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pPIC9-Fc: A Vector System for the Production of Single-Chain Fv-Fc Fusions in *Pichia pastoris* as Detection Reagents *In Vitro*

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Recombinant antibodies, especially ScFv fragments, can be applied as detection reagents and even substitute for some reagents used in immunoassays such as antibody-enzyme conjugates. For ScFv fragments, there is no such universal system available up to now. A vector system was constructed based on pPIC9- Fc, in which the hinge, C_H2 and C_H3 domains (Fc fragment) of mouse IgG1 and His-tag were cloned into the *Pichia* expression vector pPIC9. A model ScFv was introduced into pPIC9-Fc, which can bind Glutathione-S-transferase (GST) from Schistosoma japonicum, to yield the expression cassette pPIC9-ScFv-Fc. Following fermentation in a 5-liter reactor, the fusion was expressed at high levels in the methylotrophic yeast *Pichia Pastoris*, secreted as a dimeric form in the culture, and purified by Ni^{2+}-NTA column chromatography. The expression yield can reach 10–30 mg/liter of culture medium. The ScFv-Fc fusion retains the biological binding ability of the parent ScFv, and can be applied as anti-GST antibodies for the detection of GST and GST-fusion proteins. Furthermore, the successful expression and maintenance of the binding activity verify the efficacy of the vector system for use as detection reagents *in vitro*, by reacting with the specific antigens and being readily detected using general anti-mouse antibodies.

Key words: antibody engineering, detection reagent, Fc fragment, *Pichia pastoris*, single-chain Fv.

Abbreviations: GST, Glutathione-S-transferase; HRP, horse radish peroxidase; NTA, nitrilotriacetic acid; PBS, phosphate buffered saline; ScFv, single-chain Fv.

Recombinant antibodies have found many applications in various fields. Among them, the single-chain Fv (ScFv) fragment is even more favored by researchers in many different formats (*1–3*). In its fusion form, the ScFv fragment can be used as a detection reagent *in vitro* (*4–9*).

It has been reported that ScFv-enzyme fusion can be used as a detection tool by fusing ScFv to enzymes to visualize the binding of ScFv to a specific antigen (*10–13*), such as *E. coli* alkaline phosphatase-AP. This step can be replaced by using ScFv-Fc conjugates, in which the Fc fragment of immunoglobulin is genetically coupled to the parent ScFv. The combination of the Fc fragments has additional attractive virtues, compared with fusion partners such as enzymes. Joining ScFv to an Fc fragment can produce disulfide bonds to yield dimeric molecules, which may improve stability (*14*). Bivalent fusion proteins of ScFv-Fc can exhibit higher binding affinities than monovalent ScFv (*15*). Protein A and other Fc-specific reagents can simplify purification (*16*). The ScFv-Fc fusions will be useful to the rapid characterization of candidate ScFvs isolated from phage antibody libraries before conversion to full-length IgG (*17*).

Moreover, an appropriate expression system is very important. Reported ScFv-enzyme fusions were all produced in bacterial expression systems. Although ScFv-AP fusions can be directed to the perisplasmatic space in a soluble form (*10*), the oxidizing periplasm generally results in low yield (*18*). Therefore, more advanced expression systems should be adopted to improve the expression. Lower eukaryotes, such as the yeast *Pichia pastoris*, are preferred for the large-scale production of heterogenous proteins with original biological activity (*19*). This methylotrophic fungus has been shown to overexpress many different recombinant proteins under the control of the strong promoter AOX1, among which several recombinant antibodies are also included (*17*, *19–20*).

In this study, we constructed a vector system, pPIC9-Fc in *Pichia pastoris*, to provide molecules suitable for use as detection reagents. The system contains the Fc fragment of mouse IgG1 and a His-tag. As a model, an ScFv was cloned into pPIC9-Fc, which can bind Glutathione-S-transferase (GST), to yield the expression cassette pPIC9- ScFv-Fc. By fermentation, purification and verification by GST and GST-containing proteins, the ScFv-Fc fusion was produced in high levels while retaining specific antigen-binding ability, which testifies the usefulness of the vector system for application as a detection reagent *in vitro*.

