The Binding of SBI to Mycotoxins

ImmunoLin®

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HEALTH & BIOLOGICALS

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Summary

Serum-derived bovine immunoglobulin (SBI), known as Immunolin®, is a protein isolate rich in immunoglobulins for oral administration to improve immune health. SBI reduces inflammation by binding and neutralizing a variety of inflammatory antigens, improving gut epithelial barrier integrity, and maintaining gut homeostasis. In this study, fluorescence spectroscopy was used to demonstrate and quantify the binding of SBI to the fungal mycotoxins, aflatoxin G_1 (AFG_1) and aflatoxin B_2 (AFB_2). Calculated binding constants reveal SBI binds with both AFG_1 and AFB_2 in a dose dependent manner, likely through immunoglobulin-aflatoxin interactions.

Introduction

Mycotoxins are highly toxic biproducts of mold growth which commonly contaminate agricultural commodities for human consumption, including wheats, nuts, maize, spices, milk, eggs, meat, and seeds[1]. While there are more than twenty known aflatoxins (a type of mycotoxin), the prominent aflatoxins impacting the food system are aflatoxins B_1, B_2, G_1, and G_2[1]. AFB1 is the most toxic and is categorized as a group 1 carcinogen by the International Agency for Research on Cancer, followed by AFG_1, AFG_2, then AFB_2[1]. Aflatoxins mainly target the liver and cause disease or even death by mycotoxicosis. Aflatoxins are also capable of damaging the kidney, heart, testis, and brain[1]. Early symptoms of acute action level exposure include fever, malaise, loss of appetite, abdominal pain, and vomiting, while chronic exposure can suppress the immune system and have carcinogenic effects.

*"Approximately **half of people worldwide** are chronically exposed to aflatoxins through their regular food consumption"*

The United States Food and Drug Administration and the European Commission have set an action level for aflatoxins at 20 and 2 parts per billion for all foods, respectively[2,7]. Despite regulatory oversight, aflatoxin outbreaks occur, and products often test above the action level. The United States Centers for Disease Control and Prevention estimates approximately half of people worldwide are chronically exposed to aflatoxins through their regular food consumption[3]. To make matters worse, scientists predict fungal contamination of agricultural products will worsen with climate change, resulting in increased levels of aflatoxins[4]. Studies have been done to identify methods to reduce aflatoxin contamination, such as removing fungal-damaged product manually, boiling, roasting, alkaline cooking, and chloride treatments[1]. Despite efforts, there is no reliable method for aflatoxin depletion, so the US Food and Drug Administration considers aflatoxins an unavoidable contaminant of foods. Thus, any treatment that can neutralize and sequester ingested aflatoxins is of desire to consumers.

Introduction Cont'd

SBI is a purified protein powder enriched in protective immunoglobulins IgG (55%), IgM (5%), and IgA (1%) for oral administration. Previously, SBI has been shown to bind to a variety of pro-inflammatory antigens and decrease inflammatory markers in the gastrointestinal tract by neutralizing or preventing translocation of antigen across the intestinal barrier[5,6]. In this study, we used fluorescence spectroscopy to show that SBI binds aflatoxins AFG_1 and AFB_2. These data demonstrate SBI has the potential to help manage the effects of aflatoxin exposure caused by regular food consumption.

Results

Figure 1 shows the structures of aflatoxins. The structures of AFB_1 and AFB_2, as well as AFG_1 and AFG_2, differ only by a single carbon double bond which is highlighted in orange. AFGs differ from AFBs by the addition of an oxygen into a ring highlighted in red, turning the AFB's ketone into AFG's lactone. The similarities between structures suggest interactions observed between one aflatoxin and its binding partner should also exist for the other aflatoxins.

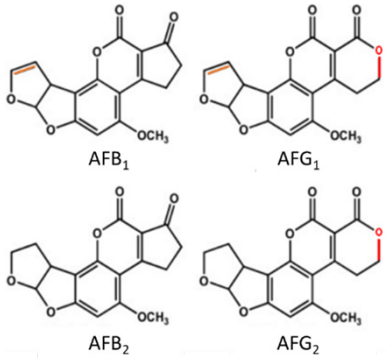

Figure 1: The structures of aflatoxins[8].

