Investigation of the Properties of Covalent Immobilized Anti-aflatoxin B1 Antibody on Membranes from Copolymer of Polyacrylamide-polyacrylonitrile

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***Abstract*:** *Aflatoxins are toxic secondary metabolites produced by a number of different fungi (Aspergillus flavus, Aspergillus parasiticus), and can be present in a wide range of food and feed commodities. The most used methods for analysis of aflatoxins are thin-layer chromatography, high-performance liquid chromatography (HPLC), electrochemical immunoanalysis and microtitre plate enzyme-linked immunosorbent assay (ELISA). Membranes from copolymer of polyacrylamide-polyacrylonitrile have been prepared. These membranes were used as a matrix for a covalent binding of polyclonal anti-aflatoxin B1 antibody. ELISA was carried out with these membranes to prove successful immobilization of the antibody. It was done comparative analysis with ELISA between standard microtiter plate and our membranes with infected peanuts.*

Keywords: *Aflatoxins, Polyclonal antibody, Immobilization, ELISA.*

Introduction

Aflatoxins are toxic fungal metabolites that contaminate a wide range of agricultural products (cereal grains, oil seeds, dried fruits, apple juice and others) and meat products from animals fed contaminated meal. Many aflatoxins are highly resistant, and survive food processing, and therefore enter the food chain and provide a threat to human health. They are listed as group I carcinogens by the International Agency for Research on Cancer (IARC). Aflatoxin B1 (AFB1) is the most significantly occurring and toxic compound. Aflatoxins are regulated in many countries and legal limits ranging from 0-50 $ng \cdot g^{-1}$ has been established for the compounds. The current maximum levels set by European Commission are 2 $ng \cdot g^{-1}$ for AFB1 and 4 $ng \cdot g^{-1}$ total aflatoxins for groundnuts, nuts, dried fruits, and cereals [1, 4, 5].

Current analysis of aflatoxins is performed by a variety of methods including mainly thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) [2], overpressured-layer chromatography, and enzyme-linked immunosorbent assay (ELISA). Chromatographic analysis is widely accepted as an official method for aflatoxin determination. Although sensitive and accurate, most of the chromatographic methods developed are laborious, expensive, time-consuming, and unsuitable for analysis of many samples; they also require sophisticated equipment and extensive clean-up procedures [7, 12].

Immunoassays provide a simple and economical alternative to instrumental methods for mycotoxin analysis. Application of immunochemical methods, especially ELISA, in the surveillance of aflatoxins is becoming more widespread, because of the sensitivity, specificity, rapidity, simplicity, and cost-effectiveness of the methods. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific aflatoxin. The direct competitive ELISA is commonly used in aflatoxin analysis [6, 8, 9].

The aim of the present study is immobilized of anti-aflatoxin B1 antibodies on membranes from copolymer polyacrylamide-polyacrylonitrile and an investigation of the properties of these membranes.

Materials and methods

Obtaining of membranes from copolymer of polyacrylonitrile-polyacrylamide and cellulose acetate butyrate

The particles from copolymer of polyacrylonitrile-polyacrylamide were obtained according to the method used in [10]. Then 0.100 g from obtained copolymer was diluted in 1 ml dimethylformamide (DMF) and the solution was stirred for 15 min on magnetic stirrer. At the same time 2.0 g of cellulose acetate butyrate was diluted in 20 ml DMF and the solution was stirred for one hour on magnetic stirred. The two solutions were mixed and stirred 2 hours. The obtained solution was placed in Petri and was dried at room temperature. The received membrane is thin, transparent and stable.

Method of oxidation of anti-aflatoxin B1 antibody

Polyclonal anti-aflatoxin B1 antibody was obtained from Sigma. The working concentration of aflatoxin B1 antibody was 0.67 $mg \cdot ml^{-1}$. The oxidation of carbohydrate moieties of antibody with periodic acid (0.04 mM in 0.05 mM acetate buffer, pH 5.0) was performed according to Zabrosky and Ogletree [11]. The unreacted periodic acid was removed with 0.025 mM ethylene glycol. The oxidized antibody was dialyzed against 50 mM phosphate buffer with pH 6.0 for 18-24 h.

Covalent immobilization of anti-aflatoxin B1 antibody on membranes from copolymer of polyacrylonitrile-polyacrylamide and cellulose acetate butyrate

The immobilization of aflatoxin B1 antibody was performed in the following manner: 3 ml of oxidized dialytic solution of antibody was added to membranes. The process was implemented for 24 hours at $t = 4°C$, in dark.

