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メタデータ	言語: eng 出版者: 公開日: 2022-08-17 キーワード (Ja): キーワード (En): 作成者: Kato, Tatsuya, Kakuta, Tatsuki, Yonezuka, Ami, Sekiguchi, Tomofumi, Machida, Yuki, Xu, Jian, Suzuki, Tohru, Park, Enoch Y. メールアドレス: 所属:
URL	http://hdl.handle.net/10297/00029087

Expression and purification of porcine rotavirus structural proteins in silkworm larvae as a vaccine candidate

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ABSTRACT

In this study, silkworm larvae were used for expression of porcine rotavirus A (KS14 strain) inner capsid protein, VP6, and outer capsid protein, VP7. Initially, VP6 was fused with Strep-tag II and FLAG tag (T-VP6), and T-VP6 was fused further with the signal peptide of *Bombyx mori* 30k6G protein (30k-T-VP6). T-VP6 and 30k-T-VP6 were then expressed in the fat body and hemolymph of silkworm larvae, respectively, with respective amounts of 330 μg and 50 μg per larva of purified protein. Unlike T-VP6, 30k-T-VP6 was *N*-glycosylated due to attached signal peptide. Also, VP7 was fused with PA-tag (VP7-PA). Additionally, VP7 was fused with Strep-tag II, FLAG-tag, and the signal peptide of *Bombyx mori* 30k6G protein (30k-T-ΔVP7). Both VP7-PA and 30k-T-ΔVP7 were expressed in the hemolymph of silkworm larvae, with respective amounts of 26 μg and 49 μg per larva of purified protein, respectively. The results from our study demonstrated that T-VP6 formed nanoparticles of greater diameter compared with the ones formed by 30k-T-VP6. Also, higher amount of VP6 expressed in silkworm larvae reveal that VP6 holds the potential for its use in vaccine development against porcine rotavirus with silkworm larvae as a promising host for the production of such multi-subunit vaccines.

Keywords Porcine rotavirus, VP6, VP7, Silkworm larvae, Vaccine

Introduction

Rotaviruses are non-enveloped viruses of the family Reoviridae that are major causes of acute gastroenteritis in infants and some species of animals [**1**]. Rotaviruses have 11 segments of double-stranded RNA (ds-RNA) as their genome, encoding several structural (VP1, 2, 3, 4, 6, 7) and non-structural proteins (NSP1, 2, 3, 4, 5, 6). The viral genome is enclosed within a triple-layered capsid, which is composed of VP2, VP6, and VP7 proteins. Additionally, VP4, a viral hemagglutinin, has been found to form spikes that project from the outer layer of VP7, a viral capsid protein. Both VP4 and VP7 are known to facilitate binding of the mature viral particles to the cell surface receptors of the host cells [**2, 3**].

Recently, live attenuated oral vaccines (Rotarix, RotaTeq, etc.) have become available for human use. They are being extensively used globally, owing to their high efficiency in providing protection against rotavirus diseases. However, instances of their lower efficiency have occurred sporadically in some countries [**4, 5**] because children in low and middle-income countries suffer from reduced immune responses, malnutrition and maternal antibodies, and the oral administration of vaccines are easily influenced by gut microbial environment, gastric acidity and breast milk antibodies [**6**]. Moreover, the reassortment of live attenuated vaccines of the wild type is often challenging for safety reasons. Therefore, developing other effective rotavirus vaccines through different strategies, such as using viral subunits or virus-like particles (VLPs), can prove to be better alternatives to the currently available live attenuated vaccines. Recently, P2-VP8 vaccine, which is composed of rotavirus VP8 fused with P2 $CD4^+$ epitope from tetanus toxin, has been developed in *Escherichia coli* expression system as an injectable vaccine and several clinical trials have also been done [**7–9**].

Previous studies have shown rotaviral capsid protein, VP6, as a potential candidate for the vaccine development [**10**]. VP6 is a major capsid protein that covers the inner capsid that is composed of VP2. The double-layered capsid particles are structurally formed when VP6 and VP2 are co-expressed in insect and plant cells [**3, 11**]. However, a single VP6 protein is produced in these hosts, while the *Escherichia coli* system produces VP6 nanoparticles or nanotubes [**12–14**]. Immunization of mice with tubular VP6, results in the production of VP6-specific IgG and IgA, as well as interferon-γ-secreting $CD4^+$ T cells in their mucosal cells and serum [**15**]. The tubular form of VP6 was seen to possess an immunostimulatory effect, similar to that of an adjuvant, as observed in the RAW 264.7 macrophage cell line [**16**].

According to some reports, immunization with VP6 expressed in plants as an oral vaccine, led to higher serum titers of VP6-specific IgG and saliva mucosal VP6-specific IgA [**17**]. Additionally, several plants (potato, corn seed, etc.) have been successfully used as hosts to express rotavirus structural proteins that can act as oral vaccines [**18, 19**]. These results demonstrate that plants are promising hosts for the preparation of rotavirus subunit vaccines. Besides plants, silkworm (*Bombyx mori*) has also been shown as a promising host for producing a subunit vaccine using a baculovirus expression system [**20, 21**]. Silkworm larvae and pupae are known to produce recombinant proteins, and a large-scale production can be effortlessly carried out by increasing the number of silkworms. Yao et al. reported that VP2, VP6, and VP7 of rotavirus could be simultaneously expressed using a multi-gene baculovirus expression system, such as MultiBac in silkworm larvae (BmMultiBac). The expressed proteins when combined, could form round nanoparticles in the hemocytes of silkworm larvae [**22**].

