Analytical Methods

Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk

Marina M. Vdovenko, Chuan-Chen Lu, Feng-Yih Yu, Ivan Yu. Sakharov

A direct competitive chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for detecting aflatoxin M1 (AFM1) was developed. To improve the sensitivity of the assay, a mixture of 3-(10-phenothiazinyl)-propane-1-sulfonate (SPTZ) and 4-morpholinopyridine (MORPH) was used to enhance peroxidase-induced CL. The concentrations of the coating anti-AFM1 antibody and the conjugate of AFB1 with horseradish peroxidase the conditions of the chemiluminescent assay were varied to optimise the condition of the chemiluminescent assay. The lower detection limit values and dynamic working range of CL-ELISA of AFM1 were 0.001 ng mL\(^{-1}\) and 0.002–0.0075 ng mL\(^{-1}\), respectively. A 20-fold dilution of milk samples prevented a matrix effect of the milk and allowed measurement of AFM1 at concentrations below than the maximum acceptable limit. Values of recovery within and between assays were 81.5–117.6% and 86–110.6%, respectively. The results of using the developed CL-ELISA to analyse samples of six brands of milk that were purchased in Taiwan revealed that AFM1 was absent from all studied samples.

1. Introduction

Aflatoxins are highly toxic, mutagenic, carcinogenic, and teratogenic compounds (Betina, 1989; Kraska et al., 2008). Aflatoxin M1 (AFM1) is a major hydroxylated metabolite of aflatoxin B1 (AFB1), which exhibits the highest toxicity among all aflatoxins. AFM1 is found in the milk and blood of animals that ingest AFB-containing feed (Battacone, Nudda, Palomba, Mazzette, & Pulina, 2009; Sabino, Purchio, & Milanez, 1995). The consumption of milk and milk products by humans, particularly by children is quite high resulting in a potential a risk of exposure to AFM1. Evidence of hazardous human exposure to AFM1 through dairy products has been reported (Galvano et al., 2001; Govaris, Roussi, Koidis, & Botsoglou, 2002; Polan, Hayes, & Campbell, 1974; van Egmond, 1989).

According to the United States Food and Drug Administration (US-FDA), the concentration of AFM1 in milk should not exceed 0.5 ng mL\(^{-1}\) (Wood, 1992). More stringent restrictions of the level of AFM1 in milk for adult consumption have been set by the European Union (0.05 ng mL\(^{-1}\)) (Commission regulation, 2004). In baby-food products this level should not exceed 0.025 ng mL\(^{-1}\). AFM1 is frequently present in commercial milk samples and dairy products, and various milk samples have been found to contain AFM1 levels greater than the maximum acceptable limit. In India almost 99% of contaminated milk samples contained more than the maximum legal level set by EU regulations and 9% of samples exceeded the limit set by the USFDA (Rastogi, Dwivedi, Khanna, & Das, 2004).

Analytical methods that combine simplicity, a high detection sensitivity and a high analytical throughput are required for the effective screening and monitoring of AFM1 in foodstuffs at ppt levels. High-performance liquid chromatography (HPLC) with a fluorescent detector and enzyme-linked immunosorbent assay (ELISA) are generally used in routine analysis (Shephard et al., 2012; Stroka & Anklam, 2002; van Egmond, 2004). Notably, HPLC is a complex and time-consuming method to imolement and it requires costly and bulky instrumentation. ELISA has none of these shortcomings. ELISA is widely accepted as the “gold standard”
Among practical of ELISA formats, the most sensitive is an assay in which the enzyme activity of peroxidase-labelled immunoreagents is determined by an enhanced chemiluminescent (CL) reaction (ECR) (Fan, Cao, Li, Kai, & Lu, 2009; Marquette & Blum, 2009; Roda & Guardigli, 2012). This reaction principle is based on the peroxidase-catalysed oxidation of luminol by hydrogen peroxide in the presence of enhancers. Some CL-ELISAs for the determination of the concentration AFM1 in milk have already been reported (Kanungo, Pal, & Bhand, 2011; Magliulo et al., 2005).

