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THE INHIBITORY EFFECT OF OCHRATOXIN A ON BOVINE CARBOXYPEPTIDASE A *IN VITRO*

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Abstract—Ochratoxin A, a mycotoxin found on maize, which causes enteritis, renal necrosis, an increase in the glycogen content of rat liver and a disturbance of the glycogen metabolism, is found to be a competitive inhibitor of carboxypeptidase A (EC 3.4.2.1). The results indicate that the inhibitory action resembles that of certain dipeptides. The inhibition constant (K_I) is 14·2 mM and the binding energy of ochratoxin A to the enzyme is estimated to be 2·5 kcal/mole. It is suggested that this toxin has an influence both on carbohydrate and protein metabolism.

OCHRATOXIN A, the major toxic metabolite produced by a strain of *Aspergillus ochraceus*, has been structurally characterized as 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-L-β-phenylalanine.[1] The toxin which has been isolated from maize[2] produces enteritis, renal necrosis, an increase in the quantity of glycogen in rat liver[3] and a disturbance of the glycogen metabolism by inhibiting the phosphorylase enzyme system.[4] The routes and time course of the metabolism of ochratoxin A were investigated by Nel and Purchase[5] and they concluded that ochratoxin A is metabolised to 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin (ochratoxin α). The hydrolysis of ochratoxin A by proteolytic enzymes was investigated by Pitout,[6] who found that the toxin was hydrolysed by carboxypeptidase A (EC 3.4.2.1). The K_m value, first order reaction constant and apparent proteolytic coefficient for the hydrolysis of ochratoxin A by carboxypeptidase A at 25° were $1{\cdot}5 \times 10^{-4}$M, $1{\cdot}1 \times 10^{-2}$ min^{-1} and 4·4, respectively.

The inhibitory effect of certain dipeptides on carboxypeptidase A was investigated by Yanari and Mitz[7] and they concluded that these dipeptides were effective competitive inhibitors of carboxypeptidase, although their apparent K_m values were substantially lower than that of carbobenzoxyglycyl-L-phenylalanine (0·03M). Dipeptides are generally poor substrates of pancreatic carboxypeptidase.[7] Since the structure of ochratoxin A resembles that of a dipeptide, this toxin might exert an inhibitory effect on the activity of carboxypeptidase A. In addition, ochratoxin α might also exert an inhibitory effect because the structure of this compound is related to benzoic acid, which inhibits the action of carboxypeptidase A.[8]

In the light of these observations it was decided to investigate the inhibitory effect of ochratoxins A and α on carboxypeptidase A activity.

MATERIALS AND METHODS

Ochratoxins A and α were prepared according to van der Merwe *et al.*[1] Both toxin solutions, which were unstable in daylight in alkaline solutions (pH 7·5),

were prepared in subdued light and kept in dark containers. Stock solutions of both toxins, 0·02M, were prepared in 0·1M NaCl–0·02M Tris buffer, pH 7·50 ± 0·02, and all dilutions of the stock solutions were made with the same buffer.

Due to the high sensitivity of ochratoxin A to pH changes, all substrate, enzyme and toxin solutions were adjusted, if necessary, to pH 7·50 ± 0·02 with 0·1N HCl or 0·1N NaOH with a Radiometer pH meter 22, fitted with an external pH scale ranging from 6·6 to 8·0.

Carboxypeptidase A (5 × crystallised, lot 800521) was purchased from Sigma Chemical Company, St. Louis. Appropriate enzyme concentrations were obtained by dilution with the 0·1M NaCl–0·02M Tris buffer, pH 7·50 ± 0·02. *N*-carbobenzoxy-glycyl-L-phenylalanine (*N*-CBZ-glyc-phe) was used as the substrate and purchased from Sigma Chemical Company. A stock solution, 0·02M, was prepared in the same buffer and all dilutions were made with this buffer. L-Phenylalanine was obtained from Eastman Organic Chemicals, New York, and a standard curve of L-phenylalanine concentration, varying from 0 to 50 μg/ml, against absorbancy readings at 570 mμ was drawn. Ninhydrin (1,2,3-Triketohydrindene) was obtained from Eastman Organic Chemicals, New York, and the reagent was prepared according to Moore and Stein.[9] All other materials used were of analytical grade.

