



ORIGINAL ARTICLE

Construction and sequencing analysis of scFv antibody fragment derived from monoclonal antibody against norfloxacin (Nor155)



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KEYWORDS

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Fluoroquinolone;
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Single chain variable fragment

Abstract Norfloxacin belongs to the group of fluoroquinolone antibiotics which has been approved for treatment in animals. However, its residues in animal products can pose adverse side effects to consumer. Therefore, detection of the residue in different food matrices must be concerned. In this study, a single chain variable fragment (scFv) that recognizes norfloxacin antibiotic was constructed. The cDNA was synthesized from total RNA of hybridoma cells against norfloxacin. Genes encoding V_H and V_L regions of monoclonal antibody against norfloxacin (Nor155) were amplified and size of V_H and V_L fragments was 402 bp and 363 bp, respectively. The scFv of Nor155 was constructed by an addition of (Gly₄Ser)₃ as a linker between V_H and V_L regions and subcloned into pPICZαA, an expression vector of *Pichia pastoris*. The sequence of scFv Nor155 (GenBank No. AJG06891.1) was confirmed by sequencing analysis. The complementarity determining regions (CDR) I, II, and III of V_H and V_L were specified by Kabat method. The obtained recombinant plasmid will be useful for production of scFv antibody against norfloxacin in *P. pastoris* and further engineer scFv antibody against fluoroquinolone antibiotics.

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1. Introduction

Norfloxacin has been reported as an antibiotic which is used to treat urinary bacterial infection in animal [3,10,33]. However, the extensive abuse of this antibiotic has caused severe food safety problems. Meanwhile, some researches indicate that low-level doses of antibiotic exposure for long periods could

result in bacteria resistance [3,11,34]. Due to the concern of antibiotic residue in animal products, maximum residue limits (MRLs) for several antibiotics have been established in many countries to protect consumers [5]. In the case of norfloxacin, the MRLs were set between 0.02 and 0.1 ppm depending on the types of the target tissues. In order to ensure human food safety and the entire ecosystem security, various chromatography methods have been developed for the determination of norfloxacin in different food matrixes [3,15,21,29]. However, these instrumental methods are time-consuming and costly, and sample preparations are demanding. During the last two decades, various immunoassay methods have been developed to detect fluoroquinolone (FQs) based on polyclonal antibody (PAb) and monoclonal antibody (MAb) [5,13,17,26]. Compared with the instrumental analysis methods, the immunoassay methods especially enzyme linked immunosorbent assay (ELISA) are more preferable for rapid screening large members of samples due to its simplicity, sensitive and inexpensive [30]. In ELISA, the most important component is antibody which binds specifically to the desired drug residues. However, PABs sometimes experience nonspecific reactivity while the more preferred MAbs require time-consuming and costly preparation and production. As a result, the preparation of high quality antibodies is still a bottleneck issue when establishing immunoassay methods [3]. Therefore, low cost and simple alternative antibody production system is of interest. Consequently, fermentation of antibody-producing yeast has been studied to produce MAb.

Single-chain fragment variable (scFv) is the smallest unit of immunoglobulin (Ig) molecule that functions in antigen-binding activities. The structure of scFv consists of variable regions of heavy (V_H) and light (V_L) chain, which are joined together by a flexible peptide linker [6,12,27] such as $(Gly_4Ser)_3$ [22] that can be easily expressed in the functional form. This allows protein engineering to improve the properties of scFv such as increase in affinity and alteration of specificity. The order of the domains can be either V_H -linker- V_L or V_L -linker- V_H and both orientations have been applied [1]. This structure remains the original specificity and full monovalent binding of the intact parent Ab [25].

To date, scFv antibodies have been successfully isolated and displayed as fragments in various expression systems such as mammalian cells, bacteria, plant cells, insect cells, and also yeast [7]. However, yeasts are frequently used as the host for heterologous gene expression to produce eukaryotic proteins [16]. During the last decades, the methylotrophic yeast, *Pichia pastoris* has become popular and successful host for expression of recombinant proteins [2,4,6,23]. The advantage is that the target proteins can be expressed as secretory forms [14]. Moreover, the production of the target proteins can be increased by high-cell-density fermentation. As a yeast cell, *P. pastoris*, can express proteins for clinic application without contamination by endotoxins [8]. Furthermore, they have the ability to perform many of the post-translational modifications usually performed in higher eukaryotes e.g. correct folding, disulfide bond formation, *O*- and *N*-linked glycosylation and processing of signal sequences [2,8,9,24,32].

The Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University, Thailand, has successfully produced MAb against norfloxacin [28]. However, the production of norfloxacin-specific recombinant antibody and the expression of scFv fragments using yeast expression system

have not yet been reported. In this study, gene encoding for V_H and V_L of MAb against norfloxacin (Nor155) was identified for future use in the study of antibody-producing methylotrophic yeast.

2. Materials and methods

2.1. Strains, plasmids, culture medium and reagents

Escherichia coli strain TOP10F⁺, pPICZ α A expression vector and ZeocinTM were purchased from Invitrogen (USA). *E. coli* TOP10F⁺ was used for all plasmid constructions. The growth medium, Luria-Bertani (LB) medium, used in shake flask experiments consisted of 5 g/L yeast extract, 10 g/L tryptone peptone and 10 g/L NaCl and 10 mg/L ampicillin. All medium components except ampicillin were sterilized by autoclaving together at 121 °C for 15 min. Ampicillin was sterilized by 0.22 μ m filtration and added to the medium immediately prior to inoculation. Low salt LB medium with ZeocinTM (25 μ g/mL) was used for screening of transformants.

2.2. Preparation of first-strand cDNA

Total RNA extraction from 5×10^6 hybridoma cells against norfloxacin (Nor155) using a NucleoSpin[®] RNA II (Macherey-Nagel) was carried out according to the manufacturer's instruction. First strand cDNA coding for the variable heavy and light chains was synthesized from the total RNA extract (approximately 1 μ g RNA) by using a first-strand cDNA synthesis kit (Fermentas).

2.3. Construction of scFv antibody gene

Here, the scFv antibody gene was mainly constructed by PCR amplification and ligation-reaction (Fig. 1A) using the following:

2.3.1. Amplification of V_H and V_L

The 1st cDNA fragments encoding the variable heavy chain (V_H) were amplified by using VHFwMH1 as forward primers and VHRwIgG1 as a reverse primer while those encoding the variable light chain (V_L) were amplified by using VLFwMk as a forward primer and VLRwKc as a reverse primer (Table 1). Each PCR reaction contains 2 μ L of 1st cDNA, 20 pmol of 5' and 3' primers, 5 μ L of 2.5 mM dNTPs, 5 μ L of Taq polymerase buffer, and 0.5 μ L of *i*-Taq DNA polymerase (Intron Biotechnology). The final volume was brought to 50 μ L with nuclease-free water. Cycling conditions were initial melt at 94 °C for 3 min followed by 30 cycles of three-step program (94 °C, 1 min; 45 °C, 1 min; and 72 °C, 2 min). The reactions were then held at 72 °C for 10 min and cooled to 4 °C [31]. Then, the amplicons were electrophoresed through 1% low-melting point agarose gel and visualized by staining with ethidium bromide. Each of the amplified DNA fragments corresponding to the predicted size was excised from the gel and was purified by using QIAquick PCR Purification Kit (QIAGEN). The purified fragments then were ligated individually into the pGEM[®] T-easy vector (Promega) and subsequently introduced into competent *E. coli* TOP10F⁺. The blue/white screening was used to select the positive clones on LB agar

