To the Graduate Council:

I am submitting herewith a dissertation written by Cheng-An Hwang entitled "Degradation of Ochratoxin A by *Acinetobacter calcoaceticus*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Technology and Science.

Frances A. Draughon

Frances A. Draughon, Major Professor

We have read this dissertation
and recommend its acceptance:

Dilip R. Sachan

Genevieve Christen

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

DEGRADATION OF OCHRATOXIN A

BY

ACINETOBACTER CALCOACETICUS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Cheng-An Hwang

May 1992

DEDICATION

This dissertation is dedicated to my parents, my wife, and my two lovely children. Without their love and support, this dissertation would not be completed.

ACKNOWLEDGEMENTS

My deepest appreciation goes to Dr. F. A. Draughon, who served as my major professor during my six-years study for M.S. and Ph.D. in the Department of Food Technology and Science, The University of Tennessee, Knoxville (UTK). My accomplishments would not be possible without Dr. Draughon's guidance, encouragement, understanding, and friendship to me and to my family. My appreciation is extended to Dr. J.L. Collins, Dr. G.L. Christen, Department of Food Technology and Science, and Dr. D.S. Sachan, Department of Nutrient, for their serving as committee members and their guidance and assistance during my doctoral study. My special thanks goes to Dr. G.L. Christen for her serving as committee member during my six-years study for the M.S. and Ph.D. in UTK.

I want to thank my fellow graduate students Randy Phebus and Brian Anthony for providing valuable assistance during my study. I wish to express thanks for the help from Erika Grandle, Chuck Payne, and staff in the Department of Food Technology and Science. I also want to thank Northern Regional Agriculture Research Laboratory of USDA for providing *Acinetobacter calcoaceticus* and *Bacillus cereus mycoides*.

ABSTRACT

Microorganisms were screened for their capability to degrade ochratoxin A (OTA). Among test microorganisms, *Acinetobacter calcoaceticus* was found to degrade OTA. The degradation of OTA by *A. calcoaceticus* was studied in an ethanol-minimal salts medium with an initial OTA concentration of 10-50 μg/mL at 25°C and 30°C. The hydrolysis of OTA by the crude enzyme isolated from *A. calcoaceticus* also was studied in a 0.1 M NaCl-0.02 M Tris buffer (pH 7.5) at 25°C. *A. calcoaceticus* was able to degrade OTA in ethanol-minimal salts medium with initial concentrations of OTA of up to 40 μg/mL. At 50 μg/mL, OTA inhibited the growth of *A. calcoaceticus* and no degradation of OTA occurred. The OTA degrading activity was found in the cell-free growth medium and in the cells. The rate of OTA removed by *A. calcoaceticus* was concentration dependent. As the OTA concentration increased, the rate of OTA removed by *A. calcoaceticus* increased. The average amounts of OTA removed by *A. calcoaceticus* in medium with an initial OTA concentration of 10, 20, 30, and 40 μg/mL were 0.1005, 0.1226, 0.1374, and 0.2262 μg/mL/hr at 30°C, respectively. At 25°C, the average amounts of OTA removed by *A. calcoaceticus* were 0.0636, 0.1172, 0.1264, and 0.2232 μg/mL/hr in medium containing 10, 20, 30, and 40 μg OTA/mL, respectively. At 25°C and 30°C, the growth of *A.*

calcoaceticus in medium containing 50 μg OTA/mL was inhibited, and OTA degradation did not occur. The rate of OTA removed by *A. calcoaceticus* in medium containing the same initial OTA concentrations of 20, 30, and 40 μg/mL was not significantly different ($p<0.05$) at 25°C and 30°C. OTA was degraded significantly by *A. calcoaceticus* during and after the log phase of cell growth at incubation temperatures of 25°C and 30°C. At 25°C, the initial rate of OTA hydrolyzed by crude enzyme of 0.2 mg protein/mL in 0.1 M NaCl-0.02 Tris buffer containing 10-60 μg OTA/mL in 10 μg/mL increments was 0.1526, 0.3215, 0.4423, 0.5903, 0.6158, and 0.6198 μg/mL/hr, respectively. The initial rate of OTA hydrolyzed by crude enzyme of 0.15 mg protein/mL was 0.1194, 0.2454, 0.3593, 0.5126, 0.5362, and 0.5581 μg/mL/hr, respectively. The initial rate of OTA hydrolyzed by crude enzyme of 0.1 mg protein/mL was 0.0550, 0.1176, 0.1502, 0.1905, 0.3087, and 0.4592 μg/mL/hr, respectively. Generally, the initial rate of OTA hydrolyzed by crude enzyme increased as the concentrations of crude enzyme and/or OTA increased. However, the initial rates of OTA hydrolyzed by crude enzyme of 0.15 and 0.2 mg protein/mL at OTA concentrations of 40, 50, and 60 μg/mL were not significantly different ($p>0.05$). Hydrolysis of OTA by the cells and crude enzyme of *A. calcoaceticus* yielded ochratoxin α. A bioassay showed decreased toxicity of OTA toward *Bacillus cereus mycoides* as OTA was hydrolyzed to

ochratoxin α by the cells and crude enzyme of *A. calcoaceticus*.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

CHAPTER I

INTRODUCTION

Generally, molds are not considered as agents of foodborne diseases but spoilage microorganisms of foods. Following the outbreak of "turkey X" disease caused by aflatoxins in 1960, numerous studies have been conducted on mycotoxins, toxins produced by toxigenic molds. Mycotoxin studies revealed the carcinogenic, mutagenic and teratogenic effects of mycotoxins in animals and humans. Among mycotoxins, ochratoxins have attracted considerable attention due to their toxic effects on biological systems.

Toxigenic strains of *Aspergillus ochraceus* Wilhelm in cereal and legume crops caused the death of ducklings, rats, and mice in South Africa (Scott, 1965) led to the isolation and chemical characterization of ochratoxins produced by *A. ochraceus* (van der Merwe et al., 1965b). Ochratoxins comprise a group of chemically related secondary metabolites produced by several species of *Aspergillus* and *Penicillium*. Among ochratoxins, ochratoxin A (OTA) attracts the most extensive study due to its occurrence and high toxicity in animals while other members of ochratoxins, ochratoxin B and C, cause little or no acute toxicity. OTA is found widely in foods of plant and animal origins, and it is toxic to cattle, swine, goats, chicken, turkey, duckling, rats, mice,

dogs, and fish. Cases of acute ochratoxicoses are characterized by nephropathy, enteritis, and immunosuppressive symptoms (Krogh et al., 1973; Prior et al., 1980; Taipia and Seawright, 1984). In most animals the primary targets of OTA are the kidneys and liver (Cole and Cox, 1981). OTA is retained in tissues of animals exposed to contaminated feedstuffs, and the highest accumulated concentrations of OTA are found in kidneys, liver, and skeletal muscle. Ochratoxin B and C have only limited toxicity to several test animals and are not considered a problem in animal feedstuffs (Vleggaar and Steyn, 1980). Under natural conditions, only OTA and, rarely, ochratoxin B have been encountered. Therefore, only OTA is considered a natural contaminant of foods and feedstuffs (Carlton, 1979). OTA contamination in foods and feedstuffs is considered a serious problem in food toxicology since OTA is capable of causing renal and hepatic carcinogenicity in mice (Kanisawa and Suzuki, 1978a,b).

Studies on microbiological degradation of OTA are not available. Although OTA has been reported to be degraded to non-toxic metabolites by rumen bacteria, the bacteria in rumen responsible for OTA degradation have not been isolated and identified. The objectives of this study were to find microorganism(s) that are capable of degrading OTA and study the mechanism of microbial degradation of OTA. The ideal microorganism(s) capable of degrading OTA should grow well

under simple incubation conditions, and the mechanism of degrading OTA is enzymatic. Results obtained from this study should provide information about the mechanism of microbial degradation of OTA and the possibility of using the microorganisms in degradation of OTA in food and feedstuffs.

CHAPTER II

LITERATURE REVIEW

Ochratoxins-Producing Fungi

Ochratoxins were the first major group of mycotoxins identified after the discovery of aflatoxins (Steyn, 1984). ochratoxin A (OTA), first isolated from *A. ochraceus* by van der Merwe et al. (1965b), is the most toxic of the ochratoxins (Chu, 1974). Other ochratoxins include the methyl and ethyl esters of OTA, ochratoxin B, and the methyl and ethyl esters of ochratoxin B. OTA are produced by several species of the genera *Aspergillus* and *Penicillium*. These molds are distributed widely in nature and have been isolated from a variety of foods and feedstuffs. *A. ochraceus* and members of the *A. ochraceus* produced significant ochratoxins in tropical and semi-tropical regions (Steyn, 1984). Scott et al. (1972) reported that *P. viridicatum* was the chief mold which grew in corn and grains stored at low temperature and was a common source of naturally occurring OTA in grains. While *A. ochraceus* and *P. viridicatum* are the main ochratoxin producers in the aspergilli and penicilli, toxin production has been recorded also from *A. alliaceus*, *A. malleus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, *A. sulphureus*, *P. cyclopium*, *P. purpurogenum*, *P. commune*, and *P. verruculosum* (Steyn, 1984).

A survey indicated that more ochratoxin-positive strains were found within *P. viridicatum* than *A. ochraceus* group (Pitt, 1987).

Influence of Environmental Factors on Ochratoxin A Formation

Several environmental factors affected the production of ochratoxins by *Aspergillus* and *Penicillium* groups (Bacon et al., 1973; Petterson and Damoglou, 1986). In natural environments, temperature and water activity are the most significant factors affecting toxin production (Bacon et al., 1973; Haggblom, 1982; Harwing and Chen, 1974). Effects of water activity (a_w) and temperature on growth and OTA production by strains of *A. ochraceus*, *P. cyclopium*, and *P. viridicatum* on agar medium, Edam cheese, and barley meal have been investigated by Northol et al. (1979). On agar medium the minimum a_w values for OTA production by *A. ochraceus*, *P. cyclopium* and *P. viridicatum* at 24°C were between 0.83-0.87, 0.87-0.90, and 0.83-0.86, respectively. At 24°C, optimum a_w values for OTA production by *A. ochraceus* and *P. cyclopium* were 0.99 and 0.95-0.99, respectively, whereas that of *P. viridicatum* was 0.95-0.99. At optimum a_w, OTA was produced by *A. ochraceus* in a temperature range of 12-37°C, whereas that for *P. cyclopium* and *P. viridicatum* was 4-31°C. Optimum temperature for OTA production by *A. ochraceus* was 31°C, whereas that for *P. cyclopium* and *P. viridicatum* was 24°C. On Edam cheese of

0.95 a_w, the minimum temperature for OTA production by *P. cyclopium* was 20°C. On barley meal, *P. viridicatum* produced maximal quantities of OTA at 0.97 a_w and could produce OTA at temperatures as low as 12°C. Production of ochratoxins in wheat and barley by *P. viridicatum* showed that the optimum a_w for both growth and OTA production was 0.97. Although growth was possible over range 0 to 31°C at a_w of 0.95, OTA was not produced between 12 to 24°C (Northol et al., 1979). In a study on the effects of the interaction between water activity and pH on mycotoxin production on a bread analogue, Lai et al. (1970) reported that *P. viridicatum* produced highest OTA on a medium containing sucrose, proline, glutamic acid or phenylalanine at pH 5.6 with 0.97 a_w at 25°C.

Chemical and Physical Properties of Ochratoxins

Ochratoxins are composed of a 3,4-dihydromethyl isocoumarin moiety linked through the 7-carboxyl group to L-β-phenylalanine by an amide bond and are classified according to biosynthetic origin as pentaketides within the group of polyketides. Ochratoxins consist of OTA, ochratoxin B, their methyl and ethyl esters, and 4-hydroxyochratoxin A (van der Merwe et al., 1965b). The basic structure of ochratoxins is shown in Fig. 1. Treatment of OTA with methanol and hydrochloric acid or other acid catalyst yields the methyl ester. Acid

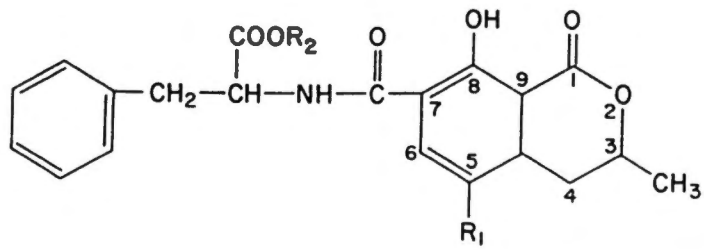


Figure 1-Chemical structure of ochratoxins.

	R_1	R_2
Ochratoxin A:	Cl	H
Ochratoxin B:	H	H
Ochratoxin C:	Cl	C_2H_5
Methyl ester of ochratoxin A:	Cl	CH_3
Methyl ester of ochratoxin B:	H	CH_3
Ethyl ester of ochratoxin B:	H	C_2H_5

Source: Wyatt, 1991.

hydrolysis of OTA was slow (30 hr reflux with 6 N hydrochloric acid) (van der Merwe et al., 1965a), but enzymatic hydrolysis by carboxypeptidase A or α-chymotrypsin occurred readily (Pitout, 1969). Hydrolysis of OTA through the amide bond yielded ochratoxin α and L-β-phenylalanine. Ochratoxin B was hydrolyzed at a faster rate than OTA.

OTA, obtained by crystallization from benzene, is a colorless crystalline compound. It has a melting point of about 90°C and contains approximately 1 mole of benzene. OTA is soluble in polar organic solvents and dilute aqueous sodium bicarbonate and is slightly soluble in water (Chu, 1974). Some physical properties of ochratoxins are shown in Table 1.

Stability of Ochratoxin A

OTA is a stable compound. It did not decrease substantially after one year storage in ethanol solution at refrigeration temperature (Chu and Butz, 1970). However, OTA is unstable in light (Neely and West, 1972). Some portion of naturally occurring OTA resisted autoclaving, the roasting procedure in the processing of coffee beans, and the procedures in brewing of beers (Chu et al., 1975). Degradation of OTA in cereal have been studied by Trenk et al. (1971). Only 36-47% and 47-48% of added OTA in rice and oatmeal were recovered after storage for 14 weeks at 4°C and 28°C, respectively. When cereals containing OTA were

Table 1-Physical properties of ochratoxins

Ochratoxin	Formula	Molecular Weight (amu[a])	Melting Point (°C)	Absorption Maxima (nm)[b]
Ochratoxin A	$C_{20}H_{18}ClNO_6$	403	169 (xylene)[c] 89-95 (benzene)	213-216; 332-334; 378-380
Ochratoxin B	$C_{20}H_{19}NO_6$	369	220-221 (methanol)	218; 318
Ochratoxin C	$C_{22}H_{22}ClNO_6$	431	NA[d]	213; 214; 333; 378
Ochratoxin D	$C_{20}H_{18}ClNO_7$	419	216-218 (benzene)	213; 314
Methyl ester ochratoxin A	$C_{21}H_{20}ClNO_6$	417	NA	213; 333; 331; 378
Methyl ester ochratoxin B	$C_{21}H_{21}NO_6$	383	134-135 (benzene)	218; 318
Ethyl ester ochratoxin B	$C_{22}H_{23}NO_6$	397	102-103 (ether)	218; 318; 364
Ochratoxin α	$C_{11}H_9ClO_5$	256	229-249 (acetone-benzene)	212-214; 217 336-338
Ochratoxin β	$C_{11}H_{10}O_5$	222	223	218; 323

[a]Atomic mass unit.
[b]Determined in ethanol solution.
[c]Solvent used in the crystallization.
[d]Not available.

Source: Chu, 1974.

autoclaved for 3 hr, 12.5-31.5% of OTA was still detected, and the amount of OTA residue depended on the moisture content of the cereal. In canning of beans, 11% of OTA was destroyed after one hour of heating at 121°C, and 57% of OTA in contaminated beans resisted a blanching, salting and heating process in tomato sauce for a one hour process (Harwing et al., 1974). Since OTA in contaminated grains may carry over to the beer, the stability of OTA in brewing has been studied. Krogh et al. (1974b) reported that 2-7% of the original amount of OTA was found in beer when highly contaminated barley was used in brewing, but no OTA was found when moderately contaminated barley was used. Chu et al. (1975) demonstrated the loss of OTA during beer brewing occurred mainly in the malt mash, boiled wort, and final fermentation steps.

Ochratoxin A in Plant Products

OTA has been isolated worldwide from corn, wheat, barley, oats, rye, sorghum, peanuts, coffee, beans, and animal feedstuffs (Yoshizawa, 1990). Natural occurrence of OTA in cereals and feedstuffs is presented in Table 2. In North America and Europe, most of the field contamination occurred in cereals, especially in corn, oats, wheat, and barley (Krogh, 1978; Shotwell et al., 1976). In Canada, OTA contamination of flour made from moldy red spring wheat was reported as 0.02 to 0.1 ppm. Rations containing the flour

Table 2-Natural occurrence of ochratoxin A (OTA) in cereals and feedstuffs

Country	Year	Commodity	No. samples examined	No. positive (% affected)	OTA level (ppb)[a]
United States	1967	Corn	283	1 (0.35)	110-150
	1968	Corn	293	3 (1.02)	83-166
	1971	Barley	127	18 (14.2)	10-40
	1973	Barley	180	23 (13.0)	10-37
	1974	Coffee beans	267	19 (7.1)	20-360
	1976	Wheat (red winter)	291	3 (1.0)	5-115
Canada	1968	Heated grains	29	18 (62.1)	30-27000
		Mixed feed	3	2 (66.7)	20-530
		Peanut	1	1 (100)	4900
		Wheat (red spring)	4	2 (50)	20-100
	1971-74	Wheat	95	6 (6.3)	30-6000
	1975-79	Barley, wheat, oat, rye	148	4 (2.7)	30-4000
		Forage	108	1 (0.9)	30-4000
		Corn	19	2 (10.5)	30-500
	1978-82	Feed (dairy cattle)	51	2 (3.9)	48-380
		Feed (beef cattle)	51	1 (2.0)	5900
		Feed (poultry)	51	1 (2.0)	140
Denmark	1973	Barley, oat (swine)	33	19 (57.6)	28-27500
	1978	Malt barley	50	3 (6.0)	9-189
Sweden	1972	Barley, oat	84	7 (8.3)	16-409.5
	1976	Barley	269	17 (6.3)	Up to 20
Yugoslavia	1971-75	Corn, wheat, Barley	47	6 (12.8)	5-90
Australia	1971-80	Goat feed	25	1 (4.0)	70000
France	1973-74	Maize	463	12 (2.6)	15-200
Poland	1974	Barley, wheat, rye, oat, maize	150	8 (5.3)	50-200
	1976	Mixed feed	203	10 (4.9)	10-50
	1975-80	Cereals	784	61 (7.8)	1100
	1981	Cereals	100	6 (6.0)	1200

Table 2-Natural occurrence of ochratoxin A (OTA) in cereals and feedstuffs (Continued)

Country	Year	Commodity	No. samples examined	No. positive & (% affected)	OTA level (ppb)*
Taiwan	1979	Chicken feed	499	215 (43.0)	
		Pig feed		(31.0)	
		Fish feed		(31.0)	
		Duck feed	3	1 (33.3)	
United Kingdom	1976-79	Barley	376	51 (13.6)	25-5000
		Wheat	101	15 (14.1)	25-2700
		Oat	46	1 (2.2)	80
		Pig meal	96	12 (12.5)	25-250
		Pig pellets	195	8 (4.1)	
		Cattle feed	318	1 (0.3)	
		Poultry feed	203	6 (3.0)	25-150
	1980-81	Turkey feed	11	11	10-130
		Broiler diet	2	2	10
		Cattle feed	11	2	10
		Pig feed	2	1	20
	1980	Maize	29	11	50-500
		Corn flour	13	4	50-500
		Soybean	25	9	50-500
		Soy products	28	6	50-500
		Cocoa beans	56	10	60-500
		Cocoa products	25	5	60-200
	1981	Breakfast cereals	243	12	5-108

*Parts per billion.

Source: Dwivedi and Burns, 1986.

subsequently caused the death of cattle (Scott et al., 1970). Extensive surveys of small grains for ochratoxins have been conducted in the United States and Canada. Among various small grains, ochratoxins were detected predominantly on wheat and barley. In the United States, 23 of 180 barley samples with OTA contamination of 0.01-0.037 ppm, 3 of 291 hard red winter wheat sample with OTA up to 0.035 ppm, 8 of 286 hard red spring wheat samples with OTA up to 0.115 ppm, and no contamination in 271 soft red winter wheat were reported (Shotwell et al., 1976). In Canada, 4 of 148 grain samples with OTA of 1-4 ppm and 6 of 315 grain samples with OTA of 0.003-0.008 ppm were reported (Prior, 1981). In the United Kingdom, OTA in moldy bread and flour from domestic environments was reported to be 0.21 mg/kg and 2.9 mg/kg, respectively (Osborne, 1980). In Italy, in a single incident that caused lethal gastroenteritis in poultry, rabbits, and dogs, a sample of moldy bread was found to be contaminated with 80 mg OTA/kg dry bread (Visconti and Bottalico, 1983). Since moldy bread and waste flour are often used as animal feed, high level of OTA in moldy bread and flour indicates that the use of moldy bread and waste flour for animal feed represents a threat to both animal and human health (Osborne, 1980; Visconti and Bottalico, 1983). No regulations for OTA in feedstuff have been established in the United States. In 1986, Sweden proposed a tolerance for OTA of 1000 μg/kg and 200 μg/kg of

feed for complete feeds for poultry and for swine, respectively (van Egmond, 1990).

Ochratoxin A in Animal Products

Although OTA is found mainly in plant products, it is frequently isolated from animal sources because of carry-over effect from contaminated feeds. Table 3 gives an indication of the extent of OTA contamination in animal tissues, animal products, and processed foods. When farm animals were fed feedstuffs containing OTA, part of the ingested OTA was retained in kidneys, liver, muscle, and fat tissue (Krogh et al., 1976a,b; Shreeve et al., 1978). OTA residues in tissues of domestic animals, including poultry and eggs, are a potential health threat to humans as OTA is known to have teratogenic effects and induce renal and hepatic tumors in mice and rats (Bendele et al., 1985; Kanisawa and Suzuki, 1978a,b; Lindenfelser et al., 1973; Purchase and van der Merwe, 1971).

In a study, pigs were exposed to dietary levels of 200, 1000, and 4000 μg OTA/kg feed for 3 to 4 months (Krogh et al., 1976b). At slaughter, the highest levels of OTA residues were found in the kidneys (50 μg/kg at the 4000 μg/kg feed level); lower levels were found in liver, muscle, and fat tissues. OTA was found in 18 out of 19 slaughtered pigs fed barley containing OTA, and the residue concentration was up to 67 μg/kg (Krogh, 1978).

Table 3-Natural occurrence of ochratoxin A (OTA) in animal tissues, animal products and processed foods

Country	Year	Commodity	No. samples examined	No. positive (% affected)	OTA level (ppb)[a]
A. Slaughtered animals					
Denmark	1972	Pig kidney	19	18	Up to 67
	1975	Pig kidney	60	21 (35)	Up to 68
Sweden	1975	Poultry muscle	14	5	4.3-29.6
	1976	Pig kidney	129	32 (25)	2-104
	1976-77	Pig kidney	34	34	32-218
		Liver	10	10	26-65
Yugoslavia	1977	Pork	12	1	5
	1979	Pig kidney	38	(6.6)	26-76
Hungary	1980-81	Pig kidney	122	(39)	5- >100
B. Animal products and processed foods					
United Kingdom	1982	Meat products	33	7	4
		Bakery products	8	3	80
		Fruits and nuts	5	1	trace
		Cheese	19	3	7
		Sugar and Confectionery	1	1	trace
Yugoslavia	1978-80	Ham	206	(28.9)	40-70
		Bacon		(18.9)	37-200
		Sausage		(12.0)	10-920

[a]Parts per billion.

Source: Dwivedi and Burns, 1986.

Generally, residues of OTA are not found in ruminants because OTA is cleaved in the forestomach by protozoal and bacterial enzymes (Galtier and Alvinerie, 1976; Hult et al., 1976). The nontoxic cleavage product of OTA, ochratoxin α, has been found in kidneys and blood of calves fed a diet containing 300 to 500 μg OTA/kg feed (Patterson et al., 1981). Two lactating dairy cows fed a ration containing 317 to 1125 μg OTA/kg feed for 11 weeks remained clinically normal throughout the feeding period (Shreeve et al., 1978). There was no obvious effect on milk production. A residue of 5 μg OTA/kg was found in the kidneys of one cow. No ochratoxin α was found in the muscle, liver, kidneys, serum, milk, and urine of the cows. However, in another study when 13.3 mg OTA/kg body weight was administrated, significant amounts of OTA (650 mg) and ochratoxin α (4500 mg) were detected in milk (Ribelin et al., 1978).

In hens fed diets containing 1 mg OTA/kg feed, the highest residue of OTA was found in the kidneys with a mean 19 μg/kg tissue (Elling et al., 1975). Liver and muscle contained much lower OTA residues, and no OTA was found in the eggs. In another study, chickens were given low levels of OTA for 341 days. OTA residues were found in the muscles, liver, and kidneys, with values ranging from 9.1 to 49.5 μg/kg (Krogh, 1976a). Hens (three week old at the beginning of the study) were fed 1 mg OTA/kg feed for five weeks (Reichmann et al., 1982). The kidneys contained 3 to

10 μg OTA/kg and the liver contained 1.5 to 2.5 μg OTA/kg. Tissue deposition and passage of OTA into eggs were studied by Juskiewicz et al. (1982). Plymouth Rock laying hens were given either single oral doses of 1, 5, or 10 mg OTA/kg body weight or fed an OTA-contaminated diet (2.5 or 10 ppm) for seven days. Residues of OTA were found in blood cells, plasma, muscle, liver, kidneys, and ovarian follicles 12 hr after a single dose of 1 mg OTA/kg body weight. The greatest amounts (8.6-8.7 ppb) were found in the kidneys and liver, and OTA levels in tissues increased with increasing dosage of OTA. In hens fed 2.5 ppm OTA, residues were detected on the second day in the liver (3.5-5.5 ppb) and kidneys (3.8-4.6 ppb) (Juszkiewicz et al., 1982). At 10 ppm in the diet, OTA residues were found in the kidneys (4.0-5.9 ppb), liver (3.8-5.2 ppb), plasma (4.0-10.2 ppb), and ovarian yolk (0.9-1.7 ppb). After four days of feeding OTA at 10 ppm, OTA was detected at 1.2 and 2.3 ppb in egg yolk and muscles, respectively (Juszkiewicz et al., 1982).

Toxicity of Ochratoxin A

The structure of ochratoxin necessary for intoxication has been studied by Chu et al. (1972). They demonstrated that ochratoxin C was not toxic after the phenolic hydroxyl group was chemically modified. Therefore, they suggested that the phenolic hydroxyl group in the dissociated form was necessary for OTA toxicity.

OTA is a nephrotoxin which is implicated in nephrotoxic syndromes in pigs (Krogh, 1978; Krogh et al., 1974a) and humans (Pavlovic et al., 1979). It has a major role in the etiology of nephritis in Scandinavian pigs (Krogh, 1990), and it is also a serious human health risk in rural areas of Scandinavia. OTA may be a causal agent of Balkan endemic nephropathy, a kidney disease with a high mortality rate in certain areas of Bulgaria, Yugoslavia, and Romania (Krogh et al., 1977). The initial toxicological effect of OTA was demonstrated in the nephron, and the proximal tubule was the primary target site. Damage of proximal tubular function was observed in swine administered OTA (Krogh et al., 1977). Susceptibility of vertebrates to OTA varied among species. Ducklings, chicks, dogs, and rainbow trouts were among the most susceptible species, while laboratory rodent animals and ruminants such as cattle and sheep appeared less susceptible (Prior and Sisodia, 1982). Experimental ochratoxicosis A was demonstrated in a variety of avians (Gilani et al., 1978), ruminants (Ribelin et al., 1978), rodents (Hood et al., 1978; Kanisawa et al., 1977), and fish (Doster et al., 1974). The effects of OTA on various biological systems such as chicken embryos (Choudhury and Carlson, 1973), zebra fish larvae (Abedi and Scott, 1969), brine shrimp (Brown, 1969), bacteria (Singer and Roschenthaler, 1978), and tissue cultures (Steyn et al.,

1975) have been studied. Acute toxicity of OTA to various animals and biological systems is shown in Tables 4 and 5.

