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DESIGN OF ANTIGEN SYNTHESIS AND PREPARATION AND CHARACTERIZATION OF SPECIFIC AND EURYTOPIC ANTIBODIES AGAINST B-GROUP AFLATOXINS

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The aim of this study was to prepare B-group aflatoxins(BGAFs) antibody with strong specificity and good eurytopicity. According to the molecular structure and active site of aflatoxin B1 (AFB_1), the BGAFs artificial antigen AFB_1-BSA was prepared by 6 methods such as oxime active ester(OAE),methylation of ammonia(MOA),mixed anhydride(MA),semi acetal(SA),epoxide(EP) and enol ether derivative(EED) and identified by UV and SDS-PAGE. Polyclonal antibodies against AFB_1(AFB_1 pAb) were prepared by immunizing New Zealand rabbits with AFB_1-BSA, and the titers of AFB_1 pAb was detected by indirect ELISA, the sensitivity of AFB_1 pAb was analyzed by indirect competitive ELISA(icELISA) and the specificity and eurytopicity of AFB_1 pAb was analyzed by cross-reactivity(CR) test. The results showed that AFB_1-BSA was synthesized successfully and the best one was OAE method among 6 synthesis methods of BGAFs artificial antigen and its conjugation ratio of AFB_1 to BSA was about 8.46:1. The immune efficacy of OAE method was the best, its AFB_1 pAb had high titers of 1:1(28×10^4)by indirect ELISA, a good sensitivity with the 50% inhibition concentration(IC_{50}) of 10.32 μg/L to AFB_1 by icELISA and a high CR to AFB_2 of 75.21%, AFG_1 of 44.13%, AFG_2 of 14.72%, AFM_1 of 16.36% and AFM_2 of 1.44%, respectively. In this study, AFB_1 pAbs with high titer, sensitivity, specificity and eurytopicity were prepared, which laid a matter and technical foundation for the establishment of BGAFs immunoassay.

Key word *B-group aflatoxins, antigen synthesis design, polyclonal antibody, characteristics analysis*
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Introduction. Aflatoxins (AFs) are a group of toxic secondary metabolites containing similar molecular structures (difuran ring and oxyheteronaphthalidone). They are produced by *Aspergillus flavus* and *Aspergillus parasiticus* through the polyketone pathway. At present, more than 20 members of the aflatoxin (AF) family have been found in food pollution. Among them, AF of group B (B group aflatoxins, BGAFs) have strong toxicity, wide pollution, and high content. Various toxic effects such as carcinogenicity, teratogenicity and immunosuppressiveness have become the main targets of food AF contamination detection (Sun D.D et.al., 2015). BGAFs include AFB_1 and AFB_2, both of which are closely related to food pollution, and both exist at the same time, mainly AFB_1, AFB_2 pollution is accompanied by AFB_1, and has a toxic additive effect (Luo, X. et. al., 2018). Therefore, there are two regulations for the maximum residue limits (MRLs) of BGAFs in foods. the current AFB1 MRL standard of foodand agricultural products in China is "GB 2761-2017 limit of fungal toxins in food" which One is that some countries including my country adopt AFB_1 MRLs, such as the current AFB_1 MRL standard of food and agricultural products in China is "GB 2761-2017 limit of fungal toxins in food" (CHINA. National Food Safety Standard Limit of mycotoxins in food. 2017), corn and its products ≤20 μg·kg^{-1}, rice and its products ≤10 μg·kg-1, wheat and its products ≤5 μg·kg-1. Second, some countries use MRLs for the total amount of BGAFs (B_1+B_2), such as EU ≤4 μg·kg^{-1}, Japan ≤10 μg·kg^{-1}, and US FDA ≤15 μg·kg^{-1}. There are many current analytical methods for food BGAFs contamination, mainly using instrumental analysis and immunoassay. In particular, immunoassay has become a technology because of its strong specificity, high sensitivity, simple operation, large-scale screening and on-site detection. Indispensable technical means, the key to establishing a BGAFs immunoassay method is to obtain excellent antibodies, and hapten design and antigen synthesis are the prerequisites for preparing excellent antibodies (Gefen T.et. al., 2015). There have been related reports on the research of BGAFs antigen synthesis methods at home and abroad (Mongkon, W. et. al., 2017, Xiao L.W, et. al., 2017), but there are no reports on the design of different hapten molecules, antigen synthesis and comparative analysis of antibody characteristics. In this study, AFB_1 was used as the starting material for the reaction. Polyclonal antibodies (pAbs) were prepared through different AFB_1 hapten molecular design and antigen synthesis methods, and their characteristics were analyzed to screen out the best hapten and antigen synthesis methods. It lays the foundation for the preparation of high-quality monoclonal antibodies of BGAFs with high sensitivity, broad recognition spectrum and strong specificity (Zhou, Y. et. al., 2007).

