JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE


DOI:10.1021/jf9045304

# Value-Added Processing of Peanut Meal: Aflatoxin Sequestration during Protein Extraction

LAUREN E. SEIFERT,[†] JACK P. DAVIS,*[,†,‡] JOE W. DORNER,[§] WILLIAM F. JAYNES,[||] RICHARD E. ZARTMAN,[||] AND TIMOTHY H. SANDERS[†,‡]

[†]Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University, and [‡]Market Quality and Handling Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Raleigh, North Carolina 27695-7624, [§]National Peanut Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Dawson, Georgia 39842-0509, and [||]Department of Plant and Soil Science, Texas Tech University, Lubbock, Texas 79409

The efficacy of a bentonite clay, Astra-Ben 20A (AB20A), to sequester aflatoxin from contaminated (~110 ppb) peanut meal during protein extraction was studied. Aqueous peanut meal dispersions (10% w/w) were prepared by varying the pH, temperature, enzymatic hydrolysis conditions, and concentrations of AB20A. After extraction, dispersions were centrifuged and filtered to separate both the water-soluble and the water-insoluble fractions for subsequent testing. Inclusion of AB20A at 0.2 and 2% reduced ($p < 0.05$) aflatoxin concentrations below 20 ppb in both fractions; however, the higher concentration of AB20A also reduced ($p < 0.05$) the water-soluble protein content. Inclusion of 0.2% AB20A did not affect protein solubility, total soluble solids, or degree of hydrolysis. Peanut meal adsorption isotherms measured the AB20A capacity to sequester aflatoxin. These results are discussed in the context of a process designed to sequester aflatoxin from contaminated peanut meal, which could enable derivatives of this high protein material to be utilized in enhanced feed and/or food applications.

**KEYWORDS:** ***Arachis hypogaea***; **peanut meal; peanut protein; sodium bentonite clay; hydrolysis**

## INTRODUCTION

Peanut meal is the nonfood-grade material remaining after the extraction of oil from peanut (*Arachis hypogaea* L.) seed (*1*). In the United States, oil is typically extracted from peanuts that are not considered suitable for human consumption due to discolored, broken, or aflatoxin-contaminated seed. Aflatoxins are toxic compounds produced by the fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (*2*). The four major naturally occurring aflatoxins are aflatoxin $B_1$ ($AfB_1$), $B_2$, $G_1$, and $G_2$ (*2*). Aflatoxin-contaminated peanuts are designated for oil production because the toxins are primarily retained in the residual meal, and the small quantity of aflatoxins that remains in the crude oil is destroyed or eliminated during the refining process (*3*). After oil extraction, the relative percentages of all peanut solids increase in the meal, including that of the protein and residual aflatoxin. Ingestion of aflatoxins from contaminated food or animal feed can lead to acute liver damage or cancer, edema, hemorrhage, alteration in digestion, and possibly death (*2*). Accordingly, the FDA has issued regulatory guidelines limiting the contamination level of aflatoxin to 20 ppb or less in foods intended for human consumption (*4*). Despite being an excellent source of protein (~45−55%), the relatively high aflatoxin concentrations typically associated with peanut meal currently limit applications within feed markets, in addition to prohibiting any food ingredient applications.

*To whom correspondence should be addressed. Tel: 919-515-6312. Fax: 919-513-8023. E-mail: jack.davis@ars.usda.gov.

Aflatoxin is an unavoidable contaminant in peanut and other crops, but it can be minimized by implementing good agricultural practices in addition to maintaining appropriate moisture and temperature conditions after harvest (*5*). However, if aflatoxin contamination cannot be prevented, a variety of physical, chemical, and biological methods have been proposed to reduce the concentration of aflatoxin and other mycotoxins in contaminated biomaterials (*6*). It is established that various types of aluminosilicate clays, or other adsorbents, can be added to animal feeds contaminated with aflatoxin or other mycotoxins to minimize the harmful effects of these toxins during feed consumption (*6*, *7*). These high-surface area, negatively charged minerals bind mycotoxins in the gastrointestinal tracts of the animals, effectively sequestering the toxins, which then pass harmlessly through the livestock (*8*). The ideal toxin−sequestrant complex should not dissociate internally and should be expelled in the animal feces (*9*). For the current work, this inexpensive solution for sequestering aflatoxin was adapted to, and evaluated in, a process design for extracting protein from peanut meal.

Peanut meal has inherently high protein and low residual oil contents, making this material an excellent target for aqueous protein extraction. As such, the effectiveness of a commercially available bentonite clay, sold under the trade name Astra-Ben 20A (AB20A), to sequester aflatoxin was evaluated during protein extraction of contaminated peanut meal. By directly adding AB20A or similar absorbents to aqueous dispersions of aflatoxin-contaminated peanut meal, the potential exists to sequester these toxins in conjunction with protein extraction.

© 2010 American Chemical Society
Published on Web 03/30/2010

Such extraction experiments relate to a processing plan ultimately designed to produce aflatoxin-free, spray-dried protein/peptide concentrates from the water-soluble extracts prepared from peanut meal dispersions. These concentrates have the potential for high-value feed and, eventually, food applications. A coproduct of this processing plan is the water-insoluble portion of the peanut meal dispersions containing the sequestered aflatoxin, which would be appropriate for enhanced feed applications as the sequestered aflatoxin should be biologically unavailable. Variables evaluated in the current work include the concentration of AB20A, pH, temperature of extraction, and protein hydrolysis conditions. Enzymatic hydrolysis of peanut meal dispersions is being studied in conjunction with this work to potentially improve protein extraction efficiency, in addition to potentially improving the nutritional and functional properties of the water-soluble extracts.

## MATERIALS AND METHODS

**Materials.** Defatted peanut meal (~110 ppb aflatoxin, 7.7% moisture) was provided by Golden Peanut Co. (Alpharetta, GA). Pepsin [enzyme commission (EC) number 232-629-3], porcine stomach mucosa (1020 units/mg protein), alcalase from *Bacillus licheniformis* (2.4 AU/g, batch 056K1213, EC 232-752-2), NaCl, and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). L-Leucine was obtained from Fluka BioChemika (Buchs, Switzerland). AB20A was provided by Prince Agri Products, Inc. (Quincy, IL). Primary and secondary $AfB_1$ antibodies and other enzyme-linked immunosorbent assay (ELISA) chemicals were also obtained from Sigma-Aldrich. Novasil Plus was obtained from Trouw Nutrition (Jackson, MS). Methanol, potassium bromide, hydrochloric acid, acetic acid, sodium hydroxide, and alkaline Norit-A decolorizing carbon were obtained from Fisher Scientific (Fair Lawn, NJ). Nitric acid was obtained from VWR International (West Chester, PA). AflaTest Developer, AflaTest columns, and mycotoxin standards were acquired from VICAM (Watertown, MA). Aflatoxin Mix Kit-M was purchased from Supelco (Bellefonte, PA).

