EVALUATION OF THE AFLATOXIN B1 ADSORPTION CAPACITY OF BENTONITE USING AN *IN VITRO* METHOD MIMICKING MONOGASTRIC GASTRO-INTESTINAL TRACT CONDITIONS

Piyarat Chansiripornchai[1*] Johanna Fink-Gremmels[2]

Abstract

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Aflatoxins are considered as unavoidable contaminants of food and animal feeds, since their production cannot be prevented successfully using current agricultural practices. The use of aflatoxin adsorbents as feed supplements is one of the most promising approaches to reduce aflatoxicoses in animals. Since there are numerous adsorbents marketed, an effective and practical method for the rapid evaluation of adsorption capacity of individual products is needed. In this study, we used an *in vitro* model designed to mimic the temperature, pH and the time of passage through the stomach and the intestinal tract of a monogastric animal, for the evaluation of aflatoxin B1 adsorption capacity of different mineral clays. The results indicate that 6 out of the 7 tested products had a high binding capacity (ranging from 98.97-100%), under the experimental conditions used. The data also demonstrate that the method allows us to differentiate those compounds with high binding capacity from those with a lesser binding capacity. Such an *in vitro* method seems useful for the rapid screening of different substances and for the pre-selection of adsorbent products that may be used as feed supplements.

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การประเมินประสิทธิภาพของเบนโทไนต์ในการดูดซับอะฟลาท็อกซินบี 1 โดยวิธีจำลองสภาวะของทางเดินอาหารแบบกระเพาะเดี่ยวภายนอกร่างกาย

    อะฟลาท็อกซินเป็นสารพิษจากเชื้อราที่มักพบปนเปื้อนในอาหารของมนุษย์และสัตว์เนื่องจากกระบวนการทางเกษตรกรรม ในปัจจุบันยังไม่สามารถป้องกันการสร้างสารพิษชนิดนี้จากเชื้อราเมื่อมีการปนเปื้อนในอาหารได้ การใช้สารดูดซับอะฟลาท็อกซิน ผสมลงในอาหารสัตว์เป็นวิธีการหนึ่งที่นิยมใช้ในการลดความเป็นพิษจากอะฟลาท็อกซินในสัตว์ เนื่องจากมีการผลิตสารดูดซับออก จำหน่ายในท้องตลาดอย่างมากมาย ดังนั้นจึงมีความจำเป็นที่จะต้องมีวิธีการที่มีประสิทธิภาพและสะดวกในทางปฏิบัติเพื่อใช้ ประเมินความสามารถในการดูดซับสารพิษของสารดูดซับแต่ละชนิด การศึกษาครั้งนี้ได้จำลองสภาวะทางเดินอาหารของสัตว์ กระเพาะเดี่ยวทั้งอุณหภูมิ ความเป็นกรดด่าง และเวลาในการผ่านของอาหารจากกระเพาะอาหารเข้าสู่ลำไส้ เพื่อประเมิน ประสิทธิภาพของเบนโทไนต์ในการดูดซับอะฟลาท็อกซินบี 1 จากผลการทดลองพบว่าสารดูดซับ 6 ใน 7 ชนิดมีประสิทธิภาพใน การดูดซับอะฟลาท็อกซินบี 1ได้ดีมาก (98.97-100%) ผลการศึกษายังแสดงให้เห็นว่าสามารถจำแนกสารดูดซับอะฟลาท็อกซินที่มี ประสิทธิภาพสูงออกจากสารดูดซับที่มีประสิทธิภาพต่ำได้โดยใช้วิธีการที่ประยุกต์ขึ้นนี้ ดังนั้นการใช้วิธีจำลองสภาวะของทาง เดินอาหารแบบกระเพาะเดี่ยวภายนอกร่างกายเพื่อประเมินประสิทธิภาพของสารดูดซับอะฟลาท็อกซินจึงเป็นวิธีการที่มี ประโยชน์ในการทดสอบประสิทธิภาพของสารดูดซับอะฟลาท็อกซินเบื้องต้นได้อย่างรวดเร็ว เพื่อนำไปประกอบการพิจารณา นำสารดูดซับชนิดนั้นๆ มาใช้เป็นสารเติมเพื่อลดพิษของอะฟลาท็อกซินในอาหารสัตว์

