Interaction of ochratoxin A with quaternary ammonium beta-cyclodextrin

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Ochratoxin A (OTA) is a widely spread nephrotoxic food contaminant mycotoxin. Unfortunately, attenuation or prevention of the toxic effects of OTA is still an unresolved problem. Molecular inclusion of OTA by cyclodextrins (CDs) results in complexes with low stability. In the human organism, OTA exists mostly in the dianionic state (OTA^2−). Therefore, our major goal was to develop a chemically modified cyclodextrin which gives a more stable complex with OTA than the previously published derivatives and which shows stronger preference towards OTA^2−. In our fluorescence spectroscopic study we demonstrate that quaternary ammonium beta-cyclodextrin (QABCD) fulfils both of these requirements. The calculated stability constant of the QABCD–OTA^2− complex was 28,840 M−1 (about 200-fold higher than that of the β-CD–OTA^2− complex). We hypothesize, that QABCD may be a suitable tool for the detoxification or prevention of the toxic effects of OTA, such complex formation may reduce its absorption from the intestine.

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1. Introduction

Mycotoxins are well known hazardous molecules, among which ochratoxin A (OTA) is a widespread food contaminant; OTA is a secondary metabolite of Aspergillus and Penicillium species (Pohland, Nesheim, & Friedman, 1992). OTA occurs in many foods (e.g. cereals, fruits, meat and dairy products) and drinks (e.g. milk, wine and coffee), as well as in animal feeds (Duarte, Pena, & Lino, 2009; Pohland et al., 1992). Chemically, it consists of a dihydroisocoumarin moiety linked with L-phenylalanine (Fig. 1). OTA is mainly nephrotoxic but numerous cell and/or animal experiments also suggest its hepatotoxic, carcinogenic, immunotoxic and teratogenic effects (Ringot, Chango, Schneider, & Larondelle, 2006). In spite of the large body of evidences, unfortunately the exact mechanism of OTA toxicity has not yet been understood in all detail. Ochratoxin A has a very high oral bioavailability (about 93%), in addition, more than 99% of OTA is albumin-bound in the circulation of humans (Hagelberg, Hult, & Fuchs, 1989; Studer-Rohr, Schlatter, & Dietrich, 2000). This is responsible for the extremely long plasma half-life of the toxin, resulting in its long-term toxicity.

Cyclodextrins (CDs) are one of the most studied molecular groups in the area of host–guest interactions; the most abundant CDs are α-, β-, and γ-CDs, which are built up from six, seven and eight glucopyranoses, respectively (Szejtli, 1988). They possess a conical structure with a hydrophobic interior and a hydrophilic exterior space. The internal cavity can entrap a wide range of guest molecules but the complex stability and selectivity can be substantially increased by appropriate chemical modifications (Dodziuk, 2008). There is a wide range of utilizations of CDs, for example to enhance solubility of active components; furthermore, analytical, pharmacological or cosmetic applications are also known. Recent studies have revealed that certain cyclodextrins are able to increase the fluorescence signal of numerous mycotoxins; for this reason their analytical application is highly acknowledged (Aghamohammadi & Alizadeh, 2007; Maragos & Appell, 2007; Maragos et al., 2008; Zhou et al., 2012). Only a few studies have been published regarding the interaction of OTA with β-, and γ-CDs and their derivatives; on the other hand, the binding affinities of these complexes are relatively low, and the inclusion of OTA in several cases gives little or no increase in its fluorescence (Amadasi et al., 2007; Hashemi & Alizadeh, 2009; Verrone et al., 2007).
Besides analytical utilization of CDs, a further very interesting application of β-CD-polypolyurethane polymer is to remove OTA from wines (Appell & Jackson, 2012). Most of the previously studied CDs show little or no preference for monoanionic or dianionic forms of OTA (Hashemi & Alizadeh, 2009). Because, in physiological environments (pH, ionic strength), OTA exists principally in dianionic (OTA$^{2-}$) form (Poór, Kunsági-Máté, Czibulya, et al., 2013; Poór et al., 2012), the identification of new CD derivatives with strong preference for OTA$^{2-}$ would be of considerable medical importance. Since CDs are non-toxic molecules, we hypothesize that, if an appropriate complex stability were achieved, CDs would be suitable candidates for detoxification applications related to OTA, even in vivo systems.

