International Food Research Journal 25(4): 1726-1732 (August 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Expression and characterization of functional single-chain variable fragment against norfloxacin in *Pichia pastoris* GS115

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Article history

Received: 4 April 2017
Received in revised form: 11 May 2017
Accepted: 13 May 2017

Keywords

Norfloxacin
Monoclonal antibody
Single-chain variable fragment
Pichia pastoris

Abstract

A functional single-chain variable fragment (scFv) of mouse monoclonal antibody against norfloxacin was expressed in yeast *Pichia pastoris* GS115, purified and characterized. Gene encoding monoclonal antibody against norfloxacin was transformed to *P. pastoris* GS115 using the pJM01 plasmid. Integration of the plasmid into the genome was verified by PCR and DNA sequencing analysis. After screening for the transformant and production of the selected scFv, the norfloxacin-binding ability was determined by an enzyme linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) analysis. Eight transformats (O1-O8) were obtained from the transformation of *P. pastoris* GS115 with PmeI-linearized pJM01. PCR products detected by gel electrophoresis confirmed that pJM01 expression cassette was integrated at *AOX1* promoter. After screening all transformants for high expression of the recombinant scFv, transformant O5 was selected for antibody production and purification. Detection of norfloxacin by ELISA with the recombinant scFv fragment was successful albeit with lower sensitivity compared to the original monoclonal antibody. In SPR analysis, our recombinant scFv fragments have the binding capability to norfloxacin equivalent to the original antibody with the average angle shift of 580 ± 133 and 505 ± 28 mDegree, respectively. The functional recombinant scFv of monoclonal antibody against norfloxacin successfully produced by methylotrophic yeast, *P. pastoris* GS115.

Introduction

Norfloxacin is an antibiotic widely used for treatment of urinary bacterial infection in consumption animals. However, the extensive abuse of this antibiotic has caused severe food safety problems. Misuse of this drug could cause drug residue in food products, resulting in drug resistance of human pathogen (Cui *et al.*, 2011; Chen *et al.*, 2014). Therefore, the residue of norfloxacin in animal products for consumption is under surveillance in many countries to ensure human food safety. Various chromatography methods have been developed for the detection of norfloxacin in different food matrixes. However, these instrumental methods are time consuming and costly which are not suitable for screening detection of a large scale of samples. To overcome these drawbacks, many immunological assays have been developed to detect antibiotics based on both polyclonal antibody (PAb) (Duan and Yuan, 2001) and monoclonal antibody (MAb) (Hu *et al.*, 2010; Jinqing *et al.*, 2011; Chusri *et al.*, 2013). Compared with instrumental analysis methods, immunoassay technology has advantages of rapid, sensitive and high throughput characteristics (Watanabe *et al.*, 2002; Kim and Kim, 2009). Since the properties of the PAb are not consistence depending on the hosts, MAb is usually preferred in the test kit development. However, the production of MAb based on hybridoma animal cell culture is generally suffered from high cost of production process (Wang *et al.*, 2007; Wang *et al.*, 2014). Recently, recombinant antibody (RAb) technology has provided an alternative approach for low-cost antibodies production with desirable affinities and specificities (Bhatia *et al.*, 2010; Chen *et al.*, 2014) and an increasing number of RAbs against fluoroquinolones has also been successfully produced