คำสำคัญ : สารดูดซับ อะฟลาท็อกซินบี 1 เบนโทไนต์ การลดความเป็นพิษ ภายนอกร่างกาย

Introduction

    Some field and storage fungi produce a variety of secondary metabolites, mycotoxins, that exert adverse effects on human and animal health. These mycotoxins can induce organ lesions and may be mutagenic, carcinogenic, immunosuppressive as well as interfering with hormonal function (Karlovsky, 1999). Among the group of mycotoxins, which represents more than 300 structurally diverse compounds, the aflatoxins have been the most intensely studied, since their discovery in the 1960s (Asao et al., 1965; Brechbuhler et al., 1967). The occurrence of these toxins in food and feed materials has caused not only adverse health effects in animals and humans, but has also resulted in economic loss, for exporting countries, due to non-marketable products (Basappa and Shantha, 1996).

Aflatoxin B_1 (AFB$_1$) and various aflatoxin analogs are commonly produced by *Aspergillus flavus* and *A. parasiticus* molds that occur in the field and during the storage of grains, cereals and nuts. Natural aflatoxins are AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$. However, following hepatic biotransformation the metabolites M_1, M_2, M_{2a}, MP$_1$, P_1, H_1 and Q_1 have been identified, whereas aflatoxicol I and II originate from bacterial metabolism (Phillips, 1999). AFB$_1$ is the most potent mycotoxins and can be both acutely toxic and carcinogenic for many animal species (Wogan and Newberne, 1967; Hendricks, 1994).

    At present, aflatoxins are considered unavoidable contaminants of food, since they cannot be prevented or eliminated by current agricultural practices (CAST, 1989). Because aflatoxin is heat stable, food and feed processing

procedures do not reduce the toxin concentration. Thus, aflatoxins are found as a contaminant of corn, peanuts, peanut butter, breakfast cereals, cornmeal, cottonseed, tortillas, animal feeds and the dairy products (including ice cream and cheese) derived from milk of toxin-exposed dairy cattle. In developed countries, grains and oil seeds that are contaminated with aflatoxins at concentrations higher than 20 ppb are condemned (Phillips, 1999). However, in lesser-developed countries with limited supplies of food, it is neither feasible, nor practical, to condemn and destroy all contaminated grains. Therefore, safe and practical chemical intervention methods for the detoxification of food and animal feeds in terms of chemoprevention against aflatoxicosis are critical needs.

In animal production, the use of mycotoxin adsorbents as feed supplements is one of the most promising approaches towards the reduction of mycotoxicoses in farm animals and towards a minimalization of carry-over of these mycotoxins from contaminated feeds into animal products (Ramos et al.,1996; Huwig et al., 2001). Different substances, including phyllosilicate minerals, zeolites, activated charcoal, synthetic resins and yeast cell-wall-derived products, have been used for the adsorption of aflatoxins *in vitro* and *in vivo* as described in numerous reports (Devegowda et al., 1996; Galvano et al., 1996; Kececi et al., 1998; Miazzo et al., 2000; Raju et al., 2000; Rosa et al., 2001). Since there are many adsorbents on the market, it is necessary to establish an effective and practical method to evaluate the aflatoxin and other mycotoxins adsorption capacity of individual adsorbents.

Our objective in this study was to determine the adsorption capacity of bentonite for AFB_1 using an in vitro method designed to mimic the conditions of a monogastric animal, which can also be used as a predictive value for humans.

Materials and methods

Materials. Seven mineral clays were obtained from commercial companies in the Netherlands. The composition of the mineral clays were labeled as

bentonites originating from different natural resources. AFB_1 standard was purchased from Sigma (St. Louis, CA, USA). All the solvents used in chromatography were of HPLC grade and all other reagents were of analytical grade.

