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Analytical Methods

Affinity maturation of single-chain variable fragment specific for aflatoxin B_1 using yeast surface display

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ABSTRACT

As aflatoxin B_1 is one of the most toxic mycotoxins, it is important to detect and to quantify aflatoxin B_1 accurately by immunological methods. To enhance aflatoxin B_1-binding affinity of the single-chain variable fragment, yeast surface display technique combined with fluorescence-activated cell sorting was applied. A randomly mutated scFv library was subjected to 4 rounds of fluorescence-activated cell sorting, resulting in isolation of 5 scFv variants showing an affinity improvement compared to the parental wild type scFv. The best scFv with a 9-fold improvement in affinity for aflatoxin B_1 exhibited similar specificity to the monoclonal antibody. Most of the mutations in scFv-M37 were located outside of the canonical antigen-contact loops, suggesting that its affinity improvement might be driven by an allosteric effect inducing scFv-M37 to form a more favorable binding pocket for aflatoxin B_1 than the wild type scFv.

1. Introduction

Aflatoxins (AFs) are fungal toxic compounds with chemical similarity (polyketide-derived furanocoumarins). Aflatoxin B_1 (AFB_1), the most potent hepatotoxic carcinogen among AFs is a secondary metabolite produced by *Aspergillus parasiticus*, *Aspergillus flavus* and *Aspergillus nomius* which can colonize naturally on plenty of raw food commodities, such as nuts, seeds and legumes during pre- and post-harvest (Sudakin, 2003). AFB_1 is a highly thermostable small molecule (MW < 400 Da) and hence very difficult to be inactivated or removed after its contamination (Raters & Matissek, 2008). Its mutagenic or carcinogenic effect on hepatocarcinoma development has been well characterized to be classified as a group I carcinogens by the International Agency for Research on Cancer (Min, Kweon, Park, Park, & Seo, 2011). Long-term intake of AFB_1 with hepatitis B virus infection is the main risk factor for the progression and development of chronic liver disease or hepatocarcinoma (Kew, 2003). Therefore, highly sensitive and specific AFB_1-detection systems which can prevent distribution of AFB_1-contaminated foods and feeds worldwide are urgently required.

AFB_1 contamination has been monitored mainly via a chromatographic or immunological method. Various chromatographic techniques including high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) have been developed for monitoring and quantifying AFB_1 (Cavaliere et al., 2007; Jaimez et al., 2000; Spanjer, Rensen, & Scholten, 2008). However, such chromatographic methods are inappropriate as an on-site detection system especially in developing countries due to time-consuming and cost-intensiveness. Therefore, the requirement for fast, portable and convenient detection tools of AFB_1 has led to the development of immunological methods such as enzyme-linked immunosorbent assay (ELISA), immuno-chromatographic strip or protein chip (Cho et al., 2005; Dorokhin, Haasnoot, Franssen, Zuilhof, & Nielen, 2011; Park, Kim, Kim, & Ko, 2014). So far monoclonal as well as polyclonal antibodies specific for AFB_1 have been used for various immunological detection modules and there are many commercialized kits (Groopman, Trudel, Donahue, Marshak-Rothstein, & Wogan, 1984; Haugen et al., 1981; Liu, Hsu, Lu, & Yu, 2013; Martin et al., 1984) which absolutely depend on their specificity and sensitivity.

However conventional monoclonal or polyclonal antibodies have some limitations in additional engineering approaches for *in vitro* affinity maturation, an increase of antibody stability, and prolonged half-life in the blood stream. It is also very difficult to produce the full-length structure of immunoglobulin G (IgG) type antibodies in various microbial systems. Besides, small sized recombinant antibodies such as single-chain variable fragment

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(scFv) or fragment antigen-binding (Fab) would be more effective as a detection probe for food toxins. In particular, scFv has been useful for AFB_1 detection, because of feasible antibody engineering tools for affinity or specificity, economical production using microorganisms such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae (S. cerevisiae)*, broad applicability for diverse detection modules. Antibody engineering technologies have been continuously advanced to improve the clinical or diagnostic functions of recombinant antibodies, such as an affinity enhancement via yeast surface display technology (Lee et al., 2010) or phage display (Hu et al., 2015), avidity increase by di- or polymerization (Zhu et al., 2010), thermal stability using nano-flow reversed-phase LC–MS (Hussack, Hirama, Ding, MacKenzie, & Tanha, 2011) or antibody solubility increase (Perchiacca, Ladiwala, Bhattacharya, & Tessier, 2012), prolonged half-life by PEGylation or increasing the affinity of fragment crystallizable region (Fc) for the Fc neonatal receptor (Patel et al., 2011). To obtain tailor-made antibodies, several technologies such as phage, ribosome, bacteria and yeast display have been developed and applied. Among them, yeast surface display technique coupled with fluorescence-activated cell sorter (FACS) enabled robust and high-throughput screening of huge library covering more than 10^9 cells and finally yielded reliable clones with high fidelity (Baek & Kim, 2014).

In a previous study, the hybridoma cell line 2C12 producing murine IgG highly sensitive and specific toward AFB_1 with nanomolar equilibrium dissociation constant value (K_D: 7×10^{-9} M) was established, even though AFB_1 is ~312.2 Da hapten that is not able to elicit an immune response without any adjuvants. The recombinant scFv against AFB_1 (scFv-WT) was cloned from cDNA of the hybridoma cell line 2C12 and the scFv-WT expressed as an inclusion body in *E. coli* cytoplasm was successfully refolded *in vitro* to recover affinity to AFB_1 (Min et al., 2011). However, its affinity was still not high enough to satisfy the most rigorous regulation detection limit of AFB_1 in foods and feeds established by European Food Safety Authority (<2 ppb). To improve the affinity of scFv-WT against AFB_1, a library containing diverse antibodies and an efficient antibody screening system was required.

