To the Graduate Council:

I am submitting herewith a dissertation written by Ronald Derike Smiley entitled "Partial Purification and Characterization of Aflatoxin Degrading Enzyme Produced by *Flavobacterium aurantiacum*". 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 Science and Technology.

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Partial Purification and Characterization of Aflatoxin B_1 Degrading Enzyme Produced by *Flavobacterium aurantiacum*

A Dissertation

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Degree

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Abstract

The ability of crude protein extracts, obtained from the enzymatic lysis of *F. aurantiacum*, to remove Aflatoxin B_1 (AB_1) and Aflatoxin B_2 (AB_2) was investigated. Crude protein extracts (800 μg/ ml total protein), prepared by enzymatic lysis (0.5 mg/ ml lysozyme) of a 48 h culture, removed 83% of AB_1 and 78% of AB_2 from aqueous solution after 24 h incubation. The buffer composition, used during the enzymatic lysis of *F. aurantiacum* affected the amount of AB_1 degraded due to the total amount of protein released by the cells. Maximum degradation (91%) was observed by crude protein extracts lysed in 50 mM Tris-HCl (pH 7.2) containing: 50 mM EDTA, 10% sucrose, 200 mM NaCl, and 0.1% (v/v) Triton X-100. As much as 78% of AB_1 was degraded, after 24 h, in aqueous solution by crude protein extracts from *F. aurantiacum* lysed in only 50 mM Tris-HCl (pH 7.2). Increasing the incubation did not result in increased degradation of either AB_1 or AB_2 by crude protein extracts. Degradation of AB_1 and AB_2 by crude protein extracts was lower than degradation observed by live cells of *F. aurantiacum*. Live cells of *F. aurantiacum* degraded 93% of AB_1 and 90% of AB_2 from aqueous solution after 24 h incubation.

The ability of crude protein extracts from *F. aurantiacum* to degrade aflatoxin B_1 (AB_1) in aqueous solution was evaluated. Crude protein extracts (800 μg/

ml total protein) degraded 75% of AB_1 in solution. Heat treated crude protein extracts (800 μg/ ml total protein) degraded 6% of AB_1 in aqueous solution. DNase I treated crude protein extracts degraded 80.5% of AB_1 in solution indicating that the removal of aflatoxin by *F. aurantiacum* is not due to non-specific binding with the bacterium's genomic DNA. Proteinase K treated crude protein extracts degraded 34.5% of AB_1 providing the most conclusive evidence yet that the degradation of aflatoxin is enzymatic. Buffer pH affected the amount of AB_1 degraded after 24 h. Maximum degradation was observed at pH 7 of those pH levels tested. Minimum degradation of AB_1 by crude protein extracts was determined to be pH 5. Acidic pH tended to be more detrimental to the enzyme responsible for degradation of AB_1 than was basic pH. The results from this work are conclusive that the degradation of AB_1 by *F. aurantiacum* is enzymatic.

The effects of pH and total protein concentration on the degradation of aflatoxin B_1 (AB_1) and B_2 (AB_2) by crude protein extracts from *Flavobacterium aurantiacum* were evaluated. Crude protein extracts (adjusted to 800 μg/ ml) degraded 25% of AB_1 at pH 5, 50% of AB_1 at pH 6, 70% of AB_1 at pH 7, and approximately 50% of AB_1 at pH 8. Of those pH values tested, maximum degradation of AB_1, by crude protein extracts was observed at pH 7. Crude protein extracts (adjusted to 800 μg/ ml) degraded approximately 60% of AB_2

at pH 5, 6, and 7 and degraded approximately 75% of AB_2 at pH 8. Of those pH values tested, maximum degradation of AB_2, by crude protein extracts was observed at pH 8. The results of this work indicate the importance of pH and on the degradation of aflatoxins by crude protein extracts from *F. aurantiacum*.

Ammonium sulfate precipitation, size exclusion chromatography, anion exchange chromatography, and membrane filtration were used to separate proteins from crude protein extracts from *F. aurantiacum*. Precipitated proteins corresponding to 60% saturation with ammonium sulfate degraded an average of 72% of AB_1 during 24 h incubation at 30°C. Some AB_1 degradation activity was observed at 20, 40, 80, and 100% saturation as well. The elution of crude protein extracts through DEAE cellulose using increasing concentrations of NaCl yielded multiple peaks. Two peaks, corresponding to pooled fraction 65-150 minutes and 155-175 minutes degraded approximately 40-45% of AB_1 from aqueous solution during 24 h incubation at 30°C. Elution of crude protein extracts through Sephadex G-125 yielded multiple peaks. The peak corresponding to pooled volumes of 100.8-109.2 mL decreased AB_1 by 63%. Two other gel filtration fractions demonstrated considerable activity. The peak corresponding to pooled volumes of 27.3-42 mL decreased AB_1 by 44.5% and the peak corresponding to pooled volumes of 81.9-88.2 decreased AB_1 38%. Ultra-filtration indicated that the protein

responsible for AB_1 degradation was between 50 and 20 kD.

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CHAPTER I

Introduction

The aflatoxins are a group of secondary metabolic products produced by *Aspergillus* spp. which are known to cause disease in humans and animals (Jarvis, 1971). Little interest in mycotoxins existed prior to an outbreak of disease in poultry in 1960 (Goldblatt, 1969). Current research has focused on the factors affecting growth of *Aspergillus* and mycotoxin production. The effects of temperature (Jarvis, 1971), relative humidity (Guo et al., 1996), pH (Buchanan and Ayres, 1975), nutritional factors (Luchese and Harrigan, 1993) and atmospheric gas composition (Landers et al., 1967) have been reported. Currently aflatoxin contamination is minimized through screening of raw materials and relying on current food processing methods to inhibit growth of aflatoxin producing molds and to remove aflatoxins already present (Parker and Melnick, 1966; Scott, 1984; Whitten, 1968).

Aflatoxin production in foods varies with environmental conditions including: temperature, relative humidity, fungicidal treatment and irrigation practices (Guo et al., 1996; Jarvis, 1971; Pettit et al., 1971). Aflatoxin can enter in the milk supply when dairy cattle ingest contaminated grain (Frobish et al., 1986). Aflatoxins have been found to exist in dairy products including: cheese, yoghurt, and infant formulas (Galvano et al., 1996). Other food

commodities known to regularly contain aflatoxins include peanuts and peanut products, corn and corn products, pistachio nuts, unrefined sugar and olive oil (Mahjoub and Bullerman, 1988; Tabata et al., 1993).

Physical, chemical and biological methods for removing aflatoxins from foods have been reported. Heating to temperatures above 200°C, microwave heating and exposure to gamma irradiation are physical methods known to remove more than 90% of aflatoxin from food (Samarajeewa et al., 1990). Several commonly used food additives that degrade aflatoxin include: hydrochloric acid (an acidulant), sulfuric acid (caramet production), sodium bicarbonate (bakery products), sodium hydroxide (vegetable oil refinement), metabisulfites (preservative), and hydrogen peroxide (bleaching agent) (Tabata et al., 1994). The first evidence for the microbial detoxification of aflatoxins was reported by Ciegler et al. (1966). Of 1,000 microorganisms tested, only *Flavobacterium aurantiacum* was capable of irreversibly removing aflatoxin from aqueous solution. In a follow up study, Hao and Brackett (1988) reported that *F. aurantiacum* could be used to remove aflatoxin from peanut milk. Little information is available concerning the exact nature of the removal of aflatoxin by *F. aurantiacum*. Line et al. (1994) demonstrated, using ^{14}C labeled aflatoxin, that aflatoxin was converted to a more water soluble product by *F. aurantiacum*. The only evidence reported that indicated that an

enzyme or enzyme system was responsible for the degradation of aflatoxin was that reported by Line et al. (1994), when it was shown that heat inactivated cells did not possess the capability of removing aflatoxins from solution.

The purpose of this research was to study the mechanism(s) by which aflatoxins are removed from aqueous solution by *F. aurantiacum*, to determine the chemical nature of the biological compound(s) responsible for removal of aflatoxins, and to develop a purification protocol in an effort to study the feasibility of using the isolated biological compound(s) for aflatoxin detoxification of foodstuffs.

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CHAPTER II

Review of Literature

Aflatoxin B_1 and B_2

Background and History of Aflatoxins

Aflatoxins are a group of toxic metabolites formed by the fungi *Aspergillus flavus, A. parasiticus* and *A. nominus* (Patterson, 1977). The aflatoxins occur in two series: aflatoxin B_1 (AB_1) and derivatives and aflatoxin G_1 (AG_1) and its derivatives (Figure 1). The letter B and G indicate blue fluorescence and green fluorescence, respectively. Members of the B series of aflatoxins are composed of bifuran coumarins fused to a lactone. Of the aflatoxins, the most important is AB_1 (Moss, 1994). AB_1 is both toxic and carcinogenic and if consumed can lead to either acute or chronic toxicity. The reported LD_{50} for AB_1 is 7 mg/kg in mice, 0.3 mg/kg in ducklings, and 0.62 mg/kg in monkeys (Gourama and Bullerman, 1995). In animal models AB_1 is the most toxic followed by aflatoxin M_1 (AM_1) , G_1, aflatoxin B_2 (AB_2) , and aflatoxin G_2 (AG_2) . Continuous feeding of AB_1 in minute quantities (15 ppm) causes liver lesions in rats (Terao and Ohtsubo, 1991). The aflatoxins can enter human foods directly through contamination of peanuts and cereal grains or indirectly through consumption of animal products or meat from animals consuming feed contaminated with aflatoxins.

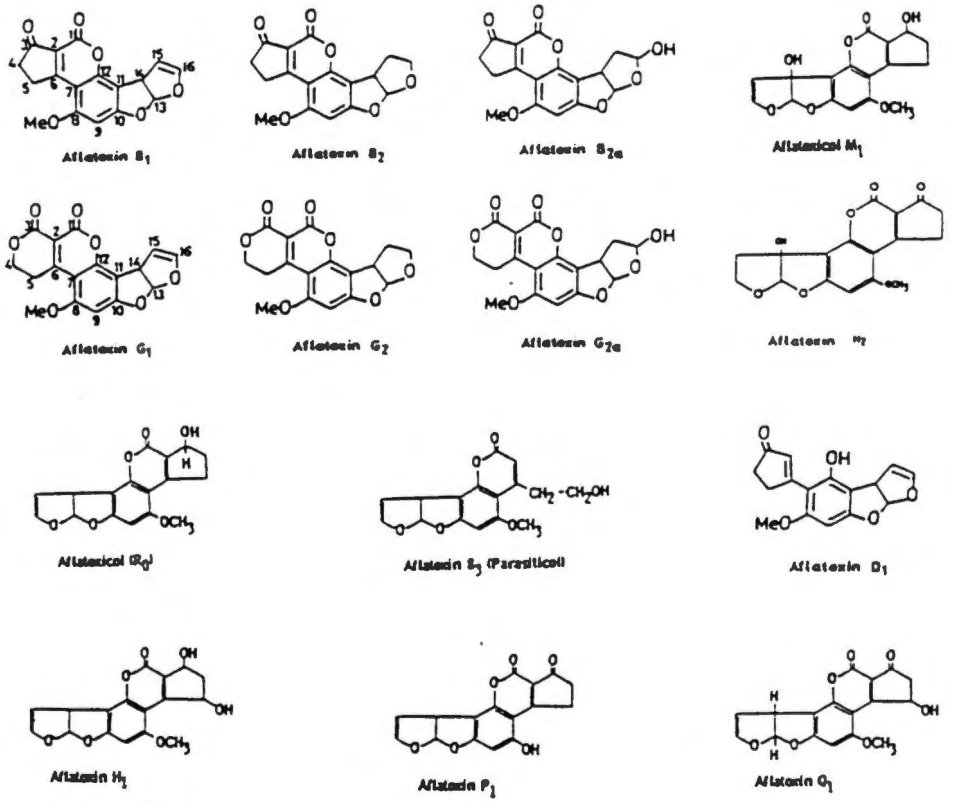

Figure 1. Structures of the various aflatoxins produced naturally or through metabolic transformations. Figure adapted from Mirocha et al. (1979).

Aflatoxin producing species of *Aspergillus* are present in the soil where peanut and cereal grains are cultivated and are known to contaminate a large variety of agricultural products (Gourama and Bullerman, 1995). Although *A. flavus* and *A. parasiticus* are soilborne organisms, differences in their occurrence on agricultural commodities exist. *A. flavus* is typically adapted to temperate climates and is most associated with the contamination of corn. *A. parasiticus* is more adapted to tropical or subtropical climates and is usually associated with the contamination of peanuts (Gourama and Bullerman, 1995). Several extrinsic parameters including temperature, pH, water activity (A_w), and atmospheric gas composition (Gourama and Bullerman, 1995) have been shown to affect aflatoxin production.

Aflatoxins were first identified as a causative agent of animal intoxication in 1960 when 100,000 young turkeys on poultry farms in south and east England died within the course of a few months (Goldblatt, 1969). Initially the causative agent was assumed to be bacteriological and was traced to a common feedmill used to supply feed to all of the poultry farms. An unknown compound was subsequently isolated, by extraction with hot methanol, from the peanut meal which was being used as a component of the poultry feed. In 1962, Nesbitt et al. resolved on alumina chromatoplates two fluorescent molecules. The molecule which fluoresced blue was termed

aflatoxin (Aspergillus **flavus** + **toxin**) B and the one fluorescing green was termed aflatoxin G. Later, two compounds fluorescing blue and two compounds fluorescing green were isolated and named Aflatoxin B_1 (AB_1), Aflatoxin B_2 (AB_2), Aflatoxin G_1 (AG_1), and Aflatoxin G_2 (AG_2). The subscripts represent the migration patterns of these compounds on thin layer chromatography plates where those labeled "1" migrate the furthest.

Occurrence of Aflatoxins in Foods

Due to the ubiquitous nature of the aspergilli, it is common to find agricultural food commodities contaminated with aflatoxins. Tabata et al. (1993) surveyed various foods in Tokyo throughout the years 1986-1990. The occurrence of aflatoxins in foods such as rice, peanuts, sugar, corn and pistachio nuts was reported (Table 1). Using a chloroform-water extraction followed by a cleanup step using Florisil column chromatography a limit of detection 0.2 ppb for AB_1, 0.1 ppb for AB_2, 0.2 ppb for AG_1, 0.1 ppb for AG_2, and 0.1 ppb for AM_1 was achieved. Although aflatoxins were found in several products, only pistachio nuts, peanuts, Brazil nuts and beans contained levels higher than the legal permissible level for Japan (10 ppb). Several foodstuffs, including edible oil, cheese, butter, and coffee beans were shown to be devoid of detectable levels of aflatoxin (Tabata et al., 1993). It was noted that although none of the cheese samples tested positive for aflatoxin during this

Table 1. Levels of occurrence of aflatoxins in foods in Tokyo between the years 1986 through 1990. Adapted from Tabata et al. (1993).

Food	No. Samples	No. Positives	Max. (ppb)			
			B_1	B_2	G_1	G_2
Rice	74	2	2.7	0.1	.09	0
Coix seed	49	5	0.6	0	0	0
Corn	181	4	0.4	0	0	0
Sugar	9	3	1.5	0.2	0	0
Peanut	149	11	21.7	5.3	22.1	6.7
Pistachio	165	5	1,382	260	306	48.3
Brazil nut	4	1	10.2	0.8	3.2	0.3

survey period, in a previous survey period (1982-1985) 16% of all the cheeses tested contained detectable levels of AM_1 (Tabata, et al., 1993).

Giridhar and Krishnamurthy (1977) surveyed un-refined peanut oil, which is commonly used as a cooking oil in parts of India, for aflatoxin contamination. The peanut oil was collected from retail shops in various regions of Andhra Pradesh. In India, peanut oil is typically used in the unrefined state due to its lower cost (Giridhar and Krishnamurthy, 1977). The number of samples testing positive for aflatoxin content greater than 100 ppb ranged from 10 to 35% depending on the region surveyed. The maximum amount of aflatoxin found in any one sample was 5,000 ppb (Gridhar and Krishnamurthy, 1977). Pal et al. (1979) investigated levels of aflatoxins in peanut oil (unrefined), refined peanut oil, peanut cake, and hydrogenated oil in Uttar Pradesh, India. They reported that approximately 67% of the unrefined peanut oil contained levels of aflatoxin ranging between 4 and 2,660 ppb. No aflatoxin was detected in refined peanut oil or hydrogenated oil, however, 70% of the peanut cakes contained aflatoxin levels between 113 and 2,250 ppb (Pal et al., 1979). These results (Giridhar and Krishnamurthy, 1977; Pal et al., 1979) indicate that retail samples of unrefined peanut oil, commonly used as a major source of cooking oil in regions of India, are invariably contaminated with significant levels of aflatoxin. Diener and Davis

(1966) screened samples of peanuts for *A. flavus* capable of producing aflatoxins. They reported that 80% of the isolates obtained produced aflatoxins to some degree with 90% producing primarily AB_1. The amount of AB_1 produced by the isolates on peanuts ranged from 10 to 12,000 ppb sample (Diener and Davis, 1966).

Production of Aflatoxins

Aflatoxin production is believed to occur from the secondary metabolism of 2 and 3 carbon fragments produced during from the primary metabolic pathways of *A. flavus, A. parasiticus* and *A. nominus* (Maggon et al., 1977; Mirocha et al., 1979; Moss, 1994; Steyn et al., 1980). Several researchers have shown that aflatoxins are derived from acetate units (Adye and Mateles, 1964; Biollaz et al., 1970; Hsieh and Mateles, 1970). The starting material for aflatoxin biosynthesis is believed to occur by the linking of acetate units to form a polyketide unit as depicted in Figure 2. Although many of the intermediary structures of aflatoxin biosynthesis have been elucidated little research has been reported about the enzymatic systems leading from the conversion of the polyketide structure to the final product. The generally accepted scheme for the biosynthesis of aflatoxins is shown in Figure 2. Aflatoxin B_1 is composed of a coumarin ring fused to a bifuran moiety and to a pentanone ring. Aflatoxin G_1 is similar to AB_1 except that a lactone ring

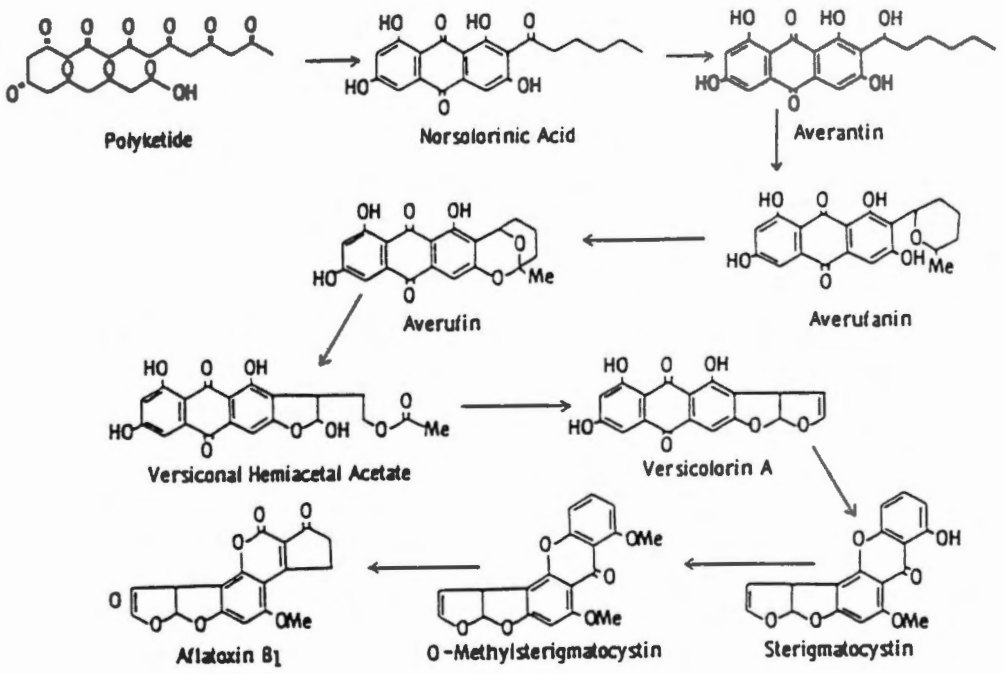

Figure 2. Biosynthesis of Aflatoxin B_1 starting with the polyketide precursor. Figure adapted from Lillehoj (1991).

replaces the pentanone. Aflatoxins M_1, B_2, and G_2 are biological transformations of either AB_1 or AG_1. Hsieh and Mateles (1970) reported on the contribution of acetate and glucose to the biosynthesis of aflatoxins by *A. flavus*. The authors used a parallel culture technique in which one flask contained ^{14}C labeled acetate (500 μmoles) and glucose (2,000 μmoles) and the other flask contained non-labeled acetate (500 μmoles) and ^{14}C labeled glucose (2,000 μmoles). It was reported that glucose uptake from the medium was approximately the same for both flasks however there was a lag period before any ^{14}C from glucose began appearing in the aflatoxin being produced. There was no lag period observed in the flask containing ^{14}C labeled acetate indicating that *A. flavus* preferentially incorporated acetate into aflatoxins. Adye and Mateles (1964) investigated the incorporation of several radiolabeled compounds into the biosynthesis of aflatoxins. They reported the relative isotopic content (RIC) (specific activity of the isolated aflatoxin / specific activity of the precursor) of ^{14}C- methionine, ^{14}C- phenylalanine, ^{14}C- tyrosine, ^{14}C- acetate, and ^{14}C- shikimate. The RIC were reported as 35.7, 8.2, 6.6, 9.9, and 0.08 respectively. The high incorporation of ^{14}C- methionine was proposed to be due to the methyl group on methionine being incorporated into the *o*-methyl group on the coumarin ring structure of aflatoxin. The incorporation of the ^{14}C- acetate agrees with the results reported by Hsieh and

Mateles (1970). The low incorporation of ^{14}C- shikimate indicates that aflatoxin biosynthesis does not occur by an aromatic pathway of biosynthesis which has been shown for coumarin ring synthesis in sweet grass.