Aim The aim of this study was to prepare B-group aflatoxins (BGAFs) antibody with strong specificity and good eurytopicity.

Materials and Methods

Main reagents, solutions and experimental animals

AFB_1, AFB_2, AFG_1, AFG_2 standard products, Singapore Pribolab product; Cationized bovine serum albumin (cBSA), goat anti-rabbit enzyme-labeled secondary antibody (GaRIgG-

HRP), American Sigma product. The diluent used in the enzyme-linked immunosorbent assay (ELISA) is 0.01 mol·L^{-1} pH7.4 phosphate buffer solution (PBS); the washing solution is PBS containing 0.5 g·L^{-1} Tween^{-20} (PBST); the blocking solution is PBST containing 50 g·L^{-1} porcine serum; the coating solution is 0.1 mol·L^{-1} carbonate buffer solution (CBS) with pH 9.6. The experimental animals were 18 male New Zealand white rabbits at the age of 2 months and weighing 1±0.2 kg. They were provided by the Experimental Animal Center of Xinxiang Medical College. They were divided into 6 groups, each with 3 rabbits.

BGAFs artificial antigen synthesis design

According to the active sites on the molecular structure of AFB1 (Figure 1), the following six methods are proposed to prepare artificial antigen AFB1-BSA (Table 1).

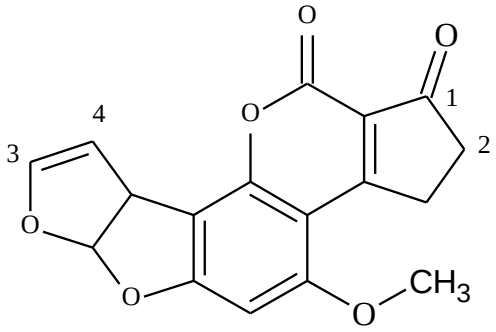
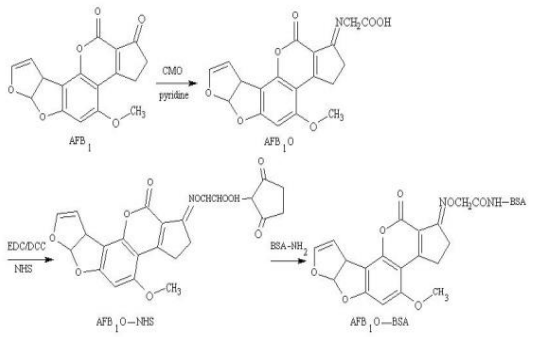
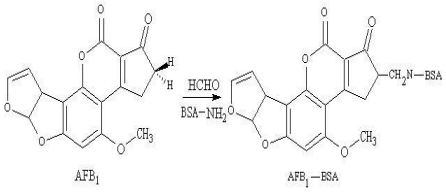
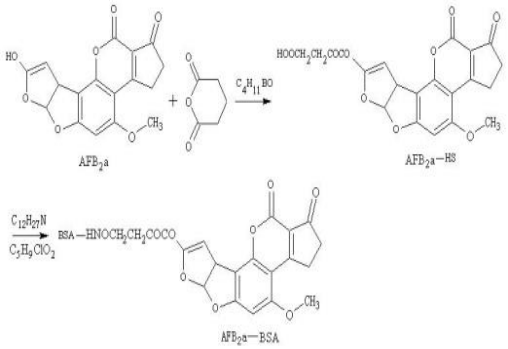
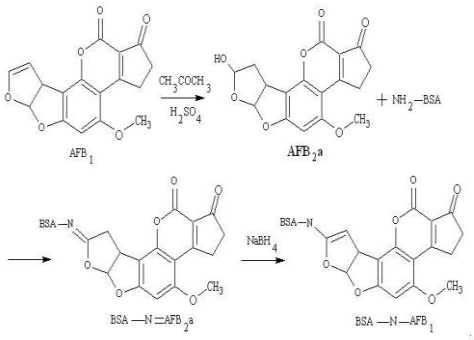
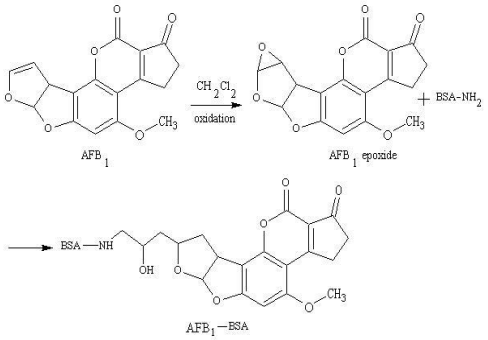
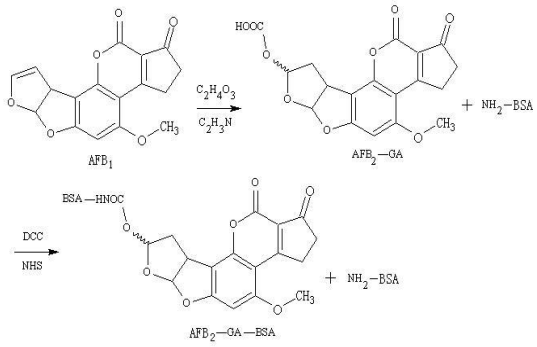


