COMPARISON OF THE CATALYTIC ACTIVITIES OF MEMBERS OF THE PREPRORICIN GENE FAMILY OF RICINUS COMMUNIS

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By

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ABSTRACT

Ribosome-inactivating proteins (RIPs) enzymatically depurinate an adenine in the ricin-sarcin loop of the 28s ribosomal RNA. Previous studies using Southern blots have indicated the presence of multiple copies of the preproricin gene in the Ricinus communis genome and a survey of a 4X draft coverage of the genome shows the presence of seven full length genes, one of which is ricin and six of which are ricin-like proteins (RLP). However, other than ricin, none of the genes have been examined for expression in the plant. The purpose of this research project was to increase our knowledge of RLPs by measuring and comparing the catalytic activities of ricin a-chain (RTA) and the a-chain of all six of the RLPs, using a rabbit reticulocyte lysate assay. The immunocrossreactivity to anti-ricin a-chain antibodies was examined using western blot. The mechanism of action was confirmed by RNA gel electrophoresis. The relative Km was determined by measuring the intensity of aniline cleavage fragments.

A rabbit reticulocyte lysate assay was used to compare the IC50 value of these toxins by measuring the decrease in production of luciferase after the addition of the toxin. While most of the toxins had an IC50 in the low nanomolar range close to 5 nM, one toxin, RLP5 was 15-fold less active with an IC50 of 75 nM. To examine the immunocrossreactivity of the RLPs, a western blot was performed using a monoclonal
anti-ricin a-chain antibody (NR-843). Only RTA, RLP3 and RLP4 could be visualized on a western blot. This indicates that not all RLPs present in the plant will react with NR-843. Using RNA gel electrophoresis combined with aniline reactivity against exposed phosphoribose backbones, it was determined that RTA and all six RLPs can deadenylate RNA in the ricin-sarcin loop. Lastly, measuring the concentration of the fragment released by the RLPs, it was determined that RTA and all six RLPs share a similar relative Km.
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CHAPTER ONE

Introduction
**Ribosome Inactivating Proteins**

Ribosome inactivating proteins (RIP) are plant proteins which inhibit protein synthesis by removal of an adenine in a conserved region, known as the ricin-sarcin loop, of most eukaryotic and some prokaryotic ribosomes (Van Damme et al, 2001). The toxic nature of a RIP was first described in 1888 by Stillmark, discussing the toxic and hemagglutinating activity of ricin. Though it was later shown that the toxic and hemagglutinating activities of ricin were separate, it was this initial discovery that allowed scientists to discover seeds with similar toxic proteins. Examples include abrin and modeccin, which come from the *Abrus precatorius* and *Adenia digitata* plants respectively (Olsnes, 2004). The mechanism by which RIPs work was elucidated in 1988 by Endo and Tsurugi. A large number of RIPs have been discovered in a variety of plant families, along with a few fungi, algae and bacteria; however they do not appear to be ubiquitous in the plant kingdom. For example the whole genome of *Arabidopsis thaliana* has been sequenced and no RIP-like sequence found (Van Damme et al, 2001).

Three types of RIPs have been described in the literature. Type I RIPs express only a single chain. This chain contains the catalytic activity. Type I RIPs are found in the plant species *Iris hollandica*, the families Poaceae, Euphorbiaceae, Cucurbitaceae, Araliaceae, and Lamiaceae and the order Caryophyllales (Van Damme et al, 2001). Type II RIPs are heterodimers and have both an a-chain and a b-chain. The a-chain shares the same catalytic activity as Type I RIPs. The b-chain has lectin activity. This moiety binds to N-galactose, N-acetylgalactosamine (Lord et al, 1994), mannose (Magnusson et al, 1991) and potentially other sugar moieties. Type II RIPs comprise the bacterial RIPs
(including Shiga and shiga-like toxins) as well as the members of plant families including but not limited to Euphorbiaceae, Cucurbitaceae, Lamiaceae, and Caprifoliaceae (Van Damme et al, 2001). The Type III RIP family is at the moment comprised of a single member, the barley protein JIP60. It differs from other RIPS as its a-chain is linked to another protein segment of similar size but unknown function via a disulfide bond (Van Damme et al, 2001). Recent thought is that most Type I RIPS evolved from a common a-chain ancestor. Type II RIPS came later on, after the fusion of the a-chain with a lectin which became the b-chain (Van Damme et al, 2001). Furthermore, it is thought that a subset of Type I RIPS are derived from later deletion of the b-chain of Type II RIPS (Van Damme et al, 2001).

Type II RIPS are the proteins which are considered to be most dangerous to people and livestock due to the binding domains present in the b-chain. Ricin and abrin are the two best known members of this class. These RIPS consist of two subunits linked together by a disulfide bridge. They are synthesized initially in the endoplasmic reticulum as preproproteins. The preproprotein consists of a signaling peptide that targets the toxin to protein bodies, an a-chain that is the catalytic component, a linker peptide and a b-chain that is the binding component. Maturation of the RIP involves removal of the signaling peptide and linker peptide, which occurs as the protein moves to the Golgi and then to either vacuoles or other storage areas within the plant. These proteins are often glycosylated, though this does not appear to be essential to activity as recombinant versions of ricin expressed in *E.coli* have the same active conformation (MIsna et al, 1993).
A problem for researchers studying Type II RIPs is that it is unclear what \textit{in vivo} evolutionary advantage they bestow. The best known activity of Type II RIPs, inactivating a ribosome via cleavage of the adenine in the ricin-sar cin loop, is one that has no known use for the plant cell proper as it would kill the cell. Type II RIPs have little to no activity against viral targets, though they have slightly stronger activity against general DNA and RNA targets. Type II RIPs are currently considered a defense against predation as they will kill animals that ingest the seeds (Van Damme et al, 2001).
The ability of RIPs to act as a glycosylase at sites other than the ricin-sarcin loop of ribosomes, even weakly, is an intriguing one. Barbieri et al (1997) showed that 52 different RIPs acted on both herring sperm DNA and polyadenylic acid. A number of these RIPs deadenylated both tobacco mosaic virus (TMV) and Bacillus phage M2 RNA, which suggests RIPs may defend the host plant against viral infection. Hudak et al (2000) suggest that RIPs may act as antivirals by binding to and degrading capped viral RNA. This appears to be largely a Type I activity with only a few Type II RIPs showing this capability and often at a much decreased rate. The ability of RIPs to depurinate guanine residues (Endo et al, 1987) remains controversial. This activity has been shown in a pokeweed antiviral protein (Hudak et al, 2002) but appears to be unique to that protein. DNA lyase and ribonuclease activity by RIPs have been reported (Roncuzzi and Gasperi-Campani, 1996, Hudak et al, 2000), but other workers have questioned the purity of the enzymes used in these studies (Barbieri et al, 2000, Day et al, 1998). Of course, a strong caveat to interpreting reports of novel biochemical functions by Type I or Type II RIP molecules is that they were evaluated in vitro. It remains unclear whether or not RIPs can replicate these functions in vivo.

A number of roles for RIPs in plants have been hypothesized. Most Type II RIPs cause generalized multi-organ failure in mammals and some insects and thus may be thought to act as a predation defense (Van Damme et al, 2001). Some Type I RIPs exhibit both antiviral and antitumor activity in vivo and are also responsible for the activation of plant defense systems (Van Damme et al, 2001). What is not known is if Type II RIPs
have a role other than predation defense *in vivo*. It is known that some exhibit a low level of antiviral activity against plant virus when exposed *in vitro*, but it is not known whether this activity is present in the plant itself. It is thought that perhaps the RIPs act as a mechanism to destroy damaged or compromised seeds to prevent dispersal of genetically flawed materials. A pathway for this activity would be for the Type II RIPs to activate the apoptotic system to kill cells infected by viruses or tumors (Van Damme et al, 2001). It would be difficult to test this theory *in vivo*, as some plants, such as *Ricinus communis*, do not express the RIP systemically (Van Damme et al, 2001).
Ricinus

*Ricinus communis* produces high concentrations of the Type II RIP, ricin, in its seeds. Up to five percent of the protein mass in the seeds can be ricin, which is produced in the endosperm of the maturing seed and contained in protein bodies (Frigerio and Roberts, 1998). *Ricinus communis* grows in both the tropical and subtropical regions of the world. In frost-free regions, it grows as a small perennial tree (up to three meters) and in the more temperate zones it grows as an annual shrub (Doan, 2004). Among its common uses, it is grown in much of the developing world, including India and Brazil, as a cash crop for its oil. Close to five million tons of castor oil is produced a year. While not grown commercially in the United States due to potential toxicity of ricin released during harvesting, a ricin free variant could allow a re-introduction of the plant for commercial growth. *Ricinus communis* can be found growing as an ornamental plant in the United States and other developed nations.

Historically, ricin has been used in traditional medicines as an abortifacient and as a poison (Lord et al, 1994). In more recent history, it was under development as a biological weapon by the United States and Canada during the First World War. It was fully developed by the United States, in conjunction with Great Britain, during World War II in the form of Compound W, ricin dust spread via cluster bomblets. Ricin has a wide variety of physiological effects. If inhaled, it causes respiratory distress followed by pulmonary edema. If injected or ingested, it causes multi-organ failure. In 1978, it was used, likely by the KGB, to assassinate Georgi Markov, a Bulgarian defector to Great Britain (Doan 2004). In 1991, members of the Patriot Council were arrested for
possession of enough ricin to kill at least 100 people. In 2003 and 2004, it was used in domestic terrorism and found in a postal processing plant in South Carolina and at the United States Senate. In 2008, a man was found in a coma in a Las Vegas hotel room and taken to the hospital. It was later determined that he was suffered ricin poisoning after ricin was found during a search of the room. The man survived and was charged with possession of a biological weapon. There is a growing concern that this toxin will be used as a weapon of mass destruction against United States military or civilian targets.

There are also beneficial uses for ricin. It is intensely studied in the area of chemotherapy, mostly by conjugating the ricin a-chain to immunofragments in an attempt to specifically target the toxic molecule to cancerous cells (Olsnes, 2004). This is among the most active area of research with regard to ricin. It appears to work better on cancers in the blood or lymph, as these cancer types are most available to the toxin, as opposed to solid cancers to which immunotoxins have poor access (Lord et al, 1994). Ricin bound to immunofragments have also been used to remove unwanted cells from bone marrow prior to transplant (Lord et al, 1994). It has the potential for research use by selectively ablating cells. The gene can be expressed along with a specific promoter designed to activate the gene in specific cell types, tissues or at specific developmental ages, allowing the researcher to study what happens in the absence of that tissue (Lord et al, 1994). Lastly, ricin vaccine research has greatly been advanced with increased knowledge of the structure and makeup of the protein (Doan, 2004). By using purified recombinant a-chain as an antigen, animals have been successfully protected against doses of ricin, indicating that it should be possible to produce similar results in humans (Doan, 2004).
**STRUCTURE AND FUNCTION OF THE RICIN TOXIN**

Ricin is the classical Type II RIP. It is synthesized as a preproprotein. It has a signal sequence that directs the initial intracellular sorting and is removed in the mature protein. It has an a-chain that is an N-glycosylase and grants the deadenylating activity for which it is known. There is a linker peptide that has been suggested to play role in sorting the preprotein into protein storage vacuoles and preventing the protein from being active if accidentally missorted to the cytoplasm (Frigerio and Roberts, 1998). Lastly, it has the lectin b-chain that provides the protein’s toxicity by enabling the binding to galactose, N-acetylgalactosamine and mannose molecules on the cell surface. It is important to note that the process by which ricin is internalized is not well characterized. It is known to bind to both clathrin coated and non-coated pits, undergoes retrograde transport from the vesicle to the Golgi apparatus and then to the endoplasmic reticulum and that the b-chain is an absolute requirement for *in vivo* toxicity. How it gets from the endoplasmic reticulum to the cytosol is unknown as ricin does not have a KDEL-like sequence, a known Golgi targeting amino acid sequence. Ricin a-chain without the b-chain that is hooked to a KDEL sequence will be functional and transported into cells, indicating that this same pathway may be used by ricin to enter the cytosol (Tagge et al, 1996). Lord and Roberts (1998) speculate that the Sec61 complex is co-opted by ricin for transport based on its known involvement in movement of misfolded proteins and studies showing that other toxins such as diphtheria toxin and cholera toxin are transported via this pathway. Its *in vivo* function, like the function of all Type II RIPs, is not known. It has been speculated to act as an anti-herbivore toxin, antifungal, or insecticide or as being
involved in the apoptosis of improperly formed seeds during development (Van Damme et al, 2001).

The mechanism of synthesis of ricin has been elucidated. The protein has a N-terminal signal peptide. This peptide directs the preproprotein from its production in the endoplasmic reticulum membrane to the lumen. It is translocated across the membrane by first having most of the signal peptide cleaved, putatively at Ser22 (Lord et al, 1994). The propeptide is N-glycosylated, to give partial protection against endo-N-acetyl glucosamidase H, though non-glycosylated proteins as expressed by *E.coli* are equivalently functional (Lord et al, 1994). Five disulfide bonds are formed (Lord et al, 1994). It should be noted that the proprotein is not active, thus if mistransportation occurs, the proprotein can be degraded without fear of intracellular damage. From here the propeptide is transported to the Golgi, where it is believed that oligosaccharide trimming occurs (Lord et al, 1994). Finally, the propetide gets transferred to vacuoles and protein bodies, where an endopeptidase removes the linker peptide and the remaining signal peptide to form mature ricin. It is believed that either the remaining signal sequence and/or the linker sequence help in the sorting process (Frigerio and Roberts, 1998). The removal of the linker peptide creates active toxin. This sequestration prevents the mature ricin toxin from killing the cell in which it is made. It is important to note that ricin appears to only be made in the endosperm of the plant during seed formation and becomes degraded quickly during the time in which the seed germinates and grows (Lord et al, 1994).