This study focused on the production of VP6 and VP7 proteins from porcine rotavirus A (KS14 strain), which is associated with swine diarrhea. Our aim is the development of vaccines to porcine rotavirus A because no commercial vaccine to porcine rotavirus A are available. Now, some live attenuated oral vaccines (Rotarix, RotaTeq, etc.) are available for human, but non-live rotavirus vaccines, for example, subunit vaccines, are demanded [**23**]. To develop vaccines to porcine rotavirus A, we try to prepare purified VP6 and VP7 in silkworm larvae as subunit vaccines. An endogenous signal peptide in silkworm, 30 kDa lipoprotein (30k6G), present as a storage protein in its hemolymph, was attached to the two VP proteins to enhance protein purification from silkworm serum [**24**]. Subsequently, the expression patterns and the resulting particle formation of purified VP6 and VP7 proteins, with or without the signal peptide, were compared and investigated. The results from the current study show that a high amount of non-glycosylated VP6 protein is obtained from silkworm larvae. Moreover, well-formed VLPs were obtained in a silkworm-based baculovirus expression system that show potential of VP6 as a vaccine candidate against porcine rotavirus in future.

Materials and Methods

Insect Cells and Silkworm Larvae

Initially, Bm5 cells were maintained at 27°C in Sf-900II medium (Thermo Fisher Scientific K. K., Tokyo, Japan) supplemented with 1% antimycotic-antibiotic solution (Thermo Fisher Scientific K. K.) and 10% fetal bovine serum (Sigma Aldrich Japan, Tokyo, Japan). The fifth instar silkworm larvae were purchased from Ehimesansyu (Ehime, Japan), and reared on an artificial diet, Silkmate S2 (Nosan, Yokohama, Japan).

Construction of Recombinant Plasmids and Baculoviruses

First, the *VP6* gene of the porcine rotavirus A (KS14 strain) was amplified by PCR for its subsequent expression of VP6 (Table 1). Primer sets Rota-VP6-F and Rota-VP6-R were used in PCR. Next, the amplified gene was inserted into pFastbac:L21>30k6G (±)-Flag-Strep-tag II-TEV-Spytag002-StuI, downstream of the Spytag002 sequence [**25**]. Each resulting plasmid was then transformed into *E. coli* BmDH10Bac to construct a recombinant *B. mori* nucleopolyhedrovirus (BmNPV) containing the VP6 gene [**26**]. VP6 was then attached with the signal peptide of 30k6G protein that encodes a 30 kDa lipoprotein in silkworms [**27**], to obtain 30k-T-VP6 (Fig. 1). 30k-T-VP6 was expressed in silkworm larvae using recombinant BmNPV.

VP6 was also expressed without a signal sequence at its N-terminus (T-VP6, Fig. 1) and was similar to the native form of VP6. PCR was performed using the primer set, Rota-VP6-F2 and Rota-VP6-R2 (Table 1). The *VP6* gene thus obtained from PCR did not have sequence encoding the signal peptide of the silkworm. The amplified DNA after phosphorylation were allowed to self-ligate. Each resulting plasmid was transformed into *E. coli* BmDH10Bac cell to construct a recombinant BmNPV.

The *VP7* gene of rotavirus A (KS14 strain) was also used to construct recombinant BmNPV for VP7 by following the above protocol. First, the primer set Rota-VP7-F and Rota-VP7-R (Table 1) was used to amplify the *VP7* gene, and the amplified gene was inserted into pFastbac:L21>30k6G(±)-Flag-Strep-tag II-TEV-Spytag002-StuI at the downstream of the Spytag002 sequence. VP7 was also attached to the signal peptide of 30k6G protein to obtain 30k-T-ΔVP7. Now, the *VP7* gene encoding its soluble domain (30k-T-ΔVP7, Fig. 1) was inserted into the vector to express VP7 in its soluble form.

Finally, the constructed plasmids were transformed into *E. coli* BmDH10Bac cells to produce recombinant BmNPV.

Additionally, the *VP7* gene containing the PA-tag sequence encoding VP7-PA (Fig. 1) was amplified via PCR using a primer set, Rota-VP7-F2 and Rota-VP7-PA-R (Table 1) to express full-length VP7. The amplified gene was inserted into pFastbac1 (Thermo Fisher Scientific K. K.), and the resulting plasmid was transformed into *E. coli* BmDH10Bac to construct recombinant BmNPV.

The extracted recombinant BmNPVs were transfected into Bm5 cells to prepare recombinant BmNPV using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer's protocol. P3 viral solution was used to infect Bm5 cells and silkworm larvae.

Expression of Each Protein in Insect Cells and Silkworm Larvae

First, recombinant baculovirus solutions were used to infect Bm5 cells in 6-well plates and were injected into silkworm larvae. Next, the suspension of insect cells was centrifuged at 20,000 × *g* to isolate the culture supernatant from cell cultures. Further, the cell pellets were suspended in the same volume of phosphate-buffered saline (PBS, pH 7.4) as the culture supernatant and then sonicated to disrupt the cells. Finally, centrifugation was performed at 20,000 × *g* to separate the soluble from the insoluble fraction.