Assessment of pH-dependent adsorption. A pH-dependent adsorption method was used as previously described by Sabater (Sabater, 2003). Seven bentonites named: bent.1, bent.2, bent.3, bent.4, bent.5, bent.6 and bent.7 were suspended in a phosphate buffer solution (CaCl2 x H_2O, 1.2 mM; KCl, 2.7 mM; KH_2PO4, 1.5 mM; MgCl2 x $6H_2O$, 1.1 mM; NaCl, 138 mM; Na_2HPO4 x $2H_2O$, 8.1 mM; pH = 7.4) to produce a final concentration of 2.5 mg of product / ml. AFB_1 standard was added to this suspension at a final concentration of 0.17 mg/ l (170 ppb). Negative controls containing the AFB_1 solution without adsorbent were also included. The mixtures were adjusted to a pH of 2.5 and incubated at 37ºC for 1 hr, with constant agitation, to simulate the gastric conditions of a monogastric animal. After taking an aliquot for analysis, the pH of the suspensions was further adjusted to 8 and the suspension was incubated at 37ºC for 3 hr, using constant agitation to simulate the conditions of intestinal passage in a monogastric animal. A second aliquot was then taken and both aliquots were immediately filtered (Mini-GF; Sartorious, Gottingen, Germany) to separate the binder. 1.5 ml of each filtrated aliquot was extracted with 8.5 ml of chloroform. The water phase was discarded and the chloroform was evaporated. The final residue was re-dissolved in 250 μl of mobile phase from which 50 μl were injected into the HPLC system.

AFB_1 detection by HPLC. A Spherisorb ODS-2, 5 μm column (4.6x 250 mm, Chrompack, The Netherlands) was connected to two high precision pumps (Gynkotekmodel 300) set at a flow rate of 0.8 ml/min and controlled by Chromeleon-GynkotecHPLC software (Softron). Fluorescence detection was performed using a FP 920 fluorescence detector (Jasco, Japan) set at 365 nm

excitation wavelength and 420 nm emission wavelength. Mobile phase consisted of acetronitrile/methanol/water (20:20:60 v:v:v) with 58.44 mg of NaCl /l.

Statistical analysis. The significance of the differences between the control and the treatment groups was determined by one-way ANOVA. Statistical significance was accepted at $p < 0.05$.

Results

An *in vitro* system designed to mimic the temperature, pH and time of passage through the stomach and the intestinal tract of a monogastric animal was applied to evaluate the AFB_1 binding capacity of 7 bentonites. AFB_1 adsorption, after the gastric (pH 2.5) and the intestinal (pH 8) phases, was assessed by HPLC determination of the unbound AFB_1 in the incubation fluid. Table 1 shows near to 100% adsorption of AFB_1 for the bent.1 to bent.6 products tested at a concentration of 2.5 mg/ml. The bent.5 showed 100% adsorption of AFB_1 in both the pH 2.5 and the pH 8 buffer aliquots (Fig.1). In contrast, bent.7 could adsorb only 10% of AFB_1. Comparable results were found in both the pH 2.5 and the pH 8 phases (Table 2). According to the results, the best mineral clays for the pH 2.5 were bent.5, 6, 2, 4, 3 and 1, and for the pH 8 were bent.5, 2, 6, 1, 3 and 4. The product bent.7 was not a good adsorbent for AFB_1 in this study.

