J Incl Phenom Macrocycl Chem (2007) 57:475–479
DOI 10.1007/s10847-006-9237-4

ORIGINAL ARTICLE

Effect of β-cyclodextrin on spectroscopic properties of ochratoxin A in aqueous solution

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Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 18 January 2007

Abstract Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by several fungal species, mainly *Aspergillus ochraceus, A. carbonarius* and *Penicillium verrucosum*. It contaminates many foodstuffs, particularly cereals and their derivatives, coffee, beer, wine and cocoa, and represents a serious health threat both to humans and animals. Spectroscopic properties of OTA solutions depend on the pH, solvent polarity and can be influenced by the presence of cyclodextrins (CDs). In this work, the effect of β-CD on spectroscopic properties of OTA in aqueous solutions has been investigated by means of absorption and steady-state fluorescence at different pHs (range 3.5–9.5). Binding constants of OTA/β-CD inclusion complexes have been determined by applying modified Benesi-Hildebrand equation. A 1:1 stoichiometry of OTA/β-CD complexes has been observed at all tested pHs.

Keywords Ochratoxin A · Aqueous solutions · β-cyclodextrin · Inclusion complexes · pH

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Introduction

Ochratoxin A (OTA), 7-(L-β-phenylalanyl-carbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-*3R*-methyl-isocumarin (Fig. 1), is a nephrotoxic, carcinogenic, hepatotoxic, teratogenic and immunotoxic mycotoxin produced by some *Aspergillus* and *Penicillium* species, mainly *A. ochraceus*, *A. carbonarius* and *P. verrucosum*. It frequently occurs as natural contaminant in various plant products and beverages (in particular cereals and derived products, wine, coffee, spices, beer, cocoa) and in feedstuffs, also at high levels. In addition, OTA has been frequently found in human milk, blood and urine indicating a diffuse and continuous human exposure to this mycotoxin [1]. The IARC (International Agency for Research on Cancer) has classified OTA as a possible carcinogen to humans (Group 2B) [2].

The availability of sensitive methods for the determination of OTA in foodstuffs and biological fluids is highly necessary in order to enable the accurate assessment of consumer exposure to this mycotoxin. Previous studies [3] showed that HPLC separation of OTA and zearalenone toxins, can be achieved in a single run by forming inclusion complexes with β-cyclodextrin (β-CD) added to mobile phase. Moreover the use of β-CD in a post-column reaction system has been developed to improve the detection limit of aflatoxins [4–6].

The interaction between cyclodextrin and organic molecules can affect the physicochemical properties of the compounds included, particularly it has been demonstrated that many organic molecules in aqueous solution show an enhancement of fluorescence when CDs are added [7–12]. This fact can be explained in a

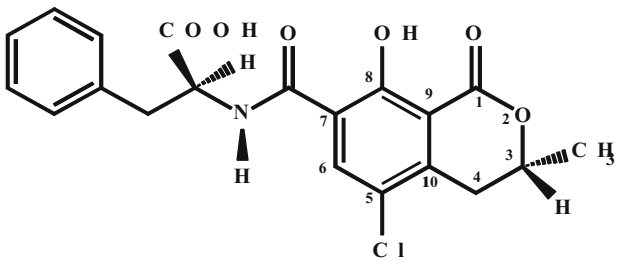

Fig. 1 Structure of Ochratoxin A

number of way: when organic molecules are included in CD cavity they are protected from fluorescence quenchers and lose rotational freedom. This loss can result in less efficient nonradiative decay and hence greater fluorescence. Also many organic molecules show significantly greater fluorescence in nonpolar than in polar environments: in the formation of inclusion complexes a relatively nonpolar environment is provided to the organic molecule and its fluorescence intensity is enhanced [8]. Fluorescence enhancement by cyclodextrin complexation can be exploited to increase the sensitivity of fluorescence-based techniques. In this work interaction between β-CD and OTA in aqueous solution at different pHs has been investigated by means of absorption and steady-state fluorescence. It was observed that a 1:1 complexation occurs between β-CD and OTA at all tested pHs. Binding constant of OTA/β-CD inclusion complexes have been determined by using a modified Benesi-Hildebrand equation.