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MATERIALS AND METHODS

pPIC9-Fc, pPIC9-ScFv-Fc Plasmid Construction—Total RNA was extracted from mouse spleen, and reverse transcribed into the first chain of cDNA using MBI Revert Aid™ H Minus M-MaLo Reverse Transcriptase. The Fc fragment (hinge, CH_2 and CH_3) of mouse IgG1 was amplified from the first chain of the cDNA by primer pairs, MO-1 (5′-GTGCCCAGGGATTGTGGT-3′) and MO-2 (5′-TTATTTACCAGGAGAGTGGGAGAGG-3′). Forty cycles of PCR were performed with incubations for 30 s at 94°C, 30 s at 50°C and 2 min at 72°C. The purified PCR fragment was initially subcloned into pMD18-T vector (obtained from TAKARA) to generate pMD18-T-Fc (All amplified PCR fragments were purified with a Qiagen Gelextraction kit and stored at –20°C for subsequent cloning.).

pMD18-T-Fc vector containing a mouse IgG1 Fc fragment was then used as a template to append *Eco*RI, *Sfi*I, *Not*I and *Avr*II, *Hin*dIII, $(His)_6$ sequences onto the 5′ and 3′ ends of Fc fragments by PCR amplification, using primer pairs MO-3 (5′-GGGAATTCAAGGGCCAGCCGGCCAGCGGCCGCGTGCCCAGGGATTGTG-3′) (The above three underlined parts are corresponding to *Eco*RI, *Sfi*I and *Not*I sites, respectively.) and MO-4 (5′-AAAACATGCCTAGG AAGCTTAAGTGGTGGTGGTGGTGGTGTTTACCAGGAGAGTGG-3′) (The above three underlined parts are corresponding to *Avr*II, *Hin*dIII and $(His)_6$ sequences, respectively.)

Before transferring this PCR fragment to the expression vector pPIC9 (maintained in our laboratory), the *Not*I site was eliminated using the Klenow fragment by filling the 5′-protruding ends of the *Not*-digested pPIC9, to give plasmid pPIC9-*Not*I minus. The plasmid pPIC9-*Not*I minus was digested with *Eco*RI and *Avr*II, and ligated to the above PCR fragment treated with *Eco*RI and *Avr*II to generate plasmid pPIC9-Fc.

The ScFv fragment, screened from a phage antibody library (constructed by Qian Feng, unpublished), can bind Glutathione-S-transferase (GST) by PCR amplification using primer pairs S1 (5′-CAACGTGAAAAAATTATTCGC-3′) and S6 (5′-GGAGTATGTCTTTTAAGTAAATG-3′). The amplified product was cleaved with *Sfi*I and *Not*I, and cloned into *Sfi*I-*Not*I-digested pPIC9-Fc to yield the expression vector pPIC9-ScFv-Fc.

The sequences of the resulting vector were confirmed by DNA sequencing (by Shanghai ShenYou Biotech, China).

Small Scale Expression of the ScFv-Fc Fusion Protein—*Pichia pastoris* strain KM71 (maintained in our laboratory) was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) and made competent as per the supplier's instructions (Invitrogen, *21*). Eighty microliters of electrocompetent cells were mixed with 10 μg of plasmid pPIC9-ScFv-Fc, which was linearized with Sal I, and electroporated by means of pulse discharge (1,500 V, 25 μF, 400 Ω; Bio-rad Gene Pulser) for 5 ms. After pulsing, 1 ml of ice cold 1 M sorbitol was immediately added to the cuvette, and the cuvette contents were transferred to a sterile microcentrifuge tube. Transformants were spread (400 μl) on MD (1.34% YNB, 4×10^{-5}% Biotin, 2% dextrose) plates, and grown at 30°C to screen for His^+ transformants according to their capacity to grow in the absence of histidine as described (Invitrogen, *21*).

