Highlights

1. A series of DNA fragments encoding the precursor or its domains of *Vibrio vulnificus* protease (VVP) were designed and expressed *in vitro* by using the cell-free translational system.
2. The N-terminal propeptide in the precursor functioned as an intramolecular chaperon promoting the folding of VVP.
3. The N-terminal propeptide was unstable and digested easily by the enzymes in the cell lysate used for the cell-free system.
4. The C-terminal domain in VVP showed to disturb the folding of VVP.

Functional analysis of the N-terminal propeptide in the precursor of *Vibrio vulnificus* metalloprotease by using the cell-free translational system

Tomoka Kawase[1]*, Fumi Miura[1], Anusuya Debnath[1], Kinuyo Imakura[1], Shin-ichi Miyoshi[1]

[1]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Kita-Ku, Okayama 700-8530, Japan

***Corresponding author**

Tomoka Kawase

Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Kita-Ku, Okayama 700-8530, Japan

Tel/Fax: +81-86-251-7968

e-mail: kawas-t@cc.okayama-u.ac.jp

Highlights

A series of DNA fragments encoding the precursor or its domains of *Vibrio vulnificus* protease (VVP) were designed and expressed *in vitro* by using the cell-free translational system.

The N-terminal propeptide in the precursor functioned as an intramolecular chaperon promoting the folding of VVP.

The N-terminal propeptide was unstable and digested easily by the enzymes in the cell lysate used for the cell-free system.

The C-terminal domain in VVP showed to disturb the folding of VVP.

Abstract

Vibrio vulnificus is a human pathogen causing fatal septicemia with edematous and hemorrhagic skin damage. The extracellular metalloprotease termed *V. vulnificus* protease (VVP) is known to be an important toxic factor eliciting the skin damage. VVP (413 aa) is composed of two domains, the N-terminal core domain having the proteolytic activity and the C-terminal domain mediating effective attachment to protein substrates. However, VVP is produced as an inactive precursor (609 aa) with the signal peptide (24 aa) and the propeptide (172 aa). In order to clarify the function of the propeptide, a series of DNA fragments encoding the precursor or its domains were designed, and the proteins were produced *in vitro* by using the cell-free translational system. The results indicated that the propeptide might function as an intramolecular chaperon promoting the folding of both N-terminal and C-terminal domains. However, the propeptide was unstable and digested easily by the enzymes in the cell lysate used for the cell-free system. Additionally, the C-terminal domain in VVP showed to disturb the folding of the N-terminal domain.

Keywords**:** *Vibrio vulnificus*, Protease**,** Propeptide, Domain, Cell-free translational system

Abbreviations:

GFP: green fluorescent protein, PCR: polymerase chain reaction, PVDF: polyvinylidene difluoride, RT-PCR: reverse transcription PCR, RTS: rapid translation system, SDS-PAGE: SDS-polyacrylamide gel electrophoresis, VVP: *Vibrio vulnificus* protease

Introduction

Vibrio vulnificus is a facultative anaerobic bacterium inhabiting in the wide range of aquatic environments from estuarine to marine water (1). However, this species is a human pathogen causing the necrotic wound infections and fatal septicemia with edematous or hemorrhagic skin damage (2). The clinical cases have been reported in many countries including USA, Japan and South Korea (1, 3). In addition, *V. vulnificus* is also known to be responsible for the epidemic and systemic infections in cultured eels (4).

V. vulnificus possess multiple virulence determinants such as polysaccharide capsule, type IV pili, hemolysin and proteases (3, 5). Among them, the 45 kDa metalloprotease termed *V. vulnificus* protease (VVP) is a major determinant of the skin damage (2). VVP is known to induce the edema formation through stimulation of histamine release (2, 6) and activation of the bradykinin-generating cascade (2, 7), and to cause hemorrhage by digestion of type IV collagen in the basal membrane (2, 8).

Although bacterial metalloprotease are divided into several families (9), VVP is known to be a member of the thermolysin family (10). The enzymes in this family are commonly elaborated as an inactive precursor having several domains, such as the N-terminal signal peptide, propeptide and the C-terminal mature enzyme (10), and the propeptide may generally function as an intramolecular chaperone supporting the folding of the mature enzyme (11, 12). In the case of VVP, the precursor is consisted of the N-terminal signal peptide (3 kDa, 24 aa), propeptide (20 kDa, 172 aa), and the C-terminal mature enzyme (45 kDa, 413 aa) (10, 13). Additionally, the mature VVP has two functional domains, the 35 kDa N-terminal domain (VVP-N) and 10 kDa C-terminal domain (VVP-C) (10, 14). The previous functional analysis revealed that the VVP-N domain is a core peptide having the proteolytic activity. On the other hand, the VVP-C domain mediates the efficient association with protein substrate including

type I collagen and elastin (14, 15); however, it has been not studied whether the VVP-C also function as intramolecular chaperone or not.

