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PAPER

A fluorescent single domain antibody against plumbagin expressed in silkworm larvae for fluorescence-linked immunosorbent assay (FLISA)

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A fluorescent single-domain antibody (fluobody), a chimera of a green fluorescent protein (AcGFP) with a single chain variable fragment antibody (scFv), against plumbagin (5-hydorxy-2-methyl-1,4-naphthoquinone; PL) was successfully expressed in the hemolymph of silkworm larvae using a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA system to develop a rapid, simple, and sensitive fluorescence-linked immunosorbent assay (FLISA). In this study, two kinds of fluobody, in which the PL–scFv was fused at the *N*-terminus (*N*-fluobody) or *C*-terminus of AcGFP (*C*-fluobody), were expressed in silkworm larvae for comparative purposes. Interestingly, both fluobodies expressed in the BmNPV bacmid DNA system retained both of their original functions as an AcGFP and a PL–scFv, although the functions of the *N*-fluobody were found to be inferior to those of *C*-fluobody when they were expressed in *Escherichia coli*. Moreover, an improvement in the limit of quantification for PL measurement was observed in FLISA (24 ng mL^{-1}) compared with conventional ELISA (0.2 μg mL^{-1}). Since both the *C*-fluobody and *N*-fluobody are useful probes for FLISA and the time-, cost-consuming refolding step required in the conventional bacterial expression system can be avoided when they are expressed in the BmNPV bacmid DNA system, the silkworm expression system is useful for expressing fluobodies when developing FLISA.

Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone; PL) is a naturally occurring bioactive compound produced by the families Plumbaginaceae, Nepenthaceae, and Droseraceae. PL has been found to have diverse pharmacological properties such as anti-cardiotonic, anti-microbial, anti-fertility, anti-atherosclerotic, anti-filarial, and anti-amyloidogenic activities.[1–6] Moreover, PL displays anti-cancer activity, which is partly mediated by the inactivation of NF-κB-regulated anti-apoptotic, proliferative, and angiogenic gene products, resulting in the commitment of cells to apoptosis.[7] Analytical methods for measuring the levels of PL have been developed using high-performance liquid chromatography (HPLC), high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD), and liquid chromatography with tandem mass spectrometry (LC-MS-MS).[8–10]

In our previous study, a monoclonal antibody against PL (MAb 3A3) and a single chain variable fragment antibody (scFv), a variable region of heavy (VH) and light (VL) chains containing a flexible peptide $(Gly_4Ser)_3$, against PL (PL–scFv; DDBJ accession number: **AB470492**) were expressed in *Escherichia coli* (*E. coli*) as a means of developing an enzyme-linked immunosorbent assay (ELISA) for serial determination of PL concentrations in PL containing plants.[11,12] Subsequently, a fluorescent single domain antibody (fluobody), a fusion protein composed of a green fluorescent protein extracted from *Aequorea coerulescens* (AcGFP), a mutant that has been codon-optimized for mammalian expression, and PL–scFv, has been constructed and expressed in *E. coli* to develop an even more simple, rapid, and sensitive immunoassay; *i.e.*, a fluorescence-linked immunosorbent assay (FLISA).[13] FLISA performed using such fluobodies can be completed rapidly because the time- and cost-consuming secondary antibody reaction and following enzyme–substrate reaction necessary for ELISA using MAb 3A3 or PL–scFv can be avoided. Moreover, an improvement in sensitivity (8-fold higher) was observed in the FLISA due to the highly sensitive fluorescence intensity of AcGFP. Thus, we demonstrated the improved utility of fluobodies for fast and sensitive

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immunoassays compared with monoclonal antibodies and scFv. So far, FLISA targeting a small molecule (hapten) has been developed for detecting two kinds of herbicide, picloram[14] and *s*-triazine,[15] and these fluobodies were expressed in the *E. coli*.

In the present study, we focused on the investigation of an alternative host to prepare a promising fluobody for FLISA because the fluobodies expressed in *E. coli* are mostly obtained as bacterial cytoplasmic inclusion bodies, which require time- and cost-consuming refolding. Therefore, we used a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA system, which employs silkworm larvae (*Bombyx mori*) as the host. The BmNPV system was first reported by Maeda *et al.* in 1985, which was used to produce human α-interferon in silkworm larvae using recombinant BmNPV.[16] Since then, it has been widely used for the expression of eukaryotic proteins having complicated structures because the protein expression level of silkworm larvae is higher than that of insect cells. Moreover, the proteins expressed in this system retain their functional structure. Due to these advantages, *B. mori* silkworm larvae infected with BmNPV have been widely used instead of insect cells. However, these traditional protein expression systems require at least 1–2 months to amplify viruses. Recently, a rapid bacmid (a baculovirus shuttle vector) system was developed for BmNPV,[17] and GFP_{uv} was successfully expressed after the one shot injection of BmNPV bacmid DNA into silkworm larvae and pupae. The bacmid can be replicated in the *E. coli* BmDH10Bac strain as a large plasmid and generate the recombinant viral DNA by site-specific transposition in *E. coli* and remains infectious in silkworm larvae. Since the replication of bacmid DNA and the transposition of the target genes of the transfer vector into the bacmid DNA can be achieved in *E. coli*, no time-consuming preparation of recombinant viruses is required.