Experimental section

Ochratoxin A (OTA) and β-cyclodextrin (β-CD) from Sigma-Aldrich were used without further purification.

A OTA stock solution (1.56 mg ml^{-1}) in toluene:acetic acid 99:1 was stored and kept in the dark at 4 °C. Aqueous stock solutions of β-CD ($3.63 \cdot 10^{-3}$ M) were prepared at different pHs (3.5, 7.5 and 9.5). OTA/β-CD solutions were obtained by drying appropriate aliquots of OTA stock solution in a stream of N_2 and by adding appropriate volumes of β-CD stock solution and then diluted with ultrapure water or buffer solutions. Phosphate and ammonium chloride buffer solutions were used at a concentration of 0.1 M. OTA/β-CD solutions were stirred by vortex for about 1 min.

UV-visible absorption spectra were recorded using a Varian Cary/3 spectrophotometer. Fluorescence measurements were realized using a Varian Cary Eclipse spectrofluorometer. Both kind of measurements were performed using a 1 cm path length quartz cell.

Results and discussion

Absorption spectrum of OTA aqueous solution at neutral pH shows two peaks at 330 nm, due to the neutral and the monoanionic form of toxin, and at 379 nm, due to the dianionic form (Fig. 2). The pKa's of carboxylic group and hydroxyl group on the isocoumarin moiety were reported to be about 4 and 7, respectively [13–16]. Therefore at pH 9.5 OTA exhibits only the absorption band at 379 nm since it exists in solution as dianion; at pH 3.5 instead the presence of the protonated form of OTA shifts the peak at 330 nm. Since the protonation of carboxylic moiety does not produce any change in the absorption spectra, such blue shift has been attributed to the protonation of phenol group [13] and thus to the neutral and monoanionic form of OTA. At intermediate pHs both monoanionic and dianionic toxin forms are present in solution; in pure water, in fact, being the toxin primarly presents in monoanionic form, the absorption spectrum shows a more intense band at 330 nm and a lower one at 379 nm. This last becomes prevalent increasing the pH of solution, which shifts the equilibrium towards the dianion form of OTA. The measured molar absorptivities for the deprotonated and fully protonated forms are 9115 M^{-1} cm^{-1} and 6600 M^{-1} cm^{-1} respectively in agreement with those reported by II'ichev [13]. Fluorescence spectra obtained exciting OTA solutions at 330 nm exhibit broad peaks at 460 nm with a shoulder at lower wavelengths, that partially overlap and that are attributable to neutral and monoanionic forms and to dianionic form respectively. Specifically the fluorescence spectra for both dianionic and monoanionic species show same peaks at around 450 nm attributed to toxin enol form [13, 17]. It has been in fact

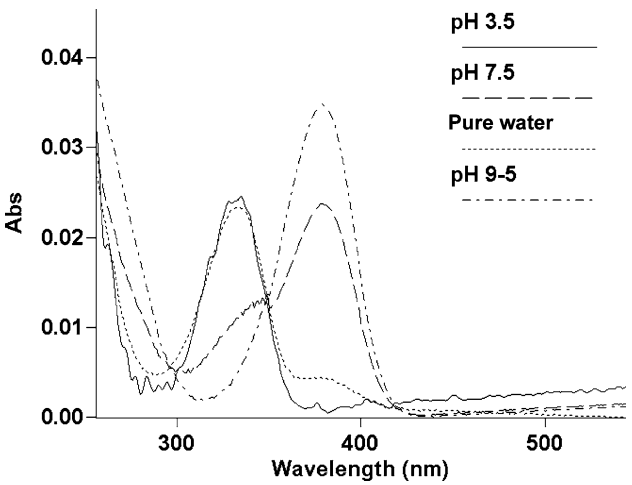

Fig. 2 Absorption spectra of OTA aqueous solutions 3.84×10^{-6} M at different pHs

