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Construction, Expression, and Characterization of a Single-Chain Variable Fragment Antibody Against 2,4-Dichlorophenoxyacetic Acid in the Hemolymph of Silkworm Larvae

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Abstract A single-chain variable fragment antibody against herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D-scFv) has been successfully expressed in the hemolymph of silkworm larvae using a rapid *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA system. Variable heavy- and light-chain domains were cloned directly from the cDNA of the hybridoma cell line 2C4 and assembled together with flexible peptide linker $(Gly_4Ser)_3$ between two domains. The yield of functional 2,4-D-scFv after purification was 640 μg per 30 ml of hemolymph, which is equivalent to 21.3 mg per liter of hemolymph. The characterization of 2,4-D-scFv using an indirect competitive enzyme-linked immunosorbent assay (icELISA) revealed that it has wide cross-reactivities against 2,4,5-trichlorophenoxyacetic acid (65.5%), 2,4-dichlorophenol (47.9%), and 2,4-dichlorobenzoic acid (26.0%), making it possible to apply 2,4-D-scFv to icELISA for detecting/determining 2,4-D and its metabolites. Judging from its cost and time requirements and its ease of handling, this BmNPV bacmid DNA expression system is more useful for expressing functional scFv than bacterial systems, which frequently require costly and time-consuming refolding.

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Abbreviations

ABTS 2	2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt
BmNPV	*Bombyx mori* nucleopolyhedrovirus
bp	Base pairs
CR	Cross-reactivities
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-D-scFv	Single-chain variable fragment antibody against 2,4-D
ELISA	Enzyme-linked immunosorbent assay
HMSS	Honeybee melittin signal sequence
HRP	Horseradish peroxidase
K_D	Dissociation constant
PBS	Phosphate-buffered saline
scFv	Single-chain variable fragment
VH	Heavy chain variable region
VL	Light-chain variable region

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) and its derivatives (Fig. 1) are some of the most widely used herbicides in agriculture. Unfortunately, they contaminate not only ground water and surface water, but also drinking water as well. Although many reports have described the harmful toxicities of 2,4-D, such as embryotoxicity, teratogenicity, and hepatotoxicity [1–5], this type of herbicide is still being used worldwide. The negative consequences of ecological contamination have increased the demand for a monitoring system for measuring water quality.

Standard qualitative and quantitative methods for measuring 2,4-D have been developed using conventional high performance liquid chromatography (HPLC) [6] and HPLC coupled with tandem mass spectrometry [7]. Although conventional qualitative and quantitative methods are available, alternative immunochemical methods may be practical. These methods include enzyme immunoassays, immunoagglutination, fluores-

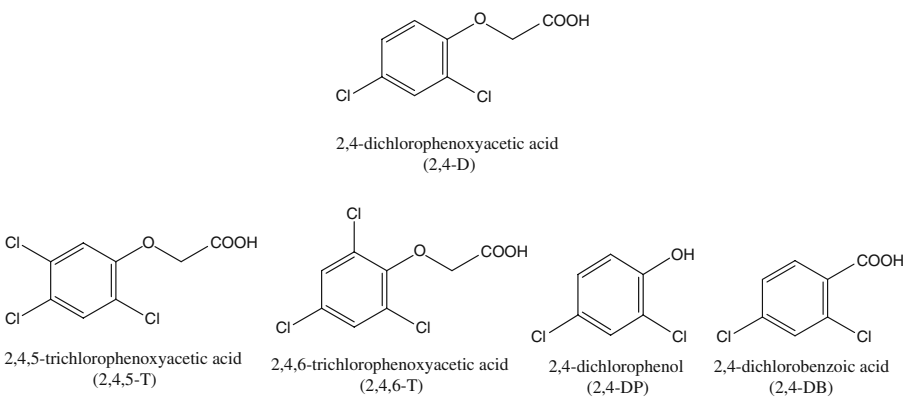

Fig. 1 Structures of 2,4-dichlorophenoxyacetic acid and structurally related compounds

cence polarization, and enzyme assays as well as antibody-based biosensor methods [8–12].

In our previous study, we produced a monoclonal antibody against 2,4-D (MAb 2C4) that was secreted from hybridoma cells prepared by fusing splenocytes with the mouse myeloma cell line, P3-X63-Ag8-653 [13]. Although monoclonal antibodies (MAb) possess more specific activity than polyclonal immunoglobulins and are suitable for large-scale production, hybridoma cell lines are not stable, especially after cryopreservation [14]. Moreover, it is time-consuming and labor-intensive to obtain MAb. These disadvantages of MAb can be overcome by using a genetically engineered antibody. Single-chain variable (scFv) antibodies, which contain variable regions of heavy (VH) and light chains (VL) together with a flexible peptide $(Gly_4Ser)_3$, have been widely used in antibody engineering as well as in research, therapeutic, and diagnostic applications, including as biosensors for the detection of target molecules [15], cytokines for immunotherapy [16], and radioisotopes for cancer imaging [17], because antibody production with bacteria is much simpler, cheaper, and faster than that with hybridomas once the recombinant antibody has been constructed.

