Interactions of mycotoxin alternariol with serum albumin and cyclodextrins

PhD thesis

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1. Introduction, aims

Alternariol (AOH) is a carcinogenic and xenoestrogenic mycotoxin produced by the *Alternaria* filamentous fungi. AOH frequently occurs as a contaminant in tomato juice, wine, and oilseeds. Human serum albumin (HSA) is the most abundant protein in the circulation. Albumin binding of exogenous and endogenous compounds may have an impact on their pharmaco- or toxicokinetic properties. Cyclodextrins (CDs) are ring-shaped oligosaccharides composed of glucose subunits. CDs have a hydrophilic external surface and a lipophilic internal cavity which makes them suitable for the formation of „host-guest" type complexes with apolar molecules such as certain mycotoxins. Previous studies have demonstrated that CD technology can suitable for the sensitization of analytical methods and for the development of new toxin binders.

In our experiments we aimed to investigate the interactions of AOH with albumin and CDs to receive detailed information about the toxicokinetic properties of the mycotoxin, and to identify novel CD-based mycotoxin binders. Binding constant were determined based on fluorescence spectroscopic studies. The binding site of AOH in albumin was tested using spectroscopic and ultrafiltration methods. The stability of AOH-CD complexes and the CD-induced enhancement in the fluorescence of the mycotoxin were investigated under acidic, physiological, and alkaline conditions. The toxin binding ability of insoluble β-CD bead polymer was tested in aqueous buffers as well as in spiked wine and tomato juice samples.

2. Methods

Fluorescence spectroscopic measurements were performed using Hitachi F-4500 fluorimeter at +25°C. The binding constant of AOH-HSA complex was determined employing three methods, based on: fluorescence quenching (λ_{ex} = 295 nm, λ_{em} = 340 nm), the albumin-induce increase in the fluorescence of AOH (λ_{ex} = 345 nm, λ_{em} = 455 nm), and the energy transfer between HSA and AOH (λ_{ex} = 295 nm, λ_{em} = 455 nm). The stability of AOH-HSA complex was evaluated employing the graphical application of Stern-Volmer equation and the Hyperquad2006 software. The species-dependent alternations regarding the albumin binding of AOH were tested using human, bovine, porcine, and rat serum albumins.

Furthermore, the effects of site markers on AOH-HSA interaction were also tested. We used many Site I (furosemide, phenylbutazone, glimepiride, indomethacin), Site II (naproxen, ibuprofen), and heme site (ethinylestradiol, methyl orange, bilirubin, and teniposide) markers, during which their impacts

on the emission signal of the AOH-HSA complex (λ_{ex} = 345 nm, λ_{em} = 455 nm) were examined in PBS (pH 7.4).

The binding site of AOH on HSA was also investigated employing ultrafiltration. In this experiment, Site I (warfarin), Site II (naproxen), and heme site (S-campthothecin) ligands were applied. Samples, containing site markers and HSA without and with 10 or 20 μM AOH, were passed through the filters with 10 kDa molecular weight cut-off value. The concentration of naproxen in the filtrate was determined with HPLC-UV, while warfarin and S-camptothecin were quantified by HPLC-FLD.

Effects of the environmental pH on the stability of AOH-CD complexes regarding native, randomly methylated, and quaternary ammonium β- and γ-CDs as well as the CD-induced increase in the fluorescence of the mycotoxin were investigated in acidic (pH 5,0), physiological (pH 7,4), and alkaline (pH 10,0) buffers, employing fluorescence spectroscopy. Furthermore, the interactions of AOH with sulfobutyl-β-CD (SBBCD), soluble SBBCD polymer, and sugammadex were determined under physiological conditions (PBS, pH 7.4). Binding constants were calculated with the graphical application of Benesi-Hildebrand equation.

In the next experiments, we tested the extraction of AOH from aqueous solutions by insoluble β-CD bead polymer (BBP). AOH removal was examined under acidic (pH 3.0 and pH 5.0), physiological (pH 7.4), and alkaline (pH 10.0) conditions. After the incubation of the mycotoxin solutions with increasing concentrations of BBP in a thermomixer, BBP was removed by pulse centrifugation, then the AOH concentration in the supernatant was quantified by HPLC-FLD. Furthermore, the influence of incubation time and temperature on the toxin extraction was tested as well. Regenerability and reusability of BBP after AOH binding were also examined. The bound mycotoxin was eluted from BBP in two consecutive steps with 50 v/v% ethanol-water mixture, then the AOH content of the supernatant was quantified by HPLC-FLD.

Our further investigations aimed to compare the interactions of AOH with β-CD (BCD) monomer, soluble BCD polymer, and BBP. Binding constants of the complexes formed with the former two CDs were calculated employing the graphical application of Benesi-Hildebrand equation based on fluorescence spectroscopic measurements, while the stability of AOH-BBP complex was determined from the bound and free fractions of AOH. Regarding the polymers, we calculated considering their BCD monomer content.

