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ORIGINAL ARTICLE

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

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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 $(Gly_4Ser)_3$ 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

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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', pPICZαA expression vector and Zeocin™ 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 μm filtration and added to the medium immediately prior to inoculation. Low salt LB medium with Zeocin™ (25 μg/mL) was used for screening of transformants.

2.2. Preparation of first-stand 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 μg 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 μL of 1st cDNA, 20 pmol of 5′ and 3′ primers, 5 μL of 2.5 mM dNTPs, 5 μL of Taq polymerase buffer, and 0.5 μL of *i-Taq* DNA polymerase (Intron Biotechnology). The final volume was brought to 50 μL 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

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