Fig.1 Molecular structure of AFB

Table1

The hapten design and antigens synthesis of AFB1

Antigen synthesis design					Antigen synthesis route	Reaction principle
Active site	Active group	Reaction method	Introduction group	Synthesis Method		
1	Carbonyl	Oximation	carboxyl	OAE		The oximation of the 1-position carbonyl group of the active site of AFB_1 to AFB_1O, the introduction of the carboxyl active group, the active ester method under the action of the coupling agent dicyclohexylcarbodiimide (DCC), the synthesis of AFB_1O and BSA in the form of a single amide bond AFB_1-BSA [7,8].
2	Active hydrogen	Mannich	Aminomethyl	MOA		Using the 2-position α-active hydrogen of AFB_1, through Mannich reaction, the α-active hydrogen and the amino group of BSA undergo an aminomethylation reaction, which is coupled in the form of Mannich base to synthesize AFB_1-BSA [9,10].

Antigen synthesis design					Antigen synthesis route	Reaction principle
Active site	Active group	Reaction method	Introduction group	Synthesis Method		
3	Hydroxyl	Acid anhydride reaction	carboxyl	MA		AFB1 is converted to AFB_2a under the action of H_2SO_4. The 2-position hydroxyl of the active site is used to react with acid anhydride. The product is a half-ester compound AFB_2a-HS. The carboxyl active group is introduced, and the coupling agent isobutyl chloroformate (IBC), AFB_1O and BSA synthesize AFB_1-BSA in the form of a single amide bond [11,12].
3	Aldehyde	Schiff	Aldehyde	SA		The condensation reaction of AFB1 under the action of H_2SO_4 produces AFB_2a with active sites of aldehyde groups, whose aldehyde groups can form unstable Schiff bases with the amino groups of BSA. Through the reduction of $NaBH_4$, the antigen AFB_2a-BSA is synthesized [13,14].
3、4	Bifuran ring	Oxidation	Hydroxyl	EP		Using dichloromethane as solvent, the double bond of AFB_1 bifuran ring is oxidized to form AFB_1 epoxide, which reacts with the primary amine of BSA to form secondary amine, introduces a hydroxyl group on the epoxide, and couples with BSA in the form of monoamide Into AFB_1-BSA [15,16].
3、4	Bifuran ring	Glycolic acid	carboxyl	EED		The molecular structure of AFB_1 contains an active site bifuran ring, which can react with glycolic acid to generate AFB_1 enol ether derivative (AFB_1-GA) with active carboxyl group, which is used to couple the carboxyl group with BSA to synthesize AFB_1-BSA [17].

BGAFs artificial antigen identification

UV Scan

Dissolve AFB_1 with methanol, prepare 1 $mg \cdot mL^{-1}$ AFB_1 solution; use volume ratio (v/v) 4:6 methanol PBS solution to dissolve BSA and AFB_1-BSA, prepare 1 $mg \cdot mL^{-1}$ BSA and AFB_1- BSA solution; UV scan at a wavelength of 200 ~ 500 nm, through the calculation formula A = εCL (where A is the absorbance value, read by the instrument; ε is the molar extinction coefficient, which is a constant value; C is the solute concentration in the solution; L is Optical path, determined by the instru-

ment), calculate the molecular binding ratio of AFB_1 and BSA (Wang Y.N. et. al., 2014).

SDS-PAGE identification

The concentration of the concentrated gel and the separating gel are selected to be 5% and 12%, the voltage is 90 v and 60 v, the sample volume is 10 μL per well, and the protein content is 10 μg per well. The UV analyzer system software calculates AFB_1 and BSA the molecular binding ratio.