Two kinds of fluobody fusing PL–scFv at the *C*-terminus of AcGFP (*C*-fluobody) or *N*-terminus of AcGFP (*N*-fluobody) with a flexible peptide linker $(Gly_4Ser)_2$ between the two domains in order to avoid structural and functional interference were used and a baculovirus expression donor vector for their expression containing the honeybee melittin secretion signal (HMSS) peptide was designed in order to enhance the secretion of fluobodies into the hemolymph of silkworm larvae.[18] The efficient expression, purification, and characterization of a fluobody against PL using a novel BmNPV bacmid DNA system and a comparison of the expression profile produced using this system in FLISA with that obtained using a bacterial system are described in this paper.

Materials and methods

Chemicals and immunochemicals

PL was purchased from Wako Pure Chemicals Industries (Osaka, Japan). HRP-labeled anti-T7-tag conjugates, DMRIE-C reagent, and the pMelBac A vector were obtained from Invitrogen (San Diego, CA, USA). HRP-labeled anti-His-tag monoclonal antibody produced by mouse was purchased from MBL International Corp. (Nagoya, Japan). The DNA polymerase and DNA restriction enzyme were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical reagent grade.

Construction of a baculovirus donor vector

The HMSS peptide used to enhance the secretion of fluobodies into the hemolymph was amplified by the PCR from the pMelBac A vector (Invitrogen) using a HMSS specific primer containing *Bam*H I and *Eco*R I restriction enzyme sites. The PCR conditions used for the amplification of HMSS were as follows: 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 5 s), and extension (72 °C, 30 s) with PrimeStar HS DNA polymerase (Takara, Kyoto, Japan). It was then purified, digested using *Bam*H I and *Eco*R I, and ligated downstream of the polyhedrin promoter of the pFastBac1 vector (Invitrogen) to generate the pFastBacMel (pFBM) vector.

Subsequently, the *C*-fluobody gene, which contains a flexible standard 10-amino acid linker $(Gly_4Ser)_2$ between the AcGFP and PL–scFv domains, AcGFP–$(Gly_4Ser)_2$–PL–scFv, and *N*-fluobody gene, which contains the same flexible linker between the PL–scFv and AcGFP domains, PL–scFv–$(Gly_4Ser)_2$–AcGFP, were amplified by PCR. In this PCR amplification, each domain was designed to contain His6-tag and T7-tag of pET28a vector for further analysis. The resultant domains were then purified, digested by *Sal* I and *Not* I, and ligated into the pFBM vector to generate pFBM/*C*-fluobody and pFBM/*N*-fluobody donor vectors for silkworm expression. The PCR conditions used for the amplification of *C*-fluobody and *N*-fluobody domains containing the His6-tag and T7-tag were as follows: 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 5 s), and extension (72 °C, 2 min) with PrimeStar HS DNA polymerase.

The primers used for the construction of the pFBM/*C*-fluobody and pFBM/*N*-fluobody were as follows: forward primer for HMSS: 5′-CGC*GGATCC***ATGAAATTCTTAGTCAAC**-3′; reverse primer for HMSS: 5′-AGC*GAATTC***CGCATAGATGTAAGAAA**-3′; forward primer for *C*-fluobody and *N*-fluobody: 5′-CGC*GTCGA-C*ACATGAGCAGC**CATCATCATCAT**-3′; reverse primer for *C*-fluobody: 5′-TTT*GCGGCCGC*CTA**CCGTTTTATTTCCAG**-3′: reverse primer for *N*-fluobody: 5′-TTT*CGGGCCGC*CTA**CTTG-TACAGCTCATCCAT**-3′. In these primers, the nucleotides coding restriction enzyme sites (*Bam*H I, *Eco*R I, *Sal* I, and *Not* I) are italicized. The nucleotides contributing to the coding sequence of the HMSS, His6-tag, *C*-fluobody, and *N*-fluobody are shown in bold.

Transposition of the pFBM/*C*-fluobody and pFBM/*N*-fluobody in *E. coli* BmDH10Bac cells

The transposition was carried out by transforming the donor pFBM/*C*-fluobody or pFBM/*N*-fluobody plasmid into *E. coli* BmDH10Bac cells. These cells contain a parent bacmid that recombines with the donor plasmid containing pFBM/*C*-fluobody or pFBM/*N*-fluobody to create an expression bacmid DNA. The resultant transformed *E. coli* BmDH10Bac cells were grown on Luria–Bertani (LB) agar plates containing kanamycin (50 μg mL^{-1}), gentamicin (7 μg mL^{-1}), tetracycline (10 μg mL^{-1}), IPTG (40 μg mL^{-1}), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (300 μg mL^{-1}). White antibiotic-resistant colonies were selected, and then the BmNPV bacmids, which were designed as BmNPV bacmid/*C*-fluobody and BmNPV bacmid/*N*-fluobody, were isolated, and their identities were confirmed by PCR using the following universal primer: M13

primer: 5′-GTTTTCCCAGTCACGAC-3′; M13 primer RV: 5′-CAGGAAACAGCTATGAC-3′.