**Sample Preparation.** Preliminary experiments were conducted to determine if aflatoxin could be sequestered from contaminated peanut meal during protein extraction. Peanut meal dispersions (10% w/w; 500−800 g total) were prepared in deionized water and adjusted to either pH 2.0 or 8.0 using 2 N HCl or 2 N NaOH, respectively. Each pH had a control (no clay) and two levels of AB20A: 0.2 (w/w) or 2% (w/w) of total dispersion, totaling six treatments. All dispersions were stirred at room temperature for 60 min. Dispersions were then fractionated as described below, and soluble and insoluble fractions were subsequently tested for aflatoxin concentration.

On the basis of the initial experiments, a concentration of 0.2% (w/w) AB20A was chosen for all subsequent testing. Two proteases, pepsin and alcalase, were evaluated for their effects on aflatoxin sequestration and protein extraction. Peanut meal dispersions (10% w/w) were equilibrated by stirring at 37 °C and pH 2.0 or 60 °C and pH 8.0, the optimal temperature and pH for pepsin and alcalase hydrolysis, respectively. Dispersions were hydrolyzed for 60 min in a water bath under constant stirring using a Heidolph Instruments overhead stirrer (Schwabach, Germany). After hydrolysis, enzymes were heat inactivated by submerging the dispersions in sealed centrifuge tubes in a water bath at 90 °C for 15 min. To separate protease effects from the temperature changes necessary for hydrolysis and protease inactivation, appropriate controls were also tested.

The food-grade proteases chosen for these experiments were commercially available. Pepsin was used at an enzyme/substrate ratio of 19000 units/g peanut meal protein, roughly 10 times higher than typically found in the human digestive tract and a concentration empirically found to give a relatively complete digestion over the 1 h test period. Alcalase was added at an enzyme/substrate ratio of 0.6 Anson units (AU)/g peanut meal protein, which again was empirically found to give a relatively complete digestion over the 1 h test period. As defined by the supplier, 1 AU is the amount of enzyme that digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product, which gives the same color with Folin−Ciocalteu phenol reagent as 1 mequiv of tyrosine per min.

**Fractionation.** Dispersions were centrifuged at 25000*g* for 20 min. The pellet (insoluble fraction) was collected for further testing. Then, the supernatant (soluble fraction) was poured through two layers of cheese cloth to exclude any extraneous insoluble matter. Filtered supernatants were then centrifuged at 8000*g* for 10 min, and the soluble fraction was collected for further testing. Soluble and insoluble fractions were frozen at −15 °C prior to analyses. All treatments were prepared in triplicate.

**Aflatoxin Detection within Insoluble Fractions.** For the first set of experiments involving two different concentrations of AB20A at pH 2.0 or 8.0, the aflatoxin concentration of the insoluble fractions was determined using the "AflaTest Procedure for Peanuts and Treenuts (0−50 ppb)" on a Series-4 VICAM Fluorometer (VICAM) according to the manufacturer's directions. A 25 g sample of the insoluble fraction was added to a blender jar along with 5 g of NaCl and 125 mL of 60% methanol:40% water and mixed on high speed for 1 min. Extracts were poured through fluted filter paper, and filtered extracts were diluted 1:1 volumetrically with deionized water and subsequently filtered through a glass microfiber filter. The filtered extract (10 mL) was passed completely through an AflaTest column at a rate of 1−2 drops/s. The column was washed twice with 10 mL of distilled water at a rate of 1−2 drops/s. The AflaTest column was eluted with 1 mL of high-performance liquid chromatography (HPLC) grade methanol at a rate of 1−2 drops/s, and the sample eluate was collected in a glass cuvette. One mL of AflaTest Developer solution was added to the eluate in a cuvette and vortexed, and the fluorescence was measured. The method is accurate in the range of 0−50 ppb. For the second set of experiments involving hydrolysis conditions and one concentration of AB20A (0.2%), aflatoxin concentrations in the water-insoluble fractions were extracted and quantified using HPLC as described previously (*10*).

**Aflatoxin Detection within Soluble Fractions.** For the first set of experiments involving two different concentrations of AB20A at pH 2.0 or 8.0, the aflatoxin concentration of the soluble fractions was determined using a modified method as described above to account for the high water content in the soluble fractions. Twenty-five milliliter samples of the soluble fractions were mixed in a high speed blender with 5 g of NaCl and 50 mL of pure methanol for 1 min. Extracts were poured through fluted filter paper, and 10 mL of filtered extracts was diluted with 20 mL of deionized water. The dilute extract was then filtered through a glass microfiber filter, and the filtered extract (9 mL) was passed completely through an AflaTest column at a rate of 1−2 drops/s. The column was washed twice with 10 mL of distilled water at a rate of 1−2 drops/s. The AflaTest column was eluted with 1 mL of HPLC grade methanol at a rate of 1−2 drops/s, and the sample eluate was collected in a glass cuvette. HPLC was used to quantify the aflatoxin concentration according to Association of Official Analytical Chemists (AOAC) official method 991.31. A KOBRA Cell (R-Biopharm Rhone Ltd., Glasgow, Scotland) was used instead of an iodine pump for postcolumn derivatization (*11*). For the second set of experiments involving hydrolysis conditions and one concentration of AB20A (0.2%), 20 mL of liquid sample was extracted with 80 mL of pure methanol, and aflatoxins were quantified by HPLC (*10*).

**Aflatoxin from Peanut Meal Adsorption Isotherms.** $AfB_1$ adsorption from aqueous peanut meal dispersions was determined using a modification of a method for corn meal dispersions (*12*). Briefly, 2 mL of water containing 3 $\mu$g of $AfB_1$ and three sequestrants [AB20A, Novasil Plus, and activated carbon (AC)] were added to 0.5 g of peanut meal in 15 mL polypropylene centrifuge tubes and equilibrated overnight on a reciprocating shaker. A 60% methanol/40% 2 M NaCl solution was added to the tubes, and the suspensions were thoroughly mixed to extract unadsorbed $AfB_1$. The tube contents were passed through 0.2 $\mu$m filters and diluted into microplates containing $AfB_1$ standards. The $AfB_1$ concentrations were measured in the microplates using ELISA techniques. Four blanks containing only peanut meal, water, and $AfB_1$ were prepared to measure initial $AfB_1$ concentrations. Six-point isotherms with three replicates of each point (i.e., 18 data points per isotherm) were prepared. Sample weights used for AB20A and Novasil Plus were 1, 2, 3, 5, 7.5, and 10 mg (0.2−2%). Greater sample weights of AC of 2.5, 7.5, 10, 15, 25, and 50 mg (0.5−10%) were needed to produce comparable $AfB_1$ adsorption. The amount of $AfB_1$ adsorbed (g $AfB_1$/kg sequestrant) was plotted against

**Table 1.** Total Aflatoxin Concentration (ppb) of Soluble and Insoluble Peanut Meal Fractions before and after Clay Treatment[a]

| sample | pH | control | 0.2% AB20A | 2% AB20A |
|---|---|---|---|---|
| soluble | 2.0 | 51.0 A | 11.6 B | 0 B |
| | 8.0 | 50.0 A | 4.8 B | 0 B |
| insoluble | 2.0 | 80.6 A | 39.8 BC | 0.9 D |
| | 8.0 | 90.7 AB | 16.3 CD | 1.5 D |

[a] All numbers are corrected for moisture content and are reported on a dry weight basis. Means within the soluble or insoluble groups followed by different letters are significantly different ($p < 0.05$).

the equilibrium $AfB_1$ concentration (mg $AfB_1$/mL) to prepare adsorption isotherms. Least-squares linear regression fits of the data were used to calculate the slope or $K_d$ values of the isotherms, which indicate the relative capacity of the three adsorbents to remove $AfB_1$ from peanut meal.

