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The Production of Recombinant Trichothecene 3-O-Acetyl Transferase and Its Protective Effects on HD11 Chicken Macrophage Cells Challenged with T-2 Toxin

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To the Graduate Council:

I am submitting herewith a thesis written by Shannon Leigh Perrin entitled "The Production of Recombinant Trichothecene 3-O-Acetyl Transferase and Its Protective Effects on HD11 Chicken Macrophage Cells Challenged with T-2 Toxin." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Judith M. Grizzle, Major Professor

We have read this thesis and recommend its acceptance:

Robert L. Donnell, Jun Lin, Kelly R. Robbins

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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The Production of Recombinant Trichothecene 3-O-Acetyl Transferase and Its Protective Effects on HD11 Chicken Macrophage Cells Challenged with T-2 Toxin

A Thesis

Presented for the

Master of Science Degree

University of Tennessee, Knoxville

Shannon Leigh Perrin

May 2008

Dedication

I dedicate this thesis to my Parents,

Jimmy and Linda Perrin.

Thank you for your unconditional love and support.

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1. LITERATURE REVIEW

The word mycotoxin comes from the Greek words *myco* meaning fungus and *toxicarious* meaning poison (Stearn, 2004). Mycotoxins, toxic secondary metabolites produced by fungi, enter the food chain through feedstuffs (Betina, 1984). The Council for Agricultural Sciences and Technology estimates that mycotoxins cause economic losses of up to 1.66 billion US dollars per year (CAST, 2003). Of these fungal toxins, the trichothecene mycotoxin, T-2 toxin, is among the most virulent (Leeson S. *et al.*, 1995). Although a number of fungal genera are capable of producing trichothecenes, *Fusarium* is the most common (Ueno, 1983). T-2 toxin occurs worldwide, but preferentially grows in cold, wet climates (Joffe, 1986). However, free trade of cereals and grains among nations has allowed climatic restrictions of mycotoxins to become obsolete.

T-2 Toxin Structure

Trichothecenes can be divided into two groups: macrocyclic and non-macrocyclic. All are characterized by a 12,13-epoxytrichothecene skeleton, and attached side chains differentiate each trichothecene (Bamburg *et al.*, 1968). The macrocyclic and non macrocyclic mycotoxins are differentiated by the presence of an ester or ether-ester bridge connecting C-4 and 15 on macrocyclic type trichothecenes that is absent in non macrocyclic types. Additionally, non-macrocyclic trichothecenes are divided into 2 subgroups: Type A and B. In this instance, the structural difference exists in the presence of a conjugated carbonyl group at the C-8 position on Type B trichothecenes which is absent in Type A trichothecenes (Leeson *et al.*, 1995). T-2 toxin, 8α-(3-

methylbutyryloxy) 4β, 15diacetoxyscirp-9-en-3α-ol, is a non-macrocyclic, type A trichothecene. Figure 1.1 shows the primary toxic component of T-2 toxin, like other trichothecenes, is the 12, 13-epoxytrichothecene skeleton followed by a double bond between C9 and C1 (Grove and Mortimer, 1969). Other examples of Type A trichothecenes include HT-2 toxin and diacetoxyscirpenol, while Type B trichothecenes include fusarenone, deoxynivalenol, and nivalenol.

Yoshizawa and coworkers (1980a) described the biotransformation of T-2 toxin in rat liver and the gastrointestinal tract. They found that T-2 toxin was quickly hydrolyzed to produce HT-2 toxin, which was then hydrolyzed to T-2 tetraol. *In vitro* trials using protein synthesis inhibition assays showed the toxicity of T-2 toxin and its derivates are, in order from greatest to least, T-2 > HT-2 > T-2 triol > T-2 tetraol > acetyl T-2 (Thompson and Wannemacher, 1986). Babich and Borenfreund (1991), using a human hepatoma cell line, found supporting evidence for this order of toxicity.

Historical Perspective

Mycotoxin poisoning has been documented in humans for centuries. During the 14th, 15th, and 16th centuries in Modern Europe people suspected of being bewitched, displayed symptoms of tremors, paralysis, spasms, seizures, permanent muscle contraction, hallucinations, manias, panics, depression and onset of gangrene. These neurological symptoms are characteristic of ergot poisoning, a mycotoxin produced by the fungus *Claviceps purpurea* and suggest those persecuted as witches might simply have been victims of mycotoxin

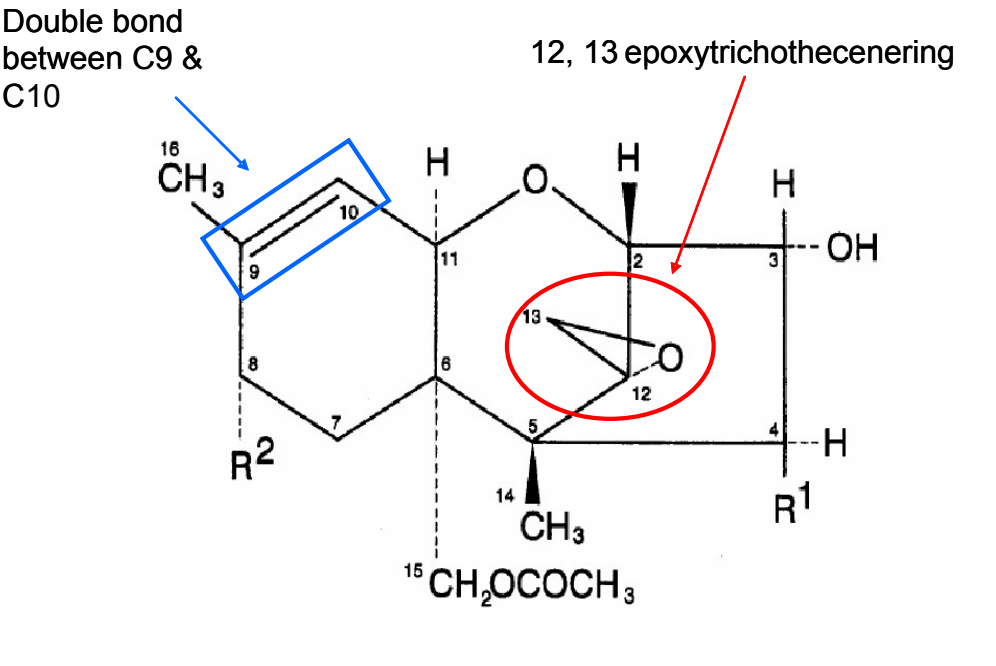

Figure 1.1 T-2 toxin structure. Toxic components are denoted by the double bond between C9 and C10, as well as, the 12, 13 epoxytrichothecene ring.

poisoning (Matossian, 1989; Leeson *et al.*, 1995). Similarly, Schoental (1983), suggested that Mozart's death was attributable to mycotoxin exposure due his poor nutritional status near the end of his life in the 18th century. Schoental (1980) also proposed that the connection between bowel cancer and beer intake was due to the use of moldy grains in the production of alcoholic substances. Specific to T-2 toxicosis: during the 1930s and 1940s the Soviet Union was affected by Russian Endemic Panmyelotoxicosis. The disease was officially called Alimentary Toxic Aleukia (ATA; Kimura *et al.*) and has been associated with *Fusarium* infected gain containing T-2 toxin (Mayer, 1953).

ATA is a potentially fatal disease that develops in four stages. The first stage, showing the caustic nature of T-2 toxin, involves inflammation of the tissues that come into direct contact with the toxin. Gingivitis, gastritis, glossitis, and esophagitis ensue, followed by nausea, vomiting, and diarrhea for a period of three to five days. In the second stage, or incubation phase, symptoms subside for two to eight weeks depending on individual resistance. During this time, T-2 toxin exerts a degenerative effect on bone marrow, and eventually leads to hemorrhagic syndrome, characterized by the appearance of hemorrhagic spots on the skin. At this point, a transition occurs to the third stage of marked sickness characterized by complete deterioration of the bone marrow resulting in agranulocytosis, necrotic angina, hemorrhagic diathesis and sepsis. Fever develops, necrosis extends the entire length of the gastrointestinal tract, and lymph nodes swell. If symptoms are diagnosed accurately, death may be

evaded. In the period of recovery, or fourth stage, necrotic and hemorrhagic symptoms may begin to diminish within two weeks, while it may take up to two months to repair the damage to the bone marrow (Mayer, 1953).

Biochemistry and Metabolism

On the molecular level, T-2 toxin inhibits protein and DNA synthesis similarly. T-2 toxin's effect on RNA synthesis is less extreme (Jeffery *et al.*, 1984). T-2 toxin inhibits proteins synthesis by interacting with peptidyl transferase located on the 60S ribosomal subunit (Wei *et al.*, 1974). Peptidyl transferase is responsible for creating the first peptide bond during initiation, but is also required for all additional steps of elongation (McLaughlin *et al.*, 1977). T-2 inhibits each step differently in a dose-dependent manner. At low concentrations, T-2 will initially stimulate initiation. However, after the first round of synthesis, further synthesis will be blocked. At high concentrations, T-2 will terminate all steps of protein synthesis (Murthy *et al.*, 1985).

T-2 exerts additional effects by acting at the cellular level. Cells most vulnerable to the action of trichothecenes are proliferative cells that contain a larger number of free polysomes found in their cytoplasmic matrix. Regenerative tissues of this type include those of hemopoietic organs, lymphoid, intestinal crypt, and cells in the bursa of Fabricius (Terao, 1983).

Laboratory Animals

In rodents, a great amount of information is available concerning the effects of T-2 toxin which has been shown to support cancer growth, and affect the immune and endocrine systems. Mice fed a diet containing 20µg/g dry diet for six weeks exhibited reduced growth, reduced food consumption, hypoplasia of bone marrow and splenic pulp, gastritis, and dermatitis (Hayes *et al.*, 1980).

Carcinogenic and Cytotoxic Activity

In mice, Corrier and Norman (1988) showed carcinogenic effects in animals intragastrically challenged with T-2 toxin for either 5 days at 2mg/kg body weight (BW), or once at 4 mg/kg BW. In the following 1 to 2 days post-T-2 toxin challenge, mice were inoculated with tumor cells. T-2 treated mice from each dosing regime displayed increased frequency of tumor development, as well as, significantly larger tumors when compared to controls. In a 16 month study, male mice fed 0, 1.5, or 3.0 parts per million (ppm) T-2 toxin developed benign pulmonary and hepatic tumors (Schiefer *et al.*, 1987). In other studies, the liver was predominantly affected, with abnormal changes reported within 24 or 48 hours following oral gavage (2 mg/kg BW T-2 toxin dose; Mollenhauer *et al.*, 1989). Histological changes were seen intracellularly within the endoplasmic reticulum heptocytes, necrotic cells within the intestine, and loss of intestinal microvilli. By day 7, 9, or 12 post-challenge, markedly affected organs were returning to normal, indicating clearance of the toxin.

Similar to mice, T-2 toxin has been shown to have carcinogenic and cytotoxic effects in rats. Schoental and coworkers (1979) reported that rats developed brain and gastrointestinal tract tumors, cardiovascular lesions, and gastric hyperkeratosis an average of 22 months after receiving up to 8 once-per-month doses of T-2 toxin (0.2 to 4.0 mg/kg of BW). An *in vitro* study using lung tissue, report that 0.1 µM T-2 toxin was cytotoxic to rat alveolar macrophages. T-2 toxin reduced cell viability and cell numbers, decreased cell volume, caused chromium release in a dose dependant manner, and caused morphological changes that led to cell lysis (Gerberick and Sorenson, 1983). T-2 toxin also caused lysis of red blood cells. Rat erythrocytes were incubated with increasing doses T-2 toxin ranging from $2.5x10^{-4}$ to $7.5x10^{-4}$ M for 4 hours. Results indicated that T-2 toxin caused hemolysis in a dose dependent manner (Segal *et al.*, 1983).

Immunological Effects

T-2 toxin has been shown to both enhance and suppress the immune system. In mice, a single 3mg/kg BW T-2 toxin dose was associated with short term mastitis resistance after inoculation with pathogenic bacteria. Mastitis resistance occurred 6 hours after T-2 toxin challenge when mice were inoculated with *Escherichia coli* and *Staphylococcus aureus* (Cooray and Jonsson, 1990). It was proposed that T-2 toxin stimulated macrophage cells, and caused immune system activation to fight the infection. However in a long term trial, Tai and Pestka (1988) showed that administration of T-2 toxin caused decreased

resistance in response to *Salmonella typhimurium*. Increased mortality was observed in mice first inoculated with *Salmonella typhimurium* and then treated with 1 mg/kg BW T-2 toxin every other day for 3 weeks. Cooray and Johnson (1990) gave a single 3mg/kg BW dose, while Tai and Pestka (1988) challenged with 1 mg/kg BW every other day for 3 weeks. The levels at which T-2 toxin was administered, duration of the studies, and whether treatment was either pre or post-bacterial inoculation contributed to these differences. Since both gram negative and gram positive bacteria were used, the type of bacteria did not appear to affect susceptibility.

Decreased resistance of T-2 toxin treated mice to *Listeria monocytogenes* was attributed to T-2 toxin's effect on reducing the population and function of T-lymphocytes and macrophage cells, and subsequent migration into sites of infection (Corrier and Ziprin, 1987). At low concentrations, T-2 toxin activated MAPKs (mitogen activated protein kinases) which regulate cell proliferation and apoptosis (Yang *et al.*, 2000). Activation of immune cell apoptosis by T-2 toxin is thought to be through up regulation of MAPKs. Similarly, T-2 toxin caused a suppression of the immune response to viral challenge. Li and coworkers (2006) used respiratory enteric orphan virus (reovirus) as a model for viral infection. Balb/C mice were injected intraperitoneally with 1.75 mg/kg BW T-2 toxin and 2 hours later were instilled with intranasal reovirus. T-2 toxin suppressed reovirus clearance from the lung and increased reovirus-induced pulmonary damage. In bronchial alveolar lavage fluid (BALF), neutrophil and lymphocyte numbers were

elevated on days 3 and 7 following T-2 toxin exposure, but macrophage numbers were elevated only 7 days post challenge. T-2 also altered cytokine production in BALF at day 3 and mucosal IgA at days 9-10 post instillation. T-2 toxin prevented clearance of the virus by suppressing leukocyte numbers, up regulating interleukin 6 (IL-6) which down regulated IFN-γ (interferon γ), and down regulated reovirus-specific IgA production. As previously discussed, Corrier and Norman (1988) suggested that along with the immunosuppressive effect of T-2 toxin to infectious disease, T-2 toxin could also render animals more susceptible to tumor growth.

Humans

Due to the lethality of T-2 toxin, little research has been performed in human subjects. Recently, T-2 toxin along with multiple other mycotoxins, was found in corn samples in the People's Republic of China where high instances of esophageal cancer have been reported (Chu and Li, 1994). Although the correlation between T-2 toxin exposure and cancer in humans seems unclear, studies in rodents have reported formation of tumors paralleling T-2 toxin administration (Schoental *et al.*, 1979; Schiefer *et al.*, 1987). An outbreak of mycotoxicosis in Kashmir Valley, India in 1987 was traced to trichothecene containing bread made from moldy grains. Twelve of 24 wheat samples were found to contain *Fusarium* mycotoxins and T-2 toxin was found in 5 of the 24 wheat samples. Winter temperatures in India vary from 0 to 25ºC. When this

was coupled with unseasonal rains in 1987, it allowed for trichothecene growth and the subsequent outbreak of widespread mycotoxicosis (Bhat *et al.*, 1989).

T-2 toxin has also been found in milk. Milk from Holstein cows treated with the equivalent of 50 ppm T-2 toxin for 15 days, had detectable concentrations of T-2 toxin, which was reported to be between 5 to 10 parts per billion (ppb;Robison *et al.*, 1979a).

Conceivably, indirect exposure to T-2 toxin may occur as a consequence to consumption of contaminated eggs or organ parts. Chi and coworkers (1978a) reported toxin passage to the egg in chickens feed a 0.25 mg/kg BW T-2 toxin. T-2 toxin cleared from within the egg within five days. Additionally, Chi and coworkers (1978b) found passage into muscle, skin and bile 12 hours following a 0.5 mg/kg BW tritium labeled T-2 toxin dose. The amount of T-2 toxin and its metabolites present in the muscle estimated by specific radioactivity was 6.7 µg T-2 toxin per 100 g tissue 12 hours post challenge which is equivalent to 67 ppb. They estimated that 1 kg muscle would contain 67 µg T-2 toxin and consumption of this amount T-2 toxin would not cause negative effects (Chi *et al.*, 1977a; Weaver *et al.*, 1978). These studies show that T-2 toxin can pass into the human food chain via egg, meat, or milk. Pestka (1995) suggested that human consumption of mycotoxin contaminated meat was more likely to cause long term heath problems than acute toxicosis.

Biological Warfare Agent

Mycotoxins have the dubious distinction of being able to cause large scale, detrimental effects to humans when used as biological warfare agents (Stark, 2005). They show potential as warfare agents because once symptoms of exposure are evident; the avenue of escape is past. Lack of explosion, odor, or availability of a detection system make mycotoxin intoxication particularly dangerous (Stark, 2005). Mycotoxin use was suspected during the Middle East War, Desert Storm. Simon and Rea (2003) used brain imaging to compare mycotoxin exposed subjects, control subjects, and subjects impaired from exposure to toxins during Desert Storm. Results showed similarities in brain scintigrams significant enough to justify further examination. Further speculations suggest that The Soviet Union used "yellow rain" on the countries of Afghanistan, Laos, and Cambodia during the cold war, and T-2 toxin has been implicated as a component of "yellow rain" (Ember, 1984; Wannemacher and Wiener, 1997) Samples from the environment and victims near the sites of chemical attacks revealed traces of T-2 toxin (Mirocha *et al.*, 1983). In another report, T-2 toxin was found in samples of yellow residue scraped from plants growing near the Phou Bia area in Cambodia in March of 1981 (Rosen and Rosen, 1982).

Occupational Hazards

T-2 toxin and other mycotoxins pose a threat to agricultural workers who may be exposed through inhalation of infected grains during harvest and milling.

Mycotoxins have been found at levels of 130 ppb in dust particles of corn, and at higher concentrations in particles of less that 11 µm in diameter (Sorenson *et al.*, 1981). As discussed earlier, T-2 toxin has been shown to be cytotoxic to rat alveolar macrophages *in vitro* (Gerberick and Sorenson, 1983). *In vivo*, the toxicity of T-2 toxin has been shown to increase twenty fold when administered to rats via inhalation verses intraperitoneal administration (Creasia *et al.*, 1990).

Poultry

Poultry suffer some of the greatest losses caused by trichothecene exposure because of their heightened sensitivity (CAST, CAST, 2003). Chi and coworkers (1977b) reported the average lethal dose to kill 50% of animals (LD_{50}) after one exposure was 5.14 mg/kg BW for day old chicks, and 4.97 mg/kg BW for 8 week old chicks. However, the LD_{50} for laying hens was 6.27 mg/kg BW T-2 toxin showing that young and growing chicks are more sensitive to T-2 toxin than adult chickens. Other species are similarly affected, with the LD_{50} values near that of chickens (Table 1). Bobwhite quail are 3 to 5 fold more resistance to T-2 toxin than other birds and laboratory animals.

T-2 toxicosis in poultry can be characterized by several physical symptoms. The first sign of which is oral lesions. Wyatt and coworkers (1972) observed oral lesions in day old chicks fed diets containing 0, 1, 2, 4, 8, and 16 µg/g diet T-2 toxin. Oral lesions developed within one week and were yellowish-white in color and caseous in texture. Over the next 2 weeks the oral lesions

Table 1. 1 LD_{50} Values of T-2 Toxin for Various Species

Species	**LD_{50} Value (mg/kg)**
8-Week Old Broiler Chicks	4.97 Oral (Chi *et al.*, 1977b)
Laying Hens	6.27 Oral (Chi *et al.*, 1977b)
Pigeons	1.7 Oral (Fairhurst *et al.*, 1987)
Bobwhite Quail	14.7 Oral (Kerston, 1998)
Rats	3.71 Oral (McKean *et al.*, 2006)

increased in size and spread over the mouth (Wyatt *et al.*, 1972). Doses of 4, 8, and 16 µg/g diet induced neural symptoms including abnormal positioning of wings, hysteroid seizures, and impaired righting reflex in chicks fed T-2 toxin from 1 day of age (Wyatt *et al.*, 1973). Egg production was also affected by T-2 toxin. Hens fed a diet containing 8 ppm diet T-2 toxin produced fewer eggs with thinner egg shells. Hatchability of fertile eggs was 20 % less when compared to controls, and feed consumption was depressed as a result of T-2 toxin consumption (Chi *et al.*, 1977c).

Chi and coworkers (1978b) challenged broiler chicks orally with 0.5 mg/kg BW tritium labeled T-2 toxin. Samples taken 0.5 to 48 hours post challenge showed T-2 toxin present in every tissue sampled 0.5 hours post challenge, with a subsequent peak at 4 hours except in muscle, skin, and bile which peaked 12 hours following exposure. T-2 toxin was found in the egg twenty-four hours following challenge with a single 0.25 mg/kg BW tritium labeled T-2 toxin dose.

Eggs (yolk and white) were found to contain, 0.7 µg T-2 toxin. In these birds, T-2 toxin cleared from egg white and yolk within five to seven days. In a second experiment by Chi and coworkers (1978b), birds were challenged 8 consecutive days with 0.1 mg/kg BW tritium labeled T-2 toxin. Radioactivity within the egg increased until day 3 and remained at that level until the last dose. T-2 toxin was found predominantly in the white, but also in the yolk and shell of the egg. In birds, dosed for 8 consecutive days, T-2 toxin had not completely cleared from eggs by day 11 following the initial dose (Chi *et al.*, 1978b).

T-2 toxin causes increased mortality in chicks infected with *Salmonella*. Chickens were fed 16 µg/g of diet and inoculated with *Salmonella*. Significantly higher mortality was observed among birds fed T-2 toxin and inoculated with *Salmonella* as compared to those only inoculated with *Salmonella* (Boonchuvit *et al.*, 1975). It was proposed that as a consequence of T-2 toxin damage, *Salmonella* bacteria were able to infect the gastrointestinal tract easier (Boonchuvit *et al.*, 1975). These results were supported by Ziprin and Elissalde (1990). Day old chicks were treated with two daily dose of 2mg/kg BW T-2 toxin. On day 6 following challenge, chicks were inoculated with *Salmonella typhimurium*. A higher rate of mortality was observed among chicks treated with T-2 toxin and inoculated with *Salmonella* when compared to *Salmonella* inoculated controls.

Spontaneous trichothecene poisoning in birds has been reported. In the Republic of Croatia, during a particularly humid season in 2002, spontaneous

poisoning of Brahma chickens took place (KonjeviÄ‡ *et al.*, 2004). Two of 10 chickens kept as a hobby died as a result of feed contaminated with *Fusarium* mycotoxins. Analysis revealed the contaminated diet contained 0.7 mg/kg T-2 toxin, 0.5 mg/kg diacetoxyscirpenol, and an unspecified amount of deoxynivalenol. Necropsy revealed no oral or esophageal lesions, but hemorrhages were found in the stomach, as was free blood in the bursa of the Fabricuis. Histopathologically, necrosis occurred in bursa of the Fabricuis, stomach, and gut. Once surviving animals were returned to an uncontaminated diet, symptoms subsided, and no additional deaths occurred.

In a study to determine the effects of trichothecenes on lymphoid cells of epithelial tissues, and the follicle-associated epithelium of the bursa of the Fabricius, day old chicks were injected with 5 mg/kg BW T-2 toxin. Chicks were sacrificed at 15 and 30 minutes post challenge, and then hourly for the next 6 hours. Initially, the central region of the bursal follicle epithelium was disturbed, and large autophagic vacuoles containing cell debris were found in the cell cytoplasm. Lipid droplets were found within the Golgi apparatus, necrotic debris within the intracellular space, and mitochondria were swollen. One and a half hours after trichothecene administration, degeneration of lymphoid cells began in the central regions and then extended into the periphery of the lymphoid follicle. Decreased bursal weight was also observed (Terao *et al.*, 1978)

Swine

Similar to avians and rodents, pigs intubated with radioactively labeled T-2 toxin showed tissue residues. Following an intubated dose of 0.4 mg/kg BW tritium labeled T-2 toxin, T-2 toxin residues of 11.5 ppb were found in muscle, 37.7 ppb in liver, and 61.4 ppb in kidney (Robison *et al.*, 1979b). Thus, dissimilar to the chicken model, the kidney appears to be a primary target of T-2 toxin in swine.

Cats

Lutsky and Mor (1981) were able to produce the symptoms of Alimentary Toxic Aleukia in cats using T-2 toxin. Male cats were given gelatinous capsules containing 0.08 mg/kg BW T-2 toxin every other day until death occurred. Cats died an average of 20.6 days following their first dose. Symptoms, analogous to ATA in humans, included leucopenia, hemorrhagic diathesis, agranulocytosis, bone marrow aplasia, and sepsis. It was concluded, cats could be used as a model for studying ATA in humans.

Multiple Mycotoxin Exposure

Multiple mycotoxins are often found growing concurrently in the environment and act together synergistically. Gigeous (1999) showed the concurrent growth of T-2 toxin and aflatoxin in soybeans grown in Tennessee. Data from North Vietnam reported corn samples containing both *Fusarium* and *Asperigillus* toxins (Wang *et al.*, 1995). Huff and coworkers (1988) investigated

the effects of multiple mycotoxins and reported that dietary T-2 and aflatoxin have additive effects. Three week old broiler chicks were fed diets containing 4.0 µg/g T-2 toxin, 2.5 µg/g aflatoxin, or both mycotoxins (4.0 µg/g T-2 toxin and 2.5 µg/g aflatoxin). Chicks fed diets containing both mycotoxins showed combined effects of each individual mycotoxin including decreased BW, increased organ weight, and decreased corpuscular volume and serum potassium levels. In another study, chicks were fed a diet containing either 2 mg/kg BW ochratoxin, 2 mg/kg BW T-2 toxin, or 2 mg/kg BW ochratoxin and 4 mg/kg BW T-2 toxin from one day of age to 3 weeks of age. Ochratoxin and T-2 toxin combined showed additive effects including decreased BW gain; increased liver, kidney, gizzard, and proventriculus weight; decreased lactate dehydrogenase activity; and increased serum triglyceride and gamma glutamyl transferase. Results of this study indicated that when combined, these two mycotoxins are more toxic than when consumed individually (Kubena *et al.*, 1988).

Similar to chickens, concurrent exposure to T-2 toxin and B_1 Aflatoxin resulted in additive effects among rats (McKean *et al.*, 2006). Rats were able to withstand a higher dose of a single mycotoxin than lower doses of two mycotoxins. Among rats simultaneous dosed with 1.86 mg/kg BW T-2 toxin and 1.36 mg/kg BW B_1 aflatoxin, a 70% mortality rate was observed. These values corresponded to ½ the LD_{50} value for each individual toxin which was 3.71 mg/kg BW T-2 toxin, and 2.71 BW mg/kg aflatoxin.