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(Leivo *et al*., 2011; Chen *et al.*, 2014,). Single chain variable fragments (scFv) are the smallest unit of immunoglobulin molecule with function in antigen-binding activities and retains the original specificity and full monovalent binding of the intact parent antibody. (Wang *et al.*, 2006; Jafari *et al.*, 2011). They are composed of a variable heavy and light chain joined by a small flexible linker peptide such as $(Gly_4Ser)_3$. Because it is functional as a single peptide, exogenous expression in various organisms is feasible, which allows protein engineering possible to improve the properties of the scFv such as increase of affinity and alteration of specificity (Kumada *et al*., 2007; Wang *et al*., 2007; Wang *et al*., 2008). The methylotrophic yeast *Pichia pastoris* has become an important expression host for the production of active recombinant proteins and many recombinant proteins have been expressed successfully in this system (Cregg *et al*., 2000; Macauley-Patrick *et al*., 2005). P. pastoris has particular industrial interest due to its powerful and tightly regulated methanol-inducible alcohol oxidase 1 *(AOX1)* promoter, an ability to perform post-translation modifications including glycosylation, disulfide-bond formation and an efficient secretion of foreign extracellular proteins. (Cregg *et al*., 2000; Jafari *et al*., 2011; Goncalves *et al.*, 2013). Importantly, *P. pastoris* expression system offers high levels of heterogeneous protein expression under high cell density and a broad range of pH and temperature condition (Li *et al.*, 2007; Potvin *et al*., 2012).

Recently, our laboratory has constructed the expression plasmid, pJM01, containing a gene encoding for scFv antibody against norfloxacin based on the pPICZαA (Mala *et al*., 2015). Therefore, the aim of the present study was to express the recombinant scFv antibody using *P. pastoris* expression system and investigate its binding characterics for further possible antibody applications.

Materials and Methods

Tranformation and selection of transformants

The expression plasmid pJM01 (Mala *et al.*, 2015) was linearized with PmeI and incorporated into *P. pastoris* GS115 (His-, Mut+, Invitrogen) chromosome by Frozen-EZ Yeast Transformation II™ (Zymo Research). Transformants was selected on YPD (1% yeast extract, 2% peptone and 2% dextrose) plates containing 100 µg/mL Zeocin™ (Invitrogen) and incubated at 30°C for 2 days. The pPICZαA plasmid (empty vector, Invitrogen) was also transformed as a negative control. Presence of synthesized scFv gene in the transformants *P. pastoris* genome was analyzed by PCR using N1 and N4 primers (Table 1). PCR components and conditions were as follows: AmpliTaq Gold® 360 Master Mix (AB Applied Biosystem), 0.5 µM of each primer, 1 cycle of 95°C for 5 min, 30 cycles of three-step program (95°C for 15 s; 55°C for 30 s and 72°C for 1.30 min). The reactions were then held at 72°C for 7 min and cooled until used at 4°C.

Table 1. Primers for scFv analysis

Primer ID	Sequence (5'-3')	Purposes
N1	GAATTCGAAGTAGAGCTGGAGGAGTCTGGG	PCR
N4	CGAGGTACCGGGAGCTCGGATACAGTTGGT	Confirmation
P1	GTTGACTCATGTTGGTATTGTGAA	Integration Confirmation
P2	GCAGCAATGCTGGCAATAGTA	
P3	AACGAAAACTCACGTTAAGGGA	
P4	CCACCACCTAGAACTAGGATATCA	
T3	ATGAGATTTCCTTCAATTTTTACTGCT	Sequencing
T4	AGAGGCTGGAGTGGGTCGCAA	
T5	CCGCCACCGCCAGATCCACCT	
T6	GCCTCCCCCAGACTCCTCCAG	

Verification of plasmid integration

Zeo^R transformant clones were cultured on YPD-zeocin plates. Chromosomal DNA of these clones was then extracted and analyzed to confirm the integration of PmeI-linearized pJM01 into the AOX1promotor of Pichia genome. Briefly, chromosomal DNA of Zeo^R transformant, pPICZαA (empty vector) and GS115 (host) was extracted by using LiOAc-SDS lysis (Looke *et al*. 2011). DNA concentration was measured by Nanodrop Lite spectrophotometer (Thermo Scientific). The quality of the extracted genomic DNA was determined by 1.0% agarose gel electrophoresis using 1 kb DNA as the marker. The following analysis methods were carried out.

(i) PCR analysis. The selected clones were analyzed by PCR as described previously using the designed P1- P4 primers (Table 1) based on the scFv sequences.

