OCHRATOXIN A LABELLED WITH ^{14}C OR ^{3}H IN THE PHENYLALANINE MOIETY

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SUMMARY

Ochratoxin A is a mycotoxin produced by Aspergillus and Penicillium moulds. The toxin is an amide formed between phenylalanine and the isocoumarin acid ochratoxin α. Ochratoxin A with high specific radioactivity can be obtained, by substitution of the natural phenylalanine for ^{14}C or ^{3}H-labelled phenylalanine.

Labelled ochratoxin A was formed by condensation of labelled phenylalanine ethylester with an anhydride derivative of ochratoxin α. The intermediate O-acetyl ochratoxin C was hydrolysed with pig liver esterase and chymotrypsin to afford ochratoxin A. The overall yield of radioactivity was 50%. Specific activities up to 500 Ci/mol and 100 Ci/mmol for ^{14}C and ^{3}H respectively were obtained.

Key words: Ochratoxin A - synthesis - pig liver esterase.

INTRODUCTION

Ochratoxin A (1) is a mycotoxin produced by several moulds from the genera Aspergillus and Penicillium. The toxin has a potent nephrotoxic effect in several animal species (1). Ochratoxin A has a widespread occurrence in cereals and is accumulated in the blood of some animals after consumption of contaminated grains. The toxin has been found in the blood of pigs and humans (2,3).

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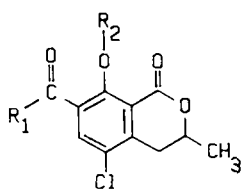
The purpose of the present study was to synthesize labelled ochratoxin A with very high specific activity to be used in binding studies with plasma proteins (4) and for whole body autoradiography studies. The commercial availability of phenylalanine labelled with ^{14}C or ^{3}H at extremely high specific activity offered the possibility to substitute the unlabelled phenylalanine moiety of the ochratoxin A molecule for the radioactive analogue. Recently such a method was published in which the N-hydroxysuccinimide derivative of ochratoxin α (2) was coupled with [^{14}C]phenylalanine methyl ester. An extremely large excess of the ochratoxin α derivative was used (5). An alternative method is presented here which can be used for synthesis of low amounts of ochratoxin using equimolar amounts of reagents with retained yield. Chang and Chu have published an alternative method in which ochratoxin A is tritiated by catalytic exchange (6).

MATERIALS AND METHODS

Ochratoxin A was isolated from Penicillium nordicum cultures grown on barley as described earlier (7). L-[U-^{14}C]phenylalanine (504



	R_1	R_2
1	Phe	H
1*	Phe*	H
2	H	H
3	CH_3COO	CH_3CO
4*	Phe*-Et	CH_3CO

Figure 1. Structures of ochratoxin derivatives. Phe represents phenylalanine and Phe-Et phenylalanine ethyl ester. Asterisk represents radioactivity.

Ci/mol) and L-[2,3,4,5,6-^{3}H]phenylalanine (130 Ci/mmol) were purchased from Amersham International plc. Pig liver esterase and α-chymotrypsin were from Sigma.

Silica gel plates were used for chromatography in the following systems: System A toluene-diethylamine (90:10) and system B toluene-dioxane-acetic acid (95:35:4). For localisation of radioactivity on the plates a Packard radiochromatogram scanner model 7201 was used. A Packard Tri-Carb model 3375 was used for liquid scintillation measurements.

EXPERIMENTAL

Ochratoxin α (2) was prepared by acid hydrolysis of ochratoxin A (1) as described by van der Merve et al. (8).

Acetic anhydride of O-acetyl ochratoxin α (3). Ochratoxin α (0.5 μmol; 128 μg) was dissolved in toluene (500 μl). Pyridine (1.5 μmol) and acetylchloride (1.5 μmol) were dissolved in toluene (50 μl) and added to the toxin. After 2 hours at room temperature the reaction was ready and the reaction mixture was filtered through glasswool in a pasteur pipette. Toluene was evaporated under a stream of nitrogen. The product was kept over night over solid KOH to remove all acetyl chloride. The product showed UV maxima at 310 nm and 340 nm. ^{1}H NMR : 1.55 (d,3H), 2.36 (s,3H), 2.40 (s,3H), 2.97 (o,2H), 4.65 (m,1H), 8.20 (s,1H).

^{14}C- and ^{3}H-Labelled phenylalanine ethyl ester. L-[U-^{14}C]-Phenylalanine (20-500 nmol) or L-[2,3,4,5,6-^{3}H]phenylalanine (1-10 nmol) were treated with ethanol (1 ml) containing 20% thionyl chloride (8:2) at room temperature overnight. The solvent was evaporated under a nitrogen stream. A solution of 1% sodium

bicarbonate (500 µl) was added and the ester was immediately extracted with diethyl ether (5x500 µl). The ether was dried over a $MgSO_4$ column and evaporated under a stream of dry nitrogen. The esterification process was checked by TLC in system A (R_f of the ester 0.65; R_f of the acid 0.00).

O-acetyl ochratoxin C (4*). To the radioactive phenylalanine ethyl ester was added 1.2 equivalents of the acetyl anhydride derivative of ochratoxin α (3) together with 1.2 equivalents of pyridine. After overnight reaction at room temperature all phenylalanine ester was consumed. The reaction was checked by TLC in system B. The product O-acetyl ochratoxin C was easily seen as a fluorescent spot under longwave UV just infront of ochratoxin C. Usually this derivative was not isolated before the next step. If isolated it was done by preparative TLC in system B.

Labelled ochratoxin A (1*). The residue from the preparation of O-acetyl ochratoxin C (4*) was dissolved in dimethyl sulfoxide (100 µl). Pig liver esterase (25 µg) and chymotrypsin (25 µg) dissolved in 0.1 M tris(hydroxymethyl)aminomethane-HCl buffer pH 7.5 (1 ml) was added and the mixture was incubated over night. The formation of ochratoxin A was monitored by TLC in system B. The buffer was acidified with 1 M HCl and ochratoxin A was extracted with several portions of chloroform. The crude product was purified by TLC in system B. Ochratoxin A was extracted from the gel with 1% of formic acid in chloroform. The final product had the same UV and fluorescence spectra and chromatographic properties as standard ochratoxin A. The concentration of the product was determined by UV absorbance (ε=6 400 M^{-1} cm^{-1}) (1) or fluorescence spectroscopy. Radioactivity was measured by liquid scintillation counting. The specific activity was calculated from these data.

RESULTS AND DISCUSSION

The yield of phenylalanine ethyl ester was between 90 and 95%. It was necessary to clean up the product by extraction from bicarbonate to increase the yield in the formation of the amide bond. The anhydride derivative of ochratoxin α (3) was unstable and was used immediately. If a large excess of the anhydride was used in the amide formation the yield decreased as some other unknown labelled product was formed. The O-acetyl ochratoxin C was never characterized. It was subjected to enzymatic hydrolysis without prior quantitation. The overall yield of these two steps varied between 50-70% in different experiments.

The methods for synthesis of labelled ochratoxin A that have been published earlier (5,6) do not describe so high specific activities as those published here. The enzymatic step used to liberate ochratoxin A in the final step seems to be the crucial thing to keep up the yield.

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