Purification of Each Protein Using Affinity Gels

T-VP6, 30k-T-VP6, and 30k-T-ΔVP7 were purified from silkworm hemolymph or the supernatant of the fat body homogenate using Strep-Tactin Sepharose (IBA GmbH,

Göttingen, Germany). First, the hemolymph was diluted 10-fold with ST buffer (100 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 8.0) and loaded onto a Strep-Tactin Sepharose column equilibrated with PBS. Next, the resin was washed with a 20-bed volume of ST buffer, and the proteins were eluted NaOH (0.1 N). The eluent was then quickly neutralized with 2 M glycine-HCl buffer when NaOH was used. The fat body was first suspended in ST buffer and disrupted via sonication during T-VP6 purification. The homogenate was then centrifuged at 10,000 × *g* to obtain the soluble fraction for further protein purification.

VP7-PA was purified from silkworm hemolymph using Anti PA-tag antibody beads (FUJIFILM Wako Chemicals, Osaka, Japan), according to the previous paper [**28**]. First, the hemolymph was diluted 10-fold with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5), loaded onto Anti PA-tag antibody beads, and equilibrated with ST buffer. Next, a 20-bed volume of TBS was used to wash the resin, and the target proteins were separated using 0.1 M glycine-HCl buffer (pH 3.0). The eluent was quickly neutralized with 1M Tris-HCl buffer (pH 9.0). Finally, the Pierce BCA Protein Assay kit (Thermo Fisher Scientific K.K.) was used to measure protein concentration following the manufacturer's instructions.

Each recombinant protein was purified from dozens of silkworm larvae several times and confirmed these properties.

Deglycosylation of Purified Protein

Briefly, glycopeptidase F (PNGase F; Takara Bio, Otsu, Japan) was used to remove *N*-glycans from the purified protein. The purified proteins were treated with PNGase F

187 under denaturing conditions, following the manufacturer's instructions. Finally, western
188 blotting was performed to analyze the purified proteins after the treatment.
189
190 **Centrifugation of Purified VP7-PA and 30k-T-ΔVP7**
191 First, purified VP7 (2 mL) was centrifuged at 122,000 × *g* for 1 h on a 20% sucrose
192 cushion (0.8 mL). Then, 1 mL (total 2 mL) of the sample fraction was collected,
193 followed by collection of a 20% sucrose fraction (0.8 mL). The pellets were then
194 suspended in 100 μL of PBS. Finally, the proteins in each fraction were analyzed by
195 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
196
197 **SDS-PAGE and Western Blot**
198 Briefly, SDS-PAGE was performed using 10% or 12% polyacrylamide gels. The
199 proteins were first stained with Coomassie Brilliant Blue. They were then transferred
200 from a gel onto a polyvinylidene fluoride membrane using the Mini Trans-Blot
201 Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) for western blot. Next, the
202 membrane was incubated with 10,000-fold diluted anti-strep-Tag II (MEDICAL &
203 BIOLOGICAL LABORATORIES, Nagoya, Japan) in TBS-T at room temperature for 1
204 h after blocking with 5% skim milk in TBS-Tween 20 (TBS-T, pH 7.6). A 10,000-fold
205 diluted goat anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody
206 (Medical & Biological Laboratories) was then used. Finally, specific proteins were
207 detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore,
208 Billerica, MA, USA) and a Fluor-S Max Multi-Imager (Bio-Rad).
209
210 **Transmission Electron Microscopy**

211 Briefly, the samples were placed onto a grid with a support film (Nisshin EM, Tokyo,
212 Japan) and negatively stained with 2.5% phosphotungstic acid. Further, transmission
213 electron microscopy (TEM) was performed using a transmission electron microscope
214 (JEM-2100F, JEOL, Ltd., Tokyo, Japan) operated at 100 kV.
215

216 # Results

217 ## Expression of VP6 and VP7 in Silkworm Larvae

218 Rotavirus structural protein, VP6, has been previously purified from the culture
219 supernatant and lysed insect cells and plants [**12, 13, 15, 29**]. In our study, we used a 30
220 kDa lipoprotein signal peptide, 30k6G, obtained from silkworm. VP6 attached to the
221 signal peptide and linked with FLAG-tag and strep-tag II (30k-T-VP6) was expressed to
222 facilitate the purification of recombinant VP6 protein in silkworm larvae. This signal
223 peptide permits the efficient secretion of expressed proteins into the hemolymph of
224 silkworm larvae [**24**]. Similarly, VP7 attached to the signal peptide and linked to FLAG-
225 tag and strep-tag II, was expressed with 30k-T-ΔVP7 (Fig. 1). The two hydrophobic
226 domains of VP7 at the N-terminus were removed for efficient secretion into the
227 hemolymph. Additionally, full-length VP7 linked with the PA-tag at its C-terminus was
228 expressed.

