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THE HYDROLYSIS OF OCHRATOXIN A BY SOME PROTEOLYTIC ENZYMES

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Abstract—Ochratoxin A, a metabolite produced by a strain of *Aspergillus ochraceus* and causing enteritis, renal necrosis, an increase of glycogen in the liver when given to rats, as well as inhibiting the phosphorylase enzyme *in vitro*, is found to be hydrolyzed *in vivo* to an iso-coumarin derivative (ochratoxin α). The object of this paper is to study the hydrolysis of ochratoxin A by enzymes trypsin, α-chymotrypsin and carboxypeptidase A. By means of thin layer chromatography on silica gel and spectrophotometric methods such as difference spectra and absorption spectra, it is found that ochratoxin A is hydrolyzed by carboxypeptidase and chymotrypsin. The apparent K_m values for the hydrolysis of ochratoxin A by carboxypeptidase and chymotrypsin at 25° are $1{\cdot}5 \times 10^{-4}$ M and 1×10^{-3} M, respectively. The first order reaction constants are $1{\cdot}1 \times 10^{-2}$ and $7{\cdot}6 \times 10^{-3}$ min^{-1}, while the apparent proteolytic coefficients are 4·4 and 0·01, respectively. These results indicate that ochratoxin A has a much greater affinity for carboxypeptidase A than for α-chymotrypsin. It is suggested that ochratoxin A is hydrolyzed *in vitro* by carboxypeptidase A and α-chymotrypsin and possibly cathepsin C from lyosomes.

OCHRATOXIN A, the major toxic metabolite produced by a strain of *Aspergillus ochraceus*, has been structurally characterised as 7-carboxy-5-chloro-8 hydroxy-3, 4-dihydro-3R-methylisocoumarin linked over its 7-carboxy group to L-β-phenyline.[1]

The LD_{50} in rats dosed per os is 20 mg/kg and the toxin produces enteritis, renal necrosis, an increase in the quantity of glycogen in the liver[2] and a disturbance of the glycogen metabolism by inhibiting the phosphorylase enzyme system.[3] The route and time course of the metabolism of ochratoxin A was investigated by Nel and Purchase[4] in view of the fact that the increase in glycogen only became evident 4 to 5 days after dosing. These authors concluded that ochratoxin A is metabolised to 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin (ochratoxin α).

In the light of these results it was decided to investigate the possible hydrolysis of ochratoxin A by proteolytic enzymes. Carboxypeptidase A (E.C. 3.4.2.1.) requires a free carboxyl group and the specificity, including stereochemical specificity, of the enzyme is determined primarily by the amino acid bearing the free carboxyl group.[5] Since this enzyme is most active on substances containing a terminal aromatic amino acid,[5] it was reasoned that ochratoxin A would be hydrolyzed by carboxypeptidase A. In addition, the activities of two other proteinases, α-chymotrypsin (E.C. 3.4.4.5) and trypsin (E.C. 3.4.4.4.) on this toxin were studied.

EXPERIMENTAL

Ochratoxin A was isolated and purified from fungal cultures as described by van der Merwe *et al.*[1]. Because ochratoxin A is unstable in daylight in alkaline solutions (pH 7·5), all ochratoxin A solutions were prepared in subdued light and kept in dark containers. A stock solution of ochratoxin A, 0·02 M, was prepared in a 0·1 M NaCl-0·02 M *Tris* buffer, pH 7·50 ± 0·02, and all dilutions of the stock solution were made with the same buffer. The concentration of diluted solutions was estimated spectrophotometrically by using a value of 7160 M^{-1} cm^{-1} for the molecular extinction coefficient.

Carboxypeptidase A (from bovine pancreas, crystalline) α-chymotrypsin (Type II: from bovine pancrease, 3 × crystallized from 4 × crystallized chymotrypsinogen; dialyzed salt-free and lyophillized) and trypsin (Type I: 1 × crystallized from bovine pancrease) were purchased from Sigma Chemical Company, St. Louis. Appropriate enzyme concentrations were obtained by dilution with the 0·1 M NaCl–0·02 M *TRIS* buffer, pH 7·50 ± 0·02. The nitrogen content of carboxypeptidase A and α-chymotrypsin were obtained from Smith and Stockell (6) and Northrop *et al.*,[7] respectively.

All enzyme and ochratoxin A solutions were adjusted, if necessary, to pH 7·50 ± 0·02 with 0·1 N HCl or 0·1 N NaOH and the pH determined with a Radiometer pH meter 22 fitted with an external meter ranging from pH 6·6 to 8·0.

Silica gel thin layer chromatoplates were prepared from Silica gel D-5 (Camag, Switzerland) to a wet layer thickness of 0·3 mm and activated at 100° for 1 hr. The solvent system contained benzene and acetic acid, analytical grade and obtainable from Merk and Co., in the ratio 80:20 (v/v).

