombinant proteins except for PMT-A showed immune responses to antisera obtained from a swine showing symptoms of PAR. Moreover, high titers of PMT-specific antibodies were raised from mice immunized with each of the recombinant proteins; however, the immunoreactivities of the antibodies to authentic PMT and heat-inactivated whole bacteria were different, respectively. In the protection study, the highest protection against homologous challenge was shown in the case of PMT2.3; relatively poor protections occurred for the other PMT fragments.**

Keywords: *Pasteurella multocida* toxin, vaccination, homologous challenge, recombinant protein

Pathogenic strains of *Pasteurella multocida*, a Gram-negative facultative bacterium, can infect various animal species; they are the causative agents of economically important diseases such as pneumonia and hemorrhagic septicemia

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in cattle, sheep, and goat; fowl cholera in chicken; and progressive atrophic rhinitis (PAR) in swine [10, 12, 33]. Zoonotic infections in humans often occur by bites or scratches by domestic animals infected with *P. multocida*. The global economic loss in animal production due to nutritional deficiency and growth retardation in early stages is because of the above-mentioned diseases caused by *P. multocida* infection; therefore, efficient vaccination has been considered the most important and desirable method for controlling these animal diseases and protecting animals [10, 18, 28].

Pathogenic strains of *P. multocida* have been classified into 5 serogroups on the basis of the capsular antigens and 16 serotypes on the basis of the somatic LPS antigens [6, 13, 25, 27]. *P. multocida* toxin (PMT), a dermonecrotic toxin, is produced mainly by serogroup D strains and is responsible for clinical symptoms of PAR in swine [11, 15, 26]. PMT has been considered as a good candidate for vaccine development. PMT, by itself, can lead to atrophy of nasal turbinates by the uncontrolled proliferation of osteoclasts and the inhibition of osteoblast activity [8, 19]. Inoculation of both purified native and recombinant PMTs without pathogen can produce all the major symptoms of atrophic rhinitis (AR) in experimentally challenged pigs [15, 21]. However, it has also been reported that native PMT is weakly immunogenic and can show stronger antigenic properties after destruction of its native structure [9, 32]. Therefore, the determination of the immunogenic epitopes of PMT that can elicit a strong and protective immune response against infection is important to develop an effective subunit vaccine, and truncated and/or partial fragments of PMT may serve as sufficiently effective immunogens that systemically induce protective immunity without exerting any cytotoxic effect in animals.

In this study, we produced recombinant proteins of the N-terminal, middle, C-terminal, and middle-C-terminal regions of PMT by using an *Escherichia coli* expression

system. We purified the recombinant proteins by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography and confirmed their antigenicity by immunoblotting with antisera obtained from a swine with PAR. The immunogenicity of these partial fragments of recombinant PMT and the protective immunity induced by them were investigated by animal vaccination and challenge experiments.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The *P. multocida* D:4 used in this study was originally isolated from a swine with AR in Korea and grown on blood agar plate for 12 h at 37°C as described previously [16, 17]. The isolate was grown overnight on Bacto brain-heart infusion (BHI) agar (Difco Laboratories, Detroit, MI, USA) at 37°C in a 5% CO_2 environment. The *E. coli* strains DH5α and BL21 (DE3) were used for gene cloning and gene expression, respectively. The plasmid vector pGEM-T (Promega Co., Madison, WI, USA) and a series of the plasmid vector pRSET (Invitrogen Co., Carlsbad, CA, USA) were used for cloning of PCR products and protein expression, respectively.

Preparation of Bacterial Outer Membranes

Bacterial envelopes were prepared by sonication and centrifugation as described previously [7]. The outer membrane was extracted with 0.5% sodium *N*-lauroylsarcosine in 0.01 M Tris-HCl (pH 8.0), and the insoluble fraction was collected by centrifugation.

Preparation of Detoxified Authentic PMT

The crude extract of authentic PMT was prepared from a *P. multocida* strain cultured in BHI medium for 24 h at 37°C as described previously [20]. Purified PMT extract was detoxified by shaking with 0.3% (v/v) formalin for 48 h at 37°C to generate the toxoid. Detoxification was confirmed by investigating PMT-induced cytopathic effects in Vero cells.