Mycotoxin Regulation

Acceptable levels of mycotoxins in food and feed grains have been regulated by various agencies for many years. Currently, 99 countries regulate mycotoxin levels which is a 30% increase from 1995 to 2003. Fortunately, this includes about 87% of the world's population. In the US, the FDA (Food and Drug Administration) has set maximum allowable mycotoxin levels in feed grains used for human and animal consumption as shown in Table 1.2 (Egmond and Jonker, 2004). Note there are no current regulations for T-2 toxin

Detoxification

Different methods for mycotoxin detoxification have been studied, however little success has been shown. A study was performed using two mycotoxin binders, 2.5 kg/ton feed of Mycofix™ (Biomin Innovative Animal Nutrition, Mexico City, Mexico) and 1.5 kg/ton of feed Zeotek™ (Nutek, Tehuacan, Mexico). Mycofix™ was able to partially protect chickens against 927 ppb T-2 toxin's detrimental effects as measured by decreased body weight gain and decreased feed consumption. Zeotek™ proved less effective that Mycofix™ which was unable to improve body weight gain or feed consumption among chickens were fed either toxin individually. When 927 ppb T-2 toxin was combined with 567 ppb ochratoxin, greater detrimental effects were observed, and neither Mycofix™ nor Zeotek™ alleviated toxic effects. Regardless of binder inclusion, the combined effects of concurrent exposure to T-2 and ochratoxin was

Table 1.2 The United States Food and Drug Administrations Regulations for Mycotoxins in Human and Animal Food

Mycotoxin	Level	Product	Animal type
Aflatoxin M1	0.5 µg/kg	Milk	human consumption
Aflatoxin B1, B2, G1, and G2	20 µg/kg	all foods except milk	human consumption
	300 µg/kg	corn, peanut products, and cottonseed meal	beef cattle, swine and poultry
Total Fumonisins	4000 µg/kg	Whole or partially degermed dry milled	human consumption
(FB1+FB2+FB3)		corn products (e.g., flaking grits, corn grits,	
		corn meal, corn flour with fat content of	
		≥ 2.25%, dry weight basis)	
	30000 µg/kg	Corn and corn by-products	Breeding ruminants,
			breeding poultry including
			lactating dairy cattle and
			hens laying eggs
Deoxynivalenol	1000 µg/kg	finished wheat products	human consumption
	10000 µg/kg	grains and grain byproducts	ruminating beef and
			feedlot cattle
			(older than 4 months)
			and for chickens

Source: (Egmond and Jonker, 2004)

additive as measured by decreased BW gain, decreased feed consumption, andblood chemistry (García *et al.*, 2003). Hydrated sodium calcium aluminosilicate (HSCAS) was fed to day-old male broiler chicks in an attempt to decrease detrimental effects of 3.5 mg/ kg diet aflatoxins (consisting of 79% B_1, 16% G_1, 4% B_2, and 1% G_2 aflatoxin), 8 mg/kg diet T-2 toxin, or 3.5 mg/kg diet aflatoxins and 8 mg/kg diet T-2 toxin until the age of 3 weeks. HSCAS, at 0.5%, provided a protective effect against the aflatoxins; however no significant effect was seen for T-2 toxin as measured by BW gain, oral lesions, and hematology. HSCAC was able to avert some signs of aflatoxicosis, such increased liver weight, when aflatoxin was combined with T-2 toxin (Kubena *et al.*, 1990). In a similar study, Kubena and coworkers (1998) tested the effects of T-Bind™ (another HSCAC compound) at 0.25 or 0.375% diet against 5 mg/kg diet aflatoxin (consisting of 79% B_1, 16% G_1, 4% B_2, and 1% G_2 aflatoxin) or 8 mg/kg diet T-2 toxin on day-old male broiler chicks. Similar to the 1990 study, T-Bind™ did not protect against T-2 toxin as measured by decreased body weight gain, decreased efficiency of feed utilization, increased mortality, and increased occurrence of oral lesions as compared to controls. However, at both dietary levels of T-Bind™ (0.25% and 0.375%) the negative effects of aflatoxin was reduced. Birds consuming T-Bind™ had higher body weight gains and feed consumption, in addition to, smaller increases in liver, kidney, heart, spleen, pancreas, and proventriculus organ weight as compared to the aflatoxin only treated controls (Kubena *et al.*, 1998).

In pigs, Schell and coworkers (1993) were able to show a protective effect against some of the negative effects of a diet naturally contaminated with aflatoxin. Pigs were fed a diet containing 800 ppb aflatoxin, as measured by high pressure liquid chromatography (HPLC), and supplemented with various clays including a palygorskite, a sepiolite, a treated calcium bentonite, or hydrated sodium calcium alumniosilicate for 4 weeks. Calcium bentonite, at 0.5%, had the greatest effect against 800 ppm aflatoxin. Pigs fed 0.5% calcium bentonite and aflatoxin improved ADG (average daily gain) and ADFI (average daily feed intake) when compared to aflatoxin controls in which ADG and ADFI were both decreased. Eight-hundred ppb aflatoxin increased AST (aspartate aminotransferase), GGT (gamma glutamyltransferase), and ALP (alkaline phosphatase) levels. Calcium bentonite, at 0.5%, prevented these increases when combined with 800 ppb aflatoxin.

Chowdhury and Smith (2005) fed a diet naturally infected with *Fusarium* to laying hens for 12 weeks and supplemented it with a 0.2% GMA (polymeric glucomannan mycotoxin absorbent; Mycosorb, Alltech Inc., Nicholasonville, KY). Mycotoxin content of the naturally infected diets was analyzed by gas chromatography and mass spectrometry. Mycotoxins present in the diets included 12.1 and 11.7 µg/g DON (Deoxynivalenol), 0.5 and 0.4 µg/g 15-acetyl-DON, and 0.6 µg/g zearalenone in contaminated diets and in contaminated diets plus GMA respectively. GMA did not prevent decreased feed consumption, but increased egg production and mass during weeks 4 and 8; increased feed

efficiency in weeks 8 to 12; and prevented a decrease in plasma glucose concentrations and amylase activity. The inconsistent increase in egg production and mass does not point to a true protective effect of GMA, which similarly can be argued to be correlated with the increase in feed efficiency during weeks 8 to 12.

Superactivated charcoal was not beneficial in protecting growing broilers against T-2 toxicosis (Edrington *et al.*, 1997). Growing broilers were fed a diet contaminated with 6 mg/kg BW T-2 toxin and supplemented with superactivated charcoal for 21 days. Birds challenged with T-2 toxin, despite superactivated charcoal supplementation, exhibited reduced body weight gain, oral lesions, reduced feed intake, and changes in hematological parameters.

As previously discussed, T-2 toxin induces PGE_2 (prostaglandin E_2)and fever (Shohami and Feuerstein, 1986). Shohami and coworkers (1987) attempted to remedy T-2 toxemia with dexamethasone, a cyclooxygenase inhibitor which interrupts arachadonic acid metabolism to PGE_2. Rats were intravenously dosed with a single 0.55 to 0.75 mg/kg BW T-2 toxin and then given dexamethasone (2 to 10 mg/kg BW) at 1, 24, and 48 hours post T-2 toxin challenge. Survival was monitored for 1 week following the initial T-2 toxin dose. All rats given T-2 toxin alone died within 21 hours of the challenge. However, only 44% mortality was observed in rats treated with dexamethasone at 1, 24, and 48 hours post T-2 toxin challenge. Dexamethasone depressed PGE_2 levels 6 hours post T-2 toxin challenge, and enhanced survival rates of rats given a

lethal dose T-2 toxin as defined in this study. Similarly, Tremel and coworkers (1985) showed reduced mortality among rats pretreated with 1.6 mg/kg BW dexamethasone and challenged 30 minutes later with 0.75 mg/kg BW T-2 toxin. Mortality was reduced 40% with dexamethasone pretreatment. Dexamethasone reduced diarrhea and pulmonary edema, but increased gastrointestinal hemorrhages.

Probiotics and Mycotoxins

Probiotics have been shown to protect subjects from the toxic effects of some mycotoxins, and thus have generated interest as a natural product useful against mycotoxicosis. For six weeks, broilers were fed a diet supplemented with *Eubacterium* which is a microbial feed supplement from the bacterial strain BBSH 797. Intestinal tissues of the broilers were protected against 10 mg/kg diet deoxynivalenol as measured by duodenum and jejunum morphology (Awad *et al.*, 2006). BBSH 797 has been shown to partially hydrolyze T-2 toxin into HT-2 toxin, a less toxic metabolite of T-2 toxin. Additionally, T-2 tetraol, and scirpentriol were transformed into their respective, less toxic deepoxy forms (Fuchs *et al.*, 2002). *In vitro* studies have shown that a commercially available probiotic supplement, Bioprofit^{TM}, can bind to Aflatoxin B1. In an *ex vivo* experiment using avian duodenal tissue, Aflatoxin B1 absorption was slowed but, not inhibited, by Bioprofit^{TM} (Gratz *et al.*, 2005). Agawane and Lonkar (2004) were able to exhibit a protective effect of a probiotic containing yeast, *Saccharomyces boulardii,* on broilers hematobiochemical profile when

challenged with 0.5 ppm Ochratoxin A (Agawane and Lonkar, 2004). Supplementation with 10 mg/kg diet *Saccharomyces boulardii* against 0.5 ppm ochratoxin A alleviated the decreased values for total proteins, albumin, and globulin while preventing increases in serum creatinine and serum glutamate pyruvate transaminase seen in ochratoxin A-only-treated birds.

Increasing T-2 toxin Toxicity

T-2 toxin toxicity may become a larger threat due to pesticide use and the recycling of poultry litter. The use of organophosphates as pesticides in crop production may increase the problems associated with T-2 toxin; as they act through the production of esterase inhibitors which block the normal metabolism of T-2 toxin and promote hydroxylation instead of hydrolysis of the molecule (Wei and Chu, 1985). In mice, the LD_{50} for the hydroxylated form of T-2 toxin, 3-hydroxyl T-2 toxin, at 4.63 mg/kg BW, is slightly higher than the LD_{50} of T-2 toxin, 5.31 mg/kg BW (Yoshizawa *et al.*, 1982). Rat liver homogenates were used to study the effects of diisopropylfluorophosphate, an esterase inhibiting organophosphate insecticide (Ohta *et al.*, 1977). Diisopropylfluorophosphate inhibited the deacetylation of T-2 toxin, thus preventing biotransformation of T-2 toxin to its less toxic derivatives (Thompson and Wannemacher, 1986). As stated previously, biotransformation of T-2 toxin occurs through hydrolysis to less toxic derivatives. It follows that T-2 toxin breaks down to the less toxic derivative HT-2 toxin which breaks down to the less toxic derivative T-2 tetraol (Yoshizawa *et al.*, 1980a; Thompson and Wannemacher, 1986). Therefore, use of

insecticides on crops contaminated with T-2 toxin could increase its toxicity by prevention of the biotransformation into these less toxic derivatives.

Eighty percent of a single dose T-2 toxin will appear in the excreta within 48 hours post challenge, while residues can remain in the tissues for many days (Chi *et al.*, 1978b; Yoshizawa *et al.*, 1980b). Exposure levels of T-2 toxin or other mycotoxins may be influenced by management practices. Kuney and Sangani (2000) reviewed management practices for chickens and determined that manure removal occurred as often as every 2-3 days to every 2 weeks in houses with concert floors. However, in houses with dirt floors, manure was removed only 2-3 times per year (Kuney and Sangani, 2000). T-2 toxin contamination of manure may accumulate in houses and would be affected by the frequency of manure removal, and thus increase the exposure risk to resident birds. Additionally, litter from chicken houses is often used for crop, grass, and vegetable production (Acosta-Martínez and Harmel, 2006). As a consequence, T-2 toxin may aerosolize and present exposure issues to humans through inhalation.

Aside from pesticide use, and recycled poultry litter, temperature has been shown to affect the toxicity of T-2 toxin. T-2 toxin was found to inhibit the growth rate of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* at a control temperature of 30ºC (Gu *et al.*, 1991). Raising the temperature to 40ºC caused a further reduction in the yeasts' growth rate which was attributed to T-2 toxin. The combination of increased temperature and T-2 toxin caused greater detrimental

effects to the yeast. Joffe (1983) reported that the 1943-1944 winters in Orenburg, Russia were warmer than average. This increased temperature coupled with frequent spring freeze thaw cycles contributed to growth of *Fusarium* mycotoxins, and led to a large outbreak of Alimentary Toxic Aleukia (Joffe, 1983). Thus, it is tempting to speculate that global warming could contribute to the toxicity of mycotoxins. Over the past century, the Earth's surface temperature has increased by 0.8°C; 75% of this increase has occurred over the last 30 years (Hansen *et al.*, 2006). T-2 toxin and other mycotoxins may pose a greater threat with the promise of a warmer future if coupled with optimal humidity.

Tri101

In 1998, Kimura and coworkers reported the isolation of trichothecene biosynthetic gene, *tri101*, found in *Fusarium* species. Trichothecene 3-0-acetyltransferase, produced by the *tri101* gene was primarily responsible for converting isotrichodermol to isotrichodermin in the T-2 toxin biosynthetic pathway (Figure 1.2). Disruption of the *tri101* gene in *Fusarium sporotrichioides* resulted in accumulation of isotrichodermol, and subsequent inhibition of trichothecene biosynthesis (McCormick *et al.*, 1999). While *tri101* has been proven to be required in T-2 toxin biosynthesis, it has also been shown to be involved in self defense against T-2 toxin (Kimura *et al.*, 1998; McCormick *et al.*, 1999). Transformed *Schizosaccharomyces pombe* cells containing the *tri101*

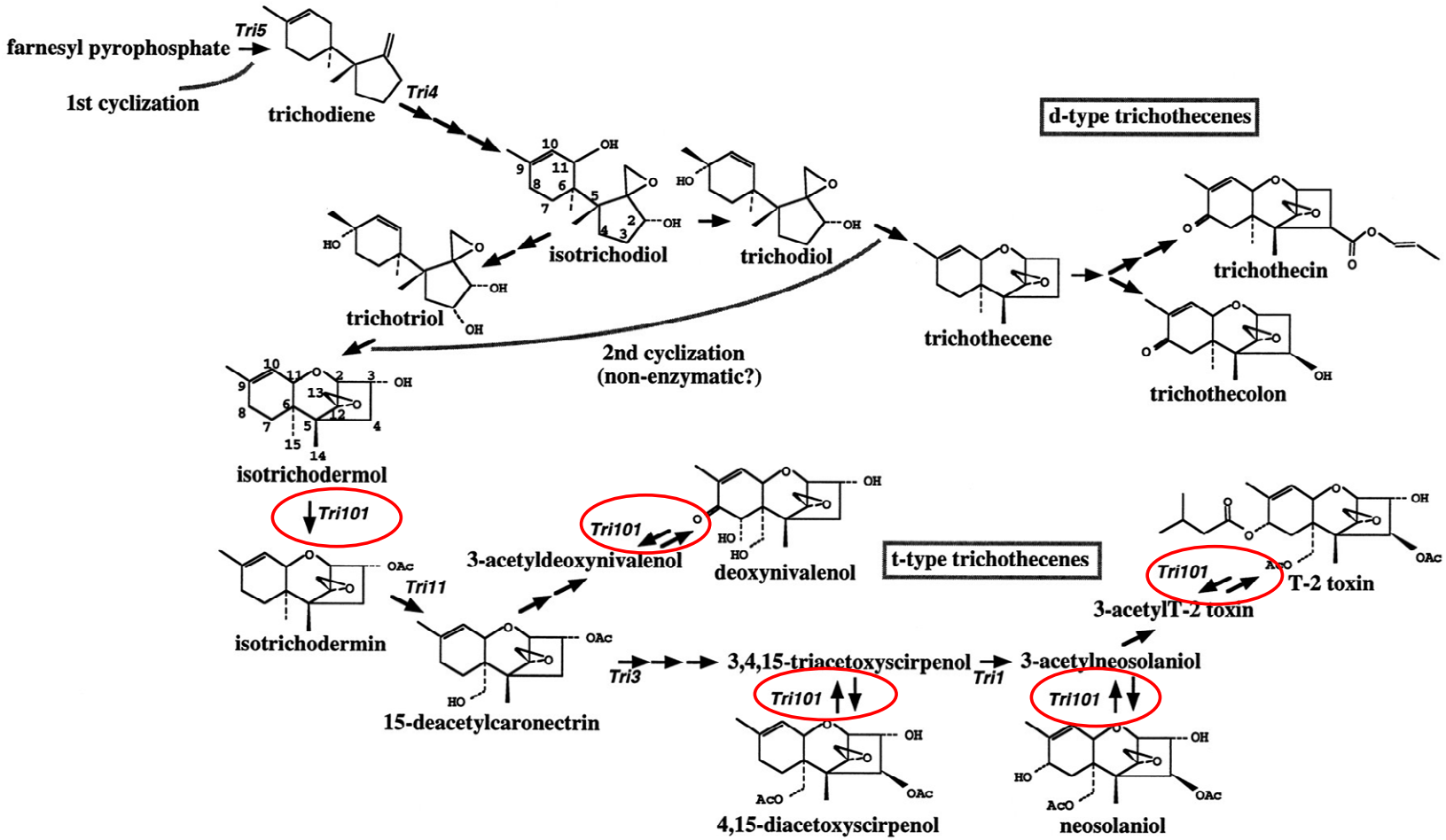

Figure 1.2 Trichothecene biosynthesis (Kimura *et al*., 1998)

gene displayed resistance against 100 µg/ml T-2 toxin (Kimura *et al.*, 1998). To determine the protective mode of action, *tri101* was cloned into an expression vector, transformed into *Escherichia coli*, and induced to obtain a crude enzyme fraction. The crude enzyme fraction was incubated with trichothecenes and acetyl CoA, and the products were analyzed by TLC (Thin Layer Chromatography) and ^{1}H NMR (Nuclear Magnetic Resonance). It was confirmed that upon contact with trichothecenes and acetyl coA, the *tri101* gene product acts as a method of self protection by inserting an acetyl group onto carbon-3 of 3-hydroxy trichothecenes (Kimura *et al.*, 1998; Figure 4). An *in vitro* study using Newcastle disease virus-infected baby hamster kidney cells was conducted to measure the effect of trichothecenes on protein synthesis (Kimura *et al.*, 1998). While it required five fold more 3-O Acetyl T-2 toxin to inhibit protein synthesis, 15 ng/ml T-2 toxin inhibited protein synthesis. Similarly, the 3-acetyl derivatives of deoxynivalenol, and diacetoxyscirpenol were one third less toxic than the 3-hydroxy derivatives. Additionally, protein synthesis was measured in a cell-free rabbit reticulocyte system. The one-time inhibitory concentration to reduce protein synthesis by 50% (IC_{50}) for T-2 toxin, DON, and DON fell between 0.3 and 1.2 µg/ml while the IC_{50} concentrations of the 3-O-acetyl derivatives were greater than 100 times higher, indicative of reduced toxicity. *Tri101* has been shown to reduce the toxicity of T-2 toxin and other trichothecenes.

The hypothesis of this research project is that *tri101* gene product may protect avians against T-2 toxin. To test this hypothesis, the objectives of the reported studies were:

1.) To isolate and purify the *tri101* gene product

2.) To test the purified protein in an *in vitro* system for protective effects against T-2 toxin

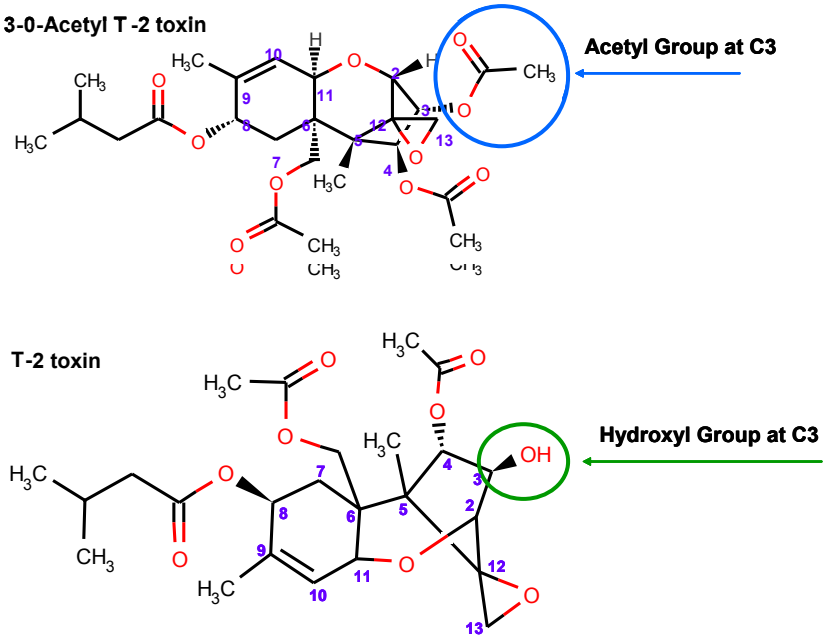

Figure 1.3 Structural difference between T-2 toxin and 3-O-acetyl T-2 toxin

References

Acosta-Martínez, V., and Harmel, R. D. (2006). Soil microbial communities and enzyme activities under various poultry litter application rates. *J Environ Qual* **35**, 1309-1318.

Agawane, S. B., and Lonkar, P. S. (2004). Effect of probiotic containing Saccharomyces boulardii on experimental ochratoxicosis in broilers: hematobiochemical studies. *J Vet Sci* **5**, 359-367.

Awad, W. A., Böhm, J., Razzazi-Fazeli, E., Ghareeb, K., and Zentek, J. (2006). Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. *Poult Sci* **85**, 974-979.

Babich, H., and Borenfreund, E. (1991). Cytotoxicity of T-2 toxin and its metabolites determined with the neutral red cell viability assay. *Appl Environ Microbiol* **57**, 2101-2103.

Bamburg, J. R., Riggs, N. V., and Strong, F. M. (1968). The structures of toxins from two strains of Fusarium tricinctum. *Tetrahedron* **24**, 3329-3336.

Betina, V. (1984). *Mycotoxins Production, Isolation, Separation and Purification*. Elsevier Science Publishing Company Inc, New York, NY.

Bhat, R. V., Beedu, S. R., Ramakrishna, Y., and Munshi, K. L. (1989). Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat production in Kashmir Valley, India. *Lancet* **1**, 35-37.

Boonchuvit, B., Hamilton, P. B., and Burmeister, H. R. (1975). Interaction of T-2 toxin with Salmonella infections of chickens. *Poult Sci* **54**, 1693-1696.

CAST (2003). Mycotoxins: Risk in Plant and Animal Systems. (A. Council of Agricultural Science and Technology, IA, Ed.).

Chi, M. S., Mirocha, C. J., Kurtz, H. F., Weaver, G., Bates, F., and Shimoda, W. (1977c). Effects of T-2 toxin on reproductive performance and health of laying hens. *Poult Sci* **56**, 628-637.

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., and Shimoda, W. (1977a). Subacute toxicity of T-2 toxin in broiler chicks. *Poult Sci* **56**, 306-313.

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., Shimoda, W., and Burmeister, H. R. (1977b). Acute toxicity of T-2 toxin in broiler chicks and laying hens. *Poult Sci* **56**, 103-116.

Chi, M. S., Robison, T. S., Mirocha, C. J., Behrens, J. C., and Shimoda, W. (1978a). Transmission of radioactivity into eggs from laying hens (Gallus domesticus) administered tritium labeled T-2 toxin. *Poult Sci* **57**, 1234-1238.

Chi, M. S., Robison, T. S., Mirocha, C. J., Swanson, S. P., and Shimoda, W. (1978b). Excretion and tissue distribution of radioactivity from tritium-labeled T-2 toxin in chicks. *Toxicol Appl Pharmacol* **45**, 391-402.

Chowdhury, S. R., and Smith, T. K. (2005). Effects of feeding grains naturally contaminated with Fusarium mycotoxins on hepatic fractional protein synthesis rates of laying hens and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *Poult Sci* **84**, 1671-1674.

Chu, F. S., and Li, G. Y. (1994). Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Appl Environ Microbiol* **60**, 847-852.

Cooray, R., and Jonsson, P. (1990). Modulation of resistance to mastitis pathogens by pretreatment of mice with T-2 toxin. *Food Chem Toxicol* **28**, 687-692.

Corrier, D. E., and Norman, J. O. (1988). Effects of T-2 mycotoxin on tumor susceptibility in mice. *Am J Vet Res* **49**, 2147-2150.

Corrier, D. E., and Ziprin, R. L. (1987). Immunotoxic effects of T-2 mycotoxin on cell-mediated resistance to Listeria monocytogenes infection. *Vet Immunol Immunopathol* **14**, 11-21.

Creasia, D. A., Thurman, J. D., Wannemacher, R. W., Jr., and Bunner, D. L. (1990). Acute inhalation toxicity of T-2 mycotoxin in the rat and guinea pig. *Fundam Appl Toxicol* **14**, 54-59.

Edrington, T. S., Kubena, L. F., Harvey, R. B., and Rottinghaus, G. E. (1997). Influence of a superactivated charcoal on the toxic effects of aflatoxin or T-2 toxin in growing broilers. *Poult Sci* **76**, 1205-1211.

Egmond, H. P. v. H. P., and Jonker, M. A. (2004). Worldwide regulations for mycotoxins in food and feed in 2003. (F. a. A. O. o. t. U. Nations, Ed.). Food and Agriculture Organization of the United Nations.

Ember, L. R. (1984). Yellow Rain. *Nature* **62**, 8-34.

Fairhurst, S., Marrs, T. C., Parker, H. C., Scawin, J. W., and Swanston, D. W. (1987). Acute toxicity of T2 toxin in rats, mice, guinea pigs, and pigeons. *Toxicology* **43**, 31-49.

Fuchs, E., Binder, E. M., Heidler, D., and Krska, R. (2002). Structural characterization of metabolites after the microbial degradation of type A trichothecenes by the bacterial strain BBSH 797. *Food Addit Contam* **19**, 379-386.

García, A. R., Avila, E., Rosiles, R., and Petrone, V. M. (2003). Evaluation of two mycotoxin binders to reduce toxicity of broiler diets containing ochratoxin A and T-2 toxin contaminated grain. *Avian Dis* **47**, 691-699.

Gerberick, G. F., and Sorenson, W. G. (1983). Toxicity of T-2 toxin, a Fusarium mycotoxin, to alveolar macrophages in vitro. *Environ Res* **32**, 269-285.

Gigeous, J. L. (1999). Relationship of late winter precipitation and mycotoxin development in soybean seeds at Ames Plantation, pp. 71. University of Tennessee, Knoxville.

Gratz, S., Mykkänen, H., and El-Nezami, H. (2005). Aflatoxin B1 binding by a mixture of Lactobacillus and Propionibacterium: in vitro versus ex vivo. *J Food Prot* **68**, 2470-2474.

Grove, J. F., and Mortimer, P. H. (1969). The cytotoxicity of some transformation products of diacetoxyscirpenol. *Biochem Pharmacol* **18**, 1473-1478.

Gu, T. S., Koshinsky, H. A., and Khachatourians, G. G. (1991). Effects of growth temperature on toxicity of T-2 toxin and roridin A to yeast. *Biotechnol Appl Biochem* **13**, 212-216.

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D. W., and Medina-Elizade, M. (2006). Global temperature change. *Proc Natl Acad Sci U S A* **103**, 14288-14293.

Hayes, M. A., Bellamy, J. E., and Schiefer, H. B. (1980). Subacute toxicity of dietary T-2 toxin in mice: morphological and hematological effects. *Can J Comp Med* **44**, 203-218.

Huff, W. E., Harvey, R. B., Kubena, L. F., and Rottinghaus, G. E. (1988). Toxic synergism between aflatoxin and T-2 toxin in broiler chickens. *Poult Sci* **67**, 1418-1423.

Jeffery, F. H., Morton, J. G., and Miller, J. K. (1984). Effects of some clinically significant mycotoxins on the incorporation of DNA, RNA and protein precursors in cultured mammalian cells. *Res Vet Sci* **37**, 30-38.

Joffe, A. Z. (1983). Environmental conditions conducive to Fusarium toxin formation causing serious outbreaks in animals and man. *Vet Res Commun* **7**, 187-193.

Joffe, A. Z. (1986). *Fusarium Species: Their Biology and Toxicology*. Wiley-Interscience, New York.

Kerston, D. (1998). The effect of T-2 mycotoxin on adult bobwhite quail (Colineus virginianus). In *Department of Animal Science*, pp. 95. University of Tennessee, Knoxville.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., and Yamaguchi, I. (1998). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. *J Biol Chem* **273**, 1654-1661.

Kimura, M., Tokai, T., Matsumoto, G., Fujimura, M., Hamamoto, H., Yoneyama, K., Shibata, T., and Yamaguchi, I. (2003). Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. *Genetics* **163**, 677-684.

Konjević, D., Srebocan, E., Gudan, A., Lojkić, I., Severin, K., and Sokolović, M. (2004). A pathological condition possibly caused by spontaneous trichotecene poisoning in Brahma poultry: first report. *Avian Pathol* **33**, 377-380.

