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Technical note

Colorimetric engineered immunoprobe for the detection and quantification of microcystins

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ABSTRACT

Microcystins (MCs) are heptapeptide toxins produced by cyanobacteria. Their global occurrence in aquatic ecosystems has prompted the development of several detection methods, including antibody-based methods. Here, we propose to apply recombinant antibody technologies to the production of a bivalent colorimetric immunoprobe (scFv-AP) made of the so-called scFv fused to the alkaline phosphatase (AP) of *Escherichia coli*. Recombinant antibody technologies allow the development of specific probes with improved properties and suitable for the detection of MCs. The fusion protein was produced in the periplasm of recombinant bacteria and was used to develop a direct competitive enzyme immunoassay for specific detection of MCs without requiring further purification. The epitope recognized by the recombinant molecule was circumscribed to a motif common to all MCs. Such a genetic approach offers many advantages over chemical cross-linking of antibodies to colorimetric enzymes and may be adaptable to the analysis of water samples and in situ detection.

1. Introduction

Cyanobacteria are ubiquitous photosynthetic microorganisms that can form blooms in surface waters. Some of them have the ability to produce in substantial amount low molecular weight bioactive compounds, such as the microcystins (MCs) which are released after cyanobacterial cell lysis or death. MCs are cyclic heptapeptides with the particular framework cyclo-(D-Ala-L-X-D-erythro-β-methylAsp-L-Y-Adda-D-Glu-*N*-methyldehydro-Ala), Adda being common to all MC congeners, and X and Y being two variable L-amino-acids. More than 90 variants have been reported among which MC-LR (whose variable positions are occupied by Leu and Arg respectively) is the most frequently detected and one of the most toxic (Pearson et al., 2010).

MCs are of great concern because of their increasing occurrence in lakes and reservoirs. They exert their toxicity by strongly inhibiting serine/threonine protein phosphatases – type 2A (PP2A) and type 1 (PP1) – that play crucial roles in dephosphorylation process of proteins. They are responsible for acute (gastrointestinal disturbances and death in humans) and chronic toxic effects which include the potential development of cancers in exposed populations (Paerl and Otten, 2013). The main routes of contamination for humans are consumption of contaminated drinking water and skin contact with surface water at recreational sites. These observations prompted the World Health Organization (WHO) to propose an upper limit for MC-LR of 1 $\mu g \cdot L^{-1}$ in drinking water. Over

Abbreviations: AP, Alkaline phosphatase; BCIP, 5-Bromo-4-chloro-1H-indol-3-yl dihydrogen phosphate; IPTG, Isopropyl β-D-1-thiogalactopyranoside; MC, Microcystin; NBT, Nitro-blue tetrazolium chloride; OVA, Ovalbumin; PBS, Phosphate buffer saline; PNPP, 4-Nitophenylphosphate.

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the past years, the increasing risks to human or animal health and the potential disruptive effects of MCs on ecosystems have led to the development of new tools for a rapid, sensitive and specific detection of MCs. Current detection methods are based on analytical and biological/biochemical methods (McElhiney and Lawton, 2005). Analytical analysis usually consists in very sensitive and specific HPLC associated with mass spectrometry, which requires highly skilled personal, expensive material and is not suitable for field applications. Bioassays with live animals are not sensitive and raise ethical concerns. PP2A or PP1 inhibition assays lack of operational stability, specificity and are not reliable. Antibodies have been considered as a relevant tool to create a wide range of immunoassays to help in the monitoring of MCs (Humpage et al., 2012). Such assays are usually developed using conventional antibodies (polyclonal or monoclonal) with several drawbacks: (i) maintaining a reproducible source of polyclonal antibodies is difficult and (ii) monoclonal antibody production is labor-intensive and these antibodies often show poor cross-reactivity against MC variants. More recently, panning of antibody phage libraries and recombinant antibody technologies have allowed the identification of anti-MC antibody fragments and their reformatting for specific applications (McElhiney et al., 2002).

Here we designed for the first time a colorimetric immunotracer made of a single chain antibody fragment (scFv) fused to an alkaline phosphatase that allows the detection of MCs in a rapid one-step competitive immunoassay. The targeted epitope was circumscribed to the Adda motif common to all MCs.