Discussion

Aflatoxins are considered as unavoidable contaminants of various feed and food commodities. The best procedure to prevent the negative effects of mycotoxins is to minimize the mycotoxin production itself, e.g. by harvesting the grain at maturity and by maintaining low moisture and low temperature during storage. This is difficult to carry out in countries with a warm and humid climate (Huwig et al., 2001). In order to avoid mycotoxicosis, several strategies have been investigated including biological, chemical and physical methods (Basappa and Shantha, 1996). Physical methods focus on the removal of mycotoxins by different adsorbents, added

to mycotoxin-contaminated diets (Ramos et al., 1996). Reduction of the bioavailability of aflatoxins in animals by the addition of dietary clay minerals has received considerable attention since the first reports using a processed montmorillonite clay (Phillips et al., 1987, 1988). The montmorillonites and the bentonites belong to a group of natural aluminosilicates (Ramos and Hernandez, 1997). The mechanism of action of these mineral clays is to bind irreversibly with the toxin in aqueous media, thereby, preventing absorption of the toxin across the intestinal wall (Schell et al., 1993). Now, new adsorbents for aflatoxins and other mycotoxins are released onto the market rapidly and thus it is necessary to determine the quality of individual adsorbent products. We therefore developed a practical and simple method to evaluate, in vitro, the aflatoxin binding capacity of mineral clays. Mycotoxin adsorbents are usually added to the feed in a concentration of 1 to 5 kg/t, which corresponds to a concentration of 1 to 5 mg/ml (Sabater, 2003). The concentration of AFB_1 used in this study (170 ppb) was much higher than the acceptable concentration of AFB_1 in animal feeds established by the US Food and Drug Administration (Basappa and Shantha, 1996). For performing AFB_1 binding assay under realistic conditions, we applied these concentrations of adsorbent and AFB_1 in our *in vitro* system.

According to our results, 6 out of 7 tested products appear to bind AFB_1 efficiently in both the pH 2.5 (98.97-100%) and the pH 8 (96.58-98.75%) phases. However, there was a different binding capacity among the tested adsorbents when tested at different pH. From the results, the binding capacity of the bent.3, 4 and 6 decreased in the pH 8 phase ($p < 0.05$). These data confirm the suitability of the applied method for the rapid screening of new products. Our results are consistent with those of Sabater (2003), who was the first to report the usefulness of such *in vitro* models under conditions of pH, temperature and time, comparable to those of a monogastric gastrointestinal tract, for evaluation of deoxynivalenol (DON) and zearalenone (ZEN) binding capacity of the adsorbents. The obvious

Table 1. AFB$_1$ *in vitro* adsorption at pH 2.5 assessed by HPLC for different mineral clays, added at 2.5 mg/ml[1]

Product	AFB$_1$ concentration (ng)	Adsorption (%)
Control	50	-
bent.1	0.52±0.01*	98.97±0.02
bent.2	0.06±0.09*	99.89±0.19
bent.3	0.29±0.17*	99.43±0.33
bent.4	0.16±0.14*	99.69±0.27
bent.5	0*	100
bent.6	0*	100
bent.7	44.31±2.81	10.77±5.68

[1]Values are the mean±SD of three independent experiments.

*Significantly different from the control ($p<0.05$, one way ANOVA)

Table 2. AFB$_1$ *in vitro* adsorption at pH 8 assessed by HPLC for different mineral clays, added at 2.5 mg/ml[1]

| Product	AFB$_1$ concentration (ng)	Adsorption (%)
Control	50	-
bent.1	0.83±0.83*	98.34±1.67
bent.2	0.63±0.56*	98.75±1.11
bent.3	1.06±0.10*	97.89±0.21
bent.4	1.71±0.21*	96.58±0.43
bent.5	0*	100
bent.6	0.72±0.27*	98.55±0.55
bent.7	46.43±1.39	6.48±2.81

[1] Values are the mean+SD of three independent experiments.

* Significantly different from the control ($p<0.05$, one way ANOVA)

advantage of the in vitro model is the ability to do rapid screening of different substances, enabling a pre-selection of reliable adsorbent products (Sabater, 2003).