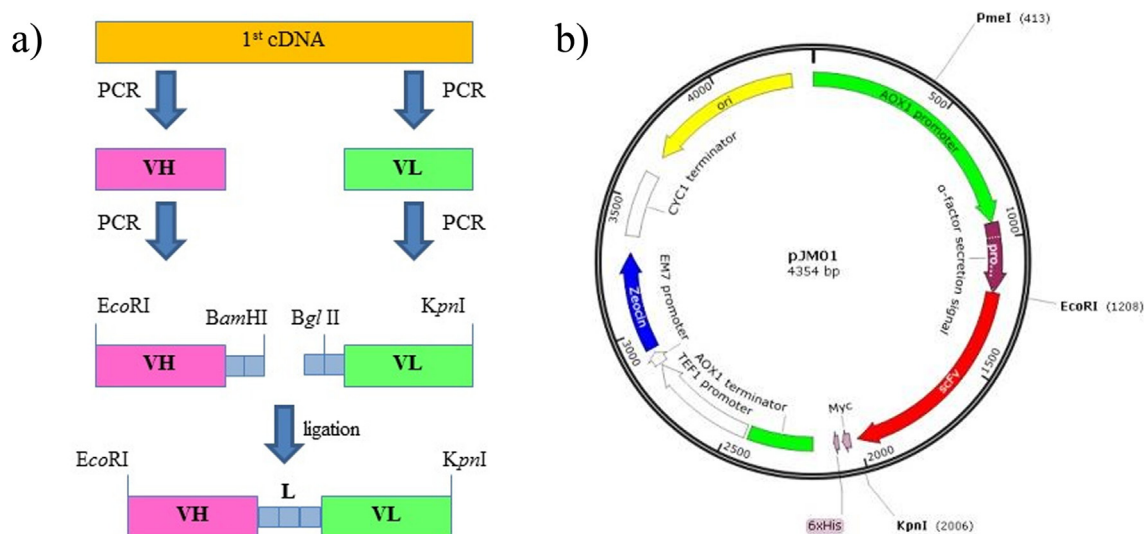


Figure 1 (a) The schematic strategies of scFv antibody construction by PCR amplification and enzymatic reaction, L; linker (Gly₄S)₃. (b) Map of recombinant vector pPICZα-scFv (pJM01). The plasmid map of the scFv containing plasmid pPICZαA, 5'AOX1: alcohol oxidase 1 promoter region induced by methanol. scFv gene encoding anti-norfloxacin gene. Zeocin: zeocin resistance gene. AOX1TT: native transcription terminator. The scFv antibody gene was inserted into the *EcoRI* and *KpnI* sites of the pPICZαA vector.

Table 1 Primers for V_H and V_L amplification, scFv construction, PCR and sequencing analysis. The underline letters represent cloning sites, *EcoRI* (GAATTC), *SacI* (GAGCTC), *BamHI* (GGATCC), *BglII* (AGATCT) and *KpnI* (GGTACC).

Primer ID	Sequence (5'-3')	Purposes	References
VHFwMH1	<u>GAATTC</u> SARGTNMAGCTGSAGSAGTC	VH amplification	[31]
VHRwIgG1	<u>GAATTC</u> AATGACAGATGGGGGTGTCGTTTTGGC	VH amplification	
VLfWmK	GGGG <u>GAGCTC</u> GAYATTGTGMTSACMCARWCTMCA	VL amplification	
VLrWkC	GGGG <u>GAGCTC</u> GGATACAGTTGGTGACAGCATC	VL amplification	
N1	<u>GAATTC</u> GAAGTAGAGCTGGAGGAGTCTGGG	VH-linker amplification	This study
N2	<u>GGATCC</u> ACCTCCGCTGAACCGCCTCCACCATAGACAGATGGGGGTGTCGTTTTGG	Linker-VL amplification	
N3	GGCGGAGGT <u>AGATCT</u> GGCGGTGGCGGATCGGACATTGTGATGACACAGTCTACA	Linker-VL amplification	
N4	CGAGGT <u>ACCGGG</u> GAGCTCGGATACAGTTGGT	Confirmation	
5'AOX1	GACTGGTTCCAATTGACAAGC		
3'AOX1	GCAAATGGCATTCTGACATCC		
TJ01F	AAGGAAGCTGCCCTGTCTTAAACCTTT		
TJ01R	TAGGTTACAAATAAAAAAGTATCAAAA		
T3F	ATGAGATTTCTCAATTTTTACTGCT	Sequencing	
T4F	AGAGGCTGGAGTGGGTCGCAA		
T5F	CCGCCACCGCCAGATCCACCT		
T6R	GCCTCCCCCAGACTCTCCAG		

Degenerate bases: R = (A/G), Y = (C/T), M = (A/C), S = (C/G), W = (A/T), N = (A/G/C/T).

plates containing 100 µg/mL ampicillin, 50 µL X-gal (20 mg/ml) and 30 µL IPTG (0.1 M) respectively.

2.3.2. Construction of V_H and V_L gene with flexible polypeptide linkers

A 15 amino acid linker encoding (Gly₄Ser)₃ was introduced behind the V_H domain and in front of the V_L domain to produce a full-length scFv which was oriented as V_H-(Gly₄Ser)₃-V_L (Fig. 1A) by using specific designed primer N1-N4 based on the V_H and V_L gene sequences as shown in Table 1. After PCR, the V_H-linker and linker-V_L amplicons were separated and purified by 1% (w/v) agarose gel electrophoresis. Afterward, the synthesized genes were cloned separately to the

pGEM® T-easy vector and was selected as above. Plasmid of the ampicillin-positive clones was extracted and analyzed by restriction enzyme digestions and electrophoresis. The plasmids containing the fragments of the specified sizes were confirmed by DNA sequencing. Finally, the transformant clones with the accurate sequence were then used to generate the expression plasmid.