Preparation of AFB1 pAb

The artificial antigens synthesized by 6 different methods were used to immunize New Zealand white rabbits. Each antigen was used to immunize 1 group, a total of 6 groups, 3 rabbits in each group. The immunization dose is calculated according to the amount of protein BSA in AFB1-BSA, each is 100 μg, the volume is 1 mL, the back is injected subcutaneously at 4 to 6 points, a total of 5 immunizations, each interval is 3 to 4 weeks, after the fifth immunization for 2 weeks, blood was collected from the ear vein, the polyantiserum was separated by centrifugation, and the polyantiserum was purified by the saturated ammonium sulfate salting-out method to prepare AFB1 pAb (Ju RH et. al., 2015).

Characteristic analysis of AFB1 pAb

Determination of potency

Indirect ELISA (Zhao HH et. al., 2015).

Sensitivity identification

Indirect competitive ELISA (icELISA) measures the half inhibitory concentration (IC50) of AFB1 pAb on AFB1 to determine sensitivity (Chen T et. al., 2014).

Specific identification

With AFB1, AFB2, AFG1, and AFG2 as inhibitors, the IC50 of each inhibitor was determined by icELISA, and the percentage of the IC50 of AFB1 pAb to AFB1 and the IC50 of other inhibitors was used as the cross-reaction rate (CR%) (Zhang C et. al., 2016), the calculation method is CR% = IC50 of AFB1 pAb to AFB1/ IC50×100 of AFB1 pAb to other inhibitors.

Results

GAFs artificial antigen identification results

UV identification

The results are shown in Figure 2. In the range of UV200-500 nm, the characteristic peak of BSA is at 278 nm, and the characteristic peak of AFB1 is at 363 nm. The artificial antigen AFB1-BSA is synthesized by 6 methods including OAE, MOA, MA, SA, EP, EED. Both contain the characteristic peaks of BSA and AFB1, indicating that the above 6 methods can synthesize artificial antigen AFB1-BSA. The calculated results of the molecular binding ratio of BSA to AFB1 (Liu, H.X. et. al., 2014) are shown in Table 2.

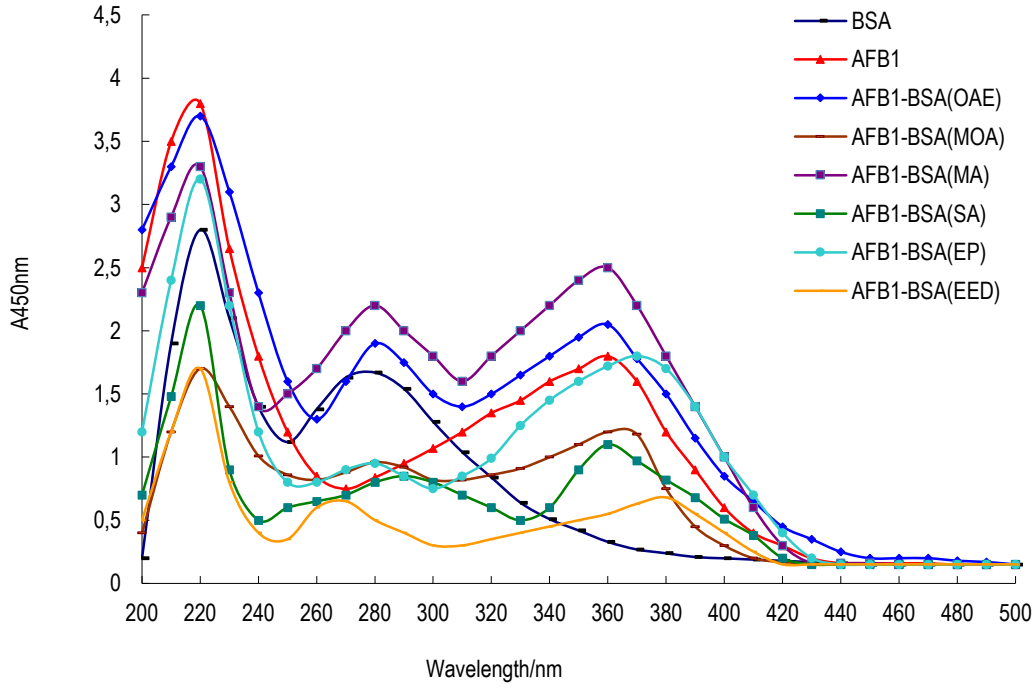


Fig.2 UV spectra of AFB_1-BSA

Table2

Molecular binding ratio of AFB_1-BSA prepared by six methods

Synthesis methods	Initial molar ratio of AFB_1 to BSA	Molecular binding ratio of AFB_1-BSA	Usage ratio of AFB_1
OAE	50:1	8.64:1	17.28
MOA	50:1	6.88:1	13.76
MA	50:1	10.78:1	21.56
SA	50:1	4.46:1	8.92
EP	50:1	6.38:1	12.76
EED	50:1	2.31:1	4.62

Note: Compared to the molecular weight of BSA and AFB_1, BSA is 66.446, AFB_1 is 312, BSA is much larger than AFB_1, so the utilization rate of BSA is 100% when the utilization ratio is calculated.