A traditional baculovirus expression system (BVES) using *Bombyx mori* nucleopolyhedrovirus (BmNPV) can take advantage of inexpensive, convenient, and high-level expression of hetelorogous proteins which provide post-translational protein modifications similar to those generated by mammalian cell expression [18]. Unfortunately, however, the traditional preparation of recombinant baculoviruses that express exogenous genes takes at least 40 days because multiple rounds of virus purification and amplification are required. Furthermore, large-scale cultivation and virus handling techniques are also required.

Recently, a novel and effective BEVS using BmNPV bacmid DNA, which is directly applicable to silkworm larvae, has been developed [19]. In this BEVS, the bacmid can be replicated in the *E. coli* BmDH10Bac strain as a large plasmid and generates recombinant virus DNA via site-specific transposition in *E. coli* and retains its infectious with silkworm larvae. Since the replication of bacmid DNA and the transposition of the target genes of the transfer vector onto the bacmid DNA are performed in *E. coli*, no time-consuming preparation of recombinant virus is required.

In this study, we report the construction, expression, purification, and characterization of 2,4-D-scFv expressed by a BmNPV bacmid DNA system. In addition, the development of an indirect competitive enzyme-linked immunosorbent assay (icELISA) using recombinant 2,4-D-scFv was demonstrated in this paper.

Materials and Methods

Chemicals and Immunochemicals

2,4-D was purchased from Wako Pure Chemicals (Osaka, Japan). Human serum albumin (HSA) was purchased from Sigma-Aldrich (Steinheim, Germany). T7-tag horseradish peroxidase (HRP) labeled conjugate and DMRIE-C Reagent were obtained from Invitrogen (San Diego, CA, USA). DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical grade.

Preparation of 2,4-D-Human Serum Albumin (2,4-D-HSA) Conjugates

1-Ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (9.0 mg) dissolved in 0.3 ml of MES buffer consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid and 0.9%

(*w*/*v*) sodium chloride was added to 2,4-D (10.8 mg) solution consisting of 1.2 ml of MES buffer and 20% (*w*/*w*) pyridine. The reaction mixture was added dropwise to 1.0 ml MES buffer containing 10.0 mg HSA and then stirred at room temperature for 7 h. Subsequently, the mixture was dialyzed against five batches of H_2O for 2 d at 4°C and then lyophilized to yield 6.8 mg of 2,4-D-HSA conjugate, which was used as a coating antigen in ELISA.

Construction of the 2,4-D-scFv Gene

The DNA and RNA manipulations were performed using standard methods. Total RNA (5 μg) was extracted from 1×10^6 hybridoma cells (2C4) using the Sepasol RNA I super reagent (Nakalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. First-strand cDNA was synthesized using random hexamer primers (Amersham Biosciences, Buckinghamshire, UK). The VH and VL genes were amplified by the polymerase chain reaction (PCR) using established antibody-specific primers [20], and the PCR products were cloned into the pGEM-T vector (Promega, Madison, WI). The cloned VH and VL genes were then transformed into *E. coli* JM109 cells. Colony PCR was used to screen for positive colonies, and the plasmids collected from the positive colonies were purified and sequenced.

The VH and VL domains were assembled and linked together in the splicing by overlapping extension PCR (SOE-PCR) to yield the full-length scFv gene. The scFv gene was constructed in a VH-linker-VL format together with a standard flexible 15-amino acid linker $(Gly_4Ser)_3$ and was then subcloned downstream of the His6-tag and T7-tag of the pET 28a expression vector (Novagen) to generate a pET28a/2,4-D-scFv plasmid.

The sequences of the VH- and VL-specific primers used for the construction of the 2,4-D-scFv gene were as follows: *BamH* I-VH: 5′-CGC CGC GGA TCC **CAG GTT CAG CTG CAG CAG TCT GGA**-3′; VH-linker: 5′-*GGA GCC GCC GCC AGA ACC ACC ACC ACC* **TGA GGA GAC TGT GAG AGT**-3′; Linker-VL: 5′-*GGC GGC GGC GGC TCC GGT GGT GGT GGT TCA* **ATT GTG ATG ACC CAG TCT**-3′; and VL-*Sal* I: 5′-CGC GTC GAC CTA **ACG TTT TAT TTC CAA CTT TGT CCC**-3′.

BamH I-VH, VH-linker, Linker-VL, and VL-*Sal* I represent a forward primer for the VH gene containing a *BamH* I site, a reverse primer for the VH gene containing a linker sequence, a forward primer for the VL gene containing a linker sequence, and a reverse primer for the VL gene containing an *Sal* I site, respectively. In the above sequences, the restriction sites are underlined, and the italicized letters indicate linker coding sequences. The nucleotides contributing to the coding sequence of 2,4-D-scFv are shown in bold.

