Smectite Clays as Adsorbents of Aflatoxin B₁: Initial Steps

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ABSTRACT

Smectite clay has been shown to sorb aflatoxin B₁ (AfB₁) in animal feed\(^1,2\) and thereby reduce its toxic influence on animals and its entrance to the human food chain. In an effort to find effective adsorbents, 39 samples proposed to adsorb aflatoxin were analyzed and classified into four groups based on their properties: coefficient of linear extensibility (COLE), pH, cation exchange capacity (CEC), organic carbon, microbial content and x-ray diffraction (XRD) spacings. A subset of 20 bentonite samples from commercial sources and reference minerals from 6 US-states and 2 sites in Mexico was selected for sorption determinations. A 10-fold difference in sorption based on the Langmuir equation was observed. Yet clay properties were mostly clustered and it is not clear which properties influence this variation. The basal spacing of AfB₁ saturated smectites exhibited greater resistance to collapse on heating than untreated smectites indicating that AfB₁ entered the interlayer galleries of the smectites. After heating the mycotoxin-clay complex the desorbed mycotoxin was altered indicating a reaction of the molecules with the clay surface. The most effective sorbent smectite samples were from three US-states (MS, ID, TX).

Key words: aflatoxin B₁, smectite, isotherm, adsorption, animal feed

INTRODUCTION

Aflatoxins are highly carcinogenic, hepatotoxic, teratogenic and mutagenetic secondary metabolites produced primarily by the fungi *Apergillus flavus* and *Aspergillus parasiticus*\(^3\). These fungi are widespread and especially a problem in warm climates.

In the US aflatoxins were found to contaminate peanuts, corn, cottonseed, grain sorghum, millet and a variety of nuts. Although aflatoxins are ubiquitous contaminants of several classes of commodities, contamination of corn likely poses the greatest risk to humans worldwide and they are considered unavoidable food and feed contaminants\(^4,5\).

Within the group of aflatoxins, AfB₁ is by far the most toxic and known for its deleterious effects on humans, poultry, livestock and other animals\(^6\). It not only does harm to animals when ingested with their feed but also poses risks to human, e.g. when the animals’ products are consumed. In addition to this, economic costs can be high when aflatoxin occurs at high concentrations in crops and renders the crop unusable.

To reduce or solve the problem of AfB₁ contamination, there have been several different approaches ranging from physical separation of contaminated kernels to chemical treatments to degrade the toxin. One of the more recently developed strategies is the attempt to prevent the adsorption of aflatoxins in feeds in the gastrointestinal tract of animals. Hydrated sodium calcium aluminosilicate (HSCAS) has been shown to be an effective sorbent of AfB₁ and reduces the negative effects of that toxin when the clay is included as a supplement in the animals’ diet\(^6,7\). The inclusion of small amounts of clay in animal feed offers an economically feasible and relatively simple way to suppress AfB₁ influences. Several companies now propose their clay products as feed additives for sorption of AfB₁ sorbents. Despite the effectiveness in AfB₁ sorption by some clays demonstrated mainly in feeding studies, there is a lack of understanding the parameters influencing the effectiveness of smectites as AfB₁ sorbents from a scientific point of view. If certain characteristics of smectites prove as reliable sorbents due to well understood mechanisms, this would allow state authorities to give seals of approval to industrial feed additives as good AfB₁ sorbents without the companies being required to carry out expensive feeding studies.

The research carried out in this study can be assigned to two different stages: (a) the screening of 39 samples to characterize their chemical and physical properties and (b) the characterization of 20 smectite samples out of the larger set as sorbents of AfB₁.

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Accordingly, the first objective of this study is to describe the range of properties of commercial sorbents offered to suppress aflatoxin in animal feed and to present the chemical and physical properties of these commercial sorbents for comparison.

The second objective of this work is to discuss the relationships among clay-sorbents’ properties and the amounts of aflatoxin they adsorb.

MATERIALS AND METHODS

Materials

Aflatoxin B₁ from Aspergillus flavus was purchased from Sigma Chemical Co. (St. Louis, MO 63118); CAS No. 1162-65-8. Acetonitrile, Chromasolv® for HPLC, gradient from Sigma Chemical Co. (St. Luis, MO 63118); CAS No. 67-56-1, were from EM Sciences; CAS 71-05-8. Benzene, GR, CAS 71-56-1, and Methanol, HPLC grade was purchased from Sigma-Aldrich; CAS No. 75-08-6.

Many samples were submitted by the Office of Texas State Chemist representing industrial products proposed as sorbents of AFB₁ and others were reference minerals from our own collection. All 39 samples were analyzed and treated as received. The smectites included in this set were from 6 different states in the United States as well as from 2 sites in Mexico. Smectites from a third site in Mexico, Laguna del Carmen, a saline lagoon in a high desert environment, and their sorption potential will be discussed in a separate paper8).