The cell-free translational system is useful to analyze functions of fragments or domains of an active enzyme or toxins (16). For instance, our group prepared some mutated *V. vulnificus* hemolysin by using the cell-free system and could identify amino acid residues essential for the activity of the toxin (17). In the present study, five DNA fragments encoding the VVP precursor (F-VVP) or its domains (M-VVP, PN-VVP, P-VVP and N-VVP) (Fig. 1) were designed and expressed *in vitro* by using the cell-free translational system. The results showed that the 20 kDa propeptide might function as an intramolecular chaperon promoting the folding of VVP and VVP-N.

Materials and Methods

In vitro *protein production*

The VVP precursor or its domains was produced *in vitro* with the rapid translation system (RTS) using the RTS 100 *E. coli* HY Kit (5 PRIME Inc., Gaithersburg, MD, USA) and the polymerase chain reaction (PCR)-amplified DNA fragments as described (17). To prepare the DNA fragments, in the first PCR, two forward primers and three reverse primers carrying the 20 or 21 bp overlap sequence for the second PCR were designed from the nucleotide sequence of the VVP precursor gene of strain L-180 (GenBank accession number, AB084580). In this nucleotide sequence, the propeptide, VVP-N and VVP-C are corresponded 76-588, 589-1530 and 1531-1830, respectively. The PCR amplification using the bacterial genomic DNA (200 ng) and KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) was carried out for 25 cycles as following: 1 min denaturation at 94 ^{o}C, 1 min annealing at an appropriate temperature, 1.5-3

min extension at 68 °C.

In the second PCR, the sequences for the T7 promoter, His6-tag and spacer peptide were added to the upstream, and the sequence for the T7 terminator was added to the down stream. Namely, the product of the first PCR (100 ng) was mixed with the RTS *E. coli* Linear Template Generation Set and His6-tag, and the admixture was subjected to the second PCR. After denaturation at 94 °C for 4 min, the reaction was carried out for 30 cycles as following: 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 2-3 min extension at 68 °C.

Each of the products of the second PCR was inserted into the plasmid vector pTA2 (Target clone™-Plus-) (Toyobo, Osaka, Japan) and transformed into *Escherichia coli* DH5α. Thereafter, the transformants obtained were cultivated in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl, pH 7.4) containing ampicillin (50 μg/ml) at 37 °C for overnight. After cultivation, each of the hybrid plasmids was purified with the Quantum Prep Plasmid miniPrep (Bio-Rad Laboratories, Hercules, CA, USA).

The purified hybrid plasmid (500 ng in 10 μl) thus obtained was mixed with 40 μl of the RTS 100 *E. coli* HY Kit, and the *in vitro* translation was carried out at 25 °C for 6 hr according to the manufacture's manual. The green fluorescent protein (GFP) was used as a positive control.

Reverse transcription PCR (RT-PCR)

Total RNA (200 ng) was extracted from the crude RTS products by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), added to the Ready-To-Go RT-PCR Kit (GE Healthcare, Buckinghamshire, England) and incubated at 42 °C for 30 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated by heat treatment at 95 °C for 5 min, and the PCR amplification was performed using an appropriate primer set for 30 cycles as follows: 30 sec denaturation at 95 °C, 30 sec annealing at 60 °C, and 2 min extension at 72 °C. Then, the PCR products were electrophoresed on a 1% agarose gel and visualized by staining with

ethidium bromide.

***Purification of the proteins produced* in vitro**

The proteins produced *in vitro* by the RTS were purified with the Capturem His-tagged Purification Miniprep Kit (Takara Bio USA, Mountain View, CA, USA). The crude RTS products (400 μl) were mixed with 400 μl of the binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 6 M urea, pH 7.4) and applied to the spin column equilibrated with the binding buffer. Thereafter, the column was washed twice with 200 μl of the binding buffer, and the proteins bound were eluted with 80 μl of the elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 6 M urea, pH 7.4). This elution step was repeated three times.

Western blotting

The crude RTS products or the purified protein preparations were treated with 10% trichloroacetic acid, and the precipitate was washed with acetone and suspended into the loading buffer containing 2% SDS and 5% 2-mercaptoethanol. This suspension was heat-treated at 100 ^{o}C for 5 min, and an aliquot of the heat-treated sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the PhastSystem using a PhastGel Gradient 10-15 (GE Healthcare).

After SDS-PAGE, the proteins separated were transferred to a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany), and then, the proteins were detected with the rabbit IgG antibody against purified VVP (18) or His6-tag (EnoGene Biotech, New York, NY, USA) followed by the secondary antibody against rabbit IgG, which was conjugated with horseradish peroxidase (Bethy Laboratories, Montgomery, TX, USA).

Protease activity

Azocasein (5 mg/ml) in 50 mM Tris-HCl buffer (pH 8.0) was used as a substrate to measure the protease activity (19). Each of the crude RTS products (20 µl) was allowed to act on azocasein (10 µl) at 30 ˚C for an appropriate period. Thereafter, the reaction was stopped by the addition of 5% trichloroacetic acid (70 µl), and the mixture was centrifuged at 3,000 x *g* for 15 min. The supernatant was withdrawn and mixed with an equal volume of 0.5 M NaOH, and the optical density at 440 nm was measured.

