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Isoleucine 61 is important for the hemolytic activity of pyolysin of *Trueperella pyogenes*

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ABSTRACT

Pyolysin (PLO) is a hemolysin secreted by *Trueperella pyogenes* (*T. pyogenes*) and is important for the pathogenicity of *T. pyogenes*. Oligomerization of PLO monomers is a critical step in the process of hemolysis. However, the mechanisms of intermolecular interaction of PLO monomers are still not clearly illuminated. In this study, two monoclonal antibodies (mAbs) against PLO, named AP-1A3 and AP-4F12, respectively, were generated firstly, of which AP-1A3 showed no or undetectable hemolysis inhibition activity against recombinant PLO (rPLO), whereas AP-4F12 could markedly inhibit the hemolytic activity of rPLO. Epitope mapping revealed that AP-1A3 recognized amino acid residues ranging from 64 to 79 of mature PLO (91–106 including the signal peptide), whereas AP-4F12 recognized amino acid residues ranging from 58 to 75 (85–102 including the signal peptide). Comparison of the amino acid sequence of two epitopes revealed that six amino acid residues ranging from 58 to 63 of PLO were associated with the hemolytic activity of PLO. Alanine scan showed that substitution of each amino acid ranging from 58 to 62 with alanine had apparent impact on the hemolytic activity of rPLO, especially for the substitution of isoleucine 61 which caused almost complete loss of hemolytic activity of rPLO. Our findings identified a region in PLO and an amino acid in that region might play important role in the process of oligomerization of PLO monomers.

1. Introduction

Trueperella pyogenes (*T. pyogenes*), formerly *Arcanobacterium pyogenes* (Yassin et al., 2011), is a ubiquitous and important opportunistic pathogen of humans and animals (Sens and Heuwieser, 2013) usually described involved in pyogenic infections (Radostits, 2007).

Pyolysin (PLO), a hemolysin secreted by *T. pyogenes*, is one of the primary virulence factors for its pathogenicity (Jost and Billington, 2005; Zhao et al., 2013). As a member of cholesterol-dependent cytolysin (CDC) family, PLO shares only 31–41% similarity in primary structure to other CDCs (Jost and Billington, 2005). Hence, the theories gained from studies on other members of CDC family, such as listeriolysin O (LLO), pneumolysin (PLY), streptolysin O (SLO), suilysin (SLY) and perfringolysin (PFO) (Giddings et al., 2003; Hotze et al., 2001; Polekhina et al., 2005; Tweten, 2005), might not be applicable for explaining the mechanisms of the virulence effect of PLO. Although previous studies had identified some regions in the structure of PLO which might be crucial for its virulence, such kind of studies are really limited (Imaizumi, 2001; Imaizumi et al., 2003; Pokrajac et al., 2013; Zhao et al., 2013).

In the current study, six amino acids, which might be related to the cytolytic activity of PLO were identified using monoclonal antibodies (mAbs), of which the isoleucine 61 (I61) is necessary for the hemolytic activity of PLO determined by alanine scan method. Our finding points out a region of amino acid position 58 to 62 and especially the I61 are essential for the hemolytic activity of PLO and might participate in the intermolecular interaction of PLO monomers.

2. Materials and methods

2.1. Preparation of wild type PLO

T. pyogenes strain HLJ-0912 (laboratory isolated) was cultured at 37 °C in Martin broth (Aobox biotechnology Co.) with 10% fetal

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Table 1
Sequence of the PCR primers used in this study.

Sequence	Targent position in *plo* gene
5-CATGCCATGGCCGGATTGGGAAACAGC-3	82
5-CATGCCATGGCGTCTGGTGTACTTGCAGT-3	232
5-CCGGAATTCCTAAATAGACTCACCCTTGACTG-3	264
5-CATGCCATGGCGCCGGTTACCAAGGATCAG-3	274
5-CATGCCATGGCGAAGCATGAACGCAAGAG-3	319
5-CCGGAATTCCTAGCGTTCATGCTTAAATACCGT-3	330
5-CATGCCATGGCGGCGTTCGATGCGAAC-3	364
5-CCGGAATTCCTAGAACGCAGAGATGTCCGAAC-3	369
5-CCGGAATTCCTATAACACGAGCGCGCCAGGAT-3	411
5-CATGCCATGGCGTCAAAGCGTCAACTG-3	649
5-CCGGAATTCCTACTTTTCAAATCCGAG-3	693
5-CCGGAATTCCTACGTTGCAGTGGTTTCAATG-3	1260

The restriction enzyme sites (*Nco*I or *Eco*RI) that were introduced in each primer are underlined. The target positions of these primers in the sequence of the *plo* gene (with nucleotides encoding signal peptide) are listed in right column.

bovine serum (FBS) for 48 h. The wild type PLO (wtPLO) was prepared from the culture supernatant of *T. pyogenes* by the procedures described previously (Ikegami et al., 2000).

2.2. Expression and Purification of recombinant PLO

Recombinant plasmid pET-30a(+)-*plo* (laboratory constructed), involving *plo* gene without the nucleotides encoding signal peptide, was transformed into *Escherichia coli* Rosetta (DE3)^{TM} competent cells. Recombinant PLO (rPLO) was expressed by inducing with isopropyl-β-D-thiogalactoside (IPTG) and then purified using nickel-charged resin. The purified protein was dialyzed against PBS with 5% glycerol at 4 °C for 48 h. The protein was quantified by Bradford method and stored at −80 °C until use.