Kubena, L. F., Harvey, R. B., Bailey, R. H., Buckley, S. A., and Rottinghaus, G. E. (1998). Effects of a hydrated sodium calcium aluminosilicate (T-Bind) on mycotoxicosis in young broiler chickens. *Poult Sci* **77**, 1502-1509.

Kubena, L. F., Harvey, R. B., Huff, W. E., Corrier, D. E., Phillips, T. D., and Rottinghaus, G. E. (1990). Efficacy of a hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and T-2 toxin. *Poult Sci* **69**, 1078-1086.

Kubena, L. F., Huff, W. E., Harvey, R. B., Corrier, D. E., Phillips, T. D., and Creger, C. R. (1988). Influence of ochratoxin A and deoxynivalenol on growing broiler chicks. *Poult Sci* **67**, 253-260.

Kuney, D. R., and Sangani, A. (2000). Crop Profile for Eggs in California. (U. S. D. o. Agriculture, Ed.).

Leeson S., G. Diaz, and Summers, J. D. (1995). *Poultry metabolic disorders and mycotoxins.* University Books, Guelph, Ontario, Canada.

Li, M., Harkema, J. R., Islam, Z., Cuff, C. F., and Pestka, J. J. (2006). T-2 toxin impairs murine immune response to respiratory reovirus and exacerbates viral bronchiolitis. *Toxicol Appl Pharmacol* **217**, 76-85.

Lutsky, I., and Mor, N. (1981). Experimental alimentary toxic aleukia in cats. *Lab Anim Sci* **31**, 43-47.

Matossian, M. A. K. (1989). *Poisons of the past: moles, epidemics and history*. Yale University Press, New Haven.

Mayer, C. F. (1953). Endemic panmyelotoxicosis in the Russian grain belt. I. The clinical aspects of alimentary toxic aleukia (ATA); a comprehensive review. *Mil Surg* **113**, 173-189.

McCormick, S. P., Alexander, N. J., Trapp, S. E., and Hohn, T. M. (1999). Disruption of TRI101, the gene encoding trichothecene 3-O-acetyltransferase, from Fusarium sporotrichioides. *Appl Environ Microbiol* **65**, 5252-5256.

McKean, C., Tang, L., Billam, M., Tang, M., Theodorakis, C. W., Kendall, R. J., and Wang, J. S. (2006). Comparative acute and combinative toxicity of aflatoxin B1 and T-2 toxin in animals and immortalized human cell lines. *J Appl Toxicol* **26**, 139-147.

McLaughlin, C. S., Vaughan, M. H., Jr., Cambell, M. H., Wei, C. M., Stafford, M. E., and Hansen, B. S. (1977). *Inhibition of Protein Synthesis by trichothecenes*. Pathotox Publishers, Inc., Park Forest South, Illinois.

Mirocha, C. J., Pawlosky, R. A., Chatterjee, K., Watson, S., and Hayes, W. (1983). Analysis for Fusarium toxins in various samples implicated in biological warfare in Southeast Asia. *J Assoc Off Anal Chem* **66**, 1485-1499.

Mollenhauer, H. H., Corrier, D. E., and Droleskey, R. E. (1989). Ultrastructural lesions induced by T-2 toxin in mice. *J Submicrosc Cytol Pathol* **21**, 611-617.

Murthy, M. R., Radouco-Thomas, S., Bharucha, A. D., Levesque, G., Pandian, S., and Radouco-Thomas, C. (1985). Effects of trichothecenes (T-2 toxin) on protein synthesis in vitro by brain polysomes and messenger RNA. *Prog Neuropsychopharmacol Biol Psychiatry* **9**, 251-258.

Ohta, M., Ishii, K., and Ueno, Y. (1977). Metabolism of trichothecene mycotoxins. I. Microsomal deacetylation of T-2 toxin in animal tissues. *J Biochem (Tokyo)* **82**, 1591-1598.

Pestka, J. J. (1995). *Fungal toxins in raw and fermented meats*. Blackie Academic & Professional, London.

Robison, T. S., Mirocha, C. J., Kurtz, H. J., Behrens, J. C., Chi, M. S., Weaver, G. A., and Nystrom, S. D. (1979a). Transmission of T-2 toxin into bovine and porcine milk. *J Dairy Sci* **62**, 637-641.

Robison, T. S., Mirocha, C. J., Kurtz, H. J., Behrens, J. C., Weaver, G. A., and Chi, M. S. (1979b). Distribution of tritium-labeled T-2 toxin in swine. *J Agric Food Chem* **27**, 1411-1413.

Rosen, R. T., and Rosen, J. D. (1982). Presence of four Fusarium mycotoxins and synthetic material in 'yellow rain'. Evidence for the use of chemical weapons in Laos. *Biomed Mass Spectrom* **9**, 443-450.

Schell, T. C., Lindemann, M. D., Kornegay, E. T., Blodgett, D. J., and Doerr, J. A. (1993). Effectiveness of different types of clay for reducing the detrimental effects of aflatoxin-contaminated diets on performance and serum profiles of weanling pigs. *J Anim Sci* **71**, 1226-1231.

Schiefer, H. B., Rousseaux, C. G., Hancock, D. S., and Blakley, B. R. (1987). Effects of low-level long-term oral exposure to T-2 toxin in CD-1 mice. *Food Chem Toxicol* **25**, 593-601.

Schoental, R. (1980). Cancer of the digestive tract and mycotoxins of Fusarium species. *Lancet* **2**, 593.

Schoental, R. (1983). Mozart's illnesses and death. *J R Soc Med* **76**, 1079-1080.

Schoental, R., Joffe, A. Z., and Yagen, B. (1979). Cardiovascular lesions and various tumors found in rats given T-2 toxin, a trichothecene metabolite of Fusarium. *Cancer Res* **39**, 2179-2189.

Segal, R., Milo-Goldzweig, I., Joffe, A. Z., and Yagen, B. (1983). Trichothecene-induced hemolysis. I. The hemolytic activity of T-2 toxin. *Toxicol Appl Pharmacol* **70**, 343-349.

Shohami, E., and Feuerstein, G. (1986). T-2 toxemia and brain prostaglandins. *Prostaglandins* **31**, 307-319.

Shohami, E., Wisotsky, B., Kempski, O., and Feuerstein, G. (1987). Therapeutic effect of dexamethasone in T-2 toxicosis. *Pharm Res* **4**, 527-530.

Simon, T. R., and Rea, W. J. (2003). Use of functional brain imaging in the evaluation of exposure to mycotoxins and toxins encountered in Desert Storm/Desert Shield. *Arch Environ Health* **58**, 406-409.

Sorenson, W. G., Simpson, J. P., Peach, M. J., 3rd, Thedell, T. D., and Olenchock, S. A. (1981). Aflatoxin in respirable corn dust particles. *J Toxicol Environ Health* **7**, 669-672.

Stark, A. A. (2005). Threat assessment of mycotoxins as weapons: molecular mechanisms of acute toxicity. *J Food Prot* **68**, 1285-1293.

Stearn, W. T. (2004). *Botanical Latin*. Timber Press; Newton Abbot: David & Charles, Portland, Ore.

Tai, J. H., and Pestka, J. J. (1988). Impaired murine resistance to Salmonella typhimurium following oral exposure to the trichothecene T-2 toxin. *Food Chem Toxicol* **26**, 691-698.

Terao, K. (1983). *The Target Organella of Trichothecenes in Rodents and Poultry*. Elsevier Science Publishers, New York.

Terao, K., Kera, K., and Yazima, T. (1978). The Effects of Trichothecene Toxins on the Bursa of the Fabricius in Day-Old Chicks. *VIRCHOWS ARCH B* **27**, 359-370.

Thompson, W. L., and Wannemacher, R. W., Jr. (1986). Structure-function relationships of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison to whole animal lethality. *Toxicon* **24**, 985-994.

Tremel, H., Strugala, G., Forth, W., and Fichtl, B. (1985). Dexamethasone decreases lethality of rats in acute poisoning with T-2 toxin. *Arch Toxicol* **57**, 74-75.

Ueno, Y. (1983). *Trichothecenes- Chemical, Biological, and Toxicological Aspects*. Elsevier Science Publishing Company, New York.

Wang, D. S., Liang, Y. X., Nguyen, T. C., Le, D. D., Tanaka, T., and Ueno, Y. (1995). Natural co-occurrence of Fusarium toxins and aflatoxin B1 in corn for feed in north Vietnam. *Nat Toxins* **3**, 445-449.

Wannemacher, R. W., Jr., and Wiener, S. L. (1997). *Trichothecene Mycotoxins*. Office of the Surgeon General, US Dept of the Army, Falls Church, Va.

Weaver, G. A., Kurtz, H. J., Bates, F. Y., Chi, M. S., Mirocha, C. J., Behrens, J. C., and Robison, T. S. (1978). Acute and chronic toxicity of T-2 mycotoxin in swine. *Vet Rec* **103**, 531-535.

Wei, C. M., Hansen, B. S., Vaughan, M. H., Jr., and McLaughlin, C. S. (1974). Mechanism of action of the mycotoxin trichodermin, a 12,13-epoxytrichothecene. *Proc Natl Acad Sci U S A* **71**, 713-717.

Wei, R. D., and Chu, F. S. (1985). Modification of in vitro metabolism of T-2 toxin by esterase inhibitors. *Appl Environ Microbiol* **50**, 115-119.

Wyatt, R. D., Colwell, W. M., Hamilton, P. B., and Burmeister, H. R. (1973). Neural disturbances in chickens caused by dietary T-2 toxin. *Appl Microbiol* **26**, 757-761.

Wyatt, R. D., Weeks, B. A., Hamilton, P. B., and Burmeister, H. R. (1972). Severe oral lesions in chickens caused by ingestion of dietary fusariotoxin T-2. *Appl Microbiol* **24**, 251-257.

Yang, G. H., Jarvis, B. B., Chung, Y. J., and Pestka, J. J. (2000). Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharmacol* **164**, 149-160.

Yoshizawa, T., Sakamoto, T., Ayano, Y., and Mirocha, C. J. (1982). 3'-Hydroxy-T-2 and 3'-Hydroxy-HT-2 TOXINS - New Metabolities of T-2 toxin, a Trichothecene Mycotoxin, in Animals. *AGR BIOL CHEM TOKYO* **46**, 2613-2615.

Yoshizawa, T., Swanson, S. P., and Mirocha, C. J. (1980a). In vitro metabolism of T-2 toxin in rats. *Appl Environ Microbiol* **40**, 901-906.

Yoshizawa, T., Swanson, S. P., and Mirocha, C. J. (1980b). T-2 metabolites in the excreta of broiler chickens administered 3H-labeled T-2 toxin. *Appl Environ Microbiol* **39**, 1172-1177.

Ziprin, R. L., and Elissalde, M. H. (1990). Effect of T-2 toxin on resistance to systemic Salmonella typhimurium infection of newly hatched chickens. *Am J Vet Res* **51**, 1869-1872.

2. PRODUCTION OF RECOMBINANT TRICHOTHECENE 3-0 ACETYL TRANSFERASE

Abstract

Mycotoxins, toxic byproducts of fungi, represent an annual US agricultural loss of $500 million to $1.5 billion dollars. Currently there are no feasible methods to effectively control this problem. T-2 toxin, produced by *Fusarium* species, is among the most toxic of mycotoxins. The *tri101* gene product, a self-defense product of *Fusarium graminearum*, is involved in the biosynthesis of T-2 toxin and converts T-2 toxin to a less toxic intermediate through acetylation at the 3-carbon. The purpose of this research project was to clone the *tri101* gene into an expression vector, pQE30, and purify the gene product. The *tri101* gene was cloned into pQE30 and transformed into JM109 *Escherichia coli* competent cells. Sequence analysis confirmed proper ligation of *tri101* into the expression vector. After induction, nitrilotriacetic acid agarose affinity chromatography was used to purify recombinant Tri101 (rTri101) protein. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) analysis confirmed the presence of a single 50 kDa protein as expected. Purified rTri101 may be used to reduce harmful effects of T-2 toxin *in vitro* and *in vivo* to reduce economic losses due to trichothecene contamination of feedstuffs.

Introduction

Mycotoxins, secondary metabolites produced by fungi, are mostly produced by *Aspergillus, Penicillium*, and *Fusarium* genus (CAST, 2003). T-2 toxin is a trichothecene mycotoxin produced primarily by *Fusarium* and grows

optimally under cold, damp environmental conditions (Lesson, 1995; Joffe, 1983). In humans and animals, symptoms of T-2 toxicosis is characterized by oral lesions, hemorrhagic lesions of the skin and organs, feed refusal, decreased body weight gain, and death (Smalley, 1973; Mayer, 1953; Chi *et al*., 1977a; Chi *et al*., 1977b). It is thought that T-2 toxin exerts its detrimental effects by inhibition of protein synthesis (McLaughlin *et al.*, 1977). In plants, T-2 toxin causes damage by inhibiting seed germination (Burmeister and Hesseltine, 1970). T-2 toxin had been identified as a biological warfare agent during the Cold War in southeast Asia (Rosen and Rosen, 1982). Currently, there are no viable treatments to alleviate problems associated with T-2 toxin exposure.

Fungal species that produce T-2 toxin have developed methods both to produce toxins and to protect themselves from the toxin (Cundliffe, 1989). Trichothecene biosynthesis, controlled by genes at 3 loci, include: the main trichothecene cluster including *tri3* through *tri14*, *tri101*, and 2 regulatory genes including *tri1* and *tri16* (Brown *et al*., 2003). Self protection lies within the *tri101* gene which has been reported in both *Fusarium graminerium* and *Fusarium sporotrichioides* (Kimura *et al*., 1998a; McCormick *et al*., 1999). Tri101, involved in both the production and 3-acetylation of trichothecene mycotoxins, is responsible for the conversion of isotrichodermol to isotrichodermin, as well as, the synthesis and acetylation of T-2 toxin, deoxynivalenol, neosolaniol, and 4, 15 diacetoxyscripenol (Figure 1.2; McCormick *et al*., 1999). Self defense is achieved by conversion of the 3-hydroxy trichothecenes to 3-O-acetyl derivatives

(Kimura *et al*., 1998a). Protective effects of *tri101*, tested *in vitro* by Kimura and coworkers (1998a), showed that the rTri101 combined with an acetyl source (acetyl CoA) and the 3-hydroxy form of T-2 toxin, deoxynivalenol, and 4, 15-diacetoxyscripenol. Acetylation of T-2 toxin, deoxynivalenol, and 4, 15-diacetoxyscripenol was confirmed by thin layer chromatography. Acetyl derivatives, tested for inhibition of protein synthesis as measured by luciferase activity in rabbit reticulocytes required a concentration 100 times greater than 3-O-hydroxy derivatives. Kimura and coworkers (1998a) also tested the ability of the trichothecene derivatives to inhibit the growth of yeast, transformed with a *tri101*-containing plasmid. Growth of the *tri101*-containing yeast was not inhibited by exposure to 3-O-acetyl T-2 toxin whereas exposure to 3-hydroxy T-2 toxin inhibited growth (Kimura *et al*., 1998a; McCormick *et al*., 1999).

The discovery of *tri101* has lead to *in vivo* and field studies to reduce the toxicity of trichothecenes. Tobacco seed germination is inhibited by 4, 15-diacetoxyscirpenol. In an attempt to alleviate this problem, *tri101*-expressing transgenic tobacco plants were constructed. Upon exposure to 4,15-diacetoxyscirpenol, tobacco plants expressing *tri101* had a significantly higher rate of seed germination as compared to controls (Muhitch *et al*., 2000). Similarly, transgenic rice plants expressing *tri101* were created by Ohsato and coworkers (2007). Root length, a parameter commonly reduced by trichothecenes, was measured in controls and *tri101* transgenic plants. *Tri101*-expressing rice plants were resistant to 40 µg/ml deoxynivalenol as measured by

root length. In an attempt to measure a change in toxicity, Manoharan and coworkers (2006) created transgenic barley plants expressing *tri101* and evaluated accumulation of deoxynivalenol in greenhouse trials. *Tri101*-transformed barley plants grown in a greenhouse were able to reduce the accumulation of deoxynivalenol, however in field trials, there was no decrease in deoxynivalenol accumulation in wild verses *tri101* expressing transgenic plants (Manoharan *et al*., 2006).

To date, no data is available concerning the application of the *tri101* gene product in any animal species. The hypothesis of this study was *tri101* gene product may protect animals against the toxic effects of T-2 toxin *in vitro.* To test this hypothesis, the objectives of this study were to:

1.) Clone *tri101* gene into expression vector pQE30

2.) Express and purify recombinant Tri101 for *in vitro* and *in vivo* studies

Materials and Methods

PCR amplification of tri101 from pUCSVtri101

Plasmid pUCSV*tri101* was obtained as a gift from Kimura and coworkers (1998a). The dried plasmid was reconstituted using 15 µL of distilled, deionized H_20 (ddH_20; this sample hereafter called reconstituted pUCSV*tri101*) and transformed into JM109 *E. coli* competent cells (Promega L2001, Madison, WI) following manufactor's instructions (Appendix A). Cells were plated on Luria Bertani (Matossian) agar plates (Fisher, BP 1426-500, Atlanta, GA) containing

ampicillin at a final concentration of 100 µg/ml (Sigma 0166-5G, St. Louis, MO) and incubated overnight at 37ºC (Appendix A). To confirm the presence of the pUCSV*tri101* in ampicillin-resistant colonies, DNA was extracted from representative colonies that were grown overnight at 37ºC in LB media plus 100 µg/ml ampillicin using a QIAprep Spin Miniprep Kit (QIAGEN 27106, Valencia, CA; (Appendix B). Agarose gel electrophoresis was performed to confirm isolation of plasmid DNA of the correct size (Appendix C). To prepare a whole cell sample (JM109 cells containing pUCSV*tri101*) for pending Polymerase Chain Reaction (PCR), 2 colonies that were grown overnight at 37ºC in LB media plus 100 µg/ml ampillicin were scraped and placed into 100 µL of distilled, deionized H20 and boiled for 10 minutes (hereafter called the whole cell sample).

To amplify the *tri101* gene, isolated plasmid DNA from pUCSV*tri101* was used as a template in a PCR. The nucleotide sequence as published in Genbank (accession number AB000874) has a coding sequence of *tri101* with 1356 base pairs from codon 135 to 1490. Unique restriction enzymes Bam HI (Promega, R602A, Madison, WI) and Hind III (Promega R604A, Madison, WI) were chosen using the NEBCutter website (http://tools.neb.com/NEBcutter2/index.php; Vincze *et al*., 2003) and added to the primers to allow proper orientation during ligation into the expression vector, pQE30 (QIAGEN 32915, Valencia, CA). Bam HI was incorporated into the 5'-end of the forward primer, and Hind III into the 5'-end of the reverse primer for PCR amplification. Primer design was based on results from the Primer 3 web

tool (http://frodo.wi.mit.edu/; Rozen and Skaletsky, 2000). Forward primer sequence was 5' AAA GGA TCC ATG GCT TTC AAG ATA CAG CTC GAC 3' and reverse primer was sequence 5' CCC AAG CTT CTA ACC AAC GTA CTG CGC ATA CTT 3' (restriction sites are underlined; Integrated DNA Technologies, Skokie, IL). The *tri101* gene was PCR amplified from both whole cell sample and from reconstituted plasmid pUCSV*tri101* using above primers (Appendix D).

Following the PCR reaction, DNA from both samples was purified using QIAquick PCR Purification Kit Protocol (QIAGEN 28104, Valencia, CA; Appendix E). Each purified product was digested with restriction enzymes BamHI and HindIII (Appendix F). Following digestion, *tri101* DNA was purified (Appendix E) and DNA band size from the 2 samples were compared by agarose gel electrophoresis (Appendix C).

Ligation of tri101 into expression vector pQE30

Tri101 prepared from the amplified whole cell sample was ligated into the pQE30 vector (QIAGEN 32915, Valencia, CA; Appendix G1). The resulting plasmid, pQE*tri101*, was transformed into JM109 competent cells (Appendix G2). Twelve transformed colonies were randomly selected from ampillicin resistant plates and inoculated into 5 mL aliquots of LB broth supplemented with ampicillin (100 µg/ml final concentration), and grown at 37ºC overnight. Plasmid DNA from pQE*tri101* was extracted using a QIASpin MiniPrep Kit (Appendix B). Agarose gel electrophoresis was used to confirm DNA from colonies (suspected to contain plasmid pQE*tri101*) were larger in size than expression vector, pQE30

(Appendix C). Sequence analysis was completed at the University of Tennessee, Knoxville Molecular Biology Resources Facility, and confirmed that the PCR product had ligated correctly into pQE30 and contained no frameshifts.

Amplification of tri101 from pQEtri101

To confirm that pQE*tri101* plasmid transformants contained the *tri101* gene of correct size, another PCR amplification was completed. Four test samples were amplified (Appendix H). Agarose gel electrophoresis confirmed successful amplification of *tri101*. To determine if restriction enzymes Bam HI and Hind III had been correctly incorporated into *tri101* by PCR, DNA was digested (Appendix I) and gel electrophoresis confirmed DNA of correct size.

Expression and Purification of rTri101

Expression

Construct JM109/pQE*tri101* cells were grown in 5mL LB media plus ampicillin (100 µg/ml final concentration; Sigma 0166-5G, St. Louis, MO) and incubated overnight at 37°C. The following morning, the 5 ml culture was added into 50mL of 37ºC LB media supplemented with ampicillin (final concentration of 100 µg/ml) and were allowed to grow to an optical density (OD) at 600 nm value was between 0.5 and 0.7 nm. At this point, a 1 ml sample was taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE; “pre-induction” sample). The remaining cell culture was induced with 2 mM (final concentration) Isopropyl-β-D-Thiogalactoside (IPTG). One mL samples were taken every hour

for 4 hours following addition of IPTG (SDS-PAGE “post- induction” samples). SDS PAGE (Appendix J) was performed to examine expression of rTri101.

To experimentally check the location of rTri101, it was expressed using a 2 hour induction with IPTG as previously described. After induction, cells were harvested by centrifugation at 4ºC for 20 minutes at 3800 x g, washed and resuspended in 5 ml of 1x phosphate buffered saline (PBS; Sigma P5493, St Louis, MO). Lysozyme (Sigma L3790, St Louis, MO) was added at 1 mg/ml and cells were lysed by sonication and centrifuged for 30 minutes at 4ºC at 15,000 x g. SDS PAGE was performed to examine the location of rTri101 either in the soluble (supernant) fraction or in the insoluble (pellet) fraction.

To predict the theoretical cellular location of the rTri101 product following induction and sonication, Signal P (www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) web tools were implemented (Bendtsen *et al.,* 2004; Nielsen *et al.*, 1997*;* Krogh *et al*., 2001; Sonnhammer *et al*., 1998). The Signal P web tool analyzes properties of amino acids within a protein sequence to predict the presence or absence of signal peptides. Signal peptides, comprised of 1 to 12 positively charged amino acids followed by region of 9 to 14 hydrophobic amino acids, are located near the N-terminal of secretory proteins (Perlman *et al*., 1983; von Heijne *et al*., 1983). TMHMM web tools analyze the properties of amino acids within a translated protein sequence to predict the presence or absence of transmembrane regions indicative of cellular location (Sonnhamer *et al*., 1998). Transmembrane regions, comprised of an

expanse of 20 or more hydrophobic amino acids in an α helix, anchor membrane bound proteins to the lipid bilayer (Nelson and Cox, 2005). Thus proteins secreted from the cell will have signal peptides, while those which are anchored in the cell membrane will have a transmembrane region.

Purification of rTri101 from inclusion bodies

For purification of rTri101 from inclusion bodies (with Empigen BB), QIAGEN's QIAExpressionist protocol was followed (Appendix K; QIAGEN, 2003). Elution fractions 2 and 3 of rTri101 purified from inclusion bodies (rTri101 IB) were combined and confirmed by SDS PAGE (Appendix J). The protein was dialyzed using a Pierce Slide-A-Lyzer 0.5 to 3 mL Dialysis cassette (Pierce 66370, Rockford, IL; Appendix L) according to protocol. Empigen (final concentration 1%) was added at this step because rTri101 precipated during dialysis. After solubilizing, rTri101 IB was sterilized by filtration through a 0.2µm syringe filter. Relative concentration of 270 µg/ml rTri101 IB was determined by Pierce BCA protein assay (Pierce 23225, Rockford, IL; Appendix M). Purified protein was aliquoted into 100 µL fractions and stored at -20ºC.

Purification of rTri101 from soluble fractions

Purification of rTri101 was completed using the soluble fraction (rTri101 SF) following the QIAExpressionist protocol (Appendix N, QIAGEN 2003). SDS PAGE (Appendix J) confirmed that rTri101 was located in the supernant after induction and sonication. Eluted fractions 2 and 3 were combined. The

concentration of 250 µg/ml rTri101 SF was determined by Pierce BCA protein assay (Appendix M) and stored at -20°C in 5 µL aliquots.

Production of crude enzyme rTri101

The protocol of Kimura and coworkers (1998) was used to produce a crude enzyme fraction of rTri101 (Appendix O) , and to replicate their experiments which showed protection of yeast cells against T-2 toxin. Location of rTri101 in the supernant fraction after sonication and centrifugation was confirmed by SDS PAGE (Appendix J). . Total protein concentration of 250 µg/ml was determined with the Pierce BCA protein assay (Appendix M) and stored at -20°C in 5 µL aliquots.

Results & Discussion

Transformation of pUCSVtri101 and amplification of tri101 from pUCSVtri101

Following transformation of pUCSV*tri101* into JM109 cells, plasmid DNA from transformants showed a band at ~ 5Kb in lanes 2 and 3 which correlated with the correct size of pUCSV*tri101* as published by Kimura and coworkers (1998a; Figure 2.1). The 3 conformational forms of DNA, supercoiled, nicked circular and linear, migrate at different rates in agarose gel electrophoresis (Thorne, 1966; e 1967). If agarose concentration is less than .92%, then supercoiled will migrat fastest followed by nicked circular, and lastly linear which migrates slowest (Grossman and Johnson, 1977). The band near 3.5 Kb Figure

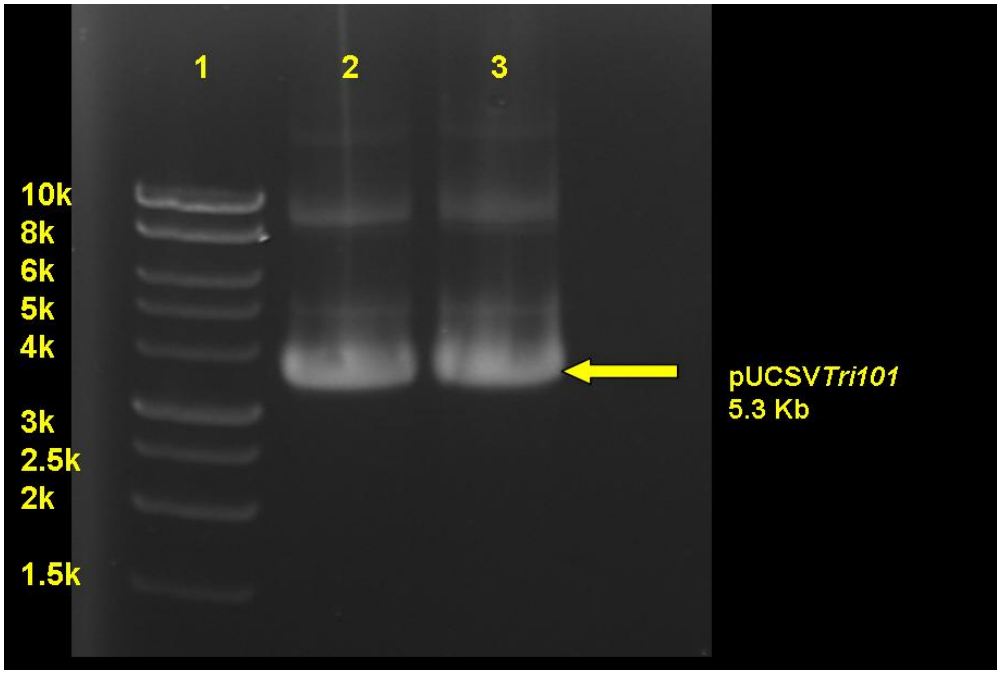

Figure 2.1 Agarose Gel Electrophoresis of plasmid DNA from JM109 cells transformed with pUCSV*tri101*. Lane 1- standard marker. Lanes 2 & 3- plasmid DNA from 2 representative colony transformants.

in 2.1 lanes 2 and 3 was supercoiled form of DNA from plasmid pUCSV*tri101* and therefore, migrated further than expected.