Time-course of fluobody expression in silkworm larvae

On the first day of the fifth instar, the silkworm larvae (21 silkworm larvae) were infected with the BmNPV bacmid/*C*-fluobody or BmNPV bacmid/*N*-fluobody. One μg of the BmNPV bacmid/*C*- or *N*-fluobody was suspended in 3 μL of DMRIE-C reagent (Invitrogen) and kept at room temperature for 45 min. Then, 50 μL of sterilized and deionized water (Nacalai Tesque, Kyoto, Japan) were added to the mixture of bacmid DNA and DMRIE-C reagent to control the infection volume easily. The resultant mixture was then directly injected into the dorsum of the larvae. The hemolymph was collected every 24 h, centrifuged at 14 000 rpm for 30 min at 4 °C, frozen immediately in liquid nitrogen, and kept at −80 °C until use. Three silkworm larvae were used per day in the time-course expression assay for each fluobody.

Indirect ELISA and the measurement of fluorescence intensity using a fluorescent microplate reader (MTP-600FE, Corona) were carried out to optimize the feeding period after infection.

Purification of fluobodies expressed in the hemolymph of silkworm larvae

Functional *C*-fluobodies and *N*-fluobodies containing the His6-tag at their *N*-termini were purified by immobilized metal ion affinity chromatography (IMAC) using His-bind resin (Novagen). The collected hemolymph (each 30 mL) was dialyzed against a binding buffer (50 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, and 10% (v/v) glycerol; pH 8.0) for IMAC.

First, 8 mL of His-bind resin were packed into the column (1 cm × 23 cm) and charged with Ni^{2+} using 50 mM $NiSO_4$ dissolved in the binding buffer. After washing the column with the binding buffer to remove unadsorbed Ni^{2+}, the dialyzed samples (30 mL) in the binding buffer were applied to resin, which was then washed with the binding buffer followed by a washing buffer (50 mM Tris–HCl, 500 mM NaCl, 50 mM imidazole, and 10% (v/v) glycerol; pH 8.0) to remove nonspecifically bound proteins. The bound proteins were then eluted with an elution buffer (50 mM Tris–HCl, 500 mM NaCl, 250 mM imidazole, and 10% (v/v) glycerol; pH 8.0) and analyzed by indirect ELISA using PL-Ova (1 μg mL^{-1}) as a coating antigen. To obtain the fluobodies at high purity, IMAC was repeatedly carried out using the same resin. The positive fraction (10 mL) obtained in the indirect ELISA was collected, dialyzed against the binding buffer, and applied to resin equilibrated with the binding buffer. The column was then washed with the washing buffer followed by the elution buffer, and the eluted samples were analyzed by indirect ELISA.

The *C*-fluobody and *N*-fluobody yields were determined according to the method of Bradford, 1976.[19]

SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out according to the methods of Laemmli and Towbin, respectively.[20,21] Protein samples were separated by 12.5% (w/v) SDS-PAGE under reducing conditions and then transferred electrophoretically onto a PVDF membrane (Millipore) at 100 V and 90 mA for 3 h in an ice water bath. The membrane was then immersed in phosphate-buffered saline containing 10% (w/v) skimmed milk (PBS-sm) for 1 h at room temperature to reduce non-specific adsorption, before being immersed in a 10 000-fold diluted solution of an HRP-labeled anti-His-tag monoclonal antibody produced by mouse (MBL International Corp.) to detect the *N*-terminal T7-tag of the fluobodies. The binding of HRP-labeled anti-His-tag monoclonal antibody was visualized by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (Sigma).

Measurement of fluorescence intensity

To compare the fluorescence intensities between *C*-fluobodies and *N*-fluobodies, the concentrations of the purified fluobodies were equalized (50 μg mL^{-1}) in PBS, which was also used as a negative control. In this assay, a black microtiter plate (FluoroNunc, MaxiSorp) was used to reduce the background fluorescence. The fluorescence intensity of each sample (50 μL $well^{-1}$) was measured with an MTP-600FE fluorescent microplate reader (Corona) at excitation and emission wavelengths of 490 nm and 530 nm, respectively.

Indirect ELISA and indirect competitive ELISA (icELISA)

Indirect ELISA was carried out to analyze the binding activity of the fluobodies to PL-ovalbumin (PL-Ova) conjugates synthesized in our previous study.[12] A 96-well immunoplate (Nunc, Maxisorb, Roskilde, Denmark) was coated with PL-Ova (1 μg mL^{-1}, 100 μL $well^{-1}$) conjugates in 50 mM carbonate buffer (pH 9) and incubated for 1 h. The plate was then washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T), before being treated with 300 μL of PBS-sm for 1 h to reduce non-specific adsorption. Subsequently, various concentrations of fluobodies (100 μL $well^{-1}$) were incubated for 1 h. The plate was then washed three times with PBS-T and incubated with 100 μL of a 5000-fold diluted solution of horseradish peroxidase (HRP)-labeled anti-T7-tag conjugates (Invitrogen) for 1 h. After washing the plate three times with PBS-T, 100 μL of 0.3 mg mL^{-1} 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate solution in 0.1 M citrate buffer (pH 4.0) supplemented with 0.003% (v/v) H_2O_2 were added to each well and incubated for 15 min. All ELISA incubation steps were carried out at 37 °C. Absorbance was measured at 405 nm with a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International).