2.3.3. Construction of recombinant scFv antibody expression plasmid

The V_H-linker and linker-V_L clones having the *BamHI*-*BglII* restriction enzyme sites and *EcoRI*-*KpnI* linearized pPICZαA vector were assembled by ligation reaction and then intro-

duced into *E. coli* TOP10F' to generate the pPICZ α A-scFv construct named pJM01 (Fig. 1B). The pJM01 was cloned in frame with the α -factor secretion signal sequence open reading frame of the pPICZ α A vector.

2.4. Verification of pJM01 plasmid

To confirm the ligation-reaction of constructed pJM01 plasmid, the following three methods were carried out.

2.4.1. PCR analysis of positive transformants

Five zeocin-resistance transformant clones were analyzed by using PCR with designed primers TJ01F/TJ01R, N1/N4, respectively (Fig. 2A) based on the pJM01 sequence map which could detect the ligation of the scFv antibody gene into the pPICZ α A, while pPICZ α A vector (Fig. 2B) was used as the negative control to compare with the positive clones. To obtain the DNA template, plasmid DNA was extracted. PCR components and conditions were as follows: AmpliTaq Gold® 360 Master Mix (AB Applied Biosystem), 0.5 μ M of each primer, 1 cycle of 95 °C for 5 min, 30 cycles of three-step program (95 °C, 15 s; 55 °C, 30 s and 72 °C, 1.30 min). The reactions were then held at 72 °C for 7 min and cooled until used at 4 °C.

2.4.2. Restriction enzyme digestion

The positive clones with the specified size according to the expected map were tested with *Eco*RI and *Kpn*I, respectively.

2.4.3. Sequencing

To consider the scFv antibody gene was cloned in frame with the α -factor secretion signal sequence. The positive clones, which showed the expected size from 2.4.1 and 2.4.2 methods were sequenced with BigDye® terminal sequencing kit and ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, USA) using four oligonucleotide primers, T03F, T04F, T05F, and T06R which were designed based on pJM01 sequence as shown in Table 1.

2.5. Identification of complementarity determining region (CDRs) and framework sequences (FR)

The obtained sequence was aligned by searching against the Genbank database for sequence homology analysis. The CDR and framework sequences were identified by comparison with IgG1 and light chain sequence database from GenBank (NCBI) or published data.

3. Results and discussion

In this study, we designed, constructed, and sequenced the pJM01 (scFv antibody) plasmid derived from MAb against norfloxacin (Nor155). Monoclonal antibody against norfloxacin clone 155 that showed high sensitivity with the limit of detection value of 1.01 ng/mL and high specificity was used as the source of genetic information. Its isotype was found to be IgG1 [28].

3.1. Construction of scFv antibody

Total RNA was extracted from MAb nor 155, and the first stand cDNA was synthesized by RT-PCR kit using total RNA as template. The V_H and V_L genes were amplified separately by PCR using the 1st cDNA template, and the lengths of V_H and V_L DNA fragments were approximately 402 bp and 363 bp, respectively (GenBank, accession no. KJ623260). These fragments were used to construct the V_H-linker and linker-V_L fragments by adding the flexible linker (Gly₄Ser)₃. Then, these fragments were cloned into pGEM® T-easy vector and identified by restriction enzyme digestion, electrophoretic analysis and DNA sequencing (data not shown). The lengths of V_H-linker and linker-V_L DNA fragments were approximately 426 bp and 380 bp, respectively. Afterward, the recombinant scFv (V_H-(Gly₄Ser)₃-V_L) fragment (800 bp) was assembled with *Eco*RI/*Kpn*I linearized pPICZ α A to generate the recombinant plasmid named pJM01, whose expected size was 4354 bp (Fig. 1B).