His^+ clones were grown in 10 ml BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% Biotin, 1% glycerol) at 30°C and 250 rpm until the culture reached $O.D._{600}$ = 2.0–6.0. The cells were then harvested by centrifugation and resuspended at a five-fold concentration in 2 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% Biotin, 0.5% methanol) to induce protein expression. The cells were incubated for 6 days at 30°C and 250 rpm, and fresh methanol was added to a total of 0.5% to maintain induction every 24 h post induction. Aliquots of culture supernatant were taken daily and examined for protein production by SDS-PAGE. Samples were also analyzed by Western blot with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (purchased from Calbiochem-Novabiochem Corporation). (refer to "Western blot analysis" below)

Large Scale Expression of ScFv-Fc Fusions—A well expressed clone was chosen for upscale expression. One liter cultures of KM71-ScFv-Fc were grown overnight in BMMY until the OD_{600} reached 2.0–6.0. The cells were collected by centrifugation and the pellet was resuspended in 250 ml BMMY medium to induce protein expression. The cells were incubated for 6 days at 30°C and 250 rpm. Fresh methanol was added to a total of 0.5% to maintain induction every 24 h post induction. After 144 h, the cells were removed by centrifugation and the supernatant was harvested and stored for further analysis.

Fermentation was carried out in a 5-liter working-volume stirred-tank reactor (B. Braun Biotech International). Basal Salts medium (3.5 liter; 4.25 ml Phosphoric acid/liter of Fermentation Basal Salts medium, 9.4 mM $MgSO_4$, 1 mM $CaSO_4$, 16.4 mM K_2SO_4, 11.4 mM KOH, 40 ml/liter glycerol) was sterilized inside the reactor. PTM1 trace salts (4.35 ml; 24 mM $CuSO_4$, 0.53 mM NaI, 19.87 mM $MnSO_4$, 0.83 mM Na_2MoO_4, 0.32 mM boric acid, 2.1 mM $CoCl_2$, 0.15 mM $ZnCl_2$, 0.23 M $FeSO_4$, 0.82 mM biotin, 5 ml/liter H_2SO_4) were added after sterilization. The fermentation mixture was inoculated with 350 ml of an overnight preculture in BMGY and maintained at 30°C. During the whole period; agitation was provided by two six-bladed impellers one-third the vessel diameter, operating at 800 rpm; aeration was constant at 0.5 liter/min/liter culture medium; pH was maintained at 5.0 by the addition of NH_4OH (28%, w/v), which was also used as a nitrogen source. Cells were grown until the glycerol was completely consumed (about 24 h), indicated by an increase in the DO to 100% (Invitrogen, *22*). Using a 50% glycerol feed containing 12 ml PTM1 trace salts/liter of glycerol feed, a glycerol-fed-batch phase was then carried out in which the biomass was bulked up to the desired level (120 to 250 g/liter wet cells) prior to induction (*23*). After the depletion of glycerol, induction was initiated. The methanol feed containing 12 ml PTM1 trace salts/liter of methanol was initiated at 1 ml/h/liter initial fermentation volume for the first two hours, and then increased in 10% increments every 30 mins to a rate of 3 ml/h/liter, which was maintained for the duration of the induction. The culture was harvested after 100 hours in methanol (*20*, *22*).

Purification of ScFv-Fc Fusions—One millimolar PMSF was added to 1 liter of the supernatant to reduce proteolysis, and the pH of the culture was adjusted to 8.0 by the

addition of 1/10 volume of 1.0 M Tris, pH 8.0. Proteins were precipitated by the addition of ammonium sulfate to 60% saturation in the cold with constant stirring over a period of 2 h (*17*, *24*). The precipitate was recovered by centrifugation, dissolved in 40 ml of 20 mM Tris/HCl pH 7.9, and dialyzed overnight against two changes of 4 liters each of 20 mM Tris/HCl pH 7.9 at 4°C. After dialysis, NaCl and imidazole were added to the sample to final concentrations of 0.5 M and 5 mM, respectively, and the sample was loaded onto a Ni^{2+}-NTA column (Pharmacia Biotech). After extensive washes with washing buffer (20 mM Tris/HCl pH 7.9, 60 mM imidazole, 0.5 M NaCl), elution buffer (20 mM Tris/HCl pH 7.9, 0.5 M imidazole, 0.5 M NaCl) was added to the column to elute the expressed fusion.