2.3. Preparation of mAbs

mAbs against rPLO were produced using a standard procedure (Imaizumi, 2001; Mazzarotto et al., 2009). Briefly, five-week-old BALB/c female mice were immunized subcutaneously with 50 μg of purified rPLO emulsified with an equal volume of Freund's complete adjuvant, then followed by two injections at 2 weeks interval with rPLO emulsified with incomplete adjuvant. Three days after the last immunization, spleen cells of the immunized mice were harvested and fused with SP2/0 mouse myeloma cells. The fused cells were cultured and selected in hypoxanthine-aminopterin-thymidine (HAT) (Sigma–Aldrich Co., LLC., China) supplemented RPMI 1640 medium. After cultivation for 3 weeks aminopterin was omitted from medium and supernatants were screened for antibody reactivity and specificity by ELISA. Cells of positive tested wells were subcloned by limiting dilution method for three times. Ascites were derived from the mice primed with a 0.5 mL adjuvant and then injected with $1–2 \times 10^6$ hybridoma cells by intraperitoneal injection. mAbs were purified from the collected ascitic fluid by Protein G or Protein A Superose (Genscript, USA) column chromatography. Isotypes of the mAbs were determined using mouse monoclonal isotyping kit (Cellway-lab, Zhengzhou, Chian).

2.4. Reactivity of the mAbs

Reactivity of the mAbs prepared was tested by western-blot method. wtPLO and rPLO were separated by 12% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in PBS at 4 °C over night, and then incubated with mAbs prepared, rabbit anti-rPLO serum (laboratory prepared, 1:1000) or anti-Histidine tag mAbs (1:3000) at 4 °C over night, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Genscript, USA, 1: 3000) or HRP-conjugated AffiniPure goat anti-mouse IgG (Genscript, USA, 1:3000) for 1 h at room temperature. Membranes were washed 3 times for 5 min with PBST after each incubation step. Detection was done using Easy See Western blot Kit (Transgen, Beijing, China) according to the manufacturer.

Table 2
Complementary oligonucleotides for synthesizing the partial *plo* gene shorter than 60 bp.

Sequence	Start location	End location
5-CATGGCGTCTGGTGTACTTGCAGTCAAGGGTGAGTCTATTGAAAATGTGCCGGTTTAAG-3	151 (232)	198 (279)
5-AATTCTTAAACCGGCACATTTTCAATAGACTCACCCTTGACTGCAAGTACACCAGACGC-3	151 (232)	198 (279)
5-CATGGCGGTACTTGCAGTCAAGGGTGAGTCTATTGAAAATGTGCCGGTTACCAAGTAAG-3	157 (238)	204 (285)
5-AATTCTTACTTGGTAACCGGCACATTTTCAATAGACTCACCCTTGACTGCAAGTACCGC-3	157 (238)	204 (285)
5-CATGGCGGCAGTCAAGGGTGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGTAAG-3	163 (244)	210 (291)
5-AATTCTTACTGATCCTTGGTAACCGGCACATTTTCAATAGACTCACCCTTGACTGCCGC-3	163 (244)	210 (291)
5-CATGGCGAAGGGTGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGTAAG-3	169 (250)	216 (297)
5-AATTCTTACTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACTCACCCTTCGC-3	169 (250)	216 (297)
5-CATGGCGGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCTAAG-3	175 (256)	222 (303)
5-AATTCTTAGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACTCCGC-3	175 (256)	222 (303)
5-CATGGCGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTAAG-3	178 (259)	225 (306)
5-AATTCTTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACGC-3	178 (259)	225 (306)
5-CATGGCGATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTACTAAG-3	181 (262)	228 (309)
5-AATTCTTAGTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATCGC-3	181 (262)	228 (309)
5-CATGGCGGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTACACGTAAG-3	184 (265)	231 (312)
5-AATTCTTACGTGTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCCGC-3	184 (265)	231 (312)
5-CATGGCGAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTACACGGTATAAG-3	187 (268)	234 (315)
5-AATTCTTATACCGTGTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTCGC -3	187 (268)	234 (315)
5-CATGGCGGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTACACGGTATTTTAAG-3	190 (271)	237 (318)
5-AATTCTTAAAATACCGTGTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACCGC-3	190 (271)	237 (318)
5-CATGGCGAAGGGTGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCTAAG-3	169 (250)	222 (303)
5-AATTCTTAGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACTCACCCTTCGC-3	169 (250)	222 (303)
5-CATGGCGGGTGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTAAG-3	172 (253)	225 (306)
5-AATTCTTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACTCACCCGC-3	172 (253)	225 (306)
5-CATGGCGGAGTCTATTGAAAATGTGCCGGTTACCAAGGATCAGCTCAAGGACGGCACCTACTAAG-3	175 (256)	228 (309)
5-AATTCTTAGTAGGTGCCGTCCTTGAGCTGATCCTTGGTAACCGGCACATTTTCAATAGACTCCGC-3	175 (256)	228 (309)

The restriction enzyme sites (*Nco*I or *Eco*RI) that were introduced in each primer are underlined. The target positions of these primers in the sequence of the plo gene (with nucleotides encoding signal peptide) are listed in right column.