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Functional characterization of Kunitz-type protease inhibitor *Pr*-mulgins identified from New Guinean *Pseudechis australis*

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ABSTRACT

Kunitz-type protease inhibitors, which consist of around 60 amino acid residues and three distinctive disulfide bridges, exhibit a broad range of physiological functions such as protease inhibitor and ion channel blocker. In this study, we identified cDNAs encoding Kunitz-type protease inhibitors, *Pr*-mulgins 1, 2 and 3, from the venom gland cDNA library of Papuan pigmy mulga snake (New Guinean *Pseudechis australis*). The deduced amino acid sequences of the *Pr*-mulgins are 92.4–99.3% identical with their orthologs in Australian *P. australis*. *Pr*-mulgin proteins were recombinantly prepared and subjected to inhibitory assays against proteases. *Pr*-mulgin 1 significantly affected matrix metalloprotease (MMP) 2; *Pr*-mulgins 2 and 3 showed potent inhibition to trypsin and plasma plasmin; and *Pr*-mulgin 2 inhibited α-chymotrypsin. *Pr*-mulgins 1, 2, and 3, however, had essentially no effect on *Drosophila* K^+ channels (*Shaker*) and rat K^+ channels (K_v 1.1).

1. Introduction

Snake venoms are rich resources of bioactive proteins including enzymes and toxins. Some of the bioactive proteins, identified from snakes, have been shown to be homologous to Kunitz-type protease inhibitor, consisting of around 60 amino acid residues with three disulfide bridges (Laskowski and Kato, 1980). For example, dendrotoxin identified from *Dendroaspis angusticeps* belongs to the family, and it blocks particular subtypes of K^+ channels with low inhibitory activities for proteases (Harvey, 2001).

Tens of Kunitz-type protease inhibitors were identified from Australian elapid snakes; five of them, mulgins 1–5, were from the cDNA and genomic sequences of Australian mulga snake (Australian *Pseudechis australis*) (St Pierre et al., 2008). However, functional characterizations of mulgins have not been reported until now.

Mulga snake, *P. australis*, has long been considered as a single species that is distributed throughout most of Australia and in a few localities of New Guinea (Cogger, 2000; O'Shea, 1996). In a recent genetic study, however, Wüster et al. (2005) identified a genetic distance between Australian and New Guinean populations, with only a few exceptions. Based on this genetic study and on the morphological observations of the Australian and New Guinean populations, the New Guinean form was classified as a separate species (Williams and Wüster, 2005), and named as Papuan pigmy mulga snake, *Pseudechis rossignolii*

Abbreviations: Bz, benzoyl; MCA, methylcoumaryl-7-amide; Boc, butyloxycarbonyl; MOC, 7-methoxycoumarin-4-yl; Dnp, 2, 4-dinitrophenyl; Cbz, carbobenzoxyl; Suc, succinyl; Ome, methoxy; BPTI, basic pancreatic trypsin inhibitor; STI, soybean trypsin inhibitor.

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(Hoser, 2000). Since this new name has not been widely adopted yet, we retain the original name, New Guinean *P. australis*, in this paper.

We have conducted a comparative study of the venom composition between Australian and New Guinean elapid snakes. In this study, we prepared the venom gland cDNA library from New Guinean *P. australis*, and isolated cDNAs encoding Kunitz-type protease inhibitors named *Pr*-mulgins 1, 2, and 3 (named after mulgins from *P. rossignolii*, see above). Recombinantly expressed and purified *Pr*-mulgin proteins exhibited novel inhibition spectrum against proteases, but they did not block rat K^+ channel (rK_v 1.1) and *Drosophila* K^+ channel (*Shaker*) activities.

2. Materials and methods

2.1. Construction of cDNA library

The snake specimen, which was identified based on its morphology, was collected in Irian Jaya, Indonesia, and maintained at the Japan Snake Institute. Construction of the snake venom gland cDNA library, subcloned into the plasmid vector $pSD64TR_{ER}$, was described previously (Inagaki et al., 2010). To isolate novel Kunitz-type protease inhibitor cDNAs, the venom gland cDNA library was used for the PCR amplification as a template. Relative locations of oligonucleotide primers were schematically represented in Fig. 1A. The initial PCRs were conducted using the vector primer (SDA; 5′-TTATGTAGCTTAGAGACT-3′) and the primer designed from the conserved sequences in the Kunitz-type protease inhibitor (Textilinin; 5′-ACTCCTCACGCTCTGGGA-3′). To isolate cDNAs for the 5′ side of the *Pr*-mulgin transcripts, an SP6 vector primer (Promega, Madison, WI) and a gene-specific primer (Kunitz-end; 5′- ATGAATTCAAGCTGGAACTGTAG-3′, the *Eco*RI site and flanking sequence are underlined) were used. The gene-specific primer was designed from the conserved sequences in the 3′ untranslated region of the transcripts of *Pr*-mulgin 1 (nt 358–374), *Pr*-mulgin 2 (nt 328–344), and *Pr*-mulgin 3 (nt 329–345). The amplified products were cloned into pCR 2.1-TOPO (Life Technologies, Carlsbad, CA). All the inserts were confirmed by sequencing using model 310 (Life Technologies) and CEQ3000 (Beckman Coulter, Brea, CA) DNA sequencers. The full-length nucleotide sequence of the *Pr*-mulgin cDNAs were identified by combining the nucleotide sequences of the overlapping clones. The cDNA sequences were subjected to a BLAST database search against sequences in GenBank.