Results and Discussion

In vitro *production of the VVP precursor or its domains*

A total of five DNA fragments encoding the VVP precursor (F-VVP) or its domains (M-VVP, PN-VVP, P-VVP and N-VVP) (Fig. 1) were prepared in the first PCR, and the sequences for the T7 promoter, His6-tag and spacer peptide were added to the upstream, and the sequence for the T7 terminator was added to the down stream in the second PCR. Thereafter, each of the DNA fragments from the second PCR was inserted into the plasmid vector pTA2, and the hybrid plasmids constructed were applied to the RTS for *in vitro* protein production.

The reaction with the RTS was carried out at 25 ºC for 6 hr, and then, total RNA was extracted from each of the crude RTS products. As shown in Fig. 2, the comparative expression of all of the DNA fragments was demonstrated by RT-PCR. The 612 bp amplicom was detected in the samples of F-VVP (lane F), M-VVP (lane M), PN-VVP (lane PN), and N-VVP (lane N), and the 554 bp amplicom was detected in the sample of P-VVP (lane P). However, western blot analysis showed that the crude RTS products from only the F-VVP and PN-VVP contained proteins detectable with the antibody against purified VVP (VVP-N and VVP-C) (Fig. 3). This indicates that the propeptide has an important role in production of the proteins.

The protease activity was also measured, but no crude RTS product was found to have the significant activity (data not shown). Likewise other bacterial metalloproteases in the thermolysin family (9, 20), the active VVP have to contain one zinc ion in the catalytic center (10, 19, 21). Therefore, the proteins produced *in vitro* may not possess the cationic ion, a prosthetic group of the enzyme.

The crude RTS products were applied to a nickel column, and the proteins bound were eluted with imidazole. Western blot analysis with the antibody against VVP revealed that, in addition to F-VVP and PN-VVP, N-VVP was also produced *in vitro* (Fig. 4). However, the amount of N-VVP produced was far less than PN-VVP, indicating that the propeptide can accelerate the folding of the proteins. The results shown in Fig. 4 also indicate negligible production of M-VVP, which suggest that the propeptide may also contribute the folding of the C-terminal domain (VVP-C) but VVP-C itself may disturb the folding process of M-VVP.

In vitro *processing of the VVP precursor or its domains*

The molecular weights of F-VVP and PN-VVP produced *in vitro* were estimated 45 kDa and 35 kDa, respectively (Fig. 4). Thus, it was speculated that the proteolytic enzymes in the *E. coli* lysate used for the RTS had digested the propeptide after the folding of the proteins. To clarify this point, by using the antibody against the His6-tag, western blot analysis of the proteins was carried out (Fig. 5). As expected, neither F-VVP nor PN-VVP was found to have the His6-tag in the molecule. In addition, the His6-tag was not detected in P-VVP, indicating the proteolytic digestion of the propeptide during *in vitro* production with the RTS. As well as F-VVP and PN-VVP, N-VVP was also found to have lost the His6-tag during *in vitro* production (Fig. 5). This suggests the proteolytic enzymes in the *E. coli* lysate can also cleave the spacer peptide.

The results shown in Fig. 5 demonstrated that the His6-tag free proteins could bind to a

nickel column. The zinc ion in the catalytic center of VVP is possible to be substituted with other cationic ions including a nickel ion (21). Therefore, the active centers of the proteins produced *in vitro* might have potentials to associate with a nickel ion of the resin in the column.

VVP is elaborated as an inactive precursor constituted of several domains, and it is activated through removal of the signal peptide and propeptide (10). Previous study by Chang et al. (13) revealed that the propeptide worked as an inhibitor/substrate for VVP, indicating the direct interaction of the propeptide with VVP. The present study clearly showed that the propeptide could function as an intramolecular chaperon promoting the folding of VVP. VVP-C, the C-terminal 10 kDa domain of VVP, is known to bind to various protein substrates or cell membrane. Therefore, VVP-C can accelerate the biological actions of VVP-N including the proteolytic action to type I collagen, type IV collagen and elastin, and the biological actions including bradykinin generation, histamine release and hemagglutination (2, 6, 14, 15, 21). However, the present study indicated that VVP-C might disturb the folding of VVP-N. Namely, production of M-VVP (VVP-N and VVP-C) was not observed, in spite of detectable production of N-VVP (VVP-N). On the other hand, the findings of the present study suggested the propeptide also supported the folding of VVP-C domain.

In conclusion, the propeptide in the VVP precursor may be an intramolecular chaperon to promote the folding of both VVP-N and VVP-C domain.

Acknowledgement

This research is partially supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Ministry of Education, Culture, Sport, Science and Technology in Japan (MEXT) and Japan Agency for Medical Research and Development (AMED).

