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ScienceDirect
Enzyme and Microbial Technology 41 (2007) 383–389
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Selection of single chain fragments against the phytopathogen *Xanthomonas axonopodis* pv. *citri* by ribosome display

Qing Yuan, Yuxian Xia, Siji Nian, Youping Yin, Yueqing Cao, Zhongkang Wang *

Genetic Engineering Research Centre, College of Bioengineering, Chongqing University, Chongqing 400030, PR China

Received 8 January 2007; received in revised form 8 March 2007; accepted 15 March 2007

Abstract

A ribosome display technique was applied to a mouse single chain variable fragment (ScFv) library to select ScFvs specific for *Xanthomonas axonopodis* pv. *citri* (Xac). The ScFv DNA library was transcribed in vitro to mRNA for ribosome display and antibody–ribosome–mRNA (ARM) complexes were produced by a rabbit reticulocyte lysate system. The O-specific lippolysaccharide (LPS) of Xac was used as an antigen to pan ARM complexes and putative ScFv-encoding genes were recovered by RT-PCR following each panning. After three rounds of ribosome display, the ScFv DNA was cloned into *Escherichia coli* TG1 for expression and the expressed products of 180 clones were analyzed by indirect ELISA. Sixty clones of those showed an antibody activity to the O-specific LPS of Xac, and three (GX13, GX44 and GX95) exhibited higher activity and specificity to Xac and no cross-reactions were observed with 10 saprophytic xanthomonads from citrus or other bacteria. The equilibrium constant (K_A) determined by BIAcore analysis for ScFvs GX13, GX44 and GX95 were $1.98 \times 10^{10}\,M^{-1}$, $1.89 \times 10^{10}\,M^{-1}$ and $3.43 \times 10^{10}\,M^{-1}$, respectively.

Keywords: Xac; O-specific LPS; Ribosome display; ScFv

1. Introduction

Recombinant antibodies are artificial construction produced by genetic engineering and one of the most remarkable molecules of these kinds is single chain variable fragment (ScFv). ScFvs, made by the association of the variable heavy and light chain region, keep the binding properties of classical antibody. The use of ScFvs is a new strategy for developing improved immunodetection tests for plant pathogens [1,2]. This technology presents several advantages compared with the conventional antibody strategy, such as facility and rapidity of use, sensibility and low cost production. Furthermore, expression of ScFvs in plants is an attractive way to control some severe plant diseases [3–5].

ScFvs can be produced by conventional hybridoma or phage display technology. Although phage display evidently represents a considerable progress over hybridoma technology, deficiencies still do exist. First, the necessary transformation step limits the library size. Secondly, selection in the context of the host environment cannot be avoided, possibly causing loss of potential candidates due to their growth disadvantage or even toxicity for *Escherichia coli*. Furthermore, difficulties in eluting phages carrying antibodies with very high affinity may be encountered [6]. Ribosome display is a novel technology for molecule evolution and antibody selection. It bases on the formation of a complex among an mRNA, a ribosome and the newly translated protein during in vitro translation [7–9]. In the ribosome display, those of the limitations of phage display are circumvented by utilizing a cell-free transcription, translation and selection system. Larger capacity and further diversity of libraries will be built and random mutations can be introduced by PCR. Using the new technology, it has been possible to select and evolve high-affinity antibodies [8].

Citrus canker caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* (Xac), a gram-negative bacterium, is a severe bacterial disease of most commercial citrus species and cultivars around the world, as well as some citrus relatives. The pathogen is the target of quarantine efforts abroad and domestics, then the development of rapid and reliable procedures for diagnosis and control of this pathogen has been an important priority [10,11]. Lippolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, and is

* Corresponding author. Tel.: +86 23 65120489; fax: +86 23 65120489.
E-mail address: zkwang5699@yahoo.com (Z. Wang).

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.03.006

suspected to be an important molecule for adhesion to and infection of plants [12]. The LPS consists of O-specific side chains, core polysaccharide and lipid A, in which the O-specific side chain determines species specificity [13,14]. For the study presented here, the O-specific LPS of Xac was used as an antigen to pan specific ScFvs against Xac by ribosome display. The selected ScFvs could be further applied to development of diagnostic reagent of Xac and research on the interaction between Xac pathogen and citrus during the phase of initial attachment-infection process.

2. Materials and methods

2.1. LPS extraction

Xac strain Gxo4, isolated from infectious leaves of sweet orange, was cultured at 28 °C for 72 h using potato dextrose agar (PDA) medium and stored in the Genetic Engineering Center of Chongqing University. The O-specific LPS of Xac was isolated and purified according to Molinaro et al. [15].

2.2. Immunization of mice

Four 6-week-old female BALB/c mice from the Animal Center of Third Military Medical University were injected intra-peritoneum with 0.5 ml suspension of Xac strain Gxo4 in 0.8% saline solution (approximately 10^8 CFU/ml) for three times at 2-week intervals. The immunized procedure lasted a period of 6 weeks. Before the mice were killed, their sera were collected from their tails for an enzyme-linked immunosorbent assay (ELISA). The spleen was removed from the immunized mouse with the highest titer, and cells were isolated for mRNA extraction.