Since *in vitro* studies are not always predictive of *in vivo* results, it is important to emphasize that the mycotoxins binding capacity of any new adsorbent should also be confirmed by in vivo experiments, before releasing the product onto the market.
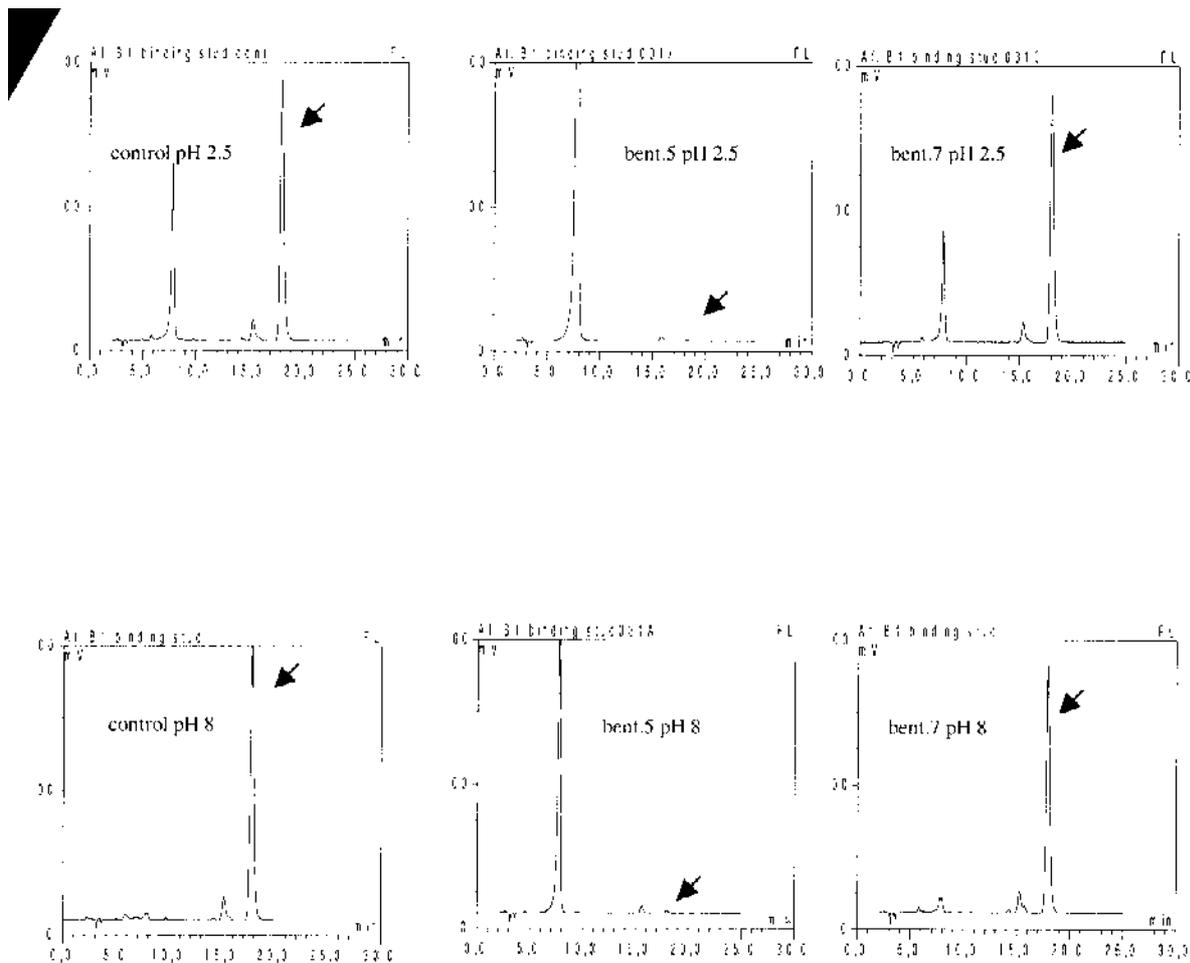
Figure 1. HPLC-chromatograms showing the detection of AFB$_1$ after incubation with different adsorbents at pH 2.5 and pH 8. The arrows show the position of the AFB$_1$ peak. These chromatograms also demonstrate that no fluorescent AFB$_1$-breakdown products were formed.

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