# Aflatoxin $M_1$ Binding to Bovine α- and κ-Caseins Demonstrated by Surface Plasmon Resonance

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## Abstract

A preliminary investigation of the potential binding interaction of isolated whey proteins and casein proteins with aflatoxin $M_1$ ($AFM_1$) using a surface plasmon resonance optical biosensor is described. The experimental conditions were restricted to facilitate a qualitative analysis that, for the first time, has demonstrated that $AFM_1$ differentially binds to α-casein and κ-casein; it has negligible interaction with β-casein and the individual whey proteins. These observations with individual casein proteins can be extrapolated to infer that such differential binding occurs in intact milk and explains the many previous reports of the heterogeneous distribution of $AFM_1$ in milk and milk products that have only indirectly proposed its affinity for casein.

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# 1. Introduction

While developing an analytical method to estimate the aflatoxin $M_1$ ($AFM_1$) content in bovine milk products, it was observed that the recoveries of spiked $AFM_1$ from milk protein concentrate and cheese were considerably lower than those from whey protein products (Wood et al., 2020). Prior literature on the apparent enrichment of $AFM_1$ in cheese, as well as ultrafiltration studies on caprine, ovine and bovine milk protein fractions, gave indirect evidence that the casein fraction binds to $AFM_1$ (Barbiroli et al., 2007; Brackett & Marth,1982; Chavarría et al., 2017; Pecorelli et al., 2020).

Several analytical techniques have been used to investigate putative protein-ligand interactions (Poor et al., 2017). Recently, optical biosensors utilising surface plasmon resonance (SPR) detection have become an important evanescent field technique for the label-free and real-time detection and characterisation of biomolecular interactions at an interface functionalised by the immobilisation of a biological recognition molecule (Homola, 2008). SPR can uniquely reveal weak binding interactions and, in pure model systems, can also determine the kinetics and thermodynamics of complex formation, albeit without necessarily identifying the binding sites.

Because of the potential for biomolecular binding interactions within complex food systems such as milk, SPR has been applied to provide information on the specificity and affinity of such interactions. SPR-based biosensors have been applied to casein (Ashley et al., 2017; Marchesseau et al., 2002; Muller-Renaud, Dupont, & Dulieu, 2005), although relatively few SPR studies have evaluated the interactions between casein and hydrophobic low molecular mass bioactive compounds (Bahri, Henriquet, Pugnière, Marchesseau, & Chevalier-Lucia, 2019; De Gobba, Møller, Rauh, Svensson, & Lund, 2020; Pacheco et al., 2020).

Many reports have proposed an affinity of $AFM_1$ for the caseins in milk and milk products, based indirectly on the observation of low $AFM_1$ recovery during quantitative analysis and the common observation of its selective concentration in cheese. However, only one study utilising equilibrium dialysis has investigated this putative interaction (Brackett & Marth, 1982).

We have therefore applied the SPR biosensor platform to directly, but qualitatively, screen for evidence of possible binding of $AFM_1$ with a panel of individual isolated milk proteins. The experimental conditions were designed specifically to determine the ligand binding partners in a dose-response manner; kinetic analysis of any binding would require different experimental conditions and was beyond the scope of this study.

# 2. Materials and methods

## 2.1. Instrumentation, reagents and biosensor surface preparation

The SPR instrument (Biacore Q), operating and data processing software and CM5 sensor chips were from GE Healthcare (Uppsala, Sweden). Reagents and surface preparation were as reported previously (Indyk, Chetikam, Gill, Wood, & Woollard, 2019). Briefly, ligand immobilisation, with either bovine serum albumin (BSA) or BSA-$AFM_1$ conjugate, was achieved by standard amine-coupling chemistry at 25 °C in sodium acetate buffer (10 mM, pH 4.4) with HBS-EP running buffer (pH 7.4).