References

1. S. Chakraborty, G.B. Nair and S. Shinoda. Pathogenic vibrios in the natural environment. Rev. Environ. Health. 12 (1997) 63-80.
2. S. Miyoshi. *Vibrio vulnificus* infection and metalloprotease. J. Dermatol. 33 (2006) 589-595.
3. M.K. Jones and J.D. Oliver. *Vibrio vulnificus*: diseases and pathogenesis. Infect. Immun. 77 (2009) 1723-1733.
4. D.L. Tison, M. Nishibuchi. J.D. Greenwood and R.J. Seidler. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. 44 (1982) 640-646.
5. M.S. Strom and R.N. Paranjpye. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microb Infect. 2 (2000) 177-188.
6. S. Miyoshi, K. Kawata, M. Hosokawa, K. Tomochika and S. Shinoda. Histamine-releasing reaction induced by the N-terminal domain of *Vibrio vulnificus* metalloprotease. Life Sci. 72 (2003) 2235-2242.
7. S. Miyoshi, H. Watanabe, T. Kawase, H. Yamada and S. Shinoda. Generation of active fragments from human zymogens in the bradykinin-generating cascade by extracellular proteases from *Vibrio vulnificus* and *V. parahaemolyticus*. Toxicon. 44 (2004) 887-893.
8. S. Miyoshi, H. Nakazawa, K. Kawata, K. Tomochika, K. Tobe and S. Shinoda. Characterization of the hemorrhagic reaction caused by *Vibrio vulnificus* metalloprotease, a member of the thermolysin family. Infect. Immun. 66 (1998) 4851-4855.
9. N.M. Hooper. Families of zinc metalloproteases. FEBS Lett. 354 (1994) 1-6.
10. S. Miyoshi and S. Shinoda. Microbial metalloproteases and pathogenesis. Microb Infect. 2

(2000) 91-98.

11. M.J. O'Donohue and A. Beaumont. The roles of the prosequence of thermolysin in enzyme inhibition and folding *in vitro*. J. Biol. Chem. 271 (1996) 26477- 26481.

12. K.S. Mclver, E. Kessler and D.E. Ohman. Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities. Microbiology, 150 (2004) 3969-3977.

13. A.K. Chang, J.W. Park, E.H. Lee and J.S. Lee. The N-terminal propeptide of *Vibrio vulnificus* extracellular metalloprotease is both an inhibitor of and a substrate for the enzyme. J. Bacteriol. 189 (2007) 6832-6838.

14. S. Miyoshi, H. Wakae, K. Tomochika and S. Shinoda. Functional domains of a zinc metalloprotease from *Vibrio vulnificus*. J. Bacteriol. 179 (1997) 7606-7609.

15. J.H. Yun, H. Kim, J.E. Park, J.S. Lee and W. Lee. Solution structure and dynamics of C-terminal regulatory domain of *Vibrio vulnificus* extracellular metalloprotease. Biochem. Biophys. Res. Commun. 430 (2013) 541-546.

16. G. Rosenblum and B.S. Cooperman. Engine out of chassis: cell-free protein synthesis and its uses. FEBS Lett. 588 (2014) 261-268.

17. S. Miyoshi, Y. Abe, M. Senoh, T. Mizuno, Y. Maehara and H. Nakao. Inactivation of *Vibrio vulnificus* hemolysin through mutation of the N- or C-terminus of the lectin-like domain. Toxicon. 57 (2011) 904-908.

18. A. Elgaml and S. Miyoshi. Role of the histone-like nucleoid structuring protein (H-NS) in the regulation of virulence factor expression and stress response in *Vibrio vulnificus*. Biocontrol Sci. 20 (2015) 263-274.

19. N. Miyoshi, C. Shimiza, S. Miyoshi and S. Shinoda. Purification and characterization of *Vibrio vulnificus* protease. Microbiol. Immunol. 31 (1987) 13-25.

20. C.C. Häse and R.A. Finkelstein. Bacterial extracellular zinc-containing metalloproteases.

1 Microbiol. Rev. 57 (1993) 823-837.

2 21. S. Miyoshi, K. Kawata, K. Tomochika and S. Shinoda. The hemagglutinating action of

3 *Vibrio vulnificus* metalloprotease. Microbiol. Immunol. 43 (1999) 79-82.

Figure and legends

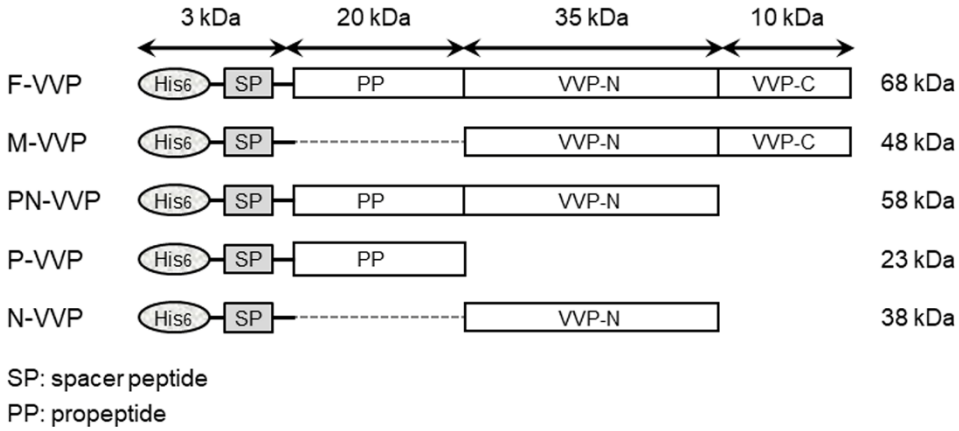


Fig. 1. The VVP precursor (F-VVP) and its domains (M-VVP, PN-VVP, P-VVP and N-VVP) designed for the RTS.

