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Original Article

Production of a monoclonal antibody against aflatoxin M1 and its application for detection of aflatoxin M1 in fortified milk

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ARTICLE INFO

ABSTRACT

Aflatoxin M1 (AFM1) is a toxic metabolite of the fungal product aflatoxin found in milk. For food safety concern, maximum residual limits of AFM1 in milk and dairy products have been differently enforced in many countries. A suitable detection method is required to screen a large number of product samples for the AFM1 contamination. In this study, monoclonal antibodies (MAbs) against AFM1 were generated using a conventional somatic cell fusion technique. After screening, five MAbs (AFM1-1, AFM1-3, AFM1-9, AFM1-11, and AFM1-17) were obtained that showed cross-reactivity with aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1) but with no other tested compounds. An indirect competitive enzyme-linked immunosorbent assay (ELISA) using a partially purified MAb and antigen-coated plates yielded the best sensitivity with the 50% inhibition concentration (IC_{50}) and the limit of detection (LOD) values of 0.13 ng/mL and 0.04 ng/mL, respectively. This indirect competitive ELISA was used to quantify the amount of fortified AFM1 in raw milk. The precision and accuracy in terms of % coefficient of variation (CV) and % recovery of the detection was investigated for both intra- ($n = 6$) and inter- ($n = 12$) variation assays. The % CV was found in the range of 3.50—15.8% and 1.32—7.98%, respectively, while the % recovery was in the range of 92—104% and 100—103%, respectively. In addition, the indirect ELISA was also used to detect AFM1 fortified in processed milk samples. The % CV and % recovery values were in the ranges of 0.1—33.0% and 91—109%, respectively. Comparison analysis between the indirect ELISA and high performance liquid chromatography was also performed and showed a good correlation with the R^2 of 0.992 for the concentration of 0.2—5.0 ng/mL. These results indicated that the developed MAb and ELISA could be used for detection of AFM1 in milk samples.

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1. Introduction

Aflatoxins (AFs) are a group of toxic substances produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* that grow on corn, wheat, rice, peanuts, and dried fruits [1,2]. AFs are considered genotoxins, teratogens, and immunosuppressants, and also have antinutritional effects [3,4]. There are four major types of aflatoxin: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Figure 1). These compounds are found in all animal feed preparation steps. Upon ingestion by animals, they are transformed into different metabolites with different levels of toxicity. Some metabolites accumulate in the body, while others are excreted via the urine, feces, or milk. The most toxic metabolite is 2, 3-epoxside-AFB1, which binds to DNA and RNA, and may result in liver cancer due to irregular protein synthesis [5,6]. In addition to AFB1, another major concern is aflatoxin M1 (AFM1), which is secreted into the milk of lactating animals. AFM1 can be found in milk at ~0.5–5% of the ingested AFB1 [7]. It is stable under normal milk pasteurization and high-temperature treatment [8,9]. AFM1 was also found in milk-derived dairy products and importantly, in human breast milk [7,10]. AFM1 is hepatotoxic and carcinogenic and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer [2,11]. To reduce the risk of aflatoxicosis, strict regulatory limits have been enforced in many countries. In China and the USA, the maximum residue limit (MRL) of AFM1 in milk is 0.5 ng/mL [12,13]. Moreover, in the European Union, the MRL of AFM1 in raw milk and milk-based products intended for adults is 0.05 ng/mL (European Commission Regulation No. 1881/2006/EC). Therefore, the contamination of AFM1 in milk products is under surveillance in many countries to ensure product safety. Chemical-based methods, such as fluorospectrophotometry, liquid chromatography coupled with tandem mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC), have been used for AFM1 determination [14,15]. However, these methods are relatively unsuitable for screening a large number of samples compared with the immunological-based methods, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assay (ICA) or lateral flow immunoassay (LFIA) [3]. LFIA is considered to be more preferable than ELISA in terms of ease of use, short assay time, and cost. But LFIA is often used qualitatively in tests that have positive or negative results based on the cut off value. By contrast, previous studies reported that ELISA can be used to semiquantitatively detect drug residues in foods within the acceptable ranges in terms of accuracy and precision [16–18]. In addition, if the antibody used in the ELISA is specific to AFM1 as well as other aflatoxins such as AFB1 and AFG1 found in foods and feeds, it might be more useful than LFIA, because the regulated MRL values are different (0.5–300 ng/mL) for different types of product. Use of LFIA with a specific cut off value is limited to only a certain MRL value. A very high sensitive LFIA with the cut off value or limit of detection of 0.02 μg/L which satisfies the EU maximum limit is not applicable in the countries where the maximum limit is set at a higher level such as 0.5 μg/L. For example, milk products containing AFM1 at 0.1 μg/L would yield a positive result and would be rejected by the LFIA with the cut off value of 0.02 μg/L although the AFM1 level of this sample is not higher than the regulated limit of 0.5 μg/L. Therefore, ELISA is probably preferred to LFIA for semiquantitative screening purpose. In this study, monoclonal antibodies (MAbs) against AFM1 were generated and used for the development of an antibody-captured competitive indirect ELISA for AFM1 detection. The reliability of the developed ELISA was also investigated.

