Biochemical Pharmacology, Vol. 18, pp 1829–1836. Pergamon Press. 1969. Printed in Great Britain

A RAPID SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CARBOXYPEPTIDASE A

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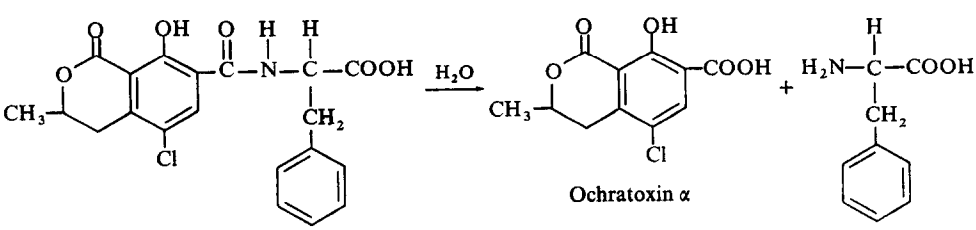
(*Received* 24 *October* 1968; *accepted* 22 *February* 1969)

Abstract—A novel method is described for the determination of the activity of carboxypeptidase A. Ochratoxin A, an isocoumarin derivative linked over its 7-carboxygroup to L-β-phenylalanine, is found to be hydrolysed *in vitro* to ochratoxin α by pancreatic carboxypeptidase. Ochratoxin A has an absorption peak at 380 mμ at pH 7·5 while ochratoxin α has only an absorption peak at 330 mμ at the same pH. When ochratoxin A is hydrolysed by means of HCl or carboxypeptidase A the absorption peak at 380 mμ disappears while a peak at 330 mμ appears. Difference and absorption spectra indicate that the decrease at 380 mμ is much more sensitive than the increase of absorption at 330 mμ. It is also found that the spectrophotometric assay method (measuring the decrease at 380 mμ) is much more sensitive than the colorimetric ninhydrin method.

THE MOST common assay method for carboxypeptidase A (EC 3.4.2.1) is based on the estimation of liberated amino acids by colorimetric ninhydrin methods.[1, 2, 3] The substrates usually employed for this assay are carbobenzoxyglycyl-L-phenylalanine, carbobenzoxyglycyl-L-tryptophan and carbobenzoxyglycyl-L-leucine. Under these assay conditions approximately 20% hydrolysis occurs within 30 min.

A spectrophotometric assay method for neutral protease from *B. subtilis* has been described by Feder.[4] Folk and Schirmer[5] also described a spectrophotometric method for assaying the activity of the porcine pancreatic carboxypeptidase A system. Since it was observed that ochratoxin A is hydrolysed by carboxypeptidase A *in vitro* to ochratoxin α,[6] it was decided to investigate this hydrolysis reaction as a possible assay method for the activity of bovine carboxypeptidase A. The enzymic reaction is:



This paper describes a spectrophotometric method for assaying carboxypeptidase A which affords certain advantages over the other methods.

EXPERIMENTAL

Ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin linked over its 7-carboxy group to L-β-phenylalanine) was isolated and purified from fungal cultures as described by Van der Merwe, Steyn and Fourie.[7] Ochratoxin α was prepared according to Van der Merwe *et al.*[7]

Ochratoxins A and α are unstable in daylight in alkaline solutions (pH 7·5) and therefore all toxin solutions were prepared in subdued light and kept in dark containers. A stock solution of ochratoxin A, 0·002 M, was prepared in a 0·1M NaCl-0·02M Tris buffer, pH 7·50 ± 0·02, and all dilutions of the stock solution were made with the same buffer. Concentrations of the diluted solutions were estimated spectrophotometrically by using a value of 7160 M^{-1} cm^{-1} for the molar absorbancy index.

Carboxypeptidase A, free of chymotrypsin and trypsin and 5 times crystallized, was purchased from Sigma Chemical Co., St. Louis. The nitrogen content of this enzyme is 15·4 per cent (see ref. 8). Appropriate concentrations were obtained by dilution with the 0·1M NaCl–0·02M Tris buffer, pH 7·50 ± 0·02.