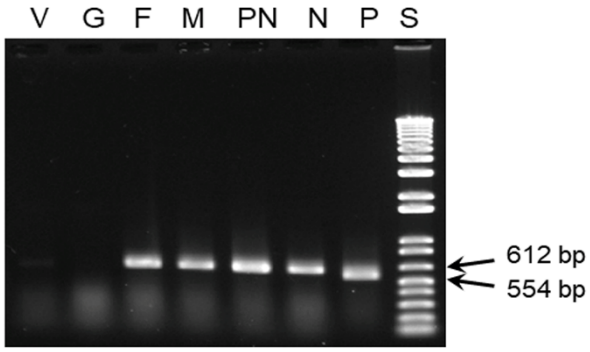


Fig 2. Agarose gel electorophoresis of the RT-PCR products. Each of the DNA fragments after the second PCR are inserted into the plasmid vector pTA2, and the hybrid plasmids constructed were applied to the RTS for *in vitro* protein production. The reaction with the RTS was carried out at 25 ^{o}C for 6 hr, and then, total RNA was extracted from each of the crude RTS products. Thereafter, RT-PCR was carried out, and the products were electropforesed on 1.0 % agarose gel. Lane V: pTA2 vector, lane G: GFP, lane F: F-VVP, lane M: M-VVP, lane PN: PN-VVP, lane N: N-VVP, lane P: P-VVP, and Lane S: 1 kb plus DNA ladder 100-12,000 bp.

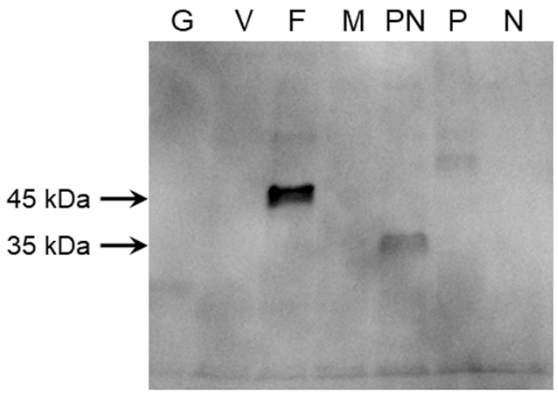


Fig 3. Western blot analysis of the crude RTS products with antibody against VVP. The crude RTS products were treated with 2% SDS and 5% 2-mercaptoethanol at 100 °C for 5 min and subjected to SDS-PAGE. Thereafter, the proteins separated were transferred to a PVDF membrane and detected with the rabbit IgG antibody against purified VVP. Lane G: GFP, lane V: pTA2 vector, lane F: F-VVP, lane M: M-VVP, lane PN: PN-VVP, lane P: P-VVP, and lane N: N-VVP.

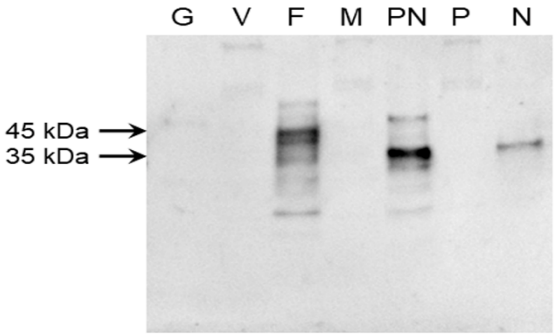


Fig 4. Western blot analysis of the purified proteins with antibody against VVP. The proteins produced *in vitro* by RTS were purified with the Capturem His-tagged Purification Miniprep Kit. Then, the proteins were treated with 2% SDS and 5% 2-mercaptoethanol at 100 °C for 5 min and subjected to SDS-PAGE. Thereafter, the proteins separated were transferred to a PVDF

membrane and detected with the rabbit IgG antibody against purified VVP. Lane G: GFP, lane V: pTA2 vector, lane F: F-VVP, lane M: M-VVP, lane PN: PN-VVP, lane P: P-VVP, and lane N: N-VVP.