Cloning of Partial PMT Fragments and Construction of Their Expression Vectors

The PMT-encoding gene was amplified by PCR from *P. multocida* D:4 isolated in Korea [16, 17]. The PMT-specific primers PMT-F (5'-CCCAAACACTGCGAATGTTTGGGG-3') and PMT-R (5'-TTC CACTGCATCCACAGCCCTTCT-3') for amplification were designed by analysis of the sequences of several PMT clones found in the Basic Local Alignment Search Tool (BLAST) sequence database (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, MD, USA). The full-length fragment of the amplified gene was confirmed by sequencing and cloned into the pGEM-T vector according to the manufacturer's instructions (Promega Co., Madison, WI, USA). The clone was digested with the following combinations of restriction enzymes: *Bam*HI–*Hin*dIII (1–483 amino acids; PMT-A), *Hin*dIII–*Hin*dIII (484–984 amino acids; PMT-B), *Hin*dIII–*Pst*I (985–1,285 amino acids; PMT-C), and *Xho*I–*Pst*I (505–1,285 amino acids; PMT2.3). The DNA fragments of PMT-A, PMT-B, PMT-C, and PMT2.3 were then cloned into the pRSET series vectors with appropriate reading frames of DNA (Fig. 1A). After sequence analysis of the clones, recombinant plasmids for the expression of each were transformed into *E. coli* BL21
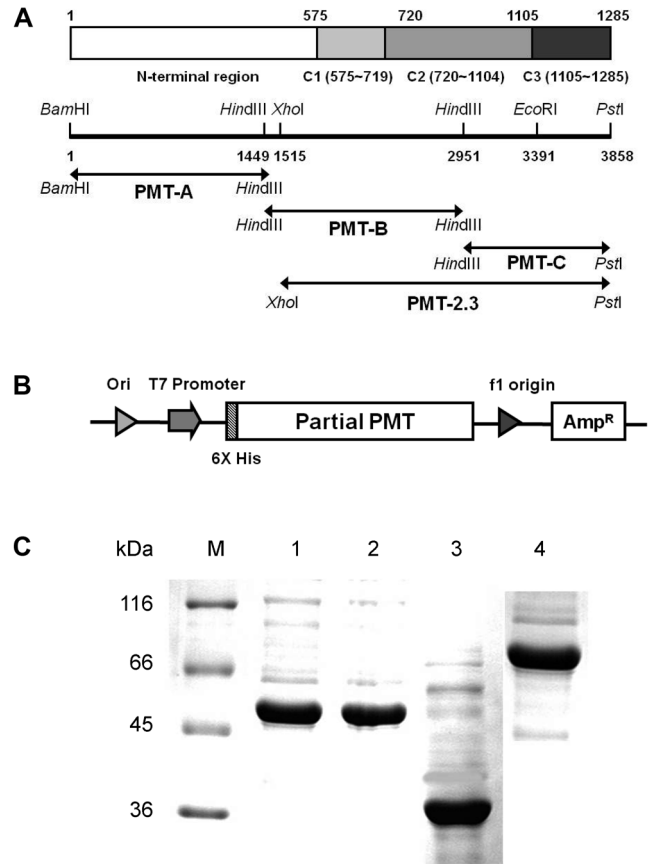

Fig. 1. Construction of expression vectors for partial PMT fragments.
A. The full length of the PMT-encoding gene (3,858 nt encoding 1,285 amino acids) was digested and then inserted into pRSET series expression vectors. **B.** The DNA fragments were used to generate recombinant proteins of the N-terminal (PMT-A; 483 amino acids), middle (PMT-B; 501 amino acids), C-terminal (PMT-C; 301 amino acids), and middle-C-terminal (PMT2.3) regions. The expression was controlled by the T7 promoter. **C.** The recombinant proteins were purified by Ni-NTA affinity column chromatography and analyzed by SDS–PAGE. M, protein molecular mass marker; lane 1, PMAT-A (53 kDa); lane 2, PMT-B (55 kDa); lane 3, PMT-C (35 kDa); lane 4, PMT2.3 (84 kDa).

(DE3). The transformants were then selected on Luria–Bertani (LB) agar plates containing ampicillin (50 μg/ml).