**Soluble Sugars.** Select soluble fractions were analyzed for sugar contents according to a previously described method (*13*).

**% Total Soluble Solids (TSS).** Approximately 2 g samples were analytically weighed in an aluminum dish and heated in a vacuum oven (VWR Scientific, Inc.) at 115 °C for 16 h. Dried samples were cooled to room temperature in a desiccator prior to final mass determination.

**Protein Solubility.** The protein concentration of water-soluble fractions was determined via the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using bovine serum albumin as the reference protein. All hydrolysates were diluted 1:20 with deionized water prior to analysis.

**Proximate Analysis.** The proximate composition of peanut meal, along with the insoluble fractions remaining after pepsin and alcalase hydrolyses, was determined in triplicate using AOCS Official Methods for protein (AOCS Ba 4d-90), moisture (AOCS Ca 2c-25), fiber (AOCS Ba 6-84), and ash (AOCS Ca 11-55) to determine their feed potential.

**Statistical Analyses.** Statistics were performed using analysis of variance to analyze the data from this randomized complete block design. Means separation was conducted using Tukey's honest significant difference test. All statistics were performed using SAS (Cary, NC).

## RESULTS AND DISCUSSION

Initial experiments tested the efficacy of AB20A at two levels, 0.2 and 2.0%, to sequester aflatoxin from pH 2.0 and pH 8.0 contaminated peanut meal dispersions during protein extraction. These two pH values were chosen because peanut proteins are best extracted at pH values above and below the isoelectric point of the proteins, which for most is near ~4.5 (*14*). Additionally, the binding mechanisms of bentonite clays, such as AB20A, have electrostatic components (*15*, *16*), making pH adjustment a logical experimental variable. The aflatoxin concentration in the soluble and insoluble fractions was adjusted for moisture content and reported on a dry weight basis (**Table 1**), as both fractions would need to be dried prior to utilization as food or feed sources. After centrifugation, higher levels of aflatoxin were detected in the water-insoluble fractions for the control samples as compared to the water-soluble fractions. Hypothetically, the sum of insoluble and soluble aflatoxin contents for either pH 2.0 or 8.0 samples should total 110 ppb aflatoxin on a dry weight basis, which was the starting concentration of aflatoxin in the dry meal. This was not observed and is attributed to experimental error in the measures, which as discussed later, was magnified by the relatively high moisture contents of both soluble and insoluble fractions. Another source of error is the potential for areas of locally high or low concentrations of aflatoxin within dry meal and/or dispersions. The addition of AB20A reduced ($p < 0.05$) or eliminated the detectable aflatoxin from both water-soluble and insoluble fractions. No pH effect was observed across any of the treatments, suggesting that AB20A sequestered the toxins when the peanut protein had a net positive (pH 2.0) or a net negative (pH 8.0) charge. Likewise, 2% addition of an AB20A analogue adsorbed at least 97% of $AfB_1$ from 10% methanol solutions adjusted to pH 3.0, 7.0, or 10.1 (*17*). Taken together, these studies suggest that hydrophobic interactions predominate over electrostatic effects in the binding mechanism of this clay. The higher concentration of AB20A was more effective in sequestering aflatoxin for both water-soluble and -insoluble fractions at either pH 2.0 or 8.0, such that the toxins were undetectable after 2% (w/w) addition for the water-soluble fractions. Similar to the current study, various bentonite clays added at 2% w/v level sequestered $AfB_1$ from artificially contaminated liquid media such that only traces remained (*18*). Aflatoxin concentrations in the AB20A-treated water-soluble samples were reduced to levels (< 20 ppb) that are suitable for use in human food products according to FDA regulations (*4*), which is an important proof of principle necessary for eventual utilization of this protein source as a food ingredient. When detectable, HPLC revealed that $AfB_1$ was the most predominant of the total aflatoxins in both soluble and insoluble fractions (data not shown).

Protein and TSS in the water-soluble fractions were quantified to further investigate the effects of adding AB20A to peanut meal dispersions (**Figure 1**). Both 2% AB20A treatments decreased ($p < 0.05$) protein solubility (**Figure 1A**). Specifically, the addition of 2% AB20A reduced protein content by 32% in the pH 2.0 and 44% in the pH 8.0 water-soluble fractions. Bentonite clays can directly bind proteins (*19*, *20*), and previous studies have also indicated that aflatoxins can bind protein (*21*, *22*). This suggests that some peanut meal protein may be bound directly to the clay and/or aflatoxin/clay complex and subsequently pulled into the insoluble fraction after centrifugation. The protein solubility data are consistent with the TSS data (**Figure 1B**), which indicate that a large component of the TSS is protein. A decrease ($p < 0.05$) in TSS was observed after the addition of 0.2 and 2% AB20A in the pH 2.0 samples and only with the addition of 2% AB20A at pH 8.0.

Because the lower AB20A concentration reduced ($p < 0.05$) the aflatoxin concentration without decreasing the soluble protein content, further experiments were conducted to determine the effects of hydrolysis with two different proteases, pepsin, and alcalase, on the efficacy of 0.2% AB20A to sequester aflatoxin in contaminated peanut meal dispersions. Enzymatic hydrolysis is an established strategy for improving the functional and nutritional properties of protein-enriched substrates (*23*). Increasing the amount of water-soluble protein via enzymatic hydrolysis was a principal objective for the current work, as these soluble extracts could potentially serve as high-value, aflatoxin-free protein/peptide concentrates after drying.

TSS in the water-soluble fractions ranged from approximately 3 to 7% and was increased ($p < 0.05$) by hydrolysis with either pepsin at pH 2.0 or alcalase at pH 8.0 (**Figure 2A**,**B**). To distinguish protease effects from the temperature changes necessary for optimum hydrolysis (37 or 60 °C) and enzyme inactivation (90 °C), appropriate controls were also tested. TSS increased approximately 80% after hydrolysis with alcalase, resulting in the highest concentration of TSS (**Figure 2B**). In general, agreement with earlier experiments (**Figure 1**), the addition of 0.2% clay, did not affect the TSS for any treatment pertaining to hydrolysis (**Figure 2A**,**B**).