Western Blot Analysis—To examine the activity of the expressed recombinant antibody, GST and several GST fusion proteins were obtained, including hCG (human chorionic gonadotropin), FGF (Fibroblast growth factor), and B_{37}, C_{21}, C_{23}, D_1, D_2 (derived from the multidrug resistance P-glycoprotein).

GST and the GST fusion proteins were resolved on SDS-PAGE along with the uninduced BL21 lysate as a negative control. The separated proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech) using a Western Blot semi-dry transfer apparatus (Bio-Rad Laboratories) with Towbin buffer (25 mM Tris, 192 mM Glycine and 3.5 mM SDS). Unreacted sites were blocked with blocking buffer (5% non-fat skim milk in PBST, phosphate-buffered saline containing 0.1% Tween-20) for 1 h. Following three washing steps, each for 10 min, with PBST buffer, the blots were incubated for 1 h at room temperature with the expressed ScFv-Fc fusion protein. The blots were also incubated with *Pichia* KM71 cells transfected with the control plasmid pPIC9 as another negative control. The samples were washed three times with PBST, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (purchased by Calbiochem-Novabiochem Corporation) diluted 1:1,000 in blocking buffer. After three washes, the membrane was developed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech.).

RESULTS

Design and Construction of the Pichia Pastoris ScFv-Fc Expression Cassette Vector—The concept was to design and construct a vector system for the production of a ScFv-Fc recombinant antibody comprising two functional cassettes, a single-chain antibody Fv (ScFv) and the mouse IgG1 Fc domain (hinge, C_H2 and C_H3) flanked by unique restriction sites (Fig. 1A). Plasmid pPIC9-ScFv-Fc for the expression of the ScFv-Fc fusions is derived from the *Pichia Pastoris* expression plasmid pPIC9 (Invitrogen). In this plasmid, the expression cassette is under the control of the strong AOX1 (alcohol oxidase) promoter for high level expression of heterologous proteins, located downstream of the *Saccharomyces cerevisiae* α-factor signal sequence, under the direction of which, the heterologous proteins are secreted into the medium (*17*).

Details of the plasmid and cloning sites are shown in Fig. 1B. First, the *Not*I site, which is at the 3′ end of the

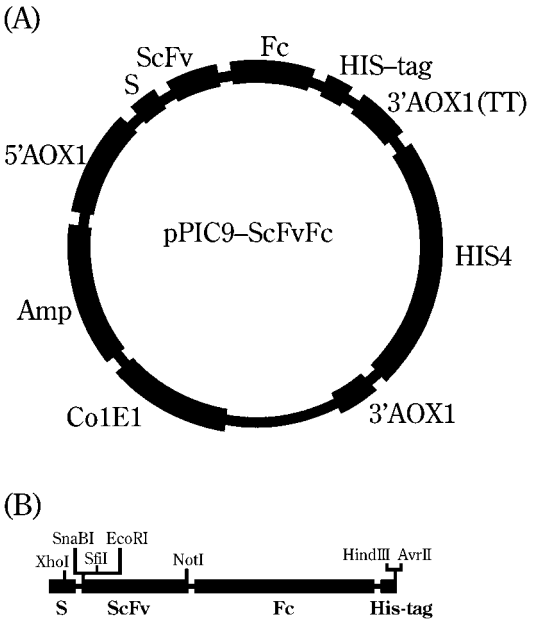

Fig. 1. **Plasmid structure.** (A) The structure of the expression plasmid pPIC9-ScFv-Fc: 5′ AOX1, alcohol oxidase 1 promoter; AOX TT, transcriptional terminator from *Pichia Pastoris* AOX1 gene; 3′ AOX1, sequences from the AOX1 gene that are further 3′ to the TT sequences; ColE1, *E. coli* origin of replication; Amp, Ampicillin resistance gene; HIS4, *Pichia* wild-type gene coding for histidinol dehydrogenase and used to complement *Pichia* his4 strains; S, an N-terminal protein secretion signal. (B) Details of the cloning sites. The cloning strategy for obtaining the expression vector is described in "MATERIALS AND METHODS."

