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ORIGINAL PAPER

Efficient expression of single chain variable fragment antibody against paclitaxel using the *Bombyx mori* nucleopolyhedrovirus bacmid DNA system and its characterizations

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Abstract A single chain variable fragment (scFv), the smallest unit of functional recombinant antibody, is an attractive format of recombinant antibodies for various applications due to its small fragment and possibility of genetic engineering. Hybridoma clone 3A3 secreting anti-paclitaxel monoclonal antibody was used to construct genes encoding its variable domains of heavy (V_H) and light (V_L) chains. The V_H and V_L domains were linked to be the PT-scFv3A3 using flexible peptide linker in a format of V_H-$(GGGGS)_5$-V_L. The PT-scFv3A3 was primarily expressed using the pET28a(+) vector in the *Escherichia coli* system, and was then further expressed by using the *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA system. Interestingly, the reactivity of PT-scFv3A3 expressed in the hemolymph of *B. mori* using the BmNPV bacmid DNA system was much higher than that expressed in the *E. coli* system. Using indirect competitive enzyme-linked immunosorbent assay (icELISA), the PT-scFv3A3 (*B. mori*) reacted not only with immobilized paclitaxel, but also with free paclitaxel in a concentration-dependent manner, with the linear range of free paclitaxel between 0.156 and 5.00 μg/ml. The PT-scFv3A3 (*B. mori*) exhibited less cross-reactivity (%) than its parental MAb clone 3A3 against paclitaxel-related compounds, including docetaxel (31.1 %), 7-xylosyltaxol (22.1 %), baccatin III (<0.68 %), 10-deacetylbaccatin III (<0.68 %), 1-hydroxybaccatin I (<0.68 %), and 1-acetoxy-5-deacetylbaccatin I (<0.68 %). With the exception of cephalomannine, the cross-reactivity was slightly increased to 8.50 %. The BmNPV bacmid DNA system was a highly efficient expression system of active PT-scFv3A3, which is applicable for PT-scFv3A3-based immunoassay of paclitaxel. In addition, the PT-scFv3A3 can be applied to evaluate its neutralizing property of paclitaxel or docetaxel toxicity.

Keywords Antibody · *Bombyx mori* nucleopolyhedrovirus bacmid DNA system · Enzyme-linked immunosorbent assay · Paclitaxel · Single chain variable fragment (scFv)

Introduction

A large number of recombinant antibodies (rAb) was developed and applied in various fields, including biotechnology [1], medicine [2], agriculture [3], and food analysis [4, 5]. rAb have attracted more attention due to some advantages over conventional polyclonal antibodies (PAb) and monoclonal antibodies (MAb), including short production time, reproducibility, and the possibility of genetic engineering. rAb fragments were predicted to share a significant part of the diagnostic market, from in vitro immunoassay to in vivo imaging [6]. Moreover, rAb can be constructed into multivalent molecules or fused with effector or reporter molecules. Engineered rAb now represent over 30 % of biopharmaceuticals in clinical trials [7].

The most popular rAb format is the single chain variable fragment (scFv), which provides opportunities for its genetic engineering to improve reactivity [5] and to construct scFv with reporter molecules [8]. Its unique advantage is based on the fact that scFv is the smallest format of

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rAb. For these reasons, an efficient expression system of scFv is a prerequisite for biopharmaceutical development.

Various expression systems have been developed and optimized to increase the amount of expressed scFv and to retain binding reactivity. Refolding of scFv from the inclusion body of the *Escherichia coli* expression system is a conventional procedure. However, the refolding process not only affects the reactivity of scFv, but also requires extensive trial and error approaches. The recovery of functional scFv from the inclusion body using the refolding procedure is often not efficient. Therefore, an alternative expression system, which provides efficient, simple, and cost-effective production of scFv, is valuable to facilitate its structural and functional analysis.

A *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA system has been developed [9] and reviewed as an efficient tool for recombinant protein expression [10]. Recombinant BmNPV bacmid can be generated by site-specific transposition. The transposase, expressed by the helper vector, transposes the Tn7 flanking cassette containing the gene of interest from the donor vector to the Tn7 attachment site in the bacmid [11]. The recombinant BmNPV bacmid can be replicated in the *E. coli* BmDH10Bac strain. Several days after *B. mori* larvae had been infected with recombinant BmNPV bacmid, recombinant protein was expressed. Without complex equipment and large time consumption, eukaryotic protein can be expressed as native and functional forms in *B. mori* larvae [10]. Moreover, BmNPV bacmid is non-infectious to humans, the expression of which can be done easily and safely.

Digoxin-specific antibody fragments (Fab) have been applied clinically as a safe and effective treatment for the management of acute and chronic digoxin poisoning [12]. With a smaller size of Fab, which diffuses into the peripheral compartments and is excreted by glomerular filtration, it exhibits superior efficacy over whole antibody for digoxin neutralization [13]. Moreover, Fab was applied to neutralize the toxicity of colchicine [14, 15] and tricyclic antidepressants [16, 17]. Previously, MAb against paclitaxel was investigated for its inhibition of paclitaxel-induced cytotoxicity, which might be a useful antidote of paclitaxel toxicity [18]. In clinical applications of paclitaxel and docetaxel, some patients suffered from their toxicities, for which evidence-based prevention and management are lacking [19, 20].