Proteolytic activities of the enzymes were determined by digestion of 2·0 ml ochratoxin A solution with 1 ml of enzyme solution, suitably diluted with the 0·1 M NaCl-0·02 M *Tris* buffer, pH 7·50 ± 0·02, at 25°. The concentration of ochratoxin A varied vetween $0{\cdot}45 \times 10^{-4}$ M to $2{\cdot}75 \times 10^{-4}$ M while the concentrations of carboxypeptidase A and α-chymotrypsin varied from 13 to 55 μg and 300 to 1000 μg protein/ml solution respectively. The decrease of absorption at 380 mμ (see below) was recorded on the time-drive attachment of the Beckman DK-2A spectrophotometer. A speed of 1 in./min was used.

Difference spectra were recorded in the Beckman DK-2A spectrophotometer as follows: the reference cell contained 2 ml of $1{\cdot}50 \times 10^{-4}$ M ochratoxin A solution and 1 ml of the buffer, while the sample cell contained 2 ml of $1{\cdot}50 \times 10^{-4}$ M ochratoxin A solution and 1 ml of enzyme solution (50 μg/ml and 300 μg/ml for carboxypeptidase and chymotrypsin, respectively).

Absorption spectra were recorded with the Beckman DK-2A ratio recording spectrophotometer.

RESULTS

By means of TLC, absorption—and difference spectra, it was shown that ochratoxin A can be hydrolyzed *in vitro* to ochratoxin α by carboxypeptidase A and α-chymotrypsin, while trypsin has no effect. The hydrolysis of the ochratoxin A is illustrated in Figs. 1, 2 and 3.

In the buffer solution at pH 7·50 ochratoxins A and α exhibit peaks in the absorption spectra at 380 mμ and 330 mμ, respectively (Fig. 2). During hydrolysis of ochratoxin A to α a decrease in absorbancy at 380 mμ or an increase at 330 mμ occurs and can be

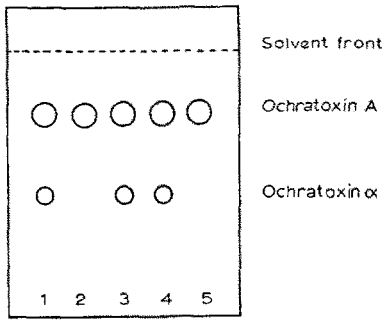

FIG. 1. A chromatogram of ochratoxin A before and after hydrolysis with carboxypeptidase A, α-chymotrypsin and trypsin. A standard mixture of ochratoxins A and α is illustrated by 1, while 2, 3, 4 and 5 represent ochratoxin A before hydrolysis and after reaction with carboxypeptidase, chymotrypsin and trypsin, respectively. Hydrolysis was performed at 25° for 5 min and the concentrations of ochratoxin A, carboxypeptidase, chymotrypsin and trypsin were $1{\cdot}5 \times 10^{-4}$ M, 50 μg/ml, 300 μg/ml and 300 μg/ml, respectively.

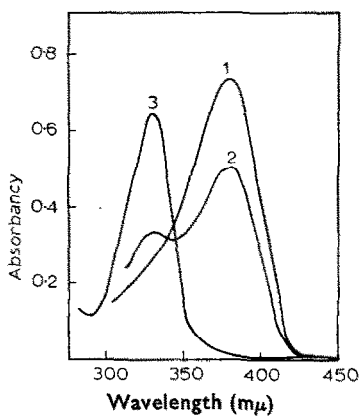

FIG. 2. The hydrolysis of ochratoxin A by proteinases. Curve 1 represents ochratoxin A ($0{\cdot}5 \times 10^{-4}$ M), curve 2 ochratoxin A ($1{\cdot}0 \times 10^{-4}$ M) treated with 50 μg/ml carboxypeptidase A at 25° for 30 min and curve 3 ochratoxin α ($1{\cdot}0 \times 10^{-4}$ M). Ochratoxin α was prepared according to van der Merwe *et al.* (ref. 1).

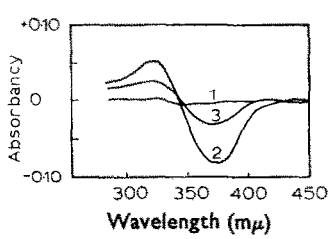

FIG. 3. The difference spectra of hydrolyzed and nonhydrolysed ochratoxin A. Curve 1 represents the baseline and curves 2 and 3 represent ochratoxin A treated with carboxypeptidase and chymotrypsin, respectively. The concentration of ochratoxin A is $1{\cdot}0 \times 10^{-4}$M while the concentration of carboxypeptidase A is 50 μg/ml and that of α-chymotrypsin 300 μg/ml.

used to monitor the proteolytic reaction. From Figs. 2 and 3 it is obvious that the decrease at 380 mμ is more sensitive than the increase at 330 mμ. This is substantiated by time-drive measurements (Fig. 4).