Sample Screening

The original set of 39 samples was screened for their chemical and physical properties using standard methods. Parameters determined for each sample were the coefficient of linear extensibility (COLE-value9), pH, CEC10), organic carbon and carbonates11) and XRD. If a smectite peak was present, the particle thickness L, was calculated using the Scherrer equation. XRD & TEM were used according to White and Dixon12).

If signs of fermentation were observed during the process of the COLE-value determination, a microbiological culture on Agar was prepared using the spread plate technique to test the sample for yeast or other living organisms13).

After this screening process 20 samples with the characteristics of smectite were examined using the AFB₁-sorption procedures described below.

Isothermal Adsorption Procedure

We used the procedure as described by Grant and Phillips9) with some small changes.

Stock solution. To prepare the stock solution acetonitrile was injected directly into the bottle as received from Sigma Chemical Co. using disposable syringe and needle. Dissolved AFB₁ was taken out using the syringe and transferred to a glass flask. To wash the AFB₁ container acetonitrile was injected several times and each time liquid was then transferred to the flask. Acetonitrile was added to the stock solution to obtain the correct volume. The flask was wrapped tightly in Al foil and stored at 0-2 °C.

Working solution. An aliquot of the stock solution was transferred into a glass flask using an Eppendorf Research Pipette and diluted with distilled water to 8 ppm. The concentration of the working solution was verified by measuring the absorbance of the 365 nm AFB₁-peak in a scan (200-800 nm wavelength) with a Beckman Coulter DU800 UV-Visible–Spectrophotometer.

Isotherms. To each 5 mL of AFB₁-solution with the concentrations 0.0, 0.4, 1.6, 3.2, 4.8, 6.4 and 8.0 ppm were added 0.1 mg smectite-sample. We used sterile FALCON® Blue Max Jr. 15 mL 17 x 120 mm polypropylene conical tubes. The concentrations were obtained by dilution of the working solution with distilled water. In order to add the small amount of clay sample to each concentration first a suspension of 10 mg clay sample per 5 mL distilled water was prepared. Then 50 µL of this 2 mg/mL suspension was transferred to the test tubes using an Eppendorf Research Pipette. The samples were prepared in duplicate. Along with the samples there were two controls consisting of 5 mL of stock solution (8 ppm) without adsorbent and 5 mL of the lowest concentration without adsorbent (0.4 ppm). After 24 hours of shaking at 200 motions/min on an orbital shaker (Cole-Parmer); the samples were centrifuged (IEC PR-7000 Centrifuge) at 51000 g for 57 min and the supernatant was transferred to the test tubes using an Eppendorf Research Pipette.

Using the linear expression of the Langmuir equation

\[
\frac{C_{eq}}{q} = \frac{1}{k_dQ_{max}} + \frac{C_{eq}}{Q_{max}}
\]

first Q\text{max} and k\text{d} were determined, where C\text{eq} is the concentration in equilibrium, q is the amount AFB₁ adsorbed, k\text{d} a distribution coefficient and Q\text{max} the maximum sorption capacity. The data were then fitted to the Langmuir equation.

XRD of AFB₁ saturated smectites

Saturation of smectite with AFB₁. To 10 mg of sample 10 mL of a 100 ppm AFB₁-solution (benzene: acetonitrile, 98: 2) was added and shaken 24 hours at 200 motions/min on an orbital shaker in 50 mL PYREX® glass test tubes with screw caps. AFB₁-concentration left in solution was determined using UV/visible spectrophotometry at \(\lambda = 348\) nm and a molar absorptivity of AFB₁ in benzene: acetonitrile \(\varepsilon = 19800\) 14).

The benzene: acetonitrile solution was directly obtained by dissolving solid AFB₁ in it, parallel to the dissolution of
Afb₁ in acetonitrile as described in detail above. The saturated smectite was mounted on a quartz plate in an aluminum frame for XRD analysis.

Along with each saturated sample control samples were prepared. The amount of sample used was 150 mg, which was first shaken 24 h in benzene: acetonitrile and then dried onto VICOR-glass slides.

Heat treatment. The heat stability of the smectite-aflatoxin complex was determined producing XRD-patterns of the saturated samples and their controls at room temperature and after heating to 100, 150, 200 and 245°C for at least 2 h.

XRD-pattern. The XRD-patterns were obtained directly after the samples were taken out of the oven or a desiccator. XRD-patterns were obtained using a Philips X-ray-diffractometer with CuKα-radiation, a graphite monochromator and a theta-compensation slit. The pattern was measured in 0.05 °2θ intervals from 2 to 32 °2θ for room temperature and 2 to 22 °2θ for all heat treatments of 100 °C and above.