2. Materials and methods

2.1. Gene synthesis and construction of expression vector

3A8 is an scFv which was selected from a human scFv phagemid library (Griffin.1 library, MRC, Cambridge, UK) after five successive rounds of pannings against MC-LR/bovine serum albumin or MC-LR/keyhole limpet hemocyanin conjugates in order to select clones capable to bind free MC-LR (McElhiney et al., 2002). A gene encoding scFv 3A8 with codons optimized for expression in *Escherichia coli* was synthesized by GeneArt (Regensburg, Germany). cDNA encoding scFv3A8 was inserted in vector pLip6/GN which is an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible vector that allows periplasmic expression of recombinant proteins in fusion with a variant of *E. coli* alkaline phosphatase (AP). The AP variant contains two mutations, D153G and D330N, and thus exhibits improved catalytic activity (Dong et al., 2012). Recombinant plasmid was cloned in XLI-blue *E. coli* strain. All molecular biology methods were carried out as in Fields et al. (2013).

2.2. Expression and identification of the scFv 3A8-AP fusion protein

Transformed bacteria were seeded in LB-Agar plates, containing 100 $\mu g \cdot mL^{-1}$ ampicillin, 50 μM IPTG, 0.3 $mg \cdot mL^{-1}$ nitro-blue tetrazolium chloride (NBT) and 0.16 $mg \cdot mL^{-1}$ 5-bromo-4-chloro-1H-indol-3-yl dihydrogen phosphate (BCIP). This allows the selection of clones that express AP fusion proteins capable of hydrolyzing the NBT–BCIP substrate resulting in brown colonies. Expression of soluble scFv3A8-AP fusion protein was carried essentially as follows: colonies were grown in 2xTY medium supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin at 37 °C until $A_{600\ nm}$ reaches 0.8. Then, bacteria were induced with 0.5 mM IPTG and the temperature was shifted to 26 °C for 16 h. Bacteria from cultures were centrifuged and the periplasm was extracted by cold osmotic shock as previously described (Fields et al., 2013). Samples were extensively dialyzed against phosphate buffer saline (PBS) (pH 7.4). Protein concentration and AP enzymatic activity of the periplasmic extracts were measured in a Beckman Coulter DxC800 automat using standard procedures. Samples were stored at −20 °C until use.

Expression of the fusion protein was analyzed after 10% SDS-PAGE electrophoresis followed by Coomassie Brilliant Blue staining or overnight passive transfer onto nitrocellulose membrane. After blocking the membrane with 5% (w/v) non-fat dry milk in PBS, pH 7.4, transferred proteins having AP activity were detected by incubation with AP substrate BCIP/NBT liquid substrate (Sigma-Aldrich).

Dot blots were carried out after direct immobilization of OVA or MC-LR/OVA conjugate (5 μg in 2.5 μL) on to nitrocellulose membrane, blocking with non-fat milk as above, followed by an incubation with periplasmic extracts (1 h at 20 °C) and staining with BCIP/NBT liquid substrate.

2.3. In silico modeling and visualization of complexes

Since no structural analysis of IGVH3A8 and IGVL3A8 had ever been experimentally performed, a three-dimensional structural model of 3A8 Fv domains was built using the Web Antibody Modeling facility (Whitelegg and Rees, 2000). Framework regions were built using highly homologous antibody templates. Complementary-determining regions (CDRs) from the Lambda chain and hypervariable loops H1 and H2 from the heavy chain were built from homologous loops of the same canonical class. The CDR H3 loop was constructed using the CONGEN conformational search procedure and, finally, the whole model was energy minimized. The rigid-body docking PatchDock server was used for epitope/paratope modeling (Schneidman-Duhovny et al., 2005). A model of the Fv 3A8 and the 3D structure of the MC-LR (Protein Data Bank entry 1LCM) were docked together with the antibody/antigen interaction input parameter with a 4.0 Ã cut-off. Observations and picture renderings were made using PyMol according to the protocol (Fields et al., 2013).

2.4. Conjugation of MC-LR to ovalbumin

0.5 mg of MC-LR (Alexis Biochemicals, San Diego, CA) was cross-linked to 0.5 mg ovalbumin (OVA) using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) in a one step coupling reaction according to the manufacturer instructions (Thermo Scientific, Rockford, IL). This process allows formation of an amide bond between any carboxyl group of MC-LR and amino group of ovalbumin and vice versa. Correct coupling was checked in a standard ELISA using AD4G2 antibody which is specific for Adda residue not involved in MC-LR/OVA interactions (Enzo Life Sciences, Villeurbanne, France).