multiple cloning sites of pPIC9, should be eliminated for the later introduction of the ScFv fragment (upstream of Fc) by digestion with *Sfi*I and *Not*I. Then the hinge, C_H2 and C_H3 domains of mouse IgG1 were cloned into the *Eco*RI and *Avr*II sites of the pPIC9-*Not*I minus vector to generate plasmid pPIC9-Fc, in which $(His)_6$ sequences were introduced by the 3′ end primer MO-4 for the convenience of later purification. *Sfi*I and *Not*I sites, which were appended by the 5′ end primer MO-3, can be used to introduce ScFv fragments from phage display vectors pCANTAB and pHEN 1. The 5′and 3′primers to amplify ScFv fragments can be either sequence-specific primers that append *Sfi*I and *Not*I sites onto the ends of ScFv genes, or universal primers of the phage display vectors, which already have in-frame *Sfi*I and *Not*I sites positioned at the ends of ScFv genes. On the other hand, the *Sfi*I and *Not*I sites can also introduce gene fragments that possess these enzyme digestion sites.

An ScFv fragment, which can bind to Glutathione-S-transferase (GST), was cloned to verify the vector system. It was screened from a phage antibody library and PCR amplified from phagemid pCANTAB 5E-GST (generated by the cloning of GST into the vector pCANTAB 5E). S1 and S6, universal primers for pCANTAB 5E, were used for amplification. The resulting plasmid pPIC-ScFv-Fc was electroporated into *Pichia* strain KM71 for expression.

Small Scale Expression and Screening of Pichia Transformants—A total of 50 colonies were screened, of which six were found to exhibit strong expression of recombinant antibody. These clones of pPIC9-ScFv-Fc His^+

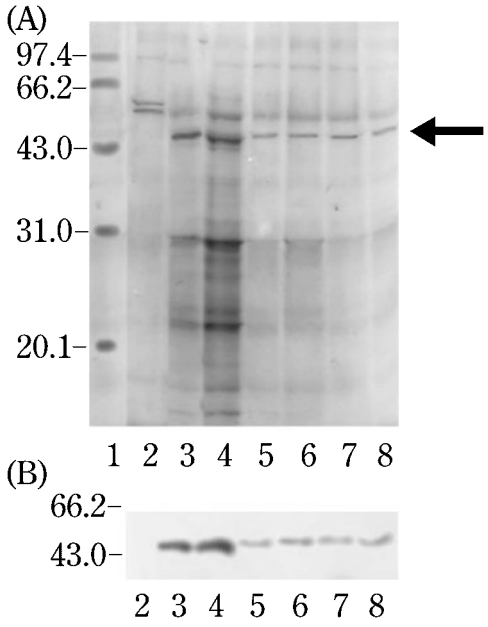

Fig. 2. **Analysis of ScFv-Fc expression in KM71.** (A) SDS-PAGE gel of expressed clones. Samples were resolved on 12% polyacrylamide gel and then stained with Coomassie Blue. Lane 1, molecular weight markers; 2, PIC9 control transformant; 3–8, pPIC9-ScFv-Fc transformants. The arrow indicates the location of the expressed protein. (B) Western blot of a duplicate of the above gel using anti-mouse IgG-HRP (horse radish peroxidase) conjugate, followed by ECL development. Molecular weight markers are indicated on left of each gel.