Purification of Recombinant Proteins from *E. coli*

An overnight culture (5 ml) of *E. coli* BL21 (DE3) harboring the recombinant plasmids grown in LB medium with ampicillin (50 μg/ml) was inoculated into 1 l of LB medium. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.8 mM) was added to induce gene expression at OD_{600} 0.7. The cells were further cultured for 12 h at 34°C with vigorous shaking, after which they were collected and resuspended in a lysis buffer [50 mM Tris-HCl (pH 8.0), 1% SDS, 10 mM EDTA; 5 ml/g of wet cells]. Subsequently, the cells were sonicated and then centrifuged (12,000 ×g, 4°C, 30 min). Insoluble materials were resuspended in a denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 8.0) and vigorously shaken for 2 h at 4°C. The supernatant was recovered by centrifugation

(10,000 ×g, 4°C, 30 min) and used for the purification of the recombinant proteins. About 1 ml of 50% Ni-NTA slurry (QIAGEN GmbH, Hilden, Germany) was added to 5 ml of the supernatant solution and mixed gently for 1 h at room temperature; the mixture was then loaded onto an empty column. The column was washed 3 times with 5 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 6.3). The recombinant protein was eluted twice with 0.5 ml of elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 5.9) and further twice with 0.5 ml of elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 4.5). Fractions were collected and analyzed by SDS−PAGE. The purified fractions were serially diluted with PBS for refolding of the protein and further analysis [16].

Vaccination and Challenge of Mice

The animal experiments were performed under the control and guidelines of the Laboratory Animal Research Center, Sungkyunkwan University. To investigate the immunogenicity of the recombinant proteins, 6- or 8-week-old female BALB/c mice were randomly selected and immunized with PBS for the negative control, detoxified authentic PMT (toxoid) for the positive control, or PMT-A, PMT-B, PMT-C, and PMT2.3, respectively. The mice were subcutaneously injected with 50 μg of each antigen emulsified with equal volumes of complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) at the first immunization (day 0) and second immunization (day 21), respectively. On the 10th day after each immunization, blood was collected from the immunized mice by tail bleeding or retrobulbar puncture using heparinized glass pipettes; the sera recovered by centrifugation were stored at –20°C for further assay. For the protection experiment, the immunized mice were intraperitoneally challenged with 10 median lethal doses (LD_{50}) of live homologous virulent *P. multocida* D:4 (4.3×10^3 CFU) 2 weeks after the second immunization. The LD_{50} was determined by the method of Reed and Muench [4]. All the challenged mice were monitored for 10 days, and their survival rates were recorded. The results were expressed as percent survival [24, 30].

Immunoreactivity and Antibody Titration

The titers of anti-PMT antibodies in the immunized mice and immunoreactivities of the partial PMT fragments with specific antibodies obtained from infected animals were determined by an ELISA. A microtiter plate was coated with 0.1 μg of each recombinant protein or detoxified authentic PMT or heat-inactivated whole bacterial cells, sealed, and incubated for 2 h at 37°C. After washing 3 times with PBS containing 0.05% Tween 20 (PBST), the plate was recoated with 200 μl of blocking solution containing 3% BSA in PBST. After a 1-h incubation at 37°C, the plate was washed 3 times with PBST and incubated overnight with 100 μl of diluted mouse sera or swine sera (1:1,000 in PBST with 1% BSA) at 4°C. The plate was then washed 3 times with PBST, and 100 μl of a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG antibody or horseradish peroxidase-conjugated goat anti-swine IgG antibody; Sigma-Aldrich Co., St. Louis, MO, USA) was added to the wells. After a 1-h incubation at 37°C, the plate was rewashed with PBST. For colorimetric detection, 50 μl of a color development solution, 3,3',5,5'-tetramethylbenzidine (TMB) dissolved in DMSO, was used. Absorbance was measured at 450 nm by using a microplate reader. All the samples were subsequently tested in duplicate [16, 17].

Statistical Analysis

Values are expressed as mean ± SD. The statistical significance of variation among different groups was determined by one-way analysis of variance (ANOVA), and the significance between each experimental group and the reference group was determined by Dunnett's test.