Proteolytic activity of carboxypeptidase A in the presence and absence of ochratoxins A and α was determined according to Neurath[10] but was slightly modified. The assay system contained 1·0 ml of substrate (varying from 0·02M to 0·002M), 0·5 ml 0·1M NaCl–0·02M Tris buffer, pH 7·5 and 0·5 ml enzyme solution (5μg protein/ml). By using a very low enzyme concentration it was possible to study the splitting of *N*-CBZ-glyc-phe in the presence of ochratoxin A without significant hydrolysis of the latter. Aliquots of 0·5 ml were removed at 5 min intervals, 1·0 ml of the ninhydrin reagent was added and the solutions were boiled in a vigorously boiling waterbath for 15 min. After cooling, the absorbancy of the solutions was read at 570 mμ in a Beckman DB spectrophotometer, using 1 mm cells. For the study of enzyme kinetics, the aliquots were removed after 30 min and subjected to the colorimetric ninhydrin analysis.

Hydrolysis of ochratoxin A in the presence of *N*-CBZ-glyc-phe was investigated by means of time-drive measurements in a Beckman DK-2A spectrophotometer. The reaction mixture contained 1·5 ml ochratoxin A ($2·5 \times 10^{-4}$M), 0·5 ml *N*-CBZ-glyc-phe ($2·5 \times 10^{-4}$M) and 0·5 ml enzyme (50 μg enzyme/ml). The control contained 0·5 ml buffer instead of 0·5 ml *N*-CBZ-glyc-phe solution. A speed of 1 in./min was used.

The equation of Lineweaver and Burk[11] was used for the calculation of the Michaelis constant (K_m) from the initial reaction velocities, where v was taken as the moles/l. of substrate hydrolysed during the first 30 min of hydrolysis. The inhibition constants (K_i) were also obtained from the Lineweaver–Burk plots according to Elkins-Kaufman and Neurath.[12]

RESULTS

The inhibitory effect of ochratoxin A on the hydrolysis of *N*-CBZ-glyc-phe is illustrated in Fig. 1. This effect is about 70 per cent after 30 min. No inhibitory effect of ochratoxin α on the enzyme was observed. Consequently, the type of inhibition by ochratoxin A was investigated and found to be substrate competitive inhibition according to the Lineweaver–Burk plots.[12] The K_m value of *N*-CBZ-glyc-phe for the

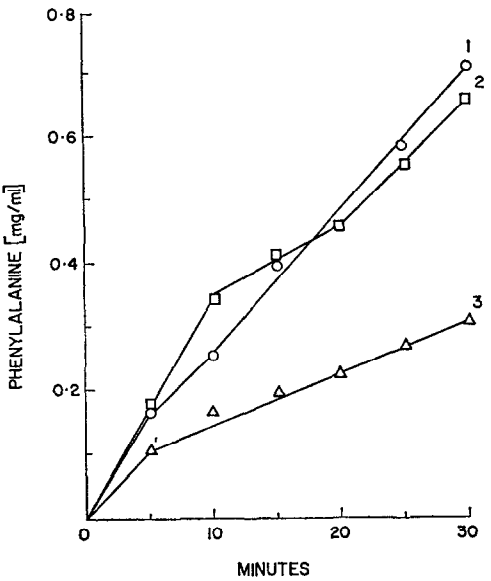

FIG. 1. The effect of ochratoxins A and α on the rate of hydrolysis of *N*-carbobenzoxy-glycyl-L-phenylalanine by carboxypeptidase A. Curves 1, 2 and 3 represent the hydrolysis of the substrate in the presence of ochratoxin α, in the absence of both toxins and in the presence of ochratoxin A, respectively. The concentrations of substrate, ochratoxins A and α were 0·02M and that of carboxypeptidase A 7 μg/ml protein solution.