(ii) Sequencing analysis. the chromosomal DNA of the selected clones were sequenced with BigDye® terminal sequencing kit and ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, USA) using four oligonucleotide primers T3-T6 (Table 1)

Expression of recombinant scFv

The validated clones were cultured in 5 mL YPGly (1% yeast extract, 1% peptone and 1% glycerol) broth overnight at 30°C, 250 rpm for 18 h. An aliquot of the dense culture was used to inoculate a fresh medium of 25 mL YPGly and the incubation was repeated. Afterward, cells were harvested by centrifugation at 3000×g for 5 min at 4°C and re-suspended thoroughly in 25 mL MMH

(1% yeast extract, 2% peptone, 1% methanol, 1.34% YNB (w/o amino acid) and 4×10^{-5}% biotin) for continuing growth under the same conditions for 72 h. Methanol was added to a final concentration of 0.5% (v/v) every 24 h to maintain induction. Culture supernatants were sampled at different time points to monitor the scFv production. Total proteins in cell-free culture supernatants were measured by Qubit® 3.0 Fluorometer (Life Technologies). Supernatant sample at the same total protein concentration of each culture was mixed with 100% trichloroacetic acid (TCA) to obtain the final concentration of 20% (v/v) in order to precipitate the total proteins. The pellets were washed twice with cold acetone to remove TCA residue. The precipitated proteins were analyzed by electrophoresis on 12% SDS-PAGE under denaturing conditions. For Western blot analysis, proteins resolved by 12% SDS-PAGE gel were transferred to Hybond-N+ membrane using a semi-dry electro-blotting apparatus (Bio-Rad, USA). The transfer was carried out for 1 h at 10V. Later, the membrane was blocked with TBST buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk powder for 1 h, and then incubated with a 1:2000 dilution of monoclonal anti-c-Myc antibody (Clone 9E10, Life Technologies) at room temperature for 2 h. The membrane was washed three times with TBST, and incubated with a 1:4000 dilution of goat anti-mouse IgG, (H+L) HRP antibody (GAM-HRP) (Life Technologies) for 1 h. After three washings with TBST, the proteins were detected with CDP-Star™ Detection Reagent. The highest expression clone was selected and stored in 15% glycerol at -75°C for further uses.

Purification of recombinant scFv

Cell-free supernatant containing soluble scFv protein was clarified through a 0.2 µM filter. Purification of scFv was carried out by immobilized metal affinity chromatography (IMAC) using a 1 mL HisTrap™ HP column according to the manufactuer's instructions.

Indirect enzyme-linked immunosorbent assay (indirect ELISA)

The dialyzed BSA conjugated norfloxacin (1µg/ml) was used to coat the 96 well plates (100 µL per well) at 4°C for overnight. The plate was washed three times with 10 mM phosphate buffer saline (PBS) pH 7.4 mixed 0.5% Tween 20 following by blocking with blocking buffer (5% (w/v) skim milk in PBS) and incubated at 37°C for 1 h. The plate was washed again. Subsequently, the purified recombinant scFv was added into the reaction well (100 µL per well) and incubated at 37°C for 2 h. Then, 100 µL of mouse anti-c-myc antibody at the dilution ratio of 1:2,000 was added. After incubation at 37°C for 2 h and washing, a GAM-HRP was added at the dilution ratio of 1:5,000 (100 µL per well). The plate was incubated at 37°C for 1 h, subsequent by washing and adding the 3, 3', 5, 5'-tetramrthylbenzidine (TMB) substrate solution (100 µL per well). After incubation in the dark at room temperature for approximately 20 min, the reaction was stopped by adding 100 µL per well of 1 M H_2SO_4. The plate was measured the optical density at 450 nm using microplate reader (Multiskan FC, type 357, Thermo Fisher Scientific).

Surface plasmon resonance (SPR)

The SPR assay was performed to measure the binding activity of the purified recombinant scFv on an Autolab SPRINGLE system. Briefly, instrument calibration and baseline adjustment was performed according to the manufacturer's manual. The gold sensor surface was saturated with the purified recombinant scFv by static mixing over the sensor surface for overnight at 4°C. The sensor surface was washed with 10 mM PBS buffer (pH 7.4) and drained before introducing the antigen solutions. Binding interactions between the antigen and antibody were allowed to proceed for a minimum period of 40 min. All SPR experiments were performed at the Scientific and Technological Research Equipment Centre (STREC), Chulalongkorn University, Thailand.