Recently, a mixture of 3-(10’-phenothiazinyl)-propane-1-sulfonate (SPTZ) in combination with 4-morpholinopyridine (MORPH) has been demonstrated to be the most efficient mixture of enhancers for peroxidase-induced CL (Marzocchi et al., 2008; Vdovenko, Della Giana, & Sakharov, 2009; Vdovenko, Demiyanova, Chemleva, & Sakharov, 2012). Under optimised conditions, the ratio of the CL intensity generated in horseradish peroxidase (HRP)-induced ECR to its background value was higher than 140,000 (Vdovenko et al., 2012). The application of these enhancers provides perspectives on the construction of sensitive CL-ELISA kits. The present work describes the ultrasensitive direct competitive CL-ELISA for determining the concentration of AFM1. To increase the sensitivity of the assay, the CL method was utilised to determine HRP activity using SPTZ and MORPH as enhancers. The developed CL-ELISA was successfully applied to determine the concentration of AFM1 in milk samples.

2. Material and methods

2.1. Chemicals and reagents

Aflatoxin M1 (AFM1) and aflatoxin B1 (AFB1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AFM1 analytical standard solution (10 μg/ml), Certified Reference Material – from Supelco (Bellefonte, PA, USA). Horseradish peroxidase (HRP, RZ 3.0) was purchased from Roche (Mannheim, Germany) and used without further purification. Sodium 3-(10’-phenothiazinyl)-propane-1-sulfonate (SPTZ) was prepared as described by (Marzocchi et al., 2008). Luminol, Tween 20, Tris, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, MO, USA), N-carboxymethyl-ethylenediamine hemi-hydrochloride (CMO), and 4-morpholinopyridine (MORPH) were from Aldrich (St. Louis, MO, USA). Black polystyrene plates (high protein binding) were obtained from Nunc (Roskilde, Denmark). 1-Ethyl-3-(3’-dime-thylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, MO, USA), N-carboxymethyl-ethylenediamine (DMF) and H2O2 (30%) were from J. T. Baker (Phillipsburg, NJ, USA). The concentration of H2O2 was estimated by measuring the absorbance using $A_{240} = 43.6$ (Kulmacz, 1986).

The polyclonal antibodies specific to AFM1 (anti-AFM1-pAb) were produced by subcutaneous immunisation of rabbits with a AFM1-CMO-BSA conjugate as described by Wang, Liu, Hsu, and Yu (2011). The purification of anti-AFM1-pAb was carried as follows: at first step saturated (NH4)2SO4 was added to the rabbit antiserum to a 35% saturation. After incubation for 1 h and centrifugation (8000 rpm for 30 min) the precipitated proteins were discarded, and the additional quantity of (NH4)2SO4 solution was added to a 50% saturation. The precipitated anti-AFM1-pAb was centrifuged and then redissolved in distilled water. The volume of the used water was equal to half of the original volume of antiserum. The anti-AFM1-pAb was dialyzed against 2 L of 0.01 M phosphate buffer with 0.15 M NaCl, pH 7.5 (PBS) for 72 h at 4 °C with two changes of the buffer. Finally, PBS was added to the obtained anti-AFM1-pAb up to the original antiserum volume. The antibody sample was stored at −20°C or lyophilized for future use.

2.2. Preparation of aflatoxin B1-CMO

The synthesis of AFB1-CMO was carried out as described by Chu, Hsia, and Sun (1977). For this, 10 mg AFB1 and 15 mg CMO were dissolved in a mixture containing 1.0 mL pyridine, 4.0 mL methanol and 1.0 mL water. After the reaction completion the mixture was gently refluxed for 2.5 h with continuous magnetic stirring, it kept at room temperature overnight. Using a rotary evaporator the reaction mixture was concentrated up to ~1 mL. To purify AFB1-CMO a thin-liquid chromatography (TLC) was carried out on silica gel plates using chloroform:methanol (9:1) in 1.5% acetic acid as an eluent. Localization of AFB1-CMO spot on the plate was detected under UV light (365 nm). Then, AFB1-CMO was removed from the TLC plate and dissolved in chloroform. Finally, AFB1-CMO was dried in the open air.

2.3. Synthesis of aflatoxin B1-HRP conjugate

AFB1 was conjugated with HRP by a carbodiimide method as follows: 1.0 mg of EDC freshly dissolved in 0.01 mL DMF and 0.8 mg of NHS in 0.01 mL DMF were added to 0.1 mL of AFB1-CMO solution (0.25 mg mL⁻¹ of DMF). The mixture was kept at room temperature for 2 h with continuous stirring. Then, 1.5 mg of HRP in 1.0 mL of 0.1 M NaHCO3, pH 8.3 was added to the AFB1-CMO solution dropwise, and the reaction solution was kept at room temperature for the next 2 h with stirring. The obtained conjugate was dialyzed against 2 L of PBS for 72 h with two changes of the buffer and stored at −20°C or lyophilized for future use.

