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Design of antigen synthesis and identification of its artificial antigen for zearalenone

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Contents

Abstract

Background: study aimed to modify the ZEN molecule and conjugate the carrier protein to prepare a complete antigen. To lay the foundation for the preparation of ZEN monoclonal antibodies. Methods: The carbonyl group at the 7 position of ZEN molecule was modified by deuteration reaction. The immunogen and antigen were synthesized by EDC method and mixed acid anhydride method, and the complete antigen was identified by UV, IR and electrophoresis. Antisera were obtained after immunization of animals, and an antiserum was designed by ELISA. Results: The immunogens were identified by UV, IR and electrophoresis, ZEN-BSA was successfully synthesized. The ratio of ZEN-BAS to ZEN was calculated to be 1 : 13. When the antibody serum was detected, the titer of the antibody reached 1:(6.4×103). Conclusion: This study demonstrated that the OAE method is preferable in preparing the ZEN. These findings lay the material and technical foundation for the preparation of anti-ZEN monoclonal antibody

Key words: ZEN, Artificial antigen, Polyclonal antibody, Performance Identification.

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1. Introduction

Zearalenone (ZEN) was produced due to the growth of Fusarium graminearum caused by improper storage or improper temperature of corn soybean meal and other feeds (Niermans et al., 2019). Most of Zen grow in plants and widely exists in food, which reduces the nutritional value of food and remains in human and animals (Rogowska et al., 2019), giving a great threat to human food safety. There are many derivatives of ZEN, including α-Zearalenol (α-ZOL), β-Zearalenol (β-ZOL), α-Zeranol (α-ZER), β-zearalenol (β-ZER) and Zearalanone (ZAN). The relationship between these five derivatives is as follows: Fusarium sp. grows in grain and produces toxic metabolite ZEN, which mainly metabolizes two products, α-ZER and β-ZER (Faisal et al., 2018), among which the main product is α-ZER, accounting for 99 % of the metabolites. After they enter the animal body, α-ZOL and β-ZOL are formed by reduction reaction, and finally ZAN is formed. ZEN has the effect of estrogen (Zheng et al., 2019), which can cause reproductive and immune diseases such as abnormal breeding function, cytotoxicity (Fig. 1).

Studies have shown that ZEN and its metabolites have estrogen activity in pigs, cattle and other animals (Zhang et al., 2018). The clinical treatment of diseases caused by a variety of mycotoxins will cause huge medical costs (Jia et al., 2014; Liu et al., 2014). The EU stipulates that the MRL of ZEN in cereals and grain products is 2 mg/kg; the MRL of ZEN in corn by-products is 3 mg/kg; the MRL of ZEN in compound feeds for piglets and young sows are 0.1 mg/kg (EC, 2007). The current ZEN MRL standard of food and agricultural products in China is “GB 2761-2017 Food Mycotoxin Limit”, which strictly stipulates that the MRL of ZEN in wheat, wheat flour, corn, and cornflour is 0.06mg/kg (CHINA, 2017). Italy stipulates that the MRL of ZEN in grains and cereal products is 0.1mg/kg (Bertuzzi et al., 2014); Australia stipulates that the MRL of ZEN in grains is 0.05 mg/kg (Tan et al., 2011) However, α-ZOL has the strongest toxicity, which is 10–20 times higher than ZEN (Yang et al., 2019; Rai et al., 2020). Therefore, single ZEN detection cannot meet the needs of food and feed industry, this has prompted immense research on detecting the total amount of ZEN and its homologues.