229 Fig. 2a illustrates the successful expression of T-VP6 and 30k-T-VP6 in silkworm
230 larvae. T-VP6 was detected in the soluble and insoluble fractions of the infected fat
231 body tissue, but not in the hemolymph. Conversely, 30k-T-VP6 was observed in the fat
232 body fractions as well as in hemolymph. These results show that the 30k6G signal
233 peptide aided the efficient expression of VP6 in the silkworm hemolymph. Also, VP7-
234 PA and 30k-T-ΔVP7 were expressed in both fat body and hemolymph fractions of

235 silkworm larvae (Fig. 2b). Interestingly, despite VP7-PA possessing two hydrophobic
236 domains at its N-terminus, it was correctly secreted into the hemolymph [30].
237
238 **Purification of VP6 and VP7 from Silkworm Larvae**
239 Affinity chromatography was used to purify T-VP6 and 30k-T-VP6 from the soluble
240 fractions of the fat body homogenate and hemolymph, respectively (Fig. 3a). The
241 molecular weight of 30k-T-VP6 was slightly higher than that of T-VP6; however, it was
242 seen as a band at approximately 45 kDa on SDS-PAGE. It was observed that the
243 expressed 30k-T-VP6 migrated to the secretory pathway of the host cells and was then
244 secreted into the hemolymph due to the presence of the 30k6G signaling peptide in
245 VP6. After PNGase F treatment, the molecular weight of 30k-T-VP6 was reduced, as
246 detected by a shift in band position on SDS-PAGE, whereas that of T-VP6 was
247 unchanged (Fig. 3b). These results indicate that *N*-glycosylation contributes to higher
248 molecular weight of the 30k-T-VP6 product via glycosylation pathway in the silkworm.
249 Moreover, VP6 is a major capsid protein of rotavirus and is known to form
250 nanoparticles or nanotubes [**12, 13**]. Therefore, we investigated the morphology of
251 purified VP6 fractions using TEM. Figure 3c shows that T-VP6 formed nanoparticles
252 with greater diameters compared to nanoparticles formed by 30k-T-VP6. These results
253 also indicate that *N*-glycosylation of 30k-T-VP6 inhibited the formation of the VLPs of
254 VP6. Furthermore, the amounts of T-VP6 and 30k-T-VP6 obtained upon purification
255 were 330 and 50 μg protein per larva, respectively. Totally, 37 mg of T-VP6 and 1.9 mg
256 of 30k-T-VP6 were purified from 112 and 37 silkworm larvae, respectively.
257 Further in our procedure, affinity chromatography was used to purify VP7-PA and
258 30k-T-ΔVP7 from the hemolymph of silkworms. SDS PAGE analysis of VP7-PA and

30k-T-ΔVP7 show bands of purified protein at approximately 40 kDa (Fig. 4a). After PNGase F treatment, deglycosylated bands of each purified VP7 were detected at a position corresponding to lower molecular weight (Fig. 4b). This result indicates that both VP7-PA and 30k-T-ΔVP7 were *N*-glycosylated within the silkworm expression system. VP7 is an outer layer protein of rotaviruses, localized in the endoplasmic reticulum (ER) [**31, 32**]. This study showed that full-length native VP7, VP7-PA, and 30k-T-ΔVP7 were secreted into the hemolymph in silkworm larvae through the secretory pathway. The purified VP7 proteins were centrifuged in a 20% sucrose cushion to investigate VP7 variants (Fig. 5a). Remarkably, VP7-PA was observed in the 20% sucrose fraction, whereas 30k-T-ΔVP7 was not seen. This result indicates that VP7-PA is morphologically different from 30k-T-ΔVP7. However, nanoparticles were not observed in either sample when examined by TEM (Fig. 5b). Corresponding to the previous studies, the results of our study show that unlike VP6, VP7 does not form ordered structures in the absence of Ca^{2+} ions [**33**]. Furthermore, the amounts of the purified VP7-PA and 30k-T-ΔVP7 reached 26 and 49 μg per larva, respectively. Totally, 0.40 mg of VP7-PA and 27 mg of 30k-T-ΔVP7 were purified from 15 and 545 silkworm larvae, respectively.

Discussion

In this study, T-VP6 and 30k-T-VP6 were successfully purified from the fat body and hemolymph of silkworm larvae, respectively (Fig. 3a). The deglycosylation assay confirmed that 30k-T-VP6 obtained from the silkworm expression system was *N*-glycosylated (Fig. 3b). In addition, T-VP6 formed nanoparticles with greater diameters compared with nanoparticles formed by 30k-T-VP6 (Fig. 3c). Therefore, these results