Construction of a Baculovirus Donor Vector

The donor vector for the expression of 2,4-D-scFv in the hemolymph of silkworm larvae was constructed by transforming the honeybee melittin signal sequence (HMSS) peptide into pFastBac 1 (Invitrogen). Briefly, the HMSS used to promote the secretion of 2,4-D-scFv into the hemolymph was amplified by PCR from the pMelBac A vector (Invitrogen), and it was then ligated downstream of the strong polyhedrin promoter of the pFastBac 1 vector (Invitrogen) in order to generate the pFastBacMel (pFBM) vector. The 2,4-D-scFv gene was then amplified by fusing it with the His6- and T7-tags from the pET28a/2,4-D-scFv plasmid by PCR using a His6-tag specific primer and a 2,4-D-scFv specific primer containing a *Sal* I and a *Not* I restriction enzyme site, respectively. It was then digested with the same restriction enzyme and ligated into the pFBM vector to generate the pFBM/2,4-D-scFv vector. The sequences of the primers used for the construction of the baculovirus

expression donor vector were as follows: forward primer for HMSS: 5′-CGC GGA TCC **ATG AAA TTC TTA GTC AAC**-3′; reverse primer for HMSS: 5′-AGC GAA TTC **CGC ATA GAT GTA AGA AA**-3′; forward primer for 2,4-D-scFv: 5′-CGC GTC GAC AC ATG AGC AGC **CAT CAT CAT CAT**-3′; and reverse primer for 2,4-D-scFv: 5′-TTT GCG GCC GCCTA **ACG TTT TAT TTC CAA CTT TGT**-3′.

In the above sequences, the nucleotides coding for restriction sites are underlined. The nucleotides belonging to the coding sequences of HMSS, His6-tag, and 2,4-D-scFv are shown in bold.

Transposition of pFBM/2,4-D-scFv in E. coli BmDH10Bac Cells

Transposition was carried out by transforming the donor plasmid, pFBM/2,4-D-scFv, into *E. coli* BmDH10Bac cells [19], which contain a parent bacmid that recombines with the donor plasmid to create expression bacmid DNA. The resultant transformed *E. coli* BmDH10Bac cells were grown on Luria–Bertani (LB) agar (10 g of Polypeptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), isopropyl-β-D-thiogalactopyranoside (40 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (300 μg/ml). White antibiotic-resistant colonies were selected, and then the BmNPV bacmid, which was designated BmNPV bacmid/2,4-D-scFv was isolated, and its sequence was confirmed by PCR using the following universal primer: M13 primer: 5′-GTT TTC CCA GTC ACG AC-3′; M13 primer RV: 5′-CAG GAA ACA GCT ATG AC-3′.

Expression of the 2,4-D-scFv Gene in Silkworm Larvae

The first day of fifth instar silkworm larvae (30 silkworm larvae) were infected with the BmNPV bacmid/2,4-D-scFv. One microgram of the BmNPV bacmid/2,4-D-scFv was suspended with 3 μl of DMRIE-C reagent (Invitrogen) and kept at room temperature for 45 min, and the resultant mixture was then directly injected into the dorsum of the larvae. After the larvae had been cultured for 144 h at 25°C, the hemolymph was collected in a microtube containing 5% sodium thiosulfate (50 μl). The hemolymph (30 ml) was collected after centrifugation at 14,000 rpm and 4°C for 30 min and used for purification.

Purification of the 2,4-D-scFv

The purification of 2,4-D-scFv was performed by cation exchange column chromatography using a TOYOPEARL CM-650M (Tosoh Corp., Tokyo, Japan) followed by immobilized metal ion affinity chromatography (IMAC) using His-bind resin (Novagen, Madison, WI, USA).

First, 30 ml of twofold concentrated starting buffer (100 mM Tris–HCl and 20% (*v*/*v*) glycerol; pH 6.5) were added to the collected hemolymph (30 ml). It was then mixed with 39 ml of starting buffer (50 mM Tris–HCl and 10% (*v*/*v*) glycerol; pH 6.5) followed by 1 ml of protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan) to produce a final volume of 100 ml. The resultant mixture was then applied to a TOYOPEARL CM-650M (15 ml; 1.1×23 cm) equilibrated with starting buffer. The bound proteins were then eluted with elution buffer (50 mM Tris–HCl, 10% (*v*/*v*) glycerol, 500 mM NaCl, and 20 mM imidazole; pH 8), which had the same composition as the binding buffer for IMAC. Subsequently, the fraction (100 ml) containing 2,4-D-scFv was collected and applied to an Ni-charged His-bind resin (8 ml; 1.6×23 cm) equilibrated with binding buffer as described

above. The column was then washed with washing buffer (50 mM Tris–HCl, 10% (*v*/*v*) glycerol, 500 mM NaCl, and 40 mM imidazole; pH 8), and the bound protein was eluted with elution buffer (50 mM Tris–HCl, 10% (*v*/*v*) glycerol, 500 mM NaCl, and 100 mM imidazole; pH 8). The fraction (95 ml) containing 2,4-D-scFv was passed through Amicon Ultra (Regenerated Cellulose with MWCO: 50,000; Millipore, Billerica, MA, USA) and concentrated using Amicon Ultra (Regenerated Cellulose with MWCO: 10,000; Millipore, Billerica, MA, USA).