According to the physical and chemical properties of ZEN, the detection method of the material can be determined mainly with thin layer chromatography (TLC), gas chromatography (GC), gas chromatography – mass spectrometry (GC-MS), etc. (Kresse et al., 2019). However, these methods cannot meet the requirements of mycotoxin determination due to their low yield, long cycle, requiring professional operation and tedious sample pretreatment (Selvaraj et al., 2015). Immunological detection technology has the advantages of simplicity, high efficiency and broad spectrum, and has gradually become one of the important methods to detect biological toxins (Xu et al., 2018; Chen et al., 2019). In this study, the method of oximation (Dong et al., 2018) was used to react with carboxymethoxylamine hemihydrochloride to modify the ketone group on the molecular formula and synthesize hapten. The artificial antigen was synthesized by EDC method and mixed anhydride method by coupling with BSA and ova. In antibody detection, horse serum was used as blocking solution for detection (Sun et al. 2014). The detection results of indirect ELISA showed that the titer of antiserum was high. This study also provides the basis for the preparation and identification of monoclonal antibodies.

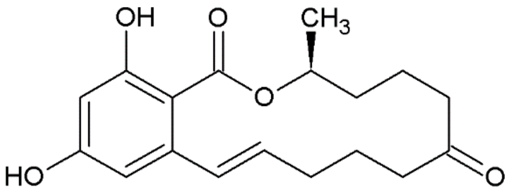

Fig. 1. The chemical structure of zearalenone

2. Materials and methods

2.1 Chemicals and Reagents. The standard of ZEN, α-ZER, β-ZER, α-ZOL, β-ZOL, ZAN and polyethylene glycol (PEG-1500) purchased from Sigma. Bovine serum albumin (BSA), chicken ovalbumin (OVA) were provided by Pierce. Goat Anti-Mouse IgG-horseradish peroxidase (IgG-HRP), phenol red was provided by Solarbio. 3,3,5,5-Tetramethylbenzidine (TMB), 1,4 dioxane, Tributylamine isobutyl chlorocarbonate, potassium bromide, RPMI-1640 medium purchased from Gibco., colchicine was provided by Shanghai Yansheng Biochemical Co., Ltd .in addition, 6-well cell and 24-well cell culture plates, cell culture bottle, 96-well microtiter plates 96 were purchased from Corning company. Hypoxantin Aminopterin and Thymidin (HAT), Hypoxantin and Thymidin (HT) were all made in-house in our laboratory.

ZEN standard solution, dilute 60% methanol with PBS to a dissolved concentration of 1 mg/mL, then dilute it to 8 ng/mL, 4 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.125 ng/mL, 0.062 ng/mL, 0.031 ng/mL, 0 ng/mL.

2.2 Equipment and Instruments. Exceed DZG-303A ultrapure water polishing system was purchased from Chengdu Kangning Special Experiment Pure Water Equipment Factory (Chengdu, China), a 303A-1 electric heating constant temperature incubator was purchased from Beijing Zhongxing Weiye Instrument Co., Ltd. (Beijing, China) N-EVAP nitrogen blower were purchased from (Organomation, USA) The infrared (IR) spectra were obtained using a Bruker Tensor 27 spectrometer (Bruker, Germany), while the ultraviolet (UV)–visible spectra were obtained with a DU-800 UV–visible spectrophotometer (Beckman-Coulter, Fullerton, CA,). JY300C electrophoresis apparatus and gel imaging system were purchased from Beijing Junyi Dongfang electrophoresis equipment Co., Ltd. (Beijing, China). In addition, a spectrophotometric microtiter reader (MULTISKAN MK3, Thermo Co., Shanghai, China) was used to measure the absorbance.

2.3 Experimental animals. 6–8 weeks old female Balb/c mice and feed provided by the Experimental Animal Center of Medical College of Zhengzhou University (Zhengzhou, China), and were given tap water and *a diet ad libitum.*