demonstrated that the monoanion undergoes an excited-state intramolecular proton transfer (ESIPT) which allows the formation of highly emissive OTA enol form [13]. Fig. 3, in fact, shows that both an intensity decrement and a blue shift of the fluorescence peak from 460 to 453 nm are recorded passing from acid to basic solutions. An opposite trend is observed exiciting OTA aqueous solutions at 379 nm, absorption maximum of the dianionic form (inset Fig. 3). In this case, as expected, the highest intensity peak, recorded at 453 nm, is observed at pH 9.5 while increasily lower intensities of the fluorescence bands are observed at lower pHs where the monoanion and neutral OTA form, absorbing at 330 nm, are mainly present. At pH 3,5 where only the protonated specie is present, no fluorescence signal is recorded exiting at 379 nm.

The addition of β-CD to pure water solutions of OTA does not change the absorption spectrum of the toxin in terms of peaks position. Changes in the band intensities were instead observed. In particular as reported in Fig. 4 a, the addition of increasing amounts of CD induces a decrease in absorbance at 379 nm and a concomitantly increase of the intensity band at 330 nm. According to this, excitation at 379 nm produces dianion fluorescence peak whose intensity decreases increasing CD concentration (Fig. 4 b). The fluorescence associated to the monoanionic specie obtained exciting at 330 nm instead increases with CD concentration. This suggests the existence of interactions between OTA and CD which promote an interconversion between the deprotonated and singly protonated form of the toxin.

The dependence of the monoanionic fluorescence enhancement on the CD concentration was used to study quantitatively the interaction between OTA and β-CD (Fig. 5). The binding constants and the stoichiometries of OTA/β-CD complexes at various pHs have been determined by means of modified Benesi Hildebrand equation [18]:

$$\frac{F_0}{F - F_0} = \frac{1}{A} + \frac{1}{AK[\mathrm{CD}]^n}$$

where K is the binding constant, F_0 is the initial fluorescence intensity of free OTA, F is the maximum fluorescence intensity of the OTA-CD inclusion complexes at the [CD] cyclodextrin concentration, A is a constant, and n is the number of binding sites. The inset of Fig. 5 reports the plot of $F_0/(F–F_0)$ vs $1/[\beta\text{-CD}]$ in pure water solutions. The linearity of the plots $F_0/(F–F_0)$ vs $1/[\beta\text{-CD}]$ obtained reflects the formation of 1:1 complex between the toxin and the β-CD. The calculated value for the binding constant evaluated by fluorescence measurements is 1570 M^{-1}. Values for association constants ranging from 350 to 1400 M^{-1} were reported for differently functionalized coumarin and β-CD [19, 20]. In order to get information about interaction of the single toxin forms with β-CD, the effect of pH on the association complex formation has been investigated. The fluorescence spectra of OTA/β-CD solutions at different pHs are showed in the Fig. 6. As reported for OTA in buffer solutions, exciting at the wavelength of absorption maximum of toxin fully protonated and monoanionic foms (330 nm), an increment of intensity and a red shift of the peak has been observed by varying pH, from basic to acid values. A concomitantly decrease in fluorescence intensity of deprotonated form has been recorded exiting at 379 nm (data not shown). Comparing the data in Figs. 2 and 6 it is possible to note that the presence of β-CD in solution produces an increment of fluorescence intensities at all tested pHs. In addition β-CD does not cause changes in absorption peaks positions and intensities (data not shown) in buffered solutions. These facts suggest that β-CD interacts with both protonated and deprotonated forms of toxin, without altering dissociation constants of the acidic moieties. The binding constants and complex stoichiometries calculated applying the modified Benesi-Hildebrand equation to the fluorescence measurements, performed at 3.5 and 9.5 pHs, are 2140 M^{-1} and 9790 M^{-1} for the fully protonated/β-CD and dianion/β-CD complexes respectively. A 1:1 stoichiometry was found for all complexes. The highest binding constant value determined for the dianion/β-CD indicates a stronger interaction between such toxin form and β-CD. This result can be explained in