Fluorescence spectroscopy was used to quantify the binding of aflatoxins to SBI. Proteins have an intrinsic fluorescence due to π to π* transitions of aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Tryptophan makes up most of the observed fluorescence because its excitation and emission spectra have the longest wavelength and lifetime. Immunoglobulins contain approximately 24 tryptophan residues, making their fluorescence easily detectible.

Results Cont'd

When excited at 280nm, the emission spectra of SBI shows a fluorescence maximum at 370nm (Figure 2A) and the ringed structure of aflatoxins around 450nm (Figure 2B and 2C). A change in intrinsic fluorescence is observed when protein binds to a small molecule, making fluorescence spectroscopy a reliable method for detecting the binding of proteins to small molecules.

SBI was used to prepare a highly purified (>95%) immunoglobulin fraction, Bovine Gamma Globulin (BGG), to confirm aflatoxins were binding to the immunoglobulins in SBI. Figure 3 shows the emission spectra of 5 µM SBI (Fig 3A) or BGG (Fig 3B) treated with increasing concentrations of aflatoxin AFG_1 (0–40 µM). The fluorescence (370nm) from the SBI or BGG in solution is reduced in a concentration-dependent manner as the concentration of AFG_1 in solution is increased. Quenching indicates the microenvironment around the fluorophores in SBI or BGG is altered, implying conformational changes in the native structures of the proteins composing SBI and BGG, likely caused by an interaction with AFG_1.

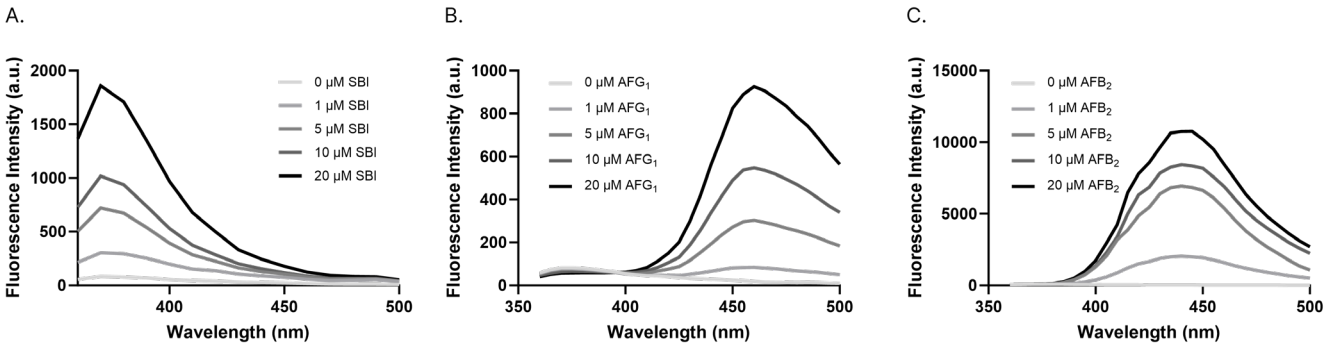

Figure 2: The intrinsic fluorescence of SBI and aflatoxins AFG_1 and AFB_2. A) The emission spectrum of increasing concentrations of A) SBI B) AFG_1 C) AFB_2 (0-20 µM) excited at 280nm.