Many of the amino acids involved in the catalytic activity of ricin have been
end, elucidated since Endo (1987) determined that ricin works by the deadenylation of A4324 in rat liver ribosomes. Since the structure of ricin has been resolved using X-ray crystallography (Montfort et al, 1987) and deletion studies (Funatsu et al, 1991, Marsden et al, 2004), a number of amino acids have come to the forefront as being involved in structure, activity, function or binding. Based on a study of 11 RIPs by Funatsu and others (1991) and other works collected in Van Damme (2001), the following amino acids are highly or absolutely conserved in the a-chain or known to be involved in function or binding: Tyr21, Phe24, Arg29, Arg48, Asp75, Asn78, Tyr80, Asn122, Tyr123, Arg134, Gly140, Ala165, Glu177, Ala178, Arg180, Glu208, Asn209, Trp211, Arg213, and Arg258 (all amino acid numbering is from the first amino acid of the a-chain, not the signal peptide). Another amino acid of importance is Cys259, which is involved in the disulfide bridge holding the a-chain and b-chain together. It is important to note that many of these are located in the proposed active site. The adenine binding site is composed of Tyr80, Tyr123, Glu177, Arg180 and Trp211, with Asn78, Arg134, Ala178, Glu208 and Asn209 being located near the active site and being involved in catalytic conformation. A particularly well characterized example is the conversion of Arg180 to glutamine which causes a 2000 fold reduction in activity (Frankel et al, 1990). While it is unknown what specific role Tyr21, Phe24, Arg29, Gly140 and Ala165 play in the activity of ricin, they are proposed to be involved in maintaining the proper conformation of the molecule. These are shown in figure 1, while figure 2 contains the structure of the ricin a-chain with the active site side chains highlighted.

While the exact mechanism of action by which ricin deadenylates the ribosome is
not known, a mechanism has been suggested by Monzingo and Robertus (1992). It is theorized that the adenine forms hydrogen bonds with Tyr80 and Tyr123. This positions Arg180 to protonate one of the nitrogens in the adenine ring, allowing the nitrogen-carbon bond between the adenine and ribose to break. Glu177 stabilizes the oxycarbonium ion on the ribose. The protonated nitrogen then attacks a water molecule and the resulting hydroxyl bonds with the carbonium of the ribose. This leaves an exposed phosphoribose backbone. This is sufficient to prevent protein synthesis by preventing the binding of elongation factors.

The lectin b-chain of ricin has two domains for the binding of carbohydrates. The first domain is a low affinity site which cannot bind N-acetylgalactosamine due to conformational constrictions. The second site is the high affinity site and can bind both galactose and N-acetylgalactose due to a lack of steric hindrance. Both sites can bind mannose (Magnusson et al, 1991). Each domain has a binding site made of five amino acids. The first domain includes Asp22 Gln35, Trp37, Asn46 and Gln47, while the second domain is made up of Asp234, Ile246, Tyr248, Asn255 and Gln256 (all numbering of amino acids is from the first amino acid of the b-chain) (Van Damme et al, 2001). Also important is Cys4, which is involved in building the disulfide bridge between the a-chain and b-chain.
UNRESOLVED ISSUES

Earlier sequencing work in this laboratory indicated high levels of variability in the preproricin gene sequence. When a 4X coverage of the Ricinus communis genome became available, the amplification primers used in the sequencing effort were BLAST searched against the genome. It became apparent that one of the primers was in fact amplifying multiple genes. Since earlier work by Halling (1985) and Tregear (1992) indicated varying number of genes and pseudo genes present in the genome one question that can be asked is: are there multiple full length homologs of the preproricin gene in the Ricinus communis genome? Some of my initial bioinformatic research work showed that there are seven genes with high levels of protein and DNA homology. One of these genes was determined to be preproricin. The other six are ricin-like proteins.

While it would have been beneficial to perform expression studies to determine if these ricin-like proteins are present in the plant, concerns regarding their potential as biothreat agents occurred. This laboratory is not set up to handle select agents and thus expression studies were not performed. Since the toxins are not dangerous unless the a-chain is active, examinations of the ricin-like proteins were concentrated on the a-chain component. The key question in regards to the a-chain of the ricin toxin is: are these proteins active? Toxin activity was defined by the toxins ability to inhibit synthesis of luciferase. Inhibition of protein synthesis is characteristic of RIP and if proteins exhibit this activity they then become more relevant from a biodefense perspective. A further question that can be asked is: if they are active, what is their relative activity to each other and what is the mechanism of that activity?
A bioinformatics approach was used to inform later wet chemistry experiments. An initial determination of the activity was performed using a cell-free coupled transcription-translation system. Further studies were performed using expressed and purified protein to determine the IC50 of each toxin, followed by the mechanism of action and the relative Km.
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Figure 1: Depiction of ricin toxin amino acid sequence. Labels are below the sequence.

Residues are shaded as below:

- **A** – Active site residues
- **A** – Residues involved in conformation or binding
- **A** – Disulfide Bond
- **A** – First Binding Domain
- **A** – Second Binding Domain
Figure 2 – Ribbon drawing of the ricin a-chain backbone with an adenine in active cleft, based on Weston et al (1994).

First to last amino acid goes from blue to orange. Sidechains of the five members of the catalytic site are green stick models. The adenine is in blue.
CHAPTER TWO

Materials and Methods
**MATERIALS**

Restriction enzymes and T4 Ligase were obtained from New England Biolabs (Ipswich, MA). All vectors and competent cells, along with ampicillin, kanamycin, Quant-it protein quantification kit, LR clonase kit, and reagents for RNA and protein electrophoresis and blotting, were obtained from Invitrogen (Carlsbad, CA). RNase-free reagents were purchased from Ambion (Austin, TX). Protein purification columns were obtained from USB Corporation (Cleveland, Ohio). Molecular grade water was purchased from Cellgro (Manassas, VA). Rabbit reticulocyte lysate (RRL), both untreated and T7 Quick Coupled Transcription Translation kits, Luciferase assay system, Cell Culture Lysis Reagent (CCLR) and Glo Lysis Buffer were obtained from Promega (Madison, WI). Ampicillin and kanamycin treated agar plates were purchased from Teknova (Hollister, CA). Gel and plasmid purification kits were purchased from Qiagen (Valencia, CA). Histidine blotting reagents, protein stains, and buffer exchange columns were purchased from Pierce (Rockford, IL). The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Monoclonal Anti-Ricin Toxin A Chain (produced *in vitro*), NR-843. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Ricin Toxin A Subunit with N-terminal Histidine Tag, Recombinant from *Escherichia coli*, NR-853.
SOFTWARE

Alignment of DNA and protein sequences was performed using Megalign (DNAstar). Analysis of DNA and protein sequence was performed using SeqBuilder (DNAstar). Modeling of new protein sequences to existing crystal structures was performed on the SWISS-Model site (Arnold et al, 2006). Sequences were mapped onto protein 1ifsA from the protein data bank (Weston, et al, 1994). Manipulation of crystal structures was performed using 3D-Mol, part of Vector NTI 10.3 (Invitrogen). Graphs were generated using Graphpad Prism (Graphpad Software). Images were cropped and manipulated using Adobe Photoshop CS2 and Adobe Illustrator CS2 (Adobe).
**CLONING**

Genes were chosen by examination of the *Ricinus communis* sequencing project at the J. Craig Venter Center (JCVI) for genes with homology to full-length preproricin. Each gene was designed to include a leader sequence that added a KpnI site, a methionine start residue and replaced the first residue of the sequence with a valine, to form a Kozak sequence for optimal expression in rabbit reticulocyte lysate. To C-terminal end a PsiI site, XhoI site and six histidine residues for metal affinity purification were added. Each gene located from the sequencing project at JCVI was purchased from Genscript (Piscataway, NJ) after optimization for maximal expression in *E.coli* and RRL. The optimized DNA sequence and protein sequence including the polyhistidine tag for each protein are shown in figures 3 though 9. The genes were received in pUC57 plasmids, along with a bacterial agar stab. Once received, a loop inoculation from the bacterial stab was plated on Luria Broth (LB) agar plates with 100 µg/ml ampicillin (Teknova). A single colony was picked from each plate and grown in 10 ml LB with 100 µg/ml ampicillin (Invitrogen) overnight at 250 rpm at 37°C. Plasmids were extracted using a Qiagen Spin Miniprep kit according to the manufacturer’s instructions.

The gene of interest was removed from the plasmid by restriction enzyme digestion under two different conditions. For the native version, 1 µg of template, 2 µl of NEB Buffer 2, 0.2 µl of 100X BSA, 1 µl of KpnI (10 units), 1 µl of PsiI (10 units), and molecular grade water to 20 µl. For the polyhistidine version 1 µg of template, 2 µl of NEB Buffer 2, 0.2 µl of 100X BSA, 1 µl of KpnI (10 units), 1 µl of XhoI (10 units), and molecular grade water to 20 µl. Restriction reactions were run for 1 hour at 37°C. 5 µl of
5X gel loading buffer was added to each reaction and loaded onto a 1% agarose gel. The gel was run for 30 minutes at 200V. The gel was visualized on a UV transilluminator and the appropriate band was extracted. The extracted gel was purified using a Qiagen Qiaquick Gel Extraction kit and eluted into a volume of 50 µl.

The gene was then ligated into a pENTR1A vector. The ligation reaction contained 2 µl of T4 Ligase Reaction Buffer, 1 µl T4 Ligase (2,000 cohesive end units), 100 ng template DNA, 100 ng of vector DNA and molecular grade water to 20 µl. The ligation reactions were run at 16°C for 10 minutes then stopped by heat inactivation at 65°C for 10 minutes. Ligation reactions were transformed into One Shot MAX Efficiency DH5α-T1 Competent Cells as directed. Briefly, 5 µl of each ligation reaction was added to a thawed One Shot tube on ice, mixed by tapping and incubated on ice for 30 minutes. The mixture was heat shocked for 30 seconds at 42°C and replaced into ice. To each tube, 250 µl prewarmed SOC medium was added. Tubes were placed in a horizontal rack and shaken at 37°C for 1 hour at 225 rpm. Both 20 µl and 100 µl were plated onto prewarmed LB agar plates containing 50 µg/ml kanamycin (Teknova) and grown overnight at 37°C. A single colony was picked from each plate and grown in 10 ml LB with 50 µg/ml kanamycin (Invitrogen) at 250 rpm overnight at 37°C. Plasmids were extracted using a Qiagen Spin Miniprep kit according to the manufacturer’s instructions.

Genes were then moved from the entry vector to the destination vector using LR clonase recombination, a process that use a mixture of integrase and exiscionase to switch genes which are between an attL sequence with a gene which is between an attR sequence (Invitrogen). A LR clonase reaction contained 100 ng entry plasmid, 150 ng
destination plasmid and TE to 8 µl at room temperature. To this, 2 µl of LR clonase
(Invitrogen) was added and incubated at 25˚C for 1 hour. Then, 1 µl of Proteinase K
solution (Invitrogen) was added and incubated at 37˚C for 10 minutes. LR clonase
reactions were transformed into One Shot MAX Efficiency DH5alpha-T1 Competent
Cells as directed. Briefly, 5 µl of each ligation reaction was added to a thawed One Shot
tube on ice, mixed by tapping and incubated on ice for 30 minutes. The mixture was heat
shocked for 30 seconds at 42˚C and replaced into ice. To each tube, 250 µl prewarmed
SOC medium was added. Tubes were placed in a horizontal rack and shaken at 37˚C for
1 hour at 225 rpm. Both 20 µl and 100 µl were plated onto prewarmed LB agar plates
containing 100 µg/ml ampicillin (Invitrogen) and grown overnight at 37˚C. Plasmids
were prepared and purified using Qiagen HiSpeed Plasmid MaxiPrep kits as directed.
Plasmid concentration was measured using a Nanodrop-1000 spectrophotometer.
Sequences were verified by Genewiz (South Plainfield, NJ).
ACTIVITY DETERMINATION

Each plasmid construct was measured as both a polyhistidine and native variant and compared to a luciferase control plus plasmid elution buffer in which no other plasmid had been added. The experiment used the TNT T7 Quick Coupled Transcription Translation kit (Promega). This kit includes a mixture that contains both T7 polymerase for the production of mRNA from the DNA template and translation equipment for the production of protein. A master mix of 0.25 µl of methionine, 10 µl of rabbit reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made and dispersed equally into tubes. Each plasmid variant was performed in triplicate. After seven minutes of incubation at 30°C, 200 ng of either plasmid in 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 200 ng of the pDEST14 vector plasmid in 10 mM Tris-Cl, pH 8.0, 1 mM EDTA or 10 mM Tris-Cl, pH 8.0, 1 mM EDTA alone was added to each tube. At 20, 25 and 30 minutes from the start of incubation, 2 µl were removed into fresh tubes. To this, 98 µl of Glo Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen. The samples were thawed on ice and 5 µl of the mixture were added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of Luciferase Assay Reagent (Promega). Data was analyzed using Graphpad Prism grouped table format (Graphpad Software). Two way ANOVA was done to assess significance.
PLASMID IC50 COMPARISON: COMPARISON OF THE INHIBITION CONSTANT OF THE
NATIVE AND POLYHISTIDINE VARIANTS OF THE RTA AND RLP5 PLASMIDS IN A RABBIT
RETICULOCYTE LYSATE TRANSCRIPTION/TRANSLATION ASSAY

Both the native and the polyhistidine version of RTA and RLP5 was measured at
8 concentrations and compared to a luciferase control to which the plasmid storage buffer
had been added. The experiment used the TNT T7 Quick Coupled Transcription
Translation kit (Promega). A master mix of 0.25 µl of methionine, 10 µl of rabbit
reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made
and dispersed equally into tubes. Each plasmid variant was performed in triplicate. The
dilution series was made in 10 mM Tris-Cl, pH 8.0, 1 mM EDTA. The samples were
incubated at 30˚C. At seven minutes a 3 µl aliquot of a 200, 100, 66, 33, 6, 3, 0.6 or 0.06
ng solution (final concentration) of each plasmid or 3 µl 10 mM Tris-Cl, pH 8.0, 1 mM
EDTA was added to each tube. A 2 µl aliquot was removed from one tube before
incubation began and 98 µl of Glo Lysis Buffer (Promega) was added, vortexed and
frozen on liquid nitrogen at 25 minutes. The samples were thawed on ice and 5 µl were
added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay
time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of
Luciferase Assay Reagent (Promega). Data was graphed with Graphpad Prism (Graphpad
Software) in xy table format using the log(inhibitor) versus response function. The
equation for the IC50 is Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))). Significance
was assessed using the extra sum of squares F-test.
**PROTEIN PURIFICATION**

In order to purify proteins based on the genes found in the *Ricinus communis* genome, the polyhistidine version of each gene was placed in a cell line designed for protein expression, BL21-A1 as directed. Briefly, 5 to 10 ng of plasmid DNA was added to a one shot vial of cells, mixed by tapping and incubated on ice for 30 minutes. The mixture was heat shocked for 30 seconds at 42°C and replaced into ice. To each tube, 250 µl prewarmed SOC medium was added. Tubes were placed in a horizontal rack and shaken at 37°C for 1 hour at 225 rpm. Both 20 µl and 100 µl were plated onto prewarmed LB agar plates containing 100 µg/ml ampicillin (Invitrogen) and grown overnight at 37°C.