Results and Discussion

Transformation to P. pastoris *GS115*

By transformation of *P. pastoris* GS115 with the PmeI-linearized pJM01, eight Zeo^R transformants (O1 – O8) were obtained on YPD/zeocin agar plates. PCR analysis of the eight Zeo^R transformants revealed the PCR product size of about 800 bp with N1 and N4 primers (Figure 1A). Because these primers annealed both upstream and downstream of the synthesized scFv gene, this result indicated that pJM01 was transformed into the genome of *P. pastoris.*

Verification of plasmid integration at the AOX1 promoter

(i) PCR analysis. The location where PmeI-linearized pJM01 expression cassette integrated the *AOX1* promoter was investigated by PCR. The P1, P2, P3 and P4 primers were specifically designed corresponding to the nucleotide of *AOX1* promoter and pJM01 regions. The P1 primer binds at upstream of the *AOX1* promoter while the P4 primer anneals

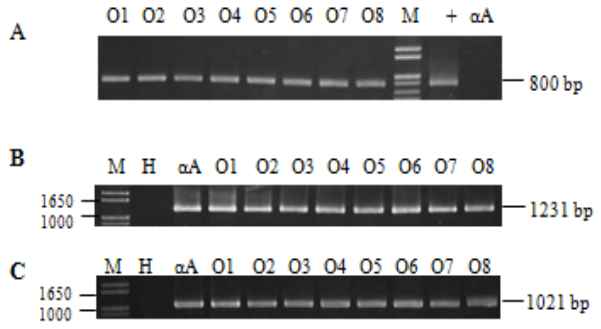

Figure 1. PCR products of Zeo^R transformant clones A) using N1 and N4 primers B) using P1 and P2 primers C) using P3 and P4 primers for amplificaton. Lane M: 1 Kb plus DNA ladder (Invitrogen). +; pJM01 as a positive control. αA; GS115 transformed with the empty vector, pPICZαA. H; untransformed GS115 strain.

at coding sequence (CDS) of the *AOX1* gene. The P2 and P3 primers were synthesized specifically for annealing at the α-factor secretion signal nucleotide region and at the ori of pJM01 region, respectively. The sequence of these primers was confirmed that they could anneal to *AOX1* promoter only. With the P1 and P2 primers, the Zeo^R transformant O1 –O8 yielded the PCR product at 1,231 bp while using P3 and P4 primer, the 1,021 bp band was detected on 1% agarose gel electrophoresis as shown in Figure 1B-C. These results revealed that PmeI-linearized pJM01 expression cassette was integrated at *AOX1* promoter of *P. pastoris* genome .

ii) Sequencing analysis. The DNA sequence of the inserted scFv gene was found to be 1161 nucleotides starting from α-factor secretion signal to stop codon (TGA) at C-terminus. This sequence has been submitted in GenBank and was assigned as No AJG06891.1. The coding sequence (CDS) of scFv expression cassette consisted of 800 nucleotides encoding a polypeptide of 266 amino acids with a predicted molecular mass of 42.50 kDa (intracellular expression including α-factor secretion signal) and 32.50 kDa (secreted expression). These results showed that the DNA sequences of all Zeo^R transformants were correct.