Extration and Quantitation of Aflatoxin B_1 and B_2

Extraction of Aflatoxins

AB_1 is typically extracted from agriculture products using either polar organic solvents (acetonitrile, methanol, or acetone) or non-polar organic solvents (chloroform or dichloromethane) (Steyn et al., 1991). In many instances water is added to the extraction buffer to facilitate extraction. In cereal foods water softens the cells making extraction more efficient (Steyn et al., 1991). Following extraction, filtration through diatomaceous earth or solid sodium sulfate is used to remove unwanted particulate material and traces of residual water (Steyn et al., 1991). The extraction process typically dilutes aflatoxins to very low levels. In order to overcome this problem, extracted aflatoxins are usually concentrated by moderate heating in a water bath under vacuum using a rotary evaporator (Steyn et al., 1991).

Detection of Aflatoxins

Aflatoxins can be detected and quantitated using thin layer chromatography (TLC) (Betina, 1985), high performance liquid

chromatography (HPLC) (Hurst et al., 1984; Pons and Franz, 1978; Takahashi, 1977), and spectroscopy (Nabney and Nesbitt, 1965). Both TLC and HPLC methods for aflatoxin analysis can be found in A.O.A.C. Official Methods of Analysis (AOAC, 1990). Nabney and Nesbit (1965) reported that all four major aflatoxins (AB_1, AB_2, AG_1, and AG_2) have an absorption maximum in the ultraviolet region at 363 nm. When known amounts of AB_1 were added to peanut meal and extracted, the values for the amount of AB_1 present as determined by spectroscopy (363nm) was calculated within 1 ppm of the amount added indicating that this method is reliable for estimating the ppm concentrations of AB_1 extracted from food products.

Betina (1985) published an extensive review on the extraction, clean-up, isolation and detection of aflatoxins by thin layer chromatography. Hurst et al. (1984) used HPLC (UV detection, λ= 363 nm) for the analysis of aflatoxins isolated from raw peanuts. This method was reported to be accurate and precise based on the percent recovery from spiked (10 ppm) samples of raw peanuts (Hurst et al., 1984). Pons and Franz (1978) also reported on the use of HPLC to detect and quantitate aflatoxins from peanut products. Using water saturated dichloromethane - cyclohexane- acetonitrile (25-7.5-1.0) as the solvent for analysis, they reported that AB_1, AB_2, AG_1, and AG_2 could be completely resolved in both standards and extracts from raw

peanuts. They also reported that AB_1, AB_2, and AG_1 peaks were resolved with no interferences, however, AG_2 was usually eluted along with a artifact. Pons and Franz (1978) compared their HPLC results with those they obtained by TLC. It was reported that there was agreement between the results obtained by HPLC and TLC. Pons and Franz (1978) reported that HPLC detection of aflatoxins was sensitive to <1 ppb when detected by absorbance and was sensitive to 0.2 ppb when detected by fluorescence.

Flavobacterium aurantiacum

Physical Characteristics and Taxonomy

Flavobacterium aurantiacum is a Gram negative rod which can typically be isolated from the soil. This organism is aerobic, nonmotile, non-sporeforming and grows over a temperature range between 5 and 30°C with an optimum near 25°C (Ciegler et al., 1966). *F. aurantiacum* gives a characteristic yellow to orange color when grown on non-selective media such as Trypticase Soy Agar (TSA) or Plate Count Agar (PCA). Pigmentation can typically be enhanced by incubation at lower temperatures. Biochemical characteristics are described in Bergy's Manual of Determinative Bacteriology (Holt, 1984).

Degradation of Organic Compounds by *F. aurantiacum*

Members within the species *Flavobacterium* have been shown to naturally degrade a wide variety of organic compounds including pesticides (Mulbry and Karns, 1989), fungicides (Saber and Crawford, 1985), and herbicides (Steiert et al., 1987). It is therefore not surprising to find a species capable of naturally degrading fungal toxins. Mulbry and Karns (1989) were successful in isolating a membrane bound hydrolase and cytosolic hydrolase from a soilborne isolate belonging to the flavobacteria capable of degrading the organophosphate insecticide parathion. Using HPLC equipped with anion exchange and affinity columns, the authors were able to achieve a fold purification of 311 and a specific activity of 0.655 IU/ mg protein. The membrane bound hydrolase was determined to be composed of a single monomeric unit with a molecular weight of approximately 35 kD. The cytosolic hydrolase was determined to be composed of a single unit with a molecular weight of approximately 43 kD. Saber and Crawford (1985) identified a soilborne isolate belonging to the genus *Flavobacterium* which could degrade the fungicide pentachlorophenol (PCP). The most efficient isolate was reported to remove 100% of the PCP (200 ppm) from aqueous solution. Using radiolabeled PCP as the sole carbon source for flavobacteria, Saber and Crawford (1985) reported that 83% of the radioactive labeled carbon was

returned as $^{14}CO_2$ and that all the chlorine was liberated as chloride ions. Steiert et al. (1987) tested a strain of *Flavobacterium*, already known to degrade PCP, for its ability to degrade other chlorinated phenols. Several of the chlorinated phenols tested were shown to be completely dechlorinated after 24 h in the presence of *Flavobacterium*. Those which were 100% dechlorinated included: PCP, 2,3,46-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, 2,3,6-trichlorophenol, 2,4,6-trichlorophenol, and 2,6-dichlorophenol. Several of the chlorinated phenols which were not degraded at all by *Flavobacterium* included: 2,4,5-trichlorophenol, 3,4-dichlorphenol, and 3,5-dichlorophenol. O'Reilly and Crawford (1989) investigated the feasibility of using polyurethane immobilized *Flavobacterium* cells for the degradation of PCP in a continuous system. It was reported that PCP concentrations as high as 300 mg/ L could be detoxified in this manner. By monitoring degradation for a period of 150 days, it was determined that 0.6% of PCP degraded per day. This was one of the first studies to indicate that *Flavobacterium spp.* could be used for detoxification on a large scale. The detoxification capabilities of the flavobacteria are not limited to just chlorinated phenols. It has been shown that *Flavobacterium* spp. can degrade other organic compounds such as the organophosphate diisopropyl fluorophosphate (Attaway et al., 1987) and the polycyclic aromatic hydrocarbon 2,6-dimethylnaphthalene (Barnsley, 1988).

Degradation of Aflatoxins by *F. aurantiacum*

The first evidence of the capability of degrading AB_1 by live cells of *F. aurantiacum* was reported by Ciegler et al. in 1966. One thousand microorganisms including yeast, molds and bacteria were screened. Of those, only *F. aurantiacum* was shown to irreversibly remove AB_1 from both solid and liquid media. When added at a level of 270 μg/ 50mL media, 30% of the AB_1 was removed after 19 h incubation by growing cells of *F. aurantiacum*. After 44 h incubation 74% of the AB_1 was removed. No further degradation of AB_1 was observed during the rest of the 68 h incubation. When AB_1 (170 μg/ 50mL) removal was tested on resting cells (stationary phase), the authors reported that 18% was removed after 16 h, 41% was removed after 41 h, and 100% was removed after 88 h. Heat inactivated (autoclaved) cells were not able to remove AB_1 from the test medium. Lillehoj et al. (1967) tested the effect of inoculum level of *F. aurantiacum* on its ability to remove AB_1 aqueous solution. They reported that an inoculum level of 11.7 Log_{10} CFU/ mL was capable of removing 49% of the original aflatoxin level after 5 min incubation, whereas, an inoculum level of 12.6 Log_{10} CFU/ mL was capable of removing 73% of the original aflatoxin level after 5 minutes. The effect of temperature on AB_1 removal using a stationary phase culture of *F. aurantiacum* was also investigated (Lillehoj et al., 1967). It was observed that 35°C was more optimal followed

by 30, 40 and 25°C. Lillehoj et al. (1967) investigated the rate of removal of AB_1 by a stationary phase culture of *F. aurantiacum* at various pH levels. At pH 6.75 AB_1 was removed at a rate of 1.3 μg/ h, at pH 5.0 the rate of removal of AB_1 was 0.6 μg/ h, and at pH 8.0 the rate of removal of AB_1 was 0.8 μg/ h (Lillehoj et al., 1967).

The degradation of AB_1 in food products by *F. aurantiacum* has been reported (Ciegler et al., 1966; Hao and Brackett, 1988; Hao and Brackett, 1989). Ciegler et al. (1966) reported that cells of *F. aurantiacum* removed 100% of AB_1 (600μg/ 50 mL) from milk after 2 h, 100% of AB_1 (700 μg/ 50 mL) from vegetable oil after 3 h, and 100% of AB_1 (700 μg/ g) from peanut butter after 4 h. *F. aurantiacum* was also shown to remove AB_1 from ground soybeans, corn and peanuts (Ciegler et al., 1966). When ground soybean was inoculated with AB_1 at a level of 400 μg/ 50 g, *F. aurantiacum* (13 Log_{10} CFU total) removed 86% of aflatoxin after 12 h at 28°C. Ground corn was inoculated with 800 μg AB_1/ 50 g and ground peanuts at 648 μg AB_1/ 50 g. The amount of AB_1 removed during the 12 h incubation period with *F. aurantiacum* was 100% for both corn and peanuts. Hao and Brackett (1989) investigated the growth and survival of *F. aurantiacum* in non-defatted peanut milk (NDPM) and partially defatted peanut milk (PDPM). The authors reported that growth of *F. aurantiacum* was slower in the NDPM than in

PDPM. In PDPM, *F. aurantiacum* reached stationary phase after approximately 36 h whereas in NDPM *F. aurantiacum* reached stationary phase after approximately 48 h. Addition of AB_1 (1 μg/ mL) did not influence the growth of *F. aurantiacum* in either of the peanut milks (Hao and Brackett, 1989). Hao and Brackett (1989) also tested the survivability of *F. aurantiacum* when inoculated into peanut milk at a level of approximately 8.7 Log_{10} CFU/ mL. They reported that populations remained constant throughout 24 h incubation. Hao and Brackett (1988) reported that *F. aurantiacum* (9 Log_{10} CFU/ mL) removed 82% of AB_1 (1 μg/ mL) from NDPM and 51% of AB_1 from PDPM after 24 h incubation. Forty percent of AB_1 was removed from phosphate buffer after 24 h. *F. aurantiacum* has also been shown to remove AM_1 from solution (Lillihoj et al., 1971). Using 5 mL aqueous solutions of AM_1 (10 μg/ mL), it was shown that *F. aurantiacum* (11 Log_{10} CFU/ mL) removed 99% of the AM_1 after 4 h incubation. Following incubation with *F. aurantiacum*, the cells were lysed by sonication, however, no AM_1 was recovered. Lillihoj et al. (1971) also evaluated the ability of *F. aurantiacum* to remove AM_1 from milk. Using a 10 mL total volume (9.9 μg AM_1/ mL) and a bacterial load of Log_{10} 11 CFU/ mL it was demonstrated that *F. aurantiacum* could remove 83% of the AM_1 with a 4 h incubation at 30°C. Line et al. (1994), using ^{14}C labeled AB_1, determined the amount of radioactivity in both

the organic and aqueous phases during chloroform extraction after reaction with *F. aurantiacum*. Using 1 mL aliquots of a 72 h cell suspension to which AB_1 was added (0.062 μg/ mL; 0.01 μCi/ mL), it was determined that only 24% of the radioactivity remained in the organic phase after 6 h. Controls, containing no *F. aurantiacum*, retained all the radioactivity in the chloroform phase. In a subsequent study, Line and Brackett (1995) investigated the effects of toxin concentration and secondary carbon source on the removal of AB_1 from phosphate buffer and microbiological media. They reported that the addition of an added nutrient source (Trypticase Soy Broth) did not affect the removal of AB_1. Using a combination of ^{14}C labeled AB_1 and non-labeled AB_1, they determined that the addition of non-labeled AB_1 did not affect the amount of radioactivity present in the aqueous phase after 72 h. The authors did note however, that there might have been competition between the labeled and non-labeled AB_1 early on in the incubation period, however, this was not monitored so there are no data available.

General Protein Purification Protocols

Preparation of Biological Extracts

The composition of Gram negative bacterial cell walls differ in complexity from those of Gram positive organisms. The Gram negative

bacterial cell wall consists of a cytoplasmic membrane, a peptidoglycan layer, a lipopolysaccharide layer, and a outer lipid bilayer membrane (Leive, 1974). Gram negative bacteria are generally considered resistant to the action of lysozyme (E.C. 3.2.1.17) as compared to Gram positive bacteria (Repaske, 1958). Lysozyme cleaves the β(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetyl-glucose-amine (NAG) which composes a major portion of the peptidoglycan layer of the cell wall (Jolles and Jolles, 1984). The peptidoglycan, of Gram negative bacteria, is physically protected from the action of lysozyme by the lipopolysaccharide and lipid bilayer portions of the outer cell membrane (Leive, 1974). Several common laboratory agents have been shown to weaken the Gram negative cell wall thus making it susceptible to the action of lysozyme (Irvin et al., 1981; Repaske, 1958; Womack et al., 1983).

In 1981, Irvin et al. reported that Tris-hydroxymethyl-aminomethane (Tris) when used as a buffering agent increased the outer membrane permeability of the Gram negative bacterium *Escherichia coli* based on the amount of alkaline phosphatase released. The effect of Tris on lysozyme sensitivity was also evaluated based upon the level of spheroplast formation by *E. coli* in the presence of both lysozyme and Tris. The extent of spheroplast formation was determined by electron microscopy (Irvin et al., 1981). The

results showed that *E. coli*, without exposure to Tris, was insensitive to lysozyme, however, those cells exposed to 0.1 M Tris formed spheroplasts upon treatment with lysozyme. Repaske (1958) reported that bacteria resistant to lysozyme could be made sensitive to its action by incorporation of EDTA in the lysis buffer. Several Gram negative organisms, including *E. coli*, *Pseudomonas aeruginosa*, *P. fluorescens*, and *Azobacter vinelandii* were tested for their susceptibility to lysozyme in the presence of EDTA and were all shown to be lysed in the presence of EDTA (Repaske, 1958). Repaske (1958) hypothesized that there may be divalent metals associated with the cell surface which interfere with the action of lysozyme. Since then, however, it has been determined that the role of divalent cations lies within the lipopolysaccharide (LPS) portion of cell wall where it helps minimize electrostatic repulsions between neighboring LPS molecules (Nikaido and Vaara, 1985). It has also been proposed that the role of Tris in cell lysis is to help displace divalent cations thus discouraging tight LPS interactions (Nikaido and Vaara, 1985). Womack et al. (1983) evaluated various detergents on the solubilization of the lipid bilayer membranes. These detergents were tested for their ability to solubilize a 0.2% dispersion of phospholipid and for their non-denaturing effects on the enzyme esterase. Triton X-100 was found to successfully solubilize lipid and was shown to not cause any enzyme denaturing when used

at levels <5.0% (Womack et al. 1983).

Ammonium Sulfate Precipitation

Ammonium sulfate is commonly used to fractionate proteins based on their solubility in solutions containing various amounts of the salt (Scopes, 1987). Ammonium sulfate precipitates proteins by binding water making it unavailable to the protein (de-solvation). Once the water is bound by the salt, hydrophobic amino acid residues which reside on the surface of the protein interact causing the proteins to precipitate. Ammonium sulfate is typically the preferred salt to be used in fractionating proteins since it is less harsh than other salts and proteins that are precipitated can usually revert back to their native state once the salt has been removed.

SDS-PAGE

Polyacrylamide gel electrophoresis is a technique which may be used to separate molecules based on size and/ or molecular weight depending on the actual parameters used in the set-up. Various texts give explicit detail of the theory of electrophoresis and the reader is referred here to several (Deutscher, 1990; Robyt and White, 1987; Scopes, 1987;) which clearly explain the theoretical principles of electrophoretic separations.

Discontinuous gel electrophoresis is a type of gel electrophoresis in which two concentrations of acrylamide are used (Scopes, 1987). The

principles of discontinuous gel electrophoresis are outlined in numerous biochemistry texts (Garfin, 1990; Robyt and White, 1987; Scopes,1987) so only a brief synopsis will be presented here. Discontinuous gel electrophoresis relies on a gel composed of two different concentrations of acrylamide. For a vertical gel, the upper acrylamide concentration is lower than the concentration of the bottom portion of the gel. The lower acrylamide concentration gel is referred to as a stacking gel. In the low acrylamide proteins are allowed to migrate relatively freely and tend to associate with one another based on molecular size. This association produces bands of proteins, based on size, within the gel. These bands are often referred to as stacks of proteins and is where the name "stacking gel" originates. Within the portion of the gel containing the higher concentration of acrylamide the proteins will be separated based on mass, charge or the mass/charge ratio. The term "separating gel" stems from fact that proteins are separated from one another within this portion of the gel.

Sodium dodecyl sulfate (SDS) is an anionic detergent commonly used to treat proteins prior to electrophoresis. The function of the SDS is to first denature the native structure of the protein so that it is linear and secondly to give the protein an overall negative charge. Since all the proteins treated with SDS have a negative charge, separation will be based solely on molecular

weight. This allows accurate assessment of the molecular weight of proteins when ran in the presence of protein molecular weight standards which have been treated in a similar manner.

Once separated, proteins entrapped within the polyacrylamide matrix must be modified in order to be visualized. On such common modification is to stain the gel with a dye which binds to the proteins. Coomassie Brilliant Blue is a commonly used dye for staining proteins (Bradford Reaction) and is available as a commercially prepared kit by BioRad (BioRad Chemical Division; Richmond, CA). Details of the reaction and specific information concerning the process are supplied with the BioRad kit (Anon., 1995). The gels are simply placed in a container with the protein staining dye for a specified period of time so that the dye may react with the protein. De-staining in methanol, ethanol, acetic acid or some other solvent is used to remove the background dye from the gel so that the protein bands may be visualized.