283 suggest that introducing *N*-glycosylation of VP6 via the silkworm glycosylation
284 pathway impedes the formation of VP6-derived VLPs. Previous reports indicate that
285 when the VP1 of mouse polyomavirus is expressed with aberrant *N*-glycosylation in
286 insect cells, VP1 remains in a monomeric form and only little formation of VLPs takes
287 place [**34**]. It has also been reported earlier that although, adeno-associated virus type 2
288 has three putative *N*-glycosylation sites, its capsid protein remains unglycosylated [**35**].
289 These results show that *N*-glycosylation of viral capsid proteins is undesirable for the
290 formation of viral particles in a virus-dependent manner. As a subunit vaccine, co-
291 administration of the subunit vaccine with some adjuvants are needed for the induction
292 of immune system in vivo to prevent the infectious-viral infection. Whereas, in the case
293 of virus-like particles, the co-administration with any adjuvant is not required because
294 virus-like particles have almost the same morphology of viruses and can solely induce
295 the host's immune system. In this study, T-VP6 form nanoparticles with diameter of
296 several dozens of nanometers such as a virus-like particle. T-VP6 is promising as a
297 virus-like particle vaccine to porcine rotavirus.
298 This study further demonstrated that T-VP6 and 30k-T-VP6 were purified at 330 and
299 50 μg protein per larva, respectively. However, the production of VP7-PA and 30k-T-
300 ΔVP7 resulted in a lower amount of 26 μg and 49 μg protein per larva, respectively. In
301 previous reports, the amount of simian rotavirus SA11 VP6 protein quantified using
302 enzyme-linked immunoassay after purification was found to be as high as 58 μg/mL in
303 the culture supernatant of insect cells [**12**]. According to some previous reports,
304 amounts as high as 400 μg/larva were obtained in *Spodoptera frugiperda*, when VP2/6
305 particles were purified by $CsCl_2$ density gradient centrifugation [**36**]. Here double and
306 triple-layered VLPs were seen [**36**]. Conversely, the amounts of VP2/6/7 particles was

307 12.7 μg/larva after purification using $CsCl_2$ density gradient centrifugation in silkworm
308 larvae [**25**]. The amounts of T-VP6 and 30k-T-VP6 obtained in this study were similar
309 to those in previous reports. Therefore, obtaining more purified proteins is possible by
310 scaling up the number of infected silkworms for potential applications in future vaccine
311 development.
312 *VP7* gene has a sequence encoding two hydrophobic domains (H1 and H2) at the N-
313 terminus of its product; however, the sequence encoding the first hydrophobic domain
314 (H1) is not translated [**31, 32**]. H2 works as a signal peptide that is removed during
315 entry into the ER. Notably, VP7 has no hydrophobic domains except H1 and H2.
316 Therefore, theoretically, it should be secreted extracellularly after the removal of H2.
317 However, VP7 is retained in the mammalian ER because of the Ile-9, Thr-10, and Gly-
318 11 sequences at the N-terminus [**32**]. This study showed the expression of VP7s in the
319 hemolymph as a secretory protein despite the presence of Ile-9, Thr-10, and Gly-11
320 sequences in the VP7s (Fig. 2). Therefore, these results show that silkworm cells have
321 diverse mechanisms for retaining mammalian cell proteins in the ER, warranting further
322 investigation.
323 In this study, T-VP6, which forms nanoparticles, and 30k-T-VP6, which does not
324 form nanoparticles, were purified from fat body and hemolymph in silkworm larvae,
325 respectively. We have to dissect silkworm larvae and collect fat body when we purified
326 nanoparticles based on VP6, whereas 30k-T-VP6 can be purified from hemolymph
327 collected from silkworm larvae easily. Collection of fat body from silkworm larvae is
328 more laborious than that of hemolymph. However, the amount of T-VP6 purified from
329 fat body is higher than that of 30k-T-VP6 purified from hemolymph.
330

Conclusion

VP6 and VP7 of porcine rotavirus A KS14 were expressed in silkworm larvae and purified from fat body and hemolymph. Especially, 330 μg of T-VP6, which has with Strep-tag II and FLAG tag at its N-terminus, was purified from a larva and formed nanoparticles with diameter of several dozens of nanometers. *N*-glycosylation in VP6 inhibited the formation of its nanoparticles. VP7-PA and 30k-T-ΔVP7, which have its native and 30k6G signal peptide at its N-terminus, respectively, were also purified from silkworm larvae and *N*-glycosylated. Sedimentation properties of two VP7s were slightly different. Totally, 37 mg of T-VP6 and 27 mg of 30k-T-ΔVP7 were purified from 112 and 545 silkworm larvae. silkworm larvae are promising host to produce subunit vaccines to porcine rotavirus.

Acknowledgement This work was supported by Research Institute of Green Science and Technology Fund for Research Project Support (2021-RIGST-21C02). in Shizuoka University.

Compliance with Ethical Standards

Conflict of interest All authors declare no conflict of interest related to this study.

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510 **Table 1** List of primers used in this study

Primer	5'-3'
Rota-VP6-F	ATGGAGGTTCTGTACTCATTGTC
Rota-VP6-R	CCCTCGAGTCACTTAACCAACATGCTTCTAATGG
Rota-VP6-F2	ATGGACTACAAGGACGACGACGAC
Rota-VP6-R2	GGTGGCGGTTTTTTAGGAG
Rota-VP7-F	ATGTATGGTATTGAATATACCACAGTTCT
Rota-VP7-R	CCCTCGAGGGGGTCACATCATACAATTCTAA
Rota-VP7-F2	CCCGGATCCATGTATGGTATTGAATATACCAC
Rota-VP7-PA-R	CCCAAGCTTTTACACCACATCATCTTCGGCACCTGGCAT GGCAACGCCTGAACCACCACCTACTCTGTAATAAAAAG CTGCAG

511

512

Figure legends

Fig. 1 Constructs of expressed VP6 and VP7. 30k6G shows the sequences encoding the signal peptide of 30k6G, a hemolymph protein of silkworm larvae. The tag sequence contains the sequences encoding Strep-Tag II and FLAG-tag

