

## **CHAPTER 8**

### **A Kinetic study into the Hydrolysis of the Ochratoxins and Analogues by Carboxypeptidase A**

This chapter comprises a collaboration between chemists and biochemists of the School for Chemistry and Biochemistry at Potchefstroom University. This chapter was submitted to the *Journal of Applied Toxicology* for publication and the results will be presented at the X International IUPAC Symposium on Mycotoxins and Phycotoxins in Brazil, 2000.

#### *Contribution made by the candidate*

The candidate was assisted by dr. Francois van der Westhuizen in the enzyme reactions and the analysis of samples. Mr. Barry Payne was responsible for the synthesis of some of the standards. The candidate was, however, responsible for the interpretation of results and the writing the publication.

## A Kinetic study into the Hydrolysis of the Ochratoxins and Analogues by Carboxypeptidase A

### ABSTRACT

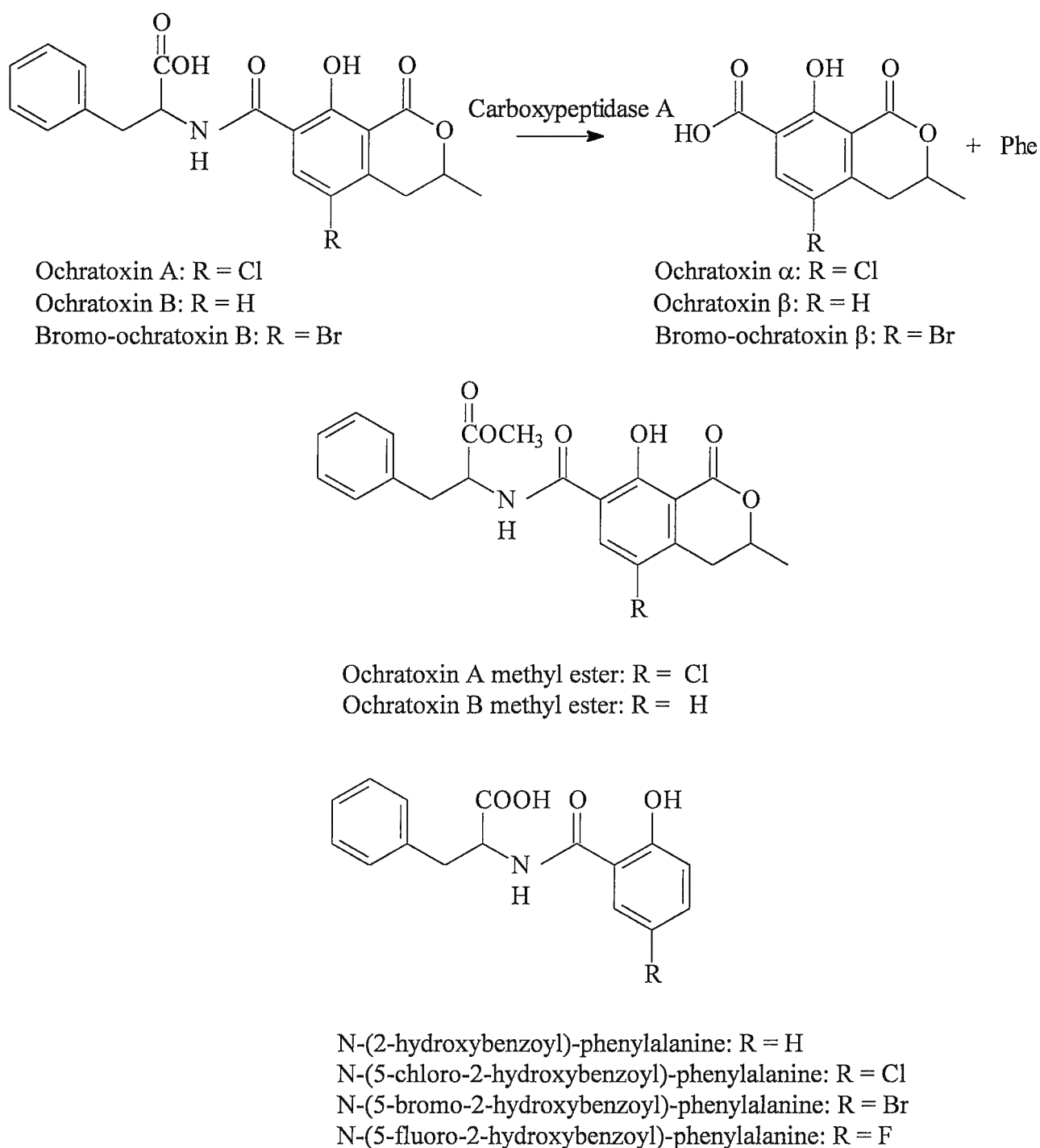
The hydrolysis of the ochratoxins and analogues by carboxypeptidase A was measured. This was done by measuring the phenylalanine formed with liquid chromatography coupled to tandem electrospray mass spectrometry. The kinetic data of ochratoxin A, ochratoxin B, the synthetic bromo-ochratoxin B were compared to the values of a number of synthesised structure analogues namely ochratoxin A methyl ester, ochratoxin B methyl ester, N-(2-hydroxybenzoyl)-phenylalanine, N-(5-chloro-2-hydroxybenzoyl)-phenylalanine, N-(5-bromo-2-hydroxybenzoyl)-phenylalanine and N-(5-fluoro-2-hydroxybenzoyl)-phenylalanine. The halogen containing analogues had lower turnovers than their des-halo analogues. There are no substantial differences in the kinetic data between the different halogen containing analogues.