Indirect ELISA using 2,4-D-HSA conjugate was carried out to investigate the presence of 2,4-D-scFv after each purification step. The yield of the purified 2,4-D-scFv was determined according to the method of Bradford [21].

Indirect ELISA Using Purified 2,4-D-scFv

Indirect ELISA was carried out to follow the presence of 2,4-D-scFv after purification and to analyze its binding to the 2,4-D-HSA conjugate. A 96-well immunoplate (Nunc, Maxisorb, Roskilde, Denmark) was coated with 2,4-D-HSA (2 μg/ml) conjugates in 50 mM carbonate buffer (pH 9.6; 100 μl/well) and incubated for 1 h. The plate was then washed and treated with 300 μl of phosphate-buffered saline (PBS) containing 10% (*w*/*v*) skimmed milk (PBS-sm) for 1 h to reduce non-specific adsorption. Subsequently, various concentrations of 2,4-D-scFv (100 μl/well) were incubated for 1 h. The plate was then washed and incubated with 100 μl of a 5,000-fold diluted solution of HRP-labeled anti- T7-tag conjugates for 1 h. For color development, 100 μl of substrate solution [0.3 mg of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt (ABTS) in 100 mM citrate buffer containing 0.003% (*v*/*v*) H_2O_2; pH 4] were added to each well and incubated for 20 min. Absorbance was measured at 405 nm with a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International).

All incubation steps of this indirect ELISA were carried out at 37°C, and PBS containing 0.05% (*v*/*v*) Tween 20 (PBS-T) was used to wash the plate three times between each step.

Indirect Competitive ELISA (icELISA) Using Purified 2,4-D-scFv

An icELISA was carried out to analyze the inhibitory activity against 2,4-D. The same procedures as were used in the indirect ELISA were used until the blocking step. After the blocked-plate had been washed three times with PBS-T, 50 μl of various concentrations of 2,4-D in 5% MeOH were incubated with 50 μl of 2,4-D-scFv (3.4 μg/ml) solution for 1 h. After competitive reaction of the 2,4-D-scFv between the free antigen, 2,4-D, and the coated antigen, 2,4-D-HSA, the 2,4-D-scFv bound to 2,4-D-HSA was combined with 100 μl of a 5,000-fold diluted solution of HRP-labeled anti-T7-tag conjugate for 1 h. After the plate had been washed, 100 μl of substrate solution were added to each well and incubated for 20 min. Absorbance was measured at 405 nm using a microplate reader. All incubation steps of this icELISA were carried out at 37°C, and PBS-T was used to wash the plate three times between each step.

The dissociation constant (K_D) was calculated to evaluate the binding affinities of the recombinant 2,4-D-scFv and its parental monoclonal antibody, MAb 2C4, according to the method of Friguet et al. [22].

To evaluate the specificity of 2,4-D-scFv, the cross-reactivities (CR) of the purified 2,4-D-scFv antibody against various compounds were calculated using the method of Weiler and Zenk [23] as follows:

$$\mathrm{CR}(\%) = \frac{\text{Concentration of } 2,4\text{−D yielding } A/A_0 = 50\%}{\text{Concentration of test compound yielding } A/A_0 = 50\%} \times 100$$

where A is the absorbance in the presence of the test compound, and A_0 is the absorbance in the absence of the test compound.

Indirect ELISA and icELISA Using Parental Monoclonal Antibody Against 2,4-D (MAb 2C4)

In both the indirect ELISA and icELISA using MAb 2C4, a 1,000-fold diluted solution of peroxidase labeled anti-mouse IgG (Organon Teknika Cappel Products), which recognizes the Fc fragment of mouse IgG was used as a secondary antibody, and the other procedures were the same as those used for the indirect ELISA and icELISA using 2,4-D-scFv mentioned above.

Recovery test of 2,4-D

To validate accuracy of this assay, five concentrations of 2,4-D (5, 10, 20, 40, and 80 μg/ml) were spiked into distilled water (0.5 ml) and the recovery was calculated by the developed icELISA using 2,4-D-scFv.

