Research Progress of Green Immunoassay for Mycotoxins

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Abstract: Mycotoxin pollution not only causes huge economic losses, but also seriously threatens the health of humans and animals. Immunoassay methods are widely used in the rapid detection of mycotoxins, but the method requires the use of toxin standards and artificial antigens. The artificial antigen synthesis is not only difficult to prepare, but also requires toxin standards as raw materials. Toxin standards are expensive and harmful to the health of producers and operators, which restricts the application and popularization of immunoanalytic methods in mycotoxin detection. If the toxic antigens and standards involved in the immune reaction is substituted with the non-toxic antigen, a green immunology test method can be established to overcome the above deficiencies. Establishing a green, fast, simple and highly sensitive immunoassay method to detect these toxins has become a hot topic in recent years. This paper reviews the application and prospect of green immunoanalysis methods based on anti-unique antibody and phage display techniques in mycotoxins.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi such as Aspergillus, Penicillium and Fusarium during their growth and reproduction[1], mainly including: aflatoxin, ochratoxin, fumonisin, deoxynivalenol, citrinin and zearalenone. Mycotoxins are widely found in nature, in a variety of crops, food and feed can be detected mycotoxin contamination. Mycotoxins are extremely harmful to humans and animals. They are carcinogenic, teratogenic and mutagenic. They can also cause liver poisoning, reproductive disorders, etc[2], and cause huge economic losses, According to statistics from the Food and Agriculture Organization of the United Nations, about 25% of the world’s grains are polluted by mycotoxins each year, and 2% of agricultural products lose their nutritional and economic value due to serious pollution, causing economic losses of tens of billions of dollars[3]. Prevention and control of mycotoxin contamination need for timely detection. The immunoassay method is widely used in the rapid detection of various mycotoxins due to its high sensitivity, strong specificity, simple operation and fast detection speed[4], but because its detection principle is a competitive antigen-antibody reaction, it is Standards and antigens for mycotoxins are required in the medium. The antigenic cause of mycotoxin is not immunogenicity, so it needs to be coupled with carrier protein to prepare artificial antigen. The synthesis of artificial antigens requires the extensive use of highly toxic mycotoxins, not only the preparation more difficult[5], and there is expensive, limited import, Defects harmful to the health of production and operation personnel, restricted the immunoassay method of application and promotion. Green immunoassay means that in toxin immunoassay, antigen synthesis methods and antibody preparation methods are selected, which are less harmful to human and the environment. using less toxic materials in place of conventional highly toxic antigen or standard, reduce the hazard to operators and environmental pollution caused by testing objects or methods[6].
This article summarizes the research results of mycotoxin green immunoassays at home and abroad in recent years from the aspects of phage display peptides and anti-idiotype antibodies replacing mycotoxin antigens, it is intended to provide a method reference for solving the mycotoxin hazards in the detection method, and also provide a useful reference for the further development of related research.

2. Research and application of phage display peptides in green immunoassay detection of mycotoxins

In 1985, Smith[7] proposed to insert foreign genes into modified phage coat protein genes, express fusion proteins containing foreign proteins or polypeptides, display them on the phage surface and maintain a specific spatial conformation, and use specific affinity to screen specific protein or polypeptide. Based on the principle of phage random peptide library technology for epitope analysis, the scientists proposed that phage display technology can be used to screen mycotoxins mimotope peptides. The method of antibodies as a ligand molecules immobilized on a support, into a phage display peptide library binding and binding site of an antibody, after a 3-4 affinity panning obtain phage polypeptide specifically binds to an antibody molecule, by DNA sequencing and analysis of the sequence obtained mimotopes, different processing sequences of fungal toxins green detection method [8].