Since the absorption of ochratoxin A is very sensitive to pH changes,[6] all ochratoxin A and enzyme solutions were adjusted, where necessary to pH 7·50 ± 0·02 with 0·1N HCl or 0·1N NaOH solutions and the pH determined with a Radiometer pH meter 22 fitted with an external pH meter ranging from pH 6·6 to 8·0.

Proteolytic activity, according to the spectrophotometric method, was determined by digestion at 25° of 2·0 ml substrate solution (ochratoxin A concentrations varying from 0·47 × 10^{-4}M to 2·75 × 10^{-4}M) with 1·0 ml of enzyme solution (concentration varying from 1200 μg to 2 μg/ml). The rate of decrease of absorbance at 380 mμ was measured with a Beckman DK-2A spectrophotometer with an absorbance range from

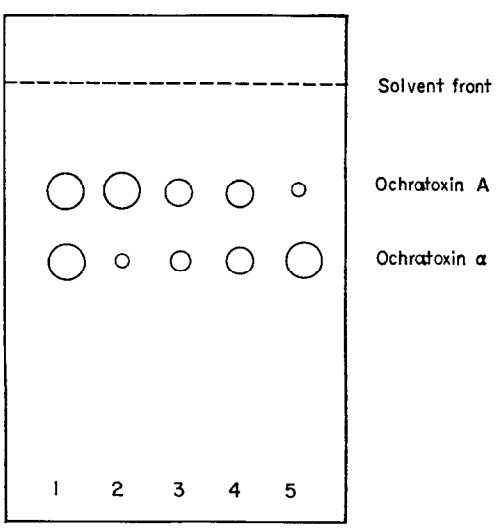


FIG. 1. A chromatogram of ochratoxin A (2·0 ml of 1·5 × 10^{-4}M) treated with different concentrations of carboxypeptidase A. A standard mixture of ochratoxins A and α is illustrated by 1, while 2, 3 and 4 represent ochratoxin A after treated with 2, 50 and 1200 μg/ml enzyme solution, respectively. Hydrolysis was performed at 25° for 180 min. The hydrolysis of the toxin with the high enzyme concentration for 48 hr at 25° is given by position 5.

—0·3 to 2·0 units, fitted with a temperature regulated cell holder and a time-drive attachment running at a speed of 1 in./min for 5 min. The reference solution contained 2 ml buffer and 1·0 ml enzyme solution. For comparison, the rate of hydrolysis was also determined according to the ninhydrin colorimetric method.[9]

