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ABSTRACT

Profilin (PROF) is a small actin-binding protein presented in apicomplexan protozoa. It was previously reported that *Neospora caninum* profilin (NcPROF) is secreted into the hemolymph of silkworm larvae regardless of the lack of an identified regular secretion signal peptide. To date, which domain is required for its secretion still remains unknown. To this end, we express a fluorescent protein (mCherry) fused with NcPROF at its N-terminus or C-terminus. Both fusion proteins were expressed and secreted into the culture supernatant from Bm5 cells or hemolymph from silkworm larvae, respectively. To further narrow down the C-terminal minimal domain required for its secretion, we constructed three truncated C-terminal domain constructions, ΔN (aa41–163), ΔN1 (aa50–163), and ΔN2 (aa144–163) respectively. All three fusion proteins were detected in the culture supernatant of Bm5 cells and silkworm hemolymph. Surprisingly, a 20-aa C-terminal α-helix domain facilitates the secretion of mCherry, allowing purification of ΔN2-mCherry from silkworm larval hemolymph by affinity chromatography. Taken together, the secretion domain from NcPROF was identified, indicating that can be utilized for the secretory expression of recombinant proteins in the future.

Keywords: Neospora caninum profilin; Secretion domain; Truncation; Protein expression

1. Introduction

Apicomplexan profilin (PROF) is one of the intrinsic proteins which locates in the apical end of tachyzoites. Profilins have been identified as small actin-binding proteins (14–17 kDa) that play multiple roles in the regulation of the polymerization of those actin filaments [1]. Interestingly, regardless of the lack of an identified secretion signal peptide, several studies have showed that *Toxoplasma gondii* PROF (TgPROF) is passively released by the parasites as a secreted protein through an unknown mechanism [2, 3]. Concurrently, our previous study proved that transmembrane protein (GP64TM) fused at the *Neospora caninum* PROF (NcPROF) at C-terminal failed to prevent it to be secreted extracellularly [4].

To date, far too little attention has been paid to the mechanism of the secretion of NcPROF. For almost 50 years, great effort has been devoted to the study of the pathway of secretion protein which classified as a classical/conventional pathway [5]. Commonly, these proteins consist of amino-terminal or terminal signal peptide conventionally transported via endoplasmic reticulum (ER) to the Golgi apparatus and eventually, to the plasma membrane or extracellular space [6]. On the other hand, for the over past 15 years, many pieces of research have proposed alternative route of secretion pathways or called as unconventional secretion where the protein can be secreted without passing the ER-Golgi and directly transported to the plasma membrane or the extracellular space [5].

In this study, a secondary structure-based domain analysis for the secretion of NcPROF was performed. Structural analyses have been performed to define the molecular basis of binding of TgPROF to TLR11, identifying two motifs, an acidic loop, and β-hairpin, that are highly conserved among different organisms of the phylum Apicomplexa [7]. Profilin of TgPROF folded into a unique central β-pleated sheet which flanked on one side by N- and C-terminal α-helices, and on the opposite side by further α-helices [1,8]. To facilitate the investigation, the fluorescent protein mCherry was fused with a series of domain-truncated

NcPROF as a reporter protein and cultured Bm5 cells or silkworm larvae were employed to ensure the appropriate expression level. From our results, we proved that a C-terminal 20-aa α-helix domain from NcPROF is required for secretion of the mCherry protein in both cultured cells and silkworm larva models. In addition, the minimal domain was then successfully applied to the secretory expression and successfully purified from silkworm hemolymph via anti-PA agarose beads affinity chromatography, indicating its possible application as a novel "signal peptide" in expressing proteins of interest in secreted forms. Altogether, this study resolved the secreted domain of NcPROF responsible for its secretion, even though the possible secretion pathway involved should be further investigated in details in the future.

2. Materials and methods

2.1. Generation of mCherry constructs fused with PA-NcPROF (Native) at N- and C- terminal.

To produce recombinant *Bombyx mori* Nucleopolyhedrovirus (BmNPV) for expressing N- or C-terminus fluorescent, mCherry that was amplified from pmCherry Vector (Cat. No. 632525, Takara, Shiga, Japan) was fused with PROF, and the vector plasmids were generated by PCR amplification from pFastBac-PA-NcPROF [4] with phosphorylated (T4 polynucleotide kinase, NEB) primers (Table 1). Amplified vectors plasmid of pFastBac-PA-NcPROF (Native) and insert amplified mCherry was self-ligated at N- and C-terminus respectively and formed a plasmid of pFastBac1_PA-mCherry-NcPROF (N-NcPROF) and pFastBac1_PA-NcPROF-mCherry (C-NcPROF). Subsequently, both constructs were transformed into *Escherichia coli* BmDH10Bac and the white colonies were identified as transformants containing each recombinant BmNPV bacmid, designating as BmNPV/Native, BmNPV/N-NcPROF and BmNPV/C-NcPROF.