2.4 Synthesis of Immunogen. According to the molecular structure and active site of ZEN, the immunogen and coating antigen were synthesized via oxime active ester (OAE) (Cha et al., 2012), synthetic semi-antigen ZEN-CMO (Fig. 2). The carboxyl group of ZENO and the amino group of BSA were coupled with a monoamide bond under the action of EDC to synthesize artificial immunogen ZEN-BSA. ZENO was dissolved in 2 mL dioxane, after which 3 mg NHS and 7 mg EDC were added, stirred at 4 °C for 4 h to prepare the hapten activation solution. Then added 1 ml of 1,4 dioxane and 45 ml of tri-n-butylamine were added respectively, and the reaction time was 20 min in ice bath, 60 µ l isobutyl chloroformate and the reaction time was 2 h in ice bath conditions. The hapten activation solution, 1 mL cBSA activation solution was added at a concentration of 20 mg/mL dropwise. The reaction solution was stirred at 4 °C for 4 h, then dialyzed with PBS at 4 °C for three days. The dialysate was changed once a day and stored at -20 °C for subsequent applications. The synthetic route of ZEN-BSA (OAE) is shown in Figure 3. The coating antigen ZEN-OVA (OAE) was prepared via the same method.

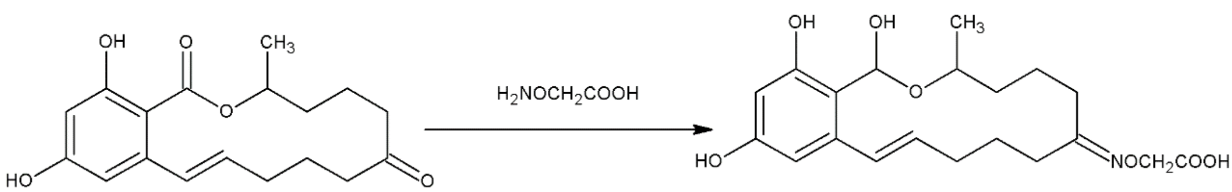

Fig. 2. ZEN-CMO synthesis route

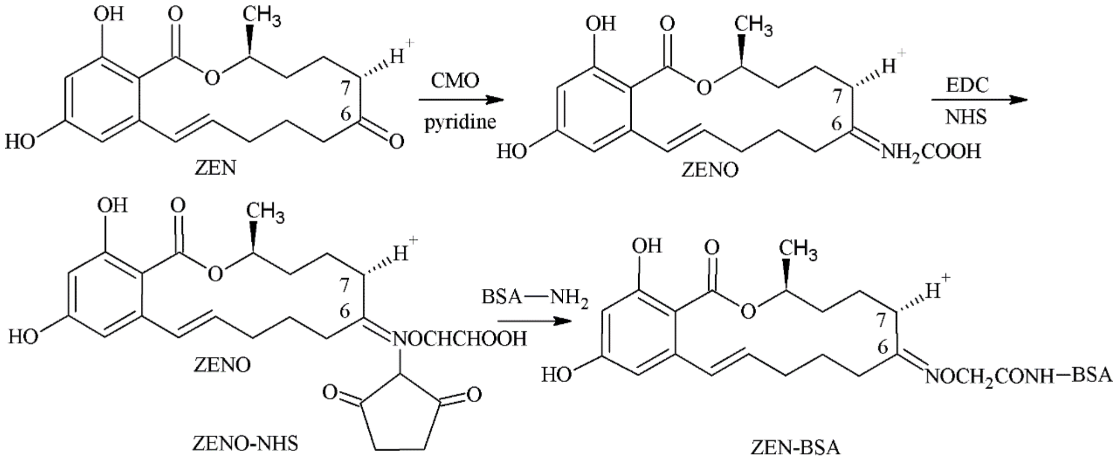

Fig. 3. Synthesis route of the ZEN-BSA by the OAE method

2.5 Identifying the Artificial Immunogen. UV identification: The PBS buffer was used as a blank control and the baseline was calibrated. Configure the concentration of BSA and OVA to 1 mg/ml. The antigen ZEN-BSA and ZEN-OVA were diluted to a protein concentration of 1 mg/ml. Dilute the standard to 20 µg/ml for ultraviolet scanning (Jiang et al., 2014). IR identification: During infrared scans, powder samples are needed. First, the artificial antigen was freeze-dried, and the dried potassium bromide was prepared. Then, the dried trace antigen and potassium bromide were mixed at the ratio of 1 : 100, and then ground. Finally, the tablet was pressed and measured on the machine (Wang et al., 2012). SDS-PAGE identification: Here, 5 % concentration gel and 12 % separation gel were selected for electrophoresis analysis. The test voltage for the concentrated gel was 100V, while that for the separation gel was 60V. Also, 10 µL/hole sample volume and 10 µg/hole protein content were used (Pedersen et al., 2006).