In recent years, researchers have used bacteriophage display technology to simulate the epitopes of various mycotoxins, established a variety of green detection analysis (see Table 1). Deng Junzhou [9] used monoclonal antibody against Deoxynivalenol (DON) to select the heptadecopeptide model epitopes of DON from phage random heptadeceptide library, and replaced DON artificial antigen to establish DON non-toxic Phage -ELISA method. The detection range of this method was 20-400 ng/mL. Liu et al [10] took the monoclonal antibody against Ochratoxin A (OTA) as the target and performed panning and screening from a random heptapeptide library. After four rounds of screening, 11 phages were found to mimic the binding of OTA to antibodies. An ELISA for detecting OTA was established using phage, with a linear range of 200-8000 pg/mL and a detection limit of 150 pg/mL. Pei Yafeng [11] took the anti-OTA monoclonal antibody as the target molecule, affinity panning and fusion of the random heptapeptide library expressed on the minor capsid protein of the filamentous M13 phage to obtain the positive phage with the mimic epitope sequence of OTA. An indirect competitive ELISA was used to establish an OTA detection method. The linear detection range was 200-1400 pg/mL, the IC50 was 974.67 pg/mL, and the detection limit was 201.33 pg/mL. Liu et al [12] used the anti-fumonisin B1 monoclonal antibody 1D11 to pan from the random cyclic heptapeptide library and obtained 3 phage peptides that can mimic FB1 binding to 1D11, and selected one with a good linear range. The chemical synthesis of the peptide was named CT-452, and a green immunoassay method based on CT-452-BSA as the coating antigen was established. The IC50 of this method is 6.06 ng/mL, and the minimum detection limit is 1.18 ng/mL. Riikka Peltomaa et al [13] used FB1 specific antibodies to screen mimotopes from the 12 peptide library, randomly selected monoclonal phage for sequence analysis and found two conservative peptide sequences. Clonal phage with vtpndtdpfr peptide sequence has high sensitivity in ELISA detection. Used for microarray; immobilize the biotinylated synthetic derivative of the mimic on a glass slide, use FB1 antibody and labeled secondary antibody in the competitive binding inhibition test of FB1, the IC50 value of the array is 37.1±2.4 ng/mL, the detection limit is 11.1 ng/mL, it has good specificity for FB1 and its structural analogue FB2. The above research shows that the simulated epitopes of mycotoxins can be obtained efficiently by phage display technology. The selected simulated epitopes can become the substitute of mycotoxins after chemical synthesis, and the green immunological analysis method (ELISA) can be established. Green immunoassay (phage-ELISA) can also be established by direct application of phages with analogue epitopes. Because different methods use different antibodies, it is difficult to compare different methods of sensitivity, specificity and other indicators. In addition, sometimes the affinity of analogue epitopes obtained by phage display screening is relatively low with antibodies [14].
Table 1 Research and application of green detection and analysis method based on mycotoxin mimic epitope

| Target analyte         | Detection method | IC50(ng/mL) | LOD(ng/mL) | Linear range (ng/mL) | Literature source |
|------------------------|------------------|-------------|------------|----------------------|-------------------|
| Deoxynivalenol         | phage-ELISA      |             |            | 20-400               | [9]               |
| Ochratoxin A           | ELISA            |             | 0.15       | 0.2-8                | [10]              |
| Ochratoxin A           | ELISA            | 0.97467     | 0.20133    | 0.2-1.4              | [11]              |
| Fumonisin B1           | ELISA            | 6.06        | 1.18       |                      | [12]              |
| Fumonisin B11          | Array-ELISA      | 37.1±2.4    | 11.1       |                      | [13]              |

3. Research and application of anti-idiotypic antibodies in mycotoxin green immunoassay detection

1974 Jerne [15] proposed immune network theory, the core is particular antigen stimulates the production of corresponding antibodies Ab1, the variable region of Ab1 binding to molecules other than the specific antigen, which itself can be used as an antigen stimulates the production of antibodies, the anti-antibody produced is called anti-idiotypic antibody Ald. Ald can be divided into four types Ab2β, Ab2α, Ab2γ, Ab2ε [16], where in Ab2β recognize antigen-binding site on the antibody Ab1, Ab2β in the conformation generated will start with Ab1 dynamic similarity antigen, i.e. Ab2β It can be used as the internal image of the antigen [17]. Thus anti-idiotypic antibody antigen involved in an immune response may alternatively establish green immunoassay. The preparation methods of anti-unique antibody Ab2β can be divided into three types, namely polyclonal Ab2β technology [18-20], monoclonal Ab2β technology [21-23] and antibody library technology [24, 25].

