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Human Single-chain Antibody Expression in the Hemolymph and Fat Body of Silkworm Larvae and Pupae Using BmNPV Bacmids

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Abbreviation: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; Bacmid, baculovirus shuttle vector; *BmNPV*, *Bombyx mori* nucleopolyhedrovirus; BmNPV-*CP*[-], cysteine protease-deficient BmNPV; BmNPV-*CP*[-]*Chi*[-], cysteine protease- and chitinase-deficient BmNPV; BSA, bovine serum albumin; bx, bombyxin; ELISA, enzyme-linked immunosorbent assay; GFP_{uv}, green fluorescent protein excitable with long-wave UV light; scFv, single-chain antibody variable region fragments.

Abstract

Seven sets of recombinant expression vectors were constructed for the expression of the human anti-bovine serum albumin (BSA) single-chain Fv fragment (scFv) 13CG2 fused to the C terminus of GFP_{uv} or bombyxin (bx) signal peptide in the hemolymph and fat body of silkworm larvae and pupae, using cysteine protease- (BmNPV-*CP*$^-$), and cysteine protease- and chitinase-deficient (BmNPV-*CP*$^-$-*Chi*$^-$) bacmids. When BmNPV-*CP*$^-$ or BmNPV-*CP*$^-$-*Chi*$^-$ bacmids were used, 16.9–18.9 mg/l (11.6–15.0 μg/larva) of scFv was expressed at 6 d.p.i., whereas wild-type BmNPV bacmid expressed only 4.4 mg/l, probably because of proteolytic degradation of the protein. The scFv yield in silkworm pupae was only 0.67–1.0 μg/pupa, which was 5.4% of that in the hemolymph of silkworm larvae. The bx signal peptide enabled the secretion of scFv into the hemolymph. Without the signal sequence, the fusion protein accumulated in the fat body and lost its biological function. The removal of GFP_{uv} significantly increased the scFv yield in the hemolymph of silkworms to 188.4 mg/l (132.4 mg/larva), which was ten times higher than that of the fusion protein.

[**Key Words:** single-chain antibody, protein expression, silkworm, BmNPV, bacmid]

Introduction

Insect cells infected with recombinant baculovirus have been used for the high-level expression of various eukaryotic gene products (1-4), partly because these insect cells are capable of a similar posttranslational modification to that occurring in mammalian cells and because of high-level protein expression. An alternative baculovirus expression system involves the use of silkworms and enables the production of recombinant proteins in silkworm larvae or pupae. Recently, a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system was developed (5). The BmNPV bacmid is a shuttle vector that can be replicated in *Escherichia coli*, cultured *B. mori* cells and silkworm larvae or pupae. This enables rapid gene expression in silkworms compared with conventional baculovirus expression systems. Moreover, cysteine protease-deficient BmNPV (BmNPV-*CP*$^-$) bacmid (6) and both cysteine protease- and chitinase-deficient BmNPV (BmNPV-*CP*$^-$-*Chi*$^-$) bacmid (7) have been developed for the efficient production of gene products from silkworms. Ability to recover intact protein using these bacmids is improved significantly compared with that of wild-type BmNPV bacmid because of significant decreases in silkworm liquefaction and proteolytic degradation of expressed proteins.

The BmNPV bacmid system is now applied to the production of antibody single-chain variable region fragment (scFv). The scFv comprises variable regions of the antibody heavy (V_H) and light (V_L) chains, which are connected by a flexible interchain linker. This minimal antigen-binding fragment, which is far smaller than full-size antibody, has attracted considerable attention: for example, as an effective reagent for tumor targeting, for radioimmunoimaging, and for the specific delivery of cytotoxic agents (8).

In this paper, we report on the expression of human scFv either as a fusion protein with GFP_{uv} or by itself, using BmNPV bacmids; in addition, we compare the amounts of expressed antibody in the hemolymph and fat body of silkworm larvae and pupae.

MATERIALS AND METHODS

Strain, plasmid and silkworm larvae *E. coli* DH10Bac, directional TOPO pENTR vector and Gateway destination vector pDEST8 were purchased from Invitrogen (Carlsbad, CA, USA). Fifth-instar hybrid Kinsyu x Syowa silkworm larvae (Ehime Sansyu, Yahatahama, Japan) were used in this study. The larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27±1^{o}C.

Construction of recombinant BmNPV bacmids Fig. 1 shows the construction of recombinant BmNPV bacmids. A human scFv (13CG2) gene was amplified from pIT2-13CG2 (9) using a 13CG2 primer set (Table 1). Polymerase chain reactions (PCRs) were performed using the following program: 3 min at 95^{o}C, 35 cycles at 95^{o}C for 15 s, 55^{o}C for 30 s and 68^{o}C for 1 min, followed by a final extension at 68^{o}C for 5 min. Amplified PCR product was purified using a GFX PCR purification kit (Biocompare Inc., San Francisco, CA) and digested by *Bgl* II and *Eco* RI restriction enzymes. Digested fragment was also purified using the GFX PCR purification kit, ligated into pBlueBacHis2/GFP_{uv} (pBH/GFP_{uv}) and digested with *Bgl* II and *Eco* RI restriction enzymes using T4 ligase (pBH/GFP_{uv}-13CG2).

To secrete expressed protein into the hemolymph of silkworms, signal sequence from bx was added to *GFP_{uv}-13CG2*. Using pBH/GFP_{uv}-13CG2 as a template, PCR was carried out

using CACC-bx-forward and pBH-reverse primers under the following conditions: 3 min at 95°C, 30 cycles at 95°C for 30 s, 57°C for 40 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The amplified DNA fragment was designated bx-GFP_{uv}-13CG2.

A DNA fragment without the bx signal sequence was also amplified using CACC-forward and pBH-reverse primers, under the same PCR conditions as above, and GFP_{uv}-13CG2 was obtained.

The two DNA fragments were cloned into pENTR using pENTR Directional TOPO Cloning Kits (Invitrogen), and pENTR/bx-GFP_{uv}-13CG2 and pENTR/GFP_{uv}-13CG2 were obtained. These products were cloned into pDEST8 using the Gateway cloning technology (Invitrogen), and pDEST8/bx-GFP_{uv}-13CG2 and pDEST8/GFP_{uv}-13CG2 were obtained, respectively. These two plasmids were then transformed into each *E. coli* DH10BacBm, DH10BacBm-*CP*$^-$ and DH10BacBm-*CP*$^-$-*Chi*$^-$, cultivated in an LB (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 20 g of agar per liter) plate containing kanamycin, gentamycin, tetracycline, 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-Gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG), and the white colony was picked up, and positive clones were selected. The BmNPV, BmNPV-*CP*$^-$ and BmNPV-*CP*$^-$-*Chi*$^-$ bacmids were used to express either 13CG2 or GFP_{uv}-13CG2 fusion protein.

13CG2 Expression in silkworm larvae and pupae BmNPV bacmid DNA was injected directly into the first day of fifth-instar silkworm larvae. Four μg of BmNPV bacmid and helper plasmid pMON7124 DNA was suspended in 5 μl of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE)-C reagent (Invitrogen) and left to stand at room temperature for 45 min. The resultant mixture was diluted to a final volume of

50 μl with PBS, and 20 μl of the mixture was then injected into the dorsum of the larvae using a syringe with a 26-gauge beveled needle. Expression of the fusion protein in silkworm larvae was confirmed using an ultraviolet illuminator.

Sample preparation for assay Hemolymph was collected at 4–8 d.p.i. by cutting the caudal leg in a tube containing 5 μl of 200-mM 1-phenyl-2-thiourea, and centrifuged at 9000 rpm for 10 min at 4^{o}C. The supernatant samples were immediately frozen at -80^{o}C for further analysis. The larval fat body was isolated by dissection under sterile conditions at 4^{o}C. It was then diluted with 25% (v/v) homogenization buffer [0.15-M NaCl, 2-mM EDTA, 2-mM dithiothreitol (DTT) and 20-mM Na_2HPO_4; pH 7.6] and sonicated on ice three times for 15 s each, with 15-s intervals, using a sonicator (VC 130PB, Sonic & Materials, Newtown, CT, USA). Finally, it was centrifuged at 13000 rpm for 30 min. The resultant supernatant was immediately frozen at -80^{o}C for further analysis.