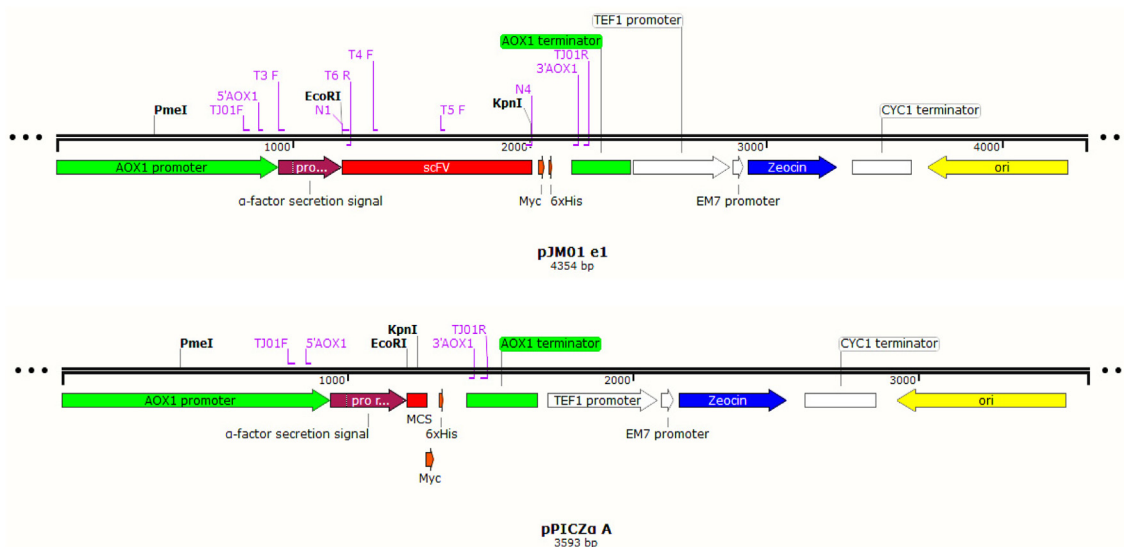


Figure 2 Schematic illustration of the strategy of linear pJM01 plasmid (a) and linear pPICZ α A vector (b).

3.2. Verification of pJM01 plasmid

To improve and facilitate the expression of the antibody fragment, V_H-linker and linker-V_L DNA fragments were inserted into the open reading frame of the *P.pastoris* expression vector pPICZαA under the regulation of *AOX1* promoter using the *EcoRI* (bp 1203–1213) and *KpnI* (1241–1246) restriction sites. At the C-terminal, the constructs were fused to a *c-myc* epitope tag and a hexahistidine-tag. Colonies were screened for inser-

tion using PCR analysis and restriction cleavage reactions. The plasmid of five zeocin-positive transformants was extracted. The presence of scFv antibody in those plasmids was tested by PCR technique with N1 and N4 primers as shown in Table 1. These primers were designed for up-stream amplification of V_H and down-stream of V_L gene. The result reveals the 800 bp (Fig. 3A) of PCR product according to the right size of the predicted restriction map of the linearized pJM01 plasmid (Fig. 2A). This information indicates