Size Exclusion Chromatography

Size exclusion chromatography, sometimes referred to as gel filtration or gel permeation chromatography, is a technique used to separate biological molecules based on size (Robyt and White, 1990). A gel filtration column is packed to the desired height with dextran beads. Dextran is a complex cross-

lined polysaccharide. Each dextran bead is porous and allows certain molecules to enter. The size of the molecules which can enter depend on the pore size which is determined by the amount of cross-linking within the polysaccharide. Separation of different size molecules occurs due to the fact that the smaller molecules will spend more time in the pores of the gel and having a greater distance to travel will elute later than larger molecules which have difficulty entering the pores.

Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) relies on the attraction of positive and negative ionic charges toward each other (Robyt and White, 1990). Protein are composed of numerous amino acid residues which may be charged positive or negative depending on the pH at which the protein is buffered. For example, lysine and arginine are positively charged at neutral pH and aspartic and glutamic acids are negatively charged at neutral pH. The overall charge of the protein will depend on pH and the number of charged residues composing the protein. There are two general types of ion exchangers, cationic and anionic.

Cation exchange resins are negatively charged. The negative charge is balanced by positively charged counter ions (Na^+ for example). When a protein, possessing an overall positive charge, is eluted through the column,

the counter ions are displaced and the protein becomes bound to the resin through electro-static interactions. Proteins possessing an overall negative charge will elute with the flow-through. Once bound, proteins may be eluted by altering the pH so that they are no longer positively charged or the ionic strength of the elution buffer is increased so that the proteins are displaced. Increasing ionic strength is typically the method of choice since altering the pH may cause irreversible denaturation of the protein. Carboxy-methyl cellulose (CMC) is commonly used as the support in cation exchange chromatography. CMC possesses numerous charged carboxyl groups which will bind proteins.

Anion exchange chromatography is performed using a solid support which is positively charged. The positive charges on the support are balanced by negatively charged counter ions (Cl^- for example). When a protein possessing an overall negative charge is eluted through the column, the counter ions are displaced and the protein becomes bound. Once bound, the protein may be eluted from the column by altering the pH so that the protein is no longer negatively charged or by increasing the ionic strength of the elution buffer so that the protein is displaced. Increasing ionic strength is the preferred method due to the adverse effects of pH on proteins. Diethylaminoethyl (DEAE)cellulose is commonly used as the solid support for

anion exchange chromatography. DEAE possesses many positively charged amine groups which proteins can bind.

Separation of charged proteins, for either anion or cation exchange chromatography, can be performed by increasing the ionic strength of the elution buffer. The increased ionic strength causes the proteins to be more attracted to the buffer than to the solid support. The weaker the ionic attraction for the support the sooner it will elute from the column. The ionic attraction of the protein is determined by the number and types of charged amino acids that the protein possess under the conditions being used.

Ultra-filtration

Ultra-filtration is a technique used to separate and/ or concentrate proteins based on molecular size (Robyt and White, 1990). The principles of ultra-filtration are similar to that of dialysis. The buffer containing a mixture of proteins is forced through a membrane under pressure from an inert gas which selectively permits certain sized molecules to pass. A variety of membranes which selectively allow molecules of different sizes are commercially available.

Protein Quantitation

The relative purity of an enzyme following various purification procedures requires a method for determining the overall protein content of

each purification fraction. Several methods for quantitating proteins are available. Regardless of the specific method used, a standard curve is prepared using known concentrations of a purified protein. The concentration of the "unknown" fractions is then determined from that standard curve. The best protein to use in generating the standard curve would be the protein that one is working with. This may be difficult especially when the protein has not been previously purified. Commonly used proteins for generating standard curves include bovine serum albumin (BSA) and gamma-globulin.

The Bradford reaction is a rapid method for protein measurement which relies on a shift in absorbance (465 nm to 595 nm) of the dye Coomassie blue when it binds to a protein (Anon., 1995). The Bradford method is considered to be highly sensitive, detecting levels as low as 1 μg. This method also has the advantages in that the reagents are commercially available (BioRad Chemical Division; Richmond, CA) and there are only a few interferences. Many proteins show the same response upon binding to Coomassie blue, so a wide range of proteins may be used in constructing standard curves for quantitating unknown concentrations of proteins.

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CHAPTER III

Removal of Aflatoxin B_1 by Crude Protein Extracts

Abstract

The ability of *F. aurantiacum* and crude protein extracts from *F. aurantiacum* to remove AB_1 from aqueous solution was investigated. Heat inactivation and Proteinase K treatment was used to determine if removal of AB_1 was associated with protein(s) present in the crude extract of the organism. *F. aurantiacum* (5 mL of 24 h culture at a population of 1.5×10^8 CFU/ mL) was capable of removing 93% of AB_1 from solution. When live cells were heat inactivated only 20% of the AB_1 was removed from aqueous solution demonstrating that AB_1 removal is not due to non-specific binding with the cell. Crude protein extracts (5 mL at 800μg/ mL), prepared from a 48 h culture, removed 75% of AB_1 from aqueous solution. Heat inactivated crude protein extracts (5 mL at 800 μg/ mL) removed approximately 6% of AB_1 from solution. Proteinase K treated crude proteins extracts (5 mL at 800μg/ mL prior to the addition of Proteinase K) removed 34.5% of AB_1 from aqueous solution. Due to decreased removal of AB_1 by heat treated and Proteinase K treated crude protein extracts, it is evident that at least one protein is involved in the removal of AB_1 from aqueous solution.

Introduction

The aflatoxins are a group of secondary fungal metabolites produced by some species of *Aspergillus* (Moss, 1994). Aflatoxins are commonly found in human food and animal feeds including: peanuts and unrefined peanut oil (Pal et al., 1979), cottonseed (Whitten, 1968), milk (Frobish et al., 1986), sugar (Tabata et al., 1993), pistachio nuts (Tabata et al., 1993) and corn (Tabata et al., 1993). Aflatoxins are known to be both toxigenic and carcinogenic and are regulated by the FDA with permissible levels being 0.5 ppb for milk and 20 ppb for all other foods (Council for Agricultural Science and Technology, 1986).

F. aurantiacum has been shown to remove 100% of aflatoxin B_1 (AB_1) from aqueous solution (Ciegler et al., 1966), 99% of aflatoxin M_1 (AM_1) from aqueous solution (Lillehoj et al., 1971), and 100% of aflatoxin G_1 (AG_1) from aqueous solution (Lillehoj et al., 1967). Hao and Brackett (1988) investigated the feasibility of using live cells of *F. aurantiacum* to remove AB_1 from peanut milk. They reported that 74% of AB_1 was removed from partially defatted peanut milk and 94% of AB_1 was removed from non-defatted peanut milk. Their work confirmed the earlier reports of the ability of *F. aurantiacum* to remove AB_1 from food products (Ciegler et al., 1966). Prior to 1994, it was not known if *F. aurantiacum* actually degraded AB_1 or if the removal of AB_1

from solution was simply due to non-specific interactions with some component of the bacterial cell such as the cell wall, DNA, or non-specific binding to some bacterial protein. Line et al. (1994) reported on the fate of ^{14}C labeled AB_1. Aflatoxin is more soluble in polar and non-polar organic solvents than in water. Line et al. (1994) monitored the amount of radioactivity associated with the organic phase and aqueous phase during solvent extraction post-incubation with live cells of *F. aurantiacum*. They reported that when AB_1 was incubated in aqueous solution without the presence of *F. aurantiacum* that virtually all of the radioactivity remained in the organic phase. However, when aqueous solutions of AB_1 was incubated with live cells of *F. aurantiacum*, 76% of the radioactivity was associated with the aqueous phase. This demonstrated that in the presence of the organism AB_1 undergoes at least one transformation which makes it more water soluble. Using day old duckling assays, Ciegler et al. (1966) showed that the products of the transformation of AB_1 by *F. aurantiacum* were no longer toxic and that no new toxigenic products were formed.

Degradation of complex organic compounds by members of the genus *Flavobacterium* is not uncommon. Flavobacteria have been shown to degrade the insecticide parathion (Mulbry and Karns, 1989), pentachlorophenol (PCP) (Saber and Crawford, 1985), chlorinated phenols (Steiert et al., 1987), and

diisopropyl fluorophosphate (Attaway et al., 1987). Presently little information is available on the exact mechanisms used to degrade such complex compounds by flavobacteria. While use of the live bacterium has shown promise in detoxifying food systems, the orange pigment normally associated with the organism may lead to undesirable organoleptic properties.

This study was undertaken to determine if AB_1 degradation, by *F. aurantiacum*, is due to a protein and to evaluate the feasibility of using crude protein extracts to remove AB_1 from aqueous solution prior to development of a purification protocol to study the mechanism(s) responsible for AB_1 degradation.

Materials and Methods

Cultures

F. aurantiacum, NRRL B-184 (USDA-ARS, Northern Regional Research Center, IL) was grown in brain heart infusion (BHI) broth (Difco; Detroit, MI) at 25°C in an orbital shaker incubator. Cells were transferred after 12 h to sterile broth and incubated an additional 24 h. Stock cultures were stored at 4°C on trypic soy agar slants (BBL; Cockeysville, MD). Stock cultures were transferred every 2 weeks following the same procedure stated above.

Degradation of AB_1 by *F. aurantiacum*

One loopful of cells from a stock culture of *F. aurantiacum* was transferred to 100 mL of BHI broth. The suspension was incubated for 12 h at 25°C in an orbital shaker incubator. After 12 h, 5 μL of the culture was transferred to 1 L of BHI broth and incubated for 48 h as described above. Following incubation, cells were pelleted by centrifugation at 10,000 x g for 10 minutes. Cells were resuspended in 50 mL sterile buffer (50 mM Tris-HCl, pH 7.2) and evenly divided into sterile centrifuge tubes. One tube was then heated in a boiling water bath for 15 minutes. Five milliliters of either live cells or heat inactivated cells was tested for degradation activity. A control tube was prepared using 5 mL Tris-HCl (pH 7.2) without any *F. aurantiacum*.

Twenty microliters of AB_1 (1,000 ppm stock in acetonitrile) were added to each test tube (20x100 mm, screw capped). The test tubes were heated to 50°C under N_2 to evaporate the acetonitrile. The tubes were then cooled (5 minutes) on ice. Five milliliters of either live cells or heat inactivated cells were then added to the appropriate test tubes. The cell/ aflatoxin suspension was then incubated at 30°C, without shaking, in the dark for 24 h. The entire procedure was replicated two more times.

Analysis of Residual AB_1

Following incubation of $AB_1$with live cells or heat inactivated cells of *F. arantiacum*, the reaction was terminated by the addition of 5 mL HPLC grade chloroform. The reaction mixture was mixed for 1 minute, on a vortex mixer, and the contents added to a 125 mL separatory flask. The test tubes were rinsed with an additional 5 mL chloroform which was subsequently added to the appropriate separatory flask. An additional 15 mL was added to each separatory flask and the flasks were shaken for 2 minutes. The organic phase (bottom phase) was collected through sodium sulfate into a 125 mL round bottom flask. The extraction was repeated again with the addition of 25 mL of chloroform and collected into the appropriate round bottom flask. The chloroform was then completely evaporated using a Rotavapor-R rotory evaporator (Buchi; Switzerland) at 55°C. The remaining aflatoxin was then dissolved in 3 mL of HPLC grade acetonitrile and was analyzed immediately by spectrophotometry.

Quantitation of Residual AB_1

Following extraction of residual AB_1, the amount remaining after incubation with live and heat inactivated cells was determined using a Hewlett-Packard 8452 diode array spectrophotometer (Hewlett-Packard; Palto

Alto, CA) by the method described by Nabney and Nesbitt (1965). Absorbance values were taken (λ=363 nm) and concentrations determined by comparing values to standard curves prepared from known concentrations of AB_1. The equation for a straight line (Y=mX + b) was determined for the standard curve and used to determine unknown concentrations of AB_1 remaining.

Preparation of Crude Protein Extracts

One liter of cells of *F. aurantiacum* was prepared as previously described. Following incubation for 48 h, cells were harvested by centrifugation and the cell pellets resuspended in 100 mL sterile buffer (50mM Tris-HCl, pH 7.2; 0.1% v/v Triton X-100; 200mM NaCl; 10% w/v sucrose w/v; 1% v/v ethyl acetate; 100mM Na-EDTA). The cells were incubated 12 h at 4°C without shaking. Following the 12 h incubation, cells were pelleted by centrifugation at 6,000 x g for 10 minutes at 4°C. The supernatant was discarded. The cells were resuspended in 25 mL sterile buffer (50mM Tris-HCl, pH 7.2; 50mM EDTA). Lysozyme (E.C. 3.2.1.17) (Sigma; Saint Louis, MO) was added at a concentration of 0.5 mg/ mL and the cell-lysozyme suspension was incubated 24 h at 4°C. Following incubation in the presence of lysozyme, cellular debris were removed by centrifugation a 20,000 x g at 4°C. The supernatant (crude protein extract) was collected and stored on ice. The total protein content was

measured and adjusted to 800 μg/ mL by dilution with 50mM Tris-HCl (pH 7.2). The crude protein extract was then equally divided into three portions (approximately 8 mL). One portion was maintained on ice, another was heated in a boiling water bath (in a screw capped test tube) for 15 minutes, and the third was treated with Proteinase K (0.1 mg/ mL) (Boehringer Mannheim; Indianapolis, IN) for 6 h at 4°C. The crude protein extract, heat inactivated crude protein extract and Proteinase K treated crude extract were tested for the ability to remove AB_1 from aqueous solution.

Degradation of AB_1 by Crude Protein Extracts

AB_1 (20 μg total) in acetonitrile was added to each of three empty test tubes (20x100 mm, screwcapped). The acetonitrile was evaporated under N_2 and 5 mL of crude protein extract, heat inactivated crude extract or Proteinase K treated crude extract was added to the appropriately labeled tube. Tubes were then incubated at 30°C for 24 h in the dark. Following incubation, the reaction was terminated by the addition of 5 mL chloroform.

Following termination of the reaction with chloroform, any AB_1 remaining was extracted and measured by spectrophotometer as previously described. A control tube, to monitor natural degradation of AB_1, was prepared which contained only AB_1 in Tris-HCl buffer (pH 7.2). The experiment was replicated two more times.

Results and Disscussion

Degradation of AB_1 by *F. aurantiacum*

The removal of AB_1 from aqueous solution by live and heat inactivated cells of *F. aurantiacum* is shown in Table 2. *F. aurantiacum* (5 mL at 1.5×10^8 CFU/ mL) removed an average of 93% of AB_1 from solution after 24 h (Table 2). In control tubes only 5.3% of AB_1 was lost which may be attributed to incomplete extraction of the AB_1 from the *F. aurantiacum*/ AB_1 mixture. Heat inactivated cultures of *F. aurantiacum* removed an average of 20.3% of AB_1 from solution after 24 h (Table 2). These results suggest that a protein, either through the direct degradation or uptake of AB_1, may be involved in AB_1 removal since proteins are known to be sensitive to heat and AB_1 degradation was reduced compared to non-heat treated *F. aurantiacum*. The amount of AB_1 removed after 24 h in this study was greater than that reported by Ciegler et al. (1966) who reported 18% degradation after 16 h and 41% degradation after 41 h. The difference in the amount of degradation between these two studies may be attributed to differences in cell concentrations, reaction volumes, and initial aflatoxin concentrations. The results for the heat inactivated cells are similar to those described by Lillehoj et al. (1967) and Line et al. (1994). Lillehoj et al. (1967) reported that most of the AB_1 could be recovered from suspensions with autoclaved cells of *F. aurantiacum*.

Table 2. Removal of AB_1 from solution by live cells[a] of *F. aurantiacum*, heat inactivated cells of *F. aurantiacum*, crude protein extract[b], heat inactivated crude protein extract, and Proteinase K treated crude protein extract from *F. aurantiacum*.

Fraction	$[AB_1]\mu g$ (initial)	$[AB_1]\mu g$ (final)	% Removal	Standard Error[c]
Control	20	19.2	5.3	1.9
F. aurantiacum	20	1.5	93.0	10.8
Heat Inactivated *F. aurantiacum*	20	16.0	20.3	2.4
Crude Extract	20	5.1	74.8	2.9
Heat Inactivated Crude Extract	20	18.9	6.3	1.0
Proteinase K Inactivated Crude Extract	20	13.1	35.0	3.11

[a] Concentration of cells was 5 mL at 1.5×10^8 CFU/ mL.
[b] Concentration of crude protein extracts was 5 mL at 800 μg/ mL.
[c] Standard error of the mean determined from 3 replications.

Likewise, Line et al. (1994) reported that autoclaved cells failed to convert radiolabeled aflatoxin to the more water soluble product observed with live cells of *F. aurantiacum*. In the studies by Lillehoj et al. (1967) and Line et al. (1994) autoclaving was used to inactivate the cells. Because of the harshness of the treatment it is difficult to conclude if the lack of removal of AB_1 was due to thermal inactivation of a protein since other changes may occur such as disrupture of the bacterial cell membrane. In this work, the thermal inactivation was much less severe. The loss of the ability to remove AB_1 from solution more conclusively points to a heat labile biological molecule such as a protein.

Ciegler et al. (1966) reported that the amount of time necessary for *F. aurantiacum* to degrade AB_1 to non-detectable levels was 88 h. This is similar to published reports for the degradation of other compounds. O'Reilly and Crawford (1989) reported that levels of PCP remained constant during the first 2 days of incubation with immobilized *Flavobacterium* but then decreased from initial levels of 70 to 20 mg/ L between days 3 and 5. This may explain why even after 24 h of incubation some aflatoxin remained.

Degradation of AB_1 by Crude Protein Extracts

Crude protein extracts from *F. aurantiacum* were prepared and evaluated for their ability to remove AB_1 from solution. After 24 h incubation, crude

protein extracts (5 mL at 1.5 x 10^8 CFU/ mL) removed an average of 74.8% of the initial AB_1 (Table 2). Heat inactivated crude protein extracts (5 mL at 800 μg/ mL) were capable of removing only 6.3% of the initial amount of AB_1 added. Likewise, a decrease in AB_1 degrading activity was observed when crude protein extracts (800 μg/ mL prior to the addition of Proteinase K) were treated with Proteinase K with approximately 35% of AB_1 being removed. Proteinase K is a non-specific protease. All protein present in the crude protein extract serves equally well as a substrate for this enzyme. Because of this, it would not be expected that Proteinase K targets only the enzyme(s) responsible for the degradation of AB_1. Given that the amount of time crude protein extracts were incubated with Proteinase K was only 6 h, it would not be expected that all AB_1 degradation activity would be lost. At present, no published work is available reporting on the ability of crude protein extracts or purified proteins to remove AB_1. The results from the heat inactivated and Proteinase K treated crude protein extracts conclusively indicate that the nature of removal of AB_1 is due to a protein and possibly enzymatic. The amount of AB_1 removed from aqueous solution by crude protein extracts was less than that by live cells of *F. aurantiacum*. This may be due to incomplete cell lysis or this may indicate that the protein(s) responsible may not be as stable once removed from the cell. It should not be overlooked that a cofactor

may be necessary for AB_1 degradation and that during cell lysis the cofactor may have been lost or sufficiently diluted to decrease activity. In some instances, enzymes are present inside the cell in an inactive form and undergo some type of modification before becoming active. This may explain the relatively long amount of time necessary to degrade AB_1 and may also explain the decreased amount of AB_1 degradation observed for crude protein extracts. Without an intact cell, any needed modifications of the enzyme(s) which degrade AB_1 may not occur.

Conclusions

Crude protein extracts, from *F. aurantiacum*, removed AB_1 from solution but not to the extent that was observed for stationary phase cells in aqueous solution. Currently, with little information available on the exact mechanisms of AB_1 degradation by *F. aurantiaucm,* it is difficult to speculate on why decreased AB_1 degradation is observed. Protease (natural proteases from inside the cell which are released upon cell death) activity is a likely candidate. The loss of an enzyme activation mechanism, which may be necessary for activation of the enzyme(s) that degradae AB_1, also explains the decreased amount of AB_1 degradation observed for crude protein extracts compared to that of stationary phase cells. The decreased AB_1 degradation noted for heat

treated cells and heat treated crude protein extracts imply that a protein and perhaps an ezyme is responsible for the degradation of AB_1. Heat treatment is known to adversly affect enzymes involved in metabolic processes leading to loss of biological activity. Crude protein extracts from *F. aurantiacum* were further tested with Proteinase K which is a non-specific protease. Decreased AB_1 degradation by crude protein extracts, following treatment with Proteinase K, verifies that the degradation of AB_1 from aqueous solution is linked to a protein and is possibly enzymatic. Numerous microbial enzymes have been purified and are currently finding use in the food industry. A purified form of the enzyme responsible for AB_1 degradation may be useful in the food industry or in the animal feed industry for removing aflatoxins.