**Key words:** Ochratoxin A, carboxypeptidase A, hydrolysis, bromo-ochratoxin B, ochratoxin B

### INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum* (van der Merwe *et al.*, 1965). OTA causes Danish porcine nephropathy (Krogh *et al.*, 1988), has an implicated role in the etiology of Balkan endemic nephropathy (Creppy *et al.*, 1993) and is a common contaminant in various food and feedstuffs including coffee (Pittet *et al.*, 1996), wine (Majerus and Ottender 1996), and beer (Speijers and van Egmond, 1993). The mechanism of action of OTA is not yet fully understood but OTA is known to induce lipid peroxidase (Omar *et al.*, 1991), to inhibit complex 3 of the electron transport chain (Moore and Truelove, 1970) and to inhibit reactions catalysed by phenylalanine-tRNA synthetase and phenylalanine-hydroxylase (Creppy *et al.*, 1984, 1990). OTA is cleaved by carboxypeptidase A to the non-toxic ochratoxin  $\alpha$  (OT $\alpha$ ) and L- $\beta$ -phenylalanine (Phe), whereas its less toxic des-chloro analogue, ochratoxin B (OTB) (Xiao *et al.*, 1995), is cleaved much quicker by this enzyme (Pitout, 1969; Doster and

Sinnhuber, 1972). The aim of the study was to investigate the role of the halogen on the dihydro isocoumarin ring of OTA in the ability of carboxypeptidase to cleave the molecule and thereby detoxify it. This was achieved by comparing the kinetics of the amide bond hydrolysis of OTA by carboxypeptidase with a number of OTA derivatives and structural analogues (**Figure 1**).



**Figure 1:** The structures of the ochratoxins and analogues

## MATERIALS

### Chemicals

OTA and ochratoxin B were extracted from *Aspergillus ochraceus* cultivated Durum wheat according to the method of Stander *et al.*, 2000. Bromo-ochratoxin B was synthesised by reaction of ochratoxin B with pyridinium perbromide hydrobromide (Steyn and Payne, 1999). The methyl esters of OTA and OTB were synthesised (methanol/hydrochloric acid) using the procedure of van der Merwe *et al.* (1965). Carboxypeptidase A (from bovine pancreas) was purchased from Boehringer Mannheim. Other reagents were purchased from Merck or Sigma. All solvents and reagents were of analytical grade.

The ochratoxins are carcinogenic compounds and should be handled with care.

### *N*-(2-hydroxy-benzoyl)-phenylalanine

Salicylic acid (1 g, 7.25 mmol) in redistilled thionyl chloride (10 ml), was heated under reflux for 2 hours. The solution was allowed to cool, and the thionyl chloride evaporated under a stream of dry nitrogen. The acid chloride in ethyl acetate (10 ml), was treated with a cold solution of NaN<sub>3</sub> (1.4 g, 23.1 mmol) in water, 10 ml, and stirred for 10 minutes on ice. The aqueous phase was extracted with ethyl acetate. The combined ethyl acetate extracts were dried (MgSO<sub>4</sub>), filtered and evaporated under reduced pressure, which yielded salicyl azide, 0.862 g (5.29 mmol, 73 %).

