Determination of the optimal conditions of anti – AFM1 antibody immobilization and AFM1 immunoassay in milk

Milka Atanasova, Nastya Vasileva, Tzonka Godjevargova

Abstract: *The optimal conditions for anti – AFM1 antibody immobilization onto magnetic nanoparticles were determined by varying the initial antibody concentration.The optimal concentration of MNPs-mAb and AFM1-fluorescent conjugate for each sample were also determined. The optimization of these conditions provides a sensitive and high selective immunofluorescent assay for AFM1 detection in milk.*

Key words*: AFM1, immunofluorescent assay, milk, antibody immobilization*

INTRODUCTION

Aflatoxins are highly toxic mycotoxins produced by some species of *Aspergillus* (*Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*), which may contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favorable to mold growth. Aflatoxin M1(AFM1) is a hydroxylated metabolite of aflatoxin B1, formed when ruminants ingest feed contaminated with aflatoxin B1. Aflatoxin M1 is then excreted in milk [5,6] and because it is relatively stable during milk pasteurization or other thermal treatments, it can also be present in milk-derived dairy products, such as cheese and yogurt [2,3].

The current maximum level set by the European Union is 0.05 μg/kg for Aflatoxin M1 in milk [1]. To minimize the occurrence of AFM1, it is essential to trace the sources of contamination using rapid, selective, sensitive and cost effective assays.

MATERIALS AND METHODS

Reagents and chemicals

Aflatoxin M1 from LKT laboratories, Acetonitrile, Bovine serum albumin (BSA) , (3-aminopropyl) triethoxysilane (APTES) and Glutardialdehyde from Sigma Aldrich. Aflatoxin M1-antibody from MyBiosource, Tween 80 from Scharlau Chemie S.A.

Immobilization of anti-AFM1antibody onto the magnetic nanoparticles

The preparation and functionalization of magnetic nanoparticles were carried out by methods described in paper [4]. 20 mg of the magnetic nanoparticles functionalized with APTES were activated with glutardialdehyde. The nanoparticles were collected with a magnet, the solution was taken out and the nanoparticles were resuspended in 5 ml glutardialdehyde solution (5 % w/v) in 50 mM phosphate buffered saline (PBS) pH 8 and incubated for two hours at room temperature with orbital agitation. Then the nanoparticles were washed once with 50 mM PBS pH 8 and five more times with 10 mM PBS pH 7.4. The nanoparticles were resuspended in 1 ml 10 mM PBS pH 7.4 containing 0,1 mg/ml anti – AFM1 monoclonal antibody and incubated for two hours at 37 °C. After that the nanoparticles were washed three times with 10 mM PBS pH 7.4 and the free active NH_2 – groups were blocked by adding 10 mM PBS pH 7.4 containing 1 % BSA and 0,05 % Tween 80. The antibody - nanoparticles were stirred for one hour at room temperature, washed three times and resuspended in the same buffer to final concentration 5 mg/ml. The same immobilization procedure was carried out using three more concentrations of the antibody: 0.5, 1 and 1.5 mg/ml.

Antibody concentration was measured before and after immobilization by the method of Bradford. The immobilized antibody concentration was calculated as the difference between the initial antibody concentration and the residual concentration after immobilization.

AFM1 immunoassay procedure

0.24 mg of the antibody – magnetic nanoparticles which provides 6 μg antibody were put in 33 microcentrifuge tubes. After that 50 μl of raw full cream cow's milk spiked with aflatoxin M1 (1.0, 2.0, 3.0, 5.0, 8.0, 10.0, 20.0, 50.0, 100.0 and 200.0 pg/ml) were added. The AFM1 standard solutions were prepared from a stock solution of aflatoxin M1 in acetonitrile with concentration 100 ng/ml. The samples were incubated 15 min at 37° C. Then 100 μl of conjugate with concentration 10, 15 and 20 μg/ml were added to each sample and incubation step was performed for 15 min at 37°C. After that, the particles were collected with a magnet, the supernatant was taken out and the residual fluorescent intensity was measured at 480/518 nm. One blank sample was prepared for each analysis as the AFM1 concentration was 0. Experimental signals were calculated by the equation:

$$Normalized\ response = (B_0 - B)/(B_0 - Bx).\ 100,\ \%$$

where ***B*** is the signal (intensity of fluorescence) measured in the presence of the increasing analyte concentrations; ***Bx*** is the signal in absence of AFM1 and ***Bo*** is the signal of the initial conjugate solution. Analysis with 1 mg MNPs-mAb (25 μg Ab) and 2 mg MNPs-mAb(50 μg Ab) were performed in the same manner.

