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Interaction of alkali and alkaline earth ions with Ochratoxin A

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ABSTRACT

The effect of alkali and alkaline earth ions on the chemical equilibrium of mono- and dianionic forms of the mycotoxin Ochratoxin A (OTA) and their bonding onto the surface of Bovine Serum Albumin (BSA) have been investigated by fluorescence spectroscopy and fluorescence polarization techniques. Our results show that alkali metal ions shift the chemical equilibrium towards formation of dianionic form of OTA. Furthermore, the alkaline earth ions can compete with BSA for binding to OTA when these ions are present in millimolar concentrations. Our data also highlight the possibility that the "free" fraction of OTA (not bound onto the surface of albumin) or at least a part of it is present in cation-bound form in body fluids. These observations are supported by stability constants and quantum-chemical calculations. Among the studied alkaline metal ions magnesium showed the highest affinity towards OTA under physiological conditions. Further research is required to analyze the potential significance of Mg^{2+}–OTA complex in cellular uptake and/or elimination of the toxin in the human body.

1. Introduction

The potent mycotoxin Ochratoxin A (OTA; Fig. 1) is produced by several *Aspergillus* and *Penicillium* fungi [1,2]. It is widespread all over the world and appears in cereals, plant and animal products, animal feeds, foods and drinks [2,3]. Previous studies suggest that chronic exposure to OTA might play a major role in development of a chronic kidney disease named Balkan Endemic Nephropathy (BEN) and is also responsible for several other adverse effects [4–6]. In aqueous solution at pH 7.4 the toxin exists in both dianionic and monoanionic forms [6,7]. In the human body more than 99% of the circulating toxin is albumin-bound [8,9] and to our current knowledge the remaining small fraction is the free, unbound OTA. Previous studies suggest that dianionic OTA binds to human serum albumin (HSA) with a very high affinity due to the formation of an ion-pair [7,10,11]. In healthy individuals plasma levels of OTA are very low: about 0.25–5.0 nmol/L nevertheless it could reach 120 nmol/L or higher concentrations in endemic areas [12–15]. Based on these data the concentration of non-protein bound toxin could be estimated to be in the pmol/L scale or in toxin exposed people may reach a few nmol/L in the blood. Rahimtula et al. hypothesize that OTA–Fe^{3+} complex could be formed in the organism, which in the presence of NADPH-cytochrome P450 reductase results in the production of OTA–Fe^{2+} complex leading to generation of hydroxyl radicals with consequent lipid peroxidation [16,17]. To our current knowledge we lack evidence on interaction of OTA with other inorganic cations and whether a potential ion binding could affect the biological behavior of the toxin. A feasible technical approach for studying the OTA–ion interactions would be fluorescence spectroscopy because Ochratoxin A is a highly fluorescent molecule [10,11,18].

In our experiments fluorescence spectroscopy and fluorescence polarization techniques were applied to investigate the effects of alkali and alkaline earth metal ions on Ochratoxin A. Fluorescence spectra suggested that the studied alkali ions (Li^+, Na^+, K^+) in our model system shifted the chemical equilibrium towards the formation of the dianioic structure of OTA. Furthermore alkaline earth ions (Mg^{2+}, Ca^{2+}, Ba^{2+})—especially Mg^{2+}—gave detectable interaction with OTA and showed differing fluorescence spectra. These observations were also supported by fluorescence polarization measurements revealing that high concentrations (in millimolar range) of alkaline earth ions were able to compete with bovine serum albumin (BSA) for Ochratoxin A binding. Stability constants and quantum-chemical calculations supported the finding that among the studied alkaline earth ions Mg^{2+} gives the most stable interaction with dianionic OTA. Free OTA is of very low concentration in the blood plasma and non-protein bound Mg^{2+} is present in hundreds of μmol/L. Therefore our investigation raises the possibility that "free" (not

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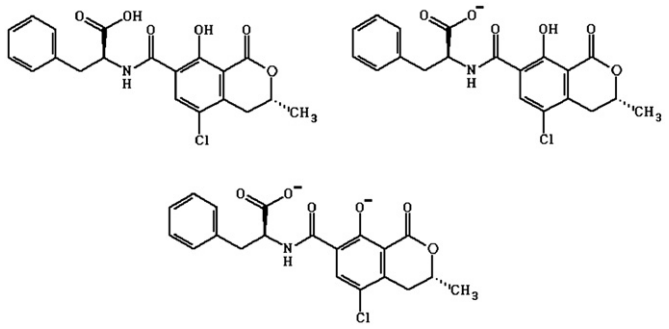

Fig. 1. Chemical structure of Ochratoxin A: nonionic (OTA), monoanionic (OTA^-) and dianionic (OTA^{2-}) forms.

protein-bound) fraction of the toxin (or a part of it) maybe present in a magnesium-bound form in body fluids. Till now, there is no information on the influence of ion–toxin interaction regarding the biological behavior of OTA (distribution, transport or other biological effects). Our investigation might be important in view of obtaining data on the physico-chemical behavior of unbound toxin for better understanding of the elimination and/or cellular interaction of free OTA in the body.

