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In vitro evaluation of binding capacity of different binders to adsorb aflatoxin

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Abstract

This study was conducted to compare the efficacy of different feed additives as mycotoxin binders *in vitro*. Four prevalent aflatoxin-sequestering agents (SAs) including two bentonite clays (common and acid activated bentonite), a yeast cell wall product and an activated charcoal product were evaluated *in vitro* to verify their capacity for binding aflatoxin B1 (AFB1). The SAs were individually mixed at two different ratios with AFB1 (1:70,000, 1:120,000) and their binding capacity indices were determined. Experimental bentonites showed high adsorption abilities, binding more than 70.00% of the available AFB1. At the 1:70,000 and 1:120,000 aflatoxin binder (AF:B) ratios, acid activated bentonite were sequestered over 87.00 and 99.00% of the AFB1, respectively. Yeast cell wall showed moderate adsorption ability at the 1:120,000 AF:B ratio, adsorbing 47.00 of AFB1. The adsorption ability of activated carbon at two AF:B ratio and yeast cell wall at 1:70,000 AF:B ratio were significantly lower than other binders. The ratio of chemisorption and binding equivalency factor were higher for acid activated bentonite compared to other sequestering agents. Based on the result of this study, it seems that acid activated bentonite could be considered efficient at sequestering the available AFB1, resulting as promising agents for use in animals diet.

Introduction

Aflatoxins are toxic secondary metabolites that affect health and performance of exposed animals. Also, undesirable effects on internal organs could cause liver and kidney lesions and hepatocellular carcinoma along with immunosuppressive effects.[1] Among aflatoxins only four of B1, B2, G1 and G2 are of the most concern and have been studied extensively.[2] Among them, AFB1 has been designated as group 1 carcinogenic compound.[3] Because of high aflatoxins contamination of animal feeds and carry over rate into blood and milk,[4,5] considerable research has been conducted to reduce their negative impacts on animal health and performance. Various strategies have been used to reduce exposure to aflatoxin in contaminated feeds.[6] Among these strategies use of sequestering agents that inhibit their absorption from the gut minimizes the carryover of these toxins into blood and milk.[7,8]

A variety of mycotoxin-sequestering agents has been tested and are currently traded throughout the world. However, only a few of them have been studied to support their effectiveness on mycotoxin binders. Among them, activated carbons, certain clays and a yeast cell wall-derived esterified glucomannan have been used for *in vitro* assays of AFB1 binding.[7,8] However, these studies did not use the successive incubation time, appropriate proportion of aflatoxin binder (AF:B) and different pH conditions of experimental binders. Thus, in this study we tested several potential sequestering agents for their ability to bind AFB1 at different AF:B ratio, incubation time and pH conditions.

Materials and Methods

Four aflatoxin-sequestering agents including two bentonite clays (common and acid activated bentonite were obtained from a local mine in South Khorasan province, Iran), a yeast cell wall product and an activated charcoal product were evaluated *in vitro*. The standard solution of AFB1 was prepared by dissolving 5.00 mg of pure toxin (Sigma-Aldrich, Munich, Germany) in 3.00 mL of acetonitrile (Ava Gostar, Tehran, Iran) for delivery into 500 mL of distilled water (approximately 10.00 µg mL^{-1}). Each sequestering agent (1.00 g) was added to a 125 mL Erlenmeyer flask containing 100 mL of 10.00% methanol

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and mixed for 30 min with a magnetic stirrer.

Binding capacity for the test sorbent was determined by adding 5.00 mL of aliquots of stock solution to 0.50% suspension of each sequestering agent. Then each sample was incubated for 2 hr in shaking incubator at 39.00 ˚C. The incubation of samples was conducted in triplicates for each sequestering agent sample. After 2-hr incubation, the mixture was centrifuged and the supernatant was obtained for analysis of residual unbound AFB1 using High-performance liquid chromatography (HPLC). The HPLC system (Perkin Elmer, Boston, USA) was equipped with ODS 5.00 µm column and fluorescence detector set (RF-551; Shimadzu, Columbia, USA). The percent adsorption of AFB1 by sequestering agents was calculated using the following equation:

$$Percent\ adsorption = (IA\text{-}RA / IA) \times 100$$

where, *IA* (ng mL^{-1}) is the initial amount of AFB1 in the digestion conical tube; *RA* (ng mL^{-1}) is the residual amount of unbound AFB1 in the conical tube after digestion procedure.

The HPLC adsorption data were used to calculate the AFB1 binding parameters. Lineal regression analysis was conducted for each sorbent. Aflatoxin binding capacity (BMax) for each sorbent was calculated from the inverse of y-intercept for the linear regression. The ratio of chemisorption (*rc*) was calculated by determining the amount of toxin bound (*Cb*) to the pellet during the capacity studies, and the amount of toxin desorbed (*Cd*).

$$rc = (Cb - Cd) / Cb$$

Binding equivalency factor (*BEF*) was determined by the following equation:

$$BEF = (BMax \times rc) / Ci$$

where, *Ci* is the amount of toxin (ng) added at the theoretical point.