Toxicity of ochratoxin A in poultry

OTA is especially important to the poultry industry (Steyn, 1984). Three outbreaks of ochratoxicosis in about 360,000 turkeys were characterized by up to 55% mortality, nephrotoxicity, and decreased consumption of feed prior to death (Hamilton et al., 1977). Outbreaks of ochratoxicosis in turkeys, laying hens, and broilers usually result in extremely high mortality (Hamilton et al., 1982). In a feeding experiment with hens (Choudhury et al., 1971), 2 and 4 mg OTA/kg feed caused highest death, morbidity, and evidence of stress of hens. Hens receiving 4 mg OTA/kg diet showed markedly depressed body weight and no sexual maturity, and hens at lower levels of OTA showed delayed sexual maturity. Egg production of hens on 1 mg OTA/kg diet was moderately decreased and that of the 2 mg OTA/kg diet groups was poor. Hens fed 4 mg OTA/kg diet had not laid eggs through one year of age. Hens receiving increased levels of OTA showed decreased efficiency in converting feed to eggs. Feeding OTA to hens also reduced the ability to hatch of fertile eggs and depressed subsequent growth of chicks for the first two weeks of life. In a study by Chang et al. (1980), turkeys were fed diets containing various doses of OTA from one day to three weeks of age. Diets

Table 4-LD_{50} of ochratoxin A in various animals

Animal	Test System and Route of Administration	LD_{50} mg/kg Body Weight
Chick	Day-old, oral intubation	2.14
	3-week-old, peroral	3.6
Turkey	Day-old Nicholas white	5.9
Cattle	5-week-old Holstein calves, oral-stomach tubing	11-25
Swine	Feeding daily	1-2
Fish	6-month old Mt. Shasta intraperitoneal	4.67
Rainbow trout	Peroral	4.7
Mice	Female, intraperitoneal	22-44.7
	Swiss mouse, Intravenous	25.7-33.9
	Swiss mouse, feeding	58.3-62.4
Rats	Female, intraperitoneal	14.3
	Male, intraperitoneal	12.6
	Female, peroral	21.4
	Male, peroral	28.0
Guinea pigs	Male, peroral	8.1
	Female, peroral	9.1
Japanese quails	Day-old, peroral	16.5

Source: Tong, 1982; Krogh, 1990.

Table 5-Acute toxicity of ochratoxin A

Biological System	Test System and Route of Administration	Acute Toxicity
Chicken embryos	Zero day injection	5-10 µg/egg
	5-day-old egg	16.95 µg/egg
	8-day-old egg	7 µg/egg
Chick	Day-old, peroral	135-166 µg/chick
Duckling	Day-old, feeding	25 µg/duckling
Zebra fish larvae	Aqueous solution	10.1 µg/mL
Bacillus subtilis	Culture medium	12 µg/mL[a]
B. megaterium	Culture medium	4 µg/mL[a]
B. cereus mycoides	Disk assay	1.5 µg/disk[a]

[a]Minimum concentration of growth inhibition.

Source: Harwing, 1974; Krogh, 1990.

containing 16 mg OTA/kg feed caused a 100% cumulative mortality at three weeks of age. Diets containing 4 and 8 mg OTA/kg feed significantly retarded growth rate, and the growth retardation was dose dependent. The size of the thymus relative to body weight was decreased significantly in diets containing 4 and 8 mg OTA/kg feed. Since the thymus is the primary determinant of cell-mediated immunity, this implied that cellular immunity in turkeys might be impaired during ochratoxicosis.

Toxicity of ochratoxin A in ruminants

Ribelin et al. (1978) investigated toxicity of OTA to ruminants. Two five-week-old Holstein calves were given single doses of 11 and 25 mg OTA/kg body weight by stomach tube. Both calves died within 24 hr. A cow which was three to six months pregnant was given 13.3 mg OTA/kg body weight by stomach tube as a single dose. This cow had difficulty in arising, diarrhea, anorexia, and abrupt cessation of milk production, all beginning one day after dosing. Although recovery was complete by day four, milk production never increased above one-third of normal during that lactation period. American La Mancha female goats treated with a daily oral dose of 3 mg OTA/kg body weight developed watery diarrhea, became dehydrated and died on day five. However goats on 1 and 2 mg OTA/kg body weight had no clinical signs of illness after 14 days. The amount of OTA required to

produced acute toxicity in ruminants made such an occurrence unlikely. However, chronic low-level intoxication was more likely to occur, and young ruminants likely suffered more severe illness than adult ruminants (Riebelin et al., 1978). The ability to hydrolyze OTA to the less toxic ochratoxin α by ruminants accounted for the reduced toxicity of OTA to ruminants (Hult et al., 1976).

Tumorigenesis of ochratoxin A

Genotoxicity tests with the *Rec* assay using *Bacillus subtilis* and the Ames test using *Salmonella typhimurium* showed that OTA had no DNA-attacking ability or mutational potential (Ueno and Kubota, 1976; Ueno et al., 1978). However, long-term feeding experiments indicated that OTA could cause renal and hepatic tumors in mice and rats (Bendele et al., 1985; Kanisawa and Suzuki, 1978a,b; Lindenfelser et al., 1973; Purchase and van der Merwe, 1971). Kanisawa and Suzuki (1978b) reported that male ddY mice fed a diet containing 40 mg OTA/kg body weight resulted in hepatic-cell tumors in five of nine treated mice within 49 weeks, and tumors were found also in nine of nine treated mice. OTA may also act as a promoter of carcinogenesis. Studies demonstrated that OTA had a synergistic effect on aflatoxin B_1 or citrinin carcinogenicity (Kanisawa, 1984; Kanisawa and Suzuki, 1978a,b).

Teratogenesis of ochratoxin A

The fetotoxic and teratogenic effects of OTA in chicken embryos (Choudhury et al., 1971; Gilani et al., 1978; Vesela et al., 1983) hamsters (Hood et al., 1976), mice (Hayes et al., 1974), and rats (Still et al., 1971) have been studied. Short and twisted limbs and neck, everted viscera, and reduced body size were observed in chicken embryo after doses of 7 μg OTA/egg was injected into chicken eggs through the air sacs after 48, 72, and 96 hr of incubation (Gilani et al., 1978). OTA was very toxic to chick embryos. The LD_{50} for chick embryos was less than 0.01 μg for six-day-old eggs injected via the air cell (Choudhury et al., 1971). Pregnant golden hamsters administered intraperitoneal injections of OTA at doses of 2.5-20 mg/kg body weight showed high prenatal mortality, fetal malformation, and diminished fetal growth (Hood et al., 1976). Malformations such as short tail, cleft lip, hydrocephalus, micrognathia, and heart defects were observed. Pregnant mice treated with OTA at a dose of 5 mg/kg body weight by intraperitoneal injection resulted in high prenatal mortality, low fetal weight, and malformation of head, eyes, face, digits, and tails of fetus (Hayes et al., 1974). Studies showed that OTA produced severe craniofacial abnormalities in test animals (Brown et al., 1976; Hood et al., 1978). OTA administered orally at levels of 6.26-25 mg/kg body weight induced fetal death and resorption in pregnant rats (Still

et al., 1971). The prenatal toxicity and teratogenicity of OTA was enhanced by coadministering citrinin and/or a low protein diet (Mayura et al., 1983; Mayura et al., 1984a,b).

Biochemical effects of ochratoxin A

One of the toxic effects of OTA in biosystems is the inhibition of protein synthesis (Heller and Roschenthaler, 1978). OTA has been reported to inhibit protein synthesis in bacterial, yeast (Boutibonnes, 1980), and mammalian cells (Roschenthaler et al., 1984). Since OTA is composed of an isocoumarin and L-phenylalanine moiety, the phenylalanine moiety competes with phenylalanine for phenylalanyl-tRNA synthetase, and thus OTA acts as a competitive inhibitor of this enzymatic process (Creppy et al., 1983,1984). Roschenthaler et al. (1984) demonstrated that mammalian phenylalanyl-tRNA synthetase had high affinity for OTA and low affinity for phenylalanine. The acute toxicity and cytotoxicity effects of OTA were reduced by increasing L-phenylalanine in diet (Creppy et al., 1980; Haubeck et al., 1981; Moroi et al., 1985). OTA may also act as an inhibitor of the mitochondrial transport system (Cole and Cox, 1981). Another toxic effect of OTA is immunosuppressive potential. The major effects of OTA on the immune response appear to be on antibody formation and, to a lesser extent, on phagocytosis (Prior and Sisodia, 1982). OTA was demonstrated to cross the placental barrier of rodents and

exert its toxic effects on the unborn fetus (Pier and McLoughlin, 1985; Thurston et al., 1986). Therefore, toxic effects of OTA on immune response is particularly important in the newborn. Studies revealed that OTA suppressed the immune response to sheep erythrocytes in BALB/c mice at oral doses as low as 0.005 μg OTA/kg body weight, and this effect was prevented by phenylalanine (Haubeck et al., 1981). Mice injected intraperitoneally daily for 50 days with 5 mg OTA/kg body weight had a significantly depressed antibody response to kill *Brucella abortus* (Prior and Sisodia, 1982).

Metabolism of Ochratoxin A

Although the pathological changes induced by OTA have been studied in some detail in several animal species, the fate of OTA *in vivo* and *in vitro* is not completely understood. Several mammalian enzyme systems biotransform OTA into isocoumarin and hydroxylated metabolites in both *in vivo* and *in vitro* systems. Galtier (1974) reported that the half-life of OTA was about 55 hr after either oral or intravenous administration in rats, and 56% of the toxin was excreted via urine and feces as free metabolites and ochratoxin α after 120 hr. Storen et al. (1982) also reported that OTA injected intraperitoneally into rats was partly metabolized to ochratoxin α and 4-hydroxyochratoxin A which appeared in the urine and feces along with unchanged OTA. The 4-hydroxyochratoxin A had no toxic effect when

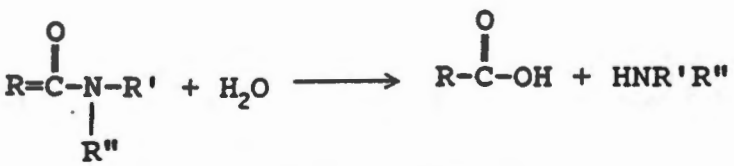
dosed to rats at 40 mg/kg body weight (Hutchison et al., 1971). OTA at 40 mg/kg body weight caused 100% mortality to rats; therefore, 4-hydroxyochratoxin A may be a detoxification product in animals dosed with OTA (Hutchison et al., 1971). Experiments with ^{14}C OTA in rats indicated that the toxin reached its highest levels in serum (90% of ^{14}C dose), liver (4.5%), and kidneys (4.4%) 30 min after intraperitoneal injection and decreased thereafter (Chang and Chu, 1977). Disc-gel electrophoresis of rat serum revealed that the toxin was predominantly bound to the serum-albumin, and the concentration of bound OTA was much higher than that of free OTA in the early stage. The higher the OTA concentration in the serum, the higher the amounts of OTA present in the liver and kidneys. Kidneys and liver contained only 1-2% of the radioactive OTA after 24 hr. Although the total amount of OTA in the liver and kidneys was eventually similar, the peak concentration of OTA in the kidneys was nearly twice as much as that in the liver. In the first 8 hr, OTA in serum, liver, and kidneys decreased exponentially and the toxin was excreted through urine in the forms of unaltered OTA and ochratoxin α or its conjugates. Only 13% of the injected radioactive OTA was excreted in the feces during the first 24 hr, and 77% of this was identified as unaltered OTA. Ochratoxin α was also found in the feces (Chang and Chu, 1977). When pigs were exposed to OTA, the toxin was found in kidneys, liver, and

fat tissues. This indicated that OTA was absorbed from the alimentary canal without prior degradation (Krogh et al., 1974b). In contrast to pigs, OTA in ruminant system was subjected to some degree of degradation before uptake by tissues. Hult et al. (1976) reported that when OTA was incubated with the contents of stomachs of cows, OTA was hydrolyzed into ochratoxin α and phenylalanine by the rumen microorganisms. This hydrolyzing effect was only found in the contents from the first stomach of the cow. They also calculated that cows should be able to degrade OTA in feed contaminated with up to 12 mg OTA/kg in 48 hr. Ochratoxin α is relatively nontoxic; therefore, ruminants are less sensitive to OTA toxicosis (Yamazaki et al., 1971).

Enzymatic Hydrolysis of Ochratoxin A

Carboxylesterases are capable of catalyzing hydrolytic reactions of the following types:

Carboxylester Hydrolysis



Carboxyamide Hydrolysis

Carboxythioester Hydrolysis

The first two reactions are equally relevant for detoxification processes (Heyman, 1980). Hydrolysis of an ester or amide bond in a toxic compound is considered to be a detoxification because of increased hydrophilicity and accelerated excretion. β-esterases are a class of carboxylesterases, and carboxypeptidase is a β-esterase. Characteristic substrates for β-esterases include esters and amides. β-esterases have been found in almost all mammalian tissues, particularly in liver, with the esterase activity toward simple aliphatic and aromatic substrates. Relatively high amounts of β-esterases are also found in proximal tubules and other regions of the kidneys. Small intestinal epithelial cells of rats contain considerable amounts of β-esterase (Heymann, 1980). Esterase activity has been found in the cell-free growth medium and on the cell surface of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1. The enzyme catalyze the hydrolysis of acetyl and other acyl groups from triglycerides and aryl and alkyl esters (Shabtai and Gutnick, 1985). Therefore, this microorganism may be able to degrade OTA.

OTA was hydrolyzed *in vitro* to an isocoumarin derivative (ochratoxin α) and L-phenylalanine by carboxypeptidase A, α-chymotrypsin, and possibly cathepsin C which was from lysosomes (Pitout, 1969). The process hydrolyzed the amide bond with the formation of ochratoxin α and phenylalanine. This enzymatic hydrolysis take place in the stomach of cows as a result of the activity of symbiotic microorganisms (Hult et al., 1976; Kiessling et al., 1986). Carboxypeptidase is most active on substances containing a terminal aromatic amino acid; this is the reason that OTA is hydrolyzed by the enzyme (Pitout, 1969). Therefore, ruminants have a lower sensitivity to OTA than other animals. The isocoumarin carboxylic acid, ochratoxin α, is very much less toxic than the parent OTA in all acute toxicity in *in vivo* and *in vitro* test systems (Yamazaki et al., 1971). Ochratoxin B was hydrolyzed six to seven times faster by carboxypeptidase than OTA (Doster and Sinnhuber, 1972). OTA was also hydrolyzed to ochratoxin α by the microbial flora in centrifugal pellet of rat caecal contents (Galtier and Alvinerie, 1976). The fraction responsible for this transformation of OTA contained protozoans mainly. The supernatant liquid was lacking in this activity. Galtier and Alvinerie (1976) found that a large proportion of OTA was lost when OTA was incubated with rat caecal contents. They suggested that non-fluorescent metabolites, not ochratoxin α, were produced from the breakdown of the

lactone group of the isocoumarin ring. However, the theory has not been studied and proven. The rumen contents of cow can hydrolyze OTA also (Hult et al., 1976). From these evidences, some researchers suggest that the initial microbial degradation of OTA is hydrolysis of the susceptible peptide bond by a peptidase, forming ochratoxin α and phenylalanine (Doster and Sinnhuber, 1972; Pitout, 1969).

Control of Ochratoxins in Foods and Feedstuffs

Strain of the mold, substrate composition, temperature, and water activity affected the production of ochratoxins by *Aspergillus* and *Penicillium* while foods and feedstuffs were stored (Lai et al., 1970; Northol et al., 1979; Petterson and Damoglou, 1986). An understanding of environmental parameters provides us with potential tools for preventing mycotoxin production in foods, feedstuffs, and the raw materials used for their manufacture. In addition to controlling the environmental factors, other approaches have been studied or suggested to prevent mycotoxin production.

Physical treatments

Usually, mycotoxins in raw agricultural commodities are contained in a very small proportion of individual seeds or kernels. Therefore, an effective and economical method of reducing the mycotoxin contents was to remove the few

contaminated seeds or kernels mechanically (Goldblatt and Dollear, 1977).

Heat treatment of OTA-contaminated raw agricultural commodities is another practice to control OTA. Some mycotoxins were destroyed at around 100°C during conventional processing of foods and feeds. More than 90% of the toxins isolated from fungi melt at temperatures above 100°C, and 70% of them have melting points in the range 150 to 250°C (Ciegler and Vesonder, 1983). Roasting or frying for up to 30 min at temperature of 150 to 200°C was shown to effectively degrade 90% of contaminated OTA (Tsubouchi et al., 1987). Although 100% degradation of added OTA was reported in coffee beans roasted at 200°C for 5 min, only 0-12% was degraded at 200°C for 10-25 min when the beans were inoculated with a toxigenic strain of *A. ochraceus*. It was suggested that the toxins deposited inside the kernels by the penetrating mycelia were protected to a greater extent than when present only on the surface in spiked sample (Tsubouchi et al., 1987). Therefore, when foods or feeds are treated with heat, mycotoxins may be protected from degradation by low heat penetration or stabilized by "binding" with food constituents of the samples.

Chemical treatments

Many chemicals were reported to destroy mycotoxins (Tong and Draughon 1985; Vandegraft et al., 1975; Wu and

Ayres, 1974). However, the chemicals able to do so without leaving deleterious residues or without the loss of nutrients appear to be limited. Wu and Ayres (1974) investigated the effect of dichlorvos, an organophosphate insecticide, on ochratoxin production. Dichlorvos at a level of 0.1 mg/100 mL yeast extract sucrose medium had no effect on the growth or ochratoxin production by either of two strains of *A. ochraceus*. At 1 mg dichlorvos/100 mL medium, growth was not affected, but OTA production was inhibited by 27% (strain NRRL 3174) and 48% (strain UGa No. 40). With dichlorvos at a concentration of 10-30 mg/100 mL medium, growth of strain UGa No. 40 was retarded by 9-20%, and production of OTA was inhibited by 72-79% and that of ochratoxin B by 62-89%. At this level of dichlorvos, growth of strain NRRL 3174 was retarded by 11-18% and production of OTA was inhibited by 50-74% and that of ochratoxin B by 49-78%. Dichlorvos also inhibited ochratoxin production by *A. ochraceus* on corn. At 0.1 mg dichlorvos/100 g corn, OTA production by either strain was not inhibited. At 1-30 mg dichlorovs/100 g corn, 25-75% and 25-83% of the OTA and ochratoxin B ordinarily produced by strain UGa No. 40 was inhibited. At the same dosage, OTA production by strain NRRL 3174 was reduced by about 25-87% and that of ochratoxin B by 50-90%.

The preservation of high moisture cereals for use as winter feed depends traditionally on reduction of the pH by lactic acid fermentation (Larsen et al., 1972). Proper use of these acids is very effective in preventing the production of mycotoxins. Vandegraft et al. (1975) reported that the addition of acetic or propionic acid into high moisture corn at a concentration of 0.1% completely inhibited the production of ochratoxins and aflatoxins.

The effects of antimicrobial food additives on growth and OTA production by strains of *A. sulphureus* NRRL 4077 and *P. viridicatum* NRRL 3711 on yeast extract sucrose broth have been investigated by Tong and Draughon (1985). Mycelium and toxin production was completed inhibited by 0.02%, 0.067%, 0.0667%, and 0.2% potassium sorbate, methyl paraben, sodium bisulfite, and sodium propionate at pH 4.5. At pH 5.5, 0.134% potassium sorbate and 0.067% methyl paraben were able to completely inhibit the mycelium and toxin production.

CHAPTER III

MATERIALS AND METHODS

Microbial Screening for Ochratoxin A Degradation

Bacteria, yeasts, and molds (Table 6) obtained from several research institutes or the Department of Food Technology and Science of The University of Tennessee, Knoxville, were screened for capability of degrading OTA by using plate assay (Ciegler et al., 1966) and test tube assay methods. The test tube assay was a modified method of the flask assay of Ciegler et al. (1966).

Plate assay

In the plate assay, a modified Czapek-Dox medium (Appendix 1), a non-fluorescent medium under UV light, was used as the growth medium for all test microorganisms. One mL of ochratoxin A (OTA) (40 μg/mL in methanol) was dispensed into petri dishes and 18 mL of medium (55°C) was added to each petri dish and mixed evenly with OTA solution. Lids of petri plates were opened partially to dissipate methanol vapor. After medium was solidified, plates were point inoculated with test microorganisms. Inoculated plates were incubated at 30°C and were examined under UV light at one day intervals for up to one week.

Table 6-List of microorganisms screened for ochratoxin A degradation

Bacteria
Acetobacter aceti
Achromobacter xerosis NRRL B-1596
Acinetobacter calcoaceticus NRRL B-551
Aeromonas hydrophila
Alcaligenes faecalis NRRL B-170
Arthrobacter oxydans
Azospirillum brasilense
Azospirillum lipoferum
Bacillus sp.
Bacillus cereus
Bacillus thuringiensis israelensis
Brevibacterium butanicum
Chromobacterium violaceum NRRL B-4369
Chromobacterium viscosum
Corynebacterium dioxydan
Escherichia coli NRRL B-3704
Flavobacterium auranticatum NRRL B-184
Flavobacterium devoran NRRL B-54
Gluconobacter oxydans
Klebsiella pneumoniae
Lactobacillus lactis
Lactobacillus plantarum C-11
Leuconostoc mesenteroides ATCC 8293
Micrococcus luteus
Microbacterium lacticum
Pediococcus pentosaceus FBB-61-6
Propionibacterium sp.
Proteus vulgaris
Pseudomonas aeruginosa
Pseudomonas fluorescens
Salmonella infantis
Selenomonas ruminantium
Spirillum volutans
Staphylococcus aureus
Streptococcus bovis
Streptococcus faecalis NRRL B-537
Xanthomonas campestris NRRL B-1459

Yeasts
Candida albican NRRL Y-477
Hansenula sp.
Kluyverdmyces fragii
Pichia membranaefaciens
Rhodotorula sp.
Saccharomyces cerevisiae ATCC 9080
Saccharomyces ellipsoides
Saccharomyces rouxii
Torulopsis magnoliae
Zygopich chevaliereii

Molds
Aspergillus niger
Aspergillus versicolor
Cladosporium
Fusarium semitectum
Geotrichum sp.
Mortierella sp.
Mucor sp.
Mucor attenvatus
Penicillium rubrium NRRL 6216
Penicillium urtical NRRL 994
Rhizopus nigricans
Spicaria violacea
Trichosporium sp.
Zygorhynchus sp.

Test tube assay

In the test tube assay, media used for growing fungi and bacteria were MY broth (Appendix 1) and modified TGY broth (Appendix 3), respectively. To 9 mL of broth was added 1 mL of OTA (20 μg/mL in 0.1 M sodium bicarbonate). Test microorganisms were inoculated. Tubes were incubated at 30°C. After 72 hr, cell-free filtrates were spotted on Redi-Plate silica gel G thin layer chromatography (TLC) plates (Fisher Scientific Co., Pittsburgh, PA). The TLC plates were developed in a solvent system containing benzene and acetic acid (9:1). After plates were developed and dried, they were observed under UV light for fluorescence. The degradation of OTA was indicated by absence of fluorescent spot or a weaker fluorescent spot than that of control on TLC plates. The degradation of OTA also was indicated by fluorescent spots that were identical to ochratoxin α which was obtained by hydrolyzing OTA with carboxypeptidase A (Sigma Chemical Co., St. Louis, MO).

Identification of *Acinetobacter calcoaceticus*

Microbial identification was performed only on test microorganisms that showed possible OTA degradation capability to confirm the identity of the microorganism. *Acinetobacter calcoaceticus* NRRL B-551 obtained from Northern Regional Agricultural Research Service of USDA (Peoria, IL) was found to degrade OTA. Identification tests

such as Gram's stain, motility examination, oxidase and catalase tests, and growth requirement were performed on this culture. Test results were compared with the characteristics of *A. calcoaceticus* described in Bergey's Manual of Systematic Bacteriology (Kriog, 1984).

Hydrolysis of Ochratoxin A by *A. calcoaceticus* in Ethanol-Minimal Salts Medium at 25°C and 30°C

A. calcoaceticus NRRL B-551 obtained from Northern Regional Agricultural Research Service of USDA (Peoria, IL) was found to degrade OTA in our preliminary studies. The degradation of OTA by this bacterium in a broth medium was studied. *A. calcoaceticus* were grown on ethanol-minimal salts medium with pH 7.5±0.02 (Appendix 2) (Shabtai and Gutnick, 1985). Flasks containing 18.8 mL of ethanol-minimal salts medium and 1 mL of 0.1 M sodium bicarbonate with OTA (Sigma Chemical Co., St Louis, MO) concentration of 0 and 0.2-1.0 mg/mL was inoculated with 0.2 mL 24-hr-old *A. calcoaceticus* to provide approximately 10^6 cells/mL and OTA concentrations of 0, 10, 20, 30, 40, and 50 μg/mL. Incubation was performed in a shaking water bath (Fisher Scientific, Pittsburgh, PA) at 25°C and 30°C in the dark. Flasks were agitated by reciprocal shaking at a rate of 90 strokes/min. At 24 hr intervals for up to 168 hr, an aliquot was withdrawn from flasks for analysis of cell numbers (by plating method) and residues of OTA (by high

performance liquid chromatography). The experiment was performed in duplicate.

A. calcoaceticus enumeration

At each sampling period, 1-mL aliquot from each sample flask was withdrawn and diluted serially with sterile 0.1% peptone water. One-tenth mL of dilution was spread-plated on pre-poured standard methods agar (Becton Dickinson and Co., Cockeysville, MD). The plates were incubated at 30°C for 48 hr.

Quantitation of ochratoxin A

At each sampling period, 0.5-mL aliquot was withdrawn from each sample flasks. The reaction between cells and OTA was terminated by adding 0.2 mL of 1 N HCl (Hult et al., 1976). The aliquot was centrifuged in a Fisher micro-centrifuge (Fisher Scientific Co., Pittsburgh, PA) at 12,400 rpm (136,000 x g) for 5 min. Supernatant was decanted and extracted with 2 mL of chloroform. The extraction was repeated three to five times until fluorescence was not detected in the water phase. Extracts were pooled and filtered through Acrodisc filter assembly (0.2 μm pore size) (Gelman Sciences, Ann Arbor, MI) and anhydrous sodium sulfate. Filtrate then was passed through a Sep-Pak silica cartridge (Waters Associates, Inc., Milford, MA). The cartridge was washed with chloroform and flushed with

nitrogen to remove residual chloroform. OTA was eluted with methanol until no fluorescence was detected in eluent. The eluent was evaporated under nitrogen and redissolved in methanol to original sample volume (Scott et al., 1971; Storen et al., 1982). Quantitation of OTA was carried out using high performance liquid chromatography (HPLC) (Waters Associates, Inc., Milford, MA) on a μ-porasil column with benzene-acetic acid-methanol (90:10:5) as eluting solvent at a flow rate of 1 mL/min. A model 420 fluorescence detector with a 340 nm excitation filter and a 440 nm emission filter (Waters Associates, Inc., Milford, MA) was used. The injection volume was 10 μL. The concentration of OTA in samples were calculated based on fluorescent intensity according to a standard curve obtained with OTA standards of 1-50 μg/mL.