Desorption experiment

Afb₁ was extracted with methanol after the heat treatments. After the last step of heating and XRD was completed, the samples were scrapped off their slides. The amount of smectite was weighted and transferred into PYREX®-glass-test tubes. Five mL of methanol as extractor was added. The amount of methanol for desorption was only half of the 10 mL 100 ppm Afb₁ solution used for saturation to ensure measurable amounts of desorbed material. After 24 h of shaking at 200 motion/min the samples were centrifuged to separate smectite from liquid. Molar absorptivity ε in methanol was 21500, according to AOAC standard method. Instead of measuring at a single position, wavelength scans were performed of the supernatant with the UV/Visible-Spectrophotometer to screen for aflatoxin desorbed from the smectite sample.

RESULTS AND DISCUSSION

Sample Screening

Table 1 and 2 summarize the results of the sample screening. The samples range in CEC from 21.7 to 101.3 cmolc/kg. Organic carbon contents are found to range from 21.7 and 74.2 cmolc/kg. Those samples need further study in order to determine the origin of the CEC and are found in the group "other mineral sorbents" (Table 1).

The 20 samples behaving like smectites were chosen to focus on characterization as Afb₁ sorbents (Table 2). All have a high CEC between 68.3 and 101.3 cmolc/kg and the XRD-characteristics of smectite. The mean crystallite dimension, L, determined using the Scherrer equation ranged from 10.3 to 25.7 nm (for untreated samples air-dried from water at room temperature on XRD-slides). Values for pH in this group were between 7.1 and 10.3 except one sample had an uncommonly low value of 4.7. The last one was included for its uniqueness in this set. Subsequent field study and observation confirmed a presumption that this low pH probably is a result of acidification caused by sulfide oxidation.

A consistency of characteristics can be observed in certain sub-groups, e.g. Mississippi (1MS, 2MS, 3MS, 4MS), Idaho (samples ending with ID) or Laguna del Carmen, Mexico (LC). When pH was plotted against CEC, L or COLE -value clustering of each sub-group can be observed which indicates the same origin of these sample sets and is important for quality assurance.

Sorption

The Langmuir equation yields an overall good fit of the different sorption values (Fig. 1). The maximum adsorption capacities obtained from this data treatment range from 0.060 (16MX) to 0.677 (8TX) mol/kg, representing a 10-fold increase in sorption capacities, as listed in table 2. Values for kd from the Langmuir equation did not show a meaningful relationship to the Afb₁ sorption and therefore are not included.

Amounts of sorption in relation to sample properties show no clear trends but are clustered instead. In contrast to a previous hypothesis by Grant and Phillips also the Ca-ion content of samples does not have a distinct influence on sorption in our study (Fig. 2). Other cations present, K⁺, Na⁺ and Mg²⁺, also seem to have no effect on the sorption ability of the untreated (as received) smectites. Preliminary data however show a reduction in sorption when smectites were K⁺ saturated, which implies that the more highly charged sites are excluded from Afb₁-sorption.

The sorption data points at the highest concentration (8 ppm) using unmodified concentration values show that 6 to 47.7 % of the Afb₁ originally present (0.4 ppm) was adsorbed by the 100 µg clay sample present. Examination of the low concentration data points shows that up to 99.8 % of the Afb₁ originally present (0.4 ppm) was adsorbed. An average of 22.2 % sorption at the 8 ppm point versus a much higher average of 47.7 % at 0.4 ppm. These observations indicate also that even if the maximum sorption capacity as calculated by Langmuir is low, the smectite still might be an effective adsorbent at low concentrations. Sorption behavior at concentration below the saturation level will be part of a further study. Chemisorption is indicated by XRD-results and by the overall good fitting of the data to the Langmuir equation.
Moles AfB1 in the mono layer (sorption capacity) mol/kg on the binding of AfB1 on smectite requires more research. was found essential for binding. The effects of this group mechanisms of action are involved in sorbing aflatoxin to supporting previous suggestions that different sites and concentrations support a more complex mechanism supporting previous suggestions that different sites and mechanisms of action are involved in sorbing aflatoxin to HSCAS and montmorillonite.