Silkworm pupae at 5–7 d.p.i. were collected and frozen in liquid nitrogen. The frozen pupae were ground in a mortar, added to 3.4 ml of homogenization buffer and sonicated on ice three times for 30 s each, with 30-s intervals, using a sonicator (VC 130PB, Sonic & Materials). They were then centrifuged at 7000 rpm for 10 min. The supernatant was frozen at -80^{o}C for future analysis.

SDS-PAGE and western blot analysis The protein content in the hemolymph and fat body was detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. SDS-PAGE was performed with 10% polyacrylamide gel using the Mini-protein III System (Bio-Rad Laboratories, Hercules, CA, USA). The respective bands were detected using a Molecular-FX multi-imager (Bio-Rad). For western blot, the samples

were boiled for 5 min before they were run on the SDS-PAGE gel. An anti-HIS G (Invitrogen) antibody was used as the primary antibody, and an anti-mouse IgG/horseradish peroxidase (HRP) antibody (GE Healthcare, Buckinghamshire, UK) was used as the secondary antibody. The immunoblot bands were visualized using ECL plus western blotting detection reagents (GE Healthcare), and analyzed using a Fluor-S/MAX multi-imager (Bio-Rad). Magic Mark XP Western Protein Standard (Invitrogen) was used as a protein molecular weight marker.

Determination of protein concentration Protein concentration was measured using a Bradford protein assay kit (Bio-Rad), with bovine serum albumin as a standard.

ELISA **13CG2 was quantified by ELISA-detectable amount having binding-ability to BSA.** One hundred μl/well of 10-μg/ml BSA was coated in a 96-well flexible assay plate overnight at 4^{o}C. 200 μl of 20% skimmed milk in phosphate buffered saline-Tween (PBST) buffer (137-mM NaCl, 10-mM phosphate, 2.7-mM KCl and 0.1% Tween 20; pH 7.4) was added to the BSA-coated plate wells for blocking, followed by incubation for 2 h at room temperature. The wells were washed three times with PBST buffer. Diluted GFP_{uv}-13CG2 fusion protein or 13CG2 protein was then added to the BSA-coated plate wells, incubated for 1.5 h at room temperature and washed three times with 200 μl of PBST. As a second antibody, 100 μl of protein A-HRP diluted 1000 times in 5% skimmed milk/PBS was added to each well, incubated for 1 h and washed three times with 200 μl of PBST. 100 μl of substrate [0.1-mg/ml 3,3',5,5'-tetramethylbenzidine (TMBZ) in 100-mM sodium acetate, pH 6.0, with 0.2% (v/v) of 30% hydrogen peroxidase] was added to each well and left at room temperature for blue-color development. The reaction was stopped by the addition of 50 μl of 1N H_2SO_4 solution. The developed color was measured at optical densities (ODs) of 450 nm and 655 nm. The value of

OD_{655} minus OD_{450} was used as a measure of the amount of 13CG2. 13CG2 expressed by *E. coli* HB2151 (pIT2-13CG2) and purified using Talon Metal Affinity Resin (Clontech, Takarabio, Shiga, Japan) was used for the calibration of 13CG2 as a standard.

RESULTS

GFP_{uv}-13CG2 Expression in the hemolymph of silkworm larvae BmNPV, BmNPV-*CP*$^-$ and BmNPV-*CP*$^-$*Chi*$^-$ bacmids with bx signal peptides were constructed for the secretion of GFP_{uv}-13CG2 fusion protein and 13CG2 scFv into the hemolymph of silkworm larvae. For expression in silkworm pupae, BmNPV, BmNPV-*CP*$^-$ and BmNPV-*CP*$^-$*Chi*$^-$ bacmids without bx signal peptides were constructed. 13CG2 scFv was expressed using a BmNPV-*CP*$^-$*Chi*$^-$ bacmid with a bx signal peptide, for comparison with the expression level of GFP_{uv}-13CG2. Seven kinds of recombinant BmNPV bacmid were constructed for the expression of GFP_{uv}-13CG2 and 13CG2 scFv in silkworm larvae and pupae (Table 2).

Expression of the GFP_{uv}-13CG2 fusion protein in the hemolymph of BmNPV bacmid/bx-GFP-13CG2-injected silkworm larvae was detected via western blot (Fig. 2A) and GFP_{uv} fluorescence (Fig. 2B). The molecular weight of the GFP_{uv}-13CG2 band was ~60 kDa (Fig. 2A), which is similar to the predicted molecular weight of the fusion protein (58 kDa). Thick GFP_{uv} fluorescent bands were observed at 4.5 and 5.5 d.p.i., although the fluorescence of the fusion band decreased and smaller degraded-protein bands were observed at 6 d.p.i. (Fig. 2B). No band was observed from mock-injected silkworm larvae hemolymph.

Comparison of GFP_{uv}-13CG2 expression in the hemolymph of silkworm larvae and pupae GFP_{uv}-13CG2 was expressed in different types of BmNPV bacmid. When BmNPV-*CP*$^-$ or BmNPV-*CP*$^-$-*Chi*$^-$ bacmids were used, thick fusion-protein bands were

observed, without small degraded molecular bands (Fig. 3A, B). When BmNPV-*CP*$^-$ or BmNPV-*CP*$^-$-*Chi*$^-$ bacmids were used, 16.9–18.9 mg/l (11.6–15.0 μg/larva) of 13CG2 was expressed at 6 d.p.i., indicating the highest scFv concentration. Wild-type BmNPV bacmid produced only 4.4 mg/l of 13CG2, probably because of proteolytic degradation of the expressed protein (Fig. 2B).

To express GFP_{uv}-13CG2 in silkworm pupae, the bx signal peptide was removed from the recombinant BmNPV bacmid construction. When wild-type BmNPV bacmid was used, proteolytic degradation of the fusion protein was observed (Fig. 4A), but when recombinant-protease-deficient, or protease- and chitinase-deficient BmNPV bacmids were used for GFP_{uv}-13CG2 the smaller degraded bands were not observed (Fig. 4A). The maximum amount of antibody expressed was 0.97 μg/pupa (Fig. 4B), which was only 6.5% of the secreted 13CG2 concentration in hemolymph.

GFP_{uv}-13CG2 Expression in the hemolymph and fat body of silkworm larvae

GFP_{uv}-13CG2 fusion protein was expressed in the hemolymph and fat body of silkworm larvae both with and without a bx signal sequence. With regard to secretion into the hemolymph of silkworms, the amount of 13CG2 expressed with the bx signal sequence was 12 times that expressed without the signal sequence (Fig. 5C). By contrast, a large amount of expressed fusion protein was observed in silkworm fat body without the bx signal sequence (Fig. 5B). The amount of 13CG2 with the bx signal sequence was five times that without the signal sequence, as estimated using ELISA, irrespective of the thick band of the latter (Fig. 5C).

13CG2 Expression in the hemolymph of BmNPV-*CP*$^-$-*Chi*$^-$ bacmid/bx-13CG2-

injected silkworm larvae 13CG2 scFv was expressed in the hemolymph of BmNPV-*CP*$^-$-*Chi*$^-$ bacmid/bx-13CG2-injected silkworm larvae. The expressed-protein molecular band was estimated to be 32 kDa (Fig. 6), which was similar to the assumed molecular weight. The amount of scFv was 188.4 mg/l (132.4 μg/larva), which was ten times higher than that of the GFP_{uv}-13CG2 fusion protein.

DISCUSSION

Single-chain antibody was expressed in the hemolymph and fat body of silkworm larvae and pupae. The amount of GFP_{uv}-13CG2 fusion protein secreted using BmNPV-*CP*$^-$ and BmNPV-*CP*$^-$-*Chi*$^-$ bacmids was four times that expressed in wild-type BmNPV bacmid. This indicates that the BmNPV-*CP*$^-$ and BmNPV-*CP*$^-$-*Chi*$^-$ bacmids are useful for maintaining the proper protein structure and biological function of antibody. Recovery of expression protein can be compromised due to potential for proteolytic degradation of target protein by a cysteine protease derived from the BmNPV. This has been a major problem in terms of the mass production of proteins of interest in silkworm larvae. The BmNPV-*CP*$^-$ bacmid system was applied to the expression of human growth hormone, and the protein was produced efficiently because of a greatly reduced degree of degradation of the expressed protein (10). Moreover, Hawtin *et al*. (11) reported that, in conjunction with the cysteine protease, AcMNPV-encoded chitinase promotes liquefaction of the host in the latter stages of infection. These findings led to the construction of an AcMNPV that was deficient in cysteine protease and chitinase. In addition, Wang *et al*. (12) constructed a chitinase-gene-deficient BmNPV mutant and reported that *B. mori* larvae infected with BmNPV *Chi*$^-$ showed clear hemolymph

and less degradation of the body after larval death. In the case of human β1,3-*N*-acetylglucosaminyltransferase production, when wild-type BmNPV bacmid was used for the secretion of protein into the hemolymph of silkworm larvae, the protein was degraded completely and its activity was not detected; in the case of BmNPV-*CP*$^-$-*Chi*$^-$ bacmid, however, activity was 62 mU/ml, which was 2.8-fold that of wild-type BmNPV bacmid (7). These examples demonstrate that the BmNPV-*CP*$^-$-*Chi*$^-$ bacmid may be an efficient tool for expressing single-chain antibody in the hemolymph of silkworm larvae.

