Received: 8 May 2020 | Revised: 3 July 2020 | Accepted: 6 July 2020

DOI: 10.1002/arch.21727

SHORT COMMUNICATION

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

Truncated reactive center loop decrease the inhibitory activity of *Antheraea pernyi* serine protease inhibitor 6

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Funding information
Weifang University Doctor Startup Fund, Grant/Award Number: 2020BS26

Abstract

Here, we assessed the effect of a systematic change in reactive center loop (RCL) length, N-terminal to the reactive center, on the inhibitory activity of the recombinant Apserpin-6. The domain prediction results indicated that the RCL is located between the amino acid numbered 359–379 at the C-terminal of Apserpin-6. The N-terminal variable region for amino acid positions P7–P1 of the RCL of Apserpin-6 was truncated or extended by residue deletion or insertion using site-directed mutagenesis. The recombinant Apserpin-6 with one or two residues insertion in RCL had no effect on prophenoloxidase (proPO) activity, whereas deletion of one or two residues in RCL lowered the efficiency of inhibition of Apserpin-6. The results of this study will facilitate the understanding of inhibition mechanism of RCL on proPO activity.

KEYWORDS

Antheraea pernyi , proPO activity, reactive center loop, serine protease inhibitor 6, variant

1 | INTRODUCTION

Insects lack adaptive immunity but have evolved effective innate immune systems against the invasion of pathogens (Lemaitre & Hoffmann, 2007). Stimulation of IMD and Toll signaling pathways to promote synthesis of antimicrobial peptides, the regulation of blood coagulation, and melanization induced by activation of prophenoloxidase (proPO) are the key innate immune processes, which are generally regulated by the protease cascades

resulting in multiple steps of protease activation (Gorman, Wang, Jiang, & Kanost, 2007; Kim et al., 2008; Lemaitre & Hoffmann, 2007). Secretion of serine protease inhibitors (serpins) is one of the strategies that limit the cascade activation in a localized level for maintaining homeostasis of the host (Kausar et al., 2018).

Serpins are a superfamily folding into a conserved tertiary structure consists nine-helices, three-sheets, and a reactive center loop (RCL) near the C-terminus, which acts as "bait" for a target protease. Serpins inhibit proteases by an irreversible suicide substrate mechanism. After binding to the target protease, serpin is cleaved at the scissile bond between residues designated P1 and P1' located in the RCL. Following cleavage, the serpin and protease form a covalently linked complex, with the cleaved RCL inserted within β-sheet A of the serpin. The conformational change causes the translocation of the protease from top to bottom and results in a stable complex without protease activity (Chu et al., 2015). Changes of RCL length caused by mutations in the distal variable region also interfere with the activity of serpins. Zhou, Carrell, and Huntington (2001) reported that deletion or insertion of residues in the distal variable region of α1-antitrypsin increased or reduced the stability of the complex.

Melanization in insects is initiated by the recognition of microbial elicitors, and then a serine protease cascade is activated to finally cleave the proPO zymogen to active PO which catalyzes the formation of quinones and melanin for killing and immobilizing microbes (Cerenius, Lee, & Soderhall, 2008; Kanost & Jiang, 2015). It has been demonstrated that serpins inhibit the proPO cascade in several insects (Chu et al., 2015; He et al., 2018; Kausar et al., 2018; Li, Ma, Lin, Zou, & Lu, 2016; Liu et al., 2015; Yang et al., 2019). In the previous studies, we reported that serine protease inhibitor-6 of *Antheraea pernyi* (Apserpin-6) inhibit proPO activity in *A. pernyi* hemolymph (Zeng et al., 2017). Here, we assessed the effect of a systematic change in RCL length, N-terminal to the reactive center, on the inhibitory activity of the recombinant serpin-6 of *Antheraea pernyi*. The variants of Apserpin-6 with residues deletion or insertion in RCL were prepared, and their abilities to inhibit proPO activation were detected. This study will facilitate the understanding of inhibition mechanism of RCL on proPO activity.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

The *A. pernyi* strain Jiaolan and the wild recombinant Apserpin-6 protein stored in our laboratory were used in this study. The larvae were maintained in a rearing chamber at 23 ± 2°C with 70 ± 5% relative humidity and fed with fresh leaves of *Quercus liaotungensis* collected from the research base of Shenyang Agricultural University (Shenyang, China).