The salicyl azide (0.862 g) in ethyl acetate (10 ml), was treated with a solution of Phe (1.5 g, 9.09 mmol) and triethyl amine (0.918 g, 9.09 mmol) in water (10 ml). The reaction was monitored by TLC (benzene/acetic acid, 5:1), and stirred for two days at ambient temperature, whereafter the organic layer was separated and 0.5 M Na<sub>2</sub>CO<sub>3</sub> (10 ml) was added to the aqueous phase, which was extracted with ethyl acetate.

The alkaline aqueous phase was acidified (1 M HCl to pH 2) and extracted with chloroform. The combined organic layer was dried (MgSO<sub>4</sub>), filtered and evaporated under reduced pressure. The resulting oil was purified by column chromatography, (silica gel, 100 g, using benzene: acetic acid, 5:1) to yield *N*-(2-hydroxybenzoyl)-phenylalanine (0.929 g, 45 %).

$\lambda_{\max}$ (MeOH) 207 ( $\epsilon$  35 600), 237 (sh) ( $\epsilon$  10 050), 300 ( $\epsilon$  4500) nm; IR: N-H (3428, 1534 cm<sup>-1</sup>), CO (1722, 1645 cm<sup>-1</sup>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)\*  $\delta$  3.28 (oc, 2H, J<sub>H-H</sub> 14.01, 5.63, 5.54

\* The following abbreviations were used in the NMR results: s = singlet; d = doublet; m = multiplet, q = quartet and oc = octet, br = broad band.

Hz), 5.06 (m, 1H), 6.72 (d, 1H,  $J_{\text{H-H}}$  7.39 Hz), 6.80 (m, 1H), 7.18 (q, 2H,  $J_{\text{H-H}}$  2.09, 7.56 Hz), 7.29 (m, 4H), 7.38 (m, 1H), 11.88 (br, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  175.69, 169.77, 161.62, 135.32, 134.77, 129.41, 128.93, 127.60, 125.71, 119.02, 118.70, 113.83, 52.95, 37.32.

### ***N*-(5-chloro-2-hydroxybenzoyl)-phenylalanine**

5-Chloro salicylic acid (1.1 g, 6.5 mmol) was coupled to Phe (1.3 g, 7.9 mmol) in a similar way as the salicylic acid reaction above. However, the crude product (1.4 g) was not cleaned by column chromatography, but was recrystallised from chloroform to yield *N*-(5-chloro-2-hydroxybenzoyl)-phenylalanine [983 mg, 4.1 mmol, 47 %, mp 172.6 °C, mp. Lit. 172-173°C (Gillman *et al.*, 1998)].

$\lambda_{\text{max}}$  (MeOH) 208 ( $\epsilon$  38 020), 312 ( $\epsilon$  3 940) nm; IR: NH (3368, 1534  $\text{cm}^{-1}$ ), CO (1722, 1632  $\text{cm}^{-1}$ ); ES-MS:  $m/z$  320/322 ( $\text{C}_{16}\text{H}_{14}\text{NO}_4\text{Cl}$  requires  $(\text{M}+1)^+$  320);  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  3.29 (oc, 2H,  $J_{\text{H-H}}$  12.73, 5.73, 5.63 Hz), 5.06 (m, 1H), 6.57 (d, 1H,  $J_{\text{H-H}}$  7.31 Hz), 6.92 (d, 1H,  $J_{\text{H-H}}$  8.9 Hz), 7.18 (m, 3H), 7.30 (m, 4H), 11.78 (s, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  174.81, 168.71, 160.32, 135.07, 134.73, 129.38, 129.05, 127.80, 125.22, 123.71, 120.32, 114.70, 52.94, 37.34.