An icELISA was also carried out to analyze the inhibitory activity of the fluobodies against PL. The same procedures that were used in the indirect ELISA were used until the blocking step. After washing the blocked-plate three times with PBS-T, 50 μL of various concentrations of PL in 10% (v/v) MeOH were incubated with 50 μL of each fluobody solution for 1 h. The plate was then washed three times with PBS-T, and the fluobody bound to PL-Ova was combined with 100 μL of a 5000-fold diluted solution of HRP-labeled anti-T7-tag conjugates for 1 h. After washing the plate three times with PBS-T, 100 μL of ABTS substrate solution were added to each well and incubated for 15 min. Absorbance was measured at 405 nm using a microplate reader.

The dissociation constant (K_D) was used to evaluate the binding affinities of the fluobodies, and the cross-reactivities (CRs) of both fluobodies against various compounds were calculated to investigate the specificity according to the method of Friguet *et al.* and Weiler and Zenk, respectively.[22,23]

Indirect FLISA and indirect competitive FLISA (icFLISA)

For the indirect FLISA, a black microtiter plate (FluoroNunc, MaxiSorp, Roskilde, Denmark) was coated with PL-Ova (1 μg mL^{-1}, 100 μL $well^{-1}$) in 50 mM carbonate buffer (pH 9) and incubated at 37 °C for 1 h. The plate was washed three times with PBS-T and then treated with 300 μL of PBS-sm for 1 h at 37 °C to reduce non-specific adsorption. Subsequently, various concentrations of fluobodies (100 μL $well^{-1}$) were incubated for 1 h at 25 °C. After washing the plate a further three times with PBS-T and adding 100 μL PBS to each well, the remaining fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a fluorescent microplate reader (MTP-600FE, Corona).

In the icFLISA, the same procedures as were used in the indirect FLISA were used until the blocking step. After washing the blocked-plate three times with PBS-T, various concentrations of PL (50 μL) in 10% (v/v) MeOH were incubated with 50 μL of purified fluobody solution for 1 h at 25 °C to observe the competition between PL and the fluobody. After washing the plate a further three times with PBS-T and adding 100 μL PBS to each well, the remaining fluorescence was measured with an MTP-600FE fluorescent microplate reader.

Results and discussion

Construction of a baculovirus donor vector and recombinant baculovirus

Baculovirus donor vectors, pFBM/*C*-fluobody and pFBM/*N*-fluobody, were successfully constructed for the expression of the *C*-fluobody and *N*-fluobody in silkworm larvae by subcloning a chimera gene encoding an His6-tag and T7-tag at the *N*-termini of the genes (1461 bp) encoding the *C*-fluobody or *N*-fluobody domain in pET28a vector. Recombinant bacmids containing the fluobody genes, BmNPV/*C*-fluobody and BmNPV/*N*-fluobody, were obtained through transposition in the *E. coli* BmDH10Bac cells developed by Motohashi *et al.*[17]

Time-course of fluobodies expression in silkworm larvae

A fluobody time-course expression assay was carried out to optimize the feeding period after the injection of bacmid DNA into silkworm. To determine the most appropriate feeding period, the hemolymph was collected every 24 h and used for indirect ELISA and measurement of fluorescence intensity using a fluorescent microplate reader (MTP-600FE, Corona).

Fig. 1 shows the indirect ELISA and fluorescence intensity results for the expression of time-course expression of both fluobodies. The absorbance at 405 nm and fluorescence intensity at 530 nm of both fluobodies increased markedly until 144 h post-infection. Each silkworm larva showed the pathological symptoms induced by the BmNPV at 144 h post-infection; *i.e.*, their bodies constricted between segments, and they writhed feebly. Since the hemolymph was secreted as the infection progressed and it was difficult to collect the hemolymph at 168 h post-infection, it was estimated that 144 h is the best feeding time after infection.

Expression and purification of fluobodies in silkworm larvae

Thirty silkworm larvae each were used for the expression of the *C*-fluobody and *N*-fluobody. Then, isolated BmNPV bacmid/*C*-fluobody and BmNPV bacmid/*N*-fluobody (1 μg) were directly injected into the dorsum of the larvae with DMRIE-C reagent (Invitrogen); and the larvae were cultured for 144 h at 25 °C; and the hemolymph was collected in the microtube containing 5% (w/v) sodium thiosulfate (50 μL), centrifuged, dialyzed, and subjected to further purification. Fig. 2 shows pictures of