Isolation of Crude Enzyme from *A. calcoaceticus*

Since the mechanism for degradation of OTA by *A. calcoaceticus* was suspected to be enzymatic, a procedure for isolation of carboxypeptidase A from bovine pancreas (Allan et al., 1963) was followed to isolate the potential enzyme(s) from growth medium of *A. calcoaceticus*. The enzyme isolation was carried out at 4°C unless otherwise specified. Ten mL of 24-hr-old *A. calcoaceticus* culture were inoculated into 2 L of ethanol-minimal salts medium in a 2.8 L Erlenmeyer flask. The flask was incubated at 30°C

under aerated condition using a stirring bar. After 72 hr, cell suspension was centrifuged in an IEC B-20A centrifuge (International Equipment Co., Needham Heights, MA) at 15,000 rpm (12,000 x g) for 20 min. The clear supernatant was collected and pooled. To the supernatant, solid finely ground ammonium sulfate was added slowly with mechanical stirring. Each addition of salt was dissolved completely before the next was added. At the same time, the solution was adjusted to pH 7.5 with 1 N NaOH. Salt addition was stopped when the solution was saturated. The precipitate (crude proteins) induced by ammonium sulfate was allowed to settle overnight. The supernatant fluid was siphoned off, and the precipitate was collected by centrifugation in an IEC B-20A centrifuge at 15,000 (12,000 x g) rpm for 20 min. The precipitate was redissolved in 20 mL distilled water. The protein suspension was dialyzed in cellulose dialysis tubing (Spectrapor Membrane Tubing, Spectra/pro4; Fisher Scientific, Pittsburgh, PA) in 500 mL distilled water for 48 hr with distilled water being changed every 12 hr. Solution obtained after dialysis contained crude enzyme protein. The concentration of crude protein was determined by Bio-Rad protein microassay procedure using bovine serum albumin as standard (Bio-Rad Labs, Richmond, CA).

Crude Enzyme Profile by Disc-Gel Electrophoresis

The protein profile of crude enzymes isolated from *A. calcoaceticus* was performed by using disc-gel electrophoresis (Cooper, 1977; Christen, 1991). The disc-gel electrophoresis was carried out in a Bio-Rad model 120 apparatus (Bio-Rad Labs, Richmond, CA). Reagents used for the electrophoresis are listed in Appendix 3. Separating gel was filled into glass tubes to within 2 cm of the top. Separating gel was allowed to polymerize undisturbed for 30 min. Stacking gel (0.1 mL) then was delivered to each tube. Tubes were exposed to fluorescent light for 30 min until polymerized. Ten μL of crude enzyme and 5 μL carboxypeptidase A (as standard) were applied to the top of stacking gel, separately, and 2 drops tracking dye were added, followed by 0.1 mL of stacking gel. Tubes were covered with parafilm and mixed by gentle inversion several times. Gel was allowed to polymerize. Electrical current of about 10 mA/tube was applied until tracking dye reached the bottom of the gels. After removal from tubes, gels were stained with brilliant blue G-perchloric acid solution (Sigma Chemical Co., St. Louis, MO) overnight. Proteins were displayed by blue bands on gels. The location of proteins was recorded by the length of proteins migrating from top of the gels.

Three sample gels (without stain) with the portion of 5.5 cm from top of the gels (portion containing proteins had

the similar location of those of carboxypeptidase A) were ground into slurry by a glass rod in a 100 mL beaker with 10 mL 0.1 NaCl-0.02 M Tris buffer (pH 7.5). The slurry was stirred constantly for 30 min and filtered through an Acrodisc filter assembly (0.45 μm pore size) (Gelman Sciences, Ann Arbor, MI). OTA was added to 3 mL of filtrate to a concentration of 10 μg OTA/mL. The change of OTA concentration in solution was monitored using the method of Pitout (1969) at 24-hr intervals for five days.

Hydrolysis of Ochratoxin A by Crude Enzyme Isolated from *A. calcoaceticus*

The method of Pitout (1969) for testing hydrolysis of OTA by carboxypeptidase was followed. Crude enzyme and OTA were diluted with 0.1 M NaCl-0.02 M Tris buffer (pH 7.5) to protein concentrations of 0.1 mg, 0.15 mg, and 0.2 mg protein/mL and OTA concentrations of 10, 20, 30, 40, 50, and 60 μg/mL solution. Reaction was carried out at 25°C. Absorbance of OTA at 380 nm was monitored by HP 8452 UV-VIS spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) at 24 hr intervals up to 192 hr. The concentration of OTA at each sampling period was determined by using a standard curve based on the absorbance of OTA at 380 nm. The experiment was performed in duplicate.

Hydrolysis of Ochratoxin A by Carboxypeptidase A

The reaction between OTA and commercially purchased carboxypeptidase A (from bovine pancreas, 37 mg protein/mL) (Sigma Chemical Co., St. Louis, MO) was studied to compare with that from crude enzyme isolate from *A. calcoaceticus*. Carboxypeptidase A and OTA were diluted with 0.1 M NaCl-0.02 M Tris buffer (pH 7.5) to concentrations of 50 μg protein/mL and 30 and 50 μg OTA/mL. OTA hydrolyzed by carboxypeptidase A at 25°C was monitored according to methods presented previously at 30 min intervals up to 4.5 hr.

Identification of Hydrolysates from Hydrolysis of Ochratoxin A by Cells and Crude Enzyme of *A. calcoaceticus*

Aliquot samples (OTA incubated with cells and crude enzyme of *A. calcoaceticus*) were filtered through an Acrodisc filter assembly (0.2 μm pore size) (Gelman Sciences, Ann Arbor, MI). Twenty μL filtrate and OTA standard were spotted on Redi-Plate silica gel G thin layer chromatography (TLC) plates (Fisher Scientific Co., Pittsburgh, PA). The TLC plates were developed in a solvent system containing benzene and acetic acid (9:1) in the dark. Plates were then dried at room temperature and observed under UV light for fluorescent spots. The R_f value of each fluorescent spot were calculated. The spots with green fluorescence were removed and extracted with methanol, and spots with blue fluorescence were extracted with methanol-

chloroform (2:1) (Storen et al., 1982). Extracts were filtered through Acrodisc filter, evaporated under nitrogen, and dissolved in methanol. Extracts was analyzed by HPLC and UV-VIS spectrophotometer to determine the retention time and maximum UV absorption. HPLC analysis was performed according to methods presented previously. Absorbance between 300-450 nm was determined by HP 8452 UV-VIS spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). The R_f values from TLC, UV spectrum from spectrophotometer, and retention time data from HPLC of sample extracts were compared with the results of OTA standard, carboxypeptidase A-hydrolyzed ochratoxin α or published research data (Cole and Cox, 1981; Pitout, 1969; Storen et al., 1982) to identify the resulting hydrolysates.

Production of Carboxypeptidase A-Hydrolyzed Ochratoxin α

Carboxypeptidase A (from bovine pancreas, 37 mg protein/mL) (Sigma Chemical Co., St. Louis, MO) and OTA were diluted with 0.1 M NaCl-0.02 M Tris buffer (pH 7.5) to a carboxypeptidase A concentration of 50 μg protein/mL and an OTA concentration of 50 μg/mL. Reaction was carried out at 30°C. The hydrolysates were subjected to TLC, HPLC, and UV-VIS spectrophotometry as describe above. The results were

used as standards to compare those obtained from using *A. calcoaceticus*.

Toxicity Test of Hydrolysates of Ochratoxin A using *Bacillus cereus mycoides*

A bioassay of OTA using *B. cereus mycoides* by the method of Borce et al. (1970) was followed. *B. cereus mycoides* was obtained from Northern Regional Agricultural Research Service of USDA (Peoria, IL). The test microorganism was inoculated first on trypticase soy agar slant for 24 hr. Three mL of antibiotic agar medium A broth (AOAC 42.203(a), 1984) (Appendix 4) was used to wash the 24 hr culture slant, and the suspension was transferred to the surface of an antibiotic agar medium A plate. Suspension was spread evenly over the entire plate using a hockey stick, and the plate was incubated at 32°C for 24 hr. The growth on agar surface was washed with 10 mL sterile 0.85% saline solution. The cell suspension was collected as working inoculum. One mL of working inoculum was inoculated into 50 mL melted agar medium A. After medium and inoculum were thoroughly mixed, 4 mL seeded agar were pipetted into petri dishes and distributed uniformly over entire surface by gently rotating the plates.

Filter paper disks (7 mm), made from No. 2 Whatman filter paper and dried at 90°C overnight, were placed on a wire mesh, and extracts from the following were applied

separately: filtrate of each 24 hr sample from *A. calcoaceticus* and 0.2 mg/mL crude enzyme incubated with 40 μg OTA/mL, *A. calcoaceticus* growth medium (without OTA added). Disks were dried with nitrogen gas and placed on seeded agar. Plates were incubated at 35°C. After 24 hr, the diameter of clear (inhibition) zones surrounding the disks were measured with a Vernier caliper to nearest one-tenth millimeter. A standard curve of inhibition zone of OTA standards was generated by the same method.

Statistical Analyses

Data were analyzed by one-way analysis of variance (ANOVA) and interactive outlier rejection (linear regression) using Statgraphics (Statistical Graphic Co., Rockville, MD). ANOVA with Tukey range test (Lyman, 1988) was used to compare cell counts and OTA concentrations with a confidence level of 95% (α=0.05). Interactive outlier rejection was used to calculate the rate of OTA hydrolyzed by cells and crude enzyme of *A. calcoaceticus* over incubation time. The overall rate of OTA hydrolysis was calculated by the regression of OTA changes over incubation time. The initial rate of OTA hydrolyzed by crude enzyme was reported when the disappearance of OTA over a given incubation time was considered linear, correlation coefficient greater than 0.95 ($r>0.95$).

CHAPTER IV

RESULTS AND DISCUSSION

Microbial Screening

None of the test microorganisms tested with the plate assay caused a color change or the disappearance of fluorescence of ochratoxin A (OTA). Fluorescence of OTA under UV light was produced from the carbon skeleton of isocoumarin ring (Bridges, 1969). Galtier and Alvinerie (1976) reported that the disappearance of fluorescence of OTA could be due to the opening of the lactone group of the isocoumarin ring when OTA was hydrolyzed by caecal contents from rats. Since none of the test microorganisms caused the disappearance of OTA fluorescence, the test microorganisms may lack the capability to break the isocoumarin ring of OTA. In the plate assay, *Selenomonas ruminantium*, *Streptococcus bovis*, and *Azospirillum brasilense* did not grow on the test medium. Since no effort such as using different media or incubation conditions was made to further test those microorganisms, their ability to degrade OTA was not ascertained. *Flavobacterium auranticatum* was reported to degrade aflatoxin B_1 causing its disappearance in modified Czapek-Dox medium (Ceigler et al., 1966). In the present research, this bacterium showed no ability to degrade OTA to nonfluorescent metabolites.

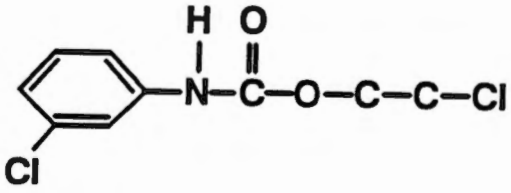
In test tube assay, only *A. calcoaceticus* gave two fluorescent spots with blue and green color under UV light on TLC plates. The green and blue fluorescent spots had a R_f value of 0.69 and 0.33, respectively, and OTA standard had a R_f value of 0.69. The two fluorescent spots on TLC plates in *A. calcoaceticus* treatments were identical in fluorescent color and R_f values to those on TLC plates from OTA hydrolyzed by carboxypeptidase A. The results obtained from TLC of *A. calcoaceticus* and pure carboxypeptidase A agreed with those of Pitout (1969). Pitout reported that carboxypeptidase A was capable of hydrolysing OTA to ochratoxin α and phenylalanine. Therefore, we hypothesized that *A. calcoaceticus* had the same capability as carboxypeptidase A for degradation of OTA, and the blue fluorescent spot which had a R_f of 0.33 on the TLC plate was ochratoxin α. Our later studies confirmed the hypothesis.

An extracellular esterase has been found both in the cell-free growth medium and on the cell surface of *A. calcoaceticus* (Shabtai and Gutnick, 1985). This esterase is capable of hydrolyzing acetyl and acyl groups from triglycerides and aryl and alkyl ester. The esterase and carboxypeptidase both belong to the class of β-esterase (Heymann, 1990). Kaufman (1967) reported that some soil microorganisms were capable of degrading phenylcarbamate herbicides (general formula, C_6H_5-NH-CO-R). Among these microorganisms, one isolate from *Acinetobacter* sp. was able

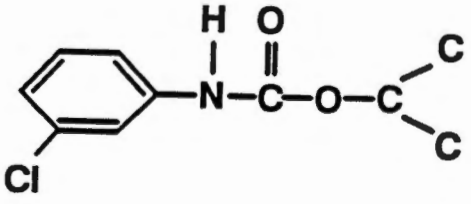
to hydrolyze two common phenylcarbamate herbicides, 2-chloroethyl-N-(3-chlorophenyl)carbamate (CEPC) and isopropyl-N-(3-chlorophenyl)carbamate (CIPC) (Fig. 2). The degradation pathways of these two herbicides by soil microorganisms have been proposed to be the hydrolysis of the molecule at the amide bond or the ester linkage (Fig. 3). Regardless of which bond was broken first, the products resulting from hydrolysis of these herbicides were the same (Kaufman, 1967). Therefore, it is reasonable to assume that the mode of action of OTA degradation by *A. calcoaceticus* was similar to that of *Acinetobacter* breakdown of CEPC and CIPC, hydrolysis of the amide bond. Results of test tube assay strongly indicated that *A. calcoaceticus* degraded OTA to ochratoxin α by cleavage of the amide bond. The identification of OTA hydrolysates (discussed later) indicated that OTA was hydrolyzed by *A. calcoaceticus* to ochratoxin α. A proposed pathway for OTA hydrolysis by *A. calcoaceticus* is presented in Fig. 4.

Identification and Taxonomy of *Acinetobacter*

The microorganism capable of degrading OTA in test tube assay was obtained from USDA labeled as *Acinetobacter calcoaceticus* NRRL B-551. This bacterium was identified to be a Gram-negative aerobic coccobacilli with twitching motility. It was oxidase negative and catalase positive and



2-chloroethyl-N-(3-chlorophenyl)carbamate (CEPC)



Isopropyl-N-(3-chlorophenyl)carbamate (CIPC)

Figure 2-Chemical structure of CEPC and CIPC.

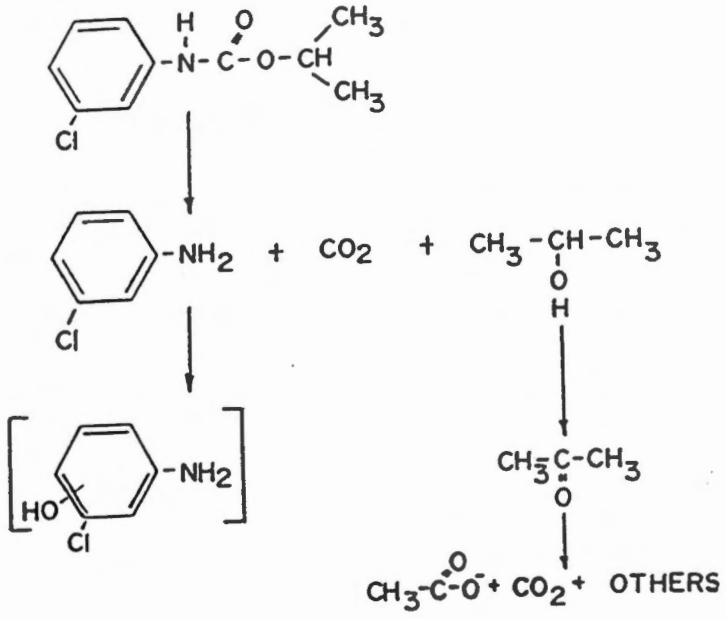
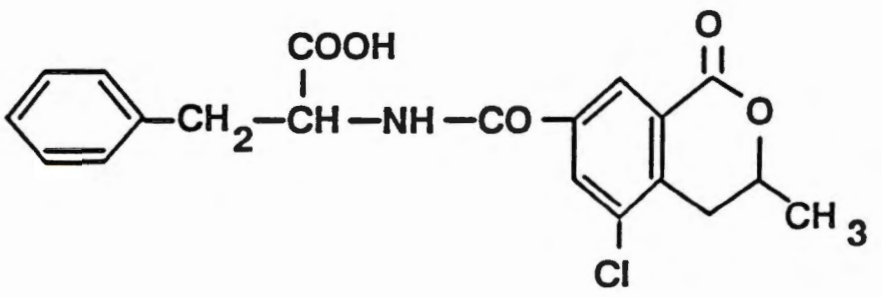
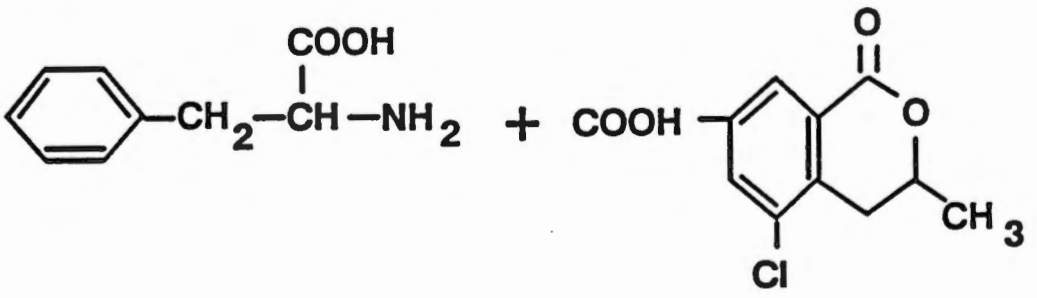


Figure 3-Proposed pathway of microbial degradation of isopropyl-N-(3-chlorophenyl)carbamate (CIPC).

Source: Kaufman, 1967.



Ochratoxin A



Phenylalanine Ochratoxin α

Figure 4-Proposed pathway of ochratoxin A degradation by *A. calcoaceticus*.

grew well in minimal salts medium containing ethanol as single carbon source. This bacterium grew on standard plate count agar to colony size 2-3 mm diameter with a cream-white color. These characteristics were compared with those of Kriog (1984) and it was concluded that the microorganism capable of degrading OTA was *Acinetobacter* sp. According to Kriog (1984), *Acinetobacter* sp., a strict aerobe, occurs naturally in soil and water. Most strains grow in a simple mineral medium containing a single carbon and energy source such as ethanol, acetate, lactate, and pyruvate and use ammonium or nitrate salts as the source of nitrogen and display no growth factor requirements. Some *Acinetobacter* sp. are capable of degrading hydrocarbons, aromatic compound such as benzoate, and alicyclic compounds such as cyclohexanol. Although acinetobacters are considered to be normally nonpathogenic, they are causative agents of nosocomial infections in debilitated individuals.

The Fate of *A. calcoaceticus* and the Degradation of OTA in Ethanol-Minimal Salts Medium at 30°C

The growth curve of *A. calcoaceticus* in ethanol-minimal salts medium without the addition of OTA at 30°C is shown in Fig. 5a. The relationship between the growth of *A. calcoaceticus* and the degradation of OTA in ethanol-minimal salts medium with an initial OTA concentration of 10-50

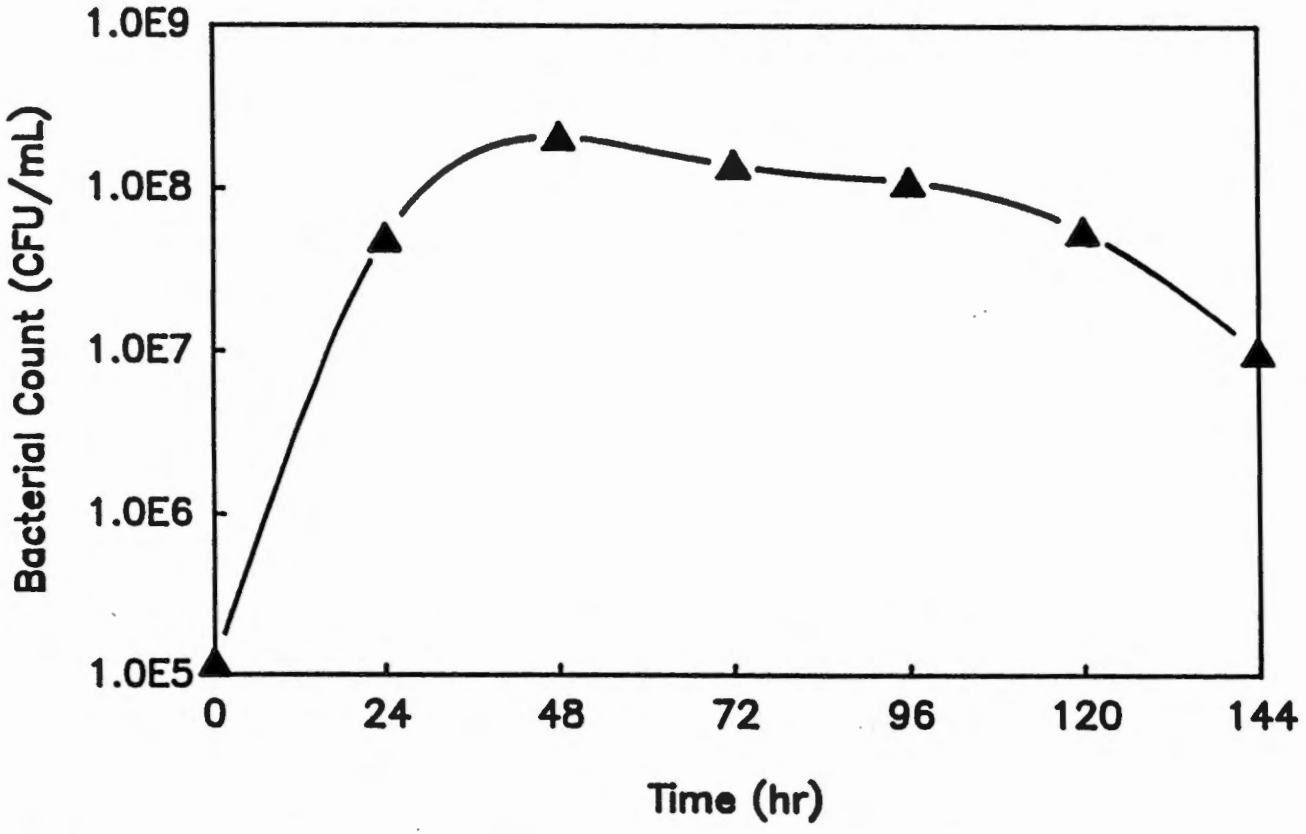


Figure 5-Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with selected concentrations of ochratoxin A at 30°C: a. Growth curve of *A. calcoaceticus* in ethanol-minimal salts medium at 30°C with no ochratoxin A added.

μg/mL at 30°C is shown in Figs. 5b-5f. The initial inoculum of *A. calcoaceticus* was approximately Log 5.5 cfu/mL. Cell numbers of *A. calcoaceticus* in growth medium with initial OTA concentration of 10-30 μg/mL reached the maximum of Log 8.8 cfu/mL at 48 hr (Figs. 5b-5d), and those in medium containing 40 μg/mL OTA reached the maximum of Log 8.5 cfu/mL at 72 hr (Fig. 5e). It is clearly demonstrated in Fig. 5f that the growth of *A. calcoaceticus* in medium was inhibited by the OTA concentration of 50 μg/mL. Comparing the growth curve of *A. calcoaceticus* in medium containing 10-40 μg/mL OTA (Figs. 5b-5e) with that containing no OTA (Fig. 5a), *A. calcoaceticus* cells decreased more rapidly after reaching the maximum in medium containing OTA. In medium with 20-40 μg OTA/mL, cells decreased after reaching the maximum, but in medium with 10 μg OTA/mL, cells increased after OTA was depleted (Fig. 5b). It appears that even OTA concentrations of 10-40 μg/mL had a toxic effect on the cells of *A. calcoaceticus* particularly at the end of the log phase. Although OTA concentration was decreased during dilution prior to plating, the OTA aliquot may have been bound to cells and inhibited the growth of *A. calcoaceticus* on the plating agar. A growth inhibitory effect of OTA on other microorganisms has been reported (Borce et al., 1970). OTA may bind to cell surfaces of *A. calcoaceticus* after cell growth reaches the end of log phase and inhibit growth.

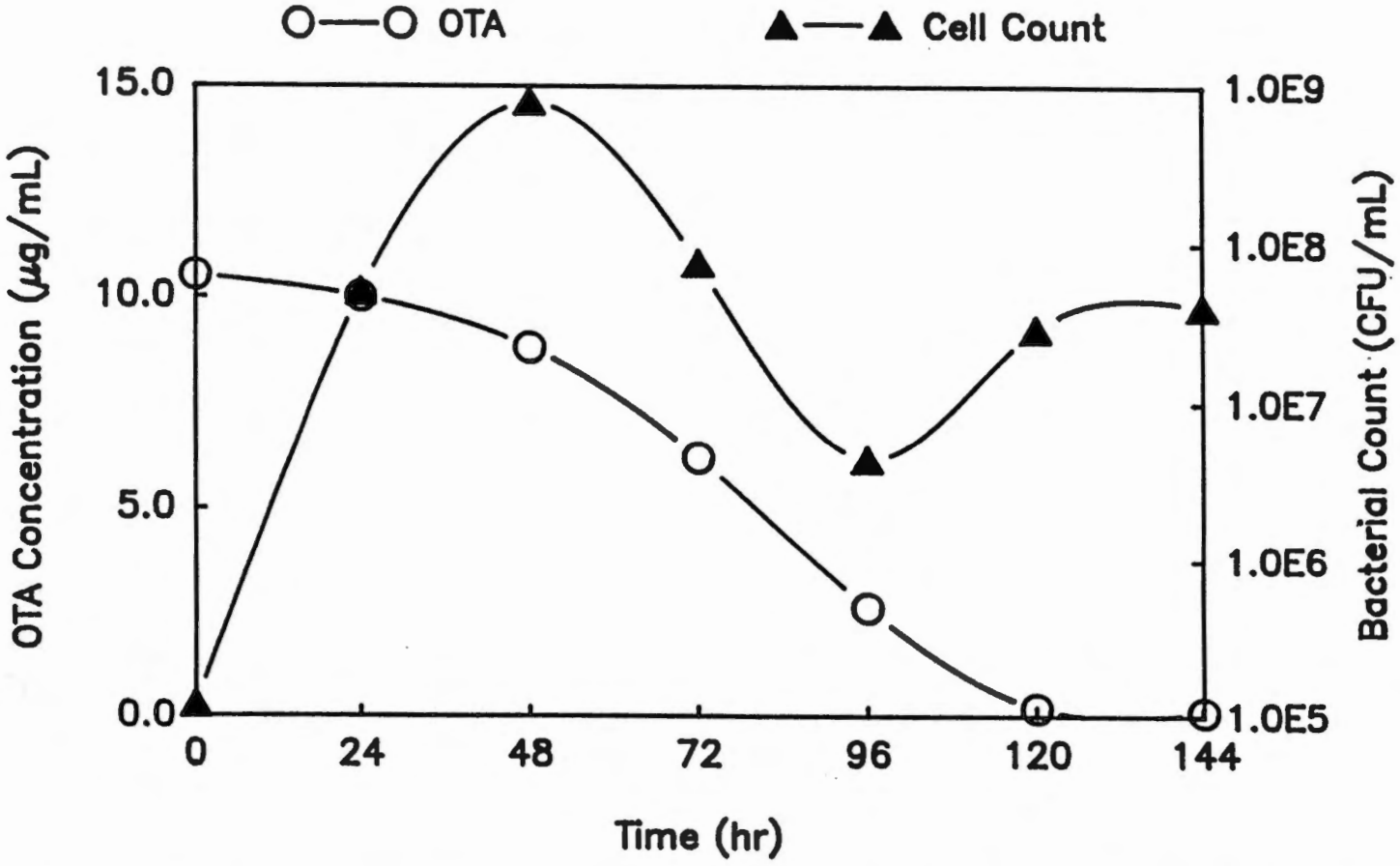


Figure 5-(Continued) b. Changes of ochratoxin A concentration and viable A. *calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 10 μg/mL.