Amplification of the *tri101* gene from whole cell pUCSV*tri101* transformed JM109 cells produced a band that migrated near the estimated size of *tri101* of 1.356 Kb (Figure 2.2, Lane 2)., and agrees reconsistuted pUCSV*tri101* product Kimura and coworkers (1998a; Figure 2.2, Lane 3). From these results, it was concluded that *tri101* had been successfully amplified and transformed into JM109 cells.

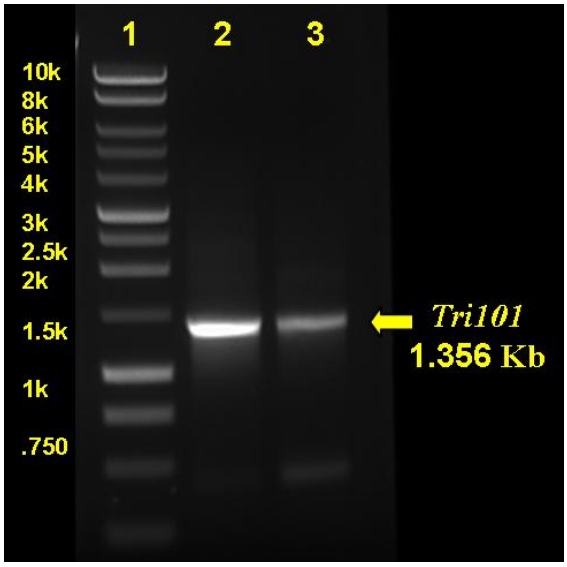

Figure 2.2 Agarose gel electrophoresis of PCR amplified *tri101*. Lane 1-standard marker. Lane 2- PCR amplified DNA from whole cell transformed JM109 cells containing pUCSV*tri101*. Lane 3- PCR amplified DNA from reconsistuted pUCSV*tri101* (Kimura *et al*., 1998a).

Ligation into pQE30 and Transformation of pQEtri101 into JM109cells

Following successful amplification of *tri101*, the gene was ligated into the expression vector, pQE30 and retransformed into JM109 cells (Figure 2.3). Gel electrophoresis of plasmid DNA from the pQE30 vector plus *tri101* showed a band of less than 3 Kb which was less that the expected size of 4.756 of pQE*tri101* (pQE30=3.4 Kb; *tri101*=1.356 Kb). Similarly, the pQE30 plasmid DNA migrated to the size analogous with something less than its expected size (3.4 Kb) of about 2 Kb. It is common in agarose gel electrophoresis for plasmid DNA to migrate further than expected. It was more important that 4 of the colonies were larger than the pQE30 expression vector control (Figure 2.3, lane

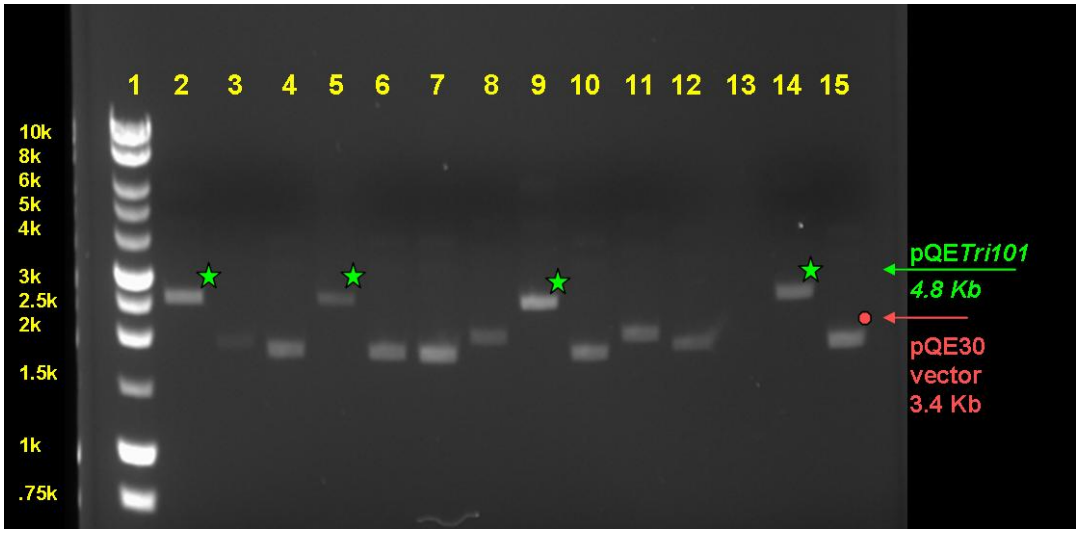

Figure 2.3 Agarose gel electroporesis pQE*tri101* transformed in JM109 cells. Lane 1- Standard marker; Lanes 2 through 12- Individual, isolated colonies; Lane 13- Blank; Lane 14- Individual, isolated colony; Lane 15 Control, expression vector pQE30. * Indicates successfully ligated plasmids. ° Indicates pQE30 control vector

15). DNA sequence analysis of DNA from Lane 14 confirmed that *tri101* had correctly ligated into pQE*tri101* and contained no frame shifts (Figure 2.4).

Amplification of tri101 from pQEtri101

Following DNA sequence analysis of DNA from Lane 14 (Figure 2.4), plasmid DNA of pQE*tri101* from lanes 2, 5, 9, and 14 were further tested by PCR to verify successful ligation of a product of correct size (Figure 2.5). Agarose gel electrophoresis showed that each of the pQE*tri101* plasmids were correctly amplified as indicated by their size of 1.356 Kb.

Test for tri101 orientation and activity of restriction enzymes in pQEtri101

To confirm proper orientation of the *tri101* gene in the pQE30 expression

ATAGAGGAGAATTAACTATGAGAGGATCGCATCACCATCACCATCACGGATCCATGGC
TTCAAGATAGGAAGAACACCCTCGGCCAGCTACCAGGCCTCCTTTCGATCTACACCCA
AATCAGTCTCCTCTACCCCGTCTCTGATCCCTCTCAATATCCCACTATTGTCAGCACCT
TCGAGCAAGGTCTTAAGCGCTTCTCCGAAGCCGTCCCATGGGTCGCAGGCCAGGTCA
AAGCCGAGGGCATTAGCGAGGGAAACACGGGAACTTCCTTTATCGTCCCTTTTGAGGA
CGTTCCTCGTGTTGTAGTGAAAGACCTCCGCGATGATCCTTCAGCGCCCACGATCGAG
GGTATGAGAAAGGCGGGATACCCTATGGCGATGTTTGACGAGAACATCATCGCGCCA
AGGAAGACGTTACCTATTGGACCTGGTACTGGCCCCGACGACCCAAAGCCTGTGATTC
TATTGCAGCTCAACTTCATCAAGGGCGGACTCATCCTCACTGTCAACGGACAGCACGG
TGCTATGGATATGGTAGGCCAAGATGCGGTGATCCGTCTACTCTCCAAGGCGTGCCGT
AACGACCCATTCACCGAAGAGGAAATGACGGCCATGAACCTCGATCGCAAGACGATA
GTTCCTTACCTTGAAAACTACACGATTGGCCCCGAGGTAGATCATCAGATTGTCAAACC
TGATGTAGCTGGTGGTGACGCTGTTCTCACGCCGGTCAGTGCAAGCTGGGCGTTCTT
CAATTCAGCCCCAAGGGCATGTCAGAGCTCAGGATGCTGCTACCAAGACTCTTGACGC
ATCAACAAAGTTCGGGTCGACTGACGATGCTCTTTCGGGTTCATCTGGAATCGGCTCT
CGCGTGGTCTCGAAAAAATCGATGGCTCTGCACCTACCGAGTTCTGCCGGCTGTGATG
CTCCACCGGAATGGGGTTTCGAAAAATACCAGGCCTTTTAAAAATGACCTACCAAAGA
CCATTGGGGAATGCCAAGAGTATGGGGAACGGTTCCGCTTTTAAAAATCGACCCGGGA
TGGCAAAAAAAAGGTTGGAGTCTTGCAAACCCAAAGTCAAGTTTTAGGGGGAGGGGAC
CTTTCCCGGTGTGTTTG

Figure 2.4 DNA sequence analysis of *tri101* with 6-Histidine tag (outlined with dashed box), Bam HI (underlined), and Hind III.

vector, DNA was sequenced. Sequence analysis proved correct orientation (Figure 2.4). To prove that the restriction enzymes were functional, DNA was digested using restriction enzymes Hind III and Bam HI. In Figure 2.6, samples in lanes 2, 3, and 5 showed 2 bands 3.4 Kb and 1.4 Kb in size. This was correct for the expression vector, pQE30 (3.4 Kb) and *tri101* (1.4 Kb). In lane 4, a single band, 5 Kb in size and indicated that only one of the restriction enzymes had correctly cut the plasmid or that the restriction enzyme sites were non-functional. Products from lanes 2, 3, and 5 were kept.

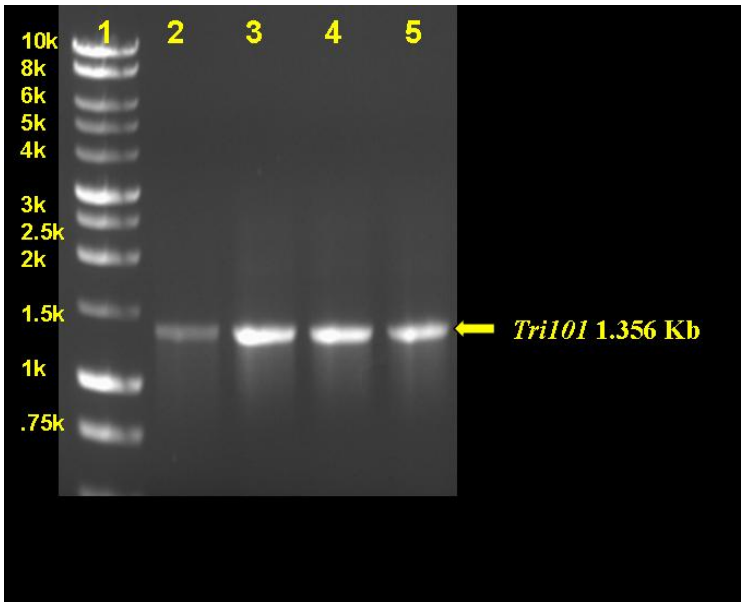

Figure 2.5 Agarose Gel Electrophoresis of PCR DNA from pQE*tri101* plasmids. Lane 1- standard marker. Lanes 2 through 6- PCR products that had indicated successful ligation of *tri101* into pQE*tri101*

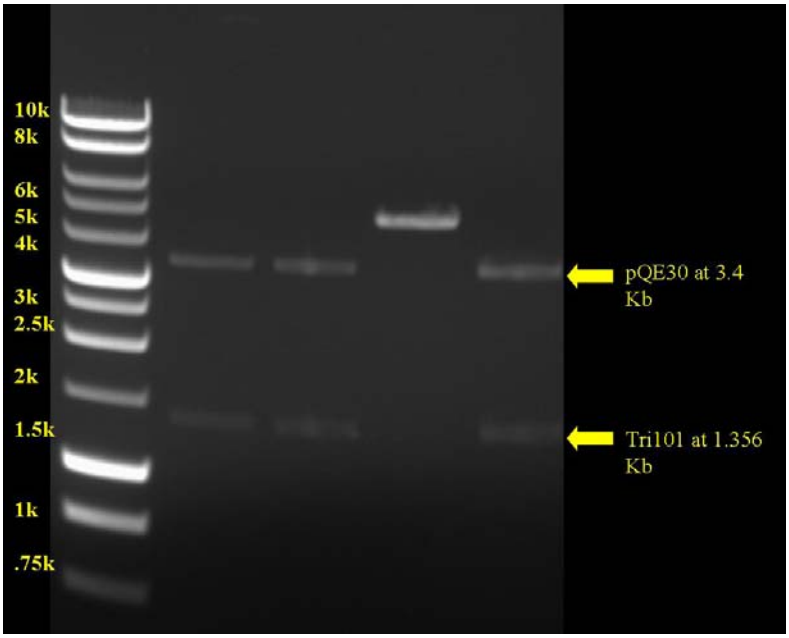

Figure 2.6 Agarose gel electrophoresis of digested pQE*tri101*. Lane 1 contains the standard marker. Lanes 2, 3, 4, and 5 contains Hind III and Bam HI digested plasmids corresponding to lanes 2, 5, 9, and 14 respectively from Figure 2.3. Note the transformant in lane 4 did not cut successfully.

Expression and Purification of tri101

Expression

Once it was established that the *tri101* gene was of correct size and sequence, experiments were begun to express the gene. Samples were collected at 0, 1, 2, 3, and 4 hours post induction with IPTG. The expected size of rTri101 was 49,600 kilodaltons (kDa; Kimura *et al*, 1998a). A protein band of the correct size was observed in the 2, 3, and 4 hour post induction samples (Figure 2.7

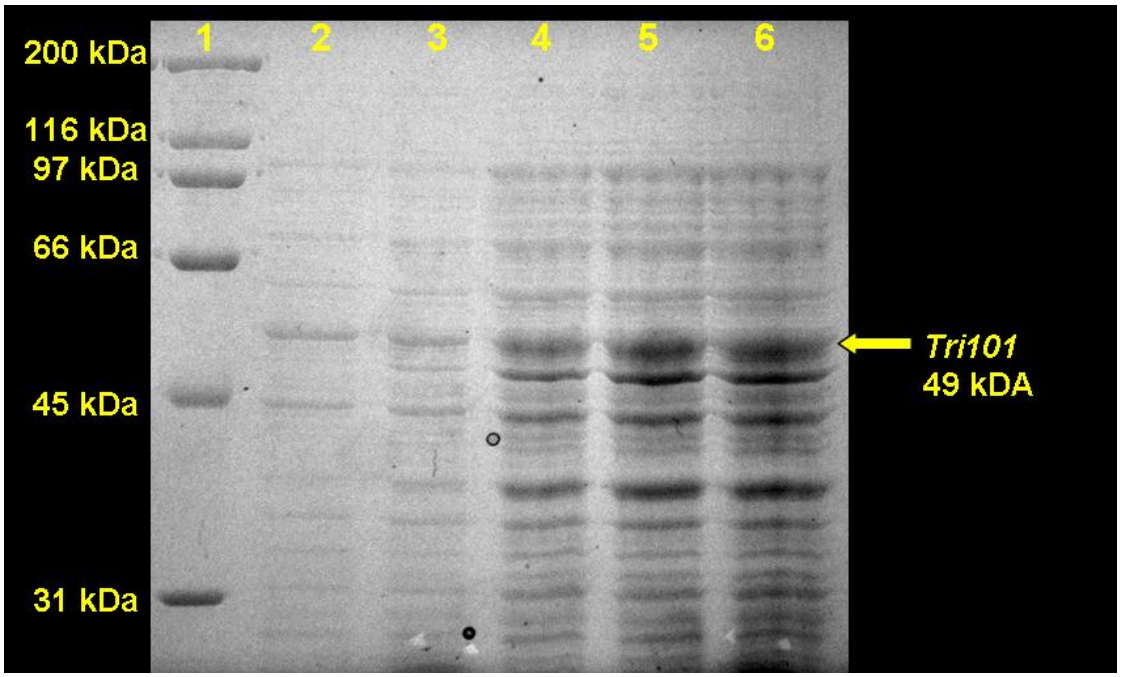

Figure 2.7 SDS PAGE results for rTri101 expression in construct JM109/pQE*tri101*. Lane 1- standard marker. Lane 2 - pre-IPTG-induced sample. Lanes 3, 4, 5, and 6- post-IPTG-induction at 1, 2, 3, and 4 hours post induction, respectively. Note that maximal induction occurred at greater than 2 hours.

Purification of rTri101 from inclusion bodies

Induction of the rTri101 in construct JM109/pQE*tri101* caused a large production of protein which was found in the insoluble or pelleted fraction (Figure 2.8). The location of rTri101 in the insoluble fraction was unexpected as Kimura and coworkers (1998a; 1998b; 2003) and Garvey and coworkers (2007) found rTri101 in the supernant or soluble fraction. Furthermore, Signal P sequence analysis did not reveal the presence of a signal peptide (Bendtsen*, et al., 2004)*. Additionally, the sequence of rTri101 did not contain any largely hydrophobic or transmembrane regions as determined by TMHMM software (Krogh *et al*., 2001;Sonnhamer *et al*., 1998). Sequence analysis showed rTri101 was cytosolic and soluble despite its location within the pellet in this study. The fact that rTri101 was found in the pellet is explained by inclusion bodies (formed by overexpression of the gene and thus made heavy enough to pellet during centrifugation). Marston (1986) reported the formation of insoluble aggregates of protein, called inclusion bodies of during the production of recombinant proteins in *E. coli*. To solubilize the rTri101 protein induced by IPTG, a mild detergent was used. Empigen BB, a mild zwitterionic detergent, was used to break the inclusion bodies (Lowthert *et al*., 1995) and was successful as SDS PAGE showed proteins of the expected size following treatment with Empigen (Figure 2.9). BCA protein assay results indicated that the total protein concentration in the eluted fractions of rTri101 IB was 270 µg/mL (Table 2.1).

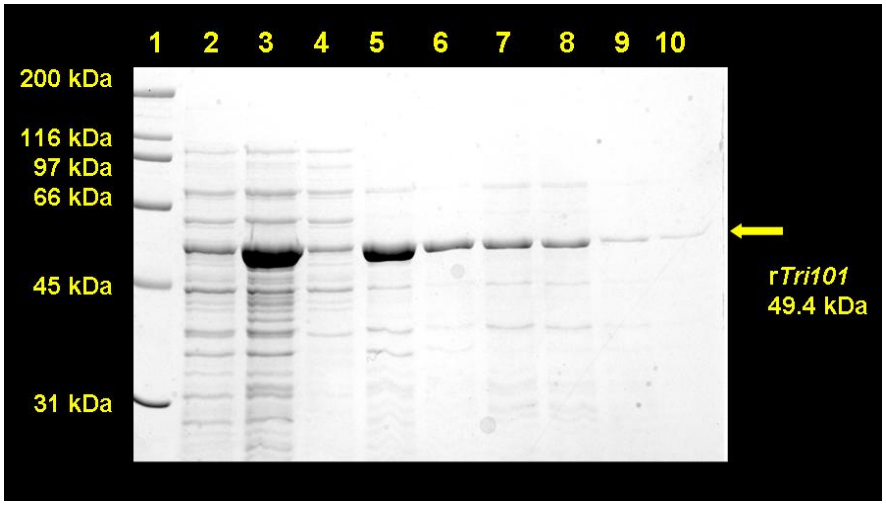

Figure 2.8 SDS PAGE gel analysis of induction, location, and wash fractions produced during purification of rTri101 IB. Lane 1- standard marker; Lane 2- pre-IPTG-induced sample; Lane 3- post-IPTG-induced sample; Lane 4- total proteins found within the supernant (soluble) fraction following sonication and centrifugation; Lane 5- total proteins found within the pellet (insoluble) fraction following sonication and centrifugation. Lanes 7 through 10- total proteins found in wash fractions 1 through 4 from the Ni-NTA column (devoid of rTri101).

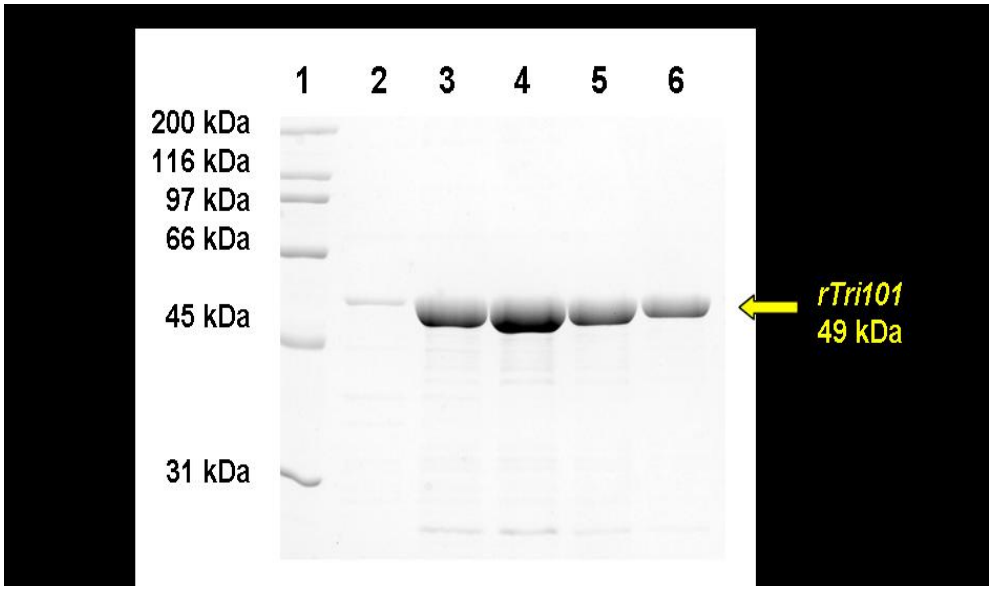

Figure 2.9 SDS PAGE of eluted rTri101 produced during purification of rTri101 IB. Lane 1- standard marker; Lanes 2 through 6- total proteins contained within the eluted fractions of rTri101 from the column.

Table 2. 1 Table 2.1 Total Protein Concentrations of rTri101 under differing purification schemes

Purification method	Concentration (µg/ml)
rTri101 purified from inclusion bodies	250 µg/ml
rTri101 purified from soluble fractions	270 µg/ml
Crude enzyme fraction rTri101	2044 µg/ml

Purification of rTri101 from soluble fractions

Recombinant Tri101 was purified from soluble fractions (rTri101 SF) which required induction with a lower concentration of IPTG (1 mM) for a shorter period of time (2 hour induction period) to both decrease the amount of protein expressed and to avoid formation of large quantities of inclusion bodies. The majority of rTri101 was found in supernant fraction which was similar to results reported by Kimura and coworkers (1998a, 1998b, 2003) and Garvey and coworkers (2007; Figure 2.10). SDS PAGE showed successful purification of rTri101 SF (Figure 2.11). The BCA protein assay results indicated that the total protein concentration in the eluted fractions of rTri101 SF was 250 µg/mL (Table 2.1).

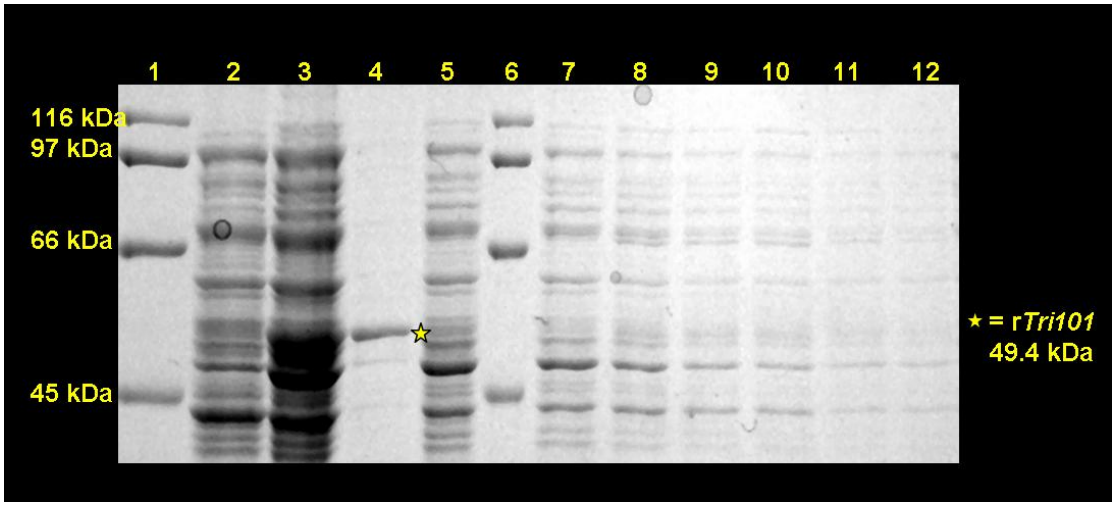

Figure 2.10 SDS PAGE analysis of induction, location, and wash fractions produced during purification of rTri101 SF. Lane 1- standard marker. Lane 2- total proteins from the pre-IPTG induced cells. Lane 3- total proteins from the post 1mM IPTG induced cells. Lane 4- total proteins found within the pellet fraction (containing rTri101) after sonication and centrifugation. Lane 5- total proteins found within the supernant fraction after sonication and centrifugation. Lane 6 contains the standard marker. Lane 7, 8, 9, 10, 11, & 12 contain total proteins from wash fractions of the column devoid of rTri101

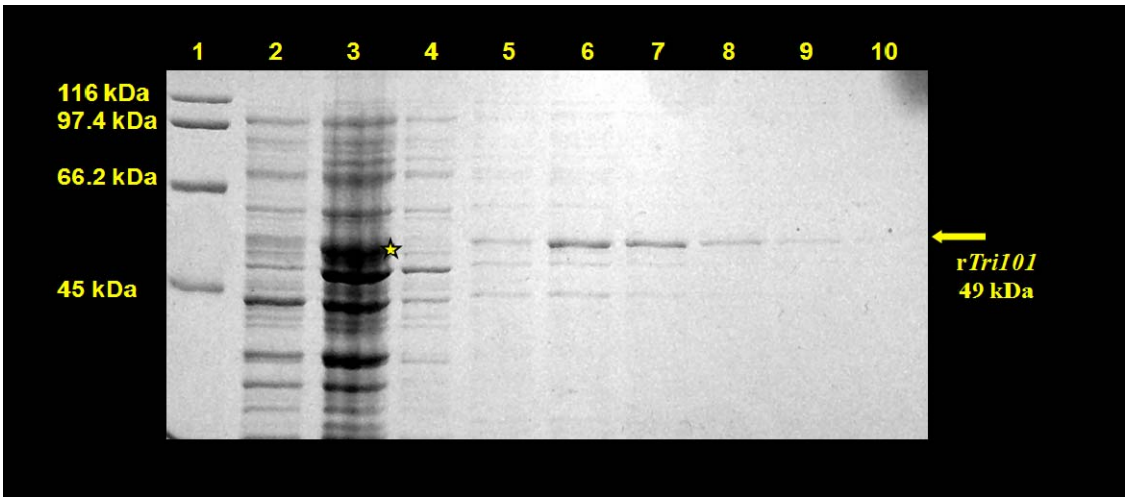

Figure 2.11 SDS PAGE of eluted rTri101 SF produced during purification. Lane 1- standard marker. Lane 2- total proteins from the pre-IPTG induced cells. Lane 3- total proteins from the post 1mM IPTG induced cells. Lane 4- total proteins from the flow through of the column, which is devoid of rTri101. Lanes 5, 6, 7, 8, 9, & 10- total proteins (including rTri101) from eluted fractions 1-7 respectively

Production of a Crude Enzyme Protein

A crude enzyme fraction of rTri101 was produced in order to confirm the work of Kimura and coworkers (1998a), who were able to show protective effects of rTri101 against T-2 toxin in an *in vitro* system using yeast cells. For the production of a crude enzyme fraction, JM109 cells containing pQETri101 was induced for 2 hours with 1 mM IPTG. SDS PAGE results indicated the protein was effectively induced, and located within the supernant (Figure 2.12). The location of rTri101 in the supernant (soluble) agreed with results previously reported Kimura and coworkers (1998a; 1998b; 2003) and Garvey and coworkers (2007). The total protein concentration of the crude enzyme protein

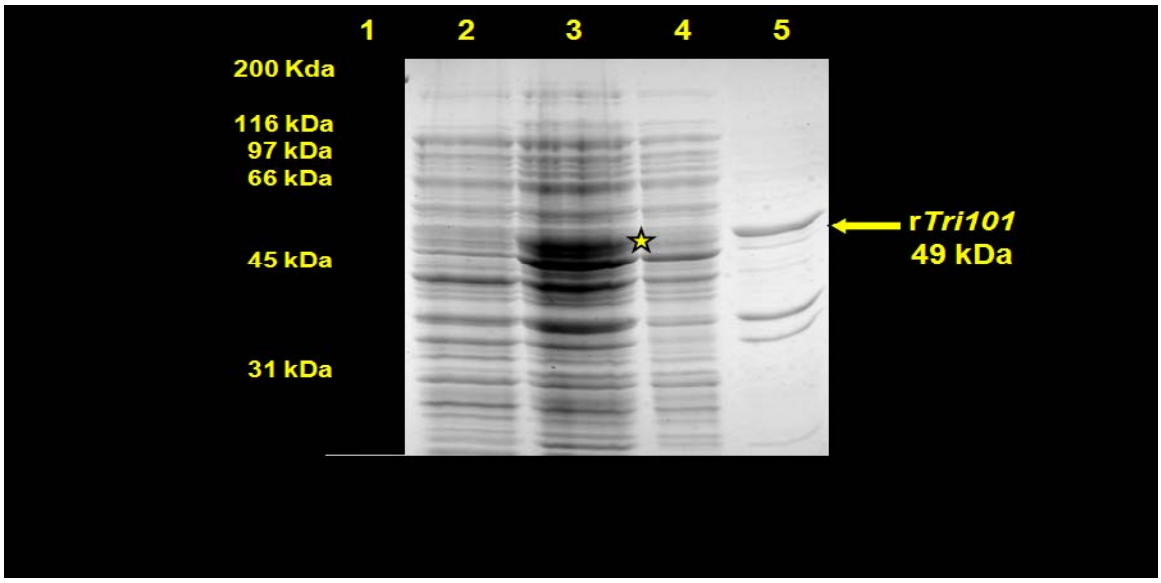

Figure 2.12 SDS PAGE of the crude enzyme fraction of rTri101. Lane 1- standard marker. Lane 2- total proteins from the pre-IPTG induced cells. Lane 3 total proteins from 2 hours post IPTG induction of cells. Lane 4- total proteins found within the supernant after sonication and centrifugation, and is devoid of rTri101 Lane 5- total proteins found within the pellet fraction after sonication and centrifugation, and contains rTri101.

was 2 mg/ml. As expected, this is much higher than either rTri101 IB or SF isolates because the crude enzyme fraction contained other cellular proteins (Table 2.1).