In the present study, our main goal was to identify a chemically modified CD which gives a more stable complex with OTA than do the previously described derivatives. Moreover, an indispensable requirement was also to show a considerably higher affinity of modified CDs towards the dianionic than the monoanionic (OTA$^{2-}$ vs. OTA$^-$) form of OTA. (2-hydroxy-3-N,N,N-trimethylamino)propyl-β-cyclodextrin chloride (QABCD) that fulfilled both of these requirements. Quaternary ammonium cyclodextrins were previously described as very suitable agents during ophthalmic formulation and permeation enhancement properties (Kis, Schoch, & Szejtli, 2003; Schoch, Bizec, & Kis, 2007). In addition, further potential applications of QABCD have also been studied (Bunke & Jira, 1998; Sebestyén, Buvári-Barcza, & Rohonczy, 2012; Wang, Cohen, Jicsinszky, & Douhal, 2012). Accordingly, the interaction between ochratoxin A and QABCD was investigated, using steady-state fluorescence spectroscopy and fluorescence polarization techniques. Furthermore, the influence of QABCD on OTA–magnesium and OTA–albumin interactions was investigated and molecular modeling studies were also performed.

### 2. Materials and methods

#### 2.1. Reagents

All reagents were of analytical grade. Ochratoxin A (OTA) was purchased from Sigma–Aldrich; 5000 µM stock solution was prepared in ethanol (Reanal, spectroscopic grade) and kept at 4°C protected from light. β-Cyclodextrin (β-CD) and (2-hydroxy-3-N,N,N-trimethylamino)propyl-β-cyclodextrin chloride (QABCD); average degree of substitution = 3.6; Fig. S1) were obtained from CycloLab Cyclodextrin Research & Development Laboratory, Ltd. Phosphate buffered saline (PBS) and 0.03 M ammonium acetate buffers (pH 5.2, 6.4, 7.4, 8.2, 9.0, respectively) were applied to examine the toxin-cyclodextrin interactions.

#### 2.2. Instrumentation

Fluorescence spectral analyses and fluorescence polarization measurements were performed using a Hitachi F-4500 fluorescence spectrophotometer and a Fluorolog $\times3$ spectrofluorimetric system. All measurements were done at 25°C in the presence of air. Fluorescence polarization ($P$) values were determined by applying the following equation:

$$
P = \frac{(I_{LV} - G \times I_{WH})}{(I_{LV} + G \times I_{WH})} \tag{1}
$$

where $I_{LV}$ and $I_{WH}$ are intensities measured at parallel and perpendicular orientations of the excitation and emission polarizer filters. $G$ is the instrumental function of the system. For calculating degree of polarization, 15 measuring points were averaged.

#### 2.3. Experimental procedures

##### 2.3.1. Fluorescence properties of OTA in the absence and in the presence of QABCD

Spectroscopic properties of nonionic and monoanionic OTA are very similar: both forms show excitation and emission wavelength maxima at 334 and 451 nm; on the other hand, dianionic OTA produces much higher fluorescence intensity and gives the corresponding maxima at 380 and 443 nm (Poór, Kunsági-Máté, Czibulya, et al., 2013; Poór et al., 2012). To investigate the influence of QABCD on the fluorescence spectra of OTA, 1 µM OTA and increasing cyclodextrin concentrations (25 µM–20 mM) were applied in pH 5.2 (for OTA$^-$) and in pH 9.0 (for OTA$^{2-}$) ammonium acetate buffers.