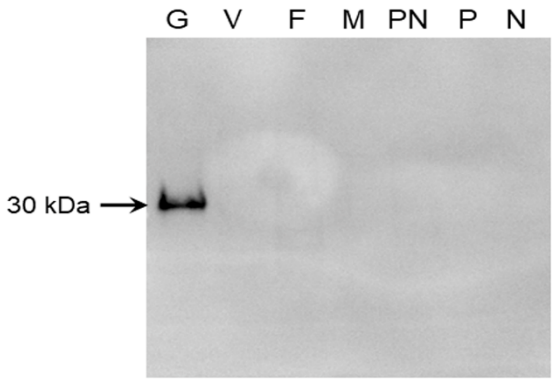
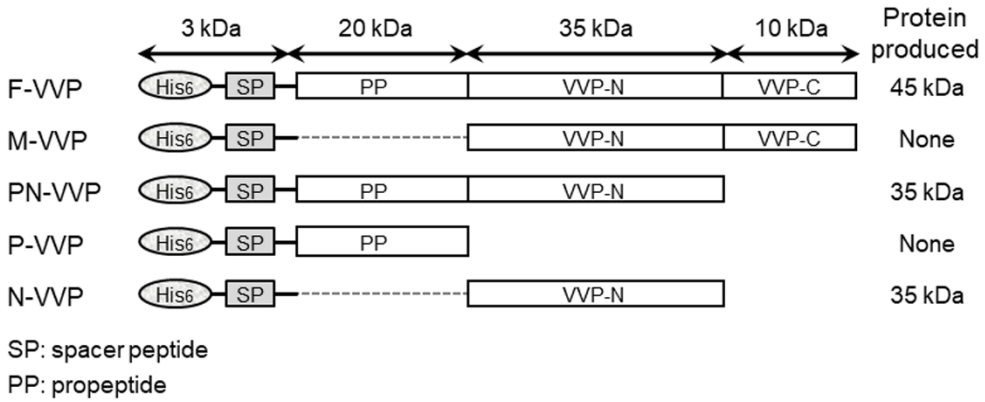


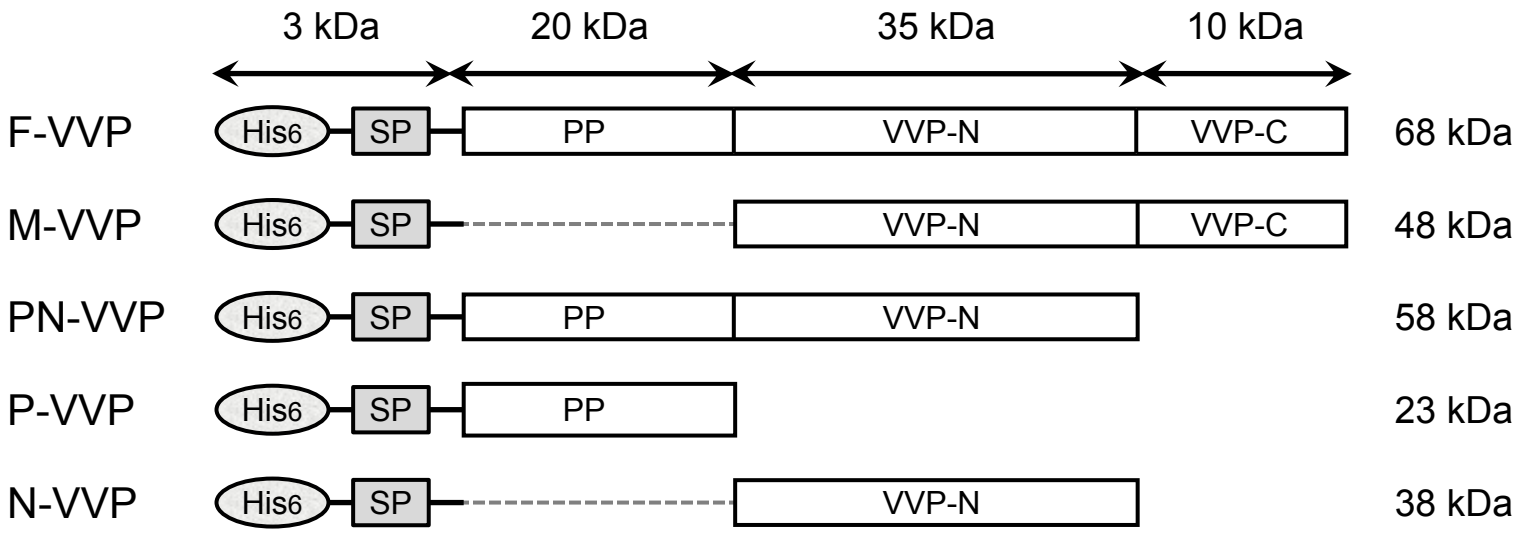
Fig 5. Western blot analysis of the purified proteins with antibody against His6-tag. The proteins produced *in vitro* by RTS were purified with the Capturem His-tagged Purification Miniprep Kit. Then, the proteins were treated with 2% SDS and 5% 2-mercaptoethanol at 100 ^{o}C for 5 min and subjected to SDS-PAGE. Thereafter, the proteins separated were transferred to a PVDF membrane and detected with the rabbit IgG antibody against His6-tag. Lane G: GFP, lane V: pTA2 vector, lane F: F-VVP, lane M: M-VVP, lane PN: PN-VVP, lane P: P-VVP, and lane N: N-VVP.

Graphical Abstract
(Kawase et al.)



1

Figure 1
(Kawase et al.)



SP: spacer peptide

PP: propeptide

Figure 2
(Kawase et al.)