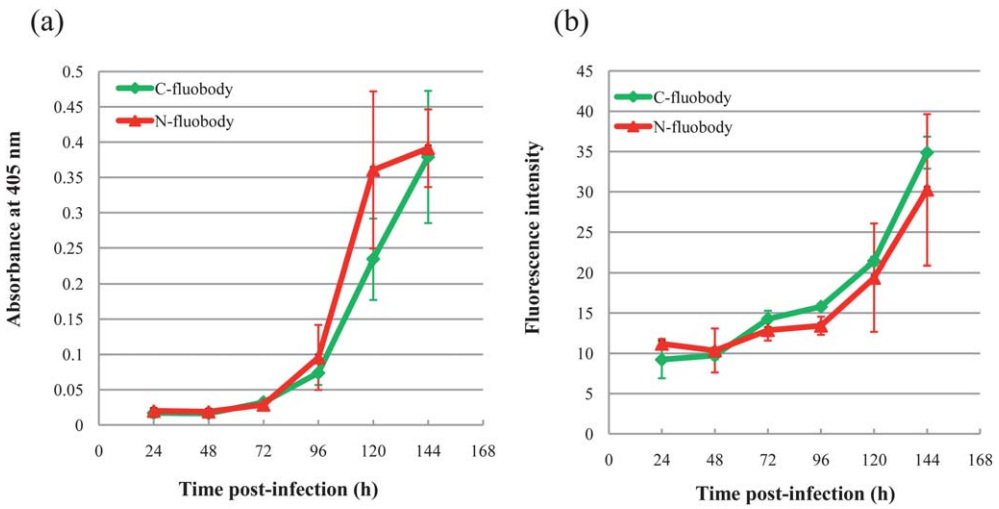

Fig. 1 Time-course of fluobodies expression in silkworm larvae. (a) Fluobody expression time-course based on indirect ELISA. Fifty μL of hemolymph were used in both indirect ELISA. (b) Fluobody expression time-course based on fluorescence intensity. Fifty μL of hemolymph were dropped into a black microtiter plate (FluoroNunc, MaxiSorp, Roskilde, Denmark), and fluorescence intensity was measured using an MTP-600FE fluorescence microplate reader.

Fig. 2 Expression analysis in silkworm larvae under a confocal fluorescence microscope. (a) Images of the silkworm larvae taken under visible light. (b) Images of the silkworm larvae taken using a confocal fluorescence microscope. In each picture, the left side shows the silkworm larvae post-infection with the BmNPV bacmid/*C*-fluobody, and the right side shows non-infected silkworm larvae.

non-infected silkworm larvae and infected silkworm larvae taken using a confocal fluorescence microscope (Nikon AZ100).

The purification of the fluobodies expressed in the hemolymph of the silkworm larvae was performed by IMAC using His-bind resin (Novagen). In this purification process, the purities of the *C*-fluobody and *N*-fluobody were estimated to be more than 80% based on Coomassie brilliant blue staining (Fig. 3a). The yields of the purified *C*-fluobody and *N*-fluobody were 1.9 mg (63.3 mg per 1 L of hemolymph) and 1.6 mg (53.3 mg per 1 L of hemolymph) per 30 silkworm larvae, respectively.

Western blot analysis showed that the hemolymph collected from the infected silkworm larvae exhibited an immunoreactive band with a molecular mass of 57 kDa, which agrees with the theoretical values for both *C*-fluobody and *N*-fluobody (56 990 Da) (Fig. 3b). The presence of fluobodies in the hemolymph indicated that the HMSS sequence incorporated into the gene construct has successfully targeted the soluble fluobodies.

Measurement of fluorescence intensity

The fluorescence intensity of the purified fluobodies (50 μL well^{-1}) was then measured at a protein concentration of 50 μg mL^{-1} using the MTP-600FE fluorescent microplate reader at an emission wavelength of 490 nm and an excitation wavelength of 530 nm. In this assay, PBS was used as both a negative control and a solvent to adjust the concentration of the fluobodies.

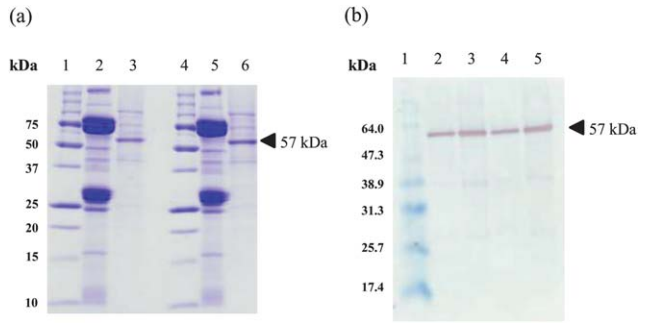

Fig. 3 SDS-PAGE and Western blot analysis of fluobodies expressed in the hemolymph of silkworm larvae. (a) SDS-PAGE analysis of fluobodies expressed in the hemolymph. Lanes 1 and 4, molecular protein marker; lane 2, hemolymph collected from infected silkworm larvae (BmNPV/*C*-fluobody); lane 3, purified *C*-fluobody (1.2 μg); lane 5, hemolymph collected from infected silkworm larvae (BmNPV/*N*-fluobody); lane 6, purified *N*-fluobody (1.2 μg). (b) Western blot analysis of fluobodies expressed in the hemolymph. Lane 1, molecular protein marker; lane 2, hemolymph collected from the silkworm larvae post-infection (BmNPV/*C*-fluobody); lane 3, purified *C*-fluobody (120 ng); lane 4, hemolymph collected from the silkworm larvae post-infection (BmNPV/*N*-fluobody); lane 5, purified *N*-fluobody (120 ng).