Conclusion

Agarose gel electrophoresis confirmed the amplification and ligation of the *tri101* into expression vector pQE30. Successful expression and purification of rTri101 was achieved from inclusion bodies, soluble fractions and crude extraction methods. Although the location of rTri101 IB was not as expected; SDS PAGE analysis of rTri101SF and crude rTri101 showed it to be in the soluble fraction and agrees with results by Kimura and coworkers (1998a; 1998b; 2003) and Garvey and coworkers (2007). The following *in vitro* experiments use each form of rTri101.

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References

Albert, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). Molecular Biology of the Cell. Garland Publishing.

Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340, 783-795.

Brown, D. W., Proctor, R. H., Dyer, R. B., and Plattner, R. D. (2003). Characterization of a fusarium 2-gene cluster involved in trichothecene C-8 modification. J Agric Food Chem 51, 7936-7944.

Burmeister, H. R., and Hesseltine, C. W. (1970). Biological assays for two mycotoxins produced by Fusarium tricinctum. Appl Microbiol 20, 437-440.

CAST (2003). Mycotoxins: Risk in Plant and Animal Systems. (A. Council of Agricultural Science and Technology, IA, Ed.).

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., and Shimoda, W. (1977a). Subacute toxicity of T-2 toxin in broiler chicks. Poult Sci 56, 306-313.

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., Shimoda, W., and Burmeister, H. R. (1977b). Acute toxicity of T-2 toxin in broiler chicks and laying hens. Poult Sci 56, 103-116.

Cundliffe, E. (1989). How antibiotic-producing organisms avoid suicide. Annual review of Microbiology 43, 207-233.

Joffe, A. Z. (1983). Environmental conditions conducive to Fusarium toxin formation causing serious outbreaks in animals and man. Vet Res Commun 7, 187-193.

Johnson, P. H., and Grossman, L. I. (1977). Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. Biochemistry 16, 4217-4225.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., and Yamaguchi, I. (1998a). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273, 1654-1661.

Kimura, M., Matsumoto, G., Shingu, Y., Yoneyama, K., and Yamaguchi, I. (1998b). The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around Tri101 and characterization of its homologue from Fusarium sporotrichioides. FEBS Lett 435, 163-168.

Kimura, M., Tokai, T., Matsumoto, G., Fujimura, M., Hamamoto, H., Yoneyama, K., Shibata, T., and Yamaguchi, I. (2003). Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. Genetics 163, 677-684.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305, 567-580.

Leeson, S., Diaz, G., and Summers, J. D. (1995). Poultry metabolic disorders and mycotoxins. University Books, Guelph, Ontario, Canada.

Lowthert, L. A., Ku, N. O., Liao, J., Coulombe, P. A., and Omary, M. B. (1995). Empigen BB: a useful detergent for solubilization and biochemical analysis of keratins. Biochem Biophys Res Commun 206, 370-379.

Manoharan, M., Dahleen, L. S., Hohn, T. M., Neate, S. M., Yu, X. H., Alexander, N. J., McCormick, S. P., Bregitzer, P., SchwarZ, P. B., and Horsley, R. D. (2006). Expression of 3-OH trichothecene acetyltransferase in barley (Hordeum vulgare L.) and effects on deoxynivalenol. Plant Science 171, 699-706.

Marston, F. A. (1986). The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem J 240, 1-12.

Mayer, C. F. (1953). Endemic panmyelotoxicosis in the Russian grain belt. I. The clinical aspects of alimentary toxic aleukia (Kimura *et al.*); a comprehensive review. Mil Surg 113, 173-189.

McCormick, S. P., Alexander, N. J., Trapp, S. E., and Hohn, T. M. (1999). Disruption of TRI101, the gene encoding trichothecene 3-O-acetyltransferase, from Fusarium sporotrichioides. Appl Environ Microbiol 65, 5252-5256.

McLaughlin, C. S., Vaughan, M. H., Jr., Cambell, M. H., Wei, C. M., Stafford, M. E., and Hansen, B. S. (1977). Inhibition of Protein Synthesis by trichothecenes. Pathotox Publishers, Inc., Park Forest South, Illinois.

Muhitch, M. J., McCormick, S. P., Alexander, N. J., and Hohn, T. M. (2000). Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Science 157, 201-207.

Nelson, D. L., and Cox, M. M. (2005). Principles of Biochemistry. W. H. Freeman and Company, New York, NY.

Ohsato, S., Ochiai-Fukuda, T., Nishiuchi, T., Takahashi-Ando, N., Koizumi, S., Hamamoto, H., Kudo, T., Yamaguchi, I., and Kimura, M. (2007). Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the Fusarium phytotoxin deoxynivalenol. Plant Cell Rep 26, 531-538.

Rosen, R. T., and Rosen, J. D. (1982). Presence of four Fusarium mycotoxins and synthetic material in 'yellow rain'. Evidence for the use of chemical weapons in Laos. Biomed Mass Spectrom 9, 443-450.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386.

Sambrook, J., and Russel, D. W. (2001). Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Smalley, E. B. (1973). T-2 toxin. J Am Vet Med Assoc 163, 1278-1281.

Sonnhammer Erik L.L, Heijne Gunnar von, and Anders, K. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology. A hidden Markov model for predicting transmembrane helices in protein sequences., pp. 175-182.

Thorne, H. V. (1966). Electrophoretic separation of polyoma virus DNA from host cell DNA. Virology 29, 234-239.

Thorne, H. V. (1967). Electrophoretic characterization and fractionation of polyoma virus DNA. J Mol Biol 24, 203-211.

3. IN VITRO STUDIES TO DETERMINE BIOLOGICAL ACTIVITY OF RECOMBINANT TRI101 SUPPLEMENTED WITH ACETYL COA ON HD11 CELLS CHALLENGED WITH T-2 TOXIN

Abstract

Experiments were conducted to test the protective effects of 3-O-acetyltransferase, the gene product of *tri101*, against challenge with T-2 toxin *in vitro*. Three-O-acetyltransferase was expressed and purified from inclusion bodies (rTri101 IB), soluble fractions (rTri101 SF), and in a crude enzyme fraction (crude rTri101). HD11 chicken macrophage cells were incubated with each isolate of rTri101 and challenged with T-2 toxin. Number of live cells per treatment were expressed as a percent of controls, set to 100%. Greatest protective effects of rTri101 were observed from isolates purified from soluble fractions. At 1nM rTri101 SF, complete protection was observed against 0.1 ppm (0.2 µM) T-2 toxin (99% rTri101 SF and T-2 toxin verses 54% T-2 toxin alone; $p<0.05$). At 2 nM rTri101 SF, partial protection was observed against 0.5 ppm (1 µM) T-2 toxin (83% rTri101 SF and T-2 toxin verses 46% T-2 toxin alone; $p<0.05$). Protective effects of rTri101 SF were limited with higher amounts of T-2 toxin, suggesting a threshold for protection. Further studies will be conducted to test for protective effects of rTri101 SF *in vivo*.

Introduction

T-2 toxin, a trichothecene mycotoxin grows optimally in cold, wet climates (Joffe, 1983). During WWII, the population of an entire region of Russia was nearly destroyed due to Alimentary Toxic Aleukia, a disease caused by T-2 toxin contamination of grain (Mayer, 1953). A more recent study reported undisclosed

amounts of T-2 toxin were found in 20% of 3490 wheat, barley, oat, rye, and corn samples from the European Union (Schothorst and Egmond, 2004). Similar to humans, health problems occurred when contaminated grains were fed to livestock. In particular, poultry exhibited sensitivity to T-2 toxin, and resulted in physical signs such as oral lesions, neural abnormalities, altered feathering, decreased weight gain, feed refusal, decreased egg production, and lethargy (CAST, 2003, Wyatt *et al*., 1972; Wyatt *et al.*, 1973a; Wyatt *et al.*, 1973b; Wyatt *et al*., 1975a; Wyatt *et al*., 1975b; Chi *et al*., 1977a; Chi *et al.*, 1977b: Chi *et al.*, 1977c). T-2 toxin caused immunosuppression rendering birds more susceptible to disease (Corrier, 1991). Poultry who show physical signs of sickness such as oral lesions cannot be slaughtered as described by the Poultry Products Inspection Act (FSIS, 2004), and thus increase monetary losses to producers.

Thompson and Wannemacher (1986) observed that addition of an acetyl group at C3 of T-2 toxin resulted in a 5 to 100 fold reduction in toxicity. Kimura and coworkers (1998a) reported *tri101* (trichothecene 3-0-acetyltransferase), found in *Fusarium graminearum*, was involved in self protection. *Tri101* functioned by adding an acetyl group to the C3 position on T-2 toxin, forming 3-O-acetyl T-2 toxin. Since then, *tri101* has been successfully inserted into transgenic rice, tobacco, and barley plants (Muhitch *et al*., 2000; Manoharan *et al*., 2006; Ohsato *et al*., 2007). In each of these studies, *tri101* was able to confer resistance to the plant against trichothecene challenge in laboratory studies. However, this resistance has yet to be shown in field studies.

The objective of this study was to investigate the ability of the purified gene product of *tri101* in an *in vitro* system for protective effects against T-2 toxin. As discussed in chapter 2, rTri101 was purified under native and denaturing conditions. A third semi purified crude enzyme fraction was also produced. Each method of purification was examined to determine optimal conditions to maintain biological activity of rTri101. Experiments were conducted with HD11 chicken macrophage cells to:

1. Determine the dose of T-2 toxin which caused 50% death of HD11 cells.
2. Test acetyl CoA
    A. Determine if acetyl CoA was toxic
    B. Determine if acetyl CoA conferred protection against T-2 toxin *in vitro*
3. Test rTri101 purified from inclusion bodies (rTri101 IB)
    A. Determine if rTri101 (IB) was toxic to HD11 cells.
    B. Determine if rTri101 IB conferred protection against T-2 toxin *in vitro* when supplemented with acetyl CoA.
4. Test rTri101 SF
    A. Determine if rTri101 SF was toxic to HD11 cells.
    B. Determine if rTri101 SF conferred protection against T-2 toxin *in vitro* when supplemented with acetyl CoA.
5. Test Crude enzyme fraction (CEP) of rTri101

A. Determine if CEP rTri101 was toxic to HD11 cells.
B. Determine if crude enzyme fraction of rTri101 conferred protection against T-2 toxin *in vitro* when supplemented with acetyl CoA.

Materials and Methods

Maintenance of the HD11 cell line

HD11 chicken macrophage cells (Beug *et al*., 1979) were maintained in RPMI 1640 media (Sigma R4130, St Louis, MO) supplemented with 5% fetal bovine serum (FBS; Invitrogen 26170-043, Carlsbad, CA) and 1% antibiotics (Sigma P4083, St Louis, MO; final concentration 50units/ml penicillin, 0.05mg/ml streptomycin, and 0.1mg/ml neomycin; hereafter called complete media). Cells were grown to confluency in a Baxter Ultra Tech incubator at 39ºC with 95% air and 5% CO_2 using Corning 25cm^2 tissue culture flasks (Fisher 1012630, Atlanta, GA). At confluency, cells were manually scraped using a cell scraper (Fisher 087732, Atlanta GA) and transferred to new flasks.

In vitro protocol

For each of the 5 experiments, the following protocol was followed. Cells near confluency were scraped, pelleted by centrifugation at 50 x g for 2 minutes, and resuspended in 2 ml of complete media. Cell concentration was calculated by trypan blue exclusion (Sigma T6146, St Louis, MO) using a 10:1 dilution of cell suspension to trypan blue. One hundred and fifty thousand cells were plated

per well into Falcon 48 well plates (0.75 cm2; Beckman Dickinson 3078, Lincoln Park, NJ) using 750 µL complete media. HD11 cells were allowed to attach for 24 hours, after which, media was removed and replaced with treatments as described below. In all experiments, cells were incubated with treatments for 18 hours. Following the 18 hour incubation, cells were manually scraped from each individual well using a 1000 µl pipette tip, pelleted by centrifugation at 14,000 rpm (16,000 x g) for 18 seconds in a Eppendorf Microcentrifuge 5415C (Fisher 05-406-2 Atlanta, GA), resuspended in 500 µL warmed (37ºC) phosphate buffered saline (PBS; Invitrogen 21600, Carlsbad, CA), repelleted by centrifugation at 14,000 rpm (16,000 x g) for 18 seconds in a Eppendorf Centrifuge 5415C (Fisher 05-406-2 Atlanta, GA), and resuspended in either 100 or 125 µL of PBS. Cells were immediately counted using trypan blue exclusion at 200x magnification using a bright field Nikon Eclipse E800 microscope (Marietta, GA). Each experiment consisted of 3 plates containing 3 replicates of each treatment per plate (except Experiments 3A and 5A which only consisted of 1 plate containing 2 replicates of each).

Experiment 1. Determine the dose of T-2 toxin which caused 50% death of HD11 cells

A. HD11 cells were plated at a density of 150,000 cells per well and challenged with:

1. 0 pppm T-2 toxin (Sigma 4887, St Louis, MO) in complete media
2. 0.1 pppm T-2 toxin in complete media

3. 0.5 pppm T-2 toxin in complete media
4. 1 pppm T-2 toxin in complete media

Experiment 2- Test acetyl CoA

A. Determine if acetyl CoA was toxic to HD11 cells

HD11 cells were plated at a density of 150,000 cells per well and treated with:

1. 0 µM acetyl CoA (Sigma P2056, St Louis, MO) in complete media
2. 5 µM acetyl CoA in complete media
3. 10 µM acetyl CoA in complete media
4. 25 µM acetyl CoA in complete media

B. Determine if acetyl CoA conferred any protective effects against T-2 toxin *in vitro*. Four experiments were conducted to test increasing doses of acetyl CoA for the protective effects against T-2 toxin. HD11 cells were plated at a density of 150,000 cells per well and treated with:

Test I- 5µM acetyl CoA verses 0.1 ppm T-2 toxin

1. Complete media
2. 5 µM acetyl CoA in complete media
3. 5 µM acetyl CoA and 0.1 ppm (0.2 µM) T-2 toxin in complete media
4. 0.1 ppm (0.2 µM) T-2 toxin in complete media

Test II 10 µM acetyl CoA verses 0.5 ppm (1 µM) T-2 toxin

1. Complete media
2. 10 µM acetyl CoA in complete media
3. 10 µM acetyl CoA and 0.5 ppm (1 µM) T-2 toxin in complete media
4. 0.5 ppm (1 µM) T-2 toxin in complete media

Test III 25 µM acetyl CoA verses 1 ppm (2 µM) T-2 toxin

1. Complete media
2. 25 µM acetyl CoA in complete media
3. 25 µM acetyl CoA and 1 ppm (2 µM) T-2 toxin in complete media
4. 1 ppm (2 µM) T-2 toxin in complete media

Experiment 3- Test rTri101 IB

A. Determine if rTri101 IB was toxic to HD11 cells.

HD11 cells were plated at 150,000 cells per well and challenged with:

1. Complete media
2. 1 nM rTri101 IB in complete media
3. 10 nM rTri101 IB in complete media
4. 100 nM rTri101 IB in complete media

B. Determine if rTri101 IB supplemented with acetyl CoA conferred any protective effects against T-2 toxin *in vitro*.

Results from experiment 3A showed that 1nM rTri101 IB was not toxic. HD11 cells were plated at a density of 150,000 cells per well and challenged with**:**

1. Complete media
2. 1 nM rTri101 IB plus 5µM acetyl CoA in complete media
3. 1 nM rTri101 IB, 5µM acetyl CoA, and 0.1 ppm (0.2 µM) T-2 toxin in complete media
4. 0.1 ppm (0.2 µM) T-2 toxin in complete media

Experiment 4- Test rTri101 SF

A. Determine if rTri101 SF was toxic to HD11 cells

HD11 cells were plated at 150,000 cells per well and challenged with:

1. Complete media
2. 1 nM rTri101 SF and 5 μM acetyl CoA, in complete media
3. 2 nM rTri101 SF and 10 μM acetyl CoA in complete media
4. 5 nM rTri101 SF and 25 μM acetyl CoA in complete media

B. Determine if rTri101 SF conferred protection against T-2 toxin *in vitro* when supplemented with acetyl CoA

Test I. 1 nM rTri101 SF and 5 μM acetyl CoA verses 0.1 ppm (0.2 μM) T-2 toxin

1. Complete media
2. 1 nM rTri101 SF plus 5μM acetyl CoA in complete media
3. 1 nM rTri101 SF, 5μM acetyl CoA, and 0.1 ppm (0.2 μM) T-2 toxin in complete media
4. 0.1 ppm (0.2 μM) T-2 toxin in complete media

Upon finding protective effects of rTri101 SF, higher levels of rTri101 SF, acetyl CoA, and T-2 toxin were examined.

Test II. 2 nM rTri101 SF and 10 μM acetyl CoA verses 0.5 ppm (1μM) T-2 toxin

1. Complete media
2. 2 nM rTri101 SF plus 10 μM acetyl CoA in complete media
3. 2 nM rTri101 SF, 10 μM acetyl CoA and 0.5 ppm (1 μM) T-2 toxin in complete media

4. 0.5 ppm (1 μM) T-2 toxin in complete media

Test III. 1 nM rTri101 SF and 5 μM acetyl CoA verses 1 ppm (2 μM) T-2 toxin

1. Complete media
2. 1 nM rTri101 SF plus 5μM acetyl CoA in complete media
3. 1 nM rTri101 SF, 5μM acetyl CoA, and 1 ppm (2 μM) T-2 toxin in complete media
4. 1 ppm (2 μM) T-2 toxin in complete media

Test IV. 5 nM rTri101 SF and 25 μM acetyl CoA verses 1 ppm (2 μM) T-2 toxin

1. Complete media
2. 5 nM rTri101 SF plus 25 μM acetyl CoA in complete media
3. 5 nM rTri101 SF, 25 μM acetyl CoA, and 1 ppm (2 μM) T-2 toxin in complete media
4. 1 ppm (2 μM) T-2 toxin in complete media

Experiment 5- Test crude rTri101

A. Determine if crude enzyme fraction of rTri101 was toxic to HD11 cells. HD11 cells were plated at 150,000 cells per well and challenged with:

1. Complete media
2. 0.2 μg/ml crude enzyme rTri101 in complete media
3. 2 μg/ml crude enzyme rTri101 in complete media
4. 20 μg/ml crude enzyme rTri101 in complete media

B. Determine if crude rTri101 conferred protection against T-2 toxin *in vitro* when supplemented with acetyl CoA. HD11 cells were plated at a density of 150,000 cells per well and challenged with:

1. Complete media
2. 0.2 µg/ml crude enzyme rTri101 plus 5 µM acetyl CoA in complete media
3. 0.2 µg/ml crude enzyme rTri101, 5 µM acetyl CoA, and 0.1 ppm (0.2 µM) T-2 toxin in complete media
4. 0.1 ppm (0.2 µM) T-2 toxin in complete media

Statistical analysis

Data were analyzed using the MIXED procedure of SAS (SAS, 2006) using a Randomized Complete Block design. Data were blocked on individual cell culture plate (random effect). Cell treatments were declared as fixed effects and the dependent variable was the number of live cells per treatment expressed as a percent of live control cells set at 100%. Treatment replicates nested within plate were declared a random effect. Differences between means were determined using Fisher's Least Significant Difference test. Significance was declared at $p<0.05$.

Results and Discussion

Experiment 1 Determine the dose of T-2 toxin which caused 50% death of HD11 cells

Incubation of HD11 cells for 18 hours in the presence of 0.1 ppm (0.2 µM) T-2 toxin, resulted in a 50% reduction in live cells when compared to controls

(53% live verses 100% live respectively; $p<0.05$; Figure 3.1). In subsequent studies, 0.1 ppm T-2 toxin was used to test for protective effects against T-2 toxin as this amount was similar to environmental levels as reported by Gigeous (1999). Calvert and coworkers (2005) similarly reported a 50% survival rate of HEp-2 human epithelial cells following a 48 hour exposure to 200 ng/ml (0.2 ppm) T-2 toxin. Time and dose trials (data not shown) using 4 to 16 ppm T-2 toxin in Phosphate Buffered Saline (PBS) for 3 and 4 hour incubations yielded unreliable results. It was concluded 18 hour incubations using 0.1 (0.2 µM) to 1 ppm (2 µM) T-2 toxin in complete media gave most consistent and repeatable data for this study.

Experiment 2 Test acetyl CoA

A.) Acetyl CoA was not toxic to the HD11 cells at any dose tested (5 µM, 10 µM, and 25 µM; Figure 3.2). The toxicity of acetyl CoA had to be established because it has been shown to be necessary for activity of 3-O-acetyltransferase *in vitro* (Kimura *et al*., 1998a; Kimura *et al*., 1998b; Kimura *et al*., 2003). To convert T-2 toxin to 3-O-acetyl T-2 toxin, Kimura and coworkers (1998a) used an approximate ratio of 1:2 T-2 toxin to acetyl CoA (0.4 mM T-2 toxin; 0.8 mM acetyl CoA) using a crude enzyme rTri101 preparation from *Fusarium graminerium*. In other studies, they were able to convert 0.5 mM T-2 toxin to 3-O-acetyl T-2 toxin using 1 mM acetyl CoA and crude rTri101 (from *Fusarium sporotrichioides*; Kimura *et al*., 1998b). More recently, Kimura and coworkers (2003) used this same 1:2 ratio (0.5 mM T-2 toxin; 1 mM acetyl CoA) to convert a

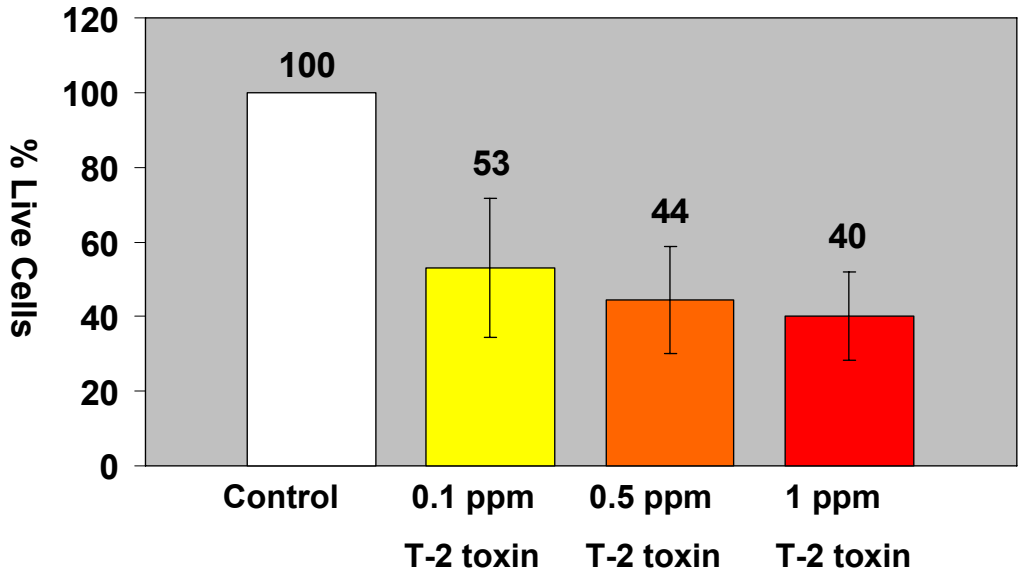

Figure 3.1 Percent live HD11 cells challenged with increasing doses of T-2 toxin. Results are expressed as percent live cells as a percent of control set to 100%.

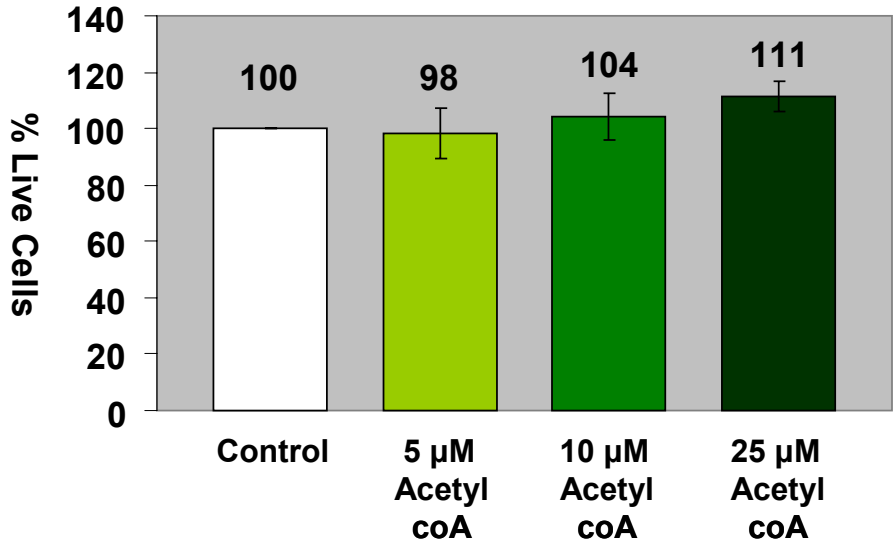

Figure 3.2 Percent live HD11cells challenged with increasing doses of acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%.

crude rTri101 preparation (from a *Fusarium* species that does not produce trichothecenes) to 3-O-acetyl T-2 toxin. These studies provided evidence that acetyl CoA was needed *in vitro* to convert T-2 toxin to the less toxic derivative, 3-O-acetyl T-2 toxin.

B.) Protective effects of acetyl CoA against challenge with T-2 toxin were not found (Figure 3.3). Cell survival when cells were incubated with 0.1 to 1.0 ppm T-2 toxin and 5 to 25 µM acetyl CoA was not different from T-2 toxin treatments ($p>0.05$).