### ***N*-(5-bromo-2-hydroxybenzoyl)-phenylalanine**

The coupling of 5-bromo salicylic acid (1 g, 4.6 mmol) and Phe (1 g, 6.1 mmol) was accomplished in a similar way as salicylic acid (See above). The only difference is that the reaction between the 5-bromo salicyl azide and the Phe (catalysed with with triethyl amine (1.22 g, 12.1 mmol)) was completed after two hours as evidenced by TLC (acetic acid/benzene, 1:5). The crude coupled product (900 mg, 2.47 mmol) was recrystallised from chloroform to obtain a yield of 43 % (735 mg, 2 mmol, m.p. 178 °C).

IR: N-H (3360, 1526  $\text{cm}^{-1}$ ), CO (1747, 1636  $\text{cm}^{-1}$ );  $^1\text{H}$  NMR (300 MHz,  $\text{CO}(\text{CD}_3)_2$ )  $\delta$  3.18 (oc, 2H,  $J_{\text{H-H}}$  14.05, 4.97, 9.42 Hz), 4.97 (m, 1H), 6.86 (d, 1H,  $J_{\text{H-H}}$  8.87 Hz), 7.28 (m, 5H), 7.52 (q, 1H,  $J_{\text{H-H}}$  2.46, 8.87), 7.97 (d, 1H,  $J_{\text{H-H}}$  2.46), 8.42 (m, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CO}(\text{CD}_3)_2$ )  $\delta$  172.24, 169.13, 160.86, 137.93, 137.23, 130.20, 129.71, 128.92, 127.24, 120.46, 116.88, 110.36, 54.28, 37.25.

***N*-(5-fluoro-2-hydroxybenzoyl)-phenylalanine**

5-Fluoro salicylic acid (500 mg, 3.185 mmol) was coupled to Phe (661 mg, 4.01 mmol) in a similar way as the salicylic acid reaction mentioned above. The only difference is that the *N*-(5-fluoro-2-hydroxybenzoyl)-phenylalanine (25.7 % yield, 248 mg, 0.818 mmol) was not cleaned with column chromatography, but recrystallised from chloroform (m.p. 162.9 °C).  $\lambda_{\text{max}}$ (MeOH) 204.1 ( $\epsilon$  17 000), 309 ( $\epsilon$  2 300) nm; IR: N-H (3368, 1534  $\text{cm}^{-1}$ ), CO (1747, 1619  $\text{cm}^{-1}$ );  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  3.29 (oc, 2H,  $J_{\text{H-H}}$  14.20, 5.63, 5.54 Hz), 5.06 (m, 1H), 6.55 (d, 1H,  $J_{\text{H-H}}$  7.34 Hz), 6.92 (m, 2H), 7.14 (m, 2H), 7.30 (m, 3H), 11.58 (s, 1H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  175.59 (s), 168.84 (d,  $J_{\text{C-F}}$  2.6 Hz), 157.83 (d,  $J_{\text{C-F}}$  1.6 Hz), 155.16 (d,  $J_{\text{C-F}}$  239.4 Hz), 135.07 (s), 129.36 (s), 129.05 (s), 127.78 (s), 122.16 (d,  $J_{\text{C-F}}$  23.2 Hz), 120.01 (d,  $J_{\text{C-F}}$  7.4 Hz), 113.60 (d,  $J_{\text{C-F}}$  26.2 Hz), 111.27 (d,  $J_{\text{C-F}}$  23.8 Hz), 52.97 (s), 37.29 (s).

**METHOD**

Kinetic data of peptide-bond hydrolysis of the ochratoxins were obtained by measuring the formed Phe. Reactions were performed using an endpoint assay and reaction mixtures contained 10 mM Tris.HCl (pH 8.0), 50 mM NaCl, 1  $\mu\text{g}$  carboxypeptidase A, and the ochratoxin analogue (ranging between 1 and 1000  $\mu\text{M}$ ). The reactions were terminated after 5 minutes using concentrated formic acid (5  $\mu\text{l}$ ), after which the mixture was deproteinated, dried under vacuum and suspended in acetonitrile/water (1:1, containing 1% formic acid). All reactions were compared to blanks, in which the enzyme was omitted, to compensate for trace amounts of Phe in the incubation mixtures. Electrospray ionisation-mass spectrometry-mass spectrometry (ESI-MS-MS) was used to detect the formed Phe and an external standard of Phe (Ring-D5) was used. This was added after termination of the reactions to quantify formed Phe. ESI-MS-MS was carried out using a VG Quattro II triple quadrupole instrument (Micromass, U.K.). The electrospray capillary was set at 3.5 kV and the cone voltage at 30 V. The source temperature was 60°C. Nitrogen was used as drying and nebulising gas. The flow rates for drying and nebulising were set at 350 L/h and 20 L/h, respectively. Data were acquired in the parent ion scan mode of operation, scanning for parent ions  $m/z$  166 (Phe) and  $m/z$  171 (Phe, ring-D5) in the first mass spectrometer and keeping the second mass spectrometer static, monitoring the collisional-induced dissociation (CID) fragment ions at