The expression of scFv fused with GFP_{uv} has been carried out in various host cells. The expression level in *E. coli* ranges from 0.1 mg/l (13) to 15 mg/l (14). Yeast was also used for this purpose, and the expression level was 2.9 mg/l (15). The expression levels in Sf 9 and human 293T kidney cells were 0.5–1.0 mg/l and 0.2–3.0 mg/l, respectively (16). In this study, the amount of scFv expressed in hemolymph of silkworm larvae was 16.9–18.9 mg/l, which is comparable to that obtained with other expression systems.

Signal peptide has an important role in the secretion of scFv fused with GFP_{uv} in the hemolymph of silkworm larvae. The expression level in hemolymph when the bx signal peptide was present was 12 times that in the absence of the signal peptide (Fig. 5C). GFP_{uv}-scFv fusion protein without the bx signal peptide was expressed in silkworm larvae to investigate whether the signal peptide is indispensable for the efficient expression of the fusion protein. GFP_{uv}-scFv accumulated in the fat body, as shown by the thick molecular band in the western blot (Fig. 5B), but its antibody amount was 0.4 μg/larva, which is only 2.7% of that expressed in the hemolymph of silkworm larvae (Fig. 5C). This indicates that scFv is activated by secretion into the hemolymph through the ER and Golgi apparatus, and is

inactive if it accumulates in cells. Its folding requires molecular chaperones in the ER and Golgi apparatus because of a structural deficiency (17). However, human granulocyte–macrophage colony-stimulating factor (hGM-CSF) without an N-terminal signal peptide has been expressed in glycosylated active form in pupae (18). The other possibility of the low expression level in the fat body may be due to be contained impure proteins or inhibitory compounds. To make clear this point, scFv-containing hemolymph was diluted two times with PBS buffer or fat body extract, SDS-PAGE and Western blot analysis, and ELISA were performed. The fat body extract showed neither any inhibitory effect nor decreasing the amount of 13CG2, but in the Western blot analysis, the band of fat body extract-containing hemolymph was slightly thick to compare with that of PBS-containing hemolymph (data not shown).

When GFP_{uv} was removed from the GFP_{uv}-13CG2 fused construct, the scFv expression level increased significantly to 188.4 mg/l in the hemolymph of silkworm larvae, which was ten times higher than that in GFP_{uv}-13CG2. In terms of scFv expression with GFP_{uv}, metabolic burden caused by fusion with GFP_{uv}, instability of mRNA or low translational efficiency might lead to the decrease in scFv antibody production.

E. coli has been reported to express 0.63–16.4 mg/l of human scFv antibody; *Saccharomyces cerevisiae* has been reported to express 1.5–4.2 mg/l; *Pichia pastoris* has been reported to express 0.4–7.3 mg/l (19). Rippmann et al. (17) expressed 40–200 mg/l of scFv antibody using a special *Proteus mirabilis* that lacked a periplasmic compartment. 32 mg/l (10^9 cells/ml) of anti-(phenyl-oxazolone) scFv antibody was produced in Sf9 cells (20). Even though a close comparison is difficult because of different scFv clones, in the present study,

the expression level of scFv antibody was 188.4 mg/l using silkworms, which is almost sixfold that of insect cell expression systems. Silkworm larvae are capable of a similar post-translational modification to that occurring in mammalian cells, and also achievable the high expression levels. The BmNPV bacmid system is a shuttle vector that can be replicated in *Escherichia coli*, cultured *B. mori* cells and silkworm larvae or pupae, which enables more rapid gene expression in silkworms compared with that in conventional expression systems. Moreover, a cysteine protease- and both cysteine protease- and chitinase-deficient BmNPV bacmids are useful for the efficient production of scFV from silkworm larvae, because of a significant decrease in proteolytic degradation of the expressed proteins. Moreover, silkworm larvae don't require any optimization of reactor performance, reactor design, and the development of appropriate media. These indicate that silkworm larvae are useful for a practical expression of scFv and scientific application in antibody production.

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Figure legends

FIG. 1. Schematic diagram of recombinant BmNPV bacmid/bx-GFP_{uv}-13CG2.

FIG. 2. Western blot (A) and GFP_{uv} fluorescence (B) of GFP_{uv}-13CG2 fusion protein expressed in the hemolymph of BmNPV bacmid/bx-GFP_{uv}-13CG2-injected silkworm larvae. The loaded-protein amounts of lanes were 275-290 μg, respectively. M and n denote molecular weight marker and mock-injected silkworm larvae hemolymph, respectively. Arrows indicate molecular weight of GFP_{uv}-13CG2.

FIG. 3. GFP_{uv} fluorescence of GFP_{uv}-13CG2 fusion protein expressed in the hemolymph of BmNPV-*CP*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected (A) and BmNPV-*CP*$^-$-*Chi*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected (B) silkworm larvae. The loaded-protein amounts in (A) and (B) were 240–310 μg and 150–225 μg, respectively. Arrows indicate molecular weight of GFP_{uv}-13CG2. GFP_{uv}-13CG2 antibody from BmNPV-*CP*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected and BmNPV-*CP*$^-$-*Chi*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected silkworm larvae hemolymph were measured using ELISA (C). WT indicates wild-type BmNPV bacmid.

FIG. 4. (A) GFP_{uv} fluorescence of GFP_{uv}-13CG2 fusion protein expressed in BmNPV bacmid/bx-GFP_{uv}-13CG2-injected, BmNPV-*CP*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected and BmNPV-*CP*$^-$-*Chi*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected silkworm pupae. The loaded-protein amounts were 65–95 μg. Arrows indicate molecular weight of GFP_{uv}-13CG2. Numbers indicate d.p.i. in (A). (B) GFP_{uv}-13CG2 antibody amounts expressed in BmNPV bacmid/bx-GFP_{uv}-13CG2-injected, BmNPV-*CP*$^-$ bacmid/bx-GFP_{uv}-13CG2-injected and BmNPV-*CP*$^-$-

Chi⁻ bacmid/bx-GFP_{uv}-13CG2-injected silkworm pupae were measured using ELISA. M and n denote molecular weight marker and mock-injected silkworm larvae hemolymph, respectively.

FIG. 5. GFP_{uv} fluorescence of GFP_{uv}-13CG2 fusion protein in the hemolymph (A) and fat body (B) of BmNPV-*CP*⁻-*Chi*⁻ bacmid/GFP_{uv}-13CG2-injected or BmNPV-*CP*⁻-*Chi*⁻ bacmid/bx-GFP_{uv}-13CG2-injected silkworm larvae. Arrows indicate molecular weight of GFP_{uv}-13CG2. Lanes 1 and 2 denote BmNPV-*CP*⁻-*Chi*⁻ bacmid/bx-GFP_{uv}-13CG2 injection and BmNPV-*CP*⁻-*Chi*⁻ bacmid/GFP_{uv}-13CG2 injection, respectively. M and n denote molecular weight marker and mock-injected silkworm larvae hemolymph, respectively. The loaded-protein amounts of (A) and (B) were 190–200 μg and 90–95 μg, respectively. GFP_{uv}-13CG2 antibody amounts from the hemolymph and fat body of recombinant-bacmid-injected silkworm larvae were measured using ELISA (C). Black and white bars denote BmNPV-*CP*⁻-*Chi*⁻ bacmid/bx-GFP_{uv}-13CG2 injection and BmNPV-*CP*⁻-*Chi*⁻ bacmid/GFP_{uv}-13CG2 injection, respectively.