SDS-PAGE identification

The results are shown in Figure 3. It can be seen that

the bands of the 6 artificial antigens AFB1-BSA lag behind the bands of BSA, indicating that the molecular weight of AFB1-BSA is greater than that of BSA, and it can be determined that the synthesis of AFB_1-BSA is successful.

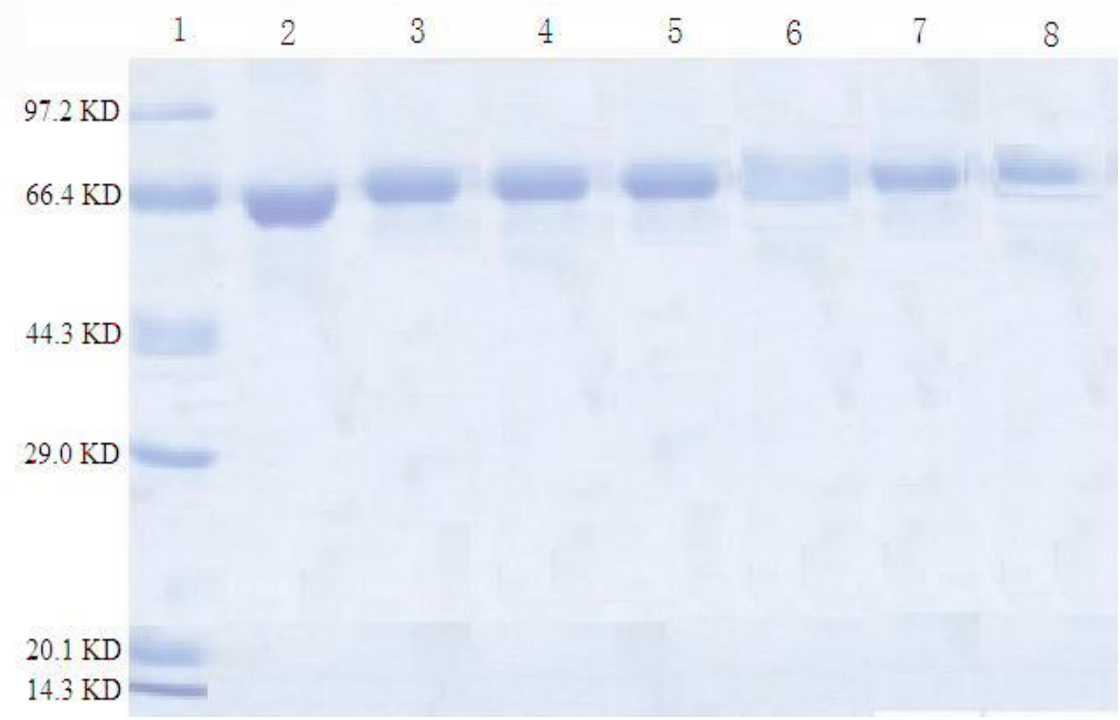


Fig.3 The SDS-PAGE photo of AFB_1-BSA

1. Maker; 2.BSA; 3. AFB_1-BSA(OAE); 4. AFB_1-BSA(MOA); 5. AFB_1-BSA(MA); 6. AFB_1-BSA(SA) ; 7. AFB_1-BSA(EP) ; 8. AFB_1-BSA(EED).

AFB1 pAb characteristic analysis

Determination of potency

The results are shown in Figure 4. It can be seen that after the immunization, one rabbit with the highest indirect ELISA titer was selected for comparison and analysis in each group. The indirect ELISA titer of the 6 immunized rabbits all reached 1: (1×10^4). It can be seen that the 6 types The AFB_1-BSA synthesized by the method has good immunogenicity. The OAE group and MA group have the best immune effect, with a titer of 1: (1.28×10^4).

