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Analysis of Ochratoxin B Alone and in the Presence of Ochratoxin A, Using Carboxypeptidase A

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A method is described for ochratoxin B analysis, which is adapted to the earlier described method of ochratoxin A analysis, using carboxypeptidase A (K. Hult and S. Gatenbeck, J. Assoc. Off. Anal. Chem. 59:128-129, 1976). The fluorescence spectra of ochratoxins A and B coincide too much to allow direct discrimination of the two compounds. A method using the differences in kinetic parameters of the enzymatic hydrolysis of the two compounds is suggested for the analysis of mixtures of the ochratoxins.

We have recently published a method for ochratoxin A analysis (3), which uses the change of fluorescence produced by hydrolysis of ochratoxin A with carboxypeptidase A. The corresponding hydrolysis of the peptide bond in ochratoxin B is possible with carboxypeptidase A (2), followed by a similar change in the fluorescence spectrum. The hydrolysis of ochratoxin B is much faster than that of ochratoxin A (2), which makes it possible to analyze ochratoxins A and B in the same sample.

MATERIALS AND METHODS

The procedure of extraction of ochratoxin B is the same as that earlier described for ochratoxin A (3). The only modification is that the volume of the 0.04 M tris(hydroxymethyl)aminomethane buffer, pH 7.50, is reduced from 5 to 2.5 ml. The amount of carboxypeptidase A, however, is the same as in the ochratoxin A analysis.

The sample must be measured immediately after the enzyme addition, as the hydrolysis is appreciable even at 0°C. The excitation spectrum is recorded from 320 to 400 nm, at 450 nm emission and 0°C. Incubation at 20°C for 60 min cleaves all ochratoxin B, and the excitation spectrum is then recorded once more at 0°C. The loss of fluorescence at 365 nm is proportional to the original concentration of ochratoxin B.

For recovery experiments, ochratoxin B dissolved in 0.04 M tris(hydroxymethyl)aminomethane buffer, pH 7.50, was added to 5-g portions of crushed barley. The following levels of ochratoxin B were used: 5, 10, 20, and 40 μg/kg of barley. Ochratoxin B was extracted and measured as described above. Standards were prepared by addition of ochratoxin B to the buffer phase of extracts from unspiked barley.

The concentrations of standard solutions of ochratoxin B in 0.04 M tris(hydroxymethyl)aminomethane buffer, pH 7.50, were determined spectrophotometrically at 365 nm by using a value of 2,700 M^{-1} cm^{-1} for the molar absorbancy index, calculated as described previously (1).

When mixtures of ochratoxins A and B were analyzed, the extractions were performed as described above, but the conditions for incubation with the enzyme were modified in the following way. After recording the excitation spectrum at zero time, the sample was incubated for 30 min at 20°C. The excitation spectrum was recorded again at 0°C, and then the incubation was continued at 37°C for 120 min before the final spectrum was determined. The loss of fluorescence was measured as the difference between the maximum, regardless of wavelength, and the fluorescence at 380 nm.

RESULTS AND DISCUSSION

Fluorescence spectra. Ochratoxin B, dissolved in 0.04 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, has an excitation maximum at 365 nm, in agreement with the published absorption maximum (1). The free isocoumarin chromophore, ochratoxin β, has an excitation maximum at 325 nm. The hydrolysis of ochratoxin B with carboxypeptidase A, giving ochratoxin β and phenylalanine, will therefore lead to a decrease of fluorescence intensity at 365 nm and an increase at 325 nm. Ochratoxins B and β have emission maxima at 435 and 425 nm, respectively. Excitation spectra were recorded at 450 nm to fit the ochratoxin A analysis (3). Ochratoxin A has a higher fluorescence intensity than ochratoxin B. At 450 nm and pH 7.50 and 8.00, the intensity of ochratoxin A is 2.08 and 1.78 times, respectively, the intensity of ochratoxin B of the same concentration.

Recovery. Recovery experiments were done in triplicate with 5, 10, 20, and 40 μg of ochratoxin B per kg of barley. The recovery was not

dependent on the amount of added ochratoxin B, and the means of three different analyses gave an average recovery of 83%. The reduced amount of buffer in the final extraction, compared to the earlier-described ochratoxin A method (3), gave a higher response to ochratoxin B in relation to background fluorescence and had no significant effect on the recovery.

The detection limit of ochratoxin B is influenced by other substances giving fluorescence in the buffer extract and for barley is equal to the detection limit of ochratoxin A (3), i.e., 4 μg/kg.

Analysis of ochratoxins A and B in the same sample. The fluorescence spectra of ochratoxins A and B coincide too much to allow direct discrimination of the two compounds. Fortunately, the kinetic parameters of the enzymatic hydrolysis (2) are different enough to allow calculation of the concentrations of the individual ochratoxins from two incubations under different conditions.

Experimental results show that it is convenient to do the first incubation so that 85 to 95% of the ochratoxin B is hydrolyzed. This is obtained after 30 min at 20°C. During this incubation it is unavoidable that 5 to 15% of the ochratoxin A is also hydrolyzed. During the second incubation, 120 min at 37°C, all remaining ochratoxins A and B are hydrolyzed. The loss of fluorescence after the first incubation (f_1) is measured. The total loss of fluorescence (f_t) between the original and the final spectra is obtained from the second incubation. The ratio f_1/f_t can be used as a measure of the amount of ochratoxin B compared to the total amount of ochratoxins A and B. If standards of pure as well as known mixtures of ochratoxins A and B are analyzed, the ratio f_1/f_t can be plotted as a function of the concentration of ochratoxin B (b_0) divided by the sum of the concentration of ochratoxin B and the concentration of ochratoxin A (a_0) multiplied by a constant k, $b_0/(a_0k + b_0)$. k is a constant expressing the ratio of fluorescence intensity for ochratoxin A to ochratoxin B.