FIG. 6. Western blot of 13CG2 expressed in the hemolymph of BmNPV-*CP*⁻-*Chi*⁻ bacmid/bx-13CG2-injected silkworm larvae at 6 d.p.i. Arrows indicate molecular weight of 13CG2. The loaded-protein concentration was 210 μg. M and n denote molecular weight marker and mock-injected silkworm larvae hemolymph, respectively.

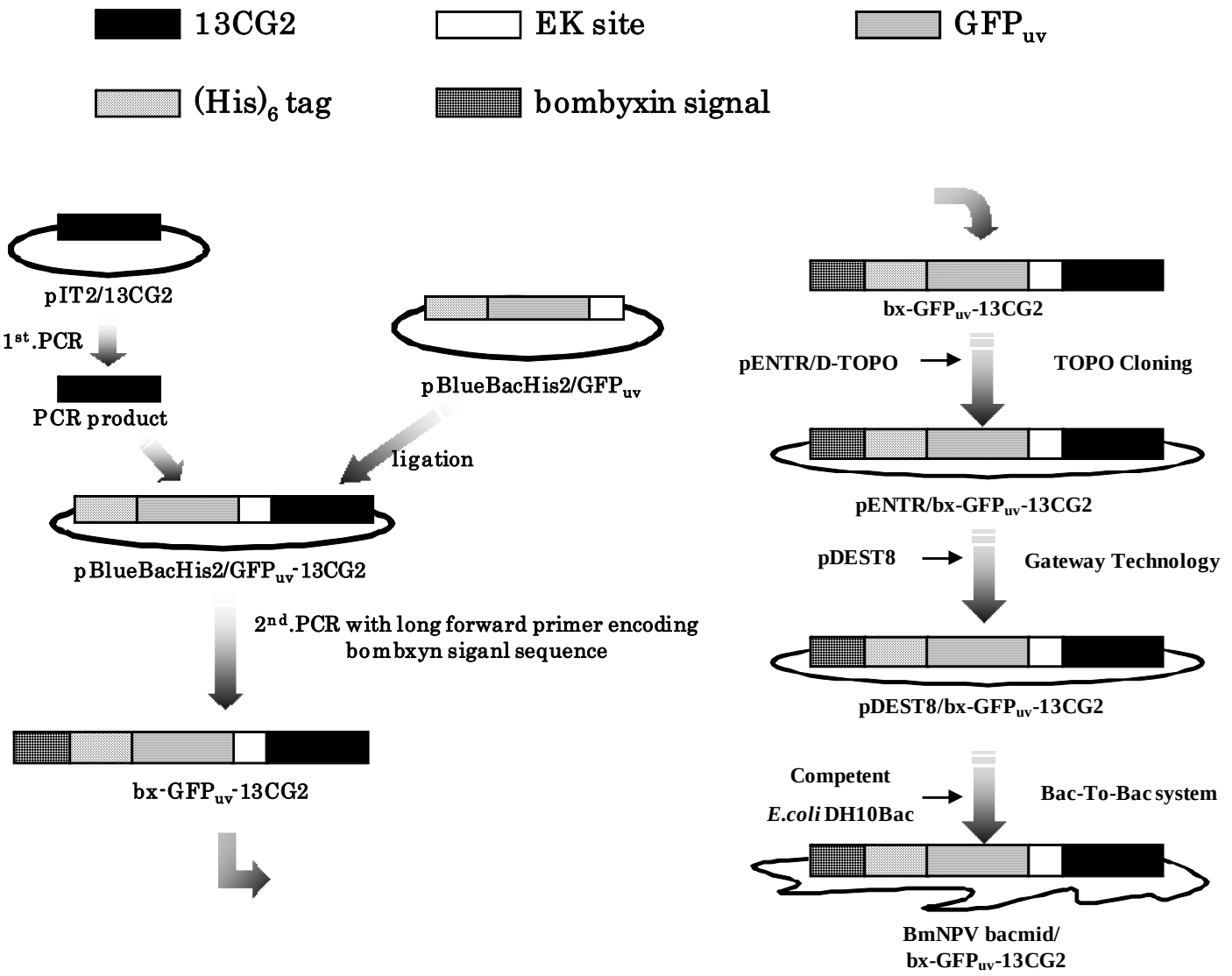
TABLE 1. Used primers set

Primer	Sequence
13CG2-forward	GAAGATCTGAGGTGCAGCTGTTGGAGTC
13CG2-reverse	CTGGAATTCCCGTTTGATTTCCACCTTGGTC
CACC-bx forward	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACAACCGCGGGGTTCTCATCATC
CACC-reverse	CACCCCGCGGGGTTCTCATCATC
pBH-reverse	ACTTCAAGGAGAATTTCCTC
M13-forward	GTAAAACGACGGCC

TABLE 2. Bacmid constructs

Name	Note
For secretion of expressed protein into silkworm larvae hemolymph	
BmNPV bacmid/bx-GFP_{uv}-13CG2	Designed for secretion of GFP_{uv}-13CG2 fusion protein into hemolymph
BmNPV bacmid-*CP*$^-$/bx-GFP_{uv}-13CG2	Designed for secretion of GFP_{uv}-13CG2 fusion protein into hemolymph using protease-deficient bacmid
BmNPV bacmid-*CP*$^-$-*Chi*$^-$/bx-GFP_{uv}-13CG2	Designed for secretion of GFP_{uv}-13CG2 fusion protein into hemolymph using protease- and chitinase-deficient bacmid
BmNPV bacmid-*CP*$^-$-*Chi*$^-$/bx-13CG2	Designed for expression of 13CG2 into hemolymph using protease- and chitinase-deficient bacmid
For expression in silkworm pupa	
BmNPV bacmid/GFP_{uv}-13CG2	Designed for expression GFP_{uv}-13CG2 fusion protein in the pupa
BmNPV bacmid-*CP*$^-$/GFP_{uv}-13CG2	Designed for expression of GFP_{uv}-13CG2 fusion protein in the pupa using protease-deficient bacmid
BmNPV bacmid-*CP*$^-$-*Chi*$^-$/GFP_{uv}-13CG2	Designed for expression of GFP_{uv}-13CG2 fusion protein in the pupa using protease- and chitinase-deficient bacmid

Fig. 1, Ishikiriyama et al.



1
2

Fig. 2, Ishikiriyama et al.