Screening and expression of recombinant scFv

Each Zeo^R tranformant was cultured and its production of recombinant scFv was monitored by Western blot analysis. Probed by mouse anti-c-myc antibody, a specific band appeared on the membrane with corresponding to the predicted recombinant scFv of about 32.67 kDa as shown in Figure 2. After 24 h of methanol induction, only O5 strain gave the specific band of the recombinant scFv antibody (Figure 2A, lane 3) while O3, O6 and O7 strains gave the noticeable band at 48 h induction. Moreover, the

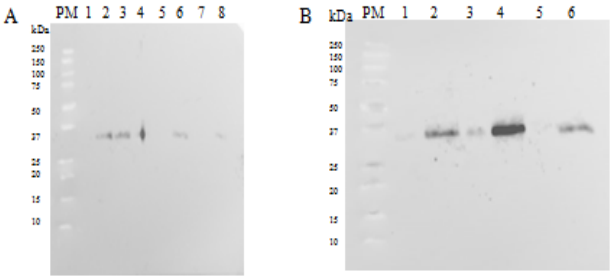

Figure 2. Western blot analysis of recombinant scFv antibody detected with mouse anti-c-myc antibody followed by GAM-HRP. A) Lane1-2: supernatant of O3 strain at 24 and 48 h induction. Lane3-4: supernatant of O5 strain at 24 and 48 h induction. Lane5-6: supernatant of O6 strain at 24 and 48 h induction. Lane7-8: supernatant of O7 strain at 24 and 48 h induction. B) Lane 1, 3 and 5: supernatant of O2, O5 and O8 strains at 48 h induction. Lane 2, 4 and 6: precipitated supernatant of O2, O5 and O8 strains at 48 h induction. Lane PM: protein marker standard (Bio-Rad).

equal volume of supernatant culture of O2, O5 and O8 strains after induction with methanol for 48 h was precipitated and subjected to the analysis. The result indicated that the precipitated supernatant at 48 h of O5 strain (lane 4) showed a specific intense band of scFv on the membrane than the specific bands of O2 (lane 2) and O8 (lane 6) as shown in Figure 2B. In the case of supernatant (without precipitatation), the supernatant of O5 revealed a faint specific band of scFv (lane 3), while those of the other clones were not detected (lane 1 and lane 5). These results showed that O5 gave the highest amount of the recombinant scFv and was selected for further studies. However, the background of detection reaction could be possibly found on blot (Figure 2A, lane 4) with many probable ways to occur such as the insufficient blocking, antibody cross-reaction with the blocking buffer and the detection reagents (ECL substrate comprised of luminal enhancer and peroxide solution) cannot be removed clearly.

Determination of recombinant scFv binding activity

(i)Indirect ELISA

The binding ability of the purified recombinant scFv to norfloxacin was assayed by an indirect ELISA and compared with that of the original MAb (MAb against norfloxacin clone 155). The result showed that the scFv bound to the antigen in a concentration-dependent manner (Figure 3A). However, the intensity of the signal was lower than that of the original MAb (Figure 3B). This might be due to the change in the structure and folding of the

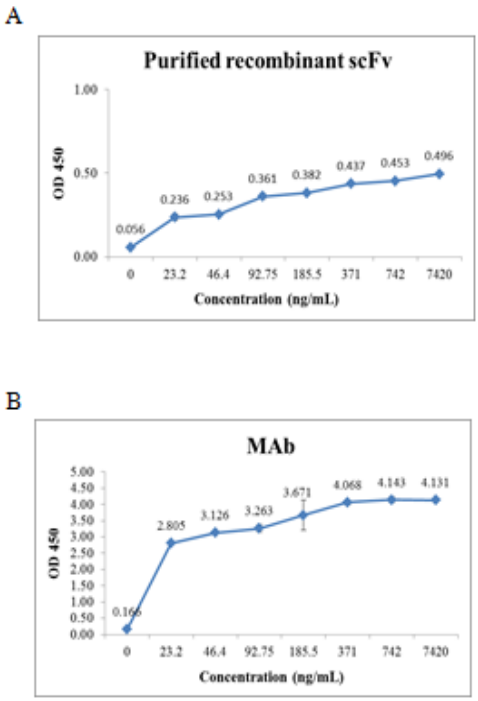

Figure 3. Comparative indirect ELISA using recombinant scFV and MAb against norfloxacin. The indirect ELISA was performed by using TMB and H_2O_2 as the substarte. The concentration of the coated norfloxacin was 1 µg/mL. A) purified recombinant scFv was subjected into the 96-well plate followed by mouse anti-c-myc antibody as primary antibody at concentration of 1:2000 and GAM-HRP as secondary antibody at the concentration of 1;5000 respectively. B) the original MAb was added into the reaction well and detected by GAM-HRP antibody as mentioned above. The reactions were measured the optical density at 450 nm. The experiments were performed three times and mean values ± SD are presented.