A single colony was picked into 25 ml LB with 100 µg/ml ampicillin and grown overnight in at 37°C. This was diluted 1:20 into 500 ml LB with 100 µg/ml ampicillin and grown for 2 hours. The culture was induced using 20% L-arabinose diluted to 0.2% in the culture and grown for 2 hours at 30°C. Cells were harvested by centrifugation at 4,000 x g for 15 minutes at 4°C. Supernatant was removed and pellets were stored frozen at -80°C.

Pellets were resuspended in 6 ml of 300 mM NaCl, 50 mM NaH₂PO₄, pH 7.5 lysis buffer. Lysozyme (USB) was added to a concentration of 1 mg/ml and incubated on ice for 30 minutes. The pellets were then sonicated using a Misonix S3000 (Misonix, NY) at power 2.5, 6 pulses of 10 seconds with 15 second rests on ice in between each pulse. DNase I was added to a concentration of a 5 µg/ml and incubated on ice for 15 minutes. The solution was then spun at 10,000 g for 30 minutes. The supernatant was
decanted into a separate tube on ice. Prep-ease Ni-TED columns (USB) were equilibrated with 4 ml of 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5 lysis buffer. The clarified supernatant was poured over the columns and allowed to drain by gravity. Each column was washed twice with 4 ml of 5 mM imidazole, 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5 lysis buffer and allowed to drain by gravity. Each column was eluted three times using 3 ml of 250 mM imidazole, 300 mM NaCl, 50 mM NaH$_2$PO$_4$, pH 7.5 lysis buffer into separate tubes. Imidazole was removed using a Zeba Desalt Spin column (Pierce). Proteins were quantified using a Quant-it kit (Invitrogen).
COOMASSIE STAIN OF SDS-PAGE

To examine the purity of the proteins, a coomassie stain was performed on a polyacrylamide gel. In order to be loaded on a gel, 200 ng of each protein was mixed with 2.5 µl of NuPAGE LDS Sample Buffer (Invitrogen), 1 µl of NuPAGE Reducing Agent (Invitrogen) and water up to 10 µl. The samples were heated at 70˚ for 10 minutes then transferred to ice for 2 minutes and briefly centrifuged. The gel used was a NuPAGE Novex Bis-Tris Mini Gel 4-12% (Invitrogen). The gel was placed in an Xcell Surelock Mini cell (Invitrogen). The first lane was loaded with 10 µl of Novex Sharp Pre-stained Protein Standard (Invitrogen), followed by RTA-BEI, RTA, RLP-1, RLP-2, RLP-3, RLP-4, RLP-5 and RLP-6. The gel was run for 15 minutes at 200V. The running buffer 1X MES Running Buffer, prepared by mixing 50 ml 20X NuPAGE MES SDS Running Buffer (Invitrogen) with 950 ml of deionized water. The outer buffer chamber contained 600 ml of buffer, while the inner chamber contained 200 ml of buffer and 0.5 ml of NuPAGE Antioxidant (Invitrogen). The gel was removed from its cassette and stained for 1 hour using GelCode Blue Safe Stain Reagent (Pierce). It was then destained using molecular grade water (Mediatech) for 1 hour. The gel was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 0.05 seconds on the white light transillumination setting.
WESTERN BLOT USING A POLYHISTIDINE DETECTING PROBE

To determine if other polyhistidine containing proteins were co-purified with the RTA/RLPs, a western blot for detecting polyhistidine containing proteins was performed using a nickel-HRP probe. In order to be loaded on a gel, 200 ng of each protein was mixed with 2.5 µl of NuPAGE LDS Sample Buffer (Invitrogen), 1 µl of NuPAGE Reducing Agent (Invitrogen) and water up to 10 µl. The samples were heated at 70˚C for 10 minutes then transferred to ice for 2 minutes and briefly centrifuged. The gel used was a NuPAGE Novex Bis-Tris Mini Gel 4-12% (Invitrogen). The gel was placed in an XCell Surelock Mini cell (Invitrogen). The first lane was loaded with 5 µl of Novex Sharp Pre-stained Protein Standard (Invitrogen), followed by RTA-BEI, RTA, RLP-1, RLP-2, RLP-3, RLP-4, RLP-5, RLP-6. The gel was run for 15 minutes at 200V. The running buffer 1X MES Running Buffer, prepared by mixing 50 ml 20X NuPAGE MES SDS Running Buffer (Invitrogen) with 950 ml of deionized water. The outer buffer chamber contained 600 ml of buffer, while the inner chamber contained 200 ml of buffer and 0.5 ml of NuPAGE Antioxidant (Invitrogen). While the gel was running, a 0.45 µm PVDF membrane (Invitrogen) was soaked in methanol for 30 seconds. It was then transferred to 1X Transfer buffer, made using 50 ml 20X NuPAGE Transfer Buffer (Invitrogen), 1 ml of NuPAGE Antioxidant (Invitrogen), 100 ml of methanol (Fisher) and 849 ml of deionized water. Four blotting pads and 2 pieces of filter paper (Invitrogen) were also soaked in 1X Transfer buffer. When the gel run was complete, it was removed from its gel and placed on a piece of filter paper. The membrane was laid on top along with another piece of filter paper. This was placed in the XCell II Blot module and loaded into
the XCell Surelock Mini cell. The module was loaded with 200 ml of 1X transfer buffer, while the outer chamber was filled with 500 ml of water. The blot was run for 45 minutes at 30V. The blot was removed from the apparatus and blocked using a 1X solution of BSA (Pierce) for 1 hour. The blot was rinsed for 10 minutes twice using 15 ml of Tris-Buffered Saline, 0.05% Tween-20 (TBST) (Pierce). The blot was incubated with a 1:5000 dilution of HisProbe-HRP (Pierce) for 1 hour. The blot was rinsed for 10 minutes four times using 15 ml of TBST. The blot was incubated in 7.5 ml of SuperSignal West Pico Substrate working solution made from equal amounts of SuperSignal West Pico Luminol/Enhancer Solution (Pierce) and SuperSignal West Pico Stable Horseradish Peroxidase Solution (Pierce), for 5 minutes. The blot was placed in a membrane sheet protector. The blot was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 15 minutes on the luminescence setting.
**Western Blot using an Anti-ricin Antibody**

To determine if the RLPs bind to an anti-ricin antibody, a western blot for detecting RTA was performed using a monoclonal antibody for ricin. In order to be loaded on a gel, 200 ng of each protein was mixed with 2.5 µl of NuPAGE LDS Sample Buffer (Invitrogen), 1 µl of NuPAGE Reducing Agent (Invitrogen) and water up to 10 µl. The samples were heated at 70˚ for 10 minutes then transferred to ice for 2 minutes and briefly centrifuged. The gel used was a NuPAGE Novex Bis-Tris Mini Gel 4-12% (Invitrogen). The gel was placed in an XCell Surelock Mini cell (Invitrogen). The first lane was loaded with 5 µl of Novex Sharp Pre-stained Protein Standard (Invitrogen), followed by RTA-BEI, RTA, RLP-1, RLP-2, RLP-3, RLP-4, RLP-5, RLP-6. The gel was run for 15 minutes at 200V. The running buffer 1X MES Running Buffer, prepared by mixing 50 ml 20X NuPAGE MES SDS Running Buffer (Invitrogen) with 950 ml of deionized water. The outer buffer chamber contained 600 ml of buffer, while the inner chamber contained 200 ml of buffer and 0.5 ml of NuPAGE Antioxidant (Invitrogen). While the gel was running, a 0.45 µm PVDF membrane (Invitrogen) was soaked in methanol for 30 seconds. It was then transferred to 1X Transfer buffer, made using 50 ml 20X NuPAGE Transfer Buffer (Invitrogen), 1 ml of NuPAGE Antioxidant (Invitrogen), 100 ml of methanol (Fisher) and 849 ml of deionized water. Four blotting pads and 2 pieces of filter paper (Invitrogen) were also soaked in 1X Transfer buffer. When the gel run was complete, it was removed from its gel and placed on a piece of filter paper. The membrane was laid on top along with another piece of filter paper. This was placed in the XCell II Blot module and loaded into the XCell Surelock Mini cell. The module was
loaded with 200 ml of 1X transfer buffer, while the outer chamber was filled with 500 ml of water. The blot was run for 45 minutes at 30V. The blot was removed from the apparatus and blocked using a 5% Blotto (Santa Cruz) solution prepared using TBST. The blot was rinsed for 10 minutes twice using 15 ml of TBST. The blot was incubated with a 1:5000 dilution of NR-843 anti-ricin monoclonal antibody (BEI) for 1 hour. The blot was rinsed for 10 minutes four times using 15 ml of TBST. The blot was incubated with a 1:20,000 dilution of goat anti-mouse secondary antibody (Zymed) for 1 hour. The blot was rinsed for 10 minutes four times using 15 ml of TBST. The blot was incubated in 7.5 ml of SuperSignal West Pico Substrate working solution made from equal amounts of SuperSignal West Pico Luminol/Enhancer Solution (Pierce) and SuperSignal West Pico Stable Horseradish Peroxidase Solution (Pierce), for 5 minutes. The blot was placed in a membrane sheet protector. The blot was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 15 minutes on the luminescence setting.
TIME COURSE: COMPARISON OF PURIFIED PROTEIN OVER 90 MINUTES

Each protein was measured at 8 time points and compared to a luciferase control to which the protein storage buffer had been added. The experiment used the TNT T7 Quick Coupled Transcription Translation kit (Promega). A master mix of 0.25 µl of methionine, 10 µl of rabbit reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made and dispersed equally into tubes. A 1 µl aliquot was removed from one tube before incubation began and 99 µl of Glo-Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen. The samples were incubated at 30°C. After 14 minutes a 1 µl aliquot was removed from each tube and 99 µl of Glo Lysis Buffer was added, vortexed and frozen on liquid nitrogen. At 15 minutes, 3 µl of 20 mM Tris-HCl, pH 7.0 or 20 mM Tris-HCl, pH 7.0 buffer containing 15 µM RTA or RTA-variant protein (1 µM final concentration) was added to each tube. A 1 µl aliquot was removed from one tube before incubation began and 99 µl of Glo Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen at 20, 25, 30, 40, 60, and 90 minutes. The samples were thawed on ice and 5 µl were added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of Luciferase Assay Reagent (Promega). Data was graphed using Graphpad Prism using the xy table format (Graphpad Software).
**Protein IC50 Comparison: Determination of the Inhibition Constant of RTA and RLPs in a Rabbit Reticulocyte Lysate Transcription/Translation Assay**

Each protein was measured at 8 concentrations and compared to a luciferase control to which the protein storage buffer had been added. The experiment used the TNT T7 Quick Coupled Transcription Translation kit (Promega). A master mix of 0.25 µl of methionine, 10 µl of rabbit reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made and dispersed equally into tubes. The dilution series was made in 20 mM Tris-HCl, pH 7.0. The samples were incubated at 30°C. At 15 minutes a 3 µl aliquot of a 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM solution (final concentration) of each protein or 3 µl 20 mM Tris-HCl, pH 7.0 was added to each tube. A 2 µl aliquot was removed and 98 µl of Glo Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen at 25 minutes. The samples were thawed on ice and 5 µl were added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of Luciferase Assay Reagent (Promega). Data was graphed with Graphpad Prism (Graphpad Software) using the xy table format and the log(inhibitor) versus response function. The equation for the IC50 is Y=Bottom + (Top-Bottom)/(1+10^(X-LogIC50)). Significance was assessed using the extra sum of squares F-test.
Comparison of Activity Between Different Purification Lots

RTA and RLP5 were measured at 8 concentrations and compared to a luciferase control to which the protein storage buffer had been added. The experiment used the TNT T7 Quick Coupled Transcription Translation kit (Promega). A master mix of 0.25 µl of methionine, 10 µl of rabbit reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made and dispersed equally into tubes. The dilution series was made in 20 mM Tris-HCl, pH 7.0. The samples were incubated at 30°C. At 15 minutes a 3 µl aliquot of a 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM solution (final concentration) of each protein or 3 µl 20 mM Tris-HCl, pH 7.0 was added to each tube. A 2 µl aliquot was removed and 98 µl of Glo Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen at 25 minutes. The samples were thawed on ice and 5 µl were added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of Luciferase Assay Reagent (Promega). Data was graphed with Graphpad Prism (Graphpad Software) using the xy table format and the log(inhibitor) versus response function. The equation for the IC50 is Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))). Significance was assessed using the extra sum of squares F-test.
HEAT INACTIVATION ASSAY: CONTROL TESTING TO ENSURE LOSS OF ACTIVITY IS DUE TO RIBOSOME INACTIVATION