In our previous study, MAb against paclitaxel (PT-MAb 3A3) was produced for analytical applications [21]. In this research, we aim to construct and express active scFv antibody against paclitaxel (PT-scFv3A3) that exhibits potential application as an antidote of paclitaxel toxicity and as an analytical method of paclitaxel. The PT-scFv3A3 in the orientation V_H-$(GGGGS)_5$-V_L was constructed using RNA of hybridoma clone 3A3 secreting MAb against paclitaxel. The PT-scFv3A3 was primarily expressed using pET28a(+) vector in the *E. coli* system and was then further expressed in the hemolymph of *B. mori* using the BmNPV bacmid DNA system. The results revealed that only PT-scFv3A3, which was expressed as an active form in the hemolymph of *B. mori* larvae, retained binding activity and specificity of its parental MAb. Therefore, it is possible to apply PT-scFv3A3 for quantitative analysis of paclitaxel using PT-scFv3A3-based indirect competitive enzyme-linked immunosorbent assay (icELISA). Furthermore, with its small size, the PT-scFv3A3 can be applied to evaluate its neutralizing activity of paclitaxel and docetaxel poisoning. The construction, efficient expression, and characterization of PT-scFv3A3 were demonstrated in this paper.

Materials and methods

Chemicals and immunochemicals

7-Xylosyltaxol (≥90.0 %) and 1-hydroxybaccatin (≥98.0 %) were obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). 1-Acetoxy-5-deacetylbaccatin I (≥98.0 %) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, P.R. China). Ovalbumin (OVA, ≥98.0 %) and baccatin III (≥95.0 %) were obtained from Sigma-Aldrich (Steinheim, Germany). Paclitaxel (≥97.0 %), docetaxel (≥98.0 %), and 10-deacetylbaccatin III (≥95.0 %) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Mouse anti-T7 tag monoclonal antibody conjugated with horseradish peroxidase (HRP) was purchased Novagen (MA, USA). Toyopearl CM-650M cation exchange resin was obtained from Tosoh Co. (Tokyo, Japan). The his-tag purification resin was purchased from Roche (IN, USA). DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemical and immunological reagents that have not been specified were commercial products of analytical grade.

Synthesis of xylosyltaxol–OVA

7-Xylosyltaxol-OVA (xyltax–OVA) conjugate, which was utilized as immobilized paclitaxel, was synthesized by periodate oxidation and reductive amination, respectively. Briefly, a solution of $NaIO_4$ (5 mg, 1 ml water) was added dropwise into a solution of 7-xylosyltaxol (10 mg, 3 ml methanol). To complete the oxidation of xylosyl group, the resultant mixture was stirred at room temperature for 1 h. Ovalbumin solution (10 mg, 6 ml), dissolved in 50 mM sodium carbonate buffer (pH 9.6), was gradually added to

the reaction mixture. The reaction mixture was continued to be stirred for an additional 5 h at room temperature. Finally, the mixture was dialyzed against distilled water (4 °C) and was lyophilized to obtain powder of xyltax–OVA conjugate (10.5 mg).

Construction of PT-scFv3A3 genes and their expression vectors

Previously, hybridoma cell (clone 3A3) secreting MAb against paclitaxel was developed [21]. The hybridoma cells were cultured in enriched RPMI 1640-Dulbecco's-Ham's F12 (eRDF; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) medium containing 10 % (v/v) of fetal calf serum (FCS; Gibco, Invitrogen, CA, USA). A week later, 1×10^6 cells of the hybridoma were processed for total RNA extraction using the Sepasol-RNA I Super G reagent (Nacalai Tesque, Kyoto, japan), the procedure for which was described in the manufacturer's instructions. First strand cDNA was synthesized using the random hexamer primer of the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK). The V_H and V_L genes were amplified by polymerase chain reaction (PCR) using V_H- and V_L-specific primers [22]. The amplified PCR products were ligated into pGEM-T vector (Promega, WI, USA), and then each ligated mixture of V_H and V_L was transformed into *E. coli* JM 109 competent cells. The colony PCR was performed to detect positive colonies of inserted V_H or V_L genes. Finally, plasmid of positive colonies was purified and sequenced (BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, CA, USA).

Specific primers for PT-scFv3A3 construction were designed from exact sequences of V_H and V_L (Table 1). The V_H gene was amplified by primers 1 and 2, whereas V_L was amplified by primers 3 and 4. The genes of V_H and V_L were linked to be in the V_H-$(GGGGS)_3$-V_L format using splicing by overlapping extension-PCR (SOE-PCR), where primers 1 and 4 were used. A full-length PT-scFv3A3 gene was subcloned into pET28a(+) expression vector (Novagen, Merck, Darmstadt, Germany). Using PT-scFv3A3 (V_H-$(GGGGS)_3$-V_L) as a template, primers 1, 6 and primers 4, 5 were used to amplify V_H and V_L, respectively. Then, the same SOE-PCR was applied to construct PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L). The obtained PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) gene was subcloned into pET28a(+), for which the N-terminal of PT-scFv3A3 was tagged with hexahistidine (His6) and T7 sequences.

Construction of a baculovirus donor vector and recombinant BmNPV bacmid

To promote secretion of expressed PT-scFv3A3 into the hemolymph of *B. mori* larvae, the honeybee melittin secretion signal (HMSS) gene was amplified from pMelBac A vector (Invitrogen) using primers 8 and 9, and was then ligated to pFastBac1 vector (Invitrogen) to generate pFastBac1Mel (Fig. 1) [23]. The PT-scFv3A3 gene with N-terminal His6 and T7 sequences was amplified from a pET28a(+)/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L), for which primers 4 and 7 were used. The resultant PCR product was digested and ligated into the downstream region of HMSS in the pFastBac1Mel vector, for which, finally, the baculovirus donor vector, pFastBac1Mel/PT-scFv3A3, was obtained.