Verification of the properties of the immobilized anti-aflatoxin B1 antibody

First it was carried out reaction for presence of immobilized antibody on membranes. The immobilized membranes were blocked with 2.5% bovine serum albumin (BSA) and stay two hours in this solution. It was prepared four dilutions of goat anti-rabbit antibody (Sigma)

marked with peroxidase – 1:100; 1:200; 1:400; 1:800 and 1:1600. The membranes were put in those solutions for one hour and then they were washed with solution of PBS and Tween 20. For visualization of the reaction was added solution of orthophenylenediamine (2.5 mg OPD, 6 ml citrate buffer, 10 μl 30% H_2O_2) and then for stopping the enzyme act was used 300 μl 10% H_2SO_4.

Furthermore was carried out qualitative analysis of the immobilized antibodies for the specific places of connection with antigens. For this reaction were used reagents and procedures from kit № R1211, Ridascreen.

ELISA method with standard kit and different blocked membranes from PAN-PAA copolymer

It was carried out competitive enzyme-linked immunosorbent assay with standard kit № R1211, Ridascreen. The kit contains microtiter plate (12 strips with 8 removable wells each) coated with antibodies against aflatoxin B1; standard solutions of aflatoxin B1; peroxidase conjugated aflatoxin B1; anti-aflatoxin B1 antibody; substrate/chromogen solution; stop solution (1N H_2SO_4).

Procedures were implemented how is described in the standard kit for two methods with membranes and without membranes. The membranes were blocked by two ways with BSA, and with Tween 20.

It was used infected peanuts for comparison between ELISA with standard microtiter plate and ELISA with our membranes. For conducting the ELISA was done previous extraction of these peanuts according to instruction in the standard kit.

Results and discussion

The membranes obtained according to the methodological part have average thickness 0.6-0.8 mm (Fig. 1).

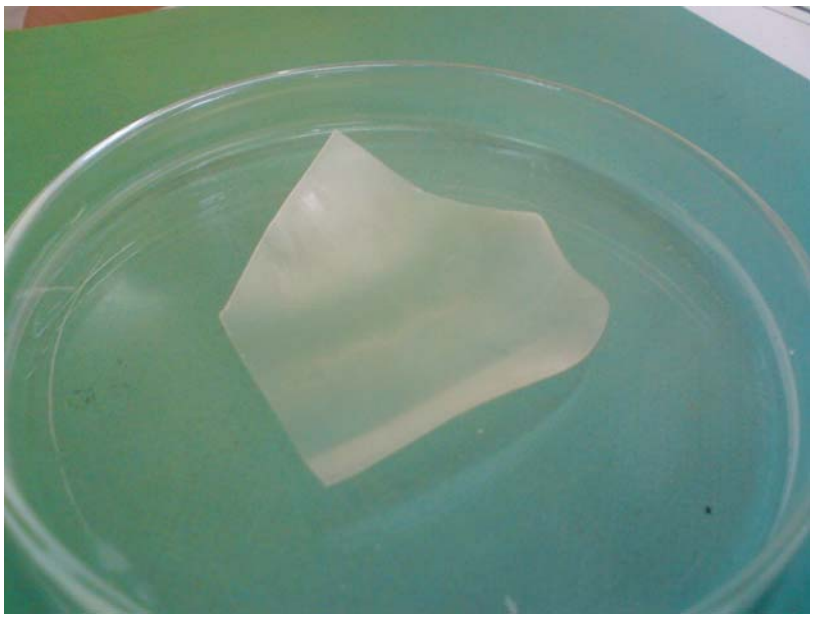

Fig. 1 Picture of obtained membrane

These membranes possess high mechanical stability which is a necessary condition to obtain the desired shape and size relevant to the requirements of the experiment. The mechanical stability of the membranes was preserved throughout the process. The membranes from copolymer were used as a matrix for covalent binding of the aflatoxin B1 antibody.

The analysis of the properties of immobilized antibody was carried out according to the methodological part. The reaction with specific goat antirabbit antibody is presented of Fig. 2.