The experiment used the TNT T7 Quick Coupled Transcription Translation kit (Promega). A master mix of 0.25 µl of methionine, 10 µl of rabbit reticulocyte lysate mixture and 200 ng of T7 luciferase plasmid per reaction was made and dispersed equally into tubes. Each protein was heat killed at 80°C for 20 minutes and then placed on ice. Non-heat treated BEI-RTA was used as a control. The samples were incubated at 30°C. At 15 minutes a 667 µM heat killed solution (final concentration) of each protein, 1µg of BEI-RTA non-heat inactivated or 20 mM Tris-HCl, pH 7.0 was added to each tube. A 2 µl aliquot was removed and 98 µl of Glo Lysis Buffer (Promega) was added, vortexed and frozen on liquid nitrogen at 25 minutes. The samples were thawed on ice and 5 µl were added to a plate kept on ice. Plates measured in a MicroLumat Plus LB 96 V with a delay time of 2 seconds and a measurement time of 10 seconds after auto-injection of 95 µl of Luciferase Assay Reagent (Promega). Data was analyzed using Graphpad Prism (Graphpad Software).
Determination of the Mechanism of Action

A reaction mixture containing 50 µl of rabbit reticulocyte lysate (Promega) and 100 ng of RTA/RLPs or 1 µl RNase-free water (Ambion) was incubated at 30˚ for 30 minutes in a PTC-1000 thermocycler (MJ Research). To this, 100 µl of RNase-free water was added. The solution was transferred to a microcentrifuge tube and 500 µl of TRIzol LS (Invitrogen) was added. The solution was incubated for 5 minutes at room temperature. To the tube 200 µl of chloroform (Alfa Aesar) was added and the solution was mixed by vigorous shaking for 15 seconds. The solution was then incubated for 15 minutes at room temperature. The tube was spun at 12,000 g for 15 minutes. The clear, aqueous phase was transferred to a fresh tube. To this, 50 µg of Glycoblue (Ambion) was added and the solution gently mixed by hand. To this, 500 µl of isopropanol (Fisher) was added and solution was chilled at -20˚ for 20 minutes. The tube was then spun at 12,000 g for 10 minutes. The supernatant was removed by pipetting. The pellets were resuspended in 50 µl of RNase-free water. From this, 20 µl was aliquoted into a pcr-tube and mixed with 20 µl of aniline acetate, pH 4.5. Aniline acetate was made by mixing 0.5 ml of aniline (Fisher) with 0.6 ml of acetic acid, glacial (Fisher) and 4 ml of RNase-free water (Personal communication, Clelia de la Peña). This mixture was incubated at 60˚ for 3 minutes. The solution was precipitated with 4 µl of 5 M ammonium acetate, 100 mM EDTA (Ambion) and 200 µl of isopropanol on dry ice for 5 minutes. The solution was spun at 12,000 g for 15 minutes. The supernatant was removed and the pellet was washed with cold 80% ethanol. The solution was incubated on dry ice for 5 minutes and spun at 12,000 g for 10 minutes. The supernatant was removed and the pellet was air-dried for 5
minutes. The pellet was resuspended in 20 µl of RNase-free water and measured on a Nanodrop-1000 (Thermo Fisher). A mixture of 500 ng of RNA, 5 µl of Novex TBE-Urea sample buffer (Invitrogen) and RNase-free water to 10 µl was incubated at 70° for 3 minutes. This solution was loaded onto a 6% TBE-Urea gel (Invitrogen) in an XCell Surelock Mini cell (Invitrogen). The outer and inner buffer chambers were loaded with 1X TBE running buffer (Invitrogen). The gel was run at 200V for 2 hours. The gel was then stained with ethidium bromide (Thermo Fisher). The gel was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 2 minutes on the UV transillumination setting with an excitation of 535 nm and emission of 600 nm.
**Determination of the Time Course for Relative Km**

A reaction mixture containing 50 µl of rabbit reticulocyte lysate (Promega) and 100 pg of BEI-RTA or 2 ng of RLP5 (Ambion) was incubated at 30˚ in a PTC-1000 thermocycler (MJ Research). At 5, 10, 15, 20 and 30 minutes a 10 µl aliquot was removed and 100 µl of RNase-free water was added. The solution was transferred to a microcentrifuge tube and 500 µl of TRIzol LS (Invitrogen) was added. The solution was incubated for 5 minutes at room temperature. To the tube 200 µl of chloroform (Alfa Aesar) was added and the solution was mixed by vigorous shaking for 15 seconds. The solution was then incubated for 15 minutes at room temperature. The tube was spun at 12,000 g for 15 minutes. The clear, aqueous phase was transferred to a fresh tube. To this, 50 µg of Glycoblue (Ambion) was added and the solution gently mixed by hand. To this, 500 µl of isopropanol (Fisher) was added and solution was chilled at -20˚ for 20 minutes. The tube was then spun at 12,000 g for 10 minutes. The supernatant was removed by pipetting. The pellets were resuspended in 20 µl of RNase-free water. From this, 10 µl was aliquoted into a pcr-tube and mixed with 10 µl of aniline acetate, pH 4.5. Aniline acetate was made by mixing 0.5 ml of aniline (Fisher) with 0.6 ml of acetic acid, glacial (Fisher) and 4 ml of RNase-free water (Personal communication, Clelia de la Peña). This mixture was incubated at 60˚ for 3 minutes. The solution was precipitated with 2 µl of 5 M ammonium acetate, 100 mM EDTA (Ambion) and 200 µl of isopropanol on dry ice for 5 minutes. The solution was spun at 12,000 g for 15 minutes. The supernatant was removed and the pellet was washed with cold 80% ethanol. The solution was incubated on dry ice for 5 minutes and spun at 12,000 g for 10 minutes. The
supernatant was removed and the pellet was air-dried for 5 minutes. The pellet was resuspended in 20 µl of RNase-free water and measured on a Nanodrop-1000 (Thermo Fisher). A mixture of 500 ng of RNA, 5 µl of Novex TBE-Urea sample buffer (Invitrogen) and RNase-free water to 10 µl was incubated at 70°C for 3 minutes. This solution was loaded onto a 6% TBE-Urea gel (Invitrogen) in an XCell Surelock Mini cell (Invitrogen). The outer and inner buffer chambers were loaded with 1X TBE running buffer (Invitrogen). The gel was run at 200V for 2 hours. The gel was then stained with ethidium bromide (Thermo Fisher). The gel was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 2 minutes on the UV transillumination setting with an excitation of 535 nm and emission of 600 nm.
Determination of the Relative Km

A reaction mixture containing 5, 15, 30, 60 100, or 120 µl of rabbit reticulocyte lysate (Promega) and 100 pg of RTA/RLP, excluding RLP5, or 2 ng of RLP5 (Ambion) was incubated at 30˚ for 10 minutes in a PTC-1000 thermocycler (MJ Research). A positive control band was created by mixing 1 µg of BEI-RTA with 50 µl of rabbit reticulocyte lysate and incubating at 30˚ for 30 minutes. This was added to 100 µl of RNase-free water. The solution was transferred to a microcentrifuge tube and 500 µl of TRIzol LS (Invitrogen) was added. The solution was incubated for 5 minutes at room temperature. To the tube 200 µl of chloroform (Alfa Aesar) was added and the solution was mixed by vigorous shaking for 15 seconds. The solution was then incubated for 15 minutes at room temperature. The tube was spun at 12,000 g for 15 minutes. The clear, aqueous phase was transferred to a fresh tube. To this, 50 µg of Glycoblue (Ambion) was added and the solution gently mixed by hand. To this, 500 µl of isopropanol (Fisher) was added and solution was chilled at -20˚ for 20 minutes. The tube was then spun at 12,000 g for 10 minutes. The supernatant was removed by pipetting. The pellets were resuspended in either 20 µl of RNase-free water for the 5 and 15 µl concentrations or in as much RNase-free water as there was rabbit reticulocyte lysate. From this, 10 µl was aliquoted into a pcr-tube and mixed with 10 µl of aniline acetate, pH 4.5. Aniline acetate was made by mixing 0.5 ml of aniline (Fisher) with 0.6 ml of acetic acid, glacial (Fisher) and 4 ml of RNase-free water (Personal communication, Clelia de la Peña). This mixture was incubated at 60˚ for 3 minutes. The solution was precipitated with 2 µl of 5 M ammonium acetate, 100 mM EDTA (Ambion) and 200 µl of isopropanol on dry ice for 5 minutes.
The solution was spun at 12,000 g for 15 minutes. The supernatant was removed and the pellet was washed with cold 80% ethanol. The solution was incubated on dry ice for 5 minutes and spun at 12,000 g for 10 minutes. The supernatant was removed and the pellet was air-dried for 5 minutes. The pellet was resuspended in 20 µl of RNase-free water and measured on a Nanodrop-1000 (Thermo Fisher). A mixture of 500 ng of RNA, 5 µl of Novex TBE-Urea sample buffer (Invitrogen) and RNase-free water to 10 µl was incubated at 70° for 3 minutes. This solution was loaded onto a 6% TBE-Urea gel (Invitrogen) in an XCell Surelock Mini cell (Invitrogen). The outer and inner buffer chambers were loaded with 1X TBE running buffer (Invitrogen). The gel was run at 200V for 2 hours. The gel was then stained with ethidium bromide (Thermo Fisher). The gel was visualized using a Kodak Image Station 2000MM (Carestream Health) and Kodak 1D Image Analysis software (Carestream Health) for 2 minutes on the UV transillumination setting with an excitation of 535 nm and emission of 600 nm. The gels were analyzed using Kodak MI Image Analysis software (Carestream Health) to generate intensity of the diagnostic fragment. Graphs were created and nonlinear regressions and Lineweaver-Burke performed using Graphpad Prism (Graphpad Software) and the Michaelis-Menten function. The equation for the Michaelis-Menten regression analysis is $Y = \frac{V_{max} \times X}{K_m + X}$. 
Figure 3 – DNA and proteins sequence of RTA

This is the optimized DNA sequence of RTA. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
This is the optimized DNA sequence of RLP1. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
Figure 5 – DNA and proteins sequence of RLP2

This is the optimized DNA sequence of RLP2. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
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0 - ----------------------------------------------- 70
5' - ATTACCCCATCTACGGTCCGGCTGGCAAGCTCCTGACCCGCTGGAGTGCTGCACATGAAATACCCGG
0 - ----------------------------------------------- 140
5' - GCTAGCGCTAGATTGTTGCGCTAGGAGCTTCTCTGGATTTAGGAACTGCACTATCCGG
0 - ----------------------------------------------- 210
5' - CTGAGCGTGAGCCCTGCGCCTGAGTGGACAAATGTGTGATTGGGTATCCTGCGGAA
0 - ----------------------------------------------- 280
5' - ATTTCTCCATCCCGGTAAACACAGGAAAGATGCGGGACGACATCACCACATCTGTTCTACCGATGTGCGGAA
0 - ----------------------------------------------- 350
5' - GTATACCTTTTGCTTTGCTGATTGGACTGAGCGCTGCTGAGACGGCTGGCTGGTTGGAA
0 - ----------------------------------------------- 420
5' - CTGGGCACCGGGTCCCGTGGAGATGCGGTATCAGGCTGGGTATATTATTAGCAAGCGGCCGCCACCCGCTGC
0 - ----------------------------------------------- 490
5' - CGACCCCTGCAGGGACTGTTCTAGCAGTGGCAGGAAGCAGCCGGCTTTGGATCTGATATCGA
0 - ----------------------------------------------- 560
5' - AGCCGAAATTGGCCACACCCTGCAATCTCCGGCCAGGCGCCCCGATACTGCTGAGATTACCTCGGA
0 - ----------------------------------------------- 630
5' - AACACGGAGGGGCAGGCTACCGGACCCATCGAGGAACGAATGAAAGCGCTTTGCGACACGGCCGATCTAGC
0 - ----------------------------------------------- 700
5' - TGCCACGCGCGACCGTGGCAATTTAAGTGTATAGTGCACTTCTGATCCGGATTGACTCTGGTTGAT
0 - ----------------------------------------------- 770
5' - GGTGTATCGCTGCCCCGCCGCGCGACGACATTGCTGGTTATAGGATCACTGCACTGCACTAATACT
0 - ----------------------------------------------- 840
5' - GAG
0 - ++++

N - MVFLKQYPINFTTAGATATQSYTTPARVSRHITLTGADVRHEIVLSNVRGLSISQRFIL
0 - +____________________________________________________________________ 60
N - IELSNAELSVTALDVNTNYVVYRA GinsayFFHPDNQDEAEAIHLPITDFPNYPYTF
0 - +____________________________________________________________________ 120
N - GNYYDRLEQLGLRENIELGTFLEISALYYSTGTQIPLTARFSIVCQMISMAAR
0 - +____________________________________________________________________ 180
N - FQYIEGEMRTIRYNSRAPPSPSVITLENSWRGLSTAIQESNEGAFASPIQLQRNGSKF
0 - +____________________________________________________________________ 240
N - NVYDVSILPIIALMVYRCAPPSSQFYKHHHHH.
0 - +____________________________________________________________________
This is the optimized DNA sequence of RLP3. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
```
5' GGTACCATGGTCTTTCTGAAAACAGTATTCGATTATACTTTACCCACCGCGGCCACCAGCAGCT
5' ++                +                                                                                   70
5' ATACCACCTTCTTTGTCGCTGCTGCGGACCATCTGACCCGCCGCTGAAGTGGCGCAATGAAATTCCGGT
5' ++                +                                                                                   140
5' GCTGGCGAACGGCCCGGCTGGTCTGCGGATTTAATCAGCGCCTTCTCGTGTGCGACGGCGAACCAGCGC
5' ++                +                                                                                   210
5' CATAGCCTTGACCCTGCCATGGAATGGAACAAATCTGCTATAGTGGTATGCTGCGGATACCAACGCT
5' ++                +                                                                                   280
5' ATTTCTTCTCCGGCGGATAGCCCCGAGATGCCGAAGCCAGTTACCCATCTGTTACCGATGCCAGAAAATCC
5' ++                +                                                                                   350
5' GTATACCTTTGGCCTTGGCGTAAAATTGATGCCCTGGAACGCTCGGCTGCTGCTGGAACATGGAA
5' ++                +                                                                                   420
5' CTGGGTATAGGGCCGCGCTGGGAAGATGCCCATCTGCGGCTGTATTATTATAGTACTGCTGCCGATCTGGC
5' ++                +                                                                                   490
5' CGACCGTTGCGCCGAGCTTTATCGTGTGGATTCAGATCGAGCGGACGCGGCTGGCACCTTGAGTATATAG
5' ++                +                                                                                   560
5' AGGCGAAAATTCGCCACCGCCATCCTCGCAACCGGCGGAGCAGCTGTCGATCTGCTGCTGGAACATGGAA
5' ++                +                                                                                   630
5' AATAGCTGGGCGCCGCTGAGGCAACCGGCAATTGAGGGGAGCAATCGAGGCGCCTTGTCGACCAGCGATTCGC
5' ++                +                                                                                   700
5' TGACGGCGCCGAGATTTGATGACAAATTATATAGTATGATGATGAGCAAACTGATTCGCTGATTCGCCGCTGAT
5' ++                +                                                                                   770
5' GGTGATACGCTTGCGCCGCGCCGAGCCACCAGTTTGGTTATAGCACTACATCACCATTACACT
5' ++                +                                                                                   840

5' MVFLKQYPIINFTTAGATAQSYTNFDIAVRSLHTGDDVRHEIPVLRNRVGLPINQRFVL
5' +++                                                                                                   60
5' VQLSNQAEHSVTMALDVTNAYVYVRAGNNAYFRRPDSDEAIAITHLFTDAQNYTFAF
5' +++                                                                                                   120
5' GGNYDRLEQLGREINLGEANPLEDIALYYSTGRIQLPTVARSFIVC IQMISEAVR
5' +++                                                                                                   180
5' FQYIEGEIRTRHRNRSCAPDSVITLSENSWRLSTAIEQESNGAFASPIQLQRNRNSKF
5' +++                                                                                                   240
5' NVYDSKLPIIAMWVCARPPSTQFYKHHHHH.
```
This is the optimized DNA sequence of RLP4. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
This is the optimized DNA sequence of RLP5. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
Figure 9 – DNA and proteins sequence of RLP6