2.2. Truncation strategies generating N- and C-terminal deletion of recombinant PA-NcPROF-mCherry (C-NcPROF)

pFastBac1-PA-NcPROF-mCherry (C-NcPROF) was used as a plasmid for truncation of N- and C-terminal. Four truncations including N- or C-terminal deletion were screened for the responsible domain for the secretion of C-NcPROF. Recombinant plasmids containing truncated C-NcPROF gene lacking 5'-coding sequence [aa41–163 (ΔN), aa50–163 (ΔN1), aa144–163 (ΔN2)] amino acid residues were prepared by PCR using each primer set (Table 1). Whereas, recombinant plasmids containing truncated C-NcPROF gene lacking 3'-coding sequence [aa1–40 (ΔC)] was also prepared by PCR using each primer set (Table 1). PCR was carried out with primers phosphorylated with T4 polynucleotide kinase (NEB), followed by self-ligation and formed a plasmid of pFastBac1-PA-ΔN NcPROF-mCherry (C-NcPROFΔN), pFastBac1-PA-ΔN1 NcPROF-mCherry (C-NcPROFΔN1), pFastBac1-PA-ΔN2 NcPROF-mCherry (C-NcPROFΔN2) and pFastBac1-PA-ΔC NcPROF-mCherry (C-NcPROFΔC). Whereas, the constructions of the BmNPV bacmid were the same as the section described above. These BmNPVs were designated as BmNPV/C-NcPROFΔN , BmNPV/C-NcPROFΔN1, BmNPV/C-NcPROFΔN2, BmNPV/C-NcPROFΔC.

2.3. Cell culture, and recombinant baculovirus preparations and infections

The cultured silkworm Bm5 cells (gifted from Prof K.S. Boo , Insect Pathology Laboratory, School of Agricultural Biotechnology, Seoul National University, Seoul, Korea) were used to prepare each recombinant BmNPV. These cells were cultivated in Sf-900II medium (ThermoFisher Scientific, Kanagawa, Japan) supplemented with 1% antibiotic-antimycotic (ThermoFisher Scientific) and 10% FBS (Gibco, Tokyo, Japan) at 27°C. Transfection of

BmNPV bacmids was done through jetPEI reagent (Polyplus-transfection Inc. New York, USA). The solutions were combined, gently mixed, and incubated for 1 h to allow the formation of complexes, which were prepared according to the manufacturer's instructions. The infected cell culture supernatant and lysate were collected at 3 d post-infection (dpi). Subsequently, around 50 µl of the recombinant baculovirus solution was injected into fifth instars of silkworm larvae (Ehime Sansyu, Ehime, Japan). Silkworm larvae were reared using Silkmate S2 (Nosan, Yokohama, Japan) as an artificial diet and kept in the environmental chamber model KCL-2000 (EYELA, Tokyo, Japan) that was controlled at 65±5% humidity and 25°C [9] for 6 to 7 d depending on the condition of infectious of the silkworm larvae [10]. The hemolymph and fat body of silkworm larvae were collected and diluted with 10-fold of phosphate buffered saline (PBS, pH 7.4) for the confirmation of the expression of each recombinant protein.

2.4. Purification using anti-PA tag beads affinity chromatography

Expressed protein from silkworm larval hemolymph was purified using anti-PA tag affinity chromatography (WAKO Pure Chemical Industries, Osaka, Japan). The 10-fold diluted hemolymph was treated with 0.5% (w/v) nonyl phenoxypolyethoxylethanol (NP-40) for overnight and centrifuged at 10,000 × g for 10 min at 4°C to remove insoluble materials. The clear supernatant was mixed with anti-PA tag affinity beads and incubated at 4°C for 24 h with gentle agitation. Next, the beads were washed 10 times with Tris-buffered saline (TBS, pH 7.6), and the bound proteins were eluted with 0.1 M glycine-HCl (pH 3) and immediately neutralized with 1.5 M Tris-HCl (pH 8.0). The elution was conducted at room temperature in a stepwise manner.

2.5. Gel electrophoresis, immunoblotting, and fluorescence analysis

The expression of the recombinant protein in the collected larval hemolymph and extract fat body was confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by 10 or 15% (w/v) acrylamide (Bio-Rad, Hercules, CA, USA). Consequently, the proteins from separating gel were transferred onto a polyvinylidene fluoride (PVDF) membrane via trans-blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). Transferred PVDF was further blocking around 1 h with skimmed milk with 5% (w/v) in Tris-buffered saline consisting of 0.1% (v/v) Tween 20 (TBST). For the western blotting purpose, the anti-PA tag and rat monoclonal antibody (NZ-1) (0.1 μg/ ml) (Wako), was incubated around 1 h followed by horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (H+L) (1:10000) (Bios Antibodies Inc. Massachusetts, USA) for another 1 h. For imaging result of the expression of recombinant proteins, molecular imager VersaDoc MP imaging systems (Bio-Rad) was used with the aid of immobilon western chemiluminescence HRP substrate (Merck Millipore, Burlington, MA, USA) around 1 min incubation. Meanwhile, for the fluorescence image of N-NcPROF, C-NcPROF and C-NcPROFΔN2, the samples were loaded on SDS-PAGE by 15% (w/v) acrylamide (Bio-Rad) with SDS buffer and was observed with a Molecular Imager FX (Bio-Rad) [11].