Bovine serum albumin, BSA-$AFM_1$ conjugate, α-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, and immunoglobulin G (IgG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The individual caseins ($\alpha_{S1}$-, $\alpha_{S2}$-, β- and κ-) were also isolated from skim milk, after acid precipitation of casein, using cation-exchange chromatography (S-Sepharose), dialysis and freeze drying. From initial aqueous stock solutions (10 mg $mL^{-1}$), each protein was individually prepared at 0–100 mg $mL^{-1}$by serial dilution in HBS-EP buffer.

## 2.2. Biosensor assay

The functionalised sensor chip was equilibrated at ambient temperature, and protein standards (100 mL) were dispensed in a 96-well microtitre plate and sealed with adhesive foil. Automated analysis was accomplished with the sequential single flow-cell addressable instrument at 25 °C, with HBS-EP as the running buffer. Individual protein standards were injected over separately designated flow cells for 120 s at 10 mL $min^{-1}$, followed by a dissociation period of 90 s. Regeneration of the sensor surface was achieved by a 5 mL injection of sodium hydroxide (5 mM) at 50 mL $min^{-1}$.

Initially, to evaluate the binding specificity qualitatively, the high-level standard (100 mg $mL^{-1}$) of each protein was sequentially injected over all three functionalised surfaces (flow cell 1, control; flow cell 2, BSA; flow cell 3, BSA-AFM1). A subsequent trial was designed to characterise the quantitative dose-response relationship, by injecting a concentration series of each protein over the BSA-$AFM_1$ surface.

The entire association, dissociation and regeneration phases of each interaction were monitored, facilitating reference sensorgram subtraction to evaluate the binding specificity. Report points acquired at the end of the association phase relative to the baseline enabled quantitation of the binding response (resonance unit, RU, where 1 RU is a change in refractive index of $10^{-6}$, equivalent to 1 pg $mm^{-2}$ protein).

# 3. Results and discussion

Optical biosensors based on SPR detection are ideally suited to revealing real-time information regarding the potential interaction of low molecular mass bioactive compounds with both the individual globular

whey proteins and the less structured caseins of milk and can monitor not only the endpoint but also the entire association and dissociation process. A direct assay format based on the interaction of immobilised $AFM_1$ with the high molecular mass proteins in solution is preferable to immobilising multiple individual proteins, and also with regard to relative molecular mass. Since a formal kinetic analysis was beyond the scope of this study, high ligand immobilisation levels ($R_L \approx 12{,}000$ RU) were targeted to reveal possible binding responses and thereby facilitate a qualitative evaluation of the binding of individual milk proteins to surface-tethered $AFM_1$.

To qualitatively evaluate binding specificity, sensorgrams for each high-level individual protein were initially acquired over all three surfaces. No reference subtraction over the nonfunctionalized control surface (flow cell 1) was required for any protein as the relative binding responses were consistently < 1 RU, an observation that confirmed the absence of non-specific binding of any milk protein to the carboxymethyldextran hydrophilic support surface. The whey proteins (a-lactalbumin, b-lactoglobulin, BSA, and IgG) were found not to bind to either the BSA-tethered surface (flow cell 2) or the active BSA-AFM1 immobilised surface (flow cell 3), with responses consistently < 2 RU. In contrast, certain caseins were found to bind with varying affinities to both BSA- and BSA-$AFM_1$-immobilised surfaces, with responses for the latter surface consistently greater than those for the former. To reveal the specific binding isotherm to $AFM_1$, sensorgrams for the three individual casein proteins (100 mg $mL^{-1}$) after subtraction of background non-specific binding from the BSA-immobilised surface (flow cell 2) are illustrated in Fig. 1.

The dose-response relationship was subsequently investigated by injecting each individual whey protein and casein protein at different concentrations (0–100 mg $mL^{-1}$) over the BSA-$AFM_1$ surface (flow cell 3), further confirming the absence of binding by the whey proteins, as illustrated in Fig. 2.