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CHAPTER IV

Degradation of AB_1 by Crude Protein Extracts

Abstract

The effects of buffer composition and lysozyme concentration were evaluated for their effectiveness at lysing *F. auranitacum.* The effectiveness of lysis was measured by the amount of degradation of AB_1 by crude protein extracts. The greatest amount of AB_1 degradation (91%) was observed when *F. aurantiacum* was lysed in buffer composed of 50mM Tris-HCl, 50mM EDTA, 10% sucrose, 200mM NaCl, and 0.1% v/v Triton X-100 at pH 7.2. Increasing the concentration of lysozyme increased the amount of AB_1 degraded by crude protein extracts from lysed cells of *F. aurantiacum*. The effects of pH, DNaseI, β-mercaptoethanol, and bovine serum albumin on removal of AB_1 by crude protein extracts was also evaluated. The presence of EDTA, β-mercaptoethanol, or BSA did not increase the amount of AB_1 degraded over that of crude extract alone. The presence of DNaseI did not decrease the removal of AB_1 from aqueous solution indicating that removal is not due to non-specific binding to the bacterium's DNA. The amount of degradation of AB_1 by live cells of *F. aurantiacum* was similar at pH 5, 6, 7, and 8 with >98% of the AB_1 being degraded. The largest amount of degradation with crude protein extracts was observed at pH 7.

Enzymatic Lysis of Gram Negative Bacteria

Bacteria can be lysed by either physical or chemical means. Physical methods usually involve either high pressure, such as the French press or shear forces, such as grinding cells in the presence of glass beads (Scopes, 1987). Chemical methods usually involve treating bacterial cells with compounds which remove the outer and inner cell wall such as a combination of detergent and lysozyme (Scopes, 1987). Gram negative bacteria are typically more difficult to lyse by enzymatic means due to the presence of a thick lipopolysaccharide outer cell membrane which prevents lysozyme from reaching the inner peptidoglycan layer (Nikaido and Vaara, 1985). The effects of various laboratory reagents and chemicals on the outer cell membrane of Gram negative bacteria has been the focus of many studies (Irvin et al., 1981; MacGregor and Elliker, 1958; Repaske, 1958; Womack et al., 1983). Irvin et al. (1981) reported the effects of Tris(hydroxymethyl)aminomethane (Tris) on the permeability of *E. coli* outer cell membranes. Irvin et al. (1981) reported that Tris increased outer cell membrane permeability and that permeability was effected by Tris concentration and exposure time. Irvin et al. (1981) reported that in the presence of Tris lipopolysaccharide (LPS), one of the components of the outer cell membrane of Gram negative bacteria, was

released into the medium. MacGregor and Elliker (1958) reported that the presence of EDTA increased the sensitivity of the Gram negative bacterium *Pseudomonas auruginosa* to quaternary ammonium compounds. It is now known that the outer membrane of Gram negative bacteria use divalent metals such as Mg^{2+} to stabilize the outer membrane (Leive, 1974). The increased sensitivity to quaternary ammonium compounds can be attributed to increased permeability of the outer cell wall due to the binding of the divalent metals which normally serve to stabilize the cell membrane. Repaske (1958) also reported on the effects of EDTA on the sensitivity of Gram negative bacteria to lysozyme. Repaske (1958) reported that EDTA increased the efficiency of lysis of both *E. coli* and *P. auruginosa*. Womack et al. (1983) investigated the effects of various detergents on the solubilization of lipid bilayer. They reported that Triton X-100 was effective at solubilizing the lipid bilayer while not demonstrating any adverse effects on enzymatic activity at low levels.

Although protein yields are typically lower when Gram negative bacteria are lysed with lysozyme, enzymatic lysis has some advantages over physical methods of lysis including: less heat produced so less thermal inactivation of enzymes, less mechanical damage to enzymes, and lower costs when equipment such as the French press is not available.

Degradation of AB_1 by *F. aurantiacum*

The degradation of AB_1 by the bacterium *F. aurantiacum* was first reported by Ciegler et al. (1966). Hao and Brackett (1988) reported that *F. aurantiacum* could be used to remove AB_1 from peanut milk. Hao and Brackett (1988) reported that non-specific binding of AB_1 to the peanut proteins may have been responsible for decreased recovery. Line et al. (1994), using ^{14}C labeled AB_1, reported that the degradation product of AB_1 was found in the aqueous phase during organic solvent extraction. As of yet, the isolation and identification of the aflatoxin degradation products has not been reported in the literature.

Effect of pH on Enzyme Activity

Enzymes have an optimum pH range for maximal activity and at pH above and below the optimum the activity decreases (Lehninger et al., 1993). Enzymes are proteins which are composed of amino acids many of which can act as weak acids or weak bases in enzyme catalyzed reactions. The decrease in enzyme activity observed when pH is outside the optimum is usually due to the ionization of a critical amino acid residue within the catalytic site. At times, the ionization of residues outside the catalytic site can lead to overall conformational changes which can also decrease activity.

The objectives of this research were to develop a standard assay for the

measurement of AB_1 degradation and to develop an enzymatic lysis procedure for the harvesting of crude protein extracts from *F. aurantiacum*.

Materials and Methods

Quantitation of AB_1 by HPLC and Spectrophotometry

A comparison, for the measurement of AB_1, by HPLC and spectrophotometry, by the method described by Nabney and Nesbitt (1965), was performed. One milligram of purified AB_1, obtained from Sigma, was dissolved in 1 mL HPLC grade acetonitrile. Standards were prepared by dilution with acetonitrile to achieve final concentrations of 20, 10, 5, and 2.5 μg/ mL. Spectrophotometric analysis was performed using a Hewlett-Packard model 8452 diode array spectrophotometer (Hewlett-Packard Co; Palto Alto, CA) (λ=363 nm). The absorbance versus concentration was plotted. HPLC analysis was performed using a Waters HPLC (Waters Associates, Inc.; Milford, MA) equipped with a Bondapak C18 column (30 cm X 3.9 mm). The flow rate was set at 1.5 mL/ minute using methanol-water (45:55) as the elution solvent. A fluorescent detector set for 360 nm excitation and 400 nm emission was used to determine peak area. The injection volume was 20 μL. Following analysis a plot of peak area versus concentration was done. Known concentrations (7.5, 12.5, 15, and 18 μg/ mL) of AB_1 were then analyzed by

both methods and the values obtained for each concentration of AB_1 by both methods were compared. Three replications were performed.

EDTA, DNaseI, β-mercaptoethanol, and BSA

Cells of *F. aurantiacum* (5 mL at 1.5 x 10^8 CFU/ mL) from a 12 h culture were transferred to 1 L of sterile BHI broth and incubated for 48 h at 25°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. Following centrifugation, cell pellets were suspended in 50 mL sterile buffer (pH 7.2) (50mM Tris-HCl, 200mM NaCl, 10% sucrose (w/v), 0.1% (v/v) Triton X-100, 0.5% ethyl acetate). Cells were incubated for 12 h at 4°C to weaken the outer cell membrane. Cells were then centrifuged at 10,000 x g for 10 minutes at 4°C. Cells pellets were suspended in 50 mL of buffer as described above. Lysozyme was added at a level of 0.5 mg/ mL and cells were lysed at 4°C for 24 h. Following cell lysis, the supernatant was clarified by centrifugation at 20,000 x g for 30 minutes. The supernatant was diluted to achieve a concentration of 800 μg/ mL and was divided into five equal portions of 10 mL. EDTA was added to one tube at a level of 0.1M (final concentration). DNaseI was added at a level of 100 μg/ mL (final concentration) to a second tube. The third tube received BSA (bovine serum albumin) at a level of 500 μg/ mL (final concentration). β-mercaptoethanol was added to the fourth tube at a level of 0.25% v/v (final

concentration). A control tube containing only sterile buffer was also prepared. For AB_1 degradation studies, 10 μL (1,000 μg/ mL stock in acetonitrile) AB_1 were added to 6 clean sterile test tubes. The tubes were heated at 55°C under N_2 to remove the acetonitrile. Five milliliters of crude extract or crude extract containing either DNaseI, BSA, EDTA, or β-mercaptoethanol was then added to the appropriately labeled test tube. The reaction was allowed to proceed for 24 h at 30°C in the dark. A control tube was prepared containing AB_1 and sterile 50mM Tris-HCl buffer (pH 7.2). After 24 h the reaction was terminated by the addition of 5 mL chloroform. The remaining AB_1, which did not degrade, was isolated by chloroform extraction using a total volume of 25 mL chloroform. Following chloroform extraction, the chloroform was evaporated, using a Rotovapor R rotary evaporator (Buchi; Switzerland) at 55°C and the remaining AB_1 was dissolved in 3 mL HPLC grade acetonitrile. AB_1 concentration was determined by spectrophotometric analysis (λ=363 nm) as previously described. The entire procedure was replicated 2 more times.

Effect of Buffer Composition on Lysis of *F. aurantiacum*

The effect of buffer composition on the lysis of *F. aurantiacum* by lysozyme was evaluated by monitoring the amount of degradation of AB_1 by crude protein extracts prepared using the various buffers. Cells, from a 12 h

culture, were transferred to 1 L sterile BHI broth and incubated for 48 h at 30°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. The cell pellets were suspended in 25 mL sterile saline solution (0.85% NaCl). The cell suspensions were then evenly distributed into five 125 mL flasks containing 20 mL of one of the following buffering systems: 5mM Tris-HCl, pH 7.2; 50mM Tris-HCl, pH 7.2; 5mM Tris-HCl, 50mM EDTA, 10% sucrose w/v, pH 7.2; 50mM Tris-HCl, 50mM EDTA, 10% sucrose w/v, 200 mM NaCl, pH 7.2; 50mM Tris-HCl, 50mM EDTA, 10% sucrose w/v, 200mM NaCl, 0.1% Triton X-100, pH 7.2. Lysozyme was added to each flask at a level of 0.5 mg/ mL and cells were lysed for 24 h at 4°C. Following lysis, the supernatant (crude protein extract) was clarified by centrifugation at 20,000 x g for 30 minutes.

The AB_1 degradation activity of the crude protein extracts prepared by enzymatic lysis using different buffers was evaluated. AB_1 (10 μg from a 1,000 ppm stock in acetonitrile) was added to individual sterile test tubes. The acetonitrile was evaporated by heating at 55°C under N_2. Five milliliters of crude extract from the different buffering systems was added to the appropriately labeled tube. The reaction was allowed to proceed for 24 h at 30°C in the dark. Following the 24 h incubation, the reaction was terminated by addition of 5 mL chloroform. The AB_1 remaining was extracted with

chloroform and quantitated using a diode array spectrophotometer (λ=363 nm) as previously stated. The entire study was replicated two more times.

Lysozyme Concentration

The effect of lysozyme concentration on the enzymatic lysis of *F. aurantiacum* was determined by measuring the amount of AB_1 degraded using equal volumes of crude extract from cells incubated with various concentrations of lysozyme. One liter of BHI broth was inoculated with 5 μL of a 12 h culture of *F. aurantiacum*. Cells were grown for 48 h at 25°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. Cell pellets were pooled and resuspended in 50 mL sterile buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% w/v sucrose, 0.1% v/v Triton X-100, pH 7.2). Cell suspensions were then evenly divided into 5 sterile 125 mL flasks. Lyophilized lysozyme was then added to each flask to obtain final concentrations of 100, 200, 300, 700, and 1,000 μg/ mL. The cells were allowed to react with the lysozyme for 24 h at 4°C. Following lysis, the supernatant (crude protein extract) was clarified by centrifugation at 20,000 x g for 30 minutes.

To evaluate the amount of AB_1 degraded by the crude protein extracts, 10 μg of AB_1 (1,000 μg/ mL stock in acetonitrile) was added to 6 sterile test tubes. The tubes were heated to 55°C under N_2 to evaporate the acetonitrile.

The tubes were cooled and 5 mL of crude extract from each of levels of lysozyme was added. The crude extracts and AB_1 were allowed to react for 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform.

Undegraded AB_1 was extracted by chloroform (25 mL total volume). The chloroform was evaporated at 55°C and the remaining AB_1 dissolved in 3 mL HPLC grade acetonitrile. Quantitation was performed using a Hewlett-Packard model 8452 diode array spectrophotometer (Hewlett-Packard Co.; Palto Alto, CA) at a wavelength of 363 nm as previously described. The entire experiment was replicated two more times.

Triton X-100, Lysozyme, and Mechanical Shear

Supernatant obtained between successive treatment of *F. aurantiacum* with Triton X-100, lysozyme, and vortexing in the presence of glass beads were evaluated for the ability to degrade AB_1 in aqueous solution. One liter of sterile BHI broth was inoculated with 5 μL of a 12 h culture of *F. aurantiacum*. The cells were grown for 48 h at 25°C in an orbital shaker incubator and harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. The cell pellets were suspended in 50 mL sterile buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, pH 7.2). Triton X-100 was then added to achieve a final concentration of 0.1% (v/v). The cells were incubated in the Triton X-

100/ buffer for 5 h. Following incubation, the cell suspension was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was removed and test for the ability to degrade AB_1. The remaining cells were resuspended in 50 mL buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, pH 7.2). Lysozyme was then added to achieve a final concentration of 0.5 mg/ mL. Lysis was allowed to proceed for 24 h. Following the 24 h incubation period, cells were pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was saved and evaluated for the ability to degraded AB_1. The cell pellet were then resuspended in 10 mL buffer as previously described. Glass beads were added at a level equal to the volume of suspended cells in a 50 mL Falcon tube. The cells and beads were vigorously vortexed for 1 minute followed by cooling on ice for 1 minute. This was repeated 4 more times. The glass beads were allowed to settle and the aqueous layer removed and centrifuged at 10,000 x g for 10 minutes at 4°C to remove any remaining whole cells and cellular debris. The supernatant was recovered and tested for the ability to degrade AB_1 in aqueous solution.

The ability to degrade AB_1 by the supernatants obtained after successive treatment of *F. aurantiacum* with Triton X-100, lysozyme and mechanical shear were evaluated. To 3 sterile test tubes, 10 μg AB_1 (1,000 μg/ mL stock in acetonitrile) was added. The tubes were heated to 55°C under N_2 to evaporate

the acetonitrile. Supernatant (crude protein extracts) (5 mL) was then added to the appropriately labeled tube. The mixture was allowed to react for 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform. Undegradaed AB_1 was extracted with chloroform. The chloroform was evaporated and the remaining AB_1 dissolved in 3 mL acetonitrile for analysis by spectrophotometry as previously described. The experiment was replicated two more times.

Effect of pH on AB_1 Degradation

The ability of stationary phase cultures and crude protein extracts to degrade AB_1 at different pH values was evaluated. One liter of BHI broth was inoculated with 5 μL of a 12 h culture of *F. aurantiacum*. The cells were grown for 48 h at 25°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. Cell pellets were resuspended in 50 mL buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% w/v sucrose, 0.1% v/v Triton X-100, 0.1% ethyl acetate, pH 7.2). Cells were incubated for 12 h at 4°C to weaken the outer cell membrane. Cells were then centrifuged at 10,000 x g for 10 minutes at 4°C. Cell pellets were suspended in 50 mL fresh buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, and 0.1% v/v Triton X-100, pH 7.2). Lysozyme was added at a level of 0.5 mg/ mL. Cells were lysed for 24 h at 4°C. The supernatant (crude protein

extract) was clarified by centrifugation at 20,000 x g for 30 minutes. Buffers were prepared as follows: pH 5.0, 50mM sodium acetate/ acetic acid; pH 6.0, 50mM HPO_4^{2-}/ $H_2PO_4^-$; pH 7.0, 50mM Tris-HCl; pH 8.0, 50mM Tris-HCl. The supernatant was divided into 5 mL aliquots which were then added to Spectrum dialysis tubing (Medical Industries, Inc.; Los Angeles, CA) which had a MWCO of 3,000 Daltons. Buffers were exchange by dialysis in excess buffer of appropriate pH for 6 h with occasional changing of the buffers. After equilibration, the final pH was checked and minor adjustments made using the appropriate acid or base as needed.

Ten micrograms of AB_1 (1,000 ppm stock in acetonitrile) were added to sterile test tubes. The tubes were heated to 55°C under N_2 to evaporate the acetonitrile. The entire contents of the dialyzed crude protein extracts from each pH was added to the appropriately labeled tube. The reaction was allowed to proceed 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform.

The remaining undegraded AB_1 was chloroform extracted. The chloroform was evaporated at 55°C under a stream of N_2 and the remaining AB_1 was dissolved in acetonitrile (3 mL) for analysis by absorbance at 363 nm as previously described. The experiment was repeated 2 additional times.

Degradation of AB_1 During One Week Storage

Aflatoxin B_1 (20 μg), from a 1,000 μg/ mL stock in acetonitrile, was incubated with either live cells or crude protein extracts from *F. aurantiacum* for up to one week to evaluate the amount of degradation which occurred. One liter of BHI broth was inoculated with 5 μL from a 12 h culture of *F. aurantiacum*. Cells were incubated for 48 h at 25°C in an orbital shaker incubator and harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. For the culture study, cells were resuspended in 50 mL sterile buffer (50mM Tris-HCL, 200mM NaCl, pH 7.2) and divided into 5 mL aliquots which were then added to 7 sterile tubes containing 20 μg of AB_1 (1,000 μg/ mL stock in acetonitrile). All the tubes were incubated at 30°C in the dark. One tube was removed each day and the amount of AB_1 degraded was determined. For the crude protein extract study, cells were grown as just described. After harvesting the cells, the cells were resuspended in 50 mL sterile buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% w/v sucrose, 0.1% v/v Triton X-100, 0.1% ethyl acetate, pH 7.2). Cells were incubated for 12 h at 4°C to weaken the outer cell membrane. Cells were then centrifuged for at 10,000 x g for 10 minutes at 4°C and re-suspended in fresh buffer (50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% w/v sucrose, 0.1% v/v Triton X-100, pH 7.2). Lysozyme was added to achieve a final concentration of 0.5 mg/ mL.

Cells were lysed for 24 h at 4°C. After lysing, the supernatant was clarified by centrifugation at 20,000 x g for 30 minutes at 4°C. The supernatant was then added in 5 mL aliquots to 7 sterile test tubes containing 20 μg AB_1. All of the tubes were then incubated at 30°C in the dark. One tube was removed each day for AB_1 degradation analysis.

At the end of each 24 h period the reaction was terminated by the addition of 5 mL chloroform. The remaining AB_1 which was not degraded was extracted using chloroform. The chloroform was evaporated and the remaining AB_1 dissolved in acetonitrile for analysis using absorbance measurements at 363 nm. This experiment was replicated 3 times, and the data presented in the next section reflect the average values obtained.