2. Methods

2.1. Materials, reagents, and animals

AFB1, AFG1, AFM1, and deoxynivalenol (DON) were purchased from Fermentek (Jerusalem, Israel) with purity above 99%. AFM1-BSA, bovine serum albumin (BSA), cinoxacin, ciprofloxacin, enoxacin, enrofloxacin, Freund's complete adjuvant
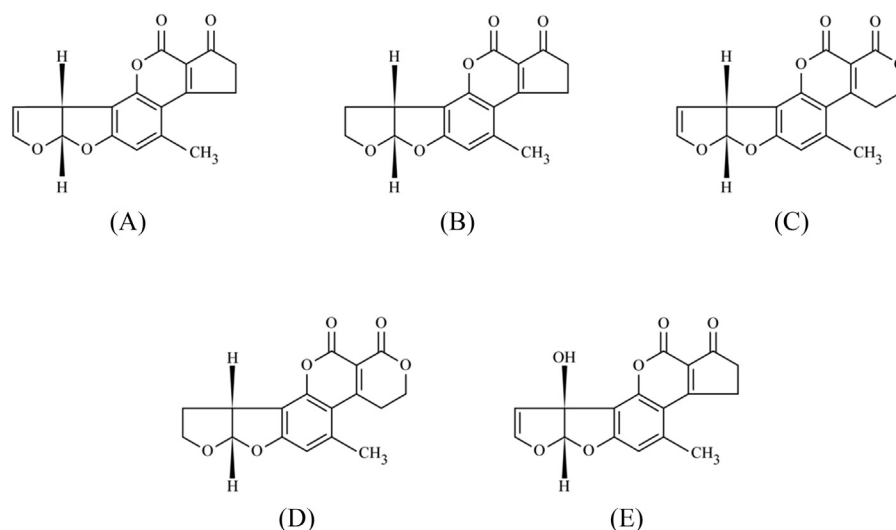

Figure 1 – Chemical structure of major aflatoxins (A) AFB1; (B) AFB2; (C) AFG1; (D) AFG2; and (E) AFM1. AF = aflatoxin.

(FCA), Freund's incomplete adjuvant (FIA), nitrofurantoin, norfloxacin, oxytetracycline, 3, 3′, 5, 5′-tetramethylbenzidine (TMB), tetracycline, and mouse MAb isotyping reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium was purchased from Biochrom AG (Berlin, Germany). Horseradish peroxidase-conjugates goat anti mouse immunoglobulin G (GAM-HRP) was purchased from Jackson Immuno (West Grove, PA, USA). Myeloma cells P3-X63Ag8 were purchased from the American Type Culture Collection (ATCC; TIB-9). Female (8-week-old) BALB/c mice (inbred strain) were obtained from the National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand.

2.2.   Production of hybridomas

Eight-week-old female BALB/C mice were immunized by intraperitoneal injection with 100 μL of AFM1-BSA (2.5 μg/mL AFM1-BSA dissolved in sterile phosphate buffer saline, PBS) and an equal volume of FCA. Three booster doses of AFM1-BSA in FIA at 2-week intervals were performed. One week after the fourth injection, antiserum was collected and checked for antibody titers by indirect ELISA. The final boost of 2.5 μg AFM1-BSA (without adjuvant) in sterilized normal saline was performed. All procedures that involved animals were approved by the Institutional Animal Care and Use Committee, the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University (Bangkok, Thailand; Protocol No. 1061001).