2.6 Preparation of ZEN pAb. Referring to the method of Kim S. H. et al. (Kim et al., 2011) and improve it. Five female BALB/c mice aged 6–8 weeks were immunized with ZEN-BSA and injected subcutaneously with 50 µg/mouse. The mice were immunized once every three weeks for five times. The antibody titer was determined by indirect ELISA. On 96 well plate, the concentration of the coated plate was determined to be 2 µg/ml by square matrix titration. The artificial antigen ZEN-OVA was diluted with CBS at 4 °C overnight, blocked with 5 % horse serum and 5 % pig serum blocking solution respectively, and incubated at 37 °C for 1 h as control, and was coated with blocking solution at 50 µL. The second antibody and Goat anti mouse IgG (diluted to the working concentration with blocking solution) were added and incubated at 37 °C for 25 min. the chromogenic solution was added for 10 min. finally, the reaction was terminated. OD450 value was determined by enzyme reader. The above steps were washed three times with 0.05 % Tween-20 in PBS every time interval of 3 min.

3. Results and discussion

Results

UV Identification. In the range of UV 220–400 nm, BSA had characteristic absorption peaks for UV at 278 nm, and ZEN had characteristic absorption peaks for UV at 236 nm, 274 nm, and 316 nm, respectively (Fig. 4). The artificial immunogen ZEN-BSA had characteristic absorption peaks for UV at 276 nm. Therefore, the absorption peak has shifted, which proves that the artificial antigen coupling is successful.

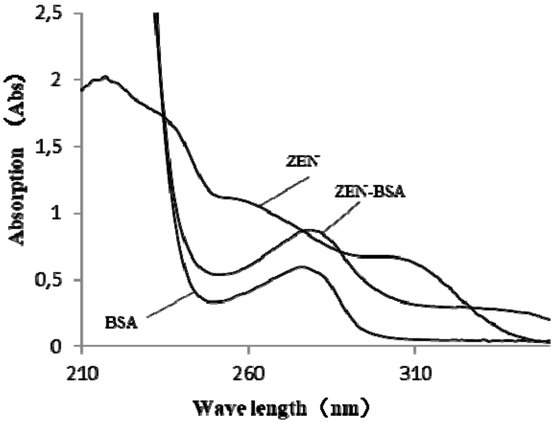

Fig. 4. UV spectra of ZEN-BSA synthesized

IR Identification. The results of the identification are shown in Figure 5 Comparing the IR of the artificial immunogen ZEN-BSA synthesized with BSA, we revealed similar IR absorption in the regions 2500–3000 cm-1 and 1000–1500 cm-1, which were the characteristic peaks produced by the amine group and amide group in BSA (Fig. 5). This finding revealed that the method successfully synthesized the artificial immunogen ZEN-BSA.