3. Results and discussion

3.1 Expression of truncation domain of the PA-NcPROF fused with mCherry at N-terminal and C-terminal

As demonstrated in Fig. 1A, the amino acid sequences of profilins from parasites have high similarity with each other. Especially, the amino acid sequence of NcPROF is almost the

same as that *T. gondii* with 95% identity (Fig. 1A). We modelled 3D structure of NcPROF via CLC sequence viewer software based on the structure of TgPROF (PDB: 3NEC) [7]. The NcPROF contains a majority of secondary structure of α-helix and β-sheets (Fig. 1B). Both helixes and strands emerge as an integral unit and formed complete a domain and properly fold for its recognized function [12]. Structure of NcPROF is quite unique, which represents as a sandwich of α-β-α, containing of 7 β-sheets in the middle of 3 α-helixes. Interestingly, the protrusion of β-sheet structure (βH) and called an acidic loop (AL) including α-helix 2 was uniquely present only at apicomplexan profilins. According to Kucera et al. [7], novel AL structure of TgPROF profilin shows a crucial function of recognition towards toll-like receptor 11 which is responsible for the secretion of interleukin (IL)-12 [13].

Initially, mCherry fused with NcPROF at the N-terminus (N-NcPROF) or C-terminus (C-NcPROF) was constructed (Fig. 2A) expressed in Bm5 cells and silkworm larvae. It is clearly shown that both constructs were expressed intra- and extracellularly in Bm5 cell and silkworm larvae. The secretion of both N-NcPROF and C-NcPROF was observed as PA-NcPROF (Fig. 2B). These results indicate that NcPROF facilitates the secretory expression of mCherry by the fusion at its N-terminus or C-terminus. It was previously reported that The PROFs from other parasites, *e.g. Babesia canis* PROF were found in the extracellular fraction [14]. Besides, we previously reported that NcPROF was secreted into the culture medium and hemolymph in Bm5 cells and silkworm larvae [4]. These results suggest that NcPROF is secreted extracellularly via an unknown unconventional protein secretion pathway. Since this protein is included in the leaderless protein group which contains no signal peptide, hence according to Reboulli [5]; leaderless protein can be translocated across the plasma membrane and are active in the extracellular medium. However, the expression of C-terminal fusion of mCherry with NcPROF produced several protein bands including also higher molecular weight bands than estimated (Fig. 2C and D). This may be caused by the N-terminal PA-NcPROF mCherry

construction was fused in front of the PA tag peptide and show several possible open reading frames from the beginning of mCherry compared with the C-terminal construct which shows only a single band form both the hemolymph and the culture supernatant at ~45 kDa. Furthermore, these results seem to be consistent with other research which found that when targeting sequence away from the N-terminus usually weakens its recognition [15]. In order to investigate evidently the secretion band for both constructions, the fluorescence images of the expressed fusion proteins were detected in Fig. 2E and further proved that N-terminal and C-terminal fused mCherry was secreted in hemolymph and culture supernatant in accordance with the western blots. Therefore, we adopted the fusion of mCherry with NcPROF at the C-terminus (C-NcPROF) in the following experiments.

3.2. Expression of truncation strategies generating N- and C-terminal deletion of recombinant PA-NcPROF-mCherry

To determine which domain of NcPROF is crucial for the secretion of mCherry in Bm5 cells and silkworm larvae, the full length of recombinant C-NcPROF was divided into truncated genes lacking either at N-terminal domain or at C-terminal domain amino acid residues which include 1–40 aa (ΔC1), 41–163 aa (ΔN), 50–163 aa (ΔN1), 144–163 aa (ΔN2) respectively (Fig. 3A). The truncated genes of C-NcPROF either at N-terminal domain or C-terminal domain was properly constructed (Fig. 3B), followed by the expression of each truncated fusion protein in Bm5 cells. Interestingly, in the case of the truncation at its C-terminal (C-NcPROFΔC), the absence of its acidic loop showed no secretion to the cultured medium (Fig. 3C). On the other hand, N-terminal truncations (C-NcPROFΔN, C-NcPROFΔN1, C-NcPROFΔN2) clearly showed the expression of each fusion protein intra- and extracellularly

in Bm5 cell (Fig. 3C). C-NcPROFΔN2 that was expressed in silkworm larval hemolymph was purified (Fig. 4B). Our results successfully identified the secretion domain of NcPROF which would help to depict the molecular basis of NcPROF involved in the infection of *N. caninum*. Furthermore, we also consider that this small "signal peptide"-like residues can be utilized as a novel signal peptide for secreting recombinant proteins of interest in eukaryotic protein expression systems. Interestingly, although this small "signal peptide"-like residues are at the C-terminus of NcPROF, these residues facilitated the secretion of mCherry by the fusion at the N-terminus of mCherry.