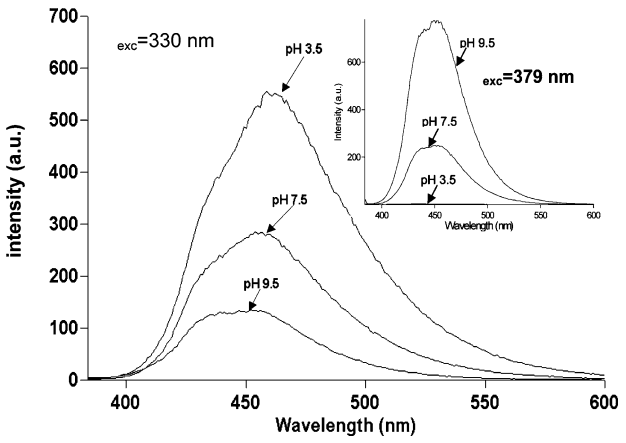

Fig. 3 Fluorescence spectra of OTA 3.84×10^{-6} M aqueous solutions at different pHs obtained exciting at 330 nm; in the inset fluorescence spectra obtained exciting at 379 nm

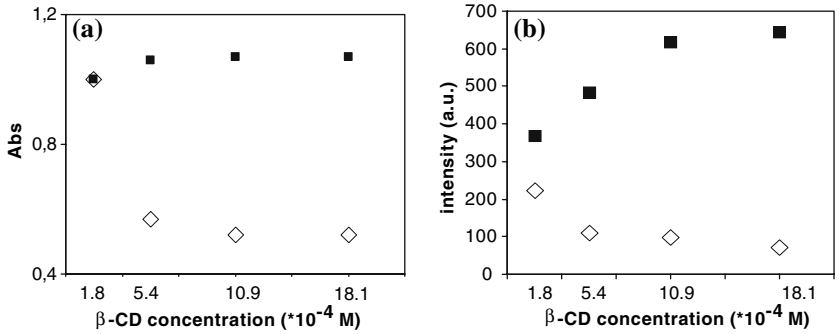

Fig. 4 Effect of β-CD concentration on OTA absorption and fluorescence spectra in pure water solutions (OTA $3.84 \cdot 10^{-6}$ M): (**a**) absorption at 379 nm (◇) and 330 nm (■); (**b**) fluorescence intensities recorded at 450 nm (λ_{exc} = 379 nm, ◇; λ_{exc} = 330 nm, ■). The absorption and fluorescence data are normalized to the corresponding values in pure water

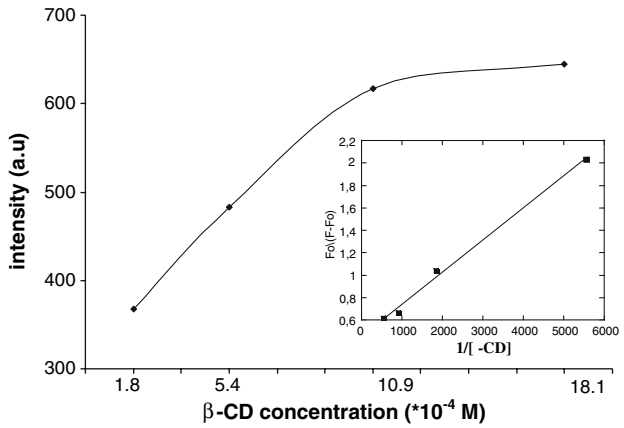

Fig. 5 Effect of β-CD concentration on fluorescence intensities of OTA 3.84×10^{-6} M pure water solutions (λ_{exc} = 330 nm, λ_{em} = 460 nm); Inset: Benesi-Hildebrand plot of $[F_0/(F-F_0)]$ vs $1/[\beta\text{-CD}]$