This is the optimized DNA sequence of RLP6. This gene was optimized for expression in E. coli and rabbit. There are KpnI, PsiI and XhoI sites for restriction ligation. The protein sequence contains a polyhistidine tail to allow for metal affinity purification.
CHAPTER THREE

Results

Part I

Bioinformatics
Computational Analysis of Ricin Gene Family Members in the Ricinus Communis Genome

Halling et al (1985), were the first to sequence the preproricin gene. A Southern blot done by the same group determined that there were potentially six members in the gene family. A later analysis done by Tregear (1992) showed eight members in the same family using a Southern blot, at least one of which was ricin, and another of which was likely ricinus communis agglutinin (RCA). More recently, a 4X coverage sequence, meaning that each base has been sequenced at least four times, has been completed at the J. Craig Venter Institute (JCVI). As of December 2007, 27 gene or gene fragments have been determined and annotated by JCVI to share some level of homology with ricin. This homology is determined using a number of methods including domain composition, protein family membership and sequence similarity, all of which are then manually curated. Of these 27 fragments, only 8 are long enough to potentially code for a full-length Type II ribosome inactivating protein (RIP). Seven of these have a coding sequence that encodes a full-length a-chain. The seven are listed with their names as designated by the JVCI naming system in table 1, and will be referred to as ricin toxin a-chain (RTA) and ricin-like protein (RLP) one through six.

An alignment of the a-chains portion of each gene along with the a-chain sequence of X03179, the preproricin gene found in Genbank, is shown in figure 10. As can be seen, one of these genes is 99% identical to the nucleotide sequence found in Genbank and exactly identical to the protein sequence of ricin, and there is ricin. The DNA identity of the ricin-like genes to the preproricin gene ranges from a low of 62.9%
to a high of 96.3%. All seven code for proteins that have an a-chain that is roughly 32 kDa in size. None of these other gene variants have been previously described in the literature.

The ricin gene family variants show a range of DNA and protein homologies to the putative ricin gene (Table 2). The protein identity of the a-chain of the ricin-like proteins to ricin ranges from a low of 52.3% to a high of 92.3%. Funatsu, et al in 1991 determined that there are 13 highly conserved amino acids by comparing the sequences of available proteins. These amino acids are Tyr21, Phe24, Arg29, Tyr80, Tyr123, Gly140, Ala165, Glu177, Ala178, Arg180, Glu208, Asn209 and Trp211. Mutation and structure studies have shown that of these conserved residues it is likely that the active cleft is formed by Tyr80, Tyr123, Glu177, Arg180, and Trp211 (Ready et al, 1991, Kim and Robertus, 1992). Additional mutational analysis has determined that a number of arginine, asparagine and aspartic acid residues play an important role in electrostatic interactions (Marsden et al, 2004). These are Arg48, Asp75, Asn78, Asn122, Arg134, Arg213, and Arg258.

A protein alignment of the RTA/RLPs with the amino acids involved activity and conformation highlighted is shown in figure 11. As can be seen, only one of the RLPs shows any variation in the sites known to be involved in either binding conformation or catalytic activity. RLP5 has 4 differences in the a-chain, one in a catalytic site (Y80N) and 3 in positions involved in conformation or electrostatic interactions (N78T, N209R and R213E). The tyrosine at position 80, along with the one at position 123, is positioned on each side of the adenine ring, holding it in position (Van Damme et al, 2001).
An image showing how the active cleft is different between RTA and RLP5 using a protein modeling program (Swiss-model) is in figures 12 and 13. Each sequence was modeled against 1ifsA, a 2.0 Å x-ray crystal model of the ricin toxin a-chain bound to adenine, using Swiss-Model (Arnold et al, 2004, Weston et al, 1994). Figure 11 shows the catalytic pocket of RTA. In this image, Tyr80 and Tyr123 form a stack around the adenine. Figure 12 shows the catalytic pocket of RLP5. As can be seen, without the tyrosine at position 80, the adenine does not appear to be fully stacked in the binding pocket. This is likely to cause RLP5 to have a lowered activity level in comparison to the other toxins.
**CLONING STRATEGY**

After determining the number of potential full-length ricin variants, gene transcripts were designed for synthesis by Genscript, a company that does custom gene synthesis. Since not all of the genes have a signal sequence, and since this sequence is used for intracellular targeting in the plant, it was excluded from the synthesized genes. The RTA sequence that was synthesized was based on the October 2007 version of the JCVI database; this has a single amino acid difference from the December 2007 version. In the October version, the amino acid at position 197 is serine, in the December 2007 version; this amino acid is a proline. This amino acid is not known to be involved in any part of the protein's activity binding or function. All other RLPs were made using the December 2007 version of the genome. Each gene was designed to include a leader sequence that added a KpnI site for cloning, a methionine residue and replaced the first residue of the sequence with a valine to form a Kozak sequence required for optimal expression in RRL. The Kozak sequence is necessary for optimal expression in mammalian systems including the rabbit reticulocyte lysate system used in most of the following experiments. To the C-terminal end a PsiI site and XhoI site were added for cloning along with six histidine residues. The PsiI site is present for removal of the polyhistidine sequence, thus allowing a native variant of the gene to be produced, while the XhoI site is for restriction cloning. The six histidine residues form a polyhistidine tag, which allows for metal affinity chromatography purification, using Ni-TED columns from USB scientific. A previous paper by Simpson et al (1995) showed that C-terminal polyhistidine tag has no effect on the function of the ricin a-chain in a cell free system. A
C-terminal tag will allow purification of only full-length protein and not deletion mutations (The QiaExpressionist, 2003). Polyhistidine tags are considered poorly immunogenic and unlikely to prevent normal protein function (Terpe et al, 2003). Additionally, the ricin toxin a-chain available through BEI contains a polyhistidine tag.

Finally, all genes were sent to Genscript for amino acid optimization. This process changed the genetic code to one more optimized for expression in E. coli primarily, while secondarily optimized for Oryctolagus cuniculus, while leaving the amino acid sequence unchanged. The genes came from the company in a pUC57 vector. The gene was then excised using KpnI and XhoI to generate a polyhistidine variant and once using KpnI and PsiI to generate a native variant. These fragments were ligated into pENTR1A from Invitrogen using T4 ligase and transformed into DH5α cells. The gene was then transferred to the vector pDEST14 using a site-specific recombination reaction sold by Invitrogen as LR Clonase. This reaction leverages lambda recombination factors to perform site-specific excision and integration. The pDEST14 contains a T7 promoter site and termination site along with a ribosome binding sequence, for optimal mammalian and bacterial expression. This plasmid was transfected into DH5α for plasmid replication and into BL-21A cells for protein production. BL-21A cells use an L-arabinose induction system, wherein the addition of L-arabinose allows production of T7 polymerase, which then transcribes the gene of interest. Genewiz sequenced all genes once in the final vector. A summary of the cloning strategy is shown in Figure 14.
Table 1 – Naming schemes used by JCVI and the new names proposed herein

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Figure 10
Figure 10
Figure 10
Figure 10 – Alignment of the a-chains of RTA/RLPs found in the JCVI castor bean sequence database with the a-chain portion of X03179, a preproricin sequence in Genbank.

A “.” indicates that the base is the same as the base in the X03179 sequence. The sequences are labeled to the right and the last base in a given line is on the left. The ruler above the sequences numbers based on X03179.
Table 2 – Percent identity between each RLP a-chain and the preproricin sequence X03179 a-chain portion in order of decreasing homology

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Figure 11
A “.” indicates that the amino acid is the same as the amino acid in the X03179 sequence.

Amino acids highlighted in orange represent amino acids involved in conformation or electrostatic interactions. Amino acids highlighted in blue represent amino acids which make up the active site.

Figure 11 – Protein alignment of ricin-like proteins.
The sequence for RTA was modeled onto an existing crystal structure (1ifsA). The protein model was hidden, showing only the amino acid side chains in the active site and their position around adenine. The two tyrosines form a stack around the adenine. 

Figure 12 – Protein model of the active site in RTA complexed with adenine.
Figure 13 – Protein model of the active site in RLP5 complexed with adenine.

The sequence for RTA was modeled onto an existing crystal structure (1ifsA). The protein model was hidden, showing only the amino acid side chains in the active site and their position around adenine. While Tyr123 forms its portion of the clamp around the adenine, the asparagine does not contain the benzene ring necessary for full stack formation.
Digest with Kpnl/Xhol
Ligate into pENTR1A
using T4 Ligase

Digest with Kpnl/PsiI
Ligate into pENTR1A
using T4 Ligase

Use LR clonase
to perform recombination

Ricin-like variant
lacZ
MCS

Amp(R)
PMB1

Ricin-like variant
attL1
Kpnl

Ricin-like variant
attL1
Kpnl

pENTR1A 6XHis
pUC origin
Xhol
attL2
Kan(R)

pENTR1A Native
pUC origin
attL2
Kan(R)

Ricin-like variant
attB2
Xhol

Ricin-like variant
attB2

T7 Terminator
bla promoter

T7 Terminator
bla promoter

pDEST14 6XHis
pBR322 origin

pDEST14 Native
pBR322 origin

Ricin-like variant
Kpnl

T7 Terminator
bla promoter

T7 Terminator
bla promoter

Kpln

attB1

T7 promoter

T7 promoter

Amp(R)
Toxin genes were received in the pUC57 plasmid. Two plasmids for each toxin were developed, one which contained a polyhistidine site at the C-terminus and one without a polyhistidine site. The pUC57 plasmid was digested with KpnI/XhoI for polyhistidine or KpnI/PsiI for the native variants. After ligation into the pENTR1A plasmid, clones were grown and plasmids purified. Each plasmid was transferred to the expression vector using LR recombination. These were then grown and purified using a maxiprep.
Part II

Protein gels and Western blots
Coomassie Stain of SDS-PAGE

In order to perform protein based experiments, it was first necessary to express and purify each of the proteins. After expression, these proteins were run on a polyacrylamide gel and stained with a coomassie based stain in order to gain a rough determination of purity and relative concentration.

After protein purification, 200 ng of each toxin was loaded onto a SDS-PAGE gel. The gel was run for 30 minutes at 200V. The gel was placed in a bath containing coomassie stain and stained for 1 hour. The gel was destained in water for 15 minutes. The gel was visualized using a CCD camera system using white light transillumination.

The results are shown in figure 15. As can be seen, each lane contains only a single band, migrating at approximately the same size as the BEI control reagent and the 30 kDa size standard. As each protein is between 30.9 to 32.0 kDa in size the bands are the appropriate size for the expected proteins. The proteins are also relatively pure, as there are no other bands detectable on the gel.
**Western Blot Using a Polyhistidine Detecting Probe**

In order to confirm that the purified proteins and detected using the coomassie stain were the proteins of interest, western blotting is used. Since each protein contained a polyhistidine tag, this was used to help specifically detect the proteins. A nickel-HRP probe was used to bind to the blot and detect the presence of any protein containing a polyhistidine group.

After protein purification, 200 ng of each toxin, along with a prestained size standard, was loaded onto a polyacrylamide gel. The gel was run for 15 minutes then transferred to a PVDF membrane for 45 minutes. The blot was then blocked with a BSA/TBST solution. The blot was incubated with HisProbe-HRP, a non-antibody nickel based probe for detecting polyhistidine groups, for 1 hour. After washing with TBST, the blot was incubated with a chemiluminescent detection solution and visualized on a CCD-camera system using luminescence detection settings.

The results are shown in figure 16. The first lane in figure 15 is a marker lane. Each lane which contains RTA or RLP shows a single band, approximately the size of the BEI control band. These bands are also approximately the size of the 30 kDa band in the marker lane. No other bands are seen, indicating that there is not likely to be any contaminating proteins containing a polyhistidine region.
WESTERN BLOT USING ANTI-RICIN ANTIBODY

In order to test how well an anti-ricin antibody would detect these RLPs, western blotting was used. A monoclonal antibody was purchased from BEI. This monoclonal is known to be able to detect both native and denatured RTA.

After protein purification, 200 ng of each toxin, along with a prestained size standard, was loaded onto a polyacrylamide gel. The gel was run for 15 minutes then transferred to a PVDF membrane for 45 minutes. The blot was then blocked with a BSA/TBST solution. The blot was incubated with BEI NR-843, a mouse monoclonal antibody, for 30 minutes. After washing with TBST, the blot was incubated with goat anti-mouse-HRP secondary antibody for 30 minutes. After washing with TBST, the blot was incubated with a chemiluminescent detection solution and visualized on a CCD-camera system using luminescence detection settings.