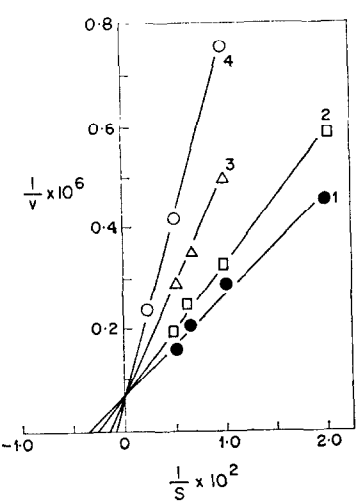

FIG. 2. Lineweaver–Burk plots for the determination of K_m and K_i values. Curve 1 represents the plot for the determination of the K_m value, while curves 2, 3 and 4 represent the effect of ochratoxin A at 0·005M, 0·02M and 0·025M, respectively. The initial velocity and substrate concentrations are represented by v and S, respectively.

enzyme was determined at the concentration level 0·020M to 0·002M. The K_i value was also determined according to the Lineweaver–Burk plot where the intercept on the $1/v$ axis, the slope and intercept on the $1/s$ axis are equal to $1/K_m(1 + I/K_i)$ $(K_m/V_{max}) \cdot (1 + I/K_i)$ and $1/V_{max}$, respectively. Since K_m is determined in the absence of the inhibitor, the value of K_i may then be calculated. The values of K_m and K_i were found to be 0·031 M and $14{\cdot}2 \times 10^{-3}$M respectively. The K_m value for *N*-CBZ-glyc-phe is 0·030M according to Yanari and Mitz.[7]

The effect of *N*-CBZ-glyc-phe on the rate of hydrolysis of ochratoxin A, as determined by the spectrophotometric assay method, is illustrated in Fig. 3. No inhibition was found to occur.

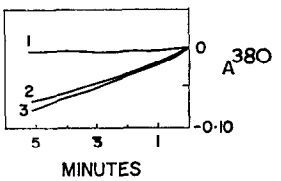

FIG. 3. Spectrophotometrical assay of the rate of hydrolysis of ochratoxin A by carboxypeptidase A in the presence and absence of *N*-carbobenzoxyglycyl-L-phenylalanine. Curve 1 represents the baseline, while curves 2 and 3 represent the hydrolysis of ochratoxin A in the absence and presence of *N*-carbobenzoxyglycyl-L-phenylalanine. The concentrations of toxin and *N*-carbobenzoxyglycyl-L-phenylalanine were $1{\cdot}5 \times 10^{-4}$M and carboxypeptidase A 50 μg/ml, respectively. A^{380} represents the absorption at 380 mμ.

The relative binding energy of ochratoxin A, to carboxypeptidase A was calculated from the K_i value by the following equation:

$$B\text{–}E = 1{\cdot}35 \log K_i - 4{\cdot}05 \qquad (1)$$

where $B - E$ and K_i are the relative binding energy and the competitive inhibitor constant, respectively. This equation was obtained by plotting log K_i values against $B - E$ on semilogarithmic paper (log 3 cycles × cm) which afforded a straight line (see Fig. 4). The $B - E$ of ochratoxin A for the enzyme was calculated to be −2·50 kcal/mol. Values for K_i and $B - E$ from various inhibitors of carboxypeptidase A are given in Table 1 and were obtained from Webb.[13, 14]