2. Materials and methods

5000 μM stock solution of Ochratoxin A (OTA; from Sigma) was prepared in spectroscopic grade 96% ethanol (Reanal) and kept at 4 °C protected from light. Bovine serum albumin (from Reanal) was used without further purification. Buffers were prepared from their corresponding acidic and basic components dissolved in tridistilled water. Tris–HCl buffer contained 100 mmol/L TRIS, pH 7.4 adjusted by 1 mol/L hydrochloric acid. Phosphate buffered saline (PBS) contained NaCl (137 mmol/L), KCl (2.7 mmol/L), Na_2HPO_4 (8 mmol/L) and KH_2PO_4 (1.5 mmol/L) in tridistilled water (pH 7.4).

Since we have shown in our previous papers that fluorescent [19–21] and quantum-chemical investigations [22] were useful tools for determination of the interactions between alkali ions and several molecules possessing aromatic moieties, these methods were chosen to analyze the ion–OTA interactions. Accordingly, Hitachi F-4500 fluorescence spectrophotometer was applied to measure excitation, emission spectra and fluorescence polarization values. Emission spectra used for calculating the binding constants (logK) were measured by Fluorolog τ3 spectrofluorimetric system (Jobin-Yvon/SPEX). The logK values of OTA-BSA and OTA-alkaline ion complexes were evaluated using Hyperquad2006 software (Protonic Software). The degree of fluorescence polarization was calculated as

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$$

where I_{VV} and I_{VH} are fluorescence intensities measured at vertical excitation polarizer setting and at vertical and horizontal emission polarizer settings respectively, while G is the actually measured optical correction factor. For calculating the degree of polarization 30 measuring points were averaged. All analyses were performed in the presence of air at +25 °C.

MOPAC2009 program (version 11.366 W) was used for the PM6 semi-empirical calculations. The PM6 method is parameterized for nearly the whole periodic system, including Li^+, Na^+, K^+ alkali and Mg^{2+}, Ca^{2+}, Ba^{2+} alkaline earth metal ions. The initial structure of the OTA (monoanionic form) was obtained from the ZINC database [23]. Geometry optimizations and determination of the interaction energies of the ochratoxin A (mono and dianionic forms) – metal ion 1:1 complexes have been done applying the aforementioned quantum chemical method. The structures of OTA and its ionic complexes are available in the electronic supplements.

3. Results and discussion

3.1. Fluorescence spectra of Ochratoxin A

Excitation spectra of monoanionic (OTA^-) and dianionic (OTA^{2-}) forms of OTA show different wavelength maxima: 340 and 380 nm, respectively. Emission λ_{max} of the variously protonated OTA forms are identical (443 nm) but the emission intensity of the dianionic structure is much higher. In PBS and Tris–HCl buffers (pH 7.4) Ochratoxin A is present mainly in dianionic form; however a detectable but considerably small amount of monoanionic OTA appears too.

3.2. Fluorescence spectra of OTA in the absence and in the presence of cations in Tris–HCl buffer

First the excitation and emission spectra of Ochratoxin A (2 μmol/L) in the absence and in the presence of 100 mmol/L cations (Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}) were studied in Tris–HCl buffer (pH 7.4). This buffer was used because TRIS is an organic base and hydrochloric acid gives only the H^+ and Cl^- ions to the system. Therefore the components of the buffer do not interfere with our investigations. In all cases chloride salts of cations were used for experimentation. Fig. 2A represents excitation spectra of OTA where in the presence of alkaline earth ions the intensities around 380 nm (excitation maximum of OTA^{2-}) increase and the fluorescence signal at 340 nm (excitation maximum of OTA^{-1}) decreases indicating that the amount of dianionic OTA becomes higher in the system. The presence of alkaline earth ions causes a shift in the excitation maxima (λ_{max} of OTA–ion complexes: Mg^{2+} = 375 nm, Ca^{2+} = 375 nm, Ba^{2+} = 377 nm) and a significant increase of fluorescence intensities were also observed (Fig. 2).

Fig. 2B shows the emission spectra of OTA in the absence and in the presence of different cations. In the case of alkali ions the emission λ_{max} of OTA did not change (443 nm) but the fluorescence signals were increasing (λ_{exc} = 380 nm) caused by the higher intensity of the dianionic form. As it was also shown by the excitation spectra, alkali earth ions shift the chemical equilibrium towards the formation of dianionic OTA. Emission spectra represent well that alkaline earth ions cause major changes in the spectra of OTA, which is represented by the shift of emission maxima (λ_{max}: Mg^{2+} = 427 nm, Ca^{2+} = 433 nm, Ba^{2+} = 439 nm) with a significant increase of the emission intensities too, mainly in the case of magnesium. These excitation and emission λ_{max} values did not change further when higher amounts of cations were added to the system.

3.3. Competition of alkaline earth ions with BSA for OTA complex formation

In this part of our studies the effects of divalent cations on the OTA-BSA system were investigated, verifying the interaction between OTA and alkaline earth ions. 2 μmol/L OTA and 3.5 μmol/L BSA concentrations were added to the system; under these environments almost the total amount of OTA is albumin-bound. In Tris–HCl buffer (pH 7.4) albumin-bound OTA has excitation and emission maxima at 395 nm and 445 nm, respectively. Cations were added to the OTA-albumin solution in 100 mmol/L concentrations. In the case of alkali earth ions excitation and emission spectra did not change (data not shown) but using alkaline earth ions the spectral changes suggested a decrease of albumin bound OTA in the samples (Fig. 3A).