AOH extraction by BBP was also tested in red wine and in tomato juice spiked with the mycotoxin. Samples were incubated in the presence of increasing BBP concentrations, then the polmer was sedimented with pulse

centrifugation. After sample preparation, AOH content of the supernatant was determined by HPLC-FLD. The mycotoxin was extracted from red wine applying dispersive liquid-liquid microextraction; however, this method was not suitable in tomato juice. Therefore, we developed a novel extraction method, employing bovine serum albumin as affinity protein. We also tested the effect of BBP on certain quality parameters of beverages, including color intensity and total polyphenol content (investigated with UV-Vis spectroscopy).

The protective effects of BCD, soluble BCD polymer, SBBCD, soluble SBBCD polymer, and sugammadex vs. the cytotoxicity of AOH were tested on HeLa cells. The cells were treated with AOH in the absence and presence of CDs for 24 h. Cell viability was evaluated based on ATP content/well, determined using luciferin-luciferase reaction.

The statistical evaluation of data was performed employing IBM-SPSS software with one-way ANOVA (and Tukey's post-hoc test).

3. Results and discussion

3.1. Evaluation of AOH-albumin interaction based on fluorescence spectroscopic measurements

In the presence of HSA, a red shift was in the fluorescence emission maximum of AOH (λ_{em}: 421 → 455 nm; λ_{ex} = 345 nm) was observed, and the fluorescence signal of the mycotoxin was significantly increased. The HSA-induced elevation in the fluorescence of AOH was considerably higher than the sum of the individual fluorescence intensities of the mycotoxin and HSA, suggesting the formation of AOH-HSA complexes. In quenching studies, AOH decreased the emission signal of HSA (λ_{em} = 340 nm) in a concentration-dependent fashion. Even low AOH concentrations induced significant quenching effects, suggesting that the binding site of AOH is close to the tryptophan moiety of HSA in position 214 (Trp-214). Furthermore, using 295 nm excitation wavelength, the energy transfer between HSA and AOH was noticed, which supports the hypothesis that the mycotoxin occupies a binding site near to Trp-214. Based on fluorescence spectroscopic studies, the binding constants of AOH-HSA complex is approximately 4×10^5 L/mol.

Human, bovine, and porcine serum albumins showed low species-dependent alternations regarding AOH-albumin interactions; however, the binding constant of AOH-RSA complex was eight-fold higher compared to the other complexes. The observed difference in the affinity of AOH-RSA and AOH-HSA complexes may cause significant toxicokinetic differences between rats and humans.

3.2. Determination of the binding site of AOH on HSA

Since the fluorescence intensity of AOH markedly increased in the presence of HSA, the displacement of the mycotoxin from albumin results in the decrease in its fluorescence emission signal at 455 nm (λ_{ex} = 345 nm). Therefore, we tested the effects of different site markers on the fluorescence of AOH-HSA complex. Site I ligands with a secondary binding sites at the heme site (glimepiride and indomethacin) induced a significant decrease in the emission intensity, similarly to certain ligands of the heme binding site (bilirubin, methyl orange, and teniposide). Based on these observations, AOH occupies Site I and/or heme site on HSA.

In ultrafiltration experiments, AOH considerably increased the amount of the Site I marker warfarin in the filtrate, while the concentrations of naproxen (Site II) and S-camptothecin (heme site) were barely affected. The high molecular weight of HSA (66.5 kDa) does not allow albumin and albumin-bound ligands to pass through the filter; therefore, the increase in the concentration of warfarin in the filtrate indicates the elevation of free warfarin concentration and the displacement of the site marker from HSA.

The latter observation was confirmed by fluorescence spectroscopic experiments: In a concentration-dependent manner, AOH decreased the fluorescence of warfarin-HSA complex (λ_{ex} = 317 nm, λ_{em} = 379 nm). Since HSA-bound warfarin exerts markedly higher fluorescence compared to the free fluorophore, the decrease in the emission signal suggests the displacement of warfarin from albumin. Therefore, it is reasonable to hypothesize that AOH occupies Site I on HSA as its high-affinity binding site.