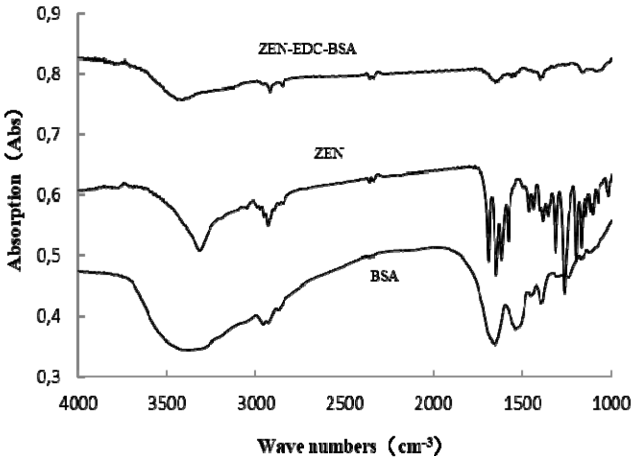

Fig. 5. IR spectra of ZEN-BSA synthesized

SDS-PAGE Identification. As shown in Figure 6, under the same conditions, the BSA is fast than the migration speed of ZEN-BSA, indicating that the molecular mass of Zen-BSA is large, indicating that the artificial antigen synthesis is successful. The gel imaging system was detected that the ZEN-BSA molecular weight was 70569.3, and the molecular weight of the BSA was 66430. Calculated, ZEN-BSA and ZEN coupling ratio of 1 : 13.

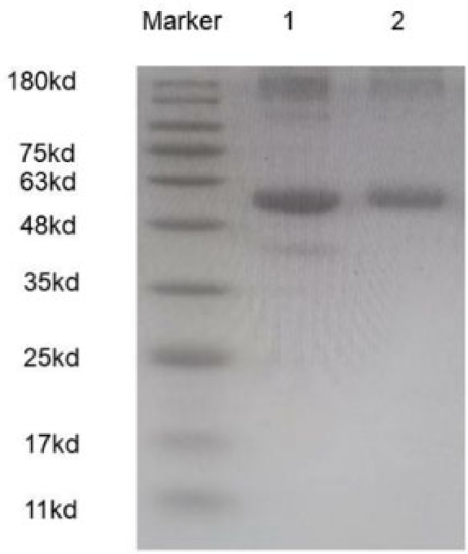

Fig. 6. SDS-PAGE identification of artificial antigen ZEN-BSA. 1 Marker; 2 BSA; 3 ZEN-BSA

3.4 ZEN pAb Characteristic Analysis. The titers of antibody in serum of mice were shown in Table 1 and table 2, and detected by ELISA, the titers of 5 mice were all 1:(6.4×103), the highest titer of horse serum blocking solution was 1:(1.024×105), and the highest titer of pig serum blocking solution was 1:(1.28×104). The negative and blank detection values of pig serum blocking solution were higher than those of horse serum blocking solution, and the background value of pig serum blocking solution was higher. which is visible, horse Serum blocking liquid is better, so the experiment is selected from the selection of horse serum as a block. Horse serum was used as blocking fluid.

Discussion

Design of ZEN immunogen synthesis method. ZEN is a small molecule substance with reactivity, but it does not have immunogenicity. There is no active group that can be directly coupled with carrier protein. Therefore, it is necessary to modify the Zen molecule through the coupling vector protein, so that it induces the body to produce immunization response reaction , it is necessary to modify Zen molecule to induce immune response by coupling carrier protein (Cao et al., 2011). In this study, the ZEN molecule was modified to synthesize a hapten by the oximation method, and an artificial antigen was synthesized after coupling with the protein. In antigen synthesis, the reaction ratio between ZEN and EDC is ZEN : EDC = 1 : 2–3, and NHS should be less than or equal to the amount of EDC. NHS acts as a catalyst to activate and protect the carboxyl group. When catalyzing esters, it must be alkaline Under the conditions. Therefore, the EDC cannot be dissolved in DDW, and it is necessary to react under the alkaline system, and the pH should be controlled between 8.0–8.4, Dicyclohexylurea precipitation will be formed when excess NHS reacts with oxime and carrier. In this experiment, in the antigen synthesis, an EDC method for immune antigen is coupled to the carrier protein BSA, and the antigenic mixed acid anhydride method is detected to be coupled to the carrier protein OVA, thereby avoiding the production of bridge resistance. The key of immunoassay technology is to synthesize the artificial antigen with good immunogenicity, so as to obtain the antibody of zearalenone with high titer and strong specificity. Therefore, the preparation of artificial antigen is one of the most critical steps in the preparation of monoclonal antibody.