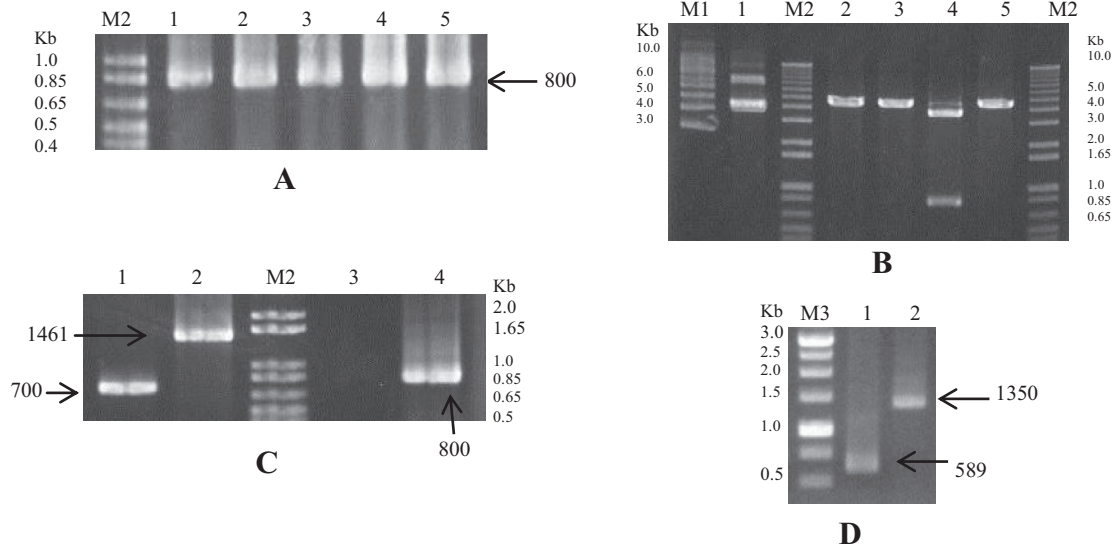


Figure 3 Electrophoretic analysis of (a) PCR product of five zeocin-positive transformants, Lane 1–5: positive transformants No. 1–No. 5, respectively, Lane M2: the 1 Kb plus DNA ladder (Invitrogen™). (b) Restriction enzyme digestion, Lane 1: intact pJM01, lane 2–5: pJM01 plasmid of transformants No. 1 digested with *EcoRI*, *KpnI*, *EcoRI/KpnI*, and *PmeI*, respectively, Lane M1: supercoiled plasmid ONESTEP Ladder (Wako Nippon GENE). (c) PCR product, Lane 1: pPICZαA, lane 2: zeocin-positive transformants No. 1. Lane 3: pPICZαA. Lane 4: zeocin-positive transformants No. 1. (d) PCR product by using 5'AOX1/3'AOX1, Lane 1: pPICZαA vector, lane 2: zeocin-positive transformants No. 1.

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1 GAATTCGAAGTAGAG CTGGAGGAGTCTGGG GGAGGCTTAGTGAAG CCTGGAGGGTCCCTG AAACCTCTCTGTGCA
1 E F E V E L E E S G G G L V K P G G S L K L S C A
76 GCCTCTGGATTCACT TTCAGTGACTATTAC ATGTATTGGTTTCGC CAGACTCCGGAAAAG AGGCTGGAGTGGGTC
26 A S G F S F S D Y Y M Y W V R Q T P E K R L E W V
151 GCAACCATTAGTGAA GGTGCCAGTTATATC TATTATTCGGACAGT GTAAAGGGACGCTTC ACCATCTCCAGAGAC
51 A T I S E G A S Y I Y Y S D S V K G R F T I S R D
226 AATGCCAGGAACACC CTGTACCTGCAAATG AGCAGTCTGAAGTCT GAGGACACAGCCATT TATTACTGTACAAGA
76 N A R N T L Y L Q M S S L K S E D T A I Y Y C T R
301 GCCTATAATAACTAC GTGTCGAAATATTTTC GATGTCTGGGGCGCA GGGACCACGGTCACC GTCTCCTCAGCCAAA
101 A Y N N Y V S K Y F D V W G A G T T V T V S S A K
376 ACGACACCCCAATCT GTCTATGGTGGAGGC GGTTCAGGCGGAGGT GGATCTGGCGGTGGC GGATCGGACATTGTG
126 T T P P S V Y G G G G S G G G S G G G S D I V
451 ATGACACTGTCTACA GCAATCCTGTCTGCA TCTCCAGGGGAGAAG GTCACAATGACTTGC AGGGCCAGTCAAGG
151 M T L S T A I L S A S P G E K V T M T C R A S S R
526 GTAAATTACATACAC TGGTTCCAGCAGAAG GCAGATCCTCCCCC AAACCCTGGATTAT GCCACATCCAACCTG
176 V N Y I H W F Q K A G S S P K P W I Y A T S N L
601 GCTTCTGGAGTCCCT GATCGCTCAGTGGC AGAGGGTCTGGGACC TCTTACTCTCTACA ATCAGCAGAGTGGAG
201 A S G V P D R F S G R G S G T S Y S L T I S R V E
676 GCTGAAGATGCTGCC ACTTATTACTGCCAG CAGTGGAGTAATAAC CCGTGGACGTTCCGGT GGAGGCACCAAGCTG
226 A E D A A T Y Y C Q W S N N P W T F G G G T K L
751 GAAATCAAACGGGCT GATGCTGCACCAACT GTATCCGAGCTCCCG GTACC
251 E I K R A D A A P T V S E L P V

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Figure 4 The nucleotide sequence and deduced amino acid sequence of the scFv (Nor155) antibody fragment. Amino acids are shaded italic in blue. The (G₄S)₃ linker between V_H and V_L is labeled in bold. The complementarity determining regions (CDRs) of V_H and V_L are predicted as CDR I (underline), CDR II (double underline), and CDR III (opened underline). This sequence has been submitted in GenBank No. [AJG06891.1](https://www.ncbi.nlm.nih.gov/nuclot/AJG06891.1).