The generation of hybridomas was carried out using conventional methods as described previously [19,20]. In brief, isolated splenocytes were fused with myeloma P3-X63Ag8 at a cell ratio of 1:3 using 50% w/v polyethylene glycol (PEG) as the fusion agent. Cells were suspended in 30 mL of RPMI 1640 medium containing 0.2 mg/mL gentamycin. The cell suspension was centrifuged at $380g$ for 5 minutes, and the cell pellet was washed with the same medium to remove PEG. The hybridomas were resuspended in hypoxanthine–aminopterine-thymidine (HAT) selective medium supplement with 20% FCS and distributed into 96-well tissue culture plates. The hybridomas were cultured at 37°C in a 5% CO_2 incubator with a suitable medium replacement interval. After 2 weeks, the culture supernatants were screened for Abs against AFM1 by an indirect ELISA. Positive cultures underwent single cell cloning for multiple rounds by limiting dilution until monoclones were achieved.

2.3.   ELISA

For indirect ELISA, 96-well plates were coated with 50 μL/well of 1 μg/mL AFM1-BSA and incubated at 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST), blocked with 300 μL of 5% skimmed milk in PBS, and incubated at 37°C for 1 hour. After washing, the plates were incubated with 100 μL/well of primary Ab (culture supernatants or antisera) for 2 hours, washed, and then incubated with secondary Ab (0.8 mg/mL at 1:10,000 dilution GAM-HRP, 100 μL/well) at 37°C for 1 hour. Enzyme assay was performed using 0.0003% TMB in 0.2 M citrate buffer, pH 4.0 and 0.34% H_2O_2 (100 μL/well) as the substrate. The reaction was allowed to occur for 10 minutes and then stopped with 100 μL/well of

1 M H_2SO_4. The absorbance at 450 nm was measured using a microtiter plate reader.

For the indirect competitive ELISA, the protocol was the same as that of the indirect ELISA with the exception that the primary Ab was added together with the free AFM1 or the competitors at the desired concentrations.

2.4.   Characterization of Ab

2.4.1.   Free AFM1 binding ability of Ab

An indirect competitive ELISA was used to screen for Abs with free AFM1 binding ability, which was determined by the % competition using the following formula:

$$\% \text{ Competition} = \frac{A_{450} \text{ without free AFM1} - A_{450} \text{ with free AFM1}}{A_{450} \text{ without free AFM1}} \times 100$$

(1)

2.4.2.   Sensitivity

The ELISA sensitivity was quantified based on the 50% inhibition concentration (IC_{50}), the concentration of the free AFM1 that resulted in a 50% reduction of the B/B_0 ratio in which B is the absorbance values obtained from the indirect competitive ELISA at different concentrations of AFM1, and B_0 is the maximum absorbance when no competitor is present. In addition to the IC_{50}, limit of detection (LOD) was also used to justify the sensitivity. The LOD was the AFM1 concentration corresponding to the point at which the mean B_0 value was reduced by three times its standard deviation. Both the IC_{50} and the LOD values were analyzed using GraphPad Prism 4.03 software (GraphPad Software, Inc., CA, USA) from the plot of % B/B_0 versus AFM1 concentration.

2.4.3.   Cross-reactivity

Cross-reactivity of the MAb was calculated using the ratio of the standard AFM1 IC_{50} with the IC_{50} of the competitors using the following formula:

$$\% \text{ Cross-reactivity} = \frac{IC_{50} \text{ of AFM1}}{IC_{50} \text{ of competitor}} \times 100 \qquad (2)$$

The IC_{50} values of the tested substances were obtained by the indirect competitive ELISA as previously described with the condition that the free AFM1 was replaced by the tested substances with varying concentration in the range of 0–4 μg/mL.