3.3. Effect of pH on the stability of AOH-CD complexes, and the CD-induced enhancement in the fluorescence of the mycotoxin

AOH possesses three phenolic hydroxyl groups. Under acidic conditions, the mycotoxin appears dominantly in its nonionic (protonated) form. However, based on the pK_a values, the nonionic, monoanionic, and dianionic forms of AOH appear at relevant concentrations in the applied pH range (pH 5.0-10.0). To characterize the interactions of AOH with CDs (native, randomly methylated, and quaternary ammonium β- and γ-CDs), fluorescence emission spectra of AOH were recorded in the presence of increasing CD concentrations (λ_{ex} = 345 nm). CDs induced a marked red shift in the emission spectrum of AOH: Its emission wavelength maxima were detected at 450-470 nm, depending on the buffer and the CD applied. Under acidic and physiological conditions, γ-CD (GCD) induced the largest increase

in the fluorescence intensity of AOH, while in alkaline buffer (pH 10.0) quaternary-ammonium-β-CD (QABCD) was the most effective from this point of view (inducing approximately 20-fold increase in the fluorescence of AOH). The fluorescence enhancement can be explained by the partial decomposition of the hydration shell around the mycotoxin molecule during the interaction of AOH with the apolar CD cavity. Thus the fluorescence quenching effects of water molecules become lower. The observed enhancement in the fluorescence of AOH can also be interesting from the analytical point of view.

At pH 5.0 and 7.4, GCD formed the most stable complex with AOH (K = 1,5 × 10^3 L/mol), while at pH 10.0 QABCD (K = 2,5 × 10^3 L/mol) and the quaternary-ammonium-γ-CD (QAGCD; K = 3,8 × 10^3 L/mol) proved to be the most effective. The stability of AOH-CD complexes was 3 to 10 times higher with the uncharged CDs in the acidic and physiological buffers than in alkaline (pH 10.0) solution. In contrast, opposite tendencies were noticed regarding QABCD and QAGCD. These observations suggest that the uncharged CDs generally prefer the nonionic form of AOH, while the positively charged tetraalkyl-ammonium side-chains of QABCD and QAGCD interact with the anionic form(s) of the mycotoxin.

Furthermore, we examined the interactions of AOH with sulfobutyl-BCD (SBBCD) derivatives (both monomer and soluble polymer) and with Sugammadex (chemically-modified GCD derivative). The stability of AOH-sugammadex complex proved to be outstandingly high (K = 2.9 × 10^4 L/mol).

3.4. Comparison of the interactions of AOH with BCD monomer, soluble BCD polymer, and BBP

Despite our observation that BCD monomer binds to AOH with low affinity, soluble BCD polymer and BBP proved to be ten-fold stronger binders (calculated based on their molar monomer content). It likely can be explained by the cooperative interactions between the BCD units.

3.5. Extraction of AOH from aqueous solutions by BBP

Extraction of AOH from aqueous solutions by insoluble β-CD bead polymer (BBP) was investigated between pH 3.0-10.0. BBP proved to be a highly effective mycotoxin binder in the pH range 3.0-7.4, the highest concentration applied (20 mg/1.5 mL) almost completely removed AOH. However, the AOH binding ability of BBP was strongly decreased at pH 10.0, even the highest BBP concentration caused only 60% decrease in mycotoxin

content. Furthermore, the effects of incubation time and temperature on the extraction were tested as well. The extraction of the mycotoxin did not show temperature dependence in the 20-40°C range. However, the incubation time strongly influenced the removal of AOH: At least 30 min incubation is required to reach the maximal mycotoxin binding. Moreover, we successfully regenerated BBP after AOH binding, employing 50 v/v% ethanol-water mixture (the bound mycotoxin were eluted from the polymer in two consecutive steps). After AOH extraction and BBP regeneration steps were repeated, the binding ability of the BBP remained unchanged, even after the second cycle.

3.6. Extraction of AOH from red wine by BBP

Removal of AOH from spiked red wine samples was tested in the presence of increasing concentrations of BBP. The mycotoxin was quantified (HPLC-FLD) in the supernatants after sample preparation. In a concentration dependent fashion, BBP reduced the amount of AOH in red wine, causing 80% decrease in its AOH content by the highest BBP concentration applied (25 mg/1.5 mL). BBP also affected the color intensity and total polyphenol content of red wine; however, the relative decrease in these parameters were lower compared to AOH removal.

3.7. Extraction of AOH from tomato juice by BBP

Removal of AOH from spiked tomato juice samples was tested in the presence of increasing concentrations of BBP. After the incubation, the remaining AOH was extracted from the supernatant employing bovine albumin as affinity protein, then the mycotoxin was quantified by HPLC-FLD. BBP decreased the concentration of AOH in tomato juice in a concentration-dependent fashion, 25 mg/1.5 mL polymer concentration caused approximately 55% reduction in the mycotoxin content. BBP did not affect the color quality of tomato juice even at the highest concentration applied; however, the relative decrease (46%) in the total polyphenol content of tomato juice was comparable with the AOH extraction.