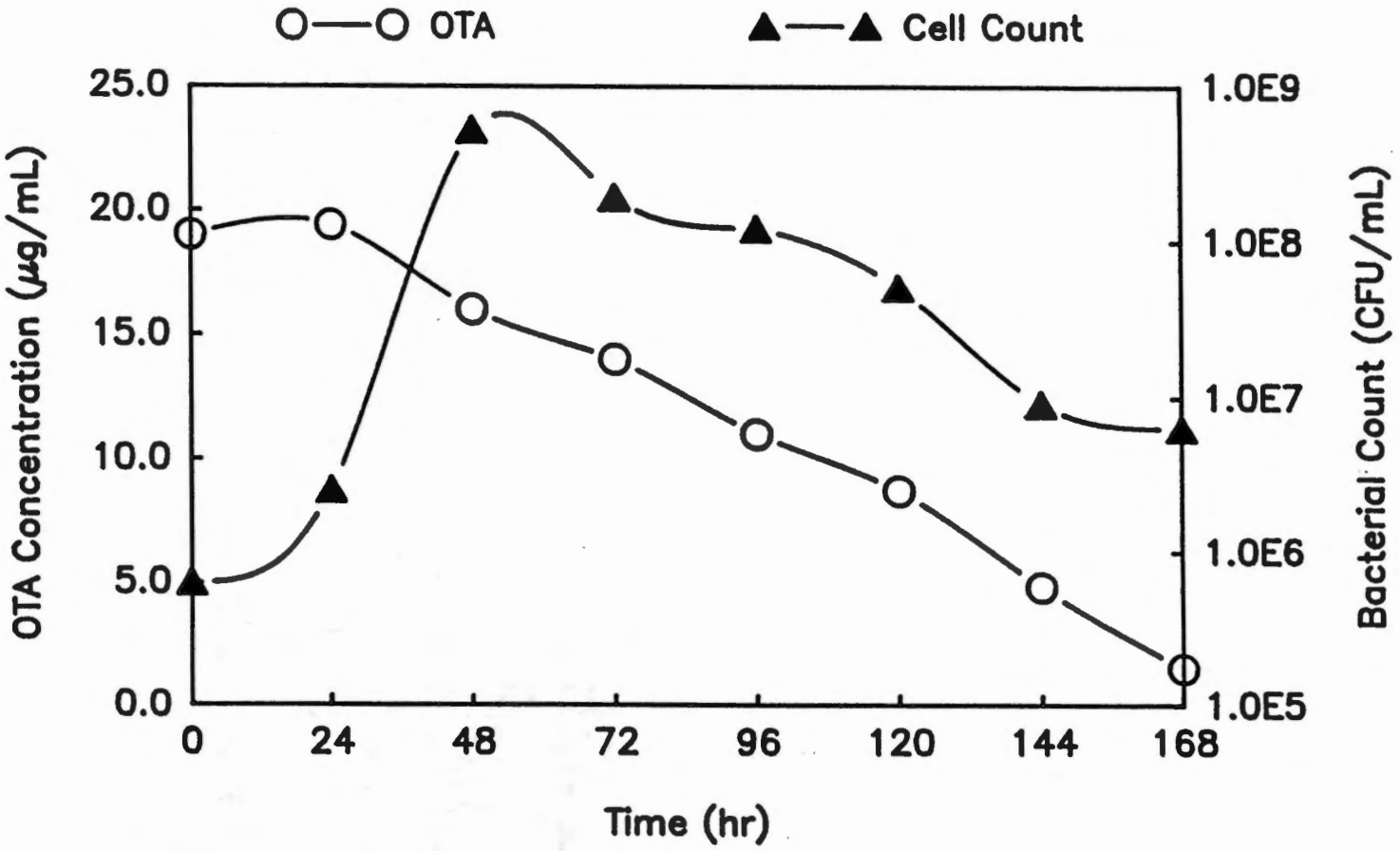


Figure 5-(Continued) c. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 20 μg/mL at 30°C.

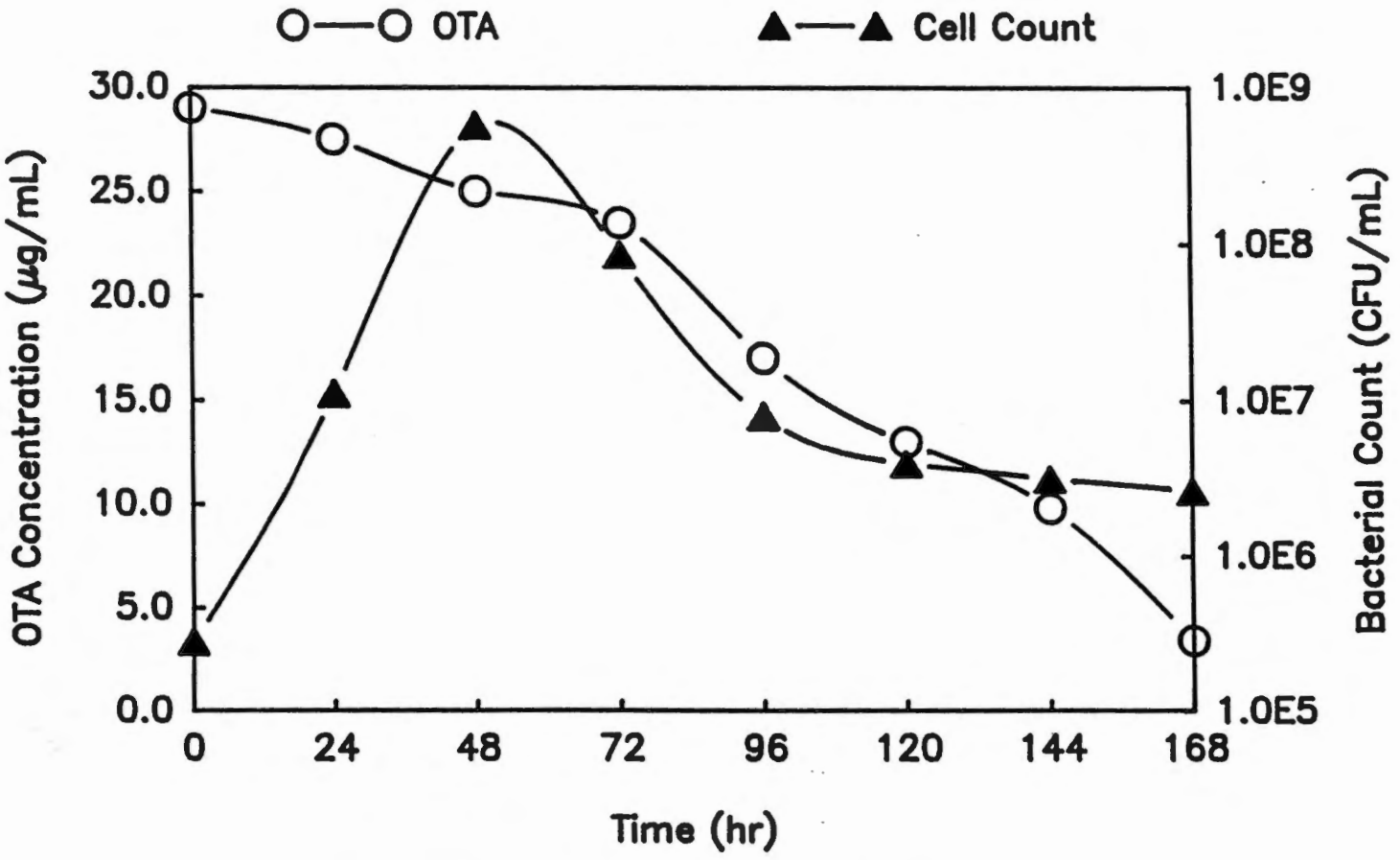


Figure 5-(Continued) d. Changes of ochratoxin A concentration and viable A. *calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 30 μg/mL at 30°C.

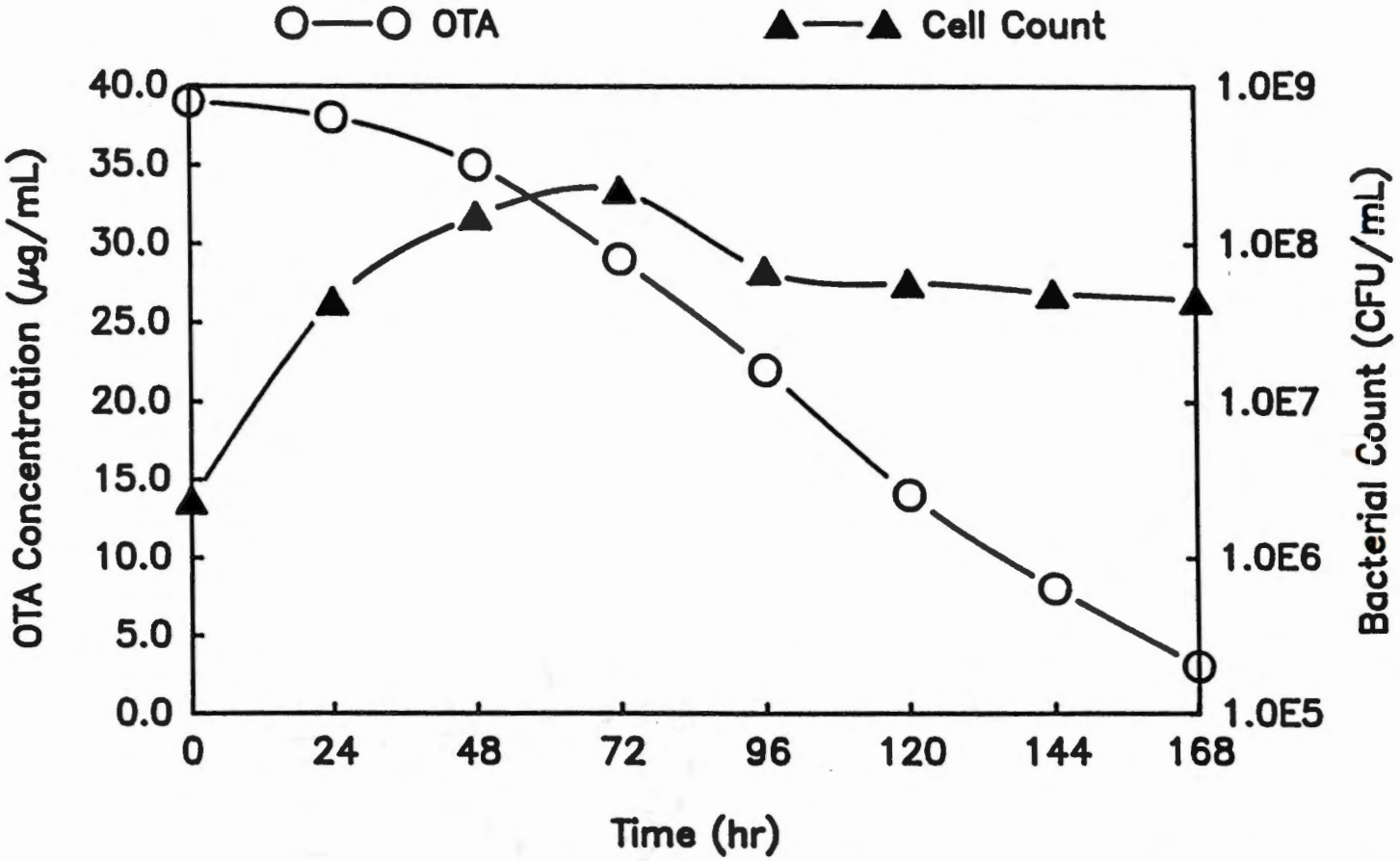


Figure 5-(Continued) e. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 40 μg/mL at 30°C.

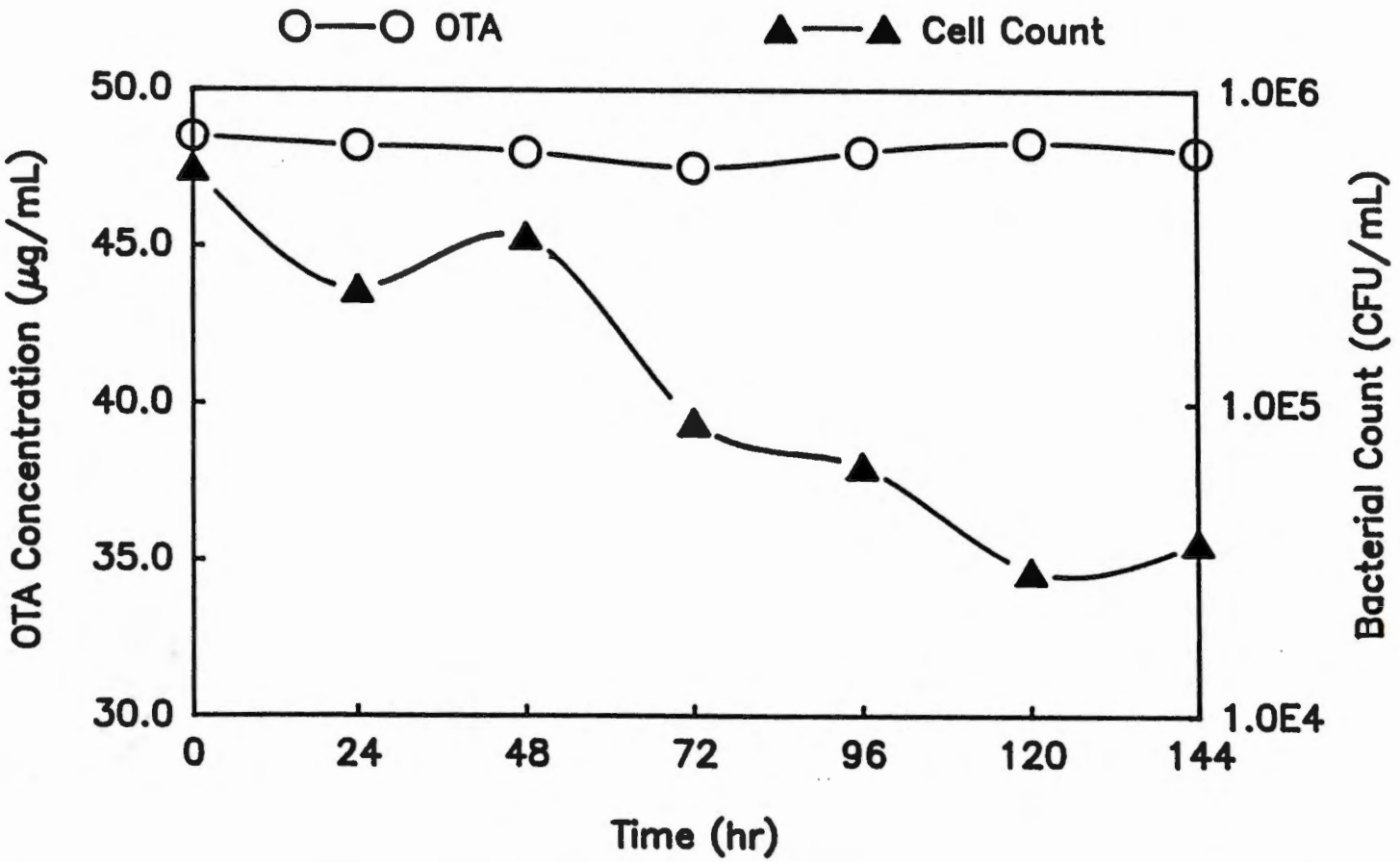


Figure 5-(Continued) f. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 50 μg/mL at 30°C.

This is supported by the fact that cells exhibited fluorescence after being washed with distilled water. The growth inhibition effect of OTA on *A. calcoaceticus* was particularly noticeable in medium containing 50 μg OTA/mL, where cell numbers decreased constantly after inoculation (Fig. 5f). Inhibition of growth of *A. calcoaceticus* by OTA was directly proportional to OTA concentration in the medium.

With initial OTA concentrations of 10-30 and 40 μg/mL, OTA decreased significantly ($p<0.05$) in the medium after 24 hr and 48 hr, respectively. In medium containing 10-40 μg OTA/mL, maximum cell numbers at different concentrations of OTA were not significantly different ($p>0.05$). Therefore, OTA degradation by *A. calcoaceticus* can be reported solely based on time. No OTA was detected in medium containing 10 μg OTA/mL after 120 hr. The average decrease of OTA was 0.1005 μg/mL/hr. In medium containing 20 μg OTA/mL, the residue of OTA was 4 μg/mL after 144-hr incubation, the average decrease of OTA was 0.1226 μg/mL/hr (Fig. 5c). In medium containing 30 μg OTA/mL, the residue of OTA was 4 μg/mL after 168-hr incubation. The average decrease of OTA was 0.1374 μg/mL/hr (Fig. 5d). In medium containing 40 μg OTA/mL, cell numbers reached maximum after 72 hr, and the decease of OTA was rapid after 48 hr. The average decrease of OTA after 48 hr was 0.2262 μg/mL/hr (Fig. 5e). In medium

containing 50 μg OTA/mL, growth of cells was inhibited and OTA was not degraded (Fig. 5f). The rate of OTA degradation by *A. calcoaceticus* growing in ethanol-minimal salts medium at 30°C increased with increasing OTA concentration up to 40 μg/mL.

The Fate of *A. calcoaceticus* and the Degradation of OTA in Ethanol-Minimal Salts Medium at 25°C

The growth curve of *A. calcoaceticus* in ethanol-minimal salts medium without the addition of OTA at 25°C is shown in Fig. 6a. The fate of *A. calcoaceticus* and the degradation of OTA in ethanol-minimal salts medium with an initial OTA concentration of 10-50 μg/mL at 25°C are shown in Figs. 6b-6f. The initial inoculum of *A. calcoaceticus* was approximately Log 6.8 cfu/mL. Cell numbers of *A. calcoaceticus* in growth medium with initial OTA concentrations of 10-30 μg/mL reached the maximum of Log 9.0 cfu/mL at 48 hr (Figs. 6b-6d). The growth patterns of *A. calcoaceticus* in ethanol-minimal salts medium with or without OTA at 25°C were similar to those at 30°C. In medium containing 10-30 μg OTA/mL, cell counts decreased slightly after inoculation but increased to the maximum after 48-hr incubation, and OTA was seen to decrease significantly ($p<0.05$) at 48 hr. As seen in *A. calcoaceticus* incubated with 40 μg OTA/mL at 30°C, the

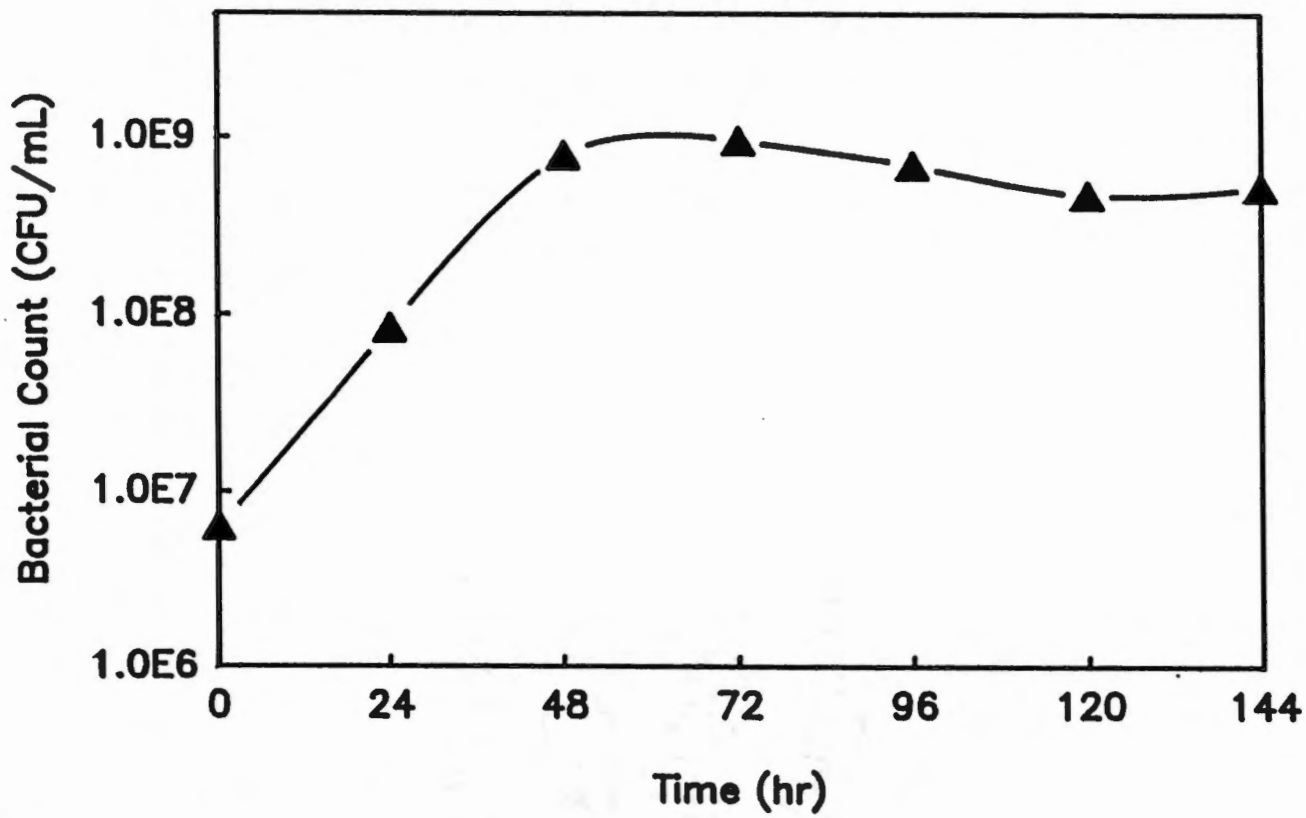


Figure 6-Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with selected concentrations of ochratoxin A at 25°C: a. Growth curve of *A. calcoaceticus* in ethanol-minimal salts medium at 25°C with no ochratoxin A added,

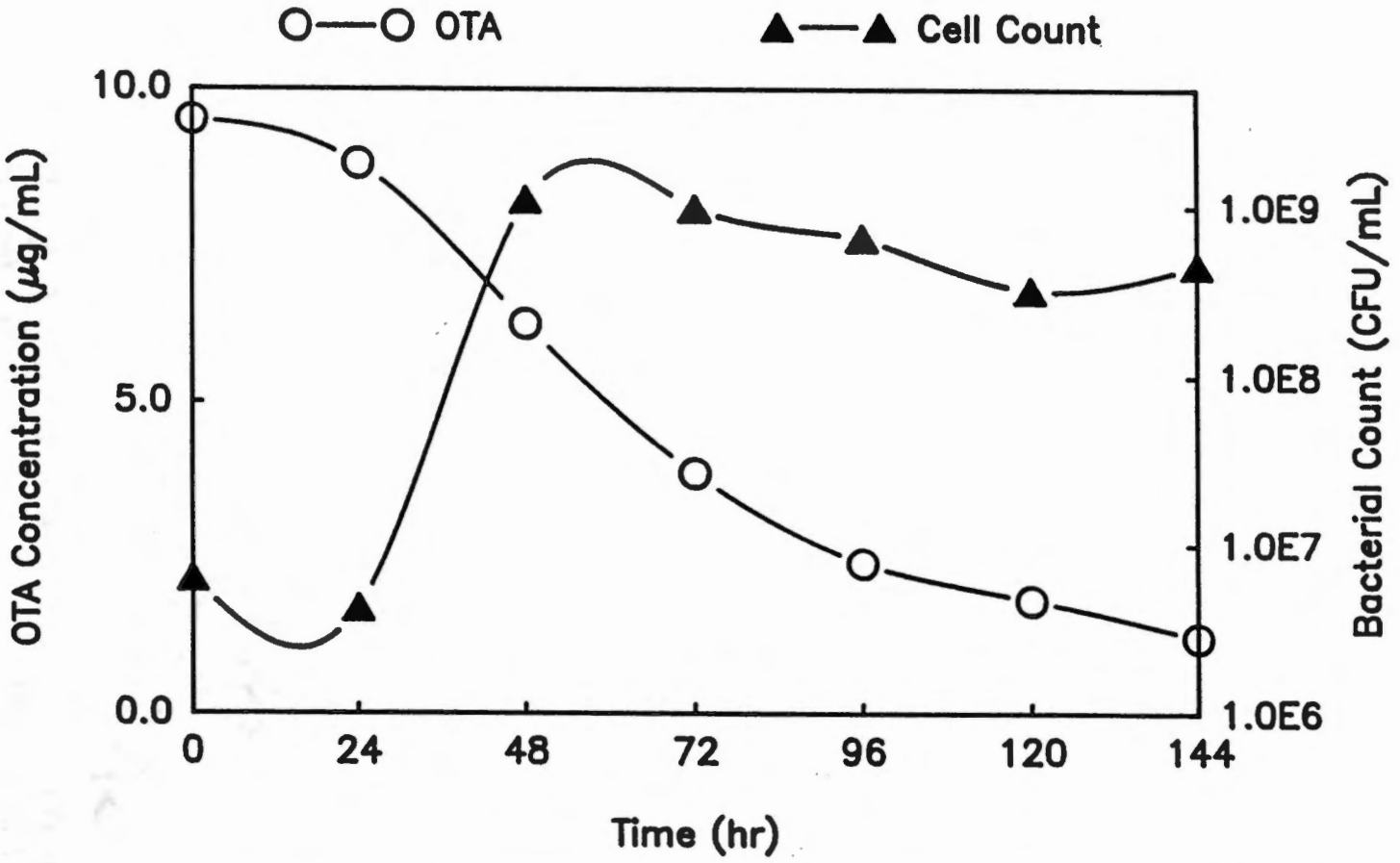


Figure 6-(Continued) b. Changes of ochratoxin A concentration and viable A. *calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 10 μg/mL at 25°C.