that all of the five positive transformants have a scFv antibody gene inserted into the pJM01 expression plasmid. In addition, we concerned about the accuracy of the nucleotide sequence of scFv antibody gene when it was cloned in frame with α -factor secretion signal in the pPICZ α A vector and translated to amino acid. For this reason, the plasmid of the zeocin-positive transformant No. 1 was selected to be digested with several restriction enzymes. In accordance with the expected map, *EcoRI*, *KpnI*, and *PmeI* were used. These enzymes have only one position of enzymatic digestion that means, after digestion completely and through electrophoresis, the digested plasmid should show the linearized plasmid. Fig. 3B shows the result bands corresponding to the correct size of 4354 bp plasmid (lanes 2, 3, and 5), while lane 4 represented the use of *EcoRI* and *KpnI* in the plasmid digestion at the same time. It showed the 800 bp of scFv antibody gene when compared with the DNA marker. Moreover, we also confirmed the plasmid of clone No. 1 by PCR with primers as described in Table 1. Fig. 3C exhibits the positive bands approximately 1461 bp of the PCR product (lane 2) by using TJ01F/TJ01R primers, approximately 800 bp of the PCR product (lane 4) by using N1/N4 primers and approximately 1350 bp by using 5'AOX1/3'AOX1 primers (Fig. 3D). As the control, these pri-

mers were used with pPICZ α A vector showing approximately 700 bp (Fig. 3C, lane 1) and 589 bp (Fig. 3D, lane 1) and any band could be detected by using N1/N4 primers (Fig. 3C, lane 3).

3.3. Sequencing analysis

The pJM01 plasmid of zeocin-positive transformant No. 1 was sequenced with specified primers (Table 1). The result showed the nucleotide sequence of scFv antibody gene with 800 nucleotides. The translation of this sequence was predicted to encode 266 amino acids with a molecular weight of 32.67 kDa including a flexible amino acid linker of (Gly₄Ser)₃ as shown in Fig. 4.

3.4. Identification of complementarity determining region (CDRs) and framework sequences (FR)

Complementarity determining regions (CDRs) are part of the variable chains in immunoglobulins (antibodies), where these molecules bind to their specific antigens. As the most variable parts of the molecules, CDRs are crucial to the diversity of

		V_H CDR I	
AAW62444	-----AQPAMAQGGKQQSGGGLVKGPGGSLKLSCAASG	FTFSDYNNMYWIRQTP	47
AAW28931	-----MAQVKLQQSGGGLVKGPGGSLKLSCAASG	FTFSSYGMWVRQTP	43
AAA83268	-----QVKLQESGGGLVKGPGGSLKLSCAASG	FTFSSYAMSWVRQTP	41
CCG26104	MDFGLSVVFLVLVKGVCQEVKLVESGGGLVKGPGGSLKLSCAASG	FTFNSYAMSWVRQTP	60
AAAL11475	-----LQPQVKLLESGGGLVKGPGGSLKLSCAASG	FTFSTYVMSWVRQTP	44
scFv	-----EFEVELEESGGGLVKGPGGSLKLSCAASG	FTFSDYNNMYWVRQTP	43
	: * : * * * * * : * * * * * : * * * * * : * * * * *		
	V_H CDR II	V_H CDR III	
AAW62444	EKRLEWVATVTDGGSHTYYPDSVKGRFTISRDNAKNNLYLQMS	SLKSEDTAMYYCARG--	105
AAW28931	DKRLEWVATISSGGSYTYYPDSVKGRFTISRDNAKNTLYLQMS	SLKSEDTAMYYCAR--	100
AAA83268	EKRLEWVATISSGGSYTYYPDSVKGRFTISRDNAKNTLYLQMS	SLRSEDTAMYYCARQD-	100
CCG26104	EKRLEWVAFISTGGS-YYYPDSVKGRFTISRDNARNILYLQMT	SLRSEDTAMYYCARG--	117
AAAL11475	EKRLEWVATITSGGDHTYYPDSMKGRFTISRDNAKNTLYLQMS	SLRSEDTTMYCVR--	102
scFv	EKRLEWVATISEGASYTYYPDSVKGRFTISRDNARNNTLYLQMS	SLKSEDTAIYYCTR	103
	: * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
	V_H CDR III		
AAW62444	----AWFAIYWGQTTTVTVS-----	SGGGSGGGSGGGSDIELTQSPA	152
AAW28931	----LGRYWGQTTTVTVS-----	SGGGSGGGSGGGSDIELTQSP	146
AAA83268	GYYPGFANWGQTTTVTVS-----	SGGGSGGGSGGGSDIELTQSPA	151
CCG26104	GDYGYFDYWGQTTTTLTVS-----	SGGGSGGGSGGGSDIELTQSPA	168
AAAL11475	GNDYGSFDYWGQTSVTVSQTVTVS----	SGGGSGGGSGGGSDIELTQSPA	158
scFv	NYVSKYFDVWAGTTVTVSSAKTTPPSVYGGGGSGGGSGGGSDI	VMTLSTAILLSASP	163
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
	V_L CDR I	V_L CDR II	
AAW62444	EKVTMTCSASSSISYIHWYQQKPGTSPKRWIYDTSK	LASGVPARFSGSGSGTSYSLTISS	212
AAW28931	EKVTITCRASSSVSYMHWFQKSGTSPKPIYDTSK	LASGVDRFSGSGSGTSYSLTISS	206
AAA83268	EEITLTCASSSVSYMHWFQKSGTSPKLLIYDTSN	LASGVDRFSGSGSGTSYSLTISS	211
CCG26104	EKVTMTCSASSSVSYMYWYQQKPRSSPKPIYLTSL	LASGVPARFSGSGSGTSYSLTISS	228
AAAL11475	EKVTMTCRASSSVFMHWYQQKARSSPKPIYGTSL	LASGVDRFSGSGSGTSYSLTISS	218
scFv	EKVTMTCRASSRVNYIHWYQQKAGSSPKPIYATSL	LASGVDRFSGSGSGTSYSLTISS	223
	* : * * * * * * * * * : : : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * :		
	V_L CDR III		
AAW62444	MEAEADAATYYCHQRSSYPYTFGGGKLEIKRA-----		244
AAW28931	MEAEADAATYYCQRSSYPYTFGGGKLEIKR-----		237
AAA83268	VEAEADAATYYCHQWSSY--TFGGGKLEIKR-----		240
CCG26104	MEAEADAATYYCQWSSNPLTFGAGTKLEIKRADA-----		262
AAAL11475	VEAEADAATYYCQWSTNPPTFGAGTKLEI-----		247
scFv	VEAEADAATYYCQWSSNPPTFGGGKLEIKRADAAPTVELPVP		267
	: * * * * * * * * * * * * * * * : * * * * * : * * * * * :		