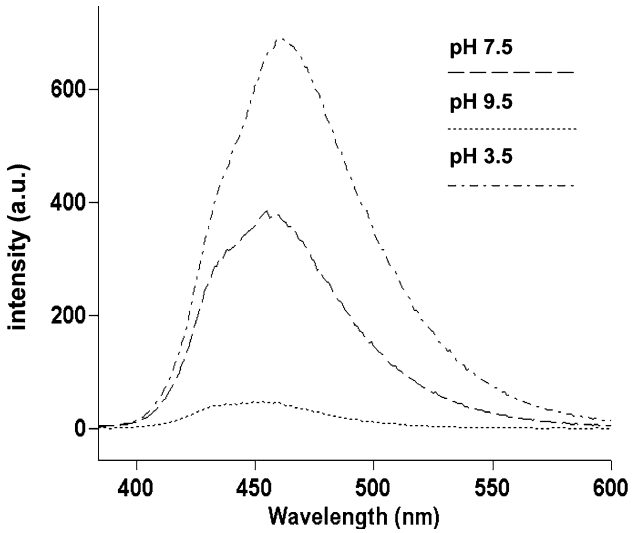

Fig. 6 Fluorescence spectra of OTA/β-CD aqueous solutions at different pHs (λ_{exc} = 330 nm), [OTA] = 3.84×10^{-6} M, [β-CD] = 10^{-4} M

terms of hydrogen bond formation between the hydroxyl groups of cyclodextrin and negative charges present on OTA moieties in particular on phenolic group. Strong phenoxide anion interaction with the protonated side chain on an aminoacid has been reported to be responsible for the high binding affinity of the dianion form of OTA to human serum albumin [21, 22]. Hydrogen bond formation, confering a partial monoanionic character to OTA, justifies also the observed shift of the monoanion/dianion equilibrium toward the monoanionic form recorded for toxin/β-CD in pure water (Fig. 4). The absence of negative charge on the phenolic moiety of the monoanionic form accounts for the lower binding constant calculated for OTA/β-CD solution at pH < 7. The higher binding constant determined for the fully protonated toxin form (pH 3.5) respect to the monoanion, can be explained with a more favorable inclusion of the neutral form in the non polar cavity of β-CD.

Conclusion

The overall results evidence the existence of interaction between β-CD and OTA at all tested pHs. A 1:1 stoichiometry for all complexes has been determined. The calculated binding constants highlight the strong interaction between the dianion toxin form and β-CD. Moreover the higher fluorescence enhancement recorded for this complex can be used to increase the sensitivity of fluorescence-based techniques for OTA detection.

References

1. WHO Food Additives Series 47, FAO Food and Nutrition Paper 74. Ochratoxin A. In: Safety Evaluation of Certain Mycotoxins in Food. pp. 281–415. WHO, Geneva (2001)