RESULTS

Expression and Purification of Partial PMT Fragments

For the construction of expression vectors for the partial PMT fragments, the full-length PMT-encoding gene was amplified by PCR, using PMT-specific primers; the PCR product was then cloned into the pGEM-T vector. The PMT-encoding gene amplified by PCR in this study contained 3,858 nucleotides (nt) encoding 1,285 amino acids, based on the sequence confirmation; the sequence of this gene is identical to the registered sequence of PMT (GenBank Accession No. X51512). The cloned gene was digested with appropriate combinations of restriction enzymes as described above and then cloned into the pRSET series expression vectors to produce recombinant proteins of the N-terminal (PMT-A; 483 amino acids), middle (PMT-B; 501 amino acids), C-terminal (PMT-C; 301 amino acids), and middle-C-terminal (PMT2.3; 781 amino acids) regions (Fig. 1A). The recombinant expression vectors for the partial PMT fragments were transformed into the expression host *E. coli* BL21 (DE3), and a large number of transformants were grown and screened on selective LB agar plates. One colony of each construct was chosen and used for the purification of the recombinant proteins. The recombinant proteins with N-terminal 6× His supplemented by the expression vector (Fig. 1B) were produced as insoluble proteins by using the *E. coli* BL21 (DE3) expression system. The recombinant proteins in insoluble state were successfully purified by Ni-NTA affinity column chromatography under denaturing conditions. The expression yield of each recombinant protein was estimated to be about 20% of the total cellular proteins (data not shown). The molecular masses of PMT-A, PMT-B, PMT-C, and PMT2.3 were ca. 53, 55, 35, and 84 kDa, respectively, as determined by SDS−PAGE and Coomassie blue staining after purification of the proteins by Ni-NTA affinity column chromatography (Fig. 1C). The molecular mass of each recombinant protein was considered to agree with the respective amino acid compositions and lengths of the proteins.

Antigenicities and Immunoreactivities of Recombinant Proteins

To investigate the antigenic properties of the partial fragments of recombinant PMT, the recombinant proteins were immunoblotted with antibodies obtained from *P. multocida*-infected animals or detoxified authentic PMT
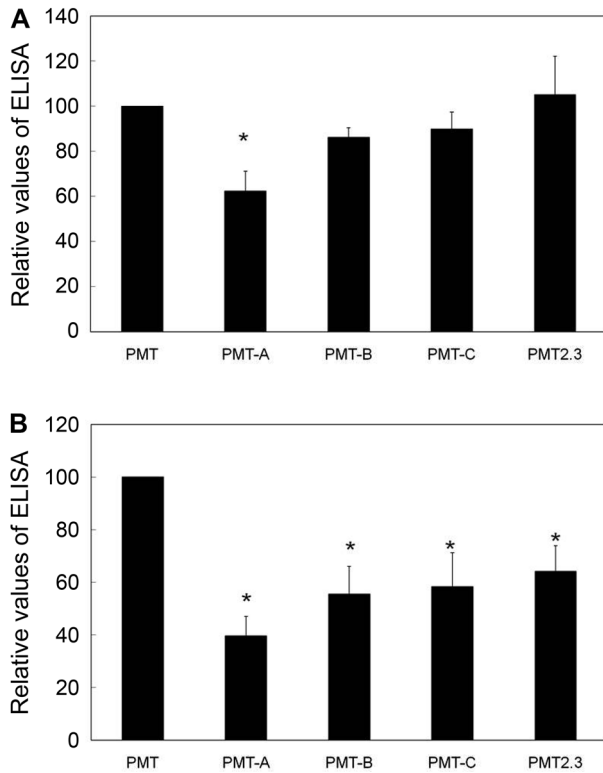
Fig. 2. Immunological detection of partial PMT fragments.
The immunoreactivities of the partial PMT fragments were measured by ELISA using antisera obtained from a swine infected with PAR (**A**) or using antisera obtained from mice immunized with detoxified authentic PMT (**B**). Relative values of ELISA were calculated by comparing with the value obtained for detoxified authentic PMT. The statistically significant difference compared with the ELISA value of authentic PMT is indicated with an asterisk.

Fig. 3. Immunoreactivities of antibodies against partial PMT fragments.
Immunoreactivities to PMT or bacterin were evaluated using the antisera of mice before immunization (Pre), after the first immunization (1st), and after the second immunization (2nd). **A**. The antibodies against partial PMT fragments were subjected to ELISA, with detoxified authentic PMT as an antigen. **B**. The antibodies against partial PMT fragments were subjected to ELISA, with heat-inactivated whole bacterial cells (bacterin) as antigens.