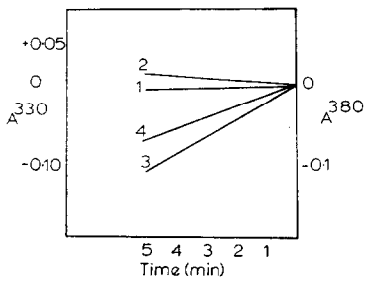

FIG. 4. Spectrophotometrical assay of the rate of hydrolysis of ochratoxin A by carboxypeptidase and chymotrypsin. Curve 1 represents the baseline, while curves 2 and 3 represent the increase at 330 mμ (A330) and decrease at 380 mμ (A380), respectively, at the same substrate ($1{\cdot}5 \times 10^{-4}$ M) and carboxypeptidase concentrations (50 μg/ml). Curve 4 represents the hydrolysis of $1{\cdot}5 \times 10^{-4}$ M ochratoxin A with 300 3μ/ml chymotrypsin.

The pH of all solutions was adjusted to $7{\cdot}50 \pm 0{\cdot}02$. The reason for this is that ochratoxin A in solution is very sensitive to pH changes due to the hydroxyl group on carbon atom no. 8. The pK_a, value of this group is found to be 7·1 and in solutions above pH 7·1 ochratoxin A exhibits on absorption peak at 380 mμ, while below pH 7·1 a peak appears at 330 mμ.

Lineweaver and Burk[8] and Eadie[9] equations were used for the calculation of the apparent Michaelis constants (K_m) from the initial reaction velocities (See Fig. 5). In order to be more accurate, v was taken as the moles/l. of substrate hydrolyzed during the first 5 min of hydrolysis (v_5) and then corrected to v_1 (moles/l. of substrate hydrolyzed during the first minute). The values of K_m for ochratoxin A are compared with that of other substrates for carboxypeptidase A[10] in Table 1. The K_m value of ochratoxin A for α-chymotrypsin is $1{\cdot}0 \times 10^{-3}$ M.

TABLE 1. THE VALUES OF THE MICHAELIS CONSTANT FOR THE HYDROLYSIS OF A FEW SPECIFIC SUBSTRATES AS WELL AS OCHRATOXIN A BY CARBOXYPEPTIDASE A AT 25°

Substrate	K_m, M
Carbobenzoxyglycyl-L-phenylalanine	0·033
Benzoylglycyl-L-phenylalanine	0·011
Benzenesulphonylglycyl-L-phenylalanine	0·014
Formyl-L-phenylalanine	0·036
Acetyl-L-phenylalanine	0·155
Chloroacetyl-L-phenylalanine	0·013
Ochratoxin A (Lineweaver–Burk plots)	0·00015
(Eadie plot)	0·00010

The first order velocity constant, k_1, which measures the rate of formation of the enzyme substrate complex for the action of carboxypeptidase and chymotrypsin on ochratoxin A was obtained by plotting log $(a/(a - x))$ against time as described by

Putman and Neurath.[11] The slope of the straight line is equal to $k/2{\cdot}303$. In this plot a and $(a - x)$ are the concentrations of the substrate at time $t = 0$ and $t = t$, respectively. Values for k_1 are given in Table 2.

TABLE 2. VALUES FOR APPARENT PROTEOLYTIC COEFFICIENT AND FIRST-ORDER REACTION CONSTANT FOR THE ACTION OF CARBOXYPEPTIDASE A ON OCHRATOXIN A AND OTHER SUBSTRATES AND α-CHYMOTRYPSIN ON OCHRATOXIN A

Enzyme	Substrate	App. proteol coeff. (C)	k_1 min^{-1}
Carboxypeptidase A	Ochratoxin A	4·4	0·11
	Carboxybenzoxyglycyl-L-phenylalanine*	12–14	
	Carbobenzoxylaanyl-L-phenylalanine*	11·0	
	Carbobenzoxyphenyl-alanine*	0·0017	
α-Chymotrypsin	Ochratoxin A	0·03	0·0076

* Values obtained from Smith.[5]