2. Monographs on the evaluation of carcinogenic risks to humans, some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, vol. 56, pp. 489–521. International Agency for Research on Cancer, Lyon (1993)
3. Seidel, V., Poglits, E., Schiller, K., Lindner, W.: Simultaneous determination of ochratoxin A and zearalenone in maize by reversed-phase high-performance liquid chromatography with fluorescence detection and β-cyclodextrin as mobile phase additive. J. Chromatogr. A **635**, 227–235 (1993)
4. Francis, O.J., Kircheneuter, G.P. Jr., Ware, G.M., Carman, A.S., Kuan, S.S.: Beta-cyclodextrin post-column fluorescence enhancement of aflatoxins for reverse-phase liquid chromatographic determination in corn. J. Assoc. Off. Anal. Chem. **71**, 725–728 (1988)
5. Capeda, A., Franco, C.M., Fente, C.A., Vazquez, B.I., Rodriguez, J.L., Prognon, P., Mahuzier, G.: Postcolumn excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis. J. Chromatogr. A **721**, 69–74 (1996)
6. Franco, C.M., Fente, C.A., Vazquez, B.I., Capeda, A., Mahuzier, G., Prognon, P.: Interaction between cyclodextrins and aflatoxins Q1, M1 and P1Fluorescence and chromatographic studies. J. Chromatogr A **815**, 21–29 (1998)
7. Baglole, K.N., Boland, P.G., Wagner, B.D.: Fluorescence enhancement of curcumin upon inclusion into parent and modified cyclodextrins. J. Photochem. Photobiol. A **173**, 230–237 (2005)
8. Wagner, B.D., MacDonald, P.J.: The fluorescence enhancement of 1-anilinonaphthalene-8-sulfonate (ANS) by modified β-cyclodextrins. J. Photochem. Photobiol. A **114**, 151–157 (1998)
9. Cosma, P., Catucci, L., Fini, P., Dentuto, P.L., Agostiano, A., Angelini, N., Monsù Scolaro, L.: Tetrakis(4-pyridyl)porphyrin supramolecular complexes with cyclodextrins in aqueous solution. Photochem. Photobiol. **82**, 563–569 (2006)
10. Fini, P., Longobardi, F., Catucci, L., Cosma, P., Agostiano, A.: Spectroscopic and electrochemical study of Rose Bengal in aqueous solutions of cyclodextrins. Bioelectrochemistry **63**, 107–110 (2004)
11. Fini, P., Loseto, R., Catucci, L., Cosma, P., Agostiano, A.: Study on the aggregation and electrochemical properties of Rose Bengal in aqueous solution of cyclodextrins. Bioelectrochemistry **70**, 44–49 (2007)
12. Agostiano, A., Catucci, L., Cosma, P., Fini, P.: Aggregation processes and photophysical properties of chlorophyll a in aqueous solutions modulated by the presence of cyclodextrins. Phys. Chem. Chem. Phys. **5**, 2122–2128 (2003)
13. Il'ichev, Y.V., Perry, J.L., Manderville, R.A., Chignell, C.F., Simon, J.D.: The pH-dependent primary photoreactions of ochratoxin A. J. Phys. Chem. B. **105**, 11369–11376 (2001)
14. Gillman, I.G., Clark, T.N., Manderville, R.A.: Oxidation of ochratoxin A by an Fe-porphyrin system: model for enzymatic activation and DNA cleavage. Chem. Res. Toxicol. **12**, 1066–1076 (1999)
15. Chu, F.S.: Studies on ochratoxins. CRC Crit. Rev. Toxicol. **2**, 499–524 (1974)
16. Uchiyama, S., Saito, Y., Uchiyama, M.: Protein-binding of ochratoxin A and its extractability from proteinous food. J. Food Hyg. Soc. Jpn. **26**, 651–657 (1985)
17. Il'ichev, Y.V., Perry, J.L., Simon, J.D.:Interaction of ochratoxin A with human serum albumin. Preferential binding of the dianion and pH effects. J. Phys. Chem. B **106**, 452–459 (2002)
18. Indirapriyadharshini, V.K., Karunanity, P., Ramamurthy, P.: Inclusion of resorcinol-based acridinedione dyes in cyclodextrins: Fluorescence enhancement. Langmuir **17**, 4056–4060 (2001)
19. Nowakowska, M., Smoluch, M., Sendor, D.: The effect of cyclodextrins on the photochemical stability of 7-amino-4-methylcoumarin in aqueous solution. J. Inclus. Phen. Macrocyc. Chem. **40**, 213–219 (2001)
20. Pitchumani, K., Velusamy, P., Srinivasan, C.: Modification of chemical reactivity upon cyclodextrin encapsulation: asymmetric bromination of chalcone and benzylideneacetone. Tetrahedron **50**, 7903–7912 (1994)
21. Perry, J.L., Christensen, T., Goldsmith, M.R., Toone, E.J., Beratan, D.N., Simon, J.D.: Binding of ochratoxin A to human serum albumin stabilized by a protein-ligand ion pair. J. Phys. Chem. B **107**, 7884–7888 (2003)
22. Perry, J.L., Il'ichev, Y.V., Kempf, V.R., McClendon, J., Park, G., Manderville, R.A., Ruker, F., Dockal, M., Simon, J.D.: Binding of ochratoxin A derivatives to human serum albumin. J. Phys. Chem. B **107**, 6644–6647 (2003)