The curve thus constructed will be a straight line (see below for the theoretical treatment), and for simplification of the procedure it is therefore only necessary to have standards of pure ochratoxins A and B.

Figure 1 shows results from two experiments with standard mixtures of ochratoxins A and B. The differences in the slopes depend on the different degrees of hydrolysis during the first incubation.

The ratio f_1/f_t gives the relative amount of ochratoxin B in the sample. The total amount

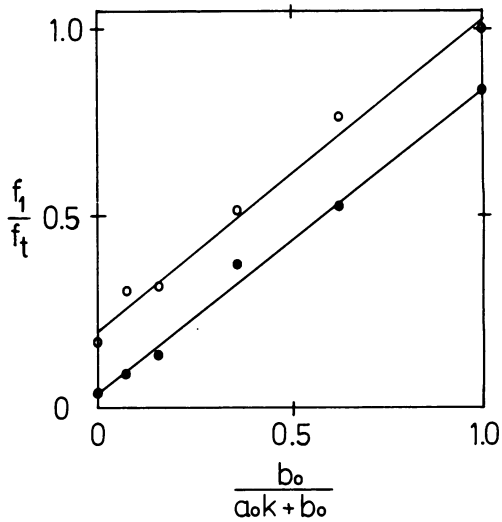

FIG. 1. *Two sets of analyses of ochratoxins A and B in the same sample, different in the degrees of hydrolysis during the first incubation. The analyses were performed in 0.04 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.00, with the total amount of ochratoxins in all points amounting to 40 μg/kg of sample, according to the described method of analysis. All points are the means of three incubations, and the lines are fitted with the least-square method. f_1 is the loss of fluorescence after the first incubation, and f_t is the total loss of fluorescence after the second incubation. a_0 and b_0 are the concentrations of ochratoxins A and B, respectively. k is a constant expressing the difference of fluorescence intensity between ochratoxin A and B.*

of ochratoxin B is calculated as the product of the ratio $b_0/(a_0k + b_0)$ and the total loss of fluorescence of the sample, f_t. The value obtained is subsequently compared with the fluorescence of a standard of known concentration. The amount of ochratoxin A is calculated in the same manner, knowing that

$$\frac{a_0}{a_0k + b_0} = 1 - \frac{b_0}{a_0k + b_0}$$

From the following theoretical approach, the resolution of ochratoxins A and B is seen to be dependent on the relative velocities of hydrolysis and the relative intensities of fluorescence (expressed by k) between the ochratoxins.

The slope of the line is dependent on the relative velocities of hydrolysis, which can be influenced by the temperature. However, the temperature influence is rather small and the change in slope is minimal. At the chosen temperature, 20°C, in the experiments shown in Fig. 1, the slopes are 0.80 and 0.82, which are

considered to be satisfactorily close to the maximal slope, 1.0. More important for the resolution is the constant, k, which is pH dependent. A lower value of k will give b_o a greater importance in the denominator in the ratio $b_o/(a_ok + b_o)$, favoring the analysis of ochratoxin B. The useful pH range for ochratoxin hydrolysis with carboxypeptidase A is between pH 7 and 8, pH 7 giving the best conditions for ochratoxin A and pH 8 giving the best conditions for ochratoxin B analyses.

Theoretical approach to the standard curve for estimating ochratoxin A and B ratios. The hydrolysis of the peptide bond in ochratoxins A and B with carboxypeptidase A follows the Michaelis-Menten equation

$$-\frac{ds}{dt} = v = \frac{V}{1 + \frac{K_m}{s}} \quad (1)$$

where t is the time, s the substrate concentration, v the observed reaction velocity, V the maximal velocity, and K_m the Michaelis constant. During the analysis, the substrate concentration (10^{-8} M) is much smaller than K_m (10^{-4} M) (2), and equation 1 can be simplified to

$$-\frac{ds}{dt} = v = \frac{V}{K_m} \cdot s \quad (2)$$

After integration between $t = 0$ to $t = t$, equation 2 gives:

$$s = s_o e^{-\frac{V}{K_m} \cdot t} \quad (3)$$

where s_o is the substrate concentration at $t = 0$. If a_o and a are the concentrations of ochratoxin A at $t = 0$ and $t = t$ and $A = V/K_m$ for hydrolysis of ochratoxin A, equation 3 gives

$$a = a_o e^{-At} \quad (4)$$

and, correspondingly, for ochratoxin B

$$b = b_o e^{-Bt} \quad (5)$$

The loss of fluorescence after the first incubation is related to the loss in concentration by a proportional expression:

$$f_1 = p \cdot [(a_o - a) \cdot k + (b_o - b)] \quad (6)$$

where p is a proportionality constant. The total loss of fluorescence is in the same manner described by

$$f_t = p \cdot [a_o \cdot k + b_o] \quad (7)$$

Combinations of equations 4 through 7 give

$$\frac{f_1}{f_t} = \frac{a_ok}{a_ok + b_o}(1 - e^{-At}) + \frac{b_o}{a_ok + b_o}(1 - e^{-Bt}) \quad (8)$$

but

$$\frac{a_ok}{a_ok + b_o} = 1 - \frac{b_o}{a_ok + b_o} \quad (9)$$

If equation 9 is substituted in equation 8

$$\frac{f_1}{f_t} = \frac{b_o}{a_ok + b_o}(e^{-At} - e^{-Bt}) + (1 - e^{-At}) \quad (10)$$

Equation 10 shows that the plot of f_1/f_t versus $b_o/(a_ok + b_o)$ gives a straight line at any given t.

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