The results are shown in figure 17. The size standard is not visible on this blot, as it does not contain any ricin homologs. The lanes containing BEI-RTA and RTA have two bands. One band is approximately 32 kDa. The other is a nondissociated homodimer commonly seen with purified RTA. Of the RLPs, only RLP3 and RLP4 were bound by the antibody. These two RLPs have the highest protein identity to RTA. The other 4 RLPs do not bind to NR-843.
Figure 15 – A coomassie stain of a 4-12% SDS-PAGE gel.

Each lane was loaded with 200 ng of RTA or RLPs. The gel was run for 30 minutes at 200V. The first lane is Novex Sharp Pre-stained Protein Standard.
Figure 16 – Western blot analysis of metal affinity chromatography purified RTA/RLP using HisProbe-HRP.

LDS-denatured, reduced samples of purified proteins were fractionated with 4-12% mini-gel SDS-PAGE. The electrophoresed proteins were transferred to PVDF membrane by electrotransfer. The blot was blocked prior to incubation with HisProbe-HRP. Immunoreactive bands were identified with chemiluminescent substrate.
Figure 17 – Western blot analysis of metal affinity chromatography purified RTA/RLP using anti-ricin antibody (NR-843).

LDS-denatured, reduced samples of purified proteins were fractionated with 4-12% mini-gel SDS-PAGE. The electrophoresed proteins were transferred to PVDF membrane by electrotransfer. The blot was blocked prior to incubation with anti-ricin antibody. The blot was washed prior to incubation with goat anti-mouse HRP secondary antibody. Immunoreactive bands were identified with chemiluminescent substrate.
Part III

Luciferase based assays
Activity Determination: Polyhistidine Tagged Plasmid versus Native Plasmid

The initial research objective was to develop an assay which would determine if both the polyhistidine tagged protein variant and the non-tagged, native variant were active. While previous studies, such as Simpson et al (1995), have shown that ricin a-chain can have a polyhistidine tag added with no negative effect on activity, it was necessary to check each protein variant to ensure that there was similarly no effect.

In this experiment a transcription/translation rabbit reticulocyte lysate (RRL) based protein expression system was used for cell free expression of the RTA/RLPs. This system contained T7 polymerase for transcription of plasmid DNA into mRNA. The system uses rabbit reticulocyte lysate, which contains all the components needed for translation of RNA into protein, such as tRNA, ribosomes, elongation factors, initiation factors and termination factors. For this experiment, luciferase plasmid, which encodes the luciferase enzyme, was incubated with RRL. After 7 minutes, the plasmid encoding the various toxin genes were added. Aliquots of the reaction mixture were withdrawn at 20, 25, and 30 minutes. The activity of luciferase was measured using a lumimometer which can detect luminescence. This allows the use of the luciferase as a measure of the activity of the RTA/RLPs. If the toxins are active, they will start to inhibit the ability of the system to synthesize more protein. This will lower the amount of luciferase made and thus lower the signal generated by the luciferase. As a control to ensure that the luciferase production is not increasing simply due to the increased production of a second plasmid, a control plasmid which encodes chloramphenicol acetyltransferase was used.
The results are shown in figure 18. The graphs depict the relative light units (RLU) along the y-axis and the three time points along the X-axis. As can been seen from the graphs, in each case both the native and polyhistidine version of each toxin was active. This activity is indicated by a lower level of luciferase activity in the treated samples. While at the 20 minute time point the luciferase activity in the treated and non-treated samples was generally the same, by 25 minutes luciferase activity was higher than in either the native or polyhistidine treated samples. It is likely that this lag in activity is due to the time it takes to transcribe and translate sufficient toxin to start inactivating ribosomes. At the 30 minute time point, the luciferase activity was very high in relation to the activity of the treated samples in all cases. In the control plasmid experiment, luciferase activity is similar in both the treated and untreated samples.
PLASMID IC50 COMPARISON: COMPARISON OF THE INHIBITION CONSTANT OF THE NATIVE AND POLYHISTIDINE VARIANTS OF THE RTA AND RLP5 PLASMIDS IN A RABBIT RETICULOCEYTE LYSAE TRANSCRIPTION/TRANSLATION ASSAY

While the previous assay indicates that both polyhistidine and native versions of the toxins are active, it does not show that they are equivalently active. An effective way to test this would be to do an IC50 experiment comparing activity of the plasmids over a range of plasmid amounts. IC50 is the concentration of a molecule which gives half maximal activity for a given assay. Comparing the activity of the native and the polyhistidine plasmid over a range of plasmid amounts will allow the determination if transcription or translation of either of these acts as a limiting factor.

This assay was performed in a similar fashion to the previous one. The native and polyhistidine versions of both the RTA and RLP5 plasmid are used. RLP5 was examined in addition to RTA in order to determine if any difference in activity due to the changes in the active site could be detected. A range of plasmid amounts from 200 ng to 60 pg was tested. The equation Y=Bottom + (Top-Bottom)/(1+10^(X-LogIC50))) was used to fit the graph and determine the IC50.

The results from this experiment are shown in figures 19, 20 and 21. These graphs represent the effects of a range of plasmid amounts for polyhistidine tailed and native plasmid represented as a function of percent luciferase control. These graphs show the characteristic sigmoidal shape of an IC50 curve. The activity of each plasmid at each point is very similar. The polyhistidine and the native versions of the proteins also have very similar IC50 values. In figure 19, the RTA native variant had an IC50 of 3.4 ng.
while the RTA polyhistidine variant had an IC50 of 2.8 ng. In figure 20, the RLP5 native variant had an IC50 of 12.2 ng, while the RLP5 polyhistidine variant had an IC50 of 12.3 ng. These two graphs indicate that there is little difference in activity between the polyhistidine and the native variant of the toxin. Figure 21 shows the difference between the RTA IC50 and RLP5 IC50. A difference of 5-6 fold exists between the two, with RLP5 being the less active toxin. This shows that the bioinformatics indication that there might be an activity difference between RTA and RLP5 due to sequence difference is indeed born out.
**TIME COURSE: COMPARISON OF PURIFIED PROTEIN OVER 90 MINUTES**

In order to perform experiments on the level of inactivation of the ribosome, it was necessary to determine the appropriate time point at which to measure the activity of the protein. This time point should be in the linear range of activity as a function of time. This is to allow differences in the activity of the various proteins to be seen, as testing at maximum activity loses discriminatory power. The methods chosen for testing this were similar to the ones in Voss et al in 2006. They performed a similar series of experiments using riproximin, a RIP derived from *Ximenia americana*, which exhibited similar toxicity to ricin at the cellular level.

The transcription/translation RRL system used in this experiment is the same as in previous experiments. This experiment used purified protein from each of the RTA/RLPs. After 15 minutes, toxin was added to a final concentration of 1 µM. At various time points, aliquots were withdrawn and frozen on liquid nitrogen. A no luciferase plasmid control was used to confirm that in the absence luciferase plasmid there is no increase in luminescence.

The results are shown in figures 22 and 23. Figure 21 is a time course graph, plotting the RLU from the luciferase reaction as a function of time. As can be seen, there is almost no increase over background of luciferase activity at the 14 minute time point. This is likely due to the time it takes to transcribe sufficient mRNA template from the plasmid to allow for efficient transcription. Luciferase activity achieves maximum levels of luminescence at 40 minutes after the initiation of incubation. In the RTA/RLP samples the luciferase achieves maximum activity at the 30-minute time point. This implies that
no more luciferase is being produced, due to irreversible inhibition by RTA or RLPs. There is almost no difference in the activity between any of the ricin variants. Ten minutes after the addition of RTA or a RLP is in the linear range and is used in subsequent experiments. Figure 22 is the same graph, in the absence of the luciferase data. While most of the RLPs cluster in activity levels similar to those of RTA, RLP5 is less active as shown by the slightly higher levels of luciferase activity at all time points.
Protein IC50 Comparison: Determination of the Inhibition Constant of RTA and RLPs in a Rabbit Reticulocyte Lysate Transcription/Translation Assay

Kinetic measurements of ricin and RTA activity vary greatly between substrates and even between different experimental setups within a substrate. The value of the $K_{cat}$ can range from 1777 m$^{-1}$ (Endo et al, 1988) to 0.01 m$^{-1}$ (Gluck et al, 1992). Thus any attempt to measure the IC50 values of each RLP should use the same system and experimental setup to control this variation. It was previously determined that 10 minutes post toxin addition, which is 25 minutes from the start of luciferase incubation, is the optimal time for determination of the IC50.

This experiment uses a similar RRL setup to previous experiments. At 15 minutes after the start of incubation, varying concentrations of RTA/RLPs are added to the RRL mixture. At 10 minutes after the addition of the toxin, aliquots are withdrawn and frozen on liquid nitrogen. The luciferase activity in these aliquots is then measured using a lumimometer. By varying the concentration of toxin added, an IC50 sigmoidal curve can be generated.

The results in figure 24 are shown as a percentage of the luciferase control. As can be seen, a sigmoidal curve representing the log of the concentration of the toxin versus the luciferase response has been generated. This curve indicates the inhibitory power of each of the toxins at any concentration along the graph. In this graph, most of the toxin variants are similarly active and have similar curves to both the BEI-RTA control and the self-generated RTA. However, RLP5 is shifted to the right, which
indicates a decrease in the activity of the toxin. The values for the IC50 are shown in Table 3. While 7 of the 8 proteins cluster around the 5 nM, RLP5 has an IC50 of 75 nM, approximately 15 fold less than the rest of the toxins. This again appears consistent with the idea that RLP5 will be less active due to changes in the active site, while the other proteins had no such changes. A previous paper (Kim and Robertus 1992) indicated that a mutation at Tyr80 may cause a decrease in activity as much as 160 fold, though using a completely different assay system. Another paper by Ready et al (1991) indicates changing Tyr80 to Phe only drops the activity 13 fold, though again using a different assay system. In general, it appears that despite changes outside the catalytic pocket and outside areas already known to be involved in the conformation of the protein, most of the RTA variants have a similar level of activity to RTA. In the one toxin which contains changes in areas involved in binding and activity, there is a decrease in the level of activity.
COMPARISON OF ACTIVITY BETWEEN DIFFERENT PURIFICATION LOTS

Purifying proteins of unknown activity has a potential problem when subsequently measuring activity. If during a single purification run the protein becomes partially inactivated, there is no way of knowing that the measured activity is correct. By comparing the activity of a protein between multiple purifications, it may be possible to identify if a protein has lowered activity due to the purification itself. This is an attempt to measure the IC50 values of RTA and RLP5 from each of 2 different purification rounds using the same system and experimental setup.

In this experiment, both RTA and RLP5 were expressed and purified in a miniprep system similar to the maxiprep system used to generate the proteins for prior experiments. After purification, an IC50 experiment was performed as above.

The results are shown in figures 25, 26, and 27. Each figure represents the activity of the luciferase as a function of increasing concentrations of toxin. This leads to the characteristic sigmoidal curve. As seen in figure 25, the difference between the activity in the two purifications of RTA were small. RTA from the 1st purification had an IC50 of 5.5 nM, while the 2nd purification had an IC50 of 6.0 nM. This is compared to the original IC50 value determined of 5.4 nM. In figure 26, a comparison of the activity between the two rounds of purification of RLP5 is shown. Again, there is little difference in the curves from the first purification and the second purification. RLP5 from the 1st purification had an IC50 of 83 nM, while the 2nd purification had an IC50 of 76 nM. The IC50 for RLP5 from the original IC50 experiments was 75 nM. Figure 27 shows the two sets of purifications together. This shows that the rightward shift in the IC50 is conserved...
between purifications. The difference in activity is approximately 15 fold.
**Heat Inactivation Assay: Control Testing to Ensure Loss of Activity is Due to Ribosome Inactivation**

Ricin is known to be inactivated by heat at 80°C for 10 minutes. To ensure that inactivation activity is due to RNA glycosylase activity and not due to buffer or purification conditions, each protein variant was heated to 80°C for 20 minutes and tested in the rabbit reticulocyte reaction.

A RRL system was used in this experiment. Each of the toxins was heated to 80°C for 20 minutes and then placed on ice. Toxin was added at 15 minutes after the incubation of the luciferase plasmid RRL mixture. After 10 minutes, aliquots were removed and frozen on liquid nitrogen. These samples were measured on a lumimometer.

The results from this experiment are shown in figure 28. This is a comparison of the luciferase activity present in each of the heat-inactivated samples, along with a control sample that was not exposed to heat. In all cases, there is no difference between any of the heat inactivated samples and the control luciferase only sample. The non-heat treated sample did show a decrease in activity. This indicates that all of these toxins are inactivated by heat, and thus unlikely to survive a hot-mash extraction process commonly used in industrialized nations for the extraction of castor oil.
Figure 18
Figure 18
Figure 18 – Determination of the activity of polyhistidine and native versions of RTA and RLPs.

A coupled transcription/translation rabbit reticulocyte lysate system was used to assay the activity of plasmids encoding native and polyhistidine variants of RTA/RLPs. Seven minutes after the addition of the luciferase plasmid to the system, plasmid encoding either the native or the polyhistidine version of a given RTA/RLP was added to the reaction mixture. Aliquots were withdrawn at 20, 25 and 30 minutes and measured using a lumimometer. At the 20 minute time point there is little difference between either variant of the plasmid and the luciferase only controls. By 25 minutes, differences appear and by 30 minutes large differences in the activity level of the treated and non-treated samples appear. All experimental samples are different from control as determined by two-way ANOVA, p<0.0001
A coupled transcription/translation rabbit reticulocyte lysate system was used to compare the activity of plasmids encoding native and polyhistidine variants of RTA. Seven minutes after the addition of the luciferase plasmid to the system, plasmid encoding either the native or the polyhistidine version of RTA was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. RTA native had an IC50 of 3.1 ng. RTA polyhistidine had an IC50 of 2.8 ng. The data was graphed is represented as a function of the control luciferase only data. Curves were not found to be significantly different as determined by extra sum of squares F-test.
A coupled transcription/translation rabbit reticulocyte lysate system was used to compare the activity of plasmids encoding native and polyhistidine variants of RLP5. Seven minutes after the addition of the luciferase plasmid to the system, plasmid encoding either the native or the polyhistidine version of RLP5 was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. RLP5 native had an IC50 of 10.6 ng. RLP5 polyhistidine had an IC50 of 11.3 ng. The data was graphed is represented as a function of the control luciferase only data. Curves were not found to be significantly different as determined by extra sum of squares F-test.
Figure 21 - Comparison of the activity of polyhistidine and native versions of RTA and RLP5.