Statistical analysis. Data were analyzed by GLM procedure of SAS (version 9.2; SAS Institute Inc., Cary, USA). Differences among groups mean were determined by the Tukey's adjustment. The significance was declared at an alpha-level of 0.05.

Results

The *in vitro* AFB1 adsorption capacity of bentonite used at different AF:B ratios are listed in Table 1. A higher binding capacity was observed at the 120,000 ratio. At the 1:70,000 and 1:120,000 AF:B ratios, bentonite was sequestered over 87.00 and 99.00% of the AFB1, respectively. No toxin was found in a ratio higher than 1:120,000. Adsorption capacity of AFB1 by different binders is presented in Table 2. A higher sequestering capacity was observed for acid activated bentonite at the 1:120,000 AF:B ratio. Common and acid activated bentonite showed high adsorption abilities, binding more than 70.00% of the available AFB1. Yeast cell wall showed moderate adsorption ability at the 1:120,000 AF:B ratio, adsorbing 47.00% of AFB1. The adsorption ability of activated carbon and yeast cell wall was significantly lower than other binders ($p < 0.05$). Also, adsorption capacity of acid activated was higher than common bentonite at different time after incubation (Table 3). The effect of incubation time on aflatoxin adsorption, BMax, rc and BEF of different bentonites are presented in Tables 3 and 4. Acidified bentonite had higher adsorption efficiency indices *versus* common bentonite ($p < 0.05$). Adsorption capacity parameters (rc and BEF) were decreased ($p < 0.05$) over the time.

Table 1. The *in vitro* AFB1 adsorption capacity of acidified bentonite used at different Aflatoxin:Binder ratios.

Initial AFB1 concentration (ng)	Binder concentration (mg)	Aflatoxin: Binder ratios	Adsorption capacity (%)
50	Control (0)	-	0.00
50	0.25	1:5,000	7.00
50	0.75	1:15,000	9.00
50	2.00	1:40,000	22.50
50	3.50	1:70,000	87.00
50	6.00	1:120,000	99.10
50	10.00	1:200,000	ND
50	37.50	1:750,000	ND
50	500	1:1,000,000	ND

ND = Not detected.

Discussion

A sizable proportion of the animal feeds are contaminated with mycotoxins that adversely affect health and performance of animals. The best strategy to prevent the mycotoxins contamination is to avoid the mycotoxin production at the time of cultivation and storage of the feed crops.[9] In many countries it is difficult to achieve this goal.[10] Therefore, in order to prevent mycotoxins poisoning, several approaches have been reported.[9] In recent years, organic and inorganic sorbent materials are used to reduce mycotoxin bioavailability in animal feeds, thus, it is necessary to evaluate the adsorption capacity of these adsorbent products. An *in vitro* practical method was used to compare the aflatoxin binding capacity of prevalent mycoadsorbents in the current experiment. According to our results, experimental common or acidified bentonites was appeared to bind AFB1 efficiently rather than other organic binders. It is well established that swelling clay especially bentonite are composed of interlayer spacing and have the external basal surfaces and edges and lead to high degree of adsorption and aflatoxins reacting at these sites.[11,12] Recent studies indicated that binding of AFB1 on interlayer surfaces of bentonite involved chemical bonding mechanisms[13] resulting into more pronounced ability to adsorb aflatoxins in the range of 90.00 - 95.00%.[10]

Table 2. The *in vitro* AFB1 adsorption capacity of various sequestering agents used at different Aflatoxin:Binder ratios.

Treatment	Initial AFB1 concentration (ng)	Binder concentration (mg)	Aflatoxin:Binder ratios	Adsorption capacity (%)
Control	50	0.00	-	0.00
Common bentonite	50	3.50	1:70,000	73.00
Common bentonite	50	6.00	1:120,000	77.00
Acidified bentonite	50	3.50	1:70,000	83.20
Acidified bentonite	50	6.00	1:120,000	93.70
Activated carbon	50	3.50	1:70,000	13.50
Activated carbon	50	6.00	1:120,000	18.00
Yeast wall	50	3.50	1:70,000	22.00
Yeast wall	50	6.00	1:120,000	47.00

Table 3. The effect of incubation time on AFB1 *in vitro* adsorption for different bentonites.

Binder	Time (hr)	Initial AFB1 concentration (ng mg^{-1})	Adsorbed AFB1	Adsorption capacity (%)
Common bentonite	3	5.76	3.43 ± 0.08	59.50 ± 1.80
	6	5.76	3.43 ± 0.01	59.40 ± 0.10
	9	5.76	3.05 ± 0.09	54.70 ± 1.90
Acidified bentonite	3	5.76	5.34 ± 0.01	92.70 ± 0.05
	6	5.76	5.39 ± 0.06	93.10 ± 0.25
	9	5.76	5.46 ± 0.07	93.70 ± 0.55

Table 4. Banding capacity parameters for experimental bentonites at different incubation time.