Fig. 2 Expression of (a) VP6 and (b) VP7 in silkworm larvae. First, silkworm larvae were infected with recombinant BmNPV containing a recombinant protein expression cassette described in Materials and Methods section. Hemolymph and fat body were collected after 4–5 d. Next, the fat body was suspended in PBS and disrupted through sonication. After that, the homogenate was centrifuged, and the supernatant and the precipitate were collected individually. Hem, Sup, and Pre denote hemolymph, supernatant of fat body homogenate, and precipitate of fat body homogenate, respectively. Arrows indicate the expressed proteins

Fig. 3 Purification of T-VP6 and 30k-T-VP6 from the fat body and hemolymph of silkworm larvae. (a) T-VP6 and 30k-T-VP6 were purified by Strep-Tactin Sepharose column chromatography as described in the Materials and Methods section. The purified proteins were analyzed using SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Asterisks indicate the purified proteins. (b) Deglycosylation of T-VP6 and 30k-T-VP6 with PNGase F, using the procedure described in the Materials and methods section. (c) TEM images of purified T-VP6 and 30k-T-VP6. The black bars represent 100 nm

Fig. 4 Purification of VP7-PA and 30k-T-ΔVP7 from the hemolymph of silkworm larvae. (a) VP7-PA and 30k-T-ΔVP7 were purified using Anti-PA-tag Antibody Beads and PA-Strep-Tactin Sepharose column chromatography, respectively, following the protocols described in Materials and Methods section. The purified proteins were analyzed using SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The asterisks (*) denote the purified proteins. (b) Deglycosylation of VP7-PA and 30k-T-ΔVP7 was performed with PNGase F treatment, as described in the Materials and Methods section

Fig. 5 Properties of VP7-PA and 30k-T-ΔVP7. (a) Centrifugation of VP7-PA and 30k-T-ΔVP7 on 20% sucrose cushion was performed, as described in the Materials and Methods section. The fractionated proteins were analyzed using SDS-PAGE. (b) TEM images of purified VP7-PA and 30k-T-ΔVP7

Fig. 1 Kato et al.

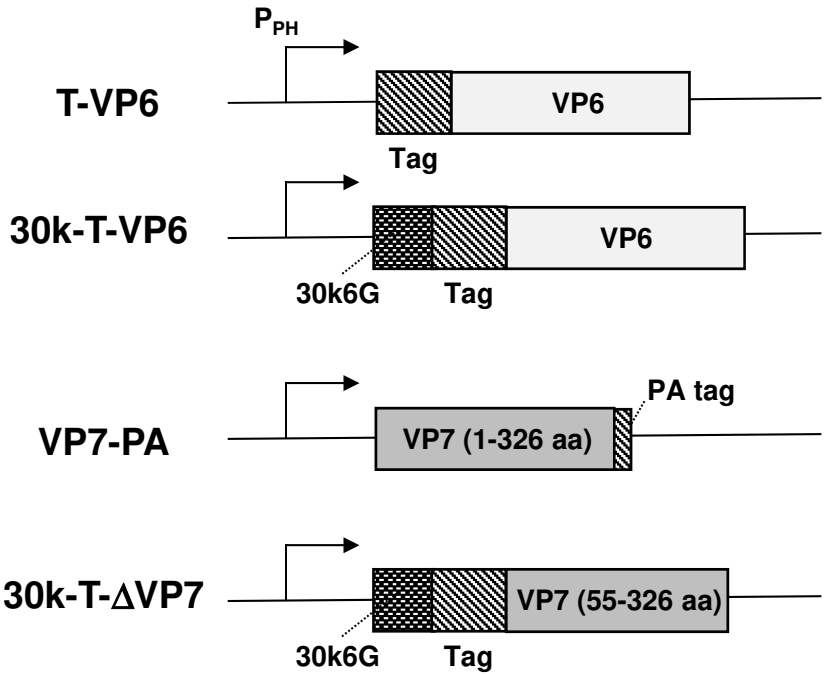
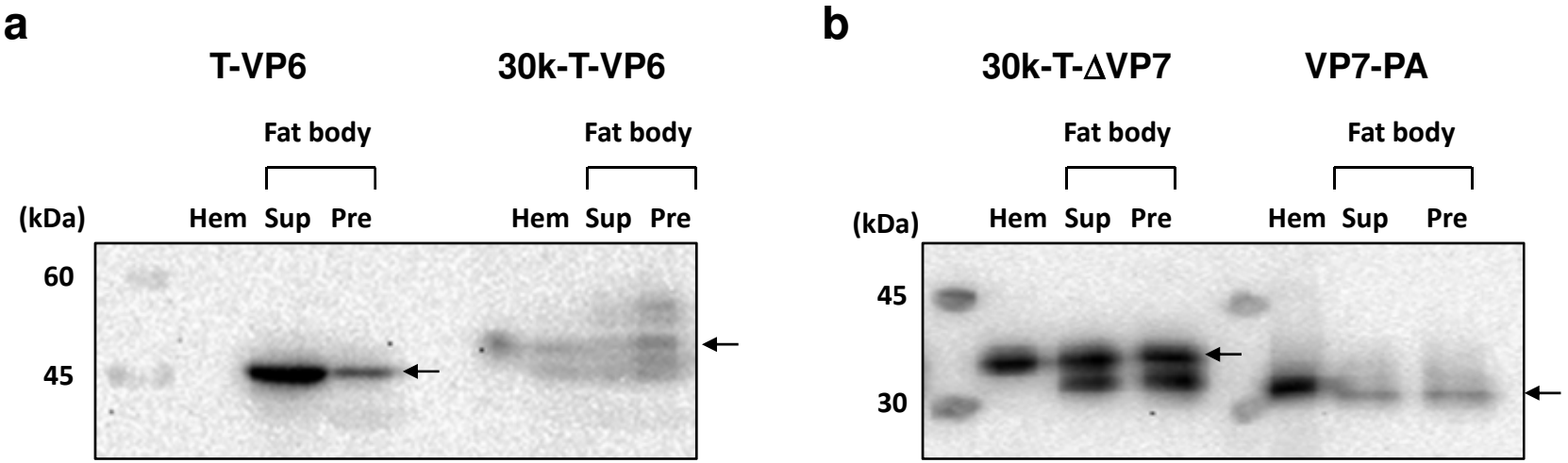