2.5.   Determination of AFM1 fortified in milk samples

Raw milk samples were fortified with AFM1 at a final concentration between 0 ng/mL and 0.8 ng/mL. Milk samples were centrifuged at $3500g$ at 4°C, and the upper fat layer was removed (adapted from [21,22]). The fortified AFM1 concentrations were measured by indirect competitive ELISA. The accuracy and precision of the analysis was evaluated by the % recovery and % coefficient of variation (CV), respectively. The analysis of six replicates of each dilution performed on 12 different lots was used to determine the intervariation of the assays, while the analysis of those obtained on a single lot were used to determine the intravariation of assays. The %

recovery and % CV was calculated using the following formulas:

$$\% \text{ Recovery} = 100 \times (\text{measured concentration/fortified concentration}) \qquad (3)$$

$$\% \text{ CV} = 100 \times (\text{standard deviation/mean}) \qquad (4)$$

In addition to raw milk, several commercially available processed milks were also tested.

2.6. Comparative analysis between ELISA by HPLC

Milk samples were fortified with AFM1 at different concentrations between 0.2 ng/mL and 5.0 ng/mL and were comparatively analyzed using the developed ELISA and HPLC. HPLC analysis was performed by the Central Laboratory Co., Ltd (Bangkok, Thailand).

3. Results and discussion

3.1. Generation of monoclonal antibody

AFM1 has a low molecular weight, and is a hapten molecule. Therefore, it was coupled to a carrier protein, BSA, before being used as an immunogen. After immunization four times at 2-week intervals, antiserum from all mice was analyzed by an indirect competitive ELISA to determine the antibody titer. The titer was defined as the reciprocal of the antiserum dilution that results in an absorbance value that is twice the background [23]. All mice responded to the immunization and yielded a high antibody titer in the range of 1:8,192,000 and 1:32,768,000 (data not shown). Generally, an immunized mouse suitable for further study should have an antibody titer of at least 1:1000 [23]. The obtained antibodies could bind with free AFM1 (data not shown), which is critical for use in an ELISA. Five fusions between splenocytes and myeloma cell lines yielded five monoclones, assigned as AFM1-1, AFM1-3, AFM1-9, AFM1-15, and AFM1-17. Isotype of all obtained MAbs was identified to be immunoglobulin G_1 (data not shown).

3.2. Characterization of MAbs

Sensitivities of the obtained MAbs were quantified in terms of IC_{50} and LOD by performing an indirect competitive ELISA. The typical response curves of the five MAbs are shown in Figure 2. The IC_{50} values were in a range of 0.02—0.04 ng/mL while the LOD values were in a range of 0.01—0.02 ng/mL. These results indicated that the obtained MAbs were sufficiently sensitive to detect AFM1 when the current MRL value was set at 0.5 ng/mL for raw milk and milk-based products. However, among the five MAbs, the MAb from clone AFM1-9 was the most sensitive, with the lowest IC_{50} and LOD values. In addition to sensitivity, specificity is also an important characteristic of the MAb, and the % cross-reactivity (%CR) is shown in Table 1. All the MAbs could bind to AFM1, as well as AFB1 and AFG1. Surprisingly, the % CR of all the MAbs to AFB1
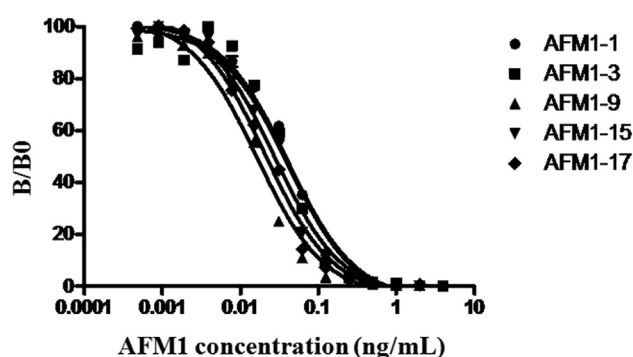

Figure 2 — Competitive inhibition curve of the obtained five monoclonal antibodies applied in an indirect competitive ELISA using 100 μL of 0.2 μg ml^{-1} AFM1-BSA as the coating antigen. In the assay, the culture supernatant of AFM1-9 was diluted at 1:1600 while those of other clones were diluted at 1:3200. The assay was performed in triplicate. AF = aflatoxin; BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay.