In contrast to TSS data (**Figure 2A**,**B**), protein solubility as measured by the BCA assay was not affected ($p < 0.05$) by hydrolysis with either of the proteases (**Figure 2C**,**D**). Hydrolysis typically results in an increase in protein solubility; however, this improvement is most obvious near the isoelectric point of a protein substrate (*24*, *25*), and isoelectric point solubility was not determined for the current treatments. The degree of hydrolysis (DH) values of soluble fractions were greater ($p < 0.05$) after hydrolysis with alcalase (42%) as compared to pepsin (15%), suggesting that alcalase more effectively digested the peanut

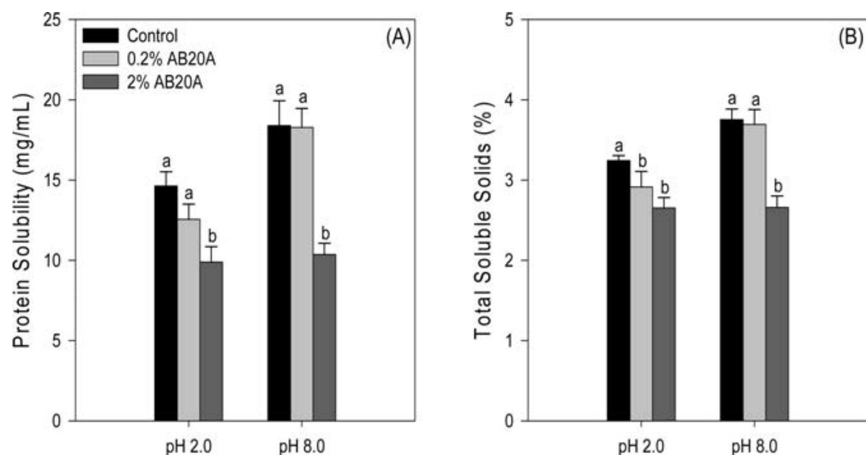


**Figure 1.** (**A**) Protein solubility and (**B**) TSS in the pH 2.0 and pH 8.0 soluble fractions at varying AB20A concentrations. Means within a group followed by different letters are significantly ($p < 0.05$) different.

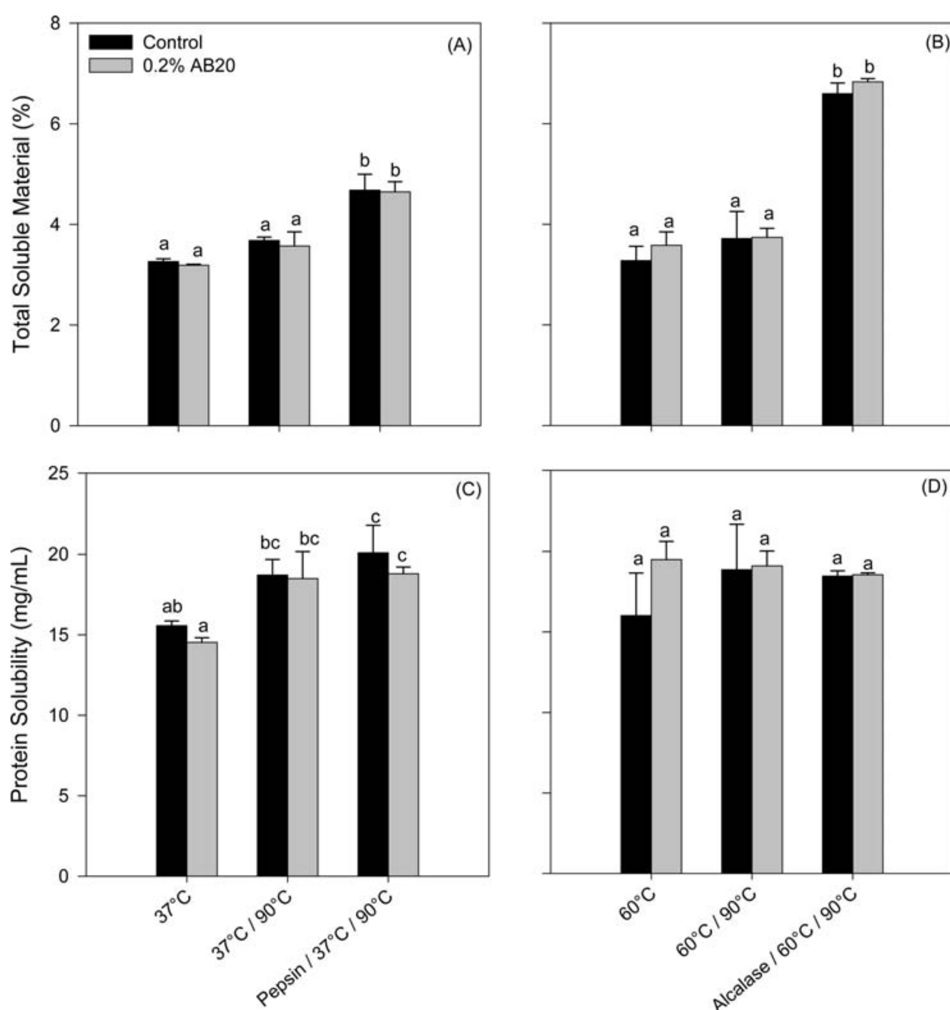


**Figure 2.** Total solids for the (**A**) pH 2.0 and (**B**) pH 8.0 soluble fractions. BCA protein for the (**C**) pH 2.0 and (**D**) pH 8.0 soluble fractions. Treatments are presented in a stepwise manner: optimal temperature for hydrolysis (37 or 60 °C), followed by the addition of the enzyme inactivation step (90 °C), and last hydrolysis with either pepsin or alcalase. Statistical significance ($p < 0.05$) was determined within the pH 2.0 or pH 8.0 treatment groups for either total solids or BCA protein.

proteins into smaller peptides and/or individual amino acids. Single amino acids and dipeptides are not detectable by the BCA assay as they do not catalyze the Biuret reaction, which is necessary for the spectrophotometric BCA assay. Therefore, any dipeptides or single amino acids generated during extensive hydrolyses are not detected by the BCA assay, which may skew protein solubility data, especially for the more extensively hydrolyzed alcalase treatment.