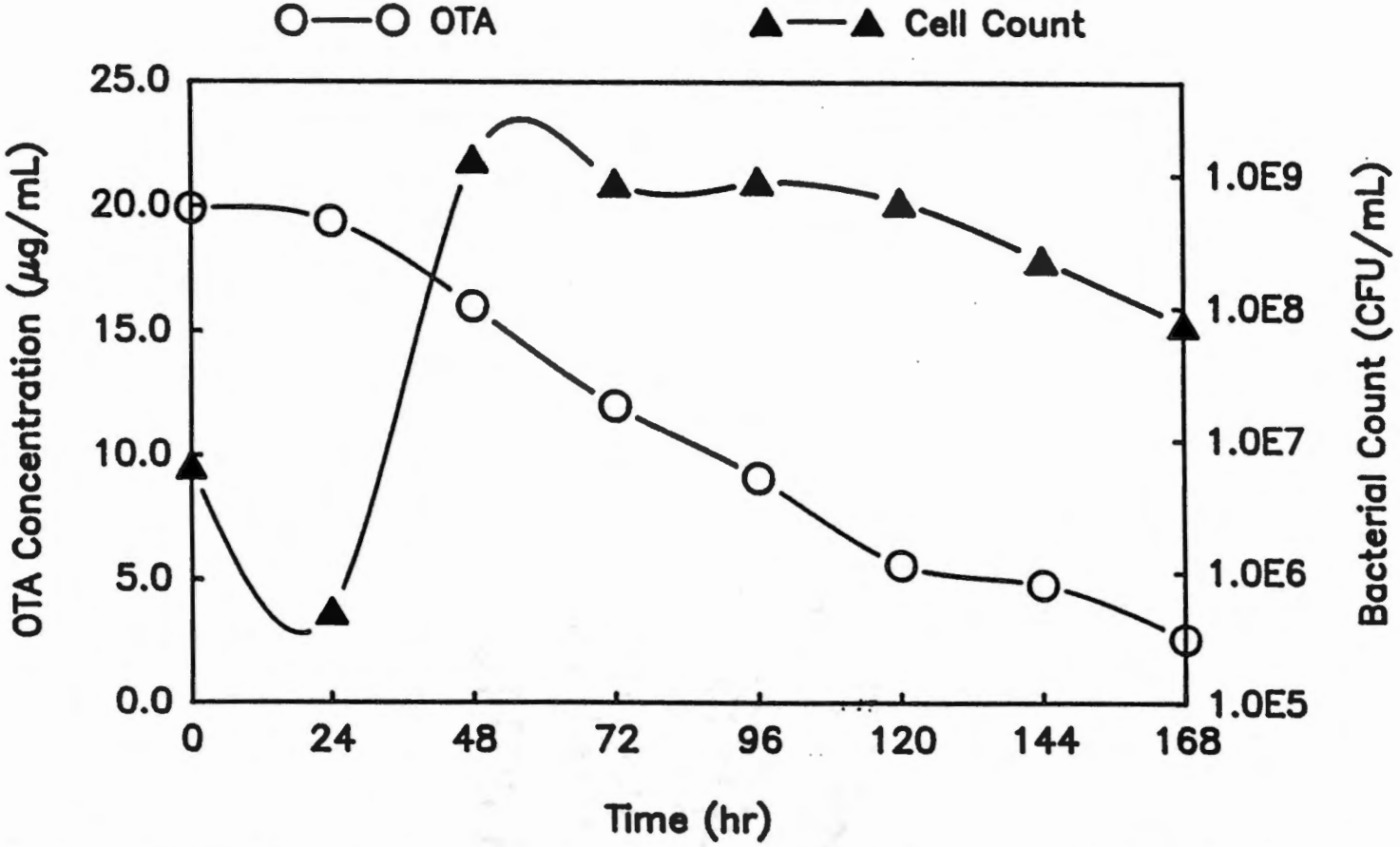


Figure 6-(Continued) c. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 20 μg/mL at 25°C.

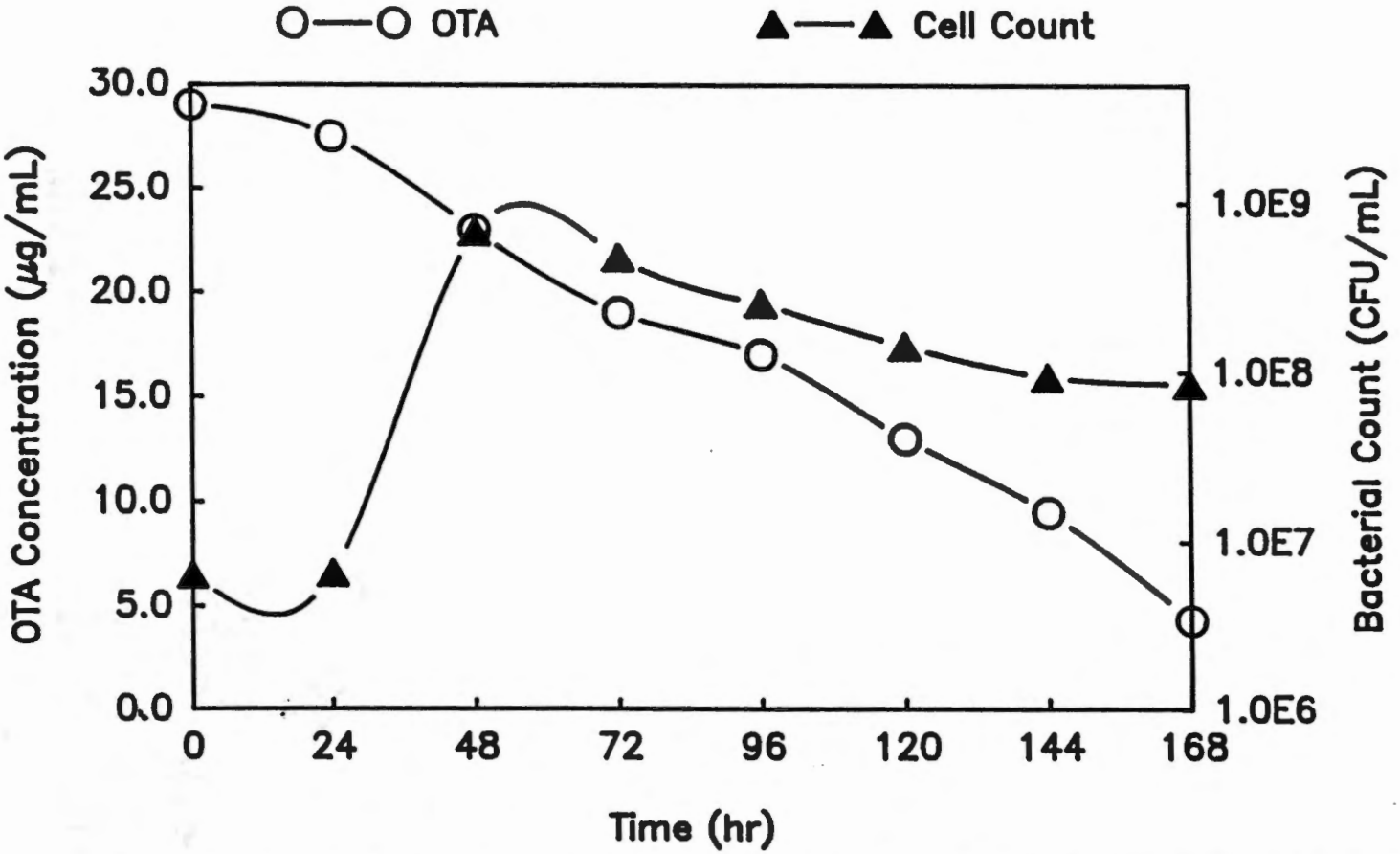


Figure 6-(Continued) d. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 30 μg/mL at 25°C.

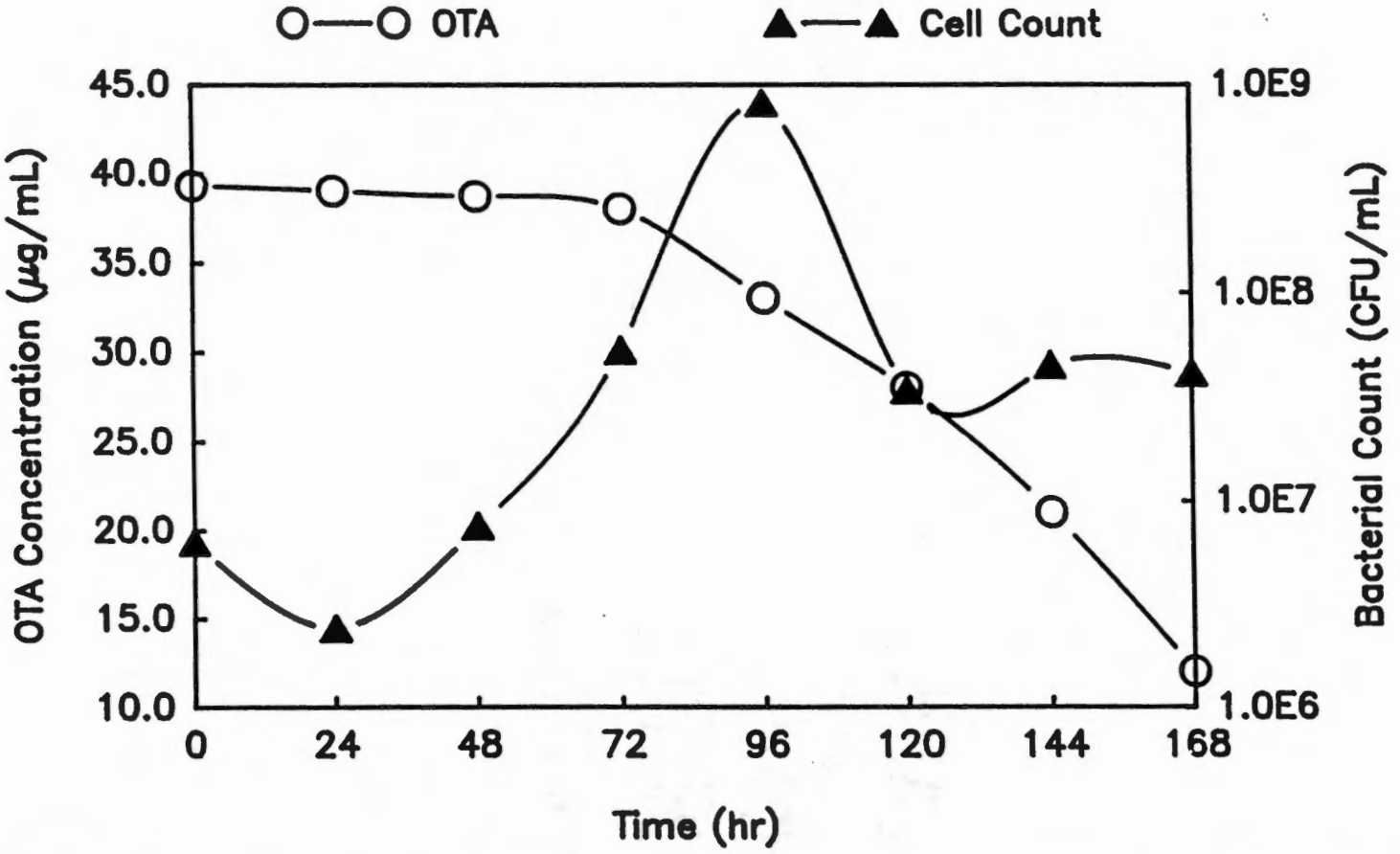


Figure 6-(Continued) e. Changes of ochratoxin A concentration and viable *A. calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 40 μg/mL at 25°C.

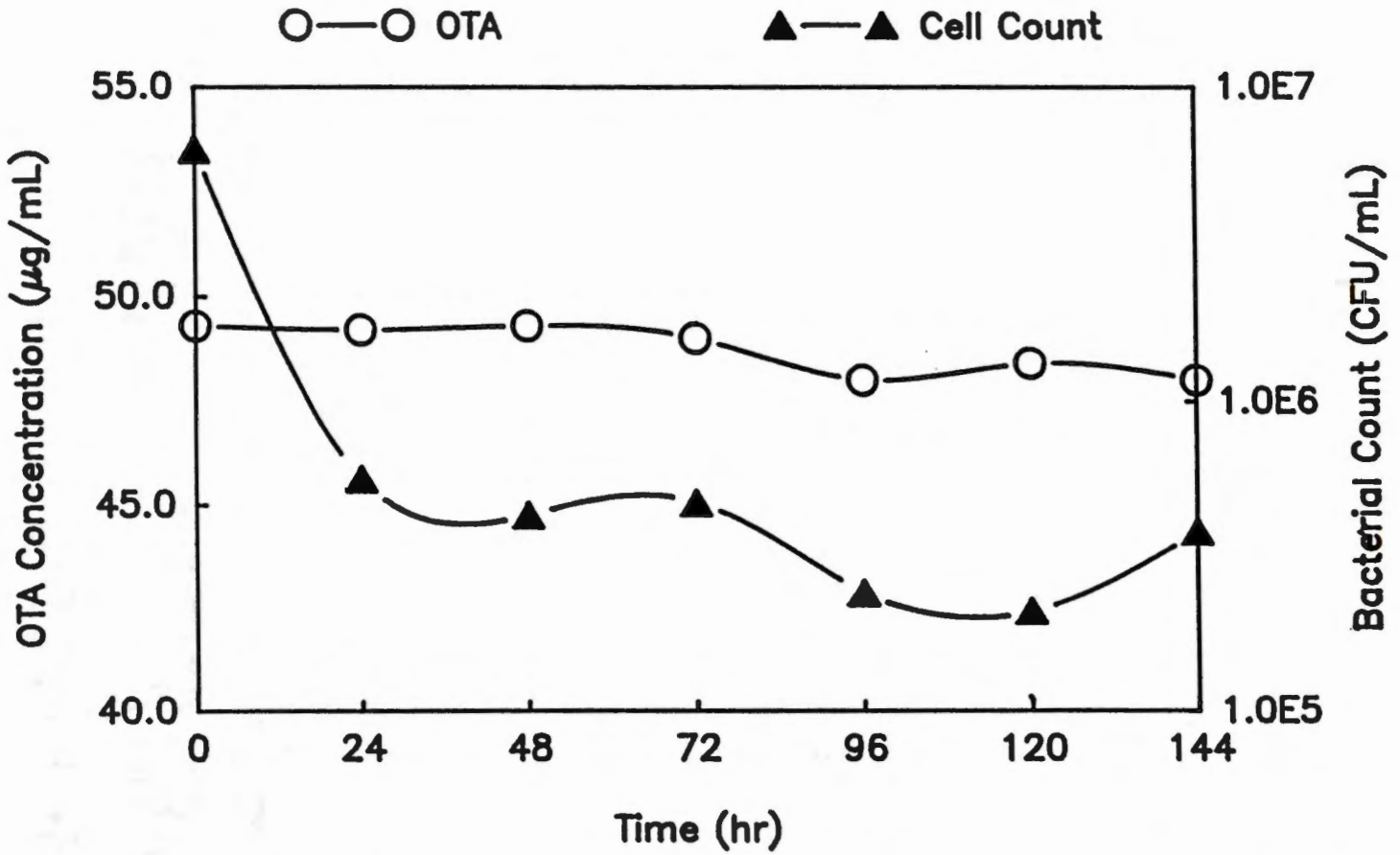


Figure 6-(Continued) f. Changes of ochratoxin A concentration and viable A. *calcoaceticus* in ethanol-minimal salts medium with an initial ochratoxin A concentration of 50 μg/mL at 25°C.

growth of *A. calcoaceticus* at 25°C in medium containing 40 μg OTA/mL was slower with a longer lag phase than those in medium containing lower concentrations of OTA. The lower incubation temperature (25°C) increased the lag phase of *A. calcoaceticus* in medium with 40 μg OTA/mL when compared to the higher incubation temperature (30°C) (Figs. 5e and 6e). In medium containing 40 μg OTA/mL, cells reached maximum numbers and decreased afterward at 96 hr (25°C) and at 72 hr (30°C), and OTA concentrations showed linear decreases ($r>0.95$) after the cells reached maximum (Figs. 5e and 6e). In medium containing 50 μg OTA/mL, the growth of *A. calcoaceticus* at 25°C was inhibited and OTA was not degraded as seen at 30°C. This finding indicated that an OTA concentration of 50 μg/mL was inhibitory to the growth of *A. calcoaceticus* both at 25°C and at 30°C. In medium containing 10 μg OTA/mL, OTA significantly decreased ($p<0.05$) at 48 hr, and the decrease was linear ($r>0.95$) thereafter. The residue of OTA in this medium was 1 μg/mL after 144 hr. The average decrease of OTA after *A. calcoaceticus* reached highest numbers (48-hr incubation) was 0.0636 μg/mL/hr (Fig. 6b). In medium containing 20 μg OTA/mL, the residue of OTA was 3 μg/mL after 144-hr incubation. The average decrease of OTA was 0.1172 μg/mL/hr (Fig. 6c). In medium containing 30 μg OTA/mL, the residue of OTA was 5 μg/mL after 168-hr incubation. The average

removal of OTA was 0.1264 μg/mL/hr (Fig. 6d). In medium containing 40 μg OTA/mL, although the cell numbers were highest only after 96-hr incubation, OTA removal was rapid after 72 hr. The average OTA removal was 0.2232 μg/mL/hr between 72-144 hr incubation period (Fig. 6e). In medium containing 50 μg OTA/mL, the growth of cell was inhibited, and OTA was not degraded. At 25°C, the rate of OTA removed by *A. calcoaceticus* was increased as the concentration of OTA in medium increased up to 40 μg/mL. OTA was degraded more rapidly ($p<0.05$) in medium containing 10 μg OTA/mL at 30°C than at 25°C. However, rate of OTA degradation in medium containing 20, 30, or 40 μg OTA/mL was not significantly different ($p<0.05$) between 25°C and 30°C at the same initial OTA concentration (Table 7).

The rate of OTA degradation by *A. calcoaceticus* increased as OTA concentration increased up to 40 μg/mL. In a preliminary study, *A. calcoaceticus* did not grow in minimal-salts medium (no ethanol added) containing OTA. This indicated that *A. calcoaceticus* was unable to use OTA as a nutrient source.

The increase in rate of OTA degradation by *A. calcoaceticus* when OTA concentrations increased suggests an enzymatic action mediated by *A. calcoaceticus*. Generally, in enzymatic reactions, an increase of substrate (OTA) at a constant enzyme concentration (microbial cells) increases

Table 7-Overall rate of ochratoxin A (OTA) hydrolyzed by *A. calcoaceticus* in ethanol minimal-salts medium at 25°C and 30°C

OTA Concentration (μg/mL)	Overall Rate of OTA hydrolysis (μg/mL/hr)[a]/Incubation Period (hr) (Correlation Coefficient)	
	Incubation Temperature (°C)	
	25	30
40	0.2232[b]/72-168 (0.99)	0.2262[b]/24-168 (0.97)
30	0.1264[b]/24-168 (0.99)	0.1374[b]/24-168 (0.97)
20	0.1172[b]/24-168 (0.99)	0.1226[b]/24-168 (0.99)
10	0.0636[b]/24-144 (0.98)	0.1005[c]/24-120 (0.98)

[a]Regression of OTA concentrations over the given incubation period.
[b-c]Means within a row followed by different superscripts are significantly different ($p<0.05$).

the velocity of the enzymatic reaction. OTA degradation occurred when *A. calcoaceticus* cells reached maximum numbers. Therefore, it can be assumed that enzyme(s) responsible for degrading OTA were produced or released during the late-log phase of cell growth. In a preliminary study, washed cells of *A. calcoaceticus* degraded OTA in 0.1 M NaCl-0.02 M Tris buffer. Perhaps the enzyme(s) responsible for degrading OTA is exocellular and/or cell-bound. Shabtai and Gutnick (1985) reported that an esterase produced by *A. calcoaceticus* RAG-1 was both exocellular and cell-bound.

Although OTA was degraded by *A. calcoaceticus* in our experiments, one cannot assume that OTA degradation by *A. calcoaceticus* in an aqueous solution will be the same as in foods or feedstuffs. Studies must be conducted with actual foodstuffs to determine optimum conditions for detoxification of OTA. In addition, the concentration of OTA is of concern since high concentrations of OTA inhibited the growth of *A. calcoaceticus* when living cells were used. Therefore, isolation of the enzyme(s) from *A. calcoaceticus* responsible for the degradation of OTA proceeded.

Protein Profile of Crude Enzyme Isolated from *A. calcoaceticus*

The crude enzyme isolated from cell-free filtrate of *A. calcoaceticus* growth medium after protein precipitation and dialysis was not separated successfully using the isoelectric precipitation method of Allan et al. (1963). They isolated carboxypeptidase A successfully from bovine pancreas. However, no precipitate was formed at pH 5.5 when crude protein (obtained from dialysis) was adjusted by addition of 0.1 N acetic acid at 0°C in our procedure. Therefore, we referred to the crude protein as crude enzyme. Since the dialysis tubing used to dialyze the crude protein had a molecular weight cutoff at 12,000-14,000, the crude protein after dialysis should contain proteins with molecular weight greater than 12,000. Disc-gel electrophoresis of crude enzyme and carboxypeptidase A showed that carboxypeptidase A had four distinct protein bands (migrating 0.5, 0.8, 4.0, and 5.5 cm/10 cm gel) and crude enzyme gave several broad protein bands (0-1.2, 2.9-3.2, 3.6-5.4, 5.5-7.1 cm/10 cm gel). Although the crude enzyme had protein bands at the same locations as those of standard carboxypeptidase A, the results provided insufficient evidence to conclude that the crude enzyme contained carboxypeptidase A. The protein profile of crude enzyme, however, indicated that some proteins in crude

enzyme did have the same molecular weight and charge as found in carboxypeptidase A. Protein bands on gels located at the same location as carboxypeptidase A were extracted with 0.1 M NaCl-0.02 M Tris buffer. The extract had OTA-degrading ability. This indicated that the proteins in crude enzyme which migrated less than 5.5 cm on 10 cm length gel possessed OTA degradation ability.

Hydrolysis of Ochratoxin A by Crude Enzyme Isolated from *A. calcoaceticus*

Hydrolysis of 10-60 μg OTA/mL by crude enzyme of 0.1, 0.15 and 0.2 mg protein/mL obtained from growth medium of *A. calcoaceticus* is shown in Figs. 7a-7f. In solutions containing 10 μg OTA/mL and crude enzyme of 0.15 and 0.2 mg protein/mL, OTA was not detected at 96 hr. It was not detected at 192 hr with crude enzyme of 0.1 mg protein/mL (Fig. 7a). In solution containing 20 μg OTA/mL, OTA was not detected at 120 hr in solutions with crude enzyme of 0.15 and 0.2 mg protein/mL (Fig. 7b). In solutions containing 30 μg OTA/mL and crude enzyme of 0.2 and 0.15 mg protein/mL, no OTA was detected at 120 hr and 144 hr, respectively, while approximately 4 μg OTA/mL was detected in solution with crude enzyme of 0.1 mg protein/mL at 192 hr (Fig. 7c). At higher the protein concentration of crude enzyme, the faster the OTA was hydrolyzed by crude enzyme. Similar results

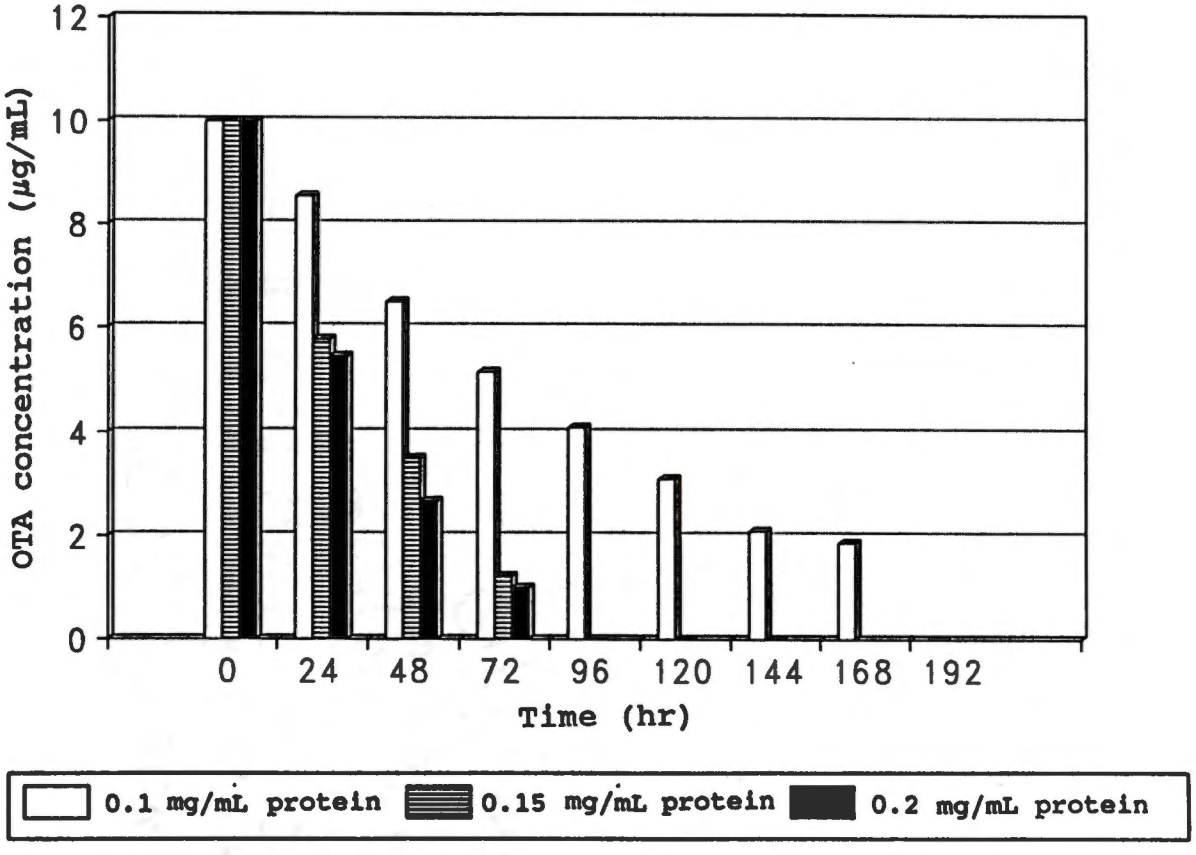


Figure 7-Decrease by hydrolysis of selected concentrations of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from *A. calcoaceticus*: a. Decrease by hydrolysis of 10 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by selected concentrations of crude enzyme isolated from *A. calcoaceticus*.

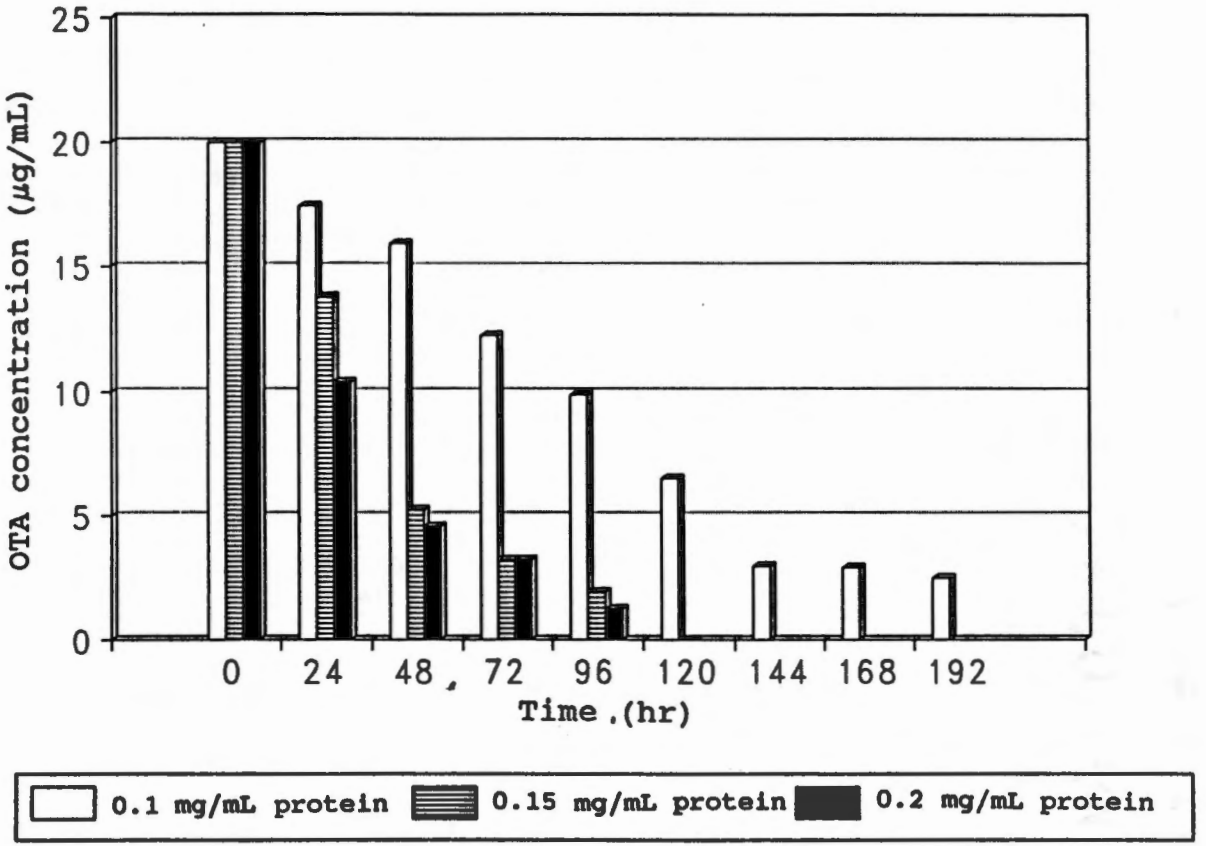


Figure 7-(Continued) b. Decrease by hydrolysis of 20 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from *A. calcoaceticus*.