##### 2.3.2. Investigation of QABCD–OTA interaction at pH 6.4, using fluorescence spectroscopy

To investigate the potential preference of QABCD towards monoanionic or dianionic OTA, pH 6.4 ammonium acetate buffer was applied because, at pH 6.4, both the monoanionic and dianionic forms of OTA co-exist in aqueous solution (Fig. 2). Fluorescence spectra reflect elevation of OTA$^{2-}$ upon addition of QABCD; therefore the corresponding binding constants ($\log K$) can be determined using fluorescence emission intensities of OTA$^{2-}$ at 380 nm excitation and 443 nm emission wavelengths. The HyperQuad 2006 program package was applied for evaluating binding constants ($\log K$) from fluorescence emission spectra, using the previously published

Fig. 1. Chemical structures of OTA (above) and QABCD (below). [QABCD is a composite mixture of statistically substituted beta-cyclodextrin isomers; therefore the depicted formula is only a representative form.]
and denotes the fluorescence emission intensity of OTA in the presence of increasing QABCD concentrations in 0.03 M ammonium acetate buffer (pH 6.4) \( \lambda_{\text{ex}} = 380 \, \text{nm}; \lambda_{\text{em}} = 443 \, \text{nm} \). Stability constants were quantified by a previously published method (Il’ichev, Perry, Rüker, Dockal, & Simon, 2002; Il’ichev, Perry, & Simon, 2002):

\[
\alpha = \frac{(P - P_b)}{\theta \times (P_b - P) + P - P_b}
\]

where \( \alpha \) is the bound fraction of the toxin, \( P \) is the polarization of the sample, \( P_1 \) and \( P_b \) are fluorescence polarization values of completely free and bound OTA, respectively. Furthermore,

\[
\theta = \frac{\varepsilon_b \times \Phi_b}{\varepsilon_f \times \Phi_f}
\]

where \( \varepsilon_b \) and \( \varepsilon_f \) are the molar absorptivities of bound and free toxin, \( \Phi_b \) and \( \Phi_f \) are the fluorescent quantum yields of the bound and free OTA. For 1:1 stoichiometry (\( n = 1 \)), the binding constant (\( K \)) can be estimated from the following equation:

\[
K = \frac{1 - \alpha}{(1 + n \times C_{\text{CD}} - \alpha \times C_{\text{OTA}})}
\]

where \( C_{\text{CD}} \) and \( C_{\text{OTA}} \) are the total concentrations of cyclodextrin and OTA, respectively.

2.3.4. Competition with magnesium ions

In order to get more information on QABCD–OTA\(^{2-} \) interaction and to confirm the calculated stability constant determined by the fluorescence polarization technique, the competition of QABCD with magnesium ions towards OTA was also investigated. PBS (pH 7.4) was used to mimic extracellular physiological conditions. Based on our previously described observation that OTA\(^{2-} \) forms a complex with magnesium ions (Poór, Kunsági-Máté, Matisz, et al., 2013), competing ability of QABCD in the presence of Mg\(^{2+} \) and OTA was investigated. 1 \( \mu \)M OTA and 15 mM MgCl\(_2\) were applied in PBS (pH 7.4). Under these conditions, OTA exists only in magnesium-bound form. Then increasing amounts (0–50 mM) of QABCD were added to the system. Due to the spectral differences between Mg\(^{2+} – \text{OTA}^{2-} \) and OTA\(^{2-} \), the spectra were decomposed into two peaks referring to the desorbed and magnesium-bound dianionic fractions. log\( K \) values have been determined by the HyperQuad program package, applying Eq. (2).

2.3.5. Effect of QABCD on OTA–albumin interaction

Since albumin is the major carrier protein of OTA, we were curious about whether QABCD might have any influence on the
OTA–albumin interaction. In order to investigate the potential influence of QABCD, the previously described model system was applied (Poór, Kunşagi-Máté, Czigula, et al., 2013; Poór et al., 2012), where 1 μM OTA and saturating amount (1.7 μM) of human serum albumin (HSA) were mixed in PBS (pH 7.4). Fluorescence excitation and emission maxima of albumin-bound OTA ($\lambda_{\text{exc}} = 393$ nm; $\lambda_{\text{em}} = 445$ nm) were used for fluorescence polarization measurements. Because OTA forms a very stable complex with HSA, in this experiment higher QABCD concentrations were applied (12.5, 25, 50, 100 and 200 mM, respectively).