(toxoid)-immunized animals. All the recombinant proteins (PMT-A, PMT-B, PMT-C, and PMT2.3) were recognized by the sera obtained from a *P. multocida*-infected swine with PAR in immunoblot analysis. In contrast, pooled sera from PBS-immunized mice and non-immunized control mice did not react with the recombinant proteins. However, the immunoreactivities of the recombinant proteins were observed to be different in multiple ELISA quantification. When the same concentration (100 ng) of each recombinant protein was used with the sera in ELISA, PMT-A (62.2%) was significantly less detectable as compared with detoxified authentic PMT (control) (Fig. 2A). PMT-B (86.2%) and PMT-C (89.9%) were also weakly detected as compared with the control; however, the difference was not significant. PMT2.3 (105.1%) was detectable at same level as compared with the control. These differences in the immunoreactivities of the recombinant proteins with the sera obtained from infected swine seem to be because of the differences in their antigenicities.

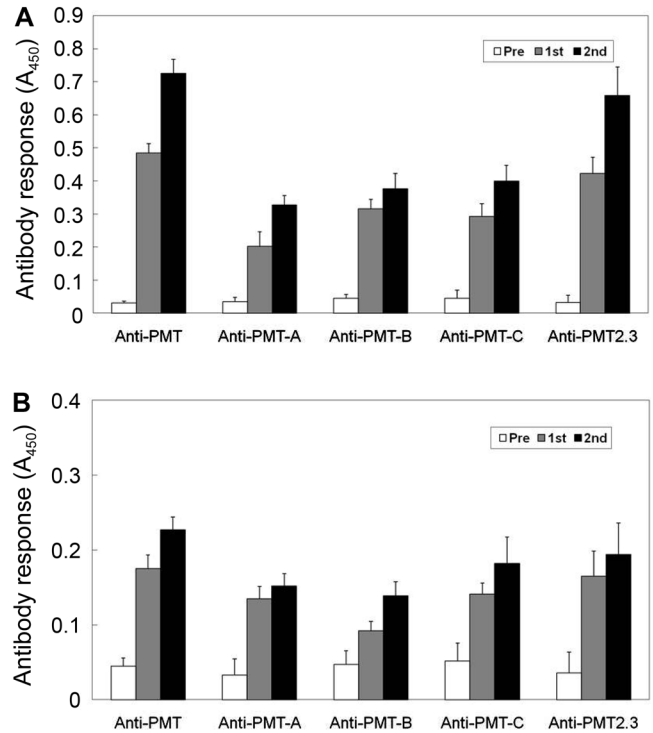In the immunoblot analysis, all the recombinant proteins were recognized by the sera obtained from detoxified authentic PMT (toxoid)-immunized mice. However, the immunoreactivities of PMT-A (39.6%), PMT-B (55.5%), PMT-C (58.2%), and PMT2.3 (64.2%) were observed to be significantly lower than the immunoreactivity of detoxified authentic PMT in multiple ELISA quantification (Fig. 2B).

Immunoreactivities of Antibodies Produced Against Recombinant Proteins
To assess the immunoreactivities of antibodies produced by the partial fragments of recombinant PMT, the antibody titers in the sera obtained from mice immunized with PMT-A, PMT-B, PMT-C, PMT2.3, and detoxified authentic PMT were measured. The recombinant proteins could raise relatively high titers of antibodies in ELISA, with detoxified authentic PMT as an antigen (Fig. 3A). The titers in all the 5 immunization groups were higher after the second immunization than after the first. However, the levels of antibody titers against PMT differed among the immunization groups. The highest titers in mouse sera (mean ΔA_{450} values were 0.485 and 0.725 after the first and second immunizations, respectively) were observed in

the PMT immunization group, and the lowest titers in mouse sera (mean ΔA_{450} values were 0.202 and 0.327 after the first and second immunizations, respectively) were observed in the PMT-A immunization group. The titers in the sera of PMT2.3-immunized mice (mean ΔA_{450} values were 0.423 and 0.658 after the first and second immunizations, respectively) were similar to those in the sera of authentic PMT. The titers in the sera of PMT-B-immunized mice (mean ΔA_{450} values were 0.315 and 0.376 after the first and second immunizations, respectively) and PMT-C-immunized mice (mean ΔA_{450} values were 0.293 and 0.399 after the first and second immunizations, respectively) were relatively higher than those in the sera of PMT-A-immunized mice, but the difference was not significant.