2.2 | Construction of Apserpin-6 variants expression plasmids

The protein sequence of Apserpin-6 was referred from National Center for Biotechnology Information (NCBI) database (GenBank: ATD87113.1). The protein spatial structure prediction was performed using SWISS-MODEL online software with template searching by Phyre2 against the SWISS-MODEL template library (https://swissmodel.expasy.org/). The template with the highest scoring crystal structure was selected for model building. Model was built according to the target template alignment using ProMod3 (Basyuni et al., 2018). Domain prediction was carried out through online software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) by searching against database CDD v3.18-55570 position-specific scoring matrix with expect value threshold 0.01 and maximum number of hits 500. The N-terminal variable region P7–P1 of the RCL of Apserpin-6 was truncated or extended by residue deletion or insertion via site-directed mutagenesis (Figure 1c). Apserpin-6 complementary DNA encoding the mature secreted protein with different RCL length were synthesized by GENEWIZ Biotechnology Co., Ltd. (Suzhou, China), then cloned into pET28a(+) expression vector by the restriction sites

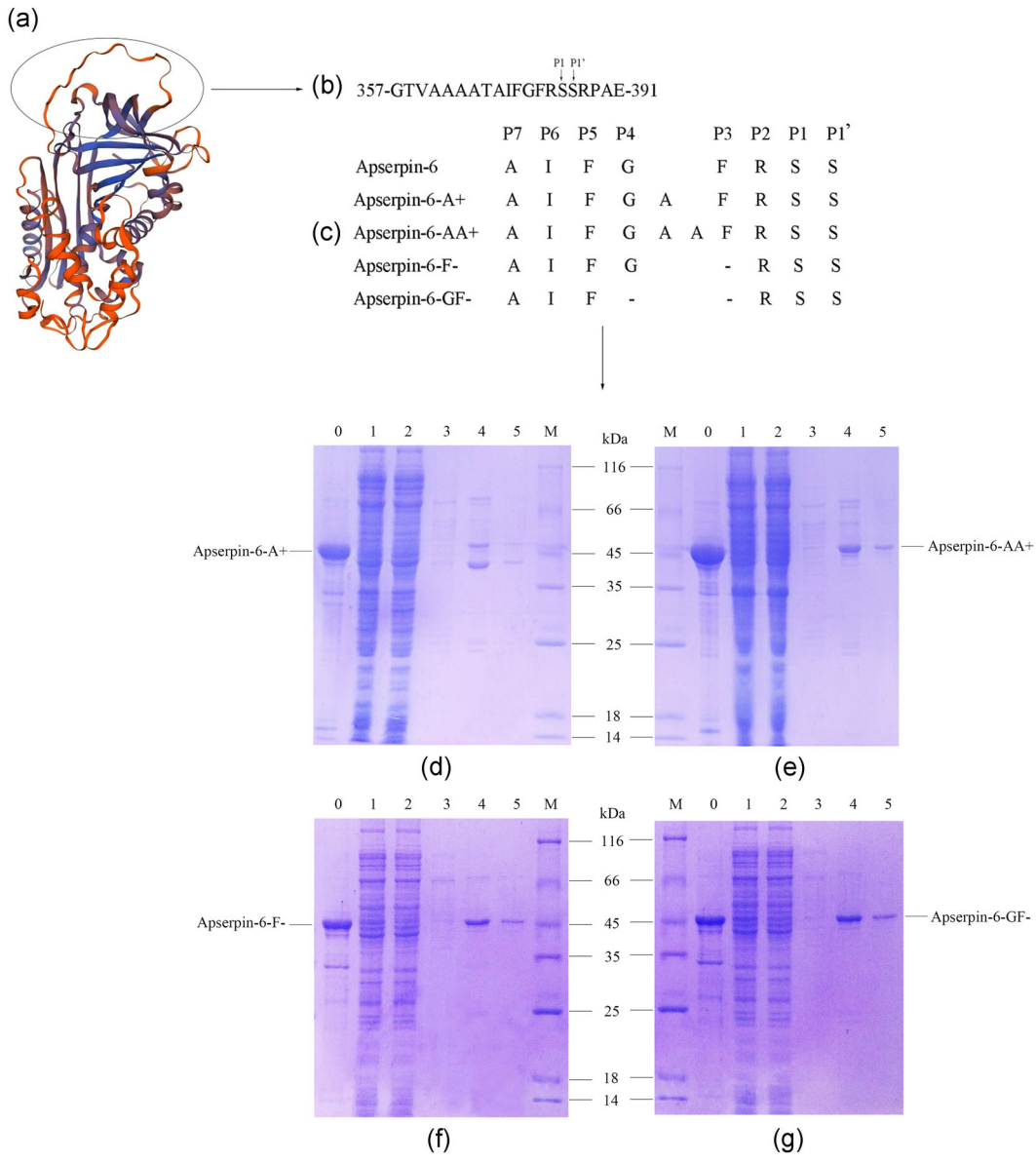

FIGURE 1 (a) The predicted 3D structure of Apserpin-6. The circle indicates the predicted RCL. (b) The predicted amino acid sequence of RCL. (c) Insertion and deletion of amino acid sites in RCL of Apserpin-6. SDS-PAGE analysis of the purified recombinant Apserpin-6 variants, (d) Apserpin-6-A+; (e) Apserpin-6-AA+; (f) Apserpin-6-F−; and (g) Apserpin-6-GF−. M, Marker. 