and AFG1 was higher than those to AFM1 which was used as the immunogen. This might be due to their similar structures. The specificity of any Ab depends on the structure of the immunogen and the ability of the immune response of the individual immunized animal. In general, a small molecule itself is not immunogenic and therefore, it must be conjugated to a carrier protein and used as an immunogen. Aflatoxins are considered to be a small molecule. Because the MAbs obtained in this study showed strong cross-reactivity to AFM1, AFB1, and AFG1, it is possible that these antibodies recognized the same epitope in all aflatoxins. A polyclonal antibody against AFM1 was produced using an AFM1-BSA conjugate as the immunogen. The obtained antibody was specific to AFM1 (100%) and could bind to other aflatoxins to a lesser degree (4%) [15]. In another study, MAbs were generated using an AFM1-BSA conjugate [24]. The obtained MAbs were specific to AFM1 and did not cross-react with AFB1, B2, G1 and G2. By contrast, Zhang et al [25] produced Abs against AFB1, B2, G1, and G2, but all five clones obtained produced Ab that cross-reacted with AFM1. However, the ability of the anti-AFM1 Ab to cross-react with other AFs is not a disadvantage because AFM1 is only found in milk not contaminated with other AFs [26]. Therefore, the detection of AFM1 in milk would not be affected by these cross-reactivities. However, this is an advantage in that the MAbs could be used to detect not only AFM1 in milk but also AFB1 and AFG1 which are usually found in other food products and feeds. Importantly, the MAbs obtained in this study did not cross-react to other tested mycotoxin and antibiotics. This result indicated that the MAbs were specific to aflatoxins.

3.3. Efficiency of AFM1 detection in raw milk by indirect competitive ELISA

MAb AFM1-19 was partially purified and used in the newly developed ELISA to determine the optimum concentration ratio of the coating primary antibody to the horseradish

Table 1 – Cross-reactivity of the obtained MAb.

Competitors	AFM1-1		AFM1-3		AFM1-9		AFM1-15		AFM1-17	
	IC_{50} (pg/mL)	CR (%)	IC_{50} (pg/mL)	CR (%)	IC_{50} (pg/mL)	CR (%)	IC_{50} (pg/mL)	CR (%)	IC_{50} (pg/mL)	CR (%)
Aflatoxins										
AFM1	40	100	36	100	17	100	18	100	22	100
AFB1	3	1328	2	1491	2	1043	1	3144	2	1393
AFG1	15	269	34	105	16	106	16	175	13	178
Mycotoxin										
Deoxynivalenol	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Antibiotics										
Tetracycline	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Oxytetracycline	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Chloramphenicol	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Ciprofloxacin	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Enrofloxacin	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02
Norfloxacin	$>10^5$	<0.04	$>10^5$	<0.04	$>10^5$	<0.02	$>10^5$	<0.03	$>10^5$	<0.02

AF = aflatoxin; CR = cross-reactivity; IC_{50} = 50% inhibition concentration; Mab = monoclonal antibody.

peroxidase conjugated secondary antibody. With the optimized conditions, the IC_{50} and LOD values were 0.13 ng/mL and 0.04 ng/mL, respectively (data not shown). Subsequently, the indirect competitive ELISA was applied to detect AFM1 fortified in raw milk samples. The milk samples must be centrifuged to separate the top lipid layer which can interfere with ELISA and only the lower clear layer was used for analyses [7]. The percentages of recovery and the coefficient of variation (CV) for both intra- and interassays are shown in Table 2. The % recovery and % CV of the intra-assay were in the range of 92–104% and 3.5–15.8%, while those of the interassay were in the range of 100–103% and 1.32–7.98%, respectively. Generally, the acceptable value of the % recovery should be between 80% and 120% [27]. Therefore, these results indicated that the accuracy and precision of the assay were suitable for the detection of AFM1 in raw milk.