Recombinant BmNPV bacmid/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was prepared by using the *E. coli* BmDH10Bac strain [9]. Ten nanograms of the pFastBac1Mel/PT-scFv3A3 were transformed into *E. coli* BmDH10Bac competent cells (100 µl), and then the transformed cells were selected in selective Luria–Bertani [LB, 1 % (w/v) polypeptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, and 1.5 % (w/v) agar] plates containing kanamycin (50 µg/ml), gentamicin (7 µg/ml), tetracycline

Table 1 List of primers used for amplification of the PT-scFv3A3s

Primer	Primer description	Sequence (5′ to 3′)[a]
P1	V_H-for-*EcoR* I	GTCGAATTCCAGGTTCAGCTGCAGCAG
P2	V_H-rev-linker 1	GGAGCCGCCGCCGCCTGAACCACCACCACCCGAGGAGACTGTGAG
P3	V_L-for-linker 1	GGCGGCGGCGGCTCCGGTGGTGGTGGTTCAGACATTGTGATGACC
P4	V_L-rev-*Not* I	TATGCGGCCGCCTAACGTTTGATTTCCAGC
P5	V_H-rev-linker 2	GGAGCCGCCGCCGCCAGAGCCACCGCCACCTGAACCACCACCACC
P6	V_L-for-linker 2	GGCGGCGGCGGCTCCGGGGGAGGGGGATCAGGTGGTGGTGGTTCA
P7	PT-scFv3A3-for-*Sal* I	CGCGTCGACACAGCAGCCATCATCATCAT
P8	HMSS-for-*BamH* I	CGCGGATCCATGAAATTCTTAGTCAAC
P9	HMSS-rev-Eco*R*I	AGCGAATTCCGCATAGATGTAAGAAA

[a] The sites of DNA restriction enzymes are shown underlined

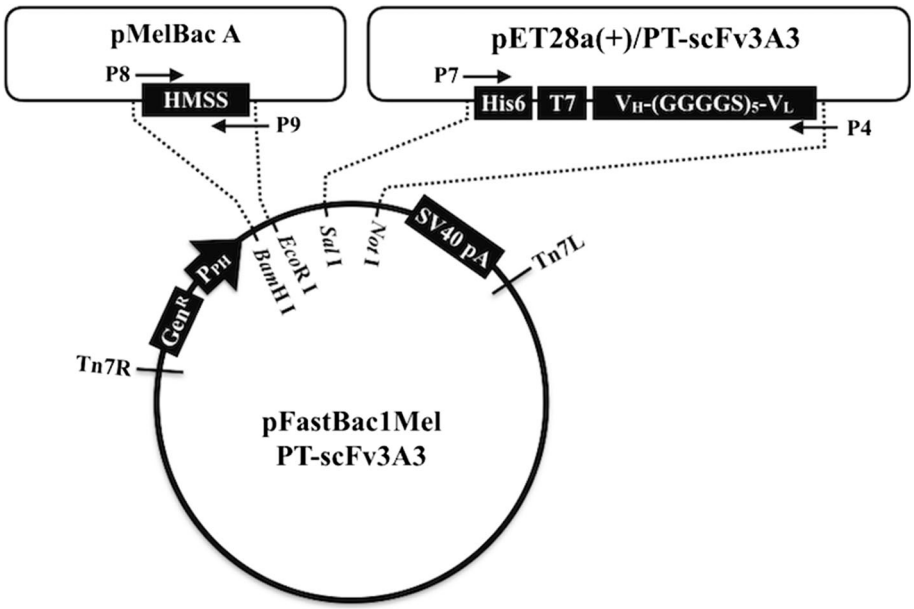

Fig. 1 Schematic representation of the construction of the pFastBac1Mel/PT-scFv3A3 donor vector. The sequence of honeybee melittin secretion signal (HMSS) was amplified from pMelBac A and subcloned into pFastBac1. The PT-scFv3A3 gene with N-terminal His6 and T7 sequences was amplified and subcloned into the downstream region of HMSS and, finally, the pFastBac1Mel/PT-scFv3A3 donor vector was obtained

(10 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) (40 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (300 μg/ml). Colony PCR using M13 universal primers was performed to screen the recombinant BmNPV bacmid in white colonies. Finally, recombinant BmNPV bacmid/PT-scFv3A3 was extracted using the QIAGEN Plasmid Maxi Kit (Valencia, CA, USA).

Expression of the PT-scFv3A3 in *E. coli*

The recombinant pET28a(+) vector with the PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) gene was transformed into the *E. coli* BL21 (DE3) strain, which was utilized as the host of expression. The cells harboring the pET28a(+)/PT-scFv3A3 construct were cultured at 37 °C (120 rpm shaking) in LB medium containing 50 μg/ml kanamycin until the optical density (OD_{600nm}) of the culture reached 0.6. Stock solution of IPTG (1 M) was added to the culture (1 mM final concentration) to induce the expression of PT-scFv3A3, and then the culture was continued shaking for 12 h. The induced cells were collected by centrifugation (8000 rpm, 5 min, at 4 °C), and then suspended in 14 ml of lysis buffer (1 mM EDTA in 50 mM Tris–HCl pH 8.0). The cells were lysed by hen egg lysozyme (1 mg/ml). Before the disruption of cells by using ultrasonication (750 watts, 20 kHz), NaCl and Triton X-100 were added to obtain final concentrations of 0.5 M and 1 % (v/v), respectively. The lysate was centrifuged at 12,000 rpm for 20 min, which was then separated to soluble and insoluble fractions (inclusion body). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was applied to evaluate the expression of PT-scFv3A3.