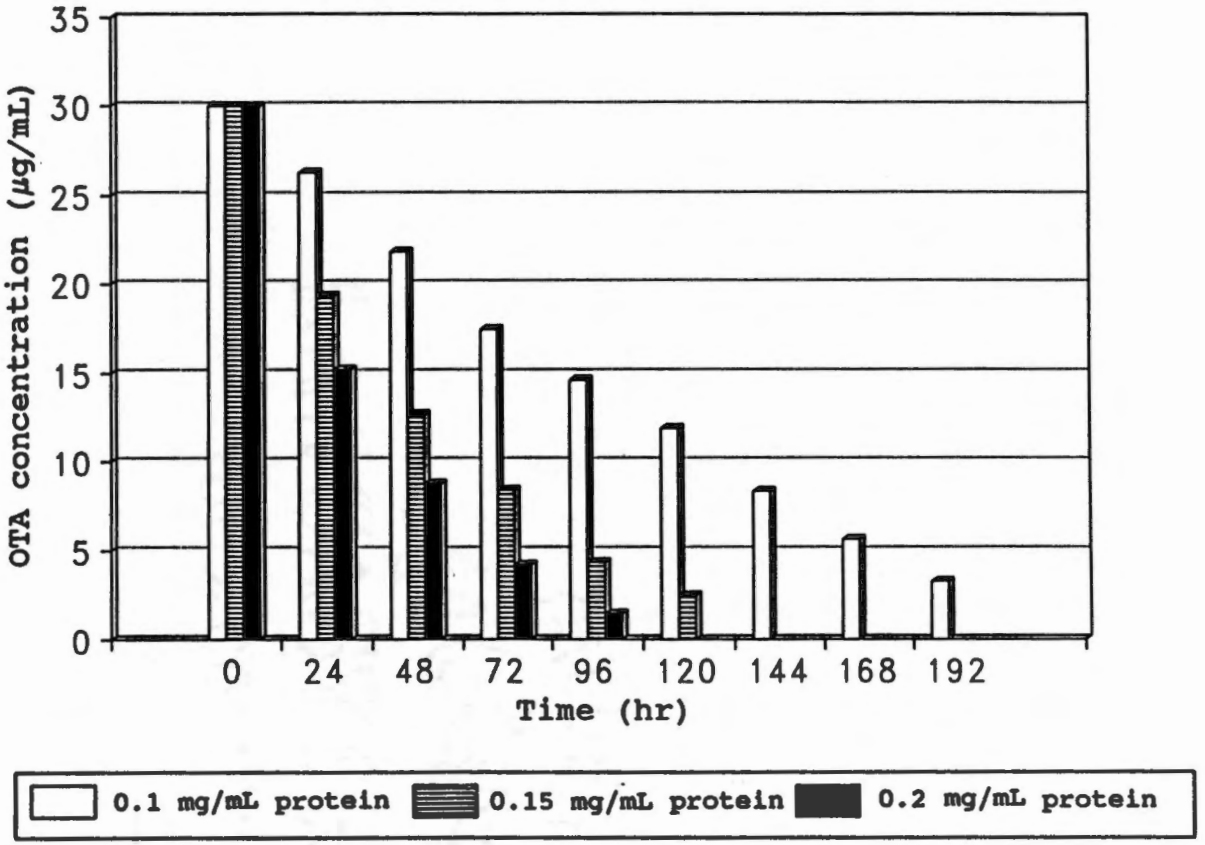


Figure 7-(Continued) c. Decrease by hydrolysis of 30 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from *A. calcoaceticus*.

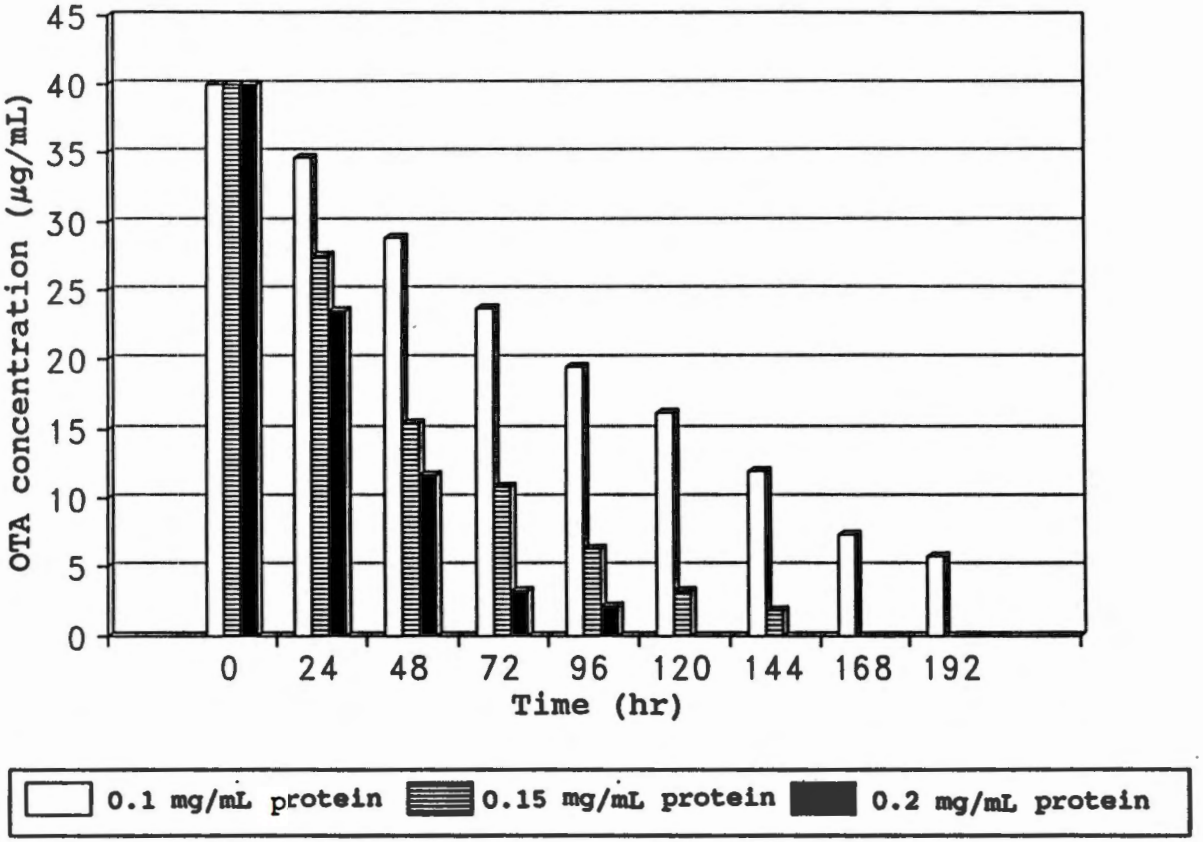


Figure 7-(Continued) d. Decrease by hydrolysis of 40 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from *A. calcoaceticus*.

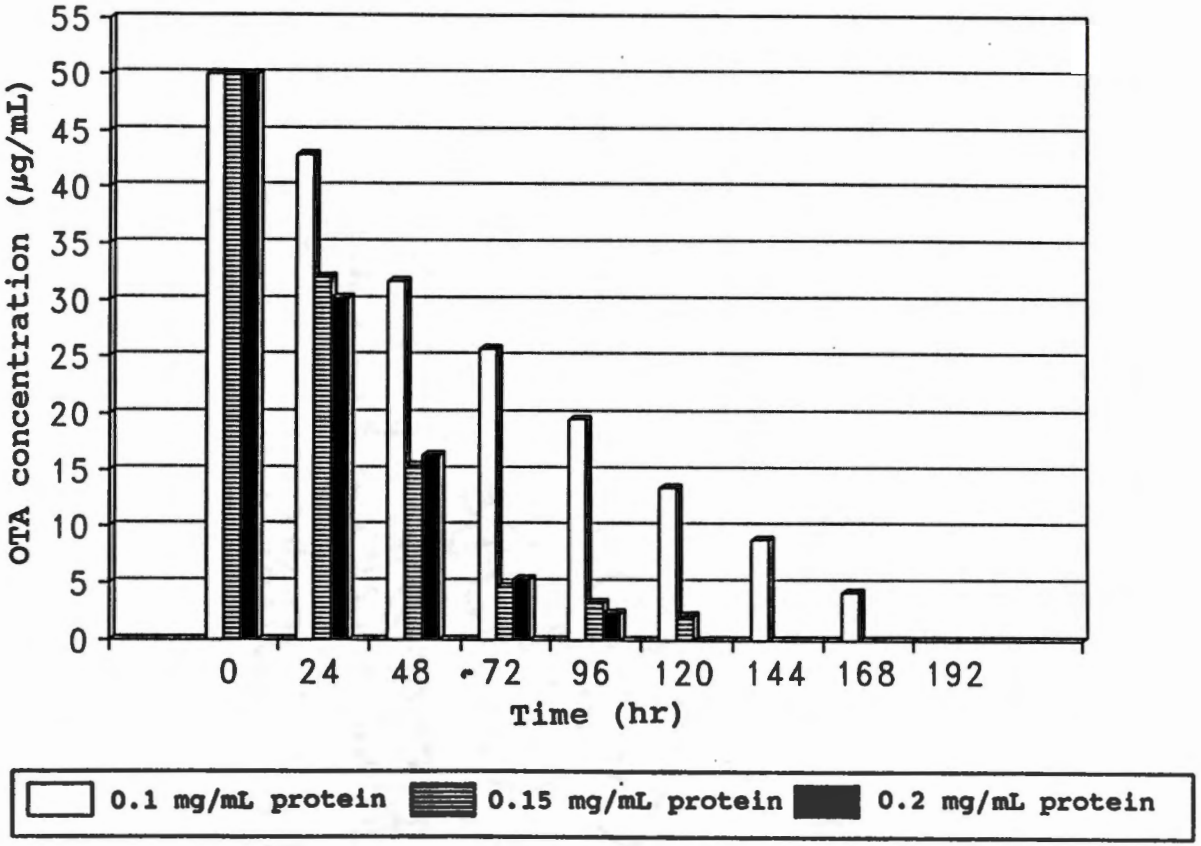


Figure 7-(Continued) e. Decrease by hydrolysis of 50 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from A. *calcoaceticus*.

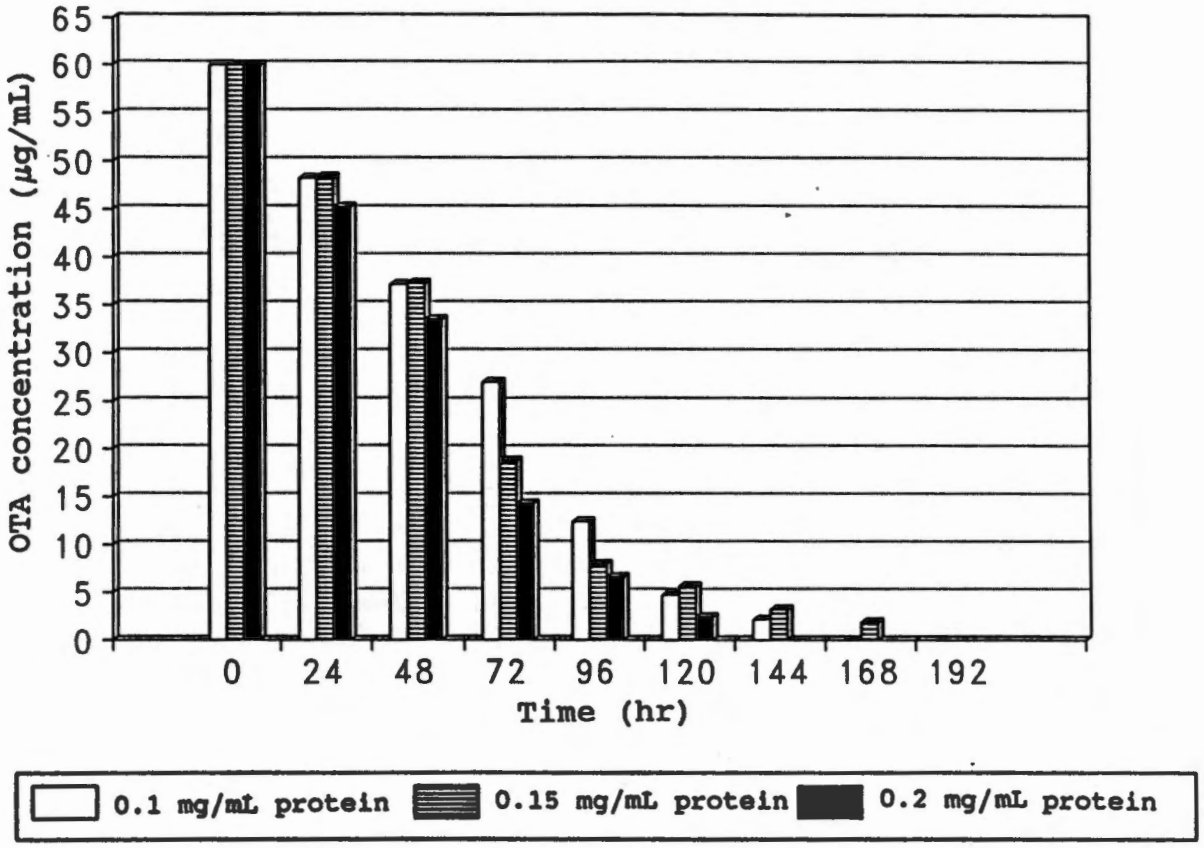


Figure 7-(Continued) f. Decrease by hydrolysis of 60 µg/mL ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by crude enzyme isolated from *A. calcoaceticus*.

were observed in OTA concentrations at 40-60 μg/mL. No OTA was detected at 120 hr in solution containing 40 μg OTA/mL and crude enzyme of 0.2 mg protein/mL, and at 168 hr with crude enzyme of 0.15 mg protein/mL. OTA remained detectable at 192 hr when crude enzyme of 0.1 mg protein/mL was added (Fig. 7d). At crude enzyme concentration of 0.2 mg protein/mL and OTA concentration of 50 μg/mL, no OTA was detected at 120 hr. In solutions containing 50 μg OTA/mL with crude enzyme concentrations of 0.15 or 0.1 mg protein/mL, OTA was not detected at 144 and 192 hr, respectively (Fig. 7e). In solutions containing crude enzyme of 0.1, 0.15, or 0.2 mg protein/mL and 60 μg OTA/mL, OTA was not detected at 168 hr, 192 hr, and 144 hr, respectively (Fig. 7f).

The rate of hydrolysis of 10-60 μg OTA/mL by crude enzyme of 0.1 mg protein/mL is shown in Fig. 8a. The rate of OTA hydrolysis was faster at higher OTA concentrations, and the disappearance of OTA over time was linear ($r>0.95$) in all OTA concentrations. OTA residues were still detected at 168 hr when initial OTA concentrations were 10-50 μg/mL. The higher concentrations of OTA, the higher rate of OTA hydrolysis by crude enzyme of 0.1 mg protein/mL was also seen when 10-60 μg OTA/mL were reacted with crude enzyme of 0.15 and 0.2 mg protein/mL (Figs. 8b and 8c). When crude enzyme concentration was 0.15 mg/mL, only trace OTA (2-3

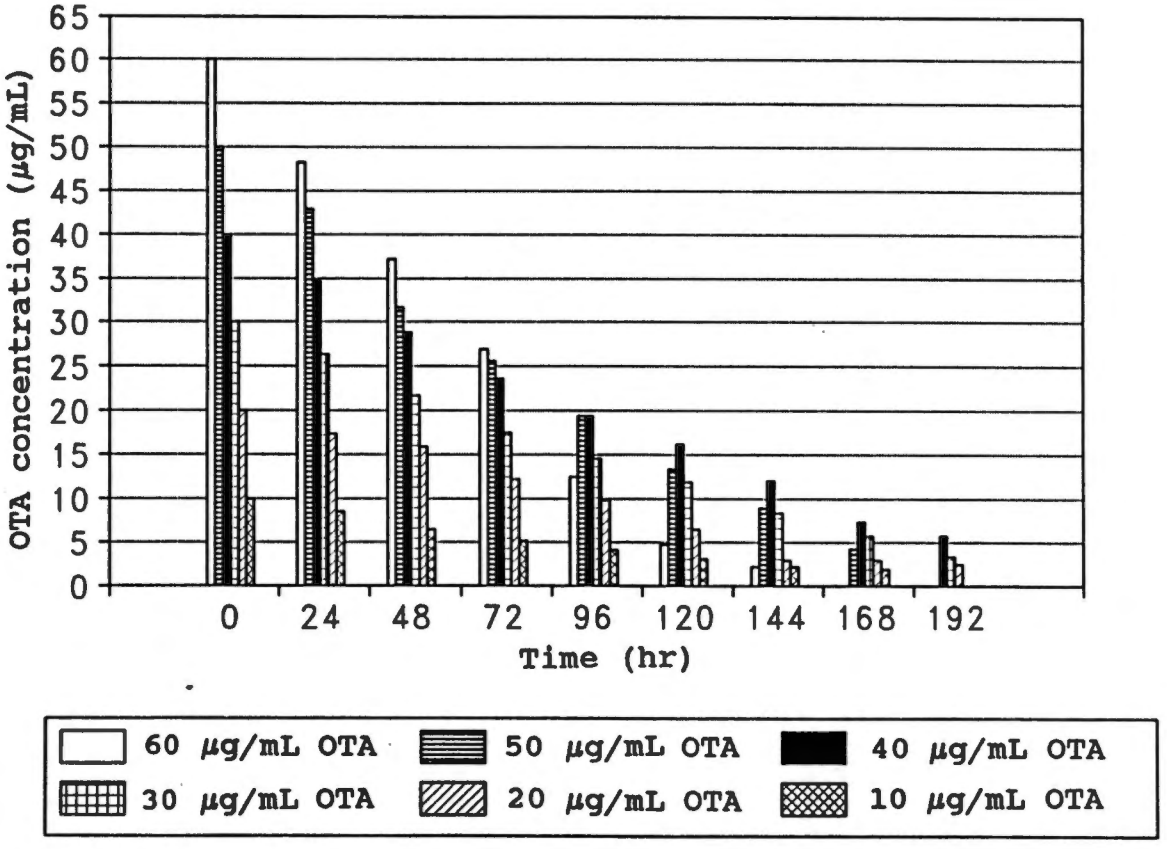


Figure 8-Decrease by hydrolysis of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by selected concentrations of crude enzyme isolated from *A. calcoaceticus*: a. Decrease by hydrolysis of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by 0.1 mg/mL crude enzyme isolated from *A. calcoaceticus*.

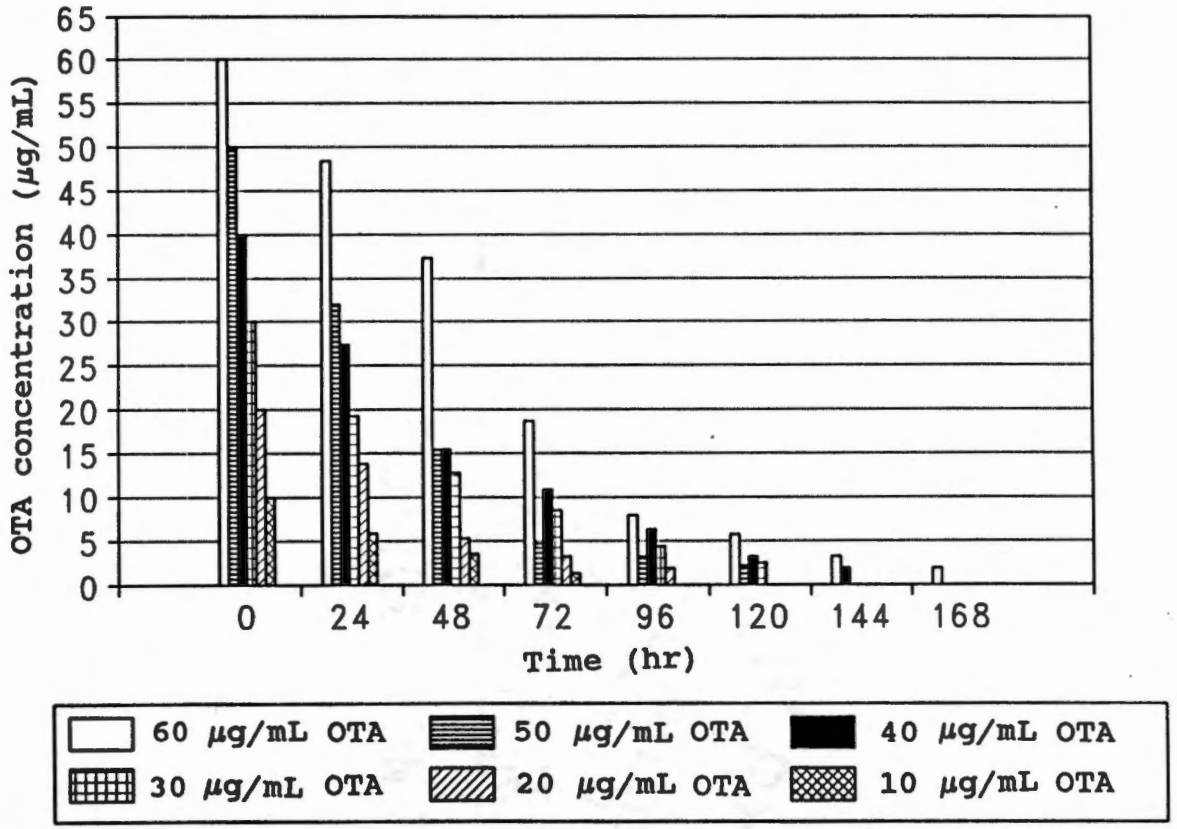


Figure 8-(Continued) b. Decrease by hydrolysis of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by 0.15 mg/mL crude enzyme isolated from *A. calcoaceticus*.

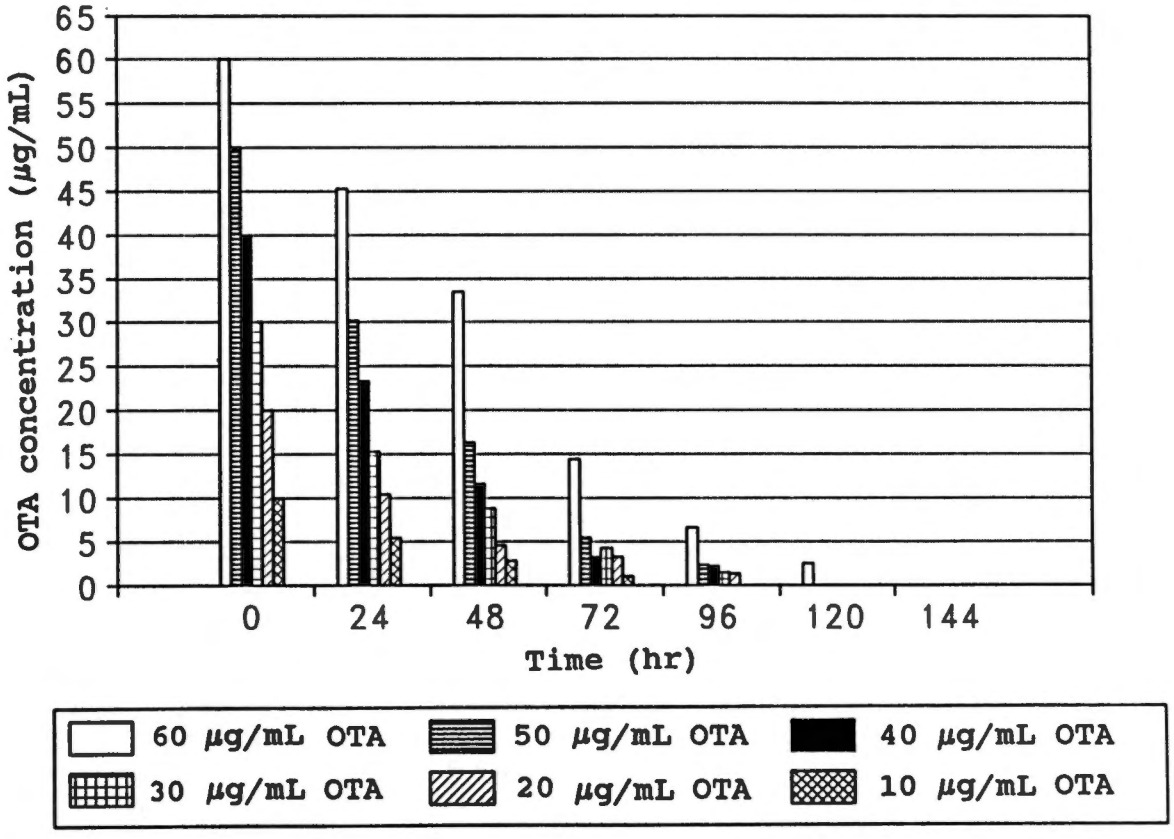


Figure 8-(Continued) c. Decrease by hydrolysis of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by 0.2 mg/mL crude enzyme isolated from *A. calcoaceticus*.

μg/mL) remained after 120 hr when initial OTA concentrations of 30, 40, and 50 μg/mL were used (Fig. 8b). In solution containing crude enzyme of 0.2 mg protein/mL and 10-50 μg OTA/ml, OTA was not detected at 120 hr (Fig. 8c). Hydrolysis of 10-60 μg/mL OTA by crude enzyme of 0.2 mg protein/mL was 24 hr and 72 hr was more rapid than that required by crude enzyme of 0.15 and 0.1 mg protein/mL, respectively. Hydrolysis dependance on crude enzyme concentrations is typical of enzymatic reactions. As OTA concentration increase, the rate of OTA hydrolysis is expected to increase until a maximum rate is reached. Rate of hydrolysis of 30 and 50 μg OTA/mL by carboxypeptidase A of 50 μg protein/mL is shown in Fig. 9. OTA was not detected after 4 hr in OTA concentration of 30 μg/mL, and only trace OTA was detected in OTA concentration of 50 μg/mL after 4.5 hr. Crude enzyme isolated from *A. calcoaceticus* hydrolyzed OTA more slowly than carboxypeptidase A did (Table 8). This was most likely due to the low concentration of OTA-hydrolyzing enzyme in the crude enzyme preparation. The hydrolysis of OTA by crude enzyme and carboxypeptidase A showed that the higher the initial OTA concentration at the same protein concentration, the faster the rate of OTA hydrolysis.