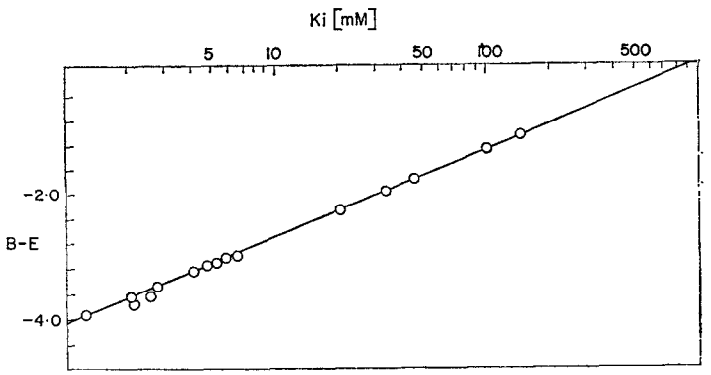

FIG. 4. A plot of relative binding energy against K_i. The values of K_i and relative binding energy were obtained from Table 1.

TABLE 1. THE RELATIONSHIP BETWEEN RELATIVE BINDING ENERGIES AND K_i OF VARIOUS INHIBITORS TO CARBOXYPEPTIDASE

Inhibition	K_i (mM)	Relative binding energy (kcal/mol)
Propionate	100	−1·36
Butyrate	5	−3·13
Valerate	2·7	−3·50
Caproinate	6·25	−3·00
Isocaproate	2·7	−3·50
Benzoate	143	−1·15
Phenylacetate	4·6	−3·18
β-Phenylpropionate	1·2	−3·97
γ-Phenylbutyrate	20	−2·31
Indoleacetate	0·78	−4·22
β-Indolepropionate	5·55	−3·06
γ-Indolebutyrate	33·3	−2·00
Benzylmalonate	4·0	−3·26
β-Cyclohexylpropionate	20·0	−2·31
β-Naphthaleneacetate	45·5	−1·82
D-Phenylalanine	2·0	−3·82
p-Nitrophenylacetate	2·5	−3·68

DISCUSSION

It is suggested by Webb[14] that a three-point attachment of the substrate on the surface of carboxypeptidase A is necessary for catalysis. The postulated enzyme sites are indicated in Fig 5. It is therefore easy to see why D-substrates cannot be hydrolysed since the peptide bonds would not be able to approach the peptidatic site.

Specific competitive inhibitors for carboxypeptidase A require only two interacting groups, and most that have been studied bind at the cationic and electrokinetic sites. Although the structure of ochratoxin A indicates no inhibition at the peptidatic site, the molecular configuration of the toxin (see Fig. 5) suggests that it exerts an inhibitory effect by binding at the cationic and electrokinetic sites. This is in accordance with the inhibitory effect of L- and D-phenylalanine and β-phenylpropionic acid, although the findings of Elkins-Kaufman and Neurath[12] showed that only the anionic forms, and not the dipolar ions, of the two amino acids are competitive inhibitors.

Ion–ion type interactions are known to be important in the binding of substrates and inhibitors to peptidases but Smith *et al.*,[8] have presented evidence that Van der Waal's forces are also involved. The inhibition constants and calculated binding energies for a number of compounds with carboxypeptidase A are given in Table 1. Smith *et al.*[8] concluded that Van der Waal's interactions of the side chains of the substrate provide a significant contribution to the total binding energy. The differences in the binding energies of inhibitors may be attributed mainly to Van der Waal's forces, and particularly to dispersion forces. Although the relative binding energy of substrate and inhibitors to carboxypeptidase can easily be calculated from the K_i values according to Smith *et al.*[8], an additional method of estimation is afforded by Fig. 5. Once the K_i value is known, the relative binding energy can be read off from the graph (Fig. 5) or the value of K_i be substituted in equation 1.