To better understand hydrolysis effects on soluble fractions, total nitrogen and sugars contents of select samples were also

determined (**Table 2**). Sample-designated controls in **Table 2** were identical to hydrolyzed samples except for enzyme treatment, that is, equivalent to the samples labeled 37 °C/90 or 60 °C/90 °C in **Figure 2** or **3**. The total soluble nitrogen increased ($p < 0.05$) after hydrolysis with either pepsin or alcalase (**Table 2**), which suggests that there was an increase in soluble peptides after enzymatic hydrolysis. Reducing sugars also react with the BCA reagent (*26*), and glucose and fructose levels were notably higher in the pH 2.0 control and pepsin samples as compared to the pH 8.0 and alcalase samples (**Table 2**). For measuring soluble protein, this could be another source of error in the BCA data. Differences in

**Table 2.** Total Nitrogen, Glucose, Fructose, and Sucrose of Soluble Peanut Meal Fractions before and after Clay Treatment[a]

| pH | enzyme | AB20A | nitrogen (%) | glucose (μg/mL) | fructose (μg/mL) | sucrose (μg/mL) |
|---|---|---|---|---|---|---|
| 2.0 | control | − | 1.6 ± 0.1 d | 610 ± 90 b | 740 ± 100 b | 6500 ± 840 c |
| 2.0 | control | + | 1.4 ± 0.1 d | 810 ± 210 a | 930 ± 220 a | 6060 ± 1440 c |
| 2.0 | pepsin | − | 4.8 ± 0.3 b | 330 ± 170 c | 420 ± 180 c | 3840 ± 450 d |
| 2.0 | pepsin | + | 4.7 ± 0.3 b | 240 ± 100 c | 330 ± 100 c | 4010 ± 370 d |
| 8.0 | control | − | 3.1 ± 0.7 c | 30 ± 10 d | 150 ± 10 d | 8440 ± 750 a |
| 8.0 | control | + | 3.3 ± 0.2 c | 30 ± 10 d | 150 ± 10 d | 8900 ± 680 a |
| 8.0 | alcalase | − | 6.1 ± 0.1 a | 230 ± 20 c | 90 ± 20 d | 7690 ± 730 b |
| 8.0 | alcalase | + | 6.0 ± 0.2 a | 230 ± 20 c | 100 ± 20 d | 7650 ± 710 b |

[a] Means within a column followed by different letters are significantly different ($p < 0.05$).

reducing sugar contents may partially explain why BCA data for pH 2.0/pepsin and pH 8.0/alcalase samples were relatively similar (**Figure 2C**,**D**), whereas soluble nitrogen was notably higher in the pH 8.0/alcalase samples as compared to the pH 2.0/pepsin samples (**Table 2**). Both pepsin and alcalase addition significantly ($p < 0.05$) affected soluble glucose, fructose, and sucrose contents (**Table 2**). While both enzymes are proteases, the potential exists for contaminating enzymes with various carbohydrate activities to modify soluble sugar content directly or indirectly. For example, alcalase was recently shown to decrease the starch molecular weight during starch isolation from rice (*27*). Peanut meal contains numerous complex carbohydrates, which could also be targets of such degradation. Accordingly, analysis of raw chromatograms found that both pepsin and alcalase addition generated numerous compounds similar to soluble sugars that were not well-resolved and likely also have reducing capacity and hence will also influence BCA data. Soluble sugar contents, which were generally not affected by 0.2% AB20A addition, are also important in future drying considerations for these preparations (*28*).

DH was not affected ($p < 0.05$) by the addition of 0.2% AB20A, which is indicative that the clay did not alter the activity of the two proteases tested. In agreement with the protein solubility data for room temperature extractions of peanut meal dispersions at pH 2.0 or 8.0 (**Figure 1A**), the BCA protein solubility was not reduced by the addition of 0.2% clay across any of the conditions necessary for protein hydrolysis (**Figure 2C**,**D**) nor

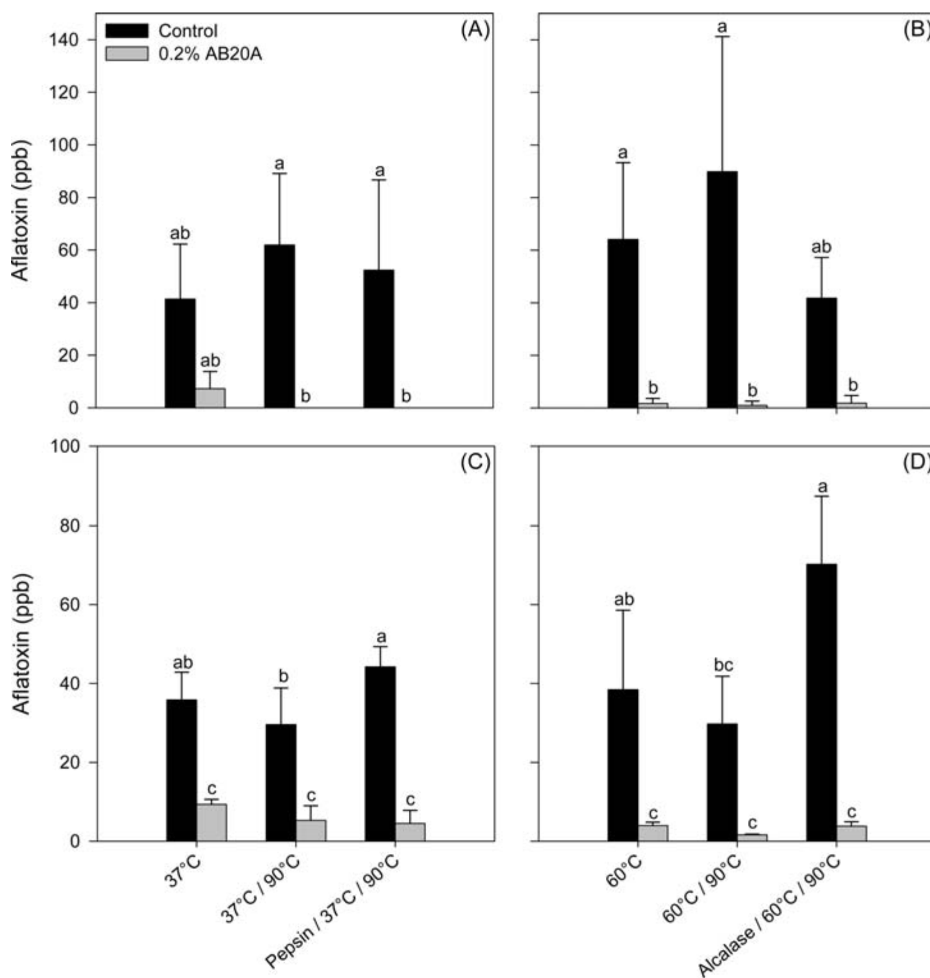


**Figure 3.** Aflatoxin concentration (ppb) of the (**A**) pH 2.0 soluble, (**B**) pH 8.0 soluble, (**C**) pH 2.0 insoluble, and (**D**) pH 8.0 insoluble peanut meal fractions before and after 0.2% clay treatment. Treatments are presented in a stepwise manner: optimal temperature for hydrolysis (37 or 60 °C), followed by the addition of the enzyme inactivation step (90 °C), and last hydrolysis with either pepsin or alcalase. All numbers are corrected for moisture content and are reported on a dry weight basis. Statistical significance ($p < 0.05$) was determined within the pH 2.0 or pH 8.0 soluble or insoluble fractions.