Experiment 3 Test rTri101 IB

A.) Recombinant Tri101 IB was purified using a mild detergent, Empigen BB (Lowhert *et al*., 1995). Preliminary results (data not shown) using 4 or 20 nM rTri101 IB with empigen yielded low livability (30% and 35% of control, respectively). It was suspected that Empigen BB had toxic effects on the HD11 cells, so a dose experiment was performed to determine Empigen BB's toxicity. Results (data not shown) indicated that empigen was highly toxic to HD11 chicken macrophage cells at 0.03%, 0.3% and 3% total volume. Therefore, Empigen BB was removed using Detergent-Out™ kit as described in Chapter 2. A dose curve of the detergent cleaned rTri101 IB showed it was not toxic at 1 nM concentration to HD11 cells (Figure 3.4).

B.) Incubation of HD11 cells in the presence of 1 nM rTri101 IB supplemented with 5µM acetyl CoA did not protect them from challenge with 0.1 ppm (0.2 µM) T-2 toxin (43% verses 100% live respectively; Figure 3.5).

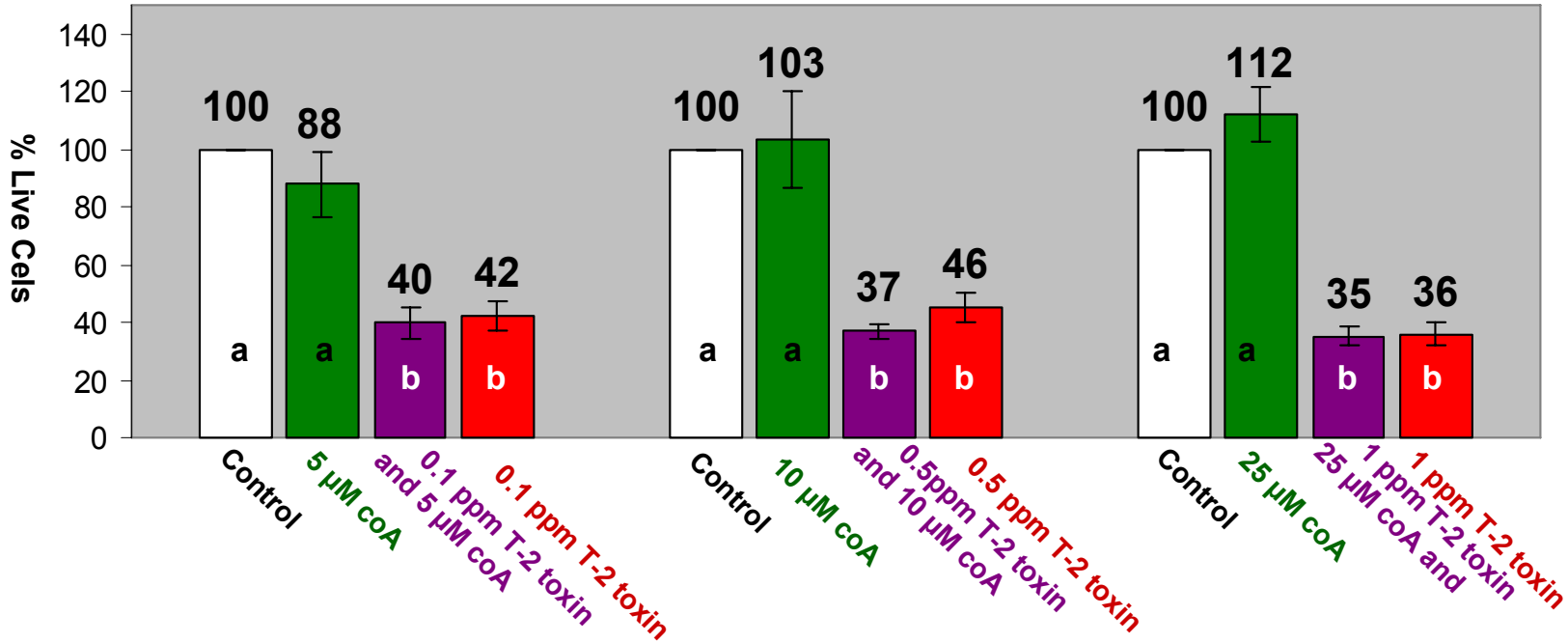

Figure 3.3 Percent live HD11cells challenged with increasing doses of T-2 toxin and acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

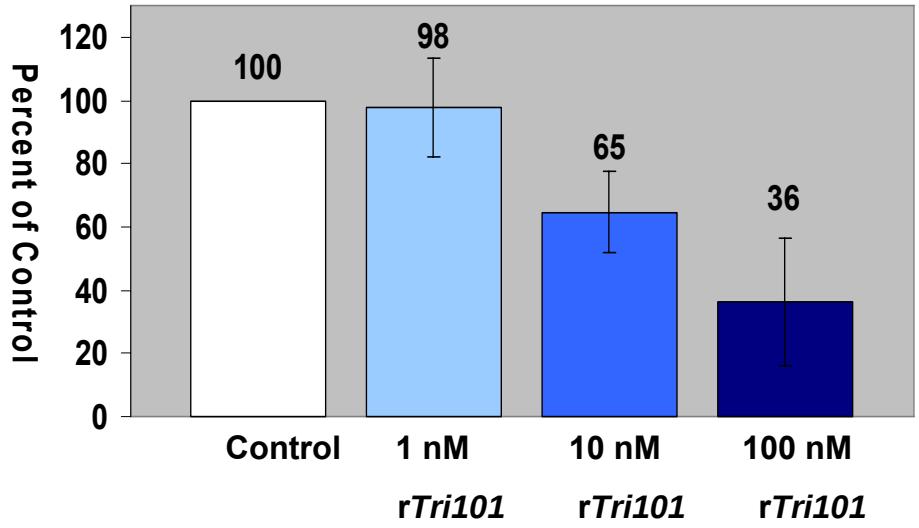

Figure 3.4 HD11 cells incubated with increasing dose of rTri101 IB, following the removal of Empigen BB. Results are expressed as percent live cells as a percent of control set to 100%.

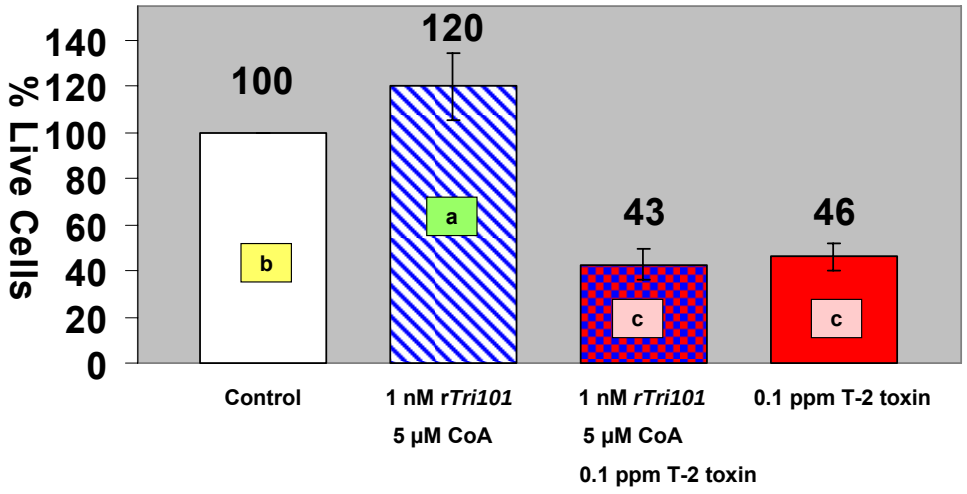

Figure 3.5 Percent live HD11 cells challenged with 0.2 µM T-2 toxin and supplemented with 1nM rTri101 IB plus 5 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

However, incubation of HD11 cells with both 1 nM rTri101 IB and 5 µM acetyl CoA did not prove to be toxic to cells (120% verses 100% live respectively; $p<0.05$). Empigen BB has been observed to be capable of solublizing proteins without altering function (Lowhert *et al*., 1995). However, during solubilization the detergent may have changed the structure, so it was no longer biologically active (Nelson & Cox, 2005). In studies reported elsewhere, T-2 toxin was converted to 3-0-acetyl T-2 toxin prior to *in vitro* studies by allowing a 1 hour incubation at 37ºC with crude rTri101 (Kimura, *et al*., 1998a). Following conversion of T-2 toxin to 3-O-acetyl T-2 toxin, both were applied to baby hamster kidney (BHK) cells and to rabbit reticulocytes in an *in vitro* translation system. The 3-O-acetyl derivative was less toxic as measured by protein synthesis inhibition for both experiments. In this study rTri101, acetyl CoA, and T-2 toxin prepared in RPMI 1640 complete media were immediately applied to HD11 cells, and incubated for 18 hours at 39ºC. Possibly, treatments should have been incubated an hour prior to application to HD11 cells for optimal protection, and to allow for the acetylation of T-2 toxin. Otherwise, the BCAprotein assay described in Chapter 2 was performed prior to removal of the detergent. During the process of Empigen removal, it was possible that the concentration of rTri101 IB was reduced, resulting in inadequate administration of rTri101 IB to the HD11 cells when challenged with T-2 toxin.

Experiment 4 Test rTri101 SF

A.) HD11 cells were incubated with increasing doses of rTri101 SF. At concentrations of 2 mM or less, rTri101 SF did not have a cytotoxic effect (Figure 3.6).

B.) Viability of HD11 cells was protected against T-2 toxin when co-incubated with rTri101 SF supplemented with acetyl CoA. Percent live cells treated with 1 nM rTri101 SF plus 5 µM acetyl CoA and challenged with 0.1 ppm (0.2 µM; Test I) T-2 toxin was not different than control cells ($p > 0.05$), and was greater than those incubated with 0.1 ppm (0.2 µM) T-2 toxin alone ($p \leq 0.05$; Figure 3.7). Thus, unlike Experiment 3, 1 nM rTri101 SF supplemented with 5 µM acetyl CoA offered complete protection against 0.1 ppm (0.2 µM) T-2 toxin.

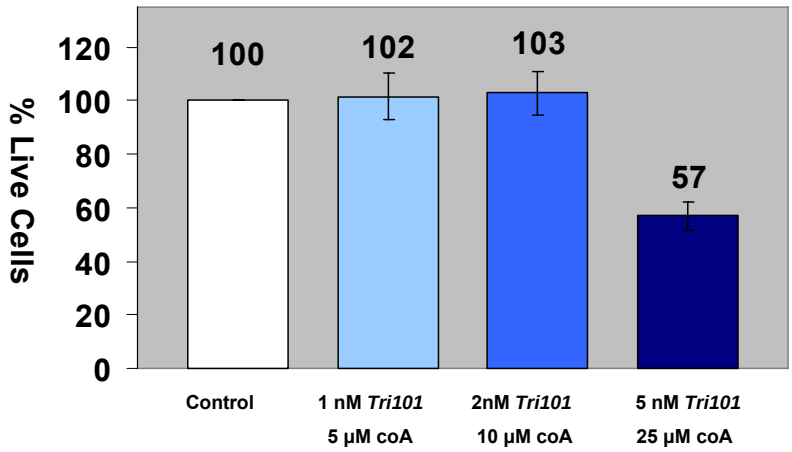

Figure 3.6 HD11 cells were incubated with increasing dose of rTri101 SF plus acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%.

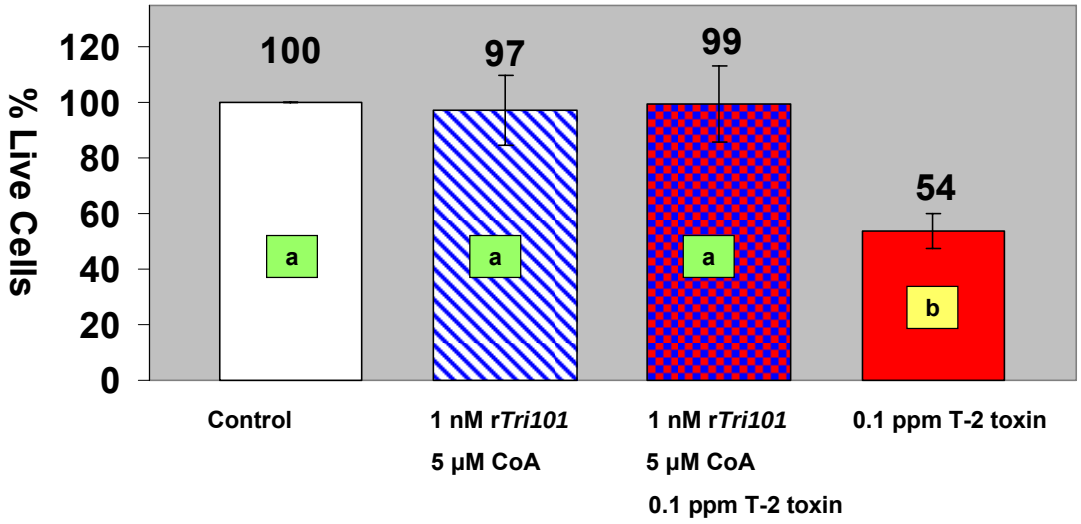

Figure 3.7 Percent live HD11 cells challenged with 1ppm (0.2 µM) T-2 toxin and supplemented with 1 nM rTri101 SF plus 5 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

At a higher challenge with 0.5 pppm T-2 toxin (Test II) partial protection was observed with 2 nM rTri101 SF supplemented with 10 µM acetyl CoA (Figure 3.8). Percent live cells challenged with 0.5 ppm (1 µM) T-2 toxin and co-incubated with 2 nM rTri101 and 10 µM acetyl CoA was less than controls (83% live verses 100% live, respectively; $p \leq 0.05$) but greater than cells challenged with 0.5 ppm (1 µM) T-2 toxin challenge alone (83% verses 46% live, respectively; $p \leq 0.05$). Results from this study are encouraging since T-2 toxin levels used in this study are similar to those found on Ames Plantation (Gigeous *et al*., 1999) and indicate rTri101 SF is biologically active.

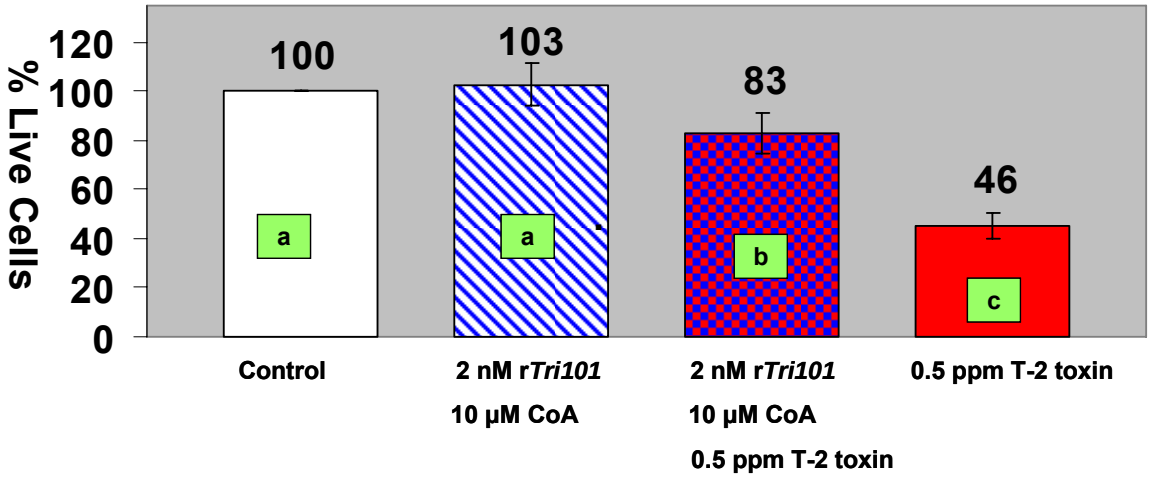

Figure 3.8 Percent live HD11 cells challenged with 0.5 ppm (1 µM) T-2 toxin and supplemented with 2nM rTri101 SF plus 10 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

In test III, HD11 cells were challenged with higher levels of T-2 toxin. Cells were challenged with 1 ppm (2 µM) T-2 toxin and supplemented with 1 nM rTri101 SF plus 5 µM acetyl CoA. At this level T-2 toxin (1 ppm), protective effects of rTri101 SF plus acetyl CoA supplementation (52% live) were not observed as compared to T-2 toxin alone (47% live; $p>0.05$; Figure 3.9). To determine if greater amounts of rTri101 SF were needed for protection at this level of T-2 toxin, the dose of rTri101 was increased 5 fold to 5 nM rTri101 SF plus a 5 fold increase in acetyl CoA, 25 µM (Test IV). When combined with 1 ppm (2 µM) T-2 toxin, 5 nM rTri101 SF plus 25 µM acetyl CoA had partial protective effects against T-2 (66% verses 36% live respectively; $p<0.05$; Figure 3.10). These results were expected because in Experiment 4A, 5 nM rTri101 SF

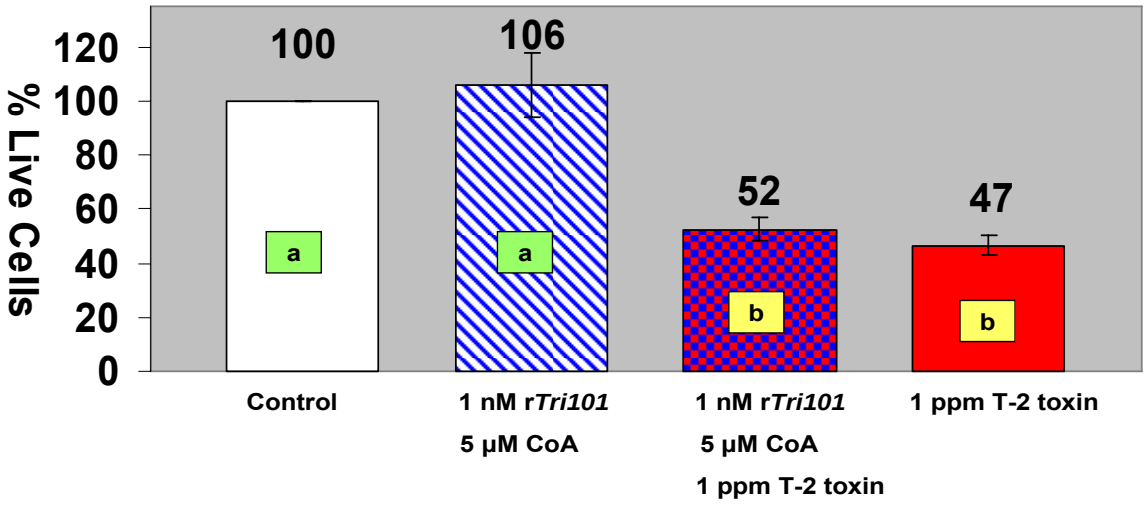

Figure 3.9 Percent live HD11 cells challenged with 1 ppm (2 µM) T-2 toxin and supplemented with 1 nM rTri101 SF plus 5 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$)

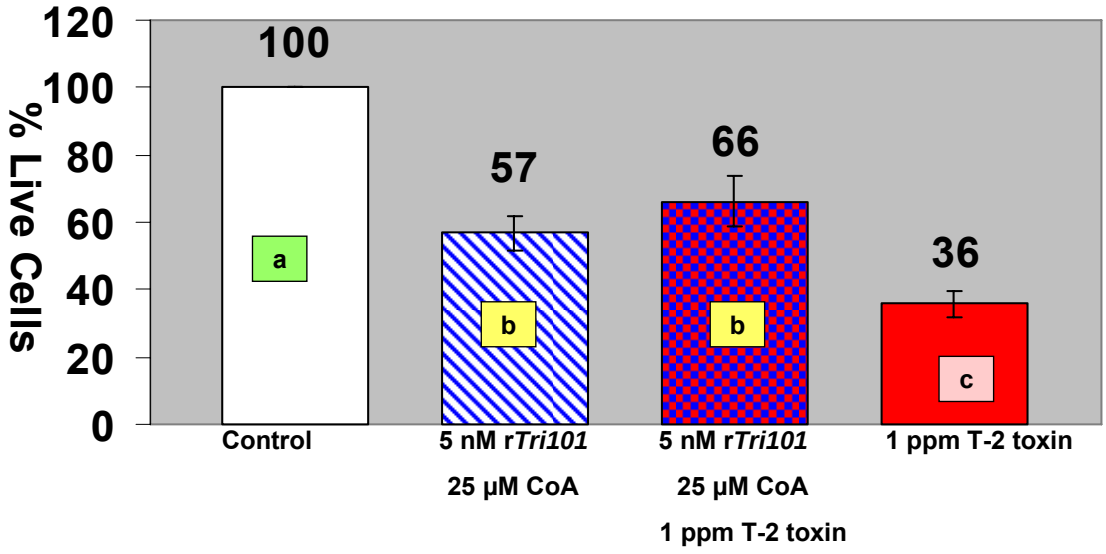

Figure 3.10 Percent live HD11 cells challenged with 1 ppm (2 µM) T-2 toxin and supplemented with 5 nM rTri101 SF plus 25 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

plus 25 µM acetyl CoA was toxic to cells (57% verses 100% live; $p<0.05$; Figure 3.6). Thus, a threshold must exist for rTri101 SF to confer protection against T-2 toxin on HD11 cells. Binding sites located on rTri101 for trichothecenes as described by Garvey and coworkers (2007) allow for hydrophobic bonding between rTri101 and trichothecenes during conversion to less toxic intermediates. Protective effects of rTri101 may be due to availability of binding sites and explain the lower level of protection observed in Test IV of this experiment.

Experiment 5 Test crude rTri101

A.) The final experiment sought to test the effects of a crude enzyme preparation of rTri101 against T-2 toxin, and to replicate Kimura and coworkers' (1998) experiment. Increasing doses of crude rTri101 were incubated with HD11 cells. At a total protein concentration of 0.2 µg/ml or less, the enzyme was not toxic (Figure 3.11). This was not representative of the amount of rTri101, as the crude preparation included multiple other proteins as was seen in Figure 2.11.

B.) Tests for protective effects of the crude rTri101 showed there was no protection against 0.1 ppm (0.2 µM) T-2 toxin (Figure 3.12). Cells treated with 0.2 µg/ml crude rTri101 plus 5µM acetyl CoA were not different than controls (83% verses 100% live respectively; $p\geq0.05$). However when cells were challenged with 0.1 ppm T-2 toxin and supplemented with 0.2 µg/ml crude

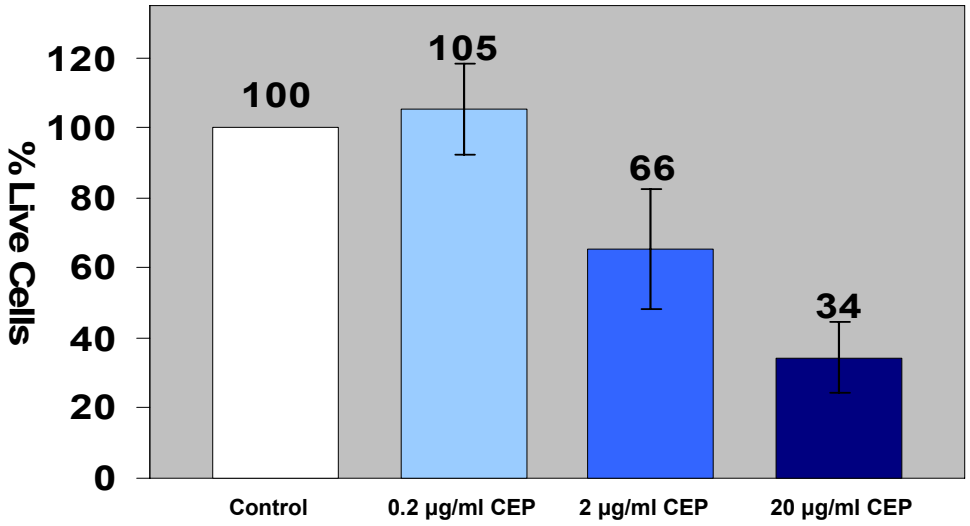

Figure 3.11 HD11 cells incubated with increasing doses of crude rTri101 Results are expressed as percent live cells as a percent of control set to 100%.

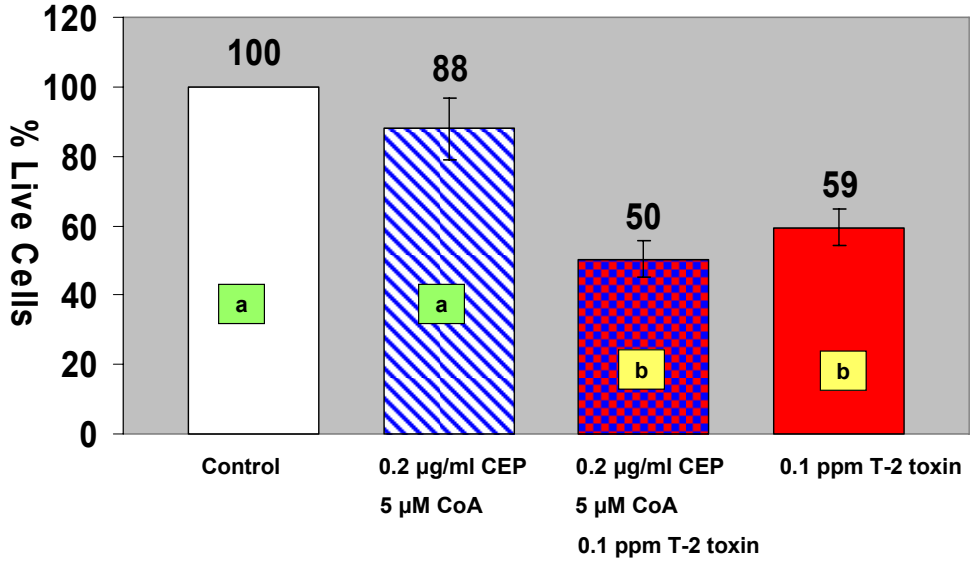

Figure 3.12 Percent live HD11 cells challenged with 0.1 ppm (0.2 µM) T-2 toxin and supplemented with 0.2 µg/ml crude rTri101 plus 5 µM acetyl CoA. Results are expressed as percent live cells as a percent of control set to 100%. Bars with uncommon letters are different ($p<0.05$).

rTri101 plus 5 µM acetyl CoA, no protection was observed, and percent live was not different than those challenged with T-2 toxin alone (50% verses 59% live respectively; $p>0.05$). Like other bacterial cells, JM109 cells used for the expression of rTri101 contain lipopolysaccharide (LPS; Amro *et al*., 2000). LPS are membrane proteins of gram negative bacterial cell walls which cause an immune response by up regulating the production of cytokines. LPS causes the activation of macrophage cells, and the production of nitric oxide which promotes antimicrobial properties of macrophages and also combines with superoxide anions to become more potent antimicrobial agents (Holst 1996; Goldsby, *et al*., 2003). LPS has been shown to up regulate nitric oxide peritoneal macrophages from C3H/He and C3H/HeJ mice and in rat Kupffer cells (Steuhr *et al*., 1985; Rietschel *et al*., 1996). In HD11 cells, following an initial endotoxin challenge, nitric oxide was induced, but in subsequent challenges, nitric oxide was no longer up regulated indicating a refractory period of endotoxin tolerance (Chang *et al*., 1996). Since the crude enzyme fraction in Experiment 5 was not purified, it contained many proteins, probably including LPS. Thus, when T-2 toxin was added to cells already activated by endotoxin, additional cell death occurred because HD11 cells unable to respond to any additional stress (Goldsby *et al*., 2003).

Conclusion

Results from these experiments indicate that biologically active rTri101 SF had significant protective effects against T-2 toxin *in vitro*. However,

trichothecene binding sites located on rTri101 may be a limiting factor in protection against higher amounts of T-2 toxin. Potentially, incorporation of the isolated gene product in poultry feeds may serve to protect animals against trichothecene poisoning. Further experiments using rTri101 will determine it's ability to provide protection against trichothecene mycotoxins *in vivo* and may prove of importance to the poultry industry.