In *T. gondii*, a unique protein secretory pathway and a protein trafficking system have been revealed, compared to those in mammalian cells [16]. This suggests that some proteins of parasites including *N. caninum* may be secreted extracellularly via this unique protein secretory pathway. However, NcPROF is also secreted extracellularly in insect cells even though no signal peptide is at the N-teminus of NcPROF. This also suggests that this secretion of the NcPROF may stem from its property as a protein in itself. In this study, we identified the amino acid residues of NcPROF for its secretion in insects and insect cells and these residues allowed mCherry to be also secreted. It is also possible that NcPROF can be used for the analysis of the unconventional protein secretion in both parasites and other eukaryotic cells. Subsequently, the secretion truncation of C-NcPROFΔN2 was successfully purified as a single band and verified through western blotting (Fig. 4B). The C-NcPROFΔN2 consists of small peptide and easily purified through anti-PA tag beads affinity chromatography compared to the others. Furthermore, the purified sample was observed under fluorescence and showed a single band similarly like band observed in western blotting (data not shown). From the outcome of our result the secretion truncation domain from *N. caninum* profilin was successfully determined and discovered. Zhang et al. [17] stated that insect cell might be inefficiently to recognized low secretion signal compared to silkworm larvae which have ability 10–100-fold high expression

and high secretory efficiency. To improve the secretion of recombinant proteins using these residues of NcPROF, its optimization by the amino acid replacement and investigation of the length is needed. Especially, a small secretion truncation domain C-NcPROFΔN2 would be helpful to become secretion agent for others recombinant protein.

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References

[1] N. Wittenmayer, M. Rothkegel, B.M. Jockusch, K.Schlüter, Functional characterization of green fluorescent protein-protein fusion proteins, Eur.J.Biochem. 267 (2000) 5247-5256. https://doi.org/10.1046/j.1432-1327.2000.01600.x.

[2] F. Yarovinsky, D. Zhang, J.F. Andersen, G.L. Bannenberg, C.N. Serhan, M.S. Hayden, S. Hieny, F.S. Sutterwala, R.A Flavell, S. Ghosh, A. Sher, TLR11 activation of dendritic cells by a protozoan profilin-like protein, Science. 308(5728) (2005) 1626-1629. https://doi: 10.1126/science.1109893.

[3] F. Plattner, F. Yarovinsky, S. Romero, D. Didry, M. Carlier, A. Sher, D. Soldati-Favre, *Toxoplasma* profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response, Cell host & Microbe. 3 (2008) 77-87. https://doi: 10.1016/j.chom.2008.01.001.

[4] H. Suhaimi, R. Hiramatsu, J. Xu, T. Kato, E.Y. Park, Secretory nanoparticles of *Neospora caninum* profilin-fused with the transmembrane domain of GP64 from silkworm hemolymph, Nanomaterials 9(593) (2019) 1-13. https://doi:10.3390/nano9040593.

[5] C. Rabouille, Special issue: membrane biology; review: pathways of unconventional protein secretion, Trends in Cell Biol. 27(3) (2017) 230-240. http://dx.doi.org/10.1016/j.tcb.2016.11.007.

[6] C. Rabouille, W. Nickel, Mechanisms of regulated unconventional protein secretion, Nat.Rev.Mol.Cell Biol.10 (2009) 148-155. https://doi:10.1038/nrm2617.

[7] K. Kucera, A.A. Koblansky, L.P. Saunders, K.B. Federick, E.M. De La Cruz, S. Ghosh, Y. Modis, Structure-based analysis of *Toxoplasma gondii* profilin: A Parasite-Specific motif is required for recognition by toll-like receptor 11, J.Mol.Biol. 403 (2010) 616-629. https://doi.org/10.1016/j.jmb.2010.09.022.

[8] I.M. Nodelman, G.D.Bowman, U. Lindberg, C.E. Schutt, X-ray structure determination of human profilin II: a comparative structural analysis of human profilin, J.Mol.Biol. 294 (5) (1999)1271-1285. https://doi.org/10.1006/jmbi.1999.3318.

[9] L. Cappellozza, S. Cappellozza, A. Saviane, G. Sbrenna, Artificial diet rearing system for the silkworm *Bombyx mori* (Lepidoptera: Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production, Appl Entomol Zool. 40(3) (2005) 405–412. https://doi.org/10.1303/aez.2005.405.

[10] M. Fukuzawa, S. Tatsuki, Y. Ishikawa, Rearing of *Ostrinia palustralis* (Lepidoptera: Crambidae) larvae with a switchover of two kinds of artificial diets, Appl Entomol Zool. 39(3) (2004) 363–366. https://doi.org/10.1303/aez.2004.363.

[11] T. Kato, T. Murata, T. Usui, E.Y. Park, Improvement of GFPuv-β3GnT2 protein production by suppressin protease in baculovirus expression system, Biosci. Biotechnol. Biochem. 67(11) (2003) https://doi:10.1271/bbb.67.2388.

[12] S. Barik, When proteome meets genome: the alpha helix and the beta strands of proteins are eschewed by mRNA splice junctions and may define the minimal indivisible modules of protein architecture, J.Biosci. 29(3) (2004) 261-273. https://doi: 10.1007/bf02702608.