2.3.6. Molecular modelling studies

Aiming to get a qualitative picture of the QABCD–OTA complex formation, molecular dynamics (MD) simulations were performed. The initial structure of the OTA molecule has been obtained from the ZINC database (Irwin & Shoichet, 2005), while the structure of the QABCD molecule was constructed by adding the three corresponding quaternary ammonium groups to the β-CD structure, the latter having been obtained from the Chenospider database. The Amber force fields, with the TIP3P explicit water model, as were later having been obtained from the Chemspider database. The Amber force fields, with the TIP3P explicit water model, as were implemented in the HyperChem 8.0 program (HyperChem Prof. 8.0, Hypercube, Inc., USA), have been used to perform the molecular dynamics simulations. The use of an explicit water model has been chosen by considering a previous detailed study related to β-CD molecules (Manunza, Deiana, Pintore, & Gessa, 1997), where it was shown, that the cyclodextrin molecule in solution mainly exists in a highly distorted structure with a major influence by the solvent, in contrast to the crystal structure which is more ordered (Amadasi et al., 2007). Therefore, the QABCD molecule has been treated as flexible in the present study. Furthermore, it is clear that the explicit consideration of the water molecules is of importance for the electrostatic interaction in the case of the QABCD–OTA$^{2-}$ complex. The MD simulations were performed for a few hundreds of ps time and at 298 K in the NVT ensemble, starting from some selected and different initial structures. The obtained final structures, after reaching equilibria, were then analysed and compared.

3. Results and discussion

3.1. Effects of QABCD on the fluorescence spectra of OTA

Previous studies clearly show that fluorescence characteristics of OTA are highly influenced by the microenvironment. Depending on the pH, OTA could be present in nonionic, monoanionic and di-anionic forms (spectral differences are detailed in 3.1.). The reported $pK_a$ values of the carboxyl and the phenolic hydroxyl groups of OTA in water are in the range of 4.2–4.4 and 7.0–7.3, respectively (Il’ichev, Perry, & Simon, 2002). Other very important parameters are the ion strength, and the type of the ions. For example, both alkali and alkaline earth metal ions are able to form complexes with OTA$^{2-}$ (Poór, Kunşagi-Máté, Matisz, et al., 2013). At pH 6.4, the monoanionic form is dominant but the dianionic form is also present. On the other hand, at pH 7.4 the dianionic OTA predominates. At pH 8.2, the amount of monoanionic form is very low and at pH 9.0 it is negligible. The addition of QABCD to 1 μM OTA solution did not modify the fluorescence spectra of the toxin (tested with 0–20 mM QABCD in ammonium acetate buffer at pH 5.2 and at pH 9.0). This result shows that the inclusion of OTA$^{-}$ and OTA$^{2-}$ by QABCD has no influence on the fluorescence behaviour of the toxin. The observed phenomenon is not surprising because recent studies highlighted that, in many cases, the inclusion of OTA by cyclodextrins is associated with little or no spectroscopic change (Amadasi et al., 2007; Hashemi & Alizadeh, 2009).

3.2. Investigation of QABCD–OTA interaction at pH 6.4, using fluorescence spectroscopy

In ammonium acetate buffer at pH 6.4, the monoanionic OTA predominates; on the other hand, the presence of QABCD (even at 25 μM concentration) results in significant increase of di-anionic OTA (Fig. 2a). Since the inclusion of OTA by the QABCD molecule did not change its fluorescence spectra, these data suggest that QABCD forms a considerably more stable complex with OTA$^{2-}$ than with OTA$^{-}$. Fig 2 shows the spectral data and molecular species distribution as the function of QABCD concentration in ammonium acetate buffer (pH 6.4). Under these conditions, the stability constant of QABCD–OTA$^{2-}$ complex (Table 1) was determined by applying Eq. (2).

