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Microcystin-LR nanobody screening from an alpaca phage display nanobody library and its expression and application

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ABSTRACT

Microcystin-LR (MC-LR) is a type of biotoxin that pollutes the ecological environment and food. The study aimed to obtain new nanobodies from phage nanobody library for determination of MC-LR. The toxin was conjugated to keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA), respectively, then the conjugates were used as coated antigens for enrichment (coated MC-LR-KLH) and screening (coated MC-LR-BSA) of MC-LR phage nanobodies from an alpaca phage display nanobody library. The antigen-specific phage particles were enriched effectively with four rounds of biopanning. At the last round of enrichment, total 20 positive monoclonal phage nanobodies were obtained from the library, which were analyzed after monoclonal phage enzyme linked immunosorbent assay (ELISA), colony PCR and DNA sequencing. The most three positive nanobody genes, ANAb12, ANAb9 and ANAb7 were cloned into pET26b vector, then the nanobodies were expressed in *Escherichia coli* BL21 respectively. After being purified, the molecular weight (M.W.) of all nanobodies were approximate 15 kDa with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified nanobodies, ANAb12, ANAb9 and ANAb7 were used to establish the indirect competitive ELISA (IC-ELISA) for MC-LR, and their half-maximum inhibition concentrations (IC_{50}) were 0.87, 1.17 and 1.47 μg/L, their detection limits (IC_{10}) were 0.06, 0.08 and 0.12 μg/L, respectively. All of them showed strong cross-reactivity (CRs) of 82.7–116.9% for MC-RR, MC-YR and MC-WR, and weak CRs of less than 4.56% for MC-LW, less than 0.1% for MC-LY and MC-LF. It was found that all the IC-ELISAs for MC-LR spiked in tap water samples detection were with good accuracy, stability and repeatability, their recoveries were 84.0–106.5%, coefficient of variations (CVs) were 3.4–10.6%. These results showed that IC-ELISA based on the nanobodies from the alpaca phage display antibody library were promising for high sensitive determination of multiple MCs.

1. Introduction

Microcystins (MCs) belong to a type of short cyclic peptides of biotoxins which produced by multiple genera of cyanobacteria, such as *Microcystis aeruginosa*, *Anabaena flosaquae*, *Oscillatoria agardhii* and *Aphanizomenon flosaquae* etc. (Rastogi et al., 2014). The hazards of MCs are injury to liver, kidney, adrenal gland and stomach, disturbing nervous systems and inducing cancer (Chen et al., 2016; Svirčev et al., 2017). Algal blooms erupt more and more frequently, they cause MCs accumulation, and threaten water resources, soil environment, agricultural products and food safety in world-wide (Brooks et al., 2016; Chen et al., 2013; Hou et al., 2017; Wells et al., 2015). So far, more than 90 MC isoforms have been isolated and identified, and the most common isoforms were MC-LR, MC-RR, MC-YR, MC-WR, MC-LW, MC-LY, MC-LF, MC-WF, MC-LA and MC-YF (Pearson et al., 2010). Among them, MC-LR was the most common isoform in waterbodies with algal blooms, also it had the strongest toxicity to human and animals, and its minimum residue limit (MRL) was 1.0 μg/L in water destined for human drinking water by World Health Organization (WHO) (Esterhuizen-Londt et al., 2017; McElhiney and Lawton, 2005).

Until now, the reported methods of MC-LR determination include liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), enzyme-linked immunosorbent assay (ELISA), phosphatase inhibition and animal experiment (Moreira

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et al., 2014). The ELISA based on antibody for MC-LR was the most popular detecting method, because of its high sensitivity, simple operation, quick reaction and low cost (Heussner et al., 2014; Liu et al., 2014). In this detecting method, how to rapidly obtain the high activity of anti-MC-LR antibody was a critical step. Although the technologies were very mature to obtain the traditional polyclonal antibody and monoclonal antibody, they could not avoid the defects of laborious antigen and adjuvant emulsifying, time-consuming immune cycles and limited antibody products from immune animals (Wang et al., 2012). Therefore, it is necessary to explore a new technology for obtaining the antigen specific antibody more simply and quickly.