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

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

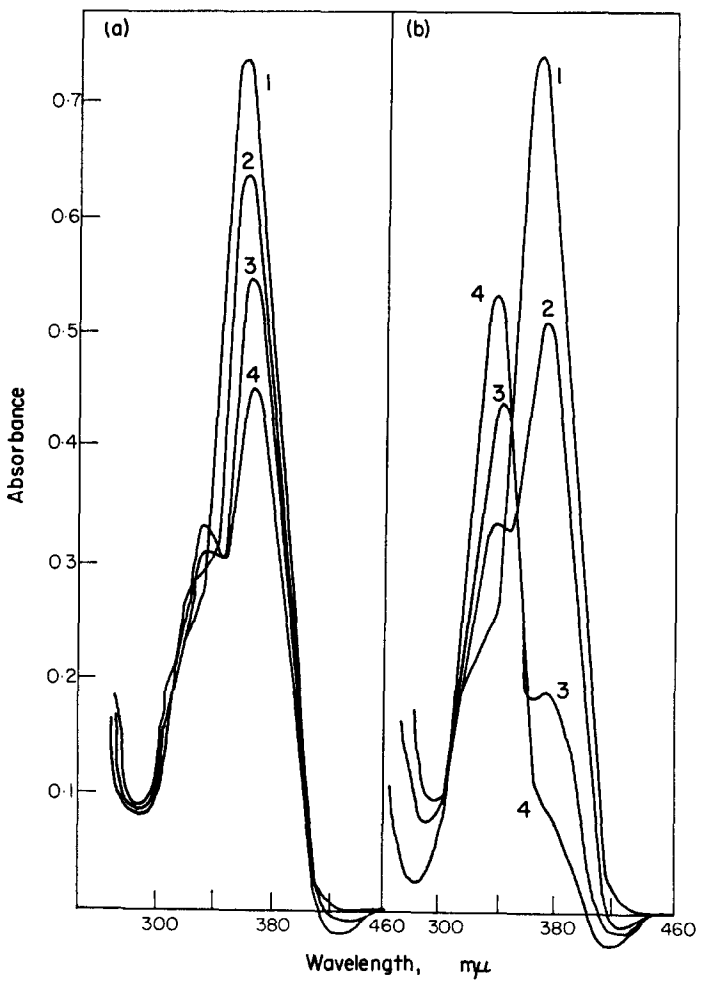


FIG. 2. The hydrolysis of ochratoxin A by three different carboxypeptidase A concentrations. In A curve 1 represents ochratoxin A (2 ml of $1{\cdot}95 \times 10^{-4}$M) before hydrolysis, curves 2, 3 and 4 (2·0 ml of $1{\cdot}0 \times 10^{-4}$M) treated with 2, 50 and 1200 μg enzyme/ml. for 180 min, respectively. In B curve 1 represents an ochratoxin A solution before hydrolysis while curves 2, 3 and 4 represent the rate of hydrolysis at 25° after 120, 1620 and 2880 min, respectively. The concentration of substrate was $1{\cdot}95 \times 10-^{4}$M and enzyme concentration was 1200 μg/ml.

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 (concentration varying from 1200 to 2 μg/ml). All absorption spectra were also recorded in the Beckman DK-2A spectrophotometer.

RESULTS

The rate of hydrolysis of ochratoxin A at 3 different enzyme concentrations is illustrated in Figs. 1–4. Absorption spectra of ochratoxins A and α showed that the former has an absorption peak at 380 mμ while the latter has an absorption peak at 330 mμ (see Fig. 2). The results from absorption and difference spectra (see Figs. 2 and 3), as well as time-drive measurements,[6] clearly illustrate that the decrease at

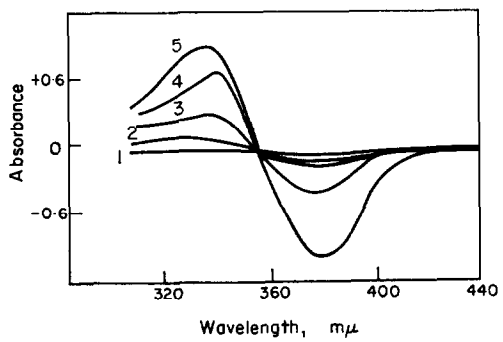


FIG. 3. Difference spectra of ochratoxin A treated with carboxypeptidase A. Curve 1 represents the baseline, curves, 2, 3 and 4 represent ochratoxin A ($1{\cdot}0 \times 10^{-4}$M) treated with 2, 50 and 1200 μg/ml of carboxypeptidase A for 180 min. respectively. Curve 5 represents ochratoxin A, treated with 1200 μg enzyme for 44 hr at 25°.

