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Anti-idiotypic nanobody-alkaline phosphatase fusion proteins: Development of a one-step competitive enzyme immunoassay for fumonisin B_1 detection in cereal

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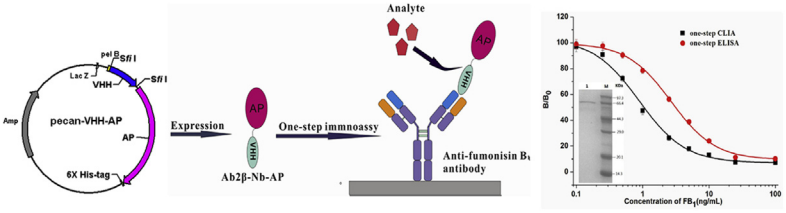
HIGHLIGHTS

- Ab2β–Nb–AP has the potential to replace chemically-coupled probes.
- Ab2β–Nb–AP is homogeneous enzyme-labelled antigen can be prepared reproducibly.
- We developed a green and rapid one-step competitive enzyme immunoassay.
- The sensitivity of one-step CLIA was 9-folds higher than two-step ELISA.

GRAPHICAL ABSTRACT



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ABSTRACT

A rapid and sensitive one-step competitive enzyme immunoassay for the detection of FB_1 was developed. The anti-idiotypic nanobody–alkaline phosphatase (Ab2β–Nb–AP) was validated by the AP enzyme activity and the properties of bounding to anti-FB1-mAb (3F11) through colorimetric and chemiluminescence analyses. The 50% inhibitory concentration and the detection limit (LOD) of colorimetric enzyme-linked immunosorbent assay (ELISA) for FB_1 were 2.69 and 0.35 ng mL^{-1}, respectively, with a linear range of 0.93–7.73 ng mL^{-1}. The LOD of the chemiluminescence ELISA (CLIA) was 0.12 ng mL^{-1}, and the IC_{50} was 0.89 ± 0.09 ng mL^{-1} with a linear range of 0.29–2.68 ng mL^{-1}. Compared with LC-MS/MS, the results of this assay indicated the reliability of the Ab2β–Nb–AP fusion protein based one-step competitive immunoassay for monitoring FB_1 contamination in cereals. The Ab2β–Nb–AP fusion proteins have the potential to replace chemically-coupled probes in competitive enzyme immunoassay systems.

1. Introduction

Fumonisin B_1 (FB_1) is the most abundant and toxic metabolite of fumonisins and is associated with human cancer [1,2]. FB_1 has been classified by the International Agency for Research on Cancer as a possible group 2B human carcinogen [3]. Concerned about the potential detrimental health effects, the United States Food and

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Drug Administration recommends that the maximum human consumption of residue limits of total fumonisins (FB_1+FB_2+FB_3) in corn and corn products is 2 ppm–4 ppm [4]. The European Union has set the maximum permissible levels for the sum of FB_1 and FB_2, which vary from 0.2 ppm to 2 ppm [5].

To efficient control of Fumonisin B_1 in cereal and cereal products, accurate and easily performed analytical methods, including instrumental techniques [6,7] and immunoassays [8–10] will be required. The instrumental techniques are precise and sensitive, but most require sophisticated equipment and complex purification steps. Immunoassays are sensitive, specific and cost effective encourage themselves to point of use tools for FB_1 determination. However, most of the previously reported immunoassays for mycotoxins use secondary antibodies as heterogeneous probes that are chemically linked with fluorescent [11] or enzymes, such as alkaline phosphatase (AP) [12] and horseradish peroxidase (HRP) [13,14]. The chemical conjugation of fluorenscent or enzymes to antibodies may result in unstable and randomly cross-linked molecules, which often reduce assay sensitivity [15]. Therefore, it is almost impossible to prepare homogeneous probes. With the rapid development of genetic engineering techniques, antibodies are fused to reporter proteins as homogeneous probes. These fusion proteins are constructed into a one-step immunoassay that avoids the use of secondary antibodies [16,17]. Many studies have reported the detection of low-molecular-weight compounds using the fusion of AP to single-chain fragments of the variable antibody region (scFv) or Nb, such as 11-deoxycortisol [18], ochratoxin A [19], and tetrabromobisphenol A [20].

In 1993, Hamers-Casterman Discovered sera of camelids contain a novel subclass of IgG antibodies that have unique functional heavy chain antibodies (HCAbs) and completely devoid of light chains [21]. nanobodies provide many unique features of antibodies that make them more useful compared with the conventional antibodies, such as ease of expression in various expression, thermostability, biophysical and easy of genetic manipulation [22,23]. Due to these advantages, many reports about environmental and biomedical applications of nanobodies are related to small molecules [24,25]. Recently, some studies on the detection of mycotoxin using nanobodies have been reported, such as ochratoxin A and Aflatoxin [26,27].