3.3. Determination of complex stabilities using fluorescence polarization

The binding of OTA to the much larger cyclodextrin molecule results in substantially increased fluorescence polarization values because the inclusion of OTA by CD causes a significant decrease in its rotational freedom (Fig. 3). Using this principle, the reaction can be easily followed by the fluorescence polarization technique. The binding constants were determined by applying Eqs. (3)–(5) at pH 7.4, 8.2 and 9.0 (Table 1). In 0.03 M ammonium acetate buffers, the increasing pH results in significant elevation of log$K$ values. The impact is understandable because, at pH 7.4 and 8.2, the monoanionic OTA is also present in the solution and it can compete with the dianionic form, despite the fact that the binding constant of QABCD–OTA$^{-}$ complex is substantially lower (log$K = 2.33 \pm 0.19$; determined in ammonium acetate buffer at pH 5.2 using Eqs. (3)–(5)). Because, at pH 9.0, OTA exists exclusively in the dianionic form, our data show the factual binding constant of QABCD–OTA$^{2-}$ complex; therefore we suggest that its log$K$ value is 4.46 (±0.09).

Thereafter, the binding constant of β-CD was compared to that of QABCD in the case of OTA$^{2-}$. Using fluorescence polarization technique, 2.16 ± 0.06 was obtained as the log$K$ value of β-CD–OTA$^{2-}$ in ammonium acetate buffer at pH 9.0. Our results underline the interesting finding that QABCD shows about 200-fold higher complex stability with OTA$^{2-}$ than does the initial native β-CD. Furthermore, QABCD shows very strong preference towards the dianionic form of OTA (approximately 130-fold higher compared to OTA$^{-}$). In addition, the determined stability constant of the QABCD–OTA$^{2-}$ complex is more than 10-fold higher than that observed in the case of the previously described most stable CD-derivative (heptakis-2,6-di-O-methyl-β-cyclodextrin; determined in an identical ammonium acetate buffer as in our experiment) (Hashemi & Alizadeh, 2009).

Recent studies have highlighted that CDs may be suitable tools for removing certain targeted or undesirable molecules from aqueous medium, e.g. from milk or water extracts (Arora & Damodaran, 2011; Ratnasooriya & Rupasinghe, 2012; Tahir & Lee, 2013). This phenomenon was also confirmed by the study of Oläh, Cserháti, and Szegi (1988), where the enhanced detoxification of industrial wastewaters by β-CD was demonstrated. In addition, Appell and Jackson (2012) further evidenced that β-CD-polyurethane polymer is able to remove OTA from wines. The above listed examples indicate that the removal of certain toxins, pesticides or other toxic
compounds from different drinks is possible with native and chemically modified CDs, suggesting a promising detoxification application of the cyclodextrin molecular group in the future.

Bearing in mind previous reports that OTA is absorbed mainly from the intestinal tract in nonionic and monoanionic forms (Galtier, 1978; Kumagai & Aibara, 1982; Roth et al., 1988) and considering our observations, we might assume that QABCD may be suitable for trapping OTA in dianionic form and in this way inhibiting the absorption of the toxin. This idea is further supported by previous studies with cholestyramine: the cholestyramine–OTA complex shows a stability constant similar to that determined for the QABCD–OTA$^{2-}$ complex (Kerkadi et al., 1999). In vivo studies verify that cholestyramine inhibits the absorption of OTA from the intestines; therefore, it enhances toxin elimination and in this way protects against OTA toxicity (Kerkadi et al., 1999; Madhyastha, Frohlich, & Marquardt, 1992).

Finally, our data show that, with ionic interaction, the inclusion of OTA by CDs can be dramatically increased. We suggest that, with
further chemical modifications of QABCD, it may be possible to achieve even higher complex stability and selectivity.