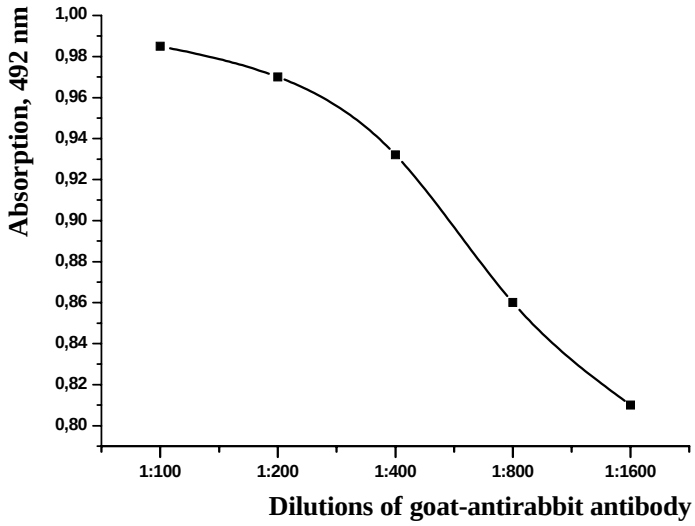

Fig. 2 Curve of different dilutions of goat-antirabbit antibody

It was observed clear difference in received absorption by different dilutions of the goat antibody whence can judge that have presence of immobilized antibody on membrane. From the data in Fig. 2 was seen that the received curve is sensitive to added conjugate goat antibody-peroxidase.

Moreover, qualitative reaction for examination of the paratope of immobilized antibody is carried out. This reaction was implemented with solution from kit № R1211, Ridascreen and from the positive result of the experiment we established that the antibody save its properties to epitope of antigen (aflatoxin B1).

From the analysis of ELISA with standard microtiter plate of the kit is obtained the standard curve which is showed on Fig. 3.

How it is seen from the graphic the received standard curve is with linear range from 1 to 50 $\mu g \cdot l^{-1}$. For comparison of the linear range was used data from Kolosova *et al.* which use monoclonal antibody for their experiments [3]. The data are showed in Table 1.

It can be seen from the table that has displacement in linear range of the polyclonal antibody from Sigma to higher values of lower limit of sensitivity.

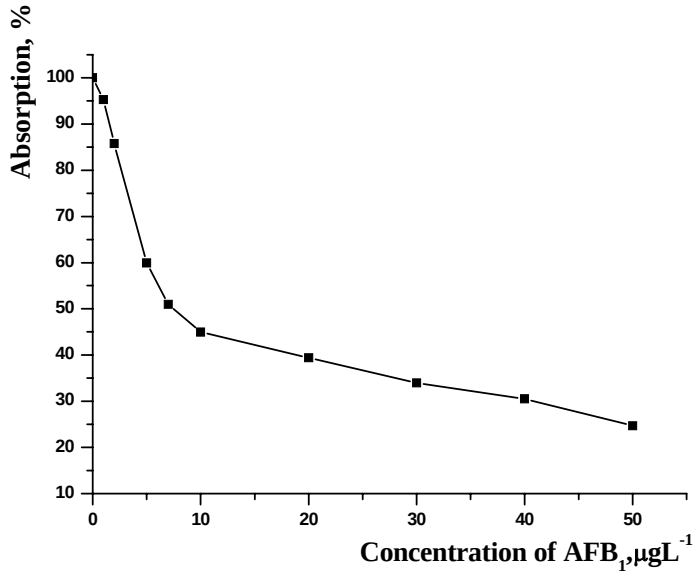

Fig. 3 Standard curve for aflatoxin B1 maked with kit № R1211, Ridascreen

Table 1. Results from the comparative analysis of the linear ranges

Analyses	Linear range, μg·l^{-1}
ELISA with standard kit № R1211, Ridascreen	1-50
ELISA with polyclonal anti-aflatoxin B1 antibodies immobilized on membranes blocked with BSA	1-50
ELISA with polyclonal anti-aflatoxin B1 antibodies immobilized on membranes blocked with Tween 20	1-50
ELISA with monoclonal antibody 34 [3]	0.1-10
ELISA with monoclonal antibody 78 [3]	0.1-1

Then the covalent immobilization of anti-aflatoxin B1 antibody on the membranes from the copolymer of polyacrylamide-polyacrylonitrile was done the ELISA with solutions from the standard kit. Before the ELISA membranes were blocked by two ways – with 2.5% BSA and with 0.2% Tween 20.

On the Fig. 4 are showed the results received from the ELISA maked with polyclonal anti-aflatoxin B1 antibody immobilized on membranes blocked with 2.5% BSA. From the graphic is seen that the linear range received with these membranes is unchanged in comparison with standard curve obtained with the standard kit – from 1 to 50 μg·l^{-1}. The values of absorption in the linear range are increased toward standard kit, e.g. by 5 μg·l^{-1} – 66%; 50 μg·l^{-1} – 37.87%.