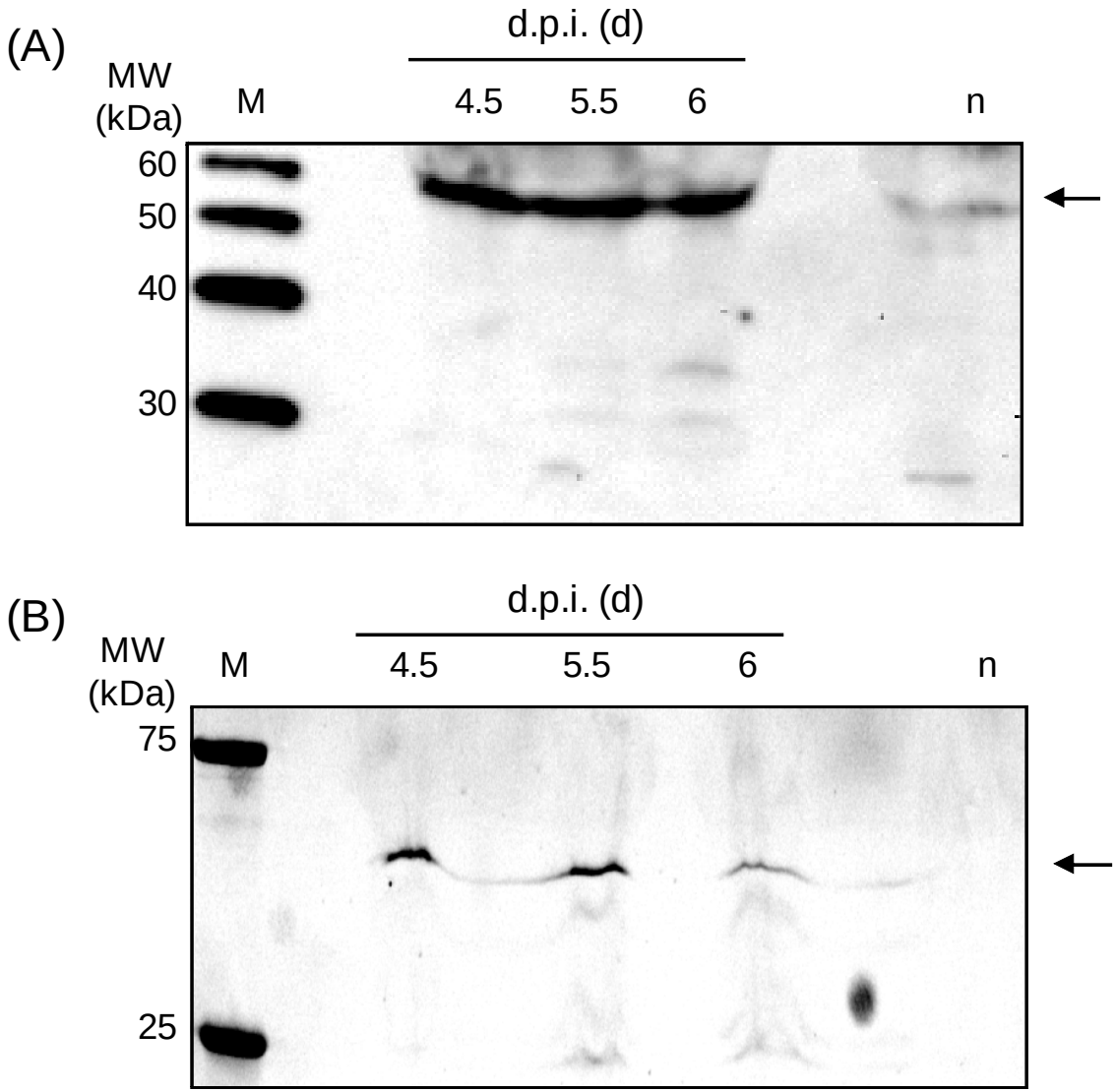


Fig. 3, Ishikiriyama et al.

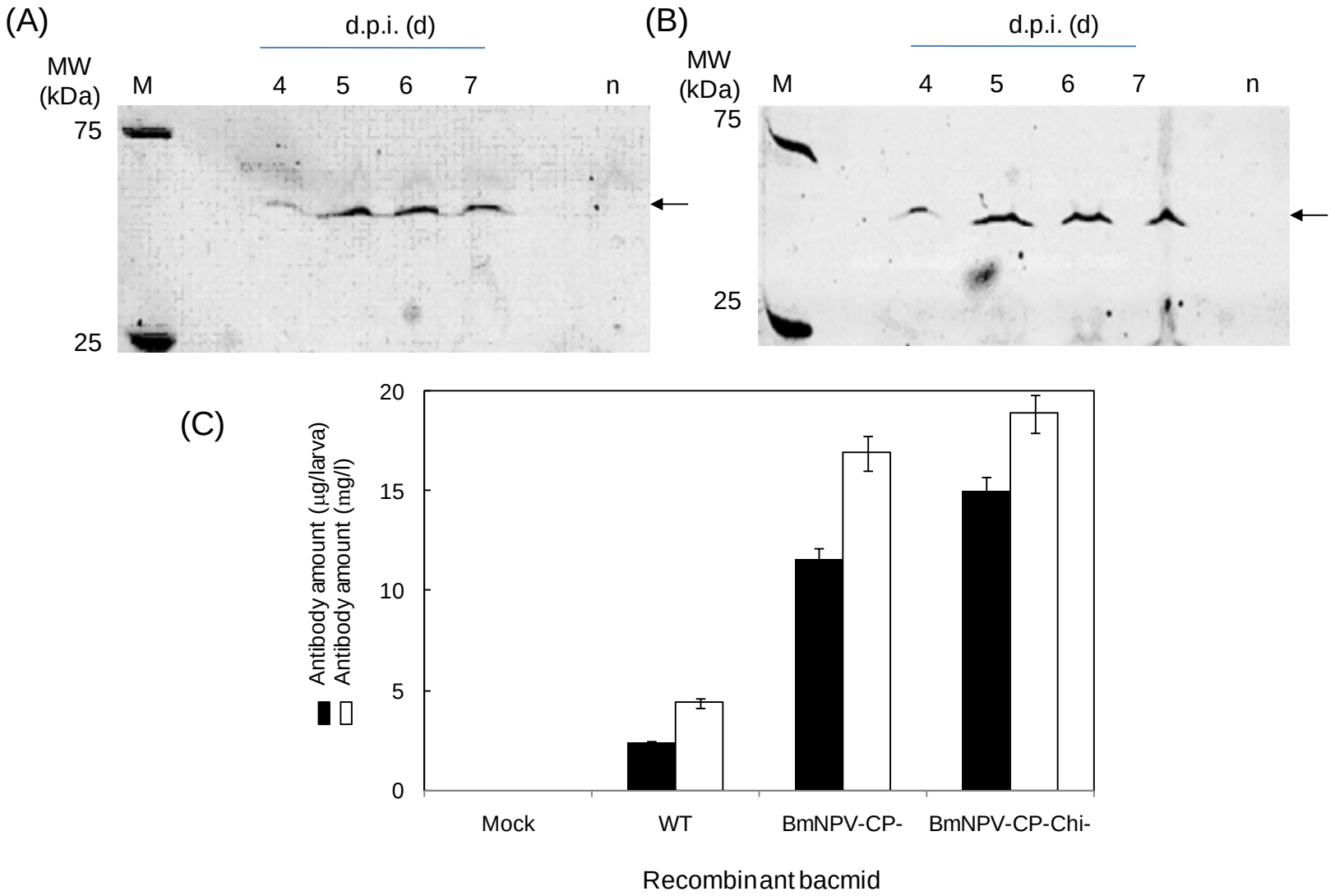


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Fig. 4, Ishikiriyama et al.