Results and Discussion

Construction of the 2,4-D-scFv Gene

The VH (342 bp) and VL domains (321 bp) were cloned from the cDNA of hybridoma cell lines that secrete MAb 2C4 by PCR using established antibody-specific primers [20] and linked together in a VH-linker-VL format with a standard flexible 15-amino acid linker $(Gly_4Ser)_3$ to yield the full-length scFv gene (708 bp) encoding 236 amino acids (Fig. 2). The scFv gene was then subcloned downstream of the His6-tag and T7-tag of the pET 28a expression vector to generate a pET28a/2,4-D-scFv plasmid. This nucleotide sequence was added to the DDBJ under accession number AB558438.

Construction of a Baculovirus Donor Vector and Recombinant Baculovirus

A baculovirus donor vector for the expression of 2,4-D-scFv in the hemolymph of silkworm larvae, pFBM/2,4-D-scFv, was successfully constructed by cloning the HMSS (63 bp) from the pMelBac A vector (Invitrogen) and the 2,4-D-scFv (708 bp) gene containing the His6-tag and T7-tag from the pET28a/2,4-D-scFv vector. A recombinant bacmid, BmNPV bacmid/2,4-D-scFv, was obtained through transposition in the *E. coli* BmDH10Bac cells developed by Motohashi et al. [19].

Expression and Purification of Recombinant 2,4-D-scFv

The isolated BmNPV bacmid/2,4-D-scFv (1 μg) was directly injected into the dorsum of the larvae with DMRIE-C reagent (Invitrogen). Each silkworm larvae showed the pathological symptoms induced by BmNPV at 144 h post-infection; i.e., their bodies constricted between segments, and they writhed feebly. As they would soon die if left as they were, the hemolymph was collected, and 2,4-D-scFv was purified using a TOYOPEAL CM-650M (Tosoh Corp.), His-bind resin (Novagen), and Amicon Ultra (Millipore). Table 1 summarizes the yield of 2,4-D-scFv. After a series of purification steps, the yield of purified 2,4-D-scFv was 640 μg per 30 ml of the hemolymph, which is equivalent to 21.3 mg per liter of hemolymph.

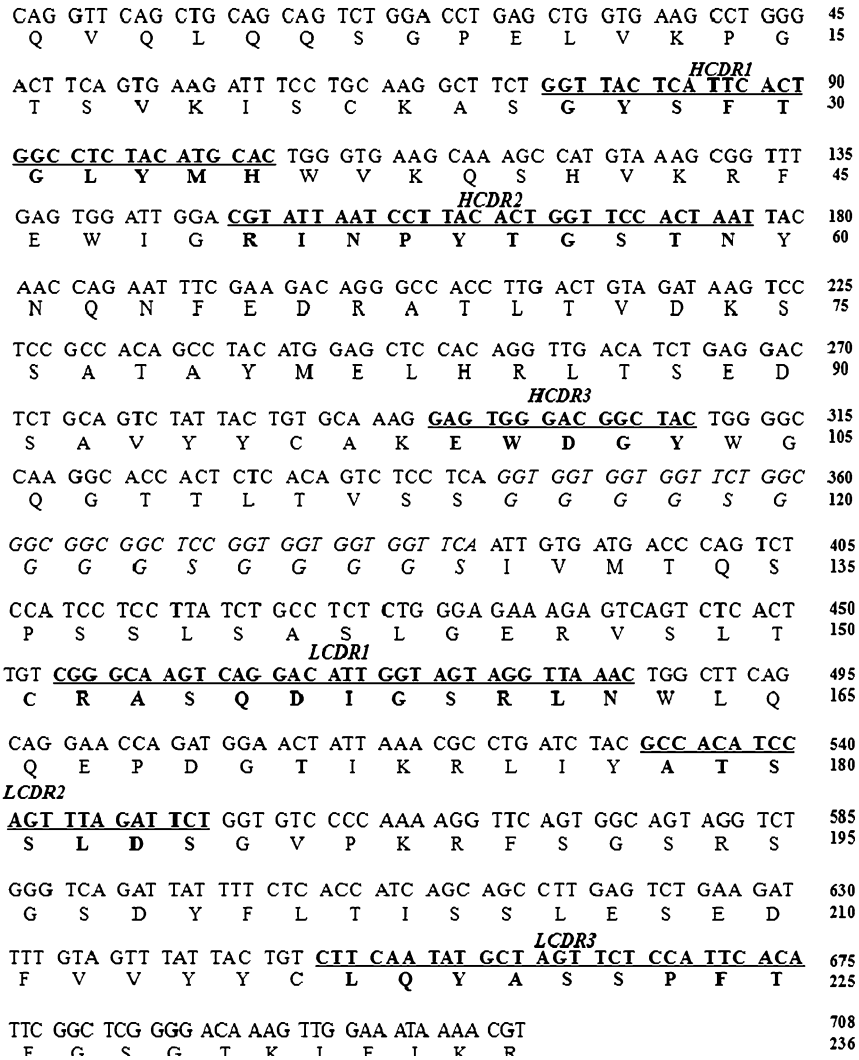

Fig. 2 Nucleotide and deduced amino acid sequences of 2,4-D-scFv. The linker fragment is shown in italics. The nucleotide sequences encoding the complementary-determining region (CDR) are *underlined*. *HCDR* and *LCDR* indicate the CDR in the heavy and light chains, respectively