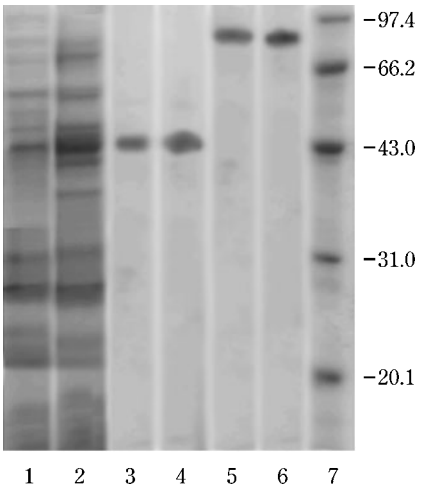

Fig. 3. **SDS-PAGE analysis of samples obtained on the large scale expression and purification of ScFv-Fc fusion.** Lane 1, culture supernatant of shake flask expression; 2, culture supernatant of 5 liter reactor fermentation; 3 and 5, 0.5 M imidazole elutes of samples by shake flask expression under reducing (lane 3) and non-reducing (lane 5) conditions; 4 and 6, 0.5 M imidazole elutes of samples by 5 liter reactor fermentation under reducing (lane 4) and non-reducing (lane 6) conditions; 7, molecular weight markers.

transformants (along with *Pichia* KM71 cells transfected with the control plasmid pPIC9) were analyzed by SDS-PAGE under reducing conditions, which showed a new band in the medium with an apparent molecular mass corresponding to the predicted size (44 kDa) of a reduced pPIC9-ScFv-Fc Antibody (Fig. 2A). Western Blotting analysis demonstrates that these bands interact with an anti-mouse IgG1 antibody (Fig. 2B). No anti-Fc reactive species were seen in *Pichia* strain KM71 cells transferred with the control plasmid pPIC9. A well-expressing clone (lane 4) was chosen for large scale expression and analysis.

Large Scale Expression of ScFv-Fc Fusions—For shake flask fermentation, 250 ml of culture medium was used to generate pPIC9-ScFv-Fc, and result is shown in Fig. 3. For fermentation in a 5-liter bioreactor, control of the methanol concentration at the induction phase is crucial (*25*). Since the Mut^S strain metabolizes methanol even more poorly than the Mut^+ strain (excess methanol in the medium should not exceed 0.3%, as described by Invitrogen), it's very important to introduce methanol very slowly to adapt the culture to grow on methanol. We have tried an initial feeding rate higher than 1 ml/h/liter initial fermentation volume such as 1.5 ml/h/liter and 2 ml/h/liter, and the pH increased sharply indicating cell lysis (data not shown). When using a rate of 1 ml/h/liter, the cells grew well by observation under microscope, showing that methanol did not reach toxic concentrations. The SDS-PAGE analysis is shown in Fig. 3.

Comparing the shake flask and bioreactor fermentation, we found that the fusion was expressed better in the latter. By scanning (Smart View 2001 by FuRi Biotech), it was estimated that, for shake flask expression, the content of the expressed protein was 10% of the total protein; for 5-liter reactor fermentation, it was 20% of the total protein. Therefore, by using the fermentation procedure, the fusion achieved a high level of expression.

Purification of the ScFv-Fc Fusion—Briefly, the purification employed ammonium sulfate precipitation of the proteins from the culture supernatants, followed by dialysis and immobilized-metal affinity chromatography. Under reducing conditions, the products isolated showed only one band at 44 kDa, indicative of high purity and integrity. Under non-reducing conditions, the ScFv-Fc protein bands have approximately twice the apparent molecular weight (88 kDa), indicating that the ScFv-Fc fusions are secreted into the medium primarily as disulfide-linked dimmers (Fig. 3). The yield after purification was about 10 mg/liter of culture for shake flask expression and 20 mg/liter of culture for 5-liter reactor fermentation, spectrophotometrically determined at 280 nm using calculated extinction coefficients (*17*, *26*). This also verifies that the production by fermentation is higher than that by shake flask expression.