When heat-inactivated whole bacterial cells (bacterin) were used as antigens in ELISA, although the sera of PMT-immunized mice showed the highest immunoreactivity to bacterin, relatively weak reactions of each antibody were observed (mean ΔA_{450} values ranged 0.092–0.175 and 0.139–0.227 after the first and second immunizations, respectively, in each group) (Fig. 3B). There were no significant differences among the groups.

Challenge of Mice and Protection Experiment
Two weeks after the second immunization, the immunized mice were intraperitoneally injected with 10 LD_{50} of live homozygous virulent *P. multocida* D:4 and were monitored for 10 days for calculation of survival rates. The results of the protection experiment are summarized in Table 1. The mice in the negative control group were not protected (0% survival) against the challenge with 10 LD_{50} of the homologous virulent strain, whereas those immunized with the heat-inactivated whole bacterial cells (bacterin) and detoxified authentic PMT (PMT in Table 1) were significantly protected against the challenge (80% and 70% survival, respectively). With regard to the recombinant proteins, the

mice immunized with PMT-A, PMT-B, and PMT-C were not protected (30%, 20%, and 30% survival, respectively); however, the mice immunized with PMT2.3 showed significant protection against the challenge (90% survival).

DISCUSSION

Pneumonia and PAR induced by *P. multocida* are widespread diseases in swine, causing reduction in feed ingestion and growth retardation [1]. Both *Bordetella bronchiseptica* and *P. multocida* are believed to be the causative agents of PAR. *B. bronchiseptica* infection, by itself, can lead to only mild and nonprogressive AR, which rarely influences the growth rate of infected swine. The toxin-producing *P. multocida* serogroup D strain is the most common strain that causes PAR with severe progressive growth retardation [9, 23].

In a previous study, inoculation of PMT alone could reproduce symptoms of PAR, and hence, PMT has been considered as an important virulent factor associated with PAR [15]. However, the molecular mechanism of virulence and action of PMT has been poorly understood; basically, the toxin is thought to bind to a putative receptor(s) on target cells, be internalized by endocytosis, and then enters the cytoplasm from the endosome [3, 14]. With regard to the biological and functional activities of PMT, two investigations with contradictory findings related to its structure have been reported. It has been suggested that the intracellular activity domain of PMT is localized to the 500 N-terminal amino acids of the protein, and the C-terminal region is essential for entry into the cells [34]. In contrast, it has been reported that the C-terminal region of PMT exerts a mitogenic effect, whereas the N-terminal region is considered as the functional domain for binding to the cell surface [2, 3, 5, 22]. Moreover, the specific residue in the C-terminal region of PMT (Cys1165), which is responsible

Table 1. Evaluation of protection conferred on immunized mice against live *P. multocida* challenge.

Group[a]	Type of vaccine	Challenge dose[b] (CFU)	Number of dead mice/number of challenged mice[c]	Survival rate (%)
1	PBS	4.3×10^3	10/10	0[*]
2	Bacterin	4.3×10^3	2/10	80[**]
3	PMT	4.3×10^3	3/10	70[**]
4	PMT-A	4.3×10^3	7/10	30[*]
5	PMT-B	4.3×10^3	8/10	20[*]
6	PMT-C	4.3×10^3	7/10	30[*]
7	PMT2.3	4.3×10^3	1/10	90[**]

[a]Mice were immunized with PBS for the negative control (group 1), heat-inactivated whole cells of *P. multocida* for the positive control (group 2), detoxified authentic PMT (toxoid; group 3), PMT-A (group 4), PMT-B (group 5), PMT-C (group 6), and PMT-2.3 (group 7).
[b]Immunized mice were intraperitoneally challenged with 10 median lethal doses (LD_{50}) of live homologous virulent *P. multocida* D:4 (4.3×10^3 CFU) 2 weeks after the second immunization.
[c]The protection index was calculated as the ratio of dead mice to total mice challenged in a group. Mice were monitored for 10 days, and their survival rates were recorded. Statistically different groups are categorized with different numbers of superscript asterisks.

for the toxic activity, has been reported [5, 31]. Recently, the crystal structure of the C-terminal region of PMT has been reported and its structure–function relationship has been predicted. The crystal structure of the C-terminal residues 575–1,285 of PMT revealed that PMT is an enzyme toxin carrying the cysteine protease-like catalytic triad dependent on the redox state of target cells and that it functions on the cytoplasmic face of the plasma membrane of target cells [14]. Therefore, structural configuration-based investigations of the immunoreactivity and protective immunity induced by PMT re considered important for the development of effective vaccines using recombinant PMT.