0, The bacterial precipitation after ultrasonication. 1, The bacterial supernatants after ultrasonication. 2, The effluents of the bacterial supernatants flow through Ni-IDA column. 3, The effluents from Ni-IDA column washed by 10 mM imidazole. 4, The effluents from Ni-IDA column washed by 250 mM imidazole. 5, The residual proteins on Ni-IDA column. Each protein sample (5 μl) mixed with 5 μl loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 5% β-mercaptoethanol, and 0.5% bromophenol blue) was subjected to 12% SDS-PAGE using a 5% (wt/vol) stacking gel and a 12% (wt/vol) resolving gel with 75 V for 30 min followed by 120 V for 1.5 hr. 3D, three dimensional; RCL, reactive center loop; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

*Bam*HI/*Xho*I to obtain Apserpin-6 variants expression plasmids which were named as Apserpin-6-A+ (one Ala insertion), Apserpin-6-AA+ (two Ala insertion), Apserpin-6-F− (Phe deletion), and Apserpin-6-GF− (Gly and Phe deletion), respectively. The DNA constructs were sequenced to confirm the correct reading frame, which included an amino-terminal hexahistidine tag.

2.3 | Expression and purification of recombinant Apserpin-6 variants

The hexahistidine-tagged recombinant Apserpin-6 variants were expressed in *Escherichia coli* strain BL21. The bacteria were grown in 1 L Luria–Bertani medium with kanamycin (50 mg/L) as a selective antibiotic at 200 rpm, 37°C. When the optical density $(OD)_{600}$ of the cultures reached 0.6, the recombinant proteins were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), with expression for 2 hr at 37°C. The bacteria were harvested by centrifugation at 12,000 rpm for 30 min.