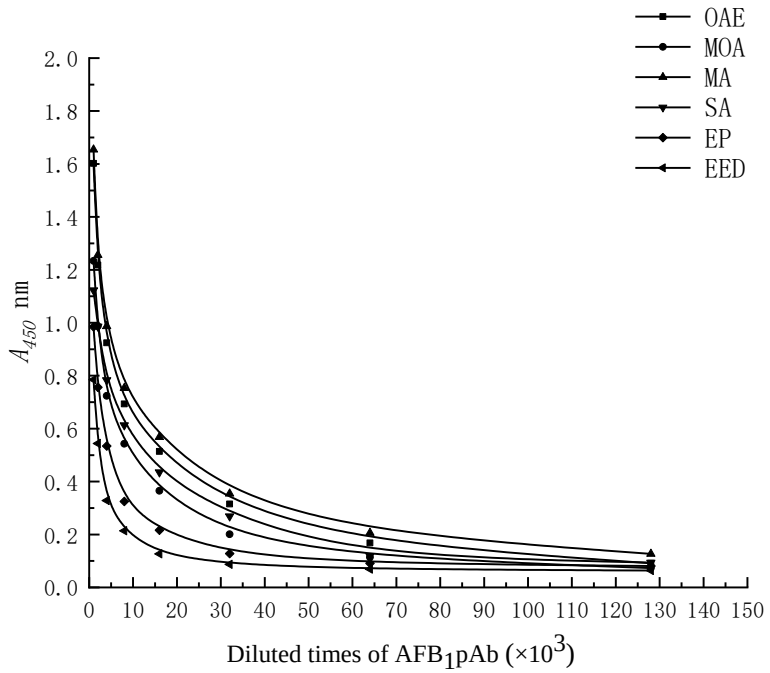


Fig.4 The indirect ELISA titer curves of AFB_1 pAb

Sensitivity analysis

The results are shown in Figure 5. It can be seen that the icELISA inhibition curve of 6 immunized rabbits has a good linear relationship. The OAE group has the best sensitivity, with an IC_{50} of 10.32 μg·kg^{-1}. The sensitivity of the other groups is inferior to that of the OAE group.

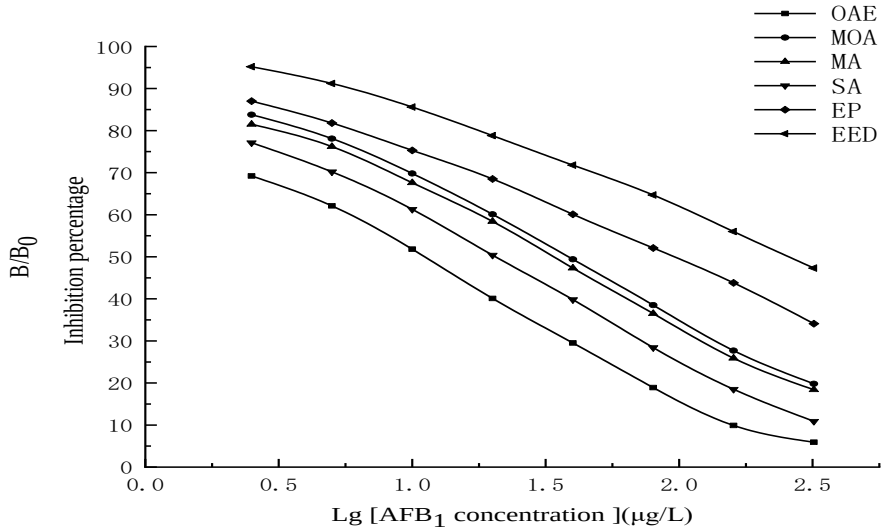


Fig.5 The sensitivity measurement of AFB_1 pAb to AFB_1 by icELISA

Table 3

The regression equation, R^2 and IC_{50} of4 AFB_1 pAb to AFB_1 by icELISA

group	Regression equation	R^2 value	IC_{50}（μg·kg^{-1}）
OAE	y＝－32.171x＋82.621	0.9916	10.32
MOA	y＝－31.822x＋99.59	0.9943	36.18
MA	y＝－31.546x＋97.263	0.9938	31.49
SA	y＝－32.875x＋92.292	0.9966	19.36
EP	y＝－25.245x＋99.481	0.9932	91.21

Specificity and broad-spectrum analysis

The results are shown in Table 4. It can be seen that the antibodies prepared by the six methods can recognize AFB_1 100%, and the OAE method has the best specificity and broad-spectrum, with an IC_{50} of 10.32 μg·kg^{-1} and a CR with AFB_2 of 86.46%; The CR with AFG_1 and AFG_2 were 44.13% and 14.72%, respectively. Antibodies prepared by other methods have good specificity and can recognize AFB_1 100%, but their sensitivity and broad-spectrum are not as good as those prepared by OAE method. The results show that the best antigen synthesis method for preparing antibodies against BGAFs with high sensitivity, strong specificity and good broad spectrum is the OAE method.