From Table 1, it is obvious that indoleacetate, indolepropionate and phenyl-

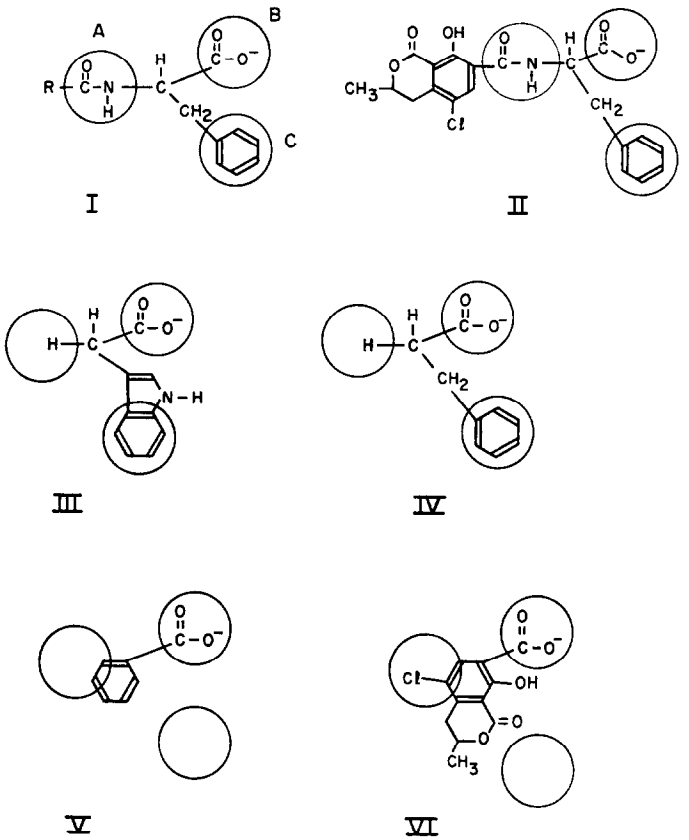

FIG. 5. Hypothetical orientation of substrate and inhibitors at the active centre of carboxypeptidase A. The enzyme sites, which are necessary for the attachment of substrates and inhibitors on the enzyme surface are represented by A, B and C. Sites A, B and C are the peptidatic site, the cationic site and the electrokinetic site, respectively. The latter site is perhaps a lipophilic region capable of reacting with alkyl or phenyl groups by dispersion forces. Configurations I, II, III, IV, V and VI are acyl-L-phenylalanine (substrate), ochratoxin A, 3-indoleacetic acid, β-phenylpropionate, benzoate and ochratoxin α, respectively. The molecular configurations are only approximate.

propionate are bound more strongly than ochratoxin A, suggesting that some steric repulsion of the latter analogue occurs. This steric repulsion is probably due to the presence of the isocoumarin moiety because ochratoxin α has no effect on the enzyme, while benzoate is an inhibitor (see Figs. 1 and 3 and Table 1).

The fact that the K_m for ochratoxin A is substantially lower than that of *N*-CBZ-glyc-phe suggests that ochratoxin A has a higher affinity for carboxypeptidase A than *N*-CBZ-glyc-phe which is corroborated by the observation that *N*-CBZ-glyc-phe has no inhibitory effect on the hydrolysis of ochratoxin A (Fig. 3). These findings suggest that ochratoxin A has a higher binding energy than *N*-CBZ-glyc-phe and should therefore be an effective competitive inhibitor of carboxypeptidase A, even though it is a poorer substrate. These findings coincide with that of Yanari and Mitz[7] who found that certain dipeptides perform as effective inhibitors although they are poor substrates. The structure of ochratoxin A

resembles that of a dipeptide in as much that it contains a peptide bond and a terminal carboxyl group.

Although ochratoxin A is the major toxin produced by *Aspergillus ochraceus*, two other toxins, with similar structure but far less toxicity, are also produced.[1] Ochratoxin B has no chloride on carbon 5, while in ochratoxin C, the carboxyl group is replaced by an ethylester group. It can therefore be anticipated that ochratoxins B and C would also inhibit carboxylpeptidase A.

In the light of these observations it is suggested that ochratoxin A exerts not only an inhibitory effect on carbohydrate metabolism, but possibly also on protein metabolism.

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