Results Cont'd

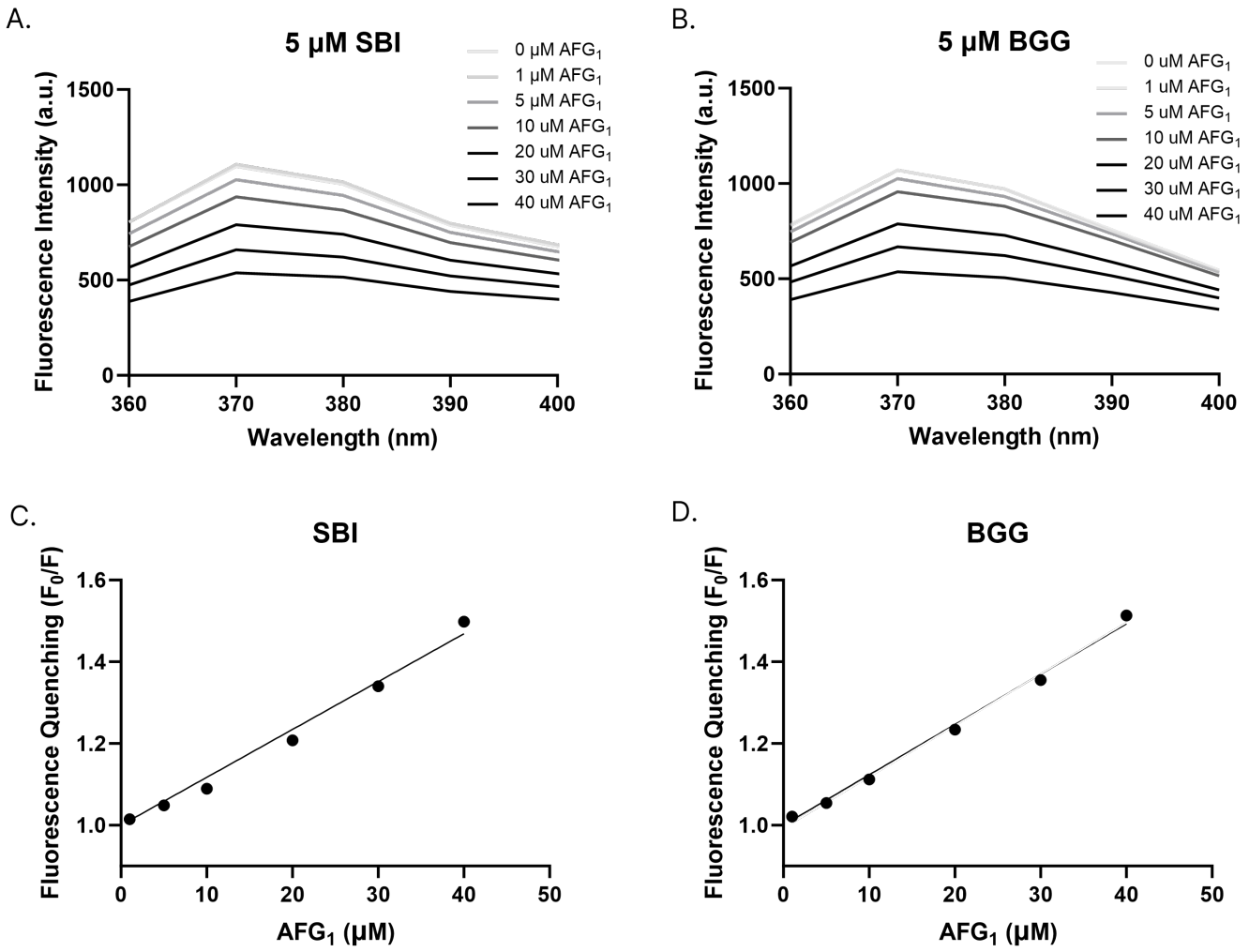

Figure 3: AFG_1 binds to SBI and BGG. A) Fluorescence intensity values of 5 µM SBI and B) 5 µM BGG titrated with increasing concentrations of AFG_1 (0-20 µM). C) Graph of Equation 1 with slope equal to the Stern-Volmer quenching constant (K_{sv}) for the interaction of AFG_1 with 5 µM SBI ($R=0.99^2$) and D) 5 µM BGG ($R=0.99^2$).

The quenching of SBI indicates SBI binds to aflatoxins and the quenching of BGG suggests immunoglobulins contained within SBI are the proteins partially responsible for the observed phenomenon. SBI does contain other serum proteins such as albumin (~10%) and transferrin (~6%). It is therefore possible these other proteins are interacting with aflatoxins and contribute to the quenching.