In this study, we investigated the antigenicity and immunoreactivity of partial fragments of recombinant PMT including its functional domains. PMT-A is a part of the N-terminal domain that predictably corresponds to the target cell-binding region; PMT-B is a part of the C1 domain that contains the membrane-targeting region and a part of the C2 domain that is responsible for putative molecular recognition; PMT-C is a part of the C3 domain that has the catalytic center responsible for cytotoxicity; PMT2.3 covers the region that was structurally determined to have enzymatic activity, by Kitadokoro *et al*. [14] (C1, C2, and C3 domain). These recombinant proteins were immunologically reactive with the antisera obtained from *P. multocida*-infected swine showing symptoms of PAR; however, PMT-A showed significantly low immunoreactivity as compared with detoxified authentic PMT, PMT-B, PMT-C, and PMT2.3 (Fig. 2A). Low immunoreactivity of PMT-A was also observed in immunological detection with the antisera obtained from mice immunized with detoxified authentic PMT (Fig. 2B). Liao *et al*. [18] demonstrated that antibody against the N-terminal portion of PMT inhibited the cytopathic effects of PMT in Vero cells, and neutralizing antibody produced against the N-terminal portion of PMT may prevent the binding of PMT to target cells and inhibit its toxic activity. According to the recently published data from Seo *et al*. [29], the shorter N-terminal fragment (residues 1–390) was immunogenic to produce protective antibody in mice and pig. On the other hand, our results suggest that the N-terminal region of PMT (residues 1–483) has relatively poor immunoreactivity to the antisera from mice immunized with authentic PMT as well as the antisera from an infected swine (Fig 2); the protection against homologous challenge was not provided by the immunization with PMT-A (Table 1). These different results seem to be due to the difference in conformation of the recombinant protein. In our study, a large amount of the recombinant proteins were produced in insoluble state in the expression process; thus these insoluble recombinant proteins were purified under denaturing conditions by using 8 M urea and then refolded by serial dilution with

PBS. The different results in immunoreactivity of the N-terminal region of PMT compared with the experiment of Seo *et al*. [29] may be explained by these purification steps, which can affect the conformational alteration of the proteins. Interestingly, despite the low immunoreactivity of PMT-A to antibodies in our study, the survival rate of mice immunized with PMT-A is similar to the mortality result of N-terminal PMT reported by Seo *et al*. [29] (30% vs. 37.5%). These mean that the quantity and quality of production of antibodies are influenced by the conformational status; however, the protective immune response is dependent on the specific antibodies raised by extremely fine epitopes, and not merely by the whole conformation of the protein.

In our study, the N-terminal, middle, and C-terminal regions of PMT could not induce effective protective immunity against *P. multocida* challenge. Although each of the partial PMT fragments could produce PMT-specific antibodies, and these antibodies reacted with both detoxified authentic PMT (Fig. 3A) and bacterin (Fig. 3B), the mice immunized with each of the partial PMT fragments failed to survive efficiently (Table 1), compared with those immunized with bacterin or detoxified authentic PMT in the homologous challenge experiment. Only PMT2.3, which contains the region of the intracellular active moiety of PMT, showed high immunoreactivity and protection. These results suggest that the epitopes involved in protective immunity are not present independently in each of the partial PMT fragments; the conformational features of PMT, especially the C-terminal region, may play a role in the production of protective antibodies.

In conclusion, the recombinant proteins of partial PMT domains produced a high level of antibodies in the immunized mice and showed specific immunoreactivities to the antisera obtained from both the swine with PAR and the mice immunized with detoxified authentic PMT. However, vaccination of mice with these partial PMT domains (PMT-A, PMT-B, and PMT-C) could not induce protective immunity against homologous challenge; only a large portion of the C-terminus including the intracellular activity of PMT (PMT2.3) could show effective protection. For the development of an effective subunit vaccine using partial PMT, further investigations based on the structural features and other virulence factors involved in *P. multocida* infection are essential. Researches on the protective potential of the protein in pigs and the detailed determination of protective epitopes are also needed.

Acknowledgments

Conflict of Interest Statement

The authors declare no conflict of interest.

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