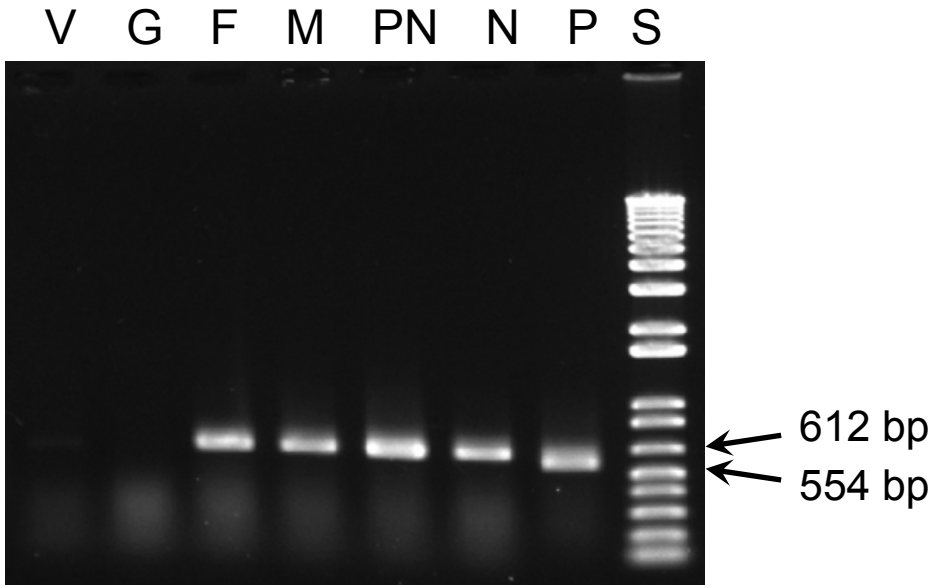


Figure 3
(Kawase et al.)

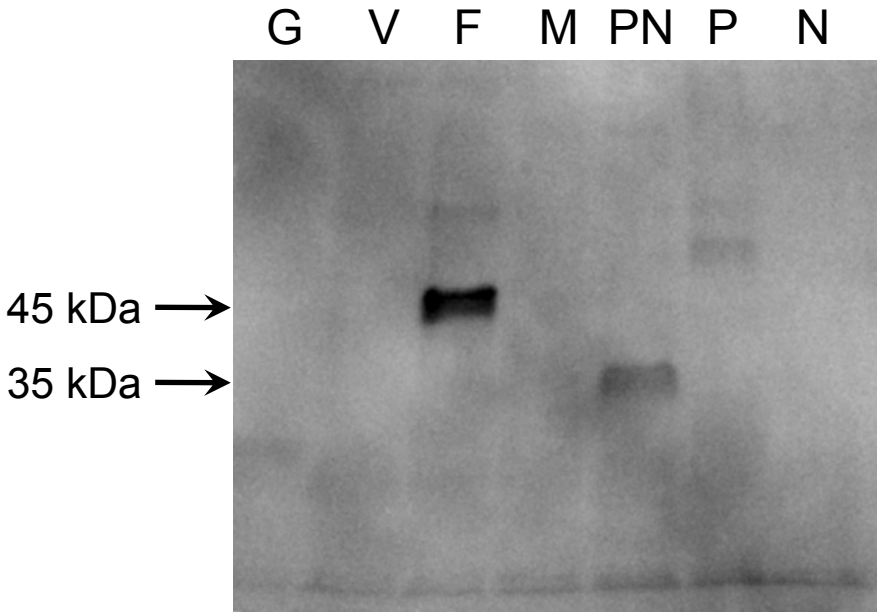


Figure 4
(Kawase et al.)