RESULTS AND DISCUSSION

Immobilization of anti-AFM1antibody onto the magnetic nanoparticles

Two main components are necessary for the development of fluorescent immunoassay for determination of a tracer antigen concentration – immobilized antibody and conjugate antigen-fluorescent dye. One of the most important conditions during the antibody immobilization is determining the optimal initial concentration. The main task is to provide optimal amount of immobilized antibody which ensures that its specific activity is unaffected. For this purpose the initial antibody concentration for immobilization on the solid phase was varied. For solid phase magnetic nanoparticles sized around 100 nm were chosen. The magnetic nanoparticles were first modified with APTES by the carbodiimide method to introduce amino groups to the surface. Covalent immobilization of the anti-AFM1 antibody onto the magnetic nanoparticles was carried out via glutardialdehyde method.

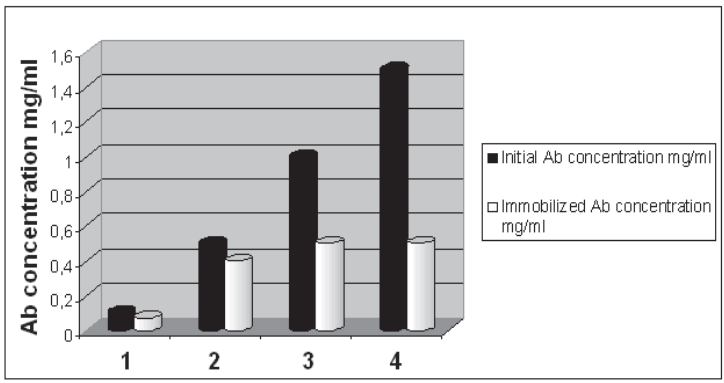

Fig. 1. Effect of initial Ab concentration on the immobilization degree

On Figure 1 the initial concentration of the antibody before immobilization and the calculated concentration of the antibody after the immobilization procedure are represented. Four experiments were carried out as the initial concentration of the antibody was varied: 1-0,1; 2-0,5; 3-1,0 и 4-1,5 mg/ml. From Figure 1 it's obvious that at the beginning with the increase of the initial antibody concentration, the immobilized antibody

concentration increases. At initial concentration 1 mg/ml the immobilized antibody is 0,5 mg/ml. With the next increase in the initial concentration the immobilized antibody remains constant. Thus the optimal initial antibody concentration for the immobilization is 1 mg/ml.

AFM1 immunoassay optimization

An immunoassay for determining AFM1 in milk was developed using the obtained MNPs-mAb. For this purpose the second component necessary for the assay was synthesized – conjugate AFM1-Flu. The synthesis of the conjugate was carried out by method described in our previous work. For the proceeding of an immune reaction it's necessary to determine its optimal conditions. Therefore the influence of the used amount of the conjugate on the normalized response of the fluorescent immunoassay was examined. Three different concentrations of the conjugate (10, 15 and 20 μg/ml) were varied and compared with three different concentrations of the MNPs-mAb (0,24; 1 and 2 mg). These amounts of MNPs-mAb provide 6; 25 and 50 μg antibody respectively. On Figure 2 the calibration curves of the immunoassay with 6 μg antibody and variable concentrations of the conjugate are represented. As can be seen the best results were obtained using 15 μg/ml conjugate, and the worst ones using 20 μg/ml