Table 4

The percent cross-reactivity of AFB1 pAb with AFB1、AFB2、AFG1、AFG2

AF	AFB_1 pAb(OAE)		AFB_1 pAb(MOA)		AFB_1 pAb(MA)		AFB_1 pAb(SA)		AFB_1 pAb(EP)		AFB_1 pAb(EED)	
	IC_{50} (μg·kg^{-1})	(%) CR	IC_{50} (μg·kg^{-1})	(%) CR	IC_{50} (μg·kg^{-1})	(%) CR	IC_{50} (μg·kg^{-1})	(%) CR	IC_{50} (μg·kg^{-1})	(%) CR	IC_{50} (μg·kg^{-1})	(%) CR
AFB_1	10.32	100	36.18	100	31.49	100	19.36	100	91.21	100	307.81	100
AFB_2	13.72	75.21	46.03	78.61	46.85	67.22	23.83	81.26	144.14	63.28	590.13	52.16
AFG_1	23.39	44.13	＞10^3	＜0.5	＞10^3	＜0.5	35.67	54.27	＞10^3	＜0.5	＞10^3	＜0.5
AFG_2	70.11	14.72	＞10^3	＜0.5	＞10^3	＜0.5	79.41	24.38	＞10^3	＜0.5	＞10^3	＜0.5
AFM	63.08	16.36	741.39	4.88	615.04	5.12	541.74	3.68	＞10^3	＜0.5	＞10^3	＜0.5
AFM	716.67	1.44	＞10^3	＜0.5	＞10^3	＜0.5	＞10^3	＜0.5	＞10^3	＜0.5	＞10^3	＜0.5

Discussion and Conclusion

About the design of BGAFs antigen synthesis method

The molecular weights of AFB_1 and AFB_2 in BGAFs are 312.27 and 314.29, respectively. They belong to small molecule haptens and have no immunogenicity. According to the theory of hapten-carrier effect, only by combining with large-molecule protein carriers to form artificial antigens can they be specific for haptens. Therefore, the design of antigen synthesis methods is very important (Zeng, H. et. al., 2014). Since the selection of different active sites and the introduction of different linking arm lengths will have a greater impact on the properties and structure of small molecules, which in turn will affect the quality of antibodies produced (Shi HY et. al., 2006). According to the molecular structure characteristics of BGAFs, this study selected the 1-position carbonyl group, 2-position active hydrogen, 3-position hydroxyl group and aldehyde group, and the difuran ring between 3-position and 4-position as the active groups. Through different chemical reaction methods, respectively introduce available carboxyl, hydroxyl, aminomethyl and other active groups to realize the coupling with carrier protein to synthesize

artificial antigens.

About the synthetic route of BGAFs artificial antigen

At present, the research on BGAFs artificial antigen synthesis method is still at the empirical level, and trial and error methods are mostly used. Although a variety of artificial antigen identification methods have been established, the immunogenicity of the artificial antigens prepared is ultimately through the effect of animal immunity. It was confirmed (Guo N. F. et. al., 2014). Based on a large number of relevant research literature, this article uses AFB_1 as the starting material for the reaction, and uses 6 methods such as OAE method, MOA method, MA method, SA method, EP method and EED method to synthesize artificial antigens, and through UV, SDS-PAGE for antigen identification and animal immunization for antibody characteristics analysis, the most ideal antigen synthesis method for the preparation of BGAFs antibody was selected by OAE method. Its advantages are that the reaction system is easy to construct, the reaction conditions are mild, the operation steps are simple, and the product yield is high. However, in terms of the advanced nature of the technical route adopted in this research, the research and application of molecular simulation technology, computer-aided technology, etc. Needs to be improved (Morita, I. 2017).

Analysis on the immune effect of BGAFs artificial antigen.