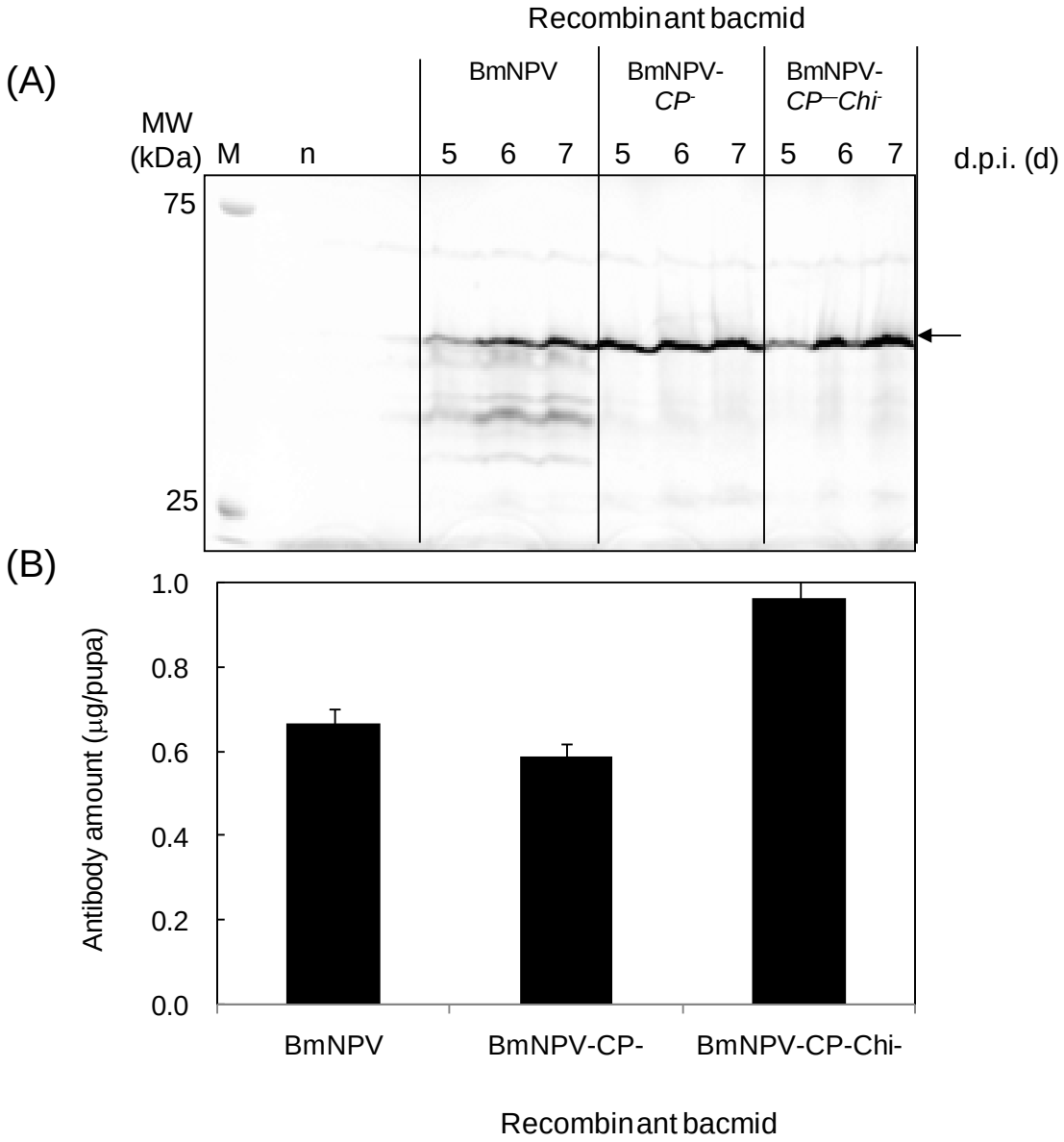
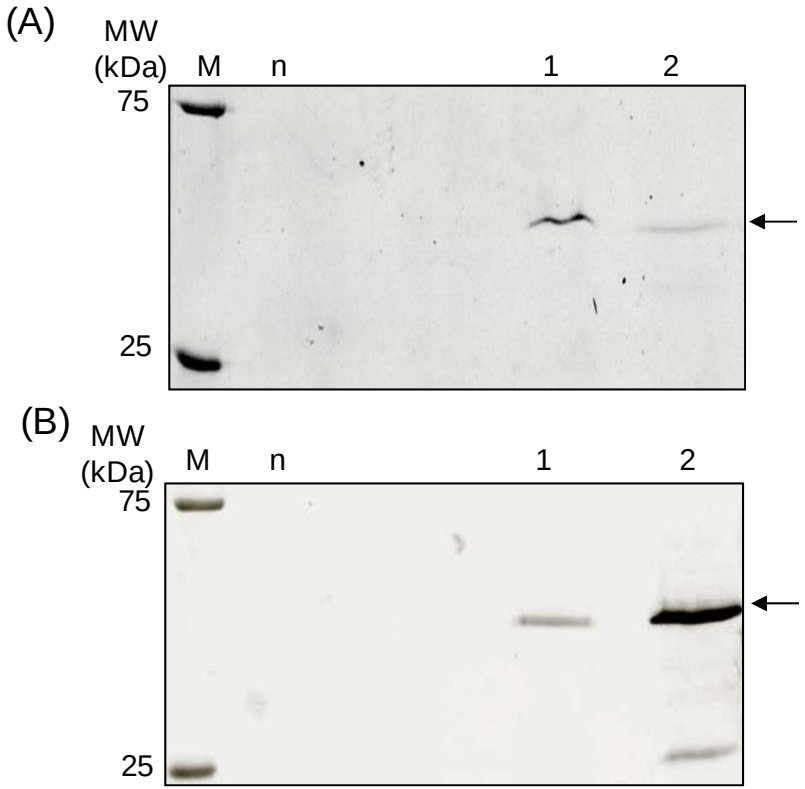


Fig. 5, Ishikiriyama et al.



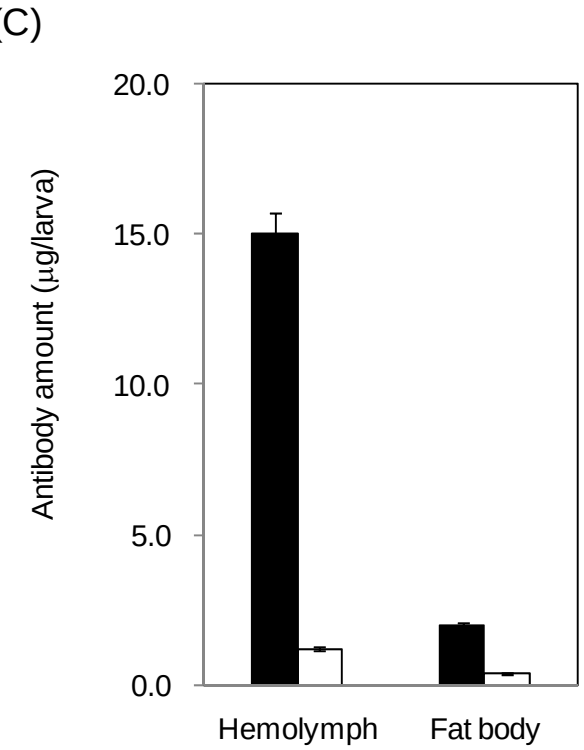
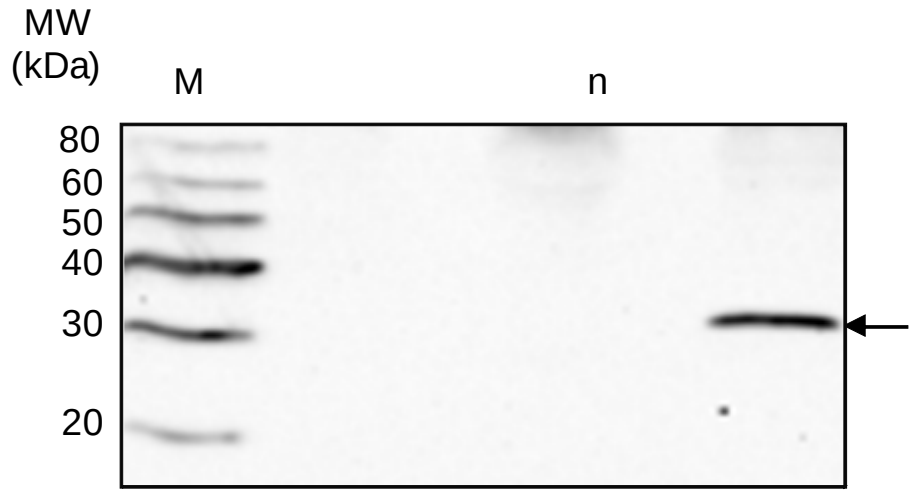


Fig. 6, Ishikiriyama et al.