In this study, a randomly mutated scFv library was constructed via error-prone PCR on the basis of scFv-WT gene and the scFv library was subjected to yeast surface display for high-throughput FACS screening. After sequential screening using flow cytometry with decrease in concentrations of the biotinylated AFB_1-BSA conjugate, 5 affinity-matured scFvs were isolated successfully and the best affinity-matured one exhibited ~9-fold-higher binding activity than scFv-WT and high specificity to other AFs in a similar level.

2. Materials and methods

2.1. Strains, plasmids and mycotoxins

The yeast strain for surface display of scFv was *S. cerevisiae* EBY100 (Invitrogen, Carlsbad, CA, USA), and *E. coli* DH5α and BL21(DE3) (Stratagene, La Jolla, CA, USA) were used for genetic manipulation and periplasmic expression of recombinant scFv, respectively. ScFv display plasmid pCTCON was donated from Prof. Yong Sung Kim at Ajou University (Chao et al., 2006; Lee et al., 2010). pET26b(+) (EMD Millipore, Darmstadt, Germany) was used for periplasmic expression of the hexahistidine tag-fused scFv. The bovine serum albumin (BSA) conjugates of AFB_1 (AFB_1-BSA conjugate), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), ochratoxin (OTA), deoxynivalenol (DON), fumonisin B_1 (FMB_1), fumonisin B_2 (FMB_2) and T-2 toxin were purchased from Fermentek Ltd. (Jerusalem, Israel). A milligram of lyophilized AFB_1-BSA conjugate was dissolved in 10 mL of 10% methanol solutions and stored at −20 °C. Biotin-XX Microscale Protein Labeling Kit (Life Technologies, Carlsbad, CA, USA) was applied to labeling the AFB_1-BSA conjugate for FACS screening.

2.2. Western blot using scFv-WT-displayed yeast cells

For construction of pCTCON plasmid displaying scFv-WT, the gene of scFv-WT was PCR-amplified using 5′-*Nhe*I and 3′-*BamH*I primers (Table 1) (Min et al., 2011). The PCR amplicon was digested with *Nhe*I and *BamH*I and then ligated into pCTCON linearized by the same restriction enzymes, resulting in the pCT-scFv-WT. The yeast cells harboring pCT-scFv-WT were induced by transferring to scFv display medium (SGCAA medium) and were cultivated at 17, 20, 23, 26 or 29 °C, respectively, to determine induction temperature for optimum display of scFv. The displayed scFv-WT proteins were released by the treatment of dithiothreitol (DTT) from outer membrane of induced cells and quantified using western blot. Displayed scFv at each temperature on the PVDF membrane (EMD Millipore, Darmstadt, Germany) was detected by the anti-c-Myc tag IgG [9E10] HRP conjugate (Abcam, Cambridge, MA, USA). Human recombinant purified His-tag c-Myc protein (~65 kDa) control (Alpha Diagnostic International, San Antonio, TX, USA) and yeast cells harboring pCTCON backbone plasmid were used as positive and negative controls, respectively. Development of transferred PVDF membrane was carried out using an Opti-4CN substrate kit (Bio-Rad, Hercules, CA, USA).

2.3. Random mutation of scFv-WT for construction of yeast library

A DNA pool with diverse scFv sequences was generated by error-prone PCR using nucleotide analogs, 8-oxo-2′-deoxyguanosine-5′-triphosphate (8-oxo-dGTP) and 2′-deoxy-p-nucleoside-5′-triphosphate (dPTP) (TriLink BioTechnologies, San Diego, CA, USA), and using HR forward and HR reverse primers (Table 1) (Chao et al., 2006). Mutation rate of error-prone PCR was modulated at 8–25 nucleotide changes in scFv-WT (~750 bp) by both a number of PCR amplification cycles (5, 10 & 20 cycles) and concentrations of 8-oxo-dGTP and dPTP (Colby et al., 2004). The error-prone PCR amplicons and pCTCON linearized by triple digestion with *Nhe*I, *BamH*I and *Sal*I were mixed at 9:1 M ratio and this mixture was transformed into *S. cerevisiae* EBY100 (10 μg DNA per 10^8 cells) based on Gietz's method (Gietz & Schiestl, 2007). Transformed cells were pooled into 100 mL of YPD medium (20 g dextrose, 20 g peptone and 10 g yeast extract in deionized-distilled H_2O (DDW) to a volume of 1 L) and the library was cultivated at 30 °C and 200 rpm for 1 h to rescue scarred cells upon transformation. Its serial diluents were plated onto selective SDCAA agar media (20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto™ casamino acids, 15 g Bacto™ agar, 5.4 g Na_2HPO_4 and 8.56 g $NaH_2PO_4 \cdot H_2O$ in DDW to a volume of 1 L) to estimate the library size. After additional cultivation for 2 h, an enriched library was transferred to 500 mL of fresh SDCAA medium, pre-incubated at 30 °C and cultivated at 30 °C and 200 rpm for 24 h. Aliquots of the over-grown library were stored under 15% glycerol at −80 °C and library stocks were prepared to contain at least 10-fold more cells than the library size which was calculated from colony counting.

2.4. Screening of high affinity scFv clones using FACS

Frozen aliquot of scFv library was inoculated into 500 mL of SDCAA medium and grew overnight to an optical density of ~7 at 600 nm (OD_{600}). This passage culture was repeated to ensure complete elimination of dead cells during storage at −80 °C. The culture, freshly re-inoculated to ~0.1 at OD_{600} in SDCAA medium was incubate at 30 °C with shaking at 200 rpm overnight and was