Purification and refolding of the PT-scFv3A3 from the *E. coli* expression system

As investigated by SDS-PAGE, the PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was expressed in the inclusion body of *E. coli*. Therefore, the inclusion body was dissolved in denaturing binding buffer (8 M urea and 0.5 M NaCl in 50 mM Tris–HCl pH 8.0), and was then subjected to a his-bind resin column equilibrated previously with the denaturing binding buffer. The non-specific bound protein was washed with 10 mM imidazole in the same buffer. Finally, His6-tagged PT-scFv3A3 was eluted with the above buffer containing 200 mM imidazole. The purity of PT-scFv3A3 was analyzed by SDS-PAGE. The concentration of the purified PT-scFv3A3 was determined by the Bradford protein assay [24].

The PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was refolded by the method described previously [25], with some modifications. One milligram of the purified PT-scFv3A3 was denatured in denaturing buffer containing 8 M urea, 0.25 M NaCl, and 5 mM 2-mercaptoethanol (β-ME) in 50 mM Tris–HCl pH 8.0 for 12 h. The denatured PT-scFv3A3 was added dropwise into 20 ml of refolding buffer [0.15 M NaCl, 1 mM EDTA, 0.5 M L-arginine, 2 mM reduced glutathione, 1 mM oxidized glutathione, and 5 % (v/v) glycerol in 50 mM Tris–HCl pH 8.0], and was dialyzed against the same buffer for 12 h. The refolded PT-scFv3A3 was dialyzed in PBS containing 5 % (v/v) glycerol, and was then concentrated by a centrifugal filter (Amicon Ultra-15, Merck Millipore, Germany). All procedures were performed at 4 °C. The concentration of refolded PT-scFv3A3 was determined by the Bradford protein assay.

Expression of the PT-scFv3A3 in the hemolymph of *B. mori* larvae

The fifth instar *B. mori* larvae were infected with recombinant BmNPV bacmid/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L). Briefly, 1 μg of the recombinant bacmid was suspended with 3 μl of DMRIE-C (Thermo Scientific, IL, USA) and then kept at room temperature for 45 min. The resultant mixture was diluted with sterilized and deionized water (50 μl; Nacalai Tesque, Kyoto, Japan), and directly injected into the dorsal node of the larvae. The injected *B. mori* larvae were cultured at 25 °C for 6 days. The hemolymph was collected into a microtube containing 5 % (w/v) sodium thiosulfate (50 μl) to avoid the oxidation of *B. mori*-derived protein. The hemolymph was centrifuged at 12,000 rpm for 20 min (4 °C). Protease Inhibitor Cocktail Kit (Thermo Scientific, IL, USA) was added to clear the hemolymph, and then the hemolymph was kept at 4 °C until the next experiment was performed.

Purification of the PT-scFv3A3 from the hemolymph of *B. mori* larvae

The hemolymph of each *B. mori* larva was analyzed directly by using indirect enzyme-linked immunosorbent assay (iELISA), and then the hemolymph containing active PT-scFv3A3 was pooled and subjected to cation exchange chromatography, and followed by Ni-affinity chromatography to obtain high purity of PT-scFv3A3.

Fifteen milliliters of cation exchanger Toyopearl CM-650M (Tosoh Co., Tokyo, Japan) were packed and equilibrated with starting buffer [10 % (v/v) glycerol in 50 mM Tris–HCl pH 8.0]. The hemolymph was centrifuged to remove aggregation, and then clear solution was subjected to cation exchanger. Unbound proteins were washed out using starting buffer, and then the bound proteins were eluted with starting buffer containing gradient concentrations of NaCl from 0 to 300 mM. Twenty milliliters of each fraction were collected and analyzed using iELISA and SDS-PAGE to evaluate presence of the PT-scFv3A3. To obtain high purity, fractions containing PT-scFv3A3 were further purified by Ni-affinity column chromatography. The fractions containing PT-scFv3A3 were pooled and dialyzed against native binding buffer [0.5 M NaCl, 10 % (v/v) glycerol, and 10 mM imidazole in 50 mM Tris–HCl pH 8.0]. The PT-scFv3A3 solution was subjected to his-tag purification resin (1 ml), which was equilibrated with native binding buffer. Unbound proteins were washed with native binding buffer (10 ml), and then bound proteins were eluted with eluting buffer [0.5 M NaCl, 10 % (v/v) glycerol, and 100 mM imidazole in 50 mM Tris–HCl pH 8.0]. The presence and purity of PT-scFv3A3 were analyzed by SDS-PAGE. The concentration of purified PT-scFv3A3 was determined by the Bradford protein assay.

SDS-PAGE and western blotting analysis of PT-scFv3A3

SDS-PAGE and western blotting analysis were applied to detect PT-scFv3A3, which was expressed in *E. coli* and *B. mori*. Protein samples were separated by 15 % (w/v) SDS-PAGE under reducing (*E. coli*) and non-reducing (*B. mori*) conditions. Electroblotting was performed to transfer proteins from SDS-PAGE to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was treated for 1 h (room temperature) with 5 % (v/v) skimmed milk in phosphate-buffered saline (S-PBS). Then, it was incubated with mouse monoclonal antibody to His6 epitope (0.5 μg/ml, Thermo Scientific, IL, USA) for 12 h at 4 °C. After unbound antibody was washed out, goat anti-mouse polyclonal antibody labeled with HRP (Abcam, Cambridge, UK) was reacted with bound antibody for 2 h at room temperature. Finally, His6-tagged PT-scFv3A3 was visualized by adding substrate solution containing 4-chloro-1-naphthol (10 mg/ml, methanol): PBS [1:9 (v/v)] and 0.01 % (v/v) H_2O_2.