The overall rate of OTA hydrolysis by crude enzyme of 0.1, 0.15 and 0.2 mg protein/mL is shown in Table 9. The

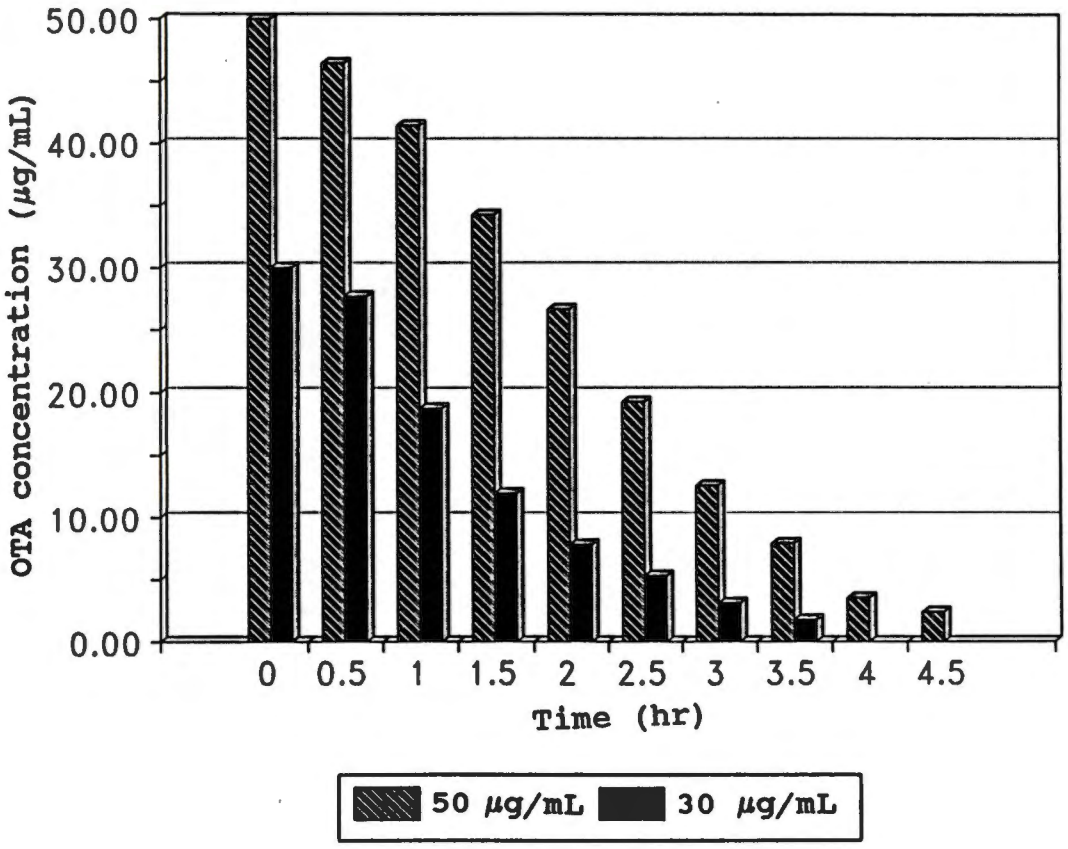


Figure 9-Decrease by hydrolysis of selected concentrations of ochratoxin A in 0.1 M NaCl-0.02 M Tris buffer by 50 µg/mL carboxypeptidase A.

Table 8-Initial rate of ochratoxin A (OTA) hydrolyzed by crude enzyme isolated from *A. calcoaceticus*and carboxypeptidase A in 0.1 M-0.02 M Tris buffer at 25°C

OTA concentration (μg/mL)	Initial Rate of OTA hydrolysis (μg/mL/hr)/Incubation hr (Correlation Coefficient)		
	Crude enzyme concentration (mg protein/mL)		
	0.10	0.15	0.20
60	0.4592^{a}/72 (0.9990)	0.5587^{a}/72 (0.9915)	0.6198^{a}/72 (0.9909)
50	0.3087^{b}/120 (0.9873)	0.5362^{a}/72 (0.9902)	0.6158^{a}/72 (0.9818)
40	0.1905^{c}/168 (0.9931)	0.5126^{a}/72 (0.9642)	0.5903^{a}/48 (0.9906)
30	0.1502^{d}/144 (0.9923)	0.3593^{b}/48 (0.9815)	0.4423^{b}/48 (0.9513)
20	0.1176^{d}/144 (0.9902)	0.2454^{c}/72 (0.9545)	0.3215^{c}/48 (0.9805)
10	0.0550^{e}/168 (0.9813)	0.1194^{d}/72 (0.9727)	0.1526^{d}/72 (0.9801)
	Carboxypeptidase A (50 μg protein/mL)		
50	10.4463^{a}/4 (0.9964)		
30	9.2256^{b}/3 (0.9907)		

[a-e]Means within a column followed by different superscripts are significantly different ($p<0.05$).

Table 9-Overall rate of ochratoxin A (OTA) hydrolyzed by crude enzyme isolated from *A. calcoaceticus* in 0.1 M NaCl-0.02 M Tris buffer at 25°C over incubation time

OTA concentration (μg/mL)	Overall Rate of OTA hydrolysis (μg/mL/hr)[a]/Incubation hr (Correlation Coefficient)		
	Crude enzyme concentration (mg protein/mL)		
	0.10	0.15	0.20
60	0.3783/144 (0.9546)	0.3623/168 (0.9010)	0.4355/120 (0.9345)
50	0.2599/168 (0.9791)	0.4008/120 (0.8630)	0.4100/96 (0.8912)
40	0.1806/192 (0.9877)	0.2561/144 (0.8925)	0.3999/96 (0.9159)
30	0.1399/192 (0.9906)	0.2227/144 (0.9321)	0.2840/96 (0.9159)
20	0.1010/192 (0.9673)	0.1949/96 (0.9052)	0.1864/96 (0.8692)
10	0.0492/168 (0.9730)	0.1194/72 (0.9727)	0.1195/96 (0.9413)

[a]Regression of OTA concentrations over the given incubation hour.

disappearance of OTA was more linear over incubation time (r value closer to 1.0) in solutions containing 10-60 μg OTA/mL with crude enzyme of 0.1 mg protein/mL than those with higher concentrations of crude enzyme. The overall rate of OTA hydrolyzed by crude enzyme increased as the concentrations of OTA and/or crude enzyme increased. In solutions containing crude enzyme of 0.2 mg protein/mL, the overall rate of OTA hydrolysis was not significantly different ($p>0.05$) among initial OTA concentrations of 40, 50, and 60 μg/mL. OTA was hydrolyzed by crude enzyme of 0.1, 0.15 and 0.2 mg protein/mL at a faster rate initially (comparing the initial rate (Table 8) with the overall rate (Table 9)). In solutions containing crude enzyme of 0.15 and 0.2 mg protein/mL, the initial rate of OTA hydrolysis was not significantly different ($p>0.05$) among OTA concentrations of 40, 50, and 60 μg/mL (Table 8).

Identification of Hydrolysates of Ochratoxin A

OTA hydrolysates obtained from OTA incubated with *A. calcoaceticus* and crude enzyme were separated on TLC plates. Two fluorescent spots, green and blue in color, appeared on TLC plates under UV light. The green fluorescent spot had a R_f value of 0.69±0.04 and was identical to the fluorescent spot given by OTA standard in color and R_f value. The blue fluorescent spot had R_f value of 0.33±0.03 and was identical

to the fluorescent spot given by ochratoxin α obtained from carboxypeptidase A hydrolysis in color and R_f value. Pure OTA and ochratoxin α had retention times of 3.19±0.32 and 4.5±0.45 min, respectively (Fig. 10). The retention times for extracts of green and blue fluorescent spots from samples (OTA with culture and crude enzyme) on TLC plates were identical to those of OTA standard and ochratoxin α. Figure 11 shows the progressive degradation of OTA by *A. calcoaceticus* at 30°C (incubation time, 0-144 hr) as the peak of OTA which appeared at approximately 3.19 min was decreased and the peak of ochratoxin α which appeared at approximately 4.5 min was increased. OTA and ochratoxin α in 0.1 M NaCl-0.02 M Tris buffer have maximum absorption at 380 nm and 340 nm, respectively (Hult and Gatenbeck, 1976). Figure 12 shows the UV spectrum for the extracts of green and blue fluorescent spots from samples on TLC plates. The blue and green fluorescent spots had maximum absorption at 340 nm and 380 nm, respectively. The absorbance of OTA at 380 nm decreased and the absorbance of ochratoxin α at 340 nm gradually increased as OTA was hydrolyzed by crude enzyme (Fig. 13). The absorbance shift from 380 nm to 340 nm due to the hydrolysis of OTA by crude enzyme was identical to those reported for some proteolytic enzymes that hydrolyzed OTA (Pitout, 1969). Therefore, an esterase or peptidase-like enzyme in the crude preparation capable of breaking the

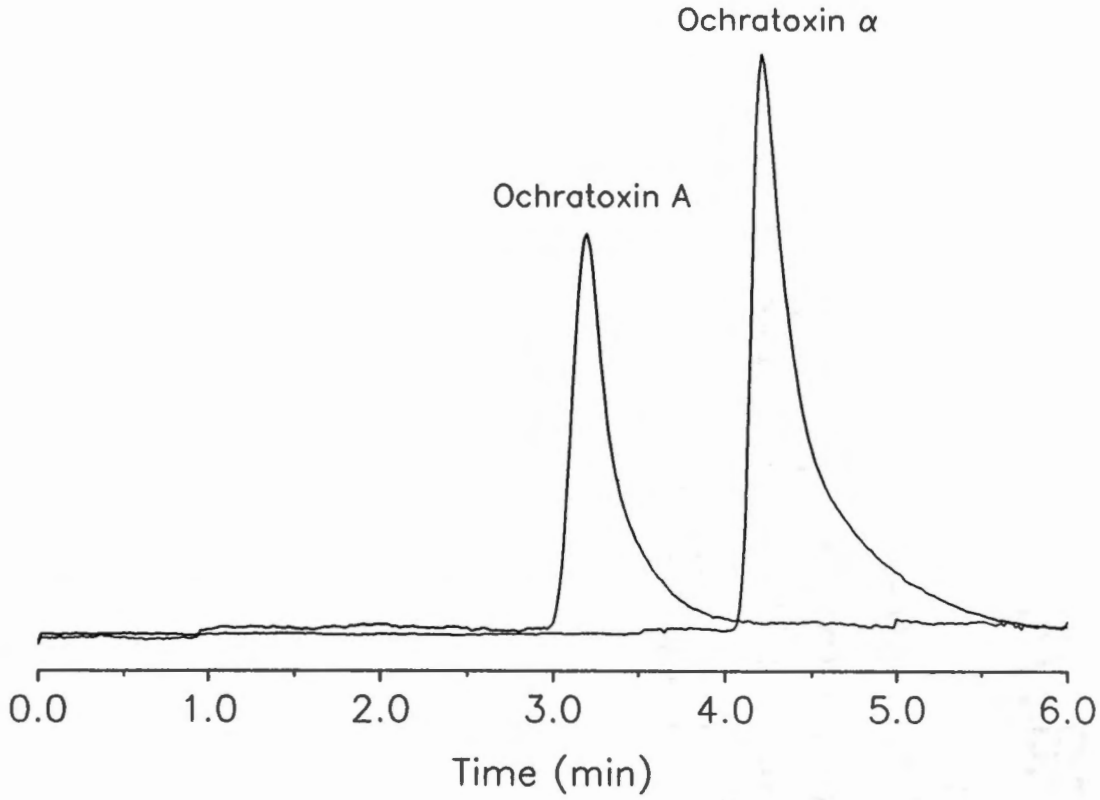


Figure 10-Fluorescence chromatogram of ochratoxin A and ochratoxin α (340 nm excitation wavelength and 440 nm emission wavelength).

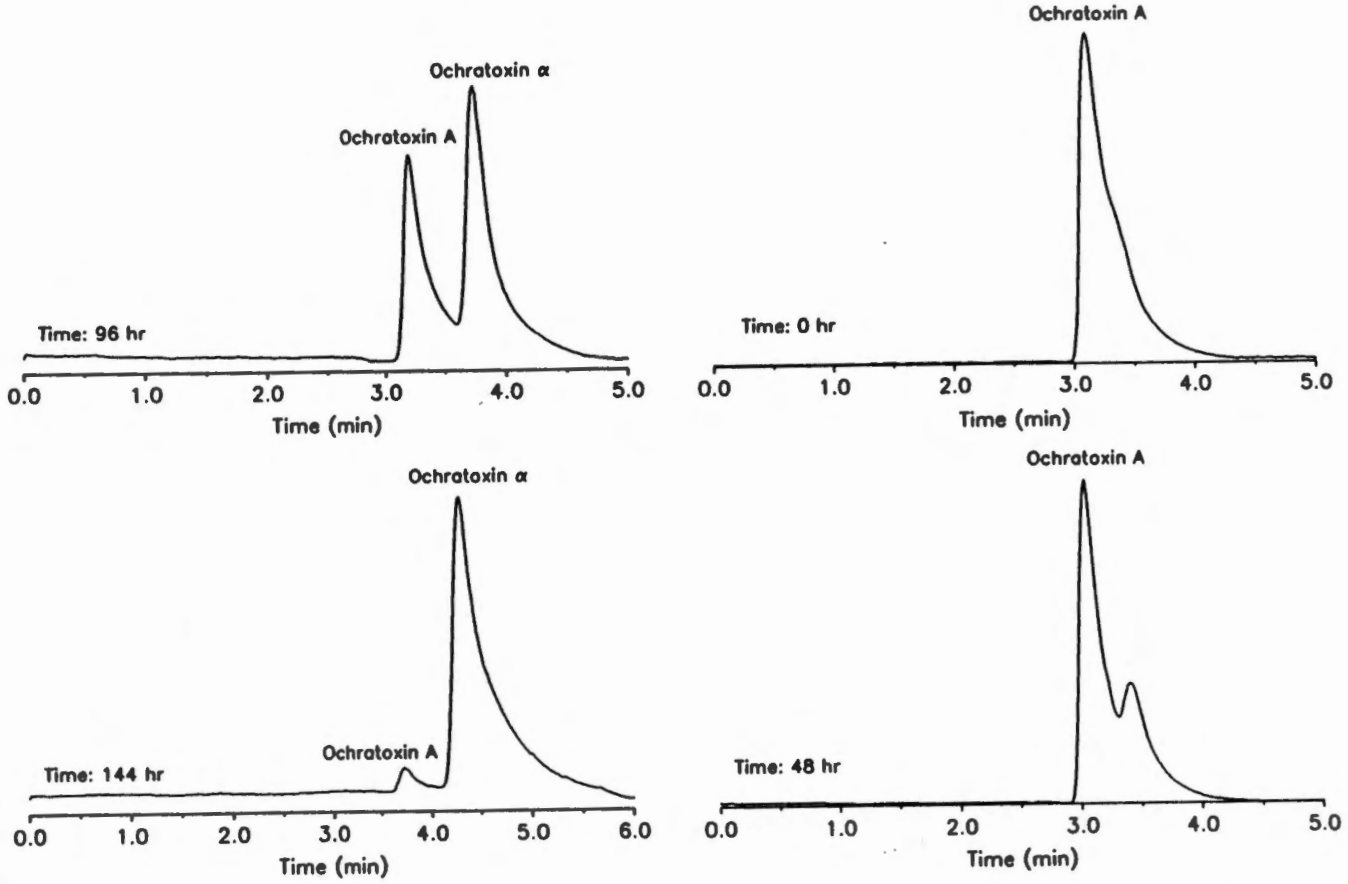


Figure 11-Progressive fluorescence chromatograms (0-144 hr) of ochratoxin A degraded by *A. calcoaceticus* (340 nm excitation wavelength and 440 nm emission wavelength).

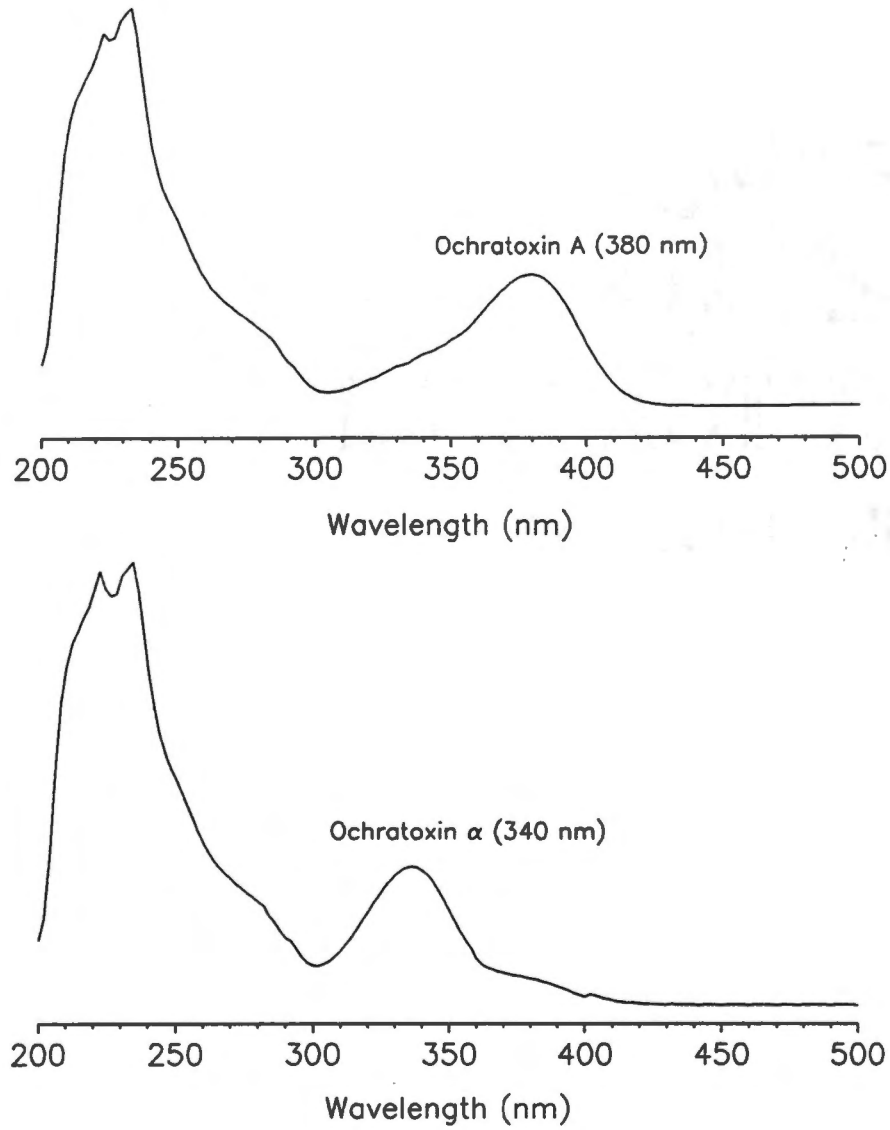


Figure 12-UV spectrum of ochratoxin A and ochratoxin α in 0.1 M NaCl-0.02 M Tris buffer.

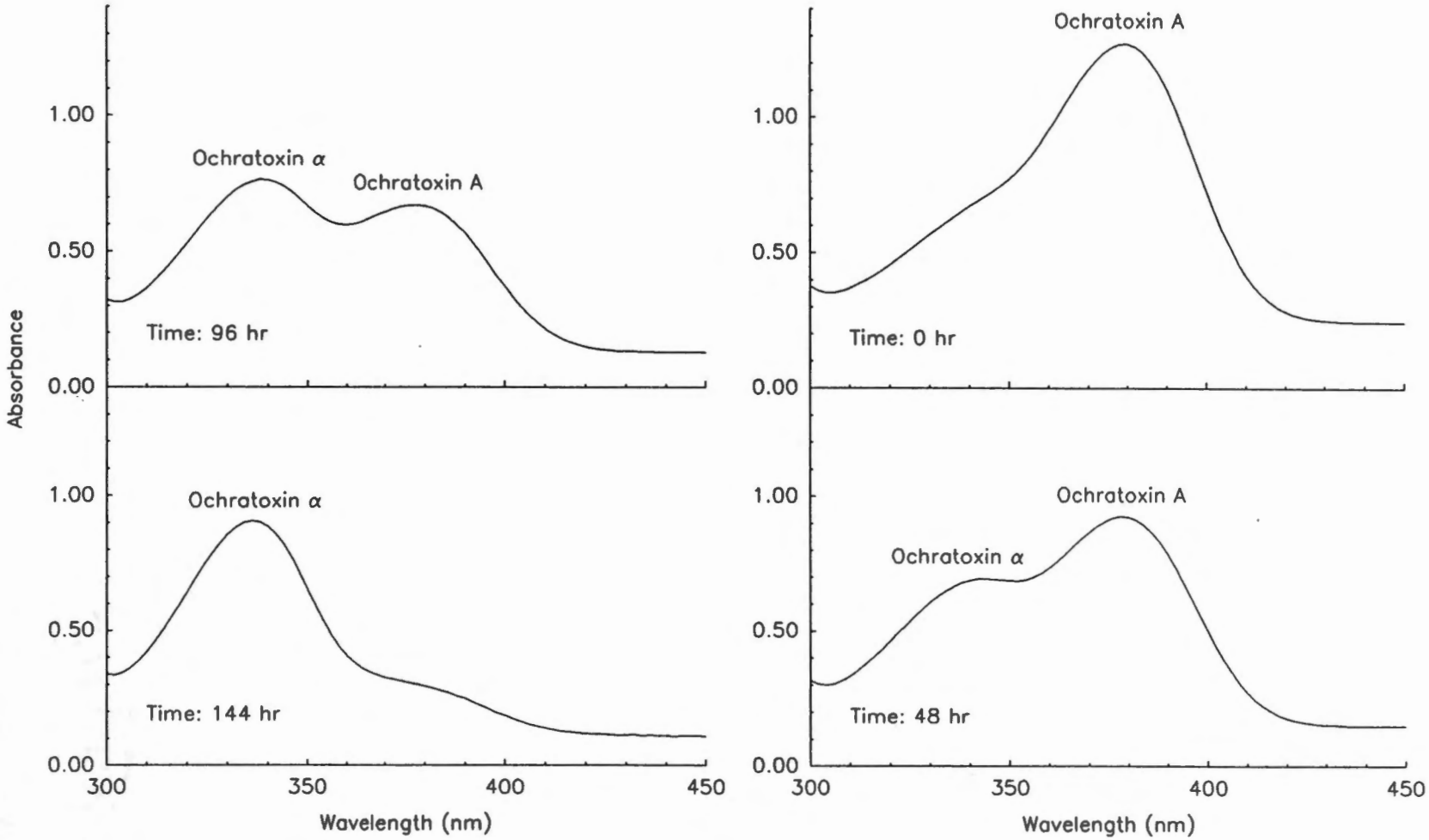


Figure 13-Progressive UV spectrum (0-144 hr) of ochratoxin A hydrolyzed by crude enzyme isolated from *A. calcoaceticus* in 0.1 M NaCl-0.02 M Tris buffer.

amide bond between isocoumarin acid and phenylalanine may be responsible for the degradation of OTA. Results obtained by HPLC, TLC, and UV-VIS spectrophotometry indicated that the OTA hydrolysate obtained from both *A. calcoaceticus* incubation with OTA and the reaction of crude enzyme with OTA was ochratoxin α. Ochratoxin α was not degraded further by *A. calcoaceticus* and crude enzyme. Therefore, it was concluded that OTA was hydrolyzed by cells and crude enzyme of *A. calcoaceticus* to yield ochratoxin α as proposed in Fig. 4.

Toxicity Test of Hydrolyzates of Ochratoxin A Using *Bacillus cereus mycoides*

The size of zone of inhibition exerted by OTA residues on *B. cereus mycoides* decreased as OTA was hydrolyzed by *A. calcoaceticus* and crude enzyme (Table 10). Gradually decreasing inhibition zones indicated that OTA was degraded and the hydrolysates resulting from the degradation were not inhibitory to growth of *B. cereus mycoides*. This indicated that OTA was hydrolyzed such that it was non-toxic to *B. cereus mycoides*. Cell-free filtrate from *A. calcoaceticus* growth medium (no OTA added) did not inhibit the growth of *B. cereus mycoides*. This simple bioassay using *B. cereus mycoides* cannot give conclusive evidence concerning toxigenicity of ochratoxin α. However a study by Yamazaki

Table 10-Corrected inhibition zone of 0.1 mL filtrate from 40 μg/mL ochratoxin A (OTA) incubated with *A. calcoaceticus* and 0.2 mg/mL crude enzyme isolated for *A. calcoaceticus* at 30°C

	Corrected Inhibition Zone[a] (mm)/OTA Concentration (μg/0.1 mL)[b]	
Incubation hr	*A. calcoaceticus*	Crude enzyme
0	4.0 / 4.3	4.1 / 4.4
24	3.8 / 4.2	3.2 / 3.7
48	3.7 / 4.1	2.3 / 2.9
72	3.4 / 3.9	2.0 / 2.6
96	2.8 / 3.3	1.2 / 1.9
120	2.3 / 2.9	0.2 / 1.0

[a]Corrected inhibition zone=diameter of inhibition zone-diameter of paper disk. Average of duplicate.
[b]Ochratoxin A concentration after reaction with *A. calcoaceticus* or crude enzyme. Based on standard curve Y=-0.967+1.13X. Correlation coefficient of 0.983. X=μg standard OTA, Y=corrected inhibition zone (mm).

et al. (1971) did show that ochratoxin α had much lower toxicity than ochratoxin A (Table 11). Although carboxypeptidase A is capable of hydrolyzing OTA to ochratoxin α, the enzyme isolated from bovine pancreas is toxic to biological systems (Sigma Chemical Co., St. Louis, MO). That *A. calcoaceticus* is capable of degrading OTA to ochratoxin α may provide a potential application for this bacterium to detoxify OTA in foods and feedstuffs.

Table 11-Toxicity of ochratoxin A and ochratoxin α on chicken embryos

	Dose (μg/egg)	Number of eggs	Number dead (%) in 24	48	72	96 hr
Ochratoxin A	100	15	10 (66.5)	15 (100)	15 (100)	-
	50	15	6 (40.0)	12 (80)	15 (100)	-
	25	15	0 (0.0)	12 (80)	12 (80)	-
	12.5	15	2 (13.3)	2 (13.3)	5 (33.3)	-
Ochratoxin α	100	10	1 (10)	3 (30)	-	3 (30)
	50	10	1 (10)	1 (10)	-	2 (20)
	25	13	1 (7.7)	1 (7.7)	-	1 (7.7)

Source: Yamazaki et al., 1971.

CHAPTER V

CONCLUSION

Acinetobacter calcoaceticus cells were able to hydrolyze ochratoxin A (OTA). A crude enzyme was isolated from the growth medium of *A. calcoaceticus* and shown to be responsible for the degradation of OTA. The end product of OTA degradation by *A. calcoaceticus* was ochratoxin α. Since hydrolysis of amide bond of OTA by peptidases yields ochratoxin α and phenylalanine, a peptidase-like enzyme may be present in the crude enzyme isolated from *A. calcoaceticus* that hydrolyzes the amide bond to yield ochratoxin α. Ochratoxin α is much less toxic in a biological system. Therefore, the degradation of OTA by *A. calcoaceticus* may be considered a form of microbiological detoxication of OTA.

This study was performed under controlled conditions and an *in vitro* test system was used. The results of this study look promising; however, they do not imply the degradation of OTA in an *in situ* system using food or feed. The findings of this study provide incentive for further study of microbiological degradation of OTA. Further studies are needed to better characterize the enzyme(s) involved in OTA degradation and to evaluate the ability of

the enzyme to detoxify OTA in foods and feeds. Further studies may also focus on increasing enzyme production by *A. calcoaceticus* through physical, chemical, or genetic manipulation and developing methodology for the enzyme purification.

LIST OF REFERENCES

Abedi, Z. H. and Scott, P.M. 1969. Detection of toxicity of aflatoxins, sterigmatocystin and other fungal toxin by lethal action on Zebra fish larvae. J. Assoc. Off. Anal. Chem. 52:963-969.

Allan, B.J., Keller, P.J., and Neurath, H. 1963. Procedures for the isolation of crystalline bovine pancreatic carboxypeptidase A. I. Isolation from acetone powders of pancreas glands. Biochem. 3:40-43.

AOAC. 1984. Antibiotics in feeds. In *Official Methods of Analysis*, pp. 813-815. Association of Official Analytical Chemists, Inc., Arlington, VA.