Results and Discussion

AB_1 Analysis by HPLC and Spectrophotometry

The effectiveness of spectrophotometric analysis of AB_1, using absorbance values at a wavelength of 363 nm was compared to analysis by HPLC. HPLC is generally the method of choice for analysis of aflatoxins from food products, however it is more expensive and time consuming than analysis by spectrophotometry. Spectrophotometric analysis is not generally suitable for the measurement of aflatoxins from food products since some food

components interfere with absorbance measurements. Since our study was done in buffered systems, spectrophotometric analysis was believed to be a suitable method for analysis of AB_1. In order to confirm this, we wanted to compare the sensitivity, precision and accuracy of spectrophotometric analysis to analysis by HPLC. Standard curves, from known amounts of AB_1, using both methods are shown in Figure 3. The sensitivity of both methods was based on the ability to detect the smallest amount of AB_1 used in this study (2.5 μg/ mL). Since both methods were able to detect AB_1 at this concentration they are comparable. The precision of both HPLC and spectrophotometric analysis of AB_1 is illustrated by the error bars around each point in Figure 3. The smaller the standard error, the lower the amount of variation between the individual observations comprising the mean value. The standard error for both the spectrophotometric anlalysis and HPLC analysis were comparable indicating that spectrophotometric analysis of AB_1, in aqueous solutions, is equally suitable to HPLC. The accuracy of spectrophotometric analysis was compared to analysis by HPLC by plotting the values of known concentrations of AB_1 (7.5, 12.5, 15, and 18 μg/ mL) versus estimated values of AB_1, as determined by HPLC or spectrophotometric analysis and is illustrated in Figure 4. The slope for each line was obtained using linear regression. Precision refers to how closely the estimated value is to

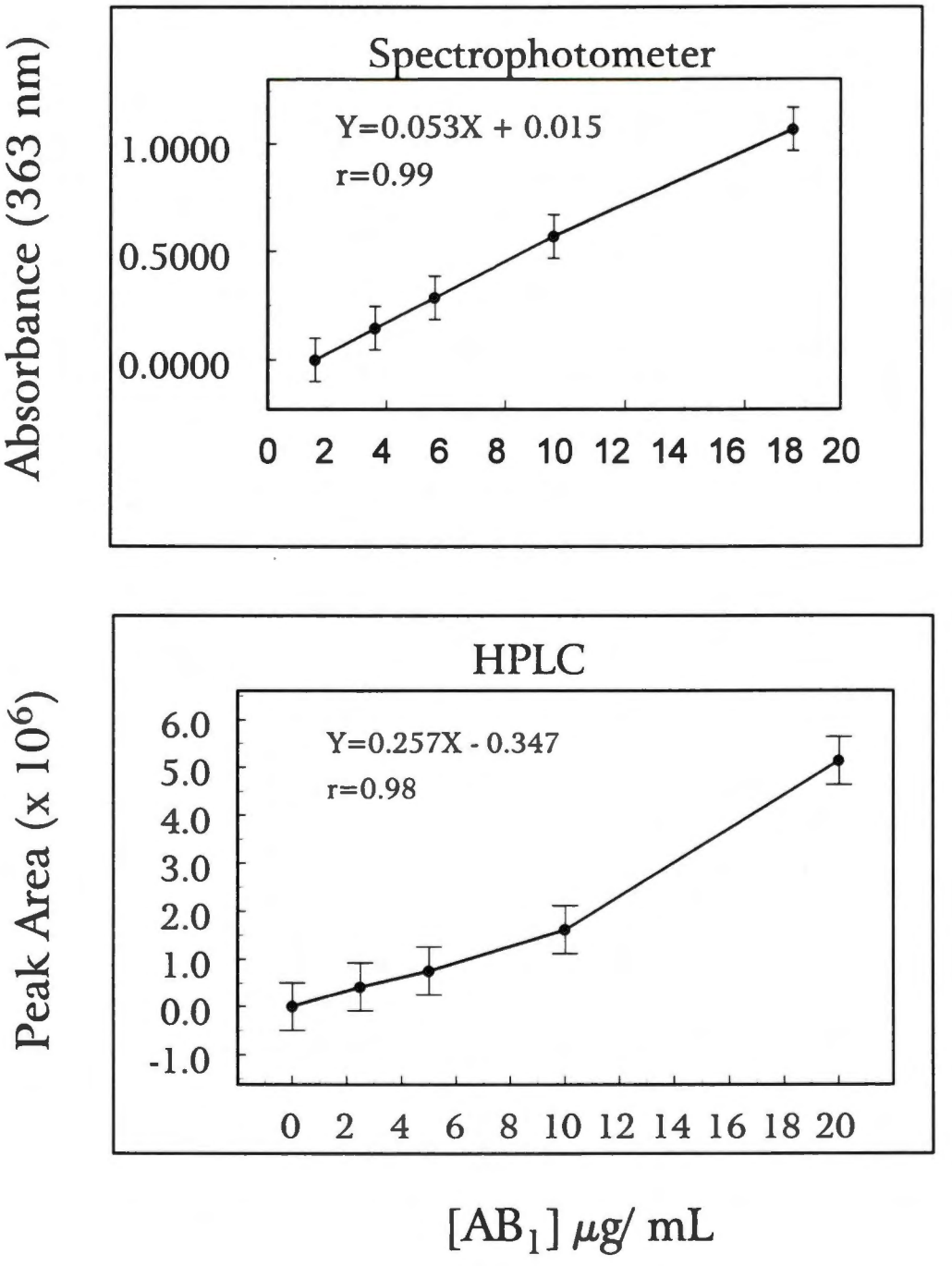

Figure 3. Standard curves of known concentrations of AB_1 by spectrophotometry (363 nm) and HPLC.

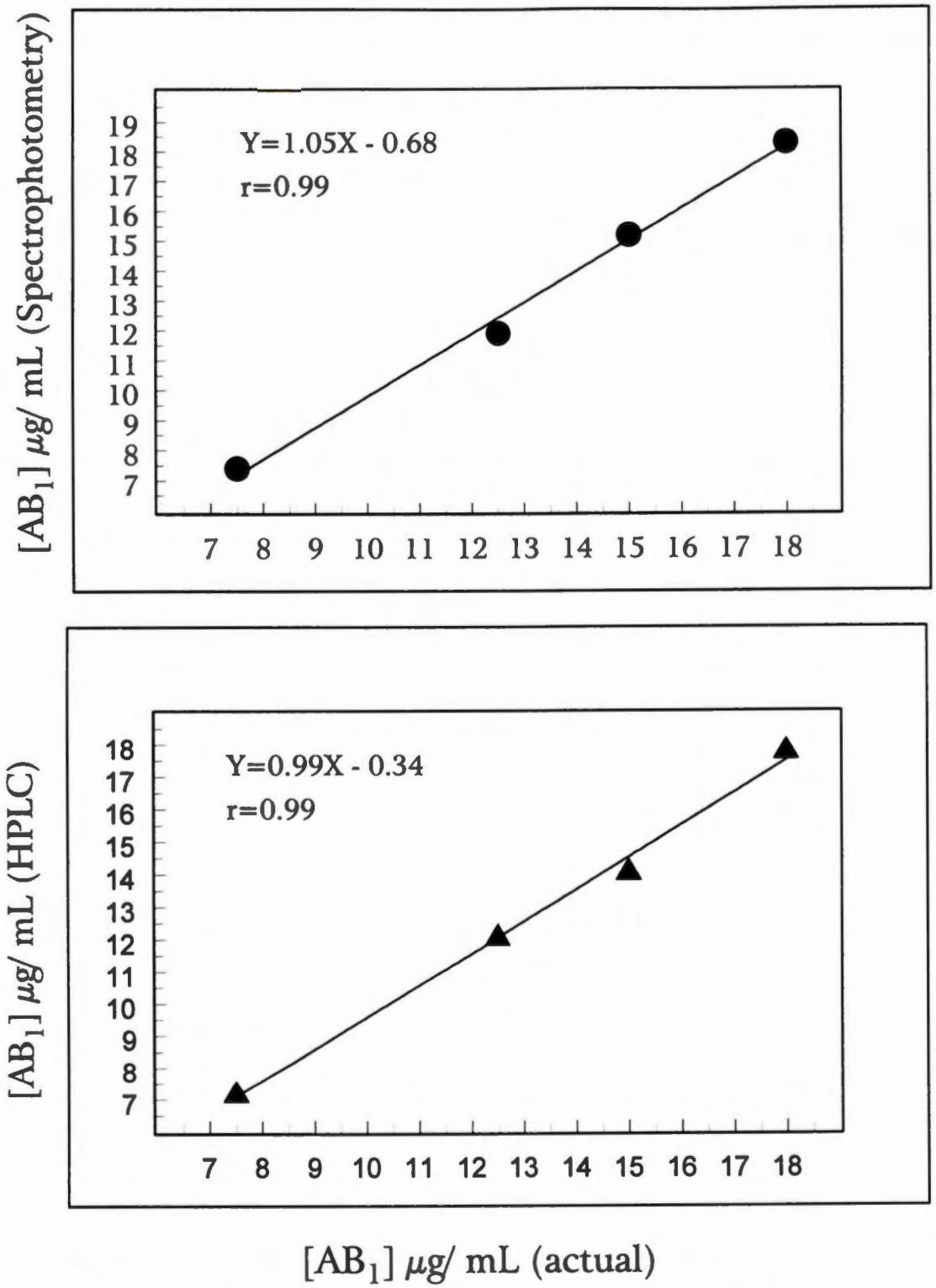

Figure 4. Plot of AB_1 concentrations as determined by HPLC and spectrophotometric analysis. Actual values (X axis) are plotted versus estimated values (Y axis).

the actual value. A slope of 1 would indicate that the actual value and estimated value was the same. The slope for estimated values of AB_1 as determined by spectrophotometric analysis was calculated as 1.04 (Figure 4). The slope for the estimated values of AB_1 as determined by HPLC was calculated as 0.99 (Figure 4). Both HPLC and spectrophotometric analysis were similar in estimating concentrations of AB_1. The results illustrated by Figure 4 suggest that both HPLC and spectrophotometry are equally accurate in determing AB_1 concentrations described by the conditions used in this experiment. Spectrophotometric analysis has been shown to be sensitive enough and equally precise and accurate as HPLC for the conditions used in this research. These results correspond to those previously presented by Nabney and Nesbitt (1965) who were able to isolate, purify and quantitate AB_1 from peanut meal using spectrophotometric analysis at a wavelength of 363 nm. It was not possible to compare molar extinction coefficient values between this work and that of Nabney and Nesbitt (1965) since their analysis was performed with methanol as the solvent and in this work acetonitrile was used as the solvent.

EDTA, DNaseI, β-mercaptoethanol, and BSA

The effects of EDTA, DNaseI, β-mercaptoethanol, and BSA were evaluated on the removal of AB_1 by crude protein extracts from *F.*

aurantiacum. Crude protein extracts (800 μg/ mL) were capable of removing approximately 93% of AB_1 (initial concentration 20 μg) from solution (Table 3). The decreased amount of AB_1 degradation observed for crude protein extracts and crude protein extracts with added EDTA, β-mercaptoethanol, DNaseI or BSA, as compared to earlier experiments, may be attributable to ommission of EDTA in the original lysis buffer. EDTA incorporated into the lysis buffer is known to enhance the enzymatic lysis of Gram negative bacteria by the chelation of divalent metals which are present in the peptidoglycan layer of the cell membrane and where they function to stabilize the membrane (Leive, 1974). Since EDTA, added to the crude protein extracts after lysis, did not enhance or inhibit the degradation of AB_1, incorporation of EDTA into the lysis buffer should not directly effect the results of any AB_1 degradation studies. EDTA is commonly added to buffers to prevent the proteolyis of proteins by metal requiring proteases. At this time no other published work is available which reports of the effects of EDTA on AB_1 degradation by *F. aurantiacum* crude protein extracts. DNaseI was added to crude protein extracts in order to cleave the genomic DNA naturally present when the cell is lysed. Aflatoxin B_1 is known to bind mammalian DNA. This experiment was conducted to determine if the disappearance of AB_1 was due to binding to the bacterium's DNA. The amount of AB_1 degraded, by crude protein extracts (5

Table 3. Effect of EDTA, DNaseI, β-mercaptoethanol, and BSA on the degradation of AB_1 in aqueous solution by crude protein extracts from *F. aurantiacum* after 24 h incubation at 30°C. (Standard errors reflect variation of the means of three replications.)

	$[AB_1]\mu g$ (Initial)	$[AB_1]\mu g$ (Final)	% Degradation	Standard Error
Control	20.0	19.2	5.0	1.1
Crude Extract	20.0	1.5	93.0	2.0
EDTA[a]	20.0	3.6	82.0	4.0
DNaseI[b]	20.0	3.9	80.5	1.1
β-mercaptoethanol[c]	20.0	2.5	87.4	1.3
BSA[d]	20.0	2.1	89.7	1.4

[a] crude extract + EDTA (0.1 M final concentration)
[b] crude extract + DNaseI (100 μg/ mL final concentration)
[c] crude extract + β-mercaptoethanol (0.25% v/v)
[d] crude + BSA (500 μg/ mL final concentration)

mL at 800 μg/ mL total protein prior to the addition of DNaseI) containing DNaseI, was approximately 81% (Table 3). Since this value is similar to that of crude protein extracts without DNaseI it appears that AB_1 is not binding to the bacterium's DNA and something else must be responsible for degradation.

β-mercaptoethanol is a reducing agent commonly added to buffers at low levels to prevent oxidation of enzymes and subsequent loss of activity. Crude protein extracts (5 mL at 800 μg/ mL total protein content) containing β-mercaptoethanol at a level of 0.25% degraded 87% of AB_1 in aqueous solution (Table 3). Since activity was not enhanced over that of crude extracts without β-mercaptoethanol it can be concluded that oxidation does not appear to cause any problems, to the enzymes responsible for the degradation of AB_1, in the reaction assay. Bovine serum albumin was added to crude protein extracts at a level of 500 μg/ mL to increase the total protein content of the crude extract. Hao and Brackett (1988) had proposed that the disappearance of AB_1 may be due to non-specific binding to proteins. Removal of AB_1 was not enhanced by the presence of BSA, indicating that the removal of AB_1 from aqueous solutions by crude protein extracts is not due to non-specific interactions with proteins (Table 3).

Effect of Buffer Composition on Enzymatic Lysis

The effect on the lysis buffer composition on the efficiency of lysis of *F. aurantiacum* was evaluated. Although spectrophotometric analysis is typically used to measure cell lysis by lysozyme (Repaske, 1958), the presence of the orange pigment possessed by this bacterium makes spectrophotometric analysis difficult. The efficiency of lysis was determined by the amount of AB_1 degraded by equal volumes of crude extract. Crude protein extracts obtained from the enzymatic lysis of *F. aurantiacum* in 5mM Tris-HCl (pH 7.2) were capable of removing approximately 79% of AB_1 from solution (Table 4). Crude protein extracts obtained from the enzymatic lysis of *F. aurantiacum* in 50mM Tris-HCl (pH 7.2) degraded approximately 89% of AB_1 from solution. Increasing the concentration of Tris by 10 fold increased the amount of degradation by an average of 10%. The increased permeability of the cell membrane of Gram negative bacteria in the presence of Tris has been reported (Irvin et al., 1981). Therefore, an increase in the amount of AB_1 degradation by crude extracts which were prepared at higher concentrations of Tris is consistent with this observation. The increased permeability may have led to increases in the release of the cell component responsible for the degradation of AB_1. Crude protein extracts obtained from the enzymatic lysis of *F. aurantiacum* in 5mM Tris-HCl, 50mM EDTA, 10% sucrose (w/v) decreased

Table 4. Effect of buffer composition on the lysis of *F. aurantiacum*[a], by lysozyme, as determined by AB_1 degradation activity[b] at 30°C for 24 h. Concentration of lysozyme added was 0.5 mg/ mL. (Standard errors reflect the variation of the means of three replicates.)

Buffer	[AB_1] μg (Initial)	[AB_1] μg (Final)	% Degradation	Standard Error
Control	10.0	9.9	1.5	0.3
5 mM Tris-HCl (pH 7.2)	10.0	1.0	78.8	1.3
50 mM Tris-HCl (pH 7.2)	10.0	1.1	89.0	1.2
5 mM Tris-HCl, 50 mM EDTA, 10% Sucrose (pH 7.2)	10.0	2.1	87.4	0.3
50 mM Tris-HCl, 50 mM EDTA, 10% Sucrose, 200 mM NaCl (pH 7.2)	10.0	1.3	89.6	0.9
50 mM Tris-HCl, 50 mM EDTA, 10% Sucrose, 200 mM NaCl, 0.1% Triton X-100 (pH 7.2)	10.0	0.9	91.0	2.1

[a] 5 mL of an equal concentration of live cells (9.6 Log_{10} CFU/ mL) was added to 125 mL of each of the buffers above.

[b] The amount of degradation was measured indirectly (AB_1 degradation activity) due to the presence of pigment which made direct spectroscopic measurement of bacterial lysis difficult.

initial levels of AB_1 by 87% (Table 4). Repaske (1958) reported that EDTA increased the efficiency of enzymatic lysis of several Gram negative bacteria. Greater release of cellular constituents in the presence of EDTA, in the lysis buffer, would ultimately lead to more degradation of AB_1. Sucrose was added to increase the osmotic strength of the buffer in order to prevent complete disruption of the cells which leads to large amounts of the orange pigment being released. Although complete disruption of the cell membrane would ultimately lead to the greater release of total cellular proteins, the pigment, which is also released and is chloroform soluble, absorbs well at 363 nm leading to erroroneous readings when AB_1 concentration is measured by spectrophotometric analysis. Crude protein extracts obtained following enzymatic lysis in 50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% sucrose (w/v) (pH 7.2), degraded approximately 90% of the original concentration of AB_1 in aqueous solution (Table 4). The increase in osmotic strength may be responsible for increases in the cell component responsible for AB_1 degradation due to the leaching of the cellular material in by osmosis. As water is removed from the cell against the osmotic gradient some dissolved cellular constituents may be removed into the medium. The highest level of AB_1 degradation was observed for crude protein extracts obtained following the enzymatic lysis in 50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% sucrose (w/v), 0.1% (v/v)

Triton X-100 (pH 7.2). When cells were lysed in this buffer the crude extract was able to degrade approximately 91% of the original AB_1. Detergents, such as Triton X-100, are responsible for solubilizing the outer membrane which is composed mainly of lipid. Once the outer membrane has been solubilized, lysozyme can react with the inner peptidoglycan layer resulting in cell lysis (Leive, 1974).

Effect of Lysozyme Concentration

The effect of lysozyme concentration on the release of the cell component responsible for AB_1 degradation was evaluated. Equal concentrations of cells of *F. aurantiacum* (10 mL at 9.3 Log_{10} CFU/ mL) were treated with 100, 200, 300, 700, and 1,000 μg/ mL lysozyme. Crude protein extracts obtained after lysis with 100μg mL lysozyme degraded an average of 31% of AB_1 in aqueous solution (Table 5). Increasing the concentration to 300 μg/ mL resulted in the degradation of approximately 55% of AB_1. The maximum amount of AB_1 degradation was observed for crude protein extracts obtained from the lysis of bacterial cells with 1,000 μg/ mL lysozyme (Table 5). Increasing the concentration of lysozyme increased the release of the cellular component responsible for AB_1 degradation. The lower amount of AB_1 degradation observed during this experiment compared to earlier experiments may be attributed to the omission of the ethyl acetate incubation. This step

Table 5. Effect of lysozyme concentration on the enzymatic lysis of *F. aurantiacum*[a] as measured by AB_1 degrading activity of crude protein extracts[b].

[Lysozyme] μg/ mL	[AB_1] μg (Initial)	[AB_1] μg (Final)	% Degradation	Standard Error[c]
Control	10.0	9.1	9.0	1.3
100	10.0	6.9	31.0	1.5
200	10.0	5.2	48.0	0.3
300	10.0	4.5	55.0	1.9
700	10.0	4.3	57.0	1.5
1,000	10.0	3.7	63.0	2.3

[a] Concentration was 10 mL at 9.3 Log_{10} CFU/ mL.

[b] Direct spectroscopic measurement of the lysis of *F. aurantiacum* could not be performed due to the presence of the orange pigment associated with this bacterium.

[c] Standard error values reflect the variation of the mean of 3 replications.

was omitted to the reduce the amount of variation that may be due to the varying amounts of the outer cell membrane removed.