3.4. Analysis of AFM1 fortified in processed milk products by indirect competitive ELISA

Different concentrations of AFM1 were fortified in various processed milk samples and subsequently quantified by an indirect competitive ELISA, as shown in Table 3. The accuracy and precision of the assay were in an acceptable range when

pasteurized nonfat milk, sterilized nonfat milk, sterilized milk, and powdered milk were used, with the exception of powdered milk sample spiked at 0.2 ng/mL. AFM1 has a melting point of approximately 299°C, so it is relatively stable under high temperatures; therefore, it cannot be destroyed by either pasteurization or sterilization processes. Consequently, milk products processed from raw milk contaminated with AFM1 still contain AFM1 [13,18,28,29]. However, the assay could not detect AFM1 spiked in all flavored milks, such as pasteurized strawberry flavored milk and pasteurized chocolate flavored milk (data not shown). This could be due to the interference of the color of the additive flavor in the absorbance measurement. Samples diluted ~10–20 times after the centrifugation step in the sample preparation could be used to reduce the matrix effect of the sample [30]. However, the sample should not be diluted in such a way that the AFM1 concentration is lower than the LOD value of the assay method. In addition, immunomagnetic nanobeads (IMNB) were recently employed in the sample preparation step for AFM1 detection using a modified LFIA. AFM1 in the sample was first captured by the IMNB and separated from the milk matrix. The captured AFM1 was then eluted from the IMNB and qualitatively detected by a conventional LFIA. This resulted in improved detection sensitivity over a conventional

Table 2 – Determination of AFM1 fortified in raw milk by indirect competitive ELISA.

Fortified concentration (ng/ml)	Intra-assay			Inter-assay		
	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)
0.80	0.83 ± 0.03	3.50	104	0.81 ± 0.01	1.32	101
0.70	0.71 ± 0.05	6.82	102	0.71 ± 0.02	2.47	101
0.60	0.62 ± 0.04	7.02	104	0.61 ± 0.01	1.86	102
0.50	0.49 ± 0.08	15.8	97	0.51 ± 0.01	2.25	101
0.40	0.40 ± 0.04	9.63	100	0.40 ± 0.01	3.51	101
0.30	0.30 ± 0.01	4.47	100	0.30 ± 0.01	3.25	100
0.20	0.18 ± 0.03	14.0	92	0.21 ± 0.02	7.98	103

AF = aflatoxin; CV = coefficient of variation; ELISA = enzyme-linked immunosorbent assay.

Table 3 – Determination of AFM1 fortified in some processed milk samples by indirect competitive ELISA.						
Fortified concentration (ng mL^{-1})	Pasteurized nonfat milk			Sterilized nonfat milk		
	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)
0.80	0.73 ± 0.01	0.73	91	0.76 ± 0.01	0.60	95
0.70	0.66 ± 0.01	1.74	96	0.66 ± 0.02	2.40	94
0.60	0.57 ± 0.01	0.09	96	0.57 ± 0.01	2.20	95
0.50	0.50 ± 0.02	4.15	100	0.46 ± 0.01	2.10	92
0.40	0.42 ± 0.01	2.86	104	0.40 ± 0.02	4.40	100
0.30	0.31 ± 0.01	0.79	103	0.27 ± 0.03	11.9	90
0.20	0.19 ± 0.02	9.24	96	0.20 ± 0.02	11.3	100
Fortified concentration (ng mL^{-1})	Sterilized fresh milk			Powdered milk		
	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)	Measured concentration (ng mL^{-1})	CV (%)	Recovery (%)
0.80	0.87 ± 0.01	0.10	109	0.82 ± 0.01	0.44	103
0.70	0.76 ± 0.01	0.35	108	0.71 ± 0.01	0.78	102
0.60	0.61 ± 0.01	1.55	102	0.59 ± 0.01	0.49	98
0.50	0.48 ± 0.01	1.94	96	0.52 ± 0.01	2.30	103
0.40	0.42 ± 0.01	3.53	105	0.41 ± 0.02	4.40	103
0.30	0.30 ± 0.02	5.37	101	0.30 ± 0.02	5.00	101
0.20	0.21 ± 0.01	5.97	103	0.20 ± 0.07	33.0	101
AF = aflatoxin; CV = coefficient of variation; ELISA = enzyme-linked immunosorbent assay.						