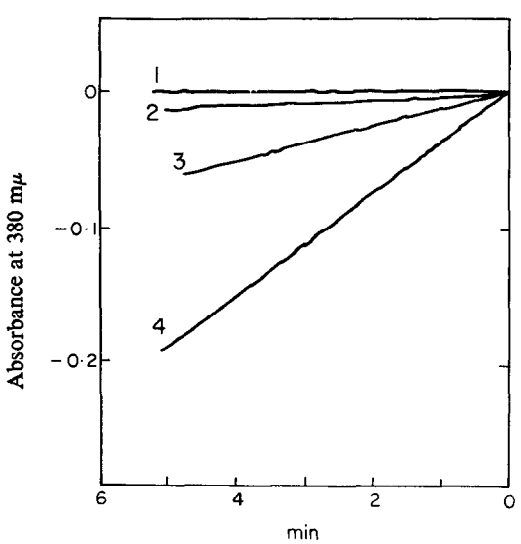


FIG. 4. Spectrophotometric assay of the rate of hydrolysis of ochratoxin A by carboxypeptidase A. Curve 1 represents the baseline, while curves 2, 3 and 4 represent the decrease at 380 mμ (A^{380}) due to the hydrolysis of a $2{\cdot}0 \times 10^{-4}$ ochratoxin A solution by 2, 50 and 1200 μg/ml carboxypeptidase.

380 mμ is an appreciably more sensitive method for assaying than the increase at 330 mμ. For comparison, the ninhydrin assay was also used to determine the rate of hydrolysis of ochratoxin A. However, no results were obtained when using the same toxin concentrations (1·5–2·75 × 10^{-4}M) as were used for the spectrophotometrical method, or at the low enzyme concentrations (2–50 μg/ml). Only the hydrolysis of ochratoxin A, at concentrations varying from 0·021M to 0·0021M, by the high enzyme concentration (1200 μg/ml) could be assayed by the ninhydrin colorimetric method.

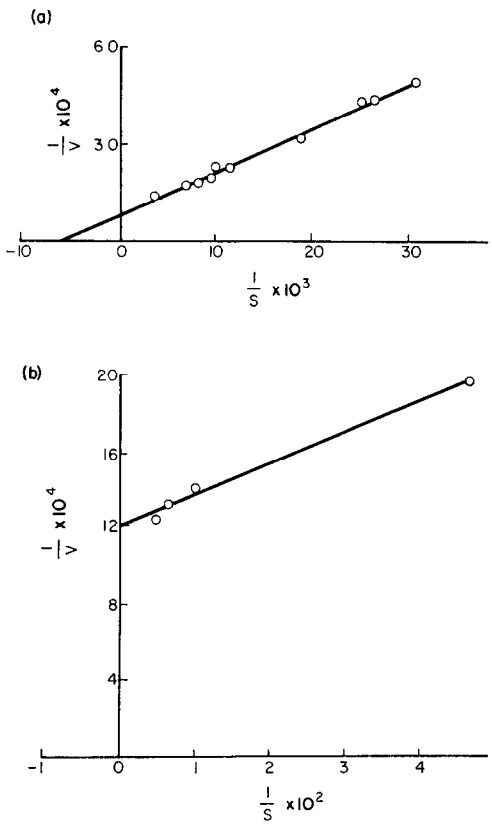


FIG. 5. Lineweaver–Burk plots for the determination of the apparent Michaelis constant (K_m). Curves A and B represent the results obtained from the colorimetric and spectrophotometric methods, respectively.

The Lineweaver–Burk equation[10] was used for the calculation of the apparent Michaelis constants from the initial reaction velocities, where v was taken as the moles/l. of substrate hydrolysed during the first 5 min (v_5) or 30 min (v_{30}) of hydrolysis in the case of the spectrophotometrical and colorimetric methods, respectively. The results are given in Fig. 5. The K_m values of ochratoxin A for carboxypeptidase A, determined according to the spectrophotometric and colorimetric methods, are 1·4 × 10^{-4}M and 1·45 × 10^{-3}M, respectively.

The integral reaction constant, k_3, or the specific rate of activation in moles/l. of

substrate hydrolysed/min/mg enzyme N/ml was determined by plotting 2·3 K_m log $(a_o/a + (a_o - a)$ against e.t (see Fig. 6) where a_o is the initial concentration at time $t = o$, a is the concentration at time $t = t$ and e is the enzyme concentration in mg N/ml.[11] The results of K_m and k_3 determinations for the hydrolysis of certain specific substrates as well as ochratoxin A by carboxypeptidase A is summarized in Table 1.