A coupled transcription/translation rabbit reticulocyte lysate system was used to compare the activity of plasmids encoding native and polyhistidine variants of RTA and RLP5. The concentration values are 200, 100, 66, 33, 6, 3, 0.6 or 0.06 ng. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. RTA native had an IC50 of 3.1 ng. RTA polyhistidine had an IC50 of 2.8 ng. RLP5 native had an IC50 of 10.6 ng. RLP5 polyhistidine had an IC50 of 11.3 ng. There is a 5-6 fold decrease in activity between RTA and RLP5. The data was graphed is represented as a function of the control luciferase only data. Curves were found to be significantly different as determined by extra sum of squares F-test, p<0.05.
A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the activity of RTA/RLPs. Aliquots were removed from the buffer only and luciferase only samples at 0 minutes. At 14 minutes, aliquots were removed from all samples. Fifteen minutes after the addition of the luciferase plasmid to the system, 1 \( \mu \text{M} \) RTA/RLPs was added to the reaction mixture. Aliquots were withdrawn at 20, 25, 30, 40, 60 and 90 minutes and measured using a lumimometer.
A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the activity of RTA/RLPs. At 14 minutes, aliquots were removed from all samples. Fifteen minutes after the addition of the luciferase plasmid to the system, 1 μM RTA/RLPs was added to the reaction mixture. Aliquots were withdrawn at 20, 25, 30, 40, 60 and 90 minutes and measured using a lumimometer. In order to better see the differences between the various toxins, the luciferase only data has been removed from this graph.
A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the IC50 of RTA/RLPs. Fifteen minutes after the addition of the luciferase plasmid to the system, 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM (final concentration) of RTA/RLPs was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. The data was graphed is represented as a function of the control luciferase only data. Curves excluding RLP5 were not found to be significantly different. Curves including RLP5 were found to be significantly different as determined by extra sum of squares F-test, p<0.0001.
Table 3 – IC50 values for RTA/RLPs

This data was generated from the dose response curves in figure 24. The IC50 is the concentration of toxin at which only 50% of the amount of luciferase activity, compared to the control, is seen.

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This experiment compares the IC50 activity of the RTA from 2 different purification rounds. A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the IC50 of RTA. Fifteen minutes after the addition of the luciferase plasmid to the system, 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM (final concentration) of RTA from two different purification experiments was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. The data was graphed as a function of the control luciferase only data. The IC50 value from the first purification of RTA was 5.5 nM The IC50 value from the second purification of RTA was 6.0 nM. Curves were not found to be significantly different as determined by extra sum of squares F-test.
This experiment compares the IC50 activity of the RLP5 from 2 different purification rounds. A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the IC50 of RLP5. Fifteen minutes after the addition of the luciferase plasmid to the system, 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM (final concentration) of RLP5 from two different purification experiments was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. The data was graphed is represented as a function of the control luciferase only data. The IC50 value from the first purification of RLP5 was 83 nM The IC50 value from the second purification of RLP5 was 76 nM. Curves were not found to be significantly different as determined by extra sum of squares F-test.
This experiment compares the IC50 activity of the RTA and RLP5 from 2 different purification rounds. A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the IC50 of RTA and RLP5. Fifteen minutes after the addition of the luciferase plasmid to the system, 667, 333, 66, 33, 6, 3, 0.6 or 0.06 nM (final concentration) of RTA and RLP5 from two different purification experiments was added to the reaction mixture. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. The data was graphed is represented as a function of the control luciferase only data. Curves including RLP5 were found to be significantly different as determined by extra sum of squares F-test, p<0.0001.
This experiment compares the activity of RTA/RLPs that have been heat inactivated. A coupled transcription/translation rabbit reticulocyte lysate system was used to measure the activity of RTA. Fifteen minutes after the addition of the luciferase plasmid to the system, 667 nM (final concentration) of RTA/RLPs that have been exposed to 80° for 20 minutes was added. Aliquots were withdrawn at 25 minutes and measured using a lumimometer. BEI-RTA that had not been exposed to heat was used as a positive control.
Part IV

RNA gel electrophoresis
**DETERMINATION OF THE MECHANISM OF ACTION**

While all of the RLPs have been shown to be active, it is important to determine the mechanism of action. The classical method of action of ricin toxin a-chain is via the cleavage of adenine from the ricin-sarcin loop. This prevents the binding of elongation factors and thus inhibits protein synthesis. By depurinating the ribosomal RNA, this leaves the phosphate backbone open to cleavage by aniline. When run on a denaturing gel, this causes the appearance of a characteristic fragment that does not appear in the absence of aniline treatment. In the absence of toxin, the aniline should have no effect and no fragment should be seen.

An experiment was designed using non-nuclease treated rabbit reticulocyte lysate. The lysate was treated with 100 ng of RTA/RLPs. After treatment with the toxins, the RNA was extracted with TRIzol and purified. This purified RNA was reconstituted with water and exposed (or not) to aniline acetate to cleave any exposed phosphate backbone. The RNA was then repurified using isopropanol and 80% ethanol and reconstituted in water. For visualization, a polyacrylamide gel was run and stained with ethidium bromide.

The results are shown in figure 29. As can be seen, in the presence of toxin and aniline, for all RLPs there is the characteristic aniline fragment. In the absence of aniline, no fragment is present. In the absence of toxin, no fragment is present, regardless of the presence or absence of aniline. This indicates that all of these proteins are acting by cleaving the adenine from the ricin-sarcin loop.
Determination of the Time Course for Relative Km

The mechanism of action of all the RLPs has been shown previously to be via the cleavage of adenine from the 28s ribosomal subunit. This allows, via aniline fragmentation, the direct measurement of a product of the enzyme. Since the product can be directly measured, this allows for the determination of the relative Km of the reaction. The Michaelis constant, Km, is a measure of the concentration at which rate of the enzymatic reaction is proceeding at half the maximal velocity. In order to determine the relative Km, it is important to first determine a time at which to measure this reaction. The time point chosen should be in the low linear range of product formation. The reason for this is to ensure that the enzyme has not been completely saturated by substrate. Two experiments were performed to measure this. Initially, the BEI control reagent was used to determine a time point. Due to the lowered activity of RLP5, a similar time course was run with RLP5 to determine a more appropriate concentration of enzyme.

To measure this, 100 pg of BEI-RTA control reagent or 2 ng of RLP5 was mixed with rabbit reticulocyte lysate and aliquots were removed at 5, 10, 15, 20 and 30 minute time points. The RNA was extracted, purified, treated with aniline acetate and repurified. The RNA was run on a gel and examined for intensity of the bands.

The results are shown in figures 30 and 31. As can be seen for BEI-RTA in figure 30, by 30 minutes significant if not maximal cutting has occurred. By 10 minutes enough cutting is present for a signal to be easily detectable by eye. In figure 31, much the same can be seen. The intensity of the bands at each time point grows and by 30 minutes the band is much the same as a positive control band (not shown).
**Determination of the Relative Km**

The mechanism of action of all the RLPs has been shown previously to be via the cleavage of adenine from the 28s ribosomal subunit. This allows, via aniline fragmentation, the direct measurement of a product of the enzyme. Since the product can be directly measured, this allows for the determination of the relative Km of the reaction. The Michaelis constant, Km, is a measure of the concentration at which rate of the enzymatic reaction is proceeding at half the maximal velocity.

To measure this, 100 pg of RTA/RLP1-4 and 6 or 2 ng of RLP5 was mixed with 5, 15, 30, 60, 100 or 120 µl of rabbit reticulocyte lysate brought up to 120 µl in RNase-free waster. A positive control band using 50 µl of rabbit reticulocyte lysate and 100 ng of BEI-RTA run for 30 minutes was placed in each gel. The RNA was extracted after 10 minutes, purified, treated with aniline acetate and repurified. The RNA was run on a gel and the intensity of the bands was measured. The equation used for the Michaelis-Menten regression analysis was $Y = \frac{V_{max} \times X}{K_m + X}$.

The results are shown in figures 32, 33 and 34. Figure 32 is a nonlinear regression of the velocity data for each RTA/RLP. Since ethidium staining is slightly different each time it is performed, the results were normalized to a positive control band in each gel. The graph shows that these curves are very similar to each other, both in shape and in range. This is more easily seen in the Lineweaver-Burke plot in figure 33. All the lines cross the X-axis, which represents $-1/K_m$, in a very tight range from -0.016 to -0.007. These are more indications that these toxins have highly similar relative Kms. Table 4 lists the relative Km values for each protein. The values range from 63.15 µl to 166.3 µl.
However as seen in figure 34, the relative activity of these is different. RLP5 required a 20-fold higher amount of enzyme to get a detectable signal. This increase in enzyme concentration has no effect on the relative Km, but shows a conserved indication that the activity of RLP5 is decreased relative to all the other members of this gene family.
Rabbit reticulocyte lysate, non-nuclease treated, was mixed with 100 ng of either RTA or RLP. Following a 30 minute incubation, the RNA was extracted, aniline treated where appropriate and purified. Lane designation is shown on the gel. The arrow indicates the fragment released by aniline treatment of the modified rRNA.
Figure 29
Rabbit reticulocyte lysate, non-nuclease treated, was mixed with 100 pg of BEI-RTA. Aliquots were withdrawn after incubation for 5, 10, 15, 20 and 30 minutes. The RNA was extracted, aniline treated where appropriate and purified. Lane designation is shown on the gel. The arrow indicates the fragment released by aniline treatment of the modified rRNA.
Figure 31 - Time Course of fragment creation by RLP5

Rabbit reticulocyte lysate, non-nuclease treated, was mixed with 2 ng of RLP5. Aliquots were withdrawn after incubation for 5, 10, 15, 20 and 30 minutes. The RNA was extracted, aniline treated where appropriate and purified. Lane designation is shown on the gel. The arrow indicates the fragment released by aniline treatment of the modified rRNA.
Figure 32 – Assessment of the relative Km of RTA/RLPs.

Rabbit reticulocyte lysate of varying concentration was incubated with either 100 pg (BEI-RTA, RTA, and RLP1-4 and 6) or 2 ng (RLP5) of toxin for 10 minutes at 30°C. rRNA was extracted, treated with aniline, purified and run on a 6% TBE-Urea polyacrylamide gel. rRNA was quantified using Kodak MI image analysis software. Intensities of the diagnostic fragment were plotted as a percentage of a positive control band. Confidence intervals for all curves showed high levels of overlap.
Table 4 – List of relative Km as determined by non-linear regression.

Nonlinear regression was performed using Graphpad Prism. The equation used for the regression analysis was \( Y = \frac{V_{\text{max}} \cdot X}{K_m + X} \). \( K_m \) represents the amount of rabbit reticulocyte lysate required to have half the \( V_{\text{max}} \). \( R^2 \) represents the goodness of fit of the regression to the data. \( K_m \) was determined from data in figure 32.

<table>
<thead>
<tr>
<th></th>
<th>( K_m ) (( \mu ))</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEI-RTA</td>
<td>101</td>
<td>0.95</td>
</tr>
<tr>
<td>RTA</td>
<td>84.5</td>
<td>0.97</td>
</tr>
<tr>
<td>RLP1</td>
<td>116.6</td>
<td>0.98</td>
</tr>
<tr>
<td>RLP2</td>
<td>103.6</td>
<td>0.98</td>
</tr>
<tr>
<td>RLP3</td>
<td>166.3</td>
<td>0.99</td>
</tr>
<tr>
<td>RLP4</td>
<td>149.5</td>
<td>0.99</td>
</tr>
<tr>
<td>RLP5</td>
<td>63.15</td>
<td>0.99</td>
</tr>
<tr>
<td>RLP6</td>
<td>103.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Figure 33 – Lineweaver-Burke representation of relative Km data.

Rabbit reticulocyte lysate of varying concentration was incubated with either 100 pg (BEI-RTA, RTA, and RLP1-4 and 6) or 2 ng (RLP5) of toxin for 10 minutes at 30°.
rRNA was extracted, treated with aniline, purified and run on a 6% TBE-Urea polyacrylamide gel. rRNA was quantified using Kodak MI image analysis software. Data was plotted as a Lineweaver-Burke for ease of differentiation of kinetic parameters.
Figure 34 - % activity at Km of BEI-RTA per 100 pg of enzyme.

Rabbit reticulocyte lysate of varying concentration was incubated with either 100 pg (BEI-RTA, RTA, and RLP1-4 and 6) or 2 ng (RLP5) of toxin for 10 minutes at 30°. rRNA was extracted, treated with aniline, purified and run on a 6% TBE-Urea polyacrylamide gel. rRNA was quantified using Kodak MI image analysis software. Activity of the enzyme at the Km of the BEI-RTA control is expressed as a function of amount of enzyme required to generate product at this concentration.
CHAPTER FOUR

Discussion
The main question that I sought to answer was: are there multiple full-length homologs of the preproricin gene in the *Ricinus communis* genome? The secondary questions are: are these proteins active, what is their relative activity to each other, and what is the mechanism of this activity?