3.8. Protective effects of CDs against the toxicity of AOH in HeLa cells

The effects of native BCD, soluble BCD polymer, SBBCD, soluble SBBCD polymer, and sugammadex on the AOH-induced viability loss were tested based on *in vitro* cell experiments. Native BCD did not exert protective effect even in the highest (more than 20-fold vs. the toxin) concentration

applied. Furthermore, soluble BCD polymer, SBBCD, and soluble SBBCD polymer only slightly alleviated the AOH-induced decrease in cell viability. In contrast, sugammadex almost completely restored cell viability, even at relatively low concentration (5-fold vs. the toxin).

4. Conclusions and new findings

AOH is a mycotoxin produced by the *Alternaria* species. It frequently appears as a contaminant in tomato, fruits, oilseed, and the corresponding processed products (wine, tomato juice, oils, etc.). Long-term exposure to AOH may result in carcinogenic and endocrine disruptor impacts. The effects of AOH have been tested in *in vivo* models; however, there were no available data regarding its plasma protein binding. The detailed characterization of AOH-albumin interactions helps the deeper understanding of the toxicokinetic properties of the mycotoxin, including species differences. Furthermore, the development of sensitive analytical methods and decontamination strategies are important to prevent the harmful effects of AOH. Thus, CD technology seems to be promising to develop new mycotoxin binders. The novel findings in my PhD thesis are the following:

- AOH forms a stable complex with HSA, its binding constant ($\log K$ = 5.6) is similar to the oral anticoagulant warfarin. Therefore, AOH-HSA interaction seems to be relevant from the toxicokinetic point of view.

- AOH occupies Sudlow's site I on HSA, and can displace warfarin from the protein in a concentration-dependent fashion.

- AOH binds to bovine and porcine albumins with similar affinity as to HSA, while the stability of AOH-RSA complex is approximately eight-fold higher.

- We developed a novel sample preparation method to extract AOH from aqueous solution and tomato juice, employing bovine serum albumin as affinity protein.

- Stability of AOH-CD complexes is highly influenced by the environmental pH and the type of the CD used. Among the first CDs tested (native, methylated, and quaternary-ammonium-β- and γ-CDs), GCD under acidic (pH 5.0) and physiological conditions while QABCD and QAGCD in alkaline solution (pH 10.0) formed the most stable ($K \sim 10^3$ L/mol) complexes with the mycotoxin.

- Among the first CDs tested (native, methylated, and quaternary-ammonium-β- and γ-CDs), QABCD increased the fluorescence of AOH (20-fold elevation) the most effectively at pH 10.0. However, at pH 5.0 and 7.4, GCD proved to be the strongest enhancer (10-fold increase).

- BBP proved to be an effective mycotoxin binder in aqueous solutions; however, it showed considerably lower binding ability at pH 10.0 than at acidic or physiological pH.

- After the extraction of AOH with BBP, the polymer can be regenerated using 50 v/v% ethanol-water mixture. The binding ability of the BBP remained unchanged, even after the second cycle.

- BBP successfully reduced the AOH content of both spiked red wine and tomato juice samples; however, in the latter beverage, less effective mycotoxin extraction was observed. As a result of BBP treatment, color intensity and total polyphenol content of red wine were also decreased; nevertheless, the relative changes in quality parameters were significantly lower compared to the relative decrease in AOH content. BBP did not change the color quality of tomato juice; however, the relative decrease in total polyphenol level was similar to the reduction of AOH content.

- Based on their molar monomer content, soluble BCD polymer and BBP bound AOH approximately ten-fold stronger compared to the BCD monomer.

- Under physiological conditions, sugammadex formed highly stable ($K = 2.9 \times 10^4$ L/mol) complex with AOH, and it almost completely abolished the AOH-induced toxicity in HeLa cells (even at five-fold concentration vs. the mycotoxin). However, BCD monomer, soluble BCD polymer, SBBCD, and soluble SBBCD polymer exerted no or only slight protective effects vs. the toxicity of AOH.

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6. List of publications

6.1. Publications related to the present PhD thesis:

Fliszár-Nyúl E, Lemli B, Kunsági-Máté S, Dellafiora L, Dall'Asta C, Cruciani G, Pethő G, Poór M, Interaction of Mycotoxin Alternariol with Serum Albumin. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 20. (2019) 2352. [**IF = 4.556**]

Fliszár-Nyúl E, Lemli B, Kunsági-Máté S, Szente L, Poór M, Interactions of Mycotoxin Alternariol with Cyclodextrins and its Removal from Aqueous Solution by Beta-Cyclodextrin Bead Polymer. BIOMOLECULES 9. (2019) 428. [**IF = 4.082**]

Fliszár-Nyúl E, Szabó Á, Szente L, Poór M, Extraction of mycotoxin alternariol from red wine and from tomato juice with beta-cyclodextrin bead polymer. JOURNAL OF MOLECULAR LIQUIDS 319. (2020) 114180. [**IF = 5.065**]

Cumulative impact factor of the publications related to the thesis: 13.703

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6.2. Oral presentations and posters related to the present PhD thesis:

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