Triton X-100, Lysozyme, and Mechanical Shear

Supernatants obtained after each successive treatment of *F. aurantiacum* with Triton X-100, lysozyme, and mechanical shear with glass beads were evaluated for the ability to degrade AB_1. Supernatant (300 μg/ mL total protein) from live cells which had been incubated with 0.1% Triton X-100 degraded an average of 17% of AB_1 (Table 6). Supernatants (900 μg/ mL total protein) from cells which had been treated with lysozyme following treatment with Triton X-100 degraded an average of 59% of AB_1 (Table 6). Supernatant (1,300 μg/ mL total protein) from cells treated with Triton X-100, lysozyme and sheared by mixing with a vortex mixer with glass beads degraded an average of 86% of AB_1 (Table 6). The low amount of degradation observed for the Triton supernatant indicates that the cell component responsible for AB_1 appears to be located within the cytosol of the cell and is not a part of the bacterial cell membrane. Subsequent increases in AB_1 degradation following treatment with lysozyme confirms this as lysozyme is responsible for removal of the inner peptidoglycan layer of the cell. Vortexing with glass beads increased the amount of degradation of AB_1 probably due to more complete removal of the cell wall leading to the complete release of the cytosol contents.

Table 6. Degradation of AB_1 by protein containing supernatants[a] obtained between the successive treatments of *F. aurantiacum* with Triton X-100, then with lysozyme, and finally by mechanical shearing[b].

	[AB_1] μg (Initial)	[AB_1] μg (Final)	% Degradation	Standard Error[c]
Control	10.0	9.4	6.0	1.8
Triton X-100	10.0	8.3	17.0	1.0
Lysozyme	10.0	4.1	59.0	0.2
Mechanical Shear	10.0	1.4	86.0	1.1

[a] The total protein content at each step was determined by Bradford Coomassie Blue Staining (BioRad Chemical Division; Richmond, CA). Total protein concentration for the Triton X-100 fraction was approximately 300 μg/ mL. Total protein content for Lysozyme fraction was approximately 900 μg/ mL. Total protein content for mechanical shear fraction was approximately 1,300 μg/ mL.

[b] Mechanical shearing was performed by vigourous mixing of cells with glass beads in a vortex mixer.

[c] n=3

Effects of pH on AB_1 Degradation

The effect of pH on the degradation of AB_1 by live cells and crude protein extracts from *F. aurantiacum* was evaluated. Live cells of *F. aurantiacum* (8.2 Log_{10} CFU/ mL) were able to degrade >98% of the initial levels of AB_1 at all pH values tested after 24 h (Figure 5). Crude protein extracts (960 μg/ mL) degraded approximately 25% of AB_1 at pH 5, 50% at pH 6, 70% at pH 7 and 50% at pH 8. The maximum amount of AB_1 degradation for crude protein extracts occurred at a neutral pH. The decreased AB_1 degradation observed at pH 6 and 8 indicate the sensitivity of the cell component to changes in pH. Lillehoj et al. (1967) reported that the rate of AB_1 removal by live cells of *F. aurantiacum* was maximum at a pH of 6.75. At pH 5, the rate of removal reduced by one half and at pH 8 the rate decreased by 0.5 μg/ mL min (Lillehoj et al., 1967). No research has been reported on the effects of pH on the ability of crude protein extracts to degrade AB_1.

Degradation During One Week Storage

The effect of prolonged incubation on the degradation of AB_1 by live cells and crude protein extracts from *F. aurantiacum* was evaluated. Live cells (8.1 Log_{10} CFU/ mL) initially decreased levels of AB_1 from 20 μg to 2.5 μg during the first 48 h of incubation (Figure 6). The amount of degradation after the first 48 h did not change during the remainder of the incubation

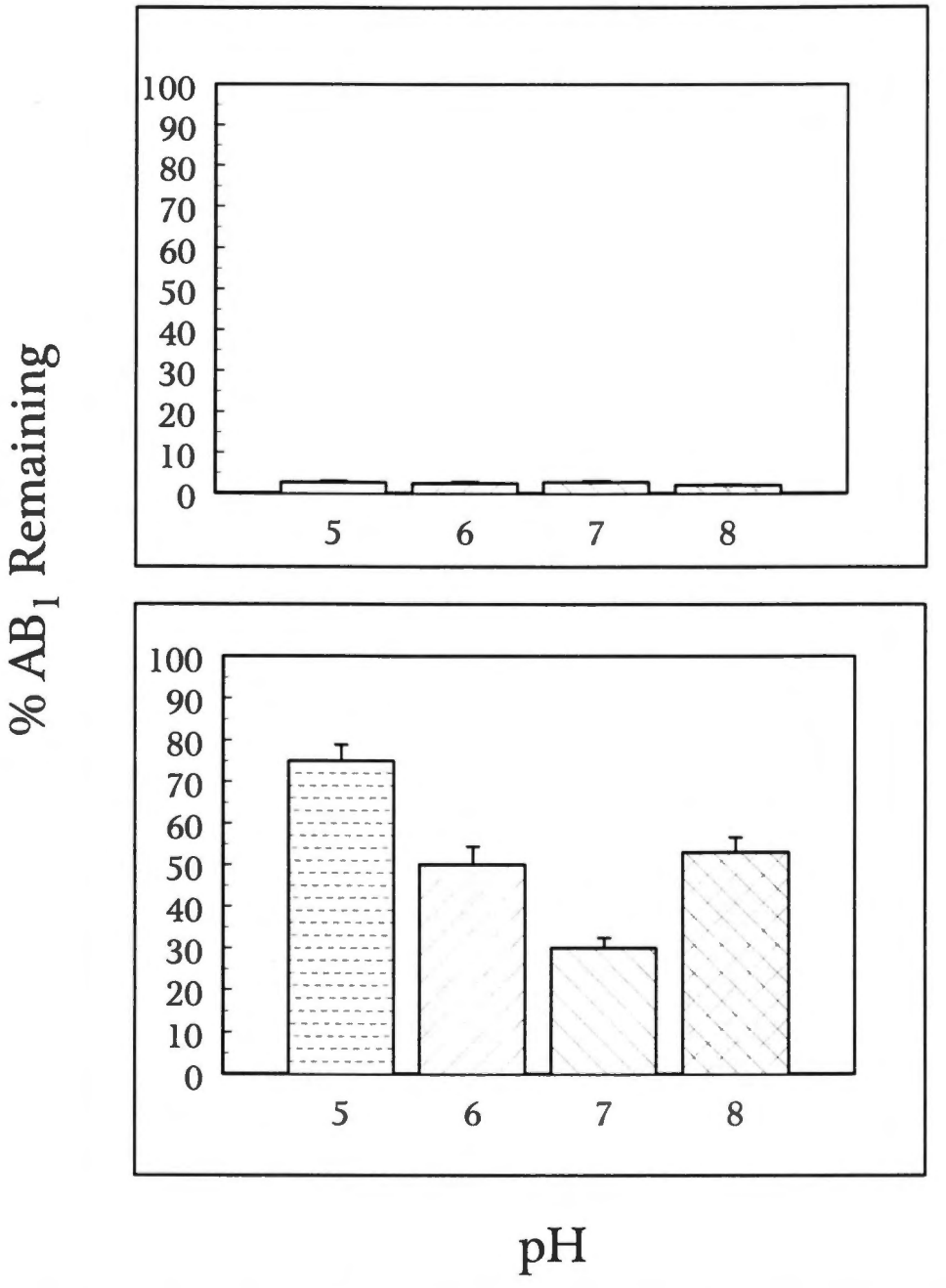

Figure 5. % Aflatoxin B_1 remaining after 24 h incubation with live cells (8.2 Log_{10} CFU/ mL) or crude protein extract (960 μg/ mL) at various pH.

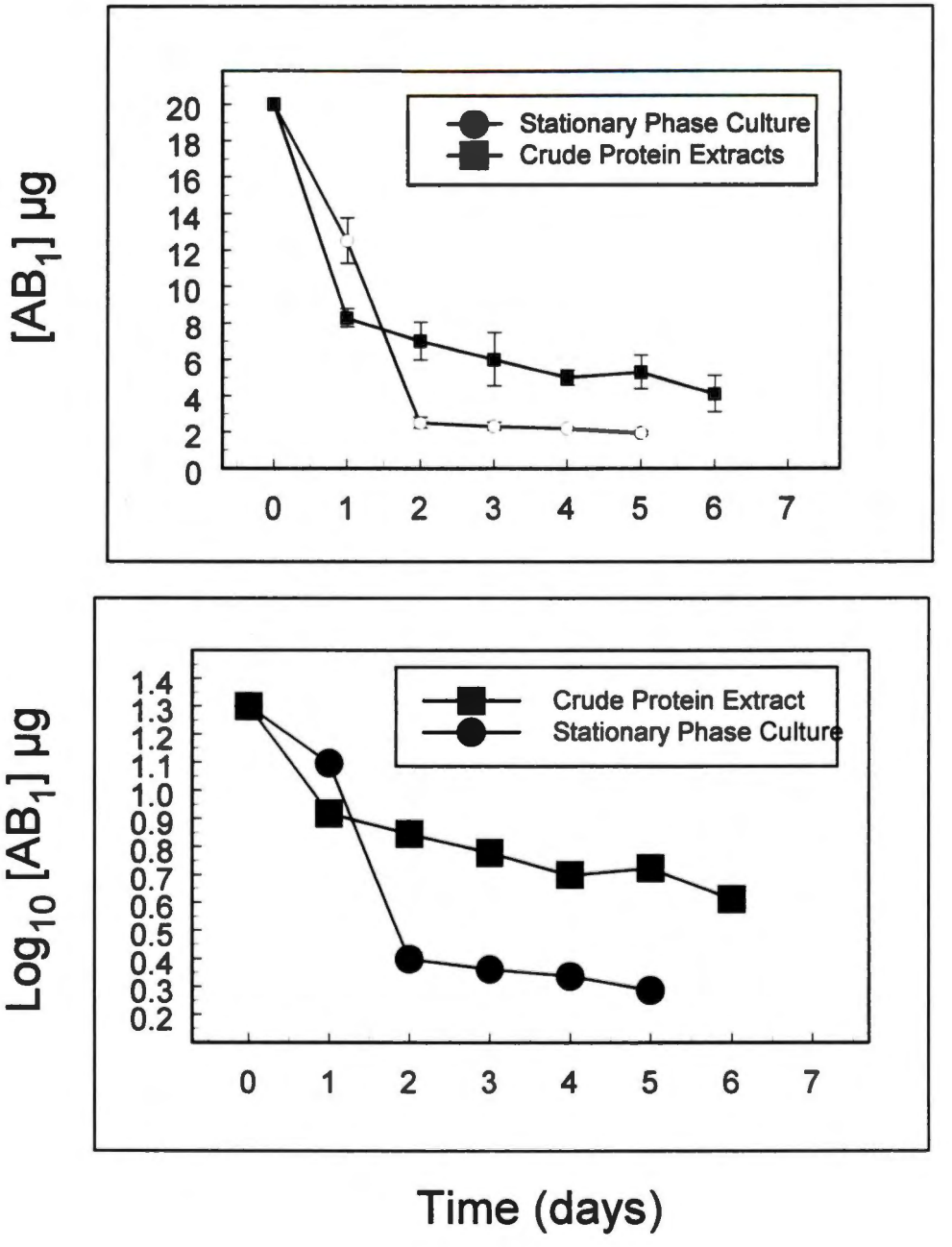

Figure 6. Degradation of AB_1 by stationary phase cultures (8.1 Log_{10} CFU/ mL) and crude protein extracts (1,000 µg/ mL total protein) during storage at 30°C for 1 week.

period. Crude protein extracts (1,100 μg/ mL) initially decreased levels of AB_1 from 20 μg to 8.0 μg during the first 24 h of incubation (Figure 6). Throughout the remainder of storage, AB_1 decreased to a final concentration of 5 μg by day 7. Crude protein extracts did not decrease AB_1 concentrations as much as live cells. Ciegler et al. (1966) had previously reported that live cells of *F. aurantiacum* were capable of degrading 100% of the initial levels of AB_1 (170 μg/ 50 mL) after 88 h. However, Ciegler et al. (1966) quantitated AB_1 using thin layer chromatography which may have been less sensitive than the spectrophotometric used in this study. Line et al. (1994) measured changes in solubility of radioactive products formed during the degradation of AB_1 by live cells of *F. aurantiacum* and reported the largest increases in aqueous solubility, for radioactive products occurred during the first 10-12 h of incubation.

Conclusions

The removal of AB_1 from aqueous solution by crude protein extracts was found to be unaffected by the presence of genomic DNA or increased concentrations of protein indicating that non-specific interactions do not play a role in the removel of AB_1 from aqueous solution . Coupled with our study on the effects of Proteinase K, it appears that the degradation of AB_1 is due to the presence of inter-cellular enzyme or enzyme complex. The AB_1

degradation by live cells was unaffected by pH during the 24 h incubation indicating that the use of the live cells as a method of detoxification could be applied to a variety of foods between the pH range of 5 through 8. The release of the cytosolic enzyme during enzymatic lysis was found to be dependent on the buffering system.

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CHAPTER V

Effect of pH and Crude Protein Extract Concentration on the Enzymatic Degradation of AB_1 and AB_2

Abstract

The ability of crude protein extracts from *F. aurantiacum* to degrade AB_1 AB_2 were investigated. Crude protein extracts were obtained from the enzymatic (lysozyme) lysis of *F. aurantiacum*. Crude protein extracts (900 μg/ mL total protein) were incubated with 10 μg of AB_1 or AB_2 at pH 5, 6, 7, and 8 for 24 h at 30°C. Undegraded aflatoxin was chloroform extracted and quantitated by spectrophotometric analysis using a wavelength of 363 nm. Approximately 25% of AB_1 and 56% of AB_2 was degraded at pH 5 during 24 h at 30°C. The degradation of AB_1 and AB_2 was similar at approximately 49 and 54% respectively at pH 6 during 24 h at 30°C. At pH 7 the degradation of AB_1 was 68% during 24 h at 30°C. The degradation of AB_2 at pH 7 was 55% during 24 h. Approximately 51% of AB_1 and 80% of AB_2 was degraded at pH 8. AB_2 degradation was greater than AB_1 degradation at pH 5, 6, and 8. AB_1 degradation was greater than AB_2 degradation at pH 7. amount of AB_2 degraded was 49%.

Introduction

The aflatoxins are a group of related secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nominus* (Moss, 1994). Aflatoxin B_1 is carcinogenic and toxigenic with demonstrated LD_{50} (mg/ kg) as follows: duckling 0.35, adult rat 7.2, sheep 1, and mouse 9 (Moss, 1994). AB_1 is considered one of the most carcinogenic naturally produced toxins known (Moss, 1994). Aflatoxin B_2 is similar in structure to AB_1 except there is no double bond in the first furan ring (Mirocha et al., 1979). AB_2 is carcinogenic but less toxic than AB_1 (Mirocha et al., 1979).

Aflatoxins have been found in many food products including milk (Frobish, 1986), cottonseed (Whitten, 1968), peanut oil (Pal et al., 1979), and corn (Tabata et al., 1993). The production of aflatoxins in foods has been shown to be influenced by pH (Buchanan and Ayres, 1975), relative humidity (Guo et al., 1996), atmospheric composition (Landers et al., 1967) and temperature (Jarvis, 1971). Buchanan and Ayres (1975) reported that at pH levels less than 6 the production of B aflatoxins was favored and at pH levels above 6 the production of G aflatoxins was favored. Maximal aflatoxin production was reported to occur at pH levels of approximately 5 (Buchanan and Ayres, 1975). The amount of AB_2 produced is typically less than AB_1. Buchanan and Ayres (1975) reported the percentages of the 4 major aflatoxins

produced at various pH. At pH 5, the levels of AB_1, AB_2, AG_1, and AG_2 were reported to be 58, 6, 39, and 3% respectively. The amount of AB_2 decreased as pH increased (Buchanan and Ayres, 1975). Landers et al. (1967) reported on the effects of atmospheric composition on the production of AB_1, AB_2, AG_1, and AG_2. Landers et al. (1967) reported that the amount of aflatoxins produced on peanut kernels stored under air (0.03% CO_2 / 21% O_2 / 79% N_2) was 154, 68, 43, and 34 μg/ g respectively. Landers et al. (1967) also reported that as levels of CO_2 increased, the production of both AB_1 and AB_2 decreased.

In 1966, Ciegler et al. demonstrated that *Flavobacterium aurantiacum* could irreversibly remove AB_1 from aqueous solution. Ciegler et al. (1966) reported that after incubation with *F. aurantiacum*, AB_1 was no longer toxic. This was the first evidence indicating microbial degradation of AB_1. Ciegler et al. (1966) reported that the amount of degradation was dependent on incubation time and initial toxin concentration. Other aflatoxins have been shown to be degraded by *F. aurantiacum* (Lillehoj et al., 1967; Lillehoj et al., 1971). Lillehoj et al. (1967) reported that both stationary and log phase cultures could remove AG_1 from aqueous solution and it was also reported that 13 Log_{10} cells could remove 340 μg of AG_1 during a 4 h incubation period. Lillihoj et al. (1971) reported that *F. aurantiacum* could remove 100% of AM_1 (initial concentration 8 μg/ mL) from aqueous solution during 4 h.

F. aurantiacum has been shown to remove various aflatoxins from aqueous solution. To date, little research is available detailing the mechanisms of aflatoxin degradation by *F. aurantiacum*. The purpose of this experiment was to evaluate the removal of AB_1 and AB_2 from aqueous solution by crude protein extracts from *F. aurantiacum* at different pH values.

Materials and Methods

Preparation of Crude Extracts

F. aurantiacum was inoculated into 1 L of sterile BHI broth from a 12 h culture. Cells were grown for 48 h at 25°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. Cell pellets were suspended in 50 mL Tris-HCl buffer (pH 7.2) containing the following: 50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% (w/v) sucrose, 0.1% (v/v) Triton X-100, and 0.1% ethyl acetate. The cell suspension was incubated at 4°C for 12 h to remove the outer cell wall. Cells were then centrifuged again at 10,000 X g for 10 minutes at 4°C. Cell pellets were suspended in 50 mL Tris-HCl buffer (pH 7.2) containing the following: 50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% (w/v) sucrose, 0.5 mg/ mL lysozyme. Cells were lysed for 24 h at 4°C. Following lysis, the supernatant (crude protein extract) was clarified by centrifugation at 20,000 x g for 30

minutes at 4°C.

pH Effects on Degradation on AB_2 by Crude Extract

The supernatant obtained from the enzymatic lysis of *F. aurantiacum* was divided into five 10 mL aliquots. The buffering agents used for enzymatic lysis were exchanged with the buffering agents needed to achieve each pH level using dialysis (3,000 MWCO). Dialysis was performed in an excess of the appropriate buffers at 4°C for 6 h. Buffers were changed hourly. The buffers consisted of the following: pH 5- 50mM sodium acetate / acetic acid, pH 6- 50mM $H_2PO_4^-$ / HPO_4^{2-}, pH 7- 50mM Tris-HCl, pH 8- 50mM Tris-HCl. The amount of dialysis tubing used was kept to a minimum to prevent dilution of the crude extract. Following dialysis the pH was checked and final adjustments were made using the appropriate acid or base as needed. The total protein content was adjusted to 900 μg/ mL using the buffer of appropriate pH. AB_2 (10 μg) was added to clean sterile test tubes. The tubes were heated to 55°C to evaporate the acetonitrile. The tubes were cooled and 5 mL of crude extract at each pH was added to the appropriately labeled tube. The crude extract and AB_2 was allowed to react for 24 h at 30°C in the dark. Following the 25 h incubation, the reaction was terminated by the addition of 5 mL chloroform. This experiment was repeated 3 times.

Analysis of Residual AB_2

The AB_2 remaining after reaction with crude protein extracts was extracted using 25 mL chloroform in a separatory flask. The organic phase was collected through sodium sulfate into a 125 mL round bottom flask. The chloroform was evaporated using a Rotovapor R rotary evaporator (Buchi; Switzerland) at a temperature of 55°C. The remaining AB_2 was dissolved in 3 mL HPLC grade acetonitrile for analysis by spectrophotometry. A Hewlett-Packard model 8452 diode array spectrophotometer (Hewlett-Packard Co.; Palto Alto, CA) using a wavelength of 363 nm was used to measure the concentrations of AB_2. Standard curves were constructed from known concentrations of AB_2.