2.3. Construction of a VH/k chain library

The mRNA was isolated from spleen cells with mRNA purification kit (Amersham, UK) according to the manufacturer's instruction. cDNA encoding the mouse VH and kappa chains were amplified by RT-PCR, respectively. Primer pairs T7/back (5′GCAGCTAATACGACTCACTATAGGAACAGACCACCATGAGGTSMARCTGCAGSAGTCWGG3′) and VH/for (5′TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC3′) were used for amplification of cDNA encoding VH chain. Primer pairs Vk/back (5′GACATTGAGCTCACCCAGTCTCCA3′) [16] and Ck/for (5′GCTCTAGAACACTCATTCC TGTTGGAGCT3′) [17] were used for amplification of cDNA encoding k-chain. A 93 bp DNA linker containing a sequence encoding $(Gly_4Ser)_3$ was amplified with primer pairs linker/back (5′GGGACCACGGTCACCGTCTCCTCA3′) and linker/for (5′TG GAGACTGGGTGAGCTCAATGTC3′) using *Taq* polymerase with 25 cycles of PCR (1 cycle is 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C). After agarose gel purification, 15 ng of VH DNA, 20 ng of linker DNA, and 50 ng of k-chain DNA were mixed with 25 μl of PCR mixture without primers and amplified for 25 cycles (1 cycle is 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C) in order to join the linker DNA with the VH and k-chain DNA. The assembled DNA was amplified in a 25 μl PCR mixture for 30 cycles (1 cycle is 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C) with primers T7/back and Ck/for.

2.4. In vitro transcription and translation

The mRNA transcripts were obtained by in vitro transcription using PCR products with T7 RNA polymerase (Promega, USA). Briefly, about 1 μg of library DNA was added to the 50 μl transcription mixture containing 40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 50 U RNase inhibitor, 0.5 mM rNTP, 20 U T7 RNA polymerase. The mixture was incubated at 37 °C for 2 h and the reaction was stopped by phenol/chloroform extraction. The transcripts were purified by 3 M LiCl, and then dissolved in diethyl pyrocarbonate (DEPC) treated water.

In vitro translation was performed in 70% nuclease-treated reticulocyte lysate (Promega, UAS). The reaction volume was 50 μl and 2 μg of purified mRNA transcripts were added to the reaction mixture containing 35 μl of rabbit reticulocyte lysate (nuclease treated), 1 μl of amino acid mixture (complete, 1 mM), 20 U RNase inhibitor. The translation was carried out at 30 °C for 30 min, then stopped by cooling on ice and adding 150 μl ice-cold PBSMB buffer (PBS with 5 mM $MgCl_2$ and 5% (w/v) BSA) immediately.

2.5. Affinity selection and RT-PCR

Microtiter plate was coated at 4 °C overnight with 100 μl of O-specific LPS solution (50 μg/ml in PBS). The coated plate was washed with PBSMT (PBS with 5 mM $MgCl_2$ and 0.05% (v/v) Tween 20) and blocked with sterilized blocking buffer PBSMB for 1 h at room temperature (RT). After being washed with PBSMT for three times, the plate was incubated on ice for at least 10 min. Then, the prepared translation products in PBSMB were added to the O-specific LPS coated wells and incubated on ice for 1 h. After three washes with ice-cold PBSTM and two washes with ice-cold PBSM, the retained ribosomal complexes were dissociated with 200 μl of EB20 buffer (PBS with 20 mM EDTA) for 10 min on ice. The mRNA was isolated from the eluted solution with RNA isolation kit (Roche, Germany), as described by the manufacturer.

The purified mRNA was recovered by RT-PCR with primers T7/back and Ck/for. The obtained DNA was used for the next round of ribosome display or cloned into *E. coli* TG1 for expression.

2.6. Cloning and expression

After selection, ScFv DNA was amplified with forward primer VHbs (5′GTCCTCG CAACTGCGGCCCAGCCGGCCATGGCCCAGGTSMARCTGCAGSAGTCWGG3′) with the *Sfi*I restriction site (underlined) and reverse primer Vkfn (5′GAGTCAT TCTGCGGCCGCTGCAGCATCAGCCCGTTT3′) with the *Not*I restriction site (underlined). The amplified DNA was digested with *Sfi*I and *Not*I, and then ligated with vector pCANTAB5E using T4 DNA ligase (Promega, USA). The ligated products were transformed into *E. coli* TG1 and soluble protein was expressed from each clone [18]. Briefly, each single clone was cultured in 5 ml of 2 × YT medium with 100 μg/ml ampicillin and 0.1% (w/v) glucose at 30 °C until they reached an absorbance of 0.6 at 600 nm. Isopropyl b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cells were incubated at 30 °C for 5–7 h with shaking at 150 rpm. The cells were pelleted and resuspended in 0.5 ml ice-cold 1× TES buffer (0.2 M Tris–HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose) and 0.75 ml ice-cold 1/4× TES buffer. After incubation on ice for 30 min, the cells were pelleted and the supernatant was retained as periplasmic extracts with the soluble ScFvs. The pCANTAB5E vector contained an additional sequence encoding the E-tag, facilitating the detection of expressed soluble ScFvs using anti-E tag monoclonal antibody.

2.7. ELISA

Indirect ELISA was performed routinely to identify the binding activity of the isolated clones. The plates were coated with 100 μl of O-specific LPS solution (50 μg/ml in PBS) overnight at 4 °C. After blocking with PBSB (PBS containing 5% (w/v) BSA), 100 μl of periplasmic extracts diluted 1:1 with PBSB were added to the antigen-coated well and then incubated for 1 h at RT. Anti-E tag antibody (1:1000, Amersham) and horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG (1:5000, Jackson) were used as primary and secondary antibody in tandem. The results of absorbance were detected using spectrophotometer at wavelength of 405 nm.

2.8. Immunoblot analysis of ScFvs expression

Periplasmic extracts concentrated by trichloroacetic acid (TCA) were subjected to SDS-PAGE on a 12% polyacrylamide gel. Pre-stained SDS-PAGE standards (Takara, Japan) were used to calibrate protein molecular size. After SDS-PAGE, the gel was transferred onto a nitrocellulose membrane using a semi-dry electroblotter (Bio-Rad, USA). The transblotted membrane was blocked for 1 h with PBSB and then incubated for 1 h at RT with anti-E tag antibody