The recombinant proteins were purified using Ni-IDA Resin (PC035; ProbeGene Life Sciences Co. Ltd., Xuzhou, China) following the manual. Briefly, the harvested bacteria were resuspended with precooled buffer D (20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 0.1% Triton-100), then lyzed via ultrasonic (600–800 W, 30 min, and 10 s each with intervals of 10 s). The supernatants by centrifugation at 1,000 rpm for 15 min were incubated with Ni-IDA column. After centrifugation, the Ni-IDA column was washed with buffer E (20 mM Tris-HCl pH 8.0, 2 M NaCl, and 0.1% TritonX-100), buffer D, buffer C1 (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% TritonX-100, and 10 mM imidazole), and buffer C2 (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% TritonX-100, and 250 mM imidazole) in sequence. The eluted proteins were dialyzed by 50 mM NaCl with 20 mM Tris (pH 8.0), and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% (wt/vol) stacking gel and a 12% (w/v) resolving gel with 75 V for 30 min followed by 120 V for 1.5 hr. The purified proteins were quantified using Bicinchoninic Acid (BCA) Protein Assay Kit (PK012; ProbeGene Life Sciences Co. Ltd., Xuzhou, China) according to the instructions as follows: 25 μl bovine serum albumin (BSA) standards (2 mg/ml) and the samples were respectively put in the microporous plates. Then 200 μl BCA reagent mixture (BCA reagent A:BCA reagent B = 50:1) was added to each microporous and incubated for 30 min at 37°C. After cooled to room temperature, OD_{562} was monitored by microplate spectrophotometer. The standard curve was constructed based on the absorbance of BSA standards. The protein concentration was calculated based on the standard curve and dilution ratio of the samples.

2.4 | proPO activity detection in *A. pernyi* hemolymph in vitro

The hemolymph was collected from 30 newly molted and well developed *A. pernyi* larvae of the fourth instar and thoroughly mixed. Then the hemolymph was centrifuged at 4°C, 1,000 rpm for 15 min to remove the hemocytes (Wang et al., 2019). Then 1 μg of the recombinant protein with 100 μl cell-free hemolymph was incubated for 30 min. The proPO activity in hemolymph was detected using Insect proPO Enzyme-Linked Immunosorbent Assay Kit (MM-245902; Meimian, China) following the manual. Briefly, the immobilized antibody was prepared via purified proPO antibody coating microplate. *A. pernyi* hemolymph incubated with recombinant Apserpin-6 proteins were added to the antibody coated micropores, then combined with horseradish peroxidase (HRP)-labeled proPO antibody to form antibody–antigen–enzyme antibody complex. With 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate, TMB was converted to blue under the catalysis of HRP, and finally into yellow under the role of acid. The absorbance of the samples at 450 nm was monitored by microplate spectrophotometer. The activity of proPO in *A. pernyi* hemolymph was determined by comparing OD_{450} of the samples to the standard curve. All data were presented as mean ± standard deviation (*SD*). Statistical differences were evaluated using Student's *t* test. The level of statistically significant difference was set at $^{**}p < .01$.

3 | RESULTS

3.1 | Construction and purification of recombinant Apserpin-6 variants

According to the sequence information of Apserpin-6 from NCBI database, the primary structure of Apserpin-6 contains 412 amino acids (Figure S1). The calculated mass was 46.48 kD. The domain prediction results indicated that there was a RCL between the amino acid 359–379 at the C-terminal (Figure S1). The predicted protein cleavage site was between the serine 374 (P1) and the serine 375 (P1'; Figure 1b). To obtain the active Apserpin-6 recombinant protein, the signal peptide (Figure S1) was removed. The calculated mass of the recombinant proteins was 45.67 kD (Apserpin-6-A+), 45.74 kD (Apserpin-6-AA+), 45.45 kD (Apserpin-6-F−), and 45.30 kD (Apserpin-6-GF−), respectively (Figure S2). SDS-PAGE results showed that there was a specific band at 45 kD after IPTG induction of all recombinant proteins (Figure 1d–g), which indicated that the recombinant proteins were about the expected size.

3.2 | proPO activity assay

The proPO activity under different Apserpin-6 variants in *A. pernyi* hemolymph was compared. As shown in Figure 2, the proPO activity had no obvious change under Apserpin-6 variants with one or two residues insertion in RCL, whereas the deletion of one or two residues in RCL lowered the efficiency of inhibition of Apserpin-6, compared with the control.