Figure 3C-D show the graph of Equation 1 (Methods) for each binding event where the y-axis is the change in fluorescence (F_0/F), the x-axis is the concentration of AFG_1, and the slope of the line is the Stern-Volmer quenching constant (K_{sv}). AFG_1-induced quenching

Results Cont'd

of SBI and BGG is linearly proportional, by value K_{sv}, to the concentration of AFG_1. When comparing the interaction of AFG_1 with both SBI and BGG, K_{sv} is similar in magnitude, instilling confidence that the immunoglobulins composing BGG are functional binders of AFG_1 in SBI.

The binding constants (K_b) were calculated using Equation 2 from the Methods section. For the interaction of AFG_1 with SBI or BGG, the value of K_b was higher for SBI than the purified immunoglobins in BGG (Table 1). This suggests a combinatorial binding event by the immunoglobulins and other proteins in SBI which yield better AFG_1 binding than immunoglobulins alone . Gibbs free energy (ΔG) was calculated using Equation 3 because of its role in the stability of the protein–ligand complexes and is shown in Table 1. ΔG was found to be negative for both interactions, which means formation of the BGG-AFG_1 and SBI-AFG_1 complexes are energetically favorable.

To test the hypothesis that SBI interacts with a variety of aflatoxins because of their structural similarity, we also examined the binding of AFB_2 (Figure 4). The increases in concentration of AFB_2 (0-40 μM) decreased the intrinsic fluorescence of SBI or BGG at 370nm (Figure 4A-B). The quenching of either SBI or BGG was again linearly dependent on the concentration of AFB_2 (Figure 4C-D) and revealed a K_{sv} in the micromolar range. The K_{sv} and K_b values for the interaction of SBI and BGG with AFB_2 were similar in magnitude to that of AFG_1, with SBI having a higher K_b, suggesting different aflatoxins have comparable affinities for immunoglobulins and SBI. The Gibb's free energies were again found to be negative (Table 1), indicating the BGG-AFB_2 and SBI-AFB_2 complex formations are favorable.

Results Cont'd

The Stern–Volmer quenching constants (K_{sv}), the binding constants (K_b), and the change in Gibbs free energy from the analysis in Figures 2 and 3 are listed in Table 1. As previously stated, the values are all similar in magnitude, in the moderate-affinity micromolar range, suggesting the interaction between the proteins and different aflatoxins would all be similar in nature. Additionally, the binding constants for the SBI-aflatoxin interactions are similar to the calculated constants of the BGG-aflatoxin interaction, which suggests the proteins composing BGG (immunoglobulins) are principally responsible for the interactions observed between SBI and aflatoxins.

Figure 5A-B show the phenomenon known as protein-induced fluorescence enhancement occurring with aflatoxins AFG_1 and AFB_2. While the concentration of both AFG_1 and AFB_2 in solution remained constant (5 µM), their intrinsic fluorescence intensity was enhanced with increasing concentrations of SBI. Aflatoxin was excited at 360nm in these assays, unlike the previous assays, to prevent the excitation and emission of SBI fluorescence which could be absorbed by aflatoxin. Not only is the fluorescent signal of SBI quenched, the fluorescence intensity of aflatoxin is also enhanced, further indicating there is a bi-molecular interaction occurring between SBI and aflatoxins.