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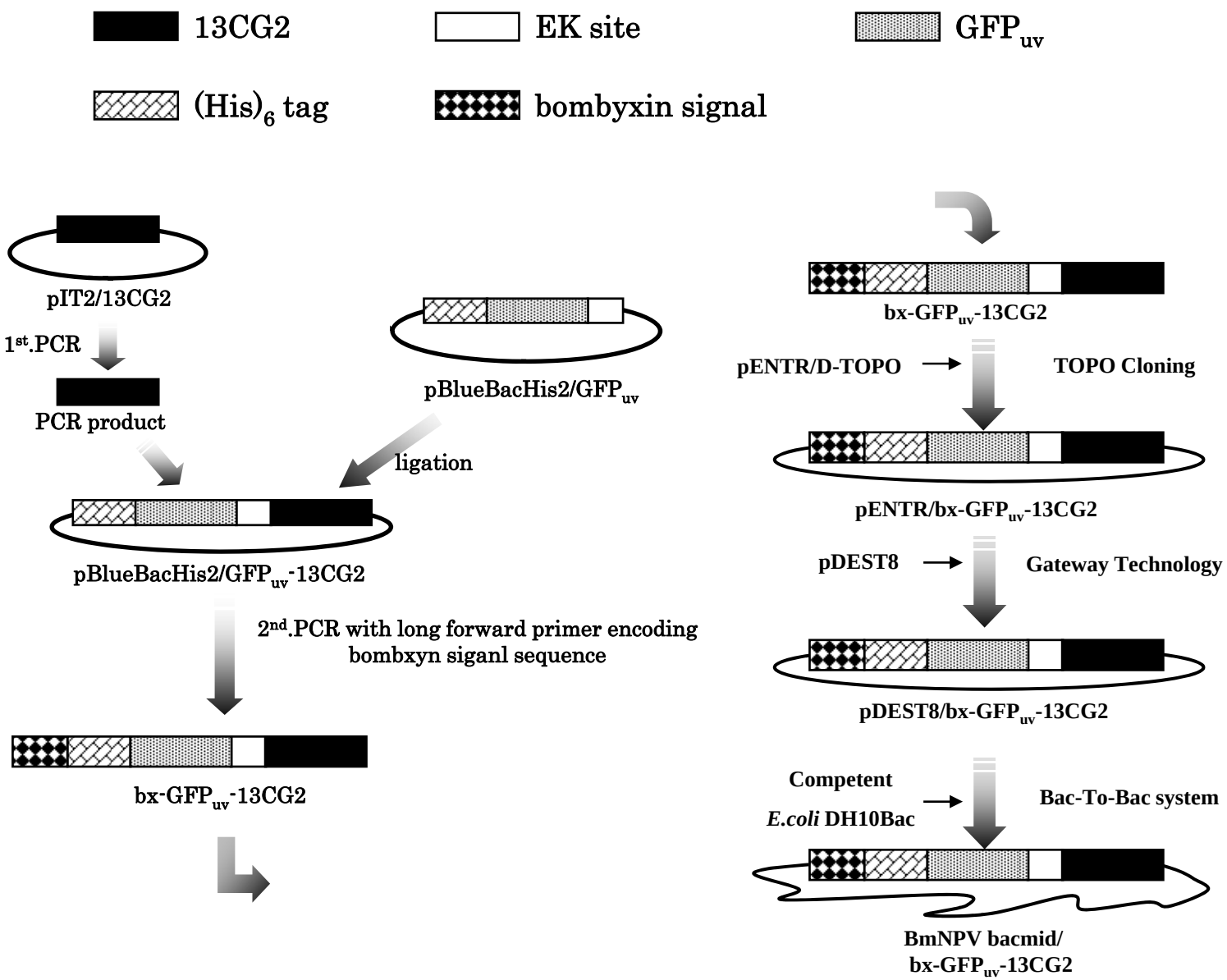
13CG2
EK site
GFPuv
(His)6 tag
bombyxin signal
pIT2/13CG2
1st.PCR
PCR product
pBlueBacHis2/GFPuv
ligation
pBlueBacHis2/GFPuv-13CG2
2nd.PCR with long forward primer encoding bombxyn siganl sequence
bx-GFPuv-13CG2
bx-GFPuv-13CG2
pENTR/D-TOPO
TOPO Cloning
pENTR/bx-GFPuv-13CG2
pDEST8
Gateway Technology
pDEST8/bx-GFPuv-13CG2
Competent
E.coli DH10Bac
Bac-To-Bac system
BmNPV bacmid/
bx-GFPuv-13CG2

Fig. 2, Ishikiriyama et al.

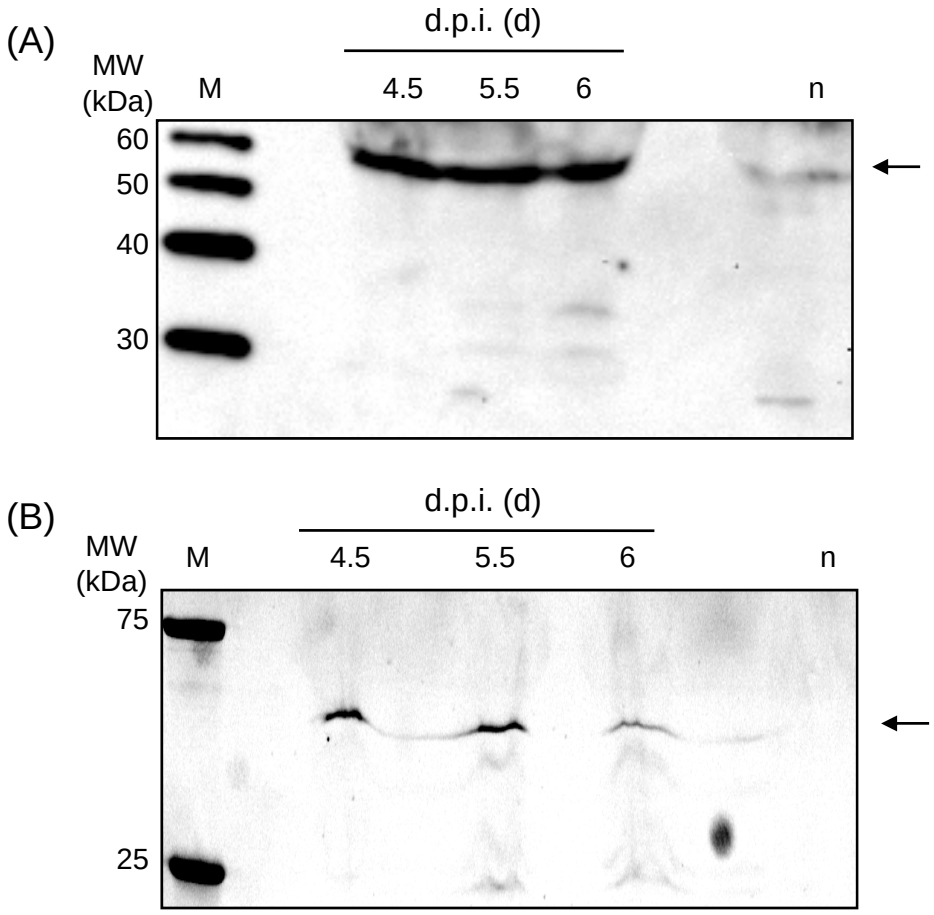

Fig. 3, Ishikiriyama et al.

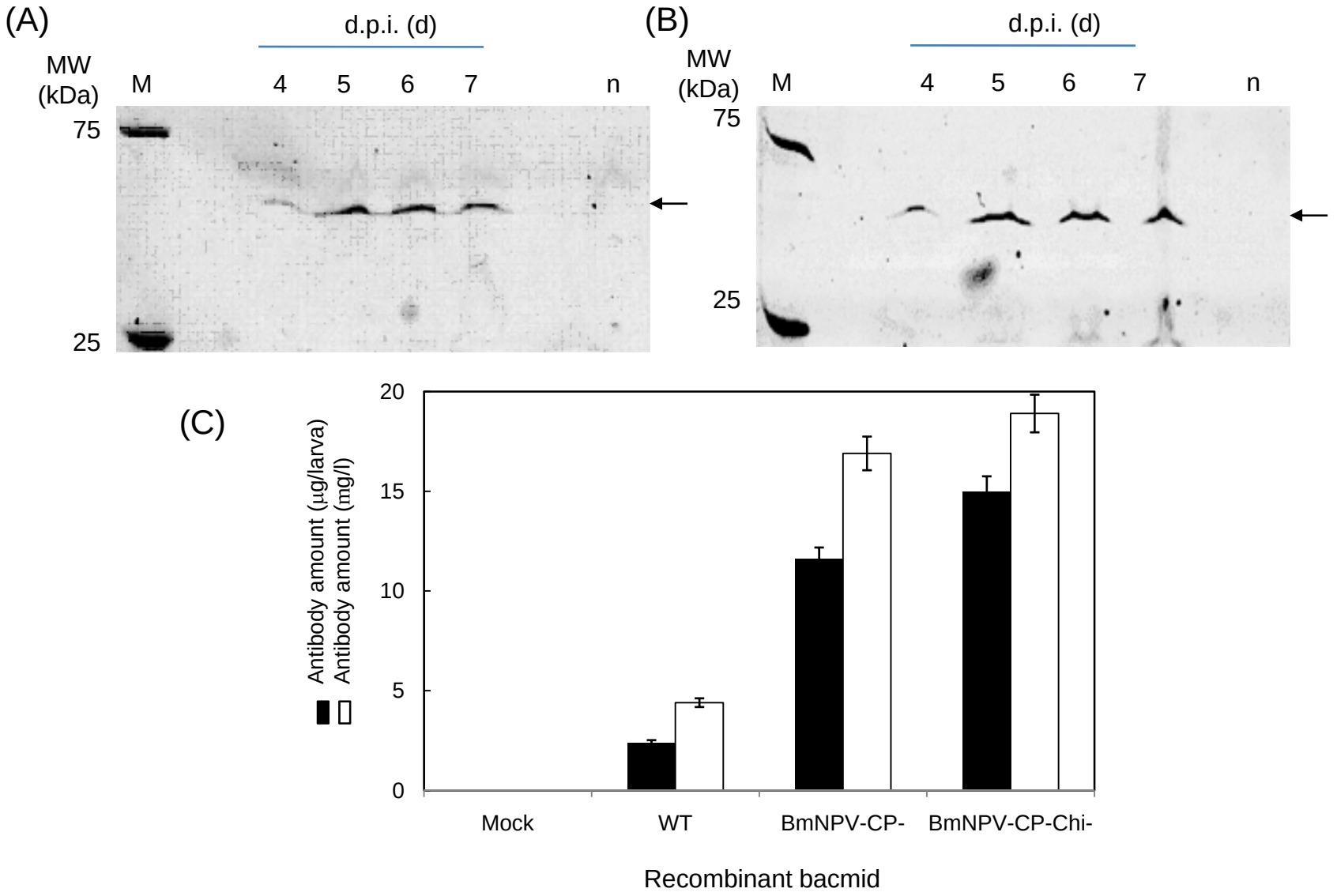

Fig. 4, Ishikiriyama et al.