FB_1 as small-molecule hapten is not large enough for direct detection and to illicit an immune response, therefore, it requires conjugation with a large carrier, such as a protein, as antigen conjugates (coating antigen or competing antigen) [28]. In the synthesized procedures, large amounts of FB_1 standard and organic solvents which are expensive and may pose a threat to human health are involved. In order to reduce hazard condition, biologically derived antigen conjugates would be a preferred reagent. For the nominal antigen, the anti-idiotypic antibody (Ab2) is the second antibody specific to idiotopes, which can bind to the paratope of the primary antibody (Ab1) [29,30]. When the idiotope recognized by Ab2 is part of the paratope of Ab1, it is said to be an Ab2β. An important feature of Ab2β is that it can represent an internal image of antigenic determinants (epitopes) and compete with the original antigen when binding the primary antibody [31]. Thus, the Ab2β nanobody works as a substitute to the original antigen and fused to reporter molecule to be used as an immunoassay label.

In our previous work, we isolated an Ab2β nanobody [32] from a naïve alpaca nanobody phage display library. In the present study, we expand a strategy for determining small molecules that are immunochemically categorized as haptens by employing an Ab2β–Nb–AP fusion protein, which is equivalently immunoreactive as the target hapten and bio-reporter. The fusion protein is used to develop a rapid, green, and sensitive one-step competitive enzyme immunoassay for the detection of FB_1 in cereal samples.

2. Materials and methods

2.1. Materials and reagents

T_4 DNA ligase and restriction enzyme S*fi*I were purchased from New England Biolabs, Inc. (Beverly, MA). Mycotoxin fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEA), aflatoxin B_1 (AFB_1), and p-nitrophenyl phosphate (pNPP) substrate were purchased from Sigma (St. Louis, MO). Disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2′-(5′-chloro)-tricyclo [3.3.1.13, 7] decan}-4-yl) phenyl phosphate (CSPD) was purchased from Roche Applied Science (Basel, CH). The vector pecan45 [33] was a generous gift from Dr. Jinny L. Liu and Dr. Ellen R. Goldman (Naval Research Laboratory, Center for Bio/Molecular Science and Engineering, Washington DC).

2.2. Cloning, expression, and purification of Ab2β–Nb–AP fusion proteins

The Ab2β nanobody specific to anti-FB1-mAb was obtained previously [32]. A recombinant plasmid encoded the Nb–AP fusion protein with a 6X His tag at its C-terminal end, and the pecan–VHH–AP was constructed as shown (Fig. 1A). Positive clones were sent to Invitrogen (Shanghai, China) for sequencing. The pecan–VHH–AP plasmid was used into *Escherichia coli* BL21 (DE3) plysS cells. A single colony of the transformants was grown, and protein expression was induced in auto-induction media [34]. Crude extracts of the cells were lysed by ultrasonic cell disruption, and the crude protein of Ab2β–Nb–AP was centrifuged at 8000 g for 10 min. The target proteins, containing a 6X His tag were purified by affinity chromatography using Ni–NTA affinity columns and were analyzed by 12% SDS-PAGE.

2.3. Measurement of the AP activity of the Ab2β–Nb–AP

2.3.1. Colorimetric analysis

Serially diluted Ab2β–Nb–AP fusion protein (50 μL) was added into a 96-well microplate, followed by the addition of 100 μL of AP buffer (pH 10.4) containing 3.8 μM pNPP, 0.01 M glycine, 50 mM $MgCl_2$, and 50 mM $ZnCl_2$. The plate was mixed and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 2 M NaOH (50 μL). The well absorbance at 405 nm was then measured on a microtiter plate reader.

2.3.2. Chemiluminometric assay

The CLIA (chemiluminescence ELISA) substrate (Roche Applied Science) that contains disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2′-(5′-chloro)-tricyclo [3.3.1.13, 7] decan}-4-yl) phenyl phosphate (CSPD) (150 μL) was added to microwells in white microplates, in which purified Ab2β–Nb–AP diluted in PBS (10 μL) was placed. The plate was mixed and incubated at room temperature for 10 min. Luminescence at 475 nm was measured using the Thermo Fluoroskan Ascent FL (Thermo Scientific).

2.4. One-step competitive immunoassay using the Ab2β–Nb–AP

One-step competitive immunoassays were performed as follows: Anti-FB_1 mAb (3F11) (2 μg mL^{-1}, 100 μL/well) was coated in microplate overnight at 4 °C and blocked with 3% skim milk in PBS (10 mM, pH 7.4) for 1 h at 37 °C. After washing with PBST (10 mM, pH 7.4, 0.5% Tween 20), a diluted Ab2β–Nb–AP (periplasmic extract) (50 μL/well) and various concentrations of FB_1 standard (from 0.1 to 100 ng mL^{-1}, 50 μL/well), both diluted with PBS (10 mM, pH 7.4) were added; the mixture in the plate was incubated at 37 °C for 30 min. After washing three times with PBST, the