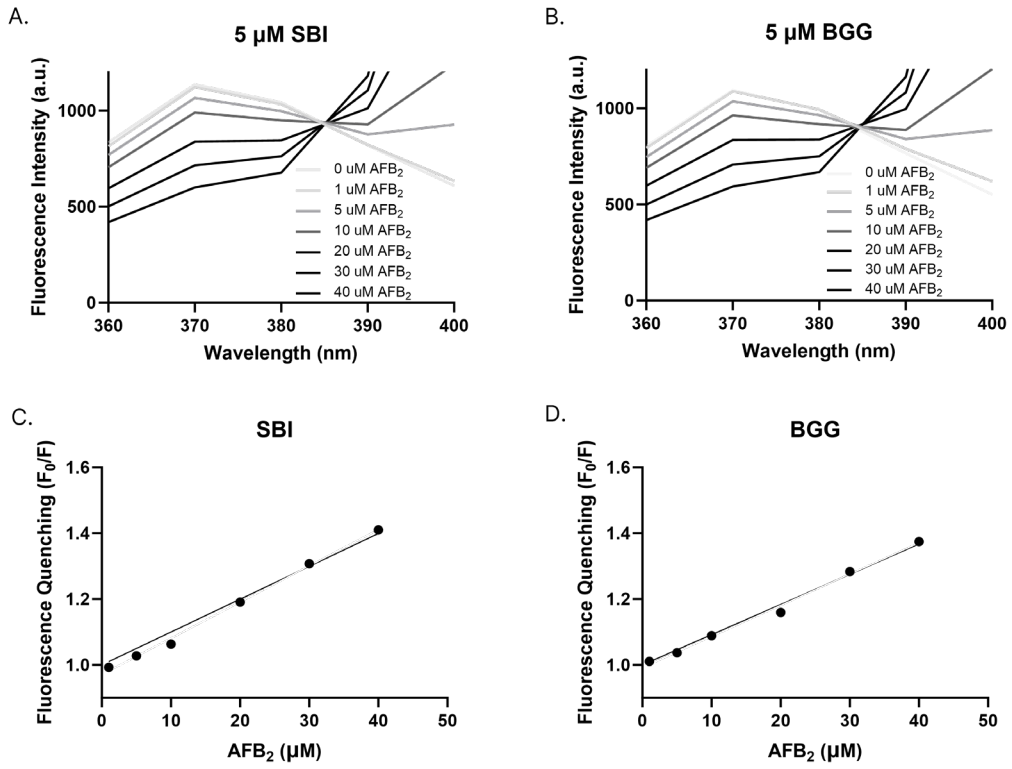

Figure 4: AFB_2 binds to SBI and BGG. A) Fluorescence intensity values of 5 µM SBI and B) 5 µM BGG titrated with increasing concentrations of AFB_2 (0-40 µM). C) Graph of Equation 1 with slope equal to the Stern-Volmer quenching constant (K_{sv}) for the interaction of AFB_2 with 5 µM SBI ($R=0.99^2$) and D) 5 µM BGG ($R=0.99^2$).

Results Cont'd

Table 1: Binding and thermodynamic parameters for complex formation.

Interaction	K_{sv} (M^{-1})	K_b (M^{-1})	ΔG (kcal mol^{-1})
AFG_1 - SBI	1.17 x 10^4	4.59 x 10^4	-6.36
AFG_1 - BGG	1.23 x 10^4	2.52 x 10^4	-6.00
AFB_2 - SBI	1.00 x 10^4	4.21 x 10^4	-7.67
AFB_2 - BGG	0.92 x 10^4	2.69 x 10^4	-6.04

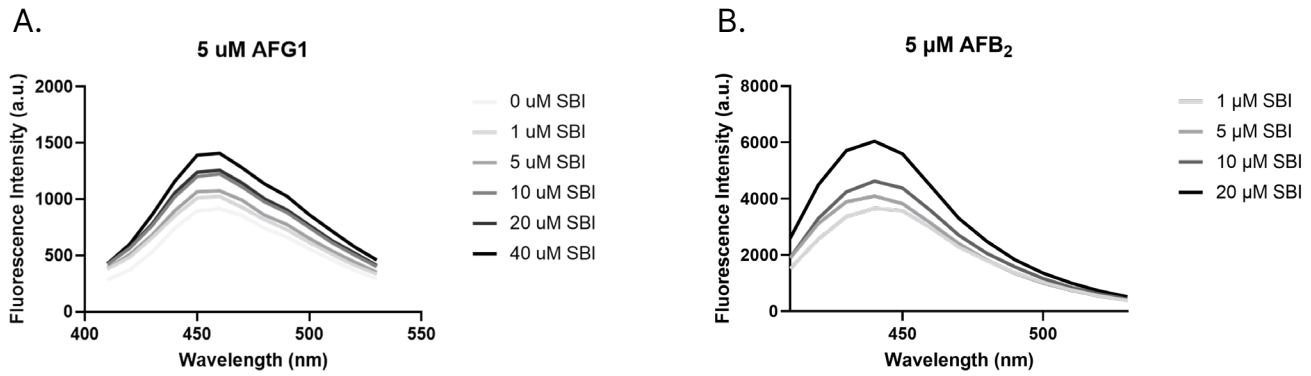

Figure 5: AFB_2 binds to SBI and BGG. A) Fluorescence intensity values of 5 µM A) AFG_1 or B) AFB_2 treated with increasing concentrations of SBI (0-20 µM).