Effect of pH on Degradation of AB_1

Crude protein extracts were prepared as described earlier in this section. The lysis buffer was removed by dialysis as previously described. Total protein concentration was adjusted to 900 μg/ mL. Final adjustments to buffers (pH 5, 6, 7, and 8) were made as described earlier. AB_1 (10 μg), from a 1,000 μg/ mL stock solution in acetonitrile, was added to sterile test tubes. The acetonitrile was evaporated at 55°C under a stream of N_2. Five milliliters of crude protein extracts, buffered at pH 5, 6, 7, or 8, were added to the appropriately labeled tubes. The tubes were incubated for 24 h at 30°C in the

dark. Following the incubation period the reaction was terminated by the addition of 5 mL chloroform. The undegraded AB_1 was extracted and quantitated as previously described for AB_2. This experiment was repicated 3 times.

Results and Discussion

Effects of pH on Degradation of AB_1 and AB_2

The effects of pH on the degradation of AB_2 by crude protein extracts, from *F. aurantiacum*, was investigated. Crude protein extracts (900 μg/ mL) degraded approximately 56% of AB_2 at pH 5 compared to approximately 25% of AB_1 at the same pH (Figure 7). At pH 6, the amount of degradation of AB_1 and AB_2 were approximately the same averaging just over 50% after 24 h incubation. At pH 7, the amount of AB_1 degradation was approximately 68% compared to 55% degradation of AB_2. This was the only pH at which the amount of degradation of AB_1 was higher than AB_2. At pH 8, almost 80% of AB_2 was degraded after 24 h compared to 51% for AB_1. To date, the only report of the effects of pH on the degradation of aflatoxin by *F. aurantiacum* was that by Lillehoj et al. (1967). Lillehoj et al. (1967) reported that the maximum amount of AB_1 degraded (1.3 μg/ mL hr) by *F. aurantiacum* occurred at pH 6.75. No research has been previously reported on the pH effects on

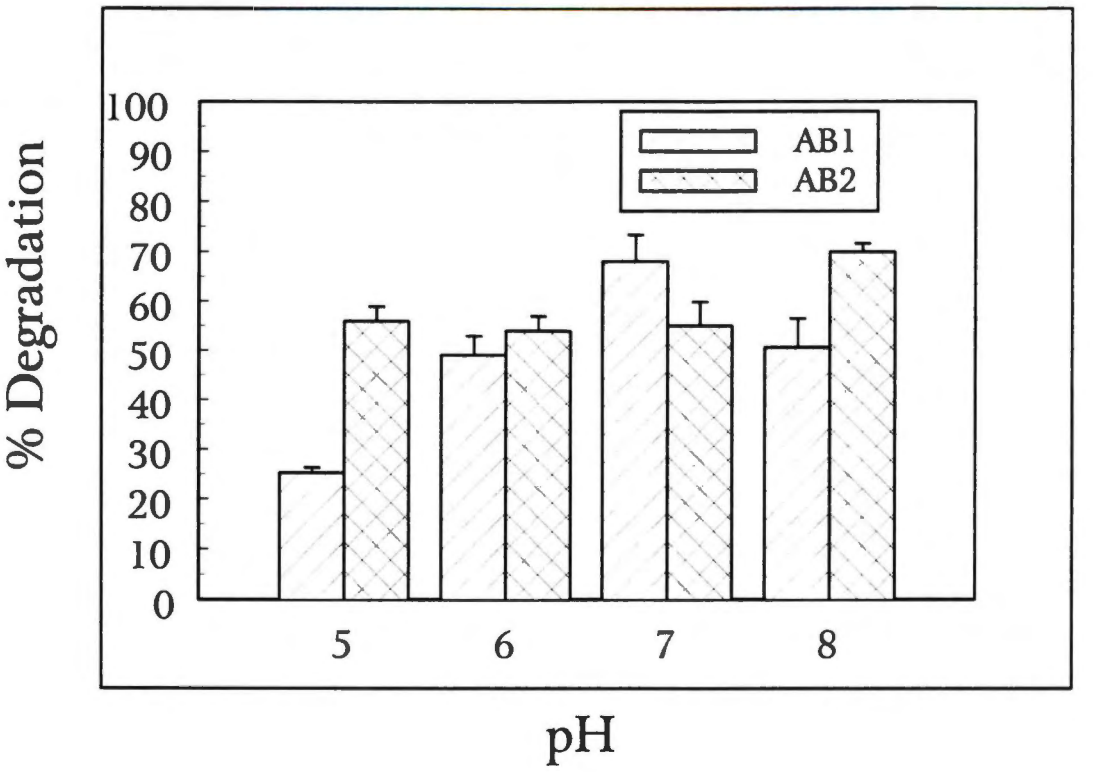

Figure 7. Degradation of AB_1 and AB_2 by crude protein extracts (900 μg/mL total protein) from *F. aurantiacum* at different pH values. Incubation time was 24 h at 30°C. Standard error bars reflect variation of the mean values for 3 replications.

degradation of AB_2 by either live cells or crude protein extracts of *F. aurantiacum*.

Conclusions

Crude protein extracts degraded AB_1 and AB_2 at pH 5, 6, 7, and 8. The maximum amount of AB_2 degradation occurred at pH 8. The maximu amount of AB_1 degraded was observed at pH 7. AB_1 and AB_2 concentrations were decreased by at least 50% at pH 6, 7, and 8. Little degradation of AB_1 was observed at pH 5. These results suggest that the degradation of AB_2 is favored over that of AB_1. These results aslo indicate that a purified form of the enzyme(s) responsible for degradation of aflatoxins may find a use in detoxification of foodstuffs at a wide range of pH values.

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CHAPTER VI

Partial Purification of Crude Protein Extracts from *F. aurantiacum* and Subsequent Degradation of AB_1

Abstract

Ammonium sulfate precipitation, size exclusion chromatography, anion exchange chromatography, and ultra-filtration were used to separate proteins from crude protein extracts from *F. aurantiacum*. Precipitated proteins corresponding to 40-60% saturation with ammonium sulfate degraded an average of 72% of AB_1 during 24 h at 30°C. Slight (<30%) AB_1 degradation activity was observed at 20, 40, 80, and 100% saturation as well. The elution of crude protein extracts through DEAE cellulose yielded multiple protein peaks. Pooled fractions of 65-150 minutes and 155-175 minutes degraded approximately 40-45% of AB_1 from aqueous solution during 24 h incubation at 30°C. Elution of crude protein extracts through Sephadex G-125 yielded multiple peaks. The peak corresponding to pooled volumes of 100.8-109.2 mL decreased AB_1 by 63%. Two other gel filtration fractions demonstrated considerable activity. The peak corresponding to pooled volumes of 27.3-42 mL decreased AB_1 by 44.5% and the peak corresponding to pooled volumes of 81.9-88.2 decreased AB_1 38%. Fractions obtained from ultra-filtration indicated that the protein responsible for AB_1 degradation was smaller than 50 kD and larger than 20 kD.

Introduction

The aflatoxins are a group of secondary fungal metabolites produced by some species of molds in genera *Aspergillus* (Moss, 1994). Aflatoxin production may occur in any food which supports fungal growth. The prevention of aflatoxins in food relies mainly on screening ingredients and raw products. Some food additives and food processing methods have been shown to decrease levels of aflatoxins in food (Hagler et al., 1982; Samarajeewa et al., 1990; Scott, 1984). Many food processing methods, shown to degrade aflatoxin, may only be suitable to certain types of food products. Although, several food additives have been shown to degrade aflatoxins their use is regulated and often may not be present at levels to sufficiently remove all aflatoxins from food. Ciegler et al. (1966) screened over 1,000 microorganisms including yeast and molds for the ability to degrade AB_1 in aqueous solution. Of those tested only one, *F. aurantiacum*, was shown to irreversibly remove AB_1 from solution. The products from the degradation of AB_1 were subsequently shown not to be toxic to day old ducklings (Ciegler et al., 1966). The amount of AB_1 degraded was shown to be dependent on the incubation time and the initial concentration of AB_1 (Ciegler et al., 1966). *F. aurantiacum* degraded 100% of AB_1 at an initial concentration of 170 μg/ 50 mL after 88 h incubation (Ciegler et al., 1966). When the concentration of

AB_1 was increased to 335 μg/ 50 mL, it took approximately 112 h for *F. aurantiacum* to degrade 91%. Ciegler et al. (1966) also demonstrated that *F. aurantiacum* could completely detoxify milk, oil, peanut butter, peanuts, and corn. In a follow up study, Lillehoj et al. (1967) reported that the degradation of AB_1 was influenced by incubation temperature and pH. The maximum amount of AB_1 was reported as 1.3 μg/ mL minute at pH 6.75. Removal of AB_1 was reported to occur faster at 35 > 30 > 40 > 25°C. Other aflatoxins have been shown to be degraded by *F. aurantiacum* (Lillehoj et al., 1967; Lillehoj et al., 1971). The complete degradation of AG_1 by F. aurantiacum was reported to occur after 240 minutes incubation (Lillehoj et al., 1967). The amount of AG_1 which was degraded was reported to 330 μg (Lillehoj et al., 1967). Lillehoj et al. (1971) reported that *F. aurantiacum* removed 35 μg/ mL AM_1 from aqueous solution. It was also reported that *F. aurantiacum* could remove AM_1 from milk (Lillehoj et al., 1971). In 1988, Hao and Brackett reported that *F. aurantiacum* removed 82% of AB_1 from non-defatted peanut milk. The amount of degradation in partially defatted peanut milk was reported to be 51% (Hao and Brackett, 1988). In 1994, Line et al. investigated the fate of ^{14}C labeled AB_1 in the presence of *F. aurantiacum*. They analyzed the amount of radioactivity associated with the aqueous and organic phases during the extraction of AB_1. Line et al. (1994) reported that the

amount of radioactivity which remained in the organic phase was only 24% after 6 h incubation with *F. aurantiacum*. This was evidence that AB_1 was being converted to a more water soluble product. Line et al. (1994) also reported that radioactive CO_2 was being produced by *F. aurantiacum* in the presence of ^{14}C labeled AB_1. At present, no published reports on the mechanisms of aflatoxin degradation by *F. aurantiacum* are available. The objective of this work was to purify the enzyme(s), produced by *F. aurantiacum*, responsible for the degradation of AB_1.

Materials and Methods

F. aurantiacum

A 1 L flask of BHI broth was inoculated with 5 μL from a 12 h culture of *F. aurantiacum*. Cells were grown for 48 h at 25°C in an orbital shaker incubator. Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. Cell pellets were suspended in 25 mL Tris-HCl (pH 7.2) containing: 50mM Tris-HCl, 50mM EDTA, 200mM NaCl, 10% (w/v) sucrose, 0.1% (v/v) Triton X-100 and 0.1% (v/v) ethyl acetate. Cells were incubated for 12 h at 4°C to weaken the outer cell membrane. Cells were then centrifuged at 10,000 x g for 10 minutes at 4°C. Cell pellets were suspended in fresh buffer as described above but with the omission of the ethyl acetate. Lysozyme was

added at a concentration of 0.5 mg/ mL and cells were lysed at 4°C for 24 h. The supernatant (crude protein extract) was obtained after centrifugation at 4°C for 30 minutes at 20,000 x g.

Ammonium Sulfate Precipitation

Crude protein extracts (approximately 25 mL) were added to a 150 mL beaker. A stir bar was added and the beaker immersed in an ice bath and placed on top of a magnetic stirrer. Ammonium sulfate (Ultrapure Bioreagent grade) (J.T. Baker, Inc.; Phillipsburg, NJ) was added at a level to achieve 20% saturation. The mixture was slowly stirred until all ammonium sulfate was dissolved (approximately 10-15 minutes). The solution was transferred to sterile centrifuge tubes and centrifuged at 25,000 x g for 30 minutes. The supernatant was collected and transferred back to the original beaker. The remaining protein pellet was suspended in 4 mL Tris-HCl (pH 7.2) buffer. The volume of the remaining supernatant was recorded and enough ammonium sulfate was added to achieve 40% saturation. After all of the ammonium sulfate was dissolved the mixture was centrifuged as previously described. The entire procedure was repeated in order to obtain 60, 80 and 100% saturation (Scopes, 1987).

The degradation of AB_1 by ammonium sulfate fractions was determined. Aflatoxin B_1 (10 μg) was added to clean sterile test tubes. The tubes were

heated to 55°C under N_2 to evaporate the acetonitrile. The tubes were cooled and 2 mL ammonium sulfate precipitated proteins (20, 40, 60, 80, and 100 % saturation) was added to the appropriately labeled tube. The precipitated protein fraction/ AB_1 was incubated for 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform.

Anion Exchange Chromatography

Crude protein extracts were separated using elution through DEAE cellulose equilibrated at pH 7.2 (Alexander et al., 1985). The resin was charged by passing 200 mL KCl (1M) through the column following by washing with 5mM Tris-HCl (500 mL). All experiments were performed at 4°C. An Econo-Pak column (BioRad; Hercules, CA) (1.5 x 12 cm, 14 cm total column height) was used to separate crude protein extracts. The column was filled to give 10 cm total bed height with DEAE cellulose (BioRad; Hercules, CA). Crude protein extracts (10 mL) were added using a transfer pipet. A constant flow rate of 1.2 mL/ minute was maintained using gravity flow using a Mariot flask apparatus (Alexander et al., 1985). The crude protein extracts were eluted onto the column using 20 mL 5mM Tris-HCl (pH 7.2). Proteins were selectively eluted using a NaCl gradient (Alexander et al., 1985). Fractions were collected in 1 minute intervals using a BioRad Model 2110 fraction collector (BioRad; Hercules, CA). Each fraction was measured for

protein content using Bradford Coomassie Blue staining (BioRad Chemical Division; Richmond, CA). Fractions were pooled based on elution profile and tested for the ability to degrade AB_1.

Aflatoxin B_1 degradation was determined on pooled anion exchange fractions. Aflatoxin B_1 (10 μg from a 1,000 μg/ mL stock in acetonitrile) was added to clean sterile test tubes. The tubes were heated to 55°C under N_2 to evaporate the acetonitrile. The tubes were cooled and 3 mL of anion exchange pooled fractions were added to the appropriately labeled tubes. The reaction was allowed to proceed for 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform.

Gel Filtration of Crude Protein Extracts

Gel filtration was performed using pre-swollen Sephadex G-125 packed into an Econo-Pak chromatography column (BioRad; Hercules, CA). The column was 1.5 by 12 cm with a total column height of 14 cm. The column was filled 10 cm with Sephadex G-125 pre-swollen in 5mM Tris-HCl (pH 7.2). A constant flow rate of 2.1 mL/ minute was maintained by gravity flow using a Mariot flask apparatus (Alexander et al., 1985). Fractions were collected in 1 minute intervals using a BioRad Model 2110 fraction collector (BioRad; Hercules, CA). Protein measurements were obtained on gel filtration fractions using Bradford Coomassie Blue staining (BioRad Chemical Division;

Richmond, CA). Fractions were pooled based on protein elution and tested for the ability to degrade AB_1.

Aflatoxin B_1 (20 μg from a 1,000 μg/ mL stock in acetonitrile) was added to clean sterile test tubes. The tubes were heated to 55°C under N_2 to evaporate the acetonitrile. The tubes were cooled and 3 mL of gel filtration pooled fractions were added to the appropriately labeled tubes. The reaction was allowed to proceed for 24 h at 30°C in the dark. After 24 h the reaction was terminated by the addition of 5 mL chloroform.

Ultra-Filtration of Crude Protein Extracts

The separation of crude protein extracts through a selectively permeable membrane was evaluated. Crude protein extracts (25 mL) were added to a Molecular/Por stirred cell (Spectrum; Houston, TX). A constant pressure of 30 psi was maintained using N_2. All separations were performed at 4°C. A selective membrane (50,000 D MWCO) was used for the first separation. Filtration was allowed to proceed until approximately 5 mL of the crude protein extract remained in the upper reservoir. The filtrate which was obtained from the 50 kD filter was then filtered through a 20 kD (MWCO) filter. Filtration was allowed to proceed until approximately 5 mL remained in the upper reservoir. Fractions (5 mL) were retained from the 50 kD filtrate, 50 kD retentate, 20 kD filtrate, and 20 kD retentate. The ability to degrade

AB_1 was evaluated for each ultra-filtration fraction.

Aflatoxin B_1 (10 μg from a 1,000 μg/ mL stock in acetonitrile) was added to clean sterile test tubes. The tubes were heated to 55°C under N_2 to evaporate the acetonitrile. Aliquots (5 mL) from the ultra-filtration fractions were then added to the appropriately labeled tubes. The reaction was allowed to proceed for 24 h at 30°C in the dark. Following the incubation period the reaction was terminated by the addition of 5 mL chloroform.

Measurement of AB_1

In order to determine the amount of AB_1 which was degraded, the amount of AB_1 remaining after reaction with each protein fraction was measured using spectrophotometry as described by Nabney and Nesbitt (1965). Following the termination of each reaction, the undegraded AB_1 was extracted using 25 mL chloroform in a separatory flask. The organic phase was collected through sodium sulfate into 125 mL round bottom flasks. The chloroform was evaporated using a Rotovapor R rotary evaporator (Buchi; Switzerland) at 55°C. The remaining AB_1 was dissolved in 3 mL acetonitrile. Analysis was performed using a Hewlett-Packard model 8452 diode array spectrophotometer (Hewlett-Packard Co.; Palto Alto, CA) using a wavelength of 363 nm. A standard curve using known amounts of AB_1 was used to determine the amount of undegraded AB_1.

Results and Discussion

Ammonium Sulfate Fractions

Crude protein extracts were fractioned using ammonium sulfate precipitation (20, 40, 60, 80, and 100% saturation). The ability to degrade AB_1 was determined for each fraction. The maximum amount of degradation which occurred after 24 h incubation was observed as 72% for the fraction corresponding to 40-60% saturation (Table 7). Other fractions demonstrated some AB_1 degradation activity ranging from 15 to 28%. The amount of total protein precipitated at each concentration of ammonium sulfate is illustrated in Figure 8. The 40-60% saturation fraction which demonstrated the most AB_1 degradation activity is also the fraction with the most protein precipitated. The 40-60% saturation fraction was also fractioned by gel filtration and ultra-filtration. No AB_1 degradation activity was observed for any fraction obtained following gel filtration or ultra filtration (data not shown since activity was lost). At present no work is reported in the literature on the purification of the enzyme(s) responsible for AB_1 degradation. The partial purification of xenobiotic degrading enzymes from flavobacteria has been reported (Mulbry and Karns, 1989). Mulbry and Karns (1989) successfully purified a parathion hydrolase from a *Flavobacterium* spp. to a single band (SDS-PAGE) of approximately 35,000 D using affinity chromatography. The fold purification

Table 7. Degradation of AB_1 by ammonium sulfate fractions obtained from the step wise addition of ammonium sulfate to crude protein extract from *F. aurantiacum*.

% Saturation	[AB_1] μg/ mL (Initial)	[AB_1] μg/ mL (Final)	% Degraded	Standard Error[a]
Control	10	9.1	9	1.4
0-20%	10	7.8	22	2.2
20-40%	10	8.3	17	4.4
40-60%	10	2.8	72	2.2
60-80%	10	7.2	28	2.1
80-100%	10	8.5	15	2.0

[a] Standard error reflects the variation of the mean of 3 replications.