SDS-PAGE analysis of the purified 2,4-D-scFv demonstrated that scFv monomer was expressed as a chimera protein containing His6- and T7-tags with a molecular mass of 29.4 kDa (molecular mass of 2,4-D-scFv: 25.6 kDa; Fig. 3). In this purification process, the purity of the purified 2,4-D-scFv was estimated at more than 95% based on Coomassie brilliant blue staining.

Indirect ELISA and icELISA Using Purified 2,4-D-scFv

The reactivity of the 2,4-D-scFv towards the 2,4-D-HSA conjugate was analyzed by indirect ELISA. A reactivity response curve was drawn by plotting absorbance against the

Table 1 Purification of 2,4-D-scFv

	Protein concentration (mg/ml)	Total volume (ml)	Total protein (mg)	Yield (%)
Hemolymph	19.1	30	573	100
CM-650M	0.51	100	51	8.9
His-bind resin	0.097	95	9.22	1.6
Amicon Ultra	0.012	53	0.64	0.12

All protein yields were calculated by the Bradford method

logarithm of 2,4-D-scFv concentration in the indirect ELISA. The concentration of 2,4-D-scFv was positively correlated with the absorbance value in a logistical manner (Fig. 4).

To analyze the inhibitory activity of 2,4-D-scFv against 2,4-D, an icELISA was carried out. 2,4-D-scFv (3.4 μg/ml) was incubated with serially double diluted concentrations of free 2,4-D on an immunoplate. Any 2,4-D-scFv binding to free 2,4-D was washed out. The 2,4-D-scFv that bound to the immobilized 2,4-D-HSA conjugates were incubated with HRP-labeled anti-T7-tag conjugates and then treated with ABTS solution. In this icELISA, the detectable range of 2,4-D for 2,4-D-scFv ranged from 0.39–50 μg/ml (Fig. 5). It became evident from this experiment that icELISA using 2,4-D-scFv displayed a twofold-higher inhibitory concentration 50% (IC50=4.5 μg/ml) than that using its parental antibody, MAb 2C4 (IC50=10 μg/ml).

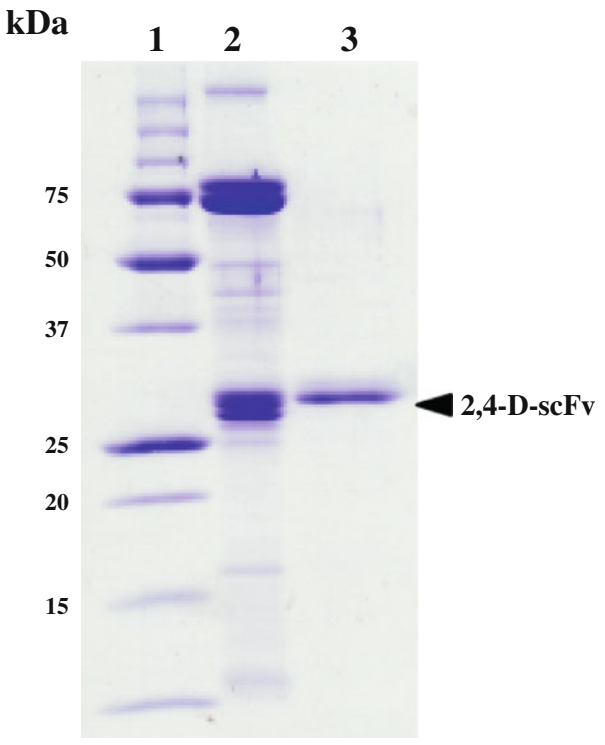

Fig. 3 SDS-PAGE analysis after 2,4-D-scFv purification. The hemolymph collected after infection was 30-times diluted with distilled water and used as a sample for lane 2. *Lane 1* molecular protein marker; *lane 2* the hemolymph after infection; *lane 3* purified 2,4-D-scFv (1.5 μg)