Guan et al.[26] immunized rabbits with the F(ab')2 fragment of anti-aflatoxin M1 (Aflatoxin M1, AFM1) monoclonal antibody, prepared polyclonal anti-idiotypic antibodies, and used it as a standard substitute for AFM1 An ELISA method was established to detect AFM1 in milk. The IC50 of the method was 2.4 μg/mL. Zhang Xun [27] and others prepared anti-idiotypic monoclonal antibodies against aflatoxin B1 (Aflatoxin B1, AFB1) using antibody F(ab'2) fragments as immunogens, and established anti-idiotypic monoclonal antibodies based The non-toxic green AFB1 ELISA analysis method detects AFB1 in flour. The intra-batch recovery rate of this method is 115.60-121.88%, and the inter-batch recovery rate is 111.89-126.98%. Monoclonal technology has the advantages of good effect, high quality and uniform and stable antibody between batches. Although anti-idiotypic monoclonal and polyclonal antibodies can establish green immunoassays for mycotoxins, the preparation of anti-idiotypic polyclonal and monoclonal antibodies is difficult and the rate of positive clones is low. From the preparation of idiotypic antibodies against the antigen to the preparation Anti-idiotypic antibodies need to be screened in large quantities at each step; antibody preparation, separation and purification is difficult, and the body can produce various types of anti-idiotypic antibodies against different idiotypes of antibodies, such as α, β, γ, and δ. The required β-type efficiency is very low. After purification and concentration, the application cost of obtaining available anti-idiotypic antibodies is very high [28], which limits its further research and development as a substitute.

3.1. Research and application of anti-idiotypic nanoantibodies in green immunoassay detection of mycotoxins

Antibody library technology is a technology that USES PCR technology to clone the full set of heavy and light chain variable region genes of antibodies, recombine the obtained target fragments into specific expression vectors, and finally transfer them into prokaryotic or eukaryotic organisms to express functional antibody molecular fragments, and obtain specific antibodies through affinity screening[29].The antibody repertoire can be divided into different display platform: phage display, ribosome display [30], yeast display [31], bacterial display [32, 33], baculovirus display[34]and the mammalian cell display[35]. Mycotoxins green immunoassay use more phage display antibody library technology. Phage display antibody library in bacteriophage vector, a phage gene encoding the antibody coupled to the coat protein, antibody - fusion protein expression casing surface of the phage,
after "adsorption - elution - amplification" and enriched in the screening process. Set specific antibodies.

Antibody is a protein molecule consisting of two heavy chains (H chain) and two light chains (L chain) with three domains, two of which are related to the recognition of antigen and constitute Fab fragment. The other domain is Fc fragment, which plays the role of assisting antibodies to recognize antigens. Nanobodies are single domains obtained by researchers from camelid animals (camels, alpacas, llamas, etc.) and some cartilaginous fishes (nurse sharks, rays, etc.) that naturally lack the heavy chain antibody variable regions of the light chain. Antibody (Variable domain of heavy chain of heavy chain antibody, VHH) [36], its structure is similar to the variable domain (VH) of conventional antibodies, consisting of four framework regions (FR) and three hypervariable regions (CDR). The three-dimensional structure of which the hypervariable region determines the ability of the antibody to recognize the antigen. Compared with traditional antibodies, the CDR3 region of Nanobodies is longer. CDR3 is the most important position for antigen recognition, accounting for 60-80% of the entire antibody. Therefore, Nanobodies can better bind to antigens, and at the same time it can recognize many common antibodies. Unrecognizable antigens are especially suitable for molecular simulation of small molecules[37]. The application of anti-idiotypic nanobodies based on phage display antibody library technology in small molecule immunoassays provides a new choice for green immunoassay methods for mycotoxins.