2.4. Determination of AFM1 by CL-ELISA

CL-ELISA was carried out using 96-wells black polystyrene plates (MaxiSorp, Nunc, Roskilde, Denmark). The plates were coated by adding into each well 100 μL of anti-AFM1-pAb (dilution 1:40,000) dissolved in PBS, and incubated at 4°C overnight. The plate was then washed using PBS with 0.05% Tween 20 (PBST) four times (ELx 50 ELISA washer from Bio-Tek instruments, USA) and blocked by adding 170 μL of PBS containing 0.1% BSA for 30 min at 37°C. The plate was washed four times with PBST. Subsequently, 50 μL of AFB1-HRP (dilution 1:20,000) in 10 mM PBS, pH 7.4 and 50 μL of AFM1 (0.00002–0.2 mg mL⁻¹) or milk sample in dilution 1/10 were added to each well. The competitive step of the assay proceeded for 1 h at 37°C. The plates were washed again as described above. Finally, 100 μL of freshly prepared substrate solution (80 mM Tris, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM MORP, and 1.75 mM H2O2) (Vdovenko et al., 2012) were added to each well and stirred. Chemiluminescence intensity was monitored after incubation for 5 min at room temperature on a Vmax automatic ELISA reader (FlexStation 3, Molecular Devices).

2.5. Preparation of spiked samples

Milk samples were purchased from Taiwanese stores. Each sample (10 mL) was centrifuged twice at 4°C at 19,500 g for 5 min. Prior to ELISA the free-fat milk was diluted 10 times using PBS.

2.6. Data analysis

Standards and samples were run in triplicates, and the mean values were processed. Standard curves were obtained by plotting the light intensity against the logarithm of the analyte concentration and fitted to a four-parameter logistic equation using the Origin 6.0 Professional software (OriginLab Corp., United States):
Y = \{(A - D) \div (1 + (x/C)^k)\} + D,

where A is the asymptotic maximum (intensity in the absence of an analyte, \(I_{\text{max}}\)), B is the curve slope at the inflection point, C is the x value at the inflection point, and D is the asymptotic minimum (\(I_{\text{min}}\), background signal).

3. Results and discussion

3.1. Optimal concentrations of coating antibody and conjugate AFM1-HRP

An assay for determining the concentration of AFM1 in milk was developed on the basis of a competitive CL-ELISA. A scheme of the direct CL-ELISA was presented in Fig. 1. The polyclonal anti-AFM1 antibody that was used in this work was produced by immunising a rabbit with the AFM1-BSA conjugate. As reported previously (Wang et al., 2011), an affinity of this antibody towards AFM1 is similar to that toward AFB1. Accordingly, in the development of the assay, the AFB1-HRP conjugate was used instead of the AFM1–HRP conjugate to reduce cost without worsening the analytical characteristics of the assay, as AFB1 is significantly less expensive than AFM1. The ability of the anti-AFM1 antibody to react with AFB1 is not a drawback of the method, as its purpose is to estimate the AFM1 content in milk samples that do not contain AFB1 as reported previously (Battacone et al., 2009; Sabino et al., 1995).

The sensitivity of competitive ELISA depends on the concentration of the capture antibody and the enzyme-labelled antigen. Therefore, a set of calibration curves for determining the concentrations of AFM1 were obtained by varying the concentrations of polyclonal antibodies and HRP conjugates. All calibration curves were of a form that was typical of competitive ELISA (data not shown). The values of \(I_{\text{C50}}\) and \(I_{\text{C20}}\), dynamic working range (\(I_{\text{C20}}\)–\(I_{\text{C50}}\)) as well as background (\(I_{\text{min}}\)) were selected as the parameters used in estimating the efficiency of the assay.