Fig. 2 Kato et al.



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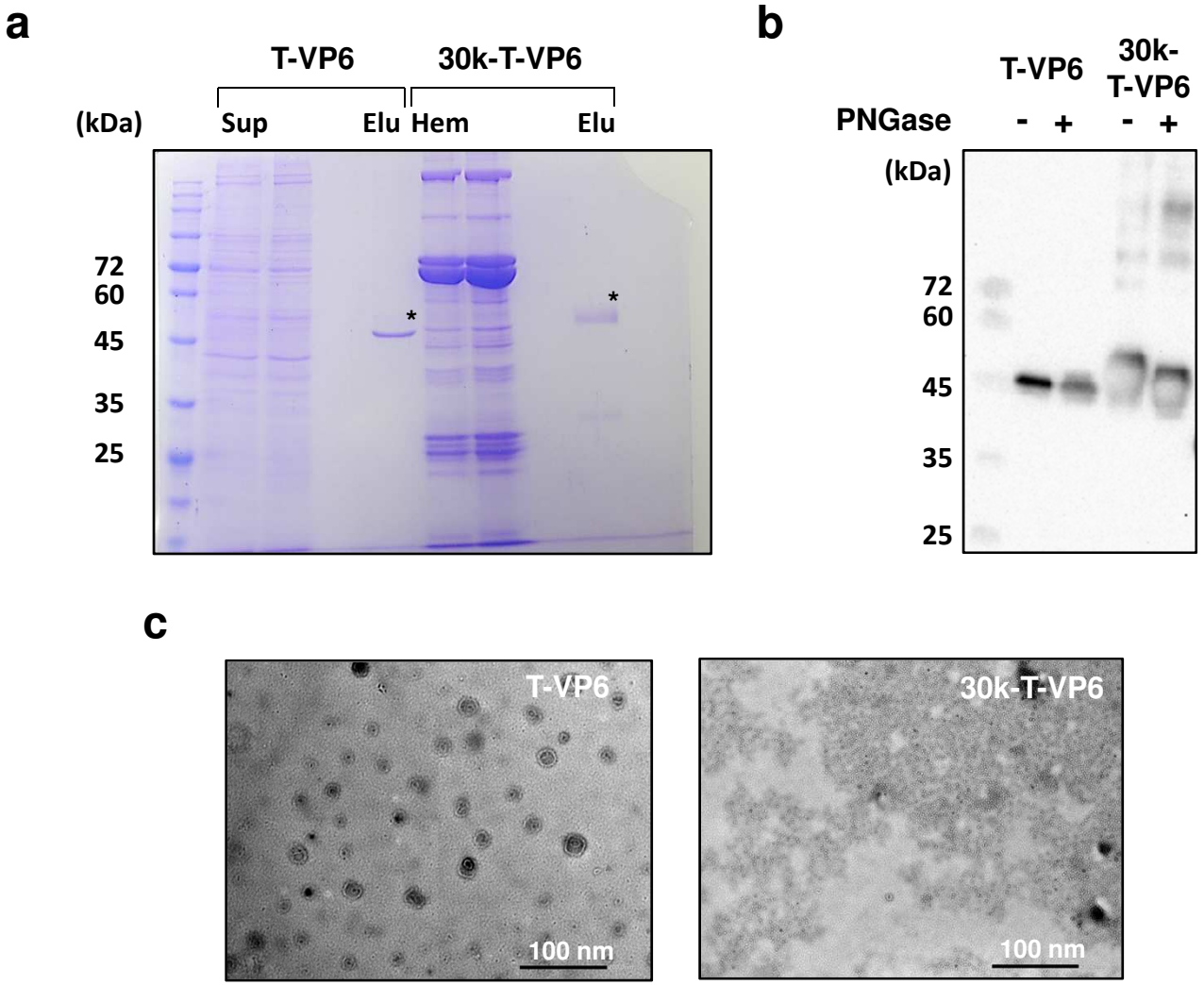