Binding Ability of the ScFv-Fc Fusion—To confirm the activity of the expressed recombinant antibody, GST and several GST-fusion proteins (hCG-human chorionic gonadotropin, FGF-Fibroblast growth factor, and B_{37}, C_{21}, C_{23}, D_1, D_2-derived from the multidrug resistance P-glycoprotein) were obtained and resolved on SDS-PAGE gels. The expressed fusion can bind them as indicated by Western blot (shown in Fig. 4). Single bands were seen with apparent molecular masses in good agreement with the predicted values.

Negative controls (uninduced BL21 lysate and *Pichia* KM71 cells transfected with the control plasmid pPIC9) were also performed. For the uninduced BL21 lysate,

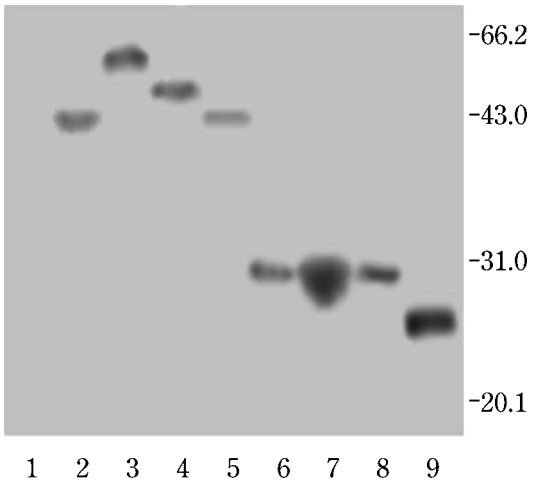

Fig. 4. **Western blot analysis of the activity of the expressed ScFv-Fc fusion.** Samples were resolved by SDS-PAGE, transferred to PVDF membranes, incubated with the expressed fusion, and then probed with HRP-conjugated anti-mouse IgG1. Lane 1, uninduced BL21 bacterial lysate; 2, FGF; 3, D1; 4, D2; 5, hCG; 6, B_{37}; 7, C_{21}; 8, C_{23}; 9, GST. The anticipated molecular weights of proteins of lane 2–9 are respectively: 42, 54, 50, 43, 30, 29 , 30, and 26 kDa.

there were no bands appearing, showing that no anti-GST reactive species were present. For the pPIC9 control transformant, when it was incubated with GST and GST fusion proteins, it did not bind to them since there were also no bands appearing (data not shown). Therefore, the ScFv-Fc fusion shows the functional activity of its maternal antibody fragment-ScFv, by specific binding capacity to GST-containing protein.

We have tried to compare the binding activities of the ScFv-Fc fusions and that of the parent ScFv. ScFv itself was cloned into several vector systems, but could not be expressed (data not shown). Therefore, we can not rule out differences in binding activity between the parent ScFv and its ScFv-Fc fusion format. And, to some degree, this shows an advantage of our vector system in gaining expression in the fusion construct ScFv-Fc. On the other hand, similar work performed before (*17*, *19*) showed the ScFv-Fc fusion to exhibit comparable activity to the parent ScFv, although it was slightly inferior. From the above data it is clear that the ScFv binding sites own the activity in the ScFv-Fc fusion format (shown in Fig. 4), which is already fit for our requirements as the detection reagent *in vitro*.

DISCUSSION

This vector system is promising for *in vitro* detection due to its attractive properties: a suitable expression system, general utility and practical applications.

The Choice of an Optimal Expression System—The methyltropic yeast *Pichia pastoris* is rapidly becoming a preferred host for the efficient expression of heterologous proteins, especially many recombinant antibody fragments (*20*, *27*). The ability to express folded secreted proteins correctly, including highly disulphide-bonded proteins (*28*), provides a distinct advantage over bacterial systems that often require laborious and inefficient procedures to denature and refold proteins expressed as insoluble inclusion bodies (*19*, *29*, *30*). In our study, it was possible to identify the monomeric and dimeric forms of ScFv-Fc under reducing and non-reducing conditions, indicating that the fusion is secreted into the medium as a bivalent molecule. However, ScFv itself can not be expressed functionally in *E. coli* (data not shown). On the other hand, the yeast system is less time-consuming and easier to manipulate than mammalian cells, and always yields higher quantities of secreted functional proteins.