References

Amro, N. A., Kotra, L. P., Wadu-Mesthrige, K., Bulychev, A., Mobashery, S., and Liu, G. Y. (2000). High-resolution atomic force microscopy studies of the Escherichia coli outer membrane: Structural basis for permeability. Langmuir 16, 2789-2796.

Beug, H., Vonkirchbach, A., Doderlein, G., Conscience, J. F., Graf, T., and Hr (1979). Chicken Hematopoietic Cells Transformed by 7 Strains of Defective Avian Leukemia Viruses Display 3 Distinct Phenotypes of Differentiation. Cell 18, 375-390.

Calvert, T. W., Aidoo, K. E., Candlish, A. G. G., Fuat, A. R. M., and Tu (2005). Comparison of *in vitro* cytotoxicity of Fusarium mycotoxins, deoxynivalenol, T-2 toxin and zearalenone on selected human epithelial cell lines. Mycopathologia 159, 413-419.

CAST (2003). Mycotoxins: Risk in Plant and Animal Systems. (A. Council of Agricultural Science and Technology, IA, Ed.).

Chang, C. C., McCormick, C. C., Lin, A. W., Dietert, R. R., and Sung, Y. J. (1996). Inhibition of nitric oxide synthase gene expression *in vivo* and *in vitro* by repeated doses of endotoxin. Am J Physiol 271, G539-548.

Chi, M. S., and Mirocha, C. J. (1978c). Necrotic oral lesions in chickens fed diacetoxyscirpenol, T-2 toxin, and crotocin. Poult Sci 57, 807-808.

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., and Shimoda, W. (1977a). Subacute toxicity of T-2 toxin in broiler chicks. Poult Sci 56, 306-313.

Chi, M. S., Mirocha, C. J., Kurtz, H. J., Weaver, G., Bates, F., Shimoda, W., and Burmeister, H. R. (1977b). Acute toxicity of T-2 toxin in broiler chicks and laying hens. Poult Sci 56, 103-116.

Chi, M. S., Mirocha, C. J., Kurtz, H. F., Weaver, G., Bates, F., and Shimoda, W. (1977c). Effects of T-2 toxin on reproductive performance and health of laying hens. Poult Sci 56, 628-637.

Corrier, D. E., and Gt (1991). Mycotoxicosis- Mechanisms of Immunosuppression. Vet. Immunol. Immunopathol. 30, 73-87.

FSIS, U. (2004). Poultry Products Inspection Act. Food Safety Inspection Service, United States Department of Agriculture.

Garvey, G. S., McCormick, S. P., and Rayment, I. (2007). Structural and functional characterization of the *TRI101* trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: Kinetic insights to combating Fusarium head blight. J Biol Chem.

Gigeous, J. L. (1999). Relationship of late winter precipitation and mycotoxin development in soybean seeds at Ames Plantation, pp. 71. University of Tennessee, Knoxville.

Goldsby, R. A., Kindt, T. J., Osborne, B. A., and Kuby, J. (2003). Cells and Organs of the Immune System. W. H. Freeman and Company, New York.

Holst, O., Ulmer, A. J., Brade, H., Flad, H. D., and Rietschel, E. T. (1996). Biochemistry and cell biology of bacterial endotoxins. FEMS Immunol Med Microbiol 16, 83-104.

Joffe, A. Z. (1983). Environmental conditions conducive to Fusarium toxin formation causing serious outbreaks in animals and man. Vet Res Commun 7, 187-193.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., and Yamaguchi, I. (1998a). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of *Tri101*. J Biol Chem 273, 1654-1661.

Kimura, M., Matsumoto, G., Shingu, Y., Yoneyama, K., and Yamaguchi, I. (1998b). The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around *Tri101* and characterization of its homologue from Fusarium sporotrichioides. FEBS Lett 435, 163-168.

Kimura, M., Tokai, T., Matsumoto, G., Fujimura, M., Hamamoto, H., Yoneyama, K., Shibata, T., and Yamaguchi, I. (2003). Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. Genetics 163, 677-684.

Lowthert, L. A., Ku, N. O., Liao, J., Coulombe, P. A., and Omary, M. B. (1995). Empigen BB: a useful detergent for solubilization and biochemical analysis of keratins. Biochem Biophys Res Commun 206, 370-379.

Manoharan, M., Dahleen, L. S., Hohn, T. M., Neate, S. M., Yu, X. H., Alexander, N. J., McCormick, S. P., Bregitzer, P., SchwarZ, P. B., and Horsley, R. D. (2006). Expression of 3-OH trichothecene acetyltransferase in barley (Hordeum vulgare L.) and effects on deoxynivalenol. Plant Science 171, 699-706.

Mayer, C. F. (1953). Endemic panmyelotoxicosis in the Russian grain belt. I. The clinical aspects of alimentary toxic aleukia (Kimura *et al.*); a comprehensive review. Mil Surg 113, 173-189.

Muhitch, M. J., McCormick, S. P., Alexander, N. J., and Hohn, T. M. (2000). Transgenic expression of the *TRI101* or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Science 157, 201-207.

Nelson, D. L., and Cox, M. L. (2005). The Three-Dimensional Structure of Proteins. W. H. Freeman and Company, New York.

Ohsato, S., Ochiai-Fukuda, T., Nishiuchi, T., Takahashi-Ando, N., Koizumi, S., Hamamoto, H., Kudo, T., Yamaguchi, I., and Kimura, M. (2007). Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the Fusarium phytotoxin deoxynivalenol. Plant Cell Rep 26, 531-538.

Rietschel, E. T., Brade, H., Holst, O., Brade, L., Muller-Loennies, S., Mamat, U., Zahringer, U., Beckmann, F., Seydel, U., Brandenburg, K., Ulmer, A. J., Mattern, T., Heine, H., Schletter, J., Loppnow, H., Schonbeck, U., Flad, H. D., Hauschildt, S., Schade, U. F., Di Padova, F., Kusumoto, S., and Schumann, R. R. (1996). Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. Curr Top Microbiol Immunol 216, 39-81.

SAS Institute Inc. (2006). SAS for Windows Service Pack 9.1.3, Cary, NC.

Schothorst, R. C., and van Egmond, H. P. (2004). Report from SCOOP task 3.2.10 "collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states". Subtask: trichothecenes. Toxicol Lett 153, 133-143.

Stuehr, D. J., and Marletta, M. A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci U S A 82, 7738-7742.

Thompson, W. L., and Wannemacher, R. W., Jr. (1986). Structure-function relationships of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison to whole animal lethality. Toxicon 24, 985-994.

Wyatt, R. D., Hamilton, P. B., and Burmeister, H. R. (1975a). Altered feathering of chicks caused by T-2 toxin. Poult Sci 54, 1042-1045.

Wyatt, R. D., Doerr, J. A., Hamilton, P. B., and Burmeister, H. R. (1975b). Egg production, shell thickness, and other physiological parameters of laying hens affected by T-2 toxin. Appl Microbiol 29, 641-645.

Wyatt, R. D., Colwell, W. M., Hamilton, P. B., and Burmeister, H. R. (1973a). Neural disturbances in chickens caused by dietary T-2 toxin. Appl Microbiol 26, 757-761.

Wyatt, R. D., Hamilton, P. B., and Burmeister, H. R. (1973b). The effects of T-2 toxin in broiler chickens. Poult Sci 52, 1853-1859.

Wyatt, R. D., Weeks, B. A., Hamilton, P. B., and Burmeister, H. R. (1972). Severe oral lesions in chickens caused by ingestion of dietary fusariotoxin T-2. Appl Microbiol 24, 251-257.

4. Conclusion

The goals of this study were to produce and isolate the *tri101* gene product, trichothecene-3-O-acetyltransferase (rTri101). Further, the aim was to determine if t*ri101* conferred protective effects against T-2 toxin on HD11 chicken macrophage cells. Experiments were performed in different chronological order than presented in this thesis. Recombinant Tri101 IB was produced first and *in vitro* experiments were conducted on it's *in vitro* protective effects. When rTri101 IB proved unsuccessful, crude rTri101 was produced similar to the protocol published by Kimura and coworkers (1998). Crude rTri101 was also observed not to confer protective effects *in vitro*. Lastly, rTri101 SF was produced. *In vitro* experiments determined that some levels of therTri101 SF conferred protective effects against T-2 toxin. The protective effects were greatest with the ratio of rTri101 to T-2 toxin was 1:200.

T-2 toxin interacts with peptidal transferase and exerts its detrimental effects by inhibiting protein, DNA, and RNA synthesis (Wei *et al.*, 1974). For this reason, T-2 toxin affects rapidly dividing cells such as those of the intestines, spleen, thymus, testes, and lymphatic system (Ueno, 1977). HD11 chicken macrophage cells were therefore well suited to test for protective effects against T-2 toxin. Poultry are sensitive to T-2 toxin. Symptoms of T-2 toxicosis in poultry include oral lesions, neural abnormalities, altered feathering, decreased body weight gain, and decreased egg production (Wyatt *et al*., 1972; Wyatt *et al.*, 1973a; Wyatt *et al.*, 1973b; Wyatt *et al*., 1975a; Wyatt *et al*., 1975b). Numerous

attempts have been made to alleviate the effects of T-2 toxin in chickens (Chowdhury and Smith, 2005; García *et al*, 2003; Kubena *et al.*, 1998; (Kubena *et al.*, 1990). However, none have been successful.

Tri101 may be the solution to the T-2 toxin problem. *Tri101* reduces the toxicity of T-2 toxin by inserting an acetyl group on C3. It was speculated the addition of this acetyl group changed the spatial arrangement of T-2 toxin's the most toxic component, the 12, 13 epoxytrichothecene ring (Grizzle, 2007; Bamburg *et al*., 1968). Therefore, T-2 toxin was rendered less toxic. The purification of rTri101 is only the first step in the long pathway to produce a feed additive or drug that may reduce the toxicity of T-2 toxin. Recombinant Tri101 protected HD11 cells against T-2 toxin. If rTri101 is successful *in vivo*, there are possibilities for its use as a therapeutic drug. Since the approval of the first recombinant protein therapeutic drug, Humulin, in 1982, 40 recombinant proteins have been approved for therapeutic use in the US (Reichert, 2003). In 2003, estimates quoted recombinant DNA technologies to represent over $32 billon dollars in the human medical biotechnology industry. This estimate is expected to grow larger by 2010 (Pavlou & Reichert, 2004). Recombinant Tri101 may ultimately serve as safe, non-invasive method to effectively control the detrimental effects of mycotoxins for humans and animals.

References

Bamburg, J. R., Riggs, N. V., and Strong, F. M. (1968). The structures of toxins from two strains of Fusarium tricinctum. Tetrahedron 24, 3329-3336.

Chowdhury, S. R., and Smith, T. K. (2005). Effects of feeding grains naturally contaminated with Fusarium mycotoxins on hepatic fractional protein synthesis rates of laying hens and the efficacy of a polymeric glucomannan mycotoxin adsorbent. Poult Sci 84, 1671-1674.

García, A. R., Avila, E., Rosiles, R., and Petrone, V. M. (2003). Evaluation of two mycotoxin binders to reduce toxicity of broiler diets containing ochratoxin A and T-2 toxin contaminated grain. Avian Dis 47, 691-699.

Grizzle, J. M. (2007). Professor, Department of Animal Science, University of Tennessee. (P. Communication, Ed.), Knoxville.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., and Yamaguchi, I. (1998). Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273, 1654-1661.

Kubena, L. F., Harvey, R. B., Bailey, R. H., Buckley, S. A., and Rottinghaus, G. E. (1998). Effects of a hydrated sodium calcium aluminosilicate (T-Bind) on mycotoxicosis in young broiler chickens. Poult Sci 77, 1502-1509.

Kubena, L. F., Harvey, R. B., Huff, W. E., Corrier, D. E., Phillips, T. D., and Rottinghaus, G. E. (1990). Efficacy of a hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and T-2 toxin. Poult Sci 69, 1078-1086.

Pavlou, A. K., and Reichert, J. M. (2004). Recombinant protein therapeutics--success rates, market trends and values to 2010. Nat Biotechnol 22, 1513-1519.

Reichert, J. M. (2003). Trends in development and approval times for new therapeutics in the United States. Nat Rev Drug Discov 2, 695-702.

Ueno, Y. (1983). Trichothecenes- Chemical, Biological, and Toxicological Aspects. Elsevier Science Publishing Company, New York.

Wei, C. M., Hansen, B. S., Vaughan, M. H., Jr., and McLaughlin, C. S. (1974). Mechanism of action of the mycotoxin trichodermin, a 12,13-epoxytrichothecene. Proc Natl Acad Sci U S A 71, 713-717.

Wyatt, R. D., Weeks, B. A., Hamilton, P. B., and Burmeister, H. R. (1972). Severe oral lesions in chickens caused by ingestion of dietary fusariotoxin T-2. Appl Microbiol 24, 251-257.

Wyatt, R. D., Colwell, W. M., Hamilton, P. B., and Burmeister, H. R. (1973a). Neural disturbances in chickens caused by dietary T-2 toxin. Appl Microbiol 26, 757-761.

Wyatt, R. D., Hamilton, P. B., and Burmeister, H. R. (1973b). The effects of T-2 toxin in broiler chickens. Poult Sci 52, 1853-1859.

Wyatt, R. D., Hamilton, P. B., and Burmeister, H. R. (1975a). Altered feathering of chicks caused by T-2 toxin. Poult Sci 54, 1042-1045.

Wyatt, R. D., Doerr, J. A., Hamilton, P. B., and Burmeister, H. R. (1975b). Egg production, shell thickness, and other physiological parameters of laying hens affected by T-2 toxin. Appl Microbiol 29, 641-645.

Appendix

Appendix A

JM109 Competent Cell Transformation Protocol

Materials

JM109 Competent Cells (Promega L2001, Madison WI)
SOC medium
LB plates with antibiotic
Falcon 17 × 100mm polypropylene culture tubes (Fisher 14-959-10B, Atlanta GA)
Bacto®-tryptone (BD Biosciences, 211705)
Bacto®-yeast extract (BD Biosciences 210929)
Ampicillin (Sigma A0166-5G); made into 50 µg/ml stock
AccuSpin MicroR MicroCentrifuge (Fisher, 13100511)
Glucose (Sigma G5767, St Louis, MO)
Petri Dishes (Fisher 0875712, Atlanta, GA)
LB Agar (Fisher BP1425-500 Atlanta, GA)
42°C gyratory water bath

Solutions

Glucose, 2M
Weigh 180.16g glucose ($C_6H_{12}O_6$; F.W. 181.16)
Add distilled water until final volume is 500ml
Filter-sterilize through a 0.2µm filter unit
Store in aliquots at -20°C.

50 mg/ml Ampicillin Stock
Measure 0.5 g Ampillicin
Add to 10 ml ddH_20
Vortex until completely mixed
Filter-sterilie through a 0.45 µm filter unit
Store in 1 ml aliquots at -20ºC

LB Agar plates supplemented with Ampicillin
500 mL dd H_20
20 g LB Agar

Autoclave for 20 minutes
Cool to 55ºC
Add 1 ml of 50 mg/mL Ampicillin stock
Pour plates

Mg2+ stock solution, 2M
Weigh 101.5g MgCl2
Weigh 123.3g MgSO4
Combine and Add distilled water until final volume is 500ml
Fillter-sterilize through a 0.2µm filter unit.

SOC medium
2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml Mg2+ stock, filter-sterilized
1ml 2M glucose, filter-sterilized
Bring to 100ml volume with distilled water.
Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg2+ stock and 2M glucose stock, each to a final concentration 20mM adjust pH to 7.0. Filter the complete medium through a 0.2µm filter unit.

Procedure

1. Chill sterile 17 × 100mm polypropylene culture tubes on ice, one per transformation (e.g., Falcon. 2059).

2. Remove frozen Competent Cells from -70°C and place on ice for 5 minutes, or until just thawed. Once the cells have thawed, pipet quickly or use chilled (4°C) pipette tips to prevent the cells from warming above 4°C.

3. Gently mix the thawed Competent Cells by tapping the tube, and transfer 50 µl to the chilled culture tube.

4. Add 1 µl pUCSV*Tri101* (the concentration was unknown) to 50 µl of Competent Cells. Move the pipette tip through the cells while dispensing. Quickly mix the tube by tapping the side.

5. Immediately return the tubes to ice for 10 minutes.

6. Heat-shock the cells for exactly 45 seconds in a water bath at exactly 42°C. Do not shake.

7. Immediately place the tube on ice for 2 minutes.

8. Add 950µl of cold (4°C) SOC medium to each transformation reaction, and incubate for 90 minutes at 37°C with shaking (approximately 225rpm).

9. For each transformation reaction, it was recommend diluting the cells 1:10 and 1:100 and plating 100µl of the undiluted, 1:10 and 1:100 dilutions on antibiotic plates. Incubate the plates at 37°C for 12-14 hours.

Appendix B
Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit

Materials

LB Broth (Fisher BP1426-500 Atlanta, GA)
Ampicillin (Sigma A0166-5G); made into 50 µg/ml stock
AccuSpin Micro R MicroCentrifuge (Fisher, 13100511 Atlanta, GA)
1.5 ml Eppendorf tubes (Fisher 05408429 Atlanta, GA)
QIAprep Spin Miniprep Kit (QIAGEN, 27106 Valencia, CA) contains:
- Buffer P1
- Buffer P2
- Buffer N3
- Buffer PB
- Buffer PE
- Buffer EB
- LyseBlue
- RNase A
- 2 ml collection tubes

Solutions

LB Broth
500 mL dd H_20
12.5 g LB Broth (powder)
Autoclave for 20 minutes
Cool to 55ºC
Add 1 ml of 50 µg/mL Ampicillin (final concentration 100 µg/ml)

Procedure

1. Grow transformed JM109 cells containing pUCSV*tri101* in LB plus ampillicin media overnight at 37ºC in a shaking water bath. Remove 1 ml of overnight culture. Centrifuge at 13,000 rpm (16,000 x g) for 1 minute at room temperature.

2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to the microcentrifuge tube.

3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex.

4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.

5. Centrifuge for 10 minutes at 13,000 rpm (~16,000xG) in a table-top microcentrifuge.

6. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

7. Centrifuge at 13,000 RPM (~16,000 x g) for 30–60 seconds. Discard the flow-through.

8. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging at13,000 RPM (~16,000 x g) for 30–60 seconds Discard the flow-through.

9. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging at 13,000 RPM (~16,000 x g) for 30–60 seconds

10. Discard the flow-through, and centrifuge at 13,000 RPM (~16,000 x g) for an additional 1 minute to remove residual wash buffer.

11. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl H_20 to the center of each QIAprep spin column, let stand for 1 minute, and centrifuge at 13,000 RPM (~16,000 x g)for 1 minute.

Note: All protocol steps should be carried out at room temperature.

Appendix C
Gel Electrophoresis

Materials

Agarose Gel (Fisher BP1356-500, Atlanta, GA))
1Kb Ladder (Promega G571A, Madison, WI)
Blue Orange 6x Loading Buffer (Promega G190A, Madison, WI)
50x Tris/Acetic Acid/EDTA (TAE) Buffer (BioRad 161-0743, Hercules, CA)
10 mg/ml Ethidium Bromide (BioRad 161-0433; Hercules, CA; wear gloves because Ethidium Bromide is a carcinogen)
Alpha Innotek FluorChemTM 5500 (AlphaInnoTek 5500, San Leandro, CA)

Solutions

1x TAE Buffer
Mix 49 parts water with 1 part 50X TAE Buffer (final concentration will be 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8.0))

Procedure

For an 0.8% concentration:

1. Measure 50 mL 1X TAE Buffer into a 125 mL glass bottle
2. Measure 0.4 g Agarose and add to 1X TAE buffer
3. Place in Microwave for 1 minute
    a. Watch solution to ensure that it does not boil over
4. Allow solution to cool on bench for 2-3 minutes
5. Add 5 µl of 10 mg/ml Ethidium Bromide
6. Mix solution by swirling
7. Pour slowly into gel tray

8. Add comb

9. Wait 15-20 minutes for gel to become firm

10. Release gel from vice

11. Place tray in electric field filled with 1X TAE buffer

12. Ensure gel is completely covered with 1X TAE buffer

13. Load reagents
    a. Load 5 µl of the 1 Kb ladder
    b. Mix 5 µl of each sample to 1 µl of Blue Orange 6x Loading Buffer

14. Place electrodes onto apparatus (positive to positive and negative to negative) so DNA will migrate toward the positive (red) node

15. Run gel100 volts of constant voltage for 30 minutes

16. Remove electrodes from apparatus

17. Remove gel

18. Take picture using transilluminating filter of the Alpha Innotek FluorChem 5500

Appendix D
PCR Reaction with pUCSVtri101

Materials

Forward primer (Integrated DNA Technologies, Skokie, IL)
Reverse primer (Integrated DNA Technologies, Skokie, IL)
Distilled, deionized H_20
Taq DNA polymerase 10x Reaction Buffer without $MgCl_2$ (Promega M190G, Madison, WI)
$MgCl_2$ (Promega A351H, Madison, WI)
dNTP (Promega C114G, Madison, WI)
Taq DNA Polymerase in Storage Buffer B (Promega M166B, Madison, WI)
0.2 ml PCR Tubes (Fisher E0030 124 260, Atlanta GA)
Eppendorf Mastercycler (Fisher S65955, Atlanta GA)

Solutions

Reaction consisted of:

5 µL whole cell pUCSV*tri101* (unknown concentration)
4 µL 5 pM/µl forward primer
4 µL 5 pM/µl reverse primer
64 µL distilled, deionized H20
10 µL Taq DNA Polymerase 10x Reaction Buffer without $MgCl_2$
10 µL MgCl2 (final concentration 1.5 mM)
2 µL dNTP
1 µL Taq DNA Polymerase in Storage Buffer B
100 µL

5 µL reconstituted pUCSV*tri101* (unknown concentration)
4 µL 5 pM/µl forward primer
4 µL 5 pM/µl reverse primer
64 µL distilled, deionized H20
10 µL Taq DNA Polymerase 10x Reaction Buffer without $MgCl_2$
10 µL MgCl2 (final concentration 1.5 mM)
2 µL dNTP
1 µL Taq DNA Polymerase in Storage Buffer B
100 µL

Procedure

Thermocycler settings started with 5 minutes at 95ºC then 33 repetitions of rotating intervals of 30 seconds at 95ºC to denature, 30 seconds at 55ºC to anneal, and 90 seconds at 72ºC for extension

Appendix E
QIAquick PCR Purification Kit Protocol

Materials

AccuSpin MicroR Microcentrifuge (Fisher, 13100511, Atlanta, GA)
QIAquick PCR Purification Kit (QIAGEN, 28106 Valencia, CA) contained:
- QIAquick Spin Columns
- Buffer PBI
- Buffer PE (concentrate)
- Buffer EV
- 2 ml collection tubes

Procedure

1. Add 500 µl of Buffer PBI to 100 µl of the PCR sample and mix.

2. Check that the color of the mixture is yellow (similar to Buffer PBI without the PCR sample). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge at 13,000 RPM (~16,000 x g) for 30–60 seconds

5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.

6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge at 13,000 RPM (~16,000 x g) for 30–60 seconds

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge at 13,000 RPM (~16,000 x g)the column for an additional 1 min.

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 µl ddH_20 to the center of the QIAquick membrane and centrifuge the column at 13,000 RPM (~16,000 x g) for 1 minute.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.

Appendix F
Digestion of tri101 from PCR amplified pUCSVtri101 using Bam HI and Hind III

Materials

ddH_20
10x Buffer E (Promega R005A, Madison, WI
Amplified, extracted, and purified *tri101* (60 ng/ml)
10 units/µL Bam HI (Promega R602A, Madison, WI)
10 units/µL Hind III (Promega R604A, Madison, WI)
1.5 ml Eppendorf Tubes (Fisher 05408129, Atlanta, GA)

Procedure

The digestion was completed using the following reaction:

37 µL distilled, deionized H_20 (ddH_20)
8 µL of 10x Buffer E (Promega R005A, Madison, WI)
35 µL of amplified, extracted, and purified 60 ng/µl *tri101*
4 µL of 10 units/µL Bam HI (Promega R602A, Madison, WI)
4 µL of 10 units/µL Hind III (Promega R604A, Madison, WI)
88 µL Total

Solutions were placed into 1.5 ml eppendorf tubes and incubated for 2 hours in a 37ºC water bath.

Appendix G1
Ligation of tri101 into pQE30

Materials

Digested (Bam HI, Hind III), purified *tri101* gene product (60 µg/ml)
pQE30 (QIAGEN 32915, Valencia, CA)
2x ligation buffer (Promega C6713, Madison, WI)
T4 ligase (Promega, M180a, Madison, WI)
1.5 ml Eppendorf tubes (Fisher 05408129, Atlanta, GA)

Procedures

The reaction consisted of:

- 2 µL of 30 ng/µl pQE30 (QIAGEN 32915, Valencia, CA)
- 2 µL digested, purified 60 ng/µl *tri101* gene product
- 5 µL of 2x ligation buffer (Promega C6713, Madison, WI)
- 1 µL of T4 ligase (Promega, M180a, Madison, WI)

  10 µL total

and was incubated at room temperature for 2 hours.

Appendix G2
JM109 Competent Cell Transformation Protocol

Corresponds to Appendix A except for the following changes:

Step 3 Gently mix the thawed Competent Cells by tapping the tube, and transfer 100 µl to the chilled culture tube. (instead of 50 µl in Appendix A)

Step 8 Add 900µl of cold (4°C) SOC medium (instead 950 µl in Appendix A) to each transformation reaction, and incubate for 90 minutes at 37°C with shaking (approximately 225rpm).