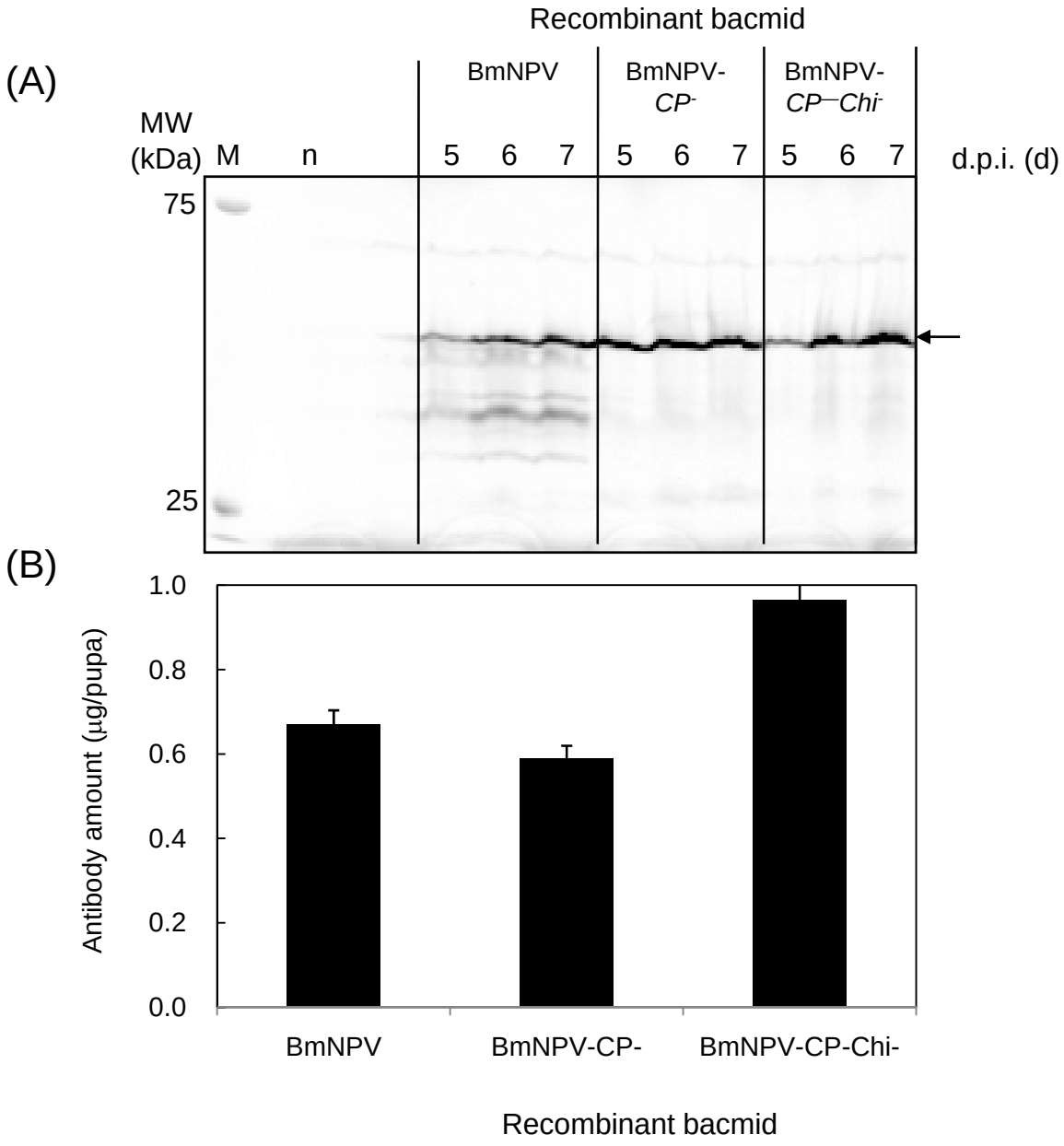

Fig. 5, Ishikiriyama et al.

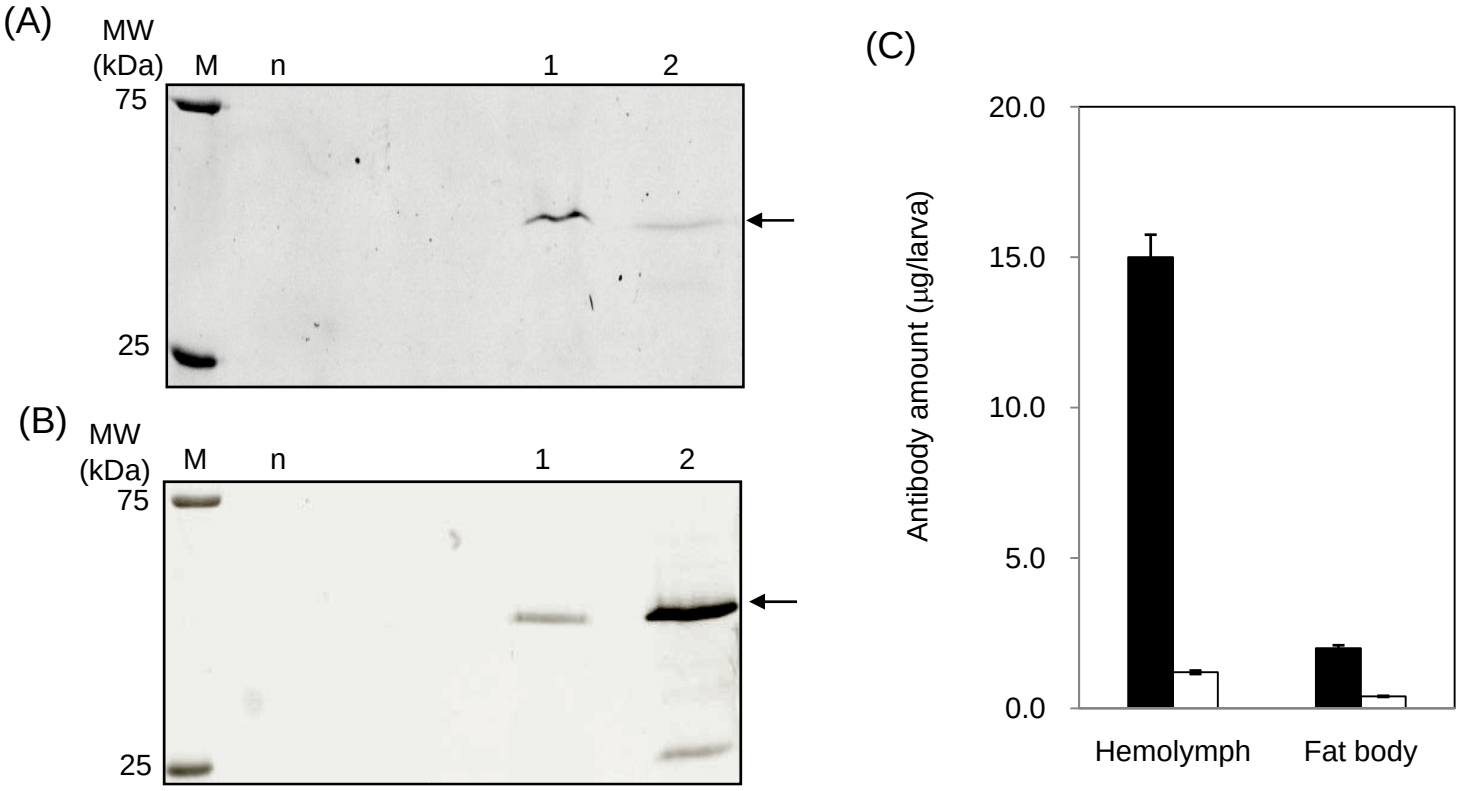

Fig. 6, Ishikiriyama et al.