The preparation of mycotoxin anti-idiotypic nanobody mainly includes: immunizing camelid animals with toxin-specific monoclonal antibodies to construct an immune nanobody library; or collecting peripheral blood of unimmunized camelid animals and constructing a natural nanobody library through genetic engineering. Then screen anti-idiotypic antibodies. In recent years, the work of researchers using anti-idiotypic nanobodies to establish a green detection and analysis method for mycotoxins is shown in Table 2. Wang et al[38] used the F(\(ab')\)2 fragment of anti-aflatoxin B1 monoclonal antibody to immunize alpaca, constructed an anti-idiotypic nanobody immune library, and obtained a strain that can interact with AFB1 by panning. Competitively bind to the idiotypic nanobody of anti-AFB1 monoclonal antibody. The Ab2\(\beta\) can induce immune response in Babl/c mice. Use this nanobody as an enzyme-labeled antigen to establish a competitive ELISA with an IC50 of 6.3 ng/mL. This method is more sensitive. Traditional ELISA has decreased. At the same time, the identification by competitive ELISA shows that anti-idiotypic nanobody is another tool for hapten simulation research. Wu Hui[39] used Zearalenone monoclonal antibody 2D3 as the target to immunize alpaca, after four rounds of affinity panning, a phage that specifically binds to the variable region of the 2D3 antibody was obtained Display Nanobody page8#. The ELISA method based on page8# has high detection sensitivity, and the IC50 value for ZEN is 0.207ng/mL. Three blank samples of corn, wheat and feed were used as substrates for the ZEN addition recovery test, and the addition recovery rate was between 71.7% and 102.2%. Wang et al[40] used anti-AFB1 monoclonal antibody 1C11 to immunize alpaca to construct an anti-1C11 nanobody phage library, from which anti-AFB1 anti-idiotypic nanobodies were obtained by affinity panning, and anti-unique nanobodies were used to replace coated antigens to establish detection. The IC50 of the AFB1 ELISA in the actual sample is 0.22 ng/mL. When the actual sample is tested, the method is consistent with the conventional ELISA test result.

Shu et al[41] took anti-FB1 monoclonal antibody (mAb) as the target, panned from the natural phage nano library, isolated an anti-idiotypic nano antibody (Ab2\(\beta\)Nb), and used Nb-ELISA to detect FB1. The IC50 of this method It is 0.95±0.12 ng/mL, the detection limit is 0.15 ng/mL, and the linear range is 0.27-5.92 ng/mL. Compared with the conventional ELISA (commercial ELISA kit), the result shows the reliability of Ab2\(\beta\)Nb as an alternative to the antigen carrier protein conjugate. Ji et al[42] used anti-OTA monoclonal antibodies as the target, and obtained anti-idiotypic nanobody phage Ald-Nb, which can recognize anti-OTA monoclonal antibodies, after four rounds of biological panning from the natural phage display nano library. The phage was established to detect OTA in cereals by fluorescent quantitative PCR. The IC50 of this method was 300 pg/mL, and the minimum detection limit was 4.17 pg/mL. Jiang Dongjian[43] established a highly sensitive and specific
immunofluorescence quantitative PCR method for the detection of DON based on phage-displayed nanobody through the obtained anti-DON idiotypic nanobody. The linear detection range of this method is 0.1-1000 ng/mL, IC50 value is (3.96±2.21) ng/mL, the lowest detection limit is 0.048 ng/mL, and it has no cross-reactivity with other mycotoxins. Wang et al[44] used anti-ZEN monoclonal antibody (mAb) as the target to screen anti-idiotypic antibodies from the natural alpaca antibody phage display library. After four rounds of panning, an anti-idiotypic antibody phage clone (Z1), the IC50 for ZEN detection by Z1 phage ELISA is 0.25±0.02 ng/mL, the linear range is 0.11-0.55 ng/mL, and the limit of detection (LOD) is 0.08 ng/mL. Xu et al [45] took anti-Citrinin (CIT) monoclonal antibody as the target, and screened a strain of anti-idiotypic antibody from the natural alpaca antibody library as a substitute for CIT hapten. The detection range of nanobody ELISA is 5.0-300.0 ng/mL, and the IC50 is 44.6 ng/mL, which is twice as high as the indirect competitive ELISA (IC50=96.2 ng/mL). Above studies indicate that anti-idiotypic antibody antigen to establish an alternative nanometer green immunological detection methods, the detection sensitivity of the high specificity of the method, the phage mimics polypeptide, anti-idiotypic antibodies based on their stable nano-independent spatial structure of the protein as compared to more suitable research and development of alternative products mycotoxins.