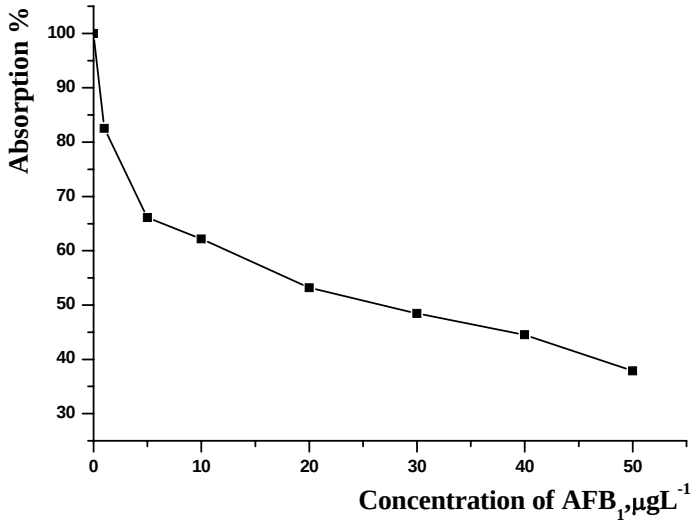

Fig. 4 Standard curve for ELISA maked with polyclonal anti-aflatoxin B1 antibody immobilized on membranes blocked with 2.5% BSA

On the next figure are showed the results received from the ELISA maked with polyclonal anti-aflatoxin B1 antibody immobilized on membranes blocked with 0.2% Tween 20.

From the graphic is seen that the linear range received with these membranes is unchanged in comparison with standard curve. The values of absorption in the linear range are increased toward standard kit, e.g. by 5 $\mu g \cdot l^{-1}$ – 74%; 50 $\mu g \cdot l^{-1}$ – 45%.

Moreover were done ELISA analysis with infected peanuts and was determined the quantity of aflatoxin B1. There are three samples with followed concentration of aflatoxin B1 – 0 $\mu g \cdot l^{-1}$, 2 $\mu g \cdot l^{-1}$ and 4 $\mu g \cdot l^{-1}$. The obtained results are given in Table 2.

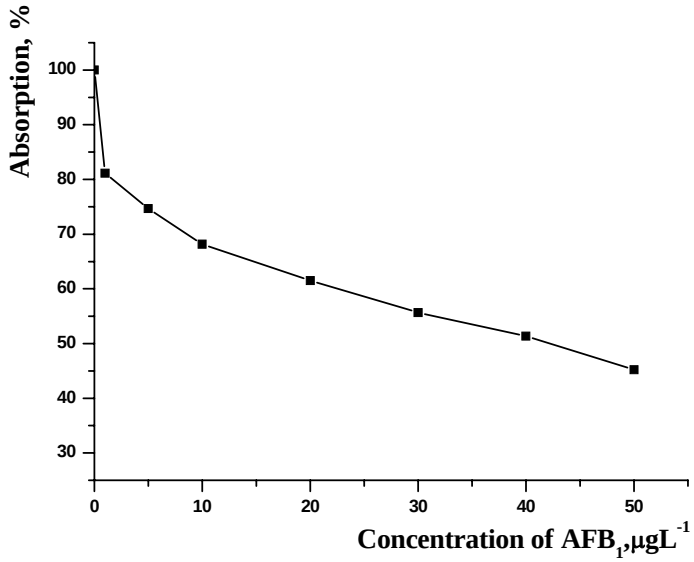

Fig. 5 Standard curve for ELISA maked with polyclonal anti-aflatoxin B1 antibody immobilized on membranes blocked with 0.2% Tween 20

Table 2. Results from the ELISA with infected peanuts

Sample $\mu g \cdot l^{-1}$	Data from ELISA with standard kit, $\mu g \cdot l^{-1}$	Data from ELISA with membranes blocked with Tween 20, $\mu g \cdot l^{-1}$
0	0.00	0.00
2	1.92	1.87
4	3.85	3.78

The obtained values for the two different ELISA are near to this which we have in the infected samples. Therefore it can be concluded that the membranes with immobilized polyclonal anti-aflatoxin B1 antibody can use in determination of aflatoxin in different infected products.

Conclusions

Membranes coated with copolymer of acrylamide and acrylonitriles were successfully obtained. It was successfully immobilized polyclonal anti-aflatoxin B1 antibody on these membranes by the investigation of a paratope it was established that the antibody save its properties to epitope of antigen.

It was defined linear range of standard ELISA kit and ELISA with immobilized antibody on membranes. In the two cases the obtained results was from 1 to 50 $\mu g \cdot l^{-1}$. However in comparison with results from others authors [3] the bottom limit of sensitivity is high.

The obtained membranes can be use in developing of immunosensor for aflatoxin B1. By the construction of immunosensor must be use more sensitive method for detection as is surface plazmon resonance.

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