Table 1
Determination of immunogen indirect ELISA by horse serum blocking solution (OD450)

Number	Dilute multiple										negative	blank
	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400		
1	2.017	1.345	0.862	0.500	0.319	0.184	0.156	0.141	0.085	0.079	0.071	0.088
2	1.746	0.875	0.480	0.325	0.177	0.147	0.115	0.090	0.071	0.065	0.067	0.065
3	1.963	1.261	0.718	0.474	0.305	0.171	0.113	0.103	0.073	0.063	0.068	0.063
4	3.764	2.927	2.347	1.905	1.492	1.149	0.713	0.448	0.386	0.367	0.065	0.060
5	2.819	1.912	1.346	0.959	0.645	0.417	0.275	0.202	0.193	0.110	0.057	0.058

Table 2
Determination of immunogen indirect ELISA results in pig serum blocking solution (OD450)

Number	Dilute multiple										negative	blank
	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400		
1	1.329	0.798	0.684	0.503	0.388	0.213	0.144	0.099	0.072	0.072	0.099	0.082
2	0.902	0.572	0.281	0.157	0.134	0.121	0.111	0.087	0.082	0.068	0.088	0.084
3	1.308	0.882	0.724	0.573	0.400	0.303	0.211	0.089	0.078	0.072	0.094	0.083
4	1.481	1.129	0.892	0.761	0.524	0.304	0.177	0.138	0.101	0.094	0.095	0.089
5	1.374	0.848	0.619	0.443	0.273	0.209	0.179	0.119	0.104	0.071	0.098	0.092

Non-specific reactions about enclosed liquid horse serum. When different animals are used as sealing fluid, because the background value is different, the sealing fluid with lower background value should be selected, so that the influence of antiserum is small, the titer is high, and the gradient is obvious. When selecting serum, it is necessary to select the animal which is far from other animals to avoid the error of test results. Meanwhile, if the blocking serum contains the target protein and has the same source as the primary antibody, the blocking serum with the same source as the secondary antibody can be used. In addition, during animal feeding, pigs mainly eat feed and grain. A small amount of mycotoxins contained in feed or grain can directly enter pigs, which may cause the problem of animal homology. Horses mainly eat grass, which directly eliminates the problem of homology. Antibodies recognize antigenic determinants and then bind to them. Pig serum and horse serum are mainly blocked by protein, and the adsorption capacity of protein is very strong. When horse serum is used as the blocking solution, the background value is lower, and the non-specific reaction is weak. It can better block the remaining antigen sites on the enzyme plate and combine with the antigen determinants on the surface, so as to achieve better blocking effect (Gaimei et al., 2013).

In regard to antibody titer. According to the current research progress, the indirect ELISA method was used and optimized (Sompunga et al., 2019). Compared with pig serum, horse serum has higher titer and better stability. The highest titer was 1:1.024×105 (Yaning, 2017).The kit prepared by the indirect ELISA method prepared by corn bibarone is applied to production, and the detection effect will be improved. Support for the development of ZEN kits, promoting the development of enzyme-linked immunization techniques to some extent, is of great significance for human food safety issues, and has certain innovation, providing technical support for farming industry.

4. Conclusions

In the immunological analysis method, the preparation of the antigen is a key part of the antibody and the establishment of an immunological test method. Through the above identification results showed that the ZEN-BSA artificial antigen and ZEN-OVA coating antigen were successfully synthesized by antigen, and horse serum has higher titer and better stability. Thus, the results of this study are successfully established immunological testing methods, which laid a foundation for the preparation of monoclonal antibody.

Ethical approval and animal rights: All experiments were conducted in accordance with the “Guidelines for the Care and Use of Laboratory Animals” of the National Institutes of Health and were approved by the Animal Ethics Committee of Henan Institute of Science and Technology. The approval number is Issue No. 2020HIST047.

Author Contributions: Conceptualization, H. Fotina and Y. Wang; software, Y. Wang; formal analysis, data curation, Y. Wang; writing-original draft preparation, Y. Wang; writing-review and editing, H. Fotina; supervision, H. Fotina. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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