Possible sites for sorption of AfB1 to the smectites include its interlayers external surfaces and edge sites. Phillips stated that the dicarbonyl system of aflatoxin was found essential for binding. The effects of this group on the binding of AfB1 on smectite requires more research. To our knowledge, this research represents the most extensive survey of smectite as AfB1 sorbents. Besides smectites, zeolites, other clays, activated charcoal, polymers and yeast products have been tested for their

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<th>pH</th>
<th>COE value</th>
<th>CEC in NaOAc [cmol/kg]</th>
<th>Crystallite dimension L [nm]</th>
<th>Maximum AfB1-sorption capacity Qmax [mol/kg]</th>
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Table 2: Smectite properties listed by increasing sorption.


cx

Figure 1: Langmuir adsorption isotherms encompassing the range of observed sample adsorption capacities.

Figure 2: Comparison of Ca2+-Ion content with sorption capacity.

XRD of AfB1 saturated smectites

The ratio of AfB1 to clay was from 7.4 to 9.8 wt%. The subsequent XRD-pattern after heat treatment showed uniform resistance to collapse of the basal spacing whereas unsaturated control samples, dried from the same matrix solution, did collapse (Fig. 3). The d-spacing of AfB1-treated samples did not collapse below 1.3 nm indicating that the AfB1-molecules entered between the layers and prevented further collapse. The difference between collapsed and uncollapsed samples at 245°C is about 0.3 nm, sufficient to allow AfB1-molecules to fit in.

Phillips et al. found that the sorption capacities of collapsed HSCAS compared to untreated HSCAS derived from isotherm experiments was decreased by more than 85%, which also indicates interlayer sorption of AfB1.

XRD-patterns also show the dissappearance of the third
Figure 3: Basal spacing of AfB$_1$-saturated and – unsaturated samples.

Figure 4: Peak intensity and disappearance of 3$^{rd}$ order peak with AfB$_1$-saturation for sample 8TX.

Desorption experiment

In the spectra from the wavelength scan an alteration of the AfB$_1$ absorbance peaks was observed. Calculated concentrations of desorbed compounds therefore refer to alteration products of AfB$_1$. In most cases the altered peaks were identified as aflatoxicol but few cases showed peaks that are still unidentified AfB$_1$-related compounds. The amount desorbed was estimated to be between 17 and 76% of the amount originally adsorbed. Regarding these differences in retention-ability of the smectites, the simplified system we used must be considered and was probably stronger than would be found in an animal’s stomach, where the solvent is aqueous. Numerous studies have been carried out by mainly animal scientists, which have shown that toxic effects were effectively inhibited in cows$^{11}$ mink$^{22}$ pigs$^{6}$, broiler chickens$^{23}$, turkey poults$^{24}$ and other animals by the inclusion of clays, especially HSCAS. Another factor that might limit the reliability of results obtained for retention-ability of the different smectites is the preceding heat treatment. Yet, the fact that heating induced alteration of sorbed AfB$_1$ to less toxic molecules like aflatoxicol suggests that AfB$_1$ reacts with the smectite surface. Doyle et al.$^{25}$ reported that aflatoxin has been found to be quite stable to heat and was not degraded until 250°C. Thus, what we found might be of interest for future research dealing with degradation of that toxin.

CONCLUSIONS

The commercial offerings of sorbents for aflatoxin include a wide array of clays, organics and mixtures. The smectite clays selected for further investigation had mostly clustered chemical properties with CEC between 68 and 101 cmol/kg and pH ranging from 7.1 to 8.8. An exceptional clay sample was pH 4.7 and field study indicated that it probably was acidified by sulfide oxidation.

Our sorption data suggest that smectites are generally suitable for AfB$_1$-sorption. However, a 10-fold difference was observed in the sorption capacity. The cause of these differences is not well understood although many smectite parameters were measured. Also there are too few first quality sorbents for the vast need on a world scale.

The clustering of sample properties made it difficult to establish their relationships to the sorption capacity. Thus samples need to be investigated with more intensive investigation of individual properties. On the 20 chosen samples more basic data are needed e.g. octahedral composition. Also more unique mineral examples are needed to test functional data.

Consistency of sample sub-groups from sorbent-providing industries with regard to sorption and other characteristic properties can be seen as reliable sources for potential sorbent additives to animal feed. NovaSil$^\text{®}$ clays that have been used in various in vivo studies and which are named 1 to 4MS in our study were reliable sorbents in vitro, too. Also samples from Idaho and Texas have been shown to be reliable smectites for AfB$_1$-sorption.

Sorption data generally fit the Langmuir-equation and theoretical maximum adsorption capacities for AfB$_1$ can be inferred.

The AfB$_1$-molecule was documented the first time directly to enter into the interlayers of the smectites by means of XRD which is in agreement with previous indirect observations. New types of data are needed on bonding between AfB$_1$ and smectites.

Upon heating of AfB$_1$ adsorbed to the smectites it was indicated, that a degradation-reaction took place. This might be of interest for future research dealing with degradation of that toxin.
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