A further advantage of *Pichia* is that it is particularly well-suited for fermentation and can reach high cell densities that may improve overall protein yields. We have compared shake-flask expression with 5-liter reactor fermentation, and found the yield of the latter to be two-fold higher than that of the former. Here the production that we obtained by fermentation can reach 20 mg/liter of culture medium, higher than in common bacterial systems after refolding of the antibodies, which is less than 10 mg/liter of culture medium (*31*, *32*), and also higher than that of mammalian cells, which is even lower (*33*, *34*). Also the medium used for fermentation is well defined and inexpensive, so that the overall production cost can be held to a low level (*20*).

The General Utility of the Vector System—By appending unique Sfi I and Not I restriction enzyme sites upstream of the Fc domain, we have made the constructed plasmid, pPIC9-Fc, a universal vector system. With these two sites, it is possible to transfer ScFv-genes selected from established phage-display systems, such as pHEN1 and pCANTAB 5E, directly into pPIC9-Fc (*35*), as well as to insert synthetic oligonucleotides coding for a specific binding peptide, both without the need for intermediate cloning steps (*10*). The usefulness of pPIC9-Fc was confirmed by cloning a ScFv-gene from phage display vector pCANTAB 5E into pPIC9-Fc to generate plasmid pPIC9-ScFv-Fc, which obtained a high expression level in *Pichia pastoris*. Furthermore, the system also facilitates purification of the fusions, by appending the C-terminally linked histidine tag, or just by protein A or G, which can bind to the Fc domain for purification (*36*).

The construction of Fc-containing fusions has been performed by many researchers in *E. coli*, yeast and mammalian cells for a variety of applications, such as complement activation (*37*), the induction of cellular cytotoxicity (*38*), investigations of the mitogenic properties of bispecific reagents (*39*), prolonging circulation half-life (*17*, *40*), and production of a greater effect on tumor tissues (*15*, *41*). However this is the first time to our knowledge that such a fusion has been utilized as a detection reagent *in vitro*. With the Fc domain, this system offers convenience for the specific detection of the reaction of the fusion with the target antigen, by binding enzyme-conjugated anti-Fc antibodies, such as an anti-mouse IgG second antibody.

The applications of the Expressed ScFv-Fc Fusion—The cloning of a model ScFv, which can bind GST, into the vector verified the application of our system in one field. The expressed fusion retains the affinity of the parent ScFv to bind to GST-containing proteins and can be used as a reagent to detect GST fusion proteins. At present, the pGEX expression vector system is one of the most

widely used systems, because high expression levels, mostly in soluble forms, can be achieved. And protein Glutathione-S-transferase (GST) can be produced in fusion with the protein of interest so that it will bind with high affinity to a glutathione affinity column, thereby enabling purification by elution from the column with reduced glutathione (*42–44*). Therefore, anti-GST antibodies are required to prove the expression of GST-containing proteins. The development and production of anti-GST antibodies will be necessary.

The major advantage of our product over that of many companies lies in the fact that our anti-GST fusion procedure is far less laborious and time-consuming without the need to immunize animals first, then purify monoclonal or polyclonal antibodies from sera. At the same time, the ScFv-Fc fusion has almost the same affinity and specificity as the original protein. Therefore, this work forms the basis for further research to make the ScFv-Fc fusion a merchandisable product as an anti-GST antibody on the market.

In conclusion, we constructed a universal vector system for the cloning and expression of recombinant antibody containing the mouse IgG1 Fc domain in *Pichia pastoris* to work as the detection reagent *in vitro*. Furthermore, the ScFv-Fc fusion that we expressed can be used as anti-GST antibodies with high specificity and at low cost.

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