PREPARATION OF ^{3}H-LABELED OCHRATOXINS

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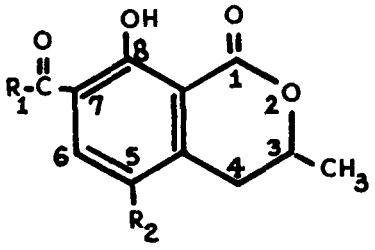
SUMMARY

^{3}H-Ochratoxins A, B, and C were obtained by tritiation of the toxins with tritiated water in acetic acid and platinum at 80° C overnight. The toxins were purified by solvent partition and thin layer chromatography with the recovery yield being 80% and the specific activity being 2.6 - 3.0 Ci/mmol. Hydrolysis of ^{3}H-ochratoxin A with carboxypeptidase A revealed that 80.9% of the tritium in ochratoxin A molecule distributed on the phenylalanine residue with only 19.1% in ochratoxin α moiety.

INTRODUCTION

Ochratoxins are a group of isocoumarins containing toxic metabolites produced by several species of Aspergillus and Penicillium. Their chemical, biological and toxicological properties have appeared in several reviews (1-4). The chemical structures of ochratoxins A, B, and C are shown in fig. 1. Ochratoxin A, 7-carboxy-5-chloro-3,4-dihydro-8-hydroxy-3-methylisocoumarin linked to L-β-phenylalanine, is most toxic among this series. The toxin is a potent nephrotoxin and causes liver damage at higher doses to test animals. The presence of this toxin in foods and feed for human and animal consumption therefore has been considered hazardous. Such consideration has been accompanied

by a demand for a radioactive toxin with high specific activity for toxicological and analytical studies. Nevertheless, the specific activity of the labeled toxin synthesized by a biosynthetic method such as supplying ^{14}C-phenylalanine, (5-7), ^{14}C-acetate, (5-7), or ^{14}C-methionine, (5,8) and Na^{36}Cl, (9) to the culture medium, has generally less than 100 μCi/mmol. In this paper, we wish to report a new method for the preparation of high specific activity labeled ochratoxins by tritiation of purified Ochratoxin A with tritiated water.



	R_1	R_2
OA	-Phe	-Cl
OB	-Phe	-H
OC	-Phe-Ethyl ester	-Cl
Oα	-OH	-Cl

Figure 1. Structures of ochratoxins A, B, C and α. OA, OB, OC and Oα represent ochratoxins A, B, C and α, respectively. Phe represents phenylalanine.

EXPERIMENTAL

Materials. Ochratoxin A (OA) was produced by Aspergillus ochraceus NRRL 3174 in rice, and was purified by Adsorbosil-5 column chromatography and Sephadex LH_{20} gel filtration as previously described (10,11). Ochratoxin α (7-carboxy-5-chloro-8-hydroxy-3-methylisocoumarin) was obtained by acid hydrolysis of purified OA with subsequent purification by passing through a Sephadex LH_{20} column (11). Adsorbosil-5 was

obtained from the Applied Science Laboratory. (State College, Pa.) All other chemicals used were either analytical grade or chemically pure.

Thin layer chromatography (TLC). Plates were coated with Adsorbosil-5 to a thickness of either 0.25 mm for analytical use or 0.5 mm for preparative work. The solvent system was benzene-acetic acid (9:1). Except if otherwise stated, the spots were located by UV or by autoradiography. For autoradiography, the developed TLC plates were air dried, covered with a layer of Handi-wrap and then placed in contact with RP-14 medical x-ray film (Kodak) in the absence of light. After an appropriate time exposure (4-18 h), the films were developed with Rapid X-ray Developer (Kodak) at 25°C for 3.5 min.

Radioactivity measurements. Except where otherwise specified, an appropriate amount of solution (generally less than 0.5 ml) or a radioactive spot scraped from a TLC plate was mixed with 10 ml of scintillation fluid (12). The solution was subjected to counting in a Packard Tricarb Model 5017 liquid scintillation spectrometer for at least 10 min.

Tritiation of Ochratoxins. Tritiation of ochratoxins was carried out by New England Nuclear (Boston, Mass.). A 90% pure ochratoxin A preparation with small amounts of ochratoxins B and C was used as the starting material. Two methods were tested.

Method A: Fifty milligrams of the toxin in 0.3 ml dry N, N-dimethylformamide was tritiated with 10 curies of tritiated water using 50 mg of 5% Rh/Al_2O_3 as the catalyst. The reaction mixture was stirred overnight at 50°C. Labile tritium was removed in vacuo, using ethanol as the solvent. After removal of the catalyst by filtration, the product was again taken to dryness in vacuo and redissolved in 10 ml of ethanol, with this product containing 57.9 mCi of radioactivity. Subsequent purification was then carried out as described below.

Method B: Tritiation was carried out using platinum as a

catalyst. Twenty-five milligrams of the toxin in 0.3 ml acetic acid was tritiated with 10 curies of tritiated water in the presence of 25 mg of platinum. The reaction mixture was stirred overnight at 80°C. Again the labile tritium and catalyst were removed. The product was redissolved in 10 ml ethanol before subsequent purification and had 650 mCi of total radioactivity.