Both commercially available and in-house isolated caseins were included to provide confirmation of binding properties, and it was further demonstrated that $\alpha_{S1}$-casein and $\alpha_{S2}$-casein bound $AFM_1$ with equivalent responses. It is apparent from these observations that, of the caseins, α-casein and κ-casein each formed a complex through interaction with immobilised $AFM_1$ in a dose–response manner, whereas the binding response of b-casein was negligible. It is also apparent that both caseins interacted with $AFM_1$ with the rapid association and dissociation characteristics typical of low affinity interactions of low molecular mass analytes with proteins. Under high ligand immobilisation levels, the isotherms for both α-casein and κ-casein were qualitatively indicative of biphasic binding to both BSA and $AFM_1$.

Whey and casein milk proteins have received attention for their ability to function as nanocarriers for low molecular mass bioactive compounds. Because of its greater hydrophobicity amongst the caseins (β > κ > α), such binding involving β-casein has recently been investigated by SPR techniques, yielding kinetic and thermodynamic information (Bahri et al., 2019; Pacheco et al., 2020). It is significant that, in

contrast to α- and κ-casein, β-casein did not bind to immobilised $AFM_1$ under the conditions of SPR analysis described in this study, an observation that indicates that forces other than hydrophobic were involved in the non-covalent interaction of the caseins with $AFM_1$.

Previous studies utilising spectroscopic and molecular modelling have reported a putative affinity between aflatoxins and both whey proteins and casein proteins, to rationalise the $AFM_1$ distribution in milk systems and its concentration in cheese products (Blanco et al., 1988; Chavarría et al., 2017), and an early study using equilibrium dialysis confirmed that $AFM_1$ associated noncovalently with casein, albeit without differentiating between the casein isoforms (Brackett & Marth, 1982). The SPR-based technique directly and unambiguously confirms the affinity of $AFM_1$ for α- and κ-casein in the model system investigated without, however, identifying the non-covalent binding site(s) involved.

# 4. Conclusions

This study has exploited an SPR-based biosensor to directly investigate the putative binding of $AFM_1$ to milk caseins that has been speculated in multiple previous studies to rationalise the heterogeneous distribution of this mycotoxin in milk and milk products. The experimental conditions facilitated a qualitative analysis confirming, for the first time, that $AFM_1$ binds specifically with α-casein and κ-casein, but that any interaction with β-casein or the whey proteins is negligible. These observations can be extrapolated to predict that such binding occurs in raw milk; future quantitative kinetic and affinity analysis may assist in characterising the interaction and provide insight into the concentration of $AFM_1$ in various milk-derived protein products.

# CRediT author statement

Harvey Indyk: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Writing original draft, Writing, review & editing. Jackie Wood: Conceptualization, Writing original draft, Writing, review & editing. Brendon Gill: Visualization, Writing, review & editing.

# Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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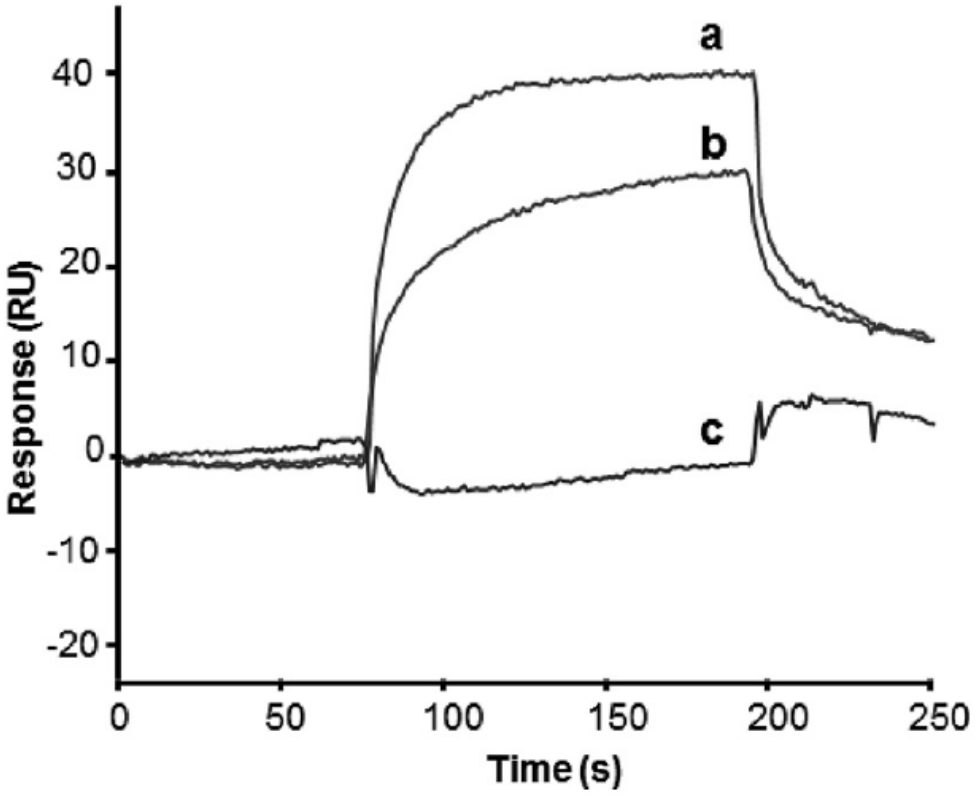