Bacon, C.W., Sweeney, J.G., Robbins, J.D., and Burdick, D. 1973. Production of penicillic acid and ochratoxin A on poultry feed by *Aspergillus ochraceus*: temperature and moisture requirements. Appl. Microbiol. 26:155-160.

Bendele, A.M., Carlton, W.W., Krogh, P., and Lillehoj, E.B. 1985. Ochratoxin A carcinogenesis in the (C57BL/6J x C3H) F_1 mouse. J. Natl. Cancer Inst. 75:733-739.

Borce, D., Grodner, R.M., Killebrew, R., and Bonner, F.L. 1970. Ochratoxin A and B confirmation by microbiological assay using *Bacillus cereus mycoides*. J. Assoc. Off. Anal. Chem. 53:616-619.

Boutibonnes, P. 1980. Antibacterial activity of some mycotxoins. Med. Sci. Biochem. Pharmacol. 8:850-851.

Bridges, J.W. 1969. Fluorescence analysis. In *Handbook of Analytical Toxicology*, I. Sunshine (Ed.), pp. 909-921. The Chemical Rubber Co., Cleveland, OH.

Brown, R.F. 1969. The effect of some mycotoxins on the brine shrimp, *Artemia salina*. J. Amer. Oil Chem. Soc. 46:119.

Brown, M.H., Szczech, G.M., and Purmalis, B.P. 1976. Teratogenic and toxic effects of ochratoxin A in rats. Toxicol. Appl. Pharmacol. 37:331-338.

Carlton, W.W. 1979. Ochratoxins. A review. In *Proceedings of Conference on Mycotoxins in Animal Feeds and Grains Related to Animal Health*, W. Shimoda (Ed.), pp. 165-287. Bureau of Veterinary Medicine, FDA, Rockville, MD.

Chang, F.C. and Chu, F.S. 1977. The fate of ochratoxin A in rats. Food Cosmet. Toxicol. 15:199-204.

Chang, F.C., Doerr, J.A., and Hamilton, P.B. 1980. Experimental ochratoxicosis in turkey poults. Poultry Sci. 60:114-118.

Choudhury, H., Carlson, C.W., and Semeniuk, G. 1971. A study of ochratoxin toxicity in hens. Poultry Sci. 50:1855-1858.

Choudhury, H. and Carlson, C.W. 1973. The lethal dose of ochratoxin for chick embryos. Poultry Sci. 52:1202-1203.

Christen, G.E. 1991. Disc-gel electrophoresis of protein. In *Food Chemisty I Lab Manual*, pp. 49-52. Department of Food Technology and Science, The University of Tennessee, Knoxville.

Chu, F.S. 1974. Studies of ochratoxins. CRC Crit. Rev. Toxicol. 2:499-524.

Chu, F.S. and Butz, M.E. 1970. Spectrophotofluorodensitometric measurement of ochratoxin A in cereal products. J. Assoc. Off. Anal. Chem. 53:1253-1257.

Chu, F.S., Noh, I., and Chang, C.C. 1972. Structural requirements of ochratoxin intoxication. Life Sci. 11:503-508.

Chu, F.S., Chang, C.C, Ashoor, S.H., and Prentice, N. 1975. Stability of aflatoxin B_1 and ochratoxin A in brewing. Appl. Microbiol. 29:313-316.

Ciegler, A., Lillehoj, E.B., and Hall, H.H. 1966. Microbial detoxification of aflatoxin. Appl. Microbio. 14:934-939.

Ciegler, A. and Vesonder, R.F. 1983. Microbial and feed toxicants: fungal toxins. In *Handbook of Food Borne Diseases of Biological Origin*, R. Miloslave, Jr. (Ed.), p. 57. CRC Press, Boca Raton, FL.

Cole, R.J. and Cox, R.H. 1981. Ochratoxins. In *Handbook of Toxic Fungal Metabolites*, R.J. Cole (Ed.), pp. 128-146. Academic Press, New York, NY.

Cooper, T.G. 1977. Electrophoresis. In *The Tools of Biochemistry*, T.G. Cooper (Ed.), pp. 194-208. John Wiley and Sons, Inc., New York, NY.

Creppy, E.E, Schlegel, M., Roschenthaler, and Dirheimer, G. 1980. Phenylalanine prevents acute poisoning by ochratoxin A in mice. Toxicol. Lett. 6:77-80.

Creppy, E.E., Nern, D., Steyn, P.S., Velggaar, R., Roschenthaler, R., and Dirheimer, G. 1983. Comparative study of the effect of ochratoxin A analogues on yeast aminoacyl-tRNA synthetases and on the growth and protein synthesis of hepatoma cells. Toxicol. Lett. 19:217-219.

Creppy, E.E., Roschenthaler, R., and Dirheimer, G. 1984. Inhibition of protein synthesis in mice by ochratoxin A and its prevention by phenylalanine. Food Chem. Toxicol. 22:883-886.

Doster, R.C. and Sinnhuber, R.O. 1972. Comparative rate of hydrolysis of ochratoxin A and B *in vitro*. Food Cosmet. Toxicol. 10:389-394.

Doster, R.C., Sinnhuber, R.O., and Pawlowski, N.E. 1974. Acute intraperitoneal toxicity of ochratoxin A and B derivatives in rainbow trout (*Salmon gairdneri*). Food Cosmet. Toxicol. 12:499-505.

Dwivedi, P. and Burns, R.B. 1986. The natural occurrance of ochratoxin A and its effect in poultry. A review. Part I. Epidemiology and toxicity. World's Poultry Sci. 42:32-47.

Elling, F., Hald, B., Jacobsen, C., and Krogh, P. 1975. Spontaneous cases of toxic nephropathy in poultry associated with ochratoxin A. Acta Pathol. Microbiol. Scand. Section A 83:739-741.

Galtier, P. 1974. The fate of ochratoxin A in the animal organism. II. Distribution in the tissues and excretion in the rat. Ann. Rech. Veter. 5:319-318.

Galtier, P. and Alvinerie, M. 1976. *In vitro* transformation of ochratoxin A by animal microbial floras. Ann. Rech. Veter. 71:91-98.

Gilani, S.H., Bancroft, J., and Reily, M. 1978. Teratogenicity of ochratoxin A in chick embryos. Toxicol. Pharmacol. 46:543-546.

Goldblatt, L.A. and Dollear, F.G. 1977. Detoxification of contaminated crop. In *Mycotoxins in Human and Animal Health*, J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman, (Ed.), p. 121. Pathotox, Park Forest South, IL.

Haggblom, P. 1982. Production of ochratoxin in barley by *Aspergillus ochraceus* and *Penicillium viridicatum*: Effect of fungal growth, time, temperature, and inoculum size. Appl. Environ. Microbiol. 43:1205-1207.

Hamilton, P.B., Huff, W.E., Harris, R.J., and Wyatt, R.D. 1977. Outbreak of ochratoxicosis in poultry. Abstr. Annu. Meet. Amer. Soc. Microbiol. 77:248-256.

Hamilton, P.B., Huff, W.E., Harris, J.R., and Wyatt, R.D. 1982. Natural occurrences of ochratoxicosis in poultry. Poultry Sci. 61:1832-1841.

Harwing, J. 1974. Ochratoxin A and related metabolites. In *Mycotoxins*, I.F.H. Purchase (Ed.), pp. 345-367. Elsevier Scientific Publishing Co., New York, NY.

Harwing, J. and Chen, Y.K. 1974. Some conditions favoring production of ochratoxin A and citrinin by *Penicillium viridicatum* in wheat and barley. Can. J. Plant Sci. 54:17-22.

Harwing, J., Chen, Y.K., and Collins-Thompson, D.L. 1974. Stability of ochratoxin A in bean during canning. Can. Inst. Food Sci. Technol. 7:288-289.

Haubeck, H.D, Lorkowski, D., Kolsch, E., and Roschenthaler, R. 1981. Immunosuppression by ochratoxin A and it prevention by phenylalanine. Appl. Environ. Microbiol. 41:1040-1042.

Hayes, A.W., Hood, R.D., and Lee, H.L. 1974. Teratogenic effects of ochratoxin A in mice. Teratology 9:93-98.

Heller, K. and Roschenthaler, R. 1978. Inhibition of protein synthesis in *Streptococcus faecalis* by ochratoxin A. Can. J. Microbiol. 24:466-472.

Heymann, E. 1980. Carboxylesterases and amidases. In *Enzymatic Basis of detoxication*, vol II, W.B. Jakoby (Ed.), pp. 291-323. Academic Press, New York, NY.

Hood, R.D., Naughton, M.J., and Hayes, A.W. 1976. Prenatal effects of ochratoxin A in hamster. Teratology 13:11-14.

Hood, R.D., Kuczuk, M.H., and Szczech, G.M. 1978. Effects of mice of simultaneous prenatal exposure to ochratoxin A and T-2 toxin. Teratology 17:25-30.

Hult, K., Teiling, A., and Gatenbeck, S. 1976. Degradation of ochratoxin A by a ruminant. Appl. Environ. Microbiol. 32:443-444.

Hutchison, R.D., Steyn, P.S., and Thompson, D.L. 1971. The isolation and structure of 4-hydroxyochratoxin A and 7-carboxyl-3,4-dihydro-8-hydroxy-3 methylisocoumarin from *Penicillium viridicatum*. Tetrahedron Lett. 43:4033-4036.

Juskiewicz, T., Piskorsha-Pliszczynska, J., and Wisniewska, H. 1982. Ochratoxin A in laying hens: Tissue deposition and passage into eggs. In *Proceeding of the 5th International IUPAC Symposium on Mycotoxins and Phycotoxins*, Vienna, pp. 122-125. IUPAC.

Kanisawa, M. 1984. Synergistic effect of citrinin on hepatorenal carcinogenesis of ochratoxin A in mice. In *Toxigenic Fungi-Their Toxins and Health Hazards*, H. Kurata and Y. Ueno (Ed.), p. 245. Elsevier Publishing Co., New York, NY.

Kanisawa, M., Suzuki, S., Lozuka, Y, and Yamazake, M. 1977. Histopathological studies on the toxicity of ochratoxin A in rat. I. Acute oral toxicity. Toxicol. Appl. Pharmacol. 42:55-64.

Kanisawa, M. and Suzuki, S. 1978a. Carcinogenicity of ochratoxin A and aflatoxin B_1 to mice. Proc. Jpn. Assoc. Mycotoxicol. 7:28-32.

Kanisawa, M. and Suzuki, S. 1978b. Induction of renal and hepatic tumors in mice by ochratoxin A, a mycotoxin. Jpn. J. Cancer Res. 69:599-604.

Kaufman, D.D. 1967. Degradation of carbamate herbicides in soil. J. Agr. Food Chem. 15:582-591.

Kiessling, K.H., Patterson, H., Sandholm, K., and Olsen, M. 1986. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. Appl. Environ. Microbiol. 47:1071-1073.

Kriog, N.R. 1984. *Bergey's Manual of Systematic Bacteriology*, N.R. Kriog (Ed.), pp. 303-305. William and Wilkins, Baltimore, MD.

Krogh, P. 1978. Causal associations of mycotoxic. Acta Pathol. Microbiol. Scand. Section A Suppl. 269:1-28.

Krogh, P. 1990. Porcine nephropathy associated with ochratoxin A. In *Mycotoxins and Animal Foods*, J.E. Smith and R.S. Henderson (Ed.), pp. 627-645. CRC Press, Inc., Boca Raton, FL.

Krogh, P., Hald, B., and Pedersen, E.J. 1973. Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Pathol. Microbiol. Scand. Section B 81:689-695.

Krogh, P., Alexsen, N.H., Elling, F., Gyrd-Hansen, N., Hald, B., Hyldgaard-Jense, J., Larsen, A.E., Madsen, A, Mortensen, H.P., Moller, T., Peterson, O.L., Ravnskow, U., Rostgaard, M., and Aalund, O. 1974a. Experimental porcine nephropathy. Acta Pathol. Microbiol. Scand. Section A Suppl. 246:1-21.

Krogh, P., Hald, B, Gertsen, P., and Myken, F. 1974b. Fate of ochratoxin A and citrinin during malting and brewing experiments. Appl. Microbiol. 28:31-34.

Krogh, P., Elling, F., Hald, B., Jylling, B., Peterson, V.E., Skadhauge, E., and Svendsen, C.K. 1976a. Experimental avian nephropathy: Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Pathol. Microbiol. Scand. Section A 84:215-221.

Krogh, P., Elling, B., Hald, B., Larsen, A.E., Lillehoj, E.B., Madsen, A., and Mortensen, H.P. 1976b. Time-dependent disappearance of ochratoxin A residues in tissues of bacon pigs. Toxicology 6:235-242.

Krogh, P., Hald, B., Plestina, R., and Ceovic, S. 1977. Balkan (endemic) nephropathy and foodborne ochratoxin A: Preliminary results of a survey of food stuffs. Acta Pathol. Microbiol. Scand. Section B 85:238-240.

Lai, M., Semeniuk, G., and Hesseltine, C.W. 1970. Conditions for production of ochratoxin A by *Aspergillus* species in a synthetic medium. Appl. Microbiol. 19:542-544.

Larsen, H.j., Jorgense, N.A. Barrington, G.P., and Niedermeier, R.P. 1972. Effect of organic acids on preservation and acceptability of high-moisture corn. J. Dairy Sci. 55:685-689.

Lindenfelser, L.A., Lillejoh, E.B., and Milburn, M.E. 1973. Ochratoxin and penicillic acid in tumorigenic and acute toxicity test with white mice. Dev. Indus. Microbiol. 14:331-336.

Lyman, O. 1988. Multiple comparisons. In *An Introduction to Statistical Methods and Data Analysis*, pp. 446-449. Pw-Kent Publishing Co., Boston, MA.

Mayura, K, Hayes, A.W., and Berndt, W.O. 1983. Effects of dietary protein on teratogenicity of ochratoxin A in rats. Toxicology 27:147-157.

Mayura, K., Parker, R., Berndt, W.O, and Phillips, T.D. 1984a. Effect of simultaneous prenatal exposure to ochratoxin A and citrinin in the rat. J. Toxicol. Environ. Health 13:553-561.

Mayura, K., Parker, R., Berndt, W.O., and Phillips, T.D. 1984b. Ochratoxin A-induced teratogenesis in rats: Partial protection by phenylalanine. Appl. Environ. Microbiol. 48:1186-1188.

Moroi, K., Suzuki, S., Kuga, T., Yamazaki, M., and Kanisawa, M. 1985. Reduction of ochratoxin A toxicity in mice treated with phenylalanine and phenobarbital. Toxicol. Lett. 25:1-4.

Neely, W.C. and West, A.D. 1972. Spectroanalytical parameters of fungal metabolites. III. Ochratoxin A. J. Assoc. Off. Anal. Chem. 55:1305-1309.

Northol, M.D., Van Egmond, H.P., and Paulsch, W.E. 1979. Ochratoxin A production by some fungal species in relation to water activity and temperature. J. Food Prot. 42:485-490.

Osborne, B.G. 1980. The occurrence of ochratoxin A in mouldy bread and flour. Food Cosmet. Toxicol. 18:615-617.

Patterson, D.S.P., Shreeve, B.J., Roberts, B.A., Berrett, S., Brush, P.J., Glancy, E.M., and Krogh, P. 1981. Effects on calves of barley naturally contaminated with ochratoxin A and groundnut meal contaminated with low concentrations of aflatoxin B_1. Res. Vet. Sci. 31:213-218.

Pavlovic, M., Plestina, R., and Krogh, P. 1979. Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. Acta Pathol. Microbiol. Scand. Section B 87:243-246.

Petterson, M. and Damoglou, A.P. 1986. The effect of water activity and pH on the production of mycotoxins by fungi growing on a bread analogue. Lett. Appl. Microbiol. 3:123-126.

Pier, A.C. and McLoughlin, M.E. 1985. Mycotoxic suppression of immunity. In *Trichothecenes and Other Mycotoxins*, J. Lacey (Ed.), p. 57. John Wiley & Sons, New York, NY.

Pitout, M.J. 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. Biochem. Pharmacol. 16:485-491.

Pitt, J.K. 1987. *Penicillium viridicatum, Penicillium verrucosum*, and production of ochratoxin A. Appl. Environ. Microbiol. 53:266-269.

Prior, M.G. 1981. Mycotoxins in animal feedstuffs and tissues in Western Canada 1975 to 1979. Can. J. Comp. Med. 45:116-119.

Prior, M.G., O'Neil, J.B., and Sisedia, C.S. 1980. Effects of ochratoxin A in growth response and residues in broilers. Poultry Sci. 59:1254-1257.

Prior, M.G. and Sisodia, C.S. 1982. The effects of ochratoxin A on the immune response of Swiss mice. Can. J. Comp. Med. 46:91-96.

Purchase, I.F.H. and van de Merwe, K.J. 1971. The long-term toxicity of ochratoxin A to rats. Food Cosmet. Toxicol. 9:681-682.

Reichmann, K.G., Blaney, B.J., Connor, J.K., and Runge, B.M. 1982. The significance of aflatoxin and ochratoxin in the diet of Australian chickens. Aust. Vet. J. 58:211-212.

Ribelin, W.E., Fukushima, K., and Still, P.E. 1978. The toxicity of ochratoxin to ruminants. Can. J. Comp. Med. 42:172-176.

Roschenthaler, R., Creppy, E.E., and Dirheimer, G. 1984. Ochratoxin A: On the mode of action of an ubiquitous mycotoxin. J. Toxicol. Toxin Rev. 3:35-39.

Scott, D.B. 1965. Toxigenic fungi isolated from cereal and legume products. Mycopathol. Mycol. Appl. 25:213-222.

Scott, P.M., van Walbeek, W., Harwig, J., and Fennell, D.I. 1970. Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin-producing strains of *Penicillium viridicatum*. Can. J. Plant Sci. 50:583-585.

Scott, P.M., Kennedy, B., and Van Walbeek, W. 1971. Simplified procedure for the purification of ochratoxin A from extracts of *Penicillium viridicatum*. J. Assoc. Off. Anal. Chem. 54:1445-1447.

Scott, P.M., van Walbeek, W., Kennedy, B., and Anyeti, D. 1972. Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J. Agr. Food Chem. 20:1103-1109.

Shabtai, Y. and Gutnick, D. 1985. Exocellular esterase and emulsan release from the cell surface of *Acinetobacter calcoaceticus*. J. Bacteriol. 161:1176-1181.

Shotwell, O.L., Goulden, M.L., and Hesseltine, C.W. 1976. Survey of U.S. wheat for ochratoxin and aflatoxin. J. Assoc. Off. Anal. Chem. 59:122-124.

Shreeve, B.J., Patterson, D.S.P., and Roberts, B.A. 1978. The 'carry-over' of aflatoxin, ochratoxin and zearalenone from naturally contaminated feed to tissues, urine and milk of dairy cows. Food Cosmet. Toxicol. 17:151-152.

Singer, U. and Roschenthaler, R. 1978. Induction of autolysis in *Bacillus subtilis* by ochratoxin A. Can. J. Microbiol. 24:563-568.

Steyn, P.S. 1984. Ochratoxins and related dihydro-isocoumarins. In *Mycotoxins: Production, Isolation, Separation and Purification*, V. Betina (Ed.), pp. 183-216. Elsevier Science Publishing Co., New York, NY.

Steyn, P.S., Vleggaar, R., DuPreez, N.P., Blyth, A.A., and Seegers, J.C. 1975. The *in vitro* toxicity of analogs of ochratoxin A in monkey kidney epithelial cells. Toxicol. Appl. Pharmacol. 32:198-203.

Still, P.E., Macklin, A.W., Bibelin, W.E., and Smalley, E.B. 1971. Relationship of ochratoxin A to fetal death in laboratory and domestic animals. Nature 234:563-564.

Storen, O., Holm, R., and Stormer, F. 1982. Metabolism of ochratoxin A by rats. Appl. Environ. Microbiol. 44:785-789.

Taipia, M.O. and Seawright, A.A. 1984. Experimental ochratoxicosis A in pigs. Aust. Vet. J. 61:219-222.

Thurston, J.R., Richard, J.L., and Peden, W.M. 1986. Immunomodulation in mycotoxicoses other than aflatoxin. In *Diagnosis of Mycotoxicoses*, J.L. Richard and J.R. Thurston (Ed.), p. 149. Martinus Nijhoff, Boston, MA.

Tong, C.H. 1982. Effects of antimicrobial food additives on growth and ochratoxin A production by *Aspergillus sulphureus* and *Penicillium viridicatum*. M.S. thesis. The University of Tennessee, Knoxville.

Tong, C.H. and Draughon, F.A. 1985. Inhibition by antimicrobial food additives of ochratoxin A production by *Aspergillus sulphureus* and *Penicillium viridicatum*. Appl. Environ. Microbiol. 49:1407-1411.

Trenk, H.L., Butz, M.E., and Chu, F.S. 1971. Production of ochratoxin in different cereal products by *Aspergillus ochraceus*. Appl. Microbiol. 21:1032-1035.

Tsubouchi, H., Yamamot, K., Hisada, K., Sakabe, Y., and Udagawa, S. 1987. Effects of roasting on ochratoxin A in green coffee beans inoculated with *Aspergillus ochraceus*. Mycopathologia 97:111-115.

Ueno, Y. and Kubota, K. 1976. DNA-attacking ability of carcinogenic mycotoxins in recombination-deficient mutant cells of *Bacillus subtilis*. Cancer Res. 36:445-451.

Ueno, Y., Kubota, K., Ito, T., and Nakamura, Y. 1978. Mutagenicity of carcinogenic mycotoxins in *Salmonella typhimurium*. Cancer Res. 38:536-542.

van der Merwe, K.J., Steyn, P.S., and Fourie, L. 1965a. Mycotoxins. Part II. The constitution of ochratoxin A, B, and C metabolites of *Aspergillus ochraceus* Wilh. J. Chem. Soc. 7083-7088.

van der Merwe, K.J., Steyn, P.S., Fouries, L., Scott, D.B., and Theron, J.J. 1965b. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature 205:1112-1113.

van Egmond, H.P. 1990. Limits and regulations for mycotoxins in raw materials and animal feeds. In *Mycotoxins and Animal Food*, J.E. Smith and R.S. Henderson (Ed.), pp. 423-426. CRC Press, Inc., Boca Raton, FL.

Vandegraft, E.E., Hesseltine, C.W., and Shotwell, O.L. 1975. Grain preservatives: Effect on aflatoxin and ochratoxin production. Cereal Chem. 52:79-84.

Vesela, D. Vesely, D. and Jelinek, R. 1983. Toxic effect of ochratoxin A and citrinin, alone and in combination, on chicken embryos. Appl. Environ. Microbiol. 45:91-93.

Visconti, A. and Bottalico, A. 1983. High levels of ochratoxin A and B in moldy bread responsible for mycotoxicosis in farm animals. J. Agric. Food Chem. 31:1122-1123.

Vleggaar, R. and Steyn, P.S. 1980. The biosynthesis of some miscellaneous mycotoxins. In *The Biosynthesis of Mycotoxins*, P.S. Steyn (Ed.), pp. 395-403. Academic Press, New York, NY.

Wyatt, R.D. 1991. Poultry. In *Mycotoxins and Animal Foods*, J.E. Smith and R.S. Henderson (Ed.), p. 579. CRC Press, Inc., Boca Raton, FL.

Wu, M.T. and Ayres, J.C. 1974. Effects of dichlorvos on ochratoxin production by *Aspergillus ochraceus*. J. Agr. Food Chem. 22:536-537.

Yamazaki, M., Suzuki, S., Sakakibara, Y., and Miyaki, K. 1971. The toxicity of 5-chloro-8-hydroxy-3,4-dihydro-3-methyl-isocoumarin-7-carboxylic acid, a hydrolyzate of ochratoxin A. Jpn. J. Med. Sci. Biol. 24:245-250.

Yoshizawa, T. 1990. Natural occurrence of mycotoxins in small grain cereals (wheat, barley, rye, oats, sorghum, millet, rice). In *Mycotoxins and Animal Food*, J.E. Smith and R.S. Henderson (Ed.), pp. 301-324. CRC Press, Inc., Boca Raton, FL.

APPENDIX

Appendix 1-Composition of modified Czapek-Dox medium, MY broth, and modified TGY broth per liter water

A. Modified Czapek-Dox medium

Sucrose	30 g	$NaNO_3$	3 g
K_2HPO_4	1 g	$MgSO_4$	0.5 g
KCl	0.5 g	$FeSO_4$	0.01 g
Yeast extract	0.05 g	Agar	20 g

Autoclave 15 min at 121°C

B. MY broth

Yeast extract	3 g	Malt extract	3 g
Peptone	5 g	Dextrose	10 g

Autoclave 15 min at 121°C. Acidify the sterile melted medium to pH 4.0 at 45-55°C with sterile 0.1 N acetic acid.

C. Modified TGY broth

Tryptone	2.5 g	Yeast extract	2.5 g
Glucose	2.5 g	KH_2PO_4	10 g

Autoclave 15 min at 121°C.

Source: Ciegler et al., 1966.

Appendix 2-Composition of ethanol-minimal salts medium per liter of water

K_2HPO_4	22.2 g	KH_2PO_4	7.62 g
$MgSO_4$	0.2 g	$(NH_4)_2SO_4$	4 g

Absolute ethanol 25 mL

Autoclave 15 min at 121°C.

Source: Shabtai and Gutnick, 1985.

Appendix 3-Electrophoresis reagents

Solution A (pH 8.6-9.0)
- 1 N HCl - 24 mL
- Tris (THAM) - 18.1 g
- Temed - 0.12 mL
- Distilled water to make 100 mL

Solution B (pH 6.6-6.8)
- 1 N HCl -48 mL
- Tris (THAM) - 5.98 g
- Temed - 0.46 mL
- Distilled water to make 100 mL

Solution C
- Acrylamide - 28.0 g
- Bis - 0.735 g
- Distilled water to make 100 mL

Solution D
- Acrylamide - 20.0 g
- Bis - 5.0 g
- Distilled water to make 100 mL

Solution E
- Riboflavin - 0.004 g
- Distilled water to make 100 mL

Solution F
- Sucrose - 40.0 g
- Distilled water to make 100 mL

Solution G
- Ammopersulfate - 0.14 g
- Distilled water to make 100 mL

Electrophoresis buffer
- Tris (THAM) - 6.0 g
- Glycine - 28.8 g
- Distilled water to make 2 L

Column Coat
- Tween 80 - 1 mL
- Distilled water - 100 mL

Dip clean tubes in solution. Remove and air dry vertically.

Appendix 3-Electrophoresis reagents (Continued)

Tracking Dye
Bromphenol Blue - 0.005 g
Distilled water to make 100 mL

Separating Gel:

Solution A	4.0 mL
Solution C	4.0 mL
Solution G	8.0 mL

Mix gently. Do not shake.

Stacking Gel:

Solution B	1.0 mL
Solution D	1.0 mL
Solution E	1.0 mL
Solution F	4.0 mL
Water	1.0 mL

Mix in foil covered container to prevent premature polymerization. Do not shake.

Source: Christen, 1991.

Appendix 4-Composition of antibiotic medium A per liter water

Pancreatic digest of gelatin	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Anhydrous glucose	1.0 g

Add 20 g agar for antibiotic agar medium A. Autoclave 15 min at 121°C.

Source: AOAC, 1984

VITA

Cheng-An Hwang was born to Chin-Long and Lin-Po Hwang in Chiayi, Taiwan, R.O.C. on March 24, 1959. He graduated from the Department of Food Science in National Chung-Shin University, Taichung, Taiwan, in June, 1984. He was married to Wen-Wen Hwang in June, 1986. In September, 1986, he began graduate studies for a Master of Science degree in the Department of Food Technology and Science in The University of Tennessee, Knoxville. He received the M.S. degree in May, 1989. At the same time, he started his doctoral program in the same department. During the course of his doctoral study, he was a graduate research assistant in the Department of Food Technology and Science. He received the Ph.D. degree in May, 1992.