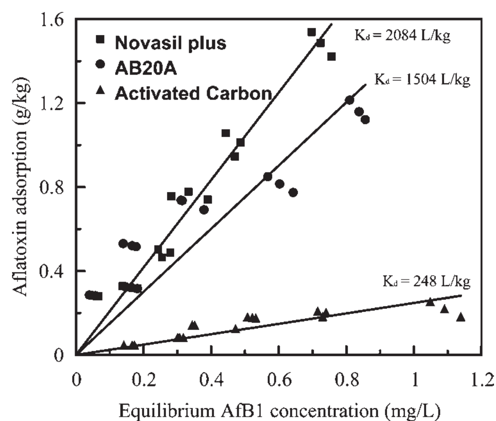


**Figure 4.** Adsorption isotherms of $AfB_1$ for AB20A, Novasil Plus, and AC from peanut meal dispersions. Three replicates of six points are shown in each adsorbent plot.

were total soluble nitrogen measures before or after hydrolysis with either pepsin or alcalase (**Table 2**). Similarly, the addition of up to 2% bentonite (w/v) did not decrease the total protein content in milk more than 5% (w/v) (*19*).

Aflatoxin contents for both soluble (**Figure 3A**,**B**) and insoluble (**Figure 3C**,**D**) fractions after the various proteolytic treatments are presented in **Figure 3**. Neither heat of processing nor hydrolysis affected aflatoxin concentrations in the soluble samples lacking AB20A at either pH value (**Figure 3A**,**B**). There was a relatively high experimental error in these measurements, especially for the soluble fractions, and this was at least partially attributable to the high moisture contents of these samples. For example, for the pH 2.0, 37 °C/90 °C soluble fraction in **Figure 4A**, the wet measures for aflatoxin were 2.2, 2.4, and 5.1, but when corrected for moisture, these numbers equated to 65, 56, and 149, respectively. As discussed previously, the sum of insoluble and soluble aflatoxin contents for any treatment should total 110 ppb aflatoxin on a dry weight basis, which was the starting concentration of aflatoxin in the dry meal. Reasons for this deviation in current measurements include high moisture contents of samples, which can magnify experimental error and/or the potential for local areas with relatively high or low aflatoxin concentrations within the dry meal or subsequent dispersions. The inclusion of 0.2% AB20A reduced ($p < 0.05$) aflatoxin to levels below 20 ppb in all water-soluble and -insoluble fractions. Aflatoxin was undetectable in the 37 °C/90 °C or pepsin-soluble samples (**Figure 3A**). Hydrolyzing samples with pepsin or alcalase did not inhibit the capacity of the clay to sequester aflatoxin in soluble fractions, which is critical for the development of edible peanut meal peptides with enhanced functional and nutritional properties. Soluble fractions with high protein and/or TSS would be most desirable for future spray-drying applications, as increased soluble solids improve drying efficiency. In conjunction with drying the soluble fractions after hydrolysis, the insoluble materials could also be dried and used as a higher grade animal feed because the aflatoxin in these fractions is sequestered and hence biologically unavailable.

Insoluble fractions of peanut meal dispersions after hydrolysis with alcalase and pepsin were analyzed for proximate composition to evaluate their potential feed values (**Table 3**). Data for these fractions were compared to those of dry peanut meal, as this is the raw material utilized for protein extraction. Note that peanut meal data are presented on a dry weight basis for effective comparison with the other fractions; however, peanut meal, which was 7.7% moisture for the current study, would likely be used without further drying for feeding applications. Insoluble fractions after pepsin hydrolysis in the presence and absence of 0.2% AB20A had lower

**Table 3.** Proximate Analyses of Peanut Meal and the Insoluble Fractions of 10% Peanut Meal Dispersions after Enzymatic Hydrolysis with Pepsin or Alcalase[a]

| sample | Kjeldahl protein (%) | fiber (%) | ash (%) |
|---|---|---|---|
| peanut meal | 54.0 A | 5.0 B | 5.3 B |
| pepsin, control | 38.0 B | 13.6 A | 2.8 C |
| pepsin, 0.2% AB20A | 41.6 B | 13.8 A | 5.8 B |
| alcalase, control | 48.1 AB | 14.0 A | 6.6 B |
| alcalase, 0.2% AB20A | 44.9 AB | 16.2 A | 9.8 A |

[a] All measurements were corrected for moisture content and are reported on a dry weight. Means within a column followed by different letters are significantly different ($p < 0.05$).

($p < 0.05$) protein contents than that of dry peanut meal (**Table 3**). While not significantly different, both alcalase insoluble fractions had 10% or greater reductions in Kjeldahl protein as compared to dry peanut meal prior to extraction. Similar to the protein and total solids data for the soluble fractions, the addition of 0.2% AB20A did not affect the Kjeldahl protein content of any insoluble fraction. The increase ($p < 0.05$) in fiber for all insoluble fractions resulted from the extraction of other components, that is, protein, simple sugars, and other water-soluble compounds, which resulted in a relative increase in fiber in the insoluble fractions. The fiber concentration within the insoluble fractions was not affected by the addition of 0.2% AB20A. The addition of 0.2% AB20A increased ($p < 0.05$) the ash content for both pepsin- and alcalase-insoluble fractions, reflecting the mineral composition of the bentonite additive. The 0.2% AB20A addition was based on wet weight of 10% w/w peanut meal dispersions, so an approximate 2% increase in ash could be logically expected upon drying insoluble fractions, and this was confirmed by the current data.

Physical adsorption by the bentonite clay is hypothesized to be responsible for the reduction of aflatoxin from the current peanut meal dispersions. The layered crystalline microstructure of clays with exchangeable cations has been demonstrated to adsorb aflatoxin (*15*, *29*). It has been hypothesized that organic substances adsorb to external or interlayer surfaces of negatively charged bentonite clays due to interactions between aflatoxin carbonyl groups and clay exchangeable cations (*15*). Clays with less charge intensity are thought to typically be more effective at absorbing aflatoxin as the increased hydration expected with higher charges may limit adsorption of hydrophobic molecules such as aflatoxins (*12*). Despite this electrostatic influence on the binding mechanism of AB20A, the capacity of AB20A to sequester aflatoxins in peanut meal dispersions was equivalent at pH 2.0 and 8.0. Furthermore, the effects of enzymatic hydrolysis were also minimal on aflatoxin sequestration at both pH 2.0 and 8.0 after treatment with pepsin or alcalase, respectively.