IMNB-based LFIA and gold-based LFIA, approximately 25 times and 50 times, respectively [31]. However, only raw milk samples were tested in this report. In addition, eight sterilized unflavored milk samples from different producers were collected from a local retail outlet in Bangkok, Thailand and checked for AFM1 concentration. The test was also performed using these samples spiked with AFM1 at 0.5 ng/mL as the internal control. The AFM1 concentrations of nonspiked samples were between 0 ng/mL and 0.14 ng/mL (Figure 3), which is lower than the MRL of 0.5 ng/mL set by the United States Food and Drug Administration but higher than the MRL of 0.05 ng/mL enforced by the European Union for milk products. However, several countries, including Thailand, have not yet established regulatory limits for AFM1 [32,33]. These AFM1 concentrations were close to the highest concentration of 0.114 ng/mL determined from 150 pasteurized milk samples from the School Milk Project in Thailand [32]. Consequently, there should be some caution in consuming these milk and milk-based products.
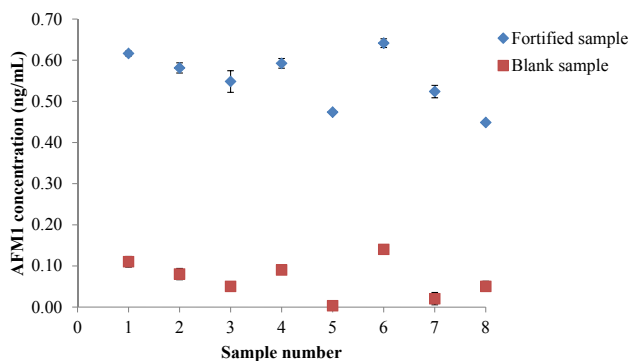
3.5. Comparison of AFM1 analysis between the developed ELISA and HPLC

A sterilized milk sample was artificially contaminated with AFM1 at different concentrations (between 0.02 ng/mL and 5.0 ng/mL). The AFM1 concentrations were then measured by both the developed ELISA and HPLC as shown in Figure 4. A linear regression analysis between both methods yielded a good correlation with an R^2 of 0.992. Moreover, a tendency of bias deviation was not observed. This indicated that the developed ELISA could be used to measure AFM1 in non-colorized milk samples. This experiment was carried out based on one milk sample fortified with AFM1 at various concentrations and the reported amount of AFM1 at each concentration was an average of six measurements. This result could be used to observe a trend of analysis results

Figure 3 – Detection of AFM1 in milk samples commercially available in a local retail outlet. The assay was performed in triplicate. AF = aflatoxin.
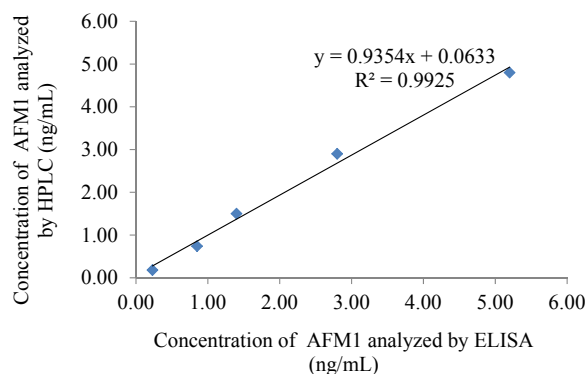
Figure 4 – Comparative measurement of AFM1 in milk samples by the developed indirect competitive ELISA and HPLC. AF = aflatoxin; ELISA = enzyme-linked immunosorbent assay; HPLC = high-performance liquid chromatography.

compared between ELISA and HPLC. Multiple samples analysis at various AFM1 concentrations should be performed to obtain more confident data. However, in general, ELISA was intended to be used as a screening detection or semi-quantitative method. The quantitative or confirmation detection of AFM1 should be based on HPLC.

3.6. Conclusion

After conventional immunization and hybridoma cell preparation and screening, MAbs against AFM1 were generated. These obtained antibodies could bind to AFM1 found in milk as well as to AFB1 and AFG1 usually found in feeds and food products. Using the optimized conditions, an indirect competitive ELISA was employed to detect AFM1 fortified in raw milk, unflavored pasteurized or sterilized milk samples with a limit of detection of 0.2 ng/mL. Most of the accuracy and precision of the assay were within an acceptable range.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2016.02.002.

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