$m/z$  120 and 125, respectively. This is achieved with argon as collision gas at a  $1.4 \times 10^{-3}$  mbar pressure in the collision cell using a collision energy setting of 12 eV.

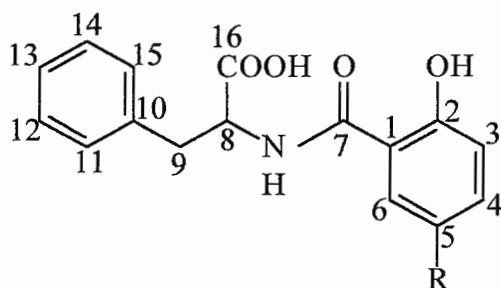
Kinetic parameters were determined using linear regression analysis of double reciprocal primary rate plots (Palmer, 1995). The averages of three primary rate plots (Lineweaver-Burk, Eady-Hofstee and Hanes plots) were calculated.

## RESULTS

An analysis of the  $^{13}\text{C}$  NMR data, presented in **Table 1** unambiguously defined the structures of the synthesised compounds. It is of importance to note the chemical shift of the halogen-bearing carbon atom C5: The fluoro substituent led to a downfield shift of *ca.* 36.1 ppm whereas the bromine substituent led to an upfield shift of *ca.* 8.7 ppm. These findings correspond to the literature values of halogen-bearing aromatic carbon atoms (Williams and Flemming, 1995). The  $^{13}\text{C}$  NMR spectrum of N-(5-fluoro-2-hydroxybenzoyl)-phenylalanine exhibited the typical characteristics of the spectrum of a fluorine bearing aromatic compound: Doublets appears at  $\delta_{\text{C}}$  156.74 and  $\delta_{\text{C}}$  153.57 ( $J_{\text{C-F}}$  239.4 Hz) which represent the fluorine bearing C5; C4 and C6 showed typical  $^2J_{\text{C-F}}$  of 23.5 Hz; C1 and C3 exhibited  $^3J_{\text{C-F}}$  of 7.4 and 6.2 Hz respectively; and C2 and C7  $^4J_{\text{C-F}}$  of 1.6 and 2.6 Hz respectively (Payne, 1998).

Hydrolysis of the ochratoxins and analogues by carboxypeptidase A using ESI-MS-MS are summarised in **Table 2**.

The enzymatic hydrolysis of the toxins could clearly be demonstrated by the formation of Phe in all cases, except in the cases of the methylated ochratoxins. Hydrolysis of the toxins progressed linearly over the period of at least 10 min. As little as 10nM Phe could accurately be detected in the reaction mixtures using this method, which allowed for very accurate measurements of the hydrolysis of the toxins. This sensitivity could not be achieved using either spectrophotometric (Hult and Gatenbeck, 1975) or HPLC-based methods (Steyn and Stander, 1999 and references cited).

**Table 1:** The proton noise decoupled  $^{13}\text{C}$  NMR data of N-(2-hydroxybenzoyl)-phenylalanine and its halogen analogues