To better understand the aflatoxin sequestering capacity of AB20A, adsorption isotherms for this material were prepared and analyzed using model peanut meal dispersions contaminated with added $AfB_1$ (**Figure 4**). Equivalent adsorption isotherms for Novasil Plus and AC were also collected and analyzed for comparison (**Figure 4**). Adsorption of $AfB_1$ from model peanut meal dispersions was slightly greater for Novasil Plus as compared to AB20A, and both adsorbed more $AfB_1$ than AC (**Figure 4**). The $K_d$ values of the clays were 6−8 times greater than the AC value and reflect the relative $AfB_1$ adsorption capacities of these adsorbents. At an equilibrium $AfB_1$ concentration of 0.15 mg/L, $AfB_1$ adsorption by the clays was about 0.34 g/kg, but only 0.05 g/kg adsorbed to AC (**Figure 4**). The addition of 1.5% (of peanut meal weight) AB20A or Novasil Plus removed as much $AfB_1$ as 10% AC. Adsorption of all of the aflatoxin from a highly contaminated feed (1000 $\mu$g $AfB_1$/kg) to 0.5% added clay would only be 0.2 g $AfB_1$/kg clay. Both AB20A and Novasil Plus are bentonites

that consist mostly of the clay mineral montmorillonite. Novasil is a commercially available hydrated sodium calcium aluminosilicate product (i.e., Na and Ca montmorillonite) that has been shown to be effective at minimizing the harmful effects of mycotoxins during animal feeding studies (*5*, *30*). Animal feeding studies have also shown that AB20A added to contaminated feed effectively prevents aflatoxin toxicity (*17*,*31*). More recently, Novasil has been demonstrated in clinical trials to be a safe and effective additive for treating humans exposed to high levels of aflatoxins (*32*, *33*). The potential of AC to sequester various toxins has been extensively studied; however, AC generally seems less effective than clay-based sequestrants at preventing the negative effects of mycotoxin exposure in livestock (*34*,*35*), which agrees with data in **Figure 4**. Soluble compounds in feed, such as proteins, can bind to AC or clay-based feed additives and limit aflatoxin binding (*12*). Aflatoxin adsorption isotherms from feed ingredients, such as corn or peanut meal, compensate for the effect of soluble feed components in blocking aflatoxin sequestration by feed additives. Adsorbents such as Novasil were previously shown to adsorb $AfB_1$ much more effectively from pure water as compared to corn meal dispersions (*12*), and a similar phenomenon was observed in the current study (data not shown). Aflatoxin adsorption measurements from corn meal dispersions, and in this case peanut meal dispersions, are thought to be more effective and conservative predictors of the capacity of an adsorbent to sequester $AfB_1$ during animal feeding than equivalent isotherms prepared from pure water (*12*). Contributing factors include the lack of competing species in pure water and the lack of a methanol extraction step in water-based isotherms (*12*). Aflatoxin solubility is greatly enhanced in aqueous methanol, which enhances the release of weakly bound toxins from adsorbents of interest. Comparison of clay products equivalent to AB20A and Novasil in a feeding study of weaning pigs exposed to high levels of aflatoxin-contaminated corn (~800 $\mu$g/kg) found that both adsorbents effectively reduced the negative effects on animal performance resulting from aflatoxin exposure (*31*). A comparison of the data in this study to published research (*17*, *31*) suggests that AB20A should effectively retain sequestered aflatoxin in the insoluble fraction of peanut meal dispersions that contain sequestered aflatoxin.

This research supports a strategy for processing and utilizing peanut meal in applications beyond animal feed or fertilizer. Aflatoxin-free, spray-dried protein/peptide concentrates generated from the soluble portion of peanut meal dispersions after enzymatic hydrolysis have the potential to be utilized as functional ingredients in food applications or as a higher quality animal feed. This strategy of adding sequestrants directly to contaminated materials during processing could also be applied to other mycotoxin-contaminated biomaterials. The insoluble materials after hydrolysis could also be dried and utilized as higher grade animal feeds because aflatoxin in these fractions is sequestered and hence biologically unavailable. Furthermore, enzymatic hydrolysis, which been shown to enhance functional and nutritional properties of numerous protein substrates, could be another strategy for adding value to the soluble fractions of peanut meal dispersions.

## ABBREVIATIONS USED

AB20A, Astra-Ben 20A; AC, activated carbon; DH, degree of hydrolysis; EC, enzyme commission; TNBS, trinitrobenzenesulfonic acid; AU, Anson units; AOAC, Association of Official Analytical Chemists; BCA, bicinchoninic acid; TSS, % total soluble solids.


## ACKNOWLEDGMENT

We thank Golden Peanut Company, LLC, for their donation of peanut meal and Prince Agri Product, Inc., for their donation of AB20A.


## LITERATURE CITED


(1) McWatters, K. H.; Cherry, J. P. Potential food uses of peanut seed proteins. In *Peanut Science and Technology*; Patee, H. E., Young, C., Eds.; American Peanut Research and Education Society, Inc.: Yoakum, TX, 1982; pp 689−736.

(2) Ellis, W. O.; Smith, J. P.; Simpson, B. K.; Oldham, J. H. Aflatoxins in food—Occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 403–439.

(3) Parker, W. A.; Melnick, D. Absence of aflatoxin from refined vegetable oils. *J. Am. Oil Chem. Soc.* **1966**, *43*, 635–638.

(4) Administration, U. S. F. a. D. Guidance for industry: Action levels for poisonous or deleterious substances in human food and animal feed. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm077969.htm (accessed September 30, 2009).

(5) Huwig, A.; Freimund, S.; Kappeli, O.; Dutler, H. Mycotoxin detoxication of animal feed by different adsorbents. *Toxicol. Lett.* **2001**, *122*, 179–188.

(6) Phillips, T. D.; Clemant, B. A.; Park, D. L. Approaches to reduction of aflatoxins in foods and feeds. In *The Toxicology of Aflatoxins*; Eaton, D. L., Groopman, J. D., Eds.; Academic Press, Inc.: New York, NY, 1994; pp 383−406.

(7) Ramos, A. J.; FinkGremmels, J.; Hernandez, E. Prevention of toxic effects of mycotoxins by means of nonnutritive adsorbent compounds. *J. Food Prot.* **1996**, *59*, 631–641.

(8) Phillips, T. D. Dietary clay in the chemoprevention of aflatoxin-induced disease. *Toxicol. Sci.* **1999**, *52*, 118–126.

(9) Diaz, D. E.; Hagler, W. M.; Hopkins, B. A.; Whitlow, L. W. Aflatoxin binders I: In vitro binding assay for aflatoxin B1 by several potential sequestering agents. *Mycopathologia* **2003**, *156*, 223–226.

(10) Sobolev, V. S.; Dorner, J. W. Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *J. AOAC Int.* **2002**, *85*, 642–645.

(11) Reif, K.; Metzger, W. Determination of aflatoxins in medicinal herbs and plant-extracts. *J. Chromatogr. A* **1995**, *692*, 131–136.

(12) Jaynes, W. F.; Zartman, R. E.; Hudnall, W. H. Aflatoxin B-1 adsorption by clays from water and corn meal. *Appl. Clay Sci.* **2007**, *36*, 197–205.

(13) Pattee, H. E.; Isleib, T. G.; Giesbrecht, F. G.; McFeeters, R. F. Investigations into genotypic variations of peanut carbohydrates. *J. Agric. Food Chem.* **2000**, *48*, 750–756.