Characterization of the PT-scFv3A3s

The reactivity of refolded PT-scFv3A3 (*E. coli*) and soluble PT-scFv3A3 (*B. mori*) against immobilized paclitaxel was evaluated by iELISA, whereas their binding to free paclitaxel and its related compounds was evaluated by icELISA. For the procedure of iELISA, a transparent 96-well plate (MaxiSorp Nunc, Roskilde, Denmark) was coated with xyltax–OVA conjugates (1 μg/ml, 100 μl/well) in coating buffer (50 mM sodium carbonate buffer pH 9.6) for 1 h. The plate was washed three times with 0.05 % (v/v) Tween 20 in 10 mM phosphate-buffered saline (T-PBS). Thereafter, the plate was treated with S-PBS (300 μl/well) for 1 h to reduce non-specific adsorption. The plate was then washed again, and then PT-scFv3A3 or hemolymph was reacted to immobilized xyltax–OVA conjugates (100 μl/well). Subsequently, the plate was washed with T-PBS thrice and incubated for 1 h with a diluted solution (1:5000) of mouse anti-T7 tag monoclonal antibody conjugated with HRP (100 μl/well). Finally, the substrate solution of 100 mM sodium citrate buffer (pH 4.0) containing 0.003 % (v/v) H_2O_2 and 0.3 mg/ml of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was added (100 μl/well) to each well after washing the plates with T-PBS. Fifteen minutes later, the absorbance at 405 nm was measured using a microplate reader (Multiskan FC,

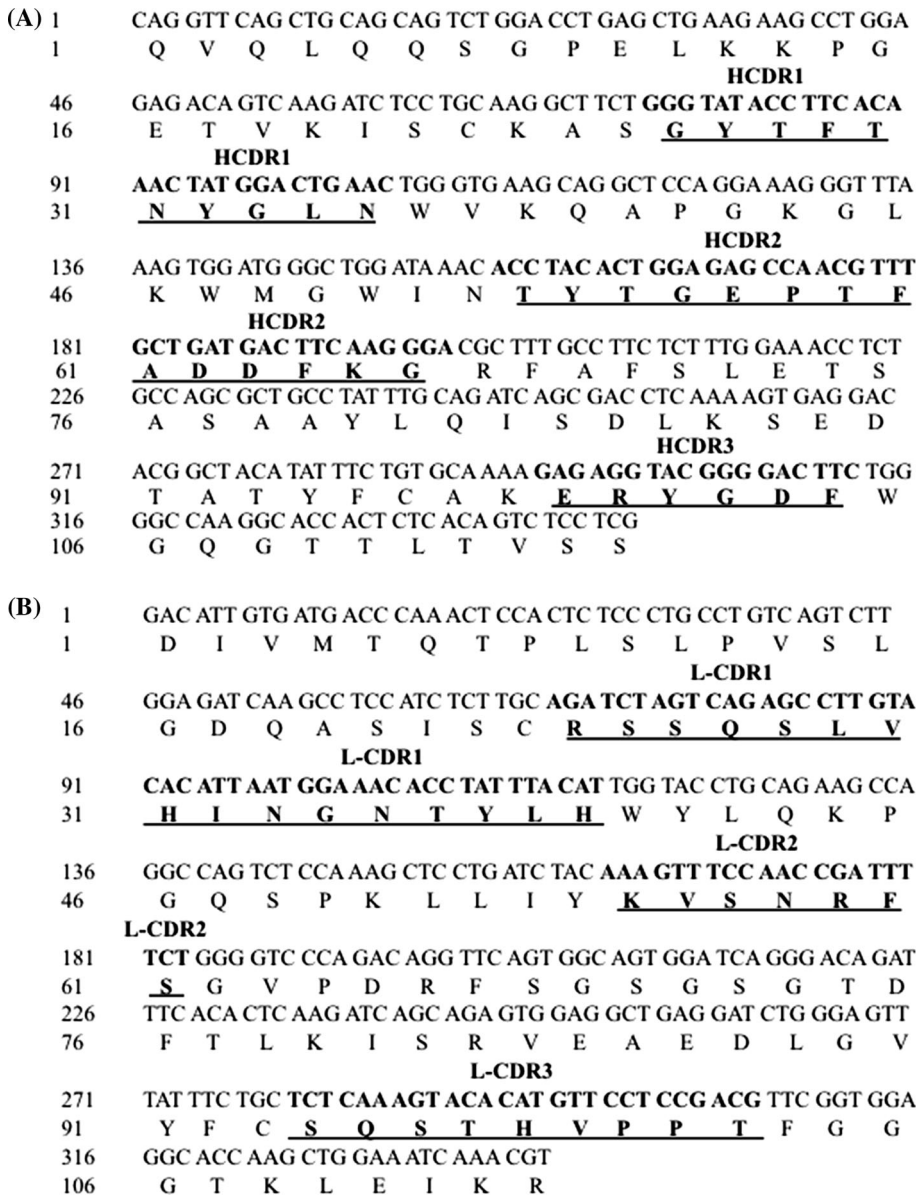

Fig. 2 The nucleotide and amino acid sequences encoding V_H (**a**) and V_L (**b**) of monoclonal antibody against paclitaxel were identified, the CDR for both the V_H and V_L domains were assigned according to the Kabat and Chothia numbering schemes (http://www.bioinf.org.uk/abs/)

Thermo Scientific). All reactions were carried out at 37 °C. Using the procedure of iELISA, serial concentrations of PT-scFv3A3 were applied to reveal whether the PT-scFv3A3 reacted with immobilized paclitaxel in a concentration-dependent manner. The results will inform the optimal concentration of PT-scFv3A3 for icELISA.

icELISA was utilized to evaluate the activity of PT-scFv3A3 against free paclitaxel and its related compounds. The same procedures used in the iELISA were used until the blocking step of S-PBS. In the competitive step, serial concentrations of free paclitaxel (50 µl/well) were applied as the PT-scFv3A3 binding competitor of immobilized paclitaxel, and then PT-scFv3A3 (50 µl/well, 6.25 µg/ml) was added. After it was well mixed, the plate was incubated for 1 h. The diluted solution (1:5000) of mouse anti-T7 Tag monoclonal antibody conjugated with HRP (100 µl/well) was added after free paclitaxel-bound PT-scFv3A3 was washed out. One hour later, the plate was washed again and substrate solution (100 µl/well) was added. The developed color was measured after 15 min of incubation.