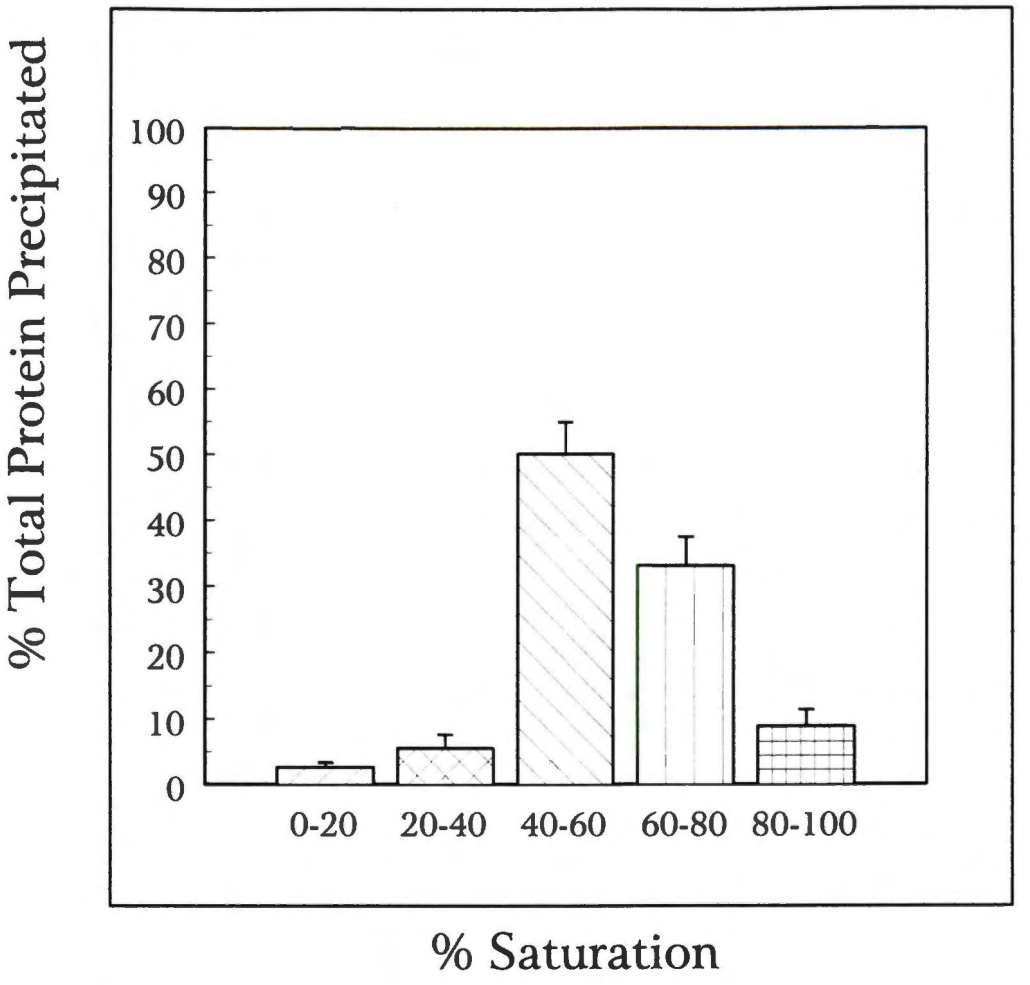

Figure 8. Percent total protein precipitated by increasing concentrations of ammonium sulfate. Levels of ammonium sulfate represented as % saturation.

was reported to be 311 and the specific activity 0.655 IU/mg protein. The stability of the parathion hydrolase over time was not mentioned.

Anion Exchange Chromatography

Crude protein extracts were fractioned using anion exchange chromatography. Proteins were eluted using increasing NaCl concentrations. The elution profile is shown in Figure 9. Protein concentration was measured using Bradford Coomassie Blue staining and absorbance values taken at a wavelength of 595 nm. Fractions were pooled as shown in Figure 10. Two fractions representing times 65-150 minutes and 155-175 minutes degraded AB_1 from initial levels of 20 μg to approximately 12 μg. Little degradation was observed for the other fractions. The amount of degradation of AB_1 was much less for fractions obtained from anion exchange chromatography than for ammonium sulfate fractions. The decreased activity may indicate that more than one protein is needed for complete degradation. Approximately 50% of the total proteins in the crude protein extracts were precipitated at the 60% saturation level with ammonium sulfate. If more than one protein is responsible for complete degradation of AB_1 it is probable that they were precipitated together and may explain why the ammonium sulfate fractions demonstrated greater activity than did the anion exchange fractions.

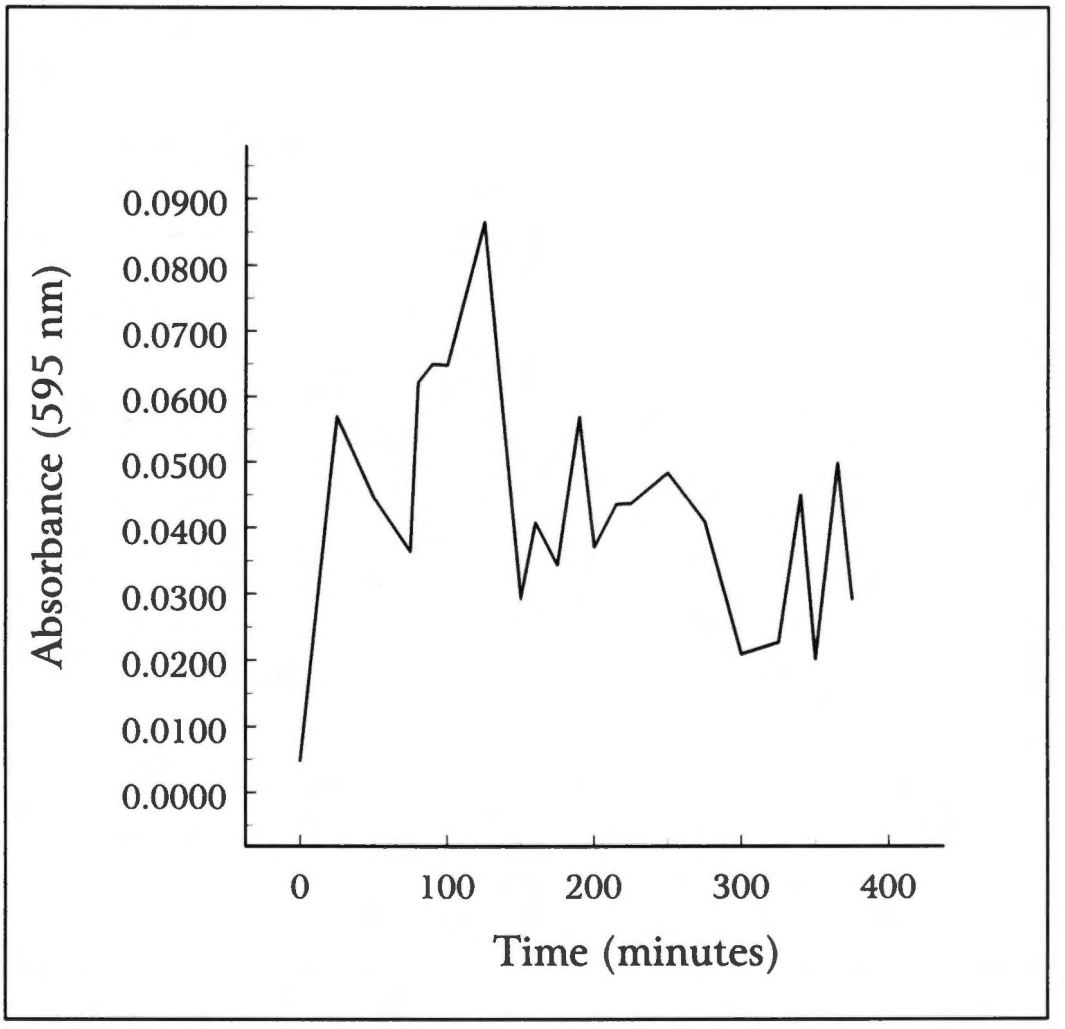

Figure 9. Elution profile for the separation of crude protein extract by DEAE anion exchange column chromatography. Elution was performed using increasing NaCl gradient (0-1 M) as described in the text.

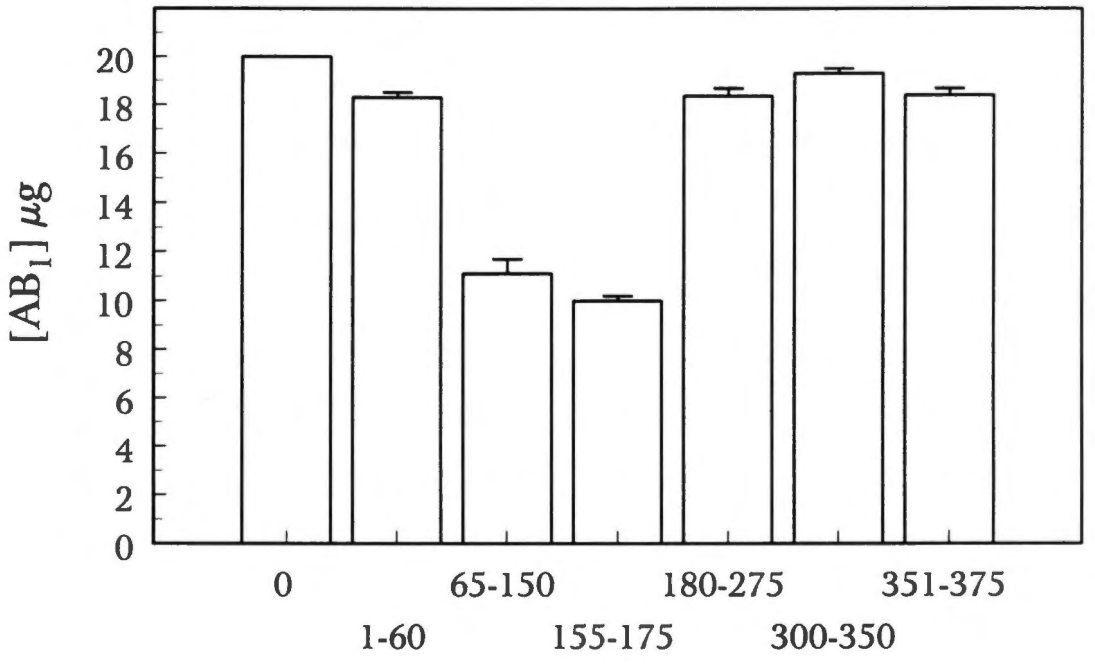

Figure 10. Aflatoxin B_1 (μg) remaining after reaction with anion exchange chromatography fractions. (Error bars represent the variation of the means of triplicate observations within one experiment.)

Gel Filtration of Crude Protein Extracts

Crude protein extracts were fractioned using gel filtration (Sephadex G-125). The elution profile is shown in Figure 11. Protein concentrations for each fraction were determined using Bradford Coomassie Blue staining. Fractions were pooled (Table 8) based on the elution profile and tested for the ability to degrade AB_1. The pooled fraction corresponding to elution volumes 100.8 through 109.2 mL degraded an average of 63.5% of AB_1 (Table 8). Other fractions demonstrated AB_1 degradation activity ranging from 23.5 to 44.5%. Since more than one fraction demonstrated AB_1 degradation activity it is possible that the enzyme responsible may be interacting with other components in the crude extract causing incomplete separation. The pooled fraction corresponding to elution volumes of 27.3 through 42 mL demonstrated considerable AB_1 degradation activity and may be indicative that more than mechanism for AB_1 is present in *F. aurantiacum*. The maximum amount of degradation of AB_1 observed for any gel filtration fraction was 63.5% which was lower than observed for the 40-60% saturation ammonium sulfate fraction but higher than the maximum amount of degradation observed for any pooled anion exchange fraction. No published reports on the separation of crude protein extracts from *F. aurantiacum* by gel filtration are currently available.

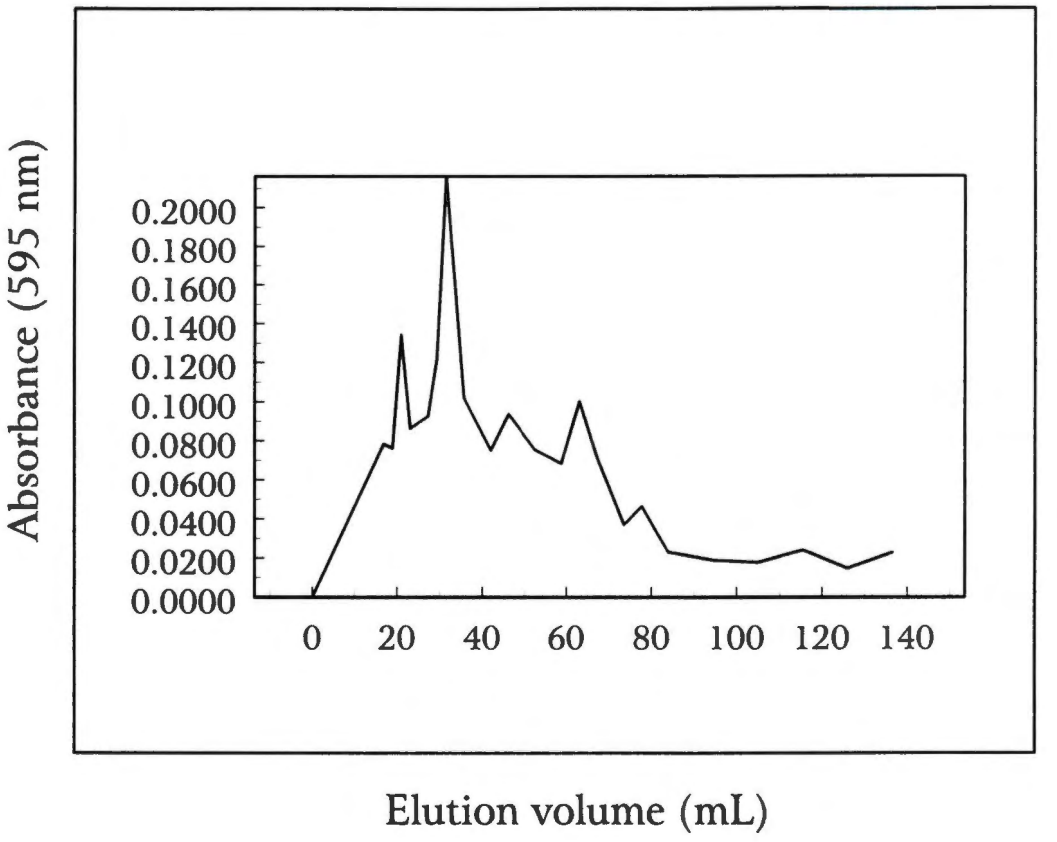

Figure 11. Elution profile of crude protein extracts using size exclusion chromatography on Sephadex G-125.

Table 8. Degradation of AB_1 by pooled gel filtration fractions, from crude protein extracts of *F. aurantiacum* using Sephadex G-125.

Pooled Fraction (mL)	[AB_1] μg (Initial)	[AB_1] μg (Final)	% Degraded	Standard Error[a]
Control	20	19.3	3.5	1.5
12.6-25.2	20	14.1	29.5	2.2
27.3-42.0	20	11.1	44.5	2.0
44.1-58.8	20	15.3	23.5	3.9
60.9-79.8	20	15.2	24.0	3.3
81.9-88.2	20	12.4	38.0	2.2
100.8-109.2	20	7.3	63.5	2.3

[a] Standard error reflects the variation of the mean of triplicate observations within one experiment.

Ultra Filtration of Crude Protein Extracts

Crude protein extracts were fractioned using ultra filtration with molecular weight cut off filters of 20,000 and 50,000 Daltons. The fractions passing through the filter and the fractions which did not pass through the filter were tested for the ability to degrade AB_1. That portion of crude protein extracts passing through the 50 kD MWCO filter (50 kD flow through) degraded an average of 52% of AB_1 from aqueous solution (Table 9). The 20 kD flow through degraded only 8% of AB_1 (Table 9). These results indicate that the enzyme responsible for degradation of AB_1 is somewhere between 20 and 50 kD in size. Currently there are no reports on the mechanism or size of the enzyme responsible for the degradation of AB_1 available.

Conclusions

Fractioning of crude protein extracts by ammonium sulfate precipitation, anion exchange chromatography, gel filtration and ultra filtration each yielded fractions capable of degrading AB_1. When these purification protocols were used in sequence all activity was lost. Several possibilities exist which might explain this observation. The presence of proteases located in the crude protein extract may be degrading the enzyme responsible for AB_1 degradation. Cofactors or a secondary enzymes which are necessary for AB_1

Table 9. Degradation of AB_1 by ultra-filtration fractions of crude protein extracts from *F. aurantiacum*.

Fraction	[AB_1] μg (Initial)	[AB_1] μg (Final)	% Degraded	Standard Error[a]
Control	10	9.9	1.0	2.3
50 kD Flow Through	10	4.75	52.5	3.3
50 kD Retained	10	6.27	37.3	4.9
20 kD Flow Through	10	4.95	8.0	2.0
20 kD Retained	10	9.2	50.5	3.6

[a] Standard error reflects the variation of the mean of 3 replications.

degradation which may be lost during the purification. Another possibility may be that the enzyme becomes less stable as it becomes more pure. One goal in the preparation of bacterial cellular extracts is to mimic the conditions present inside bacterial cell. High protein concentrations within the cell help to maintain protein complexes. Dilution of cellular extracts may disrupt and may increase the possibility of protein oxidation. Although *F. aurantiacum* may be found in the same ecological niches as aflatoxin producing mold, it would not be feasible for the bacterium to produce aflatoxin degrading enzymes in any considerable amount unless aflatoxin was present. It is possible that the enzyme(s) used by *F. aurantiacum* are present in an iactive form (zymogens) and must be enzymatically activated, possibly by cleavage of a subunit. This might explain the amount of time needed to achieve considerable aflatoxin degradation. The enzyme(s) needed to degrade aflatoxin must first be converted to an activated form. If enzymatic activation of aflatoxin degrading enzyme(s) is needed, it is possible that the enzymes which activate the aflatoxin degrading enzyme(s) are lost during the purification process and may explain the loss of activity during the purification process. Nevertheless, ammonium sulfate precipitation, gel filtration, and ultra filtration appear to be suitable as a first step in the purification of the enzyme(s) responsible for AB_1 degradation.

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Chapter VII

Summary and Conclusions

This work confirmed earlier reports that *F. aurantiacum* could degrade AB_1 in aqueous solution. This work also showed that *F. aurantiacum* could degrade AB_2 in aqueous solution. This bacterium was successfully lysed using lysozyme. Crude protein extracts from the enzymatic lysis of *F. aurantiacum* were shown to degraded both AB_1 and AB_2. Subsequent treatment of crude protein extracts with, the non-specific protease, Proteinase K revealed that the degradation of AB_1 was enzymatic. These results indicate that the use of a purified form of the enzyme responsible for AB_1 degradation, in the food industry, as a means of removing AB_1, is feasible. Degradation of AB_1 and AB_2, by *F. aurantiacum* and crude protein extracts from *F. aurantiacum*, was shown to occur over a wide range of pH values. This observation broadens the number of different products which might be detoxified either by the organism or by a purified enzyme from the organism.

Much of the characteristics of the mechanism of aflatoxin degradation still remains a mystery. To date, no reports exist indicating that the breakdown products from aflatoxin have been recovered or identified. This is an important piece of information in determining the exact mechanism of aflatoxin degradation. This work demonstrated that much of the degradation

of aflatoxin by crude protein extracts occurred within the first 24-48 h of incubation, after which degradation stopped. At this time it is not known why degradation stops after 48 h. Several possibilities exist including; presence of natural proteases from the bacterium which slowly degrade the enzyme responsible for aflatoxin degradation, product inhibition in which the product of aflatoxin degradation are very similar to the aflatoxin itself, and the presence of regulatory system which has the responsibility of activating or deactivating the enzyme needed for aflatoxin degradation. All of these possibilities should serve as ideas for future research.

Partial purification by ammonium sulfate precipitation, anion exchange chromatography, gel filtration, and ultra filtration was successful. Multiple purification steps however, resulted in a complete loss of aflatoxin degradation. The most likely reason for this observation appears to be the need of a secondary enzyme, perhaps a regulatory enzyme, or the presence of proteases. Both of these are currently under investigation. One alternative to increasing the stability of the enzyme might be the immobilization of that enzyme to a solid support which has been successful for other toxin degrading enzymes isolated from *Flavobacterium* spp.

VITAE

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