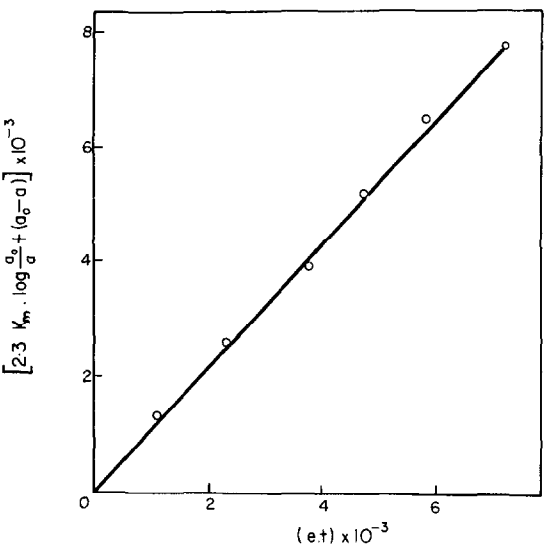


FIG. 6. Determination of the integral reaction constant (k_3). K_m represents the apparent Michaelis constant, while a_0, a, e and t represent the substrate concentration at time $t = 0$, the substrate concentration at time $t = t$, the concentration of enzyme in mg N per ml and the time in minutes, respectively. The results were obtained from the spectrophotometric method.

TABLE 1. KINETIC CONSTANTS FOR THE HYDROLYSIS OF CERTAIN SPECIFIC SUBSTRATES AND OCHRATOXIN A BY BOVINE CARBOXYPEPTIDASE A AT 25°

Substrate	K (mM)	K_3 mole/l./min/mg enzyme N/ml
Carbobenzoxyglycylphenylalanine*	33·0	2·1
Benzoylglycylphenylalanine*	11·0	2·00
Benzenesulphonylglycylphenylalanine*	14·0	0·124
Formylphenylalanine*	35·0	0·007
Acetylphenylalanine*	155·0	0·0023
Chloroacetylphenylalanine*	13·0	0·137
Carbobenzoxyglycyl-L-phenylalanine†	16·6	
N-Benzoylglycyl-L-phenylalanine†	1·8	
Carbobenzoxyglycyl-L-leucine†	33·0	
Carbobenzoxyglycyl-L-tryptophan†	7·2	
Ochratoxin A (spectrophotometrical assay)	0·14	1·1
Ochratoxin A (ninhydrin assay)	1·45	

* Snoke and Neurath[12]
† Folk and Schirmer.[5]

DISCUSSION

The enzymatic hydrolysis of ochratoxin A by carboxypeptidase A follows the general reaction kinetics exemplified by the hydrolysis of the typical substrate carbobenzoxyglycyl-L-phenylalanine.[11] The affinity of ochratoxin A for the enzyme is greater than that of carbobenzoxyglycyl-L-phenylalanine as is evidenced by a lower value of K_m (Table 1). Comparison of the K_m and k_3 values for the substrates in Table 1 provides a relative measure of their affinities and leads to the following sequence of decreasing affinities for carboxypeptidase A.

Ochratoxin A > benzoylglycylphenylalanine > carbobenzoxyglycylphenylalanine > chloroacetylphenylalanine and benzenesulphonylglycylphenylalanine > formylphenylalanine > acetylphenylalanine.

In this method the concentration of the substrate is limited between 0·45 to 2·75 $\times$ 10^{-4}M, when the Beckman DK-2A is employed which is much lower than 5 $\times$ 10^{-2}M described in the literature for other substrates.[1-3, 5] Below 0·45 $\times$ 10^{-4}M the decrease in absorbance at 380 mμ is not sensitive enough while concentrations higher than 2·75 $\times$ 10^{-4}M cannot be registered on the Beckman DK-2A.

Spectrophotometric techniques diminish many of the hazards for routine assay inherent in other methods. The advantages of the spectrophotometric method over the colorimetric method are that the former is more sensitive and shorter. The disadvantage however, is that relatively high concentrations of the enzyme are needed in comparison with the colorimetric method.[1-3]

Acknowledgement—I wish to thank Dr. I. F. H. Purchase for valuable suggestions.

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