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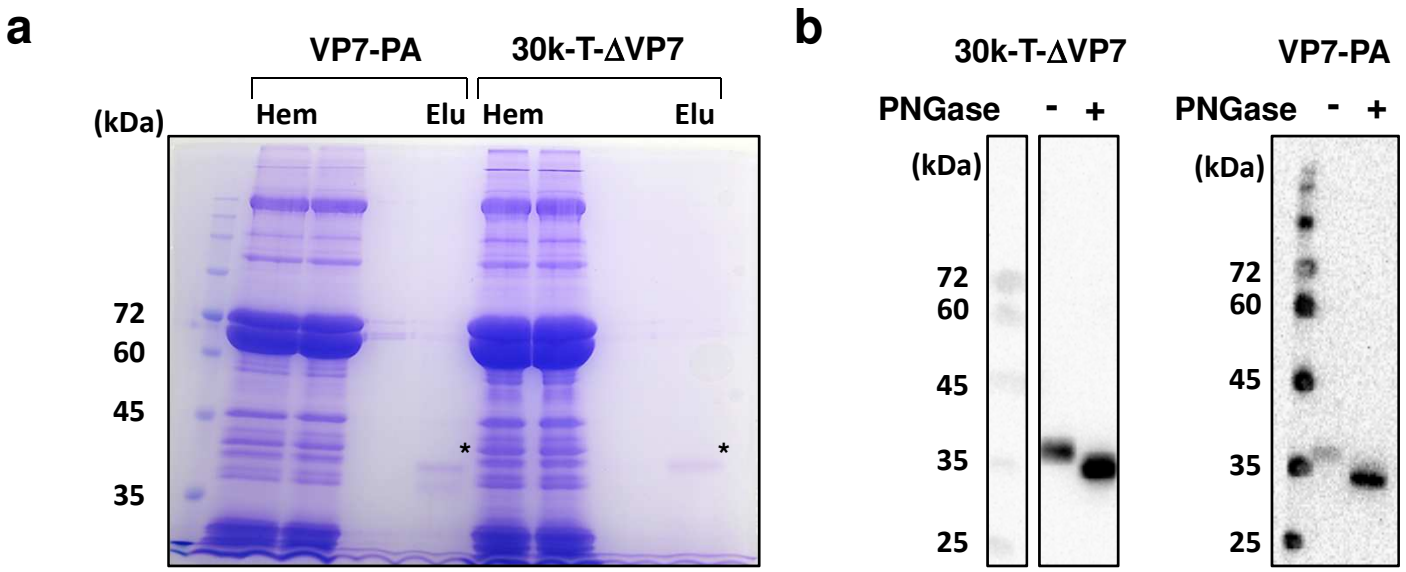
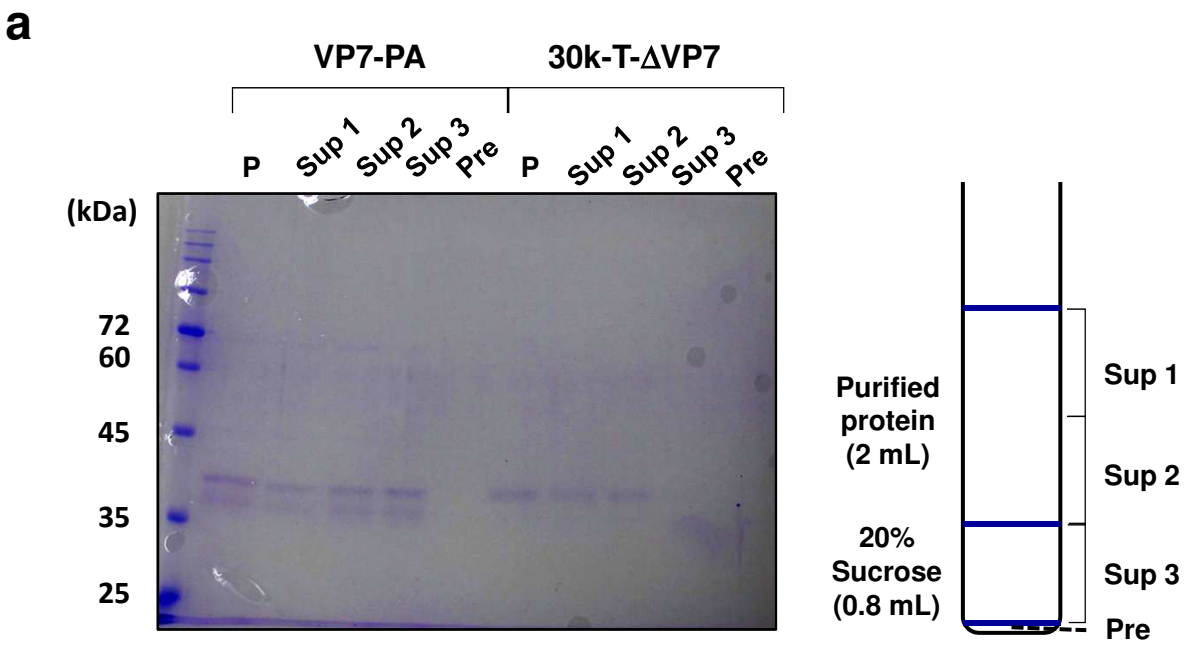
Fig. 5 Kato et al.