Purification of ^{3}H-Ochratoxins. In a typical experiment, 0.5 ml of ethanolic ^{3}H-ochratoxin solution (1.25 mg) prepared by New England Nuclear using method B was evaporated to dryness and redissolved in 25 ml of 1% $NaHCO_3$. The solution was then adjusted to pH 2.0, and extracted three times with chloroform (total 35 ml). After evaporating to dryness in vacuo to remove the chloroform, the preparation was redissolved in a small amount of chloroform (0.5 ml) and then subjected to preparative TLC using 2 Adsorbosil plates (20 x 20 cm) and developed in C_6H_6-AcOH. Ochratoxin bands were then scraped from the plates, and the individual toxin was eluted from the Adsorbosil with methanol. Identification of different ochratoxins was made by comparing with anthetic ochratoxins A, B and C prepared in our laboratory, and also by examining the spectrophotometric properties. The concentration of the purified ochratoxins was determined spectrophotometrically as described (3).

Distribution of Tritium in ^{3}H-Ochratoxin A. The distribution of tritium in OA molecule was analyzed by an enzymic method. Two milligrams of unlabelled OA mixed with a small amount of ^{3}H-OA (7.91×10^{7} cpm) and 0.7 mg carboxypeptidase A (bovine pancreas, Worthington Biochemical Co., Freehold, N.J.) was dissolved in 2 ml of NaCl (0.1 M) -Tris (0.02 M, pH 7.5) buffer and incubated at room temperature for 24 hours. After digestion, one ml of the reaction mixture mixed with 1 ml 0.02 M HCl was extracted with chloroform until no fluorescence appeared in the last chloroform extract. The chloroform extracts were then combined, concentrated and subjected to preparative TLC. Ochratoxin α band was scraped from the plate and eluted with MeOH. The total radioactivity

and Oα concentration were determined by liquid scintillation and by spectrophotometric method (3), respectively. The aqueous solution after chloroform extraction was dialyzed against 6 volumes of distilled water overnight. The total radioactivity and phenylalanine concentration in solution of the outer dialyzed bag (protein free) were determined. Phenylalanine concentration was determined by the ninhydrin method using phenylalanine as standard (13).

RESULTS AND DISCUSSION

The results of the tritiation of ochratoxins by the two described methods are summarized in Table 1. Initially, method A was tested and found that although the ^{3}H-ochratoxin recovery yield was high (83%) after purification, the specific activity was low (110 mCi/mmol).

Table 1. Preparation of ^{3}H-ochratoxins.

Method used			A	B
Ochratoxin used (mg)			50	25
Total radioactivity before purification (mCi)			57.9	650
Recovery of Radioactivity (%)[a]	Solvent extraction	$CHCl_3$ layer	83	90
		$NaHCO_3$ layer	13	5
	TLC preparation	OA[b]	36.7 (67)[c]	43.5 (70)[c]
		OB	4.3 (8)	7.0 (11)
		OC	2.7 (5)	7.0 (11)
		Oα	11.3 (20)	4.6 (7)
Specific Activity of Final Product (Ci/mmol) or (cpm/ng)[d]		OA	0.11 (97)[d]	2.99 (2,614)[d]
		OB	-[e]	2.55 (2,490)
		OC	-	2.84 (2,324)
		Oα	-	2.57 (3,571)

(a) The % of radioactivity recovery was calculated on the basis of a counting efficiency of 16%. (b) OA, OB, OC and Oα represent ochratoxins A, B, C and α respectively. (c) % of the distribution among ochratoxins. (d) Specific activity as of cpm/ng determined under the condition as described (16% counting efficiency). (e) Not determined.

Subsequently cold experiments by heating ochratoxin with 70% acetic acid (110°C for 24 hours) revealed no extensive degradation of ochratoxin. Therefore, tritiation was carried out under conditions described in method B. The exchange of tritium was excellent under those conditions. Although there appeared a slight conversion of ochratoxin A to ochratoxins B and C, the overall recovery was good. The final purified ochratoxin A had a specific activity approaching 3 Ci/mmol. The specific activities of the other ochratoxins were similar. It must be noted that although the recovery of radioactivity appeared to be low (around 60%) in the purification, ochratoxins recovery yield was high (80%). This disagreement is probably due to the fact that counting efficiency (16%) was slightly higher than it should have been. The purified ochratoxins again exhibited a single spot in TLC and had similar spectrophotometric properties as reported (3). The specific activity of the final products did not change under acidic and basic conditions followed by solvent extraction, indicating that all labile tritium had been removed.

The distribution of radioactivity in OA molecules was investigated by both acid hydrolysis (6N HCl for 24 hours at 110° C) and enzymic method. Initial tests revealed that considerable amount of radioactivity was lost in the acid hydrolysis, and the experiment was not performed further. Enzymic hydrolysis, however, gave satisfactory recovery of radioactivity. The total radioactivity recovery from TLC before purification was found to be 53, 4.9 and 31% for OA, Oα, and materials in the original spotting position (primarily phenylalanine), respectively. The distribution of tritium between phenylalanine and Oα residues was found to be 81% and 19% after purification. The result indicates that most of the tritium in OA is in the phenylalanine residue. This result agrees with the general rule for tritium exchange in which the aromatic ring is most readily tritiated by acid catalyzed exchange method.

In comparing the biosynthetic methods (5-7) for labeling

ochratoxin A with those methods just described, it is clear that method B for tritiation gave the best result. Since we did not find extensive degradation of ochratoxin A even after heating the toxin at 110°C in 70% acetic acid for 72 hours, it should be possible to obtain ochratoxins of higher specific activity (higher than 3 Ci/mmol) by a minor modification of method B such as extending the tritiation time, or by slightly increasing the reaction temperature. It must be pointed out, however, that formation of ochratoxin C has been observed under these extreme conditions. Since most radioactivity in OA was distributed in the phenylalanine residue, interpretation of experimental results should be exercised with great care if the tritiated OA is used in the in vivo metabolic studies.

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