A cross-reactivity (CR) test was performed to assess the specificity of PT-scFv3A3. In one assay, the serial concentrations of paclitaxel and related compounds were reacted individually with PT-scFv3A3, following which their comparative binding of investigated compound to

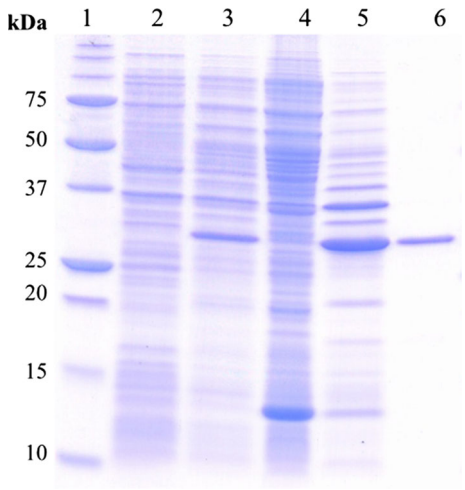

Fig. 3 SDS-PAGE analyses of PT-scFv3A3 expression in the *E. coli* system. *Lane 1* molecular weight protein marker; *lane 2* total protein before isopropyl-β-D-thiogalactopyranoside (IPTG) induction; *lane 3* total protein after IPTG induction; *lane 4* soluble fraction; *lane 5* insoluble fraction (inclusion body); *lane 6* purified PT-scFv3A3 (1 μg). Protein samples were separated on 15 % (w/v) SDS-PAGE under reducing conditions, and then the gel was stained with Coomassie Brilliant Blue

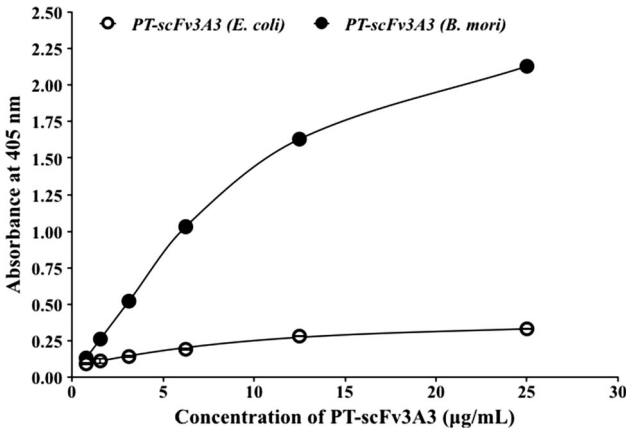

Fig. 4 Reactivity of PT-scFv3A3s against immobilized paclitaxel using indirect ELISA. The PT-scFv3A3 (*E. coli*) was expressed and refolded in vitro, respectively. PT-scFv3A3 (*B. mori*) was expressed as an active form in the hemolymph of *B. mori* larvae

paclitaxel was expressed as a percentage of cross-reactivity calculated using the following equation [26]:

$$\text{CR } (\%) = \frac{\text{IC}_{50} \text{ of paclitaxel}}{\text{IC}_{50} \text{ of investigated compound}} \times 100$$

In the equation, IC_{50} represents the concentration of the paclitaxel or investigated compound that gives 50 % inhibition of the control (the absorbance in the absence of paclitaxel or investigated compounds).

Results and discussion

Construction of PT-scFv3A3 genes and their expression vectors

The V_H and V_L domains were successfully amplified from cDNA of the hybridoma clone 3A3. The genes encoding V_H and V_L of monoclonal antibody against paclitaxel were identified, the CDR for both the V_H and V_L domains were assigned according to the Kabat and Chothia numbering schemes (http://www.bioinf.org.uk/abs/). The V_H (Fig. 2a) and V_L (Fig. 2b) sequences encode 115 and 113 amino acids, respectively. The nucleotide sequence was assigned in DDBJ as accession number LC097190. The PT-scFv3A3 was constructed with sites of DNA restriction enzymes at both ends (*Eco*R I and *Not* I), which was subcloned into the pET28a(+) expression vector. The results of DNA sequencing revealed that the PT-scFv3A3 gene was successfully constructed in the expression vector, and peptide linker was incorporated in the format V_H-$(GGGGS)_3$-V_L. Using pET28a(+)/PT-scFv3A3 (V_H-$(GGGGS)_3$-V_L) as a template, a PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) gene encoding 253 amino acids was constructed and subcloned into pET28a(+). Only pET28a(+)/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was preliminarily expressed in *E. coli*. Then, a chimeric gene encoding His6- and T7-tag, and PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was amplified from the recombinant pET28a(+) vector, and was subcloned into the downstream region of HMSS in pFastBac1Mel. When pFastBac1Mel/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) was transformed into *E. coli* BmDH10Bac competent cells, recombinant BmNPV bacmid/PT-scFv3A3 was obtained.

Expression, purification, and refolding of PT-scFv3A3 from the *E. coli* expression system

Escherichia coli BL21(DE3) harboring pET28a(+)/PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L) vectors was induced for the PT-scFv3A3 expression using IPTG. As assessed by SDS-PAGE analysis, the PT-scFv3A3 was expressed in the inclusion body (Fig. 3). The molecular sizes of chimeric PT-scFv3A3 with N-terminal His6- and T7-tags were in agreement with the theoretical molecular weight of 30443 Da, for which its isoelectric point (pI) was 8.95. The PT-scFv3A3 was purified under denatured conditions using Ni-affinity column chromatography, for which purified PT-scFv3A3 (18.2 mg) was obtained from 1 l of culture (Fig. 3).