Conclusion

Due to its high immunoglobulin content, SBI is commonly supplemented to the body to maintain gut homeostasis, to improve immune health, and for the dietary management of chronic diarrhea and loose stools caused by IBS-D and IBD. The broad specificity of SBI has been demonstrated through binding and neutralization of a variety of proinflammatory antigens, including but not limited to, microbial and pathogen associated molecular patterns like lipopolysaccharide, Candida albicans lysates, and *Helicobacter pylori* cytotoxin-associated gene A.

In this study, fluorescence spectroscopy was used to show the immunoglobulins in SBI

Conclusion Cont'd

bind aflatoxins. The similarity of BGG and SBI results indicates the interaction of SBI with aflatoxins occurs primarily through the immunoglobulins within SBI, though other serum proteins could play a role in aflatoxin binding. The negative Gibbs free energy calculation showed binding was energetically favorable, and the similarity of the calculated aflatoxin binding constants in the micromolar range imply the SBI-aflatoxin interaction is biologically relevant across different aflatoxins. The bidirectional influence on fluorescence observed with both the quenching of protein and enhancement of aflatoxin fluorescence supports the theory of a bimolecular interaction. Aflatoxins AFG_2 and AFB_1 were not tested for binding only because they differ from their structural counterparts AFG_1 and AFB_2 by presence or absence of a single bond and were predicted to behave analogously. These data demonstrate SBI has the potential to help manage the effects of aflatoxin exposure.

Methods

Materials

Aflatoxins were acquired from Millipore Sigma. SBI and BGG were produced by and acquired from Proliant Health and Biologicals.

Preparation of Stock Solutions

The stock solutions of AFB_2 and AFG_1 (3 mM) were prepared in acetonitrile with 0.1% trifluoroacetic acid and further diluted to 100 µM in 1x phosphate buffered saline pH 7.4 (PBS). SBI and BGG were diluted to 1% (w/v) in PBS, then further diluted to 10 µM prior to analysis.

Fluorescence Spectroscopy

A SpectraMax Gemini XPS spectrofluorometer, equipped with a xenon flash lamp and photomultiplier tube detector, was used to perform the fluorescence-based experiments.

Methods Cont'd

Fluorescence Spectroscopy (cont'd)

SoftMax Pro Analysis software was used to analyze the data which automatically normalizes the well absorbance to a cuvette equivalent pathlength of 1 cm. Bandwidths were set at 9nm and the read time at 15 s. The spectra were recorded using a 96-well black costar plate in 100 µL. SBI and BGG were excited at 280nm and the emission was recorded from 360-400nm or AFG_1 and AFB_2 were excited at 360nm and the emission was recorded from 410-530nm. The quenching constant (K_{SV}) was calculated by the Stern-Volmer plot using Equation 1. F_0 and F denote the intrinsic fluorescence intensities of SBI/BGG in the presence and absence of aflatoxin, respectively. [Q] represents the concentration of quencher, or aflatoxin, present.

$$\frac{F_0}{F} = K_{sv}[Q] + 1 \quad (1)$$

The binding constant (K_b) was calculated with Equation 2, where $[P]_0$ represents the protein (SBI/BGG) concentration, $[D]_0$ represents the total ligand (AFB_2 or AFG_1) concentration, and n represents the number of binding sites for the ligand.

$$log\frac{F_0-F}{F} = logK_b + n\log\left\{[D]_0 - n\frac{[P]_0(F_0-F)}{F_0}\right\} \quad (2)$$

Equation 3 was used to calculate the change in Gibbs free energy (ΔG). R (1.987 cal mol^{-1} K^{-1}) is the universal gas constant, and T is the assay temperature in Kelvin.

$$\Delta G = -RTlnK_b \quad (3)$$

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