Appendix H
PCR was completed to amplify the 4 pQE30 plasmids that accepted tri101

Appendix D with the following changes:

Use 5 µl of pQEtri101 (instead of pUCSVtri101 in Appendix D) in the reaction

Appendix I

Digestion of pQEtri101 with Bam HI and Hind III

Appendix F with the following changes:

- 13 µL distilled, deionized H_20
- 2 µL of 10x Buffer (Promega R005A, Madison, WI)
- 5 µL pQE*tri101*
- 5 µL Bam HI (Promega R602A, Madison, WI)
- 5 µL of Hind III (Promega R604A, Madison, WI)

  30 µL total

Appendix J
SDS PAGE

Materials:

Mini Protean Tetra Cell (BioRad 165-8002, Hercules CA)
44 mM Ammonium persulfate (BioRad 161-0700 Hercules, CA)
30 % bisacrylamide (BioRad 161-0436)
0.5 and 1.5 M Tris-HCl (Fisher, BP 153-1 Atlanta, GA)
Isobutanol (Fisher AA36643K7, Atlanta, GA)
1N HCl (Fisher, A485-212 Atlanta, GA)
Tris Base (Fisher, BP 152-5 Atlanta, GA)
Glycine (Fisher BP 381-5 Atlanta, GA)
SDS (Fisher BP 166500 Atlanta, GA)
≥ 99.9% Methanol (Fisher, BP 1105-4 Atlanta, GA)
99.7+% Acetic Acid $C_2H_4O_2$ (Fisher AC423220025, Atlanta, GA)
β-mercaptolethanol (Fisher BP 176-100 Atlanta, GA)
Commassie Blue R-250 (Fisher BP 161-0400 Atlanta, GA)
TEMED (Fisher BP 150-20 Atlanta, GA)
Loading Tips (Fisher 05408154, Atlanta, GA)
AccuSpin MicroR Microcentrifuge (Fisher 13100511, Atlanta GA)
Alpha Innotek FluorChemTM 5500 (AlphaInnoTek 5500, San Leandro, CA)

Solutions

44 mM Ammonium persulfate ($(NH_4)_2S_2O_8$; F.W. 228.2)
1g ammonium persulfate
10 mL dd H_2O
Aliquot into 0.5 mL fractions and freeze at -20C

Separating gel buffer- 1.5 M Tris-HCl, pH 8.8
27.23 g Tris Base ($H_2NC(CH_2OH)_3 \cdot HCl$; $C_4H_{11}NO_3 \cdot HCl$; F.W. 157.60)
80 mL dd H_2O.
Tritrate with 1N HCl to pH 8.8.
Add dd H20 to desired final volume of 150 mL

Stacking gel buffer 0.5 M Tris-HCl, pH 6.8
6 g Tris Base ($H_2NC(CH_2OH)_3 \cdot HCl$; $C_4H_{11}NO_3 \cdot HCl$; F.W. 157.60)

60 mL dd H_2O
Tritrate with 1N HCl to pH 6.8
Add dd H_20 to desired final volume of 100 ml

10X Running Buffer

Tris Base	30g
Glycine	144 g
SDS	10 g

Add dd H_2O to final volume of 1 Liter
To dilute to 1X by adding 900 mL dd H2O to 100 mL 10X Running Buffer

Staining Solution

Methanol	500 ml
Acetic Acid	100 ml
dd H_2O	400 ml
Commassie Blue R-250	2 g
Total	1 Liter

Destaining Solution

Methanol	500 mL
Acetic Acid	100 mL
dd H_2O	400 mL
Total	1 Liter

2X Loading Buffer

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2 ml
10 % SDS	4 ml
β-mercaptolethanol	1 ml
Total	9.5 ml

Water-saturated Isobutanol
Shake equal volumes of water and isobutanol ($(CH_3)_2CHCH_2OH$; F.W. 74.12) in a glass bottle
Allow to separate.
Use the top layer.
Store at -4º

Separating Gel

dd H_20	3.35 ml
1.5% Tris HCl at pH 8.8	2.5 ml
10% SDS	100 µl

Acrylamide Bis (30%)	4.0 ml
Ammonium Persulfate	40 µl
TEMED	5 µl
Total	10 ml

Stacking Gel

dd H_20	6.1 ml
0.5% Tris HCl at pH 6.8	2.5 ml
10% SDS	100 µl
Acrylamide Bis (30%)	1.3 mL

*Stop at this step (because gel will set so quickly after addition of Ammonium Sulfate and TEMED, wash the water-saturated isobutanol off the top of the cassette before adding Ammonium Persulfate to the solution)

- Decant water saturated Isobutanol from top of gel cassette
- Rinse cassette with dd H_20 at least 3 times

Ammonium Persulfate	40 µL
TEMED	10 µL
Total	10 ml

Procedure

SDS PAGE

1. Prepare 10% Separating Gel
2. Place glass cassette into casting stands
3. Load into glass cassette using 5 mL pipette
   a. Fill until about ½ to ¾ inch below top of cassette to account for stacking gel
4. Add ~ 1mL water saturated isobutanol
5. Ensure no bubbles are present
6. Allow get to set for 45 minutes
7. Prepare stacking gel

8. Add ~ 1 mL stacking gel to glass cassette to fill
9. Ensure no bubbles are present
10. Push comb into place
11. Let stacking gel set for 45 minutes
12. Meanwhile prepare samples for loading
13. Remove samples containing 2X loading buffer from -20C freezer
14. Place in floater
15. Boil for 5 minutes
16. Microcentrifuge for 10 seconds 13,000 RPM (~16,000 x g)
17. Remove glass cassettes from the gel casting stands
18. Place glass cassettes into casting frames
19. Place casting frames with glass cassettes in electrode apparatus
20. Fill portion between glass cassettes almost full with Running Buffer
    a. Ensure to fill to above beginning of gel portion, but below top of cassette
21. Add running buffer to running apparatus to fill it ~ 1/3 full
22. Remove gel comb
23. Using loading tips, 10 µL of each sample was loaded into each lane
24. Place the lid on the apparatus by positioning the positive (red) electrodes together and the negative (black) electrodes together
25. Set machine to 200 volts for 45 minutes at 3.0 Amps
26. Push start
27. Wait 45 minutes
28. Remove glass cassette

29. Decant running buffer

30. Separate the glass cassettes using plastic spatula

31. While gel is still attached to one piece of glass, cut off stacking gel portion with the spatula

32. Place gel into staining solution and shake for 1 hour at room temperature

33. Move gel to destaining solution and shake for 1 hour at room temperature

34. Move gel to distilled H_20 overnight at room temperature

35. Take picture of gel using fluorescent filter of FluorChem 5500

Appendix K
Purification of tri101 from inclusion bodies

Materials

5M NaCl (Fisher BP305-212 Atlanta, GA)
1M Imidazole (Fisher BP305-50 Atlanta, GA)
50 mM Sodium Phosphate (Fisher BP 330-1 Atlanta, GA)
6 M HCl (Fisher SA48-1 Atlanta, GA)
10% Glycerol (Fisher BP 229-1 Atlanta, GA)
30% Empigen BB Detergent (Calbiochemical 324690, San Diego, CA)
LB Broth (Fisher BP1426-500 Atlanta, GA)
LB Agar (Fisher BP1425-500 Atlanta, GA)
50 mg/ml Ampicillin (Sigma A0166-5G, St Louis, MO)
JM109 Competent Cells (Promega L2001 Madison, WI)
Ni-NTA Agarose (Qiagen 30230 Valencia, CA)
5 mL Polypropylene Columns (Qiagen 34964 Valencia, CA)
β-mercaptolethanol (Fisher BP 176-100 Atlanta, GA)
500 ml Centrifuge bottle (Fisher 14-375-355, Atlanta GA)
Distilled, deionized water
Isopropyl β-D-thiogalactopyranoside (IPTG; Sigma, I5502-1G St Louis, MO)
10X PBS (Sigma P5493, St Louis, MO)
10 mg/ml Lysozyme (Sigma L3790, St Louis, MO)
Innoculating loops (Fisher 13-075-2 Atlanta, GA)
Beckman Centrifuge (Beckman Coulter J2-HS, Fullerton, CA)
Jouan Centrifuge (ThermoScientific CR312, Atlanta, GA)
Sonic Dismembrator (Fisher 1533853, Atlanta GA)
1.5 ml Eppendorf tubes (Fisher 05408129, Atlanta, GA)
15 ml Graduated Tubes Fisher 05-538-59B, Atlanta, GA)
Falcon 5 ml Polysterene Round Bottom Tubes (Fisher 14-959-1A, Atlanta, GA)
100 O.D. x 15mm H Petri Dishes (Fisher 0875712, Atlanta, GA)
Isotemp Refridgerator (Fisher 13-986-106A, Atlanta GA)
5.7 x 93.5 mm 30 ml Nalgene Polypropylene Centrifuge Tubes (Fisher 5291C, Atlanta, GA)

Solutions

1X PBS

Dilute 1 part 10X PBS with 10 parts ddH_20

LB Broth

500 mL dd H_20
12.5 g LB Broth (powder)
Autoclave for 20 minutes
Cool to 55ºC
Add 1 ml of 50 µg/mL Ampicillin

LB Agar plates

500 mL dd H_20
20 g LB Agar
Autoclave for 20 minutes
Cool to 55ºC
Add 1 ml of 50 mg/mL Ampicillin
Pour plates

IPTG (100 mM)

40 mL dd H_20
Add 1.19 g isopropyl β-D-thiogalactopyranoside
Adjust volume to 50 ml with dd H_20
Divide in 5 ml aliquots and sterilize by filtration with 0.2 µm filter unit
Store at -20ºC (stable for 2-4 months)

Lysis buffer

50 mM Sodium Phosphate ($NaH_2PO_4 \cdot H_2O$; F.W. 137.99)
300 mM NaCL (NaCl; F.W. 58.44)
20 mM Imidazole (HNCH:CHN:CH; $C_3H_4N_2$; F.W. 68.08)
Add Deionized, dd $H_20$to bring to desired volume
Adjust pH to 8.0 using 6M HCl
Store at 4ºC

Wash Buffer

50 mM Sodium Phosphate ($NaH_2PO_4 \cdot H_2O$; F.W. 137.99)
300 mM NaCL (NaCl; F.W. 58.44)
20 mM Imidazole (HNCH:CHN:CH; $C_3H_4N_2$; F.W. 68.08)
10 % (of desired final volume) glycerol
Add dd H_20 to bring to desired volume
Adjust pH to 8.0 using 6M HCl
Store at 4ºC

Elution Buffer

50 mM Sodium Phosphate ($NaH_2PO_4 \cdot H_2O$; F.W. 137.99)
300 mM NaCL (NaCl; F.W. 58.44)
300 mM Imidazole (HNCH:CHN:CH; $C_3H_4N_2$; F.W. 68.08)
10% (of desired final volume) glycerol
Add dd H_20 to bring to desired volume

Adjust pH to 8.0 using 6M HCl
Store at 4ºC

2x Loading Buffer

2.5 ml of 0.5 M Tris HCl (pH 6.8)
2 ml of 10% Glycerol
4 ml of 10% SDS
1 ml β-marcaptoethanol
2 mg Bromophenol Blue
0.5 ml dd H_20

Procedure

Inoculation

1. Using a plastic inoculation loop, choose a single colony of JM109/pQE*tri101* from the LB plus Ampicillin agar plate

2. Inoculate colony into 5mL LB Broth with ampillicin (final concentration of 100 µg/ml)

3. Incubate overnight at 37ºC in a shaking water bath

Induction

4. Pour overnight culture into 50 mL prewarmed (37ºC) LB Ampicillin (final concentration of 100 µg/ml) media

5. Incubate culture at 37ºC for 3 hours in a shaking water bath

6. After 3 hours, check the OD using 1 mL of culture media. Use LB media as the blank. Continue incubation at 37°C until OD ≥0.5

7. Remove 2 "pre-induction" 1 mL samples
    a. Microcentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant
    c. Resuspend pellet in 50 µL 2X loading buffer
    d. Store at -20ºC until SDS PAGE

8. Add 2 mM IPTG (1 mL) to the remaining culture

9. Incubate at 37ºC for 3 hours in a shaking water bath

10. After 3 hours, remove 2 - 1mL "post induction" samples
    a. MicroCentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant
    c. Resuspend pellet in 50 µL 2X loading buffer
    d. Store at -20ºC until SDS PAGE

11. Pour remaining induced culture into 2 centrifuge bottles of equal volumes

12. Centrifuged at 4ºC at 5,000 RPM for 20 minutes (3836 x g) using the Beckman Centrifuge

13. Pour off supernant

14. Resuspended 1st pellet using 5 mL 1X PBS (room temperature). Be sure pellet is resuspended completely. Wash sides of centrifuge bottle. Transfer complete solution to the second bottle

15. Resuspend 2nd pellet using same 5mL 1X PBS suspension from bottle one

16. Transfer to 5 ml Polysterene Round Bottom Tube

17. Add 100 µL lysozyme at 50 mg/mL (final concentration 1mg/ml lysozyme)

18. Place on ice for 30 minutes

19. Meanwhile prepared ice/NaCl adding about 100 grams of NaCl to a 200 ml flask full of ice (this will keep solution extra cold during sonication)

20. Remove suspension from ice and place tube into Ice/NaCl mixture to keep cold

Sonication

21. Position sonicator probe into suspension so that it is submerged at least ½ inch, ensuring that probe does not touch sides or bottom of tube

22. Sonicate at level 3 for 1 minute

23. Rest for 2 minutes

24. Repeat 4 times (5 total)

25. Transfer solution to 30 ml polypropylene centrifuge tube

26. Centrifuge for 30 minutes at 4ºC at 10,000 RPM (15,344 x g) in the Beckman Centrifuge

Ni NTA Column

27. The supernant from centrifugication in step 26,
    a. Label tube “Soluable”
        i. Remove 50 µl for SDS PAGE
        ii. Mix with 50 µl of 2x loading buffer
    b. place in -20ºC freezer

28. Resuspend pellet from step 26, in 5 mL lysis buffer
    a. Label as “INSoluable”
        i. Remove 100 µl for SDS PAGE
        ii. Microcentrifuge for 1 minute at 13,000 RPM (16,000 x g)
        iii. Resuspend pellet in 50 µl of 2x loading buffer

29. Add 0.5 mL of 30% Empigen to the insoluable sample or pellet fraction

30. Placed on rocker in 4ºC Isotemp refrigerator overnight

31. The next morning, aliquot into 1 mL fractions and place in 1.5 ml microcentrifuge tubes

32. Microcentrifuge for 20 minutes at 13,000 RPM (~16,000 x g)

33. Meanwhile, pre-equilibrate Ni NTA Agarose using Lysis buffer
    a. Combine 1 mL Ni NTA Agarose with ~ 5mL Lysis buffer
    b. Centrifuge at 3000 for 1 minute (~1296 x g)
    c. Withdraw majority of supernant with pastuer pipette and discard (do NOT decant supernant, because agarose will pour out too)
    d. Repeat 2 additional times (3 total)

34. After microcentrifugication of insoluabe fraction is complete, there should be no pellet. Pipett all supernant into pre-equilibrated 1 ml Ni NTA solution

35. Place on rocking platforrm and mix for 1.5 hours in 4ºC

36. Label 1 mL tubes for elution 1 to 5, wash 1 to 5, and a larger tube for flow through

37. Label 11 1.5 mL tubes for SDS PAGE samples the same as Step 36. Place 50 µL 2X loading buffer in each tube plus (elution fractons 1-5, wash fractions 1-5, and flow through)

38. Aliquot 2.5 mL of Elution and Wash buffer into separate, labeled tubes

39. Fill column with lysis buffer

40. Remove tip from column and drain majority of lysis buffer from column. Replace tip when finished. Discard lysis buffer

41. Remove insoluable fraction from rocker

42. Pour the sample with Ni-NTA agarose into column

43. Allow column to settle

    a. Ni-Nta agarose will settle to bottom of the column
    b. The lysis buffer and insoluable fraction will float to top

44. Remove tip and collect flow through (Don't allow all of the lysis buffer solution to drain off. Leave a small amount, between .25 and .5 ml, above the agarose)

45. Add 1 mL Wash buffer
    a. Collect 0.3 mL fractions in each of the 5 1 mL centrifuge tubes
    b. Add rest of wash buffer as needed
    c. Don't allow the column all of the wash buffer solution to drain off. Leave a small amount, between .25 and .5 ml, above the agarose

46. Repeat step 45 with 2.5 ml of the aliquoted Elution buffer to acquire the isolated protein, rrTri101

47. Pipette 50 µL from each elution, wash, and flow through fraction tubes and place in corresponding labeled tubes with 2X loading buffer (Step 37)

48. Store remaining sample tubes at -20ºC until completing SDS PAGE

49. Run SDS PAGE (Appendix J) on samples from the pre-induced sample, the post induction sample, the supernant or soluable sample, the pellet or insoluable sample, the flow through, each of the 5 wash fractions, and each of the 5 eluted fractions.

Appendix L
Pierce Slide-A-Lyzer® Dialysis

Materials

Slide-A-Lyzer Dialysis Cassettes (Pierce 66370, Rockford, IL)
Slide-A-Lyzer Buoys (Pierce 66430 Rockford, IL)
Slide-A-Lyzer Syringe (5 ml) and 18 Gauge Needle Accessories
(Pierce 66490 Rockford, IL)
10X PBS (Sigma P5493, St Louis, MO)

Solutions

1X PBS
Dilute 1 part 10X PBS with 10 parts ddH_20

Procedure

1. Thaw rTri101 on ice
2. Remove the Slide-A-Lyzer Dialysis Cassette from its protective pouch by cutting along the dotted line.
3. Attach the hub of a hypodermic needle to the Luer-Lok® Fitting of the syringe by firmly screwing it into place.
4. Remove the protective sheath from the hypodermic needle and filled syringe with rTri101 IB sample by immersing the needle in the sample and then slowly drawing back on the syringe piston.
5. Remove cassette from the buoy. Penetrate gasket through one of the syringe ports at a corner of the cassette with the needle and inject sample. Mark the cassette corner with a permanent marker.
6. With the needle still inserted in the cassette cavity, draw up on the piston to remove air from the cavity to compress the membrane so the sample

contacts the greatest membrane surface area. Use caution to prevent the needle from contacting the membrane.

7. Remove the syringe needle from the cassette while retaining the air in the syringe. The gasket reseals and the membrane cavity has no (or minimal) air in direct contact with the sample.

8. Slip the cassette into the groove of a buoy and float this assembly in a beaker containing 800 ml of 1X PBS to dialyze:

    a. Dialyze for 2 hours in 1X PBS at 4°C;
    b. Change the 1X PBS and dialyze for another 2 hours at 4°C;
    c. Change the 1X PBS and dialyze overnight at 4°C.
    d. Use the 800 ml of 1X PBS for each buffer

9. To remove sample, fill syringe with a volume of air at least equal to the sample size.

10. Penetrate the gasket with a needle through a top, unused syringe guide port. Discharge air into cassette cavity to separate membranes, which prevents needle penetration of the membrane.

11. Rotate the Cassette until the port with the syringe is on the bottom and slowly draw back on the syringe piston to capture the dialyzed sample. Remove the syringe needle from the cassette. Discard the Slide-A-Lyzer Dialysis Cassette.

12. Store samples as -20°C until ready for BCA protein assay.

Appendix M
Pierce BCA Protein Assay

Materials

Glass tubes (Fisher 14-961-26 Atlanta, GA)
Smart Spec 3000 Spectrophotometer (Biorad, 170-2501EDU)
BCA Protein Assay Kit (Pierce 23225, Rockford, IL) contained:
BCA Reagent A, contained: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide
BCA Reagent B, contained 4% cupric sulfate
Albumin Standard Ampules, 2 mg/ml, 10 - 1 ml ampules, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide

Procedure:

Preparation of Standards and Working Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Appendix Table 2.1 as a guide to prepare a set of protein standards. Use dd H_20 to dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). There will be sufficient volume for three replications of each diluted standard.

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

(9 standards + 3 unknowns) × (2 replicates) × (2 ml) = 48 ml WR required

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.

Appendix Table 2.1 Preparation of Diluted Albumin (BSA) Standards
Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure
(Working Range = 20–2,000 µg/ml)

Vial	Volume distilled, deionized H20 (µL)	Volume & Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 µL Stock	2000
B	125	375 µL Stock	1500
C	325	325 µL Stock	1000
D	175	175 µL vial B dilution	750
E	325	325 µL vial C dilution	500
F	325	325 µL vial E dilution	250
G	325	325 µL vial F dilution	125
H	400	100 µL vial G dilution	25
I	400	0	Blank

Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Procedure Summary

1. Pipette 0.1 ml of each standard and unknown sample replicate into an appropriately labeled test tube.

2. Add 2.0 ml of the WR to each tube and mix well.

3. Cover and incubate tubes at selected temperature and time:
   - 37°C for 30 minutes (working range = 20-2,000 µg/ml)

4. Cool all tubes to room temperature

5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.

7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

Appendix N
Purification of rTri101 from soluble fractions

Use materials and solutions recipes from Appendix K with the following changes to the procedure:

Procedure

Inoculation

1. Using a plastic inoculation loop, choose a single colony of JM109/pQE*tri101* from the LB plus Ampicillin agar plate

2. Inoculate colony into 5mL LB Broth with ampillicin (final concentration of 100 µg/ml)

3. Incubate overnight at 37ºC in a shaking water bath

Induction

4. Inoculate overnight culture into 50 mL prewarmed (37ºC) LB Ampicillin (final concentration of 100 µg/ml) media

5. Incubate culture at 37ºC for 3 hours in a shaking water bath

6. After 3 hours, check the OD using 1 mL of culture media. Use LB media as the blank. Continue incubation at 37°C until OD ≥0.5

7. Remove 2 "pre-induction" 1 mL samples
    a. Microcentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant
    c. Resuspend pellet in 50 µL 2X loading buffer
    d. Store at -20ºC until SDS PAGE

8. Add 1 mM IPTG (0.5 mL) to the remaining culture

9. Incubate at 37ºC for 2 hours in a shaking water bath

10. After 2 hours, remove 2 - 1mL "post induction" samples
    a. MicroCentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant

c. Resuspend pellet in 50 µL 2X loading buffer
d. Store at -20ºC until SDS PAGE

11. Pour remaining induced culture into 2 centrifuge bottles of equal volumes

12. Centrifuged at 4ºC at 5,000 RPM for 20 minutes (3836 x g) using the Beckman Centrifuge

13. Pour off supernant

14. Resuspended 1st pellet using 5 mL 1X PBS (room temperature). Be sure pellet is resuspended completely. Wash sides of centrifuge bottle. Transfer complete solution to the second bottle

15. Resuspend 2nd pellet using same 5mL 1X PBS suspension from bottle one

16. Transfer to 5 ml Polysterene Round Bottom Tube

17. Place on ice for 30 minutes (do not add any lysozyme)

18. Meanwhile prepared ice/NaCl adding about 100 grams of NaCl to a 200 ml flask full of ice (this will keep solution extra coldl during sonication)

19. Remove suspension from ice and place tube into Ice/NaCl mixture to keep cold

Sonication

20. Position sonicator probe into suspension so that it is submerged at least ½ inch, ensuring that probe does not touch sides or bottom of tube

21. Sonicate at level 3 for 1 minute

22. Rest for 2 minutes

23. Repeat 4 times (5 total)

24. Transfer solution to 30 ml polypropylene centrifuge tube

25. Centrifuge for 30 minutes at 4ºC at 10,000 RPM (15,344 x g) in the Beckman Centrifuge

26. For the supernant from centrifugication in step 25,
    a. Label tube “Soluable”
        i. Remove 50 µl for SDS PAGE

ii. Mix with 50 µl of 2x loading buffer

27. For the pellet from step 25,
    a. Resuspend pellet in 5 mL lysis buffer
    b. Label as “INSoluable”
        i. Remove 100 µl for SDS PAGE
        ii. Microcentrifuge for 1 minute at 13,000 RPM (16,000 x g)
        iii. Resuspend pellet in 50 µl of 2x loading buffer
    c. Store in -20ºC freezer

Ni NTA Column

28. Thaw supernant (soluble fraction) on ice

29. Aliquot into 1 mL fractions and place in 1.5 ml microcentrifuge tubes

30. Microcentrifuge for 20 minutes at 13,000 RPM (~16,000 x g)

31. Meanwhile, pre-equilibrate Ni NTA Agarose using Lysis buffer
    a. Combine 1 mL Ni NTA Agarose with ~ 5mL Lysis buffer
    b. Centrifuge at 3000 for 1 minute (~1296 x g)
    c. Withdraw majority of supernant with pastuer pipette and discard (do NOT decant supernant, because agarose will pour out too)
    d. Repeat 2 additional times (3 total)

32. After microcentrifugation of soluble fraction is complete, there should be no pellet. Pipette all supernant into pre-equilibrated 1 ml Ni NTA solution

33. Place on rocking platform and mix for 1.5 hours in 4ºC

34. Label 1 mL tubes for elution 1 to 5, wash 1 to 5, and a larger, 15 ml tube for flow through

35. Label 11 1.5 mL tubes for SDS PAGE samples the same as step 34. Place 50 µL 2X loading buffer in each tube plus (elution fractions 1-5, wash fractions 1-5, and flow through)

36. Aliquot 2.5 mL of Elution and Wash buffer into separate, labeled tubes

37. Fill column with lysis buffer

38. Remove tip from column and drain majority of lysis buffer from column. Replace tip when finished. Discard lysis buffer

39. Remove soluble fraction with Ni-NTA agarose from rocker

40. Pour the sample with Ni-NTA agarose into column

41. Allow column to settle (wait 1 to 3 minutes)

    a. Ni-NTA agarose will settle to bottom of the column
    b. The lysis buffer and soluable fraction will float to top

42. Remove tip and collect flow through (Don't allow all of the lysis buffer solution to drain off. Leave a small amount, between .25 and .5 ml, above the agarose)

43. Add 1 mL Wash buffer
    a. Collect 0.3 mL fractions in each of the 5 1 mL centrifuge tubes
    b. Add rest of wash buffer as needed
    c. Don't allow the column all of the wash buffer solution to drain off. Leave a small amount, between .25 and .5 ml, above the agarose

44. Repeat step 43 with 2.5 ml of the aliquoted Elution buffer to acquire the isolated protein, rrTri101

45. Pipette 50 µL from each elution, wash, and flow through fraction tubes and place in corresponding labeled tubes with 2X loading buffer (Step 35)

46. Store remaining sample tubes at -20ºC until completing SDS PAGE

47. Run SDS PAGE (Appendix J) on samples from the pre-induced sample, the post induction sample, the supernant or soluable sample, the pellet or insoluable sample, the flow through, each of the 5 wash fractions, and each of the 5 eluted fractions.

Appendix O
Production of Crude rTri101

Use materials and solutions recipes from Appendix K with the following changes to the procedure:

Inoculation

1. Using a plastic inoculation loop, choose a single colony of JM109/pQE*tri101* from the LB plus Ampicillin agar plate

2. Inoculate colony into 5mL LB Broth with ampillicin (final concentration of 100 µg/ml)

3. Incubate overnight at 37ºC in a shaking water bath

Induction

4. Inoculated overnight culture into 50 mL prewarmed (37ºC) LB Ampicillin (final concentration of 100 µg/ml) media

5. Incubate culture at 37ºC for 3 hours in a shaking water bath

6. After 3 hours, check the OD using 1 mL of culture media. Use LB media as the blank. Continue incubation at 37°C until OD ≥0.5

7. Remove 2 "pre-induction" 1 mL samples
    a. Microcentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant
    c. Resuspend pellet in 50 µL 2X loading buffer
    d. Store at -20ºC until SDS PAGE

8. Add 1 mM IPTG (0.5 mL) to the remaining culture

9. Incubate at 37ºC for 2 hours in a shaking water bath

10. After 2 hours, remove 2 - 1mL "post induction" samples
    a. MicroCentrifuge for 1 minute at 13,000 RPM (~16,000 x g)
    b. Pour off supernant
    c. Resuspend pellet in 50 µL 2X loading buffer
    d. Store at -20ºC until SDS PAGE

11. Pour remaining induced culture into 2 centrifuge bottles of equal volumes

12. Centrifuged at 4ºC at 5,000 RPM for 20 minutes (3836 x g) using the Beckman Centrifuge

13. Pour off supernant

14. Resuspended 1st pellet using 5 mL 1X PBS (room temperature). Be sure pellet is resuspended completely. Wash sides of centrifuge bottle. Transfer complete solution to the second bottle

15. Resuspend 2nd pellet using same 5mL 1X PBS suspension from bottle one

16. Transfer to 5 ml Polysterene Round Bottom Tube

17. Add 100 µL lysozyme at 50 mg/mL (final concentration 1mg/ml lysozyme)

18. Place on ice for 30 minutes

19. Meanwhile prepared ice/NaCl adding about 100 grams of NaCl to a 200 ml flask full of ice (this will keep solution extra coldl during sonication)

20. Remove suspension from ice and place tube into Ice/NaCl mixture to keep cold

Sonication

21. Position sonicator probe into suspension so that it is submerged at least ½ inch, ensuring that probe does not touch sides or bottom of tube

22. Sonicate at level 3 for 1 minute

23. Rest for 2 minutes

24. Repeat 4 times (5 total)

25. Transfer solution to 30 ml polypropylene centrifuge tube

26. Centrifuge for 30 minutes at 4ºC at 10,000 RPM (15,344 x g) in the Beckman Centrifuge

27. The supernant from centrifugication in step 26,
    a. Label tube “Soluable”

i. Remove 50 µl for SDS PAGE
ii. Mix with 50 µl of 2x loading buffer

b. place in -20ºC freezer

28. Resuspend pellet from step 26, in 5 mL lysis buffer
    a. Label as “INSoluable”
        i. Remove 100 µl for SDS PAGE
        ii. Microcentrifuge for 1 minute at 13,000 RPM (16,000 x g)
        iii. Resuspend pellet in 50 µl of 2x loading buffer

29. Store remaining sample tubes at -20ºC until completing SDS PAGE

30. Run SDS PAGE (Appendix J) on samples from the pre-induced sample, the post induction sample, the supernant or soluable sample, and the pellet or insoluable sample.

Vita

Shannon Leigh Perrin was born on July 3, 1983, to Jimmy L. and Linda F. Perrin of Blaine, Tennessee. She received her elementary and secondary education in Rutledge, Tennessee. After graduating from Rutledge High School in 2001, Shannon enrolled the University of Tennessee College of Agricultural Sciences and Natural Resources. In 2005, she received her Bachelor of Science degree in Animal Science with a minor in Biology. Thereafter, Shannon received her Master of Science in Animal Science in May 2008.