The purpose of this research is to screen out BGAFs artificial antigen synthesis methods, and lay the material and technical foundation for the preparation of high-quality BGAFs antibodies with high sensitivity, strong specificity and broad recognition spectrum. This requires that in the design of BGAFs antigen synthesis, on the one hand, it is necessary to consider the specificity and sensitivity of the antibody to AFB_1 to meet the detection technology requirements under the AFB_1 limit standard; On the other hand, it is necessary to consider the sensitivity and broad-spectrum of the antibody to AFB_2 to meet the technical requirements for detection under the BGAFs limit standard (Xie Hui et al. 2017) used MA method to synthesize AFB_1-BSA, and screened hybridoma cell 3B9 to obtain AFB_1 mab. The antibody specifically recognizes AFB_1 with a sensitivity of 1.04 $\mu g \cdot kg^{-1}$, CR of AFB_2, AFG_1, AFG_2, and AFM_1 are 2.2%, 33.9%, 1.8%, and 5.12%, respectively, which have no CR with AMF_2 and poor broad-spectrum. Xiao Zhi et al. used SA method to synthesize AFB_1-BSA, and screened hybridoma cell 3A12 to obtain AFB_1 mab. The antibody specifically recognizes AFB_1 with a sensitivity of 6.1 $\mu g \cdot kg^{-1}$, and is compatible with CR of AFB_2, AFG_1, AFG_2, and AMF_1. They are 7.8%, 20.2%, 0.6%, and 3.68%, respectively. It has no CR with AFM_2, and it also has the problem of poor broad-spectrum.

Conclusion

The results of 6 different antigen synthesis methods and the characteristics of the antibodies produced showed that the OAE method was the best, the produced AFB_1 pAb antibody titer was high, and the indirect ELISA titer reached 1: (1.28×10^4); the sensitivity to AFB_1 was good, IC_{50} is 10.32 $\mu g \cdot kg^{-1}$; it has strong specificity and can recognize AFB_1 100%. The CR with AFB_2, AFG_1, AFG_2, AMF_1, and AFM_2 are 75.21%, 44.13%, 14.72%, 16.36% and 1.44%, respectively. The other five methods designed by this research have certain defects in varying degrees. Therefore, the author recommends that they should not be used except for research work.

In this study, based on the molecular structure characteristics of AFB_1 and the existing active sites, six BGAFs antigen synthesis methods were designed, through UV, SDS-PAGE identification and analysis of the characteristics of AFB_1 pAb produced by immunized animals, a high-titer, sensitive, specific, and broad-spectrum AFB_1 pAb was obtained. It shows that antigen synthesis design is the prerequisite for the preparation of high-quality antibodies, and the OAE method is an effective way to realize the preparation of high-quality antibodies for BGAFs, laying a material and technical foundation for the establishment of BGAFs immunoassay methods.

Author's contributions

All authors participated in this article design. Yanan WANG participated and performed writing and data collection. All authors read and approved the final manuscript. All authors contributed to the draft of the manuscript. All authors gave final approval for publication.

Conflict of interest Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Синтез та підготовка антигенів для отримання специфічних і евритопних антитів проти B-group афлатоксинів

Метою цього дослідження було вироблення антитіл до афлатоксинів групи B (BGAF) із сильною специфічністю та хорошою евритопічністю. Дослідження проводили в лабораторії безпеки та якості продуктів тваринництва Сумського НАУ, факультету ветеринарної медицини, Суми, Україна та на базі Науково-технічного інституту Хенань, Сіньсян, Китай. Відповідно до молекулярної структури та активного центру афлатоксину B1 (AFB1), штучний антиген BGAFs AFB1-BSA готували 6 ма методами, такими як метод активного ефіру оксиму (OAE), метилювання аміаку (MOA), змішаний ангідрид (MA), напівфабрикат ацеталь (SA) епоксид (EP) та похідне енолового ефіру (EED) та ідентифікували за допомогою УФ та SDS-PAGE.

Поліклональні антитіла проти AFB1 (AFB1 pAb) готували шляхом імунізації новозеландських кролів AFB1-BSA, а титри AFB1 pAb виявляли за допомогою непрямого ІФА, чутливість AFB1 pAb аналізували за допомогою непрямого конкурентного ІФА (icELISA), специфічність та еурітопічність AFB1 pAb аналізували за допомогою тесту перехресної

реактивності (CR). Результати показали, що AFB1-BSA був успішно синтезований, і найкращим був метод активного ефіру оксиму (OAE) із 6 методів синтезу штучного антигену BGAF, а його відношення кон'югації AFB1 до BSA становило близько 8,46À1. Імунна ефективність методу OAE була найкращою, його pAb AFB1 мав високі титри 1: (1,28×104) з використанням методу непрямого ІФА, чутливість з 50% концентрацією інгібування (IC50) 10,32 мкг / л до AFB1 за допомогою icELISA та високий CR до AFB2 75,21%, AFG1 44,13%, AFG2 14,72%, AFM1 16,36% та AFM2 1,44% відповідно. У цьому дослідженні були підготовлені pAbs AFB1 з високим титром, чутливістю, специфічністю та еуритопічністю, що заклало важливу та технічну основу для створення імунологічного аналізу BGAF.

Ключові слова: *афлатоксини групи В, конструкція синтезу антигену, поліклональні антитіла, аналіз, характеристики.*

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