In recent years, phage display antibody library has become a popular technology to rapidly obtain antigen specific antibody with high activity by high throughput biopanning (Zhao et al., 2016a). This technology randomly clones a lot of artificial antibody genes into a phagemid vector, then infects it to *Escherichia coli* (*E.coli*), and the antibodies are expressed on the surface of phage capsid proteins by co-expression (Qi et al., 2012; Smith, 1985). The antigen specific antibodies and their genes could be obtained by multiple rounds of library enrichment and screening, thus the antibody genes could be cloned into different expression vectors conveniently, even further for affinity maturation (Jiao et al., 2017; Pande et al., 2010; Vodnik et al., 2011; Xu et al., 2016). The reported types of antibody from the phage display antibody library were single chain variable fragment (scFv), domain antibody (DAb) and short chain peptides antibody (such as dodecapeptide and heptapeptide) (Pande et al., 2010; Vodnik et al., 2011). Zhang et al. (2012) obtained an anti-Cry1B toxin scFv from a human synthetic phage display library (Tomlinson J), and its IC_{50} was 0.84 μg/mL and the linear range of detection (IC_{20}-IC_{80}) were 0.19–1.1 μg/mL. Zhao et al. (2016b) obtained five broad-specificity phage domain antibodies for pyrethroid, and they were all capable of detecting cypermethrin, β-cypermethrin, fenvalerate and phenoxybenzoic acid simultaneously.

In natural world, some antibodies from camel, alpacas, llamas and shark are lack light chain naturally and only contain one heavy chain variable region (VHH) called nanobody (Muyldermans, 2013). Nanobody has a characteristic of low immunogenicity, strong stability, good solubility, strong penetrability and easy expression, it has been widely used in biomedicine and immunoassay field (Chakravarty et al., 2014; Liu et al., 2017; Muyldermans, 2013; Qiu et al., 2015). In this study, based on the superiorities of nanobody, we employed a large diversity and capacity of naive alpaca phage display nanobody library for screening high activity MC-LR nanobodies by biopanning, then the positive MC-LR nanobody proteins were expressed in *E.coli* BL21 by gene clone, later they were used to establish the IC-ELISA for MC-LR. Finally, we have made an assessment for the IC-ELISAs with accuracy, stability and repeatability based on nanobodies for detecting MC-LR by spiked tap water samples.

2. Materials and methods

2.1. Phage antibody library, bacterial strains, reagents and materials

Alpaca phage display nanobody library [constructed in pComb3XSS phagemid vector (ampicillin resistance, Amp^r), the capacity of the library is 2×10^9], *E.coli* TG1, KM13K07 helper phage (kanamycin resistance, $Kana^r$) were obtained from Uppark Biotechnology Co. Ltd (Chengdu, China). pET26b vector ($Kana^r$) and *E.coli* BL21 were purchased from Novagen (Germany). Microcystins (MC-LR, MC-RR, MC-YR, MC-WR, MC-LW, MC-LY and MC-LF) were purchased from ApexBio Company (USA). Anti-M13 monoclonal antibody [HRP], goat anti-mouse IgG monoclonal antibody [HRP] and anti-HIS tag monoclonal antibody [HRP] were purchased from GenScript Bio. Co.Ltd (Nanjing, China). MC-LR monoclonal antibody [immunogen was MC-LR-ovalbumin (MC-LR-OVA)] was obtained from Enzo Biochem, Inc. (USA). 2×Tap PCR Master Mix, DNA maker and protein maker were purchased from ComWin Biotech Co.,Ltd (Beijing, China). *Nco*I and *Not*I restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Inc. (Beijing, China). *N,N*-dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]-carbodimide (EDC), keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), Difco™ skim milk, Tween 20, trypsin and 3,3,5,5-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Beijing, China). Cell culture flask and 96-well plates were purchased from Corning (Beijing, China). Other reagents and materials were all purchased from GE Healthcare (Beijing, China).