Because PT-scFv3A3 (*E. coli*) was expressed as inactive forms in the inclusion body, an efficient refolding method of scFv [25] was employed to recover the reactivity of PT-

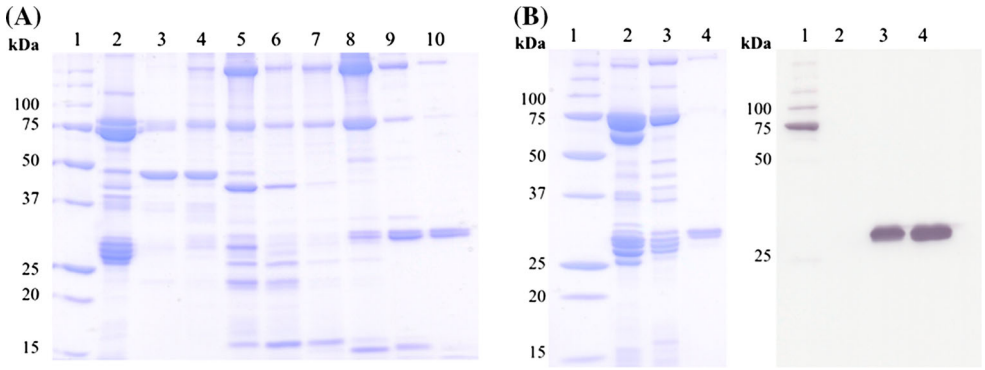

Fig. 5 Purification of PT-scFv3A3 expressed in the hemolymph of *B. mori* larvae. **a** Analysis of fractions which were collected from purification using cation exchange resin: *lane 1* molecular weight protein marker; *lane 2* hemolymph collected from infected *B. mori* larvae; *lanes 3–10* fractions of 0, 10, 20, 40, 80, 100, 200, and 300 mM NaCl in 50 mM Tris–HCl [pH 6.8, 10 % (v/v) glycerol]. **b** SDS-PAGE (*left panel*) and western blotting (*right panel*) analysis of PT-scFv3A3 expression in the hemolymph of *B. mori* larvae. *Lane 1* molecular weight protein marker; *lane 2* hemolymph collected from non-infected *B. mori* larvae (1:10 diluted in water); *lane 3* hemolymph collected from infected *B. mori* larvae (1:10 diluted in water); *lane 4* purified PT-scFv3A3 (1.5 μg), which was obtained after Ni-affinity column chromatography

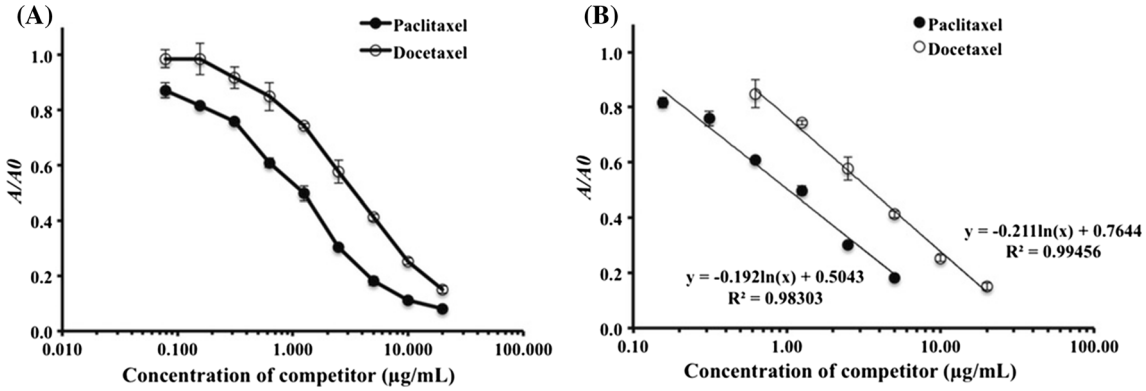

Fig. 6 The reactivity of purified PT-scFv3A3 against free paclitaxel and docetaxel using icELISA. **a** Inhibitory curve of PT-scFv3A3 by free paclitaxel and docetaxel. **b** Range of linear binding between PT-scFv3A3 and paclitaxel or docetaxel. A_0 is the absorbance with paclitaxel or docetaxel absent, and A is the absorbance with paclitaxel or docetaxel present

scFv3A3. After refolding processes, 758.6 μg of refolded PT-scFv3A3 was obtained from 1 mg of denatured PT-scFv3A3. The reactivity of refolded PT-scFv3A3s against immobilized paclitaxel was evaluated using iELISA, the result of which showed that its reactivity was too low to be characterized (Fig. 4). As refolding of PT-scFv3A3 made by *E. coli* was not efficient, which may be reflected by the complicated structure of PT-scFv3A3s, therefore, the eukaryotic expression system using *B. mori* larvae was considered to improve the reactivity of PT-scFv3A3.

Expression and purification of the PT-scFv3A3 from the hemolymph of *B. mori* larvae

Ten *B. mori* larvae were used for the expression of PT-scFv3A3 (V_H-$(GGGGS)_5$-V_L). Isolated BmNPV bacmid/PT-scFv3A3 (1 μg) was mixed with DMRIE-C transfection reagent and then injected into the dorsum of the larvae. Six days after infection, the hemolymph (approximately 1 ml) of each *B. mori* larvae was collected. All infected *B. mori* larvae secreted reactive PT-scFv3A3 into hemolymph when the reactivity was checked by iELISA (data not shown), which suggested the efficacy of transfection and expression of BmNPV bacmid/PT-scFv3A3. Overall, eukaryotic protein can be expressed as active form using a simple procedure in silkworm larvae.