As seen in Table 1, the values of background were low for all used combinations of concentrations of anti-AFM1-pAb and AFB1-HRP. When many diluted solutions were used (combination 5) the analytical parameters were not calculated because of high CV values. A comparison with the other combinations revealed that the most sensitive CL-ELISAs were obtained using combinations 1 and 2. For subsequent work, combination 2 (1:40,000/1:40,000) was chosen as optimal, because it was associated with the consumption of half as much AFB1-HRP consumption as other combinations. The lower detection limit value (LDL) equal to \(I_{\text{C10}}\) the working (linear) range (\(I_{\text{C20}}\)–\(I_{\text{C50}}\)) of the developed method were 0.001, 0.035 and 0.002–0.0075 ng mL\(^{-1}\), respectively. The coefficient of variation (CV) for determining AFM1 concentrations within the working range of the assay was 4–13% (n = 6).

The characteristics of the developed assay, in which the enzyme activity was measured using ECR with SPTZ/MORPH as enhancers, were compared with those of colorimetric ELISA (COL-ELISA) of AFM1 as described previously (Wang et al., 2011). Both assays were developed using the same immunochromatographic reagents, but the principles of detection of HRP activity differed. As shown in Table 2, the obtained results demonstrated that the LDL of CL-ELISA is 2 times lower than that of the COL-ELISA. The additional advantage of the CL-ELISA is its broader working range. Also, the replacement of the colorimetric method of measuring HRP activity with the chemiluminescent method shortened the time of such measurement and also reduced 3-folds of consumption of anti-AFM1 antibody.

3.2. Analysis of spiked and real samples

To demonstrate the practicality of the proposed CL-ELISA, AFM1 concentration in spiked milk samples was measured. Various levels of AFM1 (0.002–0.006 ng mL\(^{-1}\)) were added to an AFM1–free milk sample which was diluted to varying degrees using PBS; then, the AFM1 concentration in the obtained solutions was measured by the CL-ELISA. When the dilution of the spiked sample was 1:4, the recovery and coefficient of variation (CV) were in range 123–180% and 9.4–35%, respectively. The obtained results indicated that under the assay conditions, the milk exhibited a strong matrix effect.

One of the approaches that is widely used to prevent the matrix effect is the dilution of test samples. Accordingly, prior to the CL-ELISA, spiked milk sample was diluted by a factor of 10 and 20 times. The sample with 10-fold dilution yielded unsatisfactory recovery and CV (Table 3), whereas the 20-fold dilution yielded recovery values of 92–100%.

Analysis of 3 spiked milk samples with 20-fold dilution (Table 4) by the assay showed recovery values in the range of 82–118% and CVs (n = 4) that did not exceed 11%. Also, the values of recovery between assays obtained at the performance of the assay day by day (n = 4) were in range of 86–110.6% with CVs less than 7.7%. Therefore, the matrix effect of milk was prevented by 20-fold dilution of the milk sample.

Based on the fact that in the CL-ELISA the dilution of milk samples should be 1:20, a minimum concentration of AFM1 that may be measured in real milk samples was calculated. The lowest value of the working range of the assay in buffer solution (0.002 ng mL\(^{-1}\)) should be multiplied by the dilution factor (20) yielding 0.04 ng mL\(^{-1}\) which was lower than the maximum acceptable limits that are set in both the USA and the European Union. The obtained results demonstrate that the sensitivity and precision of the developed CL-ELISA were suitable for quantifying AFM1 in milk samples.

The developed assay was used to analyse 6 samples of milk that purchased in stores in Taiwan. Our results demonstrated that AFM1 was absent from all studied samples. This finding suggests that milk products that are sold in Taiwan are generally safe for consumers.

Fig. 1. Scheme of direct competitive CL-ELISA for determination of aflatoxin M1.
4. Conclusion

This work developed ultra-sensitive CL-ELISA for determining the concentration of AFM1 in a buffer solution. The high sensitivity of the assay was achieved using the chemiluminescent method for measuring HRP activity in the presence of SPTZ and MORPH (enhancers). The values of LDL and the dynamic working range of the CL-ELISA of AFM1 were 0.001 ng mL\(^{-1}\) and 0.002–0.0075 ng mL\(^{-1}\), respectively. Twenty-fold dilution of the milk samples completely prevented the matrix effect. The values of recovery within and between assays were 81.5–117.6% and 86–110.6%, respectively. Therefore, the developed CL-ELISA is a valuable tool for the routine quality control of milk.

Acknowledgements

The authors thank the Russian Foundation for Basic Research (11-04-92005-NNS_a) and the National Science Council of the
Republic of China (Taiwan) for Taiwan-Russia Cooperation Grant NSC-100-2923-B-040-001-MY2 for financial support.

References