3.4. Competition with magnesium ions

In the next step, the OTA–Mg²⁺–QABCD system was investigated (in PBS, pH 7.4). The OTA–magnesium complex shows excitation and emission maxima at 375 and 427 nm (with a blue shift of the emission spectrum of OTA) and gives a considerably higher fluorescence signal than does free OTA⁻²⁻ (Poór, Kunsági-Máté, Matisz, et al., 2013). Under the applied conditions (see above), OTA is presented completely in the Mg²⁺–OTA⁻²⁻ complex. Fig. 4a demonstrates that QABCD is a very effective competitor against Mg²⁺ since the addition of QABCD results in a red shift of the fluorescence spectrum of OTA and also in a significant decrease of its intensity. Under these circumstances, logK = 1.50 ± 0.01 has been determined for the formation of QABCD–OTA⁻²⁻ at room temperature. The low logK value is probably due to the exchange reaction of Mg²⁺ and QABCD towards OTA⁻²⁻. The sum of this value and the previously determined data of Mg²⁺–OTA⁻²⁻ complex (logK = 3.00 ± 0.01 in PBS at pH 7.4) (Poór et al., 2014) is 4.50, which is in good correlation with the result obtained from fluorescence polarization-based experiments (logK = 4.46).

3.5. Effect of QABCD on OTA–albumin interaction

Thereafter, the effect of QABCD on albumin binding of OTA was also investigated. OTA⁻²⁻ forms a very stable complex with HSA (logK = 7.41 at 25 °C in PBS) (Poór et al., 2012). HSA–OTA⁻²⁻ interaction is considered to be of high biological importance; therefore, in this experiment, PBS (pH 7.4) was used to mimic extracellular physiological conditions. Results are demonstrated in Fig. 4b where fluorescence polarization data of the OTA–HSA system were expressed in the presence of increasing QABCD concentrations. Since, under these conditions, both the QABCD and the albumin-bound dianionic OTA⁻²⁻ are simultaneously detected (the fluorescence wavelength maxima of the two forms are very similar: 380 → 443 nm and 393 → 445 nm, respectively), the decrease of fluorescence polarization indicates the molecular displacement of OTA⁻²⁻ from the surface of HSA (Poór, Kunsági-Máté, Czibulya, et al., 2013; Poór et al., 2012). These data show that high QABCD concentrations are needed to achieve a competing ability against OTA because of the great difference in stabilities of HSA–OTA⁻²⁻ and QABCD–OTA⁻²⁻ complexes.

3.6. Molecular modelling studies

The electrostatic potential map at a constant electron density (i.e. essentially the molecular shape and the electrostatic potential at 0.002 e/a₀³ electron density) of the structure obtained from the molecular dynamics simulations, where OTA⁻²⁻ takes its location at the lower rim of the cyclodextrin cavity, can be seen on Fig. 5. The determining interactions on the complex structure are mainly from the electrostatic interaction between the quaternary ammonium groups and the carboxyl group of OTA⁻²⁻ and the inclusion of the phenyl moiety into the apolar cavity of the cyclodextrin molecule. These results confirm our idea that the complex formation of OTA⁻²⁻ with cyclodextrins can be substantially strengthened by electrostatic interaction. Based on the molecular modelling studies, OTA⁻ and OTA⁻²⁻ ions do not behave in strongly different ways at complex formation with the QABCD cyclodextrin and they can attach to both the lower and the upper rim of the cyclodextrin cavity. However, these QABCD cyclodextrin molecules, with different positions and orientations of the quaternary ammonium groups, can show variable affinity towards OTA and its different anionic forms.

4. Conclusions

Interaction of OTA with QABCD was studied under different circumstances, using fluorescence spectroscopy. Our results show that the positively charged part of the QABCD molecule results in about 200-fold higher complex stability with OTA²⁻ than was observed in the case of β-CD. Furthermore, QABCD shows very high preference towards the dianionic form of OTA. Therefore we hypothesize that QABCD might be a suitable candidate for attenuating OTA toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.034.

References