Figure 5 Primary sequence alignment of scFv (Nor155) antibody and those of five known scFv sequences with accession number were taken from GenBank. Predicted CDR indicates the complementary-determining regions (CDRs) and framework regions that exist between the CDRs. Asterisk (*) indicates the amino acid that is the same. Hyphen (-) indicates the amino acid that is missed compared with others.

Table 2 Summary of using BLASTp searched through the GenBank database.

Accession	Definition	Amino acid	Max score	Total score	Query cover (%)	E-value	Ident (%)
AAA83268	scFv antibody, partial [<i>Mus musculus</i>]	240	326	326	94	1.00E-108	71
AAL11475	Single chain antibody 12G9, partial	247	325	325	93	3.00E-108	78
CCG26104	Anti-porcine sialoadhesin scFv antibody, partial	262	324	324	96	1.00E-107	75
AAW62444	ScFv B4 anti-pectinase antibody, partial	244	318	318	94	1.00E-105	74
AAW28931	Anti-isoketal-adduct single chain variable fragment, partial	237	315	315	94	3.00E-104	74

antigen specificities. Within the variable domain, CDR I, CDR II, and CDR III are found in the variable region of a polypeptide chain, and CDRIII is the most variable [18–20,33,34].

The complementarity determining regions (CDRs) I, II, and III of the variable region of both heavy chain (V_H) and light chain (V_L) were identified according to the method provided by Kabat [19]. The predicted CDRI, CDR II and CDR III of V_H domain were located at positions 29–37, 52–60, and 99–112 in the primary protein sequences, while the prediction of CDRI, CDR II and CDR III of V_L domain was specified at positions 170–180, 195–202, and 235–243 as depicted in Fig. 4, respectively.

The deduced amino acid sequence of the scFv Nor155 antibody gene (GenBank No. AJG06891.1) was aligned by searching against GenBank database (Fig. 5). Over 50 different scFv antibodies homologous sequence was found. Comparative analysis of the predicted amino acid sequence with the known scFv sequences is shown in Table 2. It was found that the scFv antibody was most closely related to the single chain antibody 12G9 (78% identity) followed by anti-porcine sialoadhesin scFv antibody (75% identity) and both of scFv B4 anti-pectinase antibody, and anti-isoketal-adduct single chain variable fragment (74% identity), and scFv antibody from *Mus musculus* (71% identity), respectively.

4. Conclusion

We have successfully designed, constructed, and sequenced a single-chain variable fragment (scFv Nor155) antibody derived from the monoclonal antibody against norfloxacin. The CDRs of scFv Nor155 that contribute the binding activity of antibody were predicted. Various results have been supported that the pJM01 expression plasmid contains scFv Nor155 antibody gene which is possible to be used for antibody production in methylotrophic yeast, *P. pastoris*.

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