[13] F. Yarovinsky, Innate immunity to *Toxoplasma gondii* infection, Nat. Rev. Immunol. (14) (2014)109-121. https://doi:10.1038/nri3598.

[14] R.M. Eichenberger, C. Ramakrishnan, G. Russo, P. Deplazes, A.B. Hehl, Genome-wide analysis of gene expression and protein secretion of *Babesia canis* during virulent infection identifies potential pathogenicity factors. Sci Rep. 7 (3357) (2017). https:// doi:10.1038/s41598-017-03445-x.

[15] P. Gonzalez, P. Robinet, S. Charpentier, L. Mollet, T. Normand, M. Dubois, A. Legrand, Apoptotic activity of a nuclear form of mitogaligin, a cell death protein, Biochem. Biophys. Res. Commun.(378) (2008) 816-820. https:// doi:10.1016/j.bbrc.2008.11.133

[16] L. O. Sangaré, T. D. Alayi, B. Westermann, A. Hovasse, F. Sindikubwabo, I. Callebaut, E. Werkmeister, F. Lafont, C. Slomianny, M. Hakimi, A. V. Dorsselaer, C. Schaeffer-Reiss, S. Tomavo, Unconventional endosome-like compartment and retromer complex in *Toxoplasma gondii* govern parasite integrity and host infection, Nat Commun.7(11191) (2016)1-14. https:// doi: 10.1038/ncomms11191.

[17] X. Zhang, A. Jiang, Z. Ma, Y. Xiong, J. Dou, G. Zhou, M. Qin, J. Wang, Design of signal peptide bombyxin and its effect on secretory expression efficiency and levels of *Helicobacterpylori* urease subunit B in silkworm cells and larvae, Braz. Arch. Biol.Technol. 58(3)(2015) 319-325. http://dx.doi.org/org/10.1590/S1526-8913201500013.

Figure legends

Fig. 1. Genetic sequence and molecular structure of Apicomplexan profilin. A) Several sequence alignments of Apicomplexan profilin. The top of the alignment shows the secondary structure whereas, the bottom shows the consensus and conservation of the apicomplexan profilin. GenBank accession numbers are as follow: *Neospora caninum* Liverpool, XP_003879598; *Toxoplasma gondii* ME49, XP_018638612; *Hammondia hammondi*, XP_008884750; *Besnoitia besnoiti*, PFH33071; *Sarcocystis neurona*, AGW51294; *Plasmodium falciparum 3D7*, XP_001352188; *Piliocolobus tephroscales*, XP_026306091; *Eimeria acervuline*, XP_013249165; *Babesia ovata*, GBE60705. **B**) The overall structure of *Neospora caninum* profilin. The red ribbon representations of *N. caninum* profilin in blue with the acidic loop and the β-hairpin highlighted in red.

Fig. 2. Expression of mCherry fused NcPROF in Bm5 cells and silkworm and western blot analysis. A) Construction of native NcPROF (PA-NcPROF), native NcPROF fused with mCherry at N- and C-terminal (mCherry-PA-NcPROF, PA-NcPROF-mCherry). **B**) Western blot analysis by anti-PA antibody of native PA-NcPROF, **C**) mCherry-PA-NcPROF and PA-NcPROF-mCherry expressed in and Bm5 cells, **D**) silkworm larvae. **E**) Western blot analysis by fluorescence of mCherry-PA-NcPROF and PA-NcPROF-mCherry expressed in silkworm larvae. M indicates molecular weight marker; N, mCherry-PA-NcPROF; C, PA-NcPROF-mCherry; arrows in B–E indicate the PA-NcPROF (native), mCherry-PA-NcPROF, PA-NcPROF-mCherry, respectively.

Fig. 3. Expression truncated proteins, C-NcPROFΔC, C-NcPROFΔN, C-NcPROFΔN1 and C-NcPROFΔN2 in Bm5 cells and western blot. A) Construction of C-NcPROF (FL), truncations of C-NcPROFΔC, C-NcPROFΔN, C-NcPROFΔN1 and C-NcPROFΔN2 (aa41–263 (ΔC1), aa1–40 (ΔN), aa1–49 (ΔN1), aa1–144 (ΔN2). **B**) Amplification PCR of truncations plasmid via pFastbac1_Fw and pFastBac1_Rw primers. M indicates DNA molecular weight

marker; hashes indicate DNA molecular weights of the C-NcPROF (FL), truncations of C-NcPROFΔC, C-NcPROFΔN, C-NcPROFΔN1 and C-NcPROFΔN2. **C**) Western blot analysis of the aa1–163 (FL), aa41–263 (ΔC1), aa1–40 (ΔN), aa1–49 (ΔN1), aa1–144 (ΔN2) expressed in Bm5 cell. M indicates the molecular weight marker; asterisks indicate molecular weights of the recombinant aa1–163 (FL), aa41–263 (ΔC1), aa1–40 (ΔN), aa1–49 (ΔN1) and aa1–144 (ΔN2), respectively.