Table 2 Research and application of green detection and analysis method for mycotoxins based on anti-unique nano antibodies

| Target analyte | Detection method | IC50(ng/mL) | LOD(ng/mL) | Linear range (ng/mL) | Literature source |
|----------------|------------------|-------------|------------|----------------------|-------------------|
| Aflatoxin B1   | ELISA            | 6.3         | /          | /                    | [38]              |
| Zeearalenone    | ELISA            | 0.207       | /          | /                    | [39]              |
| Aflatoxin B1   | ELISA            | 0.22        | /          | /                    | [40]              |
| Fumonisins B1  | Nb-ELISA         | 0.95±0.12   | 0.15       | 0.27-5.92            | [41]              |
| Ochratoxin A   | PCR              | 0.3         | 0.00417    | /                    | [42]              |
| Deoxynivalenol | PCR              | 3.96±2.21   | 0.048      | 0.1-1000             | [43]              |
| Zeearalenone    | ELISA            | 0.25±0.02   | 0.08       | 0.11-0.55            | [44]              |
| Citrinin       | ELISA            | 44.6        | /          | 5.0-300.0            | [45]              |

4. Conclusion and Outlook

The existence of mycotoxins seriously threatens human health and causes social and economic losses. It is of great practical significance and application value to study the substitute of toxin antigen and apply it to immunological analysis. At present, Based on the mimic epitopes of phage display peptides and the application of anti-idiotypic antibodies, both of which can be used as mimics of mycotoxin antigens in "structure recognition", specifically bind to corresponding antibodies to simulate the binding function of antigens, Realize the green immune analysis of mycotoxins and reduce the harm caused by mycotoxins. However, the application of phage display mimotopes in toxin green immunity requires some attention. For example, during panning, some false positive peptides will inevitably be obtained; peptides need to be correctly folded in vitro to simulate mycotoxins; Weak affinity with antibodies; more non-specific binding; obvious consensus sequence binding less. Aiming at the correct folding of peptides in vitro and improving the affinity, researchers have used computer software to analyze the molecular docking of antibodies and mimotopes. By replacing amino acid residues in the mimic peptides, the affinity of the antibody and mimic peptides can be increased. The stability of the peptide [46]. Researchers have also proved that the optimized mimotopes obtained by constructing the secondary peptide library is another breakthrough to solve the bottleneck of the low affinity of the mimotopes screened by the phage library with the antibody [47]. For polypeptides with more nonspecific binding, significantly less common sequence binding, and false positive, you can adjust each step of the screening, such as detergent concentration, elution method, target molecule concentration, screening rounds, library selection, and screening methods. Wait to be resolved. Secondly, the shortcomings of phage display peptides are unstable and easily degraded, which limits
their application in mycotoxin green immunity. Anti-idiotypic monoclonal and polyclonal antibodies to mycotoxins cannot be produced on a large scale due to the difficulty of preparation and high cost.

The anti-idiotypic nanobody itself has an independent protein structure, complete antigenic analog of the space conformation can be guaranteed; the anti-idiotypic nanobody only needs to be expressed in E. coli. After purification, a large number of uniform antigen substitutes were obtained which avoids the batch-to-batch difference in the traditional synthetic antigen preparation process. Currently, panning to obtain anti-idiotypic nanoantibodies with different affinities and improving detection sensitivity still require continuous exploration and research. Because nano antibodies have only one domain and their structure is relatively simple, when the sensitivity of panning Nanobodies is not high, in vitro affinity maturation methods can be used to increase their affinity and thus their sensitivity. Common methods include random mutations[48], site-directed mutagenesis[49]and rational antibody design method[50]. Anti-idiotypic nanoantibodies have the advantages of small molecular weight, simple structure, and easy genetic engineering operations. They have far-reaching and important application value in the green immunoassay of mycotoxins, and are more conducive to the development of green immunoassay kits and test strips.

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