2.2. Preparation and analyzation of MC-LR-KLH and MC-LR-BSA

Conjugating of MC-LR to KLH and BSA were performed as described by Yu et al. (2002) with some optimization. Briefly, 1 mg MC-LR toxin standards dissolved in 0.2 mL DMF, drop by drop added to 0.6 mL DMF (containing 1.5 mg EDC and 1.5 mg NHS) with gently shaking (25 rpm) for 40 min at room temperature (RT), then for overnight at 4 °C. The next day, added the mixture into the KLH or BSA solution [6 mg KLH or BSA dissolved in 6 mL 0.1 M sodium carbonate buffer (CBS, pH = 9.6)] with gently shaking (25 rpm) at RT for 4 h in dark. After that, centrifuged for 25 min at 8000 rpm and 4 °C by Amicon Ultra-4-Ultracel-3K (Millipore, USA), the supernatant pellet were resuspended by 6 mL 0.1 M sodium phosphate buffer (PBS, pH = 7.4) and centrifuged for 30 min at 8000 rpm and 4 °C, the ultrafiltration steps were repeated three times. Finally, the ultrafiltrates quantified to 6 mL by 0.1 M PBS buffer and the concentration of conjugates MC-LR-KLH or MC-LR-BSA will be determined for 1 mg/mL.

The effect of conjugates MC-LR-KLH and MC-LR-BSA were analyzed by ultraviolet full wavelength scanner (Agilent, USA) and ELISA based on MC-LR monoclonal antibody (Sheng et al., 2007). For ELISA test, MC-LR-KLH and MC-LR-BSA were diluted with PBS buffer for 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 μg/mL respectively, and coated with them 100 μL/well in 96-well plate to stand overnight at 4 °C. The negative controls were KLH and BSA, respectively. The next day, after being washed for three times with 300 μL/well of PBST solution (PBS with 0.1% Tween 20) and blocked by 300 μL/well of MPBS solution (PBS with 5% Difco™ skim milk) at 37 °C for 2 h. After being washed with PBST solution, and then MC-LR monoclonal antibody (1: 2000 diluted with CBS buffer) were added into the wells at 100 μL/well of for incubating 2 h at 37 °C. After being washed with PBST solution, 100 μL/well of goat anti-mouse IgG monoclonal antibody [HRP] (1:3000 dilution) were added for incubating 2 h at 37 °C. After being washed with PBST solution, added 100 μL/well of TMB solution, the color development were performed at RT for 20 min and stopped by 50 μL/well of 2 M sulfuric acid, the OD_{450} values were measured by an automatic microplate reader (Berthold, Germany). All tests were repeated three times and the given data were the mean value.

2.3. Amplification of alpaca phage display antibody library

Amplification of alpaca phage display antibody library were performed as described by Pírez-Schirmer et al. (2017). Total 100 μL library phage particles (10^{12} CFU/mL) were added into 10 mL *E.coli* TG1 (logarithmic phase) in 2×TY medium [16 g tryptone, 10 g yeast extract and 5 g NaCl in 1 L double distilled water (ddH_2O)], culturing for 1 h at 37 °C and 250 rpm, then 3300*g* centrifuging for 15 min at 37 °C. The cell pellets were resuspended by 10 mL 2×TY-AG liquid medium (with 100 μg/mL Amp and 1% glucose) and cultured with shaking 250 rpm at 37 °C for 2 h until cells density reached 0.6 at OD_{600}, then KM13K07 helper phages (10^{12} CFU) were added and rescued for 1 h at 30 °C and 250 rpm. The cultures were centrifuged for 30 min at 1800*g* and 30 °C, and the cell pellets were resuspended by 500 mL 2×TY-AK medium (with 100 μg/mL Amp and 50 μg/mL Kana) for culturing overnight at 30 °C and 250 rpm. The next day, after the culture centrifuged for 15 min at 10,800*g* and 4 °C, a 500 mL supernatant was obtained, and