**Fig 1. Overlaid reference subtracted sensorgrams of individual caseins (100 mg mL$^{-1}$) injected over sensor surface immobilised with BSA-AFM1 (regeneration phase removed for clarity): a, α-casein; b, κ-casein; c, β-casein**

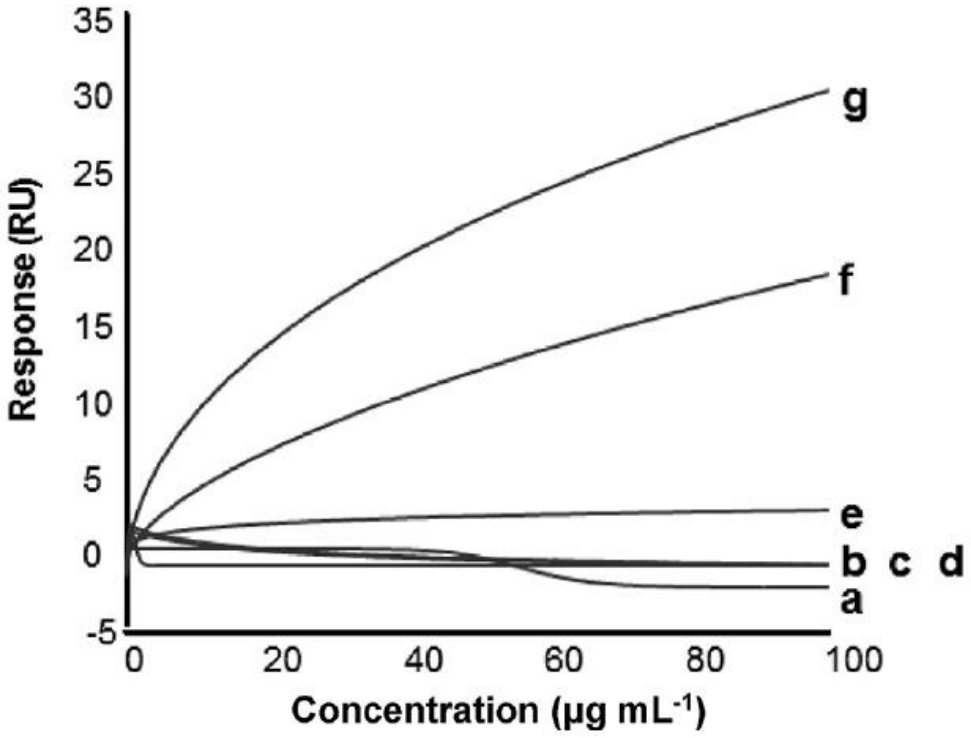


**Fig 2. Dose-response curves of individual milk proteins (0–100 mg mL$^{-1}$) injected over sensor surface immobilised with BSA-AFM1: a, α-lactalbumin; b, bovine serum albumin; c, β-casein; d, β-lactoglobulin; e, immunoglobulin G; f, κ-casein; g, α-casein**