4 | DISCUSSION

The RCL sits outside the main serpin protein structure and acts as bait for target proteases. Once the serpin binds to the protease, the P1–P1' scissile bond is cleaved, and the protease forms a covalent bond with the RCL strand (Hoffmann et al., 2006). This complex is then dragged to the opposite pole of the serpin forming an inactive complex. The length of cleaved RCL inserted into the central region of β-sheet A affecting the stability of the complex (Sun et al., 2001). The P4–P1 residues largely affect the affinity of serpins to their target enzymes (Sun et al., 2001). In this study, the length of RCL of Apserpin-6 was lengthened or shortened by deleting or inserting bases between P4–P1 residues. We chose to insert amino acid A or AA between P3 and P4 at N-terminal to extend the length of RCL, and delete amino acid F or GF at P3 and P4 to shorten RCL. The fragment was cloned into pET28a(+) plasmid vector which strictly control and express the target gene efficiently with T7 promoter for

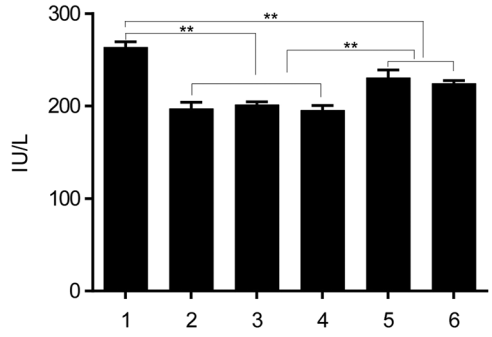

FIGURE 2 The inhibitory effect of recombinant Apserpin-6 variants on proPO activity in vitro in *Antheraea pernyi* hemolymph. 1, proPO activity in hemolymph with no additives. 2, 3, 4, 5, and 6, respectively represent the proPO activity in hemolymph with Apserpin-6, Apserpin-6-A+, Apserpin-6-AA+, Apserpin-6-F−, and Apserpin-6-GF− added. All data were presented as mean ± *SD*. Statistical differences were evaluated using Student's *t* test. The level of statistically significant difference was set at $^{**}p < .01$. proPO, prophenoloxidase; *SD*, standard deviation

prokaryotic expression, which results were consistent with the calculated mass of the recombinant proteins, indicating that the recombinant Apserpin-6 variants were successfully expressed.

The recombinant Apserpin-6 with one or two residues insertion in RCL had no effect on the proPO activity, whereas the deletion of one or two residues in RCL lowered the efficiency of inhibition of Apserpin-6 (Figure 2). The inhibitory selectivity of serpins is largely determined by the residues adjacent to the scissile bond (Li et al., 2016). We conclude that deletion of amino acid F or GF at P3 and P4 shortened the length of RCL inserted into the central region of β-sheet A, which decreased the inhibition efficiency of Apserpin-6. The results of this study indicate that the length of the serpin RCL is critical for its inhibitory activity which would facilitate the understanding of inhibition mechanism of RCL on proPO activity.

ACKNOWLEDGMENT

This study was supported by Weifang University Doctor Startup Fund (2020BS26).

AUTHOR CONTRIBUTIONS

Guobao Wang: Conceptualization (lead); Data curation (equal); Funding acquisition (lead); Methodology (lead); Project administration (lead); Validation (lead); Writing-original draft (lead); Writing-review & editing (lead). **Shuang Na:** Data curation (supporting); Formal analysis (supporting); Methodology (supporting); Project administration (supporting). **Li Qin:** Conceptualization (supporting); Data curation (supporting); Investigation (supporting); Methodology (supporting); Resources (supporting); Supervision (supporting).

DATA AVAILABILITY STATEMENT

All the data are included in our manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wang G, Na S, Qin L. Truncated reactive center loop decrease the inhibitory activity of *Antheraea pernyi* serine protease inhibitor 6. *Arch Insect Biochem Physiol*. 2020;e21727. https://doi.org/10.1002/arch.21727