(14) Basha, S. M.; Cherry, J. P. Composition, solubility, and gel electrophoretic properties of proteins isolated from Florunner (*Arachis hypogaea* L.) peanut seeds. *J. Agric. Food Chem.* **1976**, *24*, 359–365.

(15) Afriyie-Gyawu, E.; Wang, Z.; Ankrah, N. A.; Xu, L.; Johnson, N. M.; Tang, L.; Guan, H.; Huebner, H. J.; Jolly, P. E.; Ellis, W. O.; Taylor, R.; Brattin, B.; Ofori-Adjei, D.; Williams, J. H.; Wang, J. S.; Phillips, T. D. Novasil clay does not affect the concentrations of vitamins A and E and nutrient minerals in serum samples from Ghanaians at high risk for aflatoxicosis. *Food Addit. Contam.* **2008**, *25*, 872–884.

(16) Haderlein, S. B.; Weissmahr, K. W.; Schwarzenbach, R. P. Specific adsorption of nitroaromatic: Explosives and pesticides to clay minerals. *Environ. Sci. Technol.* **1996**, *30*, 612–622.

(17) Fairchild, A. S.; Croom, J.; Grimes, J. L.; Hagler, W. M. Effect of Astra-ben 20A on broiler chicks exposed to aflatoxin B1 or T2 toxin. *Int. J. Poult. Sci.* **2008**, *7*, 1147–1151.

(18) Masimango, N.; Remacle, J.; Ramaut, J. L. Role of adsorption in elimination of aflatoxin-B1 from contaminated media. *Eur. J. Appl. Microbiol.* **1978**, *6*, 101–105.

(19) Applebaum, R. S.; Marth, E. H. Use of sulfite or bentonite to eliminate aflatoxin M1 from naturally contaminated raw whole milk. *Z. Lebensm.-Unters. Forsch.* **1982**, *174*, 303–305.

(20) Di Natale, F.; Gallo, M.; Nigro, R. Adsorbents selection for aflatoxins removal in bovine milks. *J. Food Eng*. **2009**, *95*, 186–191.
(21) Barbiroli, A.; Bonomi, F.; Benedetti, S.; Mannino, S.; Monti, L.; Cattaneo, T.; Iametti, S. Binding of aflatoxin M-1 to different protein fractions in ovine and caprine milk. *J. Dairy Sci.* **2007**, *90*, 532–540.
(22) Iwaki, M.; Kumagai, S.; Akamatsu, Y.; Kitagawa, T. Aflatoxin B-1-binding proteins in primary cultured-hepatocytes of chicken-embryo—studies in-vivo and in-vitro. *Biochim. Biophys. Acta* **1993**, *1225*, 83–88.
(23) Foegeding, E. A.; Davis, J. P.; Doucet, D.; McGuffey, M. K. Advances in modifying and understanding whey protein functionality. *Trends Food Sci. Technol.* **2002**, *13*, 151–159.
(24) Beuchat, L. R.; Cherry, J. P.; Quinn, M. R. Physicochemical properties of peanut flour as affected by proteolysis. *J. Agric. Food Chem.* **1975**, *23*, 616–620.
(25) Sekul, A. A.; Vinnett, C. H.; Ory, R. L. Some functional properties of peanut proteins partially hydrolyzed with papain. *J. Agric. Food Chem.* **1978**, *26*, 855–858.
(26) Kongruang, S.; Han, M. J.; Breton, C. I. G.; Penner, M. H. Quantitative analysis of cellulose-reducing ends. *Appl. Biochem. Biotechnol.* **2004**, *113*, 213–231.
(27) Li, Y.; Shoemaker, C. F.; Ma, J. G.; Luo, C. R.; Zhong, F. Effects of Alcalase/Protease N treatments on rice starch isolation and their effects on its properties. *Food Chem.* **2009**, *114*, 821–828.
(28) Adhikari, B.; Howes, T.; Wood, B. J.; Bhandari, B. R. The effect of low molecular weight surfactants and proteins on surface stickiness of sucrose during powder formation through spray drying. *J. Food Eng.* **2009**, *94*, 135–143.
(29) Grant, P. G.; Phillips, T. D. Isothermal adsorption of aflatoxin B-1 on HSCAS clay. *J. Agric. Food Chem.* **1998**, *46*, 599–605.
(30) Pimpukdee, K.; Kubena, L. F.; Bailey, C. A.; Huebner, H. J.; Afriyie-Gyawu, E.; Phillips, T. D. Aflatoxin-induced toxicity and depletion of hepatic vitamin A in young broiler chicks: Protection of chicks in the presence of low levels of Novasil Plus in the diet. *Poult. Sci.* **2004**, *83*, 737–744.
(31) Schell, T. C.; Lindemann, M. D.; Kornegay, E. T.; Blodgett, D. J.; Doerr, J. A. Effectiveness of different types of clay for reducing the detrimental effects of aflatoxin-contaminated diets on performance and serum profiles of weanling pigs. *J. Anim. Sci.* **1993**, *71*, 1226–1231.
(32) Afriyie-Gyawu, E.; Ankrah, N. A.; Huebner, H. J.; Ofosuhene, M.; Kumi, J.; Johnson, N. M.; Tang, L.; Xu, L.; Jolly, P. E.; Ellis, W. O.; Ofori-Adjei, D.; Williams, J. H.; Wang, J. S.; Phillips, T. D. Novasil clay intervention in Ghanaians at high risk for aflatoxicosis. I. Study design and clinical outcomes. *Food Addit. Contam.* **2008**, *25*, 76–87.
(33) Wang, P.; Afriyie-Gyawu, E.; Tang, Y.; Johnson, N. M.; Xu, L.; Tang, L.; Huebner, H. J.; Ankrah, N. A.; Ofori-Adjei, D.; Ellis, W.; Jolly, P. E.; Williams, J. H.; Wang, J. S.; Phillips, T. D. Novasil clay intervention in Ghanaians at high risk for aflatoxicosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine. *Food Addit. Contam.* **2008**, *25*, 622–634.
(34) Diaz, D. E.; Hagler, W. M.; Blackwelder, J. T.; Eve, J. A.; Hopkins, B. A.; Anderson, K. L.; Jones, F. T.; Whitlow, L. W. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia* **2004**, *157*, 233–241.
(35) Edrington, T. S.; Sarr, A. B.; Kubena, L. F.; Harvey, R. B.; Phillips, T. D. Hydrated sodium calcium aluminosilicate (HSCAS), acidic HSCAS, and activated charcoal reduce urinary excretion of aflatoxin M(1) in turkey poults. Lack of effect by activated charcoal on aflatoxicosis. *Toxicol. Lett.* **1996**, *89*, 115–122.

**Received for review December 22, 2009. Revised manuscript received March 11, 2010. Accepted March 12, 2010.**