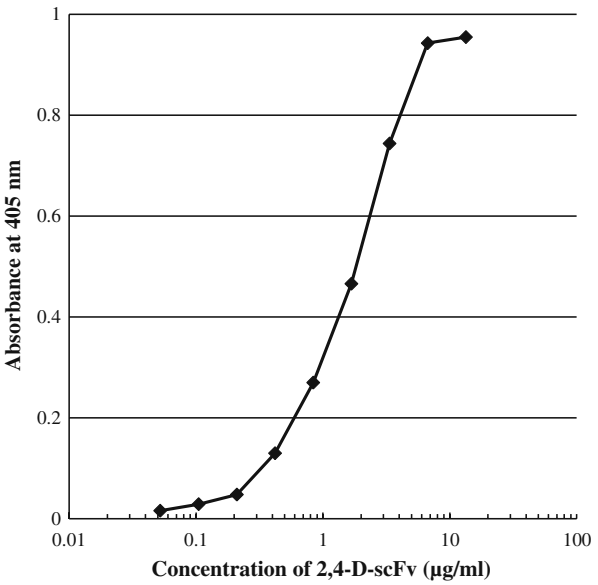

Fig. 4 Reactivity of 2,4-D-scFv against 2,4-D-HSA conjugate. To examine the reactivity of the 2,4-D-scFv, various concentrations of 2,4-D-scFv were added to the wells of a 96-well immunoplate coated with 2,4-D-HSA (2 μg/ml)

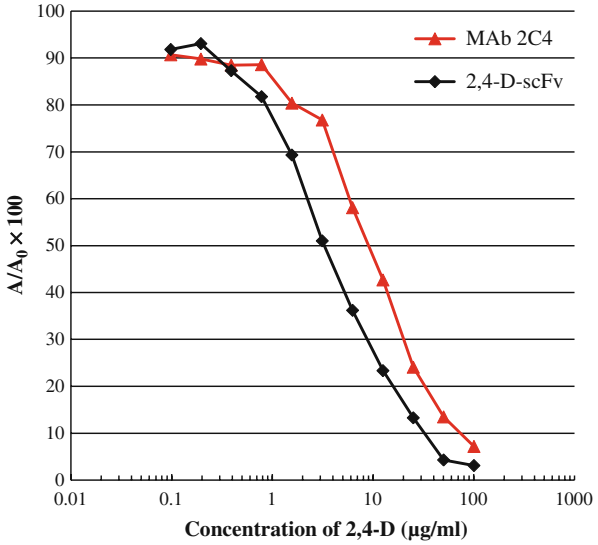

Fig. 5 Calibration curve of 2,4-D using 2,4-D-scFv and MAb 2C4. Various concentrations of 2,4-D in 5% MeOH were incubated with 2,4-D-scFv (3.4 μg/ml) or MAb 2C4 (125 ng/ml) in wells precoated with 2,4-D-HSA (2 μg/ml). After washing the plate with PBS-T, the wells were incubated with a 5,000-fold diluted solution of HRP-labeled anti-T7-tag conjugates (for 2,4-D-scFv) or a 1,000-fold diluted solution of peroxidase labeled anti-mouse IgG (for MAb 2C4). Absorbance was measured at 405 nm. The *red triangles* and *red curve* show the standard curve produced using MAb 2C4. The *black squares* and *black curve* show the results for 2,4-D-scFv. A/A_0, A_0 is the absorbance with no 2,4-D present, and A is the absorbance with 2,4-D present

Characterization of 2,4-D-scFv

The characterization of purified 2,4-D-scFv was carried out by icELISA. The specificity and binding affinity of 2,4-D-scFv were evaluated by a CR test and calculating its dissociation constant (K_D), respectively.

The CR of the 2,4-D-scFv was examined by icELISA and calculated by the method described above. Table 2 shows the CR (%) of 2,4-D-scFv as well as those of structurally related compounds. The CR of 2,4-dichlorophenol (2,4-DP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4-dichlorobenzoic acid (2,4-DB) were found to be 47.9%, 65.5%, and 26.0%, respectively, whereas other tested compounds (data not shown) did not exhibit any cross-reactivity (less than 0.05%) with 2,4-D-scFv. It is suggested that the 6-chloro group in 2,4,6-T (CR; <0.05%) and the carbonyl of the carboxyl group in 2,4-DB (26.0%) prevents them from binding to the binding cavity of 2,4-D-scFv, resulting in their low CR. Moreover, it was revealed that 2,4-D-scFv could be applied to the determination/detection of the phenoxy family of herbicides, which include 2,4,5-T, 2,4-DP, and 2,4-DB, because high CR were obtained for these compounds. The CR of 2,4-D-scFv seems to be wider than that of MAb 2C4, which was determined in our previous study [13]. It is assumed that the differences in specificity between scFv and its parental monoclonal antibody mainly depend on the molecular weight of the target hapten and its tertiary structure. The scFv against solamargine (Mw: 868.06), paeoniflorin (Mw: 480.46), and ginsenoside Re (Mw: 947.15) [24–26], all of which either contain a high molecular weight hapten (paeoniflorin and ginsenoside Re) or have a bulky tertiary structure, such as the lactone ring in the pinane skeleton of paeoniflorin, displayed almost identical CR to their parental monoclonal antibodies. Meanwhile, the scFv against 2,4-D (Mw: 221.04) and plumbagin (Mw: 188.18) [27], which contain lower molecular weight haptens and have a planar structure, showed different CR than their parental monoclonal antibodies. Taken together, the CR of a scFv against a hapten seems to depend on the hapten's molecular weight and tertiary structure.