2.2. Preparation of recombinant Pr-mulgin and mutated Pr-mulgin proteins

The cDNA fragments encoding *Pr*-mulgins were prepared by PCR using *Pr*-mulgin cDNAs as a template. As illustrated in Fig. 1A, the oligonucleotide primers for PCR were a forward primer for *Pr*-mulgins (K-Cold4-5′; 5′-GTAATACCATATGAAGGACCGTCCA-3′, the *Nde* I site is underlined) and a reverse primer for *Pr*-mulgins 1 and 2 (KAB-Cold4-3′; 5′-CTAGGATCCTAATGATGATGATGATGTGCAGCACAGGTGCG-3′, complimentary sequence corresponding to six His residues is underlined), or a reverse primer for *Pr*-mulgin 3 (KG-Cold4-3′; 5′-CTAGGATCCTAATGATGATGATGATGTGTAGCACAGGTGCG-3′, complimentary sequence corresponding to six His residues is underlined). After the amplified DNAs were digested with *Nde* I and *BamH* I, the products were subcloned into the expression vector pCold IV (Takara, Otsu, Japan). *Escherichia coli* strain Origami B (Novagen, Madison, WI) was transformed with the plasmids, and the soluble proteins were prepared by BugBuster (Novagen). Recombinant proteins were purified without chaotropic agents by Talon (Clontech, Mountain View, CA) according to the supplier's description. Buffer was exchanged to H_2O by PD-10 Desalting Columns.

The molecular weights of recombinant *Pr*-mulgin 1, 2, and 3 proteins were verified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS; Shimadzu Techno-Research, Kyoto, Japan) using AXIMA performance (Shimadzu, Kyoto, Japan). The spectra were internally calibrated using insulin B (3496.96) and insulin (5734.59), and α-cyano-4-hydroxycinnamic acid was used for matrix. Theoretical mass of *Pr*-mulgins were calculated by Protean software component of Lasergene version 8.0 (DNASTAR, Madison, WI).

To construct the expression plasmid for mutated *Pr*-mulgin 1, *Pr*-mulgin 1 SRGE, in which amino acid residues 49–52 were substituted by corresponding positions of PILP3, PCR was carried out using *Pr*-mulgin 1 cDNA as a template. The oligonucleotide primers for PCR were a forward primer for *Pr*-mulgins (K-Cold4-5′) and a reverse primer for mutation (KA-SRGE; 5′- CTCGAATTCATGCAGCACAGGTGCGTTTGCATTCCCCTCGGGACTTAAAGGT-3′, complimentary sequence corresponding to Ser-Arg-Gly-Glu residues is underlined). Using the amplified products of first PCR as a template, a second PCR was carried out using K-Cold4-5′ as a forward primer and KAB-Cold4-3′ as a reverse primer. The amplified products were subcloned into the *Nde* I and *BamH* I sites of pCold IV, and prepared the recombinant proteins in the same procedures mentioned above.

2.3. Protease assay

Bovine pancreas trypsin (T1426; Sigma–Aldrich, St. Louis, MO), bovine pancreas α chymotrypsin (C4129; Sigma–Aldrich), porcine pancreas elastase (E7885; Sigma–Aldrich), human sputum cathepsin G (SG45; Elastin products company, Owensville, MO), porcine pancreas kallikrein (K3627; Sigma–Aldrich), and human plasma plasmin (527621; EMD biosciences, La Jolla, CA) assays were performed in 85 mM Tris–HCl (pH7.5), 112.5 mM NaCl, 1.5% PEG 6000, and 15 mM $CaCl_2$. Pepsin assay was in 8 mM HCl and 16 mM $CaCl_2$. The substrates were Bz-Arg-MCA for trypsin, Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsin, Suc(Ome)-Ala-Ala-Pro-Val-MCA for elastase, Suc-Ala-Ala-Pro-Phe-MCA for cathepsin G, Cbz-Phe-Arg-MCA for kallikrein, Boc-Val-Leu-Lys-MCA for plasmin, and MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys(Dnp)–NH_2 for pepsin (PEPTIDE INSTITUE, Osaka, Japan). For comparison of inhibitory activities, BPTI (A1153; Sigma–Aldrich) and STI (202-09226; Wako Pure Chemical Industries, Osaka, Japan) were used. Human recombinant MMP activities were analyzed using a fluorimetric MMP inhibitor profiling kit, (Enzo Life Sciences International, Farmingdale, NY). After