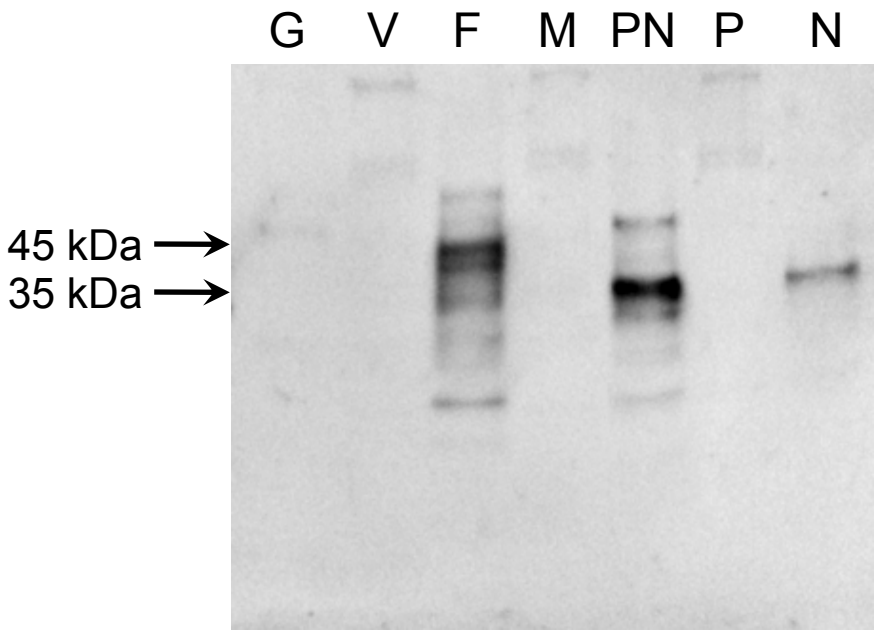
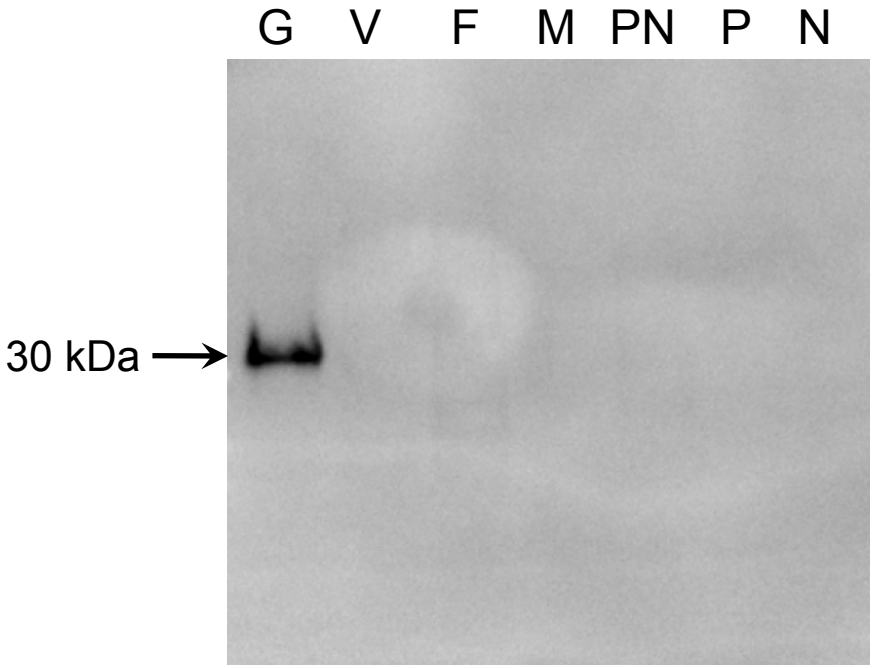
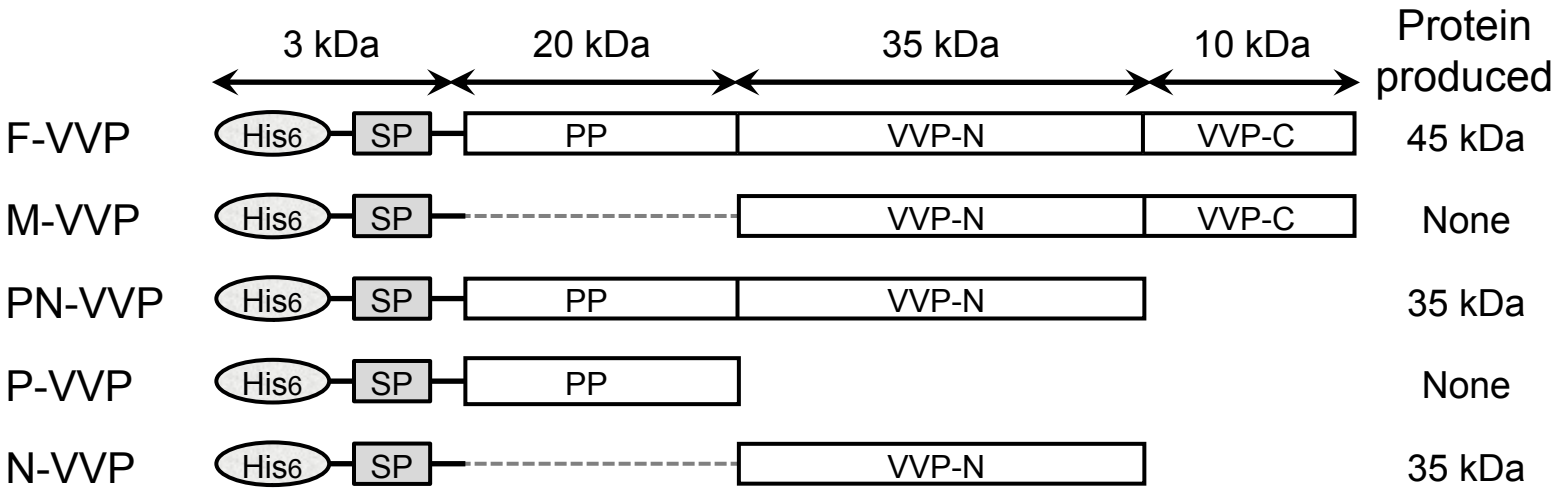


Figure 5
(Kawase et al.)



Graphical Abstract
(Kawase et al.)



SP: spacer peptide

PP: propeptide