Fig. 4. Western blot of truncated proteins, C-NcPROFΔN1 and C-NcPROFΔN2 expressed in silkworm larvae. A) Western blot analyses of aa1–49 (ΔN1), aa1–144 (ΔN2) and purified aa1–144 (ΔN2) and **B**) purified C-NcPROFΔN2 from hemolymph. M indicates the molecular weight marker; asterisks indicate molecular weights of the recombinant aa1–49 (ΔN1) and aa1–144 (ΔN2); hashes indicate the unspecific endogenous silkworm storage protein.

Fig. 1

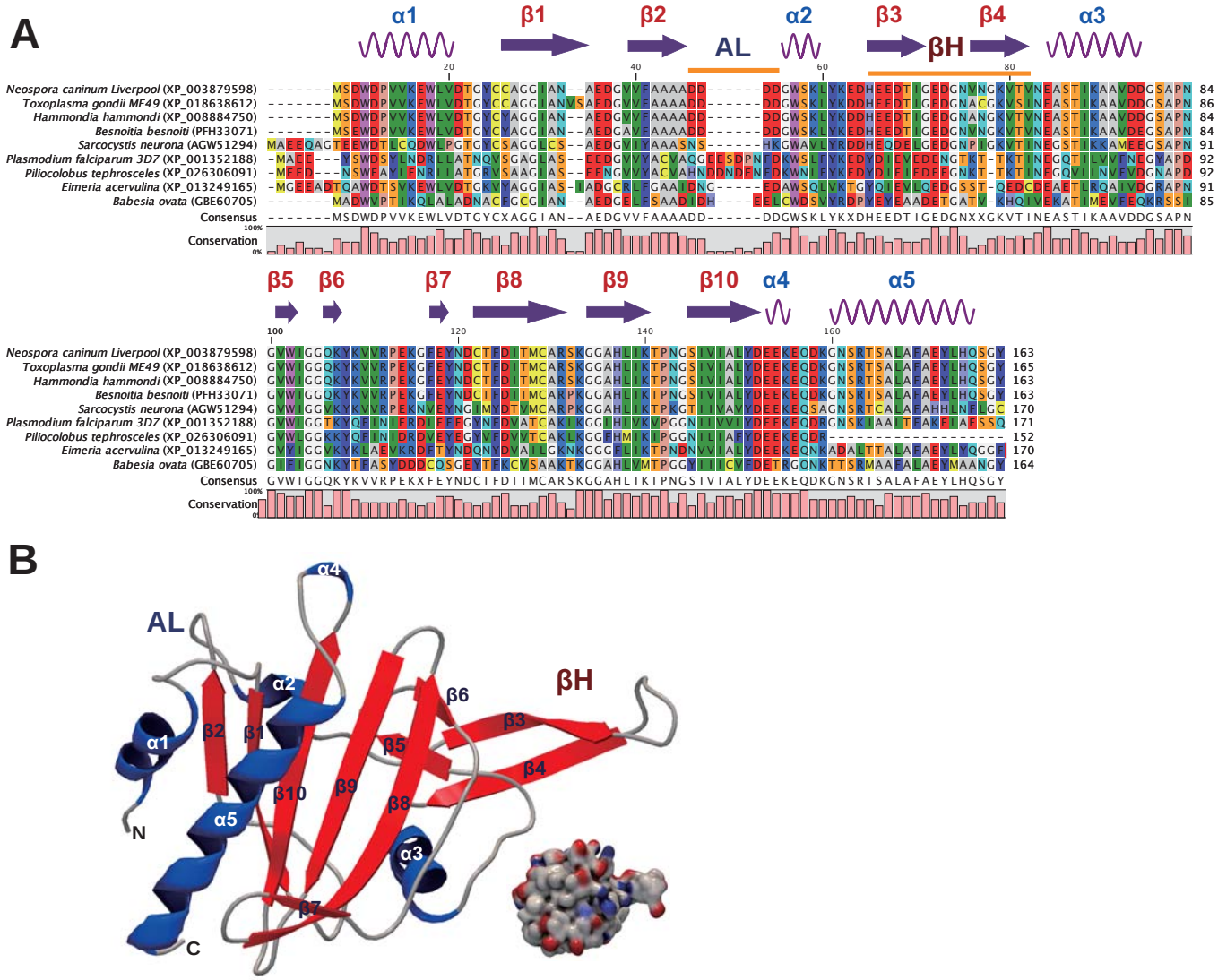

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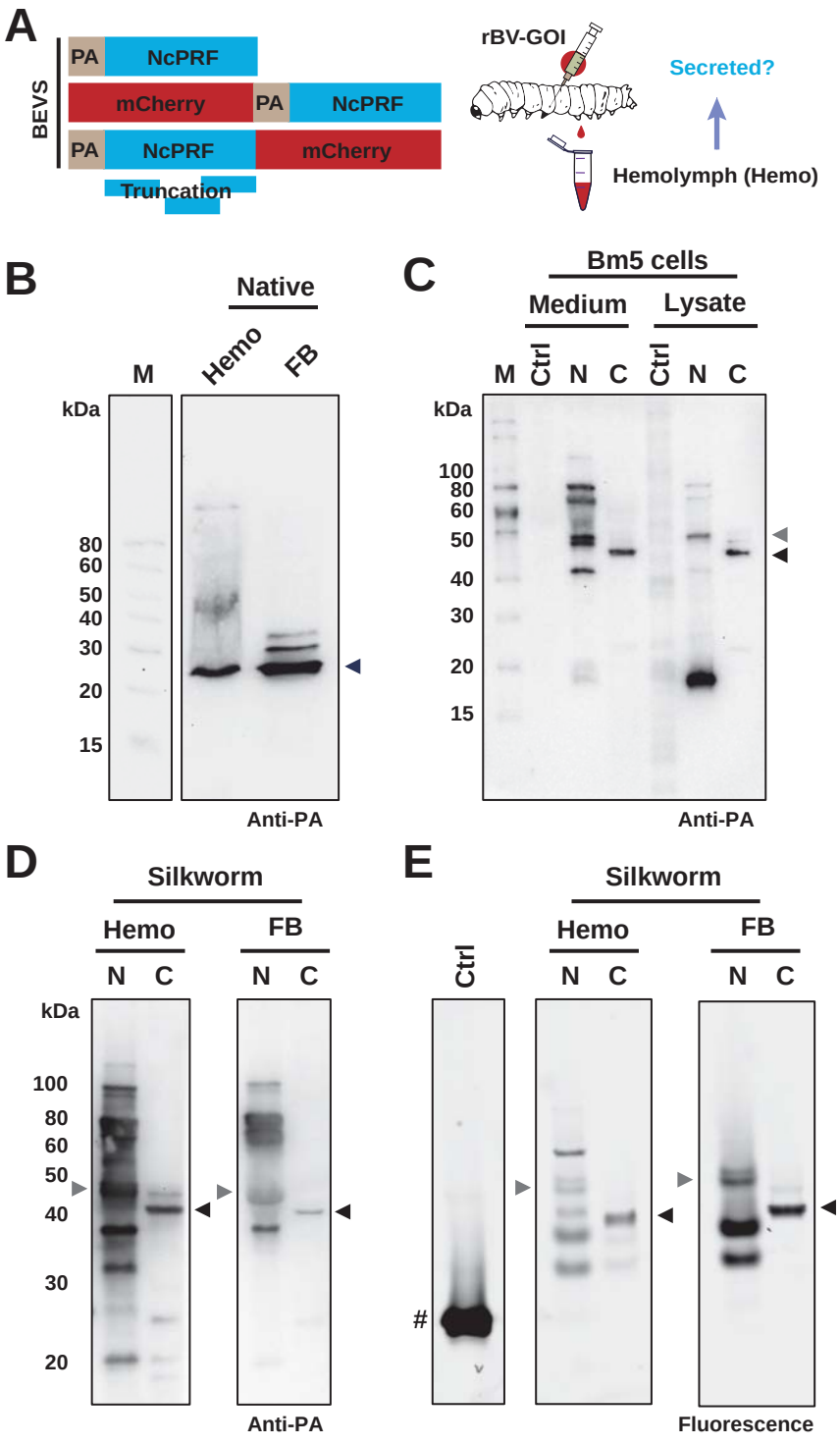
Fig. 2