recombinant scFv that affects the binding affinity (Choi *et al.*, 2004). Moreover, The O.D. of scFv was about 10 fold less than that of MAb because the indirect ELISA procedures for detection of scFv and MAb are different. For the MAb, it required only two procedures. Firstly, the norfloxacin conjugated BSA, as an antigen, is coated into ELISA plate. MAb is then applied into wells followed by enzyme linked secondary antibody (GAM-HRP) is added. Finally, a substrate is added, and enzymes on the antibody elicit a chromogenic signal. Unlike MAb, the detection of recombinant scFv required more procedures. It need primary antibody to specific binding to recombinant scFv. Moreover, secondary is required before detection. However, this may lead to nonspecific signals because of cross-reaction that the secondary antibody may bring about. For these reasons, it is also possible that the condition used in the ELISA was optimized for the original MAb. To obtain higher intensity, the analysis condition should be optimized specifically for the recombinant scFv. However, regardless of the intensity, this result confirmed that the scFv still functions in term of antigen binding ability.

Table 2. Binding activity of the purified recombinant scFv

Antibody	Antigen	SPR assay		ELISA*
(7 µg/mL)	(1 mg/mL)	Angle shift (mDegree)	Cross-reactivity (%)	Cross-reactivity (%)
Purified scFv	Norfloxacin	580±133[a]	100	-
	Enrofloxacin	394±50[b]	68	-
	Ciprofloxacin	37±19[c]	6	-
Mab	Norfloxacin	505±28[a]	-	100
	Enrofloxacin	-	-	89
	Ciprofloxacin	-	-	88

*Cross-reactivity of MAb was evaluated by using indirect ELISA. Norfloxacin at the concentration of 1 µg/mL was coated on the 96-well plate. Afterward, antigens was tested and followed by adding the GAM-HRP antibody at the concentration of 1:5000. TMB and H_2O_2 were used as a substrate. The reactions were measured the optical density at 450 nm.

(ii)Surface plasmon resonance (SPR)

The affinity of the purified recombinant scFv was also confirmed by SPR. As depicted in Table 2, a sensorgram showed the binding interaction between the purified recombinant scFv, which was immobilized on the gold surface, and the injected norfloxacin in the flow cell. The scFv and original mAb showed strong binding to norfloxacin at the average angle shift of 580±133 mDegree and 505±28 mDegree, respectively. This result indicated that there was no significant difference in the norfloxacin-binding ability between the purified recombinant scFv and the original MAb. In addition, cross-reactivity of purified recombinant scFv to other fluoroquinolones (FQs) such as ciprofloxacin and enrofloxacin was examined by SPR and compared with those of the original MAb studied by the ELISA. The comparison showed the same trend of the cross-reactivity to the other FQs. However, the SPR result of ciprofloxacin much lower than that of ELISA. It is possible that the SPR detection of an antibody which interacted with antigen is performed directly (label-free detection). Therefore, the result observation is showed the interaction between scFv and norfloxacin in real time by changing the angle. On the other hand, indirect ELISA has required enzyme antibody labeling for detection. This may lead to high nonspecific signal (cross-reactivity). These results supported that scFv is functional and possesses the desired antigen binding ability and specificity.

Conclusion

Expression of recombinant scFv antibody as a c-myc-(His)6 secreted fusion protein was achieved in the P. pastoris GS115 using plasmid pPICZαA. The produced scFv still functions in terms of antigen

binding. The genetically modified P. pastoris GS115 could be used for future antibody production and the obtained scFv fragment could be applied in an immunoassay for norfloxacin detection.

Acknowledgements

This research was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Overseas Research Experience Scholarship for Graduate Student, Graduate School, Chulalongkorn University.

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