The hemolymph was pooled and dialyzed against starting buffer, and then subjected to cation exchange resin. Unbound proteins were washed with starting buffer, and then starting buffers containing gradient concentration of NaCl were applied to elute bound proteins. Non-specific proteins were eluted much more at 0–80 mM NaCl, whereas PT-scFv3A3 was eluted at 100–300 mM NaCl (Fig. 5a). Correspondingly, the presence of PT-scFv3A3

was also detected by iELISA in fractions of 40–300 mM NaCl. Therefore, PT-scFv3A3-containing fractions (40–300 mM NaCl) were pooled and dialyzed against native binding buffer for further purification. After Ni-affinity column chromatography, PT-scFv3A3 (2.41 mg) was obtained. That is, 240 μg of purified PT-scFv3A3 was obtained from a *B. mori* larva. In general, the expression level of recombinant protein in *B. mori* larvae is higher than that in insect and animal cell culture [10]; besides, the cost of production in the silkworm larvae system is lower than that of cell culture. BmNPV bacmid DNA is a useful expression system because active scFv can be expressed in a large amount, for which purified PT-scFv3A3 can be obtained after simple two-step chromatographic technique.

The SDS-PAGE analysis revealed that purified PT-scFv3A3 (*B. mori*) was obtained when hemolymph was subjected to cation exchanger, followed by Ni-affinity resin. The purified PT-scFv3A3 presented two discrete bands in SDS-PAGE analysis (Fig. 5b, left). The final product of PT-scFv3A3 may compose of two glycosylation patterns, which was observed in the *B. mori* expression system [27] and insect cell system [28]. However, both bands were PT-scFv3A3, which were detected in western blotting using antibody against His6 epitope (Fig. 5b, right).

Characterization of the PT-scFv3A3s

In the *E. coli* expression system, although a large amount of PT-scFv3A3 was produced in insoluble fraction, in vitro refolding was inefficient for recovering the reactivity of PT-scFv3A3. When PT-scFv3A3 was expressed using recombinant BmNPV bacmid/PT-scFv3A3, its reactivity was improved abundantly (Fig. 4). This suggested that the structure of PT-scFv3A3 might be too complex to be recovered by simple in vitro refolding. Because expressed PT-scFv3A3 (*B. mori*) was modified by a post-translational process, it was secreted as active form. As the *B. mori* expression system was a useful tool for eukaryotic protein with complex structure [29, 30], only PT-scFv3A3, which was expressed in *B. mori* larvae, was further characterized.

As checked by iELISA, purified PT-scFv3A3 (*B. mori*) reacted with immobilized paclitaxel in a concentration-dependent manner (Fig. 4). The result also suggested that PT-scFv3A3 (6.25 μg/ml), which gave an absorbance of 1 (405 nm), was the optimal concentration for icELISA. Using icELISA, the results showed that the PT-scFv3A3 also reacted in a concentration-dependent manner of free paclitaxel and docetaxel. The ranges of linear binding were 0.156–5.00 and 0.625–20.0 μg/ml for paclitaxel and docetaxel, respectively (Fig. 6). Considering the small molecular size of PT-scFv3A3 and its cross-reactivity against docetaxel, the application of PT-scFv3A3 expands to the evaluation of its neutralizing activity against paclitaxel and docetaxel toxicity, because the size of the complex between PT-scFv3A3 and paclitaxel or docetaxel is approximately 31 kDa, which should be eliminated via glomerular filtration when their molecular sizes are less than 60 kDa [31]. In physiological pH, PT-scFv3A3 (pI 8.95) carries a net positive charge, for which its glomerular filtration should be increased [32]. The PT-scFv3A3 also exhibited low binding activity against paclitaxel-related compound, including 7-xylosyltaxol (22.1 %) and cephalomannine (8.50 %). With the exception of cephalomannine, the PT-scFv3A3 exhibited less cross-reactivity (%) than its parental PT-MAb 3A3 against paclitaxel-related compounds (Table 2), which may be reflected by the conformation difference between full-length PT-MAb 3A3 and PT-scFv3A3. Therefore, PT-scFv3A3-based icELISA exhibited more specific determination of paclitaxel in samples of *Taxus* spp.

Table 2 Cross-reactivity of PT-MAb3A3 and PT-scFv3A3 against structurally related compounds

Compound	Cross-reactivity (%)[a]	
	PT-MAb3A3	PT-scFv3A3
Paclitaxel	100	100
Docetaxel	70.7	31.1
7-Xylosyltaxol	31.8	22.1
Cephalomannine	6.17	8.50
Baccatin III	<0.111	<0.685
10-Deacetylbaccatin III	<0.111	<0.685
1-Hydroxybaccatin I	<0.111	<0.685
1-Acetoxy-5-deacetylbaccatin I	<0.111	<0.685

[a] The cross-reactivity was calculated using the following equation, where IC_{50} represents the concentration of the paclitaxel or investigated compound that gives 50 % inhibition of the control, which was the absorbance in the absence of paclitaxel and investigated compound: CR (%) $= \frac{IC_{50}\text{ of paclitaxel}}{IC_{50}\text{ of investigated compound}} \times 100$

Conclusion

In this study, PT-scFv3A3 was successfully constructed, expressed, and characterized. In the *Escherichia coli* expression system, active PT-scFv3A3 was not recovered from the inclusion body. However, a large amount of active PT-scFv3A3 was expressed using the BmNPV bacmid DNA system. Due to cost-effectiveness, high efficacy, and easy handling of the BmNPV bacmid DNA system, it is useful for expressing the single chain variable fragment (scFv) for various purposes.

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