It was revealed that the fluorescent intensity of the *N*-fluobody was higher than that of the *C*-fluobody (Fig. 4), despite the fluorescence intensity of the *C*-fluobody being 18-fold higher than that of the *N*-fluobody when they were expressed in *E. coli*.[13] These results raise the possibility that not only the *C*-fluobody but also the *N*-fluobody could be applied to FLISA when they are expressed in the silkworm larvae.

Indirect ELISA and indirect competitive ELISA (icELISA) using fluobodies

Indirect ELISA was carried out to investigate the binding activity of purified *C*-fluobody and *N*-fluobody to PL-Ova conjugates (1 μg mL^{-1}). A reactivity response curve was drawn by plotting the absorbance against the logarithm of fluobody concentration in the indirect ELISA. The concentration of each fluobody was positively correlated with the absorbance value in a logistical manner (Fig. 5).

The fluobodies were then applied to icELISA to analyze the inhibitory activity of fluobodies against PL. *C*-Fluobody (15.7 μg mL^{-1}) or *N*-fluobody (26.5 μg mL^{-1}) was incubated with serially double diluted concentrations of free PL on an immunoplate. Any fluobodies that had bound to free PL were washed away. The fluobodies that bound to the immobilized PL-Ova conjugates were then incubated with HRP-labeled anti-T7-tag conjugates and treated with ABTS solution to develop the color. In this icELISA, the detectable range of PL concentrations for the *C*-fluobody and *N*-fluobody ranged from 0.2–25 μg mL^{-1} (Fig. 6), indicating that they displayed the same sensitivity as PL–scFv and its parental antibody, MAb 3A3.[11,12]

Characterization of fluobodies expressed in silkworm larvae

An icELISA was carried out to investigate the specificity of the recombinant fluobodies against structurally related compounds and their binding affinity against PL.

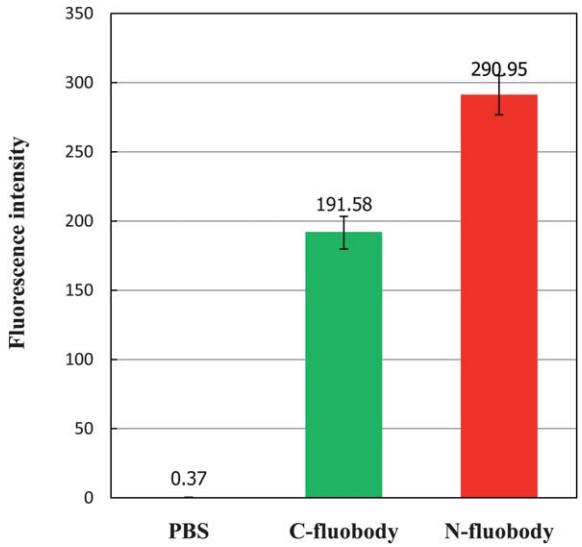

Fig. 4 Measurement of fluorescence intensity.

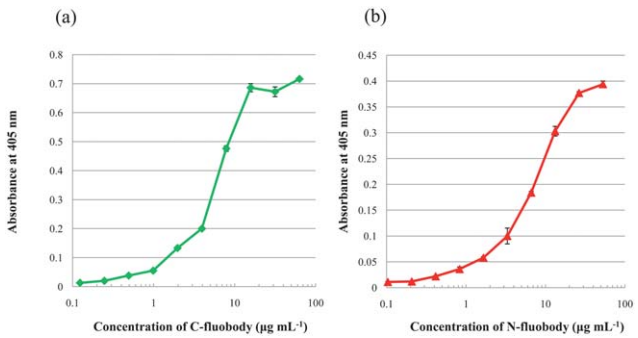

Fig. 5 Reactivity of purified fluobodies to PL–Ova conjugates in indirect ELISA. (a) Reactivity response curve of *C*-fluobody in indirect ELISA. (b) Reactivity response curve of *N*-fluobody in indirect ELISA.

The ELISA method described by Friguet *et al.* was used to estimate the dissociation constant (K_D) of the fluobodies in solution.[22] This method is equally valid for antibodies against small (hapten) and large molecular weight antigens but does not label either the antibodies or antigens involved. Briefly, various concentrations of PL were incubated with fluobody at 37 °C for 1 h until they reached equilibrium. The amount of free fluobody in the incubation mixture was then determined by ELISA, as described above. The K_D values of the *C*-fluobody and *N*-fluobody in solution were 7.42×10^{-6} M and 6.21×10^{-6} M, respectively, as determined by typical Scatchard plots. When the K_D of MAb 3A3 (6.18×10^{-6} M) was compared with those of the fluobodies, it was found that both fluobodies retained the binding affinity of their parental monoclonal antibody, MAb 3A3 (Table 1).