The overall conclusion to these broad questions is that there are seven active RLPs in the *Ricinus communis* genome. Six of the seven toxins had similar IC50 activity, with RLP5 exhibiting 15 fold less activity. All seven toxins acted via deadenylation of the 28s ribosomal subunit, as determined by the presence of the aniline fragment. All seven molecules had similar relative Kms. Only three of the seven toxins bound to a monoclonal anti-ricin a-chain antibody. These toxins have the potential to be used as biothreat agents, some of which would not necessarily be detected by ELISA or antibody neutralization assay. Specific conclusions based upon each of the unique experimental approaches used are presented in the subsequent sections.
There are seven full-length homologs of the preproricin gene in the *Ricinus communis* genome. This was determined via BLAST search comparison of the preproricin gene to the genome available on the J. Craig Venter Institute website. Table 2 shows a listing of the identities of each protein to preproricin. This protein identity ranged between a low of 52.3% to a high of 93.2%. This is not surprising as it confirms earlier reports by Tregear and Halling that the *Ricinus communis* plant codes for six to eight genes similar to preproricin. Many other Type II RIPs also express multiple toxins. Cinnamomin, from the camphor tree, produces three Type II RIPs (Yang et al, 2002). The elderberry tree produces at least two Type II RIPs with varying activities and lectin affinities (Van Damme et al, 1997). Type I RIPs code for as many as eight functional, expressed proteins (Van Damme et al, 2001). Therefore, the potential for the *Ricinus communis* plant to at least code for multiple active toxins was predictable.

All seven a-chains should be active against ribosomal targets as determined by bioinformatics. One of these toxins is ricin, and thus is known to be active. Five of the other six have no changes in any site known to be involved in binding or activity as shown in figure 10. RLP5 has four changes in amino acids important in binding and activity. One of these changes is in the active site. Previous studies (Funatsu et al, 1991, Marsden et al, 2004) have indicated that changing Asn78, Tyr80, Asn209 and Arg213 will decrease protein activity. Despite these changes, it is likely that RLP5 will remain active. None of the changes in RLP5 should be sufficient to completely remove activity; however, it is likely that these differences in the primary amino acid sequence will cause
a decrease in activity relative to the activity found in RTA. Since the multiple family members from other Type I and II RLPs have at least slightly different activities from each other, a different level of activity for one or more the RLPs would bring the *Ricinus communis* more in line with other similar plants.
WESTERN BLOT DETECTION OF RLPs

Not all proteins are bound by an anti-ricin monoclonal antibody. As can be seen in figure 16, only RTA, RLP3 and RLP4, in addition to the control toxin, are bound by monoclonal antibody NR-843 available from BEI. This raises serious issues with some of the current methods of detecting ricin contamination. These often rely on a combination of activity assays with neutralization assays. These assays use antibodies, either for neutralization or confirmation that the inactivating protein is ricin. Since many innocuous plants such as barley and wheat contain Type I RIPs, which would set off an activity assay, this antibody confirmation is crucial to confirm the presence of ricin. Given that an antibody will not necessarily detect every RLP present in the *Ricinus communis* genome, an increased risk of a false negative arises. A false negative would potentially prevent people from seeking and receiving appropriate treatment for ricin poisoning, in which prompt treatment is crucial. This would also likely inhibit forensic investigation, as investigators would begin to suspect other RIP toxins such as abrin, viscumin or cinnamomin.

There are some direct avenues of research available based on this data. The RLPs can be tested against a variety of other existing anti-ricin antibodies, both monoclonal and polyclonal. This should give some sense of the robustness of existing antibody assays to detect the variant RLPs. Epitope mapping can be done using site directed mutagenesis to determine the exact location of the existing epitope. This mapping should be directable, based on sequence comparisons, looking for regions that RLP3, RLP4 and RTA have in common, but RLP1, RLP2, RLP5, and RLP6 are different.
**LUCIFERASE BASED DETECTION OF RIBOSOME INACTIVATING ACTIVITY**

Using a cell-free transcription translation system, plasmids encoding either polyhistidine or non-polyhistidine variants of each RTA/RLP inhibit the production of luciferase. As can be seen in figure 18, all seven toxins, in both polyhistidine and native, inhibit the further synthesis of luciferase. A control plasmid encoding chloramphenicol acetyl-transferase does not cause a decrease in luciferase production, indicating that there is enough excess capacity in the system to produce both luciferase and a second protein, at least up to the level of detection of the lumimometer. This would indicate that the proteins the RTA/RLP plasmids are producing are inhibiting luciferase production. This gives credence to the earlier bioinformatics that each of these proteins is active. Additionally, this shows that adding a polyhistidine tail to each of the a-chains does not prevent them from being active. This confirms an earlier study showing that the c-terminal tail of the protein is not very involved in the activity of the protein (Simpson et al, 1995). This study stated that when c-terminal mutants were reassociated with b-chain, they took longer to translocate into the cytosol, indicating a role for the c-terminus in cell entry, but not activity.

Using a cell-free transcription translation system, plasmids encoding either polyhistidine or non-polyhistidine variants of RTA and RLP5 are equivalently active over a range of plasmid amounts. As can be seen in figure 21, both the native and polyhistidine versions of RTA and RLP5 are equivalently active. This is unsurprising as the control protein purchased from BEI contains a polyhistidine tag and is active. Additionally, studies by Simpson et al in 1995 show no difference in the activity of
polyhistidine labeled a-chains and non-polyhistidine labeled a-chains. The control protein purchased from BEI contains a polyhistidine tag. Communication with people involved in the repository indicate that there is no difference in activity between purified a-chain from the plant and recombinant polyhistidine tagged a-chain (McKee, 2007). As predicted by the bioinformatics, these plasmids are not equivalently active to each other. RLP5 is less active than RTA by approximately five-fold. While not as great a decrease in activity as might be expected, it is difficult to exactly quantify the rate at which each of these proteins is being produced in this system. Since the plasmids are optimized for expression in *E.coli* and only secondarily in rabbit, they may contain varying levels of rare codons for a mammalian expression system. This is not an issue when purified toxin is used.

Using a cell-free transcription translation system, RTA and RLPs 1-4 and 6 have similar IC50’s while RLP5 is less active. As can be seen in figure 24, all proteins produced are active. Six of the seven toxins produced and a control a-chain purchased from BEI share similar activity with IC50 values ranging from 4.8 to 6.9. RLP5 has an IC50 value of 75 nM, 15 fold less than the other RLPs. This is not surprising given its differences in amino acids involved in activity and binding. This decrease in activity is greater than that seen in the plasmid experiments. This is likely due to the fact the differences seen in this experiment are solely due to any differences in the toxin activity, not any differences in the production of the toxin *in vitro*. Again, this confirms the bioinformatics hypothesis that RLP5 will be less active due to changes in the amino acids responsible for binding and activity. This difference in activity is conserved across
multiple production runs of RLP5. When comparing two purification lots of RTA and RLP5, each protein exhibited an IC50 value similar to itself. However, the decrease in activity was conserved, in that both production runs of RLP5 were less active than both RTA production runs.

These data lend themselves to several follow-up experiments. Site-directed mutagenesis, followed by the luciferase assays, can be performed on RLP5 to determine which of its variant amino acids causes contributes most to returning the protein to RTA-like activity. Alternately, RTA could undergo site-directed mutagenesis, and see how much of a decrease each change causes in the luciferase assays. This experiment could cause some minor issues, as the large protein sequence difference between RTA and RLP5 could indicate rescue of some activities by other changes in the RLP5 sequence. However, knowing that other amino acids elsewhere in the protein may be acting to rescue the activity would be helpful in understanding the interactions of various amino acids in RTA. Yields from the purifications of each protein were poor. Comparisons of natively purified versus denaturation/renaturation purification could be performed in order to determine the ease of increase in production of each protein for future experimentation. This may also determine if any of the decrease in activity by RLP5 is due to some percentage of the protein being denatured, though this is less likely considering the data from figure 21.
MECHANISM OF ACTION OF RLPs ON RABBIT RETICULO CYTE LYSATE

Using rabbit reticulocyte lysate as a substrate, all seven toxins were able to
deadenylate the ricin-sarcin loop. As can be seen in figure 28, all toxins produced a
characteristic fragment after cleavage of the adenine and treatment with aniline. After an
RIP removes the adenine from the phosphoribose backbone, it leaves the backbone open
to attack. Aniline causes scission of the backbone, and when run on a denaturing gel,
causes the appearance of extra fragment. This does not occur in the presence of toxin but
absence of aniline, without the aniline to cleave the backbone, the fragment remains part
of the 28s rRNA. This also does not occur in the absence of toxin, regardless of the
presence or absence of aniline. This is strong evidence that the proteins are acting at the
typical site of action for an RIP. Most Type II RIPs exhibit the ribosome inactivation as
their strongest activity.
RELATIVE Km OF RLPs

All seven proteins exhibit similar relative Km values. As can be seen in table 4, all the toxins have relative Km values in the range from 63.15 µl to 166.3 µl. Km values range greatly depending on the composition of the target. RTA has a very variable Km. The type of target used has the greatest effect, as many of the small molecule imitators of the ricin-sarcin loop show much greater Km than when using ribosomes. Even when using ribosomes, the Km can range from 0.1 µM to 1.3 µM, depending on the ribosome source and purification style (Kim et al, 1992). When using small molecules are used, the Km raises significantly to 130 µM (Gluck et al, 1996), though a concomitant increase in the kcat is seen. Additionally, it again becomes clear that RLP5 is not as active as the other members of the family as it requires a 20 fold greater amount of toxin in order to see the same effect.

The next logical step would be to perform an actual Km experiment using HPLC to measure the release of adenine. Using site-directed mutagenesis on RLP5 and RTA could help determine which changes are affecting only the activity and which are affecting the Km as well. If changing RLP5s amino acids in known catalytic and functional areas back to RTA returns the activity but maintains the same Km, while changing the same amino acids in RTA to the ones present in RLP5 causes a higher Km, that may indicate that other amino acids present in RLP5 are replacing the missing amino acids function.
**Overall Conclusions**

A rabbit reticulocyte lysate assay was used to determine the IC50 value of these RLPs by measuring the production of luciferase. While RTA and RLPs 1 through 4 and 6 had IC50 values ranging from 4.8 to 6.9 nM, one toxin, RLP5, had an IC50 value of 75 nM. In a study using a monoclonal mouse anti-ricin a-chain antibody, only BEI-RTA, RTA, RLP3 and RLP4 could be visualized on a western blot. By examining an RNA polyacrylamide gel, which combined toxin activity against rabbit reticulocyte lysate ribosomes with aniline reactivity against exposed phosphoribose backbones, it was determined that RTA and all six RLPs act by cleaving an adenine in the ricin-sarcin loop. Lastly, by measuring the concentration of the fragment released by the RLPs as determined by its intensity on a polyacrylamide gel stained with ethidium bromide, it was determined that RTA and all six RLPs share a similar relative Km ranging from 63.15 µl to 166.3 µl.
POTENTIAL AVENUES FOR FURTHER RESEARCH

This study has shown that there are seven catalytically active ribosome inactivating proteins present in the *Ricinus communis* genome. This opens several pathways for further research into members of the preproricin gene family.

First, a thorough examination of expression of the ricin-like proteins should be considered. While these proteins contain either CAAT and/or TATA box structures near the beginning of the genes, there are other plant specific promoters to consider. Location specific promoters should be investigated, as other RIPS are present in a variety of plant structures not just the seeds (Van Damme et al, 2001). The leaf, stem and root are becoming the focus of studies designed to find new members of the RIP family (Kawade et al, 2008, Roy et al, 2006). Since the *Ricinus communis* plant is known to exhibit different growth patterns in different parts of the world, temperature based expression should also be considered. Stressing the plant with drought or salinity might activate the expression of certain members of the family. In *Jatropha curcas* the expression of various curcin proteins seems tied to different stresses (Qin et al, 2005). Also, maize RIP proenzymes experienced differential expression in response to drought (Bass et al, 2004). Reaction to infection by fungus or plant bacteria is a possible cause for expression. Type I RIP from the bitter melon plant gets upregulated in response to infection with *Sphaerotheca fuliginea*, a type of mildew that attacks melons (Xu et al, 2007). Time dependent or developmental expression is also a distinct possibility. Ricin itself is only expressed during the last stages of cellular endosperm differentiation. Other RIPS such as beetin (Iglesias et al, 2008) and SoRIP (Kawade et al, 2008) exhibit preferential
distribution throughout the plant.

Second, continued research into the activities of the ricin-like protein a-chains should occur. While they are all active on ribosomes, they may exhibit differential activity on other substrates. Pokeweed antiviral protein received its name for its antiviral activity. Several other plant RIPS exhibit antiviral activity, such as BBAP1 (Choudhary et al, 2008) and bryodin (Stirpe et al, 1986), against targets such as the tobacco mosaic virus. Other RIPS exhibit activity against fungal and bacterial targets (Xu et al, 2007, Vivanco et al, 1999). Generalized poly-deadenylation activity should also be examined, as this is the only other confirmed activity of ribosome inactivating proteins (Barbieri et al, 2000). Additionally, given that RTA readily reassembles with ricin b-chain, experiments on the ability of the RLPs to be cytotoxic via reassociation with the b-chain or direct injection into cells should be examined.

Lastly, an examination into the activity and specificities of the b-chain should be examined. Five of the six proteins had the complete b-chain sequenced, while RLP3 was missing a potion of the b-chain from its sequence. Despite missing a section, it did contain at least one region that contained all the amino acids considered important for one of the two b-chain binding sites. Each of these RLPs could exhibit markedly different affinities to a variety of b-chain targets such as galactose, mannose and N-acetylgalactosamine. It is not unusual in plants producing multiple Type II RIPS to have each RIP have preference to a different sugar (Van Damme et al, 2001). Mistletoe produces three different isoforms of the protein viscumin each of which has a different binding affinity to galactose and N-acetylgalactosamine. The differences found in the b-
chains could also effect how the a-chain enters the cells. Some of these proteins might be active in cell free assays but due to differences in the a-chain or b-chain have problems being taken into the cell or translocated to the cytosol.
CHAPTER FIVE

Bibliography


Hudak KA, Bauman JD, Tumer NE. Pokeweed antiviral protein binds to the cap structure of eukaryotic mRNA and depurinates the mRNA downstream of the cap. RNA (New York, N.Y 2002; 8(9):1148-59.


McKee M 12/10/2007 BEI Reagent suggestion [Personal Email]


Pena Cdl 12/3/2007 Fwd: Aniline Acid [Personal email]