Time (hr)	BMax				rc				BEF			
	Common bentonite	Acidified bentonite	*p*-value Trt	Time	Common bentonite	Acidified bentonite	*p*-value Trt	Time	Common bentonite	Acidified bentonite	*p*-value Trt	Time
3	8.50 ± 0.10	13.22 ± 0.11			0.30 ± 0.03	0.92 ± 0.01			0.18 ± 0.00	0.85 ± 0.03		
6	8.49 ± 0.10	13.38 ± 0.11	<0.01	0.42	0.32 ± 0.02	0.93 ± 0.02	<0.01	<0.01	0.19 ± 0.00	0.87 ± 0.01	<0.01	<0.01
9	7.57 ± 0.10	13.54 ± 0.09			0.11 ± 0.03	0.93 ± 0.01			0.06 ± 0.00	0.89 ± 0.01		

BMax: AFB1 binding capacity, rc: ratio of chemisorption, BEF: Binding equivalency factor, and Trt: Treatment.

In comparison with the clay sorbent, it was reported that the mechanism of AFB1binding to organic binders such as glucomannan products was shown to be Van der Waals and hydrogen bonds[14] and these attractions were reversible and depended largely on the orientation of the molecules. In agreement with our results, Moschini *et al.* reported that yeast cell wall products had a very low *in vitro* efficiency in all of tested conditions.[15] Low capacity of β-D-glucans, a major component of the inner layer of yeast cell wall, to interact with aflatoxins indicated the involvement of non-covalent bonds (adsorption) rather than real binding.[14] In contrast, the binding of AFB1 on interlayer surfaces of bentonite is chemisorption bonding mechanisms and are stronger than Van der Waals and hydrogen bonding interactions.[16]

Among the many factors that affect the absorbent capability, interaction of organic molecules with clay mineral surface chiefly depends upon the concentration of the organic molecule and clay mineral (mycotoxin: binder ratio), pH, and incubation time.[17] Based on the results, the *in vitro* efficiency of the different mineral and organic binders tested were found to be related to the AF:B ratio. The amount of the sequestering agents used were ranged between the practical dose (Table 1) and the level indicated in the *in vitro* studies (1:5,000).[15,18] A higher binding capacity was observed at the 1:120,000 ratio. At the 1:70,000 and 1:120,000 AF:B ratio bentonite was sequestered over 0.87 and 0.99 of AFB1.

Moschini *et al.* studied the effect of AF:B ratio (i.e., 1:5,000; 1:50,000 and 1:500,000) on adsorption efficacy of SAs and reported a higher sequestering capacity at the 1:500,000 AF:B ratio.[15] They found over 0.87 and 0.98 of the AFB1 adsorption capacity by sequestering agents, respectively, in water and rumen solutions.

In the present study acid activated bentonite showed higher adsorption efficiency compared to other binders. Also compared to common, acid activated bentonite had higher adsorption efficiency during the incubation time (Tables 3, 4) which indicated stronger connections in acid activated bentonite. Vekiru *et al.* showed that adsorption ability of a bentonite was influenced by the pH of the incubation media.[19] Consistent with our results in study of Chansiripornchai and Fink-Gremmels[9] six out of seven tested sequestering agents showed higher binding efficiency (98.97 - 100%) of AFB1 at the of pH 2.50. However, Gallo and Masoero reported that acid condition (pH 2.00) did not influence the amount of AFB1 recovered.[20] These authors demonstrated that this effect could be related to the limited incubation time. Desheng *et al.* showed that the maximum amount of adsorbed AFB1 was obtained from aqueous solution at pH 2.00 using a calcium montmorillonite as adsorbent.[21] Komadel suggested that at pH ≤ 3.00, the hydroxyl groups of the bentonite octahedral layer were attacked by protons' penetration in the phase and the layer started to redissolve.[22] Also, ion exchange is an important factor in

clay mineral-organic interaction. Thus, protonated clay increases the exchange reactions with the organic cations normally occupying exchange sites on the surface of the clay mineral. Some organic molecules may become cationic after adsorption at clay surface by protonation that increase adsorption capacity of clay. This act depends upon the Bronsted acidity of the clay surface. Organic molecules have the possibility of accepting protons from the clay surface. The ability of the clay surface to donate protons is determined by the nature of the mineral. Acidification of mineral could be resulted in increasing the available proton that increases protonated organic molecules.

In conclusion, our results indicated that the *in vitro* efficiency of the sequestering tested agents was related to the AF:B ratio. Our *in vitro* results ranked the adsorbents as good (bentonites), average (yeast cell wall) or poor (activated carbon). Moreover, acid activated bentonite was more effective than the tested common bentonite.

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Conflict of interest

Authors disclose no conflict of interest.

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