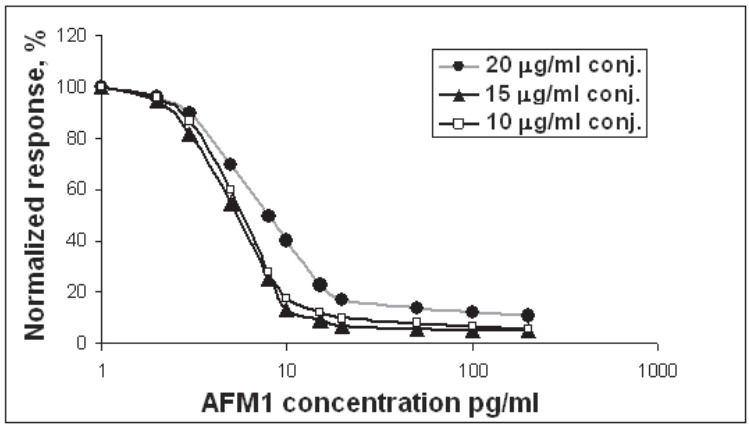

Fig. 2. AFM 1 immunoassay with 6 μg antibody

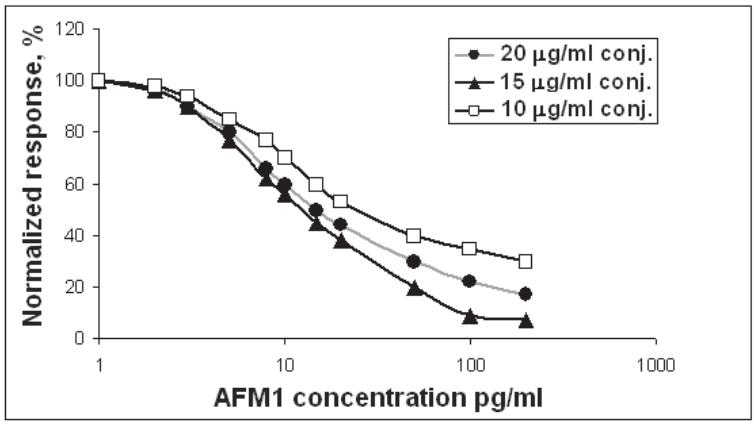

Fig. 3. AFM 1 immunoassay with 25 μg antibody

On Figures 3 and 4 the calibration curves of the immunoassays carried out at the same concentrations of the conjugate and 25 and 50 μg immobilized antibody. It's obvious that the best results were obtained at the same concentration of the conjugate. For clear comparison of the results obtained in the figures, the linear equations and limit of detection of the best curve from each figure are displayed in Table 1. Limit of detection is calculated as 90 % from the maximum normalized response of the relevant calibration curves.

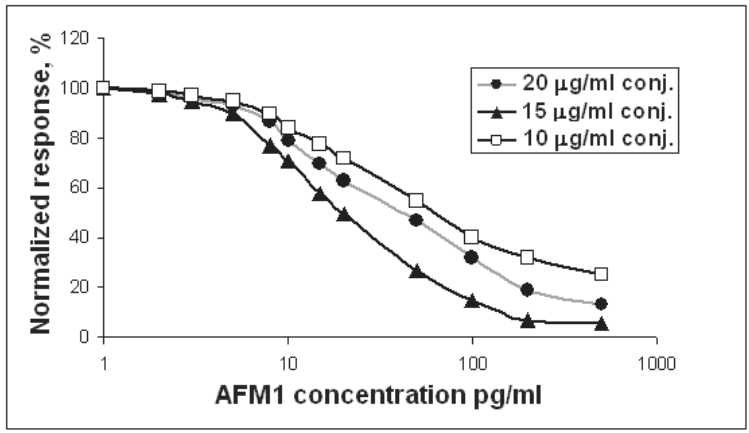

Fig. 4. AFM 1 immunoassay with 50 μg antibody

It's established that on Figure 2, the calibration curve with 25 μg antibody and 15 μg/ml conjugate had the greatest slope of the linear range. It's obvious that these are the optimal concentrations that provide the greatest sensitivity of the immunoassay. In this case the limit of detection of AFM1 in milk is also the lowest.

Table 1. Linear equations and sensitivity of the immunoassay

Antibody concentration, μg	Linear range, pg/ml	Linear equation	Limit of detection, pg/ml	R^2
6	3 – 10	y=-51,564Ln(x) + 133,07	2,5	0,996
25	3 - 100	y = -23,384Ln(x) + 111,92	2,8	0,985
50	8 - 150	y = -22,361Ln(x) + 119,92	5,0	0,979

CONCLUSION

- The optimal initial concentration of the antibody for immobilization is 1 mg/ml.
- The optimal concentration of MNPs-mAb and fluorescent conjugate are 1 mg and 15 μg/ml respectively.
- A high sensitive AFM1 immunofluorescent assay with low limit of detection was developed. The result covers the European criteria for this highly toxic mycotoxin.

REFERENCES

[1] European Commission, Commission Regulation 466/2001, Off. Eur. Comm. L77 (2001) p.7.

[2] Galvano F., V. Galofaro, A. Ritieni, M. Bognanno, A. De Angelis, G. Galvano. Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy: Second year of observation. Food Addit. Contam 18, 2001, 644-646

[3] Govaris A., V. Roussi, P. A. Koidis, N. A. Botsoglou. Distribution and stability of aflatoxin M1 during production and storage of yoghurt. Food Addit. Contam., 19, 2002, 1043-1050.

[4] Ivanova Tz. and Tz. Godjevargova. Sensitive progesterone determination using a magnetic particle based enzyme-linked immunosorbent assay. Analytical Letters. 48, 2015, 1–18

[5] Polan C. E., J. R Hayes. T. C. Campbell. Consumption and fate of aflatoxin B1 by lactating cows. J. Agric. Food Chem., 22, 1974, 635-638.

[6] van Egmond H. P. Aflatoxin M1: Occurrence, toxicity, regulation. In Mycotoxins in Dairy Products; van Egmond, H. P, Ed.; Elsevier Applied Science Publishers: London, United Kingdom, 1989; pp 11-56.

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