The CRs of the fluobodies were determined using the calculation described by Weiler and Zenk to evaluate their specificity. Table 2 shows the CRs of the fluobodies against structurally related compounds. The CRs of the *C*-fluobody were almost the same as those of *N*-fluobody, indicating that specificities of the two are similar.

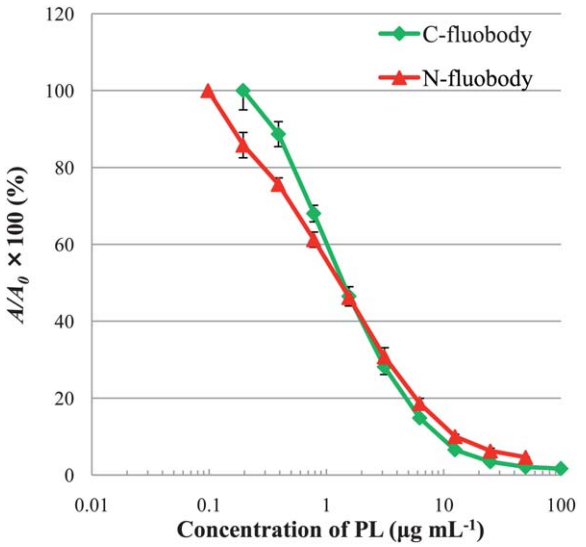

Fig. 6 PL calibration curves obtained in icELISA using the *C*-fluobody or *N*-fluobody. The green squares and green curve show the standard curve produced using *C*-fluobody (15.7 μg mL^{-1}). The red triangles and red curve show the standard curve produced when *N*-fluobody (26.5 μg mL^{-1}) was used. A/A_0, A_0 is the absorbance in the absence of PL, and A is the absorbance in the presence of PL.

Indirect FLISA and indirect competitive FLISA (icFLISA) using fluobodies

These fluobodies were then applied to indirect FLISA and icFLISA to develop a rapid, sensitive, and simple immunoassay. In this assay, the time-consuming and costly steps involving the secondary antibody and subsequent enzyme–substrate reaction could be avoided because the fluobodies had been directly fused with AcGFP, which enabled their direct detection by the MTP-600FE fluorescent microplate reader. Moreover, an improvement in sensitivity is expected in this assay system due to the detection of the fluorescence intensity of AcGFP rather than absorbance derived from an enzyme labeling secondary antibody.

An indirect FLISA was carried out to evaluate the binding reactivity of the fluobodies to PL-Ova conjugates (1 μg mL^{-1}), which were used to coat a 96-well black microtiter plate, and the remaining fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a fluorescent microplate reader (MTP-600FE, Corona). Fig. 7 shows the reactivity response curves for *C*-fluobody and *N*-fluobody in indirect FLISA. The concentrations of the both fluobodies were positively correlated with their fluorescence intensity values in a logistical manner.

Table 1 Dissociation constant (K_D) determined by icELISA

Antibody	K_D/M
MAb 3A3	6.18×10^{-6}
C-Fluobody	7.42×10^{-6}
N-Fluobody	6.21×10^{-6}

Table 2 CRs of fluobodies against structurally related compounds[a]

Compound	CRs (%)	
	C-Fluobody	*N*-Fluobody
Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)	100	100
Menadione (2-methyl-1,4-naphthoquinone)	15.0	17.1
Juglone (5-hydroxy-1,4-naphthoquinone)	6.0	6.1
Lawsone (2-hydroxy-1,4-naphthoquinone)	<0.005	0.1
Shikonin	1.1	0.9
1,4-Naphthoquinone	2.6	2.7
1,2-Naphthoquinone	6.1	4.2
Sennoside A	<0.005	<0.005
Sennoside B	<0.005	<0.005
1-Naphthol	<0.005	<0.005
1-Naphthol	<0.005	<0.005

[a] CRs were calculated using the method of Weiler and Zenk as follows: $\text{CRs}(\%) = \frac{\text{IC}_{50} \text{ for PL}}{\text{IC}_{50} \text{ for compound under investigation}} \times 100$.

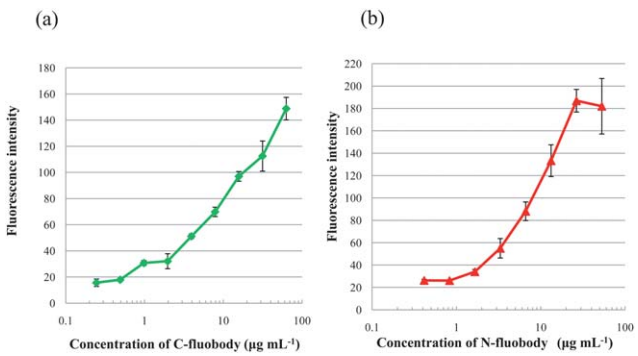

Fig. 7 Reactivity of fluobodies to PL–Ova conjugates in indirect FLISA. (a) Reactivity response curve of *C*-fluobody in indirect FLISA. (b) Reactivity response curve of *N*-fluobody in indirect FLISA.