The ELISA method described by Friguet et al. was used to estimate the dissociation constant (K_D) of 2,4-D-scFv and MAb 2C4 in solution [22]. This method is equally available for antibodies against small (hapten) and large molecular weight antigens but does not label the antibodies or antigens involved. Briefly, various concentrations of 2,4-D were incubated with 2,4-D-scFv or MAb 2C4 at 37°C for 1 h until they reached equilibrium. The amounts of free antibodies in the incubation mixture were then determined by indirect ELISA. The K_D of 2,4-D-scFv and MAb 2C4 in solution were 1.61×10^{-5} and 3.57×10^{-5} M, respectively, as determined by typical Scatchard plots. When the K_D of 2,4-D-scFv was compared with that of MAb 2C4, 2,4-D-scFv (1.61×10^{-5} M) exhibited about a

Table 2 Cross-reactivities (CR) of 2,4-D-scFv and MAb 2C4 against structurally related compounds

Compound	CR (%)	
	2,4-D-scFv	MAb 2C4
2,4-dichlorophenoxyacetic acid (2,4-D)	100	100
2,4-dichlorophenol (2,4-DP)	47.9	680
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)	65.5	<3
2,4,6-trichlorophenoxyacetic acid (2,4,6-T)	<0.05	n.d.
2,4-dichlorobenzoic acid (2,4-DB)	26.0	<3

n.d. no data

Table 3 Recovery of 2,4-D from spiked samples, as determined by icELISA

Spiked level (μg/ml)	Measured amount (μg/ml) [a]	Recovery (%)
2.5	2.626±0.224	105.0
5	5.026±0.897	100.5
10	10.703±1.226	107.0
20	21.388±0.536	106.9
40	42.257±2.576	105.6
80	78.776±3.894	98.5

[a] All values are shown as the mean±SD of triplicate samples for each level

twofold-higher binding affinity than MAb 2C4 (3.57×10^{-5} M). In our previous study, the MAb 2C4 was successfully applied in surface plasmon resonance (SPR) to detect 2,4-D in a pictogram order [12]. It is estimated that the sensitivity of SPR might be improved by using 2,4-D-scFv because of higher binding affinity of 2,4-D-scFv to 2,4-D.

The stability test of purified 2,4-D-scFv was carried out by icELISA using 2,4-D-scFv kept at 4°C for 6 months after purification. The IC50 using 2,4-D-scFv kept for 6 months (IC50=4.46 μg/ml) shows almost the same values as that using 2,4-D-scFv just after purification (IC50=4.5 μg/ml), estimating that 2,4-D-scFv expressed in hemolymph of silkworm larvae is stable.

Recovery Test

To investigate the accuracy of icELISA using 2,4-D-scFv, 2,4-D solution was spiked into distilled water at concentrations of 5–80 μg/ml, and the recovery rate of 2,4-D in this method was calculated, with recoveries close to 100% indicating good accuracy. As shown in Table 3, good 2,4-D recoveries ranging from 98.5% to 107.0% were exhibited. These results demonstrate that our icELISA using 2,4-D-scFv displays good accuracy.

Intra-, Inter-Assay Reliability

To validate the icELISA, its intra- and inter-assay reliabilities were studied. Intra- and inter-assay variability were evaluated by testing eight different 2,4-D concentration samples in

Table 4 Intra and inter-assay precision of 2,4-D analysis by icELISA

2,4-D (μg/ml)	CV (%)	
	Intra-assay (n=4)	Inter-assay (n=4)
12.50	9.5	5.7
6.25	8.5	9.4
3.13	5.0	5.8
1.56	5.5	4.5
0.78	1.1	4.6
0.39	4.4	4.4
0.20	3.2	4.6
0.10	2.6	2.7

four assays performed on the same day and on three consecutive days, respectively. The intra- and inter-assay coefficients of variation (CV) for precision were determined using the ratios of the standard deviations (SD) and means of the four assays. As shown in Table 4, the maximum intra-assay CV was 9.5%, while the inter-assay CV was 9.4%. All CV were below 10%, indicating the high reliability of icELISA using 2,4-D-scFv.

Conclusion

The construction, expression, purification, and characterization of 2,4-D-scFv were successfully pursued using our effective BmNPV bacmid DNA system in the hemolymph of silkworm larvae. The characterization of 2,4-D-scFv revealed that it could be applied to accurate and speedy immunoassays for the determination/detection of 2,4-D and its metabolites such as 2,4-DB, 2,4-DP, and 2,4,5-T, which cause environmental contamination.

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