Suhaimi et al., 2019

Fig. 3

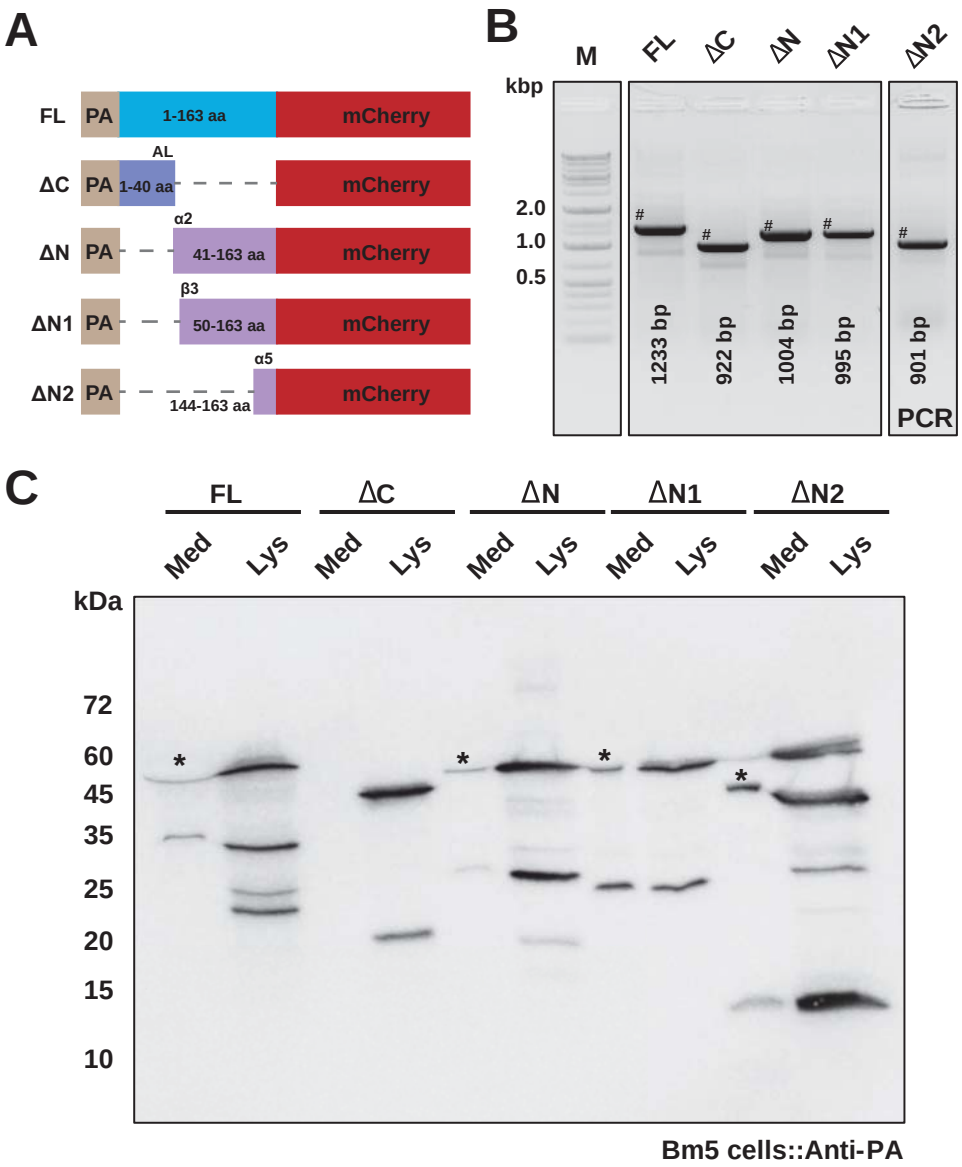


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Fig. 4

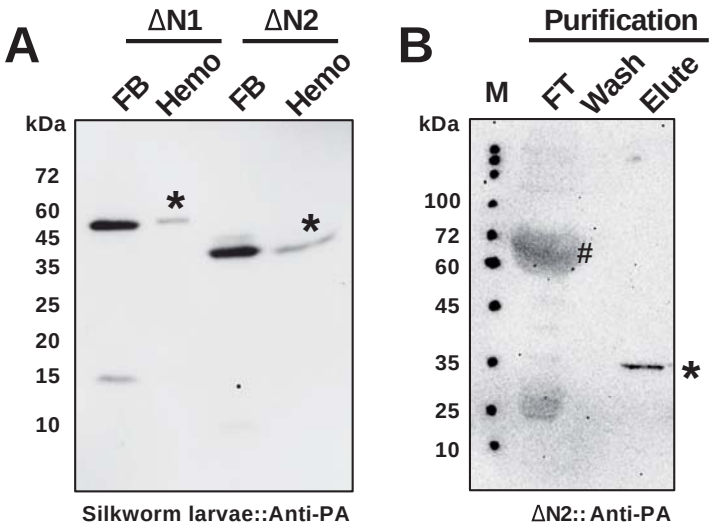

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Table 1

Primers used in this study

Name	Sequence
pFastBac1-fw	5’-tattccggattattcatacc- 3’
pFastBac1-rv	5’-acaaatgtggtatggctgatt- 3’
M13-fw	5′-cccagtcacgacgttgtaaaacg-3′
M13-rv	5′-agcggataacaatttcacacagg-3′
Profilin-fw	5’-atgtcggactgggatcccgt-3′
Profilin with stop-rv	5’-ttaatagccagactggtgaag-3′
PA-PROF-fw	5’- ggcgttgccatgccaggtgc-3’
PA-PROF-rv	5’-catgaattccgcgcgcttcg -3’
mCherry-fw	5’-gtgagcaagggcgaggaggat-3’
mCherry-rv	5’-ggcatggacgagctgtacaag-3’
PA-mCherry-fw	5’-atgggcgttgccatgccaaggtgccgaagatgatgtggtggtgagcaagggcgaggaggat-3’
PA-mCherry-rv	5’gacaacgggatcccagtccgacttgtacagctcgtccatgcc-3’
mCherry-PROF-fw	5’-ggcatggacgagctgtacaagtcggactgggatcccgttgtc-3’
C-AL-rv	5’-gtcatcgtcatcggccgccgcag-3’
C-PA-rv	5’-caccacatcatcttcggcac-3’
N-α2-fw	5’-ggatggtcaaagttgtacaaggag-3’
N1-β3-fw	5’-cacgaggaggacacaatcggaga-3’
N2-α3-fw	5’-aatgaggcctccaccattaaagctgcag-3’
N3-α5-fw	5’-aaagggaacagcaggacgtcgg-3’
C1-β4-rw	5’-gaccgtcaccttgccgttcacgttg-3’