We then analyzed the inhibitory activities of the fluobodies against PL in an icFLISA. Fig. 8 shows calibration curves for various concentrations of PL in 10% (v/v) MeOH solution. In this icFLISA, the limit of quantification was determined by the standard curve produced using various concentrations of PL and PL detection ranges of the two fluobodies ranged from 24 ng mL^{-1} to 6.3 μg mL^{-1}. In both cases, the limit of quantification for PL in FLISA (24 ng mL^{-1}) was found to be 8-fold higher than that in conventional ELISA (0.2 μg mL^{-1}), as expected. We concluded that the improvement in sensitivity was produced by the highly sensitive fluorescence of AcGFP compared with that of an enzymatic chromophore. These results indicate that sensitive immunoassays can be developed using *C*-fluobody or *N*-fluobody, providing they are expressed in silkworm larvae, although *N*-fluobodies expressed in *E. coli* cannot be used in FLISA because of their low fluorescence intensity.[13]

Intra/inter-assay precision tests

To validate the icFLISA developed using the *C*-fluobody and *N*-fluobody, their intra- and inter-assay precision values were evaluated by testing nine different PL concentration samples in three assays performed together on the same day and on three consecutive days, respectively. Intra- and inter-assay coefficients

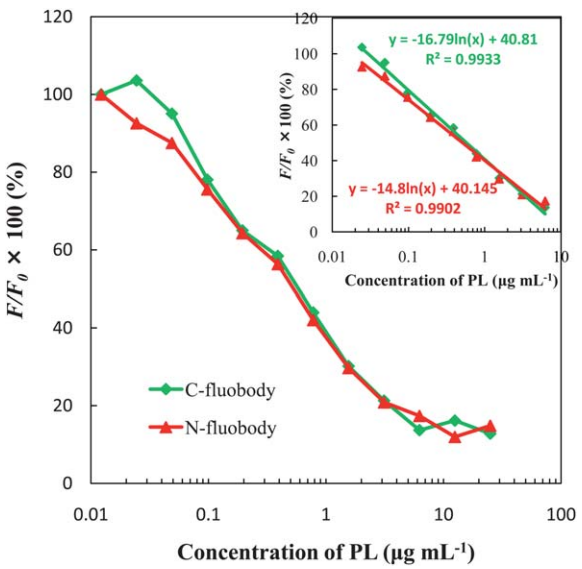

Fig. 8 PL calibration curves produced in icFLISA using the *C*-fluobody or *N*-fluobody. The green squares and green curve show the standard curve produced using *C*-fluobody (15.7 μg mL^{-1}). The red triangles and red curve show the standard curve produced when *N*-fluobody (26.5 μg mL^{-1}) was used. The inset shows the linearized calibration curve using the same experimental data. F/F_0, F_0 is the fluorescence intensity in the absence of PL, and F is the fluorescence intensity in the presence of PL.

of variation (CV) for precision were determined based on the ratios of the standard deviation (SD) and mean values from three assays. From the results shown in Table 3, the maximum intra-assay CV in the assays using *C*-fluobody and *N*-fluobody were 7.6% and 9.8%, respectively, while the maximum inter-assay CV for *C*-fluobody and *N*-fluobody were 8.2% and 12.1%, respectively. Almost all CV values were less than 10%, indicating that the icFLISA developed using fluobodies expressed in silkworm larvae is precise enough to be used in this assay.

Conclusion

Recombinant fluobodies (*C*-fluobody and *N*-fluobody) were successfully expressed in the hemolymph of silkworm larvae

Table 3 Intra- and inter-assay precision of PL determination by icFLISA using fluobodies[a]

	CV (%)			
	C-Fluobody		*N*-Fluobody	
PL/μg mL^{-1}	Intra-assay (%; $n = 3$)	Inter-assay (%; $n = 3$)	Intra-assay (%; $n = 3$)	Inter-assay (%; $n = 3$)
0.024	3.46	8.15	3.09	6.59
0.049	7.63	5.91	4.30	8.86
0.098	0.82	5.95	4.02	6.58
0.195	4.65	5.96	9.37	12.10
0.391	3.46	7.40	4.77	8.12
0.781	3.95	2.83	6.84	6.93
1.563	2.79	1.87	7.12	8.23
3.125	5.75	4.53	9.82	7.16
6.250	3.77	5.82	9.00	11.80

[a] All values are shown as the mean ± SD for three plates (five replicate wells within each plate) for each concentration for 3 consecutive days.

using a *Bombyx mori* nucleopolyhedrovirus bacmid DNA system and applied to the development of a rapid, sensitive, and simple icFLISA for detecting/determining PL in plant samples. The fluobodies expressed in this system overcome the disadvantage of time- and cost-consuming refolding because they were secreted into the hemolymph of silkworm larvae as a soluble protein. In contrast to the bacterial expression of fluobodies, the *C*- and *N*-fluobodies expressed in the BmNPV bacmid DNA system well retained their functions as a probe for FLISA in fluorescence intensity and binding affinity, making it possible to apply both fluobodies to FLISA.

Overall, the BmNPV bacmid DNA system using silkworm larvae has many advantages compared with bacterial expression systems involving *E. coli*.

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