Carbon	R = H $\delta_{\text{C}}/\text{ppm}^{\text{a}}$	R = Cl $\delta_{\text{C}}/\text{ppm}^{\text{a}}$	R = Br $\delta_{\text{C}}/\text{ppm}^{\text{b}}$	R = F $\delta_{\text{C}}/\text{ppm}^{\text{a}}$
1	118.70	120.32	120.46	120.01
2	161.62	160.32	160.58	157.83
3	113.83	114.70	116.88	113.60
4	134.77	134.73	137.23	122.16
5	119.02	123.71	110.36	155.16
6	125.71	125.22	130.20	111.27
7	169.77	168.71	169.13	168.84
8	52.95	52.94	54.28	52.97
9	37.32	37.34	37.25	37.29
10	135.32	135.07	137.93	135.07
11	129.41	129.38	128.71	129.36
12	128.93	129.05	128.93	129.05
13	127.60	127.80	127.24	127.78
14	128.93	129.05	128.92	129.05
15	129.41	129.38	128.71	129.36
16	175.69	168.71	172.24	

(a: In  $\text{CDCl}_3$ ); (b: In  $\text{CO}(\text{CD}_3)_2$ )**Table 2.** Hydrolysis of ochratoxins and analogues by carboxypeptidase A.

Toxin	$K_{\text{m}}$ ( $\mu\text{M}$ )	$K_{\text{cat}}$ ( $\text{min}^{-1}$ )	$K_{\text{cat}}/K_{\text{m}}$ ( $\text{min}^{-1} \cdot \mu\text{M}^{-1}$ )
Ochratoxin A	5.6	266	47
Ochratoxin B	36.8	2717	74
Bromo-ochratoxin B	6.6	283	43
N-(2-hydroxybenzoyl)-phenylalanine	0.8	2372	3163
N-(5-fluoro-2-hydroxybenzoyl)-phenylalanine	16.0	4276	267
N-(5-chloro-2-hydroxybenzoyl)-phenylalanine	5.5	2869	521
N-(5-bromo-2-hydroxybenzoyl)-phenylalanine	12.9	3724	289
Ochratoxin A methyl ester	Nd	-	-
Ochratoxin B methyl ester	Nd	-	-

Nd = not detected



## DISCUSSION

The relative affinity and turnover number of carboxypeptidase A for the various toxins have been determined. No hydrolysis of methylated ochratoxins was observed, which can be attributed to the position of the methyl-group. The ester moiety will clearly obstruct the ionic interaction of the substrate carboxyl group with Arg-145 on the active site of carboxypeptidase A (Pétra, 1970). OTA and bromo-ochratoxin B exhibited similar affinities and turnover numbers, which indicated that the presence of chlorine or bromine atom in the structure of the toxin had very little influence on either substrate binding or catalytic function. As was reported previously (Doster and Sinnhuber, 1972), compared to OTA which has a dissociation constant ( $K_m$ ) of 5.6 mM, carboxypeptidase A has a lower relative affinity towards ochratoxin B ( $K_m = 36.8$  mM), although the turnover of the latter is ten times higher than that of OTA. The values listed in **Table 1** compare well with those of known substrates for carboxypeptidase A, such as CBZ-glycyl-L-tryptophan ( $K_m = 6.1$  mM,  $K_{cat} = 5430 \text{ min}^{-1}$ ), although the  $K_{cat}$ -values of the ochratoxins described here are substantially lower than reported for better known substrates of the enzyme (Pétra, 1970).

In the instance of the different salicylic acids coupled to Phe (see **Table 2**), the presence of bromine and fluorine in N-(5-bromo-2-hydroxybenzoyl)-phenylalanine and N-(5-fluoro-2-hydroxybenzoyl)-phenylalanine had a more marked effect, than the chlorinated analogue which exhibited a lower  $K_m$ -value and as well as a lower turnover. It is evident from the data (see Table 1), that the catalytic efficiency ( $K_{cat}/K_m$ ) of the different salicylic acid-Phe coupled compounds are much higher than those of the different ochratoxins. As in the case with the ochratoxins the hydrolysis of halogen containing toxins was much lower compared to the analogue without the halogen group, which displayed a ten times higher catalytic efficiency. Furthermore, as in this case with the ochratoxins, there is also no substantial difference in the kinetic data between the different halogen containing analogues. This clearly indicates an effect of the presence of the halogen on the catalytic function of the enzyme, although there is no clear specificity towards the nature of the halogen present in the toxin. However, Creppy (1999) observed in preliminary tests in kidney cells that Br-OTB is more toxic than OTA; it is evident that several factors influence the toxicity of these toxins.

## ACKNOWLEDGEMENTS

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