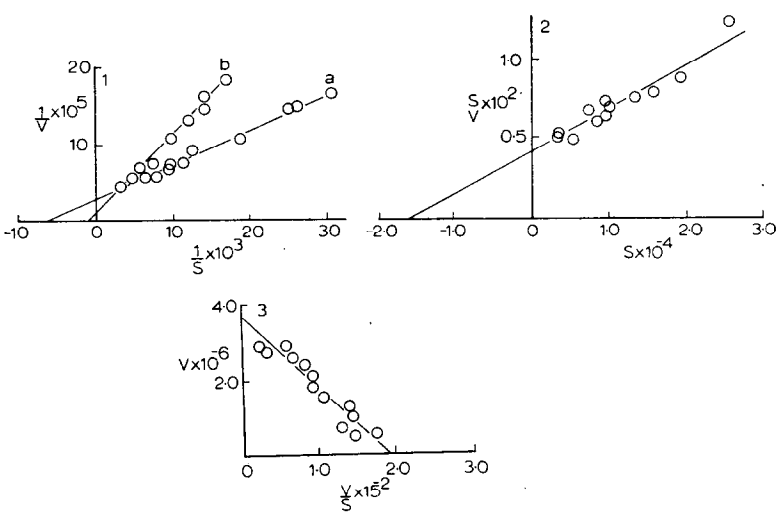

FIG. 5. Lineweaver–Burk and Eadie plots for the determination of the Michaelis constant (K_m). Curves 1 and 2 represent the Lineweaver–Burk plots while curve 3 represents the Eadie plot. The initial velocity and substrate concentrations are represented by v and S, respectively. In curve 1 the action of caroboxypeptidase and chymotrypsin are illustrated by a and b, respectively.

Certain empirical rules are of interest in interpreting the action of proteolytic enzymes.[12] The rate of action of a proteolytic enzyme is related to the structure of the substrate by the following equation:

$$C = k_1/e$$

where C is the apparent first order proteolytic coefficient, k_1 is the first order velocity constant and e the enzyme concentration in mg protein N per ml solution. A comparison of the substrates is given in Table 2.

DISCUSSION

Trypsin does not hydrolyze ochratoxin A since the specificity of this enzyme is dependent on the hydrolysis of peptide bonds of which an L-arginine or L-lysine residue contributes the carbonyl group. Replacement of either of these amino acids prevents enzymic action.[13] Although the specifiicity of pepsin favours the hydrolysis of peptide linkages in which an aromatic amino acid provides the amino group for the sensitive peptide linkage, this enzyme was not considered, since the pH optimum of its action is near pH 2. At pH values lower than 4 ochratoxin A is very poorly soluble.

Chymotrypsin acts at linkages in which the carbonyl group of tyrosine, phenylalanine, tryptophan, or, to a lesser extent, methionine is involved. In addition, it does not require the presence of free α-amino or α-carboxyl groups in its substrates.[14] Since in ochratoxin A the amino group of the phenylalanine residue is involved in the peptide bond, the hydrolysis of ochratoxin A by α-chymotrypsin is unexpected. It must be emphasised, however, that the proteolytic coefficient of chymotrypsin for ochratoxin A is low in comparison with carboxypeptidase A (See Table 2). It is possible that the α-chymotrypsin preparation was contaminated with carboxypeptidase A. The action of α-chymotrypsin (from different sources) on ochratoxin A is under further investigation.

The affinity of ochratoxin A for carboxypeptidase A is greater than that of carbobenzoxyglycyl-L-phenylalanine (a substrate used for assay purposes) as is evidenced by lower values of K_m (see Table 1). In contrast the apparent proteolytic coefficient of carboxypeptidase for ochratoxin A is lower (see Table 2). According to Smith[5] and Neurath and Schwert,[10] however, the proteolytic coefficient is not an accurate basis of comparison of hydrolysis rates. The hydrolysis of ochratoxin A by carboxypeptidase confirms that the configuration of the phenylalanine residue must be in the L-form, since the enzyme is stereochemically specific and is able to select between enantiomorphous compounds.[5, 10] It is known that carboxypeptidase A cannot hydrolyze substrates where the terminal aromatic acid is in the D-configuration.

Using isolated liver perfusion experiments, Purchase[15] observed that ochratoxin A is hydrolyzed in the liver to ochratoxin α which is excreted in the bile. This indicates that ochratoxin A, being a toxic substance, is probably absorbed by a mechanism involving pinocytosis by the lysosomes in the liver and then hydrolyzed by the proteolysis enzymes. Lysosomes contain cathepsins A, B and C where the latter enzyme acts preferentially on peptide linkages in which an L-phenylalanine or L-tyrosine provides the amino group for the peptide bond.[16] Since ochratoxin A contains an L-phenylalanine residue coupled to the isocoumarin derivative by means of its amino group, the hydrolysis of this toxin by cathespin C seems to be possible.

From the results of this work it is suggested that ochratoxin A is hydrolyzed by carboxypeptidase A and α-chymotrypsin. The possible action of cathepsin C, prepared from lysosomes, on ochratoxin A is under further investigation. The reaction of hydrolysis by these proteolytic enzymes would be as follows:

$$\text{ochratoxin A} \xrightarrow{H_2O} \text{ochratoxin } \alpha + \text{L-phenylalanine.}$$

It is interesting to note that the action of carboxypeptidase